University of Agronomic Sciences and Veterinary Medicine of Bucharest
Faculty of Animal Productions Engineering and Management

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RESEARCH ON THE ESTIMATION OF GENETIC PARAMETERS FOR PRODUCTION AND EXTERIOR TRAITS IN SUBPOPULATIONS OF THE TSURCANA BREED

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Abstract

The purpose of this work is to estimate the genetic parameters for the production and exterior traits of the 4 lines of the Turcana breed. The research was carried out on a herd of 16,196 Tsurcana sheep, structured on the 4 lines, of which: Belă, Brează, Bucălae and Oacheşă. 7 exterior traits were analyzed (conformation, wool yield, wool length, milk yield, The body weight at 60 days, The body weight at 120 days and average daily gain). For the estimation of the genetic parameters (Heritability and Genetic Correlation) the BLUP methodology was used. The heritability values recorded values from 0.116 (The body weight at 60 days in Bucălae), to 0.475 for milk yield (Belă). The values of genetic correlations achieved the full range of values, in the interval [-1, +1], with variations depending on the pair of traits and the population analyzed. Heritability and genetic correlation are useful parameters for substantiating the improvement programs of the analyzed populations.

Key words*: BLUP, exterior traits, genetic parameters, production traits.*

INTRODUCTION

The Ţurcană breed developed primarily through natural selection, becoming well adapted to a wide range of environmental conditions. Over the past 50 years, artificial selection has led to the emergence of a highly differentiated breed complex with an insular reproductive structure, comprising four subpopulations with distinct phenotypic and genetic characteristics currently undergoing consolidation (Ţurcana Brează, Bucălae, Belă, and Oacheşă). This genetic differentiation raises the need for separate breeding programs tailored to each subpopulation (Drăgănescu, 2003; 1995).

A series of studies have highlighted statistically assured differences between the four populations of the Ţurcană breed (Drăgănescu, 1979; 1994; Margetín et al., 2020). The more genetically different the populations are from each other, the more differentiated the genetic progress will be.

Results on the estimation of heritability of production traits for Valachian and Tsigai sheep were presented by Oravcová (2015). Also, Makovický (2018), obtained values within the limits of the specialized literature for the heritability of milk production and milk fat content in Valachian sheep populations.

In this analysis, the genetic differences between populations were established by the genetic determinism estimated at the level of each one. The genetic determinism of the traits expressed by heritability and genetic correlation is the basis for optimizing sheep breeding programs (El-Saied et al., 1998). The level of these indicators substantiates the genetic progress of the traits in the objective of sheep breeding.

The BLUP (Best Linear Unbiased Prediction) methodology, introduced by Henderson (1973), has become the standard tool for estimating breeding values and genetic parameters in livestock populations. Its application to sheep breeding has been validated in numerous studies, showing greater accuracy compared to classical selection methods, particularly in populations with complex family structures and multiple measured traits. The sire model, a simplified variant of the animal model, is especially useful in large-scale genetic

evaluations where full pedigree information is not available, as it allows estimation of additive genetic variance components from paternal half-sib families (Mrode & Thompson, 2005).
BLUP-based genetic evaluations have been applied to a wide range of sheep breeds and production systems across Europe. Studies on dairy breeds such as Lacaune, Awassi, and Merino-derived populations have shown that heritability estimates for milk yield, wool traits, and body weight obtained through BLUP are sufficiently stable to support selection decisions (Barillet, 2007; Komprej et al., 2012). The application of these methods to autochthonous breeds raised under extensive conditions - such as the Țurcană breed - is of particular interest, given the growing efforts to improve locally adapted genetic resources within national and European biodiversity conservation programs.
The research goal was to estimate the genetic parameters for production and exterior traits in subpopulations of the Tsurcană breed, in order to characterize them.

MATERIALS AND METHODS

Biological material studied

The research was carried out on a herd of 16,196 Tsurcană sheep, existing in the records of the Tsurcană-Dacia Sheep Breeders Association.
The total herd was structured on the 4 populations, as follows: Belă Line (4,567 females), Breză Line (1,035 females), Bucălae Line (3,288 females) and Oacheșa Line (7,306 females).
The genetic parameters were estimated for a number of seven traits included in the objective of improving the Tsurcană breed: The body weight at 60 days, The body weight at 120 days, average daily gain, wool yield, wool length, milk quantity and body conformation.
For the statistical analysis of the primary data, the following calculation methods were used: classical statistical indicators (mean, variance, standard deviation, standard error of the mean, and coefficient of variation). For the estimation of genetic parameters, the BLUP methodology (Best Linear Unbiased Prediction) was applied using the sire model for the 7 traits, according to the following biometric model:

Pi = Xibi + Ziai + ei
where:
Pi = vector of observations for character i;
Xi = Incidence matrix for fixed effects;
bi= vector of fixed effects for character i;
Zi= Incidence matrix for random effects;
ai = vector of random effects for character i;
ei= vector of residuals for character i.
To facilitate easier understanding of the BLUP equation system, it will be presented for the case of two traits, as follows (Grosu & Oltenacu, 2005):

$$\begin{bmatrix} X_1'\cdot r^{11}\cdot X_1 & X_1'\cdot r^{12}\cdot X_2 & X_1'\cdot r^{11}\cdot Z_1 & X_1'\cdot r^{12}\cdot Z_2 \\ X_2'\cdot r^{21}\cdot X_1 & X_2'\cdot r^{22}\cdot X_2 & X_2'\cdot r^{21}\cdot Z_1 & X_2'\cdot r^{22}\cdot Z_2 \\ Z_1'\cdot r^{11}\cdot X_1 & Z_1'\cdot r^{12}\cdot X_2 & Z_1'\cdot r^{11}\cdot Z_1+A^{-1}\cdot g^{11} & Z_1'\cdot r^{12}\cdot Z_2+A^{-1}\cdot g^{12} \\ Z_2'\cdot r^{11}\cdot X_1 & Z_2'\cdot r^{22}\cdot X_2 & Z_2'\cdot r^{21}\cdot Z_1+A^{-1}\cdot g^{21} & Z_2'\cdot r^{22}\cdot Z_2+A^{-1}\cdot g^{22} \end{bmatrix}$$

$$\begin{bmatrix} \tilde{b}_1 \\ \hat{b}_2 \\ \hat{a}_1 \\ \hat{a}_2 \end{bmatrix} = \begin{bmatrix} X_1'\cdot r^{11}\cdot P_1 + X_1'\cdot r^{12}\cdot P_2 \\ X_2'\cdot r^{21}\cdot P_1 + X_2'\cdot r^{22}\cdot P_2 \\ Z_1'\cdot r^{11}\cdot P_1 + Z_1'\cdot r^{12}\cdot P_2 \\ Z_2'\cdot r^{21}\cdot P_1 + Z_2'\cdot r^{22}\cdot P_2 \end{bmatrix}$$

in which, the matrices G and R contain the genotypic and environmental variances and covariances of the considered traits:

$$G^{-1} = \begin{bmatrix} \boldsymbol{S_{I_X}^2} & \boldsymbol{Cov_{I_{XY}}} \\ \boldsymbol{Cov_{I_{XY}}} & \boldsymbol{S_{I_Y}^2} \end{bmatrix}^{-1} = \begin{bmatrix} g^{11} & g^{12} \\ g^{21} & g^{22} \end{bmatrix};$$

$$R^{-1} = \begin{bmatrix} \boldsymbol{S_{i_X}^2} & \boldsymbol{Cov_{i_{XY}}} \\ \boldsymbol{Cov_{i_{XY}}} & \boldsymbol{S_{i_Y}^2} \end{bmatrix}^{-1} = \begin{bmatrix} r^{11} & r^{12} \\ r^{21} & r^{22} \end{bmatrix}$$

The G and R elements were estimated using the formulas:

1) Interfamilial genetic variance (between paternal families), for the trait X $(\boldsymbol{S_{I_X}^2})$:

$$\boldsymbol{S_{I_X}^2} = \frac{\hat{a}_i'\cdot A^{-1}\cdot \hat{a}_i + tr(A^{-1}\cdot C_{ii})}{q}$$

2) Interfamilial genetic variance (between paternal families), for the Y trait $(\boldsymbol{S_{I_Y}^2})$:

$$\boldsymbol{S_{I_Y}^2} = \frac{\hat{a}_j' \cdot A^{-1} \cdot \hat{a}_j + tr(A^{-1} \cdot C_{jj})}{q}$$

3) Additive genetic covariance between traits X and Y ($\boldsymbol{Cov_{I_{XY}}}$):

$$\boldsymbol{Cov_{I_{XY}}} = \frac{\hat{a}_i' \cdot A^{-1} \cdot \hat{a}_j + tr(A^{-1} \cdot C_{ij})}{q}$$

4) Environmental variance (residual) for trait X ($\boldsymbol{S_{i_X}^2}$):

$$\boldsymbol{S_{i_X}^2} = \frac{\hat{e}_i' \cdot \hat{e}_i + tr(B_{ii})}{n}$$

5) Environmental variance (residual) for character Y ($\boldsymbol{S_{i_Y}^2}$):

$$\boldsymbol{S_{i_Y}^2} = \frac{\hat{e}_j' \cdot \hat{e}_j + tr(B_{jj})}{n}$$

6) Environmental covariance (residual) between traits X

$$\boldsymbol{Cov_{i_{XY}}} = \frac{\hat{e}_i' \cdot \hat{e}_j + tr(B_{ij})}{n}$$

$$W = \begin{bmatrix} X_1 \oplus X_2 & Z_1 \oplus Z_2 \end{bmatrix};$$

$$B = W \cdot C \cdot W' = \begin{bmatrix} B_{ii} & B_{ij} \\ B_{ji} & B_{jj} \end{bmatrix};$$

$\oplus$ = Direct sum (Searle & Hudson, 1982)

$e_i = P_i - W \cdot \begin{bmatrix} \widetilde{b}_1 & \widetilde{b}_2 & \hat{a}_1 & \hat{a}_2 \end{bmatrix}$, e represents the error vector.

Phenotypic variances and covariances are obtained by summing the intrafamilial and intrafamilial variances and covariances, as follows:

P=G + R

$$P = \begin{bmatrix} \boldsymbol{S_{F_X}^2} & \boldsymbol{Cov_{F_{XY}}} \\ \boldsymbol{Cov_{F_{XY}}} & \boldsymbol{S_{F_Y}^2} \end{bmatrix} = \begin{bmatrix} \boldsymbol{S_{I_X}^2} & \boldsymbol{Cov_{I_{XY}}} \\ \boldsymbol{Cov_{I_{XY}}} & \boldsymbol{S_{I_Y}^2} \end{bmatrix} + \begin{bmatrix} \boldsymbol{S_{i_X}^2} & \boldsymbol{Cov_{i_{XY}}} \\ \boldsymbol{Cov_{i_{XY}}} & \boldsymbol{S_{i_Y}^2} \end{bmatrix}$$

For the character "X", the heritability was obtained according to the formula:

$$h_X^2 = 4 \times \frac{\boldsymbol{S_{I_X}^2}}{\boldsymbol{S_{F_X}^2}}$$

Estimation of genotypic correlations and their errors

The genotypic correlation between traits X and Y ($r_{G_{XY}}$), was determined based on the observational components of the intrafamilial variances and covariances, according to the formula:

$$r_{G_{XY}} = \frac{\boldsymbol{Cov_{I_{XY}}}}{\sqrt{\boldsymbol{S_{I_X}^2} \cdot \boldsymbol{S_{I_Y}^2}}}$$

The genotypic correlation error was determined based on the formula:

$$S_{r_G} = \frac{1 - r_{G_{XY}}^2}{\sqrt{2}} \cdot \sqrt{\frac{S_{h_X^2} \cdot S_{h_Y^2}}{h_X^2 \cdot h_Y^2}}$$

where:

r_G = genotypic correlation error;

$S_{h_X^2}$ = heritability error of the trait X;

$S_{h_Y^2}$ = heritability error of the trait Y;

h_X^2 = heritability of the trait X;

h_Y^2 = heritability of the trait Y.

The heritability error was estimated using the formula:

$$S_{h^2} = 4 \cdot \sqrt{\frac{2 \cdot [1 + (k-1) \cdot t]^2 (1-t)^2}{k \cdot (k-1) \cdot (q-1)}}$$

where:

$t = \frac{S_I^2}{S_F^2}$

q = number of fathers;

k = average size of father's family (n/q), n=total number of offspring.

RESULTS AND DISCUSSIONS

Average performances of the Tsurcană breed

Establishing perspective objectives in improving the Tsurcană breed requires knowing the current leve l of average performances of the traits that are part of the improvement objective, for each population analyzed. Table 1 presents the average performances of the traits in the Tsurcană Belă.

For the first character, body conformation, an average performance of 8.7861 points is observed, with a variability of 7.5468%.

For the quantity of sheared wool, the average performance was 3.6708 kg with a variability of 10.0944%., compared to 3.95 kg obtained by Rotar et al. (2025).

The third analyzed character, the length of the wool strand, recorded an average value of 27.8362 cm with a variability of 8.8471%.
For milk production per lactation, the average performance was 40.62 kg with a variability of 13.7564% compared to 58.95 kg obtained by Rotar et al. (2025).

The body weight at 60 days, the achieved performance was 15.10 kg with a variability of 11.8097%. For The body weight at 120 days, the achieved performance was 32.28 kg with a variability of 20.6271%.
The average daily gain (ADG) recorded an average of 256.1122 g with a calculated variability of 13.9494% (Table 1).

Table 1. Average performances of the traits in the Tsurcana - Belă breed

Traits	N	U.M.	MEAN ± SEM	SD	CV %	Min	Max
conformation (1)	4567	Note	8.7861 ± 0.0098	0.6632	7.5478	8	10
wool yield (2)		kg	3.6708 ± 0.0055	0.3705	10.0944	2.74	5
wool length (3)		cm	27.8362 ± 0.0364	2.4627	8.8471	23.08	34
milk yield (4)		kg	40.6232 ± 0.0827	5.5883	13.7564	30.66	55.34
body weight at 60 days (5)		kg	15.1014 ± 0.0264	1.7834	11.8097	13	22.3
body weight at 120 days (6)		kg	32.2807 ± 0.0985	6.6586	20.6271	21	103
average daily gain (7)		g	256.1122 ± 0.5287	35.7261	13.9494	114.75	366.67

For the Breazã variety of the Tsurcanã breed, the same traits were analyzed on a population of 1035 individuals registered in the breed's genealogical register with the mention Breazã population. Thus, for conformation an average score of 8.7894 was obtained with a variability of 7.3206%.
For the wool yield, a value of 3.69 kg was obtained with a variability of 12.00%. The amount of milked milk had values of 42.17 kg compared to 59.14 kg mentioned by Rotar et al. (2017).

For the amount of sheared wool, an average quantity of 3.69 kg was achieved and had a calculated variability of 12.00%, compared to 2.77 kg obtained by Nistor (2007). The length of the wool had an average of 29.22 cm per strand with a variability of 7.17% compared to 28.49 cm (Nistor, 2007). Regarding the body weight at 60 days, an average the body weight G60 of 15.40 kg and a variability of 11.69% was obtained. For the body weight at G120, an average of 31.48 kg was calculated with a variability of 7.08%. The average daily gain was 257.58 g with a variability of 15.17% (Table 2).

Table 2. Average performances of the traits in the Tsurcanã - Breazã breed

Traits	N	U.M.	MEAN ± SEM	SD	CV %	Min	Max
conformation (1)	1035	Note	8.7894 ± 0,02	0.6434	7.3206	7	10
wool yield (2)		kg	3.6952 ± 0.0138	0.4437	12.0067	2.83	5.5
wool length (3)		cm	29.2217 ± 0.0652	2.0973	7.1772	23.08	34.5
milk yield (4)		kg	42.1713 ± 0.1651	5.3124	12.5973	31.29	53.65
body weight at 60 days (5)		kg	15.4021 ± 0.056	1.8018	11.6984	13	21.5
body weight at 120 days (6)		kg	31.4872 ± 0.0693	2.2297	7.0813	22	38
average daily gain (7)		g	257.586 ± 1.2147	3.079	1.,1712	116.67	366.67

For the Bucălae variety, a score of 8.8069 was obtained for conformation with a variability of 7.768%. For the quantity of sheared wool, an average value of 3.8352 kg was obtained and a calculated variability of 11.03%. The length of the wool strand had an average value of 29.83cm with a variability of 6.074%. For the milk quantity character, an average quantity of 45.27

kg was obtained and a variability of 7.60%. For The body weight, the following were obtained: for G60, an average value of 16.37 kg with a variability of 10.90%, and for G120, an average value of 32.24 kg with a variability of 6.94%. A ADG of 260.94 g was calculated with a variability of 17.43% (Table 3).

Table 3. Average performances of the traits in the Tsurcana - Bucălae breed

Traits	N	U.M.	MEAN ± SEM	SD	CV %	Min	Max
conformation (1)	3288	Note	8.8069 ± 0.0119	0.6841	7.768	7	10
wool yield (2)		kg	3.8352 ± 0.0074	0.423	11.0294	2.95	6
wool length (3)		cm	29.8287 ± 0.0316	1.8118	6.074	23.08	34.5
milk yield (4)		kg	45.2748 ± 0.0601	3.4447	7.6085	30.68	63.28
body weight at 60 days (5)		kg	16.3716 ± 0.0311	1.7852	10.9044	13	22.6
body weight at 120 days (6)		kg	32.2449 ± 0.0391	2.2397	6.9459	25	38
average daily gain (7)		g	260.9465 ± 0.7952	45.5966	17.4735	112.9	400

By analyzing the data of the individuals from the Oacheșă Variety, an average score of 8.6748 and a variability of 7.69% was obtained. For the character of wool yield, an average quantity of 3.76 kg was obtained with a variability of 9.65%. An average length of the wool strand of 29.46 cm and a variability of 5.10% was observed. For the quantity of milk, an average of 45.37 kg was obtained with a variability of 7.088%. For the body weight at 60 days the average performance was 16.15 kg with a variability of 11.23%, and for the body weight at 120 days the average was 32.76 kg and a variability of 16.19%. For ADG an average value of 261.15 grams was obtained with a variability of 16.99%.

Table 4. Average performances of the traits in the Tsurcana - Oacheșă

Traits	N	U.M.	MEAN ± SEM	SD	CV %	Min	Max
conformation (1)	7306	Note	8.6748 ± 0.0078	0.6676	7.6953	7	10
wool yield (2)		kg	3.7638 ± 0.0042	0.3631	9.6467	2.83	6
wool length (3)		cm	29.4677 ± 0.0176	1.5046	5.1061	23.08	34.5
milk yield (4)		kg	45.3676 ± 0.0376	3.2156	7.088	32.21	62.59
body weight at 60 days (5)		kg	16.1497 ± 0.0212	1.8132	11.2274	13	22.1
body weight at 120 days (6)		kg	32.7623 ± 0.062	5.3029	16.1861	21	101
average daily gain (7)		g	261.1537 ± 0.5191	44.3715	16.9905	114.75	400

Heritability of the analyzed traits

Heritability expresses the share of the total phenotypic variability attributed to the average effect of genes.

Given that the genetic structure differs from one population to another, it results that heritability is a property of each population in particular. And in the present analysis, heritability was estimated for each of the four populations considered.

From the analysis of the heritability of morphological traits for the studied herd belonging to the Belă line, it is observed that Wool length has an intense heritability (0.44) and Milk yield also has an intense heritability (0.475).

Average daily gain ADG (0.147), wool yield (0.182) and conformation (0.147) are weakly heritable traits.

For the Bucălae herd, we note that high heritability values were calculated for the milk yield trait (0.337).

For the other analyzed traits, conformation, wool yield, G60, G120, ADG, weak heritability values were obtained.

Lazăr et al. (2022) obtained the heritability of daily milk production in the first three lactations with values between 0.015 and 0.246.

Rotar et al. (2025) reported heritability values of 0.21 for milk production and 0.34 for wool production in the Tsurcana breed. Similarly, Popa et al. (2021) estimated heritability for test-day milk production in first-lactation ewes between 0.105 at 70 days and 0.270 at 115 days of lactation.

Table 5. Heritability and heritability standard error ($h^2 \pm SE$) for the traits analyzed in the Tsurcana breed populations

Traits	Tsurcana			
	Belă	Breazăă	Bucălae	Oacheșă
conformation (1)	0.15±0.039	0.140±0.111	0.128±0.045	0.124±0.027
wool yield (2)	0.18±0.041	0.194±0.112	0.239±0.050	0.199±0.030
wool length (3)	0.44±0.05	0.270±0.112	0.231±0.050	0.236±0.031
milk yield (4)	0.47±0.051	0.337±0.112	0.254±0.051	0.291±0.034
body weight at 60 days (5)	0.26±0.041	0.176±0.112	0.116±0.044	0.118±0.026
body weight at 120 days (6)	0.36±0.047	0.183±0.112	0.118±0.044	0.293±9.034
average daily gain (7)	0.15±0.039	0.141±0.112	0.117±0.044	0.102±0.026

Heritability values for sheep

Vatankhah & Talebi (2008) reported direct heritability estimates for body weight of 0.31 ± 0.02 at birth, 0.10 ± 0.02 at weaning, and 0.19 ± 0.02 at six months of age, while maternal heritability estimates were 0.22 ± 0.02, 0.16 ± 0.02, and 0.04 ± 0.02, respectively.

In the Bucălae subpopulation, weak heritability values were recorded for most traits, ranging from 0.116 for body weight at 60 days to 0.128 for conformation. Moderate heritability values were obtained for wool yield (0.239), wool length (0.231), and milk yield (0.254).

Following the analysis of the herd of sheep belonging to the Oacheșă variety, the heritability values fluctuate within narrow limits from 0.102 for ADG to 0.293 for G120, thus being weakly heritable. We note that different heritability values were calculated for the same production character in the analyzed herds of the 4 analyzed varieties. Average heritability values of 0.27 for milk production were obtained by (Pascal et al., 2007).

Genetic correlation between the analyzed traits

The values of the genotypic correlation coefficient (r_g) indicate the existence of variable relationships in terms of intensity and direction between the traits. It is observed that some combinations of traits show positive correlations, which indicates that an increase in one character is associated with an increase in the other. Conversely, negative correlation values signal an inverse relationship between the traits. It is also noted the existence of higher intensity correlations, which suggests a stronger genetic link between these traits, while low values indicate weak relationships. The correlation error shows the degree of precision of the estimates. We can conclude that there is no strong correlation between conformation and the other analyzed traits, moreover, negative values were also obtained.

Correlations with small negative values (-0.023) Belă and at (-0.062) Breazăă between conformation and wool length and weakly positive between the other traits.

In Bucălae, conformation is negatively correlated with milk quantity (-0.18) and with G60 (-0.014). In Oacheșa, conformation is negatively correlated only with milk yield (-0.072) and positively correlated with the others, from 0.022 to G120 and 0.227 to wool length (Table 6).

Table 6. Genotypic correlation

The combination of traits	Tsurcana							
	Belă		Breazǎ		Bucălae		Oacheșă	
	r_G	S_{r_G}	r_G	S_{r_G}	r_G	S_{r_G}	r_G	S_{r_G}
conformation-wool yield	0.024	0.173	-0.131	0.469	0.042	0.191	0.023	0.127
conformation-wool length	-0.023	0.124	-0.062	0.408	0.221	0.184	0.227	0.113
conformation-milk yield	0.044	0.12	0.102	0.367	-0.18	0.181	-0.072	0.111
conformation - body weight at 60 days	0.066	0.151	0.031	0.499	-0.014	0.258	0.059	0.154
conformation-body weight at 120 days	0.015	0.134	0.034	0.49	0.208	0.245	0.022	0.111
conformation-ADG	0.118	0.187	0.06	0.552	0.188	0.247	0.143	0.161

Table 7 presents the genotypic correlations between wool yield and the other five production traits analyzed. Compared to the previous table, variations in the values of the correlation coefficients are observed, reflecting genetic differences between subpopulations. In Bela most correlations are of low to moderate intensity, but there are also stronger correlations for milk quantity with values between 0.179 in Oacheșă and 0.319 in Bucălae and for the wool length trait with values between 0.428 in Belă and 0.667 in Bucălae, indicating important genetic associations between certain traits. The presence of both positive and negative values shows that some traits evolve in the same direction, while others are antagonistic. The error associated with the coefficients indicates the level of confidence of these estimates.

The wool yield is strongly correlated in all lines with the length of the wool with limits of 0.427 in Belă and 0.667 in Bucălae.

The wool yield with the amount of milk is genetically intermediately correlated with limits of 0.179 in Oacheșă and 0.319 in Bucălae.

Wool yield and body weight at 60 days showed a moderate positive correlation in most subpopulations, with values ranging from 0.126 in Oacheșă to 0.381 in Breazǎ, while a weak negative correlation was recorded in the Bucălae line (-0.065).

Table 7. Genetic relationship between wool yield and the other 5 analyzed traits, in the Tsurcana breed

The combination of traits	Tsurcana							
	Belă		Breazǎ		Bucălae		Oacheșă	
	r_G	S_{r_G}	r_G	S_{r_G}	r_G	S_{r_G}	r_G	S_{r_G}
wool yield-wool length	0.427	0.093	0.474	0.273	0.667	0.084	0.502	0.075
wool yield - milk yield	0.259	0.103	0.237	0.301	0.319	0.13	0.179	0.09
wool yield-G60	0.229	0.131	0.333	0.381	-0.065	0.199	0.126	0.128
wool yield-G120	-0.072	0.121	0.28	0.388	0.057	0.198	-0.094	0.092
wool yield-ADG	0.069	0.172	0.129	0.468	0.103	0.197	-0.051	0.137

Note: G60 - body weight at 60 days; G120 - body weight at 120 days; ADG - average daily gain

The genetic correlation between the wool yield and G120 is weakly negative in Bela (-0.072) and Oacheșă (-0.094) and intermediately positive in Breaza (0.28) and Bucălae (0.057).

For the character wool yield and ADG it is weakly positive with limits of (0.069) at Belă and (0.129) at Breazǎ. For the Oacheșa line the correlation was negative (-0.051).

Positive genotypic correlations between wool yield and wool length were consistently observed across all four subpopulations, while negative or low values were recorded for the trait combinations wool yield – body weight at 60 days, wool yield – body weight at 120 days, and wool yield – average daily gain. In a related context, Vatankhah & Talebi (2008) reported positive genetic correlations between lamb body weight and various reproductive traits, ranging from 0.06 to 0.98.

Table 8 presents the genotypic correlations obtained between the wool length trait and milk yield, the body weight at 60 days (G60), the body weight at 120 days (G120) and average daily gain (ADG).

The correlation values between wool length and wool yield were intermediately correlated with limits between 0.061 in Brează and 0.586 in Belă line. For the correlation between wool yield and G60, intermediate values were obtained between 0.21 in Brează and 0.514 in Belă and for the Bucălae line, negative values were obtained (-0.206). The correlation between wool length and G120 is negatively correlated (-0.046) in Belă line and weakly correlated in Bucălae (0.144) and in Brează (0.362). For the Correlation between Wool Length and ADG, weakly correlated values were obtained from 0.158 in Oacheșă and 0.248 in Bucălae. A high variability of the values of the correlation coefficients is observed, from negative values to high positive values.

Table 8. Genotypic correlations between wool length and milk yield, body weight at 60 days, body weight at 120 days, and average daily gain in the Tsurcana breed

The combination of traits	Tsurcana							
	Belă		Brează		Bucălae		Oacheșă	
	r_G	S_{r_G}	r_G	S_{r_G}	r_G	S_{r_G}	r_G	S_{r_G}
wool length-milk yield	0.586	0.052	0.061	0.273	0.262	0.137	0.332	0.078
wool length-G60	0.514	0.073	0.21	0.352	-0.206	0.194	0.217	0.116
wool length-G120	-0.046	0.087	0.362	0.314	0.144	0.197	-0.13	0.086
wool length-ADG	0.175	0.12	0.218	0.389	0.248	0.189	0.158	0.126

Note: G60 - body weight at 60 days; G120 - body weight at 120 days; ADG - average daily gain

Some combinations of traits show positive correlations, which indicates a significant genetic link, but in general there are correlation values that show that there was no strong correlation between wool length and G60 except for the Belă population, and for the correlation between wool length and ADG low values were obtained, with a low correlation. Negative correlations also appear, indicating inverse relationships between traits. The correlation error must be taken into account in the interpretation of the results. Hanford et al. (2002) states that the estimate of the genetic correlation between wool the body weight and fiber length was positive (0.55).

Other authors (Naidoo et al., 2004), obtained high and positive genetic correlation values for wool yield and wool strand length from 0.45 to 0.60 in the samples tested.

Table 9. Genotypic correlations between milk yield and body weight at 60 days, body weight at 120 days, and average daily gain in the Tsurcana breed

The combination of traits	Tsurcana							
	Belă		Brează		Bucălae		Oacheșă	
	r_G	S_{r_G}	r_G	S_{r_G}	r_G	S_{r_G}	r_G	S_{r_G}
Milk yield - G60	0.683	0.051	0.515	0.245	-0.142	0.192	0.16	0.111
Milk yield - G120	0.118	0.084	0.454	0.26	-0.145	0.19	-0.151	0.08
Milk yield - ADG	0.238	0.113	0.296	0.337	-0.025	0.195	0.051	0.12

Note: G60 - body weight at 60 days; G120 - body weight at 120 days; ADG - average daily gain

Table 9 highlights the various genotypic correlations between the traits Milk quantity and G60, G120 and ADG. Thus, higher values were obtained for the correlation between milk quantity and G60 for the Belă (0.683) and Breazã (0.515) populations and weakly correlated values for Oacheșă (0.16) and negative values for Bucălae (-0.142). For the correlation between Milk quantity and G120, the results were weakly correlated for Belă (0.118) and Breazã (0.454) and negative values for Bucălae (-0.145) and Oacheșă (-0.151). The correlation between Milk quantity and ADG is weakly correlated with values between 0.051 for Oacheșă and 0.296 for Breazã and negatively correlated for Bucălae (-0.025).

Table 10. Genotypic correlations of body weight at 60 days with body weight at 120 days and average daily gain (ADG) in the Tsurcana breed

The combination of traits	Tsurcana							
	Belă		Breazã		Bucălae		Oacheșă	
	r_G	S_{r_G}	r_G	S_{r_G}	r_G	S_{r_G}	r_G	S_{r_G}
body weight at 60 day- body weight at 120 days	0.078	0.106	0.464	0.346	0.071	0.267	-0.075	0.113
body weight at 60 day - ADG	-0.051	0.15	-0.135	0.489	-0.579	0.178	-0.458	0.133

Table 10 presents the correlation values obtained by analyzing the data for the traits The body weight at 60 days and the body weight at 120 as well as ADG. Both positive and negative values were obtained. The highest positive value was obtained (0.464) between G60 and G120 in the Breazã sheep population, and the highest negative value (-0.579) was obtained between G-60 and ADG in Bucălae.

The correlation between G60 and G120 is weakly correlated with values between 0.071 in Bucălae and 0.464 in the Breazã line and negatively correlated (-0.075) in Oacheșa. For the correlation between G60 and ADG, negative values were obtained, -0.051 in the Belă Line and -0.579 in Bucălae. Mekuriaw & Haile (2014) obtained strongly positive values between 0.61 and 0.98 for the live the body weight trait.

In table 11 for the combination of the G-120 and ADG traits, we observe that only positive correlation values were obtained. High values were recorded for the Breazã (0.790) and Bucălae (0.763) populations. We can say that there is a fairly strong correlation between the two traits in the populations mentioned above. However, most of the correlation coefficient values are close to zero, which indicates weak genetic relationships between a large part of the traits.

Table 11. Genotypic correlation between the body weight at 120 days and average daily gain (ADG) in the Tsurcana breed

The combination of traits	Tsurcana							
	Belă		Breazã		Bucălae		Oacheșă	
	r_G	S_{r_G}	r_G	S_{r_G}	r_G	S_{r_G}	r_G	S_{r_G}
body weight at 120 days -ADG	0.042	0.133	0.79	0.184	0.763	0.112	0.169	0.117

The genotypic correlation error values are relatively low, suggesting a good accuracy of the estimates. Overall, the results obtained highlight the existence of relevant genetic correlations, which can be exploited in the selection process.

CONCLUSIONS

The average performances recorded across all four Tsurcana subpopulations (Belă, Breazã, Bucălae, and Oacheșă) for the seven analyzed traits fall within the breed standards and are consistent with values reported in the literature. Heritability estimates varied considerably both across traits and across subpopulations. Milk yield and wool length showed the highest heritability values (up to 0.475 and 0.44, respectively, in the Belă subpopulation), while conformation and average daily gain were weakly heritable across all four lines. These

differences confirm that heritability is a population-specific parameter and should be estimated separately for each population.

Genotypic correlations between traits covered the full range of possible values, from strongly negative to strongly positive. The most consistent association across all subpopulations was observed between wool yield and wool length, while correlations involving body weight traits were generally weak and variable. Negative correlations were identified for several trait combinations, indicating antagonistic genetic relationships that must be considered when designing selection indices.

The results provide a quantitative basis for developing differentiated breeding programs for each Tsurcana subpopulation, adapted to their specific genetic structure and production objectives

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PARTIAL RESULTS REGARDING THE ESTIMATION OF GENETIC PARAMETERS OF PRODUCTION TRAITS IN THE TSURCANA BREEDS

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Abstract

The aim of this study was to estimate the genetic parameters of production traits in the Tsurcana sheep breed, considering its four main varieties: Belă, Breazã, Bucălae and Oacheşă. The research was carried out on a total of 4,837 ewes belonging to the Association of Tsurcana Sheep Breeders "Păstorul" Arad. The analyzed traits included birth weight, weaning weight and milk yield. Statistical analysis was performed using classical descriptive statistics and the BLUP methodology applied to a sire model, in order to estimate heritability and genetic correlations among traits. The results revealed significant phenotypic differences among varieties, with the Oacheşă variety showing superior performance for birth weight and milk yield, while Breazã and Oacheşă recorded the highest weaning weights. Heritability estimates indicated moderate genetic control for all analyzed traits, with higher values for weaning weight in the Belă variety. Genetic correlations between traits were generally low to moderate, suggesting limited correlated responses to selection. These findings support the implementation of differentiated breeding programs adapted to each variety of the Tsurcana breed.

Key words*: genetic parameters, heritability, milk yield, Tsurcana sheep, varieties.*

INTRODUCTION

The sheep and goat sector play a vital role in Romanian agriculture, especially through the effective use of land in areas with natural constraints, where these species account for 80% of land valorization. Although Romania ranks 4th in the EU-28 for total herds, it only ranks 10th in meat production due to low average slaughter weights and a lack of specialized meat breeds. While domestic consumption remains low at 3.5% of total meat intake, Romania has become the EU's top exporter of live sheep, primarily serving Middle Eastern and North African markets. Government initiatives like "Choose the Sheep" and wool subsidies now aim to boost domestic demand and diversify farmer income (Grodea et al., 2018).

The Tsurcana is Romania's oldest and most dominant sheep breed, representing 40% of the national population. Often called the "peasant sheep", this polyvalent breed is prized for its profound fitness and exceptional resistance to harsh weather. Its dominance stems from an ability to thrive in diverse environments, from lowlands to alpine pastures, while enduring long transhumance routes. Highly adaptable, the Tsurcana provides varied production, including high-quality meat, resilient leather, and abundant milk, averaging 80-110 liters per lactation. Additionally, its characteristic rough white wool remains a staple for traditional textiles and Persian carpets (Udrea et al., 2021).

The Tsurcana breed comprises four distinct varieties that occupy different ecological niches and exhibit unique phenotypic traits. The Belă variety is characterized by white wool on the head and limbs, occasionally featuring small black spots near the eyes. In contrast, the Breazã variety is distinguished by a white "V-shaped" facial blaze, a trait positively correlated with superior milk production. The Oacheşă variety is identified by specific black circular patterns around the eyes. Finally, the Bucălae variety features dark extremities and face, with lambs born smoky-colored. These significant physical differences suggest these herds represent distinct subpopulations (Bîrcă et al., 2025).

The Turcana breed formed through reproductive isolation and selection for specific traits. While breeders observe notable phenotypic differences in body size and milk yield, these assessments often stem from subjective selection based on color patterns. For instance, the Breaza variety's

distinctive "V" facial marking is positively correlated with superior milk production. However, these distinctions are largely driven by local environmental conditions and the specific priorities of individual breeders, such as resistance or productivity, rather than standardized genetic benchmarks (Neață et al., 2023).

To improve a breed effectively, one must distinguish between environmental influences and genetic inheritance. Phenotypic traits often result from a combination of external factors and the animal's "inwardness" or genetic profile. While environmental effects can temporarily alter appearance or productivity, only genetic traits are passed to future generations. The use of genetic parameters, specifically heritability and genetic correlations, is essential because traditional morphological markers often have insignificant relevance for long-term improvement (Hrincă, 2017). Heritability quantifies the proportion of variation due to genetics, while genetic correlations reveal how selecting for one trait might inadvertently affect another. Without these parameters, breeding programs are essentially "shooting in the dark", providing only rough estimates rather than precise scientific management. Utilizing biological indicators like biochemical or molecular markers allows for accurate genotypic individualization, ensuring that selection leads to genuine, inheritable progress rather than mere environmental noise (Hrincă, 2017).

Recent research estimated genetic parameters using an animal model to enhance selection efficiency. A low heritability of 0.21 for milk yield and a moderate heritability of 0.34 for wool production. While the overall mean milk yield was 59 ± 0.816 kg, the Tsurcana Brează variety exhibited significantly higher performance compared to the Tsurcana Belă variety. Specifically, all ewes exceeding 100 kg of milk were from the Brează variety. For wool, males produced substantially more than females, averaging 5.37 kg. Breeding values for milk ranged from -5.65 to 5.55, indicating high genetic variation. There is currently limited recent literature comparing the specific genetic parameters across these four distinct varieties, Bucălae, Belă, Brează, and Oacheșă, simultaneously (Rotar et al., 2025).

The selection of birth weight, weaning weight, and milk yield as focal points for the Tsurcana sheep study is justified by their direct impact on the economic viability of Romanian sheep rearing. Birth weight is a critical indicator of genetic progress and selection for "precociousness", with the study recording values over 4.00 kg, significantly higher than the breed's traditional average of 3.00 kg. This initial weight provides a biological advantage for growth performances that are superior from both biologic and economic perspectives. Weaning weight, recorded at an average of 21.42 kg at 90 days, serves as a metric for the lambs' growth potential and the dams' nursing capacity. Finally, milk yield is a primary revenue driver; with an average production of 135.68 kg, it supports both lamb development and the production of dairy goods, though the study notes that low availability of qualified labor can increase exploitation costs (Dărăban et al., 2012).

From a state-of-the-art perspective, it is necessary to underline that, despite the substantial body of literature devoted to the morphological and productive characterization of the Tsurcana breed, the studies dealing with the estimation of genetic parameters of production traits at the level of its constituent varieties remain scarce and fragmentary. The majority of the existing reports either treat the breed as a single, undifferentiated genetic entity, or they focus on a single variety in isolation, without providing a comparative quantitative-genetic framework. Moreover, the few available estimates of heritability and genetic correlations refer mainly to milk yield and wool production, whereas body weight traits at birth and at weaning have been addressed almost exclusively at the phenotypic level, often through descriptive morphometric approaches. This methodological and informational gap is particularly relevant in the Romanian context, in which the Tsurcana represents the most numerous local breed and in which the design of variety-specific improvement programs presupposes an objective genetic-statistical foundation, capable of distinguishing genuine genetic differences among varieties from environmentally induced phenotypic variation. Consequently, an integrated estimation of the genetic parameters for the three productive traits considered, conducted simultaneously and comparatively in all four varieties, constitutes a

necessary preliminary step toward the rational organization of the selection activity in this breed.

In this context, the aim of the research is to estimate the genetic parameters in the Tsurcana breed for each of its varieties. Knowledge of genetic parameters is useful for optimizing breeding programs in any species of economic interest, including sheep, with the goal of maximizing annual genetic progress at the population level. It must be emphasized that the estimation of genetic parameters is not pursued here as an end in itself, but as an indispensable stage in the genetic characterization of the structure of the Tsurcana population and in the elaboration of differentiated improvement programs adapted to each of its varieties. Accordingly, the present study is conceived as a preliminary phase of a wider breeding program, aiming, on the one hand, to provide an objective genetic-statistical description of the four varieties (Belă, Breazǎ, Bucălae, and Oacheșă), and, on the other hand, to deliver the heritability values and genetic correlations required for the formulation of selection criteria, the definition of breeding objectives, and the prediction of expected genetic progress in this autochthonous breed.

MATERIALS AND METHODS

The research was carried out on a population of 4,837 Tsurcana sheep, structured into the four varieties as follows: Belă (1,547 females), Breazǎ (689 females), Bucălae (1,198 females), and Oacheșă (1,403 females), belonging to the Tsurcana Sheep Breeders Association "Pastorul Arad". The information regarding performance levels for the three considered traits (birth weight, weaning weight, and milk yield) was obtained from the aforementioned Association.

For the statistical analysis of the primary data, the following calculation methods were used:

Classical statistical indicators (mean, variance, standard deviation, standard error of the mean, coefficient of variation), the BLUP methodology, applied to an animal model, for estimating genetic parameters. For the classical statistical indicators, the following calculation relationships were used: The arithmetic mean was estimated as the ratio between the sum of the values in the sample (Xi) and their number (n), based on the formula: The variance was obtained as the sum of squared deviations between each value in the sample and the mean of the considered trait, divided by the degrees of freedom: $S^2 = (\sum(X_i - \bar{X})^2)/(n - 1)$ Obtained in this way, the variance is a value expressed in squared units, which is difficult to interpret from a biological point of view; therefore, it is more useful to return to the measurement unit of the considered trait, which involves calculating the standard deviation.

Standard deviation (s). Returning to the unit of measurement of the analyzed trait (kg) is done by extracting the square root of the variance (decoding). The obtained result is called the standard deviation or root mean square deviation: The standard error of the mean was calculated based on the following formula:

$$s\bar{X} = s / \sqrt{n}$$

The coefficient of variation (CV) is a relative measure of variability, expressed as a percentage, and shows how much one unit of standard deviation represents relative to the mean of the analyzed trait. At the sample level, the coefficient of variation is calculated based on the following relationship: The Student's t-test was used to perform comparisons between means. For the estimation of genetic parameters, the BLUP (Best Linear Unbiased Prediction) methodology was used, applied to the sire model, for the 7 traits, according to the following biometric model:

$$Pi = Xibi + Ziai + ei,$$

where:

Pi = the vector of observations for trait i; Xi = incidence matrix for fixed effects, bi = vector of fixed effects for trait i; Zi = incidence matrix for random effects, ai = vector of random effects for trait i; ei = vector of residuals for trait i. To facilitate an easier understanding of the BLUP system of equations, it will be presented for the case of two traits, as follows:

$$\begin{bmatrix} X_1' \cdot r^{11} \cdot X_1 & X_1' \cdot r^{12} \cdot X_2 & X_1' \cdot r^{11} \cdot Z_1 & X_1' \cdot r^{12} \cdot Z_2 \\ X_2' \cdot r^{21} \cdot X_1 & X_2' \cdot r^{22} \cdot X_2 & X_2' \cdot r^{21} \cdot Z_1 & X_2' \cdot r^{22} \cdot Z_2 \\ Z_1' \cdot r^{11} \cdot X_1 & Z_1' \cdot r^{12} \cdot X_2 & Z_1' \cdot r^{11} \cdot Z_1 + A^{-1} \cdot g^{11} & Z_1' \cdot r^{12} \cdot Z_2 + A^{-1} \cdot g^{12} \\ Z_2' \cdot r^{11} \cdot X_1 & Z_2' \cdot r^{22} \cdot X_2 & Z_2' \cdot r^{21} \cdot Z_1 + A^{-1} \cdot g^{21} & Z_2' \cdot r^{22} \cdot Z_2 + A^{-1} \cdot g^{22} \end{bmatrix} \begin{bmatrix} \hat{b}_1 \\ \hat{b}_2 \\ \hat{a}_1 \\ \hat{a}_2 \end{bmatrix}$$

$$= \begin{bmatrix} X_1' \cdot r^{11} \cdot P_1 + X_1' \cdot r^{12} \cdot P_2 \\ X_2' \cdot r^{21} \cdot P_1 + X_2' \cdot r^{22} \cdot P_2 \\ Z_1' \cdot r^{11} \cdot P_1 + Z_1' \cdot r^{12} \cdot P_2 \\ Z_2' \cdot r^{21} \cdot P_1 + Z_2' \cdot r^{22} \cdot P_2 \end{bmatrix}$$

where the matrices G and R contain the genotypic and environmental variances and covariances of the considered traits:

$$G^{-1} = \begin{bmatrix} \boldsymbol{S^2_{I_X}} & \boldsymbol{Cov_{I_{XY}}} \\ \boldsymbol{Cov_{I_{XY}}} & \boldsymbol{S^2_{I_Y}} \end{bmatrix}^{-1} = \begin{bmatrix} g^{11} & g^{12} \\ g^{21} & g^{22} \end{bmatrix}$$

$$R^{-1} = \begin{bmatrix} \boldsymbol{S^2_{i_X}} & \boldsymbol{Cov_{i_{XY}}} \\ \boldsymbol{Cov_{i_{XY}}} & \boldsymbol{S^2_{i_Y}} \end{bmatrix}^{-1} = \begin{bmatrix} r^{11} & r^{12} \\ r^{21} & r^{22} \end{bmatrix}$$

The elements of G and R were estimated using the following formulas: Genetic variance between families (between sire families) for trait X ($\boldsymbol{S^2_{I_X}}$):

$$\boldsymbol{S^2_{I_X}} = \frac{\hat{a}_i{}' \cdot A^{-1} \cdot \hat{a}_i + tr(A^{-1} \cdot C_{ii})}{q}$$

Genetic variance between families (between sire families) for trait Y ($\boldsymbol{S^2_{I_Y}}$):

$$\boldsymbol{S^2_{I_Y}} = \frac{\hat{a}_j{}' \cdot A^{-1} \cdot \hat{a}_j + tr(A^{-1} \cdot C_{jj})}{q}$$

Additive genetic covariance between traits X and Y ($\boldsymbol{Cov_{I_{XY}}}$)

$$\boldsymbol{Cov_{I_{XY}}} = \frac{\hat{a}_i{}' \cdot A^{-1} \cdot \hat{a}_j + tr(A^{-1} \cdot C_{ij})}{q}$$

Environmental (residual) variance for trait X ($\boldsymbol{S^2_{i_X}}$):

$$\boldsymbol{S^2_{i_X}} = \frac{\hat{e}_i{}' \cdot \hat{e}_i + tr(B_{ii})}{n}$$

Environmental (residual) variance for trait Y ($\boldsymbol{S^2_{i_Y}}$):

$$\boldsymbol{S^2_{i_Y}} = \frac{\hat{e}_j{}' \cdot \hat{e}_j + tr(B_{jj})}{n}$$

Environmental (residual) covariance between traits X and Y ($\boldsymbol{Cov_{i_{XY}}}$):

$$\boldsymbol{Cov_{i_{XY}}} = \frac{\hat{e}_i{}' \cdot \hat{e}_j + tr(B_{ij})}{n}$$

$W = [X_1 \oplus X_2 \quad Z_1 \oplus Z_2]$;

$B = W \cdot C \cdot W' = \begin{bmatrix} B_{ii} & B_{ij} \\ B_{ji} & B_{jj} \end{bmatrix}$;

$\oplus$ = Direct Sum

$$e_i = P_i - W \cdot \left[\tilde{b}_1 \quad \tilde{b}_2 \quad \hat{a}_1 \quad \hat{a}_2\right],$$

and e represents the error vector.

Phenotypic variances and covariances are obtained by summing the intra-family and inter-family variances and covariances, as follows:

$$P = G + R$$

$$P = \begin{bmatrix} \boldsymbol{S^2_{F_X}} & \boldsymbol{Cov_{F_{XY}}} \\ \boldsymbol{Cov_{F_{XY}}} & \boldsymbol{S^2_{F_Y}} \end{bmatrix} = \begin{bmatrix} \boldsymbol{S^2_{I_X}} & \boldsymbol{Cov_{I_{XY}}} \\ \boldsymbol{Cov_{I_{XY}}} & \boldsymbol{S^2_{I_Y}} \end{bmatrix} + \begin{bmatrix} \boldsymbol{S^2_{i_X}} & \boldsymbol{Cov_{i_{XY}}} \\ \boldsymbol{Cov_{i_{XY}}} & \boldsymbol{S^2_{i_Y}} \end{bmatrix}$$

For trait "X", heritability was obtained using the formula:

$$h^2_X = \frac{4 \cdot \boldsymbol{S^2_{I_X}}}{\boldsymbol{S^2_{F_X}}}$$

Estimation of genotypic correlations and their errors:

The genotypic correlation between traits X and Y ($r_{G_{XY}}$), was determined based on the observational components of intra-family variances and covariances, according to the formula:

$$r_{G_{XY}} = \frac{\boldsymbol{Cov_{I_{XY}}}}{\sqrt{\boldsymbol{S^2_{I_X}} \cdot \boldsymbol{S^2_{I_Y}}}}$$

The error of the genotypic correlation was determined using the formula:

$$S_{r_G} = \frac{1 - r^2_{G_{XY}}}{\sqrt{2}} \cdot \sqrt{\frac{S_{h^2_X} \cdot S_{h^2_Y}}{h^2_X \cdot h^2_Y}}$$

where:

S_{r_G}= the error of the genotypic correlation;

$S_{h^2_X}$= the error of heritability for trait X;

$S_{h^2_Y}$= the error of heritability for trait Y;

h^2_X= heritability of trait X;

h^2_Y= heritability of trait Y.

The error of heritability for trait X was estimated using the formula:

$$S_{h^2_X} = 4 \cdot \sqrt{\frac{[2 \cdot (1-t)^2] \cdot [1 + (k-1) \cdot t]^2}{k \cdot (k-1) \cdot (q-1)}}$$

where:

t=$S_{h^2_X}$/ 4; q = number of sires; k = average sire family size (n/q), n = total number of offspring.

RESULTS AND DISCUSSIONS

Average performances of the Tsurcana breed

In order to assess the current status of the studied population, a statistical analysis of the five considered traits was carried out. The results are presented in Tables 1-4 for each considered variety. For the Belă variety, the analysis of the results presented in Table 1 shows that the mean body weight at birth is 3.29 ± 0.021 kg, with a medium variability of 24.92%, and limits ranging between 2-5 kg.

Table 1. Average performances of production traits in the Tsurcana breed, Belă variety (original data)

Trait	n	Unit	X̄ ± Sx̄	S	CV %	Minimum limit
Birth weight	1547	kg	3.29 ± 0.021	0.819	24.92	2
Weaning weight	1547	kg	29.16 ± 0.192	7.562	25.94	17
Milk yield	1547	kg	56.26 ± 0.399	15.692	27.89	30.15

The mean weaning weight is 29.16 ± 0.192 kg, with a moderate relative variability of 25.94% and limits ranging between 17 and 37.2 kg.
The milk yield has a mean of 56.26 ± 0.399 kg, with a moderate variability of 27.89%, and limits ranging between 30.15 and 100.2 kg.
For the Breazã variety, the analysis of the results presented in Table 2 shows that the mean body weight at birth is 3.06 ± 0.022 kg, with a moderate variability of 18.98%, and limits ranging between 2.1-4.7 kg.

Table 2. Average performances of production traits in the Tsurcana breed, Breazã variety (original data)

Trait	n	Unit	X□ ± Sx□	S	CV %	Minimum limit	Maximum limit
Birth weight	689	kg	3.06 ± 0.022	0.581	18.98	2.1	4.7
Weaning weight	689	kg	29.88 ± 0.184	4.842	16.2	17.3	42
Milk yield	689	kg	56.85 ± 0.44	11.547	20.31	38.54	98.59

The mean weaning weight is 29.88 ± 0.184 kg, with a moderate relative variability of 16.20%, and limits ranging between 17.3 and 42 kg.
The milk yield has a mean of 56.85 ± 0.44 kg, with a moderate variability of 20.31%, and limits ranging between 38.54 and 98.59 kg.
For the Bucălae variety, the analysis of the results presented in Table 3 shows that the mean body weight at birth is 3.14 ± 0.02 kg, with a moderate variability of 22.36%, and limits ranging between 2 and 5 kg.

Table 3. Average performances of production traits in the Tsurcana breed, Bucălae variety (original data)

Trait	n	Unit	X̄ ± Sx̄	S	CV %	Minimum limit	Maximum limit
Birth weight	1198	kg	3.14 ± 0.02	0.701	22.36	2	5
Weaning weight	1198	kg	27.54 ± 0.075	2.607	9.47	17.3	40
Milk yield	1198	kg	60.24 ± 0.498	17.252	28.64	28.51	103.36

The mean weaning weight is 27.54 ± 0.075 kg, with a moderate relative variability of 9.47%, and limits ranging between 17.3 and 40 kg.
The milk yield has a mean of 60.24 ± 0.498 kg, with a moderate variability of 28.64%, and limits ranging between 28.51 and 103.36 kg.
For the Oacheșă variety, the analysis of the results presented in Table 4 shows that the mean body weight at birth is 3.75 ± 0.023 kg, with a moderate variability of 23.40%, and limits ranging between 2 and 5 kg.

Table 4. Average performances of production traits in the Tsurcana breed, Oacheșă variety (original data)

Trait	N	Unit	X̄ ± Sx̄	S	CV, %	Min. limit	Max. limit
Birth weight	1403	kg	3.75 ± 0.023	0.879	23.4	2	5
Weaning weight	1403	kg	29.84 ± 0.091	3.414	11.44	17.8	40
Milk yield	1403	kg	70.56 ± 0.495	18.526	26.25	34.7	104.4

The mean weaning weight is 29.84 ± 0.091 kg, with a moderate relative variability of 11.44%, and limits ranging between 17.8 and 40 kg.
The milk yield has a mean of 70.56 ± 0.495 kg, with a moderate variability of 26.25%, and limits ranging between 34.7 and 104.4 kg.
Romanian animal production, exhibits significant variations in body traits based on regional climatic conditions. Research conducted over a seven-year period indicates that sheep in North Romania generally achieve greater physical development compared to those in the East. Specifically, the study recorded the smallest mean body weight of 55.75 kg among individuals from Brăila County in East Romania. While most traits like body length and withers height reached their maximum values in Suceava County, weight and other developmental markers remain moderately correlated with local temperature and precipitation levels (Vlaic et al., 2017).

Table 5 summarizes the results regarding the average performances of the four Tsurcana varieties for each of the three analyzed traits.

Table 5. Average performances of production traits in the Tsurcana breed (original data)

Trait	Unit	Bela	Breaza	Bucalae	Oachesa
Birth weight	kg	3.29 ± 0.021	3.06 ± 0.022	3.14 ± 0.020	3.75 ± 0.023
Weaning weight	kg	29.16 ± 0.192	29.88 ± 0.184	27.54 ± 0.075	29.84 ± 0.091
Milk yield	kg	56.26 ± 0.399	56.85 ± 0.44	60.24 ± 0.498	70.56 ± 0.495

The comparative study of the four varieties of the Tsurcana breed (Belă, Brează, Bucălae, and Oacheșă) revealed significant phenotypic differences in the analyzed production traits. The Oacheșă variety recorded higher mean values for both birth weight (3.75 kg) and milk yield (70.56 kg), indicating a high productive potential and remarkable biological efficiency. Regarding weaning weight, the best performances were obtained by the Brează (29.88 kg) and Oacheșă (29.84 kg) varieties, with statistically insignificant differences between them.

The Bucălae variety, although it recorded the lowest weaning weight (27.54 kg), stood out with a relatively high average milk production (60.24 kg), thus exceeding the values recorded by the Bălă and Brează varieties. The Bălă variety showed an intermediate performance, without excelling in any of the studied production traits.

Based on the obtained results, it can be concluded that the Oacheșă variety presents the best prospects for selection aimed at increasing milk production and improving efficiency in the early stages of postnatal development.

In order to assess the statistical differences between the means of the traits for each combination of varieties, the Student's t-test was applied, and the obtained results are presented in Table 6.

The results indicate that there are highly statistically significant differences ($p<0.001$) in birth weight across all comparisons. Particularly notable is the difference between the Belă and Oacheșă varieties, suggesting a pronounced genetic differentiation.

Table 6. Testing the significance of differences (Student's t-test) for birth weight, weaning weight, and milk yield in the Tsurcana breed varieties (original data)

No.	Variety Pair	Birth weight	Weaning weight	Milk yield
1	Belă - Brează	6.51***	2.31*	0.88ns
2	Belă - Bucalae	5.10***	7.09***	6.31***
3	Belă - Oacheșă	14.92***	3.09**	22.70***
4	Brează - Bucălae	2.34***	13.67***	4.60***
5	Brează - Oacheșă	18.76***	0.25ns	17.60***
6	Bucălae - Oacheșă	19.61***	19.05***	14.61***

Regarding weaning weight, significant differences appear in all pairwise comparisons, with extremely high values between Brează and Bucălae (13.67***) and between Belă and Bucălae (7.09***).

Milk yield shows the greatest difference between Belă and Oacheșă (22.70***), while between Belă and Brează the difference is not significant (0.88ns). This suggests that the Bălă and Brează varieties have a similar milk production capacity, whereas Oacheșă stands out with a significantly different production level.

In conclusion, the statistical analysis highlighted significant differences among the Turcana breed varieties, particularly in terms of birth weight and milk yield. The Oacheșă variety shows a distinct milk production compared to the others, while the Brează and Bucălae varieties are clearly differentiated in weaning weight. These results may represent an important basis for developing differentiated selection programs by variety.

Heritability (h^2) represents the proportion of phenotypic variation that is attributed to genetic variation within a population and is an essential parameter for genetic improvement programs. Estimating heritability for productive traits allows the evaluation of the genetic selection potential, and the accuracy of this estimate is reflected by the associated standard error.

Knowledge of trait heritability is of particular importance for both the theory and practice of breeding, at least for two reasons: a) The magnitude of heritability influences all practical breeding decisions, such as: prioritizing genetic improvement or management improvement in order to increase production; choosing the breeding system; selecting the selection method and establishing the selection objective, etc. The

value of heritability is included in all formulas regarding the prediction of breeding value and the effect of selection. In the present study, heritability was obtained as the ratio between additive genetic variance and phenotypic variance, and the results are presented, for each variety, in Tables 7-11.

Table 7. Observational components of variance, heritability, and its error for three traits in the Tsurcana breed, Belă variety (original data)

No.	Trait	Phenotypic variance (S_F^2)	Interfamily variance (S_I^2)	Intrafamily variance (S_i^2)	Heritability (h^2)	Heritability error (S_(h^2))
1	Birth weight	0.33	0.012	0.321	0.144	0.069
2	Weaning weight	7.9	0.585	7.312	0.296	0.079
3	Milk yield	33.17	1.669	31.499	0.201	0.073

Table 8. Observational components of variance, heritability, and its error for three traits in the Tsurcana breed, Breză variety (original data)

Trait	Phenotypic variance (S_F^2)	Interfamily variance (S_I^2)	Intrafamily variance (S_i^2)	Heritability (h^2)	Heritability error (S_(h^2))
Birth weight	0.1	0.006	0.091	0.252	0.111
Weaning weight	5.43	0.223	5.208	0.164	0.102
Milk yield	26.59	1.2	25.393	0.18	0.104

Table 9. Observational components of variance, heritability, and its error for three traits in the Tsurcana breed, Bucălae variety (original data)

Trait	Phenotypic variance (S_F^2)	Interfamily variance (S_I^2)	Intrafamily variance (S_i^2)	Heritability (h^2)	Heritability error (S_(h^2))
Birth weight	0.2	0.011	0.192	0.213	0.08
Weaning weight	3.32	0.123	3.2	0.148	0.074
Milk yield	23.85	1.158	22.691	0.194	0.078

Table 10. Observational components of variance, heritability, and its error for three traits in the Tsurcana breed, Oacheșă variety (original data)

Trait	Phenotypic variance (S_F^2)	Interfamily variance (S_I^2)	Intrafamily variance (S_i^2)	Heritability (h^2)	Heritability error (S_(h^2))
Birth weight	0.55	0.015	0.535	0.106	0.071
Weaning weight	4.8	0.223	4.574	0.186	0.077
Milk yield	43.63	2.372	41.255	0.218	0.079

Table 11 presents, in a centralized manner, the results regarding heritability values and their associated errors for the four varieties of the Tsurcana breed.

Table 11. Heritability and its error for three traits in the Turcana breed varieties (original data)

Trait	Belă	Breză	Bucălae	Oacheșă
Birth weight	0.144 ± 0.069	0.252 ± 0.111	0.213 ± 0.080	0.106 ± 0.071
Weaning weight	0.296 ± 0.079	0.164 ± 0.102	0.148 ± 0.074	0.186 ± 0.077
Milk yield	0.201 ± 0.073	0.180 ± 0.104	0.194 ± 0.078	0.218 ± 0.079

From the analysis of the data presented in Table 11, the following conclusions can be drawn: The heritability values for birth weight indicate a moderate genetic influence, ranging between 0.106 (Oacheșă) and 0.252 (Breză). The relatively high error for Breză indicates a lower precision of the estimate. For weaning weight, the Belă variety shows the highest genetic potential ($h^2 = 0.296$), suggesting the possibility of efficient improvement through selection. The other varieties show lower heritability values. Milk yield shows relatively constant heritability values (between 0.180 and 0.218), indicating a stable genetic influence, with moderate errors, making this trait suitable for genetic selection. In conclusion, the heritability estimates for the studied traits in the Turcana breed reveal a moderate genetic influence, which justifies the use of selection for improving these traits. The Belă variety stands out with a higher genetic potential for weaning weight, while the differences in milk yield are more homogeneous among varieties.

From the standpoint of the genetic structure of the Tsurcana population, the variation of heritability among varieties is in itself a result of considerable importance, since it indicates that the additive genetic architecture of the three productive traits is not uniform across the breed. The fact that weaning weight exhibits the highest heritability in the Belă variety ($h^2 = 0.296$) and substantially lower values in the other three varieties (h^2 ranging from 0.148 to 0.186) suggests that the proportion of phenotypic variance explained by additive genetic effects depends, for this trait, on the genetic background of each variety, possibly reflecting different levels of past selection intensity and different effective population sizes. In contrast, the remarkable stability of the heritability values estimated for milk yield (h^2 between 0.180 and 0.218) indicates a comparable additive genetic determinism of lactation across all four varieties, a finding that

recommends milk production as a primary selection objective with predictable response in the entire breed. The lower heritability values obtained for birth weight, accompanied by relatively large standard errors, are consistent with the well-documented influence of maternal and environmental effects on this trait, and they argue against the use of birth weight as a standalone selection criterion. Taken together, these heritability estimates constitute an objective genetic-statistical descriptor of the four varieties, fulfilling one of the functions of the present study as a stage in the characterization of the population structure of the Tsurcana breed and providing the quantitative foundation required for the prediction of the expected genetic gain under alternative selection scenarios.

Genetic correlation between the analyzed traits

Genetic correlation (r_G) expresses the degree of association between two traits at the genetic level and is an important indicator for indirect selection in breeding programs. It allows the estimation of how selection for one trait may genetically influence other correlated traits. The accuracy of these estimates is reflected by the associated standard error. For the calculation of genetic correlation, the observational components of variance and covariance were used. The obtained results are presented in Tables 12-15 for each variety of the Turcana breed.

Table 12. Observational components of genotypic covariance, genotypic correlation, and its error for the three traits in the Tsurcana breed, Belă variety (original data)

Trait combination	Interfamily covariance (Cov_I)	Genotypic correlation (r_G)	Error of genotypic correlation (S_(r_G))
Birth weight - Weaning weight (12)	-0.005	-0.057	0.252
Birth weight - Milk yield (13)	0.017	0.12	0.291
Weaning weight - Milk yield (23)	0.025	0.026	0.219

Table 13. Observational components of genotypic covariance, genotypic correlation, and its error for the seven traits in the Tsurcana breed, Breză variety (original data)

Trait combination	Interfamily covariance (Cov_I)	Genotypic correlation (r_G)	Error of genotypic correlation (S_(r_G))
Birth weight - Weaning weight (12)	0.003	0.074	0.368
Birth weight - Milk yield (13)	0.011	0.131	0.35
Weaning weight - Milk yield (23)	0.254	0.491	0.32

Table 14. Observational components of genotypic covariance, genotypic correlation, and its error for the seven traits in the Tsurcana breed, Bucălae variety (original data)

Trait combination	Interfamily covariance (Cov_I)	Genotypic correlation (r_G)	Error of genotypic correlation (S_(r_G))
Birth weight - Weaning weight (12)	0.006	0.156	0.299
Birth weight - Milk yield (13)	0.026	0.234	0.26
Weaning weight - Milk yield (23)	0.053	0.141	0.312

Table 15. Observational components of genotypic covariance, genotypic correlation, and its error for the seven traits in the Tsurcana breed, Oacheșă variety (original data)

Trait combination	Interfamily covariance (Cov_I)	Genotypic correlation (r_G)	Error of genotypic correlation (S_(r_G))
Birth weight - Weaning weight (12)	0.004	0.079	0.369
Birth weight - Milk yield (13)	0.018	0.099	0.345
Weaning weight - Milk yield (23)	0.068	0.093	0.271

Table 16 presents, in a centralized manner, the results regarding the values of genotypic correlation and their associated errors for the four varieties of the Tsurcana breed.

Table 16. Genotypic correlation and its error for three traits in the Turcana breed varieties (original data)

Trait combination	Belă	Breză	Bucălae	Oacheșă
Birth weight - Weaning weight (12)	-0.057 ± 0.252	0.074 ± 0.368	0.156 ± 0.299	0.079 ± 0.369
Birth weight - Milk yield (13)	0.12 ± 0.291	0.131 ± 0.350	0.234 ± 0.260	0.099 ± 0.345
Weaning weight - Milk yield (23)	0.026 ± 0.219	0.491 ± 0.320	0.141 ± 0.312	0.093 ± 0.271

From the analysis of the results presented in Table 16, the following conclusions can be drawn: For the combination birth weight – weaning weight, the genetic correlations are close to zero, with slightly positive values, except for the Belă variety, where it is slightly negative (-0.057). The genetic correlation between birth weight and milk yield is positive but moderate, ranging between 0.099 and 0.234, indicating the possibility of modest simultaneous genetic progress between these traits. The correlation between weaning weight and milk yield is generally low, with a higher value for the Breză variety (0.491), which may

suggest a stronger genetic relationship between these two traits in this variety.

From the perspective of the design of breeding programs for the Tsurcana breed, the pattern of genotypic correlations reported in Table 16 has direct operational consequences. The fact that, in three of the four varieties, the genetic association between weaning weight and milk yield is weak (r_G between 0.026 and 0.141) indicates that selection applied for growth traits is not expected to generate a substantial correlated response in the dairy aptitude, and that the two breeding objectives must therefore be pursued by means of independent selection criteria or through an appropriately weighted aggregate index. The notable exception observed in the Brează variety (r_G = 0.491 between weaning weight and milk yield) is consistent with the dairy specialization historically attributed to this variety and suggests that, in this particular subpopulation, a single selection criterion may simultaneously improve both traits. The low to moderate genetic correlations between birth weight and the other two traits indicate, in turn, that an emphasis on increased birth weight, if not properly controlled, will not automatically translate into proportional improvements of weaning weight or milk yield, and that the dystocia risk associated with excessively high birth weights must be evaluated separately. Considered jointly with the heritability estimates, these genetic correlations enable the formulation of variety-specific selection indices and provide the parameters necessary for the prediction of direct and correlated genetic responses, thereby fulfilling the second function of the present study as a foundation for the development of differentiated improvement programs within the Turcana breed.

Research conducted over a seven-year period indicates that sheep in North Romania generally achieve greater physical development compared to those in the East. Specifically, the study recorded the smallest mean body weight of 55.75 kg among individuals from Brăila County in East Romania. While most traits like body length and withers height reached their maximum values in Suceava County, weight and other developmental markers remain moderately correlated with local temperature and precipitation levels (Vlaic et al., 2017).

Purebred Tsurcana lambs maintain a stable, constant growth rate during their first 90 days of life. At birth, they average 4.167 kg, with males weighing 4.295 kg and females 4.058 kg. Unlike crossbreds, purebred gender does not significantly influence weight. By 90 days, the population reaches an average of 17.275 kg. Crossing Tsurcana ewes with Blanc du Massif Central rams drastically improves results; crossbred lambs weigh 29.273 kg at three months, gaining 11.998 kg or nearly 70% more than purebreds (Borzan et al., 2023).

In the Sibiu region, purebred Tsurcana lambs reared under highland conditions registered significantly lower weaning weights compared to Suffolk crossbreeds. At the weaning age of 90 days, male Tsurcana lambs reached an average body weight of 18.58 kg, while female lambs averaged 16.69 kg. These weights were recorded after a suckling period where lambs were fed predominantly on maternal milk without supplementary feeding. In contrast, Suffolk x Tsurcana crossbreds were substantially heavier, outperforming the purebred males by 37.35% and the females by 43.02% (Popa, D. et al. 2013). Based on the research findings, the weaning weight of Tsurcana lambs, measured at 60 days, varies significantly depending on the type of birth and sex. For lambs from single births, males achieved a weaning weight of 16.458 ± 0.467 kg. Females from single births reached a lower weight of 15.380 ± 0.561 kg, representing a difference of approximately 7% compared to their male counterparts. Moreover, in the case of twin births, the weaning weights were predictably lower. Tsurcana males from twins reached 15.618 ± 0.355 kg, while twin females achieved the lowest average weight in the study at 14.493 ± 0.389 kg. Overall, the Tsurcana breed showed lower quantitative meat production performance at weaning compared to the Țigaie breed across all categories (Călin et al., 2024).

The Tsurcana sheep population in North-East Romania exhibits high milking capacity, with an average yield of 94.234 liters recorded during the first 85 days of lactation. Research confirms a strong positive correlation between suckled milk quantity and lamb growth speed, particularly during the first 15 days (r_p: 0.84) and up to 28 days postpartum. This relationship weakens as weaning approaches at 85 days,

dropping to r_p: 0.24. While ewe age influences lamb birth weight, milking capacity remains the primary driver of development. Selection for this trait is vital for improving meat production efficiency (Pascal et al., 2011). According to the breed standard, the total milk production per lactation ranges from 100 to 120 kg, while the specific milked milk yield is approximately 60 to 70 kg. Research on the Bala ecotype highlights its strong performance, with an average milked milk production of 82 kg recorded between 2016 and 2019. Although this reflects a numerical difference of nearly 12 kg above the standard's upper limit, statistical analysis confirms the Belă ecotype remains within the breed's standard performance (Puie et al., 2020).

CONCLUSIONS

The statistical analysis of the three productive traits demonstrated that the four varieties of the Tsurcana breed are characterized by distinct phenotypic profiles. The Oacheșă variety recorded the highest mean values both for birth weight (3.75 kg) and for milk yield (70.56 kg), while the Breazăand Oacheșă varieties shared the highest mean weaning weights (29.88 kg and 29.84 kg, respectively). The Student's t-test confirmed that the majority of the pairwise differences among varieties are highly statistically significant, a finding that supports the treatment of the four varieties as separate genetic-statistical subpopulations within the breed and that motivates the variety-specific estimation of genetic parameters performed in the present paper.

The heritability values estimated for the three traits indicate a moderate degree of additive genetic determination, with results ranging from 0.106 to 0.252 for birth weight, from 0.148 to 0.296 for weaning weight, and from 0.180 to 0.218 for milk yield. The highest heritability for weaning weight was recorded in the Bălă variety ($h^2 = 0.296$), which singles it out as the variety with the greatest potential for improvement of growth performance through mass selection, whereas milk yield exhibited a remarkable stability of the heritability values across all four varieties, recommending this trait as a primary selection objective with predictable genetic response throughout the breed. The lower and less precise heritability estimates obtained for birth weight argue against its use as a stand-alone selection criterion and reinforce the necessity of accounting for maternal and environmental effects in further analyses.

The estimated genotypic correlations between the analyzed pairs of traits proved to be generally low to moderate, with most values close to zero, which indicates that selection applied for one trait will not generate a substantial correlated response in the other traits and that, consequently, the corresponding breeding objectives must be pursued through independent selection criteria or through an appropriately weighted aggregate index. A notable exception was observed in the Breazăvariety, where the genetic correlation between weaning weight and milk yield reached a moderate positive value ($r_G = 0.491$), suggesting that, in this particular subpopulation, a simultaneous genetic improvement of growth and milk production is feasible and consistent with the historical dairy specialization of the variety.

Taken together, the results presented in this paper should be interpreted not as a self-contained outcome, but as a stage in the genetic characterization of the population structure of the Tsurcana breed and as the quantitative-genetic foundation required for the development of variety-specific breeding programs. The simultaneous availability of phenotypic descriptors, heritability estimates, and genotypic correlations enables the formulation of differentiated selection objectives, the definition of operationally appropriate selection criteria, and the prediction of expected genetic progress in each of the four varieties (Belă, Breazăă, Bucălae, and Oacheșă). Subsequent stages of the research will be directed toward the estimation of individual breeding values, the validation of the selection responses obtained, and the integration of the present findings into a coherent national improvement scheme for the Tsurcana breed, in agreement with the priorities of the Romanian sheep sector.

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ASSOCIATION STUDY FOR GENETIC VARIANTS LINKED WITH REPRODUCTIVE TRAITS IN ROMANIAN SPOTTED CATTLE

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Abstract

In this work, we performed a targeted candidate-gene association study to identify genetic variants linked with reproductive traits in cattle. We investigated the relationship between 348 SNPs on Axiom BovMDv3 SNP array and the following reproductive traits in cattle: calving interval (CI), gestation length (GL), age at first calving (AFC), artificial inseminations to conception (A2C), presence of twins (TW) and history of abortions (AB). The dataset included reproductive information of 587 Romanian Spotted and Romanian Brown cattle. Poisson regression was applied to test associations for CI, GL, A2C, and AFC phenotypes, whereas TW and AB traits were examined using logistic regression. The results indicate significant associations between certain SNPs and fertility-related traits. The genes SPRY1, ILDR1, SLC24A4, TANC2, LOXL2, and SLC24A4 showed the strongest associations with CI, with extremely small p-values even after correction for multiple testing. In addition, SNPs on the ACOXL, SLC4A4, ABCC9, DDR2 and TANC2 genes were significantly associated with AFC. Our findings suggest that these genes are promising candidates for future studies aimed at elucidating the mechanisms influencing bovine fertility.

Key words*: association study, candidate gene, cattle, reproductive traits.*

INTRODUCTION

Reproductive efficiency represents a foundation of sustainable and profitable dairy cattle production systems, with direct implications for herd productivity, animal welfare, and economic performance (Schwarz et al., 2024). Reproductive traits such as calving interval, gestation length, age at first calving, and conception success influence the efficiency of dairy cows, the overall management, and the replacement strategies within herds. Suboptimal reproductive performance is associated with prolonged calving intervals, increased insemination costs, and higher culling rates, ultimately reducing lifetime productivity. Moreover, it has been widely documented that reproductive performance is often negatively correlated with high milk yield (Oltenacu & Broom, 2010; Pryce et al., 2004), emphasizing the need for balanced breeding strategies that integrate both production and functional traits. Despite their importance, research consistently identifies bovine reproductive traits as having low heritability and complex genetic architecture, being influenced by numerous genes and strong environmental interactions (Berry et al., 2014; Cammack et al., 2009; Panigrahi et al., 2024). This polygenic nature makes the identification of causative genetic factors particularly challenging. Advances in bovine genomic technologies, especially the development of high-density single nucleotide polymorphism (SNP) arrays and whole genome sequencing approaches, have significantly improved the ability to detect genomic regions associated with reproductive traits. Genome-wide association studies (GWAS) have become a powerful tool

for identifying genomic variants associated with economically important traits in cattle, enabling the discovery of candidate genes involved in key biological pathways of reproductive performance (Ortega, 2018). Numerous studies have reported significant associations between SNPs and fertility related phenotypes (Chen et al., 2022; Sbardella et al., 2021; Gonzalez Berrios et al., 2024), highlighting the complex architecture of reproductive traits and validating the idea that they are controlled by a large number of loci with small individual effects (Tahir et al., 2021). Furthermore, expanding genomic research to autochthonous and dual-purpose breeds, which represent unexploited sources of genetic diversity, is essential for identifying breed specific markers and previously undetected genetic architectures that may be absent in specialized cattle breeds.

In recent years, GWAS analyses in dairy and beef cattle populations have uncovered the complex genetic architecture that governs reproductive success and have identified multiple genomic regions and candidate genes associated with both male and female fertility traits. For example, studies using SNP chip data and imputed sequence level genotypes for genetic characterization of reproduction traits in German Holstein cattle have revealed more than 2700 significant SNPs representing about 90 different genes affecting fertility in dairy cattle, with the majority of association signals on BTA6 and BTA18 and others candidate genes on BTAX (Schwarz et al., 2024). Similarly, association studies for beef bull semen attributes have identified genes and quantitative trait loci (QTL) associated with fertility traits in bulls, widely distributed across the bovine genome (Butler et al., 2022), further highlighting the complexity of fertility traits. These findings underscore the potential of incorporating genomic information into breeding programs to enhance reproductive performance through marker-assisted or genomic selection.

However, most genomic studies on cattle fertility have been conducted in specialized breeds, while locally adapted breeds remain significantly underrepresented. Romanian Spotted and Romanian Brown cattle are important dairy breeds within regional production systems, valued for their longevity, adaptability, resilience, and balanced productive traits. Nevertheless, the genetic basis of their reproductive performance has not been extensively investigated, creating a significant knowledge gap. Given that breed-specific genetic backgrounds and selection histories may influence the architecture of reproductive traits, it is essential to conduct targeted association studies in these populations. Therefore, using targeted candidate-gene association study, we aim to identify genetic variants associated with key reproductive traits in Romanian Spotted and Romanian Brown cattle. We investigate the relationship between 348 pre-selected SNPs from the Axiom BovMDv3 array and six phenotypes: calving interval (CI), gestation length (GL), age at first calving (AFC), artificial inseminations to conception (A2C), presence of twins (TW), and history of abortions (AB) in a total of 587 animals. By identifying high-priority candidate genes, this research contributes to the understanding of fertility genetics in autochthonous breeds and provides valuable insights for future genomic selection strategies.

MATERIALS AND METHODS

The study involved a total number of 587 Romanian Spotted and Romanian Brown multiparous cattle from the Research and Development Station for Bovine in Arad. All animals were maintained under uniform nutritional and veterinary protocols and were included in the Romanian Official Performance and Recording Scheme. A total of six reproduction traits were analysed: calving interval (CI), gestation length (GL), age at first calving (AFC), artificial inseminations to conception (A2C), presence of twins (TW) and history of abortions (AB).

Genotyping was performed on blood samples collected from the coccygeal vein using K3EDTA tubes. All samples were subsequently processed by IFN Schönow GmbH in Germany using the Axiom Bovine v3 microarray (Thermo Fisher Scientific), mapped against the Bos_taurus_UMD_3.1.1 reference genome. We selected a panel of 348 SNPs on the Axiom BovMDv3 SNP array, located on 156 genes that have previously been associated with reproductive traits in cattle (Table 1) and investigated the relationship between selected SNPs and the six reproductive traits.

Furthermore, we set up a quality control pipeline, which excluded markers with call rates below 0.95, minor allele frequencies (MAF) less than 0.05, and significant deviations from Hardy-Weinberg Equilibrium.

Genotype and allele frequencies were calculated using a custom R script. Departure from Hardy-Weinberg equilibrium (HWE) was assessed with the chi-square test (χ^2), and a p-value below 0.05 was considered statistically significant, indicating a potential deviation from HWE in the population. Reproductive data were analysed statistically and are presented as mean ± SD. Associations between SNP markers and the first four phenotypes (CI, GL, A2C, and AFC) were evaluated using Poisson regression, while logistic regression was applied to the last two traits (TW and AB). In all analyses, the glm function in R was used. To control the error rate, we applied the Bonferroni correction on the resulting p-values, calculating the adjusted significance threshold.

The study design, sample collection, and all experimental procedures were reviewed and approved by the Ethics Committee of the Research and Development Station for Bovine. All research activities conducted in this study complied with the European Union Directive 2010/63/EU regarding the protection of animals used for scientific purposes.

Table 1. Genes associated with reproductive traits

Gene ID	Trait	Bibliography
GC, COX17, ILDR1, KALRN, SIPA1L3, IGF2BP2, UBXN7	pregnancy rate and cow conception rate	Jiang et al., 2019
CRAMP1L, PTX4, TELO2	calving ease	Frischknecht et al., 2017
AFF1, TSPAN4, NPAS1, SLC25A48, IL9, SPOCK1, B9D1	heifer conception rate	Jiang et al., 2019
ACOXL, RAB11FIP5, SFXN5, BMP6, RREB1, CREB1, FZD5, PLEKHM3, IDH1, PIKFYVE, MAP2, FOXO6	age at first calving	Sbardella et al., 2021
EPRS	age at first service	Zhou et al., 2019
ZBTB20, BTBD11, SLC4A4, PPFIBP1	pregnancy rate	Jiang et al., 2019
GAS2L1, ASCC2	interval from first to last insemination in heifers	Frischknecht et al., 2017
ACAP2	cow conception rate	Jiang et al., 2019
FAM181A, ASB2, SLC24A4, NKX2-1, FAM188A / MINDY3, MRC1, ABCC9,	calving to first service interval	Jiang et al., 2019
CABIN1, IGLL1	non-return rate in cows	Frischknecht et al., 2017
PYGM, PLCB3	days to first service	Frischknecht et al., 2017
ATP10A, MAGEL2	gestation length and calving ease	Frischknecht et al., 2017
ADRA1B, FABP6, TANC2	Calving-difficulty	Keogh et al., 2021
TUBD1	mortality rates	Frischknecht et al., 2017
SPRY1	gestation length	Zhou et al., 2019
CPNE1, SPAG4, CTNNBL1	gestation length	Frischknecht et al., 2017
CREB1, FZD5, PLEKHM3, IDH1, PIKFYVE, MAP2, LOXL2, STC1, PELI2, TMEM260, OTX2, RNF144A, CMPK2	reproductive tract scores	Stegemiller et al., 2021
MSTN	calving interval	Keogh et al., 2021
ACAT2, APBB1, AP3B1, BCAS1, BSP3, CAST, CD14, CD40, CSNK1E, FCER1G, FSHR, FUT1, FYB, GCNT3, HSD17B12, HSD17B7, IBSP, LDB3, OCLN, PCCB, PMM2, RABEP2, TBC1D24	fertility traits	Ortega, 2018
NUF2, RGS5, RGS4, HSD17B7, DDR2, UAP1, UHMK1, FYN, TRAF3IP2, REV3L, SLC16A10, CDK19, AMD1, SLC4A1AP, SUPT7L, GPN1, ZNF512, GCKR, FNDC4, IFT172, KRTCAP3, NRBP1, PPM1G, ZNF513, SNX17, EIF2B4, GTF3C2, MPV17, UCN, TRIM54, SLC30A3, ATRAID, SLC5A6, C16H1orf174, DFFB, CEP104, LRRC47, SMIM1, CCDC27, TP73, WRAP73, TPRG1L, MEGF6, ARHGEF16, TTC28, CHEK2, HSCB, CCDC117, XBP1, ZNRF3, LOC615587, KREMEN1, EMID1, RHBDD3, EWSR1, GAS2L1, RASL10A, AP1B1, NEFH, THOC5, NIPSNAP1	number of calving	Melo et al., 2017

RESULTS AND DISCUSSIONS

In this work, we performed a targeted candidate-gene association study to identify genetic variants linked with reproductive traits in cattle. The statistical analysis employed a two-model approach to account for the specific distributional properties of the reproductive phenotypes under investigation. Traits including CI, GL, A2C and AFC were analysed using Poisson regression. Conversely, the binary outcomes of TW and AB were examined through logistic regression. Following the quality control pipeline for genotypic data, a final set of 232 SNPs was utilized for statistical analysis. Furthermore, a number of 446 animals remained after quality control, of which 415 were Romanian Spotted and 31 Romanian Brown. Due to the small number of Romanian Brown individuals left, we decided to eliminate

them from the analysis due to concerns regarding low statistical power. In conclusion, we kept a total number of 415 Romanian Spotted individuals with phenotypic and genotypic records.

For the binary traits, TW and AB, we report the numbers of individuals in the two respective classes. For TW, there were 391 individuals that never had a twin and 23 individuals that had at least one twinning event until the study was performed. Similarly, for AB, 336 individuals had zero abortions, and 79 animals had at least one occurrence of abortion. The descriptive statistics for CI, GL, AFC and A2C are reported in Table 2. On average, results for reproductive traits in 415 cows showed a calving interval of 397.27 ± 52.58 days, gestation length 284.69 ± 9.04 days, age at first calving 875.48 ± 68.80 days, and the number of artificial inseminations per conception 1.65 ± 0.88.

Table 2. Descriptive statistics for reproductive traits in Romanian Spotted cattle

Trait	Mean ± SEM	Min.	Max.
CI (days)	397.27 ± 52.58	361	514
GL (days)	284.69 ± 9.04	272	306
AFC (days)	875.48 ± 68.80	752	1037
A2C (no)	1.65 ± 0.88	1	5

Calving interval (CI), Gestation length (GL), Age at first calving (AFC), Artificial inseminations to conception (A2C).

Those results reveal a reproductive profile that aligns closely with reproductive performance for Romanian dual-purpose cattle. The mean calving interval of 397.27 ± 52.58 days is consistent with recent findings for Romanian Brown cattle, which reported an average CI of 411±38.8 days (Neamt, 2021). While these results exceed the ideal biological threshold of 365 days recommended for optimal farm efficiency, they fall within the common range for Romanian Spotted (Simmental type), which typically fluctuates up to 429 days depending on the lactation stage (Cighi et al., 2021). In contrast to the variability observed in calving intervals, the mean GL of 284.69 ± 9.04 days demonstrates remarkable physiological stability. This value is nearly identical to the average recorded in Romanian Spotted populations, where GL showed values of 283.61 and 283.24 days, respectively (Potra et al., 2020). The mean AFC of 875.48 ± 68.80 days (approximately 29 months) characterizes the studied population as semi-precocious. This finding is closely aligned with results obtained for Romanian Spotted cows, which noted an average AFC of 29 months and 14 days (Mihuta et al., 2022). The observed mean of 1.65 ± 0.88 artificial inseminations per conception (A2C) suggests good reproductive efficiency for the Romanian Spotted cattle in this study. These results demonstrate improved fertility compared to the findings of Toledo-Alvarado et al. (2017), who reported a higher mean A2C of 1.82±1.10 for Simmental cattle (Toledo-Alvarado et al., 2017). Collectively, these descriptive statistics underscore the dual-purpose nature of the Romanian Spotted breed, which prioritizes environmental resilience over the reproductive speed of specialized dairy breeds. These findings emphasize that genomic selection focusing on markers for earlier maturation and shorter post-partum intervals could significantly bridge the gap between current performance and optimal economic thresholds for the Romanian cattle sector.

The results of the Poisson regression analysis provided valuable information about how different genotypes influence the reproductive traits. Significant genomic associations were only detected for CI and AFC, suggesting that these traits possess a more discernible genetic architecture within the studied population. In total, 41 SNPs showed significant association with CI (Table 3) and 13 SNPs with AFC (Table 4). These SNPs map to various chromosomes and genes.

In Table 3 we include the most significant associations for each SNP identified for CI analysis. Each row represents the genotype with the lowest p-value and its associated coefficient, indicating the effect on the expected interval duration.

Table 3. Significant SNPs associated with Calving Interval (CI) in Romanian Spotted cattle breed

SNP ID	dbSNP ID	Gene ID	Chr	A1	A2	Genotype	P-value	Coefficient
AX_106758358	rs110865913	*SPRY1*	17	A	G	GG	1.11×10^{-15}	0.1132
AX_124377040	---	*ILDR1*	1	G	A	GG	1.28×10^{-14}	-0.1350
AX_117080166	rs109148864	*SLC24A4*	21	A	G	GG	1.87×10^{-13}	-0.0691
AX_124379423	---	*TANC2*	19	A	G	GG	1.18×10^{-13}	-0.0514
AX_106734258	rs109942542	*LOXL2*	8	T	C	TT	3.01×10^{-12}	0.0794
AX_117085492	rs110034825	*SLC24A4*	21	G	T	TT	2.03×10^{-12}	-0.0449
AX_117081263	rs132661844	*SLC24A4*	21	T	C	CC	4.58×10^{-11}	0.0423
AX_117083489	rs109820230	*SLC24A4*	21	G	A	AA	5.58×10^{-11}	0.0423
AX_117090447	rs133058937	*LOC615587*	17	G	A	AA	7.79×10^{-11}	0.0568
AX_106724830	rs41627459	*BCAS1*	13	A	G	GG	3.73×10^{-11}	-0.0420
AX_106727425	---	*ACOXL*	11	T	C	TT	3.51×10^{-11}	0.0736
AX_106740902	---	*KALRN*	1	T	C	TT	1.43×10^{-10}	0.0878
AX_117083000	rs135194517	*SLC24A4*	21	C	A	AA	3.89×10^{-10}	0.0401
AX_106752630	rs110125976	*UBXN7*	1	C	G	GG	5.06×10^{-10}	-0.0997
AX_106730474	rs29019798	*KALRN*	1	T	C	TT	2.59×10^{-10}	-0.0509
AX_115107195	---	*MAP2*	2	A	G	GG	1.02×10^{-9}	0.0396
AX_115111653	rs42889182	*IGF2BP2*	1	C	A	AA	2.96×10^{-9}	-0.0417
AX_117079758	rs133863748	*SLC24A4*	21	C	T	TT	2.18×10^{-9}	-0.0392
AX_117090789	rs110763504	*SLC24A4*	21	C	T	TT	1.97×10^{-9}	-0.0392
AX_117090924	rs109893119	*SLC24A4*	21	T	C	CC	1.71×10^{-9}	0.0392
AX_117085161	rs135732284	*SLC24A4*	21	T	G	GG	1.35×10^{-9}	0.0392
AX_168294373	rs132677621	*SLC24A4*	21	C	T	TT	1.26×10^{-9}	0.0526
AX_169407036	rs109249576	*SLC24A4*	21	A	G	GG	1.92×10^{-9}	-0.0392
AX_169408636	rs135589268	*SLC24A4*	21	A	G	GG	1.07×10^{-9}	0.0683
AX_106731399	rs108944935	*SLC24A4*	21	A	G	GG	1.97×10^{-9}	-0.0392
AX_106734241	---	*SLC4A4*	6	T	C	TT	2.29×10^{-8}	0.0977
AX_106739150	rs110404266	*DDR2*	3	C	T	TT	7.85×10^{-8}	-0.0349
AX_106743037	rs110704402	*ASB2*	21	A	G	GG	8.05×10^{-8}	-0.0552
AX_169504949	rs109703871	*SLC24A4*	21	C	T	TT	1.72×10^{-8}	-0.0370
AX_117085844	rs137491953	*SLC24A4*	21	A	G	GG	7.93×10^{-8}	0.0467
AX_117116602	rs134798153	*SLC24A4*	21	A	G	GG	1.13×10^{-8}	-0.0376
AX_117081043	rs109292204	*SLC24A4*	21	A	G	GG	1.14×10^{-8}	-0.0376
AX_117083128	rs110293188	*SLC24A4*	21	G	A	GG	1.51×10^{-8}	0.0531
AX_117086508	rs43657798	*ACOXL*	11	A	G	GG	1.61×10^{-8}	-0.0468
AX_117084106	rs136185842	*SLC24A4*	21	G	A	GG	1.79×10^{-7}	-0.0466
AX_117086824	rs29010907	*CEP104*	16	A	G	GG	1.83×10^{-7}	-0.0373
AX_106749545	rs42287687	*TTC28*	17	G	A	GG	2.19×10^{-7}	0.0497
AX_115099183	rs109516473	*BTBD11*	5	A	G	GG	1.76×10^{-7}	-0.0337
AX_185114987	rs109154186	*ZBTB20*	1	G	A	AA	1.33×10^{-7}	0.0338
AX_106743349	rs43244044	*ILDR1*	1	C	T	CC	3.99×10^{-6}	0.1540

Each row shows the SNP ID (Axiom BovMD and dbSNP ID), annotated gene (Gene ID), chromosome (Chr), alleles (A1/A2), the genotype with the most significant p-value, the p-value (P), and the Poisson regression coefficient.

The analysis of CI data revealed a significant background of genetic variants distributed across several bovine chromosomes (BTA). In total, 41 SNPs were significant at the chromosome-wide level for CI, distributed on BTA 1, 2, 3, 5, 6, 8, 11, 13, 16, 17, 19 and 21. The most significant and densely populated cluster of associations for CI was found on chromosome 21, specifically within the *SLC24A4* gene. This gene encodes a potassium-dependent sodium/calcium exchanger, which is involved in calcium homeostasis (Nayeri et al., 2016). In our study, 18 SNPs from the *SLC24A4* gene showed robust associations with CI. In different genomic studies, several variants of the *SLC24A4* gene were associated with reproduction, production, body conformation and milk urea concentration traits in cattle (Velayudhan et al., 2023; Nayeri et al., 2016; Maiorano et al., 2018; Yan et al., 2020; Ariyarathne et al., 2021). The presence of numerous significant markers in this region across different studies suggests that ion homeostasis in the reproductive tract may be an important determinant of fertility in cattle.

The most significant association ($p = 1.11 \times 10^{-15}$) with a positive coefficient of 0.1132 was found for AX_106758358 SNP (rs110865913) in the *SPRY1* gene. This gene plays a significant role in male fertility and testicular health,

particularly in protecting the integrity of the blood-testis barrier and preventing excessive inflammatory responses in the testis (Zhang et al., 2023). A previous genetic study conducted to dissect the genetic architecture related to reproductive traits in dual-purpose cattle has identified significant SNPs near the *SPRY1* gene on BTA 17 associated with gestation length (Zhou et al., 2019). By extension, variants of *SPRY1* may influence the inflammatory environment of the bovine uterus postpartum. The positive regression coefficient for the GG genotype of rs110865913 in the *SPRY1* gene suggests that this specific variant is associated with a slower return to reproductive function, potentially through suboptimal modulation of the inflammatory cascade. Integrating these findings with previous research, our results suggest that while the SPRY1 gene may not be the primary driver of the calving interval trait, it is part of the broader genomic landscape that influences calving intervals and overall reproductive efficiency.

For BTA 1, the most significant associations were identified within the *ILDR1* and *KALRN* genes. SNP AX_106740902, located on the *KALRN* gene, showed a highly significant association ($p = 1.43 \times 10^{-10}$) with a positive coefficient of 0.0878, indicating that certain genotypes at this position are linked to a prolongation of the calving interval. *KALRN* is known for its role in embryo development and has been consistently associated with pregnancy rates and conception status in U.S. Holstein populations (Yang et al., 2025). The identification of *KALRN* in the Romanian Spotted population may suggest that genetic defects in early cytoskeletal organization of the embryo may contribute to early embryonic loss, which clinically manifests as an extended calving interval.

On chromosome 13, the *BCAS1* gene was identified as a candidate locus through SNP AX_106724830. The GG genotype at this locus showed a significant reduction in the expected calving interval ($p = 3.73 \times 10^{-11}$, coefficient = -0.0420), suggesting its potential as a marker for improved reproductive efficiency. *BCAS1* has been implicated in genomic studies of bovine reproduction and was associated with fertility across different cattle populations (Ortega et al., 2017). The CI analysis identified other significant associations, involving *ZBTB20, IGF2BP2, UBXN7, MAP2, DDR2, BTBD11, SLC4A4, LOXL2, ACOXL, CEP104, TTC28, LOC615587, TANC2*, and *ASB2* genes, suggesting that the genetic architecture of CI in Romanian cattle is influenced by large-effect variants.

The results of significant genomic associations for AFC are presented in Table 4. The genetic architecture of AFC in Romanian dual-purpose breeds also exhibited significant SNP markers, where thirteen SNPs reached significance. Each entry details the genotype most significantly associated with the trait and the corresponding coefficient derived from the Poisson regression.

Table 4. Significant SNPs associated with age at first calving (AFC) in Romanian Spotted cattle breed

SNP ID	dbSNP ID	Gene ID	Chr	A1	A2	Genotype	P-value	Coefficient
AX_26649835	rs110405210	*SLC4A4*	6	G	A	AG	1.39×10^{-11}	0.0324
AX_106753905	---	*ACOXL*	11	G	T	TT	3.12×10^{-11}	0.0299
AX_106735416	---	*ABCC9*	5	T	C	TT	6.52×10^{-10}	-0.0494
AX_106739150	rs110404266	*DDR2*	3	C	T	CC	6.01×10^{-9}	-0.0387
AX_106746160	---	*TANC2*	19	T	C	TT	1.23×10^{-9}	0.0313
AX_106752999	rs41578409	*ABCC9*	5	A	G	AA	1.47×10^{-9}	0.0469
AX_106733878	---	*IBSP*	6	T	C	CT	7.98×10^{-8}	0.0316
AX_106757386	rs43480825	*AFF1*	6	T	C	TT	2.10×10^{-8}	0.1094
AX_117082950	rs109635380	*PELI2*	10	A	C	CC	2.67×10^{-8}	-0.0344
AX_124381692	rs42286695	*TTC28*	17	T	C	TT	2.24×10^{-8}	-0.0328
AX_169413127	rs133717146	*APBB1*	15	A	G	GG	3.37×10^{-8}	-0.0330
AX_106727919	rs41619788	*SLC4A4*	6	G	T	TT	3.03×10^{-7}	-0.0219
AX_115114961	rs109665442	*IGLL1*	17	T	C	CC	1.99×10^{-7}	0.0585

Each row shows the SNP ID (Axiom BovMD and dbSNP ID), annotated gene (Gene ID), chromosome (Chr), alleles (A1/A2), the genotype with the most significant p-value, the p-value (P), and the logistic regression coefficient.

The top AFC SNP was AX_26649835 in *SLC4A4* (chr6, P = 1.394×10^{-11}). Other notable associations include AX_106753905 (*ACOXL*) and two SNPs in ABCC9 (chr5): AX_106735416 and AX_106752999.

The most significant SNPs highlighted in our study was observed on BTA 6 in *SLC4A4* gene. SNP AX_26649835 (rs110405210), associated with *SLC4A4*, showed high significance (p = 1.39×10^{-11}) with a positive coefficient, reflecting an increased age at first calving for the AG genotype. The SLC4A4 locus is a well-known QTL for pregnancy rates in Holstein cows (Liang et al., 2023). The *ACOXL* gene on BTA 11, represented by SNP AX_106753905, also showed a significant association (p = 3.12×10^{-11}) with a positive coefficient on AFC (0.0299) for the TT genotype. This gene was previously found in the regions associated with AFC (Sbardella et al., 2021) and has also been identified in association with pregnancy outcome in Brahman heifers (Porto-Neto et al., 2015).

The *ABCC9* gene on BTA 5 emerged as a candidate locus for AFC. SNP AX_106735416 within this gene demonstrated a significant association (p = 6.52×10^{-10}) where the TT genotype indicating association with shorter AFC (negative coefficient = –0.0494). The *ABCC9* gene is involved in ATP-sensitive potassium channels and has been linked in previously study with fat yield in Holstein cattle (Jiang et al., 2019).

Additional significant SNPs were identified in genes with diverse biological functions, including *DDR2, TANC2, ABCC9, IBSP, AFF1, PELI2, TTC28, APBB1, SLC4A4* and *IGLL1*, highlighting the complex genetic basis of AFC.

The genomic associations identified in this study point toward several biological pathways that likely coordinate reproductive efficiency in dual-purpose cattle. A consistent pattern observed in the results is the prominent involvement of the Solute Carrier (SLC) gene family, particularly members of the SLC24 and SLC4 families, in modulating bovine reproductive efficiency. The *SLC24A4* gene, which exhibited multiple significant markers on BTA21 for CI, encodes a member of potassium-dependent sodium/calcium exchanger protein family (Nayeri et al., 2016), which is fundamental to cellular ion homeostasis. In mammalian reproduction, calcium is an important signalling molecule, and intracellular Ca^{2+} oscillations are a key signalling mechanism during oocyte maturation, activation, and fertilization (Chen et al., 2024). Given this central role of Ca^{2+} signalling, genetic variation in *SLC24A4* may influence the success of fertilization or the timing of the return to cyclicity postpartum. Similarly, the *SLC4A4* gene on BTA6, associated with both CI and AFC, encodes a bicarbonate cotransporter that plays a key role in regulating pH in the reproductive tract and oviductal fluid. Proper bicarbonate balance is essential for maintaining the microenvironment that supports sperm viability and development of the embryo in cattle (Lane & Gardner, 2000). Therefore, variation in *SLC4A4* could influence fertility traits by altering pH homeostasis during these critical reproductive stages. In this study, the genetic architecture of AFC was dominated by a signal on BTA6 involving the *SLC4A4* gene. For Romanian Spotted heifers, the AG genotype of SNP AX_26649835 was associated with a positive coefficient, indicating a delayed age at first calving. This implies that genomic differences in the homeostatic control of the oviductal environment might influence the success of the heifer's first breeding attempts, thereby determining the age at which it officially enters the productive milking herd.

CONCLUSIONS

The current targeted candidate-gene association study identified a series of genomic markers significantly linked to calving interval and age at first calving in Romanian Spotted cattle. Traits such as twinning, abortions, artificial inseminations to conception and gestation length did not yield significant signals. The recurrent prominence of genes including *SLC24A4*, *SPRY1*, and *SLC4A4* highlights that reproductive traits such as calving interval and age at first calving are controlled by fundamental biochemical regulators governing oocyte activation and uterine microenvironmental dynamics. However, the scientific utility of these results is currently constrained by technical limitations in sample size. Future research will be required, prioritizing the use of whole-genome sequence

WGS imputation to ensure that the identified SNPs are in high linkage disequilibrium with the true causal variants.

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MANAGEMENT OF GENETIC RESOURCES IN LIPIZZANER BREED NUCLEI AT SÂMBĂTA DE JOS AND BECLEAN STATE STUDS

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Abstract

The conservation of the Lipizzaner horse in Romania depends on breeding systems that maintain genetic variability while respecting established selection traditions. This paper analyzes the management of genetic resources in two Romanian state-owned Lipizzaner breeding nuclei, Sâmbăta de Jos and Beclean, by comparing current practices with those applied in earlier periods. The study is based on a critical synthesis of scientific literature and the examination of stud farm documentation, breeding regulations, and technological norms. Emphasis is placed on reproductive organization, the use of pedigree records, and practical measures aimed at maintaining genetic diversity. A comparative approach highlights continuities and changes in breeding objectives and management strategies over time. The findings identify strengths and operational constraints influencing population structure and long-term viability within the Romanian state stud farm system.

Key words*: breeding nuclei, conservation genetics, genetic resources management, Lipizzaner horse, state stud farms.*

INTRODUCTION

The Lipizzaner horse is one of the most emblematic European horse breeds, closely associated with cultural heritage, classical horsemanship, primarily managed within state-run breeding systems.

In Romania, Lipizzaner breeding has traditionally been concentrated in state-owned stud farms, at Sâmbăta de Jos and Beclean, where structured selection and reproduction practices have played a decisive role in shaping population structure.

The Lipizzan horse, considered a 'Baroque horse' (Zechner et al., 2002), today represents a cultural heritage.

The *Tradition of breeding Lipizzaner horses in Romania* has been included in the *National Inventory of Living Elements of Intangible Cultural Heritage.* (CIMEC, National Institute of Heritage, 2020)

The candidacy file was submitted by Romania for the Representative List of the Intangible Cultural Heritage of Humanity of UNESCO as part of a multinational file for the March 2020 session (Dell, 2020)

On December 1, 2022, Romania's National Commission for UNESCO welcomed the decision of the Intergovernmental Committee for the Safeguarding of the Intangible Cultural Heritage of Humanity, meeting in Rabat, Morocco, regarding the inscription on the Representative List of the Intangible Cultural Heritage of Humanity of the 'Traditions of breeding Lipizzaner horses' from 7 countries (Austria, Bosnia and Herzegovina, Croatia, Italy, Romania, Slovakia, Slovenia, and Hungary), where this heritage is actively and uninterruptedly preserved. (Romania's National Commission for UNESCO, 2022).

Like any other population of domestic animals, it can become endangered by acquiring a status of risk, under the influence of multiple factors, among which the most important and likely to manifest in the current period is economic inefficiency, compared to other populations under the same conditions (Drăgănescu, 1979; Grosu, 2003). Therefore, the Lipizzaner breed nuclei zootechnically managed by the National Forest Administration - Romsilva at the State Stud Farms of Sâmbăta de Jos and Beclean provide, in addition to their cultural significance, numerous other genetic, zootechnical, and scientific arguments, and necessarily constitute an object of genetic resource management.

MATERIALS AND METHODS

The study is based on an integrated methodological approach combining bibliographic analysis and documentary research. International scientific publications addressing genetic resource management and conservation genetics were reviewed, along with institutional documentation from the state stud farms of Sâmbăta de Jos and Beclean.
The period analysed in this study was 1920-2025.

RESULTS AND DISCUSSIONS

Across European state-managed Lipizzaner breeding nuclei, the relatively small and structurally controlled reproductive populations, typically comprising limited numbers of breeding stallions and broodmares, reflect a historical decline in horse populations followed by stabilization under conservation-oriented management, with current trends indicating the maintenance of moderate herd sizes and carefully balanced breeding structures to limit inbreeding while preserving genetic variability, and future expectations pointing towards continued reliance on controlled nucleus systems and coordinated international breeding strategies to sustain genetic resources (Zechner et al., 2002; FAO, 2007; FAO, 2015) (Table 1).

Table 1. European State Studs Lipizzan population statistics for 2025 (heads) (Lipizzan International Federation, 2026)

National Stud Farms	Stallions for breeding	Mares
Beclean, Romania	15	51
Djakovo, Croatia	25	69
Karadjeordjevo, Serbia	4	8
Lipica, Slovenia	29	142
Lipik, Croatia	6	37
Monterotondo, Italy	6	48
Piber, Austria	97	96
Sâmbăta de Jos, Romania	14	83
Szilvasvarad, Hungary	16	78
Topolcianky, Slovakia	10	57
Vucijak, Bosnia and Herzegovina	10	36
Total	232	705

The Romanian State Studs are managed since 2002 by the National Forestry Administration - Romsilva, and since 2019 through the National Forestry Administration - Romsilva - Horse Breeding Direction S.O.C.

Currently, the system is structured in 14 studs and 4 stallions depots, which are technically subordinate to the Horse Breeding Direction of the National Forestry Administration - Romsilva. The State Studs/Studfarms in Romania are elite farms whose role is to provide an optimal management of the genetic equine patrimony, by continuous genetic improvement of the cultural breeds, and the conservation of the vulnerable or endangered breeds of horses. The Stallion Depots are also elite farms positioned on the next lower level of the genetic improvement pyramid (Figure 1). These units are intended to multiply and transfer genetic progress achieved in the stud farms. An important aspect is that most state studs are in charge to manage the breeding stock of pure breeds with high risk status e.g. endangered (Hutsul, Gidran, Furioso North Star, Nonius) (Drăgănescu, 2003). In some cases they represent almost the entire livestock herd of pure bred in Romania. Romanian State Studs are also abiding by the breeding regulations applied at the moment of the breeds foundation, a true cultural vestige that conserves not only valuable genes but also a living old traditional breeding system. Experimental breeding practices were avoided in the State Studs. In the spirit to maintain the population close to the original gene pool, the old traditional breeding methods were also maintained (preserving the sire 'sire bloodlines' and 'mare families').
The national genetic patrimony breeding stock numbered then 1505 heads [733 breeding stallions used in public breeding system/ private horse breeders/owners, 94 breeding stallions (sires) and 678 broodmares (dams)]. The strategic policy for genetic improvement and conservation was maintained.

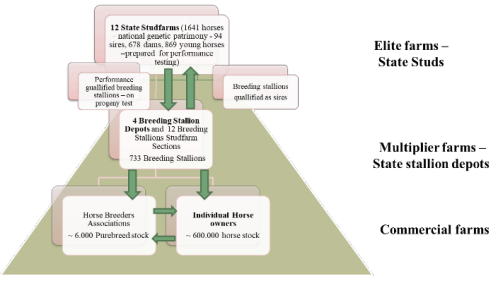


Figure 1. Horse breeding system in Romania - Genetic improvement pyramid

'Sire Bloodlines'

The small herds in the stud farms, typically comprising a maximum of 40-150 mares but with between 4 and 12 stallions, necessitated a specific breeding principle-breeding in 'bloodlines' (Drăgănescu, 1979). Thus, given the limited possibilities for exchanging breeding stock between stud farms, breeders were forced to deliberately avoid the drawbacks of inbreeding through selective breeding. Inbreeding can thus be controlled in closed populations through intra-family selection ('bloodlines'), namely by replacing each breeding animal, or at least the males, with one of their descendants (reducing the family size variance to zero) and through rotational inter-family crossbreeding, namely directing matings in such a way as to avoid close-relative matings (Drăgănescu, 1979; Drăgănescu & Grosu, 2003). The Sâmbăta de Jos and Beclean Stud Farms are not an exception to this breeding principle.

Currently, at Sâmbăta de Jos Stud Farm, seven male bloodlines are maintained at the stud farm: *Conversano*, *Favory*, *Maestoso*, *Neapolitano*, *Pluto*, *Siglavy-Capriola*, and *Tulipan*. The Pluto line was founded by the stallion Pluto I, acquired in 1936 from Atta Constantinescu's Răcăciuni estate. Another branch belonging to the same classic bloodline was imported in 1955 from Czechoslovakia through the purchase of the stallion Pluto V, born in Topolcianky.

The Siglavy-Capriola line descends from the stallion Siglavy-Capriola I, also imported from Czechoslovakia in 1930. The Conversano, Favory, Maestoso, Neapolitano, and Tulipan lines were reconstituted from the material gathered during the establishment of the State Stud at Sâmbata de Jos in 1920. The Maestoso line also has a Czechoslovak branch at Sâmbata de Jos through the stallion Maestoso XXVII, imported in 1955 (Suciu & Moldoveanu, 1975). At the same time, three broodmare daughters. At Beclean Stud Farm, eight male bloodlines are maintained farm: *Conversano*, *Favory*, *Incitato, Maestoso*, *Neapolitano*, *Pluto*, *Siglavy-Capriola*, and *Tulipan*.

Copernicus Project IC15CT96-0904

For maintaining genetic diversity within the Lipizzan breed on February 1, 1997, the International Lipizzan Federation initiated a large-scale research project at the European level - Copernicus Project IC15CT96-0904, DG 12 - CDPE, with the aim of presenting a scientific description of the phenotypic characteristics and genetic structure of the Lipizzan breed (Brem et al., 2011). In detail, the initiators of this project aimed to describe the genetic structure of the breed using DNA markers, blood group systems, polymorphic systems, morphological parameters, measurements, photographs, information regarding ancestry using pedigree records, health status, disease resistance traits, performance information, and reproductive traits (Brem et al., 2011).

The participants in this project were: the Institute for Genetics and Animal Breeding at the University of Veterinary Medicine in Vienna, the Department of Animal Husbandry at the University of Veterinary Sciences in Budapest, the Department of Animal Science at the Faculty of Biotechnology of the University of Ljubljana, the Institute for Animal Breeding at the University of Veterinary Medicine in Vienna, and the International Lipizzaner Breeders Federation (LIF). The subject of this study was the traditional breeding studs of Lipizzaner horses at Djakovo - Croatia, Lipica - Slovenia, Monterotondo - Italy, Piber - Austria, Sâmbăta de Jos and Beclean - Romania, Silvasvarad - Hungary, Topolcianky - Slovakia (Brem et al., 2011).

The arguments for the protection of the breed at the level of the European Union were as follows: the Lipizzaner breed is a pan-European breed, it also represents a cultural heritage of the continent, the scientific quality of the project, and the possibility of obtaining support from the governments of the states that manage reproduction centers of this breed (Brem et al., 2011).

The role of LIF in carrying out the project involved participation in the execution and implementation of the project: collecting information from genealogical registers on bloodlines and families, detailing the history of the breed, publishing and disseminating the results (Brem G. et al., 2011).

Among the practical aspects of this project, the following can be mentioned: describing the breed on a scientific basis and developing a target standard for this breed, developing a system to reduce inbreeding, creating a

genealogical register of the breed at the European level in a computerized system, demonstrating the genetic independence of the breed, and publishing the results (Brem et al., 2011). The study identified the existence of a 'Romanian pole' and a 'Classic pole' within the Lipizzan breed (Figure 2).

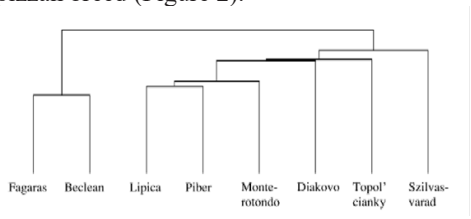


Figure 2. Dendrogram of the genetic relationships among Lipizzaner studs in Europe (Zechner et al., 2002)

The observation is based on the result of the study of the genetic relationships between the 8 important European studs in which this breed is improved. They used the algorithm, which allows the calculation of inbreeding coefficients between very large groups of individuals and obtained the matrix of the average relatedness between studs. Stud relatedness coefficients provide information about reducing the level of inbreeding by mating horses from different studs (Zechner et al., 2002).

In Romanian studs, the system of purebred breeding is applied (T. Suciu et al., 1975). The main component of a purebred improvement program is the selection plan. Since studs are actually small elite farms, consisting of 40-100 mares and 5-12 stallions, the main practical strategy for limiting inbreeding is intrafamilial selection, followed necessarily by inter-familial rotational crossing (Drăgănescu, 1979).

The application of intrafamilial selection involves going through mandatory technological stages: individualization of animals (marking by iron branding), mating control (matching of pairs), monitoring of births, recording of origin (through the genealogical register of the improved breed within the stud farm), production control (the grading operation), and expression of performance as a deviation from the family average (Drăgănescu, 1979).

The complexity and specificity of traits in the selection of purebred horses may require progressive directional selection, stabilizing selection, and regressive directional selection.

For populations whose variability and effective size are sufficiently large, progressive directional selection is most frequently applied. In stud farms that hold such populations, selection is carried out based on multiple traits simultaneously, even though the large number of traits leads to a decrease in overall selection efficiency (Drăgănescu, 1979).

Decisions regarding promotion or removal from breeding are made annually during the classification work of horses. In Romania, between 1975-2006, this was carried out based on the Instructions for the Evaluation of Breeding Horses, issued by the Ministry of Food and Agriculture, and from 2006 to the present, based on the Evaluation Criteria regarding the assessment of breeding horses, no. 894/02.11.2006 and no. 571/29.07.2008, issued by the Ministry of Agriculture and Rural Development. The main effect of selection is the change in gene and genotype frequencies in the population that is the subject of selection. This change occurs in a controlled manner and is directed in the useful direction defined within the objectives of breeding.

Marking

The individualization system accompanied by the iron branding was developed and applied according to a unitary concept after the end of the First World War, respecting the usual traditional codes established for each breed (Mărginean, 1997).

The marking tools are made of iron and reproduce in an official standard form all the specific insignia of the breed. These include letters, numbers and conventional signs.

The marking code used for the Lipizzaner breed bred at the Sâmbăta de Jos Stud Farm continued the traditional system of individualization that remains valid today: It is composed of the father's mark, the mother's mark, the stud mark, the foal number and the mare's registration number (Suciu et al., 1975) (Figure 3).

Horses must be marked only once, at weaning with the father's mark, mother's mark, stud mark, foal number. The mare registration number is applied to mares when they are promoted to the category of broodmares and receive, in the order of their entry in the herd register, the registration number (Suciu et al., 1975).

The father's mark is represented by the initial of the name of the genealogical line to which the father belongs, followed by the chronological nursery number, in Arabic numerals (Suciu et al., 1975).
The mother's mark is made up of conventional signs that represent the paternal line to which the mare belongs (Suciu et al., 1975).
The mark of the stud is represented by the initial of the name of the stud where the foal was born, regardless of the breed.
The number of foals is the order number for each offspring of a nursery stallion. It is attributed in chronological order of the date of birth to the foals, but in an uninterrupted series until the end of the father's breeding career (Suciu et al., 1975).
For the Lipizzaner breed bred in Romanian State Studs, the place of application for individualization marks in juveniles is on both sides of the body, at the place of the saddle, in the middle of an angle formed by the horizontal line that passes at a distance of 3 cm, below the spinal line and the vertical line that passes behind the thoracic angle of the back. The mother mare's matriculation number is applied to the left side of the neck, on a midline in the upper third. The father's mark and the mother's mark are applied on the left side, and the stud mark and foal number are applied on the right side (Suciu et al., 1975).

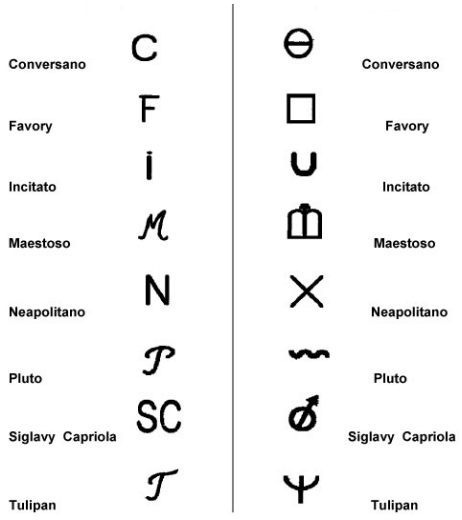


Figure 3. Conventional signs used for individualizing the Lipizzan breed by iron branding in Romania (Suciu et al., 1975)

The comparative analysis indicates continuity in fundamental breeding principles, particularly regarding line preservation and controlled mating. Differences were identified in the formalization of technological procedures across historical periods.
Parameters of genetic analysis in the population of Lipizzaner horses in Romania.
In the population of Lipizzaner horses from Sâmbăta de Jos state stud farms, in the 6.6 generations known and studied, they showed a total inbreeding of 7.12%. This resulted in an increase in homozygosity per generation of approx. 1%, a value similar to those found in other breeds of horses in Romania at that time. There were no cases of inbreeding, the observed level of inbreeding resulted primarily from the relatively high kinship itself (15% overall) of the herd (Drăgănescu & Zeneci, 1977/1978).
In two periods analyzed (1944-1953 and 1954-1963) the moderate inbreeding practiced was somewhat higher than the random one. The authors assumed possible the existence of a tendency to practice inbreeding to consolidate the existing lines and a slight tendency to subdivide the population. The actual population size, deduced from the annual increase in homozygosity, was approximative 50 individuals or 11 effective males (Drăgănescu & Zeneci, 1977/1978).
The authors concluded that the Lipizzaner bred at the Sâmbăta de Jos - Făgăraș stud farm represented a Mendelian population that has evolved relatively isolated reproductively in the last four generations and thus the name of the Romanian Lipizzaner breed is correct, reflects a reality, and the population evolves independently of other populations of Lipizzaners (Drăgănescu & Zeneci, 1977/1978).

Coat colour selection Beclean vs. Sâmbăta de Jos State Stud

From a conservation genetics perspective, the coexistence of differentiated European breeding nuclei represents a strategic advantage for long-term preservation, allowing both intra-population stability and inter-population diversity (FAO, 2007; FAO, 2015).
The identification of a distinct Romanian genetic pole supports the hypothesis of independent evolution under controlled breeding conditions and limited external influence (Zechner et al., 2002; Brem, 2011).

The Romanian Lipizzaner breeding nuclei occupy an intermediate position, combining reproductive isolation with the preservation of multiple sire lines and mare families, ensuring both genetic stability and adaptive potential (Drăgănescu, 1979; Grosu, 2003; FAO, 2013).

In Hungary (Szilvásvárad) and Slovakia (Topolčianky), breeding systems have historically allowed a higher degree of controlled genetic exchange, contributing to increased variability while preserving breed integrity (Zechner et al., 2002; Brem, 2011).

In Austria (Piber), the breeding system is characterized by a centralized structure with strong emphasis on classical sire lines and genealogical continuity (Brem, 2011). In Slovenia (Lipica), the breeding strategy remains highly conservative, focusing on maintaining the original type and genetic identity of the breed (Zechner et al., 2002).

The structure and management of Lipizzaner breeding nuclei in Romania can be better understood in the context of the broader European network of state-managed studs, including those from Austria, Slovenia, Hungary, and Slovakia, where similar conservation-oriented breeding systems have been historically maintained (Zechner et al., 2002; Brem, 2011; FAO, 2015).

Pedigree analysis remains a cornerstone of genetic management in Lipizzaner populations, providing a robust framework for tracking lineage and managing genetic relationships (Zechner et al., 2002; Brem, 2011). Despite advances in molecular tools, pedigree-based systems continue to be effective in historically structured populations (FAO, 2013; FAO, 2015).

The balance between selection and genetic diversity is a central challenge in small populations, where the effective population size is inherently limited (FAO, 2010; FAO, 2013). In Romanian Lipizzaner nuclei, this balance is achieved through rotational mating strategies and the avoidance of close-relative pairings (Drăgănescu, 1979; Grosu, 2003).

Closed breeding systems, such as those implemented at Sâmbăta de Jos and Beclean, rely on the preservation of sire lines and mare families as fundamental genetic units, ensuring both continuity and controlled variability (Suciu et al., 1975; Brem, 2011). This dual objective is critical for maintaining breed identity while preventing excessive inbreeding (Drăgănescu & Zeneci, 1977/1978; FAO, 2010).

The management of genetic resources within Lipizzaner breeding nuclei is aligned with internationally recognized principles of in situ conservation, which emphasize the maintenance of adaptive genetic variation within traditional production systems (FAO, 2007; FAO, 2015). In the Romanian context, this approach is operationalized through structured mating systems and strict pedigree control (Drăgănescu, 1979; Grosu, 2003).

From a conservation perspective, the coexistence of colour-selected and non-restricted populations enhances genetic resilience, preserving both phenotypic diversity and adaptive potential within the breed (FAO, 2010; FAO, 2015).

A comparable model is observed in the Kladruber breed, where black and grey populations are maintained in separate nuclei under strict genealogical control (Kladruby nad Labem; UNESCO Czech Heritage, 2019). This dual structure demonstrates that colour-based selection can generate stable subpopulations without compromising breed identity (FAO, 2015).

In contrast, the Sâmbăta de Jos nucleus maintains a non-restrictive selection regarding coat colour, allowing the natural predominance of grey phenotypes while conserving broader genetic variability (Nürnberg, 1993; Brem, 2011).

In the Romanian breeding system, the Beclean nucleus applies directional selection for dark coat colour, particularly black, resulting in a phenotypically uniform subpopulation. This implies controlled mating strategies that reduce the frequency of the grey allele while preserving lineage continuity (Nürnberg, 1993; Dovč et al., 2006).

Coat colour selection in Lipizzaner breeding represents a historically conditioned objective, linked to both cultural preferences and genetic mechanisms. Lipizzaners are typically born dark (black or bay), with progressive greying due to the dominant grey gene (Zechner et al., 2002; Brem, 2011).

The two breeding nuclei display complementary management approaches. Sâmbăta de Jos maintains broader phenotypic variability and a

less restrictive selection framework, while Beclean applies directional selection, particularly for coat colour, resulting in a more uniform phenotype. This dual system contributes to both stability and adaptability of the overall population.

The conservation of animal genetic resources in structured breeding systems is defined by the Food and Agriculture Organization as the management of population variability within and between populations to ensure long-term adaptability (FAO, 2007). In the case of Lipizzaner breeding nuclei from Sâmbăta de Jos and Beclean, this objective is achieved through the maintenance of closed populations combined with controlled mating strategies (Drăgănescu, 1979).

Closed breeding systems, although potentially vulnerable to inbreeding, can maintain genetic stability when supported by structured pedigree analysis and rotational mating between families (Grosu, 2003). The Romanian Lipizzaner population reflects such a model, where sire lines and mare families are preserved as fundamental genetic units (Suciu et al., 1975).

The concept of effective management of small populations emphasizes the importance of balancing selection intensity and genetic variability, particularly in elite breeding units (FAO, 2010). In Romanian state studs, the relatively small number of breeding animals necessitates strict control of mating plans to avoid the accumulation of inbreeding coefficients (Drăgănescu & Zeneci, 1977/1978).

Pedigree-based selection remains the central tool for genetic management in Lipizzaner populations, allowing breeders to trace lineage continuity and avoid close-relative matings (Zechner et al., 2002). This approach, although traditional, is still recognized as a reliable method in conservation breeding programs (FAO, 2013).

The preservation of sire lines such as *Conversano*, *Favory*, *Incitato, Maestoso*, *Neapolitano, Siglavy-Capriola, Pluto and Tulipan* ensures the continuity of historically valuable genetic traits while contributing to population structure stability (Brem, 2011). The Romanian breeding system maintains these lines in parallel, thus reducing the risk of genetic erosion.

The Copernicus Project demonstrated that European Lipizzaner populations exhibit structured genetic differentiation, with identifiable clusters such as the Romanian pole (Zechner et al., 2002). This supports the hypothesis that the Romanian population has evolved under relative reproductive isolation, maintaining distinct genetic characteristics.

The conservation of traditional breeding practices is increasingly recognized as a complementary strategy to modern genetic management, particularly in culturally significant breeds (FAO, 2015). In this context, the Romanian system represents a model of in situ conservation, where genetic resources are preserved within their historical production environment.

The integration of genealogical records, performance evaluation, and controlled reproduction ensures that selection decisions are both scientifically grounded and consistent with long-term conservation goals (R.N.P. Romsilva D.C.E.A.C. R.A.). This integrated approach is essential for maintaining both adaptive variability and breed identity.

In the context of small and historically managed populations, maintaining genetic structure stability requires the integration of both classical pedigree approaches and modern analytical frameworks, ensuring the control of inbreeding and long-term population viability (Leroy et al., 2016).

Recent pedigree-based analyses in Lipizzaner populations confirm the persistence of structured genetic clusters and support the efficiency of traditional breeding schemes in maintaining population stability (Mancin et al., 2020).

The conservation value of local breeds is not only genetic but also cultural, with traditional breeding systems representing a key component in sustaining both identity and functional diversity (Gandini & Villa, 2003).

The integration of molecular tools in conservation programs provides complementary insights to pedigree analysis, allowing a more precise characterization of genetic variability and relationships within populations (FAO, 2010).

Molecular marker studies conducted on Romanian animal populations further confirm the necessity of integrating classical and modern

approaches in genetic resource management (Socol C. T. et al., 2015).

Recent advances in molecular genetics and genomic characterization provide new perspectives for the conservation and management of small and historically valuable horse populations. Studies performed on endangered and closed populations demonstrated that pedigree analysis continues to represent an effective tool for monitoring genetic variability, founder representation and inbreeding trends, while molecular tools significantly improve the precision of population characterization and breeding management (Ablondi et al., 2018; Dell et al., 2020; Vostrá-Vydrová et al., 2016).

Current FAO recommendations emphasize that genomic characterization and cryoconservation strategies should complement traditional pedigree-based approaches in order to ensure sustainable management of animal genetic resources and preservation of long-term genetic diversity (Boettcher et al., 2022). In this context, the integration of molecular markers and genomic technologies into breeding programmes may provide valuable additional information for the conservation of structured horse populations and for optimizing mating strategies.

Emerging evidence highlighted the value of genetic markers and advanced genomic technologies in improving selection accuracy and providing a more precise assessment of genetic variability within livestock populations (Foksha et al., 2023; Davidescu et al., 2024). In conservation-oriented breeding systems, these approaches may offer important complementary support to traditional pedigree-based management of Lipizzaner breeding nuclei.

CONCLUSIONS

The management of genetic resources in Romanian Lipizzaner breeding nuclei reflects a balance between tradition and modern conservation principles.

The study confirms the existence of a distinct Romanian genetic pole within the European population.

The complementary breeding strategies applied in Sâmbăta de Jos and Beclean enhance both genetic stability and adaptive potential.

Maintaining structured breeding systems remains essential for the sustainable conservation of the breed.

The future integration of molecular genetic tools alongside pedigree-based selection may enhance the accuracy of genetic resource management and support the long-term sustainability of Lipizzaner breeding nuclei.

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ESTIMATION OF GENETIC PARAMETERS FOR LINEAR TYPE TRAITS AND SELECTION INDEX IN ROMANIAN BLACK AND WHITE DAIRY CATTLE

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Abstract

This paper presents the results of a study on the estimation of genetic parameters for exterior traits in the Romanian Black and White (BNR) cattle breed. Sixteen linear type traits and a composite Exterior Selection Index (ESI) were analysed in a population of n = 2,956 cows, evaluated using the standard linear description methodology on the 1-9 scale applied by the General Association of Cattle Breeders of Romania (A.G.C.T.R.). Descriptive statistics revealed high means for skeletal traits (stature: $\bar{X}$ = 8.888 ± 0.007; CV% = 4.43%) and elevated variability for limb and teat traits (chest width: CV% = 28.72%; hock angle: CV% = 26.53%). Heritability estimates obtained by REML, using a sire model (BLUP), ranged from h^2 = 0.10 (stature) to h^2 = 0.63 (rump width - pin bones), with a general mean of $h^2 \approx 0.18$. ESI recorded a mean of 85.544 ± 0.057 and a heritability of h^2 = 0.17. Results are broadly consistent with estimates reported in the international literature for Holstein-type breeds, with the notable exception of rump width - pin bones, which exceeded reference intervals (h^2 = 0.25-0.38), suggesting superior genetic potential for improving this trait in the analysed BNR population. The study provides baseline data for the development and optimisation of national genetic improvement programmes for BNR breed conformation

Key words*: exterior selection index, genetic parameters, heritability, linear type traits, Romanian Black and White cattle, selection*

INTRODUCTION

The Romanian Black and White breed (BNR) is one of the most important dairy cattle breeds in Romania. The assessment of exterior conformation through the linear description of type traits (Brotherstone, 1994) represents an essential tool in modern genetic improvement programmes for dairy cattle (Misztal et al., 1992). Unlike traditional overall scoring systems, linear description quantifies each anatomical trait independently on a biological scale from 1 to 9, reflecting the actual population variability between biologically defined extremes (Brotherstone & Hill, 1990). This approach enables precise estimation of genetic parameters - heritability and genetic correlations - which constitute the theoretical foundation of effective selection for conformation.

The importance of exterior traits extends beyond aesthetics: udder, limb and rump conformation are genetically correlated with productive longevity, udder health, reproductive efficiency and adaptability to mechanical or robotic milking (Williams et al., 2022; Short & Lawlor, 1992). In the context of increasing milk production intensity and the growing adoption of automated milking technologies in Romanian farms, knowledge of the genetic parameters for conformation traits is becoming a strategic priority for BNR breed improvers.

Nevertheless, the scientific literature on the estimation of genetic parameters (Harris et al., 1992) for conformation traits in the BNR breed remains limited, with most available studies focusing on production traits - milk yield and composition. International studies conducted on Holstein populations in the Czech Republic (Němcová et al., 2011), Brazil (Campos et al., 2015) and the United States (Short & Lawlor, 1992) have provided reference values for heritabilities of linear type traits (Table 1), but extrapolation of these results to the BNR population requires validation through dedicated studies, given the specific genetic and environmental charac-teristics of Romanian herds.

Table 1. Reference heritability values for linear type traits in Holstein cattle reported in the international literature

Trait	h^2 (literature)	Source
Dairy form/Angularity	0.10-0.29	Brotherstone & Hill (1990); Campos et al. (2015); Foster et al. (1989); Kőrösi et al. (2025); Lactanet (2021); Němcová et al. (2011); Short & Lawlor (1992); Sousa Junior et al. (2024); Williams et al. (2022)
Stature/Rump height	0.35-0.46	
Body depth	0.15-0.25	
Chest width	0.09-0.20	
Rump width (pin bones width)	0.25-0.38	
Rump angle	0.15-0.30	
Hock angle	0.09-0.18	
Hock quality/appearance	0.10-0.22	
Heel height	0.09-0.15	
Rear leg set (side view)	0.10-0.20	
Fore udder attachment	0.15-0.28	
Rear udder height	0.15-0.25	
Median suspensory ligament	0.10-0.20	
Udder depth	0.10-0.20	
Teat placement	0.10-0.20	
Teat length	0.25-0.38	

The objective of this study was to estimate descriptive statistics and heritabilities for 16 linear type traits and the Exterior Selection Index (ESI) in the BNR breed, integrating the results comparatively within the international literature, with a view to providing concrete recommendations for national selection programmes

MATERIALS AND METHODS

Biological material and data sources

The study was conducted on a population of n = 2,956 cows of the Romanian Black and White breed, originating from multiple farms located in different counties of Romania. Data were collected over the period 2014-2025. Exterior appraisal data were obtained from the General Association of Cattle Breeders of Romania (A.G.C.T.R.), in accordance with the official linear description methodology applied to the BNR - Holstein Friesian breed.

Conformation assessment methodology

The linear description of exterior traits was performed by A.G.C.T.R.-certified classifiers according to the European standardised linear description system for the Holstein-Friesian breed. Sixteen traits were evaluated, grouped into four sections:

- Dairy type morphoproductive characteristics: dairy form;
- Body conformation: stature, body depth, chest width, rump width (pin bones), rump angle;
- Feet and legs: hock angle, hock quality/ appearance, heel height, rear leg set (side view);
- Mammary system: fore udder attachment, rear udder height, median suspensory ligament, udder depth, teat placement, teat length.

Each trait was scored on a biological scale from 1 to 9, in which the extreme values represent biologically opposite expressions of each trait, and the value 5 represents the midpoint of the scale.

Based on the individual scores assigned to the 16 traits, the Exterior Selection Index (ESI) was calculated, expressed on a continuous scale with values theoretically ranging from 0 to 100, according to the weighting algorithm used by A.G.C.T.R.

Statistical analysis

Descriptive statistics - arithmetic mean ($\bar{X}$), standard error of the mean (SE), standard deviation (S), coefficient of variation (CV %), minimum and maximum values - were calculated for each trait using standard descriptive statistical procedures.

Variance components (sire, phenotypic and residual) and heritabilities (h^2) were estimated by REML (Restricted Maximum Likelihood) applied to a sire model (BLUP - Best Linear Unbiased Prediction). Given the large number of traits (17) and animals (2,956), canonical transformation and simultaneous diagonalisa-tion were applied. The statistical model used was:

$$\mathbf{y = Xb + Za + e}$$

where:

y = vector of observations;

b = vector of fixed effects;

a = vector of random additive genetic effects;

e = vector of residual errors;

X and Z = corresponding incidence matrices.

Fixed effects included in the model were: herd-year-season of evaluation and parity number.

Heritability was calculated according to the classical formula:

$$\mathbf{h^2 = 4V_s / V_p}$$

where V_s is the sire variance and V_p is the total phenotypic variance (Falconer & Mackay, 1996).

RESULTS AND DISCUSSIONS

The results obtained in the study are presented and analysed below, discussed in relation to data available in the international literature

Descriptive statistics of exterior traits

The descriptive statistics estimated for the 16 linear type traits and ESI in the BNR breed are presented in Table 2.

Table 2. Descriptive statistics for exterior traits in the BNR breed (n = 2,956)

Trait	N	Mean ± SE	S	CV%	Min.	Max.
Dairy form	2956	8.140 ± 0.018	0.985	12.10	2	9
Stature		8.888 ± 0.007	0.393	4.43	4	9
Body depth		7.553 ± 0.020	1.090	14.43	2	9
Chest width		5.070 ± 0.027	1.456	28.72	1	9
Rump width (pin bones)		7.459 ± 0.025	1.356	18.18	2	9
Rump angle		7.465 ± 0.022	1.211	16.22	2	9
Hock angle		5.008 ± 0.024	1.329	26.53	1	9
Hock quality		6.584 ± 0.027	1.458	22.14	1	9
Heel height		7.351 ± 0.026	1.409	19.16	2	9
Rear leg set (side view)		7.525 ± 0.023	1.260	16.75	2	9
Fore udder attachment		7.641 ± 0.021	1.166	15.25	2	9
Rear udder height		7.594 ± 0.022	1.195	15.74	2	9
Median suspensory ligament		7.513 ± 0.022	1.209	16.09	2	9
Udder depth		6.798 ± 0.027	1.461	21.49	1	9
Teat placement		5.342 ± 0.025	1.356	25.39	1	9
Teat length		5.165 ± 0.019	1.041	20.16	1	9
ESI		85.544 ± 0.057	3.117	3.64	64.055	91.559

Dairy type morphoproductive characteristics

Dairy form recorded $\bar{X}$ = 8.140 ± 0.018 and CV% = 12.10%, with values predominantly in the upper range of the scale, indicating good expression of dairy morphoproductive characteristics in the analysed population. This result suggests that the genetic component makes a substantial contribution to the phenotypic expression of this trait.

Body conformation

Stature recorded the highest mean among all analysed traits ($\bar{X}$ = 8.888 ± 0.007), with a standard deviation of S = 0.393 and a coefficient

of variation of only CV% = 4.43%, indicating a homogeneous population with values close to the upper extreme of the biological scale. This homogeneity suggests that the BNR population analysed has been subjected to intense prior selection for this trait. Similar results were reported by Němcová et al. (2011) on 78,886 first-parity Holstein cows in the Czech Republic, confirming that stature shows low phenotypic variability in intensively selected Holstein populations. Brotherstone & Hill (1990) demonstrated that the phenotypic homogeneity of body conformation traits is consistently superior to that of limb and udder traits - a trend confirmed in the present study.

Body depth ($\bar{X}$ = 7.553 ± 0.020; CV% = 14.43%) showed moderate variability, with values predominantly distributed in the upper half of the scale, suggesting a well-developed trunk on average, with acceptable individual differences. Chest width recorded the lowest mean within the group ($\bar{X}$ = 5.070 ± 0.027) and the highest variability in the entire study (CV% = 28.72%), with values covering virtually the entire scale (1-9), indicating a heterogeneous population for this trait, strongly influenced by environmental factors.

Rump width (pin bones) ($\bar{X}$ = 7.459 ± 0.025; CV% = 18.18%) and rump angle ($\bar{X}$ = 7.465 ± 0.022; CV% = 16.22%) showed high means and moderate variability.

Feet and legs

Feet and leg traits showed moderate to high phenotypic variability, with CV% ranging from 16.75% (rear leg set, side view) to 26.53% (hock angle). Means varied between $\bar{X}$ = 5.008 ± 0.024 (hock angle) and $\bar{X}$ = 7.525 ± 0.023 (rear leg set, side view).

Mammary system (udder and teats)

Udder traits showed high means and moderate variability: fore udder attachment ($\bar{X}$ = 7.641 ± 0.021; CV% = 15.25%), rear udder height ($\bar{X}$ = 7.594 ± 0.022; CV% = 15.74%) and median suspensory ligament ($\bar{X}$ = 7.513 ± 0.022; CV% = 16.09%) were situated in the upper range of the scale, with low variability.

Udder depth ($\bar{X}$ = 6.798 ± 0.027; CV% = 21.49%) showed a lower mean and higher variability, being a trait of particular importance for productive longevity (Williams et al., 2022). The high means and moderate to low variability for fore udder attachment, rear udder height and median suspensory ligament indicate a good level of morphofunctional development in the analysed population but also limited potential for rapid genetic progress through selection due to the relatively low variability. In contrast, udder depth, characterised by higher variability, offers greater opportunities for genetic improvement and represents an important selection criterion given its impact on productive longevity.

Teat traits showed moderate means and high variability: teat placement ($\bar{X}$ = 5.342 ± 0.025; CV% = 25.39%) and teat length ($\bar{X}$ = 5.165 ± 0.019; CV% = 20.16%) indicate a heterogeneous population with significant practical implications for the efficiency of mechanical and robotic milking.

Variance components and heritability of exterior traits

Heritability estimates ranged from h^2 = 0.10 to 0.63, with a general mean of $h^2 \approx 0.18$. According to the conventional classification in quantitative genetics (Falconer & Mackay, 1996), most exterior traits showed low ($h^2 < 0.20$) or moderate (h^2 = 0.20-0.40) heritability, with the exception of rump width - pin bones (h^2 = 0.63), the only value falling into the high category ($h^2 > 0.40$). These values are broadly consistent with estimates reported in the international literature for Holstein-type breeds, where the interval h^2 = 0.05-0.46 is frequently documented for linear type traits (Sousa Junior et al., 2024; Lactanet, 2021).

The estimated variance components and heritability values for each analysed trait are presented in Table 3.

Table 3. Variance components and heritability of exterior traits in the BNR breed

Trait	n	Sire variance	Phenotypic variance	Residual variance	h^2	SE of h^2
Dairy form	2956	0.047	0.873	0.826	0.22	0.068
Stature		0.003	0.140	0.137	0.10	0.065
Body depth		0.042	1.117	1.075	0.15	0.066
Chest width		0.076	1.689	1.613	0.18	0.067
Rump width (pin bones)		0.257	1.622	1.365	0.63	0.076
Rump angle		0.076	1.288	1.212	0.24	0.068
Hock angle		0.047	1.653	1.606	0.11	0.065
Hock quality		0.102	2.000	1.898	0.20	0.068
Heel height		0.054	1.826	1.772	0.12	0.066
Rear leg set (side view)		0.061	1.389	1.328	0.18	0.067
Fore udder attachment		0.040	1.284	1.243	0.13	0.066
Rear udder height		0.044	1.340	1.296	0.13	0.066
Median suspensory ligament		0.041	1.349	1.308	0.12	0.066
Udder depth		0.070	2.079	2.009	0.14	0.066
Teat placement		0.051	1.811	1.761	0.11	0.065
Teat length		0.030	1.064	1.034	0.11	0.065
ESI		0.377	9.142	8.765	0.17	0.067

Dairy type morphoproductive characteristics

Dairy form recorded a heritability of $h^2 = 0.22$ (SE = 0.068), a moderate value comparable to estimates for angularity (an equivalent concept) reported by Campos et al. (2015) ($h^2 = 0.10$-0.29) and Němcová et al. (2011) ($h^2 = 0.14$-0.25) for Holstein populations in Brazil and the Czech Republic, respectively. The moderate additive genetic variance (sire variance = 0.047) indicates an acceptable potential response to selection for improving the expression of dairy morphoproductive type in the BNR breed.

Body conformation

Stature showed the lowest heritability in the study ($h^2 = 0.10$; SE = 0.065), with an extremely low sire variance (0.003) relative to total phenotypic variance (0.140). This result may appear surprising compared to values reported in the international literature ($h^2 = 0.35$-0.46; Short & Lawlor, 1992; Lactanet, 2021), but must be interpreted in the context of the exceptional phenotypic homogeneity of the analysed population (CV% = 4.43%). As discussed in the descriptive statistics section, the intense prior selection for this trait resulted in very low total phenotypic variance, which tends to underestimate true heritability (Falconer & Mackay, 1996).

Body depth ($h^2 = 0.15$; SE = 0.066) and chest width ($h^2 = 0.18$; SE = 0.067) showed low heritabilities, consistent with estimates of 0.15-0.25 and 0.09-0.20, respectively, reported in the international literature (Němcová et al., 2011; Campos et al., 2015). The low sire variances for these traits suggest that a significant proportion of their high phenotypic variability is attributable to environmental factors - housing system, nutrition level, parity.

Feet and legs

Rump width (pin bones) recorded the highest heritability in the study: $h^2 = 0.63$ (SE = 0.076), with a sire variance of 0.257 against a phenotypic variance of 1.622. This value is clearly superior to the intervals reported in the international literature (h^2 = 0.25-0.38; Němcová et al., 2011; Campos et al., 2015), placing rump width at the top of the analysed traits in terms of the genetic contribution to phenotype. A heritability of 0.63 implies that approximately 63% of the phenotypic variability of rump width is explained by additive genetic variability, conferring an exceptionally high

potential response to selection. Short & Lawlor (1992) reported that rump width shows moderate to high heritability in American Holstein populations, noting that selection for this trait generates rapid and predictable genetic progress. The value of 0.63 may reflect a specific genetic structure of the BNR population or the effect of bulls with contrasting breeding values used in the herds included in the study, an aspect that warrants investigation through genealogical data analysis.

Rump angle showed a heritability of $h^2 = 0.24$ (SE = 0.068), a moderate value consistent with the interval $h^2 = 0.15$-0.30 reported by Short & Lawlor (1992); Němcová et al. (2011), with direct implications for calving ease and reproductive efficiency.

Hock angle ($h^2 = 0.11$; SE = 0.065) and heel height ($h^2 = 0.12$; SE = 0.066) showed low heritabilities, consistent with estimates of 0.09-0.18 reported by Němcová et al. (2011); Brotherstone & Hill (1990). These low heritabilities, combined with high phenotypic variabilities (CV% = 26.53% and 19.16%, respectively), confirm that improvement of these traits in the BNR breed is largely dependent on optimisation of environmental factors - flooring type, hoof management, mineral nutrition - rather than direct genetic selection.

Mammary system (udder and teats)

Udder traits showed low and relatively uniform heritabilities, ranging from $h^2 = 0.12$ (median suspensory ligament) to $h^2 = 0.14$ (udder depth). Although these values suggest a significant environmental influence on udder conformation, the estimated sire variances (0.040-0.070) confirm the existence of a real additive genetic component amenable to long-term improvement through selection.

Fore udder attachment ($h^2 = 0.13$; SE = 0.066) and rear udder height ($h^2 = 0.13$; SE = 0.066) show heritabilities slightly below the interval of 0.15-0.28 reported by Short & Lawlor (1992); Campos et al. (2015). Udder depth ($h^2 = 0.14$; SE = 0.066) is of particular importance for productive longevity. Williams et al. (2022) demonstrated that udder depth, together with rear udder height and teat length, represents an important genetic predictor of survival and productive longevity. The heritability estimated in the present study ($h^2 = 0.14$) is considerably lower than values reported by other authors ($h^2 = 0.41$; Kőrösi et al., 2025).

Median suspensory ligament ($h^2 = 0.12$; SE = 0.066) showed the lowest heritability among udder traits, slightly below the value of 0.22 reported by Kőrösi et al. (2025). Despite its low heritability, this trait has major practical importance due to its favourable phenotypic and genetic correlations with mastitis resistance and milking efficiency, and is therefore included in most functional selection indices used in modern Holstein breeding programmes.

Teat placement ($h^2 = 0.11$; SE = 0.065) and teat length ($h^2 = 0.11$; SE = 0.065) showed the lowest heritabilities in the entire study. The result for teat length is notable in contrast with international literature: Campos et al. (2015) reported one of the highest heritabilities for this trait ($h^2 = 0.38$), a value also supported by other authors ($h^2 = 0.36$ - Williams et al., 2022; $h^2 = 0.25$-0.32 - Short & Lawlor, 1992). The discrepancy may be attributed to: (i) the relatively small effective population size compared to reference studies; (ii) the potential effect of multiple classifiers introducing systematic variability; (iii) the specific genetic structure of the BNR population.

Exterior Selection Index (ESI)

ESI showed a heritability of $h^2 = 0.17$ (SE = 0.067), with the highest sire variance in the study (0.377) and the highest total phenotypic variance (9.142). The attenuation of ESI heritability relative to the individual heritabilities of the best-determined component traits is consistent with selection index theory (Hazel, 1943; Miglior et al., 2005), reflecting the dilution effect generated by aggregating traits with variable heritability. The use of ESI as a practical selection tool remains justified by its ability to integrate genetic information from multiple correlated traits, providing a more stable global assessment than any individual component trait taken separately (Miglior et al., 2005).

Estimation precision and implications for selection

Standard errors of heritability were relatively uniform across traits (SE = 0.065-0.076), reflecting comparable estimation precision

throughout the data set. These values are, however, higher than those reported in studies with much larger populations (SE < 0.04 in Němcová et al., 2011, n = 78,886; SE < 0.03 in Campos et al., 2015, n = 18,831), indicating that the confidence intervals of the present estimates are wider and that reported h^2 values should be interpreted with caution for traits with $SE/h^2 > 0.5$ (e.g., stature: $SE/h^2 = 0.65$).

From the perspective of selection priorities, the present study identifies rump width - pin bones ($h^2 = 0.63$) as the most promising trait for rapid genetic improvement in the BNR breed, followed by rump angle ($h^2 = 0.24$) and dairy form ($h^2 = 0.22$). Traits with low heritability - feet and legs, udder and teats - require long-term improvement strategies based on the use of proven bulls with favourable breeding values (EBV/GEBV) and simultaneous optimisation of environmental conditions, in accordance with Interbull recommendations for national genetic evaluation programmes for conformation in dairy breeds.

The relatively small effective population size (n = 2,956) compared to reference studies results in wider confidence intervals for heritability estimates. Additionally, the use of multiple classifiers may have introduced systematic variability, particularly for subjectively scored traits such as teat length. Future research should incorporate genealogical data analysis to clarify the unusually high heritability of rump width, and extend the dataset to improve estimation precision.

CONCLUSIONS

The results obtained provide a useful scientific basis for both farmers and breed improvers involved in enhancing exterior conformation in the Romanian Black and White breed. For farmers, knowledge of traits with high variability and low genetic determination - such as feet and legs traits and teat placement and length - guides management decisions towards optimising housing conditions, with more immediate and accessible effects than direct genetic selection.

For breed improvers, rump width - pin bones stands out as the most promising trait for inclusion in selection indices, due to its high genetic determination, which guarantees a predictable and rapid response to selection. Udder traits, despite their low heritability, justify sustained long-term selection given their documented role in maintaining udder health, reducing mastitis incidence and extending productive longevity - factors with a direct impact on farm profitability.

Overall, the results of this study can be used in breeding programmes to consolidate and improve the exterior conformation of the Romanian Black and White breed in line with the international standards of the Holstein breed.

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CYTOGENETIC SCREENING OF ROMANIAN BOVINE BREEDS

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Abstract

As well as the cytogenetic investigation is a tool that could be used in the genetic improvement programmes of bovine, this paper is a review about the cytogenetic screening performed in the last 15 years on 889 cows and 317 buffaloes of different breeds, raised in Romania. The karyotype study was carried out by using the short-term blood cell culture and as a result abnormalities of chromosomal structure and number were identified in 131 cows and 51 buffaloes. The effects of the abnormal configurations were represented by reproductive activity disorders, congenital and developmental malformations, freemartinism and aneuploid-heteroploidy of sex chromosomes. The necessary prophylaxis recommendations have been released for all carriers of chromosomal abnormalities.

Key words*: buffalo, cattle, chromosomal abnormalities, congenital malformations, reproductive disorders.*

INTRODUCTION

The role of the cytogenetic screening in the genetic improvement programmes of bovine, has been demonstrated since the so-called 1/29 translocation in Swedish cattle and the effects on fertility of carriers was identified (Gustavsson & Rockborn, 1964, Gusstavsson, 1971). The presence of the chromosomal abnormalities and their relationship with the degradation of reproductive performances, congenital and developmental malformations both in cattle and buffalo (Ducos et al., 2008; Nicolae et al., 2010; Perucatti et al., 2016) is very well known and therefore has become an essential condition for improving the genetic health of farm animals. For this reason, the main objective of our cytogenetic screening, was to detect the number and frequency of the abnormal configurations and remove the carriers from the herd. It is mandatory to avoid the dissemination of abnormalities in the offspring, especially when artificial inseminations are applied. The current paper is an overview about the results of the systematic chromosomal control developed for the active bovine population belonging to the main important cattle and buffalo breeds raised in our country. The goal of our study was to identify chromosomal abnormalities and their effects on the reproductive activity of the carriers, and thus to contribute to improving the genetic health of animals and, consequently, to avoid economic losses at the farm level.

MATERIALS AND METHODS

The material of cytogenetic investigation was represented by 1206 animals: 889 cows from the most common breeds in the country (Romanian Spotted, Romanian Black Spotted, Brown) and 317 buffaloes, from the Romanian buffalo breed. The analysis of chromosomes was carried out by using the short-term blood cell culture. Peripheral blood lymphocytes were cultured for about 72 hours at 38.5°C in Pb-Max (Gibco) complete medium. Two types of cell cultures were performed: without (normal cultures) and with addition of 5-bromodeuxiridine (BrdU) during the last two cell cycles in order to identify the Sister Chromatid Exchanges (SCEs). Slides from both cultures were stained with acridine orange. At least 30 metaphase plates per animal were studied under a fluorescence Nikon microscope, captured with a Photometrics Cool Snap camera, transferred on PC and processed by a specific image software. The cytogenetic

diagnosis was established taking into account the number and structure of chromosomes according to the standard normal karyotype.

RESULTS AND DISCUSSIONS

In cattle, the karyotype control (Table 1) of the 889 females revealed *normal karyotype, 2n=60, XX* for 758 females (Figure 1), 128 cases of *chromosomal instability* and 3 cases of *XX/XY chimerism.*

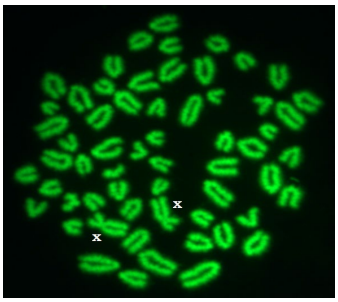


Figure 1. Cattle normal karyotype (own source)

Table 1. The results of cytogenetic investigation in cattle and buffalo breeds

Species/ Breeds	Number of animals	Cytogenetic diagnosis							
		Normal Karyotype		Chromosomal Instability		XX/XY Chimerism		XO Monosomy	
		No	%	No	%	No	%	No	%
Romanian Black Spotted	331	277	83.69	51	15.40	3	0.91		
Romanian Spotted	296	256	86.49	40	13.51				
Brown	170	147	86.47	23	13.53				
Other breeds	92	78	84.78	14	15.22				
Total cattle	**889**	**758**	**85.26**	**128**	**14.40**	**3**	**0.34**		
Buffalo	317	266	83.91	50	15.77			1	0.32
TOTAL	**1206**	**1024**	**84.91**	**178**	**14.76**	**3**	**0.25**	**1**	**0.08**

The chromosomal instability induced by the presence and frequency of many chromatidic breaks on autosomes and heterosomes, lost fragments and gaps (Figure 2) usually affect the integrity of the genetic material with serious consequences for the reproductive capacity of the carriers (Llambi & Postiglioni, 1994; Ducos et al., 2008; Nicolae et al., 2010; Perucatti et al., 2016).

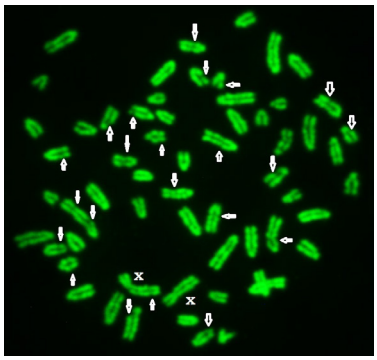


Figure 2. Cattle chromosomal instability (own source)

The ***phenotypic effects of chromosomal instability*** were expressed by reproductive disturbances (repeated inseminations, lack of heat and abortions), 4 cases of *congenital malformations* of the forelimbs (Figure 3), a case of abnormal foetus (*Schistosomus reflexus*) (Figure 6), a case of *posterior limb malformation* (Figure 9) and 3 cases of XX/XY mosaicism.

Congenital malformations of the forelimbs (Figure 3) were identified in 4 cases of *Romanian Black Spotted* calves.



Figure 3. Congenital malformation (own source)

Considering the reproductive disorders reported for their mothers, characterized by abortions and repeated inseminations, taking into account the karyotypic instability, highlighted in both

mothers and calves (Fig. 4), and knowing the possibility of the existence of toxic agents in the environment, which could have effects on the genetic material integrity (Ciotola et al., 2005; Nicolae et al., 2015), we applied the SCEs (Sister Chromatid Exchanges) test to both cows and calves.

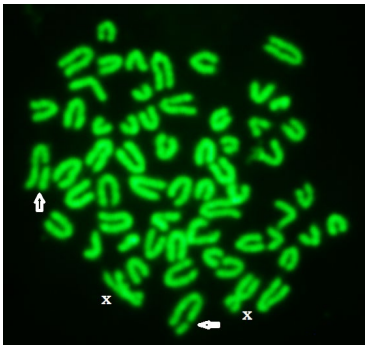


Figure 4. Abnormal configuration in calf with congenital malformation (own source)

Following the SCEs test analysis, it was observed that, if in normal situations the interchromatid exchanges/cell are approx. 6-7 SCEs/cell, in abnormal situations, such as the case of the 4 females and the 4 calves with karyotypic instability, the number of interchromatid exchanges/cell was very high, ranging between 9-13 SCEs/cell (Fig. 5). The presence of 2-3 SCEs on the same chromosome was particularly noted. This could be an indication of the damage to the integrity of the genetic material by environmental pollutants.

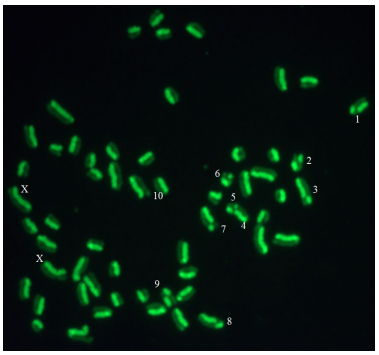


Figure 5. SCEs/ cell indicated by numbers (10) (own source)

Schistosomus reflexus (SR) was *an abnormal foetus,* with a monstrous appearance, dead at birth, described quite rarely in cattle (Azawi et al., 2012; Jana & Jana, 2013; Pandey et al., 2017) but to our knowledge, this is the first case (Figure 6) reported in Romanian Black Spotted dairy cow reared in the farm of the R & D Institute for Bovine Balotesti.

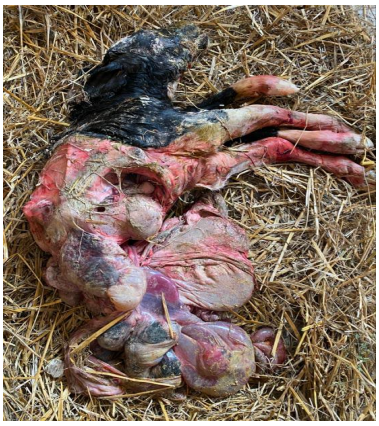


Figure 6. *Schistosomus reflexus (*own source)

Till now this kind of congenital malformation have been associated with genomic instability (Di Berardino et al., 1983; Peretti et al., 2008; Nicolae et al., 2020). To investigate the etiology of this kind of malformation we collected blood samples from both mother and foetus. The chromosomal analysis of the foetus was very difficult because the blood samples contained a lot of clots and we obtained very few good metaphases. After examination it was very clear that the sex of foetus was male. Regarding the mother, the chromosomal configuration was characterized by chromosomal fragility (Figure 7).

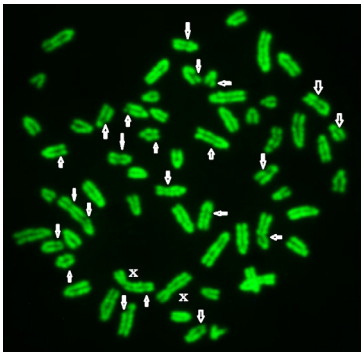


Figure 7. Chromosomal instability of the foetal SR‘s mother (own source)

For this reason, the SCEs test was applied to the mother's metaphases and the observed

interchromatid exchanges (Figure 8) were in a number much higher (9-15 SCEs/cell) than in normal situations in cows (6-7SCEs/cell). Given these results, we can state that the etiology of this congenital malformation could be of teratogenic nature, as other cases have been described before (Nicolae et al., 2020).

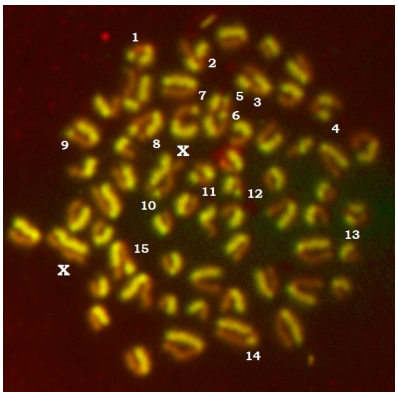


Figure 8. SCEs/ cell indicated by numbers (15) (own source)

Posterior limb malformation (Figure 9), which consists in the lack of the left posterior leg, was identified in a calf from Montbeliarde breed. In this case too, karyotype examination was performed on both the malformed calf and its mother. Following the analysis of metaphases obtained from peripheral blood lymphocyte cultures, chromosomal instability was observed in both the calf's and the mother's cells, but by far, most broken chromosomes were identified in the mother's metaphases, as can be seen in Figure 10.

To gain more information regarding the integrity of the genetic material of the two probands, a detailed analysis has been done by using the SCEs test. The obtained results, revealed a much higher number (Figure 11) of chromatid exchanges (9-15 SCEs/cell) than normal. The presence of double interchromatid exchanges on one, two or even three chromosomes in the same metaphase was very surprising. This is real evidence that the integrity of the genetic material has been very seriously affected. Trying to find out the causes of this malformation, we received information that the mother was treated with antibiotics in the first months of pregnancy.



Figure 9. Posterior limb malformation (own source)

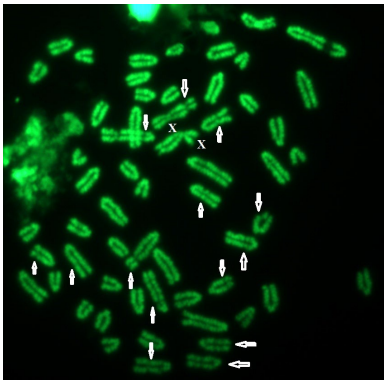


Figure 10. Chromosomal instability of the calf's mother (own source)

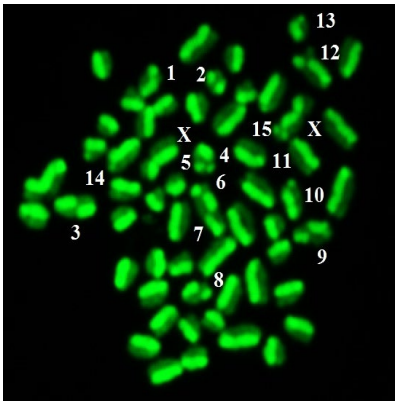


Figure 11. SCEs/cell indicated by numbers (15) (own source)

Also, the presence of aflatoxin on the farm, before and during gestation, has been reported and it is well known that this type of mycotoxin is associated with developmental and

reproductive disorders (Diaz et al., 2004; Akande et al., 2006, Aydin et al., 2008).
All this explains, once again, the teratogenic etiology of this malformation. This is, also, a warning sign for farmers who must be careful about the effects of toxic agents on females during pregnancy, in order to avoid adverse consequences for the offspring and thus, reducing economic losses at farm level.
A special chromosomal configuration ***XX/XY chimerism,*** was found in 3 Romanian Black Spotted females. This is a sex chromosome syndrome which is defined by the abnormal presence of sex chromosomes in the same organism, i.e., cells with XX and cells with XY, in different percentage (Figure 12).

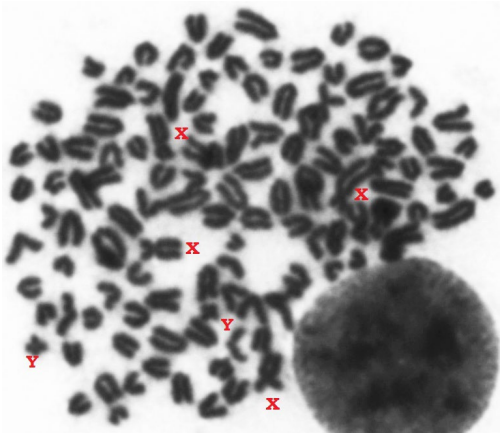


Figure 12. XX/XY Leukocyte chimerism
(one XX cell and two XY cells) (own source)

The presence of leukocyte chimerism in variable proportions, correlated with the degree of reproductive impairment is a reliable and early indicator of the diagnosis of *freemartinism* in the females. For each of our cases, 100 metaphases were examined and it was found that the proportion of XX cells varied between 15 and 35 percent and the proportion of XY cells varied between 65 and 85 percent. These configurations have as effects a serious impairment of the females' genitalia (Fig. 13) and are implicitly associated with sterility of the freemartin females. Taking into account the theoretical premises and the cases described by other authors (Basrur et al., 1970; Nicolae et al., 2001; Iannuzzi et al., 2021), their removal from the herd was recommended.



Figure 13. Abnormal freematine female genitalia (own source)

As well as the artificial inseminations in buffaloes increased, a longstanding interest was dedicated to Romanian buffalo breed too. The chromosomal analysis of 317 buffalo females give us the following results: normal karyotype (2n=50, XX) for 266 females (Figure 14) and chromosomal instability for 50 females.

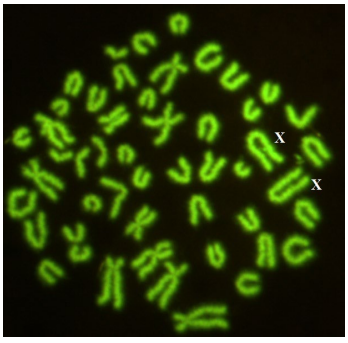


Figure 14. Buffalo normal karyotype (own source)

As can be seen in Figure 15, the damage to the chromosome structure in buffalo metaphases is evident: very clear breaks and lost fragments.

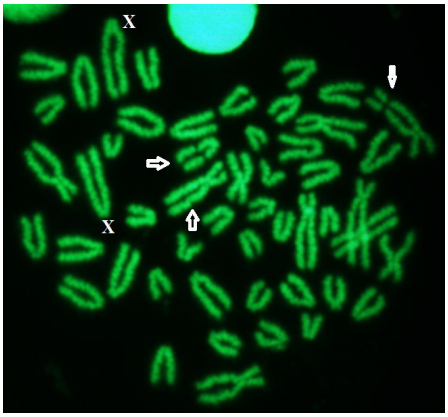


Figure 15. Buffalo chromosomal instability
(own source)

In order to investigate and identify the causes that led to this situation, the SCEs test was applied, and we observed a very high number of interchromatid exchanges/cell, ranging between 10-14 SCEs/cell (Figure 16 A, B.), compared to the number found in normal situations in buffalo (7-8 SCEs/cell). It was also remarkable the double number of SCEs on the chromosomes of the same cell. This detail draws attention to the possible damage generated by environmental pollution.

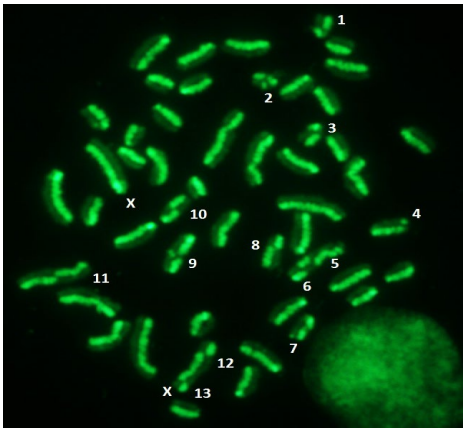


Figure 16 A. Buffalo metaphase with 13 SCEs/cell
(own source)

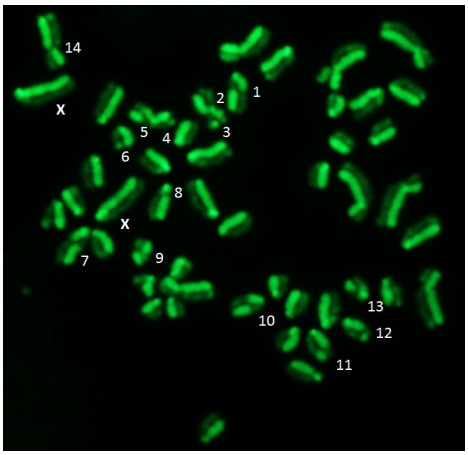


Figure 16 B. Buffalo metaphases with 14 SCEs/cell
(own source)

We also identified an aneuploid-heteroploidy of sex chromosomes **(2n=49, X0**), well known as ***X-monosomy*** or ***Turner's syndrome*****,** in a buffalo female with prominent withers and tight pelvis. Till now, only a few cases were described (Yadav et al., 1990; Prakash et al., 1992; Iannuzzi et al., 2000, Iannuzzi et al., 2021), and all were characterized by different alterations of internal genitalia and sterility.
In our case (Figure 17) in all metaphases we observed only one X chromosome and the analysis of the female's reproductive activity pointed out reproduction disorders and infertility.

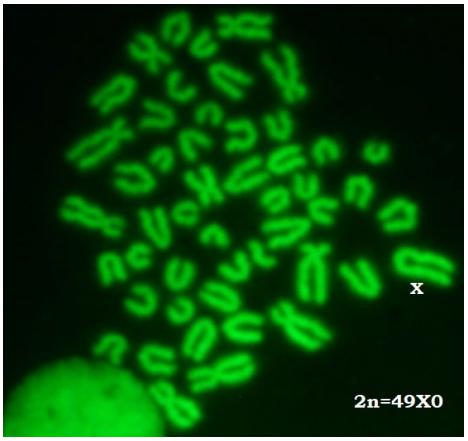


Figure 17. Buffalo X-monosomy
(own source)

For all investigated animals, chromosomal analysis bulletins have been released and appropriate prophylaxis measures have been recommended.

CONCLUSIONS

Cytogenetic investigation of 1206 females from 2 species (cattle and buffalo) of different Romanian breeds revealed *abnormal karyotype for 131 cattle and 51 buffaloes.* The large number of animals, identified with karyotypic instability, the high percentage both in cattle (14.40%) and buffalo (15.77%) as well as the effects recorded on their reproductive capacity, demonstrated once again that the damage of genetic material integrity can induce developmental and reproduction disorders of carriers. All of these, highlights that the only possibility of intervention in maintaining the genetic health of the herd is to identify abnormal genotypes and eliminate them from the population, thus avoiding the risk of dissemination in offspring. *In all cases, chromosomal analysis bulletins have been released and appropriate prophylaxis measures have been recommended*.

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GENETIC PARAMETERS FOR LITTER SIZE IN PROLIFIC BREED PALAS SHEEP

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Abstract

The aim of present study was to estimate the genetic parameters for litter size in Prolific Breed Palas sheep with individual animal model. The data consists in records of 170 ewes at first parity, 142 ewes at second parity and 63 ewes at third parity. The pedigree consisted in 345 sheep: 131 ewes, 44 rams and 170 ewes with performances at first parity. The data were from Research and Development Institute for Sheep and Goat Breeding Palas. The mean for litter size was at first lambing 1.347±0.036, at the second parity 1.458±0.041, at the third parity 1.587±0.066. The absolute breeding values of sheep at first lambing for litter size were from - 0.073 to 0.122. The relative breeding values for the best sheep were from 115.04 to 136.434. The heritability for litter size in Prolific Breed Palas sheep was 0.080. The estimation of sheep breeding values allows the choice the best sheep for litter size in Prolific Breed Palas sheep for reproduction and the genetic parameters are necessary in the breeding program of sheep breed.

Key words*: breeding values, genetic parameters, individual animal model, number of lambs.*

INTRODUCTION

The animal production depends on productive potential of animals and the number of animals (Grosu, 2019). For increase the quantitative and qualitative production of sheep it is necessary to increase the number of animals. By the breeding of sheep the meat and milk production increase. The breeding value was used for choose the sheep for reproduction.

Reproductive traits have a double importance from a breeding point of view: these traits are economically useful, thus being candidates for inclusion among breeding objectives and the reproductives traits affect the efficiency of selection (Drăgănecu and Grosu, 2003). The prolificacy could be introduced in the objective of artificial selection (Drăgănecu & Grosu, 2003).

Prolific Breed Palas is characterized by high prolificacy producing more than one lamb per birth, often twins and triplets, this breed being important for national sheep industry for improving meat production. By improving the meat production improved the farm profitability. The Prolific Breed Palas could be used for crossbreeding for increase the prolificacy for sheep breeds with low prolificacy.

The litter size was an important economic trait in sheep breeds. The litter size in sheep breed presented variability in different breeds. The environmental factors which influenced the litter size were: the farm management, the age of ewe, the year and season (Grosu et al., 2019). The importance of estimation the genetic parameters for litter size in Prolific Breed Palas was due by the characteristics of the genetic parameters: are specific for each population, are specific for each generation, for each environment. The estimation of genetic parameters was useful for estimation the breeding value of sheep. The most prolific breeds in the world were: Finnish Landrace 300% prolificacy, Romanov 250-300%, Friesian 200-225%, Bluefaced-Leicester 180-200%, Chios-Hu-Yang 200-300%, British Sheep Dary 220-300% (Bulmaga et al., 2021). In Finsheep breed the litter size was between 1-6 lambs, in Romanov 1-5, in Wadi 1-4, in Texel 1-3 lambs (Tao et al., 2021). The methods for sheep flocks were: assisted reproduction, shortening lambing period and deseasonalisation of heat and lambing (Bulmaga et al., 2021). Romania had tradition for sheep breeding. The sheep used the feed at high level. In Romanian sheep breeds, the prolificacy of local breeds was: for Merinos Palas 110-146%, Transylvanian Merinos 112-120%, Spancă 110-116%, Țigaie 104-107%, Țurcană 103-105%, Karakul 105-108% (Taftă,

1997). Teleorman Black Head had a high prolificacy 130-160% (Grosu et al., 2005). The improvement of the reproduction traits was a goal in breeding program of sheep breed. Bradford (1985) reported that the prolificacy has been used as a breeding objective in different breeding schemes despite its low heritability. In a number of breeding programs, genomic selection has been used to assign for litter size (Bolormaa et al., 2017). Crossbreeding of local breeds with Prolific Breed Palas has been the major means of genetically improving prolificacy. The prolificacy will remain an important breeding goal for sheep in local sheep breeds.

Current state of the study of reproduction traits in sheep is focused on genomic selection, the identifying the major genes which are associated with fertility, prolificacy. Inclusion of genetic information of the genes associated with reproductive ability could efficiently enhance the selection response (Abdoli et al., 2016).

For Prolific Breed Palas, it is necessary to estimate the heritability because the heritability is specific for each population, because the genetic components of variance were influenced by the frequence of genes in population and the environmental conditions of the different populations.

Because prolificacy is a trait with low heritability, in order to improve this trait will be more efficiently to select animals based on their genotype, the animals with favorable genotype.

A breeding strategy for improving the prolificacy in Prolific Breed Palas is to select sheep on genomic estimated breeding values. The methodology to estimate genomic breeding values will be Single-Step Genomic Best Linear Unbiased Prediction (ssGBLUP) to increase the accuracy of genetic evaluation of sheep. For the traits with low heritability the use of genotype in breeding values estimation may accelerate the genetic gain for young animals.

The advantage of integration major genes for high prolificacy in a breeding program was the possibility of introgression through crossbreeding, of desired mutations into local breeds which may improve prolificacy and lamb production in a short time (Olivan and Pardos, 2000). In France, England, Norway there were selection schemes for improve the litter size (Perez-Enciso, 1995).

The litter size in sheep could be increased by direct selection (Hanrahan, 1990; Schoenian and Burfening, 1990) but the gains have not been very large because of the low heritability of litter size.

The use of estimated breeding values, using BLUP (Best Linear Unbiased Prediction) methodology will accelerate genetic progress (Jansens et al., 2004).

In the breeding program in Romanian sheep breeds the genetic parameters are needed for the breeding value estimation procedure.

The Prolific Palas Breed was reproductive isolate and was selected in order to increase prolificacy rate. Breeding area of the Prolific Breed Palas was Dobrogea at Research Institute for Sheep and Goat Breeding Palas - Constanta, there are 1000 heads of sheep and 2000 in another farm in the area.

In the breeding program of Prolific Breed Palas, the breeding goal was the improvement the prolificacy this breed was specialized breed for prolificacy (over 150%), breastfeeding capacity and limitation inbreeding below 1% per generation. Breeding method was selection within breed, mating was done by rational matching of pairs by ram families.

The breed was used for increasing the prolificacy of local breeds and producing triracial hybrid lambs to achieve the priority objective of increasing meat production in sheep.

The main goal of making the Prolific Breed Palas is to obtain father rams for the hybrid ewes with high prolificacy rate.

The litter size in sheep production had an important economic value. By using the individual animal model for selection, the best animals with high breeding values increase the genetic progress for prolificacy.

The heritability of prolificacy is important and the heritability for production traits and correlation between reproductive and productive traits for implement a multi-trait index in future selective breeding programme of Prolific Breed Palas. The prolificacy was positive correlated with milk production. Knowledge of the heritability of trait is very important for the theory and practice of breeding. Almost all practical breeding decisions depend on the size of heritability: prioritizing genetic breeding or exploitation improvement in order to increase

production, choosing the breeding system, choosing the selection method, establishing the selection objective. The heritability coefficient enters into all formulas regarding the estimation of genetic progress, as well as into formulas regarding the prediction of breeding value. The heritability is a characteristic of a trait, of a population. The genetic components of variance were influenced by the frequence of genes, different in different populations and the environmental conditions of the different populations. Heritability is a property of each trait is determined by several pairs of genes with different effects.

Ziadi et al. (2024) reported that reliable estimation of genetic parameters for litter size was necessary for reach optimum rate of genetic progress.

The litter size was a trait influenced in great proportion by the environmental factors and a low proportion by genetic factors. In Romania it was necessary to have sheep breeds with high prolificacy. For improving reproduction efficiencies an important role had molecular genetics and marker-assisted selection (Deac et al., 2022). In Romania, at Research and Development Institute for Sheep and Goat Breeding Palas-Constanța was created the Prolific Breed Palas, approved as breed in 2020. The breed was created by crosses between Palas Merino sheep and rams from prolific breeds Romanov, Border Leicester, Ile-de-France, East -Friesian and Finish Landrace (Dordescu et al., 2022). By cross-breeding it was the possibility to improve quickly the prolificacy and lambs' production by incorporate the genes for high prolificacy (Gootwine, 2020).

Prolific Breed Palas had a high prolificacy, in the first lambing 143.43% and 153.80% in multiparous ewes (Dordescu et al., 2022). The total flock of Prolific Palas was 431 sheep (Dordescu et al., 2022). For increase numerical the sheep breed it was necessary to increase the prolificacy. The Prolific Breed Palas had a high milk yield which allow all lambs to be nursed. The Prolific Breed Palas was adapted for the environmental conditions from our country, the ewes are less demanding on feed and more resistant to environmental conditions. In Romania the improvement of meat production depended of reproduction traits. The prolificacy represents the number of lambs obtained from ewes that have lambed (Pascal, 2015). The genetic factors (breed, population, individual) and environmental factors (age of sheep, weight of sheep, nutrition, breeding season, rearing type, feeding level) influenced the prolificacy. Because the prolificacy was influenced by nutrition, it was necessary to assure of sheep and rams' rational feedings. For the profitability of farms must the increase the number of lambs born. By capitalizing on the lambs, increased the profitability of farms. Previous researchers estimated the genetic parameters for litter size in sheep with threshold model and linear animal model (Yadollahi et al., 2018), animal model with repeatability (Schmidova et al., 2014).

Different authors analysed the litter size using different models: repeatability model, multiple trait model, random regression model (Ziadi et al., 2025).

The BLUP (Best Linear Unbiased Prediction) methodology had more advantages: takes into account the sheep's performances data and of its offspring and relatives.

The use of the animal model that use all available sources of information and the average of more lambing can improve the population.

Breeding decisions depend on the size of heritability giving priority to genetic improvement or improving the environmental conditions in order to increase, choosing the breeding system, choosing the selection method, establishing the selection objective. The heritability enters into the formulas regarding the prediction of breeding value (Drăgănescu & Grosu, 2003).

The objective of selection was to obtain maximum genetic progress per unit of time and expense. It must be optimized by establishing several objectives, retaining the one that maximizes the annual genetic gain, with minimal costs. Meat production per female was a complex trait, made up of simpler traits: prolificacy, growth rate and offspring carcass weight. Prolificacy depends on other simple traits, such as ovulation rate. The traits that will be included in the selection objective must be taken into account three criteria: economic importance, heritability and genetic correlation with other traits. Sandu and Drăgănescu (1983) reported that the decision to accept traits in the selection objective is based on the calculation of a global indicator called the competitiveness of

the trait which will cumulate the information provided by the three parameters: economic values, heritability and genetic correlations of traits. Ollivier (1998) reported the relative economic values of production and reproduction traits. For different sheep breeds were developed maternal index. These indexes aim to enhance the efficiency of ewes flocks, making the more productive. Theodoridis et al. (2017) reported that the estimated economic values of the traits revealed their economic impact on the definition of the breeding goal for the specified production system and that milk production and fertility had the highest economic values (€ 40.30 and € 20.28 per standard deviation). The production and reproduction traits, which include milk production, prolificacy and milking speed contributed most to the breeding goal (70%), but functional traits still had a considerable share (30%) (Theodoridis et al., 2017). For realize a breeding program it is necessary to study the economic values of traits. The production and reproduction traits are the traits which can be efficiently treated through genetic improvement and other traits can be improve through managerial intervention. (Theodoridis et al., 2017). In recent studies regarding the litter size were identified the genes (NGF, AKT1, SYCP1) associated with the regulation of the development of oocytes (Tao et al., 2021).

The novelty lies in the fact that the genetic parameters for litter size in Prolific Breed Palas sheep were not estimated. The main pathways for the genetic improvement of livestock are: selection between breeds, crossbreeding and selection within breeds.

The aim of this research was the estimation the breeding values and genetic parameters using individual animal model in Prolific Breed Palas sheep. In the breeding program of Prolific Breed Palas a breeding strategy to improve litter size is the selection within breed, nucleus schemes could be a good strategy for genetic improvement. Also, the genomic selection was a breeding strategy to improve litter size. The heritability of prolificacy and production traits and the correlation between prolificacy and productive traits in Prolific Breed Palas are important for implement a multi-trait index in future breeding program.

MATERIALS AND METHODS

The data used in this study provided from Research and Development Institute for Sheep and Goat Breeding Palas-Constanța. The breeding season was planned for October-November. In the Prolific Breed Palas the ewes had lambs both in spring and throughout the year. The ewes from our study had lambs in winter and spring. The feeding was balanced.

For Prolific Breed Palas sheep breed the pedigree contended 345 sheep: 170 ewes with performances, 131 ewes and 44 rams. In the model were included the following fixed factors: the age at first lambing with 2 levels and the year of birth of ewe with 5 levels. From 229 lambs, 111 were from simple lambing and 118 lambs were from twin birth. The ewes had 65.2% simple lambing and 34.8% twin lambing.

The age of ewe at lambing was between 22 and 27 months for 84.1% from ewes and over 30 months for 15.9% from the total number of ewes with records. The prolificacy was higher for the ewes with age over 30 months, the Student test showed that were not difference signifiant between the means of samples. For year of lambing the Student test showed that were difference signifiant between means of sample ($P<0.05$).

Statistical analysis

The model used was individual animal model (Grosu et al., 2013, 2019) described as follows.

The model was:

$y_{ijk} = Y_i+A_j+a_k+e_{ijk}$

y_{ijk} = was the number of lambs;

Y_i = was the year effect (fixed effect);

A_j= age effect of ewe (fixed effect);

a = was additive genetic effect;

e = was a residual error effect.

The model could be written as:

$y=Xb+Za+e$

X = was the incidence matrices for fixed effects;

Z = was the incidence matrices for additive genetic effects;

y = was the vector of observations;

b = was the vector of the fixed effects;

a = was the vector of the additive genetic effects of sheep;

e= was the vector of error.

The mixed model-like equations were:

$$\begin{pmatrix} X'X & X'Z \\ Z'Z & Z'Z + A^{-1}K \end{pmatrix} \begin{pmatrix} \tilde{b} \\ \hat{a} \end{pmatrix} = \begin{pmatrix} X'Y \\ Z'Y \end{pmatrix}$$

$$k = \frac{1 - h^2}{h^2}$$

The variance components were estimated:

$$\sigma_e^2 = \frac{P'P - \tilde{b} * X' * P - \hat{a}' * ZP}{n - r(X)}$$

σ^2_e= was the residual variance

where:

r(x)= was the rank of matrix X which was the number of linearly independent columns, known as a degree of freedom for fitting the fixed effects of the model.

$$\sigma_a^2 = \frac{\hat{a}' * A^{-1} * \hat{a} + \sigma_e^2 * tr(A^{-1} * C_{22})}{q}$$

σ^2_a = was the additive genetic variance

where:

C_{22} = was the sub-matrix corresponding to random effects in the system of equations which was obtained after reversed throughout the system of equations:

$$C = \begin{bmatrix} X'X & X'Z \\ Z'X & Z'Z + A^{-1} * k \end{bmatrix}^{-1} = \begin{bmatrix} C_{11} & C_{12} \\ C_{21} & C_{22} \end{bmatrix}$$

The heritability for reproduction traits was estimated as:

$$h^2 = \frac{\sigma_a^2}{\sigma_a^2 + \sigma_e^2}$$

where:

h^2 = the heritability;

σ^2_a = was the additive genetic variance;

σ^2_e = was the residual variance.

The relative breeding value was:

$$BV\% = 100 + 12 * (\frac{BV_{abs} - Average\ BV_{abs}}{\sigma_{BVabs}})$$

BV% = relative breeding value;

BV_{abs} = absolute breeding value;

σ_{BVabs} = standard deviation of absolute breeding values.

RESULTS AND DISCUSSIONS

The number of lambs was important for profitability of flock in sheep breeds.

The mean for number of lambs was influenced by sheep breed. The number of lambs was influenced by maternal ability of sheep, the age of ewe, the year of calving. The milk yield in Prolific Breed Palas was 160-180 kg. The level of improvement and the degree of precocity of the breeds are correlated with the particularity of the breeds to show heat in several seasons (Pascal, 2015)

In the Table 1 were presented the statistics for number of lambs born in Prolific Breed Palas sheep in the first, second and third parity.

Table 1. The descriptive statistics for number of lambs born

Specification	Number of lambs born (first lambing)	Number of lambs born (second lambing)	Number of lambs born (third lambing)
Mean ± Standard error mean	1.347±0.036	1.458±0.041	1.587±0.066
Standard deviation	0.477	0.499	0.527
Number of ewes	170	142	63

The number of lambs born in Prolific Breed Palas breed was higher in second and third lambing than in first lambing. For the survival of lambs, it was necessary to assure optimal microclimate conditions. Prolific Breed Palas was a breed with good precocity, the young ewes could be introducing at reproduction at 7-9 months and the rams at 7-10 months (Stanciu, 2013). For young rams from the Prolific Breed Palas, Stanciu et al. (2013), reported a fecundity of 66.66-83.33% and a prolificacy of 100-122.22%, the young sheep of the Prolific Breed Palas the fecundity was 81.25% and the prolificacy 115.38%. The young ewes and young rams must to have a body weight represented 60-70% from adult sheep weight at Merinos sheep and 50-60% at prolific breeds and meat breeds sheep (Pascal, 2015). In breed sheep the reproductive process was determined by the genotype/environment interaction represented by daylight hours, temperature, humidity (Pascal, 2015). The reproduction traits depended on the farm management. The farmers organized breeding and lambing campaigns in optimal periods (Pascal, 2015). By high litter size increased in the total herd. Sharafeldin (1960) reported that the age of ewe influenced the percentage of multiple births which increased with the age of the ewe, the largest

percentage of single, twin and triplet birth was for two-, five- and six- year- old ewes. The primiparous sheep had lower prolificacy than multiparous ewes. The nutritional conditions of ewe influenced the fertility of ewe (Sharafeldin, 1960). Bulmaga et al. (2021) reported that the ewes with higher weight had higher prolificacy than the ewes with lower weight in Merinos Palas which had lower prolificacy. Pascal (2015) reported that the intensity of reproduction activity was higher in autumn season for sheep from temperate climate and that breeds as Dorset, Merinos Rambouillet, Polipay, Finish Landrace, Romanov were less exposed to seasonal influences on reproductive behaviour. For increase the profitability of farms, the farmers must know the particularities of sheep breeds for reproduction activity, reproductive biotechnologies could be applied to allow staggered parturition (Pascal, 2015). The demand for lambs' production was higher in spring in our country, fattened young and adult sheep were demanded in the foreign market.

The mean value for litter size was lower in Prolific Palas in first, second lambing and third lambing from our study than the mean obtained by Ziadi et al. (2025) reported the mean for litter size for prolific line in Tunisian Barbarine 1.43±0.57 in the first parity, 1.49±0.60 in the second parity and 1.63±0.62 in third parity. In our study the means for litter size was higher in the first three lambing than in conventional line in Tunisian Barbarine the mean was 1.08±0.28 in first parity, 1.07±0.25 in second parity and 1.06 in the third parity.

The mean for litter size in our study was closed to the value reported by Altarriba et al. (1998) for litter size of 1.35±0.48 in the Rasa Aragonesa.

Ben Salem et al. (2009) reported a lower mean of 1.32±0.47 in Noire de Thibar breed sheep than in our study. Habtegiorgis et al. (2022) reported a higher mean than in our study of 1.57 for number of lambs in Doyogena sheep.

De Vries et al. (1998) reported a mean of 1.64 for Zwartbles in first parity, 2.12 in second parity and 2.31 in third parity. The mean for number of lambs born was 1.318 in Polish Merino (Piwczynski et al., 2011).

Bromley et al. (2000) reported the mean for litter size born 1.61 in Columbia, 1.84 in Polypay, 1.63 in Rambouillet and 1.58 in Targhee.

The absolute breeding values for litter size in Prolific Breed Palas were presented in the Table 2.

Table 2. The highest absolute breeding values for litter size in Prolific Breed Palas sheep

No.	The absolute breeding values
1	0.122
2	0.121
3	0.117
4	0.108
5	0.104
6	0.100
7	0.098
8	0.068
9	0.061
10	0.058

The highest absolute breeding values for litter size in Prolific Breed Palas were between 0.058 and 0.122. The breeding values allowed to select sheep about the genetic merit.

In the Table 3 were presented the highest breeding values for litter size in Prolific Breed Palas.

Table 3. The highest relative breeding values for litter size in Prolific Breed Palas sheep

No.	The relative breeding values
1	136.434
2	136.100
3	134.763
4	131.754
5	130.417
6	129.080
7	128.412
8	118.384
9	116.044
10	115.041

The breeding value allowed estimate the genetic potential of sheep for litter size. The relative breeding values for the best sheep for litter size had the values between 115.041 and 136.434. In the sheep population there were individuals with higher prolificacy. Litter size could be improved by selection of the best sheep with the highest breeding values. By using the breeding values of sheep's, the selection lead quickly improvement

the sheep population and to achieve genetic progress. Selection using breeding values will lead to faster rates of genetic gain and flock improvement than selection based on raw data. A ram with a breeding value +0.20 produced daughters that will produce 10% more lambs than a ram with the breeding value 0.

In the Table 4 was presented the heritability for litter size in Prolific Breed Palas.

Table 4. The genetic parameters for litter size in Prolific Breed Palas

Item	Heritability
Litter size at birth	0.080±0.236

The heritability value of a trait shows us how much of the observed differences between the performances of individuals in the population are expected to be caused by difference between their breeding values.

The heritability for litter size in Prolific Breed Palas was low. The multi-trait index for milk production and prolificacy could be implemented in the breeding program of Prolific Breed Palas. The heritability could be influenced by the different environmental conditions from sheep flocks. Janssens et al. (2004) reported the heritability for litter size in sheep ranged from 0.06 to 0.13. De Vries et al. (1998) reported heritability for litter size in two prolific sheep, with repeatability model was 0.10 for Zwartbles and 0.12 for the Swifter and for multivariate model in first, second and third parity was 0.05, 0.07 and 0.10 for Zwartbles and 0.09, 0.12 and 0.09 for Swifter. De Vries et al. (2005) reported heritability for the total number of lambs born in German sheep breeds between 0.06 and 0.07.

Ziadi et al. (2025) reported heritability for litter size in Tunisian Barbarine sheep ranged from 0.04 to 0.18 for the prolific line and from 0.17 to 0.39 for the conventional line.

Piwczynski et al. (2011) reported the heritability for number of lambs born 0.25 and 0.29 estimated with a linear model and 0.054 estimated with threshold model. The heritability from our study was closed with the heritability found by Poutahmasebian et al. (2021) in Afshari sheep breed. Murphey and Thomas (2017) reported heritability for number of lambs born 0.07. Also, Sousa et al. (2000) reported the heritability 0.07 for the number of born lambs.

Murshedi et al. (2023) reported the heritability for prolificacy in Palas Prolific Line sheep was 0.177 higher than in our study.

Murshedi et al. (2024) reported heritability for prolificacy 0.235 in Palas Milk Line sheep population. The heritability for litter size in Rambouillet with linear sire model 0.16 and in Finnsheep 0.06, with animal model 0.16 in Rambouillet and 0.08 in Finnsheep, and with threshold sire model was 0.45 in Rambouillet and 0.14 in Finnsheep, and with threshold animal model 0.25 in Rambouillet and in Finnsheep 0.13 (Matos et al., 1997).

The heritability for litter size at birth in Columbia breed sheep was 0.12, in Polypay 0.12, Rambouillet 0.14, Targhee 0.01 (Okut et al., 1999). Bromley et al. (2000) reported that the heritability ranged from 0.3 to 0.11 for prolificacy traits (litter size at birth and litter size at weaning). Hanford et al. (2003) reported direct heritability 0.10 for litter size at birth and 0.07 for litter size at weaning in Targhee breed sheep.

The prolificacy influenced in a great proportion the economic efficiencies of sheep flocks. By genetic evaluation of sheep allowed to separate the influence of genetics from environmental factors.

The intensification of reproduction could be achieved by: increasing reproduction rates, early use young ewe for reproduction, organizing frequent lambing, practicing crossbreeding with prolific breeds in order to increase the reproduction rates, synchronizing the sexual cycle and grouping lambing (Pascal, 2015).

Grosu & Oltenacu (2005) reported that demographic and selection parameters together with genetic and phenotypic parameters of the selection traits determine the genetic gain achieved. The traits included in the selection objective must be chosen on economic criteria, with priority given to those that have economic importance.

Legarra et al. (2006) reported the median of the estimated economic weight for fertility, prolificacy, milk yield and longevity were 138.60 € per lambing, 40 € per lamb, 1.18 € per l, 1.66 € per year in Latxa breed and 137.66 € per lambing, 34.17€ per lamb, 0.73€ per l, 2.10 € per year for Manchega breed, consider that the fertility must include in the breeding program of sheep breed.

In the breeding program of Prolific Breed Palas a breeding strategy to improve litter size can be the nucleus schemes. Grosu & Oltenacu (2005) reported the selection plan for improvement the prolificacy was elaborate by King- the King program and that annual genetic gain was 0.03 lambs/year/ewe or 1.07% on year. The genetic gain represented the ratio between product (between heritability of the litter size and selection intensity) and generation interval. Kosgey (2004) reported that the selection of rams was realized with the criteria applied: number of lambing per year, litter size, viability of the lambs, live weight of lambs at 30 days of age and 70 days, and body conformation and the selection of ewes with the criteria applied: litter size and nursing ability. The dissemination of selected sheep is organized by the Breeding association and consists of young rams from the station and the ewes from the elite farms. The selection base remains open for include new farmers and new breeding sheep in the population under selection. The success of this program was done by the professional structure represented by Breeder's association for the management, active technical extension services and financial support (Kosgey, 2004).

Safari et al. (2005) reported that using within-breed selection in sheep the annual genetic gain was 1-2% for prolificacy, because this trait had a low heritability.

For Prolific Palas Breed will estimate a genetic gain for prolificacy will be 1.02% or 0.014 lambs/year/ewe.

We propose a breeding strategy which selection the animals by genomic breeding values. For estimating the genomic breeding value used three information sources: phenotypic informations (litter size at lambing), pedigree information (kinship between animals), genomic informations (SNP- single nucleotide polymorphism).

The model for estimation the genomic breeding value could be:

$$y_{ij} = B_i + a_j + e_{ij}$$

y_{ij} = litter size;

B_{ij} = the fixed effect;

a_j= the random effect for genetic effect;

e_{ij} = residual error.

The methodology to estimate genomic breeding values will be Single-Step Genomic Best Linear Unbiased Prediction (ssGBLUP) to increase the accuracy of genetic evaluation of Prolific Breed Palas sheep. The biometric model for estimation the genomic breeding values was realised by Misztal et al. (2009):

$$\begin{bmatrix} X' \cdot X & X' \cdot Z \\ Z' \cdot X & Z' \cdot Z + \alpha \cdot H^{-1} \end{bmatrix} \cdot \begin{bmatrix} \tilde{b} \\ \hat{a} \end{bmatrix} = \begin{bmatrix} X' \cdot y \\ Z' \cdot y \end{bmatrix}$$

$$H^{-1} = A^{-1} + \begin{bmatrix} 0 & 0 \\ 0 & G^{-1} - A_{22}^{-1} \end{bmatrix}$$

H = is the matrix which combined the genealogic information from pedigree (by A) with genomic information (by G).

The formula for genetic gain is (Grosu et al., 2019):

$$\Delta G = \frac{r_{A,I}}{T} \sigma A$$

ΔG = genetic gain;

$r_{A,I}$ = the accuracy of genetic evaluation;

T = the interval between generations;

σ_A = additive genetic variance.

The simulation selection scheme (Martin et al., 2025) will be used for Prolific Palas Breed was:

1) First step - The mating of rams with ewes (the sex ratio 1 ram/30 ewes)
2) Second step - Obtained the lambs males and females which will be genotyped all
3) Third step – the lambs were phenotyping and selected in proportion of 2% for males and 20% for females as future breeding rams and respectively future breeding ewes.

   Natality - 1.5 lambs/ewe

   Survival - 0.9

   1) 34 rams x 1000 ewes (mating)
   2) 1350 lambs

      675 lambs males + 675 lambs females will be genotyped
   3) 14 future breeding rams +135 future breeding ewes

Martin et al. (2025) reported in their simulate on study that the genetic gain increased with increasing the number of genotyped lambs and was more beneficial when heritability was low and that genotyping all the animals maximizes the genetic gain and accuracy of the breeding values.

In the breeding program of sheep, the genomic selection offers the possibility to farmers for

identify to early age the animals with high breeding values the effect was the increase the yearly genetic gain. The genetic gain depends on the accuracy of genetic evaluation, the intensity of selection, the interval between generations and the additive genetic effect (genetic variability) of the trait (Grosu et al., 2019). In genomic selection the accuracy of genetic evaluation depends on the precision of the estimation of effect of single nucleotide polymorphism and the relationship between SNP markers and genes which control the trait. The precision of estimation of SNP effects depends on the size of the reference population and the heritability of the prolificacy trait. By genomic selection, the number of candidates to selection increase, the proportion of the individual retained for reproduction decreases and the intensity of selection increases. The main advantage of genomic selection was that the parents of next generation will be selected at very early age, the selection can be realized after birth and reduced the generation interval. (Grosu et al., 2019).

CONCLUSIONS

The Prolific Breed Palas could be used for crossbreeding for increase the prolificacy for sheep breeds with low prolificacy. The genetic parameters for litter size in Prolific Breed Palas were necessary for reach optimum rate of genetic progress.

The heritability for litter size in Prolific Breed Palas population was low. In the selection of sheep, the BLUP methodology, individual animal model was a good method for the genetic evaluation of the sheep. By selection of the best sheep will increase the genetic gain. The multi-trait index for milk production and prolificacy could be implemented in the breeding program of Prolific Breed Palas. A good management could improve the reproduction traits by ensuring optimal nutrition and good microclimate conditions. The purpose of this paper was to present a methodology for estimating breeding values and genetic parameters for this breed that can be used in a breeding program that will be carried out by sheep breeder associations. For Prolific Palas Breed will estimate a genetic gain for prolificacy will be 1.02% or 0.014 lambs/year/ewe.

We propose a breeding strategy which selection the animals by genomic breeding values. For estimating the genomic breeding value used three information sources: phenotypic informations (litter size at lambing), pedigree information (kinship between animals), genomic informations (SNP- single nucleotide polymorphism).

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ASSESSMENT OF THE GROWTH ABILITIES OF LAMBS FOR MARKET REALIZATION FROM BULGARIAN SHEEP BREED FOR WOOL AND MEAT

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Abstract

The aim of the study is to assess the growth abilities of lambs until weaning of the Northeastern Bulgarian Fine-fleece breed. The animals are the property of the Scientific Center for Agriculture - Targovishte. Four groups were formed, which included 15 lambs - singles and 5 twins of both sexes. The live weight trait was recorded at birth, at 10 days, 30, 70 and 90 days. It was found that the lambs of this breed show good growth abilities until weaning. Female lambs reached an average of 24.471 kg, and males - 23.091 kg at 90 days. The average daily gain of lambs ranged from 0.148 kg to 0.306 kg for females by periods and from 0.156 kg to 0.342 kg for male lambs. The highest gain was achieved by lambs of both sexes in the period 70-90 days. The results show a good potential for compensation in the growth of lambs born as twins. The total gain achieved by twin lambs in 90 days was only 10.04% lower than singles in females, but 2.38% higher in male lambs.

Key words*: average daily gain, lambs, live weight, Northeastern Bulgarian Fine-fleece sheep breed, sex, type of birth.*

INTRODUCTION

The history of fine-fleece sheep breeding in Bulgaria reflects the general trends in the development of the agricultural sector. During the socialist period (1944-1989), sheep breeding developed rapidly and successfully. Bulgarian fine-fleece sheep breeds occupy a significant share of the total gene pool in the sector, which exceeded 10 million sheep at the end of the period. Wool production is of strategic importance for the textile industry and the foreign trade balance, together with meat production from fine-fleece farms. Trends in recent decades show a serious reduction of the fine-fleece population to a risk status. Under the new market conditions, wool production now generates more costs than income, and milk productivity cannot be relied on in fine-fleece sheep. Slavova & Staykova (2021) found that the largest relative share of income in the various productive areas of sheep breeding comes from the sale of animals for meat. Practice shows that the sale of lambs is the main source of income for farmers in the fine-fleece field. The sale of lambs immediately after weaning is a traditional practice and determines the economic results of breeding. At this age, they are ideal for the production of "light carcasses" – up to 13.0 kg, which are obtained from lambs with a live weight of up to 25,000-27,000 kg on the S-EUROP scale (Raycheva, 2005). The growth capabilities of the offspring and the potential for generating growth of fine-fleece lambs provide a chance for the preservation and sustainable development of the fine-fleece population against the background of competition from highly specialized imported breeds of sheep. European policies in the sector for the

preservation of biodiversity are in line with our strategy for the preservation and rational use of this valuable gene pool. The flock in the Scientific Center of Agriculture in Targovishte is a nucleus of the Northeastern Bulgarian Fine-fleece Sheep Breed (NEBFFB) - Shumen type. With a greatly reduced volume of the fine-fleece population, the Northeastern Bulgarian Fine-fleece breed occupies over 50% of the total volume in the direction. Studies of productivity and factors that influence the phenotypic expression of the genetic potential in fine-fleece breeds in our country were conducted by Iliev (1999, 2010); Iliev et al. (2017), Boykovski et al. (2012; 2018); Slavova (2000; 2019); Slavov (2007); Stefanova et al. (2002); Slavova et al. (2015; 2018); Stancheva et al. (2015; 2017; 2020); Staykova et al. (2013; 2022); Tzonev, T. (2014); Todorov et al. (2023). Mallick et al. (2017) investigated the influence of various factors on the weight development of Bharat Merino (BM) lambs, with the aim of improving the breeding program for the breed and increasing the economic effect of breeding. Mahala et al. (2019) established the results of changing some non-genetic factors on the growth and productivity of Avikalin sheep breed in India. Jawasreh et al. (2018) investigated the dynamics of live weight and weaning growth of Awassi lambs in Jordan. Studies on lamb meat production and ways to increase meat productivity provide the necessary recommendations for breeding work to preserve the Bulgarian fine-fleece sheep breeding. This scientific goal motivates the present study.

The aim of the study is to assess the growth abilities until weaning and the meat productivity of lambs from the Northeastern Bulgarian fine-fleece sheep breed.

MATERIALS AND METHODS

Study of the weight development of lambs from birth to weaning in the Northeastern Bulgarian fine-fleece sheep breed was the aim of the experiment. The study was conducted in the flock owned by the Scientific Center for Agriculture - Targovishte at the Agricultural Academy - Sofia during the lambing campaign 2024-2025. The experimental animals were matched by the method of analogues by sex /male, female/, type of birth/singles, twins/, age and live weight. Four groups were formed, which included 15 lambs each, born as singles and 5 twins of both sexes. Live weight was measured in the morning with an accuracy of 0.100 kg. The live weight trait was recorded at birth, at 10 days, at 30 days, at 70 days and at 90 days. The exact age at weaning in days is recorded, then equated to 90 days, for the purpose of data comparability. The animals have free access to a concentrated starter mixture with 18% crude protein, alfalfa hay and straw. Feeding and adaptation to different types of feed in the period until weaning is without quantitative restrictions. The lambs have constant access to drinking water and salt for licking. A total of 200 measurements of the live weight of the lambs from all groups were made, on the basis of which the average daily gain by periods was calculated for both sexes and for different types of birth. The data were processed using the Basic Descriptive Statistics method with the Minitab-16 software product. The reliabilities of the differences between the levels of the trait in the respective groups were established based on the distribution degree measured by Student.

RESULTS AND DISCUSSIONS

It was found that female lambs of the Northeastern Bulgarian fine-fleece breed are born with an average live weight of 4.327 kg, at 10 days they weigh an average of 5.880 kg, at 30 days - 9.242 kg, at 70 days - 18.342 kg and at 90 days an average of 24.471 kg (Table 1). The data in the table show that single female lambs achieve an 8.96% to 13.97% higher average live weight by age than lambs born as twins during the study period. The weight of twins at birth and at 10 days is significantly lower ($P<0.05$). The dynamics of the variance shows a variation in the live weight of female lambs within the range of 16.53% to 27.26% by the studied trait. In lambs born as singles, the highest variation is observed in live weight at birth (22.38%), while in twins the variation increases significantly at the last two ages (24.29% and 25.07%). This trend is probably due to the difference in the compensatory capabilities of

the individual individuals and the dynamics of the maternal effect. Female single lambs show a 10.26% higher absolute growth rate for the study period, without statistical certainty of the differences between the groups. Iliev et al., (2024) published values close to our average data on live weight at weaning for market realization of Karnobat fine-fleece lambs of both sexes (25.240 kg). Slavova et al. (2018) found a lower live weight at birth (3.541 kg) of female lambs of the Thracian fine-fleece breed, compared to our study, but at subsequent ages up to 70 days, the Thracian lambs showed higher average values for the trait and higher growth intensity. Karnobat fine-fleece female lambs were born with a lower average live weight (Staykova et al., 2023) and at one month they were equal in weight to their peers from our study. Until weaning, however, the Karnobat breed showed better growth abilities of the female offspring and outperformed the Northeastern Bulgarian lambs by 16.29%. Tzonev, T. (2014) reported a higher average weight at weaning of female lambs of the Northeastern Bulgarian fine-fleece breed by 8.07%. The author points out that single female lambs have a slightly lower birth weight (Tzonev, 2014), but at 30 and 70 days they slightly exceed the results of our study. Female twins are born with a 17.80% lower average weight than our results, but by 70 days of age they surpass the twins in our study by 6.80% (Tzonev, 2014). Staykova et al. (2025) found similar values of average live weight up to 70 days of female lambs from the Copper Red Shumen breed of sheep, which inhabits the same area - Northeastern Bulgaria. Live weight at weaning, however, is higher by 12.48% in female lambs from the Northeastern Bulgarian Fine-fleece breed.

Table 1. Live weight of female lambs up to weaning from the Northeastern Bulgarian Fine-fleece breed

Trait	Female lambs - total n = 20			Female lambs - singles n = 15			Female lambs - twins n = 5		
	x	Sx	C%	x	Sx	C%	x	Sx	C%
Live weight - birth, kg	4.327	0.212	21.58	4.497 l	0.260	22.38	3.901 l	0.342	18.67
Live weight - 10 days, kg	5.880	0.234	17.83	6.083 m	0.264	16.83	5.270 m	0.431	18.29
Live weight - 30 days, kg	9.242	0.348	16.85	9.457	0.404	16.53	8.606	0.676	17.58
Live weight - 70 days, kg	18.342	0.753	18.37	18.763	0.805	16.62	17.081	1.860	24.29
Live weight - 90 days, kg	24.471	1.020	18.61	25.101	1.071	16.57	22.590	2.522	25.07
Total gain for the whole period, kg	20.072	0.959	21.37	20.604	1.050	19.72	18.491	2.251	27.26

Significance of differences by rows – when the symbols are identical: from A to Z – P< 0.001; from a to k – P< 0.01; from l to z – P< 0.05.

The average growth rates of female lambs of the Northeastern Bulgarian fine-fleece sheep breed for the study period were respectively: 0.148 kg, 0.169 kg, 0.228 kg and 0.306 kg by periods and the total average until weaning was 0.223 kg (Table 2). The results show that single female lambs achieve from 1.77% to 26.42% higher growth at different ages, compared to twins. The highest growth is shown by singles in the period from 70 to 90 days (0.317 kg) (P<0.05). The twins give significantly lower growth from birth to 10 days (P<0.01). It was found that in terms of the total average daily gain for the entire studied period, female lambs born as twins at the end of the study were characterized by only 10.04% lower total gain. The coefficients of variation for average daily gain were high and ranged from 19.16% to 42.88%. In twin lambs, the dynamics increased after 30 days of age. The greatest variation in both types of birth of female lambs was found in the period from 70 days to weaning. This is the period in which the offspring begin to demonstrate their own growth abilities, after the gradual elimination of the effect of the different milk yield of the mothers. Slavova et al. (2018) found a higher average daily gain of Thracian female lambs in periods up to 70 days of age. The average growth of Karnobat female lambs is higher up to 70 days (Staykova et al.,

2023), in the latter period it decreases compared to our results, but the total average growth per day for the entire period is almost the same for both fine-fleece breeds. Tzonev (2014) published a slightly higher growth of single female lambs from the North-East Bulgarian fine-fleece breed up to 30 days, but then the results are similar to ours until weaning. The same author found a stronger compensatory growth in twins, which almost reached singles at weaning with a difference of only 3.6% in favor of singles (Tzonev, 2014). Copper-red Shumen female lambs are characterized by higher growth intensity up to 30 days and then show 16.14% lower average daily gain until weaning compared to our results for the Northeastern Bulgarian fine-fleece breed (Staykova et al., 2025).

Table 2. Average daily gain of female lambs up to weaning from the Northeastern Bulgarian Fine-fleece breed

Trait	Female lambs - total n = 20			Female lambs - singles n = 15			Female lambs - twins n = 5		
	x	Sx	C%	x	Sx	C%	x	Sx	C%
Gain - birth. - 10 days, kg	0.148	0.013	40.02	0.159 a	0.016	39.64	0.117 a	0.016	30.16
Gain - 10 days - 30 days, kg	0.169	0.011	29.59	0.170	0.014	32.84	0.167	0.014	19.16
Gain - 10 days - 30 days, kg	0.228	0.016	31.67	0.233	0.019	31.56	0.212	0.033	34.62
Gain - 70 days - 90 days, kg	0.306	0.028	41.18	0.317 l	0.034	41.37	0.276 l	0.053	42.88
Total average daily gain for the period, kg	0.223	0.011	21.37	0.229	0.012	19.72	0.206	0.025	27.27

Significance of differences by rows – when the symbols are identical: from A to Z – P< 0.001; from a to k – P< 0.01; from l to z – P< 0.05.

At the same ages, the average live weight values of male lambs were respectively: 4.531 kg, 6.091 kg, 9.295 kg, 16.256 kg and 23.091 kg (Table 3), with a total absolute average gain for the period of 18.561 kg. Male singles achieved a 4.11% to 28.66% higher average live weight than their twin peers at the same ages. The average weight of twins was significantly lower by the end of the first month (P< 0.05, P< 0.01, P< 0.001). Male lambs of the Karnobat Fine-fleece breed are born with a lower average live weight, but by the end of the first month they equalize in weight with their peers from our study and are weaned at an average weight 25.11% higher compared to our results (Staykova et al., 2023). Similar results were published by Slavova (2019) for the weight development of lambs of the Thracian Fine-fleece breed, which are weaned at a slightly higher live weight. Tzonev (2014) found a slightly lower live weight at birth of single male lambs of the Northeastern Bulgarian Fine-fleece breed – Shumen type, which show better growth intensity thereafter and are weaned at a higher average weight by 21.51%. In male lambs born as twins, the same trend was observed, probably as a result of environmental effects during the study period (Tzonev, 2014). In male lambs in our study, the trend for a stronger weight variance in singles up to 28.67% was maintained. It was found that male twins realized a 2.10% higher absolute growth compared to single lambs, but without statistical certainty of the differences between the groups. This shows the tendency to compensate for the lambs from multiple lambs with more intensive growth in order to realize their genetic potential, provided that the mother's milk yield allows it. Staykova et al. (2025) established a lower average live weight at birth of male lambs from the Copper Red Shumen breed of sheep, which inhabits the same area - Northeastern Bulgaria. At weaning, the local breed showed 8.08% higher live weight of male lambs (Staykova et al., 2025). Luma & Shabani (2025) studied the growth performance of the Sharri sheep breed and the Kosova sheep breed and found similar results for the former breed and significantly lower results for male lambs from the latter breed.

Table 3. Live weight of male lambs up to weaning from the Northeastern Bulgarian Fine-fleece breed

Trait	Male lambs - total n = 20			Male lambs - singles n = 15			Male lambs - twins n = 5		
	x	Sx	C%	x	Sx	C%	x	Sx	C%
Live weight - birth, kg	4.531 a	0.202	19.91	4.867 A	0.192	15.31	3.521 Aa	0.208	13.23
Live weight - 10 days, kg	6.091 l	0.323	23.69	6.561 b	0.332	19.63	4.681 lb	0.392	18.71
Live weight - 30 days, kg	9.295	0.447	21.51	9.753 m	0.523	20.77	7.920 m	0.541	15.27
Live weight - 70 days, kg	16.256	0.734	20.20	16.633	0.941	21.90	15.122	0.723	10.71
Live weight - 90 days, kg	23.091	1.120	21.75	23.340	1.441	24.15	22.381	1.160	12.58
Total gain for the whole period, kg	18.561	1.061	25.65	18.473	1.370	28.67	18.861	1.381	16.33

Significance of differences by rows – when the symbols are identical: from A to Z – P< 0.001; from a to k – P< 0.01; from l to z – P< 0.05.

The results in Table 1 and Table 3 show that female lambs by the end of the first month are characterized by a lower average live weight than males by 4.50% and 3.46%. After this age, however, female lambs in total and separately from both types of birth outperform males in total and by groups. Similar average data are also observed in terms of total absolute growth (7.53% higher for females) for the entire period, with the exception of the total growth for the period of male twins. In single male lambs, the coefficient of variation is highest at weaning (24.15%). Similar observations show the results in female twins in the last period until weaning (25.07%). Female singles show a greater diversity in live weights at birth. Călin et al. (2024) studied the potential of two sheep breeds in Romania for growth and lamb meat production - the Tsurcană breed and the Tsigaie breed. The first breed is definitely presented with lower values for average live weight by periods in lambs of both sexes, and the results for average daily growth of the animals are similar. The Tsigaie breed shows similar results to ours for average weight by periods and for average daily growth achieved by male and female lambs for the entire period of the study (Călin et al., 2024).

The results for average daily growth of male lambs of the Northeast Bulgarian Fine-fleece breed are respectively: 0.156 kg, 0.161 kg, 0.175 kg and 0.342 kg, an average of 0.207 kg for the entire period until weaning (Table 4). The highest growth is shown by male offspring in the period from 70 to 90 days (0.342 kg), but without statistical certainty of the differences. Male lambs of the Karnobat fine-fleece breed are characterized by a higher average daily growth up to 70 days, but after this age they show lower growth values than our results (Staykova et al., 2023). The twins give a significantly lower growth from birth to 10 days (P< 0.01) in our study. After the tenth day, however, we observe the opposite trend and the twin lambs realize a higher average growth until weaning, including as an absolute total average daily growth by 2.38% more than the singles for the period of the study. The coefficients of variation are high, especially for the first two periods and reach 48.65% for the birth weight of the twins. A gradual decrease follows and the variability increases again for the growth from 70 to 90 days in singles. In the latter period, lambs already rely mainly on their own potential for creating growth and mother's milk reduces its influence as a factor. At this age, the phenotypic realization of the trait depends largely on the feeding and rearing conditions on the farm. Tzonev (2014) established a higher growth of single lambs of the Northeastern Bulgarian fine-fleece breed - Shumen type up to 70 days, but until weaning the results are close to ours. Lambs born as twins also gradually increase the average growth values by periods, but cannot reach the growth of singles (Tzonev, 2014). In our study, male twins show good opportunities for compensatory growth and give a higher total average daily gain for the entire period. Staykova et al. (2025) published similar growth results for male twin lambs of the Copper Red Shumen breed, raised in the same area, but for singles and for males in general, the
local breed is distinguished by slightly higher results.

Table 4. Average daily gain of male lambs up to weaning from the Northeastern Bulgarian Fine-fleece breed

Trait	Male lambs - total n = 20			Male lambs - singles n = 15			Male lambs - twins n = 5		
	x	Sx	C%	x	Sx	C%	x	Sx	C%
Gain - birth. - 10 days, kg	0.156	0.017	47.14	0.169 a	0.019	43.88	0.116 a	0.027	48.65
Gain - 10 days - 30 days, kg	0.161	0.013	36.56	0.160	0.017	40.84	0.162	0.016	23.26
Gain - 10 days - 30 days, kg	0.175	0.011	29.21	0.172	0.014	30.97	0.180	0.021	26.52
Gain - 70 days - 90 days, kg	0.342	0.029	37.90	0.335	0.037	43.12	0.363	0.035	21.40
Total average daily gain for the period, kg	0.207	0.012	25.65	0.205	0.015	28.67	0.210	0.015	16.33

Significance of differences by rows – when the symbols are identical: from A to Z – P< 0.001; from a to k – P< 0.01; from l to z – P< 0.05.

The results in Table 2 and Table 4 show that female lambs achieve a lower average daily gain up to 10 days of age by 5.13%, then outperform males up to 70 days and again give lower results in the last period until weaning, where males dominate with 10.53%. Against the background of these dynamics in the different gender groups, the total average gain for the period of female offspring is slightly higher, but in lambs born as twins the male lambs shows a better potential for compensation in the realization of growth.

This study outlines the trends in the dynamics of live weight and growth of lambs from the Northeastern Bulgarian fine-fleece breed until weaning and market realization. The full manifestation of the genetic potential for lamb meat production can be realized only under optimal conditions of feeding and rearing of the lambs and their mothers even before conception.

CONCLUSIONS

The Northeastern Bulgarian fine-fleece sheep breed is characterized by a good growth rate of lambs until weaning. Female lambs reach an average of 24.471 kg, and males, respectively, 23.091 kg per 90 days.

The average daily gain of lambs from the Northeastern Bulgarian fine-fleece breed ranges from 0.148 kg to 0.306 kg for females by periods and from 0.156 kg to 0.342 kg for male lambs. The highest growth is achieved by lambs of both sexes in the period 70-90 days.

The results of the study show a good potential for compensation in the weight development of lambs born as twins. The total growth achieved by twin lambs in 90 days is only 10.04% lower than singles in females, but 2.38% higher in male lambs from the Northeastern Bulgarian fine-fleece breed of sheep.

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EFFECT OF CONTROLLED MILK FEEDING SYSTEMS ON GROWTH PERFORMANCE OF GOAT KIDS UNTIL WEANING

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Abstract

The duration of milk intake under artificial feeding conditions depends on the method of feeding, age, sex, and individual characteristics of the animals. The present study investigates the effect of artificial milk feeding using nipple buckets on goat kids reared in pens without their dams. The experiment was conducted under controlled feeding conditions and included goat kids from the Anglo-Nubian, Bulgarian White Dairy, and Toggenburg breeds.Throughout the study, the body weight development of the animals was monitored from birth until the weaning period (90 days). Key productive parameters were evaluated, including live body weight, average daily gain, growth rate, and related growth indicators. Differences in feeding behavior and adaptation to artificial milk feeding were identified depending on genotype and sex.The obtained results allow an objective assessment of the effectiveness of controlled artificial milk feeding systems and the degree of adaptation of goat kids to this feeding regime.

Key words: *breed, feeding, goat kids, growth, weight gain.*

INTRODUCTION

Globally, goat farming occupies a strategic position within the agricultural sector, providing high-quality dietary products and raw materials (Meza-Herrera et al., 2024). In the context of modern dairy goat farming, there is a clear trend toward increasing economic efficiency, which necessitates the implementation of various practices where the controlled feeding of kids is a fundamental element. This approach allows for the maximum utilization of produced milk for processing and provides an opportunity for reliable monitoring of the health and growth of young animals during the most critical period of their lives (Simonetti et al., 2019).

Controlled milk feeding systems (initially via nipple bottles and later using nipple buckets) are increasingly becoming the preferred method in Bulgaria, as they combine the physiological advantages of suckling with the technological discipline of rationed feeding (Delgado-Pertíñez et al., 2009; Ghibaudi & Simonetti, 2025). Despite the undeniable advantages, the adaptation of animals to this type of regime is often accompanied by stress, which varies depending on individual and breed characteristics (Toinon et al., 2022).

In Bulgaria, research on the comparative characteristics of different dairy breeds under identical artificial feeding conditions is limited. Breeds such as the Anglo-Nubian (AN), the Bulgarian White Dairy (BWD), and the Toggenburg (TG) possess different temperaments and feeding behaviors, which suggests a varying response to controlled milk intake systems.

The aim of the present study is to analyze the adaptation to controlled milk feeding, as well as its influence on the main growth parameters (live body weight and average daily gain) of goat kids from the specified genotypes. The evaluation of these parameters will allow for the optimization of technological solutions in rearing young animals, which is a key prerequisite for their development and future productivity.

MATERIALS AND METHODS

The study was carried out at the farm of the Experimental Base of the Research Institute of Mountain Stockbreeding and Agriculture in Troyan, Bulgaria. A total of 75 kids were included in the experiment: AN (n=19; 10 males and 9 females), BWD (n=36; 13 males and 23 females), and TG (n=20; 10 males and

10 females). The kids were born during the months of February and March in a herd controlled by the Association for Breeding Dairy Goats in Bulgaria. The animals were divided into three experimental groups, balanced by age.

Following birth and mandatory colostrum feeding, the kids were placed in group pens (5 animals per pen). An artificial milk feeding system was applied under the following conditions:

Feeding Method: Group feeding from buckets equipped with five individual nipples, allowing for simultaneous intake and reducing competitive stress.

Feeding Regime: Twice daily (morning and evening) with whole goat milk at a temperature of 38–39°C. After reaching 45 days of age, the kids were provided with free access (ad libitum) to alfalfa hay and a starter concentrate feed. The consumption of solid feeds was not recorded individually.

Growth Performance: The weight development of the animals was tracked from birth until weaning at 90 days of age: Live Body Weight (kg): Recorded individually at birth and every 10 days until the 90th day using an electronic scale. Average Daily Gain (g): Calculated for the specific periods.

Ethological Observations: Upon the formation of the groups, the kids were presented with a nipple bucket, and their independent interest in it was recorded. In the absence of interest, the kid's head was manually guided toward one of the nipples on the bucket. The duration of human intervention was registered. The suckling duration and the rest intervals between individual feeding episodes were also recorded. Behavioral parameters were documented using the method of direct observation and chronometry.

Statistical analysis: Data were statistically evaluated by one-way ANOVA. The Fit modelprocedure of the JMP v.7 software package was used to perform the statistical analysis (JMP Version 7, SAS Institute Inc. Cary, NC). The effects were considered to be significant at $P<0.05$; $P<0.01$ and $P<0.001$. Significant differences among the means were determined using the Tukey test ($P<0.05$). All data were expressed as mean values with pooled standard errors.

RESULTS AND DISCUSSIONS

The analysis of the growth parameters of the kids under the controlled milk feeding system shows significant dynamics influenced by the breed factor and the individual adaptive capacity of the animals.

Dynamics of Live Body Weight and Growth Intensity

The results for live body weight from birth to 90 days of age are presented in Table 1. At birth, no statistically significant differences were established between the groups, with weights ranging from 3.45 kg (AN) to 3.90 kg (TG). These baseline data are essential for objectively tracking the influence of the artificial feeding system on further development.

During the first month of life, no statistically significant differences in live body weight were observed among the kids. However, the growth rate indicates future divergences. At 10 days of age, the BWD kids showed a weight gain of 1.41 kg, which is higher than the gain in AN (1.19 kg) and TG (1.22 kg). This indicator suggests that BWD kids adapt quickly to the artificial nipple, allowing them to maintain their growth momentum after separation from the dam. At 30 days of age, the weight of the BWD reached 8.09 kg, followed by the TG at 7.98 kg, and the AN at 7.31 kg (Table 1).

After the 40th day, a clearly pronounced difference in live body weight was observed between the BWD (9.98 kg) and the AN (8.79 kg) ($P<0.05$). At 60 days of age, the superiority of the BWD increased, with their weight reaching 14.21 kg, while the TG showed values of 13.68 kg. McManus et al. (2008) reported an average daily gain for Toggenburg kids up to the 60th day of 0.112 kg, which is lower than the values established in our study for the same period. The lowest weight gain at 60 days was recorded in the AN (1.75 kg), which is nearly 30% less than the gain in the BWD (2.29 kg). In the final stage of the milk period (between the 70th and 90th day), a more pronounced differentiation in the parameters of the groups is observed. By the time of weaning at 90 days, the BWD kids reach an average weight of 20.95 kg.

Table 1. Dynamics of live weight (kg) by breed and age

Age (Days)	Indicators (kg)	AN n=19		BWD n= 36		TG n=20		Sig.
		Mean	SD	Mean	SD	Mean	SD	
Birth weight		3.45	0.59	3.69	0.91	3.90	0.44	NS
10	Weight	4.64	0.96	5.1	1.07	5.12	0.55	NS
	Weight gain	1.19	0.58	1.41	0.64	1.22	0.33	NS
20	Weight	5.92	1.03	6.49	1.24	6.43	0.61	NS
	Weight gain	1.28	0.38	1.39	0.64	1.31	0.68	NS
30	Weight	7.31	1.14	8.09	1.55	7.98	1.72	NS
	Weight gain	1.39	0.27	1.59	0.53	1.55	0.79	NS
40	Weight	8.79 ^{b}	1.22	9.98 ^{a}	1.87	9.78 $^{a, b}$	1.90	*
	Weight gain	1.48 ^{b}	0.28	1.88 ^{a}	0.71	1.81 $^{a, b}$	0.31	*
50	Weight	10.43 ^{b}	1.57	11.91 ^{a}	2.22	11.67 $^{a, b}$	1.89	*
	Weight gain	1.63	0.46	1.93	0.53	1.89	0.67	NS
60	Weight	12.18 ^{b}	1.72	14.21 ^{a}	2.52	13.68 $^{a, b}$	1.88	**
	Weight gain	1.75 ^{b}	0.22	2.29 ^{a}	0.6	1.98 ^{b}	0.80	***
70	Weight	14.01 ^{b}	1.71	16.58 ^{a}	2.68	15.89 ^{a}	1.96	***
	Weight gain	1.83 ^{b}	0.15	2.37 ^{a}	0.35	2.22 ^{a}	0.22	***
80	Weight	15.98 ^{b}	1.75	18.7 ^{a}	2.75	17.93 ^{a}	1.97	***
	Weight gain	1.97 ^{c}	0.12	2.12 ^{a}	0.17	2.04^{b}	0.18	***
90	Weight	18,00^{b}	1.73	20.95 ^{a}	2.75	20.01 ^{a}	1.96	***
	Weight gain	2.02 ^{b}	0.50	2.25 ^{a}	0.24	2.08 ^{a}	0.31	***
Absolute gain		14.55^{b}	1.37	17.26 ^{a}	2.34	16.11 ^{a}	1.78	***

Note: *- P < 0.05; **- P < 0.01; ***- P < 0.001.
^{a,b}The same letters on the same row indicate values with significant reliability.

The Toggenburg kids reach 20.01 kg. Our results are close to the values of Galina et al. (1995), who report that kids fed with whole goat milk achieve a weight gain of approximately 0.167-0.170 kg/day. At the same time, a lower live body weight is recorded for the Anglo-Nubian kids at the end of the period - 18.00 kg, which places them in a different position compared to the previous two groups. These values significantly exceed the data of Hammoud & Salem (2019), who report a birth weight of 2.54 kg and a weaning weight of only 13.12 kg for the same genotype. This superiority in our study can be attributed to the optimized feeding regime and the higher genetic potential of the animals used.

Our results regarding the weight development of the Anglo-Nubian kids (18.00 kg at 90 days of age) are similar to recent studies by Ghibaudi & Simonetti (2025), who, using a similar artificial rearing system, reported a live body weight of approximately 14 kg at 70 days of age (10 weeks). The identical levels of average daily gain in both studies (150-160 g/ day) confirm that despite the more challenging adaptation period for this genotype, under intensive management and proper technology (such as the use of nipple buckets), the kids manage to realize their genetic growth potential.

The absolute gain for the entire 90-day period reflects the final effect of the applied feeding system. For the BWD kids, a total accumulated mass of 17.26 kg was recorded, which is approximately 2.71 kg more compared to the AN group. These data indicate that controlled milk feeding from a nipple bucket has a more favorable effect on the growth of the Bulgarian White Dairy breed, while for the Anglo-Nubian breed, the same system leads to more moderate results.

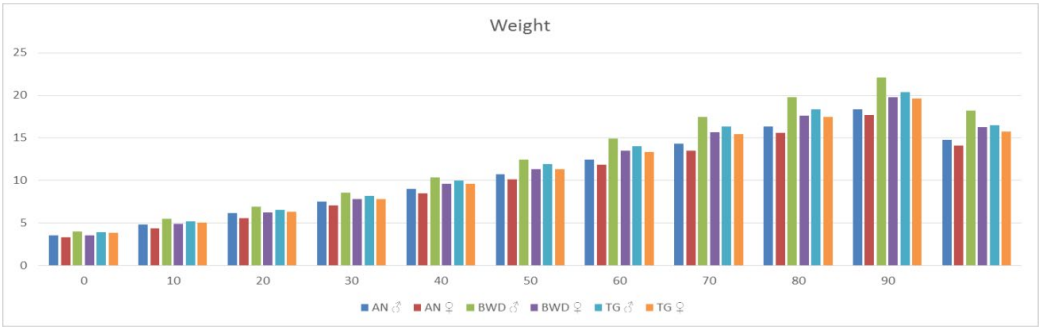


Figure 1. Dynamics of live weight (kg) in kids by breed and age

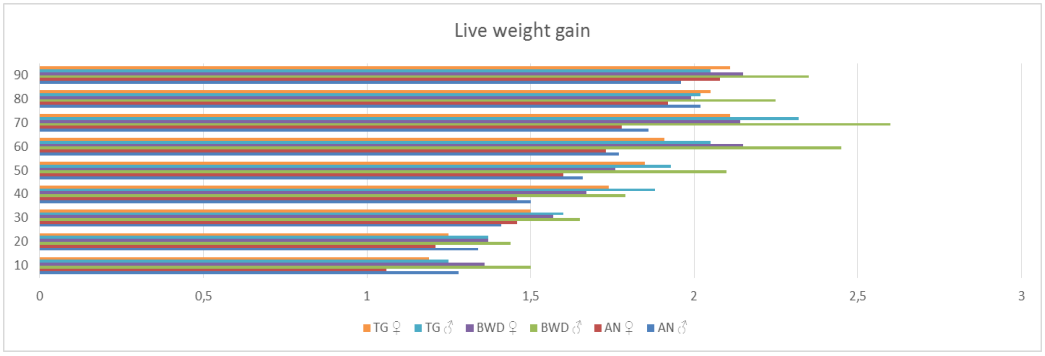


Figure 2. Dynamics of weight gain (kg) in kids by breed and age

The graphical representation of the data in Figure 1 allows for a detailed analysis of the influence of sex on the efficiency of the controlled milk feeding system.

The observed differences in weight between the sexes indicate that the conditions of pen housing and the feeding method eliminate stress factors and allow for a differentiated expression of the animals' growth capabilities.

The data analysis reveals that in all studied breeds, male kids maintain higher live body weight levels compared to females. This superiority is most pronounced in the Bulgarian White Dairy breed. In this group, male individuals reach peak values of nearly 22.00 kg by the end of the 90-day period, while females reach approximately 20.00 kg. This difference of approximately 10% in favor of the males is attributed to natural anabolic hormones, which stimulate higher feed conversion efficiency and faster muscle tissue accumulation compared to females (summarized by Never, 2015).

Figure 1 illustrates that in the early stages (up to the 20th day), the difference between the sexes is minimal. As age progresses, the divergence between male and female individuals increases progressively. Regarding the Anglo-Nubian breed, it is noteworthy that although the overall weight is lower, male kids show a better response to the feeding system after the 60th day, managing to compensate for the initial growth lag more effectively than their female counterparts. Simonetti et al. (2019) report an average weight of 14.6 ± 0.26 kg for female Anglo-Nubian kids at 12 weeks of age fed with milk replacer.

In the Toggenburg breed, male individuals reach an average live body weight of 20.01 kg, compared to their female peers (18.50 kg), which establishes this genotype as highly adaptive to the artificial milk feeding system. Our results for the Toggenburg breed are close to the values reported by Galina et al. (1995) during intensive rearing of the same breed in Mexico, confirming the genetic stability of weight gain under controlled milk intake, regardless of geographical conditions.

In the early stage (up to the 20th day) (Figure 2), the weight gain is almost uniform across all

groups, with differences in intensity based on sex and breed becoming clearly visible only after the 40th day.

The Bulgarian White Dairy kids exhibit the highest growth intensity, with male individuals reaching a gain of 2.6 kg in the interval between the 60th and 70th day. The difference in weight gain between the two sexes is most distinct here, with males outperforming females by approximately 10% at the end of the study period. Our findings correspond with our previous study (Mondeshka & Stoycheva, 2024) regarding both sex and different ages.

In the Toggenburg breed, stability and consistency are observed throughout the entire 90-day period. The males maintain a steady pace, and the females show values very close to theirs, which is an indicator of uniform weight gain within the genotype.

The Anglo-Nubian breed maintains the lowest levels of weight gain compared to the others. It is noteworthy that after the 60th day, the male kids begin to realize their capacity more effectively and improve their gain compared to their female peers, although they remains below the levels of the other two breeds.

For comparison, Simonetti et al. (2019) report an average daily gain over a 12-week period for female AN kids of 0.137 kg.

Analysis of Ethological Parameters and Adaptive Capacity

The ethological observations (Figures 3, 4 and 5) provide fundamental information on how each breed interacts with the controlled milk feeding system. The behavior of the animals during milk intake is the direct mechanism through which the growth parameters presented earlier are modulated. The observations were conducted during the adaptation of the animals to the studied system.

The most significant indicator determining milk intake is the total suckling duration. A comparison between the three figures shows that the BWD kids (Figure 3) achieve the longest period of active suckling - 178 s. This is nearly 30% more compared to the AN kids (Figure 4), whose duration is only 138 s. This greater time resource allows the BWD kids to consume a larger volume of milk per feeding, which likely underlies their higher daily gain. The Toggenburg kids (Figure 5) occupy an intermediate position with 155 s, which corresponds to their stable, yet slightly lower than BWD, growth parameters.

The time to first suckling is an indicator of the innate reflex and the animal's resilience in the external environment (Bélanger-Naud & Vasseur, 2021; Toinon et al., 2022). In the AN breed (Figure 4), this time is the longest - 60 s. In combination with the highest requirement for human help (40 s), these data indicate that Anglo-Nubian kids experience the strongest adaptive stress upon separation from the dam and transitioning to bucket feeding. This longer orientation period and dependence on staff delay the start of intensive growth, which is evident from the curve in Figure 1. Our results highlight the significance of the human factor during the initial adaptation of the kids. While Ghibaudi & Simonetti (2025) state that the success of artificial rearing depends on the "intense dedication of the operators", our study concretizes this assertion by recording the time required for assistance during the first suckling. The data show that this need for support is breed-specific. For this genotype, staff involvement is of key importance for the successful adoption of the technology.

In the AN breed, the duration of "Interruptions during suckling" is 62 s, whereas for the BWD, it is only 50 s. This indicates that kids from the BWD and TG breeds exhibit more focused and sustained feeding behavior. Their ability to consume their ration with fewer interruptions minimizes stress signals in the digestive system and optimizes the functioning of the esophageal groove, ensuring that the milk passes directly into the abomasum (Bélanger-Naud & Vasseur, 2021).

The lower ethological performance of the AN kids in our study corresponds with the findings of Ghibaudi & Simonetti (2025) regarding the difficulties in adapting this genotype to artificial feeding systems.

In conclusion, the ethological analysis proves that the controlled feeding system from a nipple bucket is most effective for breeds with a high stress-resistance threshold and a strong feeding instinct, such as the BWD and TG. The AN breed, while highly productive, requires more intensive attention from staff during the early stages of the milk period.

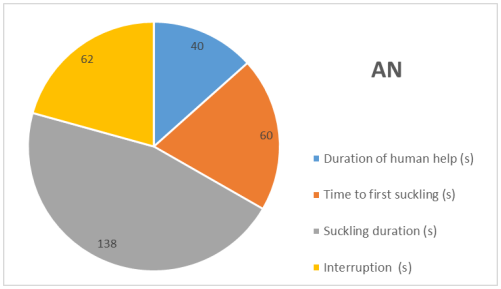


Figure 3. Ethological parameters of AN kids during feeding (s)

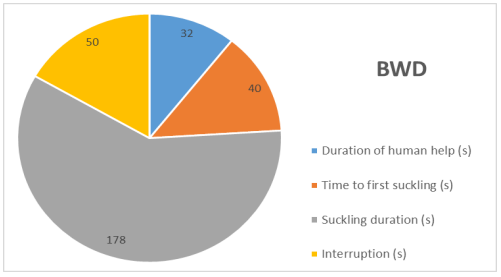


Figure 4. Ethological parameters of BWD kids during feeding (s)

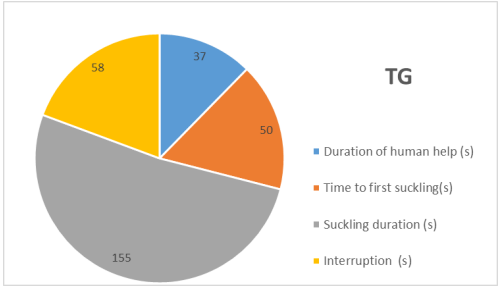


Figure 5. Ethological parameters of TG kids during feeding (s)

The study conducted highlights the role of early adaptation to the studied controlled milk feeding system as a determining factor for the future development of kids. The results demonstrate that the transition to artificial feeding is not merely a technological act, but a complex ethological process that occurs differently across individuals and genotypes (Bélanger-Naud & Vasseur, 2021; Toinon et al., 2022; Ghibaudi & Simonetti, 2025).

While in combined systems (suckling combined with milking), the growth of kids slows down by 20-33% (Hernandez et al., 2007), the system described in our study allows the kids to realize their high potential, as they receive a rationed, full-value amount of milk without competition from the milking installation.

The Bulgarian White Dairy breed demonstrates the highest technological suitability, combining stable feeding behavior with intensive growth. The Toggenburg breed proves to be adaptive and balanced, showing uniform development, which makes it suitable for the studied system with minimal human intervention. On the other hand, the Anglo-Nubian kids, despite their high genetic potential, show greater sensitivity to stress upon separation from the dam, necessitating more precise management during the first weeks of life.

Strengths and Limitations

Strengths: The study covers breeds with diverse temperaments and origins, reared under identical environmental conditions. The inclusion of measurable parameters (time to first suckling, suckling duration) allows for an understanding of the underlying causes of weight differences, rather than merely stating them. The results offer specific guidelines for farmers regarding the necessity of individual attention to different breeds during the critical adaptation period.

Limitations: Since the kids were group-housed, individual consumption of starter feed and hay was not tracked; therefore, the focus of the study is placed on the adaptation to milk feeding and the influence of the genetic factor, rather than on the efficiency of solid feed intake. The study is limited to 90 days of age. Tracking the animals at a later stage (post-weaning) would reveal whether slower-adapting kids compensate for their growth lag later on. Other behavioral reactions outside of the feeding act, which are also indicators of adaptive stress, were not recorded.

Future Directions: The current study outlines the need for follow-up research to expand the scope by including a control group (natural suckling) to compare the potential of artificial systems against the biological standard. Furthermore, systematic monitoring of health status and tracking the frequency of dietary disorders across different breeds is recommended.

CONCLUSIONS

The implementation of a controlled milk feeding system using a nipple bucket affects growth performance depending on the genotype, with the Bulgarian White Dairy kids exhibiting the highest adaptability and growth intensity.

Clearly pronounced differences in growth capacity based on sex were established; male individuals from all breeds achieved a higher live body weight, with the difference being most significant in the BWD (approximately 10% superiority for males).

Ethological analysis indicates that the Anglo-Nubian kids experience the strongest adaptive stress (the longest time to first suckling - 60 s), which requires a greater commitment from the attending staff.

The Toggenburg breed demonstrates uniformity of parameters between the sexes and stability in feeding behavior, making it highly suitable for the studied feeding system.

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TRADITIONAL AND INDUSTRIAL DAIRY PRODUCTS: COMPARATIVE ASSESSMENT OF COMPOSITION, NUTRITIONAL QUALITY AND SAFETY

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Abstract

This study aimed to compare the compositional and microbiological characteristics of traditionally and industrially manufactured dairy products and to assess whether differences in production technology are reflected in analytically measurable quality parameters. The analysis is grounded in a technological distinction between traditional and industrial production methods and focuses on dairy products originating from the South-Muntenia region. A total of 90 independent biological samples were analyzed (n = 45 per production category), covering seven product types obtained from both cow and sheep milk. The methodology is based on physicochemical and microbiological analyses performed in a university laboratory, with the support of specialized laboratories, targeting parameters relevant to compositional quality, product safety, and processing-related differences. The main quantified differences showed that traditional products had higher mean values for fat (up to 411% higher in the case of standardized industrial ovine milk), protein, dry matter, acidity, total viable count, and Escherichia coli, whereas industrial products showed higher lactose values and more uniform microbiological profiles. From a food safety perspective, Salmonella spp. and Listeria monocytogenes were absent in all analyzed samples, and aflatoxin values were below the detection limit in all cases. These results suggest that production technology may influence the final quality characteristics of dairy products, with traditional products presenting a more concentrated compositional profile and a higher degree of microbiological variability, while industrial products are distinguished by greater standardization and more controlled hygiene parameters.

Key words: *consumer perception, microbiological quality, processing technologies, traditional versus industrial.*

INTRODUCTION

In recent years, traditional dairy products have remained highly relevant in consumer preferences, especially among consumers who associate them with authenticity, local cultural identity, and traditional processing practices.

At the same time, the development of industrial dairy processing has made it possible to obtain standardized products with relatively uniform characteristics and a high degree of technological control (Bichescu & Stanciu, 2017; Stefan (Vasiliu) et al., 2024).

The two production models, traditional and industrial, differ in the way raw materials are processed, in the technologies applied, and in the level of standardization, all of which can influence the physicochemical, microbiological, and sensory characteristics of the final products.

Consumer perceptions of traditional and industrial products are shaped by several subjective factors, including personal experience, cultural values, level of information, and marketing elements. Therefore, self-reported preferences do not always reflect the actual characteristics of the products. An objective comparison between these two categories requires measurable indicators that can highlight differences in composition, physicochemical profile, and microbiological quality. In dairy products, traditional processing can lead to greater variability in the characteristics of the final products. This variability may result from the composition of the raw material, the natural microflora, the processing techniques used, the maturation period, and the storage conditions. By contrast, industrial systems aim to obtain consistent quality by controlling raw materials,

standardizing technological parameters, and monitoring the production flow. These differences can be reflected in parameters such as fat, protein, lactose, dry matter, acidity, pH, and microbiological indicators.

The study aimed to compare the compositional and microbiological characteristics of traditional and industrial dairy products and to assess whether differences in production technology are reflected in analytically measurable quality parameters. The research focuses on identifying differences between the two production systems and on outlining general trends in the compositional, physicochemical, and microbiological safety profile of the analyzed products. The results were interpreted based on experimentally determined values and statistical processing, in order to assess how production technology is reflected in the final characteristics of dairy products.

MATERIALS AND METHODS

The research was designed as a comparative study aimed at evaluating the physicochemical, microbiological, and food safety characteristics of selected industrial and traditional dairy products. The purpose of the study was to identify compositional and microbiological differences between similar products, differentiated by production technology, namely industrial or traditional processing, as well as by the animal species from which the milk originated (Petrescu-Mag et al., 2017). The study was carried out between March and April 2026 on samples of fresh milk and finished dairy products collected from the counties of Argeș, Dâmbovița, and Prahova. Traditional samples were selected to reflect products commonly available through local households and agro-food markets. Industrial samples were selected from packaged and labeled products available in national retail chains. The analyzed products were selected from both industrial and traditional sources, in order to allow a comparison between similar product categories obtained through different production technologies. The biological material analyzed consisted of fresh milk and finished dairy products obtained from cow's milk and sheep's milk. The industrial products were represented by packaged and labeled products purchased from national retail chains and originating from authorized processors. The traditional products were represented by unlabeled products obtained from individual households and agro-food markets. The products were grouped according to product type, animal species of milk origin, and production technology. In total, 14 product-species-technology categories were analyzed, as presented in Table 1. For each product-species-technology category, the analysis was based on 45 independent biological samples. Each sample was collected from a distinct production unit or individual household, ensuring that the 45 values represent independent biological observations rather than technical replicates of the same product. Samples were collected from different producers and localities across the three counties. These values were used to calculate the mean, the measure of dispersion reported in the tables, and the statistical significance of the differences between industrial and traditional products. The products included in the study are listed in Table 1.

Table 1. Dairy products analyzed and sample organization according to animal species and production technology

No.	Product analyzed	Animal species of milk origin	Production technology	No. of samples
1.	Fresh milk	Cow	Industrial	45
2.	Fresh milk	Cow	Traditional	45
3.	Fresh milk	Sheep	Industrial	45
4.	Fresh milk	Sheep	Traditional	45
5.	Burduf cheese	Sheep	Industrial	45
6.	Burduf cheese	Sheep	Traditional	45
7.	Telemea cheese	Cow	Industrial	45
8.	Telemea cheese	Cow	Traditional	45
9.	Telemea cheese	Sheep	Industrial	45
10.	Telemea cheese	Sheep	Traditional	45
11.	Urda cheese	Cow	Industrial	45
12.	Urda cheese	Cow	Traditional	45
13.	Smoked caș cheese	Cow	Industrial	45
14.	Smoked caș cheese	Cow	Traditional	45

For each product category, physicochemical parameters, microbiological parameters, and food safety indicators were evaluated. The physicochemical parameters analyzed included density, fat content, protein content, lactose content, dry matter, acidity, and pH. The microbiological parameters included total viable count and *Escherichia coli.* In addition, food safety indicators were analyzed, namely the presence of *Listeria monocytogenes*, *Salmonella* spp., and aflatoxin content. For the fresh milk samples, somatic cell count, expressed as cells/mL, was also included as a specific indicator of raw material quality.

The analyses were carried out to characterize the physicochemical, microbiological, and food safety profile of the samples included in the study. Standard reference protocols for the examination of dairy products were followed where applicable (Wehr & Frank, 2004). Physicochemical analyses were performed using a MilkoScan FT+ Fourier Transform Infrared analyzer (Foss, Denmark) for lactose determination, a Fossomatic FC somatic cell counter (Foss, Denmark) for somatic cell count, and an ELISA kit for aflatoxin M1 determination, with a limit of detection of 0.005 µg/kg. Density was determined using calibrated lactodensimeters; fat content was measured using the Gerber method; protein content was determined by the Dumas combustion method; dry matter was determined by gravimetric oven-drying; acidity was determined by Thorner titration; and pH was measured using a calibrated digital pH meter. For each analyzed product, the values of the selected parameters were determined according to the specific nature of the product and their relevance for the comparison between industrial and traditional production technologies. The physicochemical parameters determined included density, fat content, protein content, lactose content, dry matter, acidity, and pH. These indicators were used to evaluate the compositional profile of the products and to identify differences associated with the raw material and the production technology.

The microbiological parameters analyzed included total viable count and *Escherichia coli,* in order to assess the microbial load of the products. In addition, food safety indicators were evaluated, namely the presence of *Listeria monocytogenes*, *Salmonella* spp., and aflatoxin content. In the case of fresh milk, somatic cell count was also included as a specific indicator of raw material quality. The results obtained were centralized for each product, animal species, and production technology. For the quantitative parameters, the values were expressed as mean values accompanied by standard deviation or standard error of the mean, depending on the table and the statistical analysis performed. For the microbiological and food safety parameters expressed qualitatively, the results were reported using terms such as "absent" when the investigated microorganism was not detected in the analyzed samples. In the case of aflatoxin, values below the limit of detection were reported accordingly as "below the limit of detection" and were not interpreted as absolute numerical values. The data organized in this way formed the basis of the comparative analysis between industrial and traditional products, with the aim of identifying compositional, physicochemical, and microbiological differences between the two technological categories. The analyses were performed in a university laboratory and in collaboration with specialized laboratories equipped with specific instruments for physicochemical, microbiological, and food safety determinations. The determinations were carried out using standard analytical methods applied to milk and dairy products, depending on the nature of each investigated parameter (Table 2). Total viable count was determined according to ISO 4833-1:2013; Escherichia coli was enumerated using ISO 16649-2:2001; Listeria monocytogenes was detected following ISO 11290-1:2017; and Salmonella spp. was detected according to ISO 6579-1:2017.

The quantitative results were expressed in units specific to each parameter: g/cm³ for density; percentages for fat, protein, lactose, and dry matter; degrees Thorner for acidity; pH units for the acidic or alkaline reaction of the product; CFU/g for microbiological determinations; and cells/mL for somatic cell count. The qualitative results regarding the presence of *Listeria monocytogenes* and *Salmonella* spp. were expressed as "absent" when the investigated microorganisms were not detected in the analyzed samples. Aflatoxin values below the limit of detection were reported as such and

were not interpreted as absolute numerical values. For each product-species-technology category, the results obtained from the 45 analyzed samples were centralized by calculating the mean value. This method of reporting allowed the characterization of the mean level of the investigated parameters, as well as the identification of the range of variation within each product category. In the case of *Escherichia coli,* results expressed as "absent" were considered as 0 CFU/g during statistical processing. For aflatoxin, values reported below the limit of detection were retained as such and were not converted into absolute numerical values.

Table 2. Analyzed parameters and methods used

Analyzed parameter	Method used
Density	Densimeter/lactodensimeter
Fat	Gerber method
Protein	Dumas
Lactose	FTIR/MilkoScan FT+ (Foss, Denmark)
Dry matter	Gravimetric oven-drying method
Acidity	Thorner titration
pH	Digital pH meter
TVC	ISO 4833 - plate inoculation, Plate Count Agar
Escherichia coli	ISO 16649
Listeria monocytogenes	ISO 11290
Salmonella spp.	ISO 6579
Aflatoxin	ELISA
Somatic cells	Fossomatic FC (Foss, Denmark)

Statistical analysis: The data obtained were statistically processed using Google Sheets. For each analyzed parameter, the arithmetic mean and standard deviation were calculated, and the comparison between industrial and traditional products was performed by determining the absolute and percentage differences between the mean values. Prior to group comparisons, the normality of the data distribution was verified using the Shapiro-Wilk test, and the homogeneity of variances was assessed using the Levene test. The comparison of means between industrial and traditional products was carried out using Welch's t-test (Welch, 1947), a variant of Student's t-test used to compare the means of two independent groups without assuming equal variances. The threshold for statistical significance was set at $p < 0.05$, while highly significant differences were reported as $p < 0.001$. Effect sizes were calculated using Cohen's d to assess the practical significance of the differences between groups. The 95% confidence intervals (95% CI) for the mean values were also calculated to quantify the precision of the estimates. Correlations were also analyzed between selected parameters considered relevant for the compositional and technological interpretation of the products, such as acidity-pH, acidity-lactose, dry matter-fat, and dry matter-protein. Pearson correlation coefficients (Pearson, 1895) were calculated between parameters relevant to the compositional, fermentative, and microbiological interpretation of the products. The statistical processing aimed to identify comparative trends between industrial and traditional products, both within each product category and across the entire set of analyzed products. Data processing was based on the results obtained from the analyses performed on the samples included in the study.

RESULTS AND DISCUSSIONS

The analysis of compositional parameters revealed clear differences between industrial and traditional dairy products (Table 3). This category included fat, protein, lactose, dry matter, and density.

The results show that traditional products had higher mean values for fat, protein, and dry matter in all seven pairs compared, with statistically significant differences. By contrast, lactose values were higher in industrial products in six of the seven comparisons, while density showed slightly higher values in industrial products in most cases, although the magnitude of these differences was limited.

Fat content was higher in traditional products across all analyzed pairs. The differences were highly statistically significant for all products, indicating a consistent tendency for traditional variants to have a more concentrated lipid profile. The largest percentage differences were observed in fresh sheep's milk, where the traditional variant had a much higher mean

value than the industrial one, namely 7.67% compared with 1.50%. This substantial difference (411%) is explained by the fact that the industrial sheep's milk analyzed was a commercially standardized product, subjected to fat-standardization processes that reduce the original lipid content to meet specific product specifications. Normal ovine milk fat content ranges between 6% and 9%, and the traditional samples fell within this expected range. The industrial product, in contrast, had undergone partial skimming and standardization prior to packaging, which accounts for the markedly reduced fat value. This difference therefore reflects a technological distinction rather than a biological anomaly. Relevant differences were also observed in sheep Telemea cheese, where the mean fat content was 27.43% in the traditional product compared with 21.77% in the industrial product, as well as in Burduf cheese, where the values were 32.37% in the traditional product and 28.47% in the industrial product. This trend suggests that the traditional products analyzed had a higher lipid content, possibly associated with the use of less standardized raw material and with technological processes that lead to a greater concentration of solid components.

Protein content followed the same direction as fat content, with mean values being higher in traditional products in all comparisons. The differences were highly statistically significant for all analyzed pairs. For example, traditional Burduf cheese had a mean protein content of 20.70%, compared with 19.50% in the industrial variant. In smoked caș cheese, the values were 19.20% for the traditional product and 18.40% for the industrial product, while in cow's milk Telemea cheese the values were 16.83% compared with 15.97%. Although the percentage differences for protein were more moderate than those observed for fat, the trend was consistent and supports the idea of a richer compositional profile in traditional products. This aspect is important because proteins represent one of the main indicators of the nutritional value of dairy products.

Dry matter was also higher in traditional products in all analyzed comparisons. The differences were highly statistically significant, confirming the general tendency toward compositional concentration in traditional products. The most evident differences were observed in fresh sheep's milk, where dry matter was 19.47% in the traditional variant and 11.80% in the industrial variant, as well as in Burduf cheese, where the values were 56.87% in the traditional product and 51.93% in the industrial product.

The higher dry matter values observed in traditional products are consistent with the higher fat and protein values. This result supports the hypothesis that the traditional products analyzed contained a higher proportion of solid components and, consequently, had a more pronounced nutritional density compared with industrial products.

Lactose showed a different trend compared with fat, protein, and dry matter. In six of the seven comparisons, mean lactose values were higher in industrial products. The differences were highly statistically significant for all analyzed pairs. This trend is particularly evident in fermented or processed products, such as Burduf cheese, smoked caș cheese, Telemea cheese, and Urda cheese. For example, in sheep's milk Burduf cheese, mean lactose content was 0.50% in the industrial product and 0.30% in the traditional product. In smoked caș cheese, the values were 1.50% in the industrial product and 1.03% in the traditional product, while in sheep's milk Telemea cheese they were 1.20% compared with 0.93%. The lower lactose values observed in traditional products may be associated with more intense fermentative activity, in which lactose is used as a substrate by lactic microflora. The only exception was fresh cow's milk, where lactose was slightly higher in the traditional variant. This difference was limited in magnitude and should be interpreted in the context of the natural variability of raw milk and the standardization of industrial products.

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Table 3. Compositional parameters

Parameter	Product	Species	Industrial	Traditional	Dif. T vs I%	p-value	Statistical significance
Density (g/cm^3)	Burduf cheese	Sheep	1.0810 ± 0.0017	1.0805 ± 0.0021	-0.05%	p = 0.218	NS
	Smoked caș cheese	Cow	1.0585 ± 0.0003	1.0560 ± 0.0007	-0.24%	p < 0,01	**
	Fresh milk	Sheep	1.0360 ± 0.0007	1.0345 ± 0.0008	-0.14%	p < 0,05	*
	Fresh milk	Cow	1.0290 ± 0.0006	1.0290 ± 0.0011	0.00%	p = 1.000	NS
	Telemea cheese	Sheep	1.0740 ± 0.0008	1.0730 ± 0.0010	-0.09%	p = 0.10	NS
	Telemea cheese	Cow	1.0645 ± 0.0009	1.0635 ± 0.0009	-0.09%	p =0.110	NS
	Urda cheese	Cow	1.0450 ± 0.0006	1.0435 ± 0.0003	-0.14%	p < 0,05	*
Fat (%)	Burduf cheese	Sheep	28.47 ± 0.240	32.37 ± 0.530	13.70%	p < 0,001	***
	Smoked caș cheese	Cow	21.90 ± 0.111	24.67 ± 0.423	12.65%	p < 0,001	***
	Fresh milk	Sheep	1.50 ± 0.030	7.67 ± 0.215	411.33%	p < 0,001	***
	Fresh milk	Cow	3.67 ± 0.113	3.97 ± 0.078	8.17%	p < 0,001	***
	Telemea cheese	Sheep	21.77 ± 0.516	27.43 ± 0.522	26.00%	p < 0,001	***
	Telemea cheese	Cow	19.40 ± 1.107	21.43 ± 0.476	10.46%	p < 0,001	***
	Urda cheese	Cow	4.40 ± 0.141	6.10 ± 0.157	38.64%	p < 0,001	***
Lactose (%)	Burduf cheese	Sheep	0.50 ± 0.067	0.30 ± 0.071	-40.00%	p < 0,001	***
	Smoked caș cheese	Cow	1.50 ± 0.067	1.03 ± 0.087	-31.32%	p < 0,001	***
	Fresh milk	Sheep	4.50 ± 0.030	4.40 ± 0.060	-2.22%	p < 0,001	***
	Fresh milk	Cow	4.60 ± 0.060	4.67 ± 0.021	1.50%	p < 0,001	***
	Telemea cheese	Sheep	1.20 ± 0.074	0.93 ± 0.085	-22.50%	p < 0,001	***
	Telemea cheese	Cow	1.80 ± 0.060	1.53 ± 0.075	-15.00%	p < 0,001	***
	Urda cheese	Cow	3.27 ± 0.076	2.80 ± 0.077	-14.37%	p < 0,001	***
Protein (%)	Burduf cheese	Sheep	19.50 ± 0.171	20.70 ± 0.380	6.15%	p < 0,001	***
	Smoked caș cheese	Cow	18.40 ± 0.098	19.20 ± 0.242	4.35%	p < 0,001	***
	Fresh milk	Sheep	5.60 ± 0.064	5.80 ± 0.052	3.57%	p < 0,001	***
	Fresh milk	Cow	3.27 ± 0.019	3.37 ± 0.020	3.05%	p < 0,001	***
	Telemea cheese	Sheep	18.27 ± 0.108	19.13 ± 0.299	4.71%	p < 0,001	***
	Telemea cheese	Cow	15.97 ± 0.190	16.83 ± 0.200	5.39%	p < 0,001	***
	Urda cheese	Cow	12.53 ± 0.134	13.20 ± 0.158	5.35%	p < 0,001	***
Dry Matter (%)	Burduf cheese	Sheep	51.93 ± 0.265	56.87 ± 0.819	9.51%	p < 0,001	***
	Smoked caș cheese	Cow	46.40 ± 0.177	49.60 ± 0.484	6.90%	p < 0,001	***
	Fresh milk	Sheep	11.80 ± 0.165	19.47 ± 0.196	65.00%	p < 0,001	***
	Fresh milk	Cow	12.20 ± 0.060	12.87 ± 0.156	5.49%	p < 0,001	***
	Telemea cheese	Sheep	48.50 ± 0.220	51.77 ± 0.600	6.74%	p < 0,001	***
	Telemea cheese	Cow	40.87 ± 1.038	42.83 ± 0.527	4.80%	p < 0,001	***
	Urda cheese	Cow	22.53 ± 0.131	24.57 ± 0.342	9.05%	p < 0,001	***

NS – non-significant differences between means ($p > 0.05$); * – significant differences ($p < 0.05$); ** – distinctly significant differences ($p < 0.01$); *** – highly significant differences ($p < 0.001$).

In smoked caș cheese, the values were 1.50% in the industrial product and 1.03% in the traditional product, while in sheep's milk Telemea cheese they were 1.20% compared with 0.93%. The lower lactose values observed in traditional products may be associated with more intense fermentative activity, in which lactose is used as a substrate by lactic microflora. The only exception was fresh cow's milk, where lactose was slightly higher in the traditional variant. This difference was limited in magnitude and should be interpreted in the context of the natural variability of raw milk and the standardization of industrial products.

Density was practically identical between the two systems in cow's milk ($p = 1.000$), while a statistically significant difference was observed in sheep's milk (-0.14%, $p < 0.05$). This suggests that the basic parameters of raw milk are relatively uniform regardless of origin, whether industrial or traditional, and that collection and standardization processes reduce natural variability.

In semi-hard and hard cheeses, such as Telemea cheese and Burduf cheese, the differences were small, ranging from -0.05% to -0.09%, and were not statistically significant. This indicates that the manufacturing technologies used for these products are sufficiently standardized in both systems, leading to products with similar physical characteristics. Products with statistically significant differences included smoked caș cheese and Urda cheese, which are associated with less rigid manufacturing processes in traditional production, such as artisanal smoking, variability in whey drainage time (Scarlat et al., 2025), and less controlled processing temperatures. The lower density observed in the traditional variants may reflect a slightly higher water content or a less compact structure, resulting from less uniform manual processing. Therefore, density may be considered an auxiliary physicochemical parameter, useful for the general characterization of the products, but less relevant as a main indicator of compositional differences between industrial and traditional dairy products.

Overall, the analysis of compositional parameters shows that the traditional products analyzed had a more concentrated profile, reflected in higher values of fat, protein, and dry matter. By contrast, industrial products generally showed higher lactose values, which may reflect lower fermentative activity or a higher degree of technological standardization. These results support the idea that the differences between industrial and traditional products are not only technological or commercial in nature, but are directly reflected in the physicochemical composition of the analyzed products.

The analysis of acidity and pH highlights a clear differentiation between industrial and traditional dairy products (Table 4). In all seven analyzed pairs, traditional products showed higher mean acidity values compared with industrial products, and the differences were statistically significant. This trend was observed both in fresh milk and in processed products, such as Burduf cheese, Telemea cheese, smoked caș cheese, and Urda cheese. The largest absolute differences in acidity were recorded in sheep's milk Burduf cheese, where the traditional product had a mean acidity of 207.00°T, compared with 178.33°T in the industrial product. Important differences were also observed in sheep's milk Telemea cheese, with 158.67°T in the traditional variant and 139.67°T in the industrial variant, as well as in cow's milk Telemea cheese, where acidity was 123.33°T in the traditional product and 110.00°T in the industrial product. These results indicate a consistent tendency for traditional products to show a higher level of acidity.

At the same time, pH values followed an opposite trend (Table 4). Industrial products showed higher mean pH values in all comparisons, which means that traditional products had a more acidic character. For example, in Burduf cheese, the mean pH was 4.72 in the industrial product and 4.58 in the traditional product. In cow's milk Telemea cheese, the values were 5.16 for the industrial product and 4.97 for the traditional product, while in smoked caș cheese, pH was 5.46 in the industrial variant and 5.26 in the traditional variant. This relationship between acidity and pH is consistent from a physicochemical perspective. An increase in acidity is generally accompanied by a decrease in pH, reflecting the accumulation of acidic compounds formed during fermentative processes.

In the traditional products analyzed, the higher acidity values and lower pH values may suggest more intense fermentative activity or a lower degree of standardization in the technological process. The relationship with lactose supports this interpretation. In six of the seven analyzed pairs, industrial products showed higher lactose values than traditional products. This trend is important because lactose is the main fermentable substrate in milk. The reduction in lactose content observed in traditional products, together with higher acidity and lower pH, may indicate a more pronounced conversion of lactose into organic acids, especially lactic acid.

Table 4. Relationship between acidity, pH, and lactose in the analyzed products

Product	Species	Acidity I (°T)	Acidity T (°T)	pH I	pH T	Lactose I (%)	Lactose T (%)	Observed relationship
Burduf cheese	Sheep	178.33	207.00	4.72	4.58	0.50	0.30	T: acidity ↑, pH ↓, lactose ↓
Smoked caș cheese	Cow	84.33	94.00	5.46	5.26	1.50	01.03	T: acidity ↑, pH ↓, lactose ↓
Fresh milk	Sheep	21.17	23.33	6.51	6.44	4.50	4.40	T: acidity ↑, pH ↓, lactose ↓
Fresh milk	Cow	16.67	18.17	6.65	6.56	4.60	4.67	T: acidity ↑, pH ↓, lactose ↑
Telemea cheese	Sheep	139.67	158.67	4.90	4.76	1.20	0.93	T: acidity ↑, pH ↓, lactose ↓
Telemea cheese	Cow	110.00	123.33	5.16	4.97	1.80	1.53	T: acidity ↑, pH ↓, lactose ↓
Urda cheese	Cow	44.00	54.00	6.16	5.96	3.27	2.80	T: acidity ↑, pH ↓, lactose ↓

Note: I = industrial product; T = traditional product; ↑ = higher value; ↓ = lower value.

As shown in Table 4, traditional products exhibited higher acidity and lower pH values in all analyzed pairs. In most cases, this relationship was accompanied by lower lactose values, supporting the hypothesis of a more pronounced fermentative profile. The exception was fresh cow's milk, where lactose was slightly higher in the traditional variant, without altering the overall trend regarding acidity and pH. This observation is more evident in fermented or ripened products. In burduf cheese, lactose was lower in the traditional product, namely 0.30%, compared with 0.50% in the industrial product. In smoked caș cheese, lactose was 1.03% in the traditional product and 1.50% in the industrial one, while in sheep telemea cheese the values were 0.93% and 1.20%, respectively. These differences, correlated with higher acidity and lower pH, support the existence of a more pronounced fermentative profile in traditional products. Therefore, the obtained data indicate a logical relationship among the three indicators: higher acidity, lower pH, and lower lactose content. This combination is characteristic of products in which fermentative processes are more pronounced. In traditional products, this profile may be associated both with the use of a more diverse natural microflora and with less standardized technological control compared with industrial production.

Overall, the results regarding acidity, pH, and lactose highlight the existence of consistent physicochemical differences between traditional and industrial dairy products. Traditional products showed higher acidity values and lower pH values in all analyzed pairs, and in most cases, they also had a lower lactose content. This association between higher acidity, lower pH, and reduced lactose suggests a more pronounced fermentative profile of traditional products. This interpretation is supported by the fact that lactose is the main fermentable substrate in milk, and its reduction, together with increased acidity, may indicate a more intense conversion into acidic compounds, particularly lactic acid. Therefore, the analyzed traditional products appear to reflect more active or less standardized fermentative processes compared with industrial products. However, this interpretation should be made with caution, as fermentation intensity was not measured directly, but inferred based on the relationship among the analyzed parameters. The observed differences may also be influenced by other factors, such as the quality and composition of the raw material, specific microflora, ripening duration, storage conditions, and the particularities of the technological process. Thus, the data support the existence of a more pronounced acidic and fermentative profile in traditional products, without allowing these differences to be attributed exclusively to a single technological factor.

The microbiological and food safety analysis aimed to highlight the differences between industrial and traditional dairy products from the perspective of the overall microbial load, hygiene indicators, and food safety indicators. This section analyzes the results regarding the total viable count, *Escherichia coli,* somatic cell count, as well as the safety indicators represented by *Salmonella* spp., *Listeria monocytogenes*, and aflatoxin.

Table 5. Comparison of microbiological, hygiene, and food safety indicators in industrial and traditional dairy products

Parameter	Product	Species	Industrial, mean ± SEM	Traditional, mean ± SEM	Absolute difference	Difference, %	p	Trend
TVC	Burduf cheese	Sheep	41333 ± 4008	135000 ± 14746	93667	226.62%	$p < 0.001$	T > I
TVC	Smoked caş cheese	Cow	19667 ± 1947	70333 ± 7142	50666	257.62%	$p < 0.001$	T > I
TVC	Fresh milk	Sheep	28333 ± 3582	115000 ± 16619	86667	305.89%	$p < 0.001$	T > I
TVC	Fresh milk	Cow	14333 ± 1998	59333 ± 7987	45000	313.96%	$p < 0.001$	T > I
TVC	Telemea cheese	Sheep	28333 ± 2191	105026 ± 8923	76693	270.68%	$p < 0.001$	T > I
TVC	Telemea cheese	Cow	22667 ± 2004	79333 ± 6955	56666	249.99%	$p < 0.001$	T > I
TVC	Urda cheese	Cow	3633 ± 530	7933 ± 899	4300	118.36%	$p < 0.001$	T > I
E. coli	Burduf cheese	Sheep	3.33 ± 2.14	35.00 ± 11.58	31.67	950.00%	$p < 0.001$	T > I
E. coli	Smoked caş cheese	Cow	3.33 ± 2.08	15.00 ± 2.86	11.67	350.00%	$p < 0.001$	T > I
E. coli	Fresh milk	Sheep	3.33 ± 2.07	30.00 ± 8.19	26.67	800.00%	$p < 0.001$	T > I
E. coli	Fresh milk	Cow	0.00 ± 0.00	13.33 ± 4.78	13.33	NA	$p < 0.001$	T > I
E. coli	Telemea cheese	Sheep	5.00 ± 2.77	31.67 ± 4.76	26.67	533.40%	$p < 0.001$	T > I
E. coli	Telemea cheese	Cow	3.33 ± 2.07	26.67 ± 7.14	23.34	700.10%	$p < 0.001$	T > I
E. coli	Urda cheese	Cow	3.33 ± 2.12	8.33 ± 3.09	5	150.00%	$p < 0.001$	T > I
SCC	Fresh milk	Sheep	398333 ± 8091	583333 ± 39500	185000	46.44%	$p < 0.001$	T > I
SCC	Fresh milk	Cow	196667 ± 7191	336667 ± 27016	140000	71.19%	$p < 0.001$	T > I

The total viable count was higher in traditional products in all analyzed pairs, with the differences compared with industrial products being statistically significant, as shown in Table 5. This trend indicates a higher overall microbial load in products obtained by traditional methods. The largest differences were observed in sheep burduf cheese, where the traditional product recorded a mean value of 135,000 CFU/g, compared with 41,333 CFU/g in the industrial product. Important differences were also identified in fresh sheep milk, with 115,000 CFU/g in the traditional variant and 28,333 CFU/g in the industrial one, as well as in sheep telemea cheese, where the values were 105,025.91 CFU/g for the traditional product and 28,333 CFU/g for the industrial product. The higher TVC values in traditional products may be associated with the particularities of traditional processing, the use of a more diverse natural microflora, and a lower degree of standardization of production, handling, and storage conditions. By contrast, the lower values observed in industrial products may reflect stricter technological control and more standardized processing conditions. However, TVC should be interpreted as an indicator of the overall microbial load and not as a direct indicator of the presence of pathogenic microorganisms. In fermented dairy products, a higher microbial load may also include the flora specific to fermentative processes; therefore, the interpretation of this parameter should be correlated with the other microbiological and food safety indicators analyzed.

The values obtained for *Escherichia coli* highlighted clear differences between industrial and traditional products. In all analyzed pairs, traditional products showed higher mean values compared with industrial products, with the differences being statistically significant. The largest differences were observed in sheep burduf cheese, where the traditional product recorded a mean value of 35.00 CFU/g, compared with 3.33 CFU/g in the industrial product. Higher values were also observed in sheep telemea cheese, with 31.67 CFU/g in the traditional variant and 5.00 CFU/g in the industrial variant, as well as in fresh sheep milk, where the mean value was 30.00 CFU/g for the traditional product and 3.33 CFU/g for the industrial product. The presence of higher

Escherichia coli values in traditional products may indicate greater variability in hygiene-related conditions associated with processing, handling, or storage. Unlike industrial products, where the technological process is more standardized and controlled, traditional products may more strongly reflect the influence of local production conditions and hygiene practices. However, these results should be interpreted with caution. The values obtained for *Escherichia coli* are indicators of hygiene-related quality, not direct evidence of the presence of specific pathogenic agents. Therefore, the observed differences indicate a higher hygiene-related microbiological load in traditional products, without allowing a general conclusion to be drawn regarding the safety of all products in this category. The somatic cell count was analyzed only in fresh milk samples, as it is a relevant indicator for assessing the quality of the raw material. The results showed higher values in traditional fresh milk compared with industrial fresh milk, both for cow's milk and sheep's milk, with the differences being statistically significant. In fresh cow's milk, the mean SCC value was 336,667 cells/ml in the traditional variant, compared with 196,667 cells/ml in the industrial variant. For fresh sheep's milk, the difference was also evident, with a mean value of 583,333 cells/ml in the traditional product, compared with 398,333 cells/ml in the industrial product. The higher somatic cell count values in traditional milk may reflect greater variability in raw material quality, associated with differences in the physiological status of the animals, milking hygiene, collection conditions, and the level of control applied before processing. In industrial products, the lower values may be correlated with standardized collection, selection of raw material, and the application of more rigorous controls throughout the production chain. Therefore, the SCC results support the distinction between a more controlled industrial system and a more variable traditional system, without allowing direct conclusions to be drawn regarding the health status of the animals or the overall quality of all products obtained from traditional sources.

In addition to the quantitative microbiological indicators, food safety indicators were also analyzed, namely *Salmonella* spp., *Listeria monocytogenes*, and aflatoxin. These parameters were treated separately from TVC, *Escherichia coli,* and SCC, because the results were expressed predominantly qualitatively or in relation to the limit of detection. For *Salmonella* spp. and *Listeria monocytogenes*, the results indicated the absence of these microorganisms in the analyzed samples. This represents a favorable result from a food safety perspective, as these two agents are considered important indicators for assessing microbiological risks associated with food products, in accordance with the microbiological criteria established by European Commission (2005). In the case of aflatoxin, values reported below the limit of detection were retained as such and were not included in the calculation of the standard deviation or coefficient of variation. The maximum permissible levels for aflatoxins in dairy products are established by European Commission (2023). This approach was necessary because results expressed as "below the limit of detection" do not represent absolute numerical values, but indicate that the determined level was below the quantification capacity of the method used. Therefore, in the analyzed samples, the food safety indicators did not reveal relevant contamination with *Salmonella* spp. or *Listeria monocytogenes*, while aflatoxin was treated as a parameter reported in relation to the limit of detection. These results complement the general microbiological analysis, providing an additional perspective on the hygiene and safety profile of the investigated products.

The microbiological analysis highlighted higher TVC and *Escherichia coli* values in traditional products across all compared pairs, indicating a higher microbial load and a more variable hygiene and safety profile compared with industrial products. In the case of fresh milk, the higher somatic cell count values in the traditional variants also suggest greater variability in raw material quality. These results may be associated with the particularities of traditional processing, a more diverse natural microflora, and a lower degree of standardization of production, handling, and storage conditions. However, higher values of microbiological indicators should not be automatically interpreted as a lack of food

safety, especially in the case of fermented products. The absence of *Salmonella* spp. and *Listeria monocytogenes* in the analyzed samples represents a favorable result from a food safety perspective. Therefore, the analyzed traditional products present a higher and more variable microbiological profile, whereas industrial products reflect a higher level of standardization and food safety control.

The comparative analysis of industrial and traditional dairy products revealed clear differences between the two technological categories for most of the investigated parameters. The synthesized results show that traditional products presented higher mean values for acidity, fat, protein, dry matter, total viable count, *Escherichia coli,* and somatic cell count, whereas industrial products recorded higher values for pH, lactose, and, in most comparisons, density. The Pearson correlations calculated between selected parameters are summarized in Table 6.

Table 6. Summary of Pearson correlations among relevant compositional, fermentative, and microbiological parameters

Analyzed relationship	No. of correlations	Pearson r range	Predominant type of correlation	Interpretative relevance
Acidity - pH	14	-0.350 – 0.276	weak/variable	Relationship evident at the level of comparative means, but weak within groups
Acidity - Lactose	14	-0.176 – 0.226	weak/variable	Cautiously supports the fermentative interpretation
Dry matter - Fat	14	-0.289 – 0.234	weak/variable	Associates solid components with fat content
Dry matter - Protein	14	-0.330 – 0.295	weak/variable	Associates dry matter with protein content
TVC - *E. coli*	14	-0.200 – 0.137	weak/variable	Associates TVC with the hygiene-related indicator

Note: Pearson's r indicates the direction and strength of the linear relations between the analyzed parameters. Positive values indicate a direct relationship, while negative values indicate an inverse relationship.

The calculated correlations revealed variable relationships among the analyzed parameters. In particular, the acidity-pH and acidity–lactose relationships were generally weak within each product-species-technology category, indicating that the trends observed at the level of comparative means are not always expressed as strong linear relationships within groups. Therefore, Pearson correlations were used as a complementary interpretative tool, rather than as the sole argument for explaining the differences between industrial and traditional products.

Discussion: The results obtained allow clear differences to be outlined between industrial and traditional dairy products from compositional, physicochemical, and microbiological perspectives. The comparative analysis showed that traditional products generally presented higher values of fat, protein, and dry matter, suggesting a more concentrated compositional profile compared with industrial products. This tendency was consistent across all analyzed pairs and was statistically supported. From the perspective of physicochemical parameters, traditional products recorded higher acidity and lower pH values, and in most cases had a lower lactose content. The association between increased acidity, reduced pH, and lower lactose indicates a profile compatible with more pronounced fermentative processes. However, this interpretation should be viewed with caution, as fermentation intensity was not measured directly, but inferred based on the relationship among the analyzed parameters. From a microbiological perspective, traditional products showed higher TVC and *Escherichia coli* values, while in the case of fresh milk, higher somatic cell count values were observed. These results suggest a higher microbial load and greater hygiene-related variability in traditional products. By contrast, industrial products showed a more controlled profile, an aspect that may be associated with the standardization of technological processes, raw material control, and more rigorous processing conditions. The overall interpretation of the results indicates the existence of a dual profile of traditional products. On the one hand, traditional products stand out through a richer compositional profile, supported by higher values of fat, protein, and dry matter, suggesting a higher concentration of nutritional

components. The microbiological richness of traditional products, reflected in higher total viable count values, may also indicate a more diverse natural microbial community, including lactic acid bacteria with potential probiotic properties. Additionally, traditional products carry recognized cultural, heritage, and sensory value, being strongly associated with local identity and specific production practices. On the other hand, the higher values of microbiological indicators, including Escherichia coli and total viable count, and the higher somatic cell count in traditional milk, highlight the need for careful hygiene and safety control during production, handling, and commercialization. The greater variability in composition observed in traditional products is an inherent consequence of less standardized raw materials and technologies. Industrial products are characterized by a higher degree of standardization, a more controlled microbiological profile, and greater uniformity, which are clear advantages from the perspective of large-scale food safety management. Therefore, the differences between industrial and traditional products should not be interpreted unilaterally, in terms of the absolute superiority of one category over the other. Traditional products can be valued for their compositional density, technological specificity, and fermentative profile that may suggest more active microbial communities; whereas industrial products are distinguished by uniformity, predictability, and hygiene-related stability. The present study has several limitations that should be acknowledged. The samples were collected exclusively from the South-Muntenia region (counties of Argeș, Dâmbovița, and Prahova) during a limited time period (March-April 2026), and the results may not reflect the diversity of traditional dairy systems across different geographic areas, production seasons, or agro-climatic conditions. The study was observational in design; therefore, no causal inferences can be drawn regarding the mechanisms underlying the observed differences. Furthermore, the microbiological profile of the products was characterized only in terms of total viable count and selected hygiene and safety indicators; a comprehensive characterization of the microbial communities, for example through metagenomics, was beyond the scope of the present work. Overall, the results support the idea that the two product categories have distinct profiles, and their evaluation should be carried out in an integrated manner, by correlating compositional, physicochemical, microbiological, and statistical parameters.

This distribution of results indicates the existence of two distinct profiles. On the one hand, traditional products stand out through a more concentrated compositional profile, supported by higher values of fat, protein, and dry matter. On the other hand, they present higher acidity values and lower pH values, suggesting a more pronounced fermentative character compared with industrial products. The obtained results are in line with observations reported in recent literature regarding the influence of the production system on the compositional and microbiological characteristics of dairy products. In the present study, traditional products generally showed higher values of fat, protein, dry matter, and acidity, as well as lower values of pH and lactose, suggesting a more concentrated compositional profile and a fermentative evolution that may be more pronounced compared with industrial products, although this interpretation should be viewed with caution as fermentation intensity was not measured directly. This tendency is close to the conclusions formulated by Maciuc et al. (2024), who highlighted that cheeses obtained under the "mountain product" quality scheme presented favorable nutritional differences compared with conventional products, particularly through a higher content of minerals and polyunsaturated fatty acids. Although the axis of comparison is not identical, namely traditional–industrial in the present study and mountain product–conventional in the cited study, both studies support the idea that less standardized production systems, or systems closer to the traditional model, may lead to nutritional differentiation in dairy products. From a microbiological perspective, traditional products showed higher values of total viable count and *Escherichia coli* in all comparisons performed. This tendency may be associated with a lower degree of technological standardization, the particularities of traditional processing, and variations in handling, hygiene,

and storage conditions. At the same time, these results should be interpreted in the context of the nature of the analyzed products, without automatically equating higher microbial load values with a lack of food safety. The higher values of total viable count and *Escherichia coli,* without evidence of contamination with *Salmonella spp*. or *Listeria monocytogenes*, can be compared with the results reported by Di Cerbo et al. (2020) for "Burrata di Andria" PGI, where the authors found the absence of *Salmonella* spp. and *Listeria monocytogenes*, but observed differences in microbiological load, including *Escherichia coli,* depending on the use of raw or pasteurized milk. Similarly to the Italian study, the results obtained in the present research indicate that a higher microbiological load should not be automatically interpreted as a lack of food safety, but should be analyzed in relation to the nature of the product, the production technology, the degree of standardization, and the specific food safety indicators.

Therefore, comparing the present results with those reported by Maciuc et al. (2024) and Di Cerbo et al. (2020) supports the conclusion that dairy products obtained through traditional, artisanal, or less standardized systems may have a more intensely expressed compositional profile, but also greater microbiological variability, which requires the simultaneous interpretation of nutritional, fermentative, and hygiene and safety parameters. From the perspective of statistical significance, most differences between industrial and traditional products were statistically significant, with the threshold used for interpretation being $p < 0.05$. For most of the analyzed parameters, the differences were reported at $p < 0.001$, indicating a consistent separation between the two product categories. However, in the case of density, although some differences were statistically significant, their magnitude was small, suggesting more limited practical relevance compared with parameters such as fat, dry matter, acidity, or microbiological indicators.

Overall, the results support the fact that the analyzed traditional products present a more intensely expressed compositional and fermentative profile, whereas industrial products are characterized by a more standardized profile and lower values of the analyzed microbiological indicators. This synthesis integrates the main findings obtained from the detailed analysis of the compositional, physicochemical, and microbiological parameters.

The results obtained in the present study are consistent with findings reported in studies that compared raw-milk, traditional, or artisanal cheeses with products obtained under more controlled technological conditions. Rashtchi et al. (2021), in their study on raw and pasteurized Lighvan cheeses during ripening, showed that the use of raw milk was associated with more intense microbial and sensory characteristics, but also with higher levels of hygiene-related microorganisms, including coliforms and Escherichia coli. This observation is comparable to the present results, where traditional dairy products showed higher total viable count and Escherichia coli values than industrial products, suggesting greater microbiological variability in less standardized production systems. At the same time, the lower lactose values, higher acidity, and lower pH observed in traditional products in the present study support the interpretation of a more pronounced fermentative profile, similar to the differences reported between raw and pasteurized cheese systems.

A similar interpretation can be made in relation to the study by Pappa et al. (2022), who analyzed traditional hard Xinotyri cheese produced from raw or pasteurized goat milk. Their results emphasized the role of natural microbiota and ripening in shaping the microbiological and biochemical characteristics of traditional cheese.

In the present study, the higher acidity and lower pH values observed in traditional products, together with the reduction in lactose in most comparisons, suggest that traditional processing may favor a more active or less standardized fermentative evolution. However, as in the case of Xinotyri cheese, these differences should be interpreted in relation to product type, raw material, technological practices, and storage or ripening conditions. The findings are also supported by the recent characterization of traditional Sicilian Canestrato Fresco cheese reported by Pisana et al. (2025), who described a traditional raw cow's milk cheese produced by

artisanal methods and characterized by a rich microbiological profile, with high levels of lactic acid bacteria.

This perspective is relevant for the present study because it supports the idea that traditional dairy products may have a more complex microbiological profile, which can contribute to their specific compositional and fermentative characteristics. Nevertheless, this greater microbiological complexity must be interpreted together with hygiene and food safety indicators. In the present study, although traditional products had higher TVC and *Escherichia coli* values, *Salmonella* spp. and *Listeria monocytogenes* were absent in all samples, and aflatoxin values were below the detection limit. Therefore, the comparison with these studies supports the main conclusion that traditional dairy products should not be evaluated as simply superior or inferior to industrial products, but as products with a distinct profile: richer in composition, more expressive from a fermentative point of view, but also more variable microbiologically.

CONCLUSIONS

The results obtained highlight the existence of two distinct profiles of the analyzed dairy products, differentiated by production technology. Traditional products stood out through a more concentrated compositional profile, supported by higher mean values of fat, protein, and dry matter in all analyzed pairs. These characteristics indicate a higher concentration of nutritional components and reflect the influence of the raw material, natural microflora, and the particularities of traditional processing.

At the same time, traditional products showed a physicochemical profile compatible with more pronounced fermentative processes, expressed through higher acidity, lower pH, and, in most cases, lower lactose content. By comparison, industrial products were distinguished by a more standardized and controlled profile, reflected in the uniformity of the analyzed parameters and lower values of microbiological indicators, particularly TVC and *Escherichia coli.*

The statistical analysis confirmed the existence of significant differences between industrial and traditional products for most of the analyzed parameters, supporting the trends observed descriptively. However, the interpretation of these differences should be made by correlating statistical significance with the practical and technological relevance of each parameter.

The traditional and industrial dairy products should not be interpreted in terms of absolute superiority, but rather as products with different profiles. Traditional products are characterized by compositional complexity and a fermentative profile that may suggest more active microbial communities, whereas industrial products stand out through uniformity, predictability, and more rigorous microbiological control. This synthesis brings together the main findings obtained from the detailed analysis of the compositional, physicochemical, and microbiological parameters and highlights the overall differences between industrial and traditional dairy products. The present study has several limitations that should be considered when interpreting the results. Sample collection was limited to one Romanian region (South-Muntenia: Argeș, Dâmbovița, and Prahova counties) during a restricted time period, and the findings may not be generalizable to the full diversity of traditional dairy production systems in Romania or other geographic areas. The observational design of the study does not allow causal relationships between production technology and product characteristics to be established. In addition, the microbiological community of the products was not comprehensively characterized, as metagenomics or culture-independent methods were not applied. Future research should consider a wider geographic and seasonal sampling strategy, the integration of metagenomic analyses for the characterization of the microbial communities of traditional dairy products, sensory analysis with trained panels, fatty acid profiling including conjugated linoleic acid, and longitudinal studies across production seasons to better capture the natural variability of traditional production systems.

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EVALUATION OF SLAUGHTER PERFORMANCE AND CARCASS TRAITS IN POULTRY FED *Hermetia illucens* – BASED DIETS

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Abstract

The rising demand for sustainable protein sources in poultry production has highlighted insects as promising feed alternatives. This study assessed the effects of dietary inclusion of Black Soldier Fly (BSF) larvae on slaughter performance and carcass traits in broilers. Birds were assigned to a control group and three experimental groups receiving 2.0%, 3.5%, or 5.0% Hermetia illucens larvae meal. Haematological and biochemical parameters remained within physiological ranges, indicating normal health and metabolic status. Experimental lines showed improved carcass composition, with increased breast muscle weight, progressive sternum growth, and enhanced thigh and shank musculature. Combined hip and thigh weights and shank muscle mass were higher in experimental groups. These results demonstrate that BSF larvae can be safely incorporated into broiler diets, supporting growth, carcass yield, and meat quality. Furthermore, the experimental lines exhibited superior muscular development and carcass traits, suggesting their higher suitability for meat production. The findings highlight the potential of insect-based diets to enhance sustainable poultry production without compromising animal health or product quality.

Key words*: black soldier fly, broiler chickens, carcass traits, Hermetia illucens, slaughter performance.*

INTRODUCTION

Global demand for poultry products is steadily increasing due to their high nutritional value, cost-effective production, and rapid growth rate (Neeteson et al., 2023; Burnside et al., 2025). Chicken meat is recognized as an important source of high-quality protein and essential amino acids, playing a significant role in human nutrition and health. In the European Union, poultry meat remains one of the most widely consumed animal protein sources, with a continuous upward trend in demand. According to the Global Agricultural Information Network (GAIN) of the USDA, chicken consumption in the EU is projected to increase by 1.6% in 2024, confirming the sustained growth observed in recent years, when chicken meat has become one of the most accessible and frequently consumed protein sources in the region (USDA, 2026).

Poultry consumption continues to rise regardless of a country's income level, as measured by Gross Domestic Product (GDP) (OECD/FAO, 2024). However, the sustainability of poultry production is challenged by the high cost and limited availability of conventional protein sources like soybean meal and fishmeal (Syed Makhdoom et al., 2024). As a result, researchers are investigating alternative, sustainable protein sources to lessen reliance on these ingredients while maintaining animal performance and product quality. In recent years, insects have attracted considerable interest as viable alternatives to conventional feed proteins due to their high nutritional value, efficient feed conversion, and reduced environmental impact (Huis, 2013; Caisin et al., 2025). Among these, the black soldier fly (*Hermetia illucens*) has emerged as one of the most suitable species for large-scale production. Its larvae are rich in crude protein (40-60%), essential amino acids, lipids, and bioactive compounds that support animal health (Barragan-Fonseca et al., 2017; Gasco et al., 2019). Furthermore, *Hermetia illucens* larvae can be reared on a wide range of organic by-products, thereby contributing to waste valorization and supporting circular economy principles (Wang & Shelomi, 2017).

Numerous studies have evaluated the inclusion of *Hermetia illucens* larvae meal (HILM) in poultry diets, reporting comparable growth rates, feed conversion ratios, and meat quality parameters to conventional protein sources (Cullere et al., 2016; Schiavone et al., 2017). However, the impact of HILM on slaughter performance and carcass composition remains

variable and dependent on inclusion level, processing method, and the nutritional profile of the substrate used for rearing larvae (Bovera et al., 2018; Heuel et al., 2022). Understanding these effects is critical for optimizing insect-based feed formulations that meet both sustainability and production efficiency standards.

Therefore, this study aims to evaluate the slaughter performance and carcass traits of poultry fed diets containing *Hermetia illucens* larvae meal (HILM) as a replacement for conventional protein sources. The results will contribute to developing sustainable feeding strategies that maintain high carcass quality while mitigating the ecological footprint of poultry production.

MATERIALS AND METHODS

Experimental Design

The research was conducted in accordance with classical methods of experimental animal science, AOAC standards, and methodologies commonly applied in meat quality control laboratories.

The experiment was carried out at the Department of Animal Resources and Food Safety of the Technical University of Moldova. One-day-old unsexed broiler chicks the Ross-308 cross were selected as experimental material. They were randomly assigned to four groups: control group and three experimental groups, each consisting of 20 birds. The chicks were homogeneous in terms of age, body weight, and health status.

Broilers were kept under standardized environmental conditions in accordance with the Ross 308 guidelines (Aviagen, 2025). The chicks were housed in cages with wire mesh floors, equipped with adjustable feeders and nipple drinkers. Each cage was provided with an infrared lamp to maintain optimal temperature conditions.

At the beginning of the rearing period (days 1–2), the average temperature in the houses was 30-33°C. It was gradually decreased over the following days and, by the end of the experiment, it reached approximately 18-22°C (days 22-41).

The chickens were fed *ad libitum* with feed mixtures from a commercial manufacturer: starter (from day 1 to day 10 of the rearing period), grower I (from day 11 to day 24), finisher I (from day 25 to day 30), and finisher II (from day 31 until the end of the rearing period).

The control group received a basal diet, while the experimental groups were fed the same diet supplemented with different levels of *Hermetia illucens* larvae meal (HILM), as follows: 2.0%, 3.5%, and 5.0%. In the experimental diets, fish meal was replaced with the tested feed additive. Feed intake was monitored daily by weighing feed offered and refusals.

The blood was collected from birds in each group on day 41 of the study. All the hematological and serum biochemical determinations were done immediately on sample collection, following standard procedures.

Productive Performance Measurements

During the rearing period, based on the obtained production data, the following production indicators were determined: average body weight (BW, g - weekly and at the end of the production cycle); rearing period (days) and feed conversion ratio (FCR, g).

Growth performance was further assessed using the following indicators: absolute growth rate, relative growth rate, and growth intensity. Body weight gain (g/bird) was calculated as $W_2 - W_1$ where W_2 is the final body weight at the specified time point, and W_1 is the initial body weight.

Relative Growth Rate (RGR) of the animals was calculated using the following equation:

RCR = 1nW2-1nW1/t2-t1; where *W1* is the initial body weight, *W2* is the final body weight, and *t2−t1* is the duration of the period in days.

Feed intake (g/bird) was determined as the difference between the amount of feed offered and the feed residues left per group/bird. The feed conversion ratio (FCR) was estimated using the formula: *FCR = Feed intake (g)/ Weight Gain (g/d)* (FAO, 2011).

Slaughter and Carcass Evaluation

Four randomly selected broilers from each group were weighed and slaughtered after fasting for 12 h at 41 d of age. Broilers were then scalded for 90 s, picked for 30 s and weighed (dressed weight). After removal of the crop,

intestine, spleen, pancreas, bile, and reproductive organs, the rest of the body was weighed (semi-eviscerated weight), and the eviscerated weight was then determined by removing the liver, heart, gizzard, head, lung, and stomach.

Statistical Analysis

Statistical analysis was performed using Microsoft Excel (Microsoft Corp., USA). Descriptive statistics were calculated for each experimental group, including mean (M), standard deviation (SD), standard error (SE), coefficient of variation (CV), and 95% confidence intervals (95% CI). Differences among treatments were evaluated by one-way analysis of variance (ANOVA). Statistical significance was determined at $p < 0.05$, and treatment means were compared using the Least Significant Difference (LSD) test.

RESULTS AND DISCUSSIONS

Growth Performance

Initial body weights did not differ significantly among the treatment groups, confirming the homogeneity of the experimental groups at the start of the trial. This suggests that any subsequent differences observed can be attributed to the dietary treatments rather than to initial variability. The dynamics of live body weight in the experimental groups were studied at 7, 14, 21,28, 35, and 41 days of age.

Following group allocation, the mean body weights were similar across groups, indicating a high degree of baseline homogeneity and confirming that the experimental groups were comparable at the start of the study.

On day 1, no statistically significant differences were observed among treatments ($F = 0.03$, $p > 0.05$), demonstrating uniform initial conditions (Figure 1).

Throughout the experimental period, body weight increased progressively in all groups, reflecting normal growth dynamics under the applied rearing conditions. During the early phase of the trial (days 1-21), growth patterns remained comparable among treatments, and no statistically significant differences were detected ($p > 0.05$).

From day 28 onward, intergroup differences became statistically significant ($p < 0.05$), indicating that treatment composition began to influence growth performance. At this stage, birds in the EG2 group exhibited the highest mean body weight, followed by EG3, EG1, and CG.

By day 35, the EG2 group maintained the highest body weight, while EG3 showed comparable values but with greater variability. In contrast, CG and EG1 remained lower, although both groups demonstrated stable and consistent growth. The observed differences at this stage suggest that EG2 promoted more efficient weight gain compared with the other treatments.

At the final observation point (day 41), the differences among groups were highly significant ($F = 5.27$, $p < 0.01$). Birds receiving the EG2 treatment reached the highest final body weight, confirming the superior growth-promoting effect of this treatment under the conditions of the present experiment. EG3 ranked second, whereas EG1 and CG showed lower final values.

Overall, the results demonstrate that all groups exhibited steady and biologically consistent growth; however, the magnitude of weight gain differed significantly in the later stages of development. Among the tested treatments, EG2 produced the most favorable growth response, combining the highest final body weight with acceptable variability and strong statistical support.

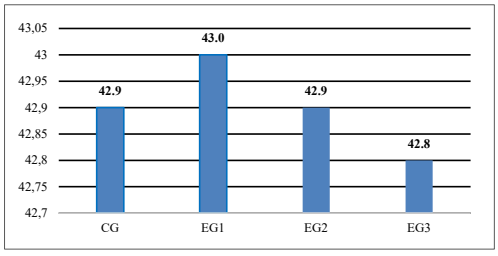


Figure 1. Broilers body weight at the start of trial, g

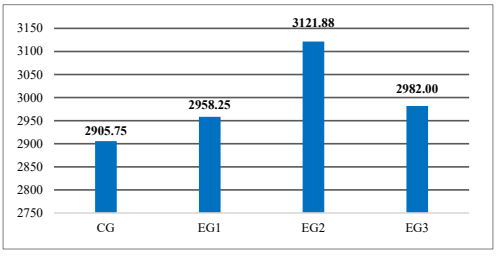


Figure 2. Broilers body weight at the end of trial, g

These findings indicate that EG2 was the most effective treatment for improving growth performance during the experimental period.

The obtained results are consistent with numerous studies investigating the use of insect-derived ingredients in poultry nutrition. In recent years, larval meal, particularly from the black soldier fly (*Hermetia illucens*), has attracted considerable attention as a sustainable and nutritionally valuable alternative protein source for livestock feed. Such ingredients are characterized by a high content of digestible protein, essential amino acids, lipids, and biologically active compounds, which can positively influence growth performance and metabolic processes in poultry (Barman & Saikia, 2024).

Experimental studies have demonstrated that the inclusion of *Hermetia illucens* larval meal in broiler diets can improve nutrient digestibility, energy utilization, and intestinal health, which ultimately contributes to improved growth performance (Dalmoro et al., 2025). In addition, insect-derived ingredients contain antimicrobial peptides and other bioactive substances that may enhance gut microbiota balance and support immune responses in poultry (Dörper et al., 2024).

The results obtained in the present experiment suggest that moderate inclusion levels of larval meal provide the most favorable effect on growth performance. In particular, the highest final body weight was observed in group EG2, which received the intermediate inclusion level (3.5% larval meal). Similar findings have been reported in other studies, where moderate dietary inclusion levels of insect meal produced optimal growth responses, while excessive inclusion did not lead to further improvements in productivity (Seyedalmoosavi et al., 2022).

Another important aspect is the relatively low coefficient of variation observed throughout the experiment, which indicates good uniformity of the birds and stable experimental conditions. This confirms that the observed differences in body weight were primarily associated with the dietary treatments rather than random variation among individuals.

Figure 3 shows the feed conversion ratio (FCR) of broilers in different experimental groups.

The control group (CG) consumed 4313.78 g of feed per bird, with an FCR of 1.48. All experimental groups exhibited reduced feed intake and improved FCR: EG1 – 4233.07 g, FCR 1.43 (−1.87% feed, +3.38% FCR); EG2 – 4186.06 g, FCR 1.34 (−2.96% feed, +9.46% FCR); EG3 – 4193.00 g, FCR 1.41 (−2.80% feed, +4.73% FCR). These results indicate that all treatments enhanced feed efficiency, with EG2 showing the greatest improvement.

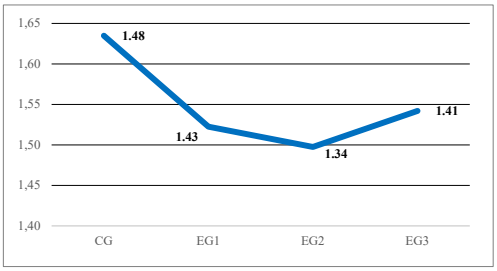


Figure 3. Feed conversion ratio of broilers, kg

Haematological and biochemical parameters

The morphological analysis of blood showed that most hematological parameters (HCT, HGB, RBC, MCH, MCV, PLT) did not differ significantly among the experimental groups.

The biochemical analysis also indicated that the majority of parameters remained within similar ranges across the studied groups.

However, statistically significant differences were observed only for alanine aminotransferase (ALT), whereas the remaining biochemical parameters did not differ significantly among groups ($p > 0.05$). According to the post hoc comparison, ALT activity was significantly higher in EG1 compared with CG, EG2, and EG3 ($p \leq 0.05$), while no significant differences were detected among CG, EG2, and EG3, which shared the same statistical grouping.

Aspartate aminotransferase (AST) activity showed only minor variation among groups, ranging from 367.50 ± 12.42 U/L in EG1 to 394.50 ± 36.38 U/L in EG3, and remained statistically comparable to CG (385.18 ± 16.62 U/L; $p > 0.05$). This indicates the absence of marked hepatocellular damage and confirms that liver function was not adversely affected by the tested treatments.

In contrast, alanine aminotransferase (ALT) demonstrated significant intergroup variation ($p < 0.05$). The highest ALT activity was recorded in EG1 (120.50 ± 2.07U/L), which differed significantly from CG (116.32 ± 1.28 U/L), EG2

(115.95 ± 0.19 U/L), and EG3 (114.15 ± 0.83 U/L). Meanwhile, EG2 and EG3 remained statistically similar to CG, indicating that only EG1 induced a modest but significant elevation in ALT activity. Despite this difference, ALT values remained within physiologically acceptable limits, suggesting only moderate modulation of hepatic metabolic activity rather than clinically relevant liver dysfunction.

Total protein (TP) and albumin (Alb) concentrations showed only minor, statistically insignificant fluctuations among groups ($p > 0.05$). Total protein values remained stable, ranging from 29.35 ± 5.62 g/L in EG3 to 30.93 ± 2.67 g/L in CG, indicating preserved protein metabolism across all treatments.

Albumin concentrations were slightly higher in EG1 (17.69 ± 2.07 g/L) and EG2 (17.78 ± 1.16 g/L) compared with CG (16.45 ± 0.66 g/L), although these differences were not statistically significant ($p > 0.05$). Nevertheless, this tendency may suggest mildly improved protein synthesis and nutritional status in these groups.

Glucose concentrations were higher in EG1 and EG2, suggesting more active carbohydrate metabolism and potentially improved energetic status. EG3 had the lowest α-amylase activity, which may indicate reduced pancreatic enzymatic activity compared with the other groups.

Uric acid concentrations were higher in EG1, EG2, and EG3 than in CG, which may reflect intensified protein turnover. However, creatinine and urea values remained stable, indicating preserved renal function.

Overall, the biochemical indicators remained within physiologically comparable limits, suggesting that all treatments were metabolically well tolerated and induced only moderate biochemical variation. Among the tested groups, EG2 maintained the most balanced biochemical profile, combining stable hepatic enzyme activity with preserved protein metabolism and no evidence of metabolic distress.

Slaughter Performance

Slaughter analysis at 41 days of age revealed no significant differences in pre-slaughter live weight among the experimental groups ($p > 0.05$), indicating that the dietary inclusion of *Hermetia illucens* larvae meal did not affect the attainment of market weight. Significant differences were observed in carcass length between the control group and EG2 ($p < 0.001$) and between the CG and EG3 ($p < 0.001$), as well as in semi-eviscerated carcass mass between EG1 and EG2 ($p < 0.05$). However, these differences were not associated with reductions in overall carcass weight or dressing percentage.

The dressing percentage ranged from 73.9% in the control group to 74.83% in the EG2 group receiving 3.5% HILM, remaining within the expected commercial range for broiler chickens (Figure 4).

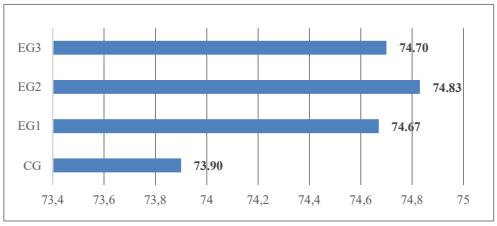


Figure 4. Dressing percentage of broilers, %
[Dressing percentage was calculated as: Dressing, % = (Fully eviscerated carcass weight/Live body weight) * 100]

Broilers in EG2 and EG3 tended to produce slightly heavier carcasses with a higher meat yield. From a production standpoint, maintaining dressing percentage above 74% is considered economically advantageous in modern broiler systems, as it reflects efficient carcass deposition relative to live mass (North & Bell, 1990; Leeson & Summers, 2005).

The absence of negative effects on carcass weight and yield indicates that skeletal development and carcass formation were not adversely influenced by the dietary treatments. Variations in carcass conformation are therefore more likely attributable to normal biological variability rather than dietary factors. The numerically higher dressing percentage observed in the group receiving 3.5% HILM may suggest an optimal inclusion level at which the nutritional advantages of insect-derived protein are effectively utilized. Black soldier fly larvae meal is characterized by a favorable amino acid profile, high digestibility, and the presence of bioactive compounds such as medium-chain fatty acids, which may enhance nutrient utilization and protein deposition.

These findings are consistent with previous studies showing that *Hermetia illucens* larvae meal can partially replace conventional protein sources without compromising growth performance or carcass traits (Huis, 2013; Gasco et al., 2019; Wang & Shelomi, 2017; Józefiak et al., 2020). From a sustainability perspective, insect-derived protein supports circular bioeconomy strategies by converting organic by-products into high-value feed ingredients, thereby reducing the environmental footprint of poultry production and dependence on imported soybean meal.

Organ Weights

The weights of carcass components, including larynx, trachea, esophagus, crop, proventriculus, gizzard, cuticle, and intestines, as well as spleen, pancreas, gallbladder, liver, heart, and lungs, were measured across different groups (CG, EG1–EG3). Minor variations were observed between CG and EG1 in most organ weights. The liver weight ranged from 58.10±3.63 g in EG1 to 63.13±1.25 g in EG2, showing minor variation compared to the control group (62.00±3.63 g).

Pancreas weight differed significantly among the experimental groups ($p < 0.05$). The control group (CG) exhibited the lowest value (4.93 g), while all experimental groups showed higher pancreas weights.

Specifically, EG1, EG2, and EG3 recorded values of 5.49 g, 5.57 g, and 5.74 g, respectively, all of which were significantly greater than the control. No significant differences were observed among the experimental groups themselves ($p > 0.05$).

The spleen showed a slight reduction in EG1 (2.78±0.20 g) and EG3 (2.87±0.31 g) compared to CG (3.63±0.38 g), whereas EG2 (3.10±0.13 g) remained closer to control values (Table 1).

Table 1. Selected internal organs

Parameter/Group	Liver (g)	Pancreas (g)	Spleen (g)
CG	62.00±3.63^{ab}	4.93 ± 0.15^{a}	3.63±0.38^{a}
EG1	58.10±3.48^{a}	5.49 ± 0.21^{b}	2.78±0.20^{b}
EG2	63.13±1.25^{b}	5.57 ± 0.38^{b}	3.10±0.12^{ab}
EG3	61.75±1.38^{ab}	5.74 ± 0.29^{b}	2.87±0.31^{b}

$^{a-c}$ - data within rows with different superscript letters are significantly different ($P < 0.05$)

These results indicate that the experimental treatments had minimal impact on liver and heart masses, while slight variations in spleen weight could reflect subtle immunological or hematopoietic responses.

The observed organ responses are consistent with recent literature reporting that insect-based diets can induce beneficial morphological adaptations without compromising health status (Gasco et al., 2019; Józefiak et al., 2020; Abd El-Hack et al., 2020; Carpentier et al., 2024).

Carcass Traits

The carcass and thigh measurements were recorded for the control group (CG) and three experimental groups (EG1–EG3) (Table 2).

Table 2. Carcass dissection parameters of broiler chickens, g

Parameter	Group			
	CG	EG1	EG2	EG3
Pre-slaughter body weight, g	3095.25 ± 158.4^{ab}	2918.00 ± 63.8^{a}	3122.75 ± 103.6^{b}	3042.50 ± 55.6^{ab}
Un eviscerated carcass weight, g	2659.00 ± 124.9^{a}	2708.00 ± 58.3^{a}	2938.75 ± 78.0^{b}	2863.00 ± 57.8^{ab}
Semi-eviscerated carcass weight, g	2488.75 ± 116.9^{ab}	2365.00 ± 64.9^{a}	2541.50 ± 44.9^{b}	2390.75 ± 48.8^{ab}
Carcass length, cm	24.25 ± 0.63^{b}	22.50 ± 1.54^{ab}	20.35 ± 0.09^{a}	20.50 ± 0.72^{a}
Eviscerated carcass weight, g	2278.25 ± 76.0^{a}	2179.00 ± 53.7^{a}	2336.75 ± 13.4^{b}	2272.75 ± 47.8^{ab}

$^{a-c}$ - data within rows with different superscript letters are significantly different ($P < 0.05$)

The analysis of slaughter traits revealed both significant and non-significant differences among the groups. Pre-slaughter body weight varied significantly ($p < 0.05$), with the highest value recorded in EG2 (3122.75 g), which differed from EG1 (2918.00 g), while CG and EG3 showed intermediate values without significant differences from either group.

A similar trend was observed for un eviscerated carcass weight, where EG2 exhibited the highest value (2938.75 g), significantly exceeding CG and EG1 ($p < 0.05$), while EG3 showed intermediate results. Semi-eviscerated carcass weight also differed significantly, with EG2 again presenting the highest value (2541.50 g), compared to the lower value in EG1 (2365.00 g),

while CG and EG3 did not differ significantly from either.

Carcass length demonstrated significant variation among groups ($p < 0.05$), with the greatest length observed in the control group (24.25 cm), whereas EG2 and EG3 showed significantly shorter carcasses (20.35 cm and 20.50 cm, respectively). EG1 presented intermediate values.

Eviscerated carcass weight followed a similar pattern to other weight-related traits, with EG2 recording the highest value (2336.75 g), significantly higher than EG1, while CG and EG3 occupied intermediate positions.

EG2 consistently demonstrated the highest values for most weight-related parameters, whereas the control group showed the greatest carcass length.

CONCLUSIONS

The inclusion of Black Soldier Fly (*Hermetia illucens*) larvae meal in broiler diets demonstrated positive effects on growth performance, carcass traits, and muscular development.

Experimental groups receiving 2.0%, 3.5%, and 5.0% *Hermetia illucens* larvae meal exhibited improved carcass composition compared to the control, with significant increases in thigh and shank muscle mass, breast muscle weight, and sternal development. Internal organ weights (liver, heart, spleen) remained within normal physiological ranges, indicating that insect-based feed did not adversely affect health or metabolic status.

The enhanced muscular development observed in experimental groups confirms that BSF larvae can serve as an effective, sustainable protein source, supporting meat yield and quality without compromising broiler health.

These findings underline the potential of insect-based diets as a sustainable strategy in poultry production, particularly for lines selected for meat performance, offering both economic and environmental benefits while maintaining animal welfare and product quality.

ACKNOWLEDGEMENTS

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EFFECTS OF RAPESEED OIL AND GROUND FLAXSEED SUPPLEMENTATION ON PRODUCTIVE PERFORMANCE AND MEAT QUALITY IN GROWING-FINISHING PIGS

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Abstract

The study aimed to evaluate the effects of dietary supplementation with rapeseed oil (RO) and ground flaxseed (GF) on the productive performance and meat quality of growing-finishing pigs (Landrace × Large White hybrid). A total of 90 pigs with an initial average body weight of 27.40 kg were randomly assigned to four dietary treatments: LC (control, without supplementation), L1 (1% RO + 1% GF), L2 (1% RO + 1.5% GF), and L3 (2% RO + 2% GF), each with three replicate pens of 7–8 animals. The experiment was conducted over two consecutive phases: Phase I – growing (25–60 kg) and Phase II – finishing (60–110 kg), with a total duration of 84 days. Productive parameters (initial and final body weight per phase, average daily gain, average daily feed intake and feed conversion ratio) and meat quality indicators (dressing percentage, backfat thickness, lean meat percentage, pH_{45} and pH_{24}, meat colour L, a*, b*, and water holding capacity) were recorded and analysed by one-way ANOVA ($p < 0.05$). The results indicated that L3 supplementation substantially improved the dietary n-6/n-3 ratio (2.2:1 vs. 22.1:1 in LC), while productive performances remained comparable across all groups ($p > 0.05$). Backfat thickness was significantly reduced in L2 and L3 ($p < 0.05$), and lean meat percentage increased linearly with supplementation level, reaching 59.80% in L3 vs. 56.80% in LC. The findings suggest that combined RO and GF supplementation represents a viable nutritional strategy for improving the dietary n-6/n-3 ratio and carcass quality of pigs without compromising growth performance.*

Key words*: ground flaxseed, growing-finishing pigs, meat quality, n-3 fatty acids, productive performance, rapeseed oil.*

INTRODUCTION

The optimization of pig diets through the inclusion of unconventional lipid sources has become a subject of growing interest in both research and commercial swine production (Zahiu et al., 2010). The current global context is characterized by significant volatility in the prices of conventional feed ingredients, particularly soybean meal and vegetable oils, which account for a substantial proportion of total production costs in intensive pig farming (Cavalcanti et al., 2026). Against this backdrop, finding sustainable, locally available, and cost-effective alternatives has become a strategic priority for the European swine sector (European Commission, 2023).

Rapeseed (*Brassica napus* L.) and flaxseed (*Linum usitatissimum* L.) represent two of the most promising oilseed crops cultivated in Romania and across Central and Eastern Europe. Rapeseed oil is characterized by a favourable fatty acid composition, with a relatively low content of saturated fatty acids, high oleic acid (C18:1, approximately 60%), and a significant proportion of linoleic acid (C18:2 n-6, ~21%) and alpha-linolenic acid (ALA, C18:3 n-3, ~9.5%). Ground flaxseed is the richest plant source of ALA among conventional feed ingredients, with ALA representing approximately 57% of total fatty acids, providing an exceptional opportunity to improve the n-6/n-3 ratio in pork (Habeanu et al., 2014).

The dietary n-6/n-3 ratio is of paramount importance not only for animal health and metabolism, but also for the nutritional value of pork for human consumption. Current dietary guidelines recommend a maximum n-6/n-3 ratio of 4:1, whereas conventional corn-

soybean meal-based diets typically deliver ratios exceeding 15:1 or even 20:1. Modifying this ratio through dietary manipulation represents one of the most effective and economically feasible strategies to produce functional pork products enriched in n-3 polyunsaturated fatty acids (PUFA) (Smet et al., 2004).

While several studies have investigated the individual effects of rapeseed oil (Daza et al., 2005; Mitchaothai et al., 2007) or flaxseed (Habeanu et al., 2014) in swine diets, data on their combined supplementation across two distinct production phases – growing and finishing – with simultaneous assessment of productive performance and carcass quality remain limited, particularly for the Landrace × Large White hybrid widely used in Romanian commercial production systems.

The novel aspect of the present study consists of the simultaneous inclusion of both ingredients at three combined dosage levels, evaluated separately for each production phase, using the genotype predominant in intensive Romanian farming.

The study tested the hypothesis that combined dietary supplementation with RO and GF improves the dietary n-6/n-3 ratio and carcass composition without negatively affecting growth performance in growing-finishing pigs. The present study aimed to evaluate the effects of three combined supplementation levels of RO and GF on average daily gain, feed intake, and feed conversion ratio during both growing and finishing phases; to assess the impact on carcass quality parameters, including backfat thickness and lean meat percentage; and to quantify the modification of the dietary n-6/n-3 ratio as a function of supplementation level.

MATERIALS AND METHODS

Animals, Housing, and Experimental Design

The experiment was conducted over a total period of 84 days, covering two consecutive production phases. A total of 90 pigs of the Landrace × Large White hybrid, weaned at 28 days and pre-selected at an average body weight of 27.40 ± 0.82 kg (Phase I entry weight), were used. Animals were in good health and were acclimatized for 10 days prior to the start of the trial.

Pigs were randomly allocated to four dietary treatment groups, each consisting of three replicate pens with 7-8 animals per pen (n = 22-23 per treatment group). The four groups were as follows: LC – control group, without lipid supplementation; L1 – 1% rapeseed oil (RO) + 1% ground flaxseed (GF); L2 – 1% RO + 1.5% GF; L3 – 2% RO + 2% GF.

The experimental design was a completely randomised block design with dietary treatment as the main factor. The pen was considered the experimental unit for productive performance traits, as feed intake was recorded at pen level.

Animals were housed in groups in climate-controlled pens (18-22°C, relative humidity 60-70%, 16 h light/8 h dark cycle). Each pen was equipped with a nipple drinker and a two-sided feeder, allowing ad libitum access to feed and water throughout the experiment. All procedures were conducted in accordance with Directive 2010/63/EU on the protection of animals used for scientific purposes and Romanian Law no. 43/2014.

Dietary Treatments and Feed Formulation

Two complete compound feed formulas were developed for each treatment group, corresponding to the two production phases: Phase I – growing (25-60 kg body weight) and Phase II – finishing (60-110 kg body weight). All diets were formulated to be approximately isoenergetic and iso-nitrogenous within each phase (ME differences between groups did not exceed 0.59 MJ/kg in Phase I and 0.59 MJ/kg in Phase II; see Table 3), meeting or exceeding the NRC (2012) nutritional requirements for growing-finishing pigs. The main ingredients were corn grain, wheat grain, barley grain, soybean meal (44% CP), wheat bran, rapeseed oil, and ground flaxseed, complemented by a standard vitamin-mineral premix (1% inclusion level for all groups).

Flaxseed was freshly ground to a particle size below 2 mm immediately before diet preparation to minimize oxidation of ALA. Rapeseed oil was added by spraying over the dry ingredient blend at a maximum mixing temperature of 40°C. The vitamin E level in the premix was adjusted to 150 mg/kg complete feed for L1 and L2, and to 220 mg/kg for L3, to provide adequate antioxidant protection for the elevated PUFA content. As a synthetic

antioxidant, butylhydroxytoluene (BHT, E321, 150 ppm) was incorporated into the premix of all groups in compliance with Regulation (EC) No 1831/2003 and Commission Regulation (EU) No 574/2011, which authorise BHT as a feed additive (functional group: antioxidants) for all animal species.

Table 1. Composition of experimental diets – Phase I: Growing (25-60 kg), % as-fed

Ingredients	LC (%)	L1 (%)	L2 (%)	L3 (%)
Corn grain	63.05	62.85	62.95	62.55
Wheat grain	8.00	7.00	7.00	6.50
Barley grain	6.50	5.50	5.00	4.50
Soybean meal (44% CP)	17.00	17.20	17.30	17.50
Wheat bran	1.00	1.00	0.80	0.50
Rapeseed oil	-	**1.00**	**1.00**	**2.00**
Ground flaxseed	-	**1.00**	**1.50**	**2.00**
Dicalcium phosphate	1.30	1.30	1.30	1.30
Calcium carbonate	1.40	1.40	1.40	1.40
Iodised salt (NaCl)	0.40	0.40	0.40	0.40
L-Lysine HCl (78%)	0.25	0.25	0.25	0.25
DL-Methionine (99%)	0.05	0.05	0.05	0.05
L-Threonine (98%)	0.05	0.05	0.05	0.05
Vitamin-mineral premix 1%	1.00	1.00	1.00	1.00
TOTAL	**100.00**	**100.00**	**100.00**	**100.00**

Table 2. Composition of experimental diets – Phase II: Finishing (60-110 kg), % as-fed

Ingredients	LC (%)	L1 (%)	L2 (%)	L3 (%)
Corn grain	67.45	66.45	66.55	66.15
Wheat grain	7.00	6.50	6.50	6.00
Barley grain	6.00	5.50	5.00	4.50
Soybean meal (44% CP)	13.00	13.30	13.40	13.50
Wheat bran	2.50	2.20	2.00	1.80
Rapeseed oil	-	**1.00**	**1.00**	**2.00**
Ground flaxseed	-	**1.00**	**1.50**	**2.00**
Dicalcium phosphate	1.10	1.10	1.10	1.10
Calcium carbonate	1.30	1.30	1.30	1.30
Iodised salt (NaCl)	0.40	0.40	0.40	0.40
L-Lysine HCl (78%)	0.20	0.20	0.20	0.20
DL-Methionine (99%)	0.03	0.03	0.03	0.03
L-Threonine (98%)	0.02	0.02	0.02	0.02
Vitamin-mineral premix 1%	1.00	1.00	1.00	1.00
TOTAL	**100.00**	**100.00**	**100.00**	**100.00**

Table 3. Nutritional value of experimental diets by phase and experimental groups

Nutritional parameter	Phase	LC	L1	L2	L3
ME (MJ/kg feed)	I	13.38	13.66	13.70	13.97
ME (MJ/kg feed)	II	13.53	13.81	13.85	14.12
Crude protein (%)	I	17.8	17.9	17.9	18.0
Crude protein (%)	II	15.6	15.7	15.8	15.9
Crude fat (%)	I	3.45	5.05	5.60	7.35
Crude fat (%)	II	3.38	5.00	5.55	7.28
Lysine (%)	I	0.97	0.97	0.97	0.98
Lysine (%)	II	0.76	0.76	0.77	0.77
Total Ca (%)	I	0.72	0.72	0.72	0.72
Total Ca (%)	II	0.63	0.63	0.63	0.63
Total P (%)	I	0.56	0.56	0.56	0.55
Total P (%)	II	0.50	0.50	0.50	0.49
LA C18:2n-6 (%)	I	1.62	2.05	2.12	2.60
LA C18:2n-6 (%)	II	1.55	1.98	2.06	2.55
ALA C18:3n-3 (%)	I	0.08	0.41	0.68	1.20
ALA C18:3n-3 (%)	II	0.07	0.38	0.65	1.17
n-6/n-3 ratio	I	20.3:1	5.0:1	3.1:1	2.2:1
n-6/n-3 ratio	II	22.1:1	5.2:1	3.2:1	2.2:1

ME = metabolisable energy; LA = linoleic acid; ALA = alpha-linolenic acid.

Measurements and analytical methods

Body weight was recorded individually at the beginning of the experiment (day 0), at the transition between phases (day 42 – end of Phase I / start of Phase II), and at the end of the experiment (day 84 – end of Phase II), using a calibrated electronic scale (± 0.1 kg accuracy). Average daily gain (ADG) was calculated separately for each phase and for the overall experimental period.

Feed intake was monitored daily at pen level by weighing the amount of feed offered and the refusals. Average daily feed intake (ADFI) was calculated per animal by dividing the total feed consumed by the number of animal-days. The feed conversion ratio (FCR, kg feed/kg body weight gain) was calculated separately for each phase.

At the end of the experiment (day 84), all 90 pigs were slaughtered at a commercial abattoir under standard conditions. The following carcass and meat quality parameters were recorded: hot carcass weight (kg); dressing percentage (%), calculated as carcass weight divided by live weight at slaughter × 100; backfat thickness (mm), measured at three anatomical points (shoulder, mid-back, and last rib) using a calliper, and averaged; lean meat percentage (%), estimated using the two-point FOM (Fat-O-Meter) method according to Commission Regulation (EC) No 1249/2008; pH at 45 minutes post-mortem (pH_{45}) and at 24 hours post-mortem (pH_{24}), measured in the *Longissimus dorsi* muscle using a portable pH meter (Testo 205); meat colour (L*, a*, b*) at 24 hours post-mortem, measured with a Minolta CR-400 colorimeter in the *Longissimus dorsi* cross-section, according to the CIE L*a*b* system; water holding capacity (WHC), assessed by the filter paper press method (Grau & Hamm, 1953, cited in Honikel, 1998).

Statistical analysis was performed using SPSS v.26 software (IBM Corp., Armonk, NY). Data were analysed by one-way ANOVA with dietary treatment as the fixed effect. Means were compared using Tukey's HSD post-hoc test. The significance level was set at $p < 0.05$. Results are presented as means ± standard error of the mean (SEM). Normality of residuals was verified using the Shapiro-Wilk test.

RESULTS AND DISCUSSIONS

Body weight dynamics and growth performance by phase

The dynamics of body weight across the two experimental phases are presented in Table 4. At the beginning of the experiment, no significant differences were recorded between treatment groups for initial body weight ($p = 0.892$), confirming successful randomisation. All animals were within the target weight range of 25–30 kg at Phase I entry.

Table 4. Body weight dynamics and productive performance by experimental phase (means ± SEM)

Parameter	Phase	LC	L1	L2	L3
Initial BW (kg)		27.42±0.85	27.38±0.91	27.44±0.78	27.36±0.88
BW end Phase I (kg)	I	60.85±1.42	60.12±1.56	59.88±1.38	59.45±1.61
BW end Phase II (kg)	II	108.60±2.15	107.85±2.32	107.40±2.08	106.90±2.44
Total weight gain (kg)		81.18±1.95	80.47±2.18	79.96±2.02	79.54±2.25
ADG Phase I (g/day)	I	802±18	802±21	792±19	780±22
ADG Phase II (g/day)	II	1132±24	1137±26	1131±22	1142±28
ADG overall (g/day)		967±16	959±18	952±17	945±19
ADFI Phase I (kg/day)	I	2.05±0.06	2.07±0.07	2.09±0.06	2.12±0.08
ADFI Phase II (kg/day)	II	2.82±0.08	2.80±0.09	2.78±0.08	2.75±0.09
FCR Phase I (kg feed/kg gain)	I	2.56±0.07	2.58±0.08	2.64±0.07	2.72±0.09
FCR Phase II (kg feed/kg gain)	II	2.49±0.06	2.46±0.07	2.46±0.06	2.41±0.08
FCR overall (kg feed/kg gain)		2.52±0.05	2.51±0.06	2.54±0.06	2.56±0.07

BW = body weight; ADG = average daily gain; ADFI = average daily feed intake; FCR = feed conversion ratio. Means within a row with no superscript letters do not differ significantly ($p > 0.05$, Tukey HSD).

During Phase I (growing, 25–60 kg), all groups showed comparable growth performance. The ADG ranged from 780 g/day (L3) to 802 g/day (LC and L1), with no statistically significant differences between groups ($p = 0.124$). The slight numerical reduction in ADG observed in

L3 during Phase I may be attributable to the adaptation period required for the higher lipid inclusion level (4% total added fat), which can temporarily reduce voluntary feed intake in pigs not previously exposed to high-fat diets. However, ADFI differences were minor and non-significant ($p = 0.510$).

During Phase II (finishing, 60–110 kg), ADG was likewise similar across all groups, ranging from 1131 g/day (L2) to 1142 g/day (L3), with no statistically significant differences ($F(3,8) = 0.26$, $p = 0.876$). FCR values in Phase II (2.41–2.49 kg feed/kg gain) did not differ significantly between groups ($F(3,8) = 1.38$, $p = 0.315$). The higher energetic density of the L3 diet compared to LC (14.12 vs. 13.53 MJ ME/kg) should be acknowledged as a potential confounding factor when interpreting Phase II performance data; however, the absence of significant differences in any productive parameter across all groups indicates that this energy difference did not produce a biologically meaningful effect under the present experimental conditions.

These findings are consistent with published data demonstrating that supplementation of pig diets with polyunsaturated fatty acid-rich oils at levels up to 4% does not impair productive performance (Habeanu et al., 2014; Dugan et al., 2004). The overall FCR values are within the expected range for the Duroc × Pietrain genotype under intensive conditions (Gomes et al., 2025).

Carcass quality and meat quality indicators

The results of carcass and meat quality evaluation are presented in Table 5. Dressing percentage was comparable across all groups (75.8–76.4%), with no significant differences ($p = 0.725$), indicating that the dietary treatments did not affect the overall efficiency of converting live weight into carcass weight.

Table 5. Carcass and meat quality indicators (means ± SEM)

Parameter	LC	L1	L2	L3
Live weight at slaughter (kg)	108.60±2.15	107.85±2.32	107.40±2.08	106.90±2.44
Hot carcass weight (kg)	82.45±1.85	82.00±1.95	81.60±1.78	81.10±2.02
Dressing percentage (%)	75.92±0.62	76.02±0.68	75.98±0.60	75.86±0.71
Backfat thickness – shoulder (mm)	22.40±0.85	21.85±0.92	20.60±0.78	19.20±0.95
Backfat thickness – mid-back (mm)	18.60±0.72	18.10±0.80	17.10±0.69	15.80±0.85
Backfat thickness – last rib (mm)	16.80±0.68	16.30±0.75	15.40±0.62	14.20±0.78
Mean backfat thickness (mm)	19.27±0.72[a]	18.75±0.80[a]	17.70±0.68[b]	16.40±0.85[c]
Lean meat %	56.80±0.85[a]	57.45±0.92[a]	58.60±0.80[b]	59.80±0.95[c]
pH_{45} Longissimus dorsi	6.42±0.08	6.45±0.09	6.48±0.08	6.50±0.10
pH_{24} Longissimus dorsi	5.62±0.05	5.65±0.06	5.68±0.05	5.72±0.06
Meat colour L* (lightness)	50.25±1.12	50.48±1.25	50.82±1.08	51.15±1.30
Meat colour a* (redness)	8.45±0.42	8.62±0.48	8.78±0.40	8.92±0.50
Meat colour b* (yellowness)	4.12±0.28	4.35±0.32	4.52±0.28	4.85±0.35
Water holding capacity (%)	71.25±1.05	71.80±1.15	72.40±1.02	72.95±1.20

Highlighted rows: parameters with statistically significant differences between groups. Different superscript letters (a, b, c) within a row indicate significant differences ($p < 0.05$, Tukey HSD). pH_{45} and pH_{24} measured in *Longissimus dorsi* muscle.

A statistically significant effect of dietary treatment was found for mean backfat thickness ($p = 0.008$). The L3 group showed the lowest mean backfat thickness (16.40 mm), significantly lower than LC (19.27 mm, $p < 0.001$) and L1 (18.75 mm, $p = 0.012$). The L2 group (17.70 mm) was also significantly lower than LC ($p = 0.031$). This reduction in subcutaneous fat deposition with increasing n-3 PUFA supplementation is supported by the known inhibitory effect of ALA and its long-chain derivatives on lipogenesis and adipocyte differentiation (Wood et al., 2008; Habeanu et al., 2014). The elevated content of ALA in L2 and L3 diets (0.65-1.17% in Phase II) likely contributed to the redistribution of energy from fat deposition to muscle protein accretion.

Lean meat percentage showed a significant linear increase with supplementation level ($p = 0.004$), ranging from 56.80% in LC to 59.80% in L3. Pairwise comparisons revealed significant differences between LC and both L2 ($p = 0.022$) and L3 ($p < 0.001$), whereas the difference between LC and L1 did not reach significance ($p = 0.412$). These results indicate that a minimum combined supplementation of 1% RO + 1.5% GF (L2) is required to produce a biologically and commercially meaningful improvement in carcass lean meat content for the Landrace × Large White genotype.

Regarding meat pH, all groups showed values within the normal range for pork (pH_{45} 6.40-6.55; pH_{24} 5.60-5.75), with no significant differences between treatments ($p > 0.05$). These values exclude the occurrence of PSE (pale, soft, exudative) or DFD (dark, firm, dry) meat defects in any of the experimental groups, confirming that the dietary supplementation did not induce metabolic stress during the perimortem period.

Meat colour parameters (L*, a*, b*) showed consistent numerical trends with increasing supplementation levels, with slight increases in redness (a*) and yellowness (b*) in L2 and L3. The increase in b* (yellowness) in L3 (4.85 vs. 4.12 in LC) is a commonly reported effect of elevated dietary PUFA in pork and is attributable to the deposition of carotenoid pigments naturally present in rapeseed oil and flaxseed (Smet et al., 2004). However, none of these colour differences reached statistical significance ($p > 0.05$), suggesting that the supplementation levels used do not produce commercially unacceptable meat colour. Water holding capacity (WHC) showed a gradual increase across treatment groups (71.25% in LC to 72.95% in L3), with no statistically significant differences ($F(3.86) = 1.84$, $p = 0.145$). These values are within the normal range for pork *Longissimus dorsi* (Honikel, 1998), and indicate that the dietary treatments did not adversely affect the ability of the muscle to retain water, which is relevant for drip loss during storage and processing yield.

CONCLUSIONS

Combined dietary supplementation with rapeseed oil and ground flaxseed did not significantly affect ADG, ADFI, or FCR in growing or finishing pigs ($p > 0.05$), confirming nutritional safety of these PUFA-rich ingredients at the tested levels.

Carcass quality improved significantly with supplementation: mean backfat thickness decreased (L2 and L3, $p < 0.05$) and lean meat percentage increased linearly, from 56.80% (LC) to 59.80% (L3), representing commercially meaningful gains under the EUROP grading system.

Meat quality parameters (pH, colour, WHC) remained within normal ranges in all groups. The L3 diet reduced the dietary n-6/n-3 ratio from 22.1:1 to 2.2:1, well within current guidelines (≤4:1). L3 (2% RO + 2% GF) is the most effective combination for improving carcass composition without compromising productive performance. Limitations include the absence of pork fatty acid profiling, use of a single genotype and production site, and residual ME differences between groups (up to 0.59 MJ/kg). Future work should address these aspects and expand to additional genotypes.

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EFFECT OF COMBINED RAPESEED OIL AND GROUND FLAXSEED SUPPLEMENTATION ON FATTY ACID PROFILE, CHEMICAL COMPOSITION, AND LIPID HEALTH INDICES OF PORK

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Abstract

The present study investigated the effects of combined dietary supplementation with rapeseed oil (RO) and ground flaxseed (GF) on the fatty acid profile, chemical composition, and lipid nutritional health indices of m. Longissimus dorsi in Landrace × Large White finishing pigs. A total of 90 pigs were assigned to four dietary treatments – LC (control, no supplementation), L1 (1% RO + 1% GF), L2 (1% RO + 1.5% GF), and L3 (2% RO + 2% GF) – monitored across two production phases: Phase I (growing, 25-60 kg) and Phase II (finishing, 60–110 kg) over 84 days. Muscle samples from m. Longissimus dorsi were analysed for chemical composition (dry matter, crude protein, intramuscular fat, ash), complete fatty acid profile by gas chromatography–FID (GC-FID), and key lipid health indices: n-6/n-3 ratio, PUFA/SFA ratio, atherogenic index (AI), thrombogenic index (TI), peroxidizability index (PI), and hypocholesterolemic/hypercholesterolemic ratio (h/H). Alpha-linolenic acid (ALA, C18:3n-3) increased 5.8-fold in L3 (3.85%) vs. LC (0.62%, $p < 0.001$). The n-6/n-3 ratio decreased from 23.4:1 to 3.6:1 in L3 ($p < 0.001$), meeting the WHO (2002, 2023) recommendation. AI and TI were significantly reduced in L2 and L3 ($p < 0.001$). Crude protein and pH were unaffected. These findings confirm that combined RO + GF supplementation effectively transforms pork into a functional food with a markedly improved cardiovascular health profile.

Key words: *atherogenic index, fatty acid profile, flaxseed, functional pork, pigs, n-6/n-3 ratio, rapeseed oil, thrombogenic index.*

INTRODUCTION

The nutritional value of pork has become a central focus in both food science research and public health policy. Beyond traditional technological quality criteria such as pH, water holding capacity, and meat colour, consumers and nutritionists increasingly demand a favourable lipid composition, particularly an optimal balance between n-6 and n-3 polyunsaturated fatty acids (PUFA). The n-6/n-3 PUFA ratio in pork derived from conventional corn–soybean meal diets typically exceeds 15:1 to 22:1, far above the maximum of 4:1 recommended by the World Health Organization (WHO, 2002) and the European Food Safety Authority (EFSA, 2010). This imbalance contributes to pro-inflammatory dietary conditions associated with cardiovascular disease, type-2 diabetes, and metabolic syndrome (Wood et al., 2008).

Rapeseed oil (*Brassica napus* L.) and ground flaxseed (*Linum usitatissimum* L.) represent the most accessible and economically feasible plant-derived sources of n-3 PUFA in Central and Eastern European swine production. Rapeseed oil provides oleic acid (~60%), linoleic acid (~21%), and alpha-linolenic acid (ALA, ~9.5%), and carries the lowest atherogenic index (AI = 0.05) among common plant lipids (Khalili Tilami & Kouřimská, 2022). Ground flaxseed is one of the richest conventional plant sources of α-linolenic acid (ALA, 18:3n-3), which typically accounts for approximately 53–57% of its total fatty acid content. Dietary inclusion of flaxseed in pig diets has been consistently shown to increase ALA deposition in muscle and adipose tissue

and to reduce the n-6/n-3 PUFA ratio in pork toward nutritionally recommended levels (Czech et al., 2023).

Recent experimental evidence has confirmed that dietary flaxseed inclusion at 5-10% significantly increases ALA and total n-3 PUFA in the m. *Longissimus dorsi* of finishing pigs, while reducing the n-6/n-3 ratio to values approaching nutritional recommendations (Bartkovsky et al., 2022). Similarly, rapeseed oil supplementation for 4-6 weeks prior to slaughter significantly decreases the n-6/n-3 PUFA ratio in pig backfat and reduces atherogenic and thrombogenic indices (Okrouhla et al., 2018). For large and medium-heavy pig breeds, Minelli et al. (2023) demonstrated that lowering the dietary n-6/n-3 ratio from 9.7:1 to 1.4:1 in Large White pigs significantly increased n-3 PUFA in the Longissimus thoracis and reduced lipid peroxidation indices. However, the synergistic effect of combining rapeseed oil and ground flaxseed across a two-phase growing-finishing system and its impact on the full spectrum of lipid health indices has not been systematically studied.

The present study aimed to: formulate and characterise iso-energetic, iso-nitrogenous experimental diets incorporating graded levels of rapeseed oil and ground flaxseed for both growing and finishing phases; determine the effect of these diets on the chemical composition and complete fatty acid profile of m. *Longissimus dorsi*; calculate and interpret lipid health indices to assess the potential of dietary manipulation in producing functional pork with improved cardiovascular value.

MATERIALS AND METHODS

Animals, housing, and experimental design

The experiment was conducted over 84 consecutive days. A total of 90 clinically healthy Landrace × Large White pigs were used, with an initial mean body weight of 27.40 ± 0.82 kg. Animals were randomly allocated to four dietary treatment groups (n = 22-23 per group), each with three replicate pens of 7-8 animals: LC – control (0.95% sunflower oil in Phase I; 0.55% in Phase II, added for iso-energetic equalisation; no n-3 PUFA sources); L1 – 1% rapeseed oil (RO) + 1% ground flaxseed (GF); L2 – 1% RO + 1.5% GF; L3 – 2% RO + 2% GF. The experimental design was a completely randomised block with dietary treatment as the main factor.

Animals were housed in climate-controlled pens (18-22°C, relative humidity 60–70%, 16 h light) with ad libitum access to feed and water. Animal welfare procedures complied with Directive 2010/63/EU and Romanian Law No. 43/2014. Two complete compound feed formulations were prepared for each treatment group corresponding to the two production phases.

Experimental diets – formulation and nutritional characterisation

All diets were formulated to be approximately iso-energetic and iso-nitrogenous within each phase, meeting or exceeding NRC (2012) requirements for growing-finishing pigs. The base ingredients were corn grain, wheat grain, barley grain, soybean meal (44% CP), and wheat bran. To minimise metabolisable energy differences between groups arising from the lipid additions, the control diet (LC) was supplemented with refined sunflower oil (0.95% in Phase I; 0.55% in Phase II), replacing an equal proportion of corn grain. Sunflower oil was chosen for LC because it is essentially devoid of ALA (<0.5%) and does not contribute to n-3 PUFA enrichment of the meat, thus serving as an energetically equivalent but nutritionally neutral lipid vehicle. Rapeseed oil and sunflower oil were added by uniform spraying over the dry blend at a maximum mixing temperature of 40°C. Flaxseed was freshly milled to a particle size below 2 mm immediately before mixing to minimise ALA oxidation. Vitamin E was included in the premix at 150 mg/kg for all groups and 220 mg/kg for group L3, while BHT (125 mg/kg) was added to all groups as an antioxidant. Nutrient values were calculated using analysed and INRA (2004) feed composition tables.

The composition of the experimental diets and their calculated nutritional values are presented in Tables 1-4.

Table 1. Ingredient composition of experimental diets – Phase I: Growing (25-60 kg), % as-fed

Ingredients	LC (%)	L1 (%)	L2 (%)	L3 (%)
Lipid supplementation level	0% RO+0% GF	1% RO+1% GF	1% RO+1.5% GF	2% RO+2% GF
Corn grain	62.10	62.85	62.95	62.55
Wheat grain	8.00	7.00	7.00	6.50
Barley grain	6.50	5.50	5.00	4.50
Soybean meal (44% CP)	17.00	17.20	17.30	17.50
Wheat bran	1.00	1.00	0.80	0.50
Rapeseed oil	-	**1.00**	**1.00**	**2.00**
Ground flaxseed	-	**1.00**	**1.50**	**2.00**
Sunflower oil	0.95	-	-	-
Dicalcium phosphate	1.30	1.30	1.30	1.30
Calcium carbonate	1.40	1.40	1.40	1.40
Iodised salt (NaCl)	0.40	0.40	0.40	0.40
L-Lysine HCl (78%)	0.25	0.25	0.25	0.25
DL-Methionine (99%)	0.05	0.05	0.05	0.05
L-Threonine (98%)	0.05	0.05	0.05	0.05
Vitamin-mineral premix 1%	1.00	1.00	1.00	1.00
TOTAL	**100.00**	**100.00**	**100.00**	**100.00**

LC = control group; L1, L2, L3 = experimental groups. RO = rapeseed oil; GF = ground flaxseed.

Table 2. Nutritional value of experimental diets – Phase I: Growing (25-60 kg)

Nutritional parameter	LC (%)	L1 (%)	L2 (%)	L3 (%)	NRC 2012 requirement
Metabolisable energy (MJ/kg)	13.65	13.66	13.70	13.97	13.40–13.80
Crude protein (%)	17.8	17.9	17.9	18.0	17.5–19.0
Crude fat (%)	4.40	5.05	5.60	7.35	-
Crude fibre (%)	2.85	2.80	2.78	2.70	max. 5.0
Lysine (%)	0.97	0.97	0.97	0.98	≥ 0.95
Met+Cys (%)	0.56	0.56	0.56	0.57	≥ 0.55
Threonine (%)	0.62	0.62	0.62	0.63	≥ 0.61
Total Calcium (%)	0.72	0.72	0.72	0.72	0.70–0.80
Total Phosphorus (%)	0.56	0.56	0.56	0.55	0.55–0.65
Linoleic acid LA (C18:2n-6, %)	2.08	2.05	2.12	2.60	-
α-Linolenic acid ALA (C18:3n-3, %)	**0.10**	**0.41**	**0.68**	**1.20**	-
n-6/n-3 PUFA ratio	**20.8:1**	**5.0:1**	**3.1:1**	**2.2:1**	≤ 10:1

Refined rapeseed oil, erucic acid < 2%. Ground flaxseed, milled to < 2 mm particle size immediately before mixing. Premix provided per kg complete feed: vitamin A 8,000 IU; vitamin D_3 1,200 IU; vitamin E 150 mg (LC, L1, L2) / 220 mg (L3); vitamin K_3 2 mg; B-complex vitamins; Fe 100 mg; Cu 10 mg; Zn 80 mg; Mn 40 mg; I 0.3 mg; Se 0.3 mg; Co 0.2 mg; phytase ≥ 500 FTU.

Table 3. Ingredient composition of experimental diets – Phase II: Finishing (60-110 kg), % as-fed

Ingredients	LC (%)	L1 (%)	L2 (%)	L3 (%)
Corn grain	66.90	66.45	66.55	66.15
Wheat grain	7.00	6.50	6.50	6.00
Barley grain	6.00	5.50	5.00	4.50
Soybean meal (44% CP)	13.00	13.30	13.40	13.50
Wheat bran	2.50	2.20	2.00	1.80
Rapeseed oil	-	**1.00**	**1.00**	**2.00**
Ground flaxseed	-	**1.00**	**1.50**	**2.00**
Sunflower oil	0.55	-	-	-
Dicalcium phosphate	1.10	1.10	1.10	1.10
Calcium carbonate	1.30	1.30	1.30	1.30
Iodised NaCl	0.40	0.40	0.40	0.40
L-Lysine HCl (78%)	0.20	0.20	0.20	0.20
DL-Methionine (99%)	0.03	0.03	0.03	0.03
L-Threonine (98%)	0.02	0.02	0.02	0.02
Vitamin-mineral premix	1.00	1.00	1.00	1.00
TOTAL (%)	**100.00**	**100.00**	**100.00**	**100.00**

LC = control group; L1, L2, L3 = experimental groups. RO = rapeseed oil; GF = ground flaxseed.

Table 4. Nutritional value of experimental diets – Phase I: Growing (25-60 kg)

Nutritional parameter	LC (%)	L1 (%)	L2 (%)	L3 (%)	NRC 2012 requirement
Metabolisable energy (MJ/kg)	13.69	13.81	13.85	14.12	13.20–13.60
Crude protein (%)	15.6	15.7	15.8	15.9	15.5–17.0
Crude fat (%)	3.86	5.00	5.55	7.28	-
Crude fibre (%)	3.20	3.15	3.12	3.05	max. 5.0
Lysine (%)	0.76	0.76	0.77	0.77	≥ 0.75
Met+Cys (%)	0.45	0.45	0.45	0.46	≥ 0.44
Threonine (%)	0.50	0.50	0.50	0.51	≥ 0.49
Total Calcium (%)	0.63	0.63	0.63	0.63	0.60–0.70
Total Phosphorus (%)	0.50	0.50	0.50	0.49	0.45–0.55
Linoleic acid LA (C18:2n-6, %)	1.82	1.98	2.06	2.55	-
α-Linolenic acid ALA (C18:3n-3, %)	**0.08**	**0.38**	**0.65**	**1.17**	-
n-6/n-3 PUFA ratio	**22.8:1**	**5.2:1**	**3.2:1**	**2.2:1**	≤ 10:1

Refined rapeseed oil, erucic acid < 2%. Ground flaxseed, milled to < 2 mm particle size immediately before mixing. Premix provided per kg complete feed: vitamin A 8,000 IU; vitamin D_3 1,200 IU; vitamin E 150 mg (LC, L1, L2) / 220 mg (L3); vitamin K_3 2 mg; B-complex vitamins; Fe 100 mg; Cu 10 mg; Zn 80 mg; Mn 40 mg; I 0.3 mg; Se 0.3 mg; Co 0.2 mg; phytase ≥ 500 FTU.

The dietary n-6/n-3 ratio decreased progressively from 20.3:1 (Phase I) and 22.1:1 (Phase II) in LC to 2.2:1 in L3 in both phases, driven by the combined ALA contribution of rapeseed oil (~9.5% ALA) and ground flaxseed (~57% ALA of total fat). This gradient ensures a clear dose-response relationship between dietary treatment and the n-3 PUFA status of muscle tissue.

Sample collection and analytical methods

At slaughter (day 84, live weight 106-109 kg), all 90 pigs were processed at a commercial abattoir. Samples of m. *Longissimus dorsi* were collected bilaterally at the level of the last rib (≈500 g per animal), vacuum-packed immediately, and stored at −20°C until analysis. Chemical composition was determined following AOAC (2019) methods: dry matter (AOAC 950.46), crude protein by Kjeldahl × 6.25 (AOAC 981.10), intramuscular fat by Soxhlet extraction (AOAC 991.36), and ash by incineration at 550°C (AOAC 942.05). Muscle pH was measured at 45 min (pH_{45}) and 24 h (pH_{24}) post-mortem using a portable pH meter (Testo 205) inserted directly into the *Longissimus dorsi*.

Fatty acid methyl esters (FAMEs) were prepared by Folch extraction followed by base-catalysed transesterification with KOH/methanol (0.5 M) and methylation with BF_3/methanol (14%). FAMEs were separated and quantified on a Shimadzu GC-2010 Plus system (FID detector; SP-2560 capillary column, 100 m × 0.25 mm × 0.2 µm). Results are expressed as g/100g total fatty acids. Individual fatty acids were identified using the Supelco 37 FAME Mix standard.

Lipid Nutritional Health Indices

The following indices were calculated from the fatty acid profile of intramuscular fat:

n-6/n-3 ratio = Σ n-6 PUFA / Σ n-3 PUFA

PUFA/SFA ratio = Σ PUFA / Σ SFA

Atherogenic Index (AI) = [C12:0 + 4×C14:0 + C16:0] / [Σ MUFA + Σ PUFA] (Ulbricht & Southgate, 1991)

Thrombogenic Index (TI) = [C14:0 + C16:0 + C18:0] / [0.5×ΣMUFA + 0.5×Σn-6 PUFA + 3×Σn-3 PUFA + (n-3/n-6)] (Ulbricht & Southgate, 1991)

Peroxidizability Index (PI) = 0.025×%MUFA + 1×%C18:2n-6 + 2×%C18:3n-3 + 4×%C20:4n-6 + 8×%C20:5n-3 + 11×%C22:5n-3 + 12×%C22:6n-3 (Erickson, 1993)

h/H (hypo/hypercholesterolemic) ratio = (C18:1n-9 + Σ PUFA) / (C14:0 + C16:0) (Santos-Silva et al., 2002)

Statistical Analysis

Data were analysed by one-way ANOVA in SPSS v.26 (IBM Corp.), with treatment group as the fixed effect. Means were compared using Tukey's HSD post-hoc test ($\alpha = 0.05$). Normality was verified by the Shapiro-Wilk test. Results are expressed as means ± SEM.

RESULTS AND DISCUSSIONS

Chemical composition of m. *Longissimus dorsi*

The chemical composition data are presented in Table 5. No significant differences were found between treatments for dry matter ($p = 0.312$), crude protein ($p = 0.845$), or ash content ($p =$

0.920), indicating that the dietary modifications did not alter the fundamental structural composition of muscle tissue. This is consistent with the meta-analytical findings of Wang et al. (2021), who confirmed across 14 studies (752 pigs) that PUFA supplementation does not significantly affect the crude protein content of the *Longissimus dorsi* ($p > 0.05$).

Table 5. Chemical composition of m. *Longissimus dorsi* (%, fresh weight; means ± SEM, n = 22–23/group)

Parameter	LC	L1	L2	L3
Dry matter (%)	26.15±0.28	25.92±0.31	25.78±0.26	25.65±0.33
Crude protein (%)	22.48±0.22	22.55±0.25	22.52±0.21	22.60±0.26
Intramuscular fat – IMF (%)	**1.92±0.12^{a}**	**1.98±0.14^{a}**	**2.10±0.11^{ab}**	**2.28±0.15^{b}**
Ash (%)	1.14±0.02	1.15±0.02	1.15±0.02	1.16±0.02
pH_{45} (45 min post-mortem)	6.42±0.08	6.45±0.09	6.48±0.08	6.50±0.10
pH_{24} (24 h post-mortem)	5.62±0.05	5.65±0.06	5.68±0.05	5.72±0.06

Different superscript letters within a row indicate significant differences ($p < 0.05$, Tukey HSD). IMF = intramuscular fat determined by Soxhlet extraction. pH_{45} and pH_{24} measured directly in m. *Longissimus dorsi*

Intramuscular fat (IMF) was significantly higher in L3 (2.28%) compared to LC (1.92%, $p = 0.032$). This moderate increase aligns with the meta-analytical weighted mean difference of +0.467% reported by Wang et al. (2021) for PUFA-supplemented pigs, and is likely attributable to stimulated hepatic lipogenesis from elevated dietary lipid intake in L3 (4% total added fat). Crude protein content was statistically equivalent across all groups (22.48–22.60%, $p = 0.845$), confirming that protein deposition efficiency was not impaired by the supplementation. pH values fell within the physiological norm (pH_{45}: 6.42–6.50; pH_{24}: 5.62–5.72), excluding PSE or DFD anomalies. Jankowiak et al. (2021) established that pH_{45} values above 6.30 are associated with higher water holding capacity and lower drip loss, a condition satisfied by all treatment groups in this study.

Fatty acid profile of intramuscular fat

The complete fatty acid profile of intramuscular fat from m. Longissimus dorsi is presented in Table 6. Dietary supplementation produced highly significant modifications in the PUFA fraction while exerting comparatively modest effects on SFA and MUFA content.

Table 6. Fatty acid profile of m. *Longissimus dorsi* intramuscular fat
(g/100 g total fatty acids; means ± SEM, n = 22-23/group)

Fatty acid	LC	L1	L2	L3
Saturated Fatty Acids (SFA)				
C14:0 (Myristic)	1.52±0.08	1.48±0.09	1.44±0.08	1.38±0.10
C16:0 (Palmitic)	24.80±0.42^{a}	24.15±0.48^{ab}	23.50±0.40^{b}	22.80±0.52^{c}
C18:0 (Stearic)	12.35±0.38	12.10±0.42	11.85±0.36	11.55±0.45
Σ SFA	**38.67±0.65^{a}**	**37.73±0.72^{ab}**	**36.79±0.62^{b}**	**35.73±0.78^{c}**
Monounsaturated Fatty Acids (MUFA)				
C16:1n-7 (Palmitoleic)	2.85±0.18	2.80±0.20	2.75±0.17	2.68±0.22
C18:1n-9 (Oleic)	40.55±0.88^{a}	41.25±0.95^{a}	42.10±0.85^{b}	43.40±1.02^{c}
Σ MUFA	**43.40±0.92^{a}**	**44.05±1.02^{a}**	**44.85±0.90^{b}**	**46.08±1.08^{c}**
n-6 Polyunsaturated Fatty Acids				
C18:2n-6 (Linoleic – LA)	14.50±0.35	14.25±0.38	14.10±0.33	13.85±0.40
C20:4n-6 (Arachidonic)	0.82±0.05	0.80±0.06	0.78±0.05	0.75±0.06
Σ n-6 PUFA	**15.32±0.38**	**15.05±0.42**	**14.88±0.36**	**14.60±0.44**
n-3 Polyunsaturated Fatty Acids				
C18:3n-3 (ALA – α-Linolenic)	0.62±0.06^{a}	1.82±0.14^{b}	2.68±0.20^{c}	3.85±0.28^{d}
C20:5n-3 (EPA)	0.05±0.01^{a}	0.12±0.02^{b}	0.17±0.02^{c}	0.24±0.03^{d}
C22:5n-3 (DPA)	0.28±0.03^{a}	0.42±0.04^{b}	0.55±0.04^{c}	0.72±0.06^{d}
Σ n-3 PUFA	**0.95±0.08^{a}**	**2.36±0.18^{b}**	**3.40±0.24^{c}**	**4.81±0.34^{d}**
PUFA Summary				
Σ PUFA (total)	**16.27±0.42^{a}**	**17.41±0.52^{b}**	**18.28±0.48^{c}**	**19.41±0.62^{d}**
PUFA/SFA ratio	**0.421±0.012^{a}**	**0.461±0.015^{ab}**	**0.497±0.014^{b}**	**0.543±0.018^{c}**

Different superscript letters (a,b,c,d) within a row indicate significant differences ($p < 0.05$, Tukey HSD). Green-highlighted section: n-3 PUFA fraction with direct experimental relevance. ALA = alpha-linolenic acid (C18:3n-3); EPA = eicosapentaenoic acid (C20:5n-3); DPA = docosapentaenoic acid (C22:5n-3).

The most significant outcome was the highly significant, dose-dependent increase in ALA content: from 0.62% in LC to 1.82% in L1, 2.68% in L2, and 3.85% in L3 ($p < 0.001$), representing a 5.8-fold enrichment in L3 vs. control. This linear dose-response pattern is consistent with the findings of Bartkovsky et al. (2022), who reported statistically significant ALA accumulation in m. *Longissimus dorsi* of pigs fed 5% and 10% flaxseed for 3-6 weeks, with the effect proportional to both inclusion level and duration. The direct dietary incorporation of ALA into muscle phospholipids in monogastric species is well established, as dietary fatty acids absorbed from the intestine are incorporated into tissue lipids largely unchanged (Okrouhla et al., 2018).

EPA (C20:5n-3) and DPA (C22:5n-3) also increased significantly across all supplemented groups ($p < 0.001$), indicating active, albeit limited, elongase and desaturase activity in the Landrace × Large White genotype. Minelli et al. (2023) similarly observed significant increases in n-3 long-chain PUFA in the *Longissimus thoracis* of Large White pigs fed a low n-6/n-3 diet, with the magnitude of transfer from diet to muscle being consistent with the 35-50% efficiency commonly reported for ALA incorporation in monogastric tissues.

Total SFA decreased significantly from 38.67% in LC to 35.73% in L3 ($p = 0.004$), driven primarily by a reduction in palmitic acid (C16:0, $p = 0.028$). This is attributed to the known inhibitory effect of elevated dietary PUFA on the activity of stearoyl-CoA desaturase (SCD) and de novo fatty acid synthesis in the liver. MUFA showed a significant increase, particularly oleic acid (C18:1n-9: 40.55% in LC vs. 43.40% in L3, $p = 0.045$), reflecting the high oleic acid content of rapeseed oil (~60% C18:1) incorporated into the diet. The PUFA/SFA ratio improved progressively from 0.421 in LC to 0.543 in L3 ($p < 0.001$), with the recommended threshold of 0.45 being surpassed from group L2 onward.

Lipid Nutritional Health Indices

The lipid health indices calculated from intramuscular fatty acid profiles are presented in Table 7.

Table 7. Lipid nutritional health indices of m. *Longissimus dorsi* intramuscular fat (means ± SEM, n = 22-23/group)

Lipid health index	LC	L1	L2	L3
n-6/n-3 PUFA ratio	23.4±1.8^{a}	6.4±0.5^{b}	4.4±0.4^{c}	3.6±0.3^{c}
PUFA/SFA ratio	0.421±0.012^{a}	0.461±0.015^{ab}	0.497±0.014^{b}	0.543±0.018^{c}
Atherogenic Index (AI)	0.612±0.022^{a}	0.568±0.024^{ab}	0.528±0.020^{b}	0.482±0.025^{c}
Thrombogenic Index (TI)	1.245±0.048^{a}	1.118±0.052^{ab}	1.012±0.044^{b}	0.895±0.055^{c}
Peroxidizability Index (PI)	78.5±3.2^{a}	94.8±4.1^{b}	108.2±3.8^{c}	128.5±5.2^{d}
h/H ratio	2.08±0.08^{a}	2.18±0.09^{a}	2.28±0.08^{b}	2.42±0.10^{c}
Σ n-3 PUFA (% total FA)	0.95±0.08^{a}	2.36±0.18^{b}	3.40±0.24^{c}	4.81±0.34^{d}

Different superscript letters within a row indicate significant differences ($p < 0.05$). AI = atherogenic index; TI = thrombogenic index; PI = peroxidizability index; h/H = hypocholesterolemic/hypercholesterolemic ratio.

The n-6/n-3 PUFA ratio was dramatically reduced in all supplemented groups: from 23.4:1 in LC to 6.4:1 in L1, 4.4:1 in L2, and 3.6:1 in L3 ($p < 0.001$). Groups L2 and L3 achieve or surpass the WHO (2002) recommendation of ≤ 4:1. For comparison, Minelli et al. (2023) achieved a muscle n-6/n-3 ratio of approximately 2.8:1 in Large White pigs fed a dietary ratio of 1.4:1, a more extreme intervention. In our system, the dietary ratio of 2.2:1 in L3 translates to a muscle ratio of 3.6:1, suggesting an approximately 39% transfer efficiency, in agreement with published values for ALA incorporation in monogastric muscle.

The atherogenic index (AI) was significantly reduced in L2 (0.528) and L3 (0.482) compared to LC (0.612; $p < 0.001$), in line with the known effect of rapeseed oil, which carries one of the lowest AI values among common plant lipids (AI = 0.05; Khalili Tilami & Kouřimská, 2022). The thrombogenic index (TI) decreased from 1.245 in LC to 0.895 in L3 ($p < 0.001$). Okrouhla et al. (2018) reported analogous significant reductions in both AI and TI in the backfat of DanBred pigs fed 40 g/kg rapeseed oil for 4–6 weeks, supporting the consistency of our findings across species, genotypes, and tissue types.

The peroxidizability index (PI) increased significantly with supplementation (78.5 in LC to 128.5 in L3, $p < 0.001$), reflecting the intrinsic oxidative susceptibility of n-3 PUFA-enriched

fat. Sobeková et al. (2023) found that 6 weeks of flaxseed supplementation significantly increased TBARS content in cardiac and brain tissue of fattening pigs ($p < 0.05$), underscoring the need for adequate antioxidant protection. In the present experiment, vitamin E was increased to 220 mg/kg NC in L3 and ethoxyquin was included in the premix of all supplemented groups; however, TBARS analysis of meat samples was not performed and is recommended for future investigations. The h/H ratio improved significantly in L2 (2.28) and L3 (2.42) vs. LC (2.08; $p = 0.002$), reflecting the combined contribution of increased oleic acid (from RO) and n-3 PUFA (from GF) to the overall cholesterol-lowering fatty acid profile.

CONCLUSIONS

The combined supplementation with rapeseed oil and ground flaxseed produced a highly significant, dose-dependent enrichment of m. Longissimus dorsi intramuscular fat with ALA (5.8-fold increase in L3 vs. LC; $p < 0.001$) and its elongation products EPA and DPA. Total n-3 PUFA increased from 0.95% to 4.81% of total fatty acids in L3. These results confirm that the Landrace × Large White hybrid possesses the metabolic capacity to incorporate dietary n-3 PUFA into muscle lipids proportionally to dietary supply.

All lipid health indices were significantly improved by the supplementation. The n-6/n-3 ratio was reduced from 23.4:1 to 3.6:1 in L3, meeting the WHO (2023) recommendation of ≤ 4:1 from group L2 onward. The AI decreased from 0.612 to 0.482 and the TI from 1.245 to 0.895 in L3 ($p < 0.001$). The h/H ratio and PUFA/SFA ratio were improved significantly in L2 and L3. Pork from L2 and L3 can be considered a functional food with a demonstrably improved cardiovascular nutritional profile.

The combination of 1% rapeseed oil + 1.5% ground flaxseed (L2) is recommended as the optimal practical level for commercial applications: it achieves an n-6/n-3 ratio of 4.4:1, surpasses the PUFA/SFA threshold of 0.45, significantly reduces AI and TI, and maintains a PI (108.2) below the critical threshold for unacceptable oxidative instability, without requiring extraordinary antioxidant interventions. The L3 combination (2% RO + 2% GF) achieves superior n-3 enrichment but necessitates strict antioxidant management (vitamin E ≥ 220 mg/kg NC, ethoxyquin ≥ 125 ppm) to mitigate oxidative risks during storage.

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EFFECTS OF OMEPOL 30 ADDITIVE ON TRYPSIN-ANTITRYPSIN SYSTEM, ENDOTOXICOSIS AND EGG PRODUCTION OF QUAILS

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Abstract

This publication presents the results of investigations carried out on 150 Japanese quails (Coturnix japonica). Investigations aimed to highlight the anti-stress and adaptive properties of phytomicrobial additive OMEPOL 30 and bioproductive potential of the quails. All three groups of birds received similar housing, veterinary and feeding conditions, the differences consisted in OMEPOL 30 adiminstration in the doses of 2.5 and 5.0 g/kg, for the experimental groups. During 10 weeks, the quails were monitored, eggs from each group were collected, the blood samples were taken for investigations at the beginning of the study, on the 35th day and the 71st day of the experiment from 5 quails randomly from each group. The tested product was well tolerated by the quails. The biochemical investigations highlighted a decrease of trypsin in the blood, as well as balanced the activity of the trypsin-antitrypsin system. In the experimental groups were found decreases in endotoxicosis indicators, such as molecules with average molecular weight and necrotic substances. Egg production indicators had higher values in the experimental groups compared to control.

Key words*: egg production, endotoxicosis, feed additive, quail, trypsin.*

INTRODUCTION

Worldwide, as well as in the Republic of Moldova, quail is of particular interest for its meat and eggs, demanded by the consumer due to their benefic potential properties (Kiwitt, 2003; Ciftci et al., 2018; Pavlicenco, 2019; Patieva et al., 2023). In addition to being exploited for eggs and meat production, the Japanese quail (*Coturnix coturnix japonica*) is frequently used for experimental research (Martynova & Kornienko, 2019; Baltag, 2020; Hatab et al., 2024).

For laying birds, the laying period, normally 12 months for quails, profoundly impacts health, welfare, and metabolism due to factors such as intense nutritional demands, elevated stress from continuous egg production, and rapid calcium mobilization for eggshell formation. The evaluation of egg production of birds is carried out by daily monitoring (Pistol, 2025). The fast development of the quail breeding sector worldwide, including in the Republic of Moldova, is explained not only by the taste, dietary and curative properties of quail eggs and meat, but also by the biological peculiarities of these birds, such as high growth rate and high egg productivity. (Kiwitt, 2003; Patieva et al., 2023). Recently, a strong correlation between egg productivity of hens and trypsin activity has been established (Vertiprakhov et al., 2023a). In birds trypsin is secreted in the pancreas. In the intestine, it is involved in the hydrolysis of proteins into peptides and amino acids, which subsequently enter the bloodstream and is distributed throughout the body. The presence of trypsin in numerous organs and tissues, as well as the presence of specific PAR receptors indicate its participation in the regulation of various physiological functions. Data obtained on the circulation of trypsin in the bodies of chickens clearly showed that its action, similar to that of hormones, is not limited to pancreatic function but is also aimed at regulating metabolism throughout the body by optimizing the free amino acid levels in various biological media (Vertiprakhov et al., 2023b). The study of the influence of biologically active substances on indices that reflect the functional state of the pancreas and the intensity of catabolic processes in the body is important for assessing the adaptive and anti-stress properties of the tested products (Putin et al., 2020). Birds are sensitive to stress, especially to long-term technological stress (Pistol, 2025). The scientific literature contains numerous data on combating stress and

enhancing immunity through the use of natural remedies, particularly those of plant origin. (Sukhorukova & Kostesha, 2010; Ciftci et al., 2018; Pavlicenco, 2019; Yehmed et al., 2023; Ashour et al., 2025; Deeb et al., 2025). In the context, the development, testing, and implementation in production of new and efficient additives obtained through microbial biotechnologies represent an important and promising scientific direction (Grigore et al., 2025). There is evidence that such products exert a beneficial effect on the productive performance of animals (Grigore, et al., 2022; Ashraf, 2022; Perricone et al., 2022).

Thus, the aim of this study was to evaluate the effect of the Omepol 30 additive on the trypsin-antitrypsin system and the phenomenon of endotoxicosis, reflected in the egg production of quails raised under poultry farm conditions.

MATERIALS AND METHODS

Object of study

The research was carried out on 150 Texas quails (The Northern Bobwhite females), staying in full egg-laying process, in conditions of the poultry farm of SRL "Mihailorina-COM", Corlăteni village, Râșcani district, Republic of Moldova. The birds used in the experiment were similar in physiological status, age, body mass and origin. The quails at the beginning of the study were 32 weeks old, the study lasted another 71 days. The quails from all three groups involved in the experiment were housed under similar conditions (housing, with the same hygienic and technological indicators, including microclimate parameters, nutrition, watering and veterinary care, which ensured their hygiene and welfare).

Feed rations

The quails were divided into three groups: the control group (CG) and the experimental groups 0.25% EG and 0.5% EG, the feed ration of which was supplemented with 0.25 and 0.5% additive, respectively. The birds in all groups were fed with the standard compound feed used on the farm, in accordance with current requirements. The additive was administered to quails with food, separately for each experimental group, according to the experimental scheme showed in Table 1.

Table 1. Scheme of administration of the Omepol 30 additive to quails

Group	No. of birds	Route of administration	Dose, g kg^{-1} feed	Dosing regimen
CG	50	-	-	-
0.25% EG	50	per os	2.5	daily
0.5% EG	50		5.0	

Development of Omepol 30 additive

The phytomicrobial feed additive, coded Omepol 30 (Barley+Carrot+ Exopolysaccharides) is composed of: dry barley biomass from beer industry wastes - 75%, dry carrot biomass from food industry wastes - 25%, exopolysaccharides of pigmented yeast *R. mucilaginosa* CNMN-Ys-10 of 30 mg g^{-1}d.w.

Barley biomass is obtained from barley wastes remaining after beer production by mixing the biomass (waste) with distilled water in the 1:1 v/v ratio, autoclaving the mixture at temperature of 115°C, 0.5 atm. for 30 minutes, then separating the supernatant (barley water extract, used for microbial biotechnologies as nutrient medium) from the sediment (barley biomass) by centrifugation at 3000 rpm for 10 minutes. The sediment is subsequently dried at 50±1°C until a constant mass is achieved. Carrot biomass is obtained from carrot wastes (peels) from the food industry, remaining after carrot processing, by repeatedly washing the peels to remove impurities, shredding them to sizes of 0.5-1.0 cm, and mixing the carrot peels with distilled water in the 1:1 v/v ratio, autoclaving the mixture at temperature of 115°C, 0.5 atm. for 30 minutes, then separating the supernatant (carrot water extract) from the sediment (carrot biomass) by centrifugation at 3000 rpm for 10 minutes, then sterilizing the supernatant (carrot water extract) in an autoclave at temperature of 115°C, 0.5 atm. for 30 minutes. The sediment (carrot biomass) is subsequently dried at 50±1°C until a constant mass is achieved. The water carrot extract, with a pH of 5.6 and a residual sugar concentration of 5.0°Bx, was used as a nutrient medium, a sole source of nutrition, for the submerged cultivation of the yeast strain *R. mucilaginosa* CNMN-Ys-10 at the temperature of 27-28°C, under shaking conditions of 200 rpm for 120 hours in order to obtain exopolysaccharides in the culture liquid. The Omepol 30 additive is obtained by mixing dry barley biomass with dry carrot biomass in the 3 (750 g):1 (250 g) ratio and gradually soaking the resulting mixture (in several rounds)

with the 4 L of culture liquid containing 8.15±1.47 g d.w. L^{-1} exometabolites from the *R. mucilaginosa* CNMN-Ys-10, in order to obtain an exopolysaccharide concentration of 30 mg g^{-1}d.w. After each round the mixture is dried at temperature of 50±1°C to remove excess moisture. Following the final round, the product was dried at the same temperature until a constant mass was reached. The concentration of exopolysaccharides in the culture liquid was determined gravimetrically by sedimentation with 96° ethyl alcohol, culture liquid: ethyl alcohol ratio 1:4 (Ma W.J. et al., 2018), obtained from culture liquid remaining after cultivated the strain *R. mucilaginosa* CNMN-Ys-10 on carrot extract nutrient medium.

Monitored parameters

During the experiment, the birds were monitored and examined continuously, according to standard procedures, and their physiological state, behavior and possible adverse reactions associated with the administration of the additive were evaluated.

Blood collection from quails was performed via the jugular vein, preferably on the right side. After firmly restraining the bird, a few feathers were plucked from the venous area, and the site was disinfected with 70% alcohol. Pressure was applied at the base of the neck to visualize the vein, after which the needle was inserted at a slight angle. Blood was slowly aspirated (approximately 1.5-2.0 mL) and promptly transferred into standard test tubes. The obtained blood serum was stored at -20°C in a freezer. Blood samples for biochemical examinations were collected in three stages: at the beginning of the study (from 5 quails randomly), at the 35th day of the experiment (first sampling) and at the end of the study, on the 71st day from the beginning of the experiment (second sampling), from 5 quails from each experimental group.

The quails were subsequently sacrificed using cervical dislocation as the stunning method. This procedure was performed by trained farm staff.

Samples collected from birds were analyzed for the determination of essential biochemical parameters, such as: trypsin, α_1-antitrypsin, α_2-macroglobulin (α_2-MG), molecules with average molecular weight (AMW), necrotic substances (NS), histidine dipeptides (carnosine). The determinations were performed by spectrophotometric methods, using commercial ELITEH kits (France). The tests were performed in the Laboratory of Biochemistry, at Nicolae Testemitanu State University of Medicine and Pharmacy, according to protocols optimized for use with the Synergy H1 Hybrid Reader multimodemicroplate spectrophotometer, (BioTek Instruments, Winooski VT, USA).

Egg production was monitored from the start of laying until the end of the experiment, the laid eggs were counted and weighed for each group separately.

Statistical indices

Statistical processing of the results was performed using the MO Excel and Statistics 9.0 software suite. The results were expressed by calculating the mean, standard deviation and confidence interval for an average of three repetitions. All differences were considered statistically significant for $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$.

RESULTS AND DISCUSSIONS

The laying quails tolerated the additive well, administered to both experimental groups simultaneously with the feed, no adverse reactions were detected. At slaughter, in order to collect samples of biological substrates (blood, liver and muscle tissue), no morphological changes in the carcasses or in the organs of the thoraco-abdominal cavity were observed. The results of the assessment of the quantity of the marker indices of the trypsin-antitrypsin system in the serum of quails are presented in the Table 2.

Table 2. Quantitative indices of the trypsin-antitrypsin system in the serum of quails from different groups

Indices	Debut	Group		
		CG	0.25% EG	0.5% EG
Trypsin, µmol L^{-1}	1.98±0.03	1 (samp.) 2.9±0.1*** 2 (samp.) 2.7±0.12	1 (samp.) 2.2±0.1** 2 (samp.) 2.3±0.04*	1 (samp.) 2.20±0.06*** 2 (samp.) 2.44±0.14
α_1-antitrypsin, µmol L^{-1}	29.3±0.8	1 (samp.) 28.92±2.42 2 (samp.) 33.43±0.35	1 (samp.) 29.93±2.60 2 (samp.) 32.92±2.20	1 (samp.) 34.93±2.34 2 (samp.) 32.75±2.14
α_2-MG, g L^{-1}	1.3±0.06	1.24±0.01 2 (samp.) 1.82±0.15**	1.12±0.06* 2 (samp.) 1.41±0.03*	1.31±0.04 2 (samp.) 1.24±0.08**

Note: * – p<0.05; ** p<0.01; *** p<0.001

The results indicate that at the first sampling, serum trypsin activity shows a significant

increasing trend compared to the initial values in all groups included in the experiment, the increase being more pronounced ($p<0.001$) in the CG by 45.5%. In the 0.25% EG and 0.5% EG the increase in serum trypsin was markedly lower compared to the initial values, constituting only 9.6-11.1% ($p<0.05$). At the same time, under the influence of the tested additive, the content of this enzyme in the experimental groups was significantly lower by 23.6-24.7% ($p<0.01$ for 0.25% EG; $p<0.001$ for 0.5% EG) compared to the CG. These trends highlight the possible anti-stress properties of the additive under the experimental conditions. This hypothesis was confirmed at the end of the experiment (second sampling), when the trypsin content in the experimental groups was lower by 9.3-13.0% compared to the CG and constituted of 2.34±0.04-2.44±0.14 μmol L^{-1}, although these values were increasing by 7.8-10.9% compared to the values from the first sampling (Table 2).

In the complex process of regulating and controlling the activity of tissues and organs involving proteolytic enzymes, enzyme inhibitors play an important role. The interactions between proteolytic enzymes and their inhibitors represent a complex biological phenomenon that largely depends on internal and external factors constantly acting on the living organism (Solcan, 2011; Pistol, 2025). The obtained results established that the serum α1-antitrypsin value in birds from the CG at the first sampling, was 28.92±2.42 μmol L^{-1}, showing an insignificant decrease of 1.4% compared to the initial value. In contrast, in the experimental groups was observed an increasing trend, with values rising by 2.0-19.1% compared to the initial value, reaching 29.93±2.60 to 34.93±2.34 μmol L^{-1}. At the same time, these values of α_1-antitrypsin in the serum of quails from the experimental groups were 3.5-20.8% higher than those recorded in the CG, but these differences were not statistically significant (Table 2).

This phenomenon can be considered as a favorable one, indicating the establishment of an adequate reactivity in the experimental quails, which allows the normal development of physiological processes involving proteolytic enzymes. At the second sampling, the level of α_1-antitrypsin in the CG was 33.43±0.35 μmol L^{-1}, in insignificant increase by 15.6% compared to the value from the first sampling, a tendency also observed in the 0.25% EG, in which the increase constituted approximately 10% compared to the previous values. In contrast, in quails from 0.5% EG (maximum dose), a slight decrease of 6.2% of this parameter was recorded, compared to the value recorded at the first sampling (Table 2). Compared to the control group at the second sampling, the content of α_1-antitrypsin in the experimental groups was insignificantly reduced, by 1.5-2.0%, that indicates the adaptive and anti-stress properties of the additive during the period of intense metabolic stress. Similar results regarding the dynamics of serum α_1-antitrypsin were also reported following the administration of the ZooBioR product to laying quails (Macari et al., 2024).

At the beginning of the research, the content of α_2-macroglobulin (α_2-MG) in quails was 1.34±0.06 g L^{-1}. At the first sampling, a negative dynamic of this indicator compared to the initial values was recorded in all groups. In the CG the decrease was 7.5%, and in the experimental groups of 2.2-16.4% (for the 0.25% EG $p<0.05$). At the second sampling, the content of α_2-MG showed a tendency to increase in quails from the CG by 46.8% ($p<0.01$) compared to the values established at the first sampling. A similar increase was observed in the 0.25% EG, where the values increased by 25.9% ($p<0.01$) compared to the first sampling. Trends in the increase of α_2-MG in the blood of animals, as a result of the administration of biologically active substances, have also been reported in other studies (Putin et al., 2020; Pistol, 2025). It is important to note that in the 0.5% EG, which consumed the maximum dose of additive, a slight decrease in α_2-MG value by 5.3% was observed at the second sampling compared with the first sampling. A comparative analysis between groups at the end of the experiment revealed a reduction of α_2-MG in the experimental groups by 22.5-31.9% compared to the CG (for the 0.25% EG $p<0.05$, for the 0.5% EG $p<0.01$). Based on the obtained results, interpreted in the context of data from the scientific literature, it can be summarized that the tested additive, administered to quails exploited for meat and egg production, significantly influences the content of the enzymes of the trypsin-antitrypsin system and

exhibits its modulating properties. However, more extensive investigations are required to establish a definitive conclusion.

In assessing the effects of feed additives on birds, it is also of interest to investigate the marker parameters of endotoxicosis, which may indicate the presence in the animal body of substances with a negative impact on general homeostasis (Mihalciuc, 2017; Rotaru, 2016). The results of the evaluation of these parameters in quails from different groups, as well as the changes induced by the administration of the additive, are presented in Table 3.

Table 3. Endotoxicosis indices in the serum of quails from different groups

Indices	Debut	Group		
		CG	0.25% EG	0.5% EG
AMW, c/u	20.50±1.36	1 (samp.) 23.34±1.16 2 (samp.) 22.32±0.90	1 (samp.) 20.46±0.99 2 (samp.) 19.31±0.75*	1 (samp.) 22.76±1.23 2 (samp.) 21.12±0.84
NS, c/u	4.15±0.37	1 (samp.) 4.31±0.43 2 (samp.) 4.14±0.09	1 (samp.) 4.61±0.37 2 (samp.) 3.51±0.38	1 (samp.) 4.46±0.35 2 (samp.) 3.99±0.18
Histidine dipeptides, μmol L^{-1}	89.95±3.60	1 (samp.) 85.96±3.73 2 (samp.) 89.08±2.36	1 (samp.) 91.76±2.76 2 (samp.) 89.55±1.63	1 (samp.) 90.20±1.74 2 (samp.) 89.28±2.00

Note: * – $p<0.05$.

The scientific literature mentions that, in industrial conditions of exploitation and animal breeding or in the case of the occurrence of pathological conditions, various disorders of metabolism and functional state of the liver, as well as of other organs and systems, may occur. These changes may lead to early tissue damage, accompanied by the formation of necrotic substances (NS), average molecular weight substances (AMW) and other compounds (Belko & Bogomoltseva, 2011; Kolberg et al., 2010). In the conducted study, it was established that the substances with average molecular weight (AMW) in the blood of quails at the beginning of the experiment constituted 20.50±1.36 c/u. At the first sampling, results in quails from the CG and 0.5% EG AMW showed a clear tendency to increase by 13.9% and 11.0%, respectively compared to the initial values. In the 0.25% EG statistically significant changes compared to the initial values were not established (Table 3). This fact highlights the potential beneficial properties of the additive tested under conditions of stress and increased metabolic demand. Comparative analysis of the serum level of AMW among groups confirmed this observation. At the first sampling the value of this parameter in the 0.25% EG was 12.3% lower than in the CG and 10.1% lower than in the 0.5% EG. Towards the end of the study, at the second sampling, the serum AMW level showed a general decreasing tendency in all groups, the CG exhibiting a 4.4% reduction compared with the first sampling. At the same stage, the AMW content in the experimental groups was lower compared with the CG, by 5.4 in the 0.5% EG and by 13.5% ($p\geq0.05$) in the 0.25% EG (Table 3). It is considered that the reduced level of AMW in the blood reflects the favorable action of the tested product, suggesting a reduction in catabolic processes in the bird body, which may also positively influence the meat quality (Rotaru, 2016; Aslam et al., 2020). Some authors have reported lower AMW values in clinically healthy young calves compared with higher values observed in enteropathies, suggesting that this parameter can serve as an integral indicator in the evolution of endogenous intoxication, reflecting the intensity of catabolic processes occurring in the body. (Belko & Bogomoltseva, 2011). In the context of these findings, other authors report that the AMW index manifests neurotoxic activity, inhibits protein biosynthesis and the activity of several enzymes, deregulates oxidative processes, induces a state of secondary immunosuppression, and exerts toxic effects on erythropoiesis as well as on the main homeostatic systems (Nazarenko & Kishkun, 2006; Mohi-Ud-Din et al., 2019).

Another marker of endotoxicosis is the biochemical index represented by necrotic substances (NS), whose values at the beginning of the experiment were 4.15±0.37 c/u. At the first sampling NS showed an increasing trend in all groups by 3.9% in the CG, 7.5% in the 0.5% EG and 11.1% in the 0.25% EG compared to the initial values (Table 3). At the same time, at the first sampling, the NS levels in both experimental groups were 3.5-7.0% higher than in the CG, although the differences were not statistically significant. At the second sampling, serum NS levels decreased compared with the first sampling in quails from all groups, which can be explained by the adaptation of the birds to the new experimental conditions. Thus, in the

CG the decrease was 14.0%, and in the 0.25% EG and 0.5% EG of 23.9% and 10.5% respectively, compared to the values from the first sampling. The lower NS values in birds from the 0.25% EG and 0.5% EG by 15.2 and 3.6% respectively, compared to the CG, indicate the beneficial effect of the additive in reducing the endotoxicosis in quails during the experimental period. Similar trends in the manifestation of endotoxicosis marker parameters (AMW and NS) were also reported in broilers that were prophylactically administered the BioR product (Putin, 2014), as well as by other authors in different pathological states (Gudumac & Prisăcaru, 2012; Muhamed, 2005; Mihalciuc, 2017; Kuzminova et al., 2023).
A special role in the evaluation of metabolism and, in particular, of anabolic processes belongs to histidine dipeptides, especially carnosine, which is considered a marker of anabolic processes in the body (Lackner et al., 2021). The conducted research determined that at the beginning of the experiment, the concentration of histidine dipeptides in quails was 89.95±3.60 μmol L^{-1}. At the first sampling, this value in the CG decreased by 4.4% compared to the beginning values.
At the same stage, the tested additive not only prevented the decline of this biochemical indicator, but on the contrary, resulted in a slight increase, by 0.3-2.0% in the experimental groups compared to the initial values. Also, at the first sampling, the level of histidine dipeptides in the experimental groups was 4.9-6.7% higher compared to the values established in the CG (Table 3). Considering two essential aspects: the use of clinically healthy birds in the experiment and the relatively constant level of the investigated indicator in blood serum, it can be noted that the increase, even insignificant, of the histidine dipeptides in the blood represents a favorable and biologically relevant result. At the second sampling, no statistically significant differences among the groups in the content of histidine dipeptides were observed, the values varying at the level of 89.08±2.36 - 89.55± 1.63 μmol L^{-1} (Table 3). Similar results, characterized by higher values of histidine dipeptides in animals treated prophylactically with various biologically active products, have been reported in other studies (Kopec et al., 2013; Kopec et al., 2020).
The above results confirm that the Omepol 30 feed additive was well tolerated by the birds, which maintained a good and uniform health throughout the experiment. In addition, the tested product exerts a positive effect on metabolic processes, demonstrating the ability to stimulate anabolic function and reducing catabolic processes. This effect is expressed through a mechanism aimed at reducing endotoxicosis parameters in the bloodstream.
Currently, the breeding and exploitation of laying birds are oriented at obtaining high-quality products with maximum economic efficiency (Covaşă & Cofas, 2016; Bobyleva, 2017; Pistol et al., 2023; Pistol, 2025; Fisinin, 2025). The synthesis of data from the scientific literature and the results of our own research on biologically active substances in birds highlight that productive indices have particular significance in the objective confirmation of the clinical, hematological, and biochemical parameters obtained during the investigation of these substances (Pistol, 2025). These indicators are also of major importance in substantiating the dose and administration regimen of the tested products. The dynamics of egg production and other indicators associated with this physiological process in quails from the CG and those under the tested additive are presented in Table 4.

Table 4. Productive and feed conversion indices in laying quails from different groups

Indices	Group		
	CG	0.25% EG	0.5% EG
Number of birds at the debut, head	50	50	50
Egg production, first day, un.	37	36	37
Laying intensity/debut, %	74.0	72.0	74.0
Average weight of an egg (debut), g	13.5±0.4	13.5±0.2	13.5±0.3
Average weight of an egg (at 35th day of the study), g	12.9±0.46	13.7±0.20	13.5±0.34
Egg production /1-71st days of the study, un.	2247	2422	2308
Average weight of an egg (at 71st day of the study), g	12.4±0.29	12.9±0.19	12.7±0.17
Average weight of an egg during the study, g	12.9±0.21	13.4±0.18	13.2±0.15
Egg mass production /period, kg	28.99	32.45	30.43
Quantity of feed consumed by birds (total study), kg	142.50	138.65	141.25
Feed consumed per 100 produced eggs, kg	6.34	5.72	6.12
Feed consumed per 10 produced eggs, kg	0.63	0.57	0.61
Feed consumed per 1 kg egg mass, kg	4.96	4.27	4.64

According to these data, the number of eggs collected on the first day of the experiment was 36-37 units per group, and the laying intensity was 72.0-74.0%, similar values for all groups. Furthermore, the egg weight on the first day of the study was practically identical in all three groups of quails involved in the experiment (Table 4).

On the 35th day of the study, the egg average weight in the experimental groups was 0.54-0.76 g, or 4.2-5.9%, higher compared to the values recorded in the CG. The correlation between the tested product and egg production in the experimental groups was maintained throughout the study. Thus, during the experiment, a total of 2247 eggs were collected from birds in the CG, corresponding to an average of 44.9 eggs per quail. This indicator was higher in the experimental groups, reaching 48.4 eggs per quail in the 0.25% EG and 46.2 eggs per quail in the 0.5% EG. In percentage terms, the level of egg production was higher by 7.8% in the 0.25% EG and by 2.9% in the 0.5% EG compared to the CG. The increase in egg productivity in the experimental groups and the lower trypsin values observed in these groups, compared with the CG, are consistent with results showing a significantly lower level of trypsin in the serum of laying hens compared with that observed in broiler chickens (Vertiprakhov et al., 2023a).

At the same time, the total egg production in the 0.25% EG exceeded that in the 0.5% EG by 114 eggs or 4.94% (Table 4). The egg average weight during the study in the experimental groups was higher by 0.29-0.47 g, or 2.2-3.6%, compared to the CG. These differences in egg weight, correlated with the total number of eggs, resulted in an increase in total egg mass production to 32.45 kg in the 0.25% EG and 30.43 kg in the 0.5% EG, compared with 28.99 kg in the CG, representing increases of 11.9% and 5.0%, respectively (Table 4). In this context, from an economic perspective, it is important to evaluate the amount of feed used in the breeding and exploitation of birds in the different groups. Thus, it was established that the amount of feed consumed for the production of 100 eggs (feed conversion) was 6.34 kg of feed in the CG, while in the experimental groups this indicator was 4.4-10.6% lower. The amount of feed consumed for the production of 1 kg of egg mass in the CG was 4.96 kg, and in the experimental groups this amount was 6.5-13.9% lower (Table 4). Comparable findings regarding the enhancement of bird productive performance through the use of various biologically active products have been documented by multiple authors (Khazaei et al., 2021; Miao et al., 2021; Pistol, 2025). The obtained results are clearly favorable and indirectly indicate the beneficial effects of the tested product on the health, welfare, and metabolic processes of quails in the experimental groups, especially the birds in the 0.25% EG, supplemented with the additive in the minimum dose.

CONCLUSIONS

In conclusion, we can mention that the Omepol 30 feed additive was well tolerated by quails. The additive demonstrated regulatory effects on the trypsin-antitrypsin system, preventing the increases in serum trypsin activity and ensuring the stability of this parameter over the course of the study. The observed serum α_1-antitrypsin levels reflect appropriate physiological reactivity, facilitating the proper functioning of proteolytic enzyme-dependent processes in experimental quails. Consequently, Omepol 30 manifests adaptive and anti-stress properties. In addition, the additive positively influences metabolic processes by stimulating anabolic activity and reducing catabolic processes, via a specific mechanism, that reduces endotoxicosis parameters in the blood, reflected by lower values of AMW and NS markers, especially during periods of increased metabolic demand. Research demonstrates that supplementing feed with the additive at 0.25% and 0.5% improves the productive performance of laying quails compared with the CG, increasing both egg number and egg mass. The maximal effect was observed at the lower dose (0.25%), accompanied by improved feed conversion efficiency, reflected in reduced feed consumption per kg of egg mass. These results highlight the potential of the additive as a practical solution for optimizing poultry production, with positive economic implications, additional validations under industrial-scale farm conditions and detailed statistical analyses needed to confirm significant differences.

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THE POTENTIAL OF MINT (*Mentha* spp.) AS A FUNCTIONAL FEED ADDITIVE IN POULTRY NUTRITION

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Abstract

The powerful antioxidant, antibacterial, and anti-inflammatory qualities of mint, a commonly grown herb, are attributed to its abundance of bioactive chemicals, particularly essential oils (such as menthol, menthone, and limonene), flavonoids, and phenolic acids. Because of its calming, digestive, and carminative properties, mint has long been employed in both traditional human medicine and the culinary arts. Its use in animal nutrition is becoming more popular as a sustainable substitute for artificial growth boosters. The potential to improve nutritional absorption, boost appetite, strengthen the immune system, and promote intestinal integrity are just a few of the many health advantages of poultry. Studies indicate that dietary mint supplementation can enhance growth performance, improve feed conversion ratio, and elevate the quality of poultry products while reducing pathogen burden in the gastrointestinal system. In order to optimise the inclusion levels of various mint preparations (such as dried leaf, essential oil, and extract) for sustainable and efficient application in contemporary poultry production systems, this abstract emphasises the need for additional research.

Key words*: broiler, essential oils, mint, phytobiotics, poultry.*

INTRODUCTION

The poultry industry plays a critical role in global food security, providing affordable sources of high-quality animal protein. However, intensive poultry production systems are often associated with challenges related to disease control, feed efficiency and animal welfare. For several decades, antibiotic growth promoters (AGPs) were routinely used to improve feed efficiency and reduce disease incidence. Nevertheless, concerns regarding antimicrobial resistance and consumer demand for antibiotic-free poultry products have led to restrictions on AGP use in many regions worldwide (Abreu et al., 2023).

As a result, considerable attention has been directed toward natural alternatives such as probiotics, prebiotics, organic acids and phytogenic feed additives. Phytogenic feed additives derived from herbs and medicinal plants are particularly attractive because they contain diverse bioactive compounds that can improve digestion, modulate gut microbiota and enhance immune responses (Oni & Oke, 2025).

Mint (*Mentha* spp.) is one of the most widely cultivated aromatic plants belonging to the *Lamiaceae* family (Mishra & Chandra, 2022). Species such as *Mentha piperita* (peppermint), *Mentha spicata* (spearmint) and *Mentha longifolia* are widely used in food, pharmaceutical and cosmetic industries due to their characteristic aroma and medicinal properties (Saqib et al., 2022). The plant contains a variety of biologically active compounds, particularly essential oils rich in menthol and menthone, which are responsible for many of its pharmacological effects (Mikaili et al., 2013).

In recent years, mint has been increasingly investigated as a phytogenic feed additive in poultry nutrition. Several studies have reported improvements in growth performance feed conversion efficiency and gut microbiota composition following dietary supplementation with mint preparations. For example, Lv et al. (2026) showed that supplementation with peppermint leaves or menthol increased body weight gain and improved feed conversion ratio in broiler chickens.

Given the growing interest in plant-based feed additives, a comprehensive synthesis of available literature is needed to evaluate the potential role of mint in poultry nutrition. Therefore, the objective of this narrative review is to summarize current knowledge on the phytochemical composition of mint, its biological activities, mechanisms of action in poultry and the effects of dietary mint supplementation on performance, health and product quality.

MATERIALS AND METHODS

The methodology used for this narrative review-type paper was established to facilitate and replicate the synthesis and use of the existing literature regarding the use of mint as a feed additive in poultry nutrition.

The literature search was conducted on five databases and repositories: MDPI, Web of Science, Google Scholar, PubMed and ScienceDirect. The period of the literature search was restricted to the last ten years to maximize the last advancements in the field of phytobiotics research, but a few older research papers were selected. Also, to be more selective were used only articles published in English.

To have a higher search sensitivity search, Boolean operators were used (AND, OR, NOT) and various search strings were formed to facilitate integration of related terms for host species, compounds and performance outcomes. The primary search strings followed these combinations: (poultry OR broiler) AND (phytobiotics OR functional feed OR plant extracts); (Mint OR Peppermint OR Mentha spp.) AND (poultry OR broiler) and (meat OR eggs); (poultry AND broiler) NOT (ruminants OR swine OR farm animals).

Articles shown on the initial search were selected based on the evaluation of the titles, abstracts and conclusions. Also, articles that lack a phytobiotics or phytogenic component or were focused primarily on synthetic growth promoters were excluded.

The selected studies were categorised thematically within the discussion framework: botanical characterisation and phytochemical composition, biological properties of mint relevant to poultry nutrition, mechanism of action of mint in poultry, effects on growth performance, intestinal health and microbiota and quality and safety of poultry products.

To conclude the schematic analysis, a dedicated section addressing limitations was incorporated also the emerging conclusions.

RESULTS AND DISCUSSIONS

BOTANICAL CHARACTERISTICS AND COMPOSITION OF MINT

Mint species belonging to the genus *Mentha* are perennial aromatic herbs characterized by square stems, opposite leaves and highly aromatic essential oils (Yosefian et al., 2023). The genus includes more than 25 species and numerous hybrids widely distributed across temperate regions of the world, the most common being: *Mentha piperita (*good mint or peppermint). *Mentha spicata* (spearmint) and *Mentha crispa (*curly mint) (Tiwari, 2016).

The biological properties of mint are primarily attributed to its rich phytochemical composition. The essential oil content of peppermint leaves typically ranges between 1% and 4% of dry matter and contains a variety of monoterpenes and other volatile compounds (Pintilia et al., 2025). Major constituents include: menthol, menthone, isomenthone, cineole, limonene, pulegone and linalool, as shown in Table 1.

Table 1. Comparative essential oils profile and biological roles of various mint species

	Mentha spicata (%)	*Mentha piperita* (%)	Biological role
Menthol	11	38-70	Antimicrobial, analgesic
Menthone	21-23	0.4-35	Digestive stimulant, metabolic precursor
Carvone	40	3.5	Pathogene inhibitor, carminative
Limonene	21	0.6-4.5	Antioxidant, gut health modulator
Pulegone	0-10	0-7	Bactericidal, hepatotoxic at high levels
1,8-Cineole	17	0.4-12	Respiratory support, anti-inflamatory
Source	(Snoussi et al., 2015)	(Hudz et al., 2023)	-

In addition to essential oils, mint contains a variety of phenolic compounds and flavonoids such as rosmarinic acid, caffeic acid, luteolin and apigenin. These compounds possess potent antioxidant activity and contribute to the plant's anti-inflammatory and antimicrobial properties (Ćavar Zeljković et al., 2021).

The concentration of these phytochemicals varies depending on several factors, including plant species, cultivation conditions, harvesting stage and extraction method. Such variability may partly explain the differences in efficacy reported among studies investigating mint supplementation in animal diets (Chrysargyris et al., 2024).

Phytochemical compounds present in mint can exert multiple biological effects relevant to poultry nutrition, including antimicrobial activity against pathogenic bacteria, stimulation of digestive enzymes and modulation of oxidative stress (Mnisi et al., 2022).

BIOLOGICAL PROPERTIES OF MINT RELEVANT TO POULTRY NUTRITION

Antioxidant activity

Oxidative stress is a major factor affecting poultry health and productivity, particularly under conditions of environmental stress such as high temperature. Phenolic compounds and flavonoids found in mint have strong antioxidant properties capable of neutralizing reactive oxygen species and protecting cellular structures from oxidative damage (Oke et al., 2024).

Experimental studies have shown that dietary peppermint supplementation can enhance antioxidant enzyme activities, including superoxide dismutase, catalase and glutathione peroxidase, while reducing lipid peroxidation markers. These antioxidant effects may contribute to improved physiological resilience in poultry, particularly during periods of heat stress (Abou Zaid et al., 2025).

Antimicrobial effects

Mint essential oils exhibit antimicrobial activity against a wide range of pathogenic microorganisms, including *Escherichia coli*, *Salmonella* spp. and *Staphylococcus aureus* (Puvača et al., 2021). The antimicrobial action of mint is mainly attributed to monoterpenes such as menthol and menthone, which disrupt microbial cell membranes and interfere with metabolic processes (Zhao et al., 2023).

Studies in broiler chickens have demonstrated that diets supplemented with peppermint or other phytogenic additives can reduce pathogenic bacterial populations in the gastrointestinal tract while increasing beneficial bacteria such as *Lactobacillus* spp. Such modulation of intestinal microbiota may improve nutrient digestion and reduce disease susceptibility (Obianwuna et al., 2024; Cojocariu et al., 2026).

Anti-inflammatory and immunomodulatory effects

Inflammation and immune responses play critical roles in poultry health and disease resistance. Mint extracts have been shown to exhibit anti-inflammatory properties by inhibiting the production of inflammatory mediators and cytokines (Ma et al., 2024).

Recent research indicates that dietary peppermint oil supplementation can enhance immune responses and improve haematological and biochemical parameters in broiler chickens. These effects suggest that mint may support immune function and contribute to improved disease resistance in poultry (Bernard et al., 2025).

MECHANISMS OF ACTION OF MINT IN POULTRY

The beneficial effects of mint supplementation in poultry diets are mediated through several physiological mechanisms.

First, essential oils present in mint stimulate the secretion of digestive enzymes such as amylase, protease and lipase, thereby improving nutrient digestion and absorption. Enhanced digestive efficiency contributes to improved growth performance and feed conversion ratio (Ghazanfari et al., 2024).

Second, the antimicrobial properties of mint essential oils help regulate the composition of intestinal microbiota. By suppressing pathogenic bacteria and promoting beneficial microbial populations, mint can improve gut health and nutrient utilization (Angane et al., 2026).

Third, antioxidant compounds in mint reduce oxidative stress and protect intestinal epithelial cells, maintaining intestinal integrity and improving nutrient absorption (Hejna et al., 2021).

Finally, mint compounds may influence metabolic processes by modulating lipid metabolism and reducing serum cholesterol and triglyceride levels. Experimental studies have

reported reductions in serum lipid levels in broilers fed diets supplemented with peppermint (Khatun et al., 2026).
Together, these mechanisms contribute to the overall beneficial effects of mint supplementation on poultry performance and health.

EFFECTS ON GROWTH PERFORMANCE

Body weight gain and feed intake

Numerous studies have investigated the effects of dietary mint supplementation on growth performance in broiler chickens. Overall, most studies report improvements in body weight gain and feed intake when mint is included at moderate levels in the diet.
For example, after Alkassar S.A. (2025) and his collaborators, supplementation with peppermint leaves at levels up to 15 g/kg diet significantly increased body weight and feed intake in broiler chickens during a 35 day feeding trial.
Similarly, another study by Asadi N. and his colleagues (2017) reported that dietary peppermint powder improved average daily weight gain and feed conversion ratio in broilers, with the optimal level identified as approximately 4.5 g/kg feed.
These improvements in growth performance are likely associated with enhanced digestive efficiency and improved gut health.

Feed Conversion Ratio

Feed conversion ratio (FCR) is a critical parameter in poultry production, reflecting the efficiency with which animals convert feed into body mass.
Several experimental studies (Salinas-Chavira et al., 2024; Reda et al., 2025; Ghazanfari et al., 2024) have demonstrated improvements in FCR following dietary supplementation with mint or mint-derived compounds. For instance, broilers receiving diets supplemented with peppermint essential oil exhibited improved feed efficiency compared with control groups.
Improved feed conversion may be attributed to enhanced nutrient digestibility and better gut microbial balance.

EFFECTS ON INTESTINAL HEALTH AND MICROBIOTA

Gut health is a key determinant of poultry productivity and disease resistance. Phytogenic feed additives such as mint can positively influence intestinal morphology and microbial populations (Shehata et al., 2022).
Studies have shown that mint supplementation increases villus height and improves intestinal barrier function, leading to enhanced nutrient absorption (Cetingul et al., 2015; Bai et al., 2023; Mustafa, 2025). Furthermore, peppermint supplementation has been associated with increased populations of beneficial bacteria such as *Lactobacillus* spp. and reduced populations of pathogenic bacteria including *E. coli* (Adaszyńska-Skwirzyńska et al., 2025; Ţuţulescu et al., 2023).
Under heat stress conditions, mint leaf powder supplementation has been shown to improve intestinal morphology and reduce physiological stress responses in broiler chickens (Al-Saadi & Al-Musawi, 2024).
These findings suggest that mint may play an important role in maintaining intestinal health under challenging environmental conditions.

EFFECTS ON CARCASS AND MEAT QUALITY

In addition to growth performance, mint supplementation may influence carcass traits and meat quality in poultry.
Research indicates that dietary peppermint supplementation can reduce abdominal fat deposition and improve carcass yield (Narimani-Rad et al., 2011; Habashy et al., 2025; Zapletal et al., 2026).
Furthermore, improvements in meat quality parameters such as reduced cooking loss and enhanced oxidative stability have been reported in broilers fed diets containing peppermint leaves or menthol (Abdel-Wareth et al., 2019, Abdel-Wareth et al., 2023; Ashraf et al., 2025).
The antioxidant properties of mint may contribute to improved meat quality by reducing lipid oxidation during storage (Kanatt Abdel-Wareth et al., 2008; Domínguez Abdel-Wareth et al., 2019; Hejna et al., 2021, Mnisi et al., 2022; Alkassar et al., 2025).

FORMS OF MINT USED AND OPTIMAL INCLUSION LEVELS

Mint can be incorporated into poultry diets in several forms, each with different concentrations of active compounds.
Dried mint leaves are the most commonly used form due to availability and cost effectiveness followed by essential oils which are highly concentrated sources of bioactive compounds.
Plant extracts are standardized preparations used in experimental studies and aqueous solutions are added to drinking water (Tafrihi et al., 2021; Hutsol et al., 2023; Gavarić et al., 2025).
The choice of preparation influences the bioavailability of active compounds and the overall efficacy of supplementation.
Determining the optimal inclusion level of mint in diets for poultry remains challenging due to variations in plant species, preparation methods and experimental conditions (Abdelli et al., 2021; Ștef et al., 2025; Urban et al., 2025).
Studies have reported beneficial effects with peppermint leaf inclusion levels ranging from approximately 5 to 15 g/kg diet (Ma et al., 2024; Abdel-Wareth & Lohakare, 2023).
Other experiments using mint leaf powder have reported positive effects at levels between 0.6% and 1.8% of the diet (Ahmed et al., 2016; Abdel-Wareth & Lohakare, 2023; Alkassar et al., 2025).
Excessive inclusion levels, especially for essential oils and hydro-alcoholic extracts may negatively affect feed intake due to strong aroma or potential toxicity of certain compounds, highlighting the need for careful dose optimization (Rais et al., 2025; Kolygas et al., 2025; Nayak et al., 2025).

LIMITATIONS AND RESEARCH GAPS

Despite the promising results found in the literature, several limitations remain regarding the use of mint in poultry nutrition.
First, the phytochemical composition of mint varies widely among species and cultivation conditions, which may lead to inconsistent results across studies.
Second, many experiments have been conducted under controlled laboratory conditions rather than commercial production environments.
Third, long-term studies evaluating the effects of mint supplementation on poultry health, productivity and economic efficiency are limited.
Finally, interactions between mint and other dietary components, including probiotics and enzymes, remain poorly understood.
Addressing these gaps will be essential for developing standardized recommendations for the use of mint as a feed additive in poultry production.

CONCLUSIONS

Mint (*Mentha* spp.*)* represents a promising phytogenic feed additive with a high potential to improve poultry health and productivity.
Its rich content of essential oils, phenolic compounds and flavonoids contributes to a wide range of biological activities, including antimicrobial, antioxidant and anti-inflammatory effects.
Evidence from experimental studies indicates that dietary supplementation with mint can enhance growth performance, improve feed conversion efficiency, support intestinal health and improve meat quality in poultry. Furthermore, mint may help mitigate the negative effects of environmental stressors such as heat stress.
However, variability in mint species, preparation methods and inclusion levels highlights the need for further research to standardize its application in poultry diets. Future studies should focus on large-scale commercial trials, dose optimization and synergistic interactions between mint and other feed additives.
Overall, mint represents a sustainable and natural alternative to antibiotic growth promoters, contributing to more environmentally friendly and consumer-acceptable poultry production systems.

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INFLUENCE OF TURMERIC *(Curcuma longa* L.) POWDER ON GROWTH PERFORMANCE AND INTESTINAL HISTOMORPHOMETRY IN BROILERS

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Abstract

Natural herbal products are increasingly recognized as potent and cost-efficient alternatives to antibiotic growth promoters, improving growth performance and disease resistance in livestock and poultry. The main compound-curcumin is a lipophilic polyphenol derived from the rhizomes of Curcuma longa, has been highlighted for its beneficial effects, making it a promising feed additive. This study evaluated the effects of dietary turmeric supplementation on growth performance in broilers, as well as selected histomorphometric parameters, including intestinal villus height, width, surface area, and villus crypt depth. A total of 24 male Ross 308 broilers were assigned to two treatments over 42 days: a control group receiving a basal diet and an experimental group fed the same diet supplemented with 1% turmeric powder. The results showed that turmeric supplementation notably increased villus height, width, and surface area ($p<0.001$). These structural changes enhanced the absorptive capacity of the intestinal mucosa and improved nutrient transport, leading to significant ($p<0.05$) increases in body weight (BW), feed intake (FI), and feed conversion ratio (FCR).Overall, our findings indicate that turmeric can be applied as a natural alternative to conventional growth promoters in broiler production.

Key words*: chicken meat, growth promoters, intestinal morphology, turmeric.*

INTRODUCTION

In recent decades, antibiotics have been used, on a large scale, in the prevention and treatment of many diseases in humans and animals. Their use, continuously and repeatedly, determines drug resistance by disrupting the balance of the intestinal microbiota, a process manifested by changes in nutrient metabolism, the loss of integrity in intestinal mucosa, invasion of pathogens, and the disruption of immunity (Gomaa, 2020; Ramirez et al., 2020).

In addition, antibiotics triggers various secondary effects on body (i.e., dysbiosis, allergies, liver and kidney toxicity, emesis, and diarrhea) (Bercik et al., 2014; Mostafa et al., 2014; Kesavelu et al., 2023). Following the adoption of Regulation (EC) No 1831/2003, the European Union progressively phased out antibiotic growth promoters, with the definitive ban entering into force on 1 January 2006. This regulatory measure stimulated increasing interest in the identification and evaluation of alternative feed additives, particularly phytochemicals and other plant-derived bioactive compounds. Phytochemicals are promising alternatives to antibiotic growth promoters, improving animal performance, nutrient availability, gut microbiota balance, immunity, and feed conversion ratio (FCR) efficiency. (Lillehoj et al., 2012; Gadde et al., 2017; Rahman et al., 2022). Phytochemicals can be used in solid, dry, and ground form or as extracts (crude or concentrated) and can also be classified according to the process used to obtain the active ingredients in essential oils (EOs)-which are lipophilic substances obtained by cold extraction or steam/alcohol distillation) and oleoresins (extracts derived from non-aqueous solvents) (Gadde et al., 2017). The main bioactive phytochemicals are polyphenols, whose composition and concentration vary with

plant source, origin, environmental conditions, storage, and processing. In recent years, phytochemicals have been used as natural growth promoters in ruminants, pigs, and poultry. A wide variety of herbs and spices (e.g., turmeric, thyme, oregano, rosemary, marjoram, garlic, ginger, green tea, black cumin, coriander, and cinnamon) have been used in poultry as alternatives to antibiotic growth promoters (AGPs) (Gadde et al., 2017; Stratakos et al., 2017). In addition to herbs and spices, various EOs (e.g., thymol, carvacrol, cinnamaldehyde, eugenol, coriander, star anise, ginger, garlic, rosemary, turmeric, basil, cumin, lemon, and sage) have been used individually or in the form of blends to strengthen animal health and performance (Gadde et al., 2017). A few studies have been reported the use of EOs in poultry diets. Thus, the use of thymol and star anise EOs improved FCR. Also, the use of turmeric alone or mixed with capsicum (bell pepper) (Lee et al., 2013; Kim et al., 2013; Moharreri et al., 2022) has led to an increase in the resistance of birds to enteric diseases such as coccidiosis and necrotic enteritis. A mixture of phytonutrients based on carvacrol, cinnamaldehyde, and Capsicum oleoresin has been shown to enhance innate immunity and reduce the adverse effects of enteric pathogens (Lee et al., 2010; Kim et al., 2019), being approved for the first time in the EU as a botanical feed additive to improve the growth performance of broiler chickens. It resulted in increased body weight (BW) and decreased FCR and mortality (Bravo et al., 2008; Kim et al., 2019). The beneficial promoting effects of phytochemicals are attributed to their antimicrobial and antioxidant properties. Additionally, their inclusion in the diet leads to the stabilization of the intestinal microbiota and the reduction of toxic microbial metabolites in the intestine due to their direct antimicrobial properties on various pathogenic bacteria, which leads to the relief of intestinal problems and immune stress, consequently improving growth performance (Kim et al., 2015). Another significant advantage of dietary phytochemical supplementation is its capacity to attenuate oxidative stress while enhancing antioxidant defense mechanisms in different tissues, thereby contributing to improved overall health status (Settle et al., 2014). In addition, phytochemicals exert notable immunomodulatory effects by stimulating the proliferation of immune cells, regulating cytokine responses, and promoting higher antibody titers (Lee et al., 2010; Lee et al., 2017). Recent meta-analytic evidence further strengthens these observations, demonstrating that curcumin supplementation significantly increases daily weight gain (DWG) and improves FCR, without affecting feed intake (FI), highlighting its role as an efficient natural growth promoter. Moreover, curcumin has been shown to enhance antioxidant defenses by increasing enzymatic activity and lessening lipid peroxidation markers (e.g., malondialdehyde and hydroperoxides), thereby mitigating oxidative stress in broilers (Hernández-García et al., 2025). Administration of mixtures of phytochemicals has been evidenced to exert synergistic effects in reducing the negative consequences of enteric infections.

For example, supplementing broiler chicken diets with a mixture of *Curcuma longa*, *Caspicum annuum* (pepper), and *Lentinus edodes* improved BW gain and increased serum profilin antibody titer, as well as decreased fecal excretion of oocysts of *E. acervulina*, compared to control chicks fed only a basal diet or a basal diet supplemented only with Capsicum plus Lentinus (Lee et al., 2010). In addition, a number of bioactive compounds of curcumin in turmeric, such as demethoxycurcumin, bisdemethoxycurcumin, and tetrahydrocurcuminoids (Kiuchi et al., 1993; Sadeghi et al., 2012; El-Saadony et al., 2023), have antioxidant, anti-inflammatory, and nematocidal activities (Alia et al., 2006), protective effects against mutagenicity and hepatocarcinogenicity induced by aflatoxins (Ahmadi, 2010; Dai et al., 2022), and against coccidiosis (Lee et al., 2010; Yadav et al., 2020). Although turmeric and curcumin have shown beneficial effects in humans and animals, their impact on intestinal morphology, metabolism, and nutrient absorption in broilers remains insufficiently explored.

Starting from these considerations, the objective of the present study was to evaluate the effects of supplementing the diet of broilers with turmeric powder on the morphology of the intestine, with the final aim of clarifying a new perspective on the possible mechanisms through which curcumin exerts its effects as a potential

promoter of their growth. In approaching this topic, we have considered the many advantages of using solid forms of plants, such as availability, easy and practical application, and lower costs compared to different EOs formulas.

MATERIALS AND METHODS

Experimental design and biological material

The experiment was carried out on 24 one-day-old male Ross 308 broiler chicks obtained from a local commercial hatchery. Upon arrival, the birds were randomly distributed into two experimental groups: a control group (LC) and a treated group (LTu). Each group consisted of 12 birds, arranged in two replicates of six chicks each. The broilers were maintained under controlled environmental conditions, with temperature, humidity, ventilation, and general management practices adjusted according to standard broiler-rearing requirements. The birds received a basal compound feed, while feed and drinking water were provided ad libitum throughout the experimental period. A continuous 24-hour lighting program was applied.

The growing period lasted for 42 days, divided into three phases: the starter phase from day 0 to 10, the grower phase from day 11 to 35, and the finisher phase from day 36 to 42.

Throughout the experimental period, feed and water were provided ad libitum. The birds received compound feed (CF) formulated according to the requirements of the main broiler growth phases. The starter feed contained 2850 kcal/kg metabolizable energy (ME) and 21.00% crude protein (CP), the grower feed contained 3050 kcal/kg ME and 19.00% CP, and the finisher feed contained 3080 kcal/kg ME and 17.20% CP.

From day 1 to day 42, the control group received the basal compound feed, whereas the experimental group was fed the same basal diet supplemented with 1% turmeric powder. Feed and water were provided ad libitum throughout the experimental period.

Histomorphometric analysis of the intestine

At 42.5 days of age, the broilers were slaughtered following stunning, and small-intestine samples were collected immediately after evisceration. The tissue fragments were fixed in 10% neutral buffered formalin to preserve tissue architecture and prevent post-mortem degradation. Subsequently, the samples were processed according to standard histological protocols, embedded in paraffin, and sectioned at 5 µm using a Leica RM 2125 RT manual rotary microtome.

For the histological study, the sections were stained by the Mallory trichrome staining method (Bancroft, 1990). The mounting of the sections was done with Canada balsam (anhydrous medium). The histomorphometric analysis was carried out using an Olympus CX41 light microscope fitted with a 10× eyepiece and 10×, 40×, and 100× optically corrected objectives. Image acquisition and measurements were performed with Quick Photo Micro 2.2 software. The evaluated intestinal parameters were villus height, villus width, villus surface area, villus perimeter, and crypt depth. Villus height was defined as the linear distance between the villus apex and the villus–crypt junction. Villus width was measured at the central portion of the villus, whereas villus area and perimeter were obtained by tracing the villus contour. Crypt depth was determined as the distance from the crypt mouth to the muscularis mucosae.

Statistical analysis

The statistical analysis of the results obtained by histomorphometry was carried out using the IBM SPSS 23 package (SPSS Inc., Chicago, IL, USA). All data was tested for normality using the Shapiro-Wilk test, and differences between the experimental variants were made with the Student test. Results were presented as the mean and standard error of the mean (mean±SEM), while the differences were considered to be statistically significant at $p<0.05$, respectively.

RESULTS AND DISCUSSIONS

At the end of the growing period, the body weight of the chicks from the experimental group (LTu) was significantly higher at 2776.17±303.72 g (mean ± standard error of the mean) compared to that of the chicks from the control group (LC) at 2489.00±70.092 g, indicating a difference of approximately 10%. The broilers in the control group (LC) exhibited the lowest intake of combined feed throughout the experiment (4368.00±579.828 g), while the highest consumption of combined feed was

observed in the broilers in the experimental group (LTu) at 4726.00±14.14 g. In the experimental group (LTu), where the feed was supplemented with turmeric, a FCR of 1.72±0.009 was recorded, while the control batch registered a value of 1.81±0.447. The difference is 4.42%, with statistical significance ($p<0.05$) (Table 1).

In the control group (LC), broilers fed only the basal compound diet showed preserved intestinal mucosal architecture, with no apparent histological abnormalities or morphometric deviations observed in the examined sections (Figure 1 A, B).

Table 1. Effect of turmeric supplementation on BW, FI, and FCR in broiler chickens (mean ± standard error of the mean)

Specification	LC	LTu	P values
BW (g)	2489.89 ± 242.81[a]	2776.17 ± 303.72[b]	p<0.05
FI (g)	4368.00 ± 579.83[a]	4726.00 ± 14.14[b]	p<0.05
FCR (kg CM/kg gain)	1.81 ± 0.45[a]	1.73 ± 0.01[b]	p<0.05

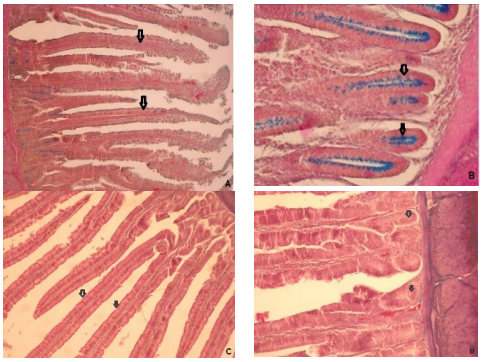


Figure 1. Intestinal mucosa: A, B - LC. intestinal villi (100x) and intestinal glands (200x) (arrows); C. D - LTu –intestinal villi (100x) and intestinal glands (200x) (arrows) (Mallory trichrome staining)

Intestinal villi resulted in an average height of 1245.82±152.08 µm, an average width of about 105.38±12.47 µm, a surface area of 108554.02±26761.47 µm^2, perimeter of 2642.97±254.92 µm and the average depth of the crypts was about 262.42±55.40 µm (Table 2). In individuals of the experimental group (LTu), supplementing the basic diet with 1% turmeric powder led to an increment of the intestinal villi (Figure 1C, D) and of the histomorphometric parameters, compared to the control group (LC). Thus, in LTu chickens, the height of the intestinal villi was 1794.21±315.52 µm, with an average surface of 242152.27±55309.69 µm^2 and a perimeter of 3624.32±644.82 µm, with significant differences between individuals from this group and the control group ($p<0.001$) (Table 2, Figure 2). The villi have a rectangular appearance; they have a much larger average width (150.64±20.04 µm), compared to the control group (LC), where the average width was 105.38 ± 12.47 µm. Between the two groups, the differences were significant ($p<0.001$) (Table2, Figure 2). Turmeric supplementation produced only a numerical increase in crypt depth, from 262.42 ± 55.40 µm in the control group to 278.97 ± 41.24 µm in the experimental group, without reaching statistical significance ($p>0.05$) (Table 2, Figure 3).

Table 2. Effect of turmeric dietary supplementation on height, area, perimeter, width of intestinal villus and crypts depth in 42-day-old broiler chickens (mean ± SD)

Specification	N	LC 40	LTu 40	P values
Intestinal villus	Height (µm)	1245.82 ±152.08[a]	1794.21 ±315.52[b]	p<0.001
	Area (µm^2)	108554.02 ±26761.47[a]	242152.27 ±55309.69[b]	p<0.001
	Perimeter (µm)	2642.97 ±254.92[a]	3624.32 ±644.82[b]	p<0.001
	Width (µm)	105.38 ±12.47[a]	150.64 ±20.04[b]	p<0.001
Crypt depth (µm)		262.42 ±55.40[a]	278.97 ±41.24[a]	p>0.05

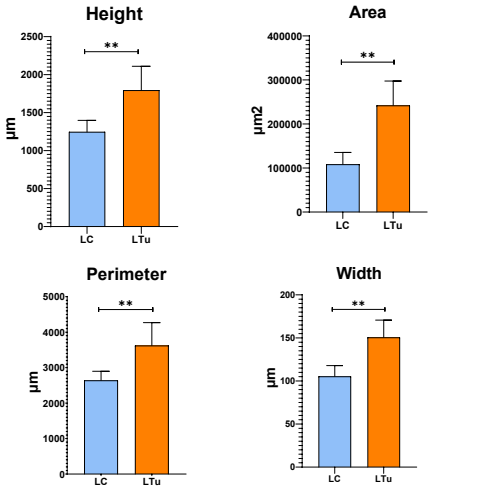


Figure 2. Graphic representation of the height, area, perimeter and width of intestinal villus at LC and LTu (**p<0.001)

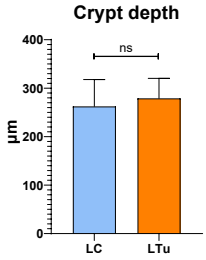


Figure 3. Graphic representation of the crypts depth at LC and LTu (ns – insignificant p>0.05)

Curcumin has been stated to have a number of biological activities, such as anti-inflammatory, antioxidant (Gandhi et al., 2011; Sharifi-Rad et al., 2020; Saadony et al., 2023) antimicrobial (Araujo and Leon, 2001; Sharifi-Rad et al., 2020; Saadony et al., 2023), anticoagulant, antidiabetic, and antiulcer (Kepinska-Pacelik et al., 2023).

Also, it could improve digestibility and metabolism, prevent biliary disorders and anorexia in humans and farm animals (Al-Sultan and Gameel, 2004; Chattopadhyay et al., 2004). Although various studies have been conducted on the use of turmeric and its active compounds, curcumin, as an antioxidant and immune enhancer in humans, animals, and broilers (Kumari et al., 2007; Gandhi et al., 2011; Sureshba et al., 2023), the effect of curcumin on intestinal morphology, as well as its association with metabolism and nutrient absorption, in broilers is a topic still being explored.

Numerous studies (Platel and Srinivasan, 2000; Durrani et al., 2006; Kumari et al., 2007; Hafez et al., 2022) showed that curcumin dietary supplementation has a significant positive effect on WG and feed efficiency at a marketable age (42 days), thus validating the positive effect of curcumin on broiler growth performance.

Recent studies have shown that curcumin supplementation enhances intestinal structure, improves gut barrier function, and modulates microbiota composition, thereby supporting nutrient absorption and immune response in poultry (Zhengyu Xu et al., 2024*)*.

These findings are consistent with recent evidence from the scientific literature, demonstrating that curcumin supplementation improves growth performance by increasing daily WG and reducing FCR ratio, while simultaneously enhancing intestinal morphology (↑ villus height and VH/CD ratio, ↓crypt depth) and antioxidant status (↑ SOD, CAT, GSH-Px; ↓ MDA), indicating improved nutrient absorption and reduced oxidative stress (Hernandez-Garcia et al., 2025*)*.

These findings are further supported by recent study indicating that curcumin acts as a multifactorial growth promoter by improving feed efficiency, modulating lipid metabolism, optimizing intestinal integrity, and exerting antioxidant, antimicrobial, and immunomodulatory effects, particularly under stress conditions (Fouad et al., 2025).

The results of our study show that supplementing the basic diet with 1% turmeric powder led to improvements in growth performance, FI, and FCR (kg NC/kg gain) in the individuals of the experimental group (LTu) related to the individuals of the control group (LC).

The efficiency on growth performance could be due to the larger surface area of intestinal villi (Raiput et al., 2013), and this positive effect is influenced by anti-inflammatory, antioxidant, and antibacterial activity (Chattopadhyay et al., 2004; Bondar et al., 2023) or the prebiotic-like effect of curcumin (Niamsa and Sittiwet, 2009). Also, Emadi and Kermanshahi (2007) reported that *Curcuma longa* improved FCR in broilers, and these beneficial effects could be due to augmented secretion of enzymes such as amylase, trypsin, chymotrypsin, and lipase (Platel and Srinivasan, 2000). Supplementation of the basic diet with curcumin causes a decrease in abdominal fat in a manner dependent on the concentration of curcumin, an aspect due to its influence on adipocyte apoptosis, or the withdrawal of glucose from the blood. De Beer (2008) believes that greater fat mobilization may be due to stimulation of the thyroid hormone T4. Dietary curcumin has been shown to lower cholesterol and stimulate fat digestion (Hong, 2022). Additionally, Gandhi et al. (2011) and Kumari et al. (2007) previously reported that curcumin significantly decreased total cholesterol, which could be due to inhibition of the active enzyme hepatic 3-hydroxyl-3-methylglutaryl CoA-reductase (HMGCR), which governs cholesterol synthesis in the liver (Galib et al., 2011). Thyroid hormones play an important role in the regulation of fat metabolism, and plasma concentrations of these hormones could be potential indicator of metabolic activity and physiological response in

commercially farmed birds (Melesse et al., 2011). Raiput et al. (2013) demonstrate that curcumin supplementation in broiler chicken diets led to increased intestinal villi height and width, which may be due to increased epithelial cell turnover in all segments of the small intestine. Consequently, the increase in intestinal villi is associated with mitotic activation of stem cells in the intestinal mucosal epithelium (Samanya and Yamauchi, 2002; Bondar et al., 2023). Intestinal epithelial cells are initially originated in the crypts and migrates along the surface of the villi upwards towards their tips.

In the present study, supplementing the basic diet of broilers with turmeric 1% led to an increase in the height ($p<0.001$) and width of the villi ($p<0.001$), their surface area ($p<0.001$), as well as the depth of the crypts ($p>0.05$).

Many studies have confirmed that the intestine can react morphologically and adapt by increasing the length and/or by increasing the height or decreasing the villi in response to external factors related to dietary changes (Žikic et al., 2008; Peric et al., 2010). The increase in the height of the villi enhances the absorption capacity of the intestinal mucosa, a consequence of the increase in the absorption surface, the expression of microvilli at the apical pole of the absorbing epithelial cells, and, implicitly, the nutrient transport systems at their level (Pluske et al., 1996; Rajput et al., 2013).

Crypt depth reflects the proliferative activity of the intestinal epithelium and is often associated with the rate of mucosal renewal. Increased crypt depth may occur when epithelial replacement is intensified, particularly under conditions involving villus epithelial loss, inflammatory stimulation, or pathogen-derived toxins. However, in the present study, the increase observed in the turmeric-supplemented group was numerical and not statistically significant, indicating that 1% turmeric powder did not induce a marked alteration of crypt architecture. This finding should therefore be interpreted as a mild morphometric tendency rather than a clear biological effect. In contrast, Rajput et al. (2013) observed a reduction in crypt depth in curcumin-supplemented broilers, which may indicate improved mucosal stability and a reduced need for epithelial regeneration. The potential beneficial effects of turmeric may also be linked to its role in digestive physiology, as turmeric supplementation has been reported to stimulate bile acid secretion and enhance the activity of digestive enzymes such as lipase, amylase, and proteases, which are directly involved in nutrient digestion and metabolic efficiency (Hong et al., 2022).

Curcumin also improves liver function and reduces serum triglycerides, LDL, and blood glucose concentrations (Emadi and Kermanshahi, 2007; Gandhi et al., 2011). However, Desphpande et al. (1998) reported decreased BW accompanied by hepatotoxic effects, expressed by focal necrosis, in mice and rats fed turmeric or an ethanolic extract of turmeric at high doses and for prolonged periods of time. Similar aspects are also described by Kandarkar et al. (1998), who mention processes of coagulative necrosis, accompanied by parenchymal regeneration, using turmeric or turmeric extract in doses considered necessary for anticancer activity.

CONCLUSIONS

Adding turmeric 1% to broiler chicken diets resulted in expanded BW, FI, FCR ($p<0.05$), height, width, villi area perimeter ($p<0.001$), and crypt depth ($p>0.05$) in the experimental group (LTu) compared to the control group (LC). Villi size increases the intestinal mucosa's absorption capacity due to the absorption surface, microvilli at the apical pole of the absorbing epithelial cells, and implicitly, nutrient transport systems at their level. Increased intestinal villi are linked to mitotic activation of intestinal mucosa epithelium stem cells, resulting in deeper intestinal crypts. The histological changes and histomorphometric analysis of this study's data suggest turmeric may promote growth in broiler feed.

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ESTIMATES OF METHANE EMISSIONS FROM DAIRY CATTLE BASED ON MANURE MANAGEMENT SYSTEMS

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Abstract

This paper aimed to estimate methane (CH_4) emissions from manure produced by dairy cattle in Romania between 2016 and 2025. Data on the dairy cattle population were obtained from the National Institute of Statistics, while manure-related greenhouse gas (GHG) emissions were estimated using Tier 2 methodologies provided by the International Panel on Climate Change Guidelines (IPCC, 2019). Gross energy intake (GE), digestible energy (DE), and volatile solid excretion (VS) were calculated using national reference values, while the methane-producing capacity (B_0) and methane conversion factors (MCF) were based on IPPC (2019) default values. At the national level, methane emissions from dairy cattle manure decreased from 6.618 Gg CH_4 yr^{-1} (165.455 t CO_2-eq) in 2016 to 6.054 Gg CH_4 yr^{-1} (151.350 t CO_2-eq) in 2025. These results indicate a gradual reduction in methane emissions, mainly driven by the decline in dairy cattle numbers, and highlight the importance of accurate country-specific data for improving greenhouse gas inventories and supporting effective mitigation strategies in the dairy sector.

Key words*: dairy cattle, manure management, methane emissions.*

INTRODUCTION

Methane (CH_4) is a potent greenhouse gas, with a global warming potential (GWP) of 28–34 times higher that of carbon dioxide over a 100-year time frame (Forster et al., 2021; Manono, 2026). Its atmospheric concentration has more than doubled since pre-industrial times, largely driven by anthropogenic activities, with livestock production identified as one of the principal global sources (Skeie et al., 2023).

Worldwide, livestock systems constitute a major source of anthropogenic methane (Nugrahaeningtyas et al., 2024; Xie et al., 2025), mainly through enteric fermentation and manure handling (Popa et al., 2025).

Usually, practices of manure management strongly influence methane emissions, as anaerobic decomposition of volatile solids in stored manure can lead to substantial CH_4 production (IPCC, 2006; IPCC, 2019). The magnitude of these emissions depends on factors such as manure storage type, temperature, retention time, and the biochemical characteristics of the manure itself (Im et al., 2020; Cheng et al., 2022).

Manure composition is influenced by the type of animal (beef or dairy), production stage, production type, and type of feeding (Symeon et al., 2025). Accurate estimation of methane emissions from manure is therefore essential for national greenhouse gas inventories and guiding the development of effective mitigation strategies within the livestock sector. In the livestock sector, dairy cattle represent a significant emission source due to their high feed intake, intensive production systems, and the large quantities of manure they generate (Stolarski et al., 2025).

In the European Union, agriculture accounts for about 54% of total methane emissions, with dairy cattle responsible for about 42% through enteric fermentation and 8% from manure production. Enhancing the precision of emission estimates has therefore become a central priority under the EU's 2050 climate-neutrality objectives (EEA, 2022).

Given these considerations, updated and accurate estimates of methane emissions from dairy cattle manure are essential for Romania's climate reporting obligations and for identifying mitigation opportunities in the dairy sector.

By integrating national data on cattle populations with refined calculations of gross energy intake, digestible energy, and volatile solid excretion, this study improves emission estimates and highlights trends relevant to

policy development and sustainable livestock management.
In this context, the present study aims to quantify CH_4 emissions from the dairy cattle sector in Romania, with a focus on manure handling and management systems.

MATERIALS AND METHODS

The dairy cattle population used in this paper to estimate CH_4 emissions was reported by the National Institute of Statistics (INS, 2025).
For accuracy, methane (CH_4) emissions from manure were calculated for an experimental Romanian Black and Spotted dairy cattle herd at the Research and Development Institute for Bovine Balotesti during the 2022-2025 period.
Methane emissions from manure handling and management systems were estimated using the Tier 2 methodology, following the 2019 Refinement to the 2006 IPCC Guidelines for National Greenhouse Gas Inventories (IPCC, 2006; IPCC, 2019), and applying the corresponding equations:

CH_4 emissions from manure management (10.22)

$$CH_{4\ Manure} = \sum_{(T)}\left[\left(EF_{(T)} * N_{(T)}\right)/10^6\right]$$

where:
Emissions = methane emissions from enteric fermentation, Gg CH_4/yr^{-1};
$EF_{(T)}$ = emission factor for the defined livestock population, kg CH_4 /$head^{-1}/yr^{-1}$;
$N_{(T)}$ = the number of head of livestock species/category T in the country;
T = species/category of livestock.

CH_4 emission factor from manure management (10.23)

$$EF_{(T)} = (VS_T * 365) * \left[B_{0(T)} * 0.67 * \sum_{S,k}(MCF_{S,k}/100) * AWMS_{(T,S,k)}\right]$$

where:
$EF_{(T)}$ = annual CH_4 emission factor for livestock category T, kg CH_4 /$animal^{-1}/year^{-1}$;
$VS_{(T)}$ = daily volatile solid excreted for livestock category T, kg dry matter/$animal^{-1}/day^{-1}$;
365 = basis for calculating annual VS production, $days/year^{-1}$;
$B_{0(T)}$ = maximum methane producing capacity for manure produced by livestock category T, m^3 CH_4/kg^{-1} of VS excreted;
0.67 = conversion factor of m^3 CH_4 to kilograms CH_4;
$MCF_{(S,k)}$ = methane conversion factors for each manure management system S by climate region k, percent;
$AWMS_{(M,S,k)}$ = fraction of livestock category T's manure handled using animal waste manure management system S in climate region k, dimensionless.

Volatile solid excretion rates (10.24)

$$VS = \left[GE * (1 - DE/100) + (UE * GE)\right] * \left[(1 - ASH)/18.45\right]$$

where:
VS = volatile solid excretion per day on a dry-organic matter basis, kg VS/day^{-1};
GE = gross energy intake, MJ /day^{-1};
DE% = digestibility of the feed in percent (e.g. 60%);
(UE∗GE) = urinary energy expressed as fraction of GE. Typically, 0.04 GE was considered urinary energy excretion by ruminants (reduced to 0.02 for ruminants fed with 85 percent or more grain in the diet);
ASH = the ash content of feed calculated as a fraction of the dry matter feed intake (0.08 for cattle);
18.45 = conversion factor for dietary GE per kg of dry matter (MJ kg^{-1}).
The manure emissions were expressed as Gg CH_4 /yr^{-1} and t CO_2-eq.
The animal waste manure management system (AWMS) was a solid storage.
The specific values for GE, DE, VS, and EF were obtained either from recently published research articles (Enculescu et al., 2025) or calculated directly in this study (Table 1).
The parameters B_0 (0.24 m^3 CH_4/kg VS), MCF (2 %), ASH (8%) and AWMS (1) were adopted from the default values recommended by the IPCC (2006; 2019).
UE∗GE was calculated by multiplying the urinary energy coefficient (0.04 for ruminants), expressed as a fraction of gross energy, with the gross energy intake (GE).

The gross energy intake (GE) was calculated based on the following equation:

GE (kcal/kg) = 5.72 ∗ GP + 9.5 ∗ GB + 4.79 ∗ CelB + 4.17 ∗ SEN (Stoica, 1997)

where:
GE = gross energy intake;
GP = crude protein;
GB = crude fat;
CelB = crude fibres;
SEN = non-nitrate extractable substances.
The percentage of digestible energy (DE) of raw energy was calculated according to the following relation:

DE % = (DE/GE) ∗ 100

Emission factor (EF) of 5.51 kg/head/year was considered for dairy cows with an average body weight of 650 kg, average milk yield of 15.00 kg/head/day and a diet (kg/head/day) consisting of 25-30 kg silage maize, 5 kg alfalfa hay, and 6 kg concentrates. Emission factors (EF) of 6.20 kg/head/year, 6.61 kg/head/year, 6.80 kg/head/year, and 6.90 kg/head/year were considered for dairy cows with an average body weight of 650 kg, average milk yield between 16.30-16.55 kg/head/day and a diet (kg/head/day) consists of 28 kg silage maize, 7 kg alfalfa hay, and 6 kg concentrates.
To quantify methane emissions (t CO_2-eq) from dairy cattle manure, the basic formula of the IPPC Tier 2 approach was applied:

$$Emissions = N_{(T)} * (EF_{manure}/1000) * GWP_{CH4}$$

where:
Emissions = methane emissions from manure management of dairy cattle, t CO_2-eq;
$N_{(T)}$ = the number of head of livestock species/category T in the country;
$EF_{(T)}$ = emission factor for the defined livestock population, kg $CH_4/head^{-1}/yr^{-1}$;
$GWP_{100\ CH4}$ = the global warming potential of CH_4 in 100 years;
1 kg CH_4= 25 kg CO_2-eq (Forster et al., 2007).

Table 1. The values used for calculations of methane emission from dairy cows' manure management systems

	GE[1], MJ/head/day	DE[2], MJ/head/day	DE, %	ASH[3], %	VS[4], kg dry matter/ head/day	EF[5], kg CH_4/head/ yr^{-1}	Source
The value used for the number of dairy cattle from Romania							
2016-2025	282.85	200.12	70.75	8	4.69	5.51	Enculescu et al., 2025
The value used for the number of dairy cattle from the Research and Development Institute for Bovine Balotesti							
2022	310.27	217.00	69.93	8	5.28	6.20	Enculescu et al., 2025
2023	312.34	211.93	67.85	8	5.63	6.61	Enculescu et al., 2025
2024	314.20	210.44	66.97	8	5.80	6.80	Enculescu et al., 2025
2025	329.00	223.30	67.87	8	5.9	6.93	Own calculation

[1]GE=gross energy intake; [2]DE=digestible energy; [3]ASH=ash content; [4]VS=volatile solid excretion; [5]EF = emission factor.

RESULTS AND DISCUSSIONS

Table 1 summarises the values used to estimate methane emissions from manure management systems in dairy cattle. For the national dairy herd (2016-2025), GE averaged 282.85 MJ head/day, with a corresponding VS output of 4.69 kg/head/day and an EF of 5.51 kg CH_4/head/year. In contrast, the Romanian and Black Spotted dairy cattle reference herd showed higher GE intakes (310-329 MJ head/day) and greater VS excretion rates (5.28-5.90 kg head/day), which consequently led to increased emission factors (6.20-6.93 kg CH_4/head/year). These differences reflect variations in feeding intensity, productivity, and management practices between herds. The evolution of methane emissions from the Romanian dairy cattle sector over the 2016-2025 period is presented in Table 2.
The number of dairy cows shows a continuous decline from 1.201.132 head in 2016 to 1.077.589 head in 2023, followed by a slight recovery in the 2024-2025 period.
This reduction in the national herd size directly drives the downward trend in total methane emissions, which decrease from 6.618 Gg CH_4/year in 2016 to 5.938 Gg CH_4/year in 2023, representing an overall reduction of approximately 10%. CO_2-equivalent emissions show a similar downward trend, decreasing from 165.455 t CO_2-eq to 148.450 t CO_2-eq over

the same period. The modest increase in 2024-2025 reflects the slight rise in animal numbers during these years. The results indicate that methane emissions from the Romanian dairy sector have decreased substantially over the past decade.

This reduction is not due to changes in emission intensity, but rather to the progressive decline in the national dairy herd.

The slight rebound in 2024-2025 suggests a stabilisation of the sector, with proportional effects on methane output.

Table 2. Summary of methane emissions estimated from the Romanian dairy cattle sector between 2016 and 2025

Years/Specification	Dairy cows, heads/year	Tier 2	CH_4 emission/head/year	
			Gg[2] CH_4/yr^{-1}	*t CO_2-eq*[3]
2016	1.201.132		6.618	*165.455*
2017	1.168.975		6.441	161.025
2018	1.170.076		6.447	161.175
2019	1.147.399		6.322	158.050
2020	1.130.638	EF[1]: 5.51	6.230	155.750
2021	1.118.342		6.162	154.050
2022	1.104.215		6.084	152.100
2023	1.077.589		5.938	148.450
2024	1.079.600		5.949	148.725
2025	1.098.915		6.054	151.350

[1]EF = emission factor; [2]Gg CH_4/yr^{-1} = gigagrams CH_4/year; [3]t CO_2-eq = tonne CO_2 equivalent.

Table 3 summarises the methane emissions generated by the Romanian Black and Spotted dairy cows' reference herd during 2022-2025.

The emission factors (EF) applied to this herd range from 6.20 to 6.90 kg CH_4/head/year, values that are higher than the national Tier 2 EF of 5.51 kg CH_4/head/year used for the national Romanian dairy herd.

These higher EF values reflect the higher gross energy intake, higher volatile solids excretion, and slightly higher milk yields. The diet provided (28 kg maize silage, 7 kg alfalfa hay, and 6 kg concentrates) supports increased productivity and consequently increases the methane generation potential from manure.

Despite the higher emission factors, the absolute methane emissions remain extremely low, ranging from 0.527 to 0.555 Gg CH_4/year (13.175 t CO_2-eq-13.875 t CO_2-eq), due to the very small herd size (77-85 head).

Table 3. Summary of methane emissions estimated from the Romanian Black and Spotted dairy cows

Years/Specification	Dairy cows, heads/year	Tier 2	CH_4 emissions/head/year	
			Gg[2] CH_4/yr^{-1}	*t CO_2-eq*[3]
2022	85	EF:6.20	0.527	13.175
2023	84	EF:6.61	0.555	13.875
2024	80	EF:6.80	0.544	13.600
2025	77	EF:6.90	0.531	13.275

[1]EF = emission factor; [2]Gg CH_4/yr^{-1} = gigagrams CH_4/year; [3]t CO_2-eq = tonne CO_2 equivalent.

Overall, the results of the present study suggest that the dynamics of methane emissions in the Romanian dairy cattle sector are influenced primarily by herd evolution, with the reductions in animal numbers being the main driver of the decline in total emissions during the analysed period. The differences observed between the national herd and the Romanian Black and Spotted cows reference herd highlight the major role of energy intake, ration digestibility, and volatile solids excretion in determining emission factors. Popa et al. (2017) reported a decreasing trend in CH_4 emissions in a buffalo farm, driven both by herd size reduction and by the transition to an organic farming system that improved manure management practices.

These findings are relevant for the dairy sector, suggesting that management interventions can significantly contribute to emission reductions, complementing the effects of herd structure.

Furthermore, the study conducted by Popa et al. (2021) demonstrated that feeding strategies represent an essential tool for reducing methane and CO_2 equivalent emissions from manure management systems. Ration variants rich in green forage generated the lowest emissions,

whereas diets with higher proportions of concentrates resulted in increased emissions from both enteric fermentation and manure.

These results showed that ration digestibility, composition, and structural balance directly influence the methanogenic potential of manure, complementing international observations on the role of forage quality in shaping emission dynamics.

International literature supports this integrated perspective. Benchaar & Hassanat (2019) showed that higher digestibility of corn silage can increase manure-derived methane emissions due to greater volatile solids excretion and higher degradability, while Van Gastelen et al. (2023) demonstrated that certain dietary modifications can reduce enteric CH_4 without increasing manure methane.

Additionally, Hanafiah et al. (2021) highlighted the influence of animal age and manure type on methanogenic potential, emphasising the importance of biological characteristics and post-excretion management. Therefore, methane emission reduction cannot be attributed to a single factor, but rather results from the interaction among herd size, production level, ration composition and digestibility, volatile solids excretion, and manure management practices.

An effective mitigation strategy must integrate all these components, avoiding the transfer of emissions from one source to another and ensuring a coherent, sustainable approach tailored to the specific conditions of Romanian dairy farms.

However, several limitations should be acknowledged. Firstly, the current study relied on Tier 2 IPCC methodology, which, although robust, depends on assumed or default values for certain parameters (e.g., B_0, MCF) that may not fully capture farm-specific variability. Additionally, the analysis focuses primarily on herd size, feeding characteristics, and manure management, without incorporating broader system-level factors such as feed quality, climatic conditions, or long-term changes in genetic potential.

The limited availability of detailed farm-level data, particularly for smaller herds, may also constrain the precision of emission estimates.

Future research should integrate direct measurements of manure characteristics, longitudinal monitoring of feeding practices, and farm-specific emission coefficients to refine national inventories and support more targeted mitigation strategies. Incorporating such detailed information improves the accuracy of national greenhouse gas inventories and supports more effective mitigation strategies aligned with EU climate policies.

CONCLUSIONS

This study shows that methane emissions from dairy cattle manure handling systems in Romania have declined mainly due to reductions in herd size, while emission factors remained strongly influenced by feeding practices and production intensity. Applying the IPCC (2019) Tier 2 methodology improved the accuracy of national estimates and highlighted the importance of system-specific data for inventory refinement and for developing effective, sustainable manure management strategies.

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DIET WITH APPLE POMACE IS ABLE TO COUNTERACT OXIDATIVE STRESS IN SPLEEN OF PIGLETS EXPERIMENTALLY CONTAMINATED WITH *Escherichia coli* LIPOPOLYSACCHARIDE DURING POST-WEANING PERIOD

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Abstract

The spleen plays an essential role in the immune and circulatory system, contributing to the body's defense against infections through the filtering of blood-borne pathogens and antibody production. Oxidative stress, the imbalance between free radicals and antioxidants can affect spleen causing inflammation, cell damage and at the long-term different spleen disorders. In the present study we investigated the effect of a diet including apple pomace to mitigate the oxidative stress and the impairment of antioxidant enzymes (catalase-CAT, superoxide dismutase-SOD and glutationperoxidase-GPx) activity in piglets after weaning experimentally challenged with E. coli lipopolysaccharide (LPS), a well-known inducer of oxidative stress and inflammation. Twenty-four piglets were randomly allocated to four experimental diets: group 1 and 2 received a normal diet for postweaning period while group 3 and 4 received a diet including 5% of apple pomace. In the day 20 with 24h before slaughtering the groups 2 and 4 were challenged with LPS at 80µg/kg b.w. In the day 21 piglets were slaughtered and samples of spleen were collected to determined oxidative and antioxidative stress markers. Results showed that E. coli LPS produced a significant increase of lipid, protein and DNA oxidation and reduce antioxidant enzymes activity. Apple pomace alone did not stimulate splenic antioxidant enzyme activities, but in LPS-challenged piglets, the diet with apple pomace attenuated partially the reduction in SOD, CAT, and GPx activity as well as the increase of oxidative markers, suggesting a protective effect on antioxidant defences under inflammatory oxidative conditions.

Key words*: apple pomace, oxidative stress, piglets, post-weaning.*

INTRODUCTION

The post-weaning period is widely recognized as a critical biological physiological stage in pig production, marked by abrupt separation from the sow, dietary and environmental change, social mixing, and immature digestive and immune function (Taranu et al., 2022). These converging stressors frequently impair feed intake and gut barrier integrity, favour dysbiosis, and increase susceptibility to opportunistic infections, with downstream consequences that include systemic inflammation and oxidative stress (Cui et al., 2024; Dufourny et al., 2021; Yan et al., 2024).

Uncontrolled oxidative stress with an overproduction of free radicals (ROS) that overcomes the body's antioxidant defense system is a key element of post weaning inflammation and must be taking into account when formulating nutritional strategies for this period (Huang et al., 2022; C. Li et al., 2020).

Within this context, the spleen is a relevant target organ. Beyond its classical hematological function (hematopoietic organ), the spleen is a central secondary lymphoid tissue that integrates innate and adaptive immune responses, filters blood-borne antigens, and supports immune cell activation and antibody production (Pistol et al., 2023). In young animals when exposed to inflammatory stimuli, splenic redox homeostasis is especially important because excessive oxidative pressure can compromise immune cell viability and function, promote macromolecular damage, and amplify inflammatory signaling. Key enzymatic defense in this system includes superoxide dismutase, catalase, and glutathione peroxidase,

which act sequentially to detoxify superoxide and hydrogen peroxide and to limit propagation of lipid and protein oxidation (Huang et al., 2023; Y. Li et al., 2020). Measuring these enzymes therefore provides a functional readout of the spleen's capacity to buffer oxidative challenge during the post-weaning transition.

Interest in agroindustry by-products as functional feed ingredients has increased in parallel with circular-economy objectives, and apple pomace is a prime example (Van Zanten et al., 2019; Yafetto et al., 2023). Generated in large amounts by juice and cider processing, apple pomace retains substantial quantities of bioactive compounds, including polyphenols, dietary fibre, and residual micronutrients (Anghel, 2024; Ao et al., 2022; Dufourny et al., 2021; Taranu et al., 2023). These components have been associated with modulation of gut microbiota, reinforcement of intestinal barrier function, and attenuation of inflammatory tone, all of which are highly relevant to post-weaning physiology (Ao et al., 2022; Dufourny et al., 2021; Guo et al., 2022; Taranu et al., 2023). From a redox perspective, apple-derived polyphenols are reported to directly scavenge reactive species and, importantly, to influence cellular antioxidant programs that govern enzymatic defense (Huang et al., 2022). Across animal models and feeding studies, apple pomace or apple polyphenol preparations have been linked to improved antioxidant status, including increases in superoxide dismutase and glutathione peroxidase activity and/or preservation of catalase activity under stress conditions, accompanied by reduced indices of lipid peroxidation (Ao et al., 2022; Chedea et al., 2019; Huang et al., 2023).

Other reports show that pomace and polyphenol-based interventions can normalize antioxidant enzyme activities when they are suppressed by challenge conditions rather than indiscriminately stimulating them in healthy tissue, a pattern consistent with a stress-mitigating, homeostasis-restoring role (Cui et al., 2024; Q. Li et al., 2020; Y. Li et al., 2020). These observations provide a rationale for focusing on superoxide dismutase, catalase, and glutathione peroxidase as sensitive endpoints when evaluating apple pomace in weaned piglets.

Lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, is a well-established trigger of systemic innate immune activation and oxidative stress through pathways that enhance cytokine production, increase reactive oxygen species generation, and disrupt antioxidant enzyme function (Bonetti et al., 2024; C. Li et al., 2020). The LPS challenge is relevant to the post-weaning stage, when piglets face heightened exposure to enteric pathogens and endotoxin translocation risk due to barrier instability (Yan et al., 2024; Zhang et al., 2021). LPS has been used experimentally to mimic weaning stress and to study different possibilities for countering it. Therefore, evaluating dietary apple pomace against an acute *E. coli*-LPS challenge allows assessment of whether this agri-food by-product can preserve splenic antioxidant defences and limit oxidative damage in a setting that mimics key elements of post-weaning inflammatory pressure.

MATERIALS AND METHODS

Animals and Diets

Twenty-four TOPIGS-40 weaned piglets (21 days of age; initial body weight 7.26 ± 0.42 kg), individually identified by ear tags, were randomly allocated to four experimental groups (n = 6/group) and maintained on their respective dietary treatments for 21 days. The first group (Control group) received a standard complete diet based on corn and soybean meal, according to NRC (2012) for the weaning period. The second group (LPS group) received the same control diet and was challenged intraperitoneally with *E. coli*-LPS at a dose of 80µg/kg body weight, 24h before slaughter (day 20). The third group (apple pomace group-AP) fed a complete diet including 5% apple pomace obtained from a local apple juice producer from Voinești, Dâmbovița County, Romania. The fourth group (apple pomace plus LPS group-AP+LPS) received the same 5% apple pomace diet and was likewise challenged intraperitoneally with LPS (80 µg/kg body weight) 24 h before slaughter.

The proximate composition (dry matter, crude protein, fat, crude fibres and ash were determined with ISO methods (SR EN ISO 5983-2:2009; SR ISO 6492:2001; SR EN ISO

6865:2002: SR EN ISO 2171: 2010), using the semiautomatic Kjeltec Auto 2300 system); minerals were performed by atomic absorption spectrometry after microwave digestion and mineralization with nitric acid (Regulation (EC) no. 152/2009 STAS 9597/16-86STAS 9597/17-86).

Animals were housed at the experimental facility of the National Research-Development Institute for Animal Biology and Nutrition, Balotești, Romania, with two pens assigned to each treatment group (3 piglets/pen).

At the end of the 21-day experimental period, piglets were fasted and euthanized by electrically stunned in compliance and spleen samples were collected on ice and stored at –80°C until analysis. All procedures were conducted with efforts to minimize suffering at slaughter with applicable ethical standards and regulations (EU Council Directive 98/58/EC, Romanian Law 43/2014, and approval of the Ethics Committee of the National Research and Development Institute for Animal Biology and Nutrition, Balotești, Romania).

Determination of polyphenols content of Apple Pomace

Polyphenols from apple pomace were extracted with 80% acetone using a sample-to-solvent ratio of 1:7 (w/v) at 37°C under continuous shaking (Thermo Fisher, Biosan, USA) for 20h. Total polyphenols concentration was then quantified from acetonic extract by using an UV-Vis Spectrophotometer Specord 205 (Analytik Jena, GMbH, Germany) and Folin-Ciocalteu reagent. The absorbance was read at 750nm and the concentration was calculated on a gallic acid curve calibration (r^2 =0.9985) as described by Chedea et al., 2017. The results are expressed as gallic acid equivalent (GAE)/g of apple pomace.

The polyphenolic profile (individual polyphenolic compounds) of the acetone extract was characterized by high-performance liquid chromatography (HPLC) using a Jasco Chromatograph (Jasco Corporation, Tokyo, Japan) equipped with Lichrosorb RP-C18 column (25 x 0.46 cm) and UV/Vis detector, based on retention times and UV detection (270 nm) as described by Vlassa et al., 2022 (Vlassa et al., 2022). Results are expressed as mg gallic acid equivalent per 100 ml extract (mg GAE/ml). All determinations were performed in triplicates.

Determination of total antioxidant capacity

Total antioxidant capacity (TAC) was assessed using an ABTS radical cation-based assay namely 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) as described by Marin et al. 2017 (Marin et al., 2017). Absorbance was recorded at 732 nm immediately after H_2O_2 addition (A0) and again after 3 min of incubation at 37°C (A3) using a Uvikon 923 double-beam spectrophotometer (Kontron Instruments, Zurich, Switzerland). The percentage inhibition of ABTS radical cation formation was converted to Trolox equivalent antioxidant capacity (TEAC) using a Trolox calibration curve (0-2.5 mM). Results were expressed as mM TEAC/g and were used as an index of the sample antioxidant capacity.

Antioxidant Enzyme Activity

The activities of the antioxidant enzymes glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD) in spleen tissue were determined using commercial assay kits from Cayman Chemical Company (Ann Arbor, MI, USA), as previously described (Marin et al., 2024). GPx and CAT activities were expressed as nmol/min/mL, while SOD activity was expressed as U/mL.

Assessment of Lipid Peroxidation

Lipid oxidation was assessed in spleen tissue lysates prepared in PBS, as previously described (Marin et al., 2024). Briefly, 200 µL of tissue lysate was mixed with 26 µL of 0.5 N HCl and 260 µL of TBA-TRIS reagent, then incubated at 95°C for 60 min. The reaction was terminated by cooling the samples on ice for 10 min, and absorbance was measured at 532 nm using a Varioskan™ LUX microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). Results were expressed as nmol/mL thiobarbituric acid-malondialdehyde (TBARS-MDA).

Assessment of Protein Oxidation

Protein oxidation was evaluated spectrophotometrically (560 nm excitation wavelength and 585-595 nm emission wavelength) by measuring protein carbonyls following derivatization with 2,4-dinitrophenylhydrazine, according to the protocol described in Marin et al., 2024 (Marin et al., 2024). Results were expressed as nmol carbonyls/mg protein.

Assessment of DNA Oxidation

DNA oxidation was determined by an immunoenzymatic assay using a DNA/RNA Oxidative Damage ELISA kit (Cayman Chemical Company, Ann Arbor, MI, USA) as described by Marin et al. (Marin et al., 2017). Absorbance was measured at 420 nm, and results were expressed as pg/mL 8-hydroxy-2′-deoxyguanosine (8-OHdG).

Statistical analysis

For spleen biochemical analyses, the individual piglet was considered the experimental unit. Data are presented as mean ± standard error of the mean (SEM). Statistical differences among experimental groups were evaluated by one-way analysis of variance (ANOVA) for all analysed parameters. When a significant overall treatment effect was detected by ANOVA ($p < 0.05$), post hoc pairwise comparisons between group means were performed using Tukey's multiple comparisons test in GraphPad Prism (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSIONS

Polyphenolic concentration in apple pomace

The result of the Folin Ciocalteu analysis revealed a concentration of 4.80 mg total polyphenols /100 g of apple pomace. Table 1 showed that the pomace is rich in catechin with anti-inflammatory and antioxidant role, ferulic acid, sinapic acid and luteoil known for their calming inflammatory and antioxidant role as well as the neuroprotective action.

Table 1. Polyphenolic composition of the investigated apple pomace

Polyphenolic compounds	Total Polyphenols (mg GAE/100 g)
Galic acid	0.48
Catechin	2.55
Chlorogenic acid	1.14
Vanillic acid	0.68
Caffeic acid	1.35
Syringic acid	0.16
p-Coumaric acid	0.95
Ferulic acid	10.61
Sinapic acid	4.38
Rutin	0.00
Quercetin	0.00
Luteolin	3.13
TOTAL	25.44

No significant differences were recorded between the control and experimental feed in terms of dry matter, crude protein, crude fat, cellulose and ash. But, the dietary inclusion of apple pomace increased by 19.9% the ADF fiber level and minerals (+8.5% Fe, +10.8% Zn and +11.36% Mn), compared to the control diet. Polyphenols such as caffeic acid (15.57 vs 0112 mg % g), coumaric acid (16.88 vs 0.34 mg % g), ferulic acid, sinapic acid, Rutin, Quercetin, Luteolin were also significantly increased in the diet containing apple pomace.

Effects of Dietary Apple Pomace and LPS Challenge on Splenic Antioxidant Enzyme Activity

The activities of glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD) were evaluated in spleen tissue from all experimental groups (Figure 1). Overall, *E. coli* LPS challenge was associated with reduced antioxidant enzyme activity, while dietary apple pomace modulated these responses attenuating the reduction under inflammatory conditions.

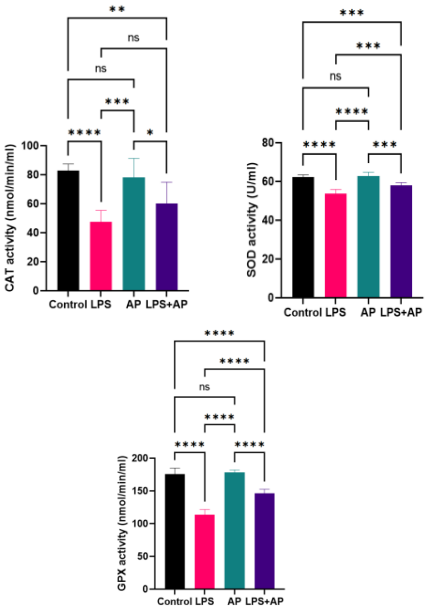


Figure 1. Effects of dietary apple pomace and LPS challenge on splenic antioxidant enzyme activities (CAT, SOD, and GPx) in piglets after weaning. They were fed for 21 days with a Control diet (Control and LPS groups) or AP diet (AP and LPS+AP groups). On day 20, piglets from LPS and LPS+AP groups were challenged with 80 µg LPS/b.w. The results are displayed as mean ± SEM. *=indicates a significant difference ($p < 0.05$); "ns" indicates no significance ($p > 0.05$)

Compared with the Control group, LPS administration significantly reduced CAT activity by 42.69% ($p < 0.0001$). Although, in the AP+LPS group, apple pomace managed to increase CAT activity over that of the group LPS (26.95%), this increase did not reach statistical significance in the Tukey post hoc test ($p = 0.1626$), probably due to the pronounced variability of the values. However, CAT activity in the AP+LPS group remained significantly lower than in the Control group ($p = 0.0047$) and the AP group ($p = 0.0346$). No significant difference was observed between the Control and AP groups ($p = 0.8676$).

A similar overall pattern was observed for SOD activity. Relative to the Control group, LPS significantly decreased SOD activity by 13.88% ($p < 0.0001$). In contrast to CAT, apple pomace inclusion significantly attenuated the LPS-induced decrease in SOD, with SOD activity in the AP+LPS group being 8.25% higher than in the LPS group ($p = 0.0006$). Nevertheless, SOD activity in the AP+LPS group remained significantly lower than in both the Control ($P = 0.0010$) and AP ($p = 0.0005$) groups. SOD activity did not differ between the Control and AP groups ($p = 0.9629$).

GPx activity was also significantly affected by LPS challenge, showing a 35.37% decrease in the LPS group compared with the Control group ($p < 0.0001$). Dietary apple pomace significantly attenuated this reduction, as GPx activity in the AP+LPS group was 28.79% higher than in the LPS group ($p < 0.0001$). However, GPx activity in the AP+LPS group remained significantly lower than in the Control and AP groups (both $p < 0.0001$). No significant difference was observed between the Control and AP groups ($p = 0.9266$).

Taken together, these results indicate that the diet with apple pomace alone did not significantly modify splenic GPx, CAT, or SOD activity under basal conditions, while under LPS-induced oxidative stress it significantly improved SOD and GPx activity, with a non-significant upward trend for CAT.

The post-weaning period is a critical stage in piglets, during which dietary transition, social stress, and environmental changes can increase inflammation and oxidative stress, particularly under the effect of pathogenic endotoxins. Apple pomace is a relevant functional agri-food by-product because it provides polyphenols and fibre with potential redox- and inflammation-modulating effects, while also supporting circular feed-use strategies (Van Zanten et al., 2019). The *E. coli*-LPS challenge used here was deemed appropriate to test whether dietary apple pomace could preserve splenic antioxidant defences under acute inflammatory oxidative stress, as suggested by previous work on apple-derived polyphenols and other polyphenol-rich by-products in piglets (Guo et al., 2022; Huang et al., 2022; Pistol et al., 2023).

In the present study LPS suppressed splenic GPx, CAT, and SOD activities, indicating impaired enzymatic antioxidant defences in the spleen during post-weaning inflammatory stress.

Because these enzymes act sequentially to neutralize reactive oxygen species and limit oxidative damage, their reduction reflects diminished antioxidant buffering capacity. This pattern is consistent with LPS-challenged piglet studies reporting decreased antioxidant enzyme activity together with increased oxidative stress markers (Cui et al., 2024; Huang et al., 2023; Xu et al., 2019). Apple pomace alone did not significantly alter splenic GPx, CAT, or SOD activity under basal conditions, whereas under LPS challenge it improved SOD and GPx activity and showed a non-significant upward trend for CAT. This indicates a stress-responsive protective effect, which is compatible with the concept that polyphenol-rich ingredients preserve enzyme function under oxidative challenge by lowering reactive oxygen species burden and supporting redox-sensitive pathways. The partial recovery observed here (significant for SOD and GPx, weaker for CAT) is suggestive given the tissue- and enzyme-specific nature of antioxidant responses and aligns with reports of organ-specific pomace effects and selective improvements in LPS models depending on tissue and sampling time (Chedea et al., 2019; Huang et al., 2023; C. Li et al., 2020; Xu et al., 2019).

This protective effect is compatible with the polyphenol-rich composition of apple pomace and with evidence that apple polyphenols can enhance antioxidant capacity through direct radical-scavenging and redox-regulated mechanisms; although signalling pathways were not measured here, the preservation of splenic

SOD and GPx activity is consistent with findings reported for apple polyphenols, fermented apple pomace, and related by-product-based antioxidant strategies (Ao et al., 2022; Guo et al., 2022; Huang et al., 2022; Vlassa et al., 2022).

Effects of Dietary Apple Pomace and LPS Challenge on Splenic Lipid Peroxidation (TBARS-MDA)

Splenic lipid peroxidation, assessed by TBARS-MDA, was significantly affected by *E. coli*-LPS challenge and modulated by dietary apple pomace inclusion (Figure 2). Compared with the Control group, LPS administration significantly increased TBARS levels by 66.84% ($p < 0.0001$), indicating enhanced lipid peroxidation under inflammatory oxidative stress conditions. Dietary apple pomace significantly attenuated the LPS-induced increase in TBARS-MDA. In the AP+LPS group, TBARS-MDA levels were 26.44% lower than in the LPS group ($p < 0.0001$), suggesting that apple pomace attenuates partially lipid oxidation damage in the spleen of piglets that received the pomace.

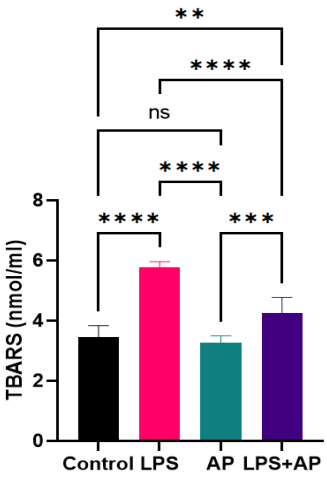


Figure 2. Effects of dietary apple pomace and LPS challenge on splenic TBARS-MDA marker in piglets after weaning. They were fed for 21 days with a Control diet (Control and LPS groups) or AP diet (AP and LPS+AP groups). On day 20, piglets from LPS and LPS+AP groups were challenged with 80µg LPS /b.w. The results are displayed as mean ± SEM. *= indicates a significant difference ($p < 0.05$); "ns" indicates no significance ($p > 0.05$)

However, TBARS values in the AP+LPS group remained significantly higher than those in both the Control group ($p = 0.0028$) and the AP group ($p = 0.0004$).

The diet with apple pomace alone (AP group) did not significantly affect TBARS levels compared with the Control group ($p = 0.7765$). Lipid peroxidation is a key downstream consequence of oxidative stress, as reactive oxygen species attack polyunsaturated fatty acids of cellular membrane and generate secondary products such as TBARS/MDA. The increase in splenic TBARS after LPS challenge indicates measurable lipid oxidative injury beyond enzymatic antioxidant suppression, consistent with reports in weaned piglet challenge models where LPS elevates TBARS/MDA in plasma and/or tissues (He & Wang, 2026; Huang et al., 2023; Q. Li et al., 2020). Apple pomace significantly reduced TBARS in LPS-challenged piglets, but values remained above Control and AP, indicating partial protection. This response is supported by studies showing reduced lipid peroxidation following antioxidant dietary supplementation in LPS-related piglet models, including rice bran oil, benzoic acid plus essential oils, postbiotics plus essential oils, and olive oil cake extract (He & Wang, 2026; Yan et al., 2024; Zhang et al., 2021). Fruits pomace evidence is also relevant, grape pomace reducing lipid peroxidation in piglet organs, (Chedea et al., 2019) and fermented apple pomace improving antioxidant indicators and reduced plasma MDA in weaned pigs (Ao et al., 2022; Vlassa et al., 2022). The reduction in TBARS may reflect direct radical scavenging by apple pomace polyphenols and indirect preservation of antioxidant defenses, limiting propagation of lipid peroxidation; the lack of difference between AP and Control supports absence of a pro-oxidant effect under basal conditions.

Effects of Dietary Apple Pomace and LPS Challenge on Splenic Protein Oxidation

Splenic protein oxidation, assessed by protein carbonyl content, was significantly affected by *E. coli*-LPS challenge and modulated by dietary apple pomace inclusion (Figure 3). Compared with the Control group, LPS challenge significantly increased protein carbonyl levels by 18.29% ($p < 0.0001$), indicating enhanced

protein oxidative damage under inflammatory conditions.

The diet with apple pomace prevented the increase in protein oxidation induced by LPS. In the AP+LPS group, protein carbonyl level was reduced by 17.27% relative to the LPS group ($p < 0.0001$). Moreover, protein carbonyl values in the AP+LPS group did not differ significantly from those in the Control group ($p = 0.0959$) or the AP group ($p = 0.8066$), indicating normalization of this oxidative stress marker.

Apple pomace alone (AP group) did not significantly affect protein carbonyl content compared with the Control group ($p = 0.4409$), with values being 1.32% lower than in Controls.

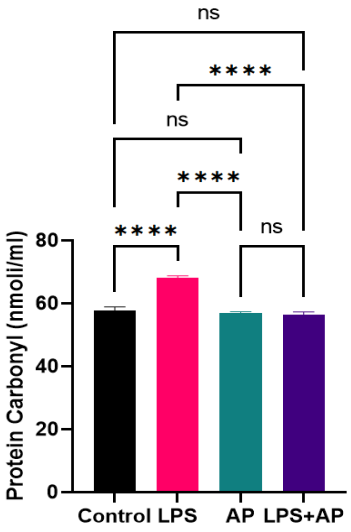


Figure 3. Effects of dietary apple pomace and LPS challenge on splenic Protein carbonyl marker in piglets after weaning. They were fed for 21 days with a Control diet (Control and LPS groups) or AP diet (AP and LPS+AP groups). On day 20, piglets from LPS and LPS+AP groups were challenged with 80µg LPS /b.w. The results are displayed as mean ± SEM. *= indicates a significant difference ($p < 0.05$); "ns" indicates no significance ($p > 0.05$)

Protein carbonyls are established markers of oxidative protein damage. LPS significantly increased splenic protein carbonyls, indicating that endotoxin-induced oxidative stress affected protein integrity in addition to lipids, consistent with inflammatory reactive oxygen species overproduction and with reports in LPS-challenged piglets showing increased oxidative damage alongside impaired antioxidant defenses (C. Li et al., 2020; Yin et al., 2014).

Apple pomace effectively prevented the LPS-induced increase, as protein carbonyl values in the AP+LPS group were reduced versus LPS and did not differ from Control or AP, suggesting stronger protection for protein oxidation than for some endpoints where recovery was partial. This is biologically meaningful because protein oxidation can impair enzyme activity, membrane transport, and immune cell function, which are relevant for splenic immune competence during post-weaning stress. The finding aligns with evidence that polyphenol-rich interventions reduce macromolecular oxidation during inflammatory challenge, despite marker- and tissue-dependent responses (Chedea et al., 2019; Huang et al., 2022).

The reduction in protein carbonyls likely reflects decreased reactive oxygen species availability through direct scavenging and improved enzymatic antioxidant protection, consistent with concurrent preservation of SOD and GPx, while the lack of effect in the AP group supports a challenge-dependent mechanism.

Effects of Dietary LPS Challenge on Splenic DNA Oxidative Damage

Splenic DNA oxidative damage, assessed by 8-hydroxy-2'-deoxyguanosine (8-OHdG) level, was significantly affected by *E. coli*-LPS challenge and modulated by dietary apple pomace (Figure 4). Compared with the Control group, LPS administration significantly increased 8-OHdG level by 61.76% ($p < 0.0001$), indicating marked DNA oxidative damage under inflammatory oxidative stress conditions.

The diet included apple pomace significantly attenuated the LPS-induced DNA oxidative damage. In the AP+LPS group, 8-OHdG level was reduced by 25.10% relative to the LPS group ($p < 0.0001$). However, 8-OHdG value in the AP+LPS group remained significantly higher than those in both the Control group ($p = 0.0243$) and the AP group ($p = 0.0019$), indicating partial protection of apple pomace against LPS-induced DNA oxidation. Apple pomace alone (AP group) did not significantly affect 8-OHdG level compared with the Control group ($p = 0.6010$), with values being 8.52% lower than in Controls.

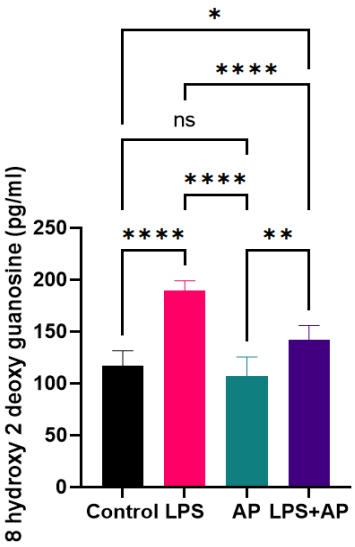


Figure 4. Effects of dietary apple pomace and LPS challenge on splenic DNA oxidation in piglets after weaning. They were fed for 21 days with a Control diet (Control and LPS groups) or AP diet (AP and LPS+AP groups). On day 20, piglets from LPS and LPS+AP groups were challenged with 80µg LPS /b.w.
The results are displayed as mean ± SEM. *= indicates a significant difference ($p < 0.05$); "ns" indicates no significance ($p > 0.05$)

8-OHdG is a marker of oxidative DNA damage. The increase in splenic 8-OHdG after LPS challenge indicates that oxidative injury extended to nucleic acids. A broader pattern reported in LPS-challenged piglets, showed that inflammatory challenge elevates oxidative damage markers together with impaired antioxidant defenses (Cui et al., 2024; Novais et al., 2020). Apple pomace significantly reduced 8-OHdG in LPS-challenged piglets, but values remained above Control and AP, indicating partial protection and matching the partial recovery pattern observed for TBARS and antioxidant enzymes. This is compatible with evidence that polyphenol-rich feeds limit oxidative damage in challenged animals, with efficacy influenced by tissue, marker, and challenge severity (Chedea et al., 2019; Cui et al., 2024). In this context, reducing splenic 8-OHdG is relevant because DNA oxidative damage can affect immune cell viability and function. The reduction likely reflects combined reactive oxygen species scavenging and preservation of antioxidant defenses, consistent with improved SOD and GPx, while the lack of difference between AP and Control indicates that apple pomace did not disturb basal DNA redox status.

Effects of Dietary Apple Pomace and LPS Challenge on Splenic Total Antioxidant Capacity

Splenic total antioxidant capacity (TAC) was significantly affected by *E. coli*-LPS challenge and modulated by dietary apple pomace inclusion (Figure 5). Compared with the Control group, LPS administration significantly reduced TAC by 2.29% ($p < 0.0001$), indicating a decrease in overall antioxidant defence capacity under inflammatory oxidative stress conditions.

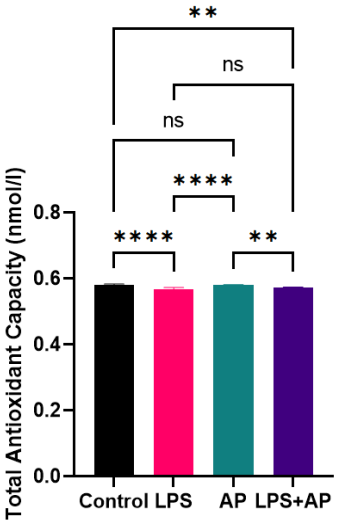


Figure 1. Effects of dietary apple pomace and LPS challenge on splenic TCA in piglets after weaning. They were fed for 21 days with a Control diet (Control and LPS groups) or AP diet (AP and LPS+AP groups). On day 20, piglets from LPS and LPS+AP groups were challenged with 80µg LPS /b.w.
The results are displayed as mean ± SEM. *= indicates a significant difference ($p < 0.05$); "ns" indicates no significance ($p > 0.05$)

The diet with apple pomace partially attenuated the LPS-induced reduction in TAC. In the AP+LPS group, TAC was 0.90% higher than in the LPS group; however, this increase did not reach statistical significance in the Tukey post hoc test ($p = 0.0931$). TAC values in the AP+LPS group remained significantly lower than those in both the Control group ($p = 0.0033$) and the AP group ($p = 0.0069$), indicating incomplete recovery of total antioxidant

capacity. Apple pomace alone (AP group) did not significantly affect TAC compared with the Control group ($p = 0.9982$), with values differing by only 0.12%. TAC provides an integrated estimate of antioxidant defense, reflecting combined enzymatic and non-enzymatic contributions. LPS significantly reduced splenic TAC, indicating reduced global antioxidant buffering capacity, consistent with reports linking decreased TAC with increased oxidative damage and depressed antioxidant enzymes in LPS challenge models (Jerez-Bogota et al., 2025; Jerez-Bogota et al., 2023).

In contrast to clearer effects observed for SOD, GPx, TBARS, and protein carbonyls, apple pomace produced only a modest, non-significant increase in TAC in LPS-challenged piglets, and TAC remained lower than in Control and AP. This suggests limited recovery of the total antioxidant pool under acute challenge conditions, which is plausible because TAC integrates multiple antioxidant systems and may be less responsive to partial enzyme recovery, particularly if low-molecular-weight antioxidants remain depleted after LPS exposure.

This endpoint-specific responsiveness aligns with the literature, where several interventions increase TAC in challenged piglets but responses vary by matrix, tissue, timing, and intervention type (Y. Li et al., 2020; Yan et al., 2024). Evidence from apple-derived interventions and fruit pomace studies supports antioxidant benefits in piglets, although direct spleen-specific TAC responses under LPS challenge remain limited (Ao et al., 2022; Jerez-Bogota et al., 2025). The non-significant TAC recovery may reflect the severity and timing of the acute LPS challenge, where ongoing oxidative demand can continue to deplete non-enzymatic antioxidants despite partial preservation of enzymatic defenses. Overall, the TAC data support a protective but limited effect of apple pomace on global splenic antioxidant capacity, consistent with partial recovery rather than complete normalization under acute inflammatory oxidative stress.

CONCLUSIONS

Taken together, the results obtained herein support a coherent interpretation, LPS impaired splenic antioxidant defense and increased oxidative damage to lipids, proteins, and DNA, while dietary apple pomace (at 5% inclusion rate) mitigated these effects to different extents depending on the endpoint. The strongest protection was observed for protein oxidation, partial protection for antioxidant enzymes, TBARS, and 8-OHdG, and only limited recovery for TAC, indicating a marker-specific protective profile under acute challenge conditions. These findings support apple pomace as a promising functional feed ingredient and agri-food by-product for improving oxidative stress resilience in weaned piglets, while highlighting the need for further studies on dose, duration, and mechanisms of action.

Further studies should define optimal inclusion levels, feeding duration, and the molecular mechanisms involved.

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OPTIMIZING POST-EMERGENCE HERBICIDE PROGRAMS FOR RAGWEED *(Ambrosia artemisiifolia)* AND ASSOCIATED BROADLEAF WEEDS IN SUNFLOWER

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Abstract

Ambrosia artemisiifolia is one of the most problematic weeds in sunflower cultures, with major agronomic, environmental and public health implications. A post-emergence, double application experimental field trial was conducted in western Romania in a tribenuron-tolerant sunflower hybrid. The control of Halauxifen-methyl and Halauxifen-methyl+Tribenuron-methyl mixtures with or without the adjuvant isodecyl alcohol ethoxylate against A. artemisiifolia, Cirsium arvense, Persicaria maculosa and Hibiscus trionum was visually evaluated during five assessment dates. Crop phytotoxicity was evaluated as well, which remained low and transient throughout the trial. Treatment efficacies were species-dependent and against A. artemisiifolia, a single application of halauxifen-methyl at 1 L/ha achieved the highest efficacy, reaching complete control at 14 and 50 days after application. In contrast, halauxifen-methyl at 1 L/ha+tribenuron-methyl at 45 g/ha+90% isodecyl alcohol ethoxylate at 0,1% V/V consistently provided the strongest and most consistent suppression of C. arvense, P. maculosa, and H. trionum, while simultaneously remaining among the most efficient treatments against ragweed. These findings indicate that optimized post-emergence herbicide treatment can provide effective broadleaf weed management in Tribenuron-tolerant sunflower while maintaining acceptable crop safety.

Key words*: chemical weed control, halauxifen methyl, herbicide efficacy, sunflower, tribenuron methyl.*

INTRODUCTION

Sunflower, *Helianthus annuus* L., is one of the most important oilseed crops in Romania, a country that ranks among the leading sunflower producers in the European Union (Stoicea et al., 2022). High and stable yields in this crop depend largely on effective weed management, as sunflower is frequently infested by a broad spectrum of dicotyledonous weed species that compete directly with the crop for light, water, and nutrients (Jursík et al., 2020; Máčajová et al., 2022; Pinke et al., 2010). Among the most problematic species are *Ambrosia artemisiifolia* L., *Cirsium arvense* L., *Persicaria maculosa* Gray, and *Hibiscus trionum* L. (Anastasiu et al., 2024; European Commission. Joint Research Centre., 2021; Stoicea et al., 2022). Their presence in sunflower fields reduces yield, affects crop uniformity, contributes to the persistence of the soil seed bank, and increases the risk of herbicide resistance development (Knolmajer et al., 2025; Pinke et al., 2010; Vink et al., 2012). Under these conditions, the development of selective and efficient weed control programs becomes essential.

Synthetic auxin herbicides represent an important class of compounds used in the post-emergence control of broadleaf weeds. These herbicides induce severe physiological and morphological disturbances in susceptible plants, ultimately leading to their death (Grossmann, 2010; Morderer, 2018).

Halauxifen-methyl is a next-generation synthetic auxin herbicide with systemic activity, absorbed through the leaves and translocated to meristematic tissues, where it mimics the action of indole-3-acetic acid and disrupts normal plant growth processes (Ludwig-Müller et al., 2021; McCauley et al., 2020; Xu et al., 2022). This results in tissue curvature, growth abnormalities, chlorosis, necrosis, and finally the death of the target weed. Previous studies have shown that halauxifen-methyl is effective against a wide range of annual and perennial broadleaf weeds, including species with reduced sensitivity to older herbicides (Mehdizadeh et al., 2020;

Morderer, 2018; Xu et al., 2022; Zimmer et al., 2018).

Tribenuron-methyl, a sulfonylurea herbicide classified in HRAC Group B, has a different mode of action, inhibiting acetolactate synthase and thereby blocking the biosynthesis of branched-chain amino acids essential for cell division and plant survival (Bozic et al., 2012; Busi et al., 2018; Yu & Powles, 2014). In sunflower, its use is restricted to tribenuron-methyl-tolerant hybrids, but when combined with halauxifen-methyl it may broaden the weed control spectrum and reduce the risk of resistance development by targeting distinct biochemical pathways (Máčajová et al., 2022; Xu et al., 2022; Zimmer et al., 2018). In practice, the efficacy of such mixtures may be further influenced by the use of adjuvants, which improve retention, spreading, foliar penetration, and systemic uptake of active ingredients (HERA, 2009; Mehdizabeh et al., 2020; Talmage, n.d.). This is particularly relevant when target weed species have waxy or pubescent leaves, or when applications are carried out under less favorable environmental conditions.

Although several herbicides are currently registered for sunflower protection, comparative data regarding the efficacy of different rates, active ingredient combinations, sequential applications, and the role of adjuvants in optimizing weed control remain limited. Recent work also shows that the effect of adjuvants depends strongly on their influence on spray properties, droplet behavior, and retention, rather than on a universal increase in efficacy (Basílio et al., 2024). This gap is also especially important under current agricultural conditions, where increasing pressure from herbicide-tolerant or resistant weed species and European policies aimed at reducing chemical inputs require more precise and sustainable treatment strategies (European Commission. Joint Research Centre, 2021; Jursík et al., 2020; Stoicea et al., 2022). This topic also fits within the broader European regulatory framework for invasive alien species management (Branquart et al., 2016).

Therefore, the present study was undertaken to identify effective and reliable weed control strategies against major invasive broadleaf species, with particular emphasis on *A. artemisiifolia*, in sunflower. Beyond its agronomic relevance, common ragweed also has a well-documented public-health impact because of its highly allergenic pollen, which contributes substantially to seasonal allergy burden across Europe (Lake et al., 2017; Schaffner et al., 2020).

The present study extends previous research by comparatively evaluating halauxifen-methyl and tribenuron-methyl under several application strategies, including reduced doses, higher doses, sequential treatments, and the addition of a 90% isodecyl alcohol ethoxylate adjuvant. The objectives were to assess herbicide efficacy against the main invasive broadleaf weeds present in the experimental field, to evaluate crop selectivity in sunflower, and to determine the contribution of the adjuvant to treatment performance. Through this approach, the study aims to provide applied information relevant to the optimization of herbicide programs and to the integration of these strategies into sustainable weed management systems targeting *A. artemisiifolia*.

MATERIALS AND METHODS

1. *Site description and crop establishment*

The field experiment was conducted in 2024 near Hodoș village, Timiș County, Romania (45°40′29″N, 21°48′58″E), on a clay loam soil with 39% sand, 33% clay, and 26% silt. Soil pH was 7.09, organic matter content was 10.2%, humus exceeded 6%, and cation exchange capacity ranged from 22 to 26 meq 100 g^{-1}. The area is characterized by a temperate-continental climate, with mean annual precipitation of approximately 600 mm and mean temperatures of 20-24°C during the main crop-development period.

Seedbed preparation included disking, harrowing, and leveling. Before sowing, a compound NPK fertilizer (15:15:15) was incorporated at 300 kg ha^{-1}. No additional phytosanitary treatments were applied during the growing season. The tribenuron-methyl-tolerant sunflower hybrid SUVEX (KWS), selected for compatibility with the Express® herbicide system, was sown on 15 April 2024 at 70 cm row spacing, targeting a final density of 62,000 plants ha^{-1}. This compatibility is

associated with ALS-based tolerance to tribenuron-methyl, enabling selective post-emergence use of sulfonylurea herbicides in the crop.

2. ***Experimental design and treatments***

The experiment was established as a randomized complete block design (RCBD) with four replications. Eight treatment variants were evaluated, including an untreated control. The treatment structure was designed to compare reduced-rate mixture programs, a sequential reduced-rate mixture, a higher-rate single-application mixture, a halauxifen-methyl reference treatment, and a standard mixture-based program supported by adjuvant.

Two application timings were used: application A on 15 May 2024 and application B on 21 May 2024. Application B was used only for the sequential treatment (T5).

The experimental design compared reduced-rate, sequential, reference, and standard mixture-based herbicide treatments under field conditions. To improve foliar uptake and support treatment efficacy, a 90% isodecyl alcohol ethoxylate adjuvant was included in the relevant mixture-based treatments (Mehdizabeh et al., 2020). The treatment structure is summarized in Table 1.

Table 1. The applied treatments used during this trial

Trt.	Timing	Active Ingredient	Dosage	
T1	A	halauxifen-methyl +tribenuron-methyl	25	g/ha
	A	90% isodecyl alcohol ethoxylate	0.1	% V/V
T2	A	halauxifen-methyl + tribenuron-methyl	30	g/ha
	A	90% isodecyl alcohol ethoxylate	0.1	% V/V
T3	A	halauxifen-methyl +tribenuron-methyl	40	g/ha
	A	90% isodecyl alcohol ethoxylate	0.1	% V/V
T4	A	halauxifen-methyl +tribenuron-methyl	50	g/ha
	A	90% isodecyl alcohol ethoxylate	0.1	% V/V
T5	A	halauxifen-methyl + tribenuron-methyl	25	g/ha
	A	90% isodecyl alcohol ethoxylate	0.1	% V/V
	B	halauxifen-methyl + tribenuron-methyl	25	g/ha
	B	90% isodecyl alcohol ethoxylate	0.1	% V/V
T6	A	halauxifen-methyl	1	L/ha
T7	A	halauxifen-methyl	1	L/ha
	A	tribenuron-methyl	45	g/ha
	A	90% isodecyl alcohol ethoxylate	0.1	% V/V
T8	-	UNTREATED CONTROL	-	-

Reduced-rate mixture treatments T1-T3 consisted of halauxifen-methyl + tribenuron-methyl applied once at total rates of 25, 30, and 40 g ha^{-1}, respectively, each combined with 90% isodecyl alcohol ethoxylate at 0.1% V/V. T4 consisted of a single application of the same mixture at 50 g ha^{-1} plus adjuvant. T5 was a sequential reduced-rate program in which the 25 g ha^{-1} mixture plus adjuvant was applied twice at a six-day interval. T6 was the halauxifen-methyl reference treatment applied alone at 1 L ha^{-1}. T7 consisted of halauxifen-methyl at 1 L ha^{-1} combined with tribenuron-methyl at 45 g ha^{-1} and adjuvant at 0.1% V/V. T8 was the untreated control. These combinations, doses and rates were selected according to technical recommendations in order to optimize efficacy and limit phytotoxicity (EPPO, 2022).

Each plot measured 21 m^2 (3 × 7 m) and consisted of four sunflower rows. A 1m buffer was maintained between plots and around the experimental perimeter to reduce spray drift. Evaluations were made in the central plot area to minimize edge effects.

3. ***Herbicide application***

Herbicides were applied post-emergence when sunflower plants were at 4-6 true leaves (Stefanic et al., 2023). The initial weed density was assessed before herbicide application using quadrat counts and converted to plants/m^2. The added values were 58.86 plants/m^2 for *A. artemisiifolia*, 8.57 plants/m^2 for *C. arvense*, 6.29 plants/m^2 for *P. maculosa*, and 6.86 plants/m^2 for *H. trionum*. At the time of application, the target weed species were at 3-8 true leaves. Treatment applications were performed using a portable compressed-air boom sprayer equipped with six flat-fan nozzles, calibrated to deliver 250 L ha^{-1} at a constant pressure of 500 kPa. The adjuvant used in the study was a 90% isodecyl alcohol ethoxylate formulation applied at 0.1% V/V in the relevant treatments. Applications were performed under favorable agrotechnical and meteorological conditions, and the second sequential treatment was applied under comparable field conditions.

4. ***Crop safety and weed-control assessments***

Crop selectivity and weed-control efficacy were assessed visually after treatment application. Crop safety was evaluated through phytotoxicity scoring, crop vigor, and visible morphological injury. Phytotoxicity was rated on a 0-100% scale, where 0 indicated no visible crop injury and 100 indicated complete crop destruction. Crop vigor was rated on the same scale, where 100% indicated optimal plant development and uniformity. Leaf and stem deformation symptoms were recorded separately to characterize transient treatment-related responses.

Weed control efficacy was estimated visually as percentage reduction in weed presence relative to the untreated control. Visual control ratings were determined for the dominant broadleaf weeds present in the field: *Ambrosia artemisiifolia*, *Cirsium arvense*, *Persicaria maculosa*, and *Hibiscus trionum*. All assessments were based on four field replicates. Assessments were conducted at 3, 7, 14, 28, and 50 days after application (DAA), matching the evaluation schedule presented below.

5. ***Statistical Analysis***

Statistical analyses were performed to evaluate the effects of herbicide treatments, the influence of evaluation time, and the interaction between these two experimental factors on crop response and weed-control efficacy.

The experimental design was analyzed as a two-factor structure, with treatment as Factor A and evaluation time as Factor B. Two-way analysis of variance (ANOVA) was used to assess the main and interaction effects of treatment and evaluation time on phytotoxicity, crop vigor, incidence and severity of leaf and stem deformation, and weed-control efficacy for *A. artemisiifolia*, *C. arvense*, *P. maculosa*, and *H. trionum*. When significant differences were detected, means were compared using the Least Significant Difference (LSD) test and Duncan's multiple range test to identify statistically distinct groups among treatments and evaluation times. Significance thresholds were set at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$. All datasets fulfilled the assumptions of normality and homogeneity of variance, and no data transformation was required. To complement variance analysis, cluster analysis based on Euclidean distance was used to group treatments and evaluation times according to biological similarity. In addition, factor contribution analysis was used to estimate the relative contribution of treatment, evaluation time, and their interaction to the total observed variability, thereby highlighting the relative importance of each experimental factor.
All statistical analyses were conducted using STATISTICA 13.3 software (StatSoft, USA), while descriptive statistics and graphical outputs were prepared in Microsoft Excel. Results are presented as mean values ± standard error.

RESULTS AND DISCUSSIONS

The present study shows that the agronomic value of post-emergence herbicide programs in tribenuron-tolerant sunflower should be assessed not only by peak efficacy against a single target weed, but by the combined balance of crop selectivity, persistence of visible crop response, and consistency of suppression across the full broadleaf spectrum present in the field. Under the conditions of this experiment, all tested programs avoided persistent crop injury, but they differed clearly in the breadth and stability of weed control. This is especially important in sunflower, where early weed interference has direct consequences for crop performance. Recent work has shown that even short delays in weed removal can reduce plant height, head diameter, and 1000-kernel weight, and that the beginning of the critical period of weed control may occur around the 4-6 leaf stage (Savić et al., 2025; Stefanic et al., 2023). This closely matches the application window used here and reinforces the agronomic importance of timely post-emergence intervention (Stefanic et al., 2023).

1. ***Phytotoxicity assessment***

A first important outcome of the study is the favorable crop tolerance of the tested hybrid. Table 2 presents the mean phytotoxicity values

visually assessed across four field replications for each treatment.

Two-way ANOVA showed that both treatment, Factor A, and evaluation time, Factor B, had a highly significant effect on phytotoxicity, with $F(7,148) = 11.03$, $p < 0.001$ and $F(4,148) = 27.34$, $p < 0.001$, respectively. The interaction between treatment and evaluation time was also significant, indicating that the crop response varied depending on the time of assessment. Student's test, compared with the untreated control T8, showed that T1, T2, and T3 did not induce statistically significant phytotoxicity, while T4 and T5 caused only slight and biologically acceptable visual effects. The highest phytotoxicity values were recorded in T6 and T7, at about 10%, indicating a more intense physiological response than in the other treatments.

In terms of evaluation time, phytotoxicity reached its maximum at the first assessment, 17 May, where it reached 3.75%, and then declined progressively to 0% at the final evaluation. This pattern indicates that the visual symptoms were concentrated in the earliest period after herbicide application and diminished as the crop recovered.

Table 2. Mean phytotoxicity values recorded after herbicide application

Treatment	Phytotoxicity (%)				
	3 DAA	7 DAA	14 DAA	28 DAA	50 DAA
T1	0	0	0	0	0
T2	0	0	0	0	0
T3	0	0	0	0	0
T4	5	0	0	0	0
T5	5	0	0	0	0
T6	10	5	0	0	0
T7	10	5	0	0	0
T8	0	0	0	0	0

Cluster analysis supported the same trend. T1, T2, T3, and T8 formed a group without visible symptoms, T4 and T5 formed an intermediate group with slight responses, and T6 and T7 formed a distinct group with more evident symptoms. Likewise, the first assessment date was clearly separated from the later evaluations, while the last three observation dates were grouped by the absence of symptoms. Although the differences among treatments and evaluation dates were statistically significant, phytotoxicity remained low and transient and disappeared completely by 50 days after application. These results indicate good crop selectivity and a strong recovery capacity of the tested sunflower hybrid. Similar temporary responses to synthetic auxin herbicides, including halauxifen-methyl, have also been reported in the literature, without long-term negative effects on crop development when treatments are applied at the appropriate growth stage and under suitable agronomic conditions (Grossmann, 2010; Morderer, 2018; Zimmer et al., 2018)

2. ***Crop vigor assessment***

Visual assessment of crop vigor showed that this parameter remained at a very high level throughout the entire observation period. The mean values of four replications per treatment are presented in Table 3.

Throughout the entire assessment period, all treated variants, T1-T7, maintained constant values of 100%, indicating very good tolerance of the sunflower crop to the applied treatments and the absence of any visible negative effect on plant development. The only reduction in crop vigor was recorded in the untreated control, T8, where values declined to 95% and then to 85% at the last two evaluations.

Table 3. Mean crop vigour values recorded after herbicide application

Treatment	Crop Vigor (%)				
	3 DAA	7 DAA	14 DAA	28 DAA	50 DAA
T1	100	100	100	100	100
T2	100	100	100	100	100
T3	100	100	100	100	100
T4	100	100	100	100	100
T5	100	100	100	100	100
T6	100	100	100	100	100
T7	100	100	100	100	100
T8	100	100	100	95	85

Analysis of variance showed that both treatment and evaluation time had a significant effect on crop vigor, and the interaction between the two factors was also significant. Student's test confirmed that the treated variants maintained a very high level of crop vigor, without visible reductions during the assessment period, whereas the untreated control showed a progressive decline in this parameter toward the end of the observation period. Cluster analysis supported the same

pattern, grouping the treated variants in an area characterized by high and stable vigor values, clearly distinct from the response of the untreated control.

These results show that the observed statistical differences were mainly driven by the decline in crop vigor in the untreated control, under conditions where weed competition was not controlled. From a biological point of view, the crop maintained an optimal level of vigor in all treated variants, confirming the very good selectivity of the tested herbicides and the good tolerance of the sunflower hybrid used in the experiment. Compared with the untreated control, the results also support the indirect role of herbicide intervention in maintaining crop physiological status and competitiveness through the reduction of weed pressure.

3. *Morphological symptoms induced by herbicide treatments*

To assess the morphological response of the crop to the applied treatments, leaf and stem deformation symptoms were recorded in terms of both severity and incidence. The values presented in Table 4 and Table 5 represent the mean of the four replications for each treatment and evaluation time, while the statistical interpretation was based on the full set of individual observations.

The highest leaf deformation values were recorded in T6 and T7 at 3 days after application, where they reached 10%, and declined to 5% at 7 days after application. In T4 and T5, symptoms were weakly expressed, with values of 5% at 3 days after application, and then disappeared completely. No leaf deformation was observed in T1, T2, T3, or T8 during the entire assessment period. In terms of incidence, symptoms were recorded only at 7 days after application, when T6 and T7 reached 100%, while all other treatments remained at 0%. At the later evaluations, incidence values were 0% for all treatments, showing that these symptoms were slight and transient under field conditions. Statistical analysis confirmed highly significant differences among treatments and evaluation times for both leaf deformation severity and incidence, $p < 0.001$. Stem deformation symptoms were observed only in some treatment variants at the first evaluation times. T4 and T5 reached values of 5% at 3 days after application, whereas higher values, 10%, were recorded in T6 and T7. At 7 days after application, deformation persisted only in T6 and T7, with values of 5%, and by 50 days after application all values had returned to 0%. The incidence data followed the same pattern. Thus, at 7 days after application, all plants in T6 and T7 showed slight stem deformation, whereas all other treatments recorded an incidence of 0%.

Table 4. Leaf deformation severity and incidence (%) at different evaluation times

Treatment	Leaf deformation			Leaf deformation incidence	
	3 DAA	7 DAA	50 DAA	7 DAA	50 DAA
T1	0	0	0	0	0
T2	0	0	0	0	0
T3	0	0	0	0	0
T4	5	0	0	0	0
T5	5	0	0	0	0
T6	10	5	0	100	0
T7	10	5	0	100	0
T8	0	0	0	0	0

At the final evaluation, stem deformation incidence was 0% in all experimental variants. Statistical analysis showed highly significant differences among treatments and evaluation times, $p < 0.001$, indicating that both treatment type and evaluation timing significantly influenced the occurrence of stem deformation. Comparison with the untreated control showed very significant differences in T6 and T7 and significant differences in T4 and T5, whereas T1, T2, and T3 did not differ significantly.

Table 5. Stem deformation severity and incidence (%) at different evaluation times

Treatment	Stem deformation			Stem deformation incidence	
	3 DAA	7 DAA	50 DAA	7 DAA	50 DAA
T1	0	0	0	0	0
T2	0	0	0	0	0
T3	0	0	0	0	0
T4	5	0	0	0	0
T5	5	0	0	0	0
T6	10	5	0	100	0
T7	10	5	0	100	0
T8	0	0	0	0	0

Cluster analysis confirmed this separation by distinguishing variants without symptoms from those in which stem deformation was more evident. Factor contribution analysis also

showed that treatment had a greater influence than evaluation time on stem deformation variability. Overall, the maximum values remained low, the symptoms declined over time, and their effects were reversible, confirming the good tolerance of the sunflower hybrid to the applied treatments.

Phytotoxicity and deformation symptoms were confined to the early post-application period and disappeared at later assessments, indicating that visible crop responses were transient rather than persistent. Even the strongest early responses, observed in T6 and T7, did not translate into lasting loss of crop performance. This pattern is consistent with previous studies describing short-lived auxinic symptomatology after post-emergence herbicide application, including temporary deformation, chlorosis, or growth disturbance, without evidence of sustained injury when treatments are applied at appropriate crop stages and under suitable agronomic conditions (Grossmann, 2010; Jursík et al., 2020; Morderer, 2018; Zimmer et al., 2018). From a practical standpoint, these results indicate that early visual stress should not automatically be interpreted as agronomically unacceptable when recovery is rapid and complete.

4. ***Herbicide efficacy on target weed species***

Herbicide efficacy was evaluated against the four main broadleaf weed species identified in the experimental field, namely *A. artemisiifolia*, *C. arvense*, *P. maculosa*, and *H. trionum*. Across all four species, efficacy generally increased from 7 to 14 days after application and then remained stable or changed only slightly by the final evaluation, indicating that treatment response became more pronounced after the first post-application interval.

The comparative analysis of the four weed species revealed clear differences among the tested variants in both control efficacy and the ability of the treatments to maintain this effect over time. This interpretation is consistent with current crop-weed competition frameworks, which emphasize that agronomic response depends not only on the presence of individual weeds, but also on the composition, persistence, and aggressiveness of the competing weed community (Savić et al., 2025).

Treatment ranking was species-dependent. Against *A. artemisiifolia*, the highest species-specific efficacy was recorded in T6, where control reached 95.0% at 7 DAA and 100.0% at both 14 and 50 DAA. T7 also performed strongly against this species, with values of 86.25%, 97.5%, and 97.5%, respectively. The comparative evolution of treatment efficacy across the four evaluated weed species is illustrated in Figure 1.

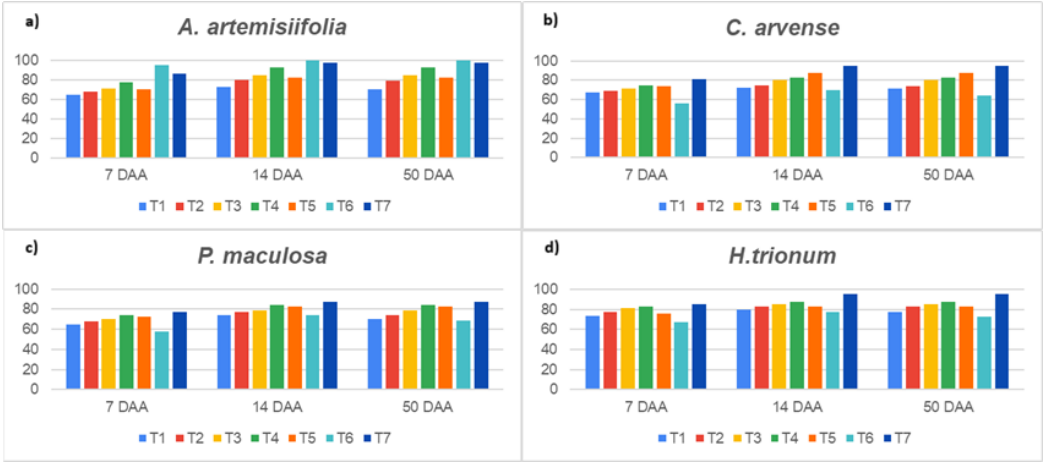


Figure 1. Comparative herbicide efficacy (%) against *A. artemisiifolia*, *C. arvense*, *P. maculosa*, and *H. trionum* at 7, 14, and 50 days after application. Values represent the mean of four field replications for each treatment

The values shown represent the mean efficacy recorded across the four field replications for each treatment at the selected evaluation timings. These dates were chosen because they reflect the main stages of post-emergence treatment performance, namely the fully expressed control phase, the persistence of efficacy over time, and the final level of weed suppression.

In contrast, T7 provided the strongest and most stable suppression of the other three species, reaching 95.0% against *C. arvense*, 87.5% against *P. maculosa*, and 95.0% against *H. trionum* at the later assessments. T4 and T5 also maintained high efficacy across several species, particularly against *C. arvense* and *P. maculosa*. T1 and T2 remained at intermediate levels, while T6, despite its excellent performance against *A. artemisiifolia*, showed weaker final control of *C. arvense*, *P. maculosa*, and *H. trionum*, with 63.75%, 68.75%, and 72.5%, respectively. The reduced-rate treatments T1, T2 and T3 should therefore be interpreted primarily as high selectivity but lower intensity control options. Their main strength was the absence of visible phytotoxicity and the preservation of maximal crop vigor throughout the experiment. However, their efficacy remained consistently below that of the stronger mixture-based treatments, particularly against the more difficult broadleaf species. From a management perspective, these treatments may be useful where crop safety is prioritized and weed pressure is moderate, but the present data do not support treating them as broadly equivalent alternatives to T4, T5, or T7 for robust spectrum-wide suppression.

Statistical analysis supported these differences. Treatment had a highly significant effect on efficacy in all four species, with $p < 0.001$ in each case. Evaluation time was also significant, reflecting the increase in efficacy after application and the stabilization of treatment response over time. The treatment × time interaction was significant as well, showing that treatment performance changed across evaluation dates. For *H. trionum*, the interaction was significant at $p < 0.01$, while for the other species it was highly significant, $p < 0.001$. Student's test confirmed that all herbicide treatments were significantly more effective than the untreated control. For *A. artemisiifolia*, efficacy means ranged from 63.3% to 90.4%, with T7, T5, and T4 grouped among the most effective treatments. For P. maculosa, T7 reached a mean efficacy of 84.2%, followed by T4, T5, and T3.

Cluster analysis supported the same general trend and showed a clear separation among treatments according to their efficacy level. For *A. artemisiifolia*, the clustering pattern indicated the clearest separation among treatments, distinguishing the untreated control from the most effective variants and from those with intermediate or lower efficacy. The upper part of the dendrogram, from Figure 2 was associated with treatments showing the highest and most persistent control, whereas the remaining active variants were grouped at lower efficacy levels.

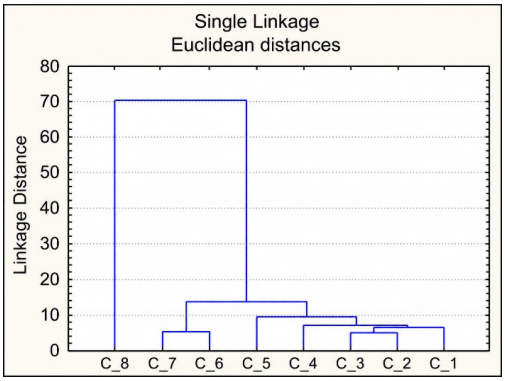


Figure 2. Dendrogram of herbicide treatments grouped according to the similarity of their efficacy in controlling *A. artemisiifolia* where C_1 = T1, C_2 = T2, C_3 = T3, C_4 = T4, C_5 = T5, C_6 = T6, C_7 = T7, and C_8 = T8, the untreated control

This trend was in agreement with the efficacy data, which placed T6 and T7 at the top of the response range and identified T4 as the strongest mixture-based treatment below them. For *C. arvense*, the untreated control formed a distinct cluster, T6 was separated as the least effective herbicide treatment, and the remaining variants were grouped within a higher-efficacy class. For *P. maculosa*, cluster analysis distinguished a high-efficacy group including T7, T4, T5, and T3, an intermediate group represented by T2 and T1, and a low-efficacy group represented by T6. For *H. trionum*, the untreated control and T7 were

clearly differentiated, while the remaining herbicide-treated variants were grouped at an intermediate efficacy level.

In *C. arvense*, *P. maculosa*, and *H. trionum*, factor contribution analysis showed that treatment explained most of the observed variability, with values of 96.9%, 98.4%, and 98.4%, respectively, whereas the contribution of evaluation time remained low, between 0.8% and 2.0%. These statistical results indicate that the herbicide program itself was the main determinant of control efficacy.

Overall, T4, T5, and especially T7 provided the most favorable results by combining high efficacy with good crop selectivity. T4, based on a single application of halauxifen-methyl + tribenuron-methyl at a total rate of 50 g/ha, provided a favorable balance between weed control and crop tolerance. Although it did not surpass T7, T4 clearly outperformed the lower-rate programs and, in several cases, approached the upper performance range of the strongest treatments. This makes T4 a practically relevant option because it achieved a favorable balance between crop safety and efficacy without requiring a sequential application strategy. In sunflower systems where strong broadleaf suppression is required, but a less intensive mixture-based treatment is preferred, T4 may represent a useful compromise.

T5 (halauxifen-methyl + tribenuron-methyl 25 g/ha + adjuvant), applied sequentially, maintained good efficacy and, in some of the evaluated species, provided better persistence of the control effect, suggesting that this strategy may be useful under conditions of high infestation pressure or staggered weed emergence. This may be particularly relevant where a single application does not adequately control weeds that emerge later, as emergence patterns can strongly influence the timing and persistence of post-emergence control (Marschner et al., 2024).

T6, corresponding to halauxifen-methyl applied at the registered dose, was highly effective against *A. artemisiifolia*, but its weaker performance against the other species indicates a narrower control spectrum than the variants based on active ingredient mixtures. This result is also important from a resistance-management perspective. Even when a single-active treatment performs very well against ragweed, more diversified post-emergence treatment strategies remain a safer agronomic option when resistance is developing in parallel. Recent work has shown that common ragweed is capable of evolving diverse ALS-resistance mechanisms within and among field populations, reinforcing the value of treatment structures that reduce dependence on a single mode of action (Busi et al., 2018; Knolmajer et al., 2025; Loubet et al., 2023). More recent field evidence has also documented broader multi-resistance patterns in common ragweed populations (D'Amico et al., 2024). In line with integrated weed-management strategies, these treatments should be combined with complementary agronomic and mechanical measures such as crop rotation, inter-row cultivation, field hygiene, and reduction of the soil seed bank, so that chemical control remains an effective but not isolated component of a durable control strategy (Kalogiannidis et al., 2022; Naeem et al., 2022).

The limitations of the present study should also be acknowledged. The experiment was designed to assess post-emergence weed-control efficacy and visible crop tolerance, but did not include yield, thousand-seed weight, or seed-quality measurements. However, the evaluation period up to 50 DAA covered the critical period of weed control in sunflower, during which weed interference can strongly affect yield components. Therefore, the clear differences in early and sustained weed suppression observed among treatments remain agronomically relevant, as effective weed control during this interval supports the protection of crop development and yield components.. Additional work is needed to determine whether the broad-spectrum efficacy advantage of T7 translates into improved productivity under field conditions and whether the strong ragweed control observed with T6 has any distinct effect on final crop output.

CONCLUSIONS

The control of problematic broadleaf weeds in sunflower remains a major agronomic challenge, especially where high infestation pressure and the need to reduce chemical inputs require more precise and flexible herbicide strategies.

This study showed that effective chemical control of the main broadleaf weeds in sunflower, namely *A. artemisiifolia*, *C. arvense*, *P. maculosa*, and *H. trionum*, can be achieved through optimized combinations of halauxifen-methyl and tribenuron-methyl. The results showed that high efficacy and good crop selectivity were not limited to standard rates only. Reduced-rate mixtures and sequential applications also gave satisfactory results when treatments were applied within the optimal window, namely when the sunflower crop had good tolerance and the weeds were still at early growth stages. This agrees with previous studies showing that optimized lower-dose herbicide strategies can still contribute meaningfully to ragweed suppression and more sustainable control approaches (Wang et al., 2022).

The trial also showed that some minor and transient physiological responses, such as slight leaf or stem deformation, may occur after application, but these did not affect overall crop performance. This confirms that treatment efficacy should be interpreted together with crop tolerance when new herbicide strategies are evaluated.

Another important result was the positive role of the isodecyl alcohol ethoxylate adjuvant, which improved uptake and helped maintain high control efficacy, especially in the strongest treatment variants. This suggests that adjuvant-supported treatments may offer greater flexibility for optimizing post-emergence weed control while maintaining a high level of performance.

Overall, the results support the integration of chemical control into broader weed-management systems. This is in line with recent ragweed reviews showing that long-term suppression of *A. artemisiifolia* requires diversified and integrated management strategies rather than reliance on a single control approach (Knolmajer et al., 2025). The most promising treatments, especially T4, T5, and T7, may be used successfully in sunflower fields heavily infested with problematic broadleaf weeds. Their effect can be strengthened further when combined with complementary agronomic practices such as crop rotation, inter-row cultivation, mulching, and early field hygiene.

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ENHANCEMENT OF NAPIER GRASS SILAGE QUALITY AND AEROBIC STABILITY USING *Lactobacillus* spp.

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Abstract

This study investigated the effects of Lactobacillus spp. on Napier grass silage quality, dry matter loss (DM loss), and aerobic stability. The experiment employed a 3 × 3 factorial design in a completely randomized design (CRD), with three levels (0, 1×10^5, and 1×10^6 cfu/g fresh weight) of Lactobacillus buchneri and Lactobacillus plantarum. The silage was ensiled for 24 days. The following parameters were analyzed: DM loss, fermentation characteristics, nutritive values, fiber fractions and anaerobic microbial profiles. Subsequently, the silage was exposed to air for 2, 4, and 6 days to determine aerobic stability characteristics and microbial profiles. The results showed that the highest inoculation LB level improved silage aerobic stability but increased DM loss and negatively affected fermentation quality. However, co-inoculation with the highest levels of both lactic acid bacteria resolved the DM loss problem and improved overall fermentation quality while maintaining enhanced aerobic stability compared with sole inoculation of LB. In conclusion, co-inoculation with the highest levels of lactic acid bacteria improved dry matter recovery, fermentation quality, and aerobic stability of Napier grass silage.

Key words*: aerobic stability, Lactobacillus buchneri, Lactobacillus plantarum, Napier grass, molasses.*

INTRODUCTION

Optimal silage fermentation is fundamentally dependent on the characteristics of the forage, particularly its moisture levels, buffering capacity, and the availability of water-soluble carbohydrates and buffering capacity (Wilkinson, 2005). While adding molasses boosts energy, the remaining sugars can trigger aerobic deterioration by fostering the growth of yeasts and mold once the silage is exposed to oxygen (Guan et al., 2002; Moon, 1983). Consequently, silage quality deteriorates due to aflatoxin contamination from mold proliferation once the silage is exposed to air (Ferrero et al., 2019).

Lactobacillus buchneri produces acetic acid, which inhibits the proliferation of aerobic microorganisms (Jatkauskas et al., 2025). However, the use of LB has been associated with DM losses in the form of CO_2 (Jin, et al., 2024; Oude Elferink et al., 2001), resulting in economic losses (Goeser et al., 2015) and affecting silage fermentation quality. Previous studies have demonstrated that inoculating LB in combination with LP can address DM loss and improve corn silage quality (Huisden et al., 2009). However, the application of LB combined with LP in molasses-treated Napier grass has not been confirmed, and the appropriate concentration (cfu/g fresh weight) of both bacterial species for this purpose is unclear.

This study hypothesized that combinations of both bacterial species at various levels can increase acetic acid content, decrease DM loss, improve fermentation quality, and enhance the aerobic stability of silage. The objectives were to evaluate the inoculation of LB combined with LP at various concentrations (cfu/g fresh forage weight) in molasses-treated Napier grass silage, specifically examining its effects on DM loss, fermentation characteristics, and aerobic stability.

MATERIALS AND METHODS

Experimental Design

A 3×3 factorial arrangement within a completely randomized design was implemented to evaluate the treatments in this research. Two factors were studied: *Lactobacillus plantarum* (LP) and *Lactobacillus buchneri* (LB). Each factor

consisted of three inoculation rates: 0, 1×10^5, and 1×10^6 cfu/g of fresh grass weight.

Ensiling process

Napier grass, harvested from the Suranaree University of Technology farm in Thailand, has a dry weight of approximately 25% (DM). Forage preparation involved chopping the Napier grass into 1-2 cm segments, followed by the incorporation of molasses at a rate of 5% on a fresh-weight basis. Napier grass was treated with lactic acid bacteria according to the factorial design described above. The total volume of inoculants was adjusted to 2% of fresh forage weight by adding deionized water and was mixed uniformly into the chopped, molasses-treated Napier grass. Each treatment was replicated four times, with 500 g of fresh forage weight per replicate. The silage was sealed in polyethylene bags, stored at 25°C, and ensiled for 24 days.

Chemical analysis

Dry matter content was assessed using a freeze-drying machine. The percentage of dry matter loss was calculated by subtracting the %DM content after ensiling from the %DM content prior to ensiling. The dried samples were ground using a laboratory blender to achieve an approximate particle size of 1 mm. Water-soluble carbohydrate analysis was conducted through sugar extraction, following the method outlined by Chow and Simon (2004). The sugar content of the extracted solution was evaluated using the procedure described by Dubois et al. (1956). Nutrient compositions, including crude protein, fat, and ash, were analyzed using standard methods according to the AOAC (1995). Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF) and Acid Detergent Lignin (ADL) were determined using detergent fiber analysis according to Van Soest et al., (1991). A 10× dilution was prepared to assess the forage buffering capacity following the method of Playne and McDonald (1966), as well as to determine NH_3-N, pH, lactic acid, and volatile fatty acids (VFAs). A well-homogenized sample of 50 g fresh weight was diluted tenfold by adding 450 mL of deionized water and thoroughly blended using a laboratory blender. The concentrations of lactic acid and volatile fatty acids (VFAs) were analyzed using a gas chromatography instrument (Agilent 7890B GC) with an Agilent CP-Sil 5 CB column (0.32 mm × 25 m fused silica), following the procedure described in the Agilent application note for lactic acid and C2–C7 VFA analyses. NH_3-N concentration was determined using a method adapted from Weatherburn (1967).

Aerobic stability

The silage sample was collected on day 24 of ensiling to determine aerobic stability characteristics, as described by Ashbell et al. (1987; 1991). The silage was exposed to air for 2, 4, and 6 days. A 10× dilution of each sample (as described above) was prepared to measure pH and aerobic bacteria (including yeast, lactate-assimilating yeast, and mold). WSC loss was calculated by subtracting the WSC content at day 24 of ensiling from the WSC content measured after 2, 4, and 6 days of air exposure.

Microbial enumerations

A series of 10-fold serial dilutions ranging from 10^{-1} to 10^{-10} was prepared. From each dilution, 0.1 ml is onto the appropriate culture medium using a pipette. The pour-plate (pour-on) technique was applied to ensure even distribution and thorough mixing of the inoculum with the medium. Enumeration of *Lactobacillus* spp.: Lactobacillus MRS agar was used as the selective culture medium. The medium was prepared by dissolving 67.17 g of MRS agar powder in 1000 ml of distilled water and heating until completely dissolved. The medium was sterilized by autoclaving at 121°C. After cooling to approximately 60°C, cycloheximide was added at a concentration of 0.4 g per 1000 ml of agar and mix thoroughly. Enumeration of *Clostridium* spp.: Reinforced Clostridium Medium (RCM) was prepared by dissolving 30.50 g of RCM powder in 1000 ml of distilled water and heating until a rolling boil was achieved. The medium was sterilized by autoclaving at 121°C. Once cooled to 60°C, the medium was supplemented with D-cycloserine (200 mg dissolved in 20 ml phosphate buffer per 1000 ml of agar) and methyl red (50 mg per 1000 ml of agar) and mixed thoroughly. Enumeration of *Enterobacterium* spp.: Violet Red Bile Glucose Agar (VRBGA) was used as the culture medium. The agar was prepared by

suspending 38.53 g of powder in 1000 ml of distilled water and heating until fully dissolved. This medium should not be sterilized by autoclaving.

Statistical Analysis

Statistical analyses were performed using SPSS version 23. To meet ANOVA assumptions, the normality of data was assessed using the Shapiro-Wilk test ($p > 0.05$). For variables that exhibited non-normal distribution, data transformations (e.g., logarithmic or square root transformations) were applied to achieve skewness and kurtosis values near zero. Specifically, the Box-Cox transformation (1964) was employed for fermentation parameters when standardized residuals failed to meet the assumptions of normality and homogeneity of variance.

Data for all parameters (excluding microbial profiles) were subjected to a two-way Analysis of Variance (ANOVA) using SPSS version 23 (IBM Corp, 2016). The statistical model was designed to evaluate the main effects of LP inoculation, LB inoculation, and their interaction (LP × LB). Treatment means were compared using Tukey's Honestly Significant Difference (HSD) test, with statistical significance declared at $p < 0.05$.

Regarding microbial profiles, due to the lack of experimental replication, these data were not subjected to statistical analysis. Instead, the results are presented as **descriptive data** to provide a general overview of the microbial community, though we recognize this limits the ability to draw definitive statistical conclusions.

RESULTS AND DISCUSSIONS

Silage Fermentation

This study examined the impact of inoculating two species of lactic acid bacteria at varying levels on the fermentation quality of Napier grass silage with molasses addition. The findings indicated that the combination of both bacterial species, especially when LB was inoculated at the highest level, adversely affected silage fermentation quality compared with the combination using the highest levels of both bacterial species. Notably, there was a significant accumulation of acetic acid due to the high-level of LB inoculation (Table 2). As previously reported, LB can anaerobically convert lactic acid to acetic acid (Oude Elferink et al., 2001). Therefore, a high-level inoculation of LB led to an increased conversion of lactic acid to acetic acid under anaerobic conditions. These results align those of Kung et al. (2018), who noted that LB inoculation resulted in higher acetic acid levels and lower lactic acid levels.

LP is classified as a homofermentative lactic acid bacterium (Salvetti et al., 2012) and is recognized for its rapid and substantial production of lactic acid (Kung Jr., 1998; Muck & Kung, 1997; Oliveira et al., 2017). Higher inoculation levels of LP enable a more rapid pH decline in silage, which inhibits the metabolism of epiphytic lactic acid bacteria, including LP, and suppresses other anaerobic microorganisms through acidic conditions more quickly than without LP inoculation. Therefore, the addition of LP was expected to solve the problem of reduced lactic acid levels caused by the anaerobic degradation of lactate to acetic acid by LB. However, the study found that LP did not solve the problem as expected in this case. The study showed that increasing the level of LP along with different levels of LB did not increase the lactic acid levels. Higher levels of LP inoculation resulted in lower lactic acid content than treatments without LP inoculation (Oliveira et al., 2017).

Table 1. Nutritional composition and microbiological characteristics of Napier grass with/without 5% molasses added by fresh weight

Items	Napier grass added molasses	Napier grass
DM (% FW)	22.2	26.2
CP (% DM)	8.3	8.4
Fat (% DM)	3.5	2.8
Ash (% DM)	10.8	9.5
NDF (% DM)	55.2	70.0
ADF (% DM)	30.1	39.3
ADL (% DM)	8.5	10.3
WSC (g WSC/kg DM)	164.7	62.9
BC (meq NaOH/kg DM)	86.5	90.0
LAB (log_{10} cfu/kg FW)	9.4	9.9
ENT (log_{10} cfu/kg FW)	9.4	9.8
CLOS (log_{10} cfu/kg FW)	7.5	8.0

Note: DM = Dry Matter, FW = Fresh weight, BC = Buffering capacity, LAB = Lactic acid bacteria, ENT = *Enterobacteria* spp., CLOS = *Clostridium* spp.

Inoculation with the highest level of LB, combined with varying amounts of LP, did not lead to a significant reduction in lactic acid

concentration as LP levels increased. This resulted in a higher pH, which was influenced by the highest level of LB (LB6) in this study. Consequently, there was a notable increase in acetic acid content whereas lactic acid levels remained low (Table 2).

Thus, the highest LB inoculation level triggered secondary anaerobic fermentation. Elevated pH facilitates the fermentation processes of both LB and epiphytic lactic acid bacteria also promoting the metabolism of undesirable anaerobic microorganisms (Oliveira et al., 2017; Wilkinson, 2005). The residual WSC results of this study were consistent with the findings of Huisden et al. (2009), who studied inoculants containing LB for corn silage. They found that forage treated with the LB inoculant had lower residual WSC than the control group, which resulted from more complete utilization of forage WSC for lactic acid fermentation by lactic acid bacteria in the inoculant-treated forage.

The residual WSC results of this study were significantly influenced by the main effects of LB and LP, as indicated in Table 2. The lowest residual WSC was observed in silage inoculated with the highest level of LB, where WSC was largely used for fermentation and lactic acid synthesis. During secondary anaerobic fermentation triggered by high LB levels, anaerobic microorganisms consume WSC.

The NH_3-N results were similarly affected by LB and LP. As the levels of lactic acid bacteria increased, NH_3-N levels decreased. The addition of molasses resulted in a high abundance of WSC, and inoculation at greater levels of both species facilitated more extensive WSC fermentation than at lower levels. This led to rapid lactic acid production and a swift drop in silage pH, which inhibits plant proteolytic enzymes during the initial ensiling phase and suppresses proteolytic bacteria during both the initial ensiling and secondary anaerobic fermentation phases (Oliveira et al., 2017; Rooke & Hatfield, 2003). As a result, the reduction of NH_3-N in silage increased with higher levels of LP, since the pH decreased more quickly than with lower inoculation levels of either species.

The highest silage pH was observed in silage inoculated with the greatest amount of LB, which correlated with the significant presence of acetic acid. This finding aligns with the meta-analysis by Kleinschmit and Kung (2006). Acetic acid, produced through LB fermentation, is a weaker acid than lactic acid, which is predominantly generated by LP fermentation (Kleinschmit & Kung, 2006; Muck et al., 2018). Inoculation with the highest level of LB did not effectively suppress secondary anaerobic fermentation by undesirable microorganisms, such as *Clostridium* spp. and *Enterobacteria*, compared to the untreated control (LP0LB0). This was evidenced by the butyric acid results, which revealed notable levels of butyric acid on day 24 of ensiling in silage inoculated with high levels of both lactic acid bacterial species. This situation arose because LP has a slow growth rate at pH levels above 5 during the early stages of silage (Kung, 1998). Generally, the two main groups found in silage are *Saccharolytic* and *Proteolytic clostridia*.

Clostridia grow optimally at a pH of 7.0-7.4, and wet forage conditions before ensiling are conducive to Clostridia growth (McDonald et al., 2010). Therefore, the initial ensiling conditions and high moisture content of the forage promoted Clostridia proliferation. An increase in silage pH promotes secondary anaerobic fermentation, which activates undesirable anaerobic microorganisms. *Saccharolytic clostridia* can degrade lactic acid into acetic and butyric acid (McDonald et al., 2010).

Theoretically, inoculation with the highest level of LP should produce more lactic acid than inoculation at lower levels. Consequently, *Saccharolytic clostridia* would have more substrate (lactic acid) available for conversion into butyric acid than treatments with lower LP levels.

Microbial enumeration of Napier grass silage supplemented with molasses on day 24 of ensiling (Table 1) was used to support the fermentation results observed at this stage. The silage was inoculated with various treatment combinations involving two lactic acid bacterial species at different levels. As previously described, LP grows slowly at higher pH; therefore, clostridia were not immediately suppressed by acidic conditions during the early stage of ensiling. In addition,

inoculation with the highest LB levels resulted in higher silage pH values than other treatment combinations. Moreover, the highest level of LP produced greater amounts of lactic acid, which served as a substrate for *Saccharolytic clostridia*. Consequently, higher *Clostridium* spp. counts were observed in treatments combining LP with the highest LB levels.

Although the silage pH values in these treatment combinations were insufficient to inhibit undesirable anaerobic bacterial fermentation compared with the untreated silage or other inoculation treatments, the butyric acid concentrations at day 24 of ensiling remained within the recommended range established by the Agriculture and Food Development Authority (Teagasc) (Kung et al., 2018).

The standard recommendation for optimum silage butyric acid concentration specifies that levels should not exceed the range of approximately 10-40 g of butyric acid/kg DM silage. Despite the silage pH values of both treatment combinations (LP5LB6, LP6LB6) falling within the recommended standard range (not exceeding pH 4.5 and remaining below pH 3.7, which is necessary to inhibit fermentation by undesirable anaerobic microorganisms), the treatment combinations of both lactic acid bacterial species did not significantly affect the overall silage fermentation quality. This can be attributed to the fact that all silage analytical parameters remained within recommended standards. However, the combined treatment with the highest level of LB resulted in a positive effect on silage fermentation quality compared to the untreated control group for both lactic acid bacteria species.

Nutritional value of Napier grass silage after 24 days

The nutritional value of silage was influenced by inoculation with both lactic acid bacterial species, primarily as a consequence of silage DM loss. This loss of DM directly lead to a reduction in the nutritive value. As previously noted, the lowest DM loss at the highest LB inclusion level was achieved when it was combined with LP (LB6LP5 and LB6LP6). This improvement was attributed to the acidic conditions established by LP inoculation, which inhibited LB metabolic activity. Consequently, the combination of the highest LB level with varying LP levels resulted in a higher silage DM content after 24 days of ensiling than treatments without LP. These effects were observed when both lactic acid bacterial species were inoculated into Napier grass supplemented with molasses and ensiled for 24 days, the treatment combinations did not significantly affect the fiber fractions NDF and ADF but did significantly increase ADL content. This is expected because lactic acid bacteria cannot ferment forage fibers due to their lack of fibrinolytic enzymes (Dewar et al., 1963; Pahlow et al., 2003). Therefore, lactic acid bacteria inoculation did not reduce the NDF or ADF content of the silage. However, inoculation with both bacterial species affected silage DM loss, as the lost dry matter consisted of substrates that could be fermented by anaerobic silage microorganisms.

As a result, NDF and ADF tended to increase but without statistical significance, whereas ADL showed statistically significant differences due to the decrease in fermentable fractions. Additionally, the crude protein content of silage was affected by the inoculation of the two bacterial species, demonstrating a significant LB x LP interaction. The crude protein content of the silage decreased as the LP level increased when mixed with uninoculated LB. This was likely due to the slower pH decline during initial fermentation compared to other treatment groups (as explained above), which may have allowed proteolytic clostridia to ferment forage crude protein into other products during early fermentation (Oliveira et al., 2017). The crude protein content of the silage also decreased with increasing DM loss. These results were confirmed by DM loss measurements, where the highest losses occurred with sole LP inoculation, resulting in a lower crude protein content. Furthermore, at the highest level of LB inoculation, the silage crude protein content increased with increasing LP levels, consistent with improved dry matter recovery.

Aerobic stability characteristics of Napier grass silage after exposure to air for 2, 4, and 6 days

Silage from each treatment combination was exposed to air for 2, 4, and 6 days after opening

the sealed plastic bags at 24 days of ensiling to evaluate aerobic deterioration characteristics, including silage pH, DM loss, residual water-soluble carbohydrates (WSC), and microbial populations. Overall, the treatment combinations improved the aerobic stability of the silage following air exposure.

Sole inoculation with LP resulted in the highest silage pH after 2 days of air exposure compared with the other treatment combinations. This result agrees with previous studies indicating that LP inoculation leads to lower acetic acid production and higher residual WSC concentrations, thereby increasing susceptibility to aerobic deterioration relative to treatments combined with LB (Tabacco et al., 2011; Weinberg et al., 2002).

After 4 days of air exposure, silage inoculated with 1×10^5 cfu LB/g fresh forage weight (LB5) in combination with various levels of LP exhibited only minor increases in pH; however, the treatment combined with 1×10^5 cfu LP/g fresh forage weight (LP5) showed a higher silage pH than the other combinations. In addition, treatments inoculated with the highest level of LB combined with different levels of LP rarely exhibited increases in silage pH after 4 days of air exposure.

Furthermore, inoculation with the highest level of LB combined with various levels of LP (LP0LB6, LP5LB6, and LP6LB6) markedly improved the aerobic stability of the silage. In contrast, combinations containing lower LB levels failed to enhance aerobic stability after 6 days of air exposure. Consequently, the highest LB level combined with different LP levels resulted in the lowest silage DM losses and the lowest silage pH values on day 6 of air exposure compared with treatments containing lower LB levels.

This was due to the highest concentration of acetic acid at the bag opening in the highest LB treatment combinations compared to the other treatments. As is well established, acetic acid inhibiting the proliferation of yeast and mold during air exposure (Muck et al., 2018).

Therefore, inoculation at the highest LB level in molasses-treated Napier grass improved silage aerobic stability after 6 days of air exposure. After 6 days of air exposure, residual WSC in the highest LB inoculation level remained more stable than that in lower LB levels due to the inhibition of yeast and mold growth by acetic acid (Muck et al., 2018). This was confirmed by aerobic microbial profiles 6 days after air exposure (Table 3), which demonstrated lower counts of yeast, lactate-assimilating yeast, and mold at the highest LB inoculation level.

CONCLUSIONS

The experimental results indicate that sole inoculation with *Lactobacillus buchneri* (LB) at its highest level negatively impacts silage quality. This decline is primarily due to the conversion of lactic acid into acetic acid, which raises the silage pH beyond ideal levels. While inoculating with both LB and *Lactobacillus plantarum* (LP) at varying levels initially resulted in lower fermentation quality compared to non-inoculated silage, the overall fermentation products remained within recommended standard ranges, suggesting that combined inoculation does not significantly impair the final product.

The study highlights a critical trade-off regarding dry matter (DM) loss and stability. While the highest level of LB inoculation alone caused the most significant DM loss, the combination of high-level LB with LP effectively mitigated this depletion. Furthermore, this dual-species treatment at maximum levels enhanced aerobic stability, maintaining silage integrity for up to 6 days after air exposure, a performance comparable to the high-level LB treatment alone.

In conclusion, for Napier grass silage supplemented with 5% molasses, the simultaneous inoculation of both bacterial species at their highest levels is the most effective strategy. This approach optimizes the balance between fermentation quality and dry matter preservation while ensuring robust aerobic stability during the feed-out phase.

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Table 2. Effects of treatment combination on the nutritional value of Napier grass silage on day 24

Items	LB0			LB5			LB6			*P - value*		
	LP0	LP5	LP6	LP0	LP5	LP6	LP0	LP5	LP6	LB	LP	LBxLP
DM (%)	23.42^{ab}	21.61^{b}	22.40^{ab}	24.07^{a}	22.78^{ab}	22.97^{ab}	22.26^{b}	23.71^{ab}	23.41^{ab}	NS	NS	< 0.01
CP (%)	9.01^{ab}	8.54^{cd}	8.20^{d}	9.05^{ab}	8.66^{de}	9.13^{a}	8.72^{abc}	9.14^{bc}	9.12^{a}	< 0.01	NS	< 0.01
EE (%)	3.49	2.11	2.02	3.46	3.91	1.58	3.51	2.56	1.50	NS	NS	NS
Ash (%)	12.24	12.09	11.49	12.02	11.3	11.28	11.82	12.05	12.10	NS	NS	NS
NDF (%)	60.04	60.45	59.65	59.88	60.56	60.94	59.40	60.8	61.31	NS	NS	NS
ADF (%)	34.62	34.41	35.79	34.36	34.73	35.26	33.46	35.75	35.54	NS	NS	NS
ADL (%)	9.40^{a}	9.52^{a}	9.40^{a}	7.24^{ab}	9.10^{a}	9.25^{a}	6.09^{b}	9.41^{a}	8.13^{ab}	< 0.01	< 0.01	< 0.05
DM loss (%)	10.77^{ab}	17.66^{a}	13.08^{a}	8.81^{b}	13.21^{a}	12.48^{a}	15.18^{a}	10.72^{ab}	10.81^{ab}	< 0.05	< 0.05	< 0.01
pH	3.80	3.78	3.78	3.83	3.83	3.80	3.88	3.96	3.87	< 0.01	NS	NS
NH_3-N (% of total N)	13.03	9.62	8.12	12.08	8.39	8.88	9.01	6.60	6.55	< 0.05	< 0.01	NS
Residue WSC (g /kg DM)	23.74	25.78	28.23	24.58	24.83	29.49	21.91	19.81	21.63	< 0.01	< 0.01	NS
Lactic acid (g/kg DM)	70.87^{b}	62.78^{b}	55.94^{b}	73.74^{a}	60.17^{b}	56.36^{b}	67.07^{b}	53.40^{b}	63.83^{b}	NS	< 0.01	< 0.05
Acetic acid (g/kg DM)	21.06	24.66	22.58	24.29	21.40	21.51	32.94	46.43	41.05	< 0.01	NS	NS
Propionic acid (g/kg DM)	5.57	6.82	8.49	4.95	6.99	12.33	5.86	9.91	13.85	NS	< 0.01	NS
Butyric acid (g/kg DM)	10.36	10.36	10.65	10.13	11.08	10.60	10.11	13.39	13.23	< 0.01	< 0.01	NS

Note: LB0, LB5 & LB6 – *LB* (0, 1×10^{5}, and 1×10^{6} cfu/g fresh forage weight), LP0, LP5 & LP6 = *LP* (0, 1×10^{5}, and 1×10^{6} cfu/g fresh forage weight), NS = non-significant

^{a,b} within the same row, different letters represent significant differences.

Table 3. Effects of treatment combination on residual WSC and pH in air-exposed Napier grass silage

Items	LB0			LB5			LB6			*P - value*		
	LP0	LP5	LP6	LP0	LP5	LP6	LP0	LP5	LP6	LB	LP	LBxLP
pH at 2 days	3.94^{c}	8.25^{a}	8.04^{b}	3.86^{cd}	3.82^{d}	3.82^{d}	3.87^{cd}	3.92^{c}	3.89^{cd}	< 0.01	< 0.01	< 0.01
residual WSC (g/kg DM)	25.86^{a}	19.05^{bc}	25.97^{a}	18.06^{bc}	17.11^{c}	22.84^{ab}	20.95^{abc}	20.47^{bc}	17.80^{bc}	< 0.05	NS	< 0.05
pH at 4 days	7.20^{a}	8.80^{a}	8.67^{a}	4.53^{c}	7.58^{a}	4.85^{b}	3.89^{e}	3.97^{d}	3.93^{ed}	< 0.01	< 0.01	< 0.01
residual WSC (g/kg DM)	26.58^{a}	17.71^{b}	11.35^{b}	21.31^{ab}	20.35^{ab}	20.13^{ab}	16.70^{b}	17.87^{b}	18.21^{b}	< 0.05	NS	< 0.05
pH at 6 days	8.28^{a}	8.87^{a}	8.84^{a}	8.00^{a}	9.10^{a}	8.00^{a}	4.20^{b}	3.70^{b}	4.24^{b}	NS	NS	< 0.05
residual WSC (g/kg DM)	26.97^{a}	19.60^{ab}	18.30^{ab}	16.90^{ab}	16.41^{ab}	19.10^{ab}	13.41^{b}	19.40^{ab}	19.76^{a}	< 0.01	NS	< 0.05

Note: LB0, LB5 & LB6 – *LB* (0, 1×10^{5}, and 1×10^{6} cfu/g fresh forage weight), LP0, LP5 & LP6 = *LP* (0, 1×10^{5}, and 1×10^{6} cfu/g fresh forage weight), NS = non-significant

$^{a-d}$ within the same row, different letters represent significant differences

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CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITIES OF THYME (*Thymus vulgaris*) AND TEA TREE (*Melaleuca alternifolia*) ESSENTIAL OILS

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Abstract

Essential oils are explored as natural alternatives to synthetic additives because of their diverse biological activities and potential use in poultry production. This study evaluated the chemical composition and in vitro biological properties of thyme (Thymus vulgaris) essential oil (TEO) and tea tree (Melaleuca alternifolia) essential oil (TTEO) as potential bioactive candidates for broiler nutrition. Gas chromatography–mass spectrometry (GC–MS) analysis showed that TEO was dominated by thymol (52.84%), whereas TTEO was characterized mainly by terpinen-4-ol (37.14%). Antimicrobial activity against Escherichia coli, Salmonella typhimurium, Listeria monocytogenes, and Clostridium perfringens assessed by broth microdilution and disc diffusion assays, showed stronger inhibition by TEO, MICs of 7.5 µg/mL against S. typhimurium and C. perfringens, while TTEO exhibited MICs of 7.5 µg/mL against L. monocytogenes and C. perfringens, 15 µg/mL against S. typhimurium, and 62.5 µg/mL against E. coli. Antioxidant activity, determined by the DPPH assay, was higher for TEO (65.48% at 0.09 mg/mL), whereas TTEO reached 56.02% at 17 mg/mL Both oils showed modest anti-inflammatory effects and relevant in vitro bioactivity, with thyme oil showing superior antimicrobial and antioxidant performance.

Key words*: antimicrobial activity, DPPH radical scavenging, GC–MS profiling, Melaleuca alternifolia essential oil, phytogenic feed additives, Thymus vulgaris essential oil.*

INTRODUCTION

Essential oils (EOs) are complex mixtures of volatile compounds derived from aromatic plants and have been extensively investigated because of their diverse biological properties (Bakkali et al., 2008). In recent years, interest in the use of natural compounds as alternatives to synthetic products has increased substantially, largely driven by the demand for safer solutions for both human health and the environment (Burt, 2004). In poultry production, the interest in EOs has expanded beyond their conventional use as flavouring agents, as they are increasingly regarded as multifunctional phytogenic candidates capable of simultaneously modulating microbial pressure, oxidative stability, and inflammatory status (Movahedi et al., 2024; Toader et al., 2025). Such a multifunctional profile is particularly relevant in modern production systems, where nutritional strategies are expected not only to support performance, but also to contribute to gut health, product quality, and reduction of reliance on conventional antimicrobial inputs (Toader et al., 2025; Toader et al., 2024). For this reason, the integration of chemical characterization with biological testing is essential for establishing meaningful structure-activity relationships and for identifying which oils are most suitable for future application as functional additives in broiler nutrition.

Thyme (*Thymus vulgaris*) essential oil (TEO) is particularly valued for its high content of phenolic constituents, especially thymol and carvacrol, which are largely responsible for its antimicrobial and antioxidant activities (Marchese et al., 2016). Likewise, tea tree (*Melaleuca alternifolia*) essential oil (TTEO) is characterized by the presence of terpinen-4-ol and other terpenes that contribute to its antimicrobial and anti-inflammatory effects (Carson, Hammer, & Riley, 2006). Importantly, the biological efficacy of an essential oil cannot be predicted solely from its botanical origin, because its activity depends on chemotype, the relative abundance of dominant constituents, and potential synergistic or antagonistic interactions among minor compounds (Benomari et al., 2023). The antioxidant potential of these oils is

important for limiting oxidative processes, while their antimicrobial and anti-inflammatory properties also support their considerable potential in animal production, in addition to applications in the food, pharmaceutical, and cosmetic industries (Miguel, 2010).

Therefore, this study aimed to investigate the chemical composition and *in vitro* biological activities of TEO and TTEO, with particular emphasis on their antimicrobial, antioxidant, and anti-inflammatory properties, to determine their suitability as natural functional additives with prospective use in broiler production.

MATERIALS AND METHODS

In vitro evaluation of antimicrobial activity

The microdilution broth method was used to evaluate the antimicrobial susceptibility, which involved testing serial 2-fold dilutions of the EO in liquid growth medium distributed into 96-well microtiter plates (Balouiri, Sadiki, & Ibnsouda, 2016). Each well was inoculated with a standardized microbial suspension containing EO of interest, adjusted to a turbidity equivalent to the 0.5 McFarland scale, and further diluted in the same growth medium. Following inoculation, the 96-well microtiter plates are incubated under conditions suitable for the microorganism being tested. The minimum inhibitory concentration (MIC) was selected as the lowest concentration of antimicrobial agent that completely inhibited microbial growth in the wells.

In vitro antimicrobial testing was performed against the bacterial strains *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 14028), *Listeria monocytogenes* (ATCC 19114), and *Clostridium perfringens* (ATCC 13124). A 10^{-2} dilution of fresh bacterial culture, standardized to 0.5 McFarland (1.5×10^8 CFU/ml), was used for the assay (Balouiri et al., 2016). The reference strains (ATCC) were initially revived through 24h incubation in Brain Heart Infusion (BHI) broth (Oxoid, CM1135) at 37°C, followed by subsequent transfer to BHI agar for 24 hours at 37°C. Each bacterial strain was then adjusted to an optical density (OD) equivalent to a 0.5 McFarland standard using BHI broth. Subsequently, bacterial suspensions were dispensed into flat-bottomed 96-well microtiter plates, with a total working volume of 200 µl per well. Using an electronic Brand Transferpette pipette (15–300 µl), 100 µl of bacterial suspension were added to each well. Test samples, diluted from a stock solution, were applied to the wells at concentrations ranging from 7.5 µg/ml to 4000 µg/ml Agar plates were covered and incubated overnight at 37°C. Post-incubation, the optical OD was quantified at 540 nm via an ELISA Microplate Absorbance reader (BIORAD PR 1100). All tests were conducted in triplicate, with bacterial suspensions in BHI serving as positive controls. To complement MIC results, the disc diffusion assay was performed according to a previously reported study and readapted for essential oils (Balta, Marcu et al., 2021). Briefly, 100µl of *L. monocytogenes* (ATCC 19114), *S. enterica typhimurium* (ATCC 14028), *E. coli* (ATCC 25922) bacterial cultures were accurately spread onto Tryptone Soya Agar yeast extract (TSAYE Oxoid, UK), and *Campylobacter jejuni* (NCTC 11168) was plated on Blood Agar Base Nr. 2 (BAB, Oxoid Ltd., UK) supplemented with 5% (vol/vol) horse blood (Aquilant Scientific N.I.). Next, each EO was diluted in tenfold increments from 100% to 0.50% (v/v) in DMSO, and 20µl of corresponding antimicrobial concentration was pipetted on 6 mm paper discs (Oxoid, UK). The antimicrobial activity of each essential oil was evaluated with a calliper by measuring the diameter of the inhibition zone around the disc for each concentration.

Determination of volatile compounds from EOs

The analysis of the EOs was performed by Gas Chromatography-Mass Spectrometer (GC-MS) with a Thermo Electron system consisting of a Focus GC chromatograph equipped with DB-5MS capillary column (25 m × 0.25 mm; 0.25 µm film thickness) and TriPlus auto sampler, connected to a Polaris Q ion trap mass detector. Helium (noble gas) was used as carrier gas at a flow rate of 1 mL/min. The injector and temperature detectors were set up at 250°C. The oven temperature was programmed from 60ºC (3 min) to 200ºC at a rate of 10ºC/min, then from 200ºC to 240ºC at a rate of 12ºC/min. The mass spectra were recorded at 70 eV (EI) and were scanned in the range 35-450 *m*/*z*. The injection volume was 1 µL from 1:100 hexane dilution. Identification of the volatile components was carried out using Xcalibur® software and NIST

11 Mass Spectral Library, an alkane standard solution for GC (C8-C20 in hexane, Sigma Aldrich Co., St. Louis, USA) being used for retention indexes (RI/tR) determination (Marinas et al., 2023). The relative percentage (%) content of each component was calculated based on the GC-peak areas without any correction factors. - Mass Spectrometry (GC-MS).

Anti-inflammatory activity determination

The erythrocytic suspension was prepared through the method previously described, with minor modifications, where the blood samples were collected in heparinized vacutainers and centrifuged at 3000 rpm for 10 minutes (Gunathilake, Ranaweera, & Rupasinghe, 2018). Following the centrifugation, the supernatant was discarded, and the erythrocyte pellet was washed with an equal volume of isotonic sodium chloride (0.9%) solution. This centrifugation and washing process was repeated three times. Subsequently, the erythrocyte pellet was reconstituted as a 40% suspension in isotonic Phosphate-Buffered Saline (PBS, pH 7.4). The protocol was approved and licenced by the Bioethics Committee from the University of Life Sciences "King Mihai I" from Timisoara (No.56/7.07.2021).

The heat-induced hemolysis test was performed according to the method earlier described by Okoli et al. (2008), incorporating modifications (Gunathilake et al., 2018; Okoli et al., 2008). Different concentrations (i.e. 10 µl/ml, 20 µl/ml, 40 µl/ml, 80 µl/ml, and 160 µl/ml) of essential oil (extract) were dissolved in 5 ml isotonic PBS (pH 7.4) (RemedLab, Bucharest, Romania), to which 100 µl erythrocyte suspension was added. After gentle mixing, samples were incubated at 54°C for 20 minutes in a water bath. Following incubation, samples were centrifuged at 2500 rpm for 3 minutes, and the absorbance of the supernatant was detected at 540 nm using a UV/Vis spectrophotometer (Specord 205-Analytik Jena AG, Germany). A control sample consisted of PBS and 100 µl erythrocyte suspension. The percentage inhibition of hemolysis was calculated using the following formula:

(%) Inhibition of Hemolysis = 100 - A1/A2 × 100

where: A1 = absorbance of the test sample, and A2 = absorbance of the control sample.

Protein denaturation evaluation was performed as previously described with small modifications [13]. Different concentrations (i.e. 10 µl/ml, 20 µl/ml, 40 µl/ml, 80 µl/ml, and 160 µl/ml) of essential oils were added to 1 ml of 1% egg albumin (Oxford Lab Fine Chem LPP, Maharashtra, India) including 4 ml of PBS (pH 6.4). The mixture was gently mixed and incubated in a water bath at 37°C for 15 minutes. Subsequently, it was heated at 70°C for 5 minutes. After cooling, turbidity was measured at 660 nm optical density using a UV/VIS spectrophotometer (Optima SP-3000, Tokyo, Japan). The control sample consisted of PBS without extract.

(%) Inhibition of denaturation = 100 - A1/A2 × 100

where: A1 = absorbance of the control sample, and A2 = absorbance of the test sample.

Antioxidant capacity using the DPPH [1,1-diphenyl-2-picrylhydrazyl] radical scavenging activity assay

The DPPH assay was also performed as previously described with small modifications (Ciulca et al., 2021). In order to describe the antioxidant capacity of TTEO and TEO, a stock methanolic solution was prepared by dissolving 1 ml of essential oil in 10 ml of methanol (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). From this stock solution, multiple dilutions were prepared, and from each dilution, 3 ml were taken and combined with 1 ml of 0.3 mM DPPH solution, followed by incubation in the dark for 30 minutes. Absorbance was quantified at 517 nm using a UV/VIS spectrophotometer (Specord 205; Analytik Jena AG, Jena, Germany). Each sample was analyzed in triplicate, and the mean values were recorded. The control sample was also prepared, where the extract was replaced by methanol, keeping the same concentrations and volume of DPPH solution. Ascorbic acid was used as a positive control at a concentration of 0.016 mg/ml in methanol, provided by Lach-Ner (Neratovice, Czech Republic).

The radical scavenging activity (RSA) was calculated according the following formula:

$$(\%)\ \text{RSA} = (\text{A control} - \text{A samples}) / (\text{A control}) \times 100$$

where: A control = absorbance value of the control sample and A sample = absorbance value of the tested extracts.

Statistical analysis

All assays were conducted in triplicate, and data are presented as mean ± standard deviation (SD); comparative evaluation of biological activities of the tested EOs was performed based on the recorded experimental responses.

RESULTS AND DISCUSSIONS

Chemical profile of EOs

Chemical profiling of TEO was evaluated by GC-MS which detected 29 volatile compounds as presented in Table 1. Thymol emerged as the most abundant constituent, representing 52% of its chemical content. This was followed by p-Cymene, which accounted for 18.54%, β-(E)-Caryophyllene at 7.33%, and γ-Terpinene at 5.71%. linalool and carvacrol were identified in lesser quantities, each comprising less than 3% of the oil. We also detected 23 volatile compounds present in trace amounts, ranging from 0.05% to 1.26%, respectively.

A chromatographic analysis characterized TEO volatile compounds by depicting their distribution patterns through prominent peaks that appeared in the chromatogram (Figure 1). Thymol served as the principal volatile component of Thyme EO as it produced a major peak at retention time (tR) 11.89 minutes which represented 99% of the total relative abundance. The next most prevalent compound was β-(E)-Caryophyllene that appeared at 13.62 tR and reached a 35% relative abundance level. Carvacrol and γ-Terpinene, displayed tR of 11.97 and 8.16 minutes, and relative abundances of 14%, while p-Cymene was observed at a 7.61 tR with a 29% relative abundance. The remaining volatile compounds within the Thyme EO exhibited lowered detection thresholds because their concentrations were detected under 5% relative abundance.

Similarly, GC-MS analysis of TTEO revealed a different composition, identifying 34 volatile compounds (Table 2). Predominantly, Terpinen-4-ol was identified as the principal constituent, comprising 37.14% of the oil's composition. This was closely followed by g-Terpinene, constituting 20.11%, and α-Terpinene at 11.23%. Among the detected volatile compounds in the oil analysis were α-Terpinolene, p-Cymene, Eucalyptol, α-Muurolene, γ-Cadinene, α-Terpineol, Aromadendrene, and α-Pinene, totaling 4% to 1% of the overall chemical composition. The remaining 29 compounds were discerned at concentrations below 1%. The subsequent chromatographic examination confirmed the quantity distribution of volatile substances in TTEO.

The chromatogram (Figure 2) indicated that Terpinen-4-ol produced the strongest peak at a tR of 10.24 minutes because it represented the overwhelming distribution. The g-Terpinene peak appeared at an 8.18 tR position with a relative abundance level amounting to 63%. γ-Cadinene (14.84 tR) was one of several distinctive compounds together with α-Muurolene (14.54 tR), Alloaromadendrene (13.84 tR), α-Terpineol (10.36 tR), α-Terpinolene (8.65 tR), and α-Terpinene (7.45 tR) that made up a total 45% to 5% of the constituents. The chromatogram also revealed other compounds within the oil, albeit at lower relative abundances.

Recent meta-analytical research corroborates that EOs are potential growth promoters in poultry farming, indicating that their inclusion in feed could lead to improved productivity (Irawan, Hidayat, Jayanegara, & Ratriyanto, 2021). The main effects of EOs on poultry production and growth promotion from the recent findings is depicted in Figure 1.

Table 1. Volatile compounds identified in TEO

tR (min)	RI calculated	Compound	CAS	%
5.93	926	a-Thujene	2867-05-2	0.54
6.02	931	a-Pinene	80-56-8	0.31
6.30	946	Camphene	79-92-5	1.26
6.97	983	b-Pinene	127-91-3	0.77
7.61	1019	p-Cymene	99-87-6	18.54
7.73	1026	Eucalyptol	470-82-6	0.98
8.16	1051	g-terpinene	99-85-4	5.71
8.34	1061	cis-Sabinene hydrate	15537-55-0	0.09
8.65	1079	α-Terpinolene	586-62-9	0.14
8.83	1090	Linalool	78-70-6	2.33
9.63	1139	Camphor	76-22-2	0.77
9.98	1161	Borneol	507-70-0	1.10
10.11	1170	Terpinen-4-ol	562-74-3	0.80
10.31	1182	α-Terpineol	98-55-5	0.11
10.89	1221	Thymol methyl ether	1076-56-8	1.06
11.03	1230	Carvacrol methyl ether	6379-73-3	0.90
11.89	1289	Thymol	89-83-8	52.84
11.97	1295	Carvacrol	499-75-2	2.41
12.58	1339	α-Cubebene	17699-14-8	0.10
12.96	1367	α-Copaene	3856-25-5	0.17
13.62	1417	β-(E)-Caryophyllene	87-44-5	7.33
13.69	1422	β-Copaene	18252-44-3	0.05
14.02	1448	n.i.		0.10
14.12	1456	Alloaromadendrene	25246-27-9	0.05
14.23	1465	n.i.		0.05
14.26	1467	γ-Muurolene	30021-74-0	0.12
14.53	1488	Germacrene D	23986-74-5	0.22
14.75	1506	n.i.		0.11
14.83	1513	γ-Cadinene	39029-41-9	0.59
14.96	1524	n.i.		0.05
15.67	1583	Caryophyllene oxide	1139-30-6	0.31
16.28	1636	τ-Cadinol	5937-11-1	0.10
				100.00
		Total identified		**99.69**

[1] n.i.- not identified

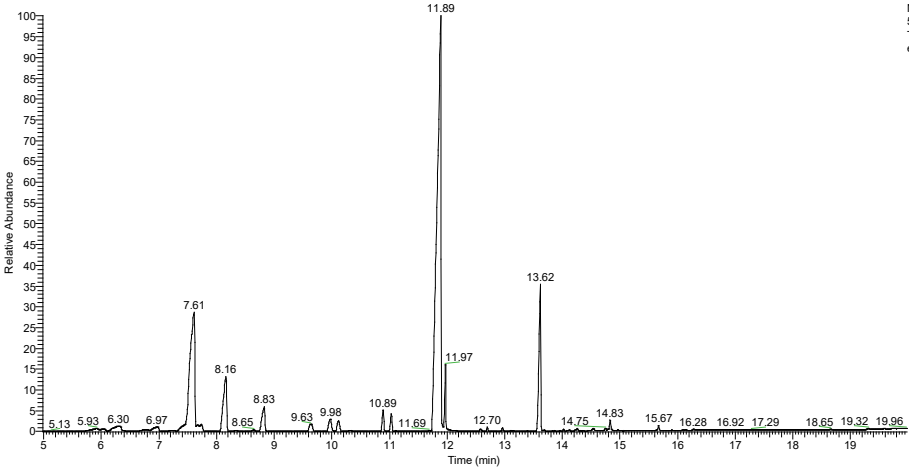


Figure 1. GC chromatogram of TEO

Table 2. Volatile compounds identified in TTEO

tR (min)	RI calculated	Compound	CAS	%
5.91	925	α-Thujene	2867-05-2	0.70
6.03	932	a-Pinene	80-56-8	1.08
6.72	969	Sabinene (β-Thujene)	3387-41-5	0.56
6.96	982	b-Pinene	127-91-3	0.43
7.23	997	α-Phellandrene	99-83-2	0.41
7.45	1009	α-Terpinene	99-86-5	11.23
7.59	1017	p-Cymene	99-87-6	3.20
7.73	1026	Eucalyptol	470-82-6	3.18
8.18	1052	g-Terpinene	99-85-4	20.11
8.65	1079	a-Terpinolene	586-62-9	3.70
8.82	1089	trans-Sabinene hydrate	17699-16-0	0.08
9.23	1114	cis-2-p-Menthen-1-ol	29803-82-5	0.15
9.53	1133	trans-2-p-Menthen-1-ol	29803-81-4	0.18
10.24	1178	Terpinen-4-ol	562-74-3	37.14
10.36	1185	α-Terpineol	98-55-5	2.49
10.57	1199	cis-Piperitol	16721-38-3	0.09
12.56	1336	α-Cubebene	17699-14-8	0.09
12.91	1361	α-Ylangene	14912-44-8	0.14
12.96	1365	α-Copaene	3856-25-5	0.32
13.25	1386	n.i.		0.09
13.43	1399	α-Gurjunene	489-40-7	0.82
13.58	1411	β-Caryophyllene	87-44-5	0.50
13.68	1419	n.i.		0.12
13.84	1432	Aromadendrene	489-39-4	2.21
13.94	1440	n.i.		0.21
14.12	1454	Alloaromadendrene	25246-27-9	0.77
14.23	1463	γ-Muurolene	30021-74-0	0.88
14.45	1481	Germacrene D	23986-74-5	0.54
14.54	1489	α-Muurolene	10208-80-7	3.07
14.84	1513	γ-Cadinene	39029-41-9	2.63
14.89	1518	δ-Cadinene	483-76-1	0.71
14.97	1524	n.i.		0.34
15.37	1558	n.i.		0.17
15.48	1567	n.i.		0.13
15.59	1576	Spathulenol	6750-60-3	0.16
15.68	1584	Globulol	51371-47-2	0.35
15.78	1592	Viridiflorol	552-02-3	0.31
16.11	1621	Ledol	557-27-5	0.14
16.15	1625	Cubenol (1-epi)	19912-67-5	0.28
16.31	1639	τ-Cadinol	5937-11-1	0.33
				100.00
		Total identified		**98.94**

[1]n.i. - not identified

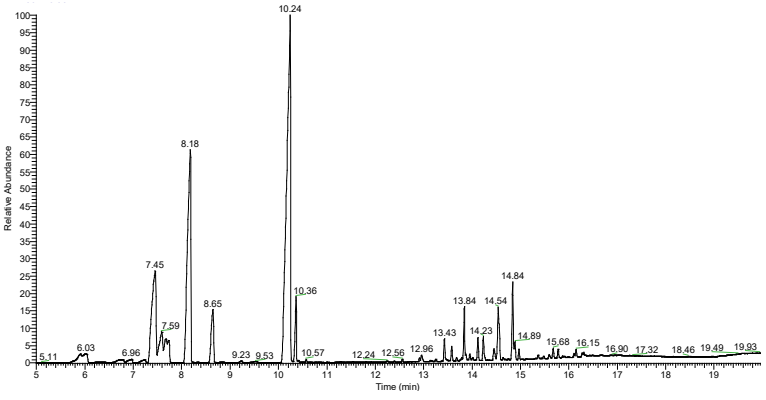


Figure 2. GC chromatogram of TTEO

Antimicrobial activity of EOs

First, we have determined the overall MIC values of all EOs are presented in Table 3. TEO had a more efficient inhibitory effect against *S. typhimurium* and *C. perfringens* at sub-inhibitory concentrations at an MIC of 7.5 µg/ml. Our results demonstrated that the TTEO had the same MIC value against *L. monocytogenes* and *C. perfringens* at 7.5 µg/ml. In addition, the MIC of TEO on *E. coli* and *L. monocytogenes*, was higher (015 µg/ml). The MIC of TTEO on *S. typhimurium* MIC was at 15 µg/ml, however, when used against *E. coli* an MIC of 62.5 µg/ml was recorded. These results clearly indicate and efficient antimicrobial activity of EOs against all bacterial pathogens investigated. These MIC findings were further supported by the disc diffusion method, which revealed clear inhibitory zones (Figure 3 and Figure 4) against all four examined pathogens.

First, *C. jejuni* NCTC 11168, as shown by the differential inhibitory zones measured in millimetres (mm), had a pronounced response across the various concentrations. Specifically, the zones of inhibition spanned from 58.75 mm at the highest concentration (100%) to 7.85 mm at the lowest concentration (1%) (Figure 3A). In comparison, TTEO exhibited a lesser antimicrobial effect, with the largest zone of inhibition observed at 24.11 mm for the highest concentration and 8.10 mm at 1 % (Figure 4A). When we assessed the antimicrobial effect against *L. monocytogenes* NCTC 4885, (Figure 4B) the TEO had a higher inhibitory zone effect ranging from 28.40 mm at 100% concentration, to 11.20 mm at 25% concentration. However, the TTEO had a reduced efficacy, producing inhibitory zones of 13.45 mm, 7.5 mm, and 6.8 mm for concentrations ranging from 100% to 50% (Figure 3B).

Moreover, the inhibitory action of both EOs against *E. coli* was comparable, yielding the largest inhibition zones of 21.43 mm for TEO and 17.41 mm for TTEO, with the smallest of 11.93 mm and 8.36 mm, respectively (Figure 3C and Figure 4C.). Smaller inhibitory zones were observed when testing both EOs against *Salmonella enterica* serovar *Typhimurium* (ATCC 14028), of 9.63 mm for TEO and 7.68 mm for TTEO at 100% concentration (Figure 3D and Figure 4D). Conclusively, these results confirm the MIC efficacy of EOs and underlines their potential use as antimicrobial agents against all pathogens investigated.

Table 3. Antimicrobial activity of TTEO and TEO, MIC method

EO	Concentration µg/mL	*E. coli*	*S. typhimurium*	*L. monocytogenes*	*C. perfringens*
TTEO					
	4000	0.107	0.177	0.109	0.113
	2000	0.174	0.277	0.130	0.117
	1000	0.291	0.385	0.163	0.149
	500	0.383	0.477	0.197	0.184
	250	0.587	0.502	0.222	0.256
	125	1.039	0.817	0.241	0.307
	62.5	**1.062***	0.973	0.314	0.314
	31	1.096	1.025	0.352	0.374
	15	1.220	**1.061***	0.438	0.396
	7.5	1.271	1.123	**0.472***	**0.418***
TEO					
	4000	0.233	0.155	0.122	0.175
	2000	0.252	0.166	0.195	0.191
	1000	0.298	0.198	0.212	0.210
	500	0.397	0.210	0.271	0.291
	250	0.414	0.258	0.285	0.338
	125	0.497	0.391	0.361	0.371
	62.5	0.594	0.410	0.393	0.397
	31	0.616	0.486	0.395	0.420
	15	**0.717***	0.506	**0.413***	0.422
	7.5	1.082	**0.654***	0.479	**0.474***
Control		1.072	1.101	0.477	0.485

*Results expressed as optical density, MIC values are highlighted with asterisk

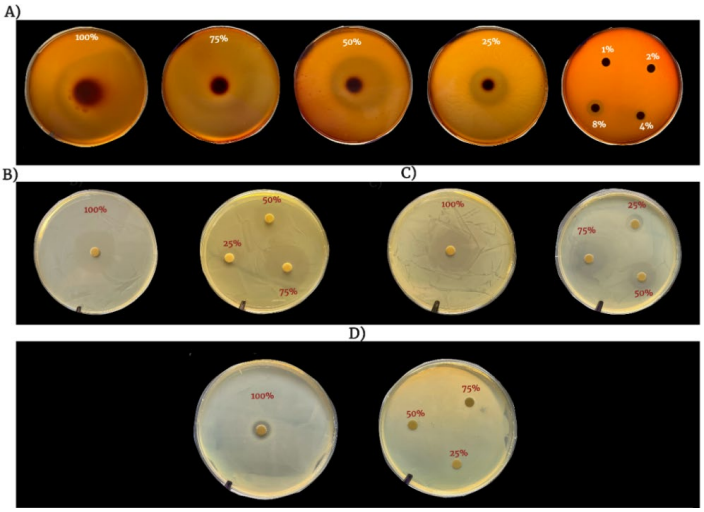


Figure 3. Antimicrobial activity of TEO visualized through disc diffusion assay (original source). This figure presents representative images of Petri dishes illustrating the zones of inhibition at varying concentrations of TEO against four pathogens: A) *C. jejuni*; B) *L. monocytogenes*; C) *E. coli*; D) *S. typhimurium*. Each panel (A-D) demonstrates the differential susceptibility of the pathogens to TEO, highlighting its concentration-dependent antimicrobial efficacy

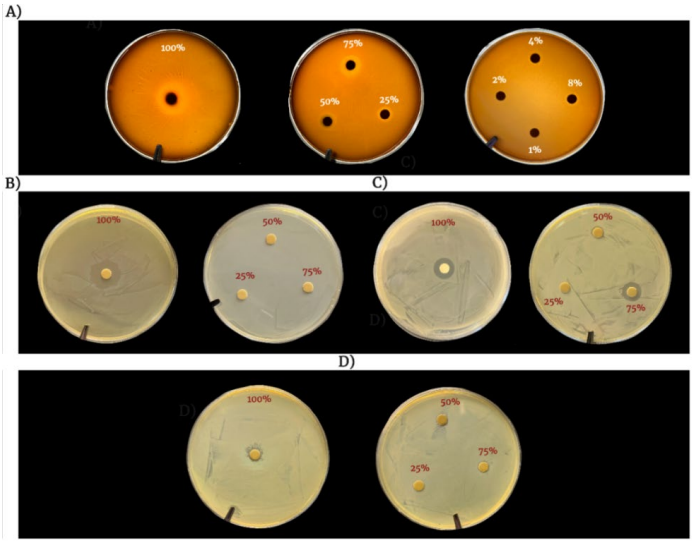


Figure 4. Inhibitory zones elicited by TTEO across varied concentrations, as observed in Disc diffusion assays (original source). Displayed are representative Petri dish images that delineate the antimicrobial effect of TTEO against: A) *C. jejuni*; B) *L. monocytogenes*; C) *E. coli*; D) *S. typhimurium*. Each subsection (A-D) details the pathogen-specific response to different concentrations of TTEO

Membrane lysis evaluation

Next, we have investigated the ability of EOs to prevent hemolysis upon exposure to heat. We have observed that TEO and TTEO, at their maximum tested concentration of 160 µl/ml. Specifically, the protective effect of dexamethasone was 47.49%, while TTEO provided a protection level of 5.04% and TEO oil offered a much lower protection level of just 0.05%. The results of membrane lysis assessment contributed by both EOs are depicted in Table 4. Conclusively, these results show that EOs can inhibit haemolysis by protecting the red blood cells membranes. The protective mechanism might require further investigation.

Table 4. Anti-inflammatory activity of TTEO and TEO

Concentration of EO	OD values	Hemolysis (%)	Hemolysis inhibition (%)
TTEO			
10 µl/ml	2.341	111.16	-
20 µl/ml	2.297	109.07	-
40 µl/ml	2.252	106.90	-
80 µl/ml	2.198	104.37	-
160 µl/ml	2.000	94.96	5.04
TEO			
10 µl/ml	2.491	118.28	-
20 µl/ml	2.394	113.68	-
40 µl/ml	2.310	109.69	-
80 µl/ml	2.200	104.47	-
160 µl/ml	2.105	99.95	0.05
Negative Control	2.106	100	-
Positive Control	1.003	52.50	47.49

[1]Negative Control was represented by PBS; Control Positive with dexamethasone.

Antioxidant capacity

To further improve our knowledge on the efficacy of these EOs we have studied their ability to reduce oxidative stress. The antioxidant activity according to DPPH assay of TTEO and TEO are represented in Table 5. The antioxidant capacity of Tea Tree and Thyme essential oils, quantified by their IC50 values (the concentration required to inhibit 50% of the target radical), varies depending on the assay used and the specific chemical profile of the oil. Lower IC50 values represent higher antioxidant potency.

Tea Tree Essential Oil (*Melaleuca alternifolia*) (TTEO) demonstrates significant potency, particularly in scavenging primary free radicals. Thyme oil (TEO) performance is strongly linked to its high phenolic content (thymol and carvacrol). Its IC50 values vary significantly by plant region and species, in this case show a higher antioxidant activity than TTEO.

Table 5. Antioxidant activity of activity of TTEO and TEO

Sample	IC50 DPPH assay
Tea tree (*Melaleuca alternifolia*) - TTEO	147 µg/ml
Thyme (*Thymus vulgaris*) - TEO	125 µg/ml

DPPH free radical neutralization activity related to ascorbic acid

DISCUSSIONS

Recent *in vitro* molecular data indicated that TTEO incorporated in nanoliposomes (TTONL), enhanced their antibacterial activity against different *E. coli* strains (Bo et al., 2023). The MIC and MBC of TTONL for *E. coli* were about 4-fold lower than those of diluted EO. The *in vivo* application of TTONL nanoliposomes in broiler chickens challenged with *E. coli* demonstrated promising results and alleviated clinical symptoms and intestinal lesions while significantly reducing mRNA expression of pro-inflammatory markers like NLRP3 and NF-kB in the duodenum and cecum (Bo et al., 2023). Similar observations regarding the inclusion of Thyme (Almremdhy & Al-Khafaji, 2020; Belali et al., 2024; Hassan et al., 2024; Hassanin et al., 2024; Naderiboroojerdi et al., 2022; Noruzi et al., 2022; Olfati & Hosseini, 2022; Puvača et al., 2022; Vlaicu et al., 2023) and Tea tree (Lv et al., 2022; Qu et al., 2019; Wang et al., 2025; Wang et al., 2024; Yang et al., 2022; Yu et al., 2025; Yuan et al., 2023) EO were previously reported which also suggested their role as growth promoters, as antimicrobials, and as inducers of antioxidant and anti-inflammatory effects with a effective impact on poultry health and meat quality. The effects of TTEO are primarily attributed to its main bioactive compound, terpinen-4-ol, alongside other significant constituents such as γ-terpinene and α-terpinene (≈ 40%, 23%, and 10%), respectively) (Lv et al., 2022; Qu et al., 2019; Y. Wang et al., 2025;

Yachao Wang et al., 2024; Yang et al., 2022; Yu et al., 2025; Yuan et al., 2023). The antimicrobial efficiency of both EOs is linked to its membrane-interacting mechanism which modifies the membrane permeability and membrane fluidity until the microbial cells become leaky and eventually die (Hassanzadeh et al., 2024; Lv et al., 2022; Salinas-Chavira & Barrios-García, 2024). TTEO has a remarkable antibacterial effect, as previously described against antibiotic resistant strains of *E. coli*, *Enterococcus* species and *Staphylococcus aureus* (Iseppi, Mariani, Benvenuti, Truzzi, & Messi, 2023). Moreover, our results show that TTEO has an anti-inflammatory through reduced inflammatory markers. These anti-inflammatory effects occurs through various mechanisms starting with lowering IL-2, IL-6, IL-17 and TNF-α while decreasing NLRP3 mRNA expression, but inhibiting Caspase-3 and Bax and boosting Bcl-2 anti-apoptotic gene activity (Bo et al., 2023; Lv et al., 2022; Wang et al., 2025; Wang et al., 2024). The treatment shaped the immune state of broilers through immune modulation that included $CD^{3+}CD^{8+}$ T-cell subset growth together with reduced expression of inflammatory cytokines (IFN-γ, TNF-α, IL-1β), which collectively reduced systemic inflammation (Wang et al., 2025).

In our study, the TEO also has robust antimicrobial properties similar to observations from other studies, emphasizing its beneficial impact on many poultry pathogens including *E. coli*, *S. typhimurium*, *C. perfringens*, *L. monocytogenes* and *C. jejuni* (Balta et al., 2021; Sakkas & Papadopoulou, 2017; Stratakos et al., 2018). For example, thymol and carvacrol act as the main active components which disrupt microbial membranes to induce membrane permeability leaks and cause impairment of vital metabolic functions (Kachur & Suntres, 2020; Sakkas & Papadopoulou, 2017). *In vitro* tests using subinhibitory MIC concentrations demonstrated that TEO affects the growth rate of *Salmonella enterica*, serovar Enteritidis, confirming its ability to suppress bacterial growth at low concentrations (Hassanzadeh et al., 2024). Other results exposed its ability to inhibit the activity of virulence genes (*hilA*, *spv*, *sefA*, *invA*) and other virulence factors which are vital for its pathogenicity (Hassanzadeh et al., 2024). *In vivo*, TEO encapsulated particles improved performance in broiler chicks after the finisher period and prevented *Salmonella* infection (Olfati & Hosseini, 2022). In a pilot study, the antibacterial properties of TEO, at 5% concentration, reduced *Enterobacteriaceae* and *Salmonella* Derby infection by 88.9% concomitantly with a reduction in *Mammaliicoccus lentus* by 77.7% (Galgano et al., 2023). All these data suggests that TEO is practical replacement alternative for standard chemical disinfectants which the poultry industry can be used to address poultry litter management. TEO, at 200 mg/kg, was reported to reduce the harmful effects of aflatoxin B1 in broiler diets and significantly enhance BW/BWG and FCR compared to other treatments (Fawaz et al., 2022).

The antioxidant effect of TTEO identified in our study was also confirmed in earlier studies which described its role in enhancing SOD and CAT enzyme activity and simultaneously reducing MDA productions (Qu et al., 2019; Wang et al., 2025; Wang et al., 2024). The primary active compounds of terpinen-4-ol along with other monoterpenes act as antioxidants to efficiently reduce oxidative stress along with inflammatory responses which was documented in previous studies involving broiler chickens and quails (Deminicis et al., 2024; Khan et al., 2023; Qu et al., 2019; Wang et al., 2025; Wang et al., 2024). Our research data matches previous observations showing antioxidative effects because it revealed better meat quality together with decreased drip loss. The antioxidant capacity of TEO, evidenced by neutralization of free radicals (DPPH assay), closely mirrors previous findings. Similarly to TTEO, the antioxidant activities of thymol and carvacrol from TEO function by directly reacting with free radicals and simultaneously strengthening endogenous antioxidant defenses through SOD, CAT and GPx (Saim et al., 2024) enhancement (Deeb et al., 2024; Ghalandari et al., 2022; Gümüş et al. 2023).

The antimicrobial properties of TTEO extended its beneficial impact on preventing common spoilage bacteria *S. aureus* and *E. coli* thereby increasing poultry meat shelf life. In broilers, experiments with other essential oils support this trend. Jimoh et al. (2024) gave broilers various spice EOs in drinking water and found that drip loss ranged from 4.5% in control birds down to

1.5% in the cinnamon EO group (Jimoh et al., 2024).

CONCLUSIONS

Both EOs effectively inhibited the growth of bacterial pathogens (*S. typhimurium, E. coli*, *C. perfringens*, *L. monocytogenes*, including *C. jejuni*), confirming their potential use as natural antimicrobials in broiler feed. Additionally, TEO demonstrated a more efficient antioxidant capacity over TTEO, suggesting an enhanced potential in combating oxidative stress in poultry. Our results indicate a protective effect of both EOs against protein denaturation and erythrocyte membrane lysis suggesting anti-inflammatory benefits.

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MICROBIOLOGICAL SAFETY OF PROTEIN FEED RAW MATERIALS IN THE FARM-TO-FORK CHAIN

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Abstract

This study evaluated the microbiological safety of soybean and sunflower meals within the "Farm-to-Fork" chain at a Romanian feed mill over two consecutive years. A total of 185 analyses were conducted at reception and from stock to quantify yeast and mold contamination. Results showed high compliance, with mean fungal loads (400-553.3 cfu/g) remaining significantly below reference literature averages (865-1,040 cfu/g) and representing only 8% of the safety threshold. Sunflower meal exhibited higher vulnerability, with a 2,000 cfu/g peak in the first year leading to the rejection of 4.7% of batches. While Aspergillus remained the dominant genus (>60% prevalence in stock), a marked decline in Penicillium was observed in the second year. The 100% positivity rate in second-year stock samples (mean 528.5 cfu/g) underscores the dynamic nature of storage. These findings demonstrate that while protein meals harbor endemic microflora, rigorous reception protocols and controlled storage are essential for mitigating latent toxigenic risks in animal feed production.

Key words*: food safety, soybean meal, yeasts and molds, feed safety.*

INTRODUCTION

The fundamental pillar of the "Farm-to-Fork" strategy lies in the recognition that animal health and human food safety are inextricably linked to the quality of livestock nutrition. Within this framework, the microbiological safety of protein-rich raw materials like soybean and sunflower meals-serves as a critical determinant of the entire food supply chain's integrity (Lapusneanu et al., 2021; Matei & Pop, 2022). These materials are inherently susceptible to colonization by a diverse spectrum of pathogenic microorganisms, including toxigenic molds and enteric bacteria, which can infiltrate the production cycle at any stage from cultivation and harvest to industrial processing and onsite farm storage (Chatterjee & Abraham, 2018; Matei et al., 2024; Völkel et al., 2011).

Sunflower meal, while a valuable protein and fiber source in ruminant and poultry diets, is highly susceptible to contamination by yeasts and molds due to its hygroscopic nature and the presence of residual lipids. Fungal colonization, predominantly by species of *Aspergillus*, *Penicillium*, and *Rhizopus*, occurs frequently when the meal is stored under conditions of high relative humidity or inadequate aeration. The proliferation of these microorganisms not only leads to the degradation of essential amino acids and the oxidation of residual fats-resulting in rancidity and reduced palatability-but also poses a critical risk of mycotoxin accumulation. Specifically, the fibrous matrix of the sunflower hulls can harbor fungal spores, complicating the decontamination process and compromising the overall hygienic quality of the final compound feed (Streit et al., 2012; Alali & Ricke, 2012).

Soybean meal, a primary protein source in livestock nutrition, is highly susceptible to contamination by yeasts and molds during storage and processing. This fungal proliferation is often driven by high moisture content and inadequate ventilation, leading to the deterioration of the meal's nutritional profile and the potential synthesis of mycotoxins. While yeasts primarily impact the sensory characteristics and palatability of the feed, the presence of molds, particularly from the *Aspergillus* and *Penicillium* genera, poses a significant biosafety risk. Such contamination not only reduces the bioavailability of essential amino acids but also introduces chemical instability through the degradation of lipids and

proteins within the soybean matrix (Lapusneanu et al., 2023; Marin et al., 2013; Świątkiewicz et al., 2021).

The objective of this study was to evaluate the microbiological safety of protein-rich feed raw materials by quantifying yeast and mold contamination. Specifically, soybean and sunflower meal samples were analyzed over two consecutive years (2023 and 2024), with data collected from a commercial feed mill in Romania. The assessment targeted two critical stages of the supply chain: the initial reception of raw materials and the subsequent storage phase within the facility's inventory (stock). The soybean meal samples analyzed in this study originated from South America (primarily Brazil), while the sunflower meal was sourced from local domestic production (Romania).

MATERIALS AND METHODS

To determine the yeast and mold content in soybean and sunflower meal at the reception stage, 54 and 21 analyses were performed, respectively, during the 2023 year, followed by 73 and 16 analyses in 2024 year. Regarding the protein raw materials held in stock, 10 analyses for both meal types were conducted in 2023, while 7 analyses were carried out for each in 2024.

The safety of the protein raw materials was evaluated based on the Total Fungal Count (TFC), which represents the combined number of yeasts and molds per gram of sample. Microbiological analysis for the enumeration of yeasts and molds was performed in accordance with the SR ISO 21527-2:2009 standard. This horizontal method is specifically designed for products with a water activity less than or equal to 0.95 - including grains, cereals, and flours - utilizing a colony-counting technique. The microbiological analysis followed the horizontal method for the enumeration of yeasts and molds, specifically adapted for oilseed by-products (soybean and sunflower meals). Unlike whole cereal grains, these protein-rich matrices require careful homogenization to ensure the release of fungal spores from the fibrous and lignified structures of the meal particles. A representative 10g portion of each meal sample was diluted in 90mL of buffered peptone water. Due to the high absorptive capacity of sunflower meal, the initial suspension was allowed to stabilize before performing serial decimal dilutions.The microbial load, expressed as colony-forming units (CFU) per gram or milliliter, was calculated from plates yielding countable ranges. When required by the analytical objectives, yeasts and molds were recorded as distinct populations. Statistical processing of the resulting data involved the determination of range (minimum and maximum values) and the calculation of central tendency and variance estimators (Krnjaja et al., 2017; Lapusneanu et al., 2025a). Specifically, the arithmetic mean ($\bar{x}$) and standard deviation (s) were derived for all positive samples.

The fungal load thresholds adopted in this study, while aligned with national legislation, are fundamentally grounded in the broader European regulatory framework. Specifically, Regulation (EC) No 183/2005 on feed hygiene mandates that feed business operators implement procedures based on HACCP principles to control microbiological hazards. At this time, EU legislation does not specify a numerical limit for total yeasts and molds for protein feed raw materials.

RESULTS AND DISCUSSIONS

For the quantitative determination of TFC in soybean and sunflower meal, determinations were carried out on samples taken in two consecutive years, both for the raw materials received and for those in the unit's stock. In accordance with Order No 249 of March 31, 2003, the maximum permissible total number of potentially toxin-producing fungal species (*Aspergillus, Penicillium, Fusarium, Alternaria*) is 1000 cfu/g.

The results obtained, along with the statistical significance of the values established for the TFC in soybean meal, are presented in Table 1.

In 2023, soybean meal samples collected at the reception stage (n=54) exhibited a positivity rate of 81.4%, with a mean fungal load of 446.5 ± 216 cfu/g. During 2024, the proportion of positive samples decreased slightly to 76.7% (n=73), corresponding to a lower mean value of 409.8 ± 199.4 cfu/g. These figures represent independent batches analyzed upon arrival at the facility in each respective year, rather than a longitudinal monitoring of the same lots. This

indicates a marginal reduction in both the prevalence and the average fungal concentration in 2024 compared to 2023, likely due to variations in environmental conditions during the harvest and transit of distinct raw material shipments. Regarding the samples obtained from the unit's stock, the mycological analysis revealed a mean concentration of 400 ± 122.4 cfu/g in the first year (with 90% of samples testing positive) and an increased load of 528.5 ± 292.7 cfu/g in the second year (with 100% positivity). The notable rise in both the mean value and the standard deviation in the second year's stock suggests a higher degree of variability and a potential intensification of fungal proliferation during storage, influenced by the hygroscopic nature of the meal and fluctuating warehouse microclimates. Overall, the mean values established across both years remained centered around 400 cfu/g. These results highlight a relatively low TFC; however, it should be noted that a mean of approximately 400 cfu/g represents 40% of the maximum allowable limit (1000 cfu/g).

The mycological screening identified three potentially toxigenic fungal genera: *Aspergillus*, *Penicillium*, and *Mucor*. The distribution of these genera within the total analyzed samples is illustrated in Figure 1, which underscores that *Aspergillus* was the most frequently isolated genus.

Table 1. Results on the total fungal number in soybean meal from reception and stock

Specification		2023						2024					
		n	Positive (%)	Min. (cfu/g)	Max. (cfu/g)	x̄	s	**n**	Positive (%)	Min. (cfu/g)	Max. (cfu/g)	x̄	s
Soybean meal	Recep.	54	81.4	100	900	446.5	216	73	76.7	200	900	409.8	199.4
	Stock	10	90	200	600	400	122.4	7	100	200	900	528.5	292.7

n - number of samples analyzed; x̄ - mean; s - standard deviation; Min. - minimum value identified; Max. - maximum value identified.

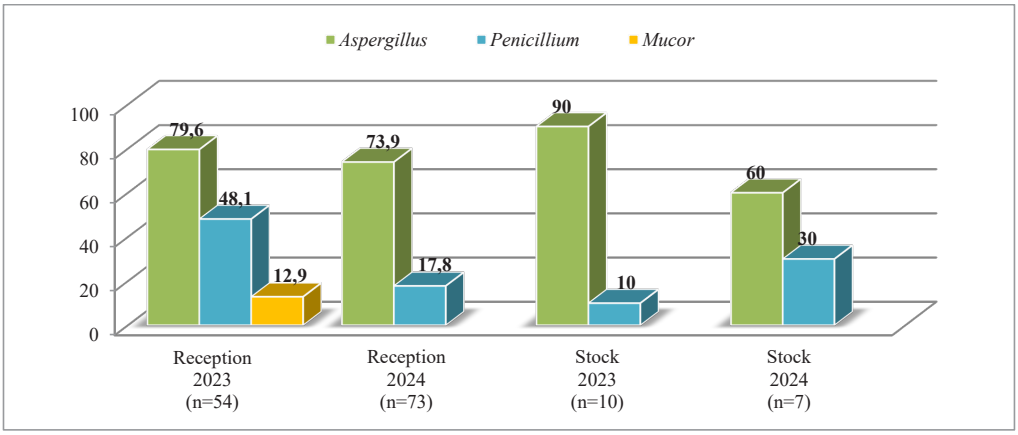


Figure 1. Proportion (%) of potentially toxigenic fungal genera in soybean meal on reception and stock

The graphical representation illustrates the distribution of fungal genera isolated from the soybean meal samples. At the reception stage during the 2023 year, *Aspergillus* was identified in 79.6% of the samples, followed by *Penicillium* (48.1%) and *Mucor* (12.9%). In 2024, a general decline in fungal isolation frequencies was observed: *Aspergillus* was detected in 73.9% of the samples, while *Penicillium* showed a marked decrease, falling to 17.8%. Regarding the prevalence of potentially toxigenic fungi in the stored samples, *Aspergillus* remained the dominant genus, though its incidence varied significantly between the two periods, decreasing from 90% in 2023 to 60% in 2024.

The results of the quantitative determinations of the total number of fungi in sunflower meal from reception and stock of the studied unit are presented in Table 2.

Table 2. Results on the total fungal number in sunflower meal from reception and stock

Specification		2023						2024					
		n	Positive (%)	Min. (cfu/g)	Max. (cfu/g)	x̄	s	n	Positive (%)	Min. (cfu/g)	Max. (cfu/g)	x̄	s
Sunflower meal	Recep.	21	71.4	200	2000	553.3	467.3	16	58.7	200	800	422.7	183.5
	Stock	10	70	200	900	550	272.3	7	100	300	900	685.7	247.8

Sunflower meal samples collected at the reception stage during the 2023 year showed a positivity rate of 71.4%, with a maximum TFC of 2,000 cfu/g. In comparison, during the 2024 year, 58.7% of the samples tested positive, with a maximum TFC of 800 cfu/g. Relative to 2023, when the mean fungal load was 553.3 cfu/g, a decrease in the average concentration to 422.7 cfu/g was observed in 2024.

Analyses conducted during the reception phase in 2023 revealed that 4.7% of the total samples exceeded the maximum allowable TFC limit by 1,000 cfu/g, necessitating the rejection of the respective batches. Regarding the sunflower meal retrieved from the stock, the mean fungal count was 550 cfu/g in 2023 (with a 70% positivity rate) and 687.7 cfu/g in 2024, where all seven analyzed samples (100%) yielded positive results.

The distribution of potentially toxigenic fungal genera identified in the sunflower meal over the two-year study period is presented in Figure 2, highlighting the presence of two main genera: *Aspergillus* and *Penicillium*. For the samples collected at reception in 2023, *Aspergillus* showed an incidence of 66%, followed by a slight decrease to 62.5% in 2024. In the case of the *Penicillium* genus, it was identified in 52.3% of the samples analyzed at reception during the 2023 year, while in 2024 year, the isolation rate significantly dropped to 12.5% of the analyzed samples.

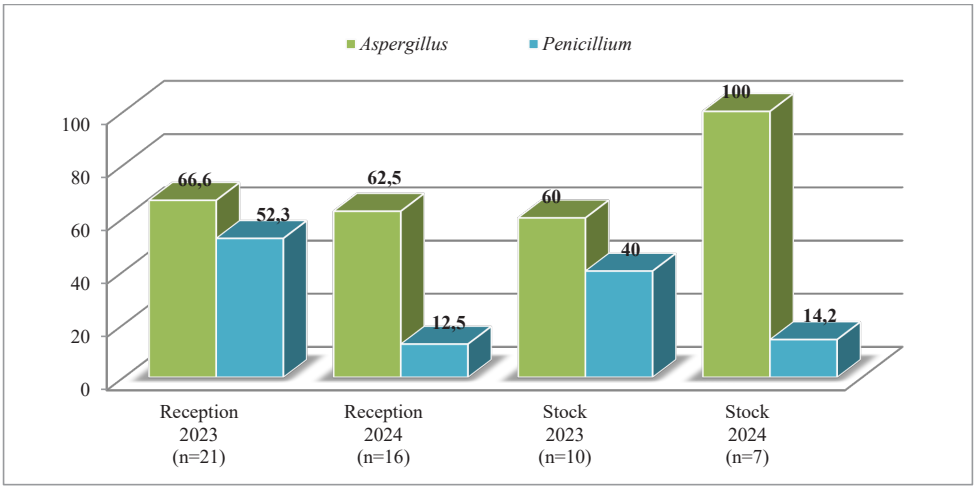


Figure 2. Proportion (%) of potentially toxigenic fungal genera in sunflower meal on reception and stock

Regarding the sunflower meal samples retrieved from the unit's stock, the incidence of the *Aspergillus* genus exceeded 60% during both years of the study, confirming its dominance within the storage microflora. In contrast, the isolation frequency for the *Penicillium* genus

showed significant annual variation, being considerably higher in 2023 (40%) compared to 2024 (14.2%).

In other studies, which have investigated microbiological contamination of feed raw materials, the presence of *Aspergillus* genus has been identified in over 50% of analyzed samples. The mycological profile identified in this study is consistent with broader taxonomic assessments of protein-rich feed matrices. Although our current results highlight the prevalence of certain toxigenic genera, they complement previous findings, in which the genera *Cladosporium* and *Fusarium* were also isolated from soybean and sunflower meal (Lăpușneanu et al., 2023; Lapusneanu et al., 2025a; Lapusneanu et al., 2025b). The occasional presence of these additional genera highlights the complex microbial succession in oilseed byproducts and confirms that the "farm-to-fork" chain remains susceptible to a diverse range of fungal contaminants, depending on seasonal and regional variations.

The literature states that *Aspergillu*s fungi are the main producers of aflatoxins and ochratoxin A (Kim et al., 2012; Marin et al., 2013; Völkel et al., 2011).

In the case of soybean meal, the results of the present study indicate a superior hygienic status compared to the reference literature. While previous reports established an average fungal load of 865 cfu/g, with maximum values of up to 3,400 cfu/g (Lapusneanu et al., 2021), our results obtained during the reception stage indicate significantly lower average values, of 446.5 ± 216 cfu/g in 2023 and 409.8 ± 199.4 cfu/g in 2024. The maximum concentration recorded for soybean meal was 900 cfu/g, suggesting that these batches had a high microbial load. Although specific data on suppliers' processing conditions were not available for this study, such results are consistent with the known impact of industrial procedures. It is possible that the low initial microbial loads observed are related to the standard heat treatments typically used during solvent extraction, which may lead to a reduction in fungal colonization in oilseed by-products.

In contrast, sunflower meal proved to be more susceptible to fungal colonization compared to soybean meal. During the reception stage, our data recorded a maximum TFC value of 2,000 cfu/g in 2023, which led to the rejection of 4.7% of the analyzed batches (n = 21) for exceeding the limit of 1,000 cfu/g. During this period, the average fungal load was 553.3 ± 467.3 cfu/g, which decreased to 422.7 ± 183.5 cfu/g in 2024. Although the maximum value is slightly higher than the 1,900 cfu/g reported in previous comparative studies, our overall average remained well below the reference average of 1,040 cfu/g (Lapusneanu et al., 2021). This increased susceptibility may be linked to the specific physical structure of sunflower meal; its high fiber content and lignified particles provide a larger surface area for spore attachment and moisture retention, facilitating fungal growth (Canibe et al., 1999). Furthermore, the observed increase in stock contamination levels during 2024, reaching an average of 685.7 ± 247.8 cfu/g, suggests that environmental fluctuations within the warehouse, such as localized increases in relative humidity, play a more decisive role in fungal proliferation than the initial load upon receipt.

In addition, storage data revealed a notable trend in 2024, when 100% of the sunflower meal and soybean meal samples tested positive for fungal contamination. For soybean meal, the average load increased to 528.5 ± 292.7 cfu/g, while sunflower meal reached a higher average of 685.7 ± 247.8 cfu/g. The observed increase in both mean values and standard deviations during the storage phase, particularly the rise to a maximum of 900 cfu/g in both matrices, suggests that post-receipt conditions may facilitate the reactivation of latent fungal spores. Although no direct monitoring of the microclimates in the storage facilities was performed, these results are consistent with the known hygroscopic nature of protein flours. It is possible that localized fluctuations in humidity and temperature within the storage facility create microenvironments favorable to fungal proliferation, even when initial loads at reception are relatively low.

CONCLUSIONS

The microbiological monitoring of protein-rich feed raw materials over a two-year period provides critical insights into the safety dynamics of the "Farm-to-Fork" chain.

The consistent isolation of *Aspergillus* (dominant in both years) and *Penicillium* identifies the primary biological hazards within the chain. Although the fungal counts remained within safe limits, the presence of these potentially toxigenic genera poses a latent risk for mycotoxin synthesis, requiring integrated mycological and toxicological surveillance.

The study confirms that while protein feed raw materials are inherently colonized by a "background" microflora, a robust management system based on rigorous reception testing and optimized storage can successfully mitigate microbiological risks. These findings contribute to the strengthening of feed safety protocols, ensuring the integrity of the food chain from the very first link.

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CONTROL OF *Eriochloa villosa* (Thunb.) Kunth, AN EMERGING THREAT TO SILAGE CROPS: A POT CULTURE STUDY

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Eriochloa villosa, an invasive Poaceae weed species, is an increasingly problematic pest in agricultural fields. In this study, the efficacy of 19 herbicide treatments applied in three replicates as formulations in various application rates and presence or absence of adjuvants was evaluated against E. villosa in an outdoor pot trial located in Giarmata, Romania. Three visual assessments were carried out every week after the single post-emergence application and inferential statistical analyses showed significant effects of treatments, assessment dates and their interactions ($p < 0.001$). Efficacy increased over time for most treatments, although those based on Propaquizafop, Clethodim and Cycloxydim provided the highest control, several variants already reaching total control by the second assessment, for both homologated rates as well as higher doses. Quizalofop-P-ethyl and Fluazifop-P-butyl also provided high control, while the others only reached a moderate amount of control. Our findings indicate that out of the tested formulations, those based on ACCase inhibitors performed the best, showing promising option for management strategies of E. villosa, although field trials are still needed to further investigate their effect in natural conditions.

Key words: *chemical control, phytotoxicity, Poaceae, post-emergence, treatment performance.*

INTRODUCTION

Eriochloa villosa (Thunb.) Kunth 1829, commonly known as the woolly cupgrass, is a grassy weed species part of the Poaceae family, native to East Asian countries (Shhagapsoev et al., 2018). It was first officially reported as an invasive species in the 1940s in the United States of America and by the 1980s deemed a problematic species of economic importance (Darbyshire et al., 2003). In Romania, *E. villosa* is increasingly present in agricultural fields, competing intensively with other crops and native flora, with both researchers and farmers raising awareness of its detrimental effects (Lele et al., 2025).

Although there are several studies that focus on the control of *E. villosa* published before year 2000, many of those tested herbicides are no longer approved for usage and, although a surge of new papers are being recently published on *E. villosa*, only a fraction of these focus on its chemical control (Lele et al., 2025). As such, it is necessary to assess the efficacy of as many prospective herbicides against *E. villosa* to develop a proper management program that could prevent its spread and diminish its economic impact.

Due to the negative impact herbicides have upon environmental and human health, the creation of new active ingredients, as well as formulae and premix improvements of already known, reliable approved herbicides was necessary in order to create more sustainable and efficient management programs (Parven et al., 2025). Many of those formulations were not tested against *E. villosa*, which creates a relevant knowledge gap (Lele et al., 2025).

While it may be found on disturbed land as well, *E. villosa* impact is most relevant on cultivated farmland and, as such, selecting herbicides that are safe for crop application as well as efficient is pivotal (Lele et al., 2025). Several modern herbicidal agents tested against *E. villosa* are part of the lipid synthesis (ACCase) inhibitor group (HRAC 1) and are generally efficient in controlling early developmental stages of this species (Cui et al., 2021; Guo et al., 2014; He et al., 2013; Lele et al., 2025). Another relevant HRAC group is 2, with acetolactate synthase (ALS) enzyme inhibitors. Although one of the most known active ingredients tested against it, Nicosulfuron, is becoming less effective against certain populations due to developed tolerance (Hinz & Owen, 1996; Ma et al., 2018; Song, 2022). Although Glyphosate, part of Group 9 (5-

enolpyruvylshikimate-3-phosphate, or ESPS, synthase Inhibitors), can be very effective against *E. villosa* even when mature, though it is not suitable for most crop applications because of its non-selective nature, so it is preferred for ruderal weed populations control or genetically modified resistant crop cultures (Nurse et al., 2015; Owen, 2008).

For this experimental trial, several types of herbicides and adjuvants were selectively chosen in order to gain a deeper understanding of the interaction between them and *E. villosa* in a controlled setting in a practical screening trial. A notable mention is that these are often used against weeds in important crops such as corn, sunflower, soybeans and more.

Several already known and tested active ingredients were chosen, whether tested in numerous studies or only once. Nicosulfuron based treatments were used in these trials due to its extensively studied effect against *E. villosa*, making it relevant as a point of reference in comparison to newly tested active ingredients (Lele et al., 2025; Ma et al., 2018). Three other active ingredients, all part of HRAC Group 1, Clethodim, Quizalofop-P-ethyl, Fluazifop-P-butyl, that were previously tested in two trials based in China were also chosen for their reported success in their control and modern composition, as well as to further study their effect in different conditions (Lele et al., 2025).

Two of the treatments consisted of mixes of pre-emergent and post-emergent active ingredients: S-metolachlor (HRAC 15, pre-emergent) in only one treatment, and Dicamba (HRAC 4, pre- and post-emergence), Mesotrione (HRAC 27, pre- and post-emergence) and Nicosulfuron in both. These are characterized by their efficacy both as pre- and post-emergence herbicides and were chosen specifically to observe their effect on *E. villosa*, as numerous farmers choose to apply mixed substances to protect their cultures as early as possible against a larger spectrum of weed species, which created a need to know of their reliability as a prospective choice (Carles et al., 2017; Morokhovets et al., 2024; Young & Hart, 1999).

Three HRAC group 1 herbicides (Propaquizafop and Cycloxydim) were chosen due to their successful control of other species within the same taxonomic family, as well as because no other research was performed to test the efficacy against *E. villosa* (Ohta et al., 2025; Raju, 2010; Willoughby & Forster, 2022).

We chose to study various dosage rates to observe their evolution in time as well as the response of *E. villosa*. Adjuvants *Silwet Gold* and *Dash* were chosen for several reasons, most important being that they are often commercialized and sold together with the treatment products that they are paired with. Adjuvants are also important in trials as observing treatments with or without them is relevant for management strategies or further studies (Brankov et al., 2025).

RStudio (version 2026.01.0, Build 392), based on the open-access R programming language, was used to conduct all data analyses and create figures due to its ready availability, wide community support and vast popularity among researchers, as well as because of its highly reliable data analysis, customizable data representations and visualizations (Del Toro et al., 2022; R Core Team, 2024).

The main objective of our study is to assess the control of 19 treatments that consist of various doses and adjuvants against *E. villosa*, with an aim to fill existing knowledge gaps within the field. We evaluate several modern active ingredients and commercial premixes never before tested against this species and published officially. The trial took place outdoors in Giarmata, Romania, using pots for a more controlled environment and 3 repetitions. Efficacy was visually assessed every week after the application of the treatments (7, 14 and 21 DAA), a timing based on common practices. Factors that affected the final rating for each treatment included the presence of chlorosis, necrosis and stunted growth. We hypothesize that every chemical treatment will present a significantly higher efficacy than untreated control. While several active ingredients present in this trial were not tested against *E. villosa* in the past, they are well appreciated in other trials against grasses and it is hypothesized that they will show additional differences when an adjuvant or higher doses are used within groups of the same active ingredient compared to lower application rates during the first and second assessment dates, at 7 DAA and 14 DAA. For the end of the trial, we hypothesize that the most efficient active ingredients will reach high control, regardless of dosage or adjuvant use.

MATERIALS AND METHODS

The experimental trial was located in Giarmata, Timiș County, Romania, in outdoor conditions. Environmental data on temperature, humidity and precipitation during the trial were acquired via DTN LLC and collected from May 16th 2023 to July 21st 2023 for this area (Figure 1).

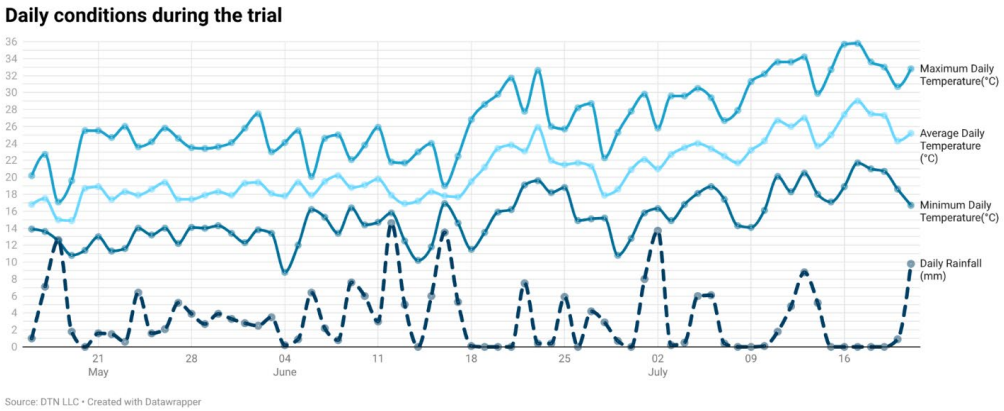


Figure 1. Visual representation of the maximum daily temperatures, average daily temperatures, maximum daily temperatures as well as daily rainfall during the period of the trial, starting from the 16th of May until the 21st of July. Each dot along the lines represents a day. Created using datawrapper.de

In May, temperatures were an average of 17.7°C, a minimum average of 12.9°C and a maximum average of 23.6°C. Rainfall averaged 3.5 mm per day, with a total of 54.3 mm. In June, temperature averaged at 19.9°C, with an average minimum of 14.5°C and an average maximum of 25.4°C. Rainfall averaged 3.4 mm per day, with a total of 102.4 mm. In July, temperatures averaged at 24.6°C, with a minimum average of 17.8°C and a maximum average of 31.4°C. A graphic visualization of the weather data may be seen in figure 1.

Seeds were collected in 2022 from infested fields surrounding Topolovățu Mic village, Timiș county, Romania and properly stored in glass jars covered by a breathable textile in dry conditions at constant room temperature. They were not treated prior to usage. Before sowing, black plastic pots with a height of 15 cm and diameter of 20 cm were prepared and filled with an equal combination of mixed *TS3 Fine Klasmann* and *BIO Plantella START* commercial seedling soils. Besides naturally occurring water from precipitation, the pots were watered as necessary for a uniform development of all plant individuals, every 2 to 3 days and natural direct sunlight was available until 2 to 3 PM, until the area was shaded by neighboring buildings, thus maintaining a more appropriate temperature for the potted plants, as black pots are susceptible to overheating.

On May 16th 2023, 5 seeds were sown per pot at 2 cm depth and all pots were watered afterwards. Emergence started on May 23rd and by May 29th all final seedlings emerged. Each pot contained an average of 3 to 5 seedlings by the start of the application timing, which was conducted on June 19th, when the seedlings' developmental stages ranged from 3 leaves to one emerging tiller. The herbicide treatments were applied at a rate of 200 L/ha using a battery powered hand sprayer with a cone nozzle at 1.2 bar pressure. The application volume per pot was calculated based on the pot surface area of 314.16 cm^2 (with 6.283 ml needed per pot), delivered at 1.1 seconds of application at 35 cm above the pot. The recipient was cleaned between each treatment application. The subsequent assessments each took place on June 26th, July 3rd and July 10th. The complete list of treatments, products, active ingredients and applied rates are presented in Table 1.

Table 1. List of all 20 treatments, condensed into two adjoined tables

Trt. No.	Active Ingredient(s)	Dose	Trt. No.	Active Ingredient(s)	Dose
1	**Propaquizafop 100 g/l**	1 l/ha	**12**	**Clethodim 120 g/l**	1 l/ha
2	**Propaquizafop 100 g/l**	1.5 l/ha	**13**	**Clethodim 120 g/l**	1.5 l/ha
3	**Propaquizafop 100 g/l**	2 l/ha	**14**	**Clethodim 120 g/l**	2 l/ha
4	**Propaquizafop 100 g/l**	1.5 l/ha	**15**	**S-metolachlor 960 g/l**	1.5 l/ha
	adjuvant	0.1 l/ha		**Dicamba 312,5 g/kg**	0.5 kg/ha
5	**Quizalofop-P-ethyl 50 g/l**	1.5 l/ha		**Mesotrione 150 g/kg**	
6	**Quizalofop-P-ethyl 50 g/l**	2 l/ha		**Nicosulfuron 100 g/kg**	
	adjuvant	0.1 l/ha	**16**	**Cycloxydim 100 g/l**	2 l/ha
7	**Nicosulfuron 40 g/l**	1.5 l/ha		**adjuvant**	2 l/ha
8	**Nicosulfuron 40 g/l**	1 l/ha	**17**	**Cycloxydim 100 g/l**	4 l/ha
	adjuvant	0.1 l/ha	**18**	**Cycloxydim 100 g/l**	1 l/ha
9	**Fluazifop-P-butyl 150 g/l**	1 l/ha		**adjuvant**	1 l/ha
10	**Fluazifop-P-butyl 150 g/l**	1 l/ha	**19**	**Dicamba 312,5 g/kg**	0.6 kg/ha
	adjuvant	0.1 l/ha		**Mesotrione 150 g/kg**	
11	**Fluazifop-P-butyl 150 g/l**	1.3 l/ha		**Nicosulfuron 100 g/kg**	
			20	Control	-

The control of 8 active ingredients and 2 adjuvants under commercial formulations was tested against *E. villosa* during this trial. The herbicidal formulation mixes used were Propaquizafop 100 g/l, Quizalofop-P-Ethyl 50 g/l, Nicosulfuron 40 g/l, Fluazifop-P-Butyl 150 g/l, Clethodim 120 g/l, S-metolachlor 960 g/l , Dicamba 312.5 g/kg + Mesotrione 150 g/kg + Nicosulfuron 100 g/kg together and Cycloxydim 100 g/l. The adjuvants were *Silwet Gold* and *Dash*. Adjuvants were used according to popular usage trends in the local area. Each herbicidal product was tested in one or several rates with or without a compatible adjuvant None of the Clethodim treatments were combined with adjuvants. We used approved doses, as well as higher doses, in order to observe their effect on *E. villosa* and the time required for them to reach full control.

The experimental setup consisted of 3 repetitions of 20 treatment variants, with a total of 60 experimental pots. Each pot was treated separately to avoid drifting, with the sprayer being thoroughly washed between each variant. Treatment number 20 was the control and left untreated. Treatments and doses are represented in Table 1, and the European Union non-approved rates highlighted and noted in the description, and should not, under normal circumstances, be applied on normal fields. Pot placements were systematic, in a grid layout of 3 rows by 20 columns, with variants in a succeeding sequence. Given that randomization was not employed, replicates were included as a random effect in the statistical linear mixed effects model, which ensures that treatment comparisons were made within rather than across replicates. A likelihood ratio test confirmed the replicate effect was statistically significant (χ^2=28.607, df=1, p<0.001), which indicates that between replicate variance was partitioned from the residual error. While this approach does not fully compensate for the lack of randomization, it represents a post-hoc statistical control for the systematic placement used.

Control assessments were carried out visually across 3 different timings: 7, 14 and 21 days after application. Signs of chlorosis, necrosis, bleaching, distortion and stunted growth were taken into consideration for a final assessment score ranging from 0 to 100 based on the percentage of the affected area, the latter being considered complete control and the plants dead. A statistical analysis was done using RStudio. A preliminary data visualization was completed by creating boxplots representing the comparison between treatments by day of assessment using the "ggplot2" package (Wickham, 2016). A linear mixed-effects model was used, which takes into consideration both the main factors (treatment and assessment date) and the random environmental variation, represented by the 3 replicates. A Type III ANOVA was done to determine whether there were significant effects or interactions between factors. We chose this particular analysis partly due to the heterogeneous nature of our biological data, but mainly because of the statistically significant interactions between the treatments and days of assessment (Kuznetsova et al., 2017; Satterthwaite, 1946). The analysis was implemented using the "lme4" and

"lmerTest" R packages using Satterthwaite's approximation for degrees of freedom for mixed-effects models.

To identify which treatments differed significantly, the estimated marginal means was calculated and adjusted for each treatment to represent their performance as though all other factors were constant. Every treatment is compared to all others for each of the 3 assessment days. To avoid risks of false positives and keep a high level of confidence, the Šidák adjustment was applied to *p*-values (with α=0.05) (Sidak, 1967). This analysis was performed using the "emmeans" R package (Lenth & Piaskowski, 2025).

The estimated marginal means efficacy percentages were represented numerically in a table using the "gt" package. For graphic representations, treatments were organized into groups of statistical significance so that if the difference is not statistically significant, they are assigned the same letter. This was represented via "ggplot2" using the "multcomp" and "multcompview" R packages as basis for data analysis (Hothorn et al., 2008).

RESULTS AND DISCUSSIONS

Results

A Type III analysis of variance (ANOVA) was performed to assess the statistical significance of the results by treatment, assessment date and ultimately, by their interaction.

The ANOVA confirmed our hypothesis that there are significant differences between the 20 treatments (F = 112.74, $p < 0.001$), as well as between the 3 assessments taken at 3 separate dates after the treatment application (F = 1577.18, $p < 0.001$). A significant interaction was also observed between treatment types and assessment timings (F = 12.74, $p < 0.001$), meaning the relative treatment efficacy is very dependent on the time duration between the time of application and date of assessment (Table 2). The model used accounted for variation or block effects. Because these two factors present significant differences among themselves, as well as within their interaction, the results are statistically significant and will be described as well as further statistically investigated.

Treatment types by herbicidal substance are described in this section as qualitative categories for an easier understanding of the active ingredients and their effect against *E. villosa*, though they were not included as statistical factors due to dosage and rate heterogeneity among them. Treatment means data and their visualization as boxplots are found in Table 3 and Figure 2, respectively. Means are represented by the results of the mixed-effects model statistical analysis, with a pooled standard error of 3.9.

Table 2. ANOVA of the estimated marginal mean (EMM) efficacies of the 20 treatments across the assessment days

Type III ANOVA							
Experimental Factor	Sum Sq	Mean Sq	Df1	Df2	F-value	P-value	Sig
Treatment	79610.55	4190.03	19	118	112.74	1.58×10^{-66}	***
Assessment Day (DAA)	117234.44	58617.22	2	118	1577.18	7.31×10^{-86}	***
Treatment x DAA	17987.79	473.36	38	118	12.74	6.11×10^{-27}	***

Experimental Factors: `Treatment` represents the 20 application variants; `Assessment day (DAA)` represents the time intervals (7, 14, 21 days). Significance levels (a = 0.05): *** p< 0.001, ** p<0.01, * p<0.05, p<0.1; `ns`=not significant (p> 0.05). Degrees of freedom calculated using Satterthwhaite's approximation for mixed effects models.

Values represent the predicted efficacy resulting from a linear mixed-effects model using the Satterthwaite approximation for degrees of freedom. EMMs are adjusted for the fixed effects of treatments, assessment dates and their interaction.

Table 3. Estimated marginal means of the efficacy of the 20 treatments over the 3 assessment dates

Mean Efficacy (%) per Treatment (*DAA = Days after application; 1-20: Treatments*)																			
DAA	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
7	43.3	33.3	30	36.7	13.3	43.3	10	16.7	13.3	16.7	30	36.7	23.3	36.7	30	56.7	66.7	23.3	26.7	0
14	100	100	96.7	100	63.3	96.7	26.7	40	56.7	53.3	86.7	96.7	100	100	60	100	100	100	76.7	0
21	100	100	100	100	86.7	100	53.3	76.7	93.3	90	96.7	100	100	100	76.7	100	100	100	93.3	0

Note: Values are estimated marginal means. Pooled Standard Error (SE) = 3.9

The values are derived from a linear mixed effects model used in the ANOVA. They are adjusted for the fixed effects of treatments and assessment days as well as by their interaction (which is significant at (p < 0.001). Data shows that the efficacy of the treatments changed significantly over time.

Propaquizafop 100 g/l was used in 4 treatments (T1 through T4). During the first assessment (7DAA), the best treatment was T1, with 43.3% control. During the second assessment (14DAA), every treatment provided a control of over 96.7%, with T1, 2 and 4 reaching full control. By the time of the last assessment, all Propaquizafop treatments reached full control.
Quizalofop-P-ethyl 50 g/l was used in 2 treatments (T5 and T6). There were notable differences between them throughout the trial, with T6 reaching 43.3% control during the first assessment, 96.7% during the second and eventually 100% by the last, while T5 reaching only up to 86.7% control on the last assessment.
Nicosulfuron 40 g/l was used in 2 treatments (T7 and T8). While there were differences between treatments, neither achieved full control, with a maximum efficacy of 76.7% at 21DAA, for T8.
Fluazifop-P-butyl 150 g/l was used in 3 treatments (T9 through T11). Their efficacy was between 13.3% and 30% during the first assessment, between 53.3% and 86.7% during the second and finally between 90% and 96.7% on the last, with T11 having the highest efficacy of all, especially during the first two assessments.

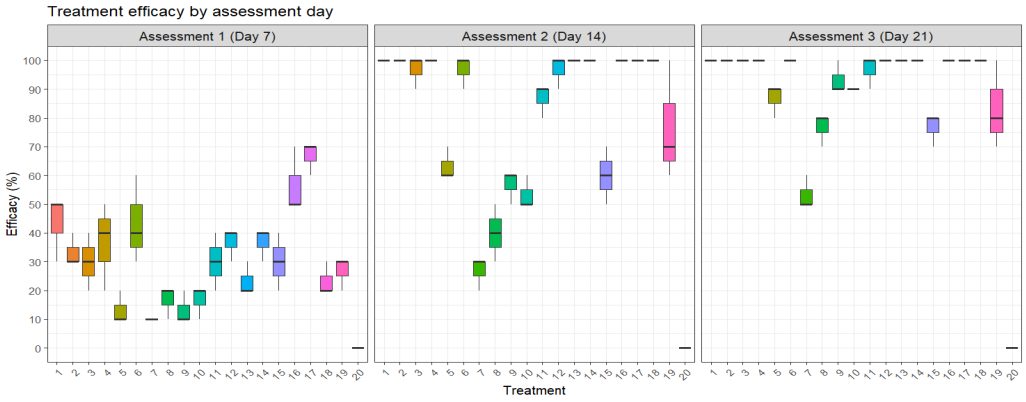


Figure 2. Boxplot graphic of the efficacy of all treatments (from 1 to 20, x-axis, horizontal) by day of assessment (7, 14 and 21 days after the treatment application, y-axis, vertical). An upward evolution is observed in most treatments except for 20, the control

Clethodim 120 g/l was used in 3 treatments with an efficacy of between 23.3% and 36.7% during the first assessment (7DAA), followed by a significant increase starting with the second assessment, of between 96.7% and 100%. By the last assessment, all treatments reached full control. Overall, T14 provided the best control throughout the assessments.
Cycloxydim 100 g/l was used in 3 treatments (T16 through T18). Efficacies ranged from 23.3% to 66.7% during the first week, with T17 having the highest efficacy at this time, followed by full control on all subsequent assessments.
Significance groups were determined using a mixed-effects model inference with multiplicity-adjusted pairwise comparisons using the Šidák correction. Two groups are considered statistically different when the adjusted *p*-value characteristic of a certain pair is lower than α=0.05. Thresholds between groups are visually represented in Figure 3 by using letters. If two treatments share at least one letter, they are not significantly different, while two treatments that fall within completely different letters groups are considered statistically different, as they pass the α=0.05 threshold.
On the first assessment date, 7 days after the application, grouping by letter ranges from A through H, as the herbicides behave very differently from one another. Letter H contains the treatment T17 with the most efficient control against *E. villosa* at this time. In group G, Treatments T1, T6 and T16 (both in G and H), the second most successful treatment group. On the other hand, letter A contains the treatments with the least amount of control, with results that were not significantly different even compared to the control (also part of A).

On the second assessment date, 14 days after the application, significance groups are reduced as a result of less significant differences between treatments. Group H disappears and G takes its place as the one containing the most efficient treatments, with T1 through T4, T6, T12 - T14 and T16 - T18 presenting the highest efficacies. T11 is part of both groups F and G. Group B containing T7 and T8 is the least efficient treatment, besides the control, A.

During the last assessment date, 21 days after the application, many groups are converged due to increasingly more similar effects against the target weed species, with treatments T1 - T4, T6, T11 - T14 and T16 - T18 are part of letter group D, with the best results, while the following most efficient, though part of both groups C and D, are T5, T9, T10 and T19. B remains the weakest treatment at this date as well, though at this time only T7 remains part of the group.

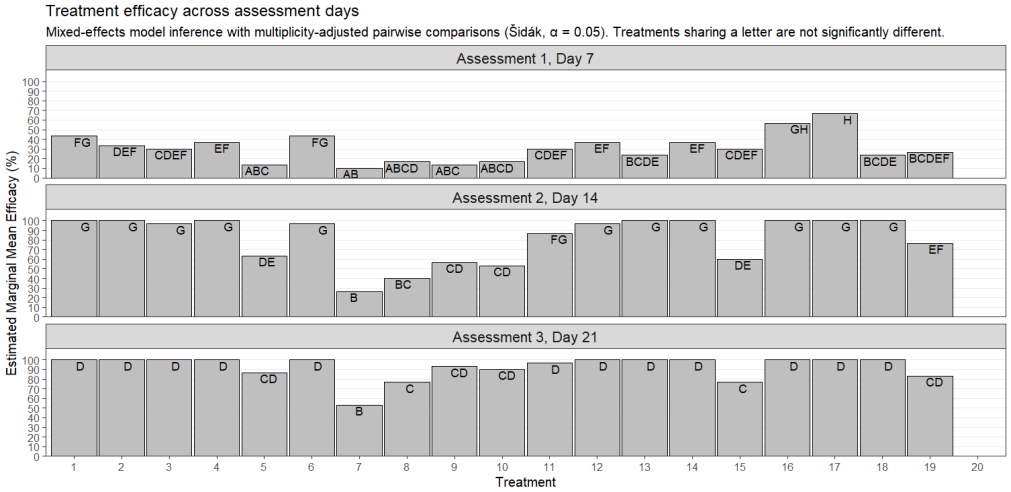


Figure 3. Estimated marginal mean efficacy of the 20 treatments over the 3 assessment dates (7, 14 and 21 days after application). The predicted mean efficacy based on a linear mixed effects model accounting for the two factors as well as environmental variation represented by replicates is visualized in bars. Statistical groupings were determined by the Sidak adjusted pairwise comparisons, where ($\alpha = 0.05$) and treatments with the same letter in the same assessment day are not significantly different. Treatment 20 is the control

Discussions

In this study, we demonstrated the efficacy of several herbicide treatments against the invasive weed species, *E. villosa,* in a pot trial experiment.

By the end of the experimental period, Propaquizafop, Clethodim and Cycloxydim treatments showed excellent results against the studied weed species as soon as the second assessment date, and full control by the last assessment date. They are approved for use on both soybean and sunflower crop cultures, though not in corn. Results provided by the Clethodim application are similar to that of another published trial, albeit under a different formulation (He et al., 2013).

Quizalofop-P-Ethyl and Fluazifop-P-Butyl had excellent control over *E. villosa*, both with over 95% control when at the highest application rate. They are approved for use on sunflower crop cultures. Treatments 15 and 19, comprising mixes of pre- and post-emergence herbicidal ingredients, provided good control, of between 76.7 and 83.3% at 21DAA. They are approved for use on sunflower and corn fields.

Treatments that included Nicosulfuron only performed the poorest, with a range of 53.3% and 76.7% (lower rate combined with an adjuvant) at 21DAA, though still within an acceptable control range. This active ingredient is approved for use on corn. While certain Asian populations were revealed to have become resistant to Nicosulfuron, it is not known whether this is the case for Romanian ones, as no prior investigations on the subject were conducted up to now (Ma et al., 2018).Differences between the treatments using ACCase inhibitors (Propaquizafop, Quizalofop-

P-ethyl, Fluazifop-P-butyl, Clethodim and Cycloxydim) and the ALS Nicosulfuron are significant, even though both modes of action are well known to affect seedling development and growth (Jhala et al., 2026; Whitcomb, 1999). A potential explanation for this difference may be due to the tolerance of *E. villosa* to Nicosulfuron, which was already documented in scientific literature, and which operates on two levels, its inherent biochemical capacity to partially hydroxylate it via its microsomes, and an evolutionarily acquired tolerance which was observed in certain populations following excessive Nicosulfuron applications (Hinz & Owen, 1996; Jhala et al., 2026; Lele et al., 2025; Ma et al., 2018; Song, 2022).

A caveat to the interpretation of our study is the presence of limitations such as those imposed by the nature of the experiment, as the number of weed individuals in each pot were low in number, and so, their growth stage heterogeneity could have had an important effect upon the results (Ogunkunle & Beckett, 1988). Another is the fact that randomization was not used for this trial, although measures were taken for all of the pots to be in the same condition. While most were of similar growth stages, they still varied between 3 to 5 leaves at the application timing, which is known to lead to different responses to herbicides, especially if tillers have emerged, as well, which was the case for several individuals (Barros et al., 2007). Another significant factor is that the experiment was not conducted in a greenhouse, but outside, in an open area, and placed in a non-randomized manner, in order from the first to the last treatment, with replicates in rows. While the pots were regularly watered and maintained, increasingly hot summer temperatures present by the end of the trial are very likely to have enhanced the later effect of herbicides, possibly in slightly unequal measure due to placement, which lead to an additional stress the plants had to face (Varanasi et al., 2016). These limitations might explain the differences between certain groups of active ingredients where the higher dosage had an initial lower effect upon *E. villosa*, such as for Propaquizafop, though quickly reaching the same level of statistical significance.

Despite abovementioned limitations, the overall trend of the treatments was very clear, with mostly excellent results, especially for the never before tested herbicidal ingredients Propaquizafop and Cycloxydim which, regardless of environmental or experimental factors, showed a decisively strong control effect throughout the trial for all application rates, with or without an adjuvant. These consistently positive results may be explained by the fact that they strongly target grassy species such as Poaceae members and have such a strong effect upon this family that they are not even approved for use on corn, as it would destroy the crop alongside the grassy weeds. Clethodim showed similarly excellent results.

Great control is also shown by the highest rates of Quizalofop-P-Ethyl and Fluazifop-P-Butyl, results that are similar to that of the two previous studies that tested their efficacy against this same species, which noted the full control of early applications (Guo et al., 2014; He et al., 2013). Their lower performance may be explained by the fact that the growth stages of the weed individuals in this trial were higher than in the original paper. The difference in efficacy between T5 (86.7%) and T7 (100%) may be due to the combination of higher application rate and the addition of the adjuvant, but since both treatments differ simultaneously in both application rate and adjuvant presence, the experimental design does not allow the two effects to be separated statistically.

Corn approved herbicidal ingredients Nicosulfuron, showed results similar to that of previous research, being highly dependent on application timing and the growth stage of the weed species. As they are specifically created for use on corn, these ingredients do not exhibit the same strong effect as the ones above, though often perform well in double application combinations (Lele et al., 2025).

T19 performed better than T15, and while T19 had two active ingredients, S-metolachlor is a strictly pre-emergent herbicide and had no effect upon the already emerged and growing young plants. In any case, care should be taken that herbicide dosage is not exceeded for applications, as their effect upon cultivated crops can seriously stunt their development, as well as may have a negative impact on the environment.

Even though corn is one of the most sensitive crops when competing against *E. villosa* for

resources, we chose to study new herbicides that are only approved to use on sunflower and soybean fields for several reasons. The main reason was the fact that most are either understudied or not officially studied at all against this species, and we decided this knowledge gaps are worth investigating further. Another reason is the fact that we believe it is important to adopt integrated control methods, which include crop rotations including dicots such as the sunflower and soybean in weed management plans, with the double effect of a stronger herbicide applied and the disruption of the normal development timings of weeds such as *E. villosa* by the yearly crop change. Our findings are relevant in the light of newer and harsher regulations imposed by various countries, which may force farmers to change their preferred herbicides in favor of newer, more environmentally friendly ones. Data strongly suggests that regardless of the doses we used, the results are still satisfying, and so reducing the application rate may also be a viable economic choice farmers could adopt, as all treatments applied below the maximum approved rate had efficacies of over 76.7% and up to 100% control. Although this trial revealed relevant information regarding the chemical control potential of the tested active ingredients, it is still a controlled pot trial and thus should be followed by larger scale field trials, so that the response of crops to both the competition against *E. villosa*, and to the application rates may be studied and better understood. Future field based studies are especially important as the heterogeneity of the studied weed species is greatly reduced, especially if the trial grounds are carefully chosen for their population density homogeneity. Of equal importance is that application rates, as well as the addition or absence of adjuvants, are further studied, especially in experimental designs that carefully consider statistical analyses that allow regression modelling, which may be of great use in predicting the effect of even smaller doses and in planning weed management protocols.

CONCLUSIONS

In our pot trial study, the efficacy of several herbicidal ingredients applied as commercial products with or without adjuvants was studied against *E. villosa* in an outdoor environment. Overall, the most efficient herbicides were those belonging to the HRAC Group 1, the ACCase Inhibitors, regardless of dosage or the addition of adjuvants. Propaquizafop, Clethodim and Cycloxydim, never tested against *E. villosa* in an experimental trial, performed the best, with some treatments reaching full control as soon as the second assessment. While not approved for use on corn, one of the most susceptible crops to resource competition against *E. villosa*, they are approved on sunflower and soybean fields. They may be used successfully both as part of a management plan including crop rotation, as well as offering flexibility in herbicide usage alongside those used on monocot crops such as corn and cereals.

The results of this study should be considered indicative of their potential rather than fully predictive of their performance in fields. Future studies should include a broader range of dosages and adjuvants, as well as both pre- and post-emergence herbicides in field conditions. Competition dynamics studies between *E. villosa* and crop cultures are also necessary for their better understanding and development of more effective management strategies.

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POST-EMERGENCE CONTROL OF *Eriochloa villosa* (Thunb.) Kunth IN SILAGE CORN

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Abstract

Weed interference in agricultural fields poses an important challenge globally due to direct competition with vulnerable crops such as corn. Our study is focused on the chemical control of Eriochloa villosa, commonly known as the woolly cupgrass, an emerging invasive Poaceae species in Romania that germinates in waves and was reported to significantly reduce corn yield. We conducted a post-emergence experimental trial in which six herbicidal formulations were tested against E. villosa in a field located near Buzas, Timis County, Romania. In this practical screening, three visual assessments were carried out and data statistically analysed. Our findings revealed that treatments, assessments dates and their interactions were significant, and treatments based on Tembotrione 44 g/l and Mesotrione 75 g/l + Nicosulfuron 30 g/l provided the highest efficacy, with over 90% control 24 days after the application, while other treatments provided only moderate control. The use of these post-emergence active ingredients is immediately applicable in agricultural field management strategies by farmers and other specialists, and the results establish an important foundation for future research of E. villosa.

Key words*: weed suppression, chemical control, phytotoxicity, post-emergence, Poaceae.*

INTRODUCTION

Segetal weed species have long negatively impacted agricultural crops, though numerous control techniques were used to cope with their pressure. While chemical control existed in rudimentary form since antiquity in the form of salts, plant extracts, mineral and organic mixes, the creation of synthetic herbicides revolutionized agriculture (Horvath et al., 2023). Despite their popularity and efficacy, extensive and irresponsible herbicide usage led to severe ecological impact and health issues in humans as well as animals, and often to the development of herbicide resistance in numerous weed species (Heap, 2014; Hongoeb et al., 2025; Pogurschi et al., 2024; Pratt et al., 1997). In response to legislative measures taken against harmful substances to human health and the environment, numerous herbicides were banned, which led to the need to optimize management strategies using newer alternatives (Mallory-Smith & Roma-Burgos, 2020).

Corn, *Zea mays* L., is one of the most relevant crop species due to its high adaptability, genetic variability and high yield potential, with a yearly production of over 1 billion metric tons globally (García-Lara & Serna-Saldivar, 2019). Despite these traits, corn is still very susceptible to the presence of weed species in early developmental stages due to high weed density, allelopathy and resource competition (Casimero et al., 2022; Horvath et al., 2023; Meissle et al., 2010; Soltani et al., 2016; Vila-Aiub et al., 2008). Due to these factors, losses, especially during early stages of crop development, are significant and consistent, leading to serious economic strain (Cerrudo et al., 2012).

Weed biodiversity within crop fields may vary greatly depending on location, season and management strategies, although recently, a loss in species richness was noted in Europe, but not in abundance (Petit et al., 2016). *Eriochloa villosa* (Thunb.) Kunth (woolly cupgrass) is an invasive species whose populations germinate in waves throughout the season and are often abundantly found in infested corn fields, which can lead to significant competitive pressure and yield loss of over 80% (Darbyshire et al., 2003; Lele et al., 2025). In our experimental field trial, this species had the highest and most homogenous presence in the plots, occurring in

all of them. *E. villosa* is the focus of our study as its response to newer herbicides is important to evaluate to better manage its fast spread.
Although *E. villosa* had the largest population in this particular trial, it was not the only species encountered and generally, herbicides with a broader control spectrum may still be preferred for fields that present both grassy and broadleaf weed species in order to properly manage the wider variety, so our herbicide choice was based on this aspect (Kushwah, 2020; Susha et al., 2018).
Our objective was to assess the chemical efficacy of 7 herbicide treatment variants against the most abundant species encountered in the trial field, *E. villosa*. We hypothesize that herbicide efficacy against *E. villosa* would significantly differ among treatments, but that they will achieve higher efficacy than the untreated control. We also hypothesize that efficacy will increase after each evaluation, with the highest control by the last assessment, although efficacy increase rate will depend on the herbicide, with some showing faster improvement than others. As the herbicide used in T5 clearly labels high efficacy against *E. villosa*, we expect it to achieve great control. No phytotoxicity is expected in corn, as all chosen herbicides are within recommended rates.

MATERIALS AND METHODS

The experimental trial was located on a field near Buziaș town, in Timiș County, Romania, in open field conditions. The location was chosen for its homogenous *E. villosa* populations, ensuring representative results. Environmental and weather data on temperature, precipitation and humidity were acquired from DTN LLC, collected from May 16th 2024 to June 8th 2024 for this area (Figure 1). Soil data was collected using a standard soil sampler tube at 0.5 m below the surface and texture analyses performed at OSPA Timiș laboratories.
During May, temperatures averaged 18.5°C, with the minimum temperatures averaging at 12.7°C and maximum temperatures averaging at 24.5°C. Rainfall averaged 2.00 mm per day with a total of 31.2 mm during the period of the trial.

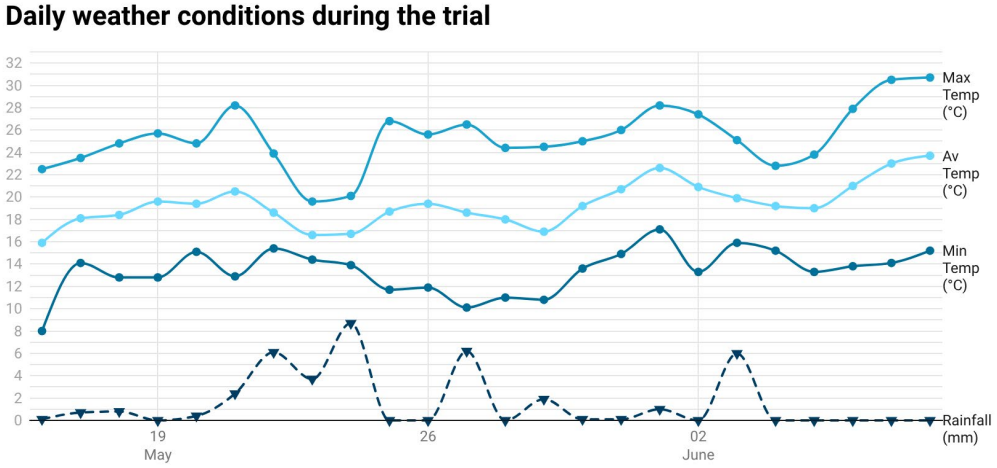


Figure 1. Visual representation of minimum daily temperatures, average daily temperatures and maximum daily temperatures throughout the trial, represented by continuous lines with one dot along for each day. Daily rainfall is represented by a dashed line, with triangles along the line representing each day. Graph created with datawrapper.de

During June, temperatures averaged at 21.2°C, with minimum temperatures averaging at 14.7°C and maximum temperatures averaging at 27.1°C. Rainfall averaged at 0.87 mm per day, with a total of 7.00 mm during the trial period. The field was not irrigated.

Pedological laboratory analyses of particle sizes presented the following soil texture properties: 13.5% coarse sand, 28% fine sand, 30.3% silt, 28.2% colloidal clay and 47.6 physical clay. According to these analyses, the soil is classified as clay. The pH was 6.51 with a percentage of 9.5% organic matter.

Corn variety Stine 9734-G was sown on May 2nd and presented, on average, 3 true leaves at the date of application. Crop yield analyses were not carried out as only the efficacy of the treatments *E. villosa* was included in the final analysis, and the results would not reflect the reality of the field due to the presence, albeit heterogeneous, of other weed species.

The weed developmental stage at the application timing did not exceed 4 true leaves for most individuals, and only a fraction presented grown tillers. Although multiple weed species were present in this field, we chose to focus on *E. villosa* only due to the heterogeneous nature of the other weed populations, which disallowed representative or reliable statistical analyses.

The experimental trial design is a randomized complete block design (RCBD), with 7 treatments including the control (T1) and 6 replicates. One-meter paths between each row of replicates were kept for easy access to each plot. Plots were sized at 5 x 3 m (15 m^2) each. Treatments consisted of 6 commercially formulated herbicides and one adjuvant for T6 only as per product indications. Each treatment was applied once, as post-emergence.

The complete list of treatments, along with formulations and dosages, is represented in Table 1.

Table 1. Complete list of treatments used during this trial

Trt. no.	Active Ingredient(s)	Rate of application
1	-	-
2	S metolachlor 960 g/l	1.5 l/ha
3	Nicosulfuron 40 g/l	0.8 l/ha
4	Dicamba 312,5 g/kg + Mesotrione 150 g/kg + Nicosulfuron 100 g/kg	0.6 kg/ha
5	Tembotrione 44 g/l	2.25 l/ha
6	Mesotrione 75 g/l + Nicosulfuron 30 g/l	2 l/ha
7	Tembotrione 345 g/l + Thiencarbazone 68 g/l	0.29 l/ha
	Adjuvant (esterified rapeseed oil)	2 l/ha

They are represented by the control (T1), one pre-emergence type treatment (T2) and 5 post-emergence treatments (T3-T7). All were applied as post-emergence, in one timing. All except T3 were applied at the maximum allowed rate.

Nicosulfuron was chosen as it was often used against *E. villosa* in experimental trials in the past (Arnold et al., 2005; Lele et al., 2025). The lower than allowed dosage was used as the maximum dose is intended for applications of fields where *Sorghum halepense* L. Pers. is present, which was not the case. Newer active ingredients such as Tembotrione, Mesotrione and Thiencarbazone were chosen for this trial because they contain modern active ingredients, all registered after the year 2000, as well as because no research papers reported the response of *E. villosa* populations in a controlled trial against any of them. All treatments contained post-emergence formulations, with the exception of treatment 2 (containing S-metolachlor) which, although a pre-emergent herbicide, it is often used in combination mixes by farmers and was included in this post-emergence application to observe any possible effect on the crop and on already emerged weed species under field conditions. Several months after the final assessment, this active ingredient was banned from use in the European Union based on regulation (EU) 2024/20, though we will still present the results as other countries may still use formulations using this active ingredient (European Commission, 2023).

The application took place on May 16th 2024. It started at 7:12 PM and ended at 7:46 PM. Air temperature during the application averaged 20.8°C while soil temperatures averaged 23.8°C. Humidity averaged 40.3%. Wind speeds averaged 3.53 meters per second (minimum 1.4 and maximum 6.6). The application was completed using an experimental grade backpack sprayer with compressed air at 2.6 bars, a spraying band 3 m wide with 6 flat fan type nozzles spaced 50 cm between each other and the application performed at 50 cm above the ground to reduce drift.

The chemical control against *E. villosa* was visually assessed taking into consideration symptoms of chlorosis, bleaching, necrosis and stunted growth or deformation. We used percentages from 0 (no effect) to 100 (all dead) in increments of 10 for each plot during every assessment date. Crop phytotoxicity was visually assessed according to these guidelines as well. The assessments were carried out during three different dates, at 9 (May 25th), 18 (June 2nd) and 24 (June 8th) days after the first application.

RStudio version 2026.01.0 (Build 392) was used for data analysis and graph creation within this experimental trial. Several packages were used to facilitate handling of data, statistical analyses

as well as table and figure creation. The packages “dplyr”, “tidyr” and “tibble” were used to handle data and organize the code; “car”, “emmeans” and “multcomp” and “multcompview” were used for extended statistical analyses that were not available in base RStudio; while “ggplot2” and “gt” were used in the creation of publication grade visual representations (Fox & Weisberg, 2019; Graves et al., 2026; Hothorn et al., 2008; Iannone et al., 2026; R. Lenth & Piaskowski, 2025; Müller & Wickham, 2026; Wickham, 2016; Wickham, François, et al., 2026; Wickham, Vaughan, et al., 2026).

Treatments, assessment dates and replicates were taken into consideration as factors. Descriptive statistics analyses were performed to gain a better understanding of the data and factor interaction. Following the descriptive statistics analyses, normality was evaluated using the Shapiro-Wilk test on the response values and model residuals, as well as a visual evaluation using Q-Q plots (Shapiro & Wilk, 1965). Homogeneity of variances among treatments was determined using Levene’s test. These analyses were chosen due to their suitability for small to medium sample sizes (Yang & Berdine, 2021).

A Type II blocked analysis of variance (ANOVA) based on an ordinary least squares (OLS) linear model was applied, with assessment date (DAA) and treatment as categorical factors, replicate as a fixed block, and their two-way interactions included (Efficacy ~ Replicate + DAA (Days after application) + Treatment + DAA:Treatment)); this model was chosen as it is recommended for unbalanced factorial designs (Langsrud, 2003; Shaw & Mitchell-Olds, 1993).

Based on the ANOVA results, post-hoc comparisons were performed using estimated marginal means (EMMs) with Šidák adjusted pairwise comparisons. Compact letter groupings were created at $\alpha = 0.05$ and confidence intervals reported at 95% adjusted confidence level (R. V. Lenth, 2016; Sidak, 1967).

Effect sizes were analysed to determine which factors or interactions were the strongest drivers of variation in efficacy. Additionally, comparisons of treatments to control were carried out for a clear statistical view of the most efficient treatments.

RESULTS AND DISCUSSIONS

Results

In total, 10 species were found in the experimental field trial: *Hibiscus trionum* L. (31 plots), *Xanthium strumarium* L. (14 plots), *A. artemisiifolia* L. (9 plots), *Convolvulus arvensis* L. (6 plots), *Echinochloa crus-galli* (L.) P. Beauv. (5 plots), *Rubus caesius* L. (4 plots), *Cirsium arvense* (L.) Scop. (1 plot), *Polygonum aviculare* L and *Polygonum maculosa* Gray (both in 1 plot each). Due to their low representation and low frequency across and within plots, these species were excluded from the statistical analyses.The only species considered in this trial was *E. villosa*, and raw mean efficacy values were calculated for all treatments and visually represented in Table 2 and Figure 2.

Table 2. Visual representation of the raw mean efficacy of the treatments against *E. villosa*, with DAA = Days after application. The values are expressed as % efficacy

Mean treatment efficacy (%) against *Eriochloa villosa*							
Assessment day	Treatment						
	1	2	3	4	5	6	7
9 DAA	0	20	40	56.7	81.7	61.7	36.7
18 DAA	0	38.3	60	66.7	91.7	90	46.7
24 DAA	0	40.0	55	61.7	93.3	98.3	48.3

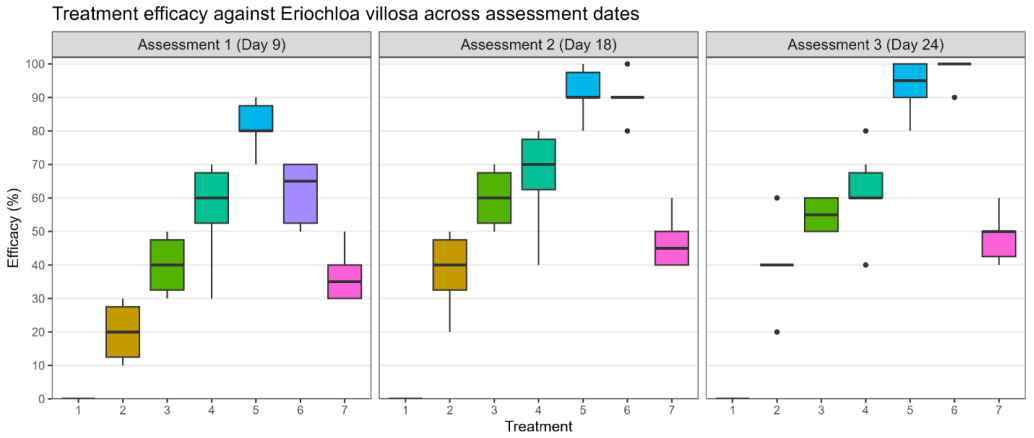


Figure 2. Boxplot representation of raw mean efficacies of all treatments against *E. villosa* across three assessment dates

Treatment 2, although a pre-emergence herbicide, was applied post-emergence in order to assess any potential effect upon already germinated and emerged weed seedlings. Its efficacy was low overall, reaching 38.3% by the second assessment and up to 40% by the third. Field observations indicated that established individuals were not visibly affected, and the control was associated with newly emerged seedlings, which presented clear signs of chlorosis. Treatment 3, a grass selective herbicide, reached up to 60% control against *E. villosa* at the second assessment, although efficacy declined by the third, settling on 55%. Treatment 4 performed similarly to T3, reaching a control of 66.7% at the second assessment and dipping in efficacy by the third, settling on 61.7%. Treatment 5 provided high control throughout the trial against *E. villosa*, with 81.7% by the first assessment, reaching 91.7% by the second assessment and 93.3% by the last. Treatment 6 provided excellent control by the final assessment, reaching 98.3%, although control was lower compared to T5 during the first two assessments. Treatment 7 provided moderate control, reaching 46.7% by the second assessment, though stagnating at 48.3% by the third and final assessment date. No signs of crop phytotoxicity symptoms were visible on corn throughout the trial, therefore the data, which was consistently 0 for every plot, is not presented.

Shapiro-Wilk normality tests were performed on both the raw mean efficacy values and the residuals of the final model. Raw mean efficacy values deviated significantly from normality ($W = 0.9388$, $p = 2.295 \times 10^{-5}$), while the residuals of the final model did not significantly differ from a normal distribution ($W = 0.9799$, $p = 0.05771$). Levene's test resulted in $F = 1.477$, $p = 0.105$, which was not significant at $\alpha = 0.05$, indicating that the homogeneity of variances was acceptable across groups.

Diagnostic plots supported the normality test results presented above.

The residuals were distributed around 0 without a clear pattern in the residuals vs fitted plot, while in the normal Q-Q plot, most residuals followed the reference line closely, only presenting small deviations at the tails, indicating that residual normality was broadly acceptable for the chosen model (Figure 3).

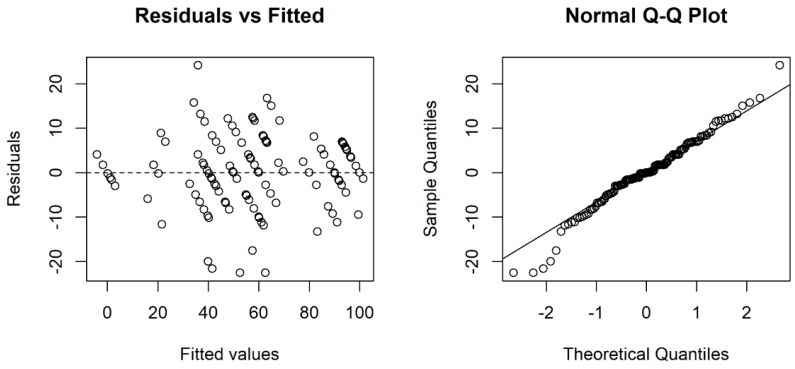


Figure 3. Residuals vs fitted and Normal Q-Q plot visual assessments of the model assumptions

A type II blocked OLS ANOVA model that included main effects as well as their two-way interaction was used to evaluate the efficacy of treatments (α=0.05). Assessment dates (DAA) and treatments had a significant effect on efficacy, while replicates did not. The DAA x Treatment was also statistically significant (Table 3).

Table 3. Blocked OLS ANOVA

Blocked OLS ANOVA (Type II) (*Efficacy (%) ~ Replicate + DAA + Treatment + DAA x Treatment*)					
Effect	Sum Sq.	Df	F value	Pr (>F)	Sig
Replicate	692.0635	5	1.7503	0.1301	*ns*
DAA	5530.1487	2	34.9659	0	***
Treatment	100349.2063	6	211.4947	0	***
DAA x Treatment	2936.5097	12	3.0945	0	***
Residuals	7907.9365	100	NA	NA	*ns*

Note: Significance levels (Sig): *** p < 0.001; ** p < 0.01; * p < 0.05; ns p ≥ 0.05.

We performed post-hoc comparisons based on the estimated marginal means (EMMs) with Šidák correction in order to further examine differences within each assessment date for *E. villosa*. The resulting compact letter display (CLD) showed considerable differences among treatments across all assessments. Treatments sharing at least one letter were not considered significantly different, while treatments assigned to completely different letter groups differed significantly (α=0.05). These results are visually represented in Figure 4.

At the first assessment date, 9 days after the application, the highest efficacy against *E. villosa* was recorded by Group E (T5), which was significantly different from all other treatments, followed by Group D (T4 and T6) and Group C (T3 and T7).

At the second assessment date, 18 days after the application, several treatments shifted statistical groups as efficacy increased over time. The highest efficacies were associated with T5 and T6 (Group E), which were significantly different from all other treatments, followed by T3 and T4 (Group D).

By the third and final assessment date, 24 days after the application, the treatment statistical groups shifted again, becoming more compact. Group D (T5 and T6) contained the most efficient treatments and differed significantly compared to all others. Group C (T3, T4 and T7) contained the following most efficient treatments, partially overlapping with Group B (T7, T3, T2).

Overall, the most effective treatments in this trial were T5 and T6, with a difference of over 30% between these and the following treatments, T3, T4 and T7, which showed moderate efficacy against this species. Images of the experimental plots can be found in figure 6, where each treatment is represented in two pictures, one overall, and one closer to the ground. The significant treatment effect as well as the significant Treatment:DAA interaction confirmed that herbicide efficacy greatly differed among treatments and changed over time.

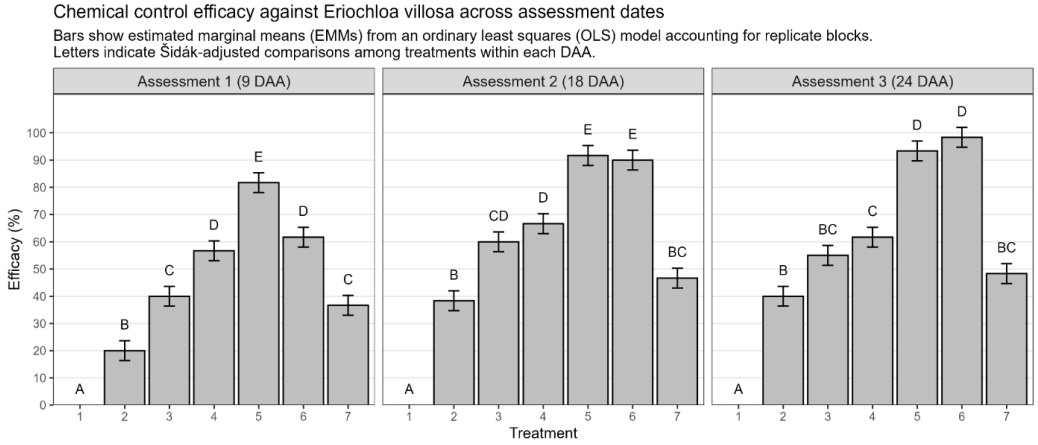


Figure 4. Estimated marginal mean efficacy of the treatments across assessments. Bars represent the predicted mean efficacy based on a type II blocked OLS ANOVA model and it accounts for the treatment, assessment date as well as their interaction ($\alpha = 0.05$). Treatments with the same letter within the same assessment are not significantly different

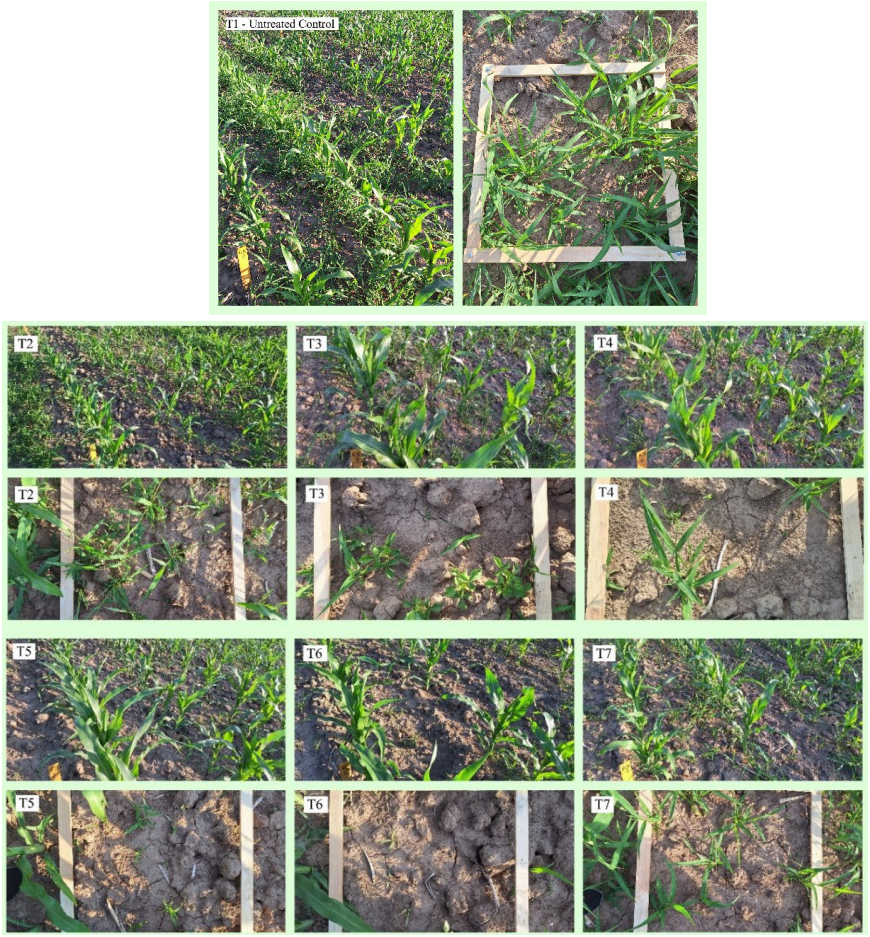


Figure 5. Representative plot images of all treatments at 24 DAA

Discussions

The response of *E. villosa* to 6 different herbicide formulations was studied in this experimental field trial, several of which were not officially tested against this species and reported in research papers before. Only reports of S-metolachlor and Nicosulfuron being used against this species have been published before, with varied results (Lele et al., 2025). Treatments 5 and 6 provided excellent control against *E. villosa*, while treatments 3, 4 and 7 only provided moderate control against it. As expected in our hypothesis, it showed a low response towards treatment 2 most likely because it was a pre-emergence herbicide applied as post-emergence. Our trial suggests only a limited number of these herbicides provided strong, reliable control of this species, leading to a direct relevant choice of treatments in infested corn fields.

Our hypotheses proved to be mostly correct, as all treatments were more effective than untreated control and their efficacy increased across assessment dates, although they slightly dropped for T3 and T4 after the second assessment. The efficacy of all treatments evolved differently throughout the trial, despite some of them reaching similar final results. This difference in efficacy is important from the perspective of prospective management choices because even though the final result is similar, early suppression of weeds reduces early competitive pressure for corn, when it is the most vulnerable (Horvath et al., 2023). Corn did not show any phytotoxic symptoms following the application, likely because all treatment doses were within approved safety thresholds.

As this was a field trial under natural, typical environmental conditions, several variables may have influenced the results. The weed population was abundant, as the field was selected specifically for its high and relatively homogeneous *E. villosa* infestation. Even so, the individual plants' growth stage did not exceed 4 true leaves and only a small proportion developed tillers by the time of application. The trial geographical location was that of a typical agricultural field subjected in a temperate continental climate, making it easy to replicate in other areas with similar climatic and pedological conditions, even in other countries or continents. Average temperatures during the trial were approximately 2°C higher than the historical mean from the past 30 years in this area, while rainfall in June was half of the historical amount for the corresponding study time period, which could have affected the response of *E. villosa* to the selected herbicides due to increased drought and heat stress. Regardless, these field variables reflect common types of variability under natural agricultural conditions.

E. villosa response to Nicosulfuron is known to vary depending on factors such as application timing, weather conditions, growth stage and possible genetic resistance to this active ingredient, as well as the number of applications, where it was often recommended as a double application. Considering the conditions of the experimental field, its design, the application timing and the population abundance, our results of treatment 3 are in line with previous studies (Lele et al., 2025). While T5 and T6 showed a very high efficacy, T7 only provided moderate control, even with the addition of the adjuvant. This contrast suggests that formulation differences and active ingredient combinations, species substance uptake and translocation can significantly influence their performance, even for herbicides that appear promising based on their composition.

Overall, our findings of previously tested herbicide formulations or active ingredients fit within existing knowledge on *E. villosa* control and add new, relevant information, especially for farmers in regions with similar environmental conditions, as they may contribute to the immediate improvement of management strategies against this problematic species. These results also present a starting point in choosing experimental designs for further chemical control studies.

Future studies of this difficult to control species may include multiple trial years, multiple dosages of the same active ingredient or formulation and multiple applications throughout the experimental period. In this type of research, other weed species are often included and studied if their populations are abundant and homogeneous enough for reliable statistical analyses. Advanced herbicide trials also include the study of crop yield quantity and content analyses for a better understanding of

the interaction between the crop and competing weeds. The biochemical response of *E. villosa* against the most efficient herbicides is a relevant aspect of understanding the mechanisms of herbicide efficacy and the pathways of active ingredient action within the metabolism of this species. Local genetic analysis may be required in the future, as weed tolerance becomes an increasingly important management aspect, with widely used active ingredients like Nicosulfuron becoming less effective as populations adapt to repeated use.

CONCLUSIONS

Weed control became an increasingly relevant challenge in modern agriculture, both because numerous widely used herbicides were banned and many weed species, like *E. villosa*, started exhibiting genetic tolerance to widely used alternatives in certain populations. Despite this, research focused on its management declined in the past two decades, which resulted in relatively few studies on modern herbicides with more sustainable formulations.

In this trial, we studied the efficacy of several modern herbicides against *E. villosa* and obtained very satisfactory results for two of them, treatments 5 and 6, which provided over 90% control in field conditions. Even though the other treatments only provided moderate control, the results nonetheless offer a useful insight into the most promising formulations for post-emergence applications. These results also help identify future research directions which may include testing lower doses, alternative mixtures of the same active ingredients, different or multiple application timings and most importantly, the interaction between these factors with the weed species and the infested crop.

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EVOLUTION OF PROTEIN METABOLISM AND BODY MASS OF QUAILS UNDER THE INFLUENCE OF PHYTOMICROBIAL ADDITIVE OMEPOL 30

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Abstract

This research presents the effects of phytomicrobial additive OMEPOL 30 on markers of protein metabolism in blood serum and the evolution of body mass of laying quails. A total of 150 quails were divided into groups: control group and two experimental groups with 50 birds in each one. The control group was fed with basic combined feed, the experimental groups - with basic combined feed supplemented with 0.25 and 0.5% of additive during 71 days. The results indicated that the product was well tolerated by laying quails, strengthening their health status. In addition, modulation of protein metabolism was inferred from changes in marker parameters: the significant increase ($p<0.01$) of total proteins, albumin and creatinine in the experimental groups, as well as the tendency to increase urea and decrease uric acid in the blood, which attested to the intensification of protein metabolism. In conclusion, we can mention that tested product improves metabolic health of quails, which was also proven by stopping the loss of body mass of birds during intensive egg laying.

Key words*: additive, creatinine, quail, protein, urea.*

INTRODUCTION

Breeding and utilization of animals, including poultry, involve measures aimed to maintain their health and welfare. Thus, animal husbandry, an important branch of the national economy, currently, faces with two essential problems: firstly, the increasing demand for animal products at the national and global levels, and secondly, the responsibility of animal owners to provide products that are of high quality, harmless, and accessible to consumers (Macari et al., 2014; Baltag, 2020). At the same time, in recent years, animal products with dietary and curative properties have become increasingly popular. Among these an important place belongs to products obtained from quails, namely eggs and meat (Pavlicenco, 2019; Khazaei et al., 2021). It is worth mentioning that the Japanese quail (*Coturnix japonica*), a domestic variety Japanese quail raised for meat and especially for eggs, prescribed in dietary nutrition, is widely distributed and utilized in most countries in the world (Ahmed et al., 2024). Simultaneously, it should be noted that the breeding and exploitation of quails for meat and eggs production under intensive conditions can be compromised by the impact of stress factors, which necessarily reflect on their health and productive potential. Over the past decades, in order to reduce and prevent the consequences of technological stress, livestock breeders have used different categories of compounds, including biologically active ones, administered to animals through different routes. These substances are selected for their anti-stress, adaptive and growth-stimulating properties (Simeanu, 2004; Galip & Seyidoolu, 2012; Pavlicenco, 2019; Pistol, 2025; Hamprakorn et al., 2025). The problem of intensifying and improving productive performance with the help of growth biostimulants has been studied for a long time (Georgescu et al., 1995). In addition, these authors were among the first to address issues related to the health implications and regulations of the use of biostimulants in animal husbandry. At the same time, international regulatory authorities are gradually imposing restrictions on the use of hormonal substances and antibiotics for this purpose. Currently, an increasing number of researchers are investigating new growth

stimulants of natural origin for animals (Ahmed et al., 2024; Maţencu, 2024; Hamprakorn et al., 2025). The purpose of this research was to evaluate the impact of phytomicrobial feed additive Omepol 30 on the protein metabolism and body mass evolution of adult quails, exploited under poultry farm conditions.

MATERIALS AND METHODS

Object of study

This study was conducted in poultry farm conditions, SRL "MIHAILORINA-COM", Rîşcani district, Republic of Moldova. The research object was a flock of 150 adult quails, breed - Texas universal, divided into three groups: control group (CG), experimental group with 0.25% additive (0.25% EG), experimental group with 0.5% additive (0.5% EG) and the phytomicrobial feed additive, coded Omepol 30 (Barley+Carrot+Exopolysaccharides). The Omepol 30 additive is composed of: dry barley biomass from beer industry wastes - 75%, dry carrot biomass from food industry wastes - 25%, exopolysaccharides of pigmented yeast *R. mucilaginosa* CNMN-Ys-10 of 30 mg g^{-1}d.w. Barley biomass is obtained from barley wastes remaining after beer production by mixing the biomass (waste) with distilled water in the 1:1 v/v ratio, autoclaving the mixture at temperature of 115°C, 0.5 atm. for 30 minutes, then separating the supernatant (barley water extract, used for microbial biotechnologies as nutrient medium) from the sediment (barley biomass) by centrifugation at 3000 rpm for 10 minutes. The sediment is subsequently dried at 50 ± 1°C until a constant mass is achieved. Carrot biomass is obtained from carrot wastes (peels) from the food industry, remaining after carrot processing, by repeatedly washing the peels to remove impurities, shredding them to sizes of 0.5-1.0 cm, and mixing the carrot peels with distilled water in the 1:1 v/v ratio, autoclaving the mixture at temperature of 115°C, 0.5 atm. for 30 minutes, then separating the supernatant (carrot water extract) from the sediment (carrot biomass) by centrifugation at 3000 rpm for 10 minutes, then sterilizing the supernatant (carrot water extract) in an autoclave at temperature of 115°C, 0.5 atm. for 30 minutes. The sediment (carrot biomass) is subsequently dried at 50 ± 1°C until a constant mass is achieved. The water carrot extract, with a pH of 5.6 and a residual sugar concentration of 5.0°Bx, was used as a nutrient medium, a sole source of nutrition, for the submerged cultivation of the yeast strain *R. mucilaginosa* CNMN-Ys-10 at a temperature of 27-28°C, under shaking conditions of 200 rpm for 120 hours in order to obtain exopolysaccharides in the culture liquid. The Omepol 30 additive is obtained by mixing dry barley biomass with dry carrot biomass in the 3:1 ratio and gradually soaking the resulting mixture (in several rounds) with the required volume of culture liquid containing exopolysaccharides from *R. mucilaginosa* CNMN-Ys-10, in order to obtain an exopolysaccharide concentration of 30 mg g^{-1}d.w. After each round the mixture is dried at temperature of +50±1°C to remove excess moisture. Following the final round, the product was dried at the same temperature until a constant mass was reached.

Feed rations

The Omepol 30 additive was administered to quails with food, separately for each experimental group, according to the experimental scheme showed in Table 1.

Table 1. Scheme of administration of the Omepol 30 additive to quails

Group	No. of birds	Route of administration	Dose, g kg^{-1} feed	Dosing regime
CG	50	-	-	-
0.25% EG	50	per os	2.5	daily
0.5% EG	50		5.0	

The birds used in the experiment were similar in terms of breed, age, physiological status, and body weight. In addition, the quails from all three groups involved in the experiment were housed in the same shelter, obviously with the same hygienic and technological indicators, including microclimate parameters, nutrition, watering and veterinary care, which ensured their hygiene and welfare. During the experiment, quails were monitored and examined to assess their health status, as well as possible adverse reactions to the studied product.

Monitored parameters

During the study, the behavior of quails and any possible adverse reactions following the use of the additive were assessed. Blood samples for biochemical examination were collected in three stages: at the beginning of the study, randomly, from 5 quails out of 150, at the 35th day of the experiment (first sampling) and at the end of the study, on the 71st day from the beginning of the experiment (second sampling), 5 quails from each group. In the blood serum collected from the birds, the essential biochemical parameters of protein metabolism were determined, such as total proteins, albumins, urea, uric acid, creatinine, some liver markers: ALT and AST transaminases, total bilirubin and its fractions, glucose content.

These tests were performed in the Laboratory of Biochemistry, at Nicolae Testemitanu State University of Medicine and Pharmacy, according to protocols optimized for use with the Synergy H1 Hybrid Reader multimode-microplate spectrophotometer, (BioTek Instruments, Winooski VT, USA) (Gudumac et al., 2010).

The evolution of the quail body mass was determined during the study by individual weighing of the birds at the beginning, 35th and 71st days of the experiment.

Statistical indices

Statistical processing of the results was performed using the MO Excel and Statistics 9.0 software suite. The results were expressed by calculating the mean, standard deviation and confidence interval for an average of three repetitions. All differences were considered statistically significant for $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$.

RESULTS AND DISCUSSIONS

Investigation of metabolism, including key parameters of basal metabolism, is a priority in testing new remedies (Putin et al., 2020; Khazaei et al., 2021; Pistol, 2025).

The dynamics of protein metabolism indicators in quails during the application of the Omepol 30 additive is presented in Table 2. According to the obtained results, the level of total proteins in blood serum decreased in CG at the first sampling by approximately 10% ($p<0.05$) compared to the beginning of the experiment, a similar trend also was established in 0.5% EG (-2.4%) compared to the beginning of the experiment. In contrast, in 0.25% EG at the first sampling, the analyzed parameter increased by 4.1% compared to the initial values. The tested additive favored an increase in total protein levels in both experimental groups at the first sampling, by 8.2-15.3% compared to the CG (for 0.25% EG, $p<0.05$). At the end of the study (second sampling), a positive dynamic in total protein levels was recorded in all groups compared to the first sampling. Comparison among groups showed higher values of the investigated parameter in both experimental groups compared to the CG, with an increase of 6.5-15.2% (for 0.25% EG, $p<0.01$) (Table 2). Similar trends of increased protein content in the blood of animals, under the influence of biologically active compounds, have been reported in several scientific studies (Ahmed et al., 2024; Olgun et al., 2024; Pistol, 2025; Elbaz et al., 2025).

The serum albumin level at the first sampling in the CG, showed a significant decreased tendency of 16.1% ($p<0.05$) compared to the initial values, the tendency that was also established in the 0.5% EG (-12.5%) compared to the initial values. At the same time, the investigated parameter at the 0.25% EG, in contrast, showed a slight increase of 1.8% compared to the initial values. It is worth noting that albumin serum level in the blood at the first sampling in the experimental groups has a tendency to increase by 4.2-21.3% compared to the CG (for 0.25% EG $p<0.01$). At the second sampling, serum albumin levels in the experimental groups showed a slight tendency to decrease by 0.7-3.1% compared with the CG; however, no statistically significant differences were observed (Table 2). The close values of this parameter indicate a uniform physiological state of the liver in all groups, highlighting the absence of adverse reactions at the level of this organ in quails from the experimental groups. The concentration of urea, the final product of protein metabolism, at the end of the experiment was higher in the experimental groups by 6.3-13.6% compared to the control, but the differences were not statistically

significant (Table 2). Higher values of urea in the blood serum were also reported in broilers treated with BioR and Catosal (Macari et al., 2014; Putin, 2014).

The values of uric acid, resulting from the decomposition of purines in the serum of quails, at the first sampling, had a tendency to increase in birds from the CG and 0.25% EG compared to the initial values. At the end of the experiment (second sampling), the values of uric acid decreased in all groups compared to the initial values. At the same time, a decrease of uric acid of 1.9-5.8% was attested, in the experimental groups compared to the CG, indicating a more efficient and appropriate metabolism in the birds that consumed the additive (Table 2).

Table 2. Dynamics of protein metabolism indices in the blood serum of quails from different groups

Parameter	Debut	Group		
		CG	0.25% EG	0.5% EG
Total proteins, g L^{-1}	45.41±0.86	1 (samp.) 40.98±1.33* 2 (samp.) 45.14±1.74	1 (samp.) 47.25±1.34* 2 (samp.) 52.00±0.7**	1 (samp.) 44.32±1.63 2 (samp.) 48.07±0.98
Albumins, g L^{-1}	16.38±0.79	1 (samp.) 13.75±0.46* 2 (samp.) 15.62±0.55	1 (samp.) 16.68±0.5** 2 (samp.) 15.14±0.21	1 (samp.) 14.33±0.67 2 (samp.) 15.51±0.16
Urea, mmol L^{-1}	9.31±0.27	1 (samp.) 9.50±0.35 2 (samp.) 9.07±0.17	1 (samp.) 10.31±0.69 2 (samp.) 10.30±0.45	1 (samp.) 9.45±0.35 2 (samp.) 9.68±0.42
Uric acid, µmol L^{-1}	391.8±12.6	1 (samp.) 404.0±22.9 2 (samp.) 390.96±12.5	1 (samp.) 415.4±18.51 2 (samp.) 368.23±10.6	1 (samp.) 387.46±6.36 2 (samp.) 383.47±8.58
Creatinine, µmol L^{-1}	223.±10.4	1 (samp.) 175.5±9.4** 2 (samp.) 178.2±4.4	1 (samp.) 210.39±29.9 2 (samp.) 209.6±3.71***	1 (samp.) 169.16±4.6** 2 (samp.) 192.90±4.86*
Glucose, mmol L^{-1}	5.99±0.12	1 (samp.) 5.77±0.05 2 (samp.) 5.97±0.14	1 (samp.) 6.18±0.24 2 (samp.) 5.92±0.13	1 (samp.) 5.83±0.06 2 (samp.) 6.03±0.08

Note: * - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$

The average serum creatinine value at the first sampling had a negative dynamics compared to the debut in all groups. Thus, this parameter decreased significantly ($p<0.01$) at the CG by 21.5% and in the 0.5% EG by 24.3% compared to the debut value. In the 0.25% EG this decrease was less than 5.8%, with no statistically significant differences compared with the reference value (Table 2). Thus, it can be noted that the tested additive, in the minimum dose of 0.25%, prevented the decline in creatinine level. At the end of the experiment (second sampling), the creatinine concentration in the CG and 0.5% EG increased, compared with those recorded at the first sampling, whereas in the 0.25% EG the values remained practically constant (-0.4%).

Comparative analysis of creatinine concentration among groups at the end of the study established that this index in the experimental groups was significantly ($p<0.001$) higher than the control of 17.6% in the 0.25% EG and of 8.2% ($p<0.05$) in the 0.5% EG (Table 2). This phenomenon is considered beneficial and reflects the muscle mass of the animals (Nazarenko & Kishkun, 2006). Several recent studies have reported higher serum creatinine levels in birds following treatment with various bioactive compounds (Pavlicenco, 2019; Putin et al., 2020; Pistol, 2025). The intensively exploited animal organism requires substantial energy support to ensure the normal development of metabolic processes. During the first sampling, the blood glucose concentration in quails showed a slight decrease compared with the initial values: by 3.7% in the CG and 2.7% in the 0.5% EG. In contrast, quails in the experimental group consumed 0.25% additive (0.25% EG), this parameter increased by 3.2%, although the differences were not statistically significant. By the end of the experiment (second sampling), the glucose levels were practically identical across all groups, ranging among 5.92±0.13 - 6.03±0.08 mmol L^{-1}. These results indicate the absence of adverse physiological reactions in the birds to the tested additive (Table 2).

The role of liver in protein metabolism is well established, and the importance of liver markers in characterizing this process and assessing the effects of tested products on the animal organism is incontestabil. The activity of ALT and AST transaminases, the concentration of bilirubin and its fractions in the serum of quails from different groups are presented in Table 3. The activity of ALT enzyme at the beginning of the research was 42.62 ± 2.53 u L^{-1}. The ALT activity at the first sampling had a moderate tendency to decrease by 6.6-8.0% in the CG and 0.5% EG compared to the initial values, and in the 0.25% EG it remained practically constant of 42.09 ± 3.03 u L^{-1}, compared to the reference value. At the end of the experiment (second sampling) the ALT enzyme activity showed an increasing trend in

all studied groups: CG of 10.5%, 0.25% EG of 7.8% and 0.5% EG of 30.8% compared to the values established at the first sampling, for 0.5% EG the difference being statistically significant ($p<0.001$) (Table 3). At the same time, at the end of the experiment, the ALT activity in the experimental groups was insignificantly higher by 3.1% in the 0.25% EG and significantly ($p<0.05$) by 22.2% in the 0.5% EG, compared to the CG (Table 3). The evaluation of the aspartate aminotransferase (AST) activity during the study, as well as between the groups, did not identify the existence of manifestations that would indicate a negative impact of the tested product on the body and especially on the functional state of the liver. However, some divergences in the physiological limits are established. Thus, the AST value in the blood of quails from the CG at the first sampling, denoted a true tendency ($p<0.01$) of increase to 33.27 ± 1.18 u L^{-1}, compared to the initial values, which is 19.2% more (Table 3).

Table 3. Liver marker indices in quail serum from different groups

Parameter	Debut	Group		
		CG	0.25% EG	0.5% EG
ALT, u L^{-1}	42.62±2.53	1 (samp.) 39.81±2.77 2 (samp.) 44.00±1.15	1 (samp.) 42.09±3.03 2 (samp.) 45.37±2.51	1 (samp.) 39.23±1.31 2 (samp.) 51.33±1.87*
AST, u L^{-1}	27.91±1.05	1 (samp.) 33.27±1.2** 2 (samp.) 33.33±1.04	1 (samp.) 25.78±0.47 2 (samp.) 28.44±1.45*	1 (samp.) 27.52±1.01 2 (samp.) 28.00±2.34
Total bilirubin, µmol L^{-1}	36.88±2.17	1 (samp.) 33.97±0.86 2 (samp.) 30.57±2.20	1 (samp.) 29.12±2.85 2 (samp.) 34.94±2.03	1 (samp.) 30.09±3.50 2 (samp.) 46.10±2.10***
Direct bilirubin, µmol L^{-1}	23.57±1.86	1 (samp.) 22.05±1.56 2 (samp.) 21.14±2.39	1 (samp.) 22.86±2.67 2 (samp.) 20.52±2.58	1 (samp.) 22.29±2.93 2 (samp.) 31.43±2.26*
Indirect bilirubin, µmol L^{-1}	13.31±1.29	1 (samp.) 11.92±0.76 2 (samp.) 9.43±1.10	1 (samp.) 6.26±0.7*** 2 (samp.) 14.42±1.22*	1 (samp.) 7.80±1.17* 2 (samp.) 13.08±0.70

Note: * - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$

In contrast, birds from the experimental groups at the first sampling, showed a slight decrease in AST activity by 1.4-7.6% compared to the beginning of the experiment, which indicates that the tested additive exhibits a hepatoprotective effect, with the 0.25% minimum concentration ensuring a maximal effect (Table 3).

The AST activity analysis at the end of the study (second sampling) is of considerable importance in highlighting the action of the tested product on quails, and especially on the liver function. The data of this enzyme at the second sampling, compared to the values established at the first one, exhibits a tendency to increase in all studied groups. In the 0.25% EG, the increase was maximal, up to 28.44 ± 1.45 u L^{-1} by 10.3%. In the CG and 0.5% EG groups, insignificant increases were also observed, reaching 33.33 ± 1.04 and 28.00 ± 2.34 u L^{-1}, respectively (Table 3).

At the second sampling, AST activity in the CG was 33.33 ± 1.04 u L^{-1}, and in the 0.25% EG and 0.5% EG of 28.44 ± 1.45 and 28.00 ± 2.34 u L^{-1}, respectively, which represents a decrease by 14.7-16.0% compared to the control group, for the 0.25% EG this decrease being significant ($p<0.01$) (Table 3). The minimal of the AST activity in the experimental groups can be classified as positive and indicates the beneficial impact of the tested product on the functional state of the liver in experimental quails. Similar effects in the blood of birds and animals were established when quails (Alagawany et al., 2021; Negm et al., 2025) and rabbits (Mohamed et al., 2025) were treated with biologically active compounds.

The total bilirubin level in birds in the CG decreased to 33.97 ± 0.86 µmol L^{-1} at the first sampling by 7.9% compared to the initial values. The decrease in total bilirubin by 18.4-21.0% compared to the initial values was also observed in the experimental groups, where this decrease was significant ($p<0.05$) (Table 3). At the same time, compared to the control at the first sampling a significant decrease ($p\geq0.05$) of total bilirubin in the experimental groups by 11.4-14.3% was established (Table 3). At the second sampling, the total bilirubin level in the CG decreased to 30.57 ± 2.20 µmol L^{-1} by 10.0% compared to the values recorded at the first sampling. In the experimental groups the total bilirubin content increased by 19.9-53.2% compared to the values from the first sampling, and constituted 34.94 ± 2.03-46.10 ± 2.10 µmol L^{-1}, for the 0.5% EG the difference being significant ($p<0.01$). At the same time, these values are of 14.3-50.8% higher than the values in the CG at the second sampling, significant ($p<0.001$) for the 0.5% EG. Significant differences in total bilirubin content in the CG and 0.25% EG were not observed (Table 3).

Similar effects in serum bilirubin levels under the influence of biologically active substances have also been reported in animals (Mațencu, 2019).

At the first sampling, the level of direct bilirubin (conjugated, metabolized in the liver) has a moderate tendency to decrease to 22.05 ± 1.56 µmol L^{-1} compared to the initial values in all groups, with a maximum (-6.4%) in birds in the CG. The decreased values in the experimental groups were more moderate of -3.01% in the 0.25% EG and of -5.4% in the 0.5% EG (Table 3). At the second sampling direct bilirubin in the CG constituted 21.14 ± 2.39 µmol L^{-1} by 4.12% lower compared to the first sampling, and in the 0.25% EG of 20.52 ± 2.58 µmol L^{-1}, decreasing by 10.2% compared to the reference value. In quails in the 0.5% EG, with the maximum dose of the tested product, direct bilirubin was significantly higher than the CG and 0.25% EG values ($p<0.05$) (Table 3). The minimum of direct bilirubin values in the 0.25% EG, after the second sampling, is a beneficial phenomenon, and indicates that the additive in this concentration contributes to strengthening the functional state of the liver.

A valuable parameter in the evaluation of the functional state of the liver is also indirect bilirubin (free, unconjugated). At the first sampling, a decreasing trend in indirect bilirubin was observed compared with the initial values in all three groups. In the CG the decrease was 10.4% and the indirect bilirubin value was 11.92±0.76 µmol L^{-1} (Table 3). In the experimental groups at the first sampling, the decreases in indirect bilirubin compared to the initial values were significant ($p<0.01$) for the 0.25% EG and ($p<0.05$) for the 0.5% EG, and the values were 6.26±0.71 and 7.80±1.17 µmol L^{-1}, respectively. These values are also significantly lower, ($p<0.001$) for the 0.25% EG and ($p<0.05$) for the 0.5% EG, compared to the CG value (Table 3). At the second sampling, in birds from the CG, a significant decrease ($p<0.05$) of this parameter was recorded, being of 20.9% compared to the first sampling. At this stage, the level of indirect bilirubin in the bloodstream was significantly higher ($p<0.05$) in the 0.25% EG group (Table 3).

The obtained results indicate that the additive in the minimum dose of 0.25%, beneficially influences the metabolic health of quails in states of stress and high metabolic demands, especially liver function, intervening as a natural adaptogenic product.

In the complex assessment of the influence of biologically active compounds on farm animals and poultry, it is very important to monitor essential zootechnical parameters, such as: body weight, egg laying intensity, daily gain, viability, feed conversion, etc. (Balanescu et al. 2019; Khazaei et al. 2021; Miao et al. 2021; Macari et al., 2024; Pistol, 2025). According to the cited sources the animal productive parameters are essential in evaluating and explaining the results obtained in the process of hematological and biochemical investigations for establishing the optimal dose and administration regimen of the tested products.

In our study, the effect of the Omepol 30 phytomicrobial additive on body mass indices in quails during the full egg-laying process was investigated. At the beginning of the study, the body mass of quails from different groups did not differ significantly and varied between 291.3 ± 5.5 and 292.2 ± 5.7 g (Table 4).

Table 4. Influence of the Omepol 30 additive on productive indices of quails

Indices	Group		
	CG	EG 0.25%	EG 0.5%
Nr. quails /debut, head	50	50	50
Body mass/debut, g	292.2±5.7	291.3±5.5	291.5±7.0
Body mass at 35 days, g	261.7±7.1**	278.8±9.5	268.4±6.6**
Total gain /debut - 35 days, g/head	-31.12	-12.50	-23.11
Nr. quails /at 71 days, head	40	42	41
Body mass at 71 days, g	265.8±8.6	294.0±6.6**	285.6±8.8
Total gain /35 - 71 days, g/head	-26.4	2.70	-5.9
Average daily gain /period of 71 days, g	-	0.038	-
Viability, %	80.0	84.0	82.0

Note: ** - $p<0.01$

At 35 days the body mass dynamics had a negative expression, but with different values. During this period, birds in the CG and 0.5% EG lost an average of 31.12 and 23.11 g respectively ($p<0.01$), in body weight compared to the beginning of the study, and in the 0.25% EG this decrease of body mass was less pronounced and constituted 12.50 g (Table 4). At the end of the experiment (71 days) the

body mass of the quails increased compared to the 35th day in all groups. In the CG the average mass was 265.8 ± 8.6 g, insignificantly higher (1.6%) compared to the value at 35th day. In the 0.25% EG and 0.5% EG in the same period the body mass was 294.0 ± 6.6 and 285.6 ± 8.8 g with increases of 5.5 and 6.4% compared to the reference values (Table 4). Thus, we can mention that at the end of the experiment, the body mass of the quails from the 0.25% EG and 0.5% EG was 10.6 ($p<0.01$) and 7.5% higher respectively compared to the quails from the CG. It should be noted that the minimum concentration of 0.25% is the only one that ensures at the end of the experiment a positive gain in the weight of the birds of 2.70 g/head, and a maximum flock viability of 84%. In the CG and 0.5% EG during the experiment, the quails lost body mass, the total gain being negative, and the viability of the flock were 80 and 82% respectively (Table 4). The beneficial impact of biologically active products of different nature on the body mass and viability of birds has been established by several authors (Balanescu et al., 2019; Pavlicenco, 2019; Ahmed et al., 2024; Pistol, 2025).

CONCLUSIONS

On balance, supplementation of the quail ration, exploited in poultry farm conditions for meat and eggs, with the Omepol 30 feed additive, obtained from residual biomass of barley and carrot from the food industry and exopolysaccharides of pigmented yeast *Rhodotorula mucilaginosa*, does not induce adverse reactions, has a positive effects the health and welfare of birds. The additive in the concentration of 0.25 and 0.5% significantly influences the basal metabolism, especially protein metabolism, by increasing the content of total proteins and albumins in the blood, also reflected in increasing urea and decreasing uric acid levels. The additive significantly increases the concentration of creatinine in serum, a beneficial phenomenon, correlated with the body muscle mass and influences the functional state of the liver, directly involved in the development of metabolic processes, by modifying the concentration of ALT, AST and bilirubin. Despite that, during the experimental period, the values of the indices evaluated in the serum were within the limits of physiological norms. The positive influence of the additive, in the 0.25% concentration on the protein metabolism of quails and the functional state of the liver is also confirmed by the flock viability and the dynamic of the body mass of quails, exploited in poultry farm conditions.
The scale-up of the additive use requires further research to optimize the concentration range, as well as the period and duration of administration, in order to maximize quail performance.

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ASSESSMENT AND CLASSIFICATION OF MINERAL OIL CONTAMINATION IN FEED AND MILK IN RELATION TO LEGISLATIVE BENCHMARKS

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Abstract

Contamination of animal feed and food with mineral oil hydrocarbons (MOH), particularly the MOSH (saturated) and MOAH (aromatic) fractions, is becoming an increasingly important issue for food safety due to the absence of harmonized and binding legislative limits at the European level. This study aims to assess and classify MOH contamination in animal feed and milk in relation to current legislative benchmarks, combining a literature-based approach with an applied analysis. Different relevant references were reviewed to evaluate the current regulatory framework and its limitations. In addition, 32 feed samples and 16 milk samples collected from farms in North-Eastern Romania were analyzed for MOSH and MOAH contamination levels using previously validated LC-GC-FID methodology. The results were assessed in relation to existing guideline values and further interpreted through hypothetical regulatory scenarios. A classification framework for contamination levels was proposed, highlighting a high proportion of non-compliant samples. The findings emphasize the need for legislative measures and improved monitoring across the entire agri-food chain.

Key words*: contamination, food safety, legislative benchmarks, mineral oil.*

INTRODUCTION

The presence of mineral oil hydrocarbons (MOH) in a wide range of matrices, along with their potential toxicity for both animal and human organisms, has attracted increasing scientific attention across multiple fields, including food products, animal feed, and other consumer goods (Pirow et al., 2019; Nestola, 2022; Moret et al., 2025). Although MOH have been detected in the environment for more than three decades (Grob et al., 1991), their occurrence was initially not considered a major concern. However, as toxicological studies have highlighted their potential for bioaccumulation and possible carcinogenic effects, the perception of MOH has shifted, and they are now recognized as contaminants of growing concern (Grob, 2018).

In Europe, recent incidents involving MOH contamination have raised concern among consumers, particularly following the detection of mineral oil hydrocarbons in various food products (Foodwatch, 2019). However, uncertainties regarding actual exposure levels and associated health risks persist, highlighting the need for a more consistent legislative approach. MOH exhibit variable toxicity, with the aromatic fraction (MOAH) being of particular concern due to its potential genotoxic effects (Grob, 2018; Pirow et al., 2019; Nestola, 2022; Srbinovska et al., 2022).

Since 2008, following the identification of elevated levels of MOH contamination in certain food products and food contact materials, the perception of these contaminants has undergone important changes. A key event was the detection of MOH contamination in batches of sunflower oil exported from Ukraine to several European Union countries, which led to the introduction of temporary maximum limits of 50 mg/kg (Biedermann & Grob, 2009; EFSA, 2012). In response to such incidents, the European Food Safety Authority (EFSA, 2012) published in 2012 a scientific opinion on the presence of MOH in food, highlighting the need for further investigations and more effective control measures, particularly in the context of limited data for a comprehensive risk assessment (EC, 2017). This milestone marked

a turning point in both research and regulatory approaches, prompting European authorities to initiate monitoring programs and to establish indicative, rather than binding, reference values.
Current European Union legislation and international recommendations provide guidance on acceptable levels of mineral oil hydrocarbons (MOH); however, their implementation remains largely at the discretion of individual Member States. As of July 2024, EU legislation concerning MOH, particularly MOAH, is still under development, with a preliminary draft regulation, indicative and non-binding in nature, proposed for MOAH in food, with the objective of its future integration into the European Union Contaminants Regulation (EU) 2023/915 (EC, 2023; Sante, 2025).
Although several European countries have attempted in recent years to implement stricter measures to control MOH contamination, most of these initiatives have faced many limitations and have not been translated into binding legislative standards, remaining instead as scientific references for future regulatory developments. The absence of clearly defined and enforceable limits restricts the ability of both producers and authorities to effectively prevent contamination, while the establishment of mandatory regulations could contribute to improved public health protection and support the maintenance of a safe and compliant agri-food sector. The MOSH/MOAH issue continues to evolve, particularly due to the involvement of key regulatory bodies such as the European Food Safety Authority and the European Commission. Since the publication of the EFSA scientific opinion in 2012 and its subsequent updates (EFSA, 2012; EFSA et al., 2019; EFSA 2023), legislative discussions have become more structured and application-oriented. However, many gaps remain, including the lack of maximum limits across all product categories and the inconsistency between recommended guideline values and enforceable legislation. Although MOH contamination may affect multiple stages of the agri-food chain, the present study specifically focuses on feed and milk contamination in relation to current legislative benchmarks.
This study addresses the issue of mineral oil hydrocarbon (MOH) contamination in the context of feed and food safety, with a particular focus on existing legislative limitations. The main objective is to provide an integrated approach combining a review of the current regulatory framework with an applied assessment of MOSH and MOAH contamination levels in feed and milk. Based on experimental data and existing legislative benchmarks, the study further aims to develop a classification framework for contamination levels, allowing for a structured interpretation of compliance and potential risk.

MATERIALS AND METHODS

This paper combines a literature review and an applied experimental evaluation aiming to provide a comprehensive perspective on mineral oil hydrocarbon (MOH) contamination and its legislative implications. The literature review focused on assessing the current regulatory framework and toxicological aspects related to MOH, based on specific scientific publications and institutional reports. Sources were systematically selected from the main scientific databases and official documents addressing MOH contamination in feed and food, as well as related risk assessment and legislative developments. Togheter, an applied study was conducted using contamination data obtained from selected samples along the feed and dairy sector. The experimental component aimed to assess the levels of MOSH and MOAH contamination in relation to existing legislative benchmarks.

Literature review

A comprehensive literature review was conducted to assess mineral oil hydrocarbon (MOH) contamination, with particular emphasis on toxicological aspects and current legislative limits. In order to capture regulatory developments and new legislative trends, specific studies and reference reports addressing MOH contamination in food and feed, as well as its implications for food safety, were selected (Figure 1).
The literature search covered publications and official documents published between 2008 and 2025, with particular attention given to studies published after the EFSA scientific opinion issued in 2012. The sources included scientific publications accessed through major online

databases such as PubMed, ScienceDirect, Web of Science, Scopus, CAB Direct or Google Scholar, as well as institutional resources from the association institution's documentary fund, such as the Anelis Plus Database.

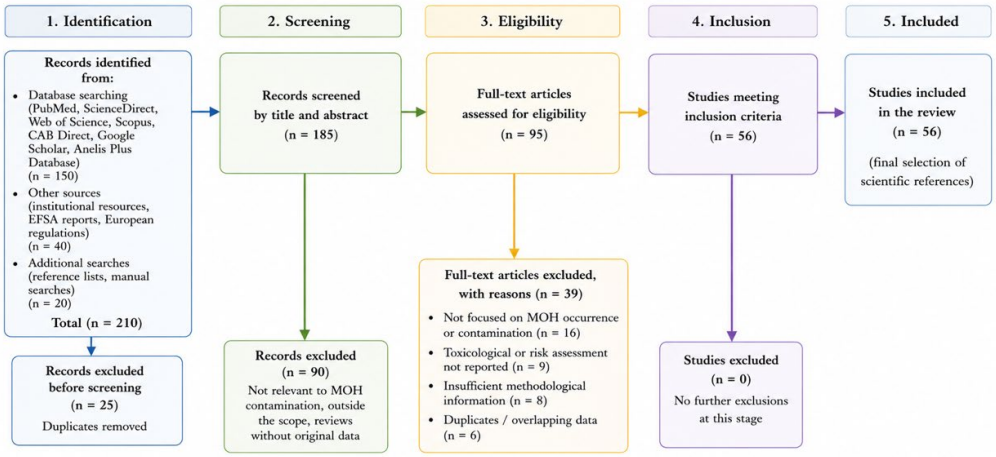


Figure 1. PRISMA flow diagram of the literature selection process addressing MOH contamination in food and feed (original)

In addition, authoritative documents such as the assessments issued by EFSA and other relevant European regulations were taken into account.

To ensure comprehensive coverage of the topic, various combinations of keywords were used during the search process, such as: "feed and food safety", "mineral oil hydrocarbon", food "contaminant", "MOSH", "MOAH", "exposure and risk assessment". The inclusion criteria considered studies focused on MOH occurence, toxicological assessment, food and feed contamination, risk asessment, and legislative aspects related to the feed and milk production chain. Duplicate publications, studies lacking sufficient methodological informationm and papers not directly related to MOH contamination were excluded from the final selection. Following the processes of identification, screening and evaluation, a total of 56 scientific references were selected and included in this study.

Data sources and analytical methodology

An applied study was conducted on a total of 32 feed samples and 16 milk samples collected from different crop farms and dairy farms in North-Eastern Romania (Table 1). The samples were analyzed to determine the levels of MOSH and MOAH contamination in order to assess their compliance with current indicative legislative limits. The sampling procedures and analytical methods applied for both feed and milk followed the protocols described in previous studies. The contamination data used in the present study were derived from experimental investigations previously carried out by the authors. Detailed information on the origin of the samples, sampling techniques and analytical procedures have been reported in previous works (Matei & Pop, 2023; Matei et al., 2024a; 2024b) and were adapted to the objectives of the present study.

Table 1. Identification and coding of the analyzed feed and milk samples

Samples					
Name	Code	Name	Code	Name	Code
Feed samples				Milk samples	
Mountain pasture F-MP	F-MP-A	Corn grains F-C	F-C-A	Milk 1	M1-A
	F-MP-B		F-C-B		M1-B
Natural hay F-NH	F-NH-A		F-C-C	Milk 2	M2-A
	F-NH-B		F-C-D		M2-B
Alfalfa hay F-AH	F-AH-A	Triticale F-T	F-T-A	Milk 3	M3-A
	F-AH-B		F-T-B		M3-B
	F-AH-C	Soya F-S	F-S-A	Milk 4	M4-A
	F-AH-D		F-S-B		M4-B
Corn silage F-CS	F-CS-A		F-S-C	Milk 5	M5-A
	F-CS-B		F-S-D		M5-B
	F-CS-C	Sunflower meal F-SS	F-SS-A	Milk 6	M6-A
	F-CS-D		F-SS-B		M6-B
Alfalfa silage F-AS	F-AS-A	Combined feed F-CF	F-CF-A	Milk 7	M7-A
	F-AS-B		F-CF-B		M7-B
Brewer's spent grain F-BSG	F-BSG-A		F-CF-C	Milk 8	M8-A
	F-BSG-B		F-CF-D		M8-B

Data interpretation and classification approach

The results obtained from previous experimental studies were compared with currently available reference values. In order to better assess the potential impact of future legislative developments, this analysis was based on hypothetical scenarios that consider the potential tightening of proposed legislative limits at European Union level (Bratinova & Hoekstra, 2019; Bratinova et al., 2023). These scenarios enabled the identification of non-compliant samples and the estimation of the proportion of samples that would require additional control measures. Based on this comparative analysis, a classification framework for MOH contamination levels was developed. The proposed classification consists of grouping the analyzed samples into distinct contamination ranges, based on their relative position with respect to existing legislative reference values. This classification allows a structured interpretation of contamination severity, facilitating the differentiation between compliant, borderline and non-compliant samples.

The classification system can support both risk assessment and decision-making processes by providing a practical tool for assessing contamination levels in feed and milk. Additionally, it enables the extrapolation of current results under more stringent regulatory scenarios, providing insight into potential future compliance challenges.

RESULTS AND DISCUSSIONS

MOH in feed and milk

The presence of mineral oil hydrocarbons (MOH) in the feed and milk production chain is a major concern, having been reported in both plant and animal-derived products (Srbinovska et al., 2023; EFSA, 2012). Contamination can occur at several stages of the production and processing chain of feed and milk, either through direct anthropogenic sources or through migration from materials used during processing (Lăpușneanu et al., 2023; Ciobotaru et al., 2024; Gucianu et al., 2023; Manoliu et al., 2024), transport or packaging (Van Heyst et al., 2020). According to the scientific literature, MOH levels and composition vary considerably depending on the food matrix and the source of contamination (Bratinova & Hoekstra, 2019). Animal products, such as milk and meat, are considered particularly vulnerable due to the potential transfer of MOH from contaminated feed (Bauwens et al., 2022). In addition, vegetable oils, fish products and cereals have also been identified as among the most affected categories, with direct implications for food safety and public health (EFSA, 2012).

Recent studies indicate that air pollution is an important source of MOH contamination, resulting from industrial, agricultural and urban activities (Van Heyst et al., 2020). However, evidence regarding the exclusive contribution of environmental sources to food contamination remains limited, especially for the MOAH fraction, which tends to undergo rapid oxidation when exposed to atmospheric conditions (Menegoz Ursol et al., 2022). Many of the observed contamination patterns may also be influenced by technological factors, including processing conditions and material interactions. Previous studies have shown that technological interventions, such as the incorporation of artificial additives, can alter the physicochemical properties of food products, indirectly affecting their safety profile (Flocea et al., 2024; Gucianu et al., 2024). These findings are consistent with previous research highlighting the influence of technological factors on the quality and safety of animal-derived products (Ciobotaru et al., 2024). In addition to environmental and technological sources, food contact materials, particularly packaging systems, represent an important pathway for contamination. Recent advances in food product development (Manoliu et al., 2025; Coșarcă et al., 2025) and smart packaging technologies have been increasingly focused to improve food safety and traceability. Innovative approaches, such as the development of functional food products and the implementation of smart packaging systems, contribute to enhanced product stability and consumer protection (Anchidin et al., 2024a; 2024b). However, these technological advancements may also introduce new challenges related to material composition and potential contaminant migration (Davidescu et al., 2025).

In terms of animal production, due to their lipophilic nature, MOH have the ability to

accumulate in the adipose tissues of animals, while feeding contaminated feed is considered a primary route of exposure (Grob, 2018). In addition, the MOSH fraction is known to accumulate in the liver and other organs, while MOAH present carcinogenic potential due to their structural similarity to polycyclic aromatic hydrocarbons (PAH) (Nestola, 2022). The presence of contaminants in animal derived products is not limited to MOH, as previous studies have reported the accumulation of various toxic elements in animal tissues, emphasizing the broader context of food safety risks (Boișteanu et al., 2023; Boișteanu et al., 2024; Ciobanu et al., 2020).

Since 2011-2012, the potential toxicity of mineral oil hydrocarbons (MOSH and MOAH) has attracted the attention of regulatory authorities, leading to the initiation of investigations into their presence in the food sector (EFSA, 2012). In the absence of sufficient data to support a comprehensive risk assessment, regulatory bodies established temporary reference limits. These limits have been periodically recommended and updated, and are referred to in documents such as the European Union Recommendation 2017/84 (EC, 2017); guidelines issued by the German Federal Ministry for Food, Agriculture and Consumer Protection (BMEL, 2020), as well as proposals from the Consumer Protection Consortium of the Federal States (LAV) and the German Food Federation (BLL, 2022).

Germany has been one of the most active countries in addressing MOH contamination. In 2011, the German Federal Ministry of Food and Agriculture (BMEL) and the German Federal Institute for Risk Assessment (BfR) published legislative proposals and scientific opinions on the risk assessment associated with MOSH and MOAH, highlighting the need for a clearer regulatory framework.

Various measures aimed at establishing maximum acceptable levels of MOH contamination have been initiated over time, one of the most notable being the Joint Research Centre (JRC) Guidance Document. This document, developed by Bratinova & Hoekstra (2019), proposed a procedural, step-by-step set of measures necessary to maintain acceptable level of MOSH and MOAH contamination in different products. However, despite these coordinated efforts, several challenges persist, including the lack of harmonisation of analytical protocols, the absence of validated and standardised methods and variability in data interpretation. These limitations complicate the integration of analytical results and undermine the reliability of contamination control (Bratinova et al., 2023).

Legislative proposals and expert opinions regarding reference levels for MOSH and MOAH contamination, presented in Table 1, reflect the evolution of regulatory actions over time. Although some of these proposals have not been officially implemented, more recent initiatives, such as the JRC Guide (Bratinova & Hoekstra, 2019) or documents developed at the level of the European Commission (2022) and EFSA (2023), continue to be used as reference models for establishing safety limits. The data presented in Table 2 were processed based on the work of Menegoz Ursol (2022).

Between 2011 and 2017, the German Federal Ministry of Food and Agriculture (BMEL) issued three additional legislative ordinances proposing various recommended maximum limits for MOSH and MOAH contamination in food. In parallel, the European Union, through Commission Recommendation 84/2017, requested the collection of additional data, with a particular focus on the most toxic MOAH fractions. According to the scientific literature, an important milestone in the development of legislative criteria for MOSH and MOAH was reached in 2019 with the publication of the JRC technical reports (JRC Guidelines), prepared by Bratinova & Hoekstra (2019) under the auspices of the European Commission. This guideline included recommendations for the detection and quantification of MOH, along with indicative limits, and has since become a reference framework for monitoring these contaminants.

In Germany, control authorities within the Working Group of the Federal States on Consumer Protection, together with the Food Federation, suggested the use of reference safety levels developed as part of a joint data collection project initiated in 2016. Although these limits were not based on toxicological data or exposure assessments, they were considered sufficiently protective for a wide range of food products (Menegoz Ursol, 2022).

In May 2022, the Standing Committee on Plants, Animals, Food and Feed, Section Novel Foods

and Toxicological Safety of the Food Chain of the European Commission issued specific recommendations on the maximum levels of MOAH permitted in food, setting limits raging from 0.5 mg/kg to 2 mg/kg, depending on the fat content of the products (EC, 2022). Although the Commission recommends the withdrawal or recall of products exceeding these limits, these requirements are not legally binding and remain at the discretion of Member States in terms of their application (Srbinovska et al., 2023; EC, 2022; Bratinova et al., 2023).

Table 2. Legislative proposals on MOSH/MOAH reference levels

YEAR	AUTHORITY / LEGISLATIVE PROPOSAL	MAXIMUM LIMIT (mg/kg)	REFERENCES
2011	Ordinance I of the German Federal Ministry of Food and Agriculture (BMEL)	MOSH < 0.6 MOAH nd (< 0.15)	Menegoz Ursol, 2022; Luisi, 2019
2011	Opinion of the German Federal Institute for Risk Assessment (BfR)	MOSH $C_{10–16}$ < 12.0 MOSH $C_{16–20}$ < 4.0	
2012	European Food Safety Authority (EFSA)	-	EFSA, 2012
2013	Ordinance II of the German Federal Ministry of Food and Agriculture (BMEL)	MOAH nd (< 0.15)	Menegoz Ursol, 2022; Luisi, 2019
2014	Ordinance III of the German Federal Ministry of Food and Agriculture (BMEL)	MOSH $C_{20–35}$ < 2.0 MOAH $C_{16–35}$ < 0.5	
2017	Ordinance IV of the German Federal Ministry of Food and Agriculture (BMEL)	MOAH < 0.5	
	Provisions of the Scientific Committee of the Belgian Federal Agency for the Safety of the Food Chain (FASFC) on action thresholds	MOSH $C_{16–35}$ = 5.0 – 150.0 MOAH $C_{16–35}$ < 0.5	
2019	JRC Technical Reports "Guidance on sampling, analysis and reporting of data for the monitoring of mineral oil hydrocarbons in food and food contact materials"	MOSH & MOAH 0.5 mg/kg (< 4 % CF) 1.0 mg/kg (> 4 % CF) 2.0 mg/kg (fats)	Bratinova & Hoekstra, 2019
2020	Provisions of the German Food Federation (BLL) on reference levels	MOSH = 4.0–13.0	Menegoz Ursol, 2022
	Ordinance V of the German Federal Ministry of Food and Agriculture (BMEL)	MOAH < 0.5	
2021	State Working Group for Consumer Protection (LAV) & German Food Federation (BLL)	MOSH = 13.0 MOAH < LOQ	
2022	European Commission, Standing Committee on Plants, Animals, Food and Feed, Section Novel Foods and Toxicological Safety of the Food Chain	MOSH & MOAH 0.5 mg/kg (< 4 % CF) 1.0 mg/kg (> 4 % CF) 2.0 mg/kg (fats))	EC, 2022; Bratinova et al., 2023
2023	European Food Safety Authority (EFSA) European Commission (EC) ▪ draft regulation aimed at integrating MOAH limits into the European Regulation on food contaminants (EU) 2023/915	MOSH & MOAH 0.5 mg/kg (< 4 % CF); 1.0 mg/kg (> 4 % CF; ≤ 50 % CF) 2.0 mg/kg (fats) > 50 % CF)	EFSA, 2023
2025	Revision 7 of the SANTE PLAN 2023/2345 (draft not adopted or endorsed by the European Commission) released by the Directorate-General for Health & Food Safety	specific limits of 2 mg/kg are planned for cocoa beans and cocoa mass	Sante D.G., 2025
2027		1 mg/kg MOAH, cocoa powder 4 mg/kg MOAH, cocoa butter (Jan. 1st, 2027)	
2028		2 mg/kg MOAH, cocoa beans and mass (Jan. 1st, 2028)	
2030		2 mg/kg MOAH, cocoa beans and mass (Jan. 1st, 2030)	

CF=crude fat.

On the other hand, in 2023, the European Food Safety Authority (EFSA, 2023) published a draft scientific opinion on the risk assessment of mineral oil hydrocarbons. The limited availability of information on the occurrence of MOAH in food led to the development of two predictive scenarios. EFSA continued to classify MOAH as having a potential toxicological risk, while MOSH were not considered hazardous to human health (EFSA, 2023). EFSA also emphasized the importance of collecting additional data on MOAH and their long-term effects on feed and food safety, as well as human health. The assessment was carried out through a public consultation process and is intended to support the development of stricter and better-founded future legislation. The scientific opinion provided is expected to support EU Member

States in the correct management of associated risks.

More recently, regulatory efforts have intensified. However, many of these provisions remain under discussion and have not yet been formally adopted. Current draft scenarios suggest the introduction of specific maximum levels for certain food matrices, with progressively stricter limits planned over time, reflecting an ongoing process of regulatory consolidation (Sante, 2025).

For the analysis of the research results, a classification system was developed to categorize contamination levels into five ranges for MOSH (normal, low, moderate, high, very high) and four ranges for MOAH (normal, low, moderate, high). Although current recommendations generally indicate a maximum acceptable level of 0.5 mg/kg for MOH contamination, a more detailed classification may provide a clearer understanding of the distribution and severity of contamination in feed and milk. It is important to note that values exceeding the threshold of 0.5 mg/kg can be considered "non-compliant" from the perspective of current recommendations. These ranges allow for a more nuanced characterization of contamination levels and a more precise assessment of contamination trends. In this way, we not only distinguish between compliance or non-compliance, but also supports the possibility of assessing potential risks.

The results on MOSH and MOAH contamination levels were presented separately for roughage and for concentrate and compound feeds (Figure 2 a,b). This separate visualization allowed highlighting the variations in contamination levels depending on the type of feed, considering the specific characteristics of each category. Roughage (e.g. hay, silage) and concentrate and compound feeds differ in both composition and technological processing. These technological and compositional differences may influence their susceptibility to contamination, as reported in previous studies (Matei et al., 2024; Matei & Pop, 2022).

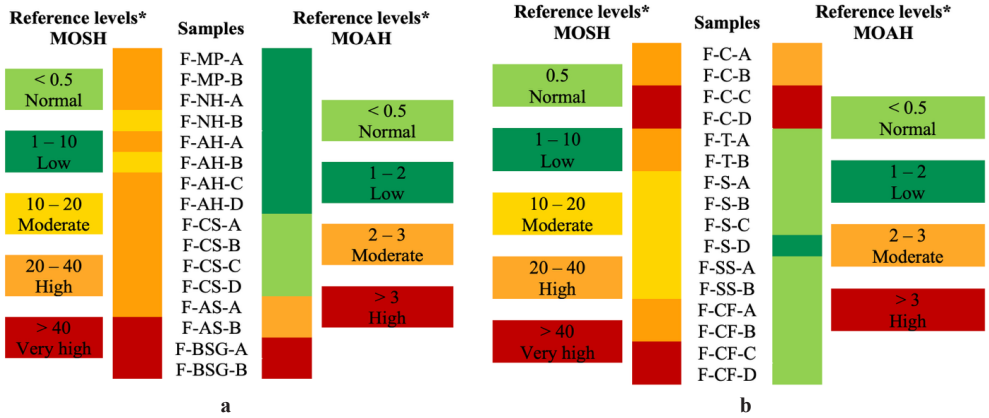


Figure 2. Proposed classification system for MOSH and MOAH contamination levels: a-bulk feeds; b-concentrated and compound feeds
(*current proposals; EU-RL=European Commission Recommended Limits (ScoPAFF) for foods with ≤ 4% fat; performance criteria for MOSH/MOAH analysis: LOQ < 0.5 mg/kg; values are the average of 2 replicates/sample)

The results of the analysis of feed samples indicate an alarming situation regarding MOSH contamination. Over 75% of the samples showed moderate to high levels of contamination, while more than 20% were classified as highly contaminated, far exceeding the current reference limits (0.5 mg/kg). Very high levels of contamination were particularly evident in samples from the F-BSG (brewer's grain) and F-CF (compound feed) categories, suggesting that the technological processes involved in their production and handling may be major sources of contamination. Samples from the F-CS (corn silage), F-AH (alfalfa hay) and F-C (corn grains) categories also showed high levels of contamination. These findings could be associated with the use of agricultural

machinery, as well as inadequate storage conditions, which may facilitate the transfer and accumulation of contaminants.

Regarding the MOAH fraction, the majority of the samples analyzed fell within the reference limit of 0.5 mg/kg, which is encouraging, given the higher toxicological concern associated with this fraction. Over 75% of the bulk feed samples showed normal (25%) or low contamination limits, ranging between 1-2 mg/kg (50% of the samples). At the same time, approximately 70% of the concentrate feed samples recorded normal contamination levels, below the reference limit of 0.5 mg/kg. Although the majority of the samples fell within the recommended limits, approximately 25% were classified as marginally non-compliant, exceeding the threshold of 2 mg/kg. These findings indicate the need for further investigations to identify and eliminate potential sources of contamination, although the indications point more towards technological contamination of these feeds. Feed contamination is a multifactorial issue, involving not only chemical contaminants such as MOH, but also microbiological hazards and mycotoxins. Previous studies have highlighted the presence of aflatoxins in feed obtained in feed mills, emphasizing the importance of control measures throughout the feed production chain (Lăpușneanu et al., 2024).

In the case of milk, MOSH contamination levels were generally acceptable, as approximately 94% of the samples analyzed fell within normal (6.25%) or low (87.5%) categories. A more concerning situation was observed for MOAH contamination, where only 50% of the samples fell within the normal (25%) or low (25%) categories. Meanwhile, 12.5% of the samples exhibited moderate contamination levels, and 37.5% showed high levels of contamination.

Milk contamination with MOSH and MOAH reflects a mixed situation in relation to established food safety limits. In the case of the MOSH fraction, the majority of the samples analyzed (94%) fell within the safety limits: 6.25% of the samples recorded normal contamination levels, while 87.5% showed low levels (Figure 3).

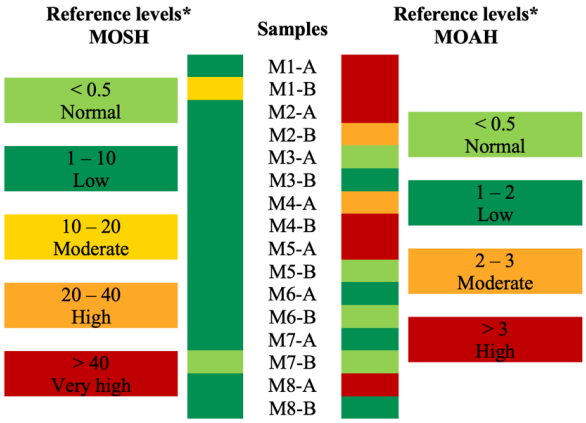


Figure 3. Proposed classification system for MOSH and MOAH milk contamination levels:
(*current proposals; EU-RL=European Commission Recommended Limits (ScoPAFF) for foods with ≤ 4% fat;
performance criteria for MOSH/MOAH analysis: LOQ < 0.5 mg/kg; values are the average of 2 replicates/sample)

In contrast, for the MOAH fraction, the situation was more concerning. Only half of the samples fell within the normal (25%) or low (25%) categories. At the same time, 12.5% of the samples showed moderate contamination levels, and 37.5% exceeded the thresholds considered acceptable and were classified as highly contaminated. Given the higher toxicity associated with MOAH and their potential to cause adverse health effects, these results may highlight a considerable risk to food safety. These findings are consistent with previous studies highlighting the vulnerability of dairy products to contamination and quality variations. Analyses of RASFF notifications over the past two decades have demonstrated the recurrent occurrence of contamination incidents in dairy products, emphasizing the importance

of risk management (Postolache et al., 2020). In addition, farm management systems have been shown to significantly influence the hygienic quality of milk, which may also impact their susceptibility to contamination (Rațu et al., 2023).

CONCLUSIONS

The consistent exceedance of the reference limits established for MOSH and MOAH in the majority of the samples analysed suggests an urgent need for intervention at both regulatory and agricultural practice levels, underlining the importance of harmonising legislative standards at European level to reduce associated risks.

The results highlight an alarming situation for feed safety, with potentially severe implications for animal and consumer health. Given the high proportion of non-compliant samples, it is essential to prioritize stricter legislative measures, as well as investments in technological solutions to reduce contamination.

The contamination distribution observed for the MOAH fraction shows that contamination levels are relatively better controlled compared to MOSH. However, given the toxicological concern associated with this fraction, even low contamination levels may require strict monitoring and maintenance of rigorous control measures. The results obtained from milk analysis also highlight the need for stricter preventive measures throughout the production, storage and processing chain to limit sources of MOAH contamination.

In order to improve food safety and effectively control MOH contamination, it is essential to establish clear and legally binding limits for the MOSH and MOAH fractions, based on the latest toxicological data. It is also recommended to implement a monitoring and reporting system across the entire agri-food sector, with a focus on reducing technological sources of contamination. International collaboration to harmonize regulations, support research into innovative methods to reduce contamination and educate consumers are also necessary measures that could contribute to reducing public health risks and increasing confidence in food safety.

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THE ROLE OF L-HISTIDINE IN BROILER PRODUCTIVE PERFORMANCE, IMMUNITY, AND MEAT QUALITY

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Abstract

L-histidine is an essential amino acid involved in protein synthesis, acid-base regulation, antioxidant protection, immune function and skeletal muscle metabolism in broiler chickens. Interest in histidine has increased because modern broiler genotypes have high breast muscle accretion, while reduced-protein feeding programs require precise amino acid supply. This article evaluates current scientific evidence on dietary L-histidine and related carnosine precursors in broilers, with an emphasis on productive performance, immune and antioxidant status, carcass traits, breast meat quality, and practical feed formulation. The literature indicates that histidine supply influences muscle dipeptide deposition and may support growth, feed conversion, breast yield, oxidative stability of meat, and tolerance to metabolic stress. However, responses depend on genotype, age, sex, dietary protein level, amino acid balance, beta-alanine availability and environmental conditions. Therefore, histidine should be considered both an indispensable amino acid and a functional nutrient in precision broiler nutrition.

Key words*: amino acid metabolism, antioxidant capacity, broiler chickens, carnosine, L-histidine.*

INTRODUCTION

Broiler production has become one of the most efficient systems for converting plant-based feed ingredients into animal protein. This efficiency is strongly dependent on accurate amino acid nutrition, because modern broilers have high rates of lean tissue deposition and relatively low tolerance to amino acid imbalances. Among the indispensable amino acids, L-histidine has received less attention than lysine, methionine, threonine and valine, although it participates in several physiological processes that are directly linked to performance and meat quality. Histidine is required for protein synthesis, haemoglobin formation, tissue growth, regulation of acid-base balance, synthesis of histamine and formation of histidine-containing dipeptides such as carnosine and anserine (Moro et al., 2020).

The practical importance of histidine in poultry nutrition is increasing for three main reasons. First, reductions in dietary crude protein are widely promoted to decrease feed costs, nitrogen excretion and environmental impact. These programs are successful only when limiting amino acids are supplied with sufficient precision, and recent work has emphasised that the energetic cost of amino acid catabolism and the balance among individual amino acids must be considered carefully in broilers (Woyengo et al., 2023; Cho et al., 2024; Macelline et al., 2025). Second, high breast yield genotypes may have increased sensitivity to changes in amino acid supply, especially when amino acids affect muscle metabolism and postmortem quality. Third, histidine is the precursor of carnosine, a dipeptide with antioxidant and buffering properties that can improve muscle oxidative stability and meat shelf life (Hu et al., 2009; Cong et al., 2017a; Baldi et al., 2021).

In poultry muscle, carnosine and anserine are relevant because they can buffer hydrogen ions during anaerobic metabolism, chelate transition metals and reduce lipid oxidation. Therefore, nutritional strategies that increase muscle carnosine content may improve meat quality, particularly in breast muscle, where high metabolic activity and rapid postmortem pH decline can contribute to quality defects. Studies with histidine, beta-alanine and carnosine have demonstrated that muscle dipeptide

concentration can be modified by diet, but the magnitude and biological relevance of this modification vary among experiments (Haug et al., 2008; Kai et al., 2015; Kralik et al., 2015a; Lackner et al., 2021; Qi et al., 2021).

The objective of this paper is to review and synthesise the available evidence regarding the role of L-histidine in broiler chickens, focusing on growth performance, immune regulation, antioxidant status, carcass characteristics and meat quality. Special attention is given to histidine-to-lysine ratios, histidine and beta-alanine as carnosine precursors, in ovo amino acid strategies, reduced-protein feeding and the practical implications of histidine in modern broiler feed formulation.

MATERIALS AND METHODS

This paper was prepared as a narrative literature review of peer-reviewed studies related to histidine, carnosine, beta-alanine, amino acid balance and reduced-protein nutrition in broiler chickens. The literature was selected to cover the following topics: histidine metabolism, histidine-containing dipeptides, growth performance, carcass and breast yield, meat oxidative stability, breast muscle quality, immune and antioxidant responses, ingredient amino acid digestibility and low-protein feeding programs. The main databases consulted were ScienceDirect, PubMed, Google Scholar and Web of Science. Search terms included L-histidine, broiler, chicken, carnosine, anserine, beta-alanine, histidine-to-lysine ratio, meat quality, oxidative stability, immune response, reduced-protein diet, standardised ileal amino acid digestibility and nitrogen excretion.

Studies were considered relevant when they addressed broiler chickens directly or provided mechanistic information applicable to poultry nutrition and muscle biology. Direct broiler experiments were prioritised for practical conclusions. Studies in other species were used only when they provided useful information on histidine metabolism, carnosine functions or antioxidant mechanisms and were interpreted cautiously. Particular emphasis was placed on research published during the last decade because of changes in broiler genetics, carcass yield, feed ingredient use and the expanding interest in low-protein diets supplemented with crystalline amino acids. The final reference list includes 30 sources that together support the major areas of the paper: histidine metabolism, broiler performance, meat quality, immunity, ingredient digestibility and sustainable feed formulation.

The literature search covered publications from 2008 to 2025, with the main emphasis on studies from 2015 onwards. An initial screening of titles and abstracts was performed across the four databases using the search terms listed above. Records were excluded if they: (i) did not involve poultry or did not provide mechanistic information relevant to poultry; (ii) focused exclusively on laying hens or turkeys without applicable data for broilers; (iii) were conference abstracts, letters or non-peer-reviewed sources; or (iv) were not available in full text. From an initial pool of approximately 120 records identified by the search strategy, 60 were screened at the full-text level, and 30 were selected for inclusion in the final reference list based on direct relevance to the topics covered in this review.

RESULTS AND DISCUSSIONS

HISTIDINE METABOLISM AND NUTRITIONAL SIGNIFICANCE IN BROILERS

Histidine metabolism and physiological functions

Histidine is an indispensable amino acid for poultry because broilers cannot synthesise it in sufficient quantities to meet the requirements of rapid growth. In addition to its structural role in body proteins, histidine is involved in several metabolic pathways. It contributes to haemoglobin synthesis, tissue repair, acid-base regulation and methyl group metabolism. Histidine can be decarboxylated to histamine, a compound involved in immune responses, vascular function and gastric secretion. The imidazole ring of histidine gives it specific buffering properties, which are important in tissues where rapid changes in pH occur. These biochemical functions explain why histidine deficiency may affect not only body weight gain, but also blood physiology, immunity and muscle quality (Moro et al., 2020).

One of the most important routes of histidine utilisation in broilers is the synthesis of histidine-containing dipeptides. Carnosine is formed from beta-alanine and histidine, while anserine is produced through methylation of carnosine. These dipeptides are particularly abundant in skeletal muscle. The limiting substrate for carnosine synthesis may vary with species, tissue and diet. In poultry, both histidine and beta-alanine availability can influence carnosine and anserine concentrations, although some studies suggest that beta-alanine may be especially important when histidine is adequate (Haug et al., 2008; Kai et al., 2015; Qi et al., 2021).

Carnosine is relevant to broiler nutrition because it performs several functions in muscle. It can buffer hydrogen ions, reduce oxidative damage, inhibit lipid peroxidation and react with reactive carbonyl compounds. These properties are valuable because the broiler breast muscle has high economic importance and is vulnerable to quality deterioration during storage. Research on poultry and other animals confirms that increased carnosine deposition can be associated with improved oxidative stability and better meat quality traits (Hu et al., 2009; Cong et al., 2017a; Kralik et al., 2018; Li et al., 2024). Therefore, histidine should be evaluated not only as a growth-limiting amino acid, but also as a nutrient that can influence postmortem muscle metabolism.

Dietary histidine requirements and histidine-to lysine ratio

Amino acid requirements in broilers are commonly expressed relative to digestible lysine because lysine is closely related to lean tissue deposition and is often used as the reference amino acid in ideal protein systems. Histidine has historically been considered less limiting than methionine, lysine, threonine or valine in corn-soybean meal diets. However, the relative importance of histidine increases when diets contain alternative ingredients, low levels of crude protein or high additions of crystalline amino acids. Franco et al. (2017) estimated optimal ratios of digestible histidine to digestible lysine for broilers and showed that histidine can influence both performance and breast yield when other amino acids are adequately supplied.

More recent work directly evaluated dietary histidine titration in female Cobb 500 broilers. Nelson et al. (2024) reported that histidine-to-lysine ratios in the range of approximately 33% to 38% supported desirable responses in feed conversion and breast yield. These results are important because they provide practical ratio targets for modern broiler genetics. They also indicate that histidine may become a limiting amino acid in high-performance feeding systems, particularly when crude protein is reduced and the diet is formulated close to minimum amino acid specifications.

The optimum histidine level is unlikely to be universal. Differences in strain, sex, age, growth rate, environmental temperature, health status and ingredient composition can change the response. Lackner et al. (2022a) demonstrated that different histidine-to-lysine ratios affected performance, meat quality and the occurrence of breast myopathies. This finding is particularly relevant for modern broilers because breast myopathies are linked to rapid growth, high breast muscle accretion and altered muscle metabolism. Although histidine cannot be considered a single solution for such problems, an adequate histidine supply may support metabolic stability in breast muscle.

Histidine, beta-alanine and carnosine deposition in muscle

Several studies have examined whether dietary histidine, beta-alanine or carnosine can increase carnosine concentration in broiler meat. Haug et al. (2008) showed that histidine supplementation increased carnosine and anserine concentrations in broiler muscle, confirming that diet can alter the pool of bioactive dipeptides in poultry meat. Kai et al. (2015) also reported that dietary histidine influenced carnosine and anserine levels in broiler muscles. These early results created interest in using histidine as a nutritional strategy to produce meat with improved functional and antioxidant properties.

The role of beta-alanine must also be considered because carnosine synthesis requires both beta-alanine and histidine. Qi et al. (2021) reported that beta-alanine and L-histidine supplementation affected growth performance, meat quality, carnosine content and the expression of enzymes related to carnosine metabolism. Lackner et al. (2021) similarly

evaluated histidine and beta-alanine supplementation and found effects on carnosine concentration, growth performance and meat quality. These studies indicate that combined supplementation may be more effective than histidine alone in some feeding conditions, especially when the other precursor becomes limiting.

Research in slow-growing chickens has provided additional evidence that carnosine precursor supplementation can modify meat biochemical composition. Suwanvichanee et al. (2022) showed that beta-alanine and L-histidine supplementation affected carnosine synthesis, meat quality and protein structure in Korat chickens. Promkhun et al. (2023) further demonstrated that dietary carnosine precursors changed biochemical compounds and metabolomic profiles in the same type of slow-growing chicken. Although slow-growing genotypes differ from commercial broilers, these studies support the broader concept that histidine-related metabolism can modify muscle composition and meat quality.

Dietary carnosine supplementation has also been tested directly. Hu et al. (2009) observed effects of carnosine on growth performance, carcass characteristics, meat quality and oxidative stability in broiler chickens. Cong et al. (2017a) reported that carnosine supplementation improved aspects of meat quality and antioxidant capacity, while Cong et al. (2017b) associated carnosine supplementation with changes in growth performance, meat quality, antioxidant capacity and muscle fibre characteristics. These findings are consistent with the biochemical role of carnosine as an antioxidant and pH-buffering compound.

Effects on growth performance and feed efficiency

Growth performance responses to histidine depend on whether the basal diet is deficient, marginal or adequate in histidine. When histidine is clearly deficient, supplementation can improve body weight gain, feed intake and feed conversion because histidine becomes limiting for protein synthesis. When histidine is adequate, further supplementation may have smaller or inconsistent effects on performance but can still influence muscle dipeptide concentrations and meat quality. This explains why different experiments report variable responses to histidine or carnosine supplementation.

Nelson et al. (2024) provided direct evidence that titrating histidine in modern broilers can improve economically important performance traits. Franco et al. (2017) also demonstrated that the histidine ratio affects performance and breast yield when evaluated within an ideal protein framework. These results support the inclusion of histidine in least-cost formulation matrices, especially when formulating diets with reduced crude protein or alternative feed ingredients.

Carnosine and its precursors may also affect performance indirectly by improving oxidative status, metabolic efficiency or muscle integrity. Hu et al. (2009), Cong et al. (2017b) and Kralik et al. (2018) reported beneficial or potentially beneficial responses in growth performance and carcass traits after supplementation with carnosine, histidine or related amino acids. However, the performance response is not always proportional to the increase in muscle carnosine. Therefore, the most reliable justification for histidine supplementation remains meeting the amino acid requirement, while additional functional benefits should be considered as complementary advantages.

Immune response and antioxidant status

Histidine and histidine-containing dipeptides are linked to immune function and antioxidant defence. Histidine can influence histamine synthesis and immune signalling, while carnosine can reduce oxidative stress through radical scavenging, metal chelation and protection against lipid peroxidation. In rapidly growing broilers, oxidative stress may arise from high metabolic activity, heat stress, inflammation or tissue damage. Nutritional strategies that strengthen antioxidant defences can therefore contribute to health and performance stability.

In ovo amino acid nutrition is an emerging strategy for supporting early development. Keshtkaran et al. (2024) reported that in ovo injection of L-carnosine affected physiological indexes in neonatal broiler chickens. Yehia et al. (2025) showed that in ovo-fed amino acids influenced muscle and liver metabolome in

broiler chickens, with implications for early metabolic programming. These findings suggest that histidine-related pathways may be relevant not only during the feeding period but also during embryonic and neonatal development.

The antioxidant effects of histidine-related compounds are also supported by work on muscle oxidative stability. Baldi et al. (2021) examined the role of histidine dipeptides in postmortem acidification of broiler muscles, showing the relevance of these compounds to muscle metabolism after slaughter. Cong et al. (2017a), Hu et al. (2009) and Kopec et al. (2020) reported that dietary carnosine, L-histidine or β-alanine can influence antioxidant capacity, histidine-dipeptide deposition and oxidative stability in chicken tissues. Immune and antioxidant responses should be interpreted with caution because they are influenced by stress level and experimental conditions. In healthy broilers raised under low challenge, the measurable effect of histidine supplementation on immune markers may be limited. Under heat stress, disease challenge, high stocking density or oxidised feed conditions, histidine and carnosine may become more important. Future studies should evaluate immune outcomes together with oxidative biomarkers, growth performance and meat quality to identify the conditions under which histidine has the greatest functional value.

Carcass characteristics, breast yield and breast muscle quality

Breast yield is one of the most important economic traits in broiler production. Because breast muscle is rich in protein and represents a major sink for amino acids, even small changes in amino acid balance may influence carcass value. Histidine has been associated with breast yield responses in ideal amino acid ratio studies. Franco et al. (2017) and Nelson et al. (2024) demonstrated that histidine-to-lysine ratio can affect breast yield, indicating that histidine should not be ignored when optimising diets for high breast meat production.

Breast muscle quality is also an important issue in modern broilers. Rapid growth and high breast yield have been associated with myopathies such as wooden breast and white striping, as well as changes in pH, water-holding capacity and texture. Lackner et al. (2022a) specifically evaluated histidine-to-lysine ratios in relation to performance, meat quality and breast myopathies. Although breast myopathies have multifactorial causes, amino acid nutrition can influence muscle metabolism, antioxidant status and tissue repair, and histidine may contribute through carnosine synthesis and acid-base regulation.

The quality of broiler meat is determined by pre-slaughter muscle metabolism and postmortem biochemical changes. Histidine-containing dipeptides may affect both. Baldi et al. (2021) showed that these dipeptides are involved in postmortem acidification, a process closely related to ultimate pH and meat quality. Kralik et al. (2015a) reported that dietary histidine, hybrid line and gender influenced meat quality and carnosine concentration. These results show that histidine effects interact with genetic and biological factors, and that recommendations should consider the production system and target market.

Meat oxidative stability and shelf-life

Oxidative stability is a key component of meat quality because lipid and protein oxidation reduce colour stability, flavour, nutritive value and consumer acceptance. Poultry meat contains unsaturated fatty acids and can be sensitive to oxidative deterioration during processing and storage. Carnosine and anserine are valuable because they can inhibit oxidation reactions and may contribute to shelf-life extension. Studies by Hu et al. (2009), Cong et al. (2017a) and Kralik et al. (2015b) demonstrated that carnosine or its precursors can improve oxidative stability in chicken meat.

Kralik et al. (2015b) reported that beta-alanine and L-histidine affected carnosine concentration and oxidative stability of chicken meat. Kralik et al. (2018) further evaluated amino acids in relation to growth performance, carcass characteristics, meat quality and carnosine concentration. These results confirm that nutritional manipulation of carnosine precursors can be a practical strategy to improve the value of poultry meat, especially where oxidative stability is important for fresh meat display, processed products or extended storage.

Recent alternative feeding strategies may also influence meat bioactive compounds. Grassi et al. (2025) examined bioactive compounds in the

breast meat of broilers fed black soldier fly wholemeal. Insect meals may contribute nutrients and bioactive compounds, but their amino acid composition and digestibility must be carefully evaluated. Such studies are relevant because future poultry feeding systems may use more alternative protein sources, making it necessary to monitor histidine supply, carnosine deposition and meat quality at the same time.

Histidine in reduced-protein and sustainable broiler diets

As described above, reduced-protein diets are widely adopted, and histidine can become deficient or imbalanced in such programs. Histidine becomes important in this context because feed formulation may rely more heavily on crystalline amino acids and alternative ingredients. Woyengo et al. (2023) reviewed strategies for low-protein broiler diets and emphasised the need to balance essential and nonessential amino acids while maintaining performance. Cho et al. (2024) also reported that low-protein diets supplemented with amino acids can influence growth performance and nitrogen excretion.

The economic and metabolic cost of amino acid catabolism is another important factor. Excess amino acids are deaminated and used for energy, producing nitrogen waste and increasing metabolic heat. Macelline et al. (2025) highlighted the cost of amino acid catabolism for energy utilisation in broiler chickens. From this perspective, the goal is not simply to add more amino acids but to provide the correct amount and ratio. Histidine supplementation should therefore be integrated into an ideal protein approach that considers lysine, sulfur amino acids, threonine, valine, isoleucine, arginine, phenylalanine plus tyrosine and other potentially limiting amino acids.

Reduced-protein diets may also alter immune and oxidative status because they change nitrogen metabolism, gut fermentation and ingredient composition. Kareem et al. (2025) evaluated reduced-protein diets with varying ingredient combinations, while Kandel et al. (2025) examined the impact of soybean meal and field pea inclusion on growth performance. These studies illustrate that ingredient choices affect more than crude protein concentration. They also modify amino acid profiles, digestibility, fibre level and antinutritional factors. Therefore, a reduced-protein diet can be successful only if digestible histidine and other amino acids are accurately supplied.

Ingredient digestibility and feed formulation implications

Accurate formulation requires reliable values for standardised ileal amino acid digestibility. Histidine content and digestibility differ among feed ingredients, and this variation becomes more important when alternative protein sources are used. De Almeida et al. (2025) reported values for nitrogen-corrected apparent metabolizable energy and standardised ileal amino acid digestibility of distillers dried grains with solubles. Iqbal et al. (2025) investigated the standardised ileal amino acid digestibility of rice protein meal. Such studies are important because ingredients like DDGS, rice protein meal, field peas, insect meals and other plant proteins may change the supply of digestible histidine relative to lysine.

Feed additives may also influence nutrient utilisation. Kumar et al. (2025) evaluated nutrient density diets with emulsifier supplementation and showed that formulation strategies can interact with nutrient availability and energy utilisation. Although emulsifiers do not directly supply histidine, improved fat digestion and energy use can alter the balance between energy intake and amino acid deposition. In precision feeding, histidine recommendations should be interpreted together with metabolizable energy, digestible amino acid values, feed intake and the expected growth curve.

Practical least-cost formulation should include digestible histidine as an active nutrient constraint, especially in low-protein diets or diets containing nontraditional protein sources. If histidine is not constrained, the diet may meet crude protein and lysine specifications but remain marginal in histidine. This risk is higher when soybean meal is reduced and replaced with cereals, DDGS, rice protein meal, field peas or insect-derived ingredients. Therefore, the formulation matrix should include analysed or reliable tabular values for total histidine, standardised ileal digestible histidine and the ideal histidine-to-lysine ratio.

In ovo supplementation and early metabolic programming

The embryonic and neonatal periods are critical for broiler development because digestive capacity, immune function and muscle satellite cell activity are being established. In ovo nutrition aims to provide nutrients directly to the embryo before hatch, supporting early development and post-hatch adaptation. Keshtkaran et al. (2024) showed that in ovo injection of L-carnosine affected physiological indexes of neonatal broiler chickens, suggesting that histidine-related compounds can influence early metabolic status.

Yehia et al. (2025) reported that in ovo-fed amino acids changed the muscle and liver metabolome of broiler chickens. This is relevant because the liver is central to amino acid metabolism, antioxidant defence, and energy regulation, while muscle is the main target tissue for meat production. If histidine-related pathways are modified early in life, they may influence later growth and resilience. However, in ovo supplementation requires careful control of dose, injection site, timing and solution osmolarity. More research is needed before practical recommendations can be made for commercial hatcheries.

Summary of main literature sources

Table 1 below provides a brief summary of key references identified in this research, including the main outcomes reported for specific dietary interventions.

Table 1. Summary of key studies on L-histidine, beta-alanine and carnosine in broiler chickens and related species

Reference	Species/ Genotype	Dietary Intervention	Main outcomes reported
Haug et al. (2008)	Broiler	L-histidine supplementation of commercial feed	Increased carnosine and anserine in muscle
Hu et al. (2009)	Broiler	Dietary carnosine supplementation	Improved growth, carcass traits, meat quality, oxidative stability
Kai et al. (2015)	Broiler	Dietary L-histidine levels	L-histidine influenced carnosine and anserine in muscle
Kralik et al. (2015a)	Broiler (hybrid, sex)	Dietary L-histidine, hybrid line, gender	Meat quality and carnosine concentration are affected by all three factors
Kralik et al. (2015b)	Broiler	β-Alanine + L-histidine supplementation	Increased muscle carnosine; improved oxidative stability
Franco et al. (2017)	Broiler	L-histidine: Lysine ratio titration (ideal protein)	Optimal L-histidine: Lysine ratio estimated; breast yield and performance affected
Cong et al. (2017a, b)	Broiler	Dietary carnosine supplementation	Improved meat quality, antioxidant capacity, and muscle fibre characteristics
Kralik et al. (2018)	Broiler	Amino acid (L-histidine) supplementation	Growth, carcass, meat quality and carnosine all positively influenced
Kopec et al. (2020)	Broiler	Carnosine, L-histidine, β-Alanine supplementation	Antioxidant characteristics of breast meat and blood improved
Baldi et al. (2021)	Broiler	Histidine-dipeptide role in postmortem metabolism	Histidine-dipeptides regulate postmortem acidification and ultimate pH
Lackner et al. (2021)	Broiler	L-histidine + β-Alanine supplementation	Carnosine deposition, growth performance and meat quality were affected
Qi et al. (2021)	Broiler	β-Alanine + L-histidine supplementation	Growth, meat quality, carnosine and carnosine-related enzyme mRNA changed
Lackner et al. (2022a)	Broiler	L-histidine: Lysine ratio (multiple levels)	Performance, meat quality and breast myopathy incidence influenced
Lackner et al. (2022b)	Broiler	L-histidine + β-Alanine at multiple ages	Blood plasma metabolome altered; age-dependent responses identified
Suwanvichanee et al. (2022)	Korat chicken (slow-growing)	β-Alanine + L-histidine supplementation	Carnosine content, meat quality and protein secondary structure modified
Promkhun et al. (2023)	Korat chicken (slow-growing)	Carnosine precursors in the diet	Jejunal metabolomic profile and biochemical compounds changed
Keshtkaran et al. (2024)	Neonatal broiler	In ovo L-carnosine injection	Physiological indices of neonatal chicks were affected
Nelson et al. (2024)	Cobb 500 female broiler	L-histidine titration (22-42 d phase)	L-histidine: Lysine ~33-38% optimal for feed conversion ratio and breast yield
Yehia et al. (2025)	Broiler	In ovo amino acid feeding	Muscle and liver metabolome altered; early metabolic programming demonstrated

Knowledge gaps and future research

Despite growing interest, several knowledge gaps remain. First, the histidine requirement for modern broilers should be refined by age, sex, genotype and production objective. Studies such as those by Nelson et al. (2024), Franco et al. (2017) and Lackner et al. (2022a) provide important ratio estimates, but more work is needed under different dietary protein levels, environmental temperatures and health conditions. Second, the interaction between histidine and beta-alanine requires further clarification. Research by Qi et al. (2021), Lackner et al. (2021), Suwanvichanee et al. (2022) and Promkhun et al. (2023) indicates that both precursors influence carnosine synthesis, but the most cost-effective supplementation strategy is still uncertain.

Third, the relationship between histidine supply, breast myopathies and meat quality deserves more attention. Carnosine may improve buffering capacity and oxidative stability, but breast myopathies are complex conditions involving vascular, metabolic, oxidative and structural changes. Histidine should therefore be studied together with antioxidants, sulfur amino acids, arginine, glycine, creatine precursors and management factors. Fourth, more research is needed on histidine in low-protein diets because the benefits of reducing crude protein depend on complete amino acid balance and accurate digestibility values (Woyengo et al., 2023; Cho et al., 2024; Macelline et al., 2025).

Finally, future studies should combine performance measurements with metabolomics, transcriptomics and meat quality evaluation. Lackner et al. (2022b) demonstrated that histidine and beta-alanine supplementation changed the blood plasma metabolome of broiler chickens at different ages. Such approaches can help identify biomarkers of histidine adequacy and explain why some birds respond more strongly than others. Better understanding of these mechanisms will allow nutritionists to use histidine more precisely in sustainable broiler production systems.

Practical recommendations

Based on the current literature, broiler diets should be formulated using digestible histidine values rather than total histidine or crude protein alone. Histidine should be included as a nutrient constraint when crude protein is reduced, when soybean meal is partially replaced, or when alternative protein ingredients are used. A digestible histidine-to-lysine ratio close to the range reported in recent broiler studies may be used as a practical starting point, but the exact target should be adjusted according to genotype, age, sex, feed intake and expected breast yield (Franco et al., 2017; Lackner et al., 2022a; Nelson et al., 2024).

Supplementation with histidine, beta-alanine or carnosine may be considered when the goal is to increase muscle carnosine concentration or improve oxidative stability of meat. However, the economic value of this strategy depends on ingredient prices, product positioning and measurable improvements in shelf life or quality. In standard broiler production, the first priority should remain meeting the digestible histidine requirement. In premium meat programs or systems with high oxidative challenge, additional carnosine precursor supplementation may provide added value.

Feed manufacturers should also update ingredient matrices with recent digestibility data for DDGS, rice protein meal, insect meals, field peas and other alternative ingredients. Without accurate digestibility values, histidine supply can be overestimated or underestimated. Routine formulation should evaluate histidine together with lysine, methionine plus cysteine, threonine, valine, isoleucine and arginine, particularly in reduced-protein diets. This approach supports productive performance while reducing nitrogen excretion and improving sustainability.

CONCLUSIONS

L-histidine is an essential amino acid with important nutritional and functional roles in broiler chickens. Beyond its contribution to protein synthesis, histidine is involved in acid-base balance, immune regulation, antioxidant protection and the synthesis of carnosine and anserine in skeletal muscle. The reviewed literature shows that adequate histidine supply can support growth performance, feed efficiency, breast yield, meat oxidative stability and postmortem muscle metabolism. Histidine becomes especially important in modern feeding programs based on reduced crude protein,

alternative ingredients and precision amino acid formulation.

The most consistent practical message is that histidine should be considered within the ideal protein concept and formulated on a digestible basis. Dietary histidine-to-lysine ratio, beta-alanine availability, genetic line, sex, age and dietary protein concentration all influence the response. Histidine and its related dipeptides also have potential as nutritional tools for improving meat quality and oxidative stability, but more research is needed to define cost-effective supplementation strategies. Future studies should integrate performance, immune markers, antioxidant status, metabolomic responses, breast myopathy evaluation and meat quality traits to establish precise recommendations for sustainable broiler production.

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AN ANALYSIS OF THE OWNER'S DECISION-MAKING ROLE IN DOG AND CAT NUTRITION FROM A PSYCHOSOCIAL, ECONOMIC, AND SCIENTIFIC PERSPECTIVE

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Abstract

One of the most accessible and effective ways to support the well-being of pets is to pay close attention to their nutrition, which has led to a significant increase in pet owners' awareness and demands regarding the food they feed their pets. Although the first scientifically based nutritional recommendations for dogs and cats appeared in the mid-1980s, they were not officially standardized until 2006, with the publication of Nutrient Requirements of Dogs and Cats by the National Research Council. The literature consistently highlights a discrepancy between owners' perceptions of "healthy" nutrition and the physiological reality of animals, a discrepancy emphasised by the process of humanisation of pets. This paradigm shift has contributed to the development of an extremely dynamic and segmented market, but has also generated significant nutritional risks associated with misinformation and the projection of human needs onto animal physiology.

Key words*: pet food, companion animal nutrition, pet owner perception, nutritional guidelines, animal physiology.*

INTRODUCTION

The global pet food market is undergoing a phase of rapid expansion, reaching an Estimated value of approximately €136.5 billion in 2026 (Statista, 2026; Fortune Business Insights, 2026). Euromonitor International also forecasts that the total global pet market will reach approximately €174.8 billion by 2025 (Matos, 2026), a trend directly linked to the increase in the number of pets - specifically dogs and cats - in households worldwide. This trend is driven in particular by the demographic segments generically referred to as Millennials (1981-1996) and Gen Z (1997-2012) (Krishna et al., 2025), who are redefining industry standards by shifting from passive acceptance of attributes such as "complete and balanced nutrition" to a rigorous analysis of food safety, supply chain transparency, and ingredient sustainability. However, this phenomenon of "premiumisation" and "humanisation" (anthropomorphism) (Puri et al., 2025) poses significant nutritional risks, resulting from the projection of human ethical values onto animal physiology. This discrepancy manifests itself through the adoption of alternative diets, such as plant-based or vegan diets for species with specific biological requirements (carnivores), which can lead to deficiencies in essential amino acids and metabolic imbalances if not grounded in strict scientific protocols.

MATERIALS AND METHODS

This study was based on a literature review methodology; therefore, an approach was used that took into account multiple criteria and factors analyzing the intersection between pet owners' behavior, the dynamics of the global pet food market, and scientifically-based nutritional requirements.

To investigate industry trends, data provided by market intelligence leaders such as the Mintel Group, Innova Market Insights, and GlobalPETS were utilised. An important methodological note is that access to this data was achieved through summaries and press releases available to the general public. Therefore, certain scores and complex numerical datasets were synthesized from these public versions, as we did not have access to premium full-access commercial reports.

Data on the pet population and ownership rates at the global and regional levels were extracted from constantly updated sources, such as the

2026 World Population Review and FEDIAF reports.

Articles published in prestigious academic journals (e.g., Animals, Frontiers in Veterinary Science, Agriculture, British Journal of Nutrition) were analysed, with a particular focus on recent publications from 2024-2026, to ensure the scientific information is up-to-date.

By synthesizing these sources, the research aims to balance the consumer's subjective perception, as measured by market studies and surveys, with the clinical reality revealed by nutritional research documented in experimental studies testing digestibility and the functioning of the intestinal metabolome.

RESULTS AND DISCUSSIONS

In a paper published in 2004, which reports on data recorded up to that early stage in the development of the pet food sector, Jürgen Zentek shows that, according to estimates by the European Pet Food Industry Federation (FEDIAF), the number of households owning pets had reached 55 million in 2004. Data for Western European EU countries indicated that the cat population was larger than that of dogs, with approximately 47 million cats and 41 million dogs recorded.

A factor often neglected in analyses comparing the situation from over 20 years ago with that of today is the change in the European Union's geographical scope; consequently, estimates are based on fewer countries, specifically the 15 member states as of 2004. FEDIAF compiles annual statistics to track the distribution and growth in the number of pets owned; thus, the most recent data showing the distribution of dogs and cats in the major European countries is presented in Table 1.

The evolution of the relationship between humans and pets (particularly dogs and cats) reflects a profound shift in the ontological paradigm within contemporary European society. Recent studies conducted in Spain, Romania, and across Europe highlight a clear transition from functional utilitarianism to a relationship based exclusively on affinity and emotional attachment (Rombach et al., 2026; Hica et al., 2026).

Historically, the presence of animals in human habitats was justified by their role as "tools for survival." Dogs, for example, performed clear functions such as assisting in hunting, protecting property, or providing traction in extreme weather conditions, with the ultimate goal of facilitating food procurement or travel over long distances (Azkona, 2025; Volsche et al., 2025).

Table 1. Pet ownership statistics in European countries, 2026

Country	Dogs (million)	Cats (million)
United Kingdom	12.7	11.9
Germany	10.6	15.2
Spain	9.3	5.9
Italy	8.8	10.2
Poland	8.1	7.3
France	7.6	14.9
Ukraine	4.9	7.6
Romania	4.2	4.4
Portugal	2.8	2.0
Hungary	2.8	2.4

Source: World Population Review, 2026

As living conditions improved and basic needs were met, this utilitarian function was replaced by a psychosocial one.

In this context, the phenomenon of anthropomorphism - defined as the attribution of mental states, emotions, and human social roles to animals - has become the primary driver of owners' behavior. This shift in perspective is also reflected in legislative developments.

A prime example is Spain's Law 17/2021 (Ley 17/2021, de 15 de diciembre), which amended the legal status of animals, transforming them from "objects or movable property" into "sentient beings" (seres sintientes). This legal recognition establishes the animal as a family member with protected rights, a status that dictates new standards of ethics and welfare (Egaña-Marcos et al., 2025).

This "humanization" of pets has direct implications for the economic dynamics of the pet food market (Rombach et al., 2026). Choosing food is no longer simply a matter of resource management, but an act of emotional responsibility. The owner, perceiving the animal as an equal, tends to project their own nutritional and ethical demands onto it. Thus, the demand for personalized diets, "human-grade" ingredients, and radical transparency in the production process becomes an extension of

the deeply emotional behavior developed within the new urban family ecosystem (FEDIAF 2025; Innova Market Insights, 2026).

For a product to be labeled “human-grade”, all ingredients must be suitable for human consumption, and the product must be manufactured, packaged, and stored in facilities that comply with human food safety standards, as defined by the AAFCO (Association of American Feed Control Officials) and FDA regulations.

Conventional "feed-grade" food is produced in feed mills where health regulations are tailored to the livestock sector, while "human-grade" food is processed exclusively in facilities inspected and authorized for human consumption. This distinction is essential because, according to critical analyses, a product loses its status as “human-grade” as defined by AAFCO the moment it enters a compound feed processing facility, even if the original ingredients were of superior quality.

The ingredients and raw materials used in conventional pet food allow for the utilization of slaughterhouse by-products and meat and bone meal, but food produced to standards specific to human consumption requires the exclusive use of meat and organs from animals slaughtered specifically for human consumption, thus ensuring superior biological safety and rigorous traceability.

From a psychological perspective and as a result of the changing mindset of pet owners, who are now more often categorized as “pet parents” (German, 2015), studies show that for this group, choosing a product processed to human standards represents more of an ethical necessity and a form of preventive medicine, intended to reduce the risks associated with industrial-grade ingredients (Tanprasertsuk et al., 2026).

From a nutritional point of view, ileal digestibility studies (Hendriks et al., 2013) demonstrate that diets based on human-grade ingredients offer increased amino acid bioavailability, often exceeding the 85-90% threshold (Oba et al., 2019). This efficiency is attributed to gentle processing and low ash content, standards considered exceptional in pet nutrition; however, to validate its undeniable clinical superiority, extensive longitudinal studies must be conducted. Until then, choosing this type of food remains a form of proactive preventive medicine, based on the logic of human food safety now applied to pets.

New tendencies in pet nutrition are driving a transition among pet owners toward grain-free diets. This trend is based on consumer perceptions of biologically appropriate nutrition (Petrescu et al., 2023), where the exclusion of carbohydrates derived from grains is interpreted as a way to align the diet of companion animals with the ancestral nutritional profile of carnivorous species.

Recent research indicates that grain-free diets offer a better profile of essential amino acids due to their high meat content, but this advantage is counterbalanced by the risk of severe mineral imbalances. In particular, exceeding legal limits for manganese in insect-based diets and the inadequate calcium-to-phosphorus ratio in over a quarter of the products analyzed underscore that excluding grains does not automatically mean increased nutritional safety (Zhang et al., 2025).

Although pet owners tend to attribute superior biological value to ingredients sourced from the wild, associating “ancestral” diets with nutritional purity and ethical principles, this preference often overlooks the major clinical risks arising from the bioaccumulation of toxic elements in tissues. Recent research confirms an increased incidence of contamination in game meat used in the human food supply chain (Ciobanu et al., 2022; Boișteanu et al., 2024), a protein source that is now increasingly common in pet food as well.

Conversely, another market that is the subject of intense research is that of vegetarian or vegan pet owners.

Research on this market segment shows that vegetarians and vegans often extend their compassion for animals that are slaughtered beyond their own consumption, feeling a deep moral discomfort when purchasing meat products for their dogs and cats. Despite this, it is encouraging to note that most vegetarians refuse to impose their lifestyle on animals, acknowledging their different biological needs.

There is a strong desire within the vegan community to switch pets to a plant-based diet, but a study conducted on a large sample of over 3,000 people shows that the actual implementation rate is extremely low, at just

1.6% of the studied population (Dodd et al. 2019). This demonstrates that some pet owners may, above all, be responsible, fearful of causing nutritional deficiencies or health problems for their carnivorous companions, which outweighs their desire to align the animal's diet with their ethical values.

From a biological perspective, it is perfectly natural to have serious reservations about feeding carnivores - descendants of wolves or wild cats - a diet consisting exclusively of plants.

From a legal and scientific perspective, AAFCO and FEDIAF neither prohibit nor require the use of meat in dog and cat food, but both organizations enforce extremely rigorous standards with minimum and maximum values for nutrients (proteins, amino acids, fatty acids, calcium, phosphorus, vitamins, etc.). For a manufacturer to be able to label a food as "complete and balanced," the product must meet those values, regardless of the source of the raw materials.

Studies observe that many veterinary organizations that follow AAFCO guidelines warn that a plant-based food approved only on paper (through formulations) is extremely risk-prone (Kanakubo et al., 2015; WSAVA, 2026). Without actual testing of the food on animals, there is no guarantee that the cat or dog can actually absorb those synthetic vitamins and minerals added to the plant-based formula.

Regarding the factors influencing the choice of pet food, recent studies and publications highlight a hierarchy of priorities dominated by palatability, which received the highest importance score of 4.6 out of 5. This preference is explained by owners' desire to obtain immediate emotional gratification through the animal's visible satisfaction during feeding, according to Euromonitor, 2025. Nutritional quality ranks nearly as high, as noted in the studied publications, reflecting a paradigm shift toward preventive nutrition and the long-term health of dogs and cats (Mintel, 2025).

The criterion regarding specific ingredients is closely linked to consumers' need for safety and supply chain transparency (Matei et al. 2024, Toukabri et al., 2025), while the importance placed on freshness indicates a growing trend toward avoiding ultra-processed products (Mintel, 2025). The novelty lies in the area of price and promotions, which recorded the lowest influence score, being considered a secondary factor in the purchasing decision, as modern pet owners prioritize the emotional bond and the animal's well-being over financial savings (Euromonitor, 2025).

The environmental sustainability of the pet food production chain is also an important factor for some pet owners who consider environmental safety when purchasing a particular type of food (Nicholles and Knight, 2025). Regarding this concern, a study that examined the carbon footprint of conventional dog and cat food in the U.S. and assessed the use of water, agricultural land, fossil fuels, phosphates, and biocides concluded that the total environmental impact attributed to the production of this food is between 25% and 30% (Okin et al., 2017, Oven et al., 2022). The solution would be to use new sources of protein; until now, these were considered to be insect-based proteins, but efforts are now being made to introduce cultured meat to the market. The technology behind it involves multiplying animal cells in vitro, in a controlled and sterile environment (using a bioreactor), without the need to slaughter animals.

At the European level, the United Kingdom was the first European country to approve the use of cultured meat in pet food in July 2024, followed by the commercial launch of the first product of this type in February 2025 (Meatly, 2025; Oven et al., 2022).

The 2024 YouGov study provides a clear picture of the Romanian public's attitudes toward cultured meat. The proportion of respondents in Romania who would be willing to try cultured meat "at least once" is 35%, while 53% stated that they "would never eat" cultured meat. However, perceptions shift when it comes to using cultured meat in pet food; in this case, respondents in Romania are somewhat more open to introducing cultured meat into their dog's or cat's diet if it is nutritionally safe and sustainable. It should be noted that the numerical scores and rankings presented represent a synthesis of trends extracted from the summaries and official press releases of the cited sources, with the full data subject to the premium access policies of market research providers.

By comparing the hierarchy of priorities described above, where quality and specific ingredients are the primary drivers of purchasing decisions, with the requirements of pet owners in Romania, a study confirms that scientifically grounded food safety (Petrescu et al., 2025) is the strongest driver of trust (42.3%) among urban residents.

CONCLUSIONS

Avoiding metabolic or nutritional risks caused by fashionable but unbalanced diets requires the implementation of urgent measures to filter and validate information coming from online sources. In this context, educating pet owners on how to select scientifically based information sources in the field of animal nutrition becomes a priority.

Given the exponential growth in pet ownership and Romanian owners' adoption of super-premium or even human-grade diets, Romania must support the development of its own market through investments in combined feed mills dedicated exclusively to dogs and cats. Currently, Romania has a major trade deficit in the pet food sector, and high-standard local production would ensure not only superior ingredient traceability but also alignment with global sustainability and food safety requirements.

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THE USE OF MEDICINAL PLANTS-BASED PHYTOACTIVE SUBSTANCES AS ALTERNATIVE INPUTS IN CATTLE FORAGE AND TREATMENT

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Abstract

Medicinal plants are used throughout the world both to treat diseases and to maintain the general health of both humans and animals. The aim of this work is to analyze the trends for research on the use of medicinal plants and extracts for the health of cattle. A structured analysis was performed in the Web of Science database with a combination of keywords between years 2020-2025, which enabled the assessment of multiple bibliographic networks with VOSviewer. Due to antibiotic-resistant microorganisms, attempts are being made to use medicinal plants with immunoregulatory (815 articles), antimicrobial (820 articles), antifungal (815 articles), and anti-inflammatory effects (816 articles) to sustain the animal by strengthening natural defense mechanisms. Most medicinal plants contain alkaloids, minerals, vitamins, flavonoids, saponins, which contribute to numerous physiological processes in the animal body. By supplementing the rations of cows with phytoactive substances, there is a visible improvement in the defense mechanism, digestibility of nutrients and an increase in milk production. These approaches can be used to partially or complete replacement of conventional treatments and have a dual role for both the health of the animal and increased yield of main products.

Key words*: active substances, animal health, defense mechanism, multiple benefits, type of action.*

INTRODUCTION

Throughout history, medicinal plants have represented a valuable source of therapeutic agents in the treatment of various pathologies and are still used today due to their benefits (Zhao et al., 2022). Herbal medicines have been used since ancient times due to their accessibility, effectiveness, and low cost (Shahrajabian et al., 2020) and represent a traditional method of treating various conditions (Michel et al., 2020). Of all vascular plants existing globally, approximately 10% are used for medicinal purposes, with approximately 500,000 species (Salmerón-Manzano et al., 2020) and according to IUCN and WWF, approximately 80,000 species of flowering plants are used for therapeutic purposes (Michel et al., 2020). Medicinal plants represent a natural option for supporting and improving animal health (Rochfort et al., 2008).

Animal health is the essential factor that has determined the reduction or in some countries the prohibition of antibiotics administered in animal feed as a result of the occurrence of residues in both milk and meat (Kholif et al., 2024). In order to reduce antibiotics, plant-based alternatives derived from medicinal plants are increasingly integrated into animal feed (Wang et al., 2024).

Medicinal plants contain a large number of chemical compounds, which is why many drugs have been obtained or derived from them (Grover et al., 2002). These compounds are naturally present in plants and the curative properties of medicinal plants result from these biological components (Ahad et al., 2021).

Bioactive compounds are found in different parts of plants such as: stems, roots, leaves, seeds and fruits (Sun and Shahrajabian, 2023) and give plants antioxidant, anti-inflammatory and antimicrobial properties (Iommelli et al., 2025; Dar et al., 2023). Various chemical compounds, such as flavonoids, vitamins, minerals, saponins and tannins, are present in plants, and they can improve the productive performance of animals through changes in the metabolism of the digestive tract (Alem, 2024).

Natural volatile compounds contribute to improving digestion, modifying rumen function, reducing methane production, and improving milk and meat quality in ruminants (Ramdani et al., 2023). Polyphenols are the main compounds of natural bioactive substances derived from plants. Their composition and concentration vary depending on numerous factors, and in recent years they have been used in the nutrition of ruminants, birds and pigs (Lillehoj et al., 2018). The aim of the paper is to analyze research trends regarding the use of medicinal plants and extracts for cattle health. This scientific article aims to contribute to the update of research in the zootechnical field and to the correlation of international databases on the benefits of medicinal plants used in cattle feeding, as well as to outline future research directions regarding their applicability.

MATERIALS AND METHODS

In this article, we analyzed the literature on the effects of phytoactive substances from medicinal plants used in the treatment and feeding of cattle. All extracted databases were further analyzed with VOSviewer (Arruda et al., 2022; Bukar et al., 2023) as follows. The bibliographic search was performed in the Web of Science Core Collection, with the filter for publishing years of 2020-2025, using keywords including cow, cattle, medicinal plants, anti-inflammatory, dairy cows, phytoactive substance. The keywords used for searches are found in the diagrams in Figures 1-5. In order to see the current interest of the subject, combinations of keywords containing the word cow or cattle with each of the specific keywords were used, namely: antifungal – 815 articles, anti-inflammatory – 816 articles, antioxidant – 817 articles, antimicrobial – 820 articles and immunoregulatory substances – 815 articles. To create graphical representations, a minimum number of occurrences of a keyword was used, namely 20 or 10 depending on the appearance of studies in this field and the thresholds were set to a value that not load the image and not produce confusions on the general trends. Out of a total of 2,298 keywords identified in the analyzed articles, 118 met the selection threshold required for map generation in VOSviewer (for one of the five representative maps). This approach was necessary to evaluate the large and well-founded directions in this field that provide a basis for comparison, in addition, the Bibliographic network produced by VOSviewer that visually indicates the position of a term in research and its importance, brings benefits to identify less studied niche areas. This second result is necessary for the design of future research and the realistic evaluation of the basis for comparison.

RESULTS AND DISCUSSIONS

Bibliografic analysis performed using VOSviewer highlighted several distinct keyword groups regarding the use of phytoactive substances with antifungal effects (Figure 1).

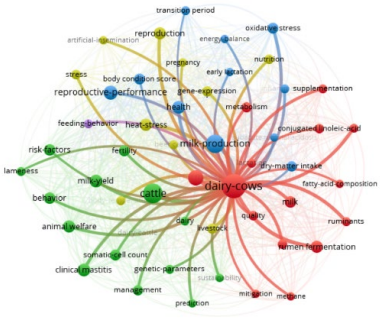


Figure 1. Antifungal bibliographic analysis and correlation with associated fields

The main keywords in the form of different clusters are, dairy cows, milk production, health, reproduction, and reproductive performance. These form strong links between them, highlighting the interdependence between the use of phytoactive substances, the animal's health status, but also productive and reproductive parameters. These clusters also represent main concepts from which secondary keywords are derived, reflecting the complexity and multidimensional nature of research on the use of antifungal phytoactive substances in cattle breeding systems. The proximity of the terms milk production and metabolism to the central node suggests a major interest in the direct effects of nutritional strategies on productive performance, while terms such as methane and sustainability, positioned peripherally, reflect emerging directions oriented towards environmental impact.

Performance is closely linked to health, behavior, risk factors, well-being and stress because these factors directly influence the metabolic efficiency, immune system functioning and adaptive capacity of animals (Chebel et al., 2016) which together have a major impact on milk production and reproductive performance (Llonch et al., 2018). Reproductive performance, fertility, lactation, milk production form closely correlated clusters, suggesting that lactation and milk production have an impact on fertility and reproductive performance (Eldawy et al., 2021; Bello et al., 2012; Nebel and McGilliard, 1993) emphasizing the need for integrated nutritional and management strategies. These indicate that scientific research on antifungal phytoactive substances is integrated into a broad context due to the links between them. Visual interpretation of the map reveals the grouping of terms into four distinct thematic areas that are differentiated by color codes, which define the architecture of current research. The red cluster represented by animal health and performance represents the central core of the network. It highlights the critical interdependence between the administration of phytoactive substances and the optimization of health status, directly correlated with productivity and reproductive performance parameters.

The map represented by anti-inflammatory substances highlights the keyword dairy cows (Figure 2), representing a cluster that forms the most networks, but the central location of the performance cluster suggests that although dairy cows represent the main key topic of the research, performance is the key concept through which the effects of nutritional and management interventions are evaluated.

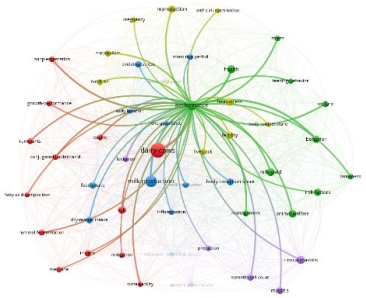


Figure 2. Anti-inflammatory bibliographic analysis and correlation with associated fields

The close association between milk production and the concepts related to transition period and oxidative stress suggests the interest of research into nutritional interventions, including the use of phytoactive substances with anti-inflammatory effects with a role in supporting and adapting metabolism in early lactation (Kumprechtová et al., 2022).

The interconnection of the term performance with aspects related to well-being and eating behavior highlights the integrative nature of performance which is influenced by biological, nutritional and management factors (Cooke et al., 2020).

Terms such as sustainability, stress, genetic parameters, growth performance, well-being are positioned on the periphery of the network but are interconnected both with each other and with the other keywords. Their position suggests that sustainability and genetics issues are being progressively integrated into studies on dairy cow performance and health (Brito et al., 2021; VandeHaar et al., 2016). These terms in the VOSviewer map indicate subthemes with low frequency but potential for development.

The graphic configuration of the map reflects the complexity of the interactions between inflammation, metabolism and productivity, being structured into five major thematic areas, namely: The Red Cluster is represented by the Thematic Core (Dairy Cows): This constitutes the central pillar of the analysis, having the highest density of nodes and connections. The Green Cluster represents Performance and Intervention Evaluation: Centrally positioned, this cluster acts as an outcome indicator, grouping together key concepts. The Blue Cluster represents Transition Physiology and Oxidative Stress: Focuses on metabolic challenges specific to the critical transition period. The Yellow Cluster represents Well-being and Eating Behavior: Integrates the ethological and management dimensions. The Purple Cluster represents Genetics and Sustainability (Emerging Directions): Represents niche areas with a currently lower keyword frequency, but with a steady increase in the specialized literature

In the graphic representation made with the help of VOSviewer, several distinct groups of keywords were highlighted reflecting the main research directions associated with the use of

phytoactive substances with antioxidant properties (Figure 3).

Performance is the centrally located cluster that interconnects with most keywords. It forms close links with oxidative stress, quality, transition period, health, early lactation, highlighting the determining role of these factors in optimizing performance in dairy cows (Cardoso et al., 2021).

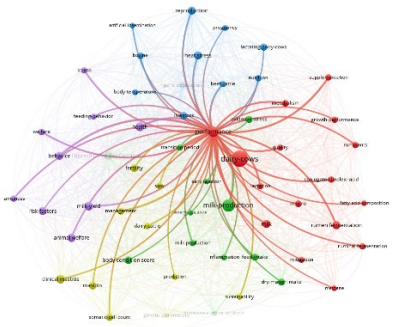


Figure 3. Antioxidant bibliographic analysis and correlation with associated fields

The proximity of the terms to the central cluster suggests that "performance" is analyzed mainly in the context of metabolic adaptation but also of maintaining health in critical stages of the production cycle (Caixeta and Omontese, 2021). Milk production, an important cluster, presents a close link with energy balance, lactation, sustainability, because this association reflects the research interest in optimizing milk production through technological interventions (Gheorghe-Irimia et al., 2023) that support both productive performance and the sustainability of livestock operations (Gaworski, 2021).

Risk factors are interconnected with animal welfare, reproductive performance, health, fertility, metabolism, feeding behavior, suggesting that risk factor management is essential for maintaining metabolic and reproductive health but also for ensuring animal welfare (Licitra et al., 2021).

Welfare and breeding performance form a distant link, being located on the periphery of the map but interconnected with other central terms, suggesting that welfare and breeding performance indicate the current orientation towards more sustainable and animal-oriented production systems (Losacco et al., 2025).

The map highlights the multidimensional role of antioxidants, grouping research into four directions of interest that define the metabolic resilience of animals: The Red Cluster represented by Performance and Systemic Health: Represents the dominant cluster and main connection node of the map. The Green Cluster stands for Technological Optimization and Energy Balance: It focuses on the applied side of nutrition. The Blue Cluster represents Risk Factor Management: Groups research that explores the interface between metabolism, well-being and reproductive function. The Yellow Cluster represents Sustainable Production Systems: Reflecting current management trends

The graph associated with the use of phytoactive substances with antimicrobial properties presents a slightly different structure in several respects (Figure 4). Although correlations are observed between most of the keywords, the bond density is lower, indicating a thematic diversity focused on certain sub-themes.

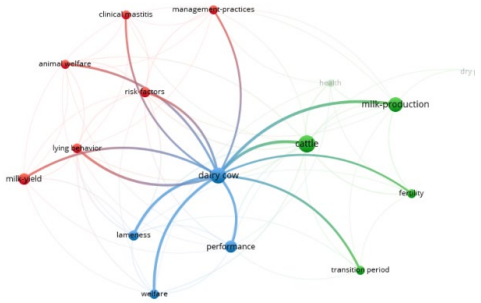


Figure 4. Antimicrobial bibliographic analysis and correlation with associated fields

The cluster represented by dairy cows is interconnected with performance, behavior, milk production and management practices, highlighting the multidimensional nature of the research. This interconnection suggests that studies on the use of phytoactive substances with antimicrobial properties are oriented not only towards productive parameters but also towards optimizing growth conditions and behavioral parameters (Priyashantha et al., 2026).

The graphic representation highlights a single link between mammary rest period and milk production, which suggests a specific thematic

association, indicating that the mammary rest period is predominantly analyzed in the context of the impact on productive performance, without being extensively integrated into other thematic directions (Manriquez et al., 2019). The configuration of this map emphasizes the transition from curative treatments to preventive management and animal welfare, being structured as follows:

The Red Cluster represented by Management and Breeding Practices: Represents an extensive field that correlates dairy cow exploitation systems with their behavior. The Green Cluster represents Breast Health and Prevention: This thematic grouping focuses on a critical point in production: the mammary rest period. The Blue Cluster represents Performance and Subject Centricity.

The bibliometric analysis performed using VOSviewer highlighted several distinct groups of keywords related to the use of phytoactive substances with immunoregulatory effects (Figure 5). The strongest relationship highlighted in the graphic representation is observed between dairy cows and fertility.

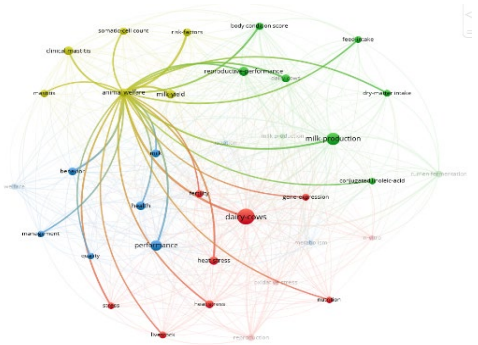


Figure 5. Immunoregulatory bibliographic analysis and correlation with associated fields

Relevant connections are also identified with terms such as gene expression, performance, milk production, animal welfare and ruminal fermentation, suggesting the multidimensional nature of the research. This network structure indicates that fertility is a central element in studies dedicated to dairy cows, being correlated with both physiological and biochemical mechanisms as well as with productive and physiological parameters (de Souza Fonseca et al., 2022; Roche et al., 2017).

Nutrition, although located on the periphery of the network, forms close relationships with most keywords, representing an integrating factor, correlated with productive, metabolic and health parameters.

The fifth map illustrates the complexity of the immune response and its integration into the animal's systemic physiology, being defined by the following clusters: The Red Cluster represents Fertility and Uterine Physiology: It constitutes the central pillar and most robust area of the map. The Green Cluster represents Physiological and Biochemical Mechanisms: It groups fundamental research on internal processes. The Blue Cluster represents Integrative Nutrition: Although positioned peripherally, this cluster correlates dietary strategies with health parameters and metabolic profile. The Yellow Cluster represents Risk Management and Behavioral Performance: Located slightly peripherally, but characterized by a dense network of connections.

CONCLUSIONS

Medicinal plants represent an important resource of bioactive compounds with antioxidant, anti-inflammatory, antimicrobial, immunoregulatory properties, which can contribute to maintaining health and improving the productive performance of cattle.

The integration of medicinal plants in cattle nutrition is considered a promising alternative to the use of antibiotics, in the context of current concerns regarding the occurrence of residues in animal products and the development of antimicrobial resistance.

The bibliometric analysis reveals a close correlation between the research of phytoactive compounds in bovine zootechnics and indicators of health, productivity and reproductive performance. The study of these substances, recognized for their antioxidant, antimicrobial and immunoregulatory valences, is approached in an integrated way, targeting the metabolic balance and physiology of dairy cows.

The integrated analysis of the maps: Antioxidant, Immunoregulatory, Anti-inflammatory, demonstrates a major paradigm shift in current research. Scientific interest has shifted from treating clinical conditions to early

modulation of metabolism and the immune system.

In most maps, the onset of lactation and the mammary dormancy period appear as major connection hubs.

A significant number of clusters, in maps (2, 4 and 5) position animal behavior and management practices as key elements in the network. Even if they sometimes appear slightly peripheral, the large number of links they form demonstrates that behavior monitoring and welfare assurance are recognized as essential tools for early detection of disease risks (such as mastitis) and for maintaining productivity.

The peripheral but emerging positioning of topics such as ecological footprint (methane emissions), genetic selection for resilience and sustainability of production systems clearly shows where the future of research is heading. These bibliometric niches indicate that future livestock performance will only be validated if it is ecologically sustainable and genomically substantiated.

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EFFECTS OF A SYNBIOTIC AND A POLYPHENOL-ELECTROLYTE COMPLEX ON BROILER PERFORMANCE AND MEAT QUALITY

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Abstract

Feed additives are widely used in broiler farms to support intestinal health, reduce oxidative stress, and optimize productive performance and meat quality. The study was designed to evaluate the impact of administering these products, either individually or in combination, on broiler chickens. The study evaluated the individual and combined effects of a multi-strain symbiotic (E) and a polyphenol-electrolyte complex (NC), on productive performance, carcass yield, breast muscle oxidative stability, and meat quality of Ross 308 broilers raised under commercial summer conditions. A total of 40,000 day-old chicks were distributed into four sections of 10,000 birds each (Avicola Smirna SRL, Ialomita County, Romania; June 10-July 17, 2024; 38 days). Groups were: C (control), E (synbiotic 0.5 kg/t feed), N (polyphenol-electrolyte complex, 0.5 kg/t feed), and E+N (both products). Mean house temperature was 28.6 ± 3.4°C. Group E+N achieved the highest final body weight (2,528 ± 67 g; +6.5% vs C, $p < 0.001$), lowest FCR (1.56 vs 1.62, $p < 0.001$), and lowest mortality (2.51 vs 3.48%, $p < 0.05$). Breast muscle MDA was lowest in E+N (0.58 nmol/g; −36.3% vs C, $p < 0.001$), while SOD and CAT activities were highest ($p < 0.001$ vs C). Carcass yield improved to 75.5% in E+N (vs 73.4% in C; $p < 0.01$). EBI improved by +16.0% in E+N vs C (259.5 vs 223.8; $p < 0.001$). Combined supplementation represents an effective antibiotic-free strategy to counteract heat stress-related performance losses and improve meat antioxidant status in summer commercial broiler production.

Key words*: broiler chickens, feed additives, growth performance, meat quality, economic efficiency.*

INTRODUCTION

The progressive intensification of commercial broiler production has substantially improved growth rate and feed efficiency, but has also increased susceptibility to two major production challenges: intestinal dysbiosis and oxidative stress (Sugiharto, 2016; Yegani & Korver, 2008). In summer production cycles, these challenges are compounded by heat stress, which suppresses feed intake, impairs antioxidant capacity, disrupts intestinal barrier integrity, and increases mortality, reducing the productive and economic efficiency of commercial flocks (Lara & Rostagno, 2013; Mangan & Siwek, 2024). The restriction of antibiotic growth promoters in the European Union since 2006 (Regulation EC No 1831/2003) has made the identification of effective non-antibiotic feed additives addressing these challenges a priority for the poultry industry.

Synbiotics – defined as combinations of prebiotics and probiotic microorganisms with a demonstrable intestinal health benefit – are among the most studied non-antibiotic strategies in broiler nutrition. The commercial synbiotic used contains *Bacillus subtilis* DSM 17299 (1×10^9 CFU/g), *Lactobacillus acidophilus* NCIMB 30184 (1×10^8 CFU/g), fructooligosaccharides (FOS, 15% w/w), and mannan-oligosaccharides (MOS, 10% w/w). It acts by colonising the intestinal mucosa, competitively excluding pathogenic species, enhancing villus height-to-crypt depth ratio, and improving nitrogen and amino acid retention (Brisbin et al., 2008; Yegani & Korver, 2008; Tufan & Bolacali, 2017).

The polyphenol-electrolyte product is specifically formulated for use during thermal stress. It contains a blend of bioavailable plant polyphenols (grape marc, rosemary and oregano extracts; minimum 8% total polyphenol content by Folin–Ciocalteu method) combined with potassium chloride and sodium bicarbonate,

which together support electrolyte balance, activate the Nrf2/ARE antioxidant pathway, neutralise reactive oxygen species (ROS) generated during heat stress, and maintain voluntary feed intake at elevated ambient temperatures (Hu et al., 2019; Mangan & Siwek, 2024). Polyphenol-based additives have been shown to reduce malondialdehyde (MDA) concentrations in broiler breast muscle by 15-30% and to increase superoxide dismutase (SOD) and catalase (CAT) activities significantly under heat stress conditions (Untea & Panaite, 2020; Dominguez et al., 2019).

The two products target mechanistically distinct and non-overlapping biological pathways. The synbiotic (S) modulates intestinal microbiota composition and epithelial morphology through direct luminal colonisation, competitive exclusion of pathogenic species, and enhancement of the villus height-to-crypt depth ratio, thereby improving nutrient digestibility and intestinal barrier integrity (Brisbin et al., 2008; Abdel-Wareth et al., 2021). The polyphenol-electrolyte complex (PEC) acts systemically by activating the Nrf2/ARE antioxidant pathway in response to oxidative challenge, reducing cellular reactive oxygen species (ROS) accumulation, protecting myofibrillar protein integrity, and supporting electrolyte homeostasis at tissue level (Hu et al., 2019; Mangan & Siwek, 2024). These mechanisms operate at different biological levels and are functionally independent: synbiotics exert no primary antioxidant activity, and polyphenol-electrolyte product have no direct effect on intestinal microbiota composition (Sugiharto, 2016; Dominguez et al., 2019). Beyond the absence of redundancy, a positive interaction between these pathways can be anticipated: the improvement of villus surface area and enterocyte functionality by symbiotic product increases the absorptive capacity available for polyphenol uptake, potentially enhancing the systemic bioavailability of PEC's active components; conversely, the reduction of oxidative damage to enterocytes mediated by PEC may sustain the intestinal microenvironment required for efficient synbiotic colonisation (Untea & Panaite, 2020). While individual effects of synbiotics and polyphenol-electrolyte products on broiler performance have been documented separately (Akbarian et al., 2016; Bromfield et al., 2024), published data on their simultaneous administration with comprehensive evaluation of productive performance, carcass characteristics, breast muscle oxidative stability, and economic efficiency under commercial summer conditions remain limited. The hypothesis of the present study was therefore that combined administration of product S and product PEC would produce additive or synergistic effects on all evaluated parameters, exceeding the benefits of either product alone. The objective was to evaluate these effects in Ross 308 broilers at Avicola Smirna SRL over a full 38-day summer production cycle.

MATERIALS AND METHODS

Animals, housing, and experimental design

The study was conducted in a commercial farm setting under normal agricultural management practices in accordance with Council Directive 2007/43/EC and Romanian national legislation (Government Ordinance No. 37/2002). As a commercial observational study with no invasive procedures, formal ethical approval from an institutional animal ethics committee was not required under Romanian legislation. All procedures were conducted with the approval of the farm owner and in compliance with applicable animal welfare standards.

The experiment was conducted at the commercial broiler farm Avicola Smirna SRL, Ialomita County, Romania, during the summer production cycle (June 10-July 17, 2024; 38 days). The trial was carried out within a single climate-controlled production house (2,000 m²) equipped with automated tunnel ventilation (8 variable-speed ridge fans, 12,000 m³/h each; 12 exhaust fans, 40,000 m³/h each), 70 evaporative cooling pad panels (1.8 m × 0.9 m each), gas radiant heating with sensor-based control, and 8 temperature + 4 relative humidity sensors connected to a centralised climate management computer (Fancom F-37 Poultry Controller). Recorded mean house temperature over the production cycle was 28.6 ± 3.4°C (range 21.2-38.8°C), with temperatures exceeding 28°C on 22 out of 38 days. Mean relative humidity was 58.4 ± 6.2%. Mean Temperature-Humidity Index (THI) was 79.8 ± 4.2 (calculated as THI = dry-bulb T°C − [0.55 − 0.0055 × RH%] × [dry-

bulb T°C − 14.5]), exceeding the critical threshold of 72 on 28 days of the cycle, confirming exposure to moderate-to-severe heat stress for a substantial portion of the production period (Lara & Rostagno, 2013; Saeed et al., 2019).

A total of 40,000 Ross 308 day-old chicks (sexed: 51% male, 49% female; sourced from MACH hatchery, Czech Republic) were distributed into four experimental sections of 10,000 birds each (Table 1), separated by solid floor-to-ceiling partition panels to prevent cross-contamination of feed and water between sections. Stocking density was 20 birds/m². Birds were housed on rice-husk litter (initial depth 8 cm) with ad libitum access to feed (chain feeding system, Roxell Mealmatic) and water (nipple drinkers, 1 per 10 birds) throughout the cycle.

The study was conducted as a commercial observational trial in which each section of 10,000 birds constituted one experimental unit per treatment. The authors acknowledge that the absence of independent house-level replication is a limitation of this design, and that between-section statistical comparisons at the bird level may be influenced by section-level management factors. Results should be interpreted in the context of a well-controlled commercial setting and not as a fully replicated experimental design.

Table 1. Experimental design (single house, summer cycle June 10-July 17, 2024)

Group	n (birds)	Supplementation
C - Control	10,000	Basal diet, no additive
S	10,000	Basal diet + Synbiotic (0.5 kg/t feed, days 1–38)
PEC	10,000	Basal diet + polyphenol-electrolyte complex (0.5 kg/t feed, days 1–38)
S+PEC	10,000	Basal diet + Synbiotic + Polyphenol-Electrolyte Complex

The two proudcts were incorporated into the feed at 0.5 kg/tonne throughout all three phases. In Group S+PEC, both products were added simultaneously at their respective inclusion rates.

Diets and feeding programme

All sections received the same three-phase basal diet (Table 2) formulated and manufactured by a commercial factory from Romania. The diet was formulated to cover the nutritional requirements of Ross 308 broilers under heat stress conditions, incorporating electrolyte supplementation, anhydrous betaine, and organic acid blend to support osmoregulation and feed intake during the summer cycle. Feed composition was verified by proximate analysis at reception using AOAC (2016) reference methods. Raw material nutritive value was monitored by NIR spectroscopy (Foss NIRS DS2500) at the food compound.

Table 2. Composition and calculated nutritional characteristics of basal diets for Ross 308 broilers

Ingredient/ Nutritional indicator	Starter (d 1-10)	Grower (d 11-25)	Finisher (d 26-38)
Ingredients (%)			
Corn	51.85	55.40	59.10
Wheat	3.50	3.00	2.50
Soybean meal	33.80	28.20	23.40
Sunflower meal	0.00	2.00	3.50
Sunflower oil	4.95	6.25	6.78
Dicalcium phosphate	1.94	1.74	1.54
Calcium carbonate	1.42	1.42	1.52
Sodium chloride	0.24	0.24	0.24
Sodium bicarbonate	0.18	0.14	0.10
DL-Methionine 99%	0.29	0.23	0.18
L-Lysine HCl 78%	0.23	0.25	0.22
L-Threonine 98%	0.12	0.10	0.08
L-Valine 98%	0.06	0.04	0.03
Vitamin-mineral premix	1.18	0.76	0.65
Phytase (750 FTU/kg product)	0.01	0.01	0.01
NSP-enzymes (xylanase + beta-glucanase)	0.02	0.02	0.02
Coccidiostat	0.05	0.05	0.00
Antioxidant (BHA)	0.02	0.02	0.02
Citric / fumaric acid blend	0.08	0.07	0.05
Bentonite	0.01	0.01	0.01
Choline chloride	0.05	0.05	0.05
TOTAL	**100.00**	**100.00**	**100.00**
Nutritional parameters			
Metabolisable energy, ME (kcal/kg)	2,995	3,052	3,110
ME : crude protein ratio (kcal/% CP)	133.7	152.6	172.8
Crude protein, CP (%)	22.41	19.99	18.00
Crude fat (%)	7.52	8.81	10.08
Crude fibre (%)	3.22	3.08	2.94
Crude ash (%)	5.68	5.25	4.92
Dry matter (%)	88.05	87.74	87.52
Lysine	1.20	1.06	0.96
Methionine	0.57	0.49	0.41
Methionine + cysteine	0.87	0.78	0.69
Threonine	0.80	0.71	0.63
Tryptophan	0.24	0.21	0.18
Valine	1.01	0.90	0.80
Isoleucine	0.87	0.77	0.68
Arginine	1.40	1.25	1.11
Calcium, Ca (%)	0.99	0.91	0.87
Available phosphorus, avP (%)	0.48	0.42	0.38
Total phosphorus (%)	0.70	0.63	0.56

Betaine and acidifier included for thermal stress protection.

Data collection and measurements

Body weight (BW) was measured weekly on a random sample of 50 birds per section (0.5% of each section) at days 0, 7, 14, 21, 28, 35, and 38 (slaughter day). Average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR = total feed consumed / total live-weight gain) were calculated per section per phase. Mortality was recorded individually by the farm veterinarian and expressed as cumulative percentage per section. At slaughter (day 38), 20 birds per section (10 male + 10 female, random selection) were individually weighed before and after processing. Carcass yield, dissected anatomical region weights (breast muscle, thighs + drumsticks, wings, back + neck), abdominal fat pad weight, and internal organ weights (liver; heart + gizzard) were recorded as percentages of live slaughter weight. Breast muscle samples (pectoralis major, right side) from the 20 slaughter birds per section were pooled by section, transported chilled (4°C), and analysed within 24 hours at an accredited laboratory (Laborex 2000 SRL, Bucharest; SR EN ISO/IEC 17025:2018, Certificate LI 1160) for: proximate composition (moisture, crude protein, crude fat, mineral ash) per AOAC (2016); caloric value by adiabatic bomb calorimetry; fatty acid profile by GC-FID per ISO 12966-2:2011; amino acid profile by ion-exchange chromatography per ISO 13904:2005; malondialdehyde (MDA) as a marker of lipid peroxidation by the thiobarbituric acid reactive substances (TBARS) method (Buege & Aust, 1978); superoxide dismutase (SOD) activity by the xanthine oxidase inhibition assay (Marklund & Marklund, 1974); and catalase (CAT) activity by the UV-spectrophotometric method (Aebi, 1984). Economic efficiency was assessed using the European Efficiency Factor (EEF = [BW (kg) × (100 − mortality%) / (FCR × slaughter age)] × 100) and European Broiler Index (EBI = EEF × carcass yield / 100).

Statistical analysis

Data are expressed as mean ± SD. Differences between groups were assessed by one-way ANOVA followed by Tukey's HSD post-hoc test. Significance levels were significat $p < 0.05$ (*), distinct significant $p < 0.01$ (**), high significant $p < 0.001$ (***). All tests were two-tailed. Analyses performed in IBM SPSS Statistics v.25.

RESULTS AND DISCUSSIONS

Thermal conditions during the production cycle

The recorded microclimate data confirmed that birds experienced genuine heat stress throughout the summer cycle. Mean house temperature was 28.6 ± 3.4°C, with temperatures exceeding 28°C – the critical thermal threshold for Ross 308 beyond which feed intake and growth rate are suppressed (Lara & Rostagno, 2013) – on 22 of 38 production days. The mean THI of 79.8 exceeded the moderate-stress threshold of 72 on 28 days, reaching levels consistent with severe stress (THI > 84) on several consecutive summer days. Saeed et al. (2019), in a comprehensive review of heat stress effects in commercial poultry, documented that sustained exposure to THI > 79 for periods exceeding 6 hours/day results in measurable reductions in ADG (8-18%) and FCR deterioration (5-12%) relative to thermoneutral conditions. The productive results recorded in Group C of the present study are consistent with these published effects, confirming the validity of the thermal challenge and providing the biological context within which the additive effects of symbiotic and Polyphenol-Electrolyte Complex are to be interpreted.

Body weight and average daily gain

Mean body weight and ADG data are presented in Table 3. Initial chick weight was uniform across sections (42.2-42.3 g; $p > 0.05$). Significant between-group differences emerged from day 14 onward. At day 14, Group S showed the greatest advantage (+20.2 g vs C; $p < 0.05$), Group S+PEC the highest absolute BW (+30.8 g vs C; $p < 0.05$), while Group PEC showed an intermediate but non-significant advantage (+11.6 g; $p > 0.05$). This pattern of earlier BW response in Group S versus Group PEC during the prestarter phase is consistent with the faster onset of synbiotic intestinal colonisation effects (Brisbin et al., 2008), while the polyphenol component exerts its primary effects during periods of peak oxidative challenge, which intensify progressively as ambient temperatures increase through the grower and finisher phases. By day 38, Group S+PEC achieved 2,528 ± 67 g ($p < 0.001$ vs C 2,374 ± 69 g), Group S 2,448 ± 65 g ($p < 0.05$), and Group PEC 2,431 ± 69 g ($p < 0.05$).

Table 3. Body weight (g/bird) and average daily gain (g/bird/day) by group and interval (mean ± SD)

Interval	Group C	Group S	Group PEC	Group S+PEC	Significance
Body weight (g/bird)					
Day 0	42.3±1.3	42.2±1.4	42.3±1.3	42.2±1.4	NS
Day 7	186.4±8.8	190.2±8.4	188.5±8.6	192.7±8.3	NS
Day 14	451.6±16.2	471.8±15.4	463.2±15.8	482.4±15.1	*
Day 21	864.3±25.4	899.7±23.6	884.6±24.8	917.5±24.1	**
Day 28	1,518±40.2	1,572±38.4	1,555±41.0	1,608±39.6	**
Day 35	2,138±57.4	2,211±54.8	2,192±58.6	2,276±56.2	*S,PEC; ***S+PEC
Day 38 (slaughter)	2,374±68.6	2,448±65.2	2,431±69.4	2,528±66.8	*S,PEC; ***S+PEC
ADG (g/bird/day)					
Days 1–11	21.3 ± 1.1	21.6 ± 1.2	21.3 ± 1.0	21.3 ± 1.0	NS
Days 12–26	38.7 ± 1.8	39.7 ± 1.7	41.9 ± 1.9	41.9 ± 1.9	* PEC, S+PEC vs C
Days 27–38	59.3 ± 2.4	60.6 ± 2.2	62.6 ± 2.5	62.6 ± 2.6	NS
Overall d 1–38	94.1 ± 3.5	95.7 ± 3.4	98.5 ± 3.8	98.5 ± 3.7	* PEC, S+PEC vs C

NS = not significant; *p < 0.05; **p < 0.01; ***p < 0.001 vs Group C (one-way ANOVA, Tukey HSD). n = 50 birds/section at each timepoint. Overall ADG = (BW at slaughter − BW at placement) / 38 days.

The +6.5% BW improvement in Group S+PEC falls within the 4–8% range documented for synbiotic supplementation in commercial broilers (Sugiharto, 2016), and the +2.4% improvement in Group PEC is consistent with the 65–84 g BW gains per bird reported by Mangan & Siwek (2024) for polyphenol-based interventions in heat-stressed broilers.

Quintana-Ospina et al. (2023), analysing commercial performance data from over 95 million Ross 308 AP broilers under tropical conditions (mean temperature 28-32°C), reported final BW of 2,050-2,150 g at day 35 in non-supplemented flocks. Adjusted for the additional 3 growth days, our Group C BW of 2,374 g is consistent with this range, confirming that the thermal challenge at Avicola Smirna produced comparable performance outcomes to documented heat-stress conditions in the published literature. Bromfield et al. (2024), evaluating next-generation Bacillus-based probiotics in Ross 308, reported day-21 BW of 847 g in their best-performing formulation, comparable to the 864-917 g range across our groups at the same timepoint, supporting the biological plausibility of the data recorded.

Feed intake, specific consumption (FCR), and mortality

ADFI, FCR, and mortality are presented in Table 4. ADFI during the prestarter and grower phases did not differ significantly among groups ($p > 0.05$). During the finisher phase (days 27-38), however, Groups PEC and S+PEC recorded significantly higher ADFI than Group C (+5.2 g/bird/day and +7.8 g/bird/day, respectively; $p < 0.05$ and $p < 0.01$), while Group S did not differ from control. This selective ADFI improvement in PEC-supplemented groups is mechanistically consistent: the potassium chloride and sodium bicarbonate components of PEC counteract the electrolyte depletion caused by increased respiratory evaporation during heat stress, maintaining cellular hydration and voluntary feed intake at elevated temperatures (Saeed et al., 2019). FCR was significantly improved in Group S (1.58; $p < 0.05$ vs C 1.62) and Group S+PEC (1.56; $p < 0.001$), but not in Group PEC alone (1.61; $p > 0.05$).

This suggests that despite higher finisher-phase ADFI, PEC alone did not improve nutrient utilisation efficiency sufficiently to reach statistical significance at FCR level – the FCR benefit was primarily driven by the synbiotic component improving intestinal morphology and nutrient digestibility, consistent with Tufan & Bolacali (2017). Cumulative mortality was significantly reduced only in Group S+PEC (2.51% vs 3.48%; $p < 0.05$), saving approximately 97 birds per 10,000 per cycle. Van der Klein et al. (2024) reported significantly reduced mortality in DFM-treated flocks under summer heat stress, supporting the role of synbiotics in protecting intestinal barrier integrity against heat stress-induced permeability increases.

Table 4. Average daily feed intake (g/bird/day), FCR, and cumulative mortality (%) by group (mean ± SD)

Parameter	Group C	Group S	Group PEC	Group S+PEC
ADFI d 1–11 (g/bird/day)	22.8±1.4	23.4±1.5	23.6±1.3	24.2±1.4
ADFI d 12–26 (g/bird/day)	84.6±3.5	86.1±3.2	87.4±3.6	88.8±3.4
ADFI d 27–38 (g/bird/day)	110.4±4.6	112.8±4.3	115.6±4.8	118.2±4.5
ADFI overall (g/bird/day)	74.8±3.3	76.4±3.0	77.8±3.4	79.5±3.2
FCR (kg feed/kg gain)	1.62±0.04	1.58±0.03	1.61±0.04	1.56±0.03
Cumulative mortality (%)	3.48±0.31	2.84±0.26	3.12±0.28	2.51±0.23

ADFI finisher and overall: *S, **S+PEC vs C ($p < 0.05$ and $p < 0.01$); S vs C NS. FCR: *S vs C ($p < 0.05$); ***S+PEC vs C ($p < 0.001$); PEC vs C NS. Mortality: *S+PEC vs C ($p < 0.05$); S and PEC vs C NS.

Slaughter yield and carcass characteristics

Carcass yield and part weights are presented in Table 5. Group S+PEC produced the highest carcass yield (75.5 ± 0.6%; $p < 0.01$ vs C 73.4%), followed by Group PEC (74.6 ± 0.7%; $p < 0.05$) and Group S (74.0 ± 0.6%). Breast muscle proportion was highest in Group S+PEC (32.6 ± 0.7%; $p < 0.001$ vs C 30.1%), with significant improvements also in Groups S and PEC ($p < 0.01$ and $p < 0.05$ respectively). Abdominal fat was significantly reduced in Groups PEC and S+PEC ($p < 0.05$ and $p < 0.01$), consistent with the inhibition of lipogenic enzymes by polyphenols documented by Dominguez et al. (2019) and Untea & Panaite (2020). Internal organ weights did not differ among groups ($p > 0.05$), confirming the absence of supplementation-induced hypertrophy. The carcass yield range of 73.4-75.5% is consistent with Suliman et al. (2023), who reported 72.4-74.8% for Ross 308 at 35 days, with the slight upward shift in our study attributable to the additional 3 days of growth and methodological inclusion of neck in the carcass weight. The breast muscle improvement in Groups PEC and S+PEC (31.2 and 32.6% vs 30.1%) is consistent with the finding of Hu et al. (2019) that polyphenol supplementation in heat-stressed broilers reduces cortisol-mediated muscle protein catabolism, thereby supporting anabolic muscle deposition relative to live weight.

Table 5. Carcass yield and dissected part weights (% of live slaughter weight) by group (mean ± SD)

Parameter (%)	Group C	Group S	Group PEC	Group S+PEC
Carcass yield	73.4±0.7	74.0±0.6	74.6±0.7	75.5±0.6
Breast muscle	30.1±0.9	30.9±0.8	31.2±0.8	32.6±0.7
Thighs + drumsticks	30.2±0.7	30.5±0.6	30.7±0.7	31.1±0.7
Wings	9.8±0.4	9.7±0.3	9.6±0.4	9.5±0.3
Back + neck	19.0±0.5	18.8±0.5	18.5±0.5	17.9±0.5
Abdominal fat pad	1.88±0.19	1.76±0.17	1.71±0.18	1.58±0.16
Liver	2.51±0.20	2.46±0.18	2.43±0.19	2.39±0.17
Heart + gizzard	2.76±0.16	2.73±0.14	2.70±0.15	2.67±0.14

Carcass yield: *PEC, **S+PEC vs C; S vs C NS. Breast muscle: *S, **PEC, ***S+PEC vs C. Abdominal fat: *PEC, **S+PEC vs C. All other parameters NS ($p > 0.05$).

Table 6. Breast muscle proximate composition, oxidative stability indices, and selected quality parameters by group (mean ± SD)

Parameter	Group C	Group S	Group PEC	Group S+PEC
Proximate composition	74.2 ± 0.5	74.0 ± 0.5	73.8 ± 0.5	73.5 ± 0.6
Moisture (%)	74.5±0.6	74.2±0.5	74.1±0.6	73.8±0.7
Crude protein (%)	21.6±0.4	22.2±0.4	21.9±0.4	22.8±0.4
Crude fat (%)	2.22±0.22	2.08±0.20	2.05±0.21	1.84±0.18
Mineral ash (%)	1.13±0.06	1.14±0.05	1.15±0.06	1.16±0.06
Caloric value (kcal/100 g)	112.6±3.4	113.8±3.2	113.4±3.5	115.1±3.3
Oxidative stability	8.62 ± 0.18	8.72 ± 0.17	8.85 ± 0.19	9.01 ± 0.20
MDA (nmol/g)	0.91±0.09	0.76±0.08	0.68±0.07	0.58±0.07
SOD activity (U/mg protein)	5.84±0.41	6.52±0.44	7.08±0.38	7.73±0.42
CAT activity (U/mg protein)	11.8±1.0	13.2±0.9	14.6±1.1	16.4±0.9
Fatty acid profile				
n-6/n-3 PUFA ratio	6.62±0.44	6.34±0.41	6.18±0.38	5.91±0.36
PUFA/SFA ratio	0.79±0.06	0.82±0.05	0.84±0.06	0.88±0.05
Amino acids (g/100 g protein)				
Lysine	8.54±0.20	8.68±0.19	8.61±0.21	8.92±0.20
Methionine	2.41±0.09	2.48±0.08	2.45±0.09	2.55±0.09

MDA (malondialdehyde): *S, **PEC, ***S+PEC vs C. SOD (superoxide dismutase): *S, **PEC, ***S+PEC vs C. CAT (catalase): *S, **PEC, ***S+PEC vs C. Crude protein: *S, ***S+PEC vs C. Crude fat: *PEC, **S+PEC vs C. n-6/n-3: *S, **S+PEC vs C. Lysine: *S+PEC vs C. All other parameters NS.

Table 7. European Efficiency Factor (EEF) and European Broiler Index (EBI) by group

Parameter	Group C	Group S	Group PEC	Group S+PEC
BW at slaughter (g)	2,374	2,448	2,431	2,528
Mortality (%)	3.48	2.84	3.12	2.51
FCR	1.62	1.58	1.61	1.56
Age at slaughter (d)	38	38	38	38
Carcass yield (%)	73.4	74.0	74.6	75.5
EEF	305.2	322.8	316.4	343.7
EBI	223.8	239.0	235.8	259.5

*S vs C ($p < 0.05$); PEC vs C NS; ***S+PEC vs C ($p < 0.001$).

Breast muscle oxidative stability and meat quality

Proximate composition and oxidative markers are presented in Table 6. The most significant finding in this section was the pattern of oxidative stability, with MDA progressively decreasing and SOD/CAT activities progressively increasing from Group C to S+PEC, consistent with the expected mechanistic effects of both additives on the antioxidant system. MDA was lowest in Group S+PEC (0.58 ± 0.07 nmol/g; −36.3% vs C 0.91 nmol/g; $p < 0.001$) and Group PEC (0.68 nmol/g; −25.3% vs C; $p < 0.01$), while Group S showed an intermediate reduction (0.76 nmol/g; −16.5%; $p < 0.05$). The greater MDA reduction in Group PEC versus Group S confirms that the antioxidant mechanism in this study was primarily mediated by polyphenol component, consistent with Nrf2/ARE pathway activation documented by Hu et al. (2019) and Dominguez et al. (2019). SOD and CAT activities followed the inverse pattern, being highest in Groups PEC and S+PEC ($p < 0.01$ and $p < 0.001$ vs C), confirming upregulation of endogenous antioxidant enzyme synthesis. These results represent the first simultaneous documentation of MDA, SOD, and CAT responses to combined synbiotic + polyphenol supplementation in a commercial Ross 308 summer production cycle.

Crude protein was significantly higher in Groups S (22.2%; $p < 0.05$) and S+PEC (22.8%; $p < 0.001$) versus Group C (21.6%), while crude fat was reduced in Groups PEC and S+PEC ($p < 0.05$ and $p < 0.01$). These values are consistent with Suliman et al. (2023), who reported breast muscle CP of 21.8-22.6% and crude fat of 1.8–2.5% for Ross 308 at 35 days. The n-6/n-3 PUFA ratio improved from 6.62 in Group C to 5.91 in Group S+PEC ($p < 0.01$), approaching the WHO recommended maximum of 4:1 for meat products and consistent with the fatty acid profile findings of Suliman et al. (2023). Lysine content in S+PEC breast muscle (8.92 g/100 g protein; $p < 0.05$ vs C 8.54) reflects improved protein deposition efficiency mediated by synbiotic-enhanced intestinal amino acid absorption (Tufan & Bolacali, 2017).

Economic efficiency

EEF and EBI values are presented in Table 7. Group S+PEC achieved the highest EEF (343.7) and EBI (259.5), improvements of +12.6% and +16.0% respectively versus Group C (EEF 305.2; EBI 223.8; $p < 0.001$). Group S showed significant improvements (EEF 322.8; EBI 239.0; $p < 0.05$ vs C). Group PEC did not reach statistical significance for EEF or EBI versus control (316.4 and 235.8; $p > 0.05$), despite significant improvements in carcass yield and breast muscle oxidative stability, reflecting the FCR neutrality of Polyphenol-Electrolyte Complex as a standalone additive. At commercial scale, Group S+PEC generated approximately 1,540 kg additional live weight per 10,000-bird section vs Group C (value: ~7,700 lei at prevailing ex-farm prices), with FCR savings of approximately 3,100 kg feed (~6,510 lei), for a gross benefit of ~14,210 lei per section per cycle. After subtracting additive costs (~2,850 lei), the net economic benefit of S+PEC supplementation is estimated at ~11,360 lei per 10,000-bird section per cycle.

Freitas et al. (2025) reported EBI values of 300-320 for well-managed European commercial flocks under temperate conditions without supplementation. Our Group C EBI of 223.8 is below this range, reflecting the documented depressive effect of sustained summer heat stress on cumulative flock performance (Saeed et al., 2019; Mangan & Siwek, 2024). The S+PEC improvement of +16.0% demonstrates that combined supplementation partially but substantially compensates for heat stress-related performance losses, reducing the productivity gap relative to temperate benchmarks. This finding has practical importance for commercial producers in continental European climates, where summer heat stress is expected to intensify in frequency and severity under climate change projections.

CONCLUSIONS

The present commercial observational study demonstrated that individual and combined administration of synbiotic and polyphenol-electrolyte complex to Ross 308 broilers significantly improved growth performance, feed conversion ratio, carcass yield, breast muscle oxidative stability, and economic efficiency over a 38-day summer production cycle characterised by sustained heat stress (mean THI 79.8; temperatures above 28°C on 22 of 38 days). The following specific conclusions are drawn:

The synbiotic (0.5 kg/t feed) improved FCR by 2.5% (1.58 vs 1.62; $p < 0.05$ vs C), enhanced breast muscle crude protein (+2.8%; $p < 0.05$ vs C), and increased SOD activity (+11.6%; $p < 0.05$ vs C), consistent with its synbiotic mechanism of improving intestinal health and nutrient absorption.

Polyphenol-electrolyte complex (0.5 kg/t feed) significantly improved final body weight (+2.4%; $p < 0.05$ vs C), maintained higher finisher-phase ADFI (+5.2 g/bird/day; $p < 0.05$), reduced breast muscle MDA by 25.3% ($p < 0.01$ vs C), and increased SOD and CAT activities ($p < 0.01$ vs C), consistent with its polyphenol-mediated Nrf2/ARE antioxidant activation mechanism under heat stress.

Combined administration (Group S+PEC) produced the best results across all evaluated parameters: final BW +6.5% ($p < 0.001$), FCR −3.7% ($p < 0.001$), carcass yield +2.1 pp ($p < 0.01$), breast muscle protein +1.2 pp ($p < 0.001$), MDA −36.3% ($p < 0.001$), EBI +16.0% ($p < 0.001$), and lowest mortality (2.51%; $p < 0.05$) versus the unsupplemented control.

The net economic benefit of combined S+PEC supplementation was estimated at approximately 11,360 lei per 10,000-bird section per summer cycle, after deducting additive costs.

The study has limitations inherent to its single-house commercial observational design (one section per treatment, no independent house-level replication). Future research should validate these findings in a multi-house replicated design and include intestinal morphology, microbiota composition, and blood oxidative status biomarkers to further characterise the mechanisms of action.

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EFFICIENCY OF USING LOCAL FEED RESOURCES IN TURKEY HYBRID NUTRITION

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Abstract

Feeding represents a key determinant of productive efficiency in turkey production systems, directly influencing growth performance, production costs, and overall sustainability. This study evaluated several dietary formulations designed for an interline turkey hybrid developed within the gene bank of INCDCSZ Braşov. The diets were optimized in terms of nutrient levels and incorporated local feed resources (peas, maize, wheat, sunflower meal) alongside medicinal plants with immunostimulatory potential (nettle, garlic, calendula). The results showed that balanced formulations based on local raw materials and supplemented with 2-4.5% medicinal plants ensured an optimal balance between productive performance, animal health, and economic efficiency. These feeding strategies also reduced reliance on imported feed ingredients and limited vulnerability to market price fluctuations. Although the average unit cost of the feed increased moderately, economic analysis indicated that the potential biological and preventive health benefits may partially compensate for these additional costs.

Key words*: economic efficiency, feed conversion ratio, local feed resources, medicinal plants, turkey hybrids.*

INTRODUCTION

The importance of feeding in meat production

Feeding represents the major component of any poultry production system, consistently accounting for 60-70% of total production costs. This reality is well documented in the scientific literature (Donohue & Cunningham, 2009; Zampiga et al., 2021). In the case of turkeys raised for meat production, the levels of crude protein (CP) and metabolizable energy (ME) are key determinants of growth performance, carcass quality, and the economic efficiency of production (Ognik et al., 2021).

In the current context, characterized by global fluctuations in feed raw material prices (FAO, 2024), diet optimization is no longer solely a matter of biological performance, but also of economic sustainability and efficient integration into the modern food chain, where product quality and requirements related to food safety and packaging are becoming increasingly important (Davidescu et al., 2025; Curea et al., 2023).

Numerous experimental studies have demonstrated the independent and significant effects of metabolizable energy (ME) and crude protein (CP) on turkey performance. For example, Sell et al. (1985) showed that both increasing protein and energy levels influence daily weight gain and slaughter yield; however, the additional benefits tend to plateau once certain nutritional thresholds are exceeded. More recent studies confirm that adjusting dietary energy levels affects not only growth rate but also meat quality (color, cooking losses), thereby directly linking feeding strategies to the commercial value of the final product (Abouelezz et al., 2019; Lungu et al., 2024; Moise et al., 2025).

Reference nutritional recommendations remain those published by the National Research Council (NRC, 1994); however, these have been expanded and updated by more recent reviews highlighting the importance of diet formulation not only in terms of total protein but also digestible amino acids (Lee et al., 2023; Konieczka et al., 2021). Targeted supplementation with essential amino acids-particularly lysine, methionine + cystine, and threonine-allows productive performance to be maintained

at moderate CP levels, while simultaneously reducing feed costs and nitrogen excretion (Abouelezz et al., 2019).

Moreover, the literature indicates that feed form and processing play an essential role in determining feed conversion efficiency. Pelleting and optimization of particle size increase feed intake and digestibility, aspects that are particularly important in young birds. In addition, the ratio between ME and CP can affect the yield of specific anatomical parts: inadequate ratios may reduce breast meat proportion, even when body weight gain appears satisfactory (Gous et al., 2023).

Thus, the scientific literature emphasizes that the efficiency of compound feeds in turkeys is not determined exclusively by protein or energy supply, but by a combination of factors: optimal levels of ME and CP, the profile of digestible amino acids, feed form, and appropriate adaptation to the different growth phases (NRC, 1994; Rostagno et al., 2017; Abdollahi et al., 2013; Aviagen Turkeys, 2022). All these aspects must be evaluated together with the unit cost of the finished feed, since nutritionally adequate formulations that are excessively expensive cannot be applied in practice (Donohue & Cunningham, 2009; OECD-FAO, 2023).

Contribution of local feed resources

The use of on-farm feed resources represents a fundamental strategy for reducing costs and increasing the sustainability of poultry farms (OECD-FAO, 2023; Mottet & Tempio, 2017). Locally available raw materials (maize, wheat, peas, and sunflower) form the basis of many compound feed formulations used in turkey production, providing both energy and protein supply (NRC, 1994; Ravindran, 2013).

Maize is one of the most widely used cereals in poultry feeds due to its high starch content and superior metabolizable energy value (NRC, 1994; Rostagno et al., 2017). At the same time, it contains relatively low levels of crude protein and essential amino acids, particularly lysine and tryptophan, which requires its combination with protein sources (Watson & Ramstad, 2003; Leeson & Summers, 2001; Rostagno et al., 2017). Under Romanian conditions, maize also benefits from wide availability and relatively competitive prices, making it a key ingredient in feed formulations (INSSE, 2023; OECD-FAO, 2023).

Wheat, compared with maize, has a higher protein content and provides appreciable amounts of amino acids such as methionine and tryptophan, although it supplies slightly lower metabolizable energy (Day et al., 2006; Shewry, 2009; NRC, 1994). In addition, the relatively high levels of antinutritional factors (arabinoxylans, NSP - non-starch polysaccharides) may reduce digestibility, which is why the use of exogenous enzymes is often recommended (Cowieson et al., 2006; Choct, 2006). Nevertheless, locally produced wheat remains an important resource for diversifying the cereal base and balancing feed costs (OECD-FAO, 2023).

Peas represent an alternative protein source to soybean meal, containing approximately 22-25% crude protein and a favorable starch composition (NRC, 1994; Rostagno et al., 2017). However, the relatively low level of methionine and the presence of certain antinutritional factors (tannins, trypsin inhibitors) require limiting the inclusion rate or applying technological treatments. Studies indicate that peas can partially replace soybean meal without compromising poultry performance, thereby reducing both feed costs and dependence on imported protein sources (Tufarelli et al., 2018).

Sunflower (seeds or meals) provides a valuable contribution of protein (28-38% CP depending on processing) and crude fiber, but it is deficient in lysine, which requires correction through the use of synthetic amino acids or other lysine-rich ingredients (González-Alvarado et al., 2007; NRC, 1994). In Romania, sunflower meal is widely available as a co-product of the oil industry, making it economically attractive for use in feed formulations (OECD-FAO, 2023; INSSE, 2023).

The role of medicinal plants in turkey nutrition

The integration of medicinal plants into poultry feed formulations represents an innovative approach aimed at addressing current requirements for reducing the use of synthetic additives and increasing the sustainability of poultry production (Windisch et al., 2008; Greathead, 2003). Phytonutrients derived from plants such as nettle (*Urtica dioica*), garlic (*Allium sativum*), and calendula (*Calendula officinalis*) provide a range of nutritional and functional benefits, with positive effects on productive performance, animal health, and the quality of the products obtained (Amad et al.,

2011; Toghyani et al., 2011; Trifunschi et al., 2017; Surai et al., 2019).

Nettle is an important source of vitamins (A, C, K), minerals (Fe, Ca, Mg), and bioactive compounds with antioxidant and immunostimulatory properties (Bhusal et al., 2022; Chrubasik et al., 2007). In poultry diets, nettle contributes to the reduction of oxidative stress and the stimulation of immune responses, which may lead to greater disease resistance and more efficient nutrient utilization (Surai et al., 2019; Windisch et al., 2008).

Garlic contains organosulfur compounds (allicin, diallyl disulfide) with antimicrobial, antifungal, and immunomodulatory effects (Block, 1993; Amagase et al., 2001; Golubkina et al., 2024). In poultry feeding, the inclusion of garlic in diets has been associated with improved feed conversion, reduced mortality, and an improved lipid profile of meat (Karangiya et al., 2016; Toghyani et al., 2011).

Calendula is rich in carotenoids (lutein, zeaxanthin), flavonoids, and volatile oils with anti-inflammatory and antioxidant effects (Mishra et al., 2024; Preethi et al., 2009). In poultry nutrition, calendula contributes to improving skin and egg yolk quality by intensifying pigmentation, while also supporting overall health through the reduction of inflammatory and oxidative processes (Makinde, 2022; Surai et al., 2019).

Numerous studies have confirmed the beneficial effects of medicinal plants on productive performance and poultry health. For example, Ognik et al. (2021) demonstrated that supplementing turkey diets with plant extracts can modulate immune status and oxidative stress markers. Moreover, these authors highlighted the role of amino acids and plant-derived bioactive compounds in supporting intestinal health, suggesting a synergistic interaction between conventional nutrition and phytotherapeutic supplementation (Surai et al., 2019; Zhai et al., 2018; Lungu & Moise, 2025; Usturoi et al., 2023; Usturoi et al., 2025).

From a practical perspective, the partial replacement of synthetic additives with phytotherapeutic resources offers the advantage of reducing costs associated with veterinary treatments and producing meat with superior organoleptic properties, which is better appreciated by consumers. In addition, the use of medicinal plants in poultry nutrition aligns with current trends in the European Union aimed at reducing antibiotic use and promoting natural solutions for maintaining animal health (European Commission, 2019).

Economic rationale: cost-efficiency analysis

Feeding strategies for meat turkeys must be evaluated not only from the perspective of the biological value of feeds but also in terms of economic efficiency. Considering that feed represents the main component of production expenses (Donohue & Cunningham, 2009; Biofeed, 2020), it is essential to identify formulations that provide the best balance between nutritional parameters and the unit cost of the finished feed.

Cost-efficiency analysis involves comparing productive performance (average daily gain, feed conversion ratio, and slaughter yield) with the costs associated with producing each kilogram of feed. This approach allows the evaluation of the "real cost of performance" and the selection of formulations that, at a moderate cost, can ensure results comparable to or even superior to more expensive alternatives (Donohue & Cunningham, 2009; Pesti & Choct, 2023).

In recent years, numerous studies have shown that reducing the overall level of crude protein, combined with targeted supplementation of essential amino acids, does not compromise productive performance but reduces feed cost and nitrogen excretion, thereby contributing to both economic and environmental sustainability (Strifler et al., 2023; Woyengo et al., 2023; Lee et al., 2023). In this context, economic analysis goes beyond the simple comparison of ingredient prices, requiring the integration of biological results into the calculations.

In the Romanian market, where the prices of soybean meal and synthetic additives are strongly influenced by imports and fluctuations in international markets, the use of local resources (maize, wheat, peas, sunflower) and medicinal plants reduces dependence on external raw materials and provides farmers with greater financial stability.

From the farmers' perspective, the most relevant indicators in the economic evaluation of feeding strategies are the unit cost of the finished feed, the feed conversion ratio, the cost per kilogram of meat produced, and the added value of the final product, including meat quality and

consumer acceptability. These aspects are becoming increasingly important in the context of current requirements regarding food safety and the sustainability of the food supply chain (Pesti & Choct, 2023).

MATERIALS AND METHODS

Experimental design

The experimental design was developed to test the hypothesis that the use of feed resources available on small farms (peas, maize, wheat, sunflower) and the differentiated integration of medicinal plants into compound feed formulations can improve productive performance, with a favorable impact on production costs and the sustainability of farming systems.

Experimental conditions

The experimental groups were established immediately after hatching, using poults originating from the same hatchery and from parent flocks of the same age in order to minimize genetic influences on the results. The testing period lasted 20 weeks, during which growth parameters, mortality, and feed conversion were monitored.

Experimental structure

The interline turkey hybrid obtained by crossing the Diana and White Large breeds was used, as this combination is known to express heterosis effects, leading to superior growth performance, feed conversion efficiency, and disease resistance compared with pure breeds (Crawford, 1990; Besbes, 2009).

Four experimental groups were established as follows:

- Control (M) - standard diet, without the addition of medicinal plants;
- Group 1 (G1) - diet supplemented with 4.5% medicinal plants (nettle 3%, garlic 1%, calendula 0.5%);
- Group 2 (G2) - diet supplemented with 3% medicinal plants (nettle 1.5%, garlic 1%, calendula 0.5%);
- Group 3 (G3) - diet supplemented with 2% medicinal plants (nettle 0.5%, garlic 1%, calendula 0.5%).

Growth phases

Turkey feeding was organized into four technological phases (starter, grower 1, grower 2, and finisher). This approach reflects age-specific nutritional requirements:

- during the starter phase, protein requirements are high in order to support the development of organs and muscle tissue;
- subsequently, protein requirements decrease, but maintaining adequate energy and mineral intake remains essential for achieving the final body weight and ensuring carcass quality.

The four technological growth phases were as follows:

- starter (0-7 weeks): compound feed containing 24% crude protein (CP);
- grower 1 (8-11 weeks): diets containing 22% CP and variable levels of medicinal plants (0-4.5%);
- grower 2 (12-15 weeks): diets containing 20% CP and variable levels of medicinal plants (0-4.5%);
- finisher (16-20 weeks): diets containing 17% CP and variable levels of medicinal plants (0-4.5%).

The aim was to evaluate the effect of medicinal plant inclusion while maintaining a constant protein level, with moderate variations in energy density and structural fraction.

Integration of medicinal plants and justification of inclusion levels

The diets were formulated based on locally available feed resources (maize, wheat, peas, sunflower meal, soybean meal), supplemented with mineral additives (calcium carbonate, salt) and dried, ground medicinal plants.

For each growth phase, four distinct formulations were developed (Control + G1 + G2 + G3), resulting in a total of 12 experimental diets. These formulations were balanced to ensure a constant crude protein level, with the only differences deriving from the proportion of medicinal plants included (Tables 1, 2, 3, and 4). Three levels of medicinal plant inclusion (4.5%, 3%, and 2%) were tested, compared with a control diet without plant supplementation. The selection of these inclusion levels was based on literature indicating beneficial effects of nettle, garlic, and calendula on digestibility, immune status, and meat quality (Rahimi et al., 2011; Bhusal et al., 2022). However, these effects are dose-dependent: excessively high inclusion levels may reduce palatability or affect nutritional balance, whereas moderate inclusion levels may exert stimulatory effects on intestinal health and metabolic processes.

Statistical analysis of the data

The experimental data were statistically processed using a one-factor model, in which the experimental factor was the nutritional treatment (Control, G1, G2, G3). The productive parameters evaluated (final body weight, average daily gain, feed conversion ratio, and viability) were expressed as the arithmetic mean (X) and the standard error of the mean (Sx). Differences between means were assessed using one-way analysis of variance (ANOVA). When significant differences were identified, pairwise comparisons of means were performed using Tukey's post-hoc test. The level of statistical significance was set at $p < 0.05$, with differences considered highly significant $p < 0.01$ and very highly significant at $p < 0.001$.

Table 1. Required feed formulations

Specification		CP (%)	MP* (%)	Nettle (%)	Garlic (%)	Calendula (%)
Starter		Compound feed containing 24% CP				
Grower 1 (8-11 weeks)	Control (Diet 1)	22.00	0.00	0.00	0.00	0.00
	Group 1 (Diet 2)	22.00	4.50	3.00	1.00	0.50
	Group 2 (Diet 3)	22.00	3.00	1.50	1.00	0.50
	Group 3 (Diet 4)	22.00	2.00	0.50	1.00	0.50
Grower 2 (12-15 weeks)	Control (Diet 5)	20.00	0.00	0.00	0.00	0.00
	Group 1 (Diet 6)	20.00	4.50	3.00	1.00	0.50
	Group 2 (Diet 7)	20.00	3.00	1.50	1.00	0.50
	Group 3 (Diet 8)	20.00	2.00	0.50	1.00	0.50
Finisher (16-20 weeks)	Control (Diet 9)	17.00	0.00	0.00	0.00	0.00
	Group 1 (Diet 10)	17.00	4.50	3.00	1.00	0.50
	Group 2 (Diet 11)	17.00	3.00	1.50	1.00	0.50
	Group 3 (Diet 12)	17.00	2.00	0.50	1.00	0.50

*MP - medicinal plants; Diet ingredients (feed resources available from household farms): peas; maize; wheat; sunflower.

RESULTS AND DISCUSSIONS

Feed formulations used

The analysis of the diets corresponding to the three technological phases highlights the maintenance of crude protein levels according to age-specific requirements (22% in Grower 1, 20% in Grower 2, and 17% in Finisher), while the inclusion of medicinal plants led to adjustments in energy density and in the structural fraction of the diets (Tables 2-4). In all phases, the groups supplemented with phytotherapeutic additives showed slightly lower metabolizable energy values and a moderate increase in crude fiber content compared with the control, thereby implicitly modifying the ME/CP ratio. Although moderate, these variations may influence nutrient utilization efficiency and voluntary feed intake, particularly during stages characterized by high growth intensity or by the optimization of final tissue deposition. Therefore, the interpretation of productive performance should be conducted in the context of these nutritional adjustments and not solely in terms of the effects of the bioactive compounds present in the medicinal plants.

Table 2. Compound Feed Formulations - Grower 1 Phase (8-11 weeks)

Raw materials	Control (Diet 1)	Group 1 (Diet 2)	Group 2 (Diet 3)	Group 3 (Diet 4)
	%	%	%	%
Wheat	30.18	22.56	25.19	27.08
Maize	25.00	25.00	25.00	25.00
Sunflower seeds	26.04	29.48	28.03	27.14
Soybean meal	10.00	9.70	10.00	10.00
Peas	5.00	5.00	5.00	5.00
Calcium carbonate	3.39	3.37	3.38	3.38
Salt	0.39	0.40	0.40	0.40
Nettle	0.00	3.00	1.50	0.50
Garlic	0.00	1.00	1.00	1.00
Calendula	0.00	0.50	0.50	0.50
Total	100	100	100	100
ME (MJ/kg)	11.05	10.49	10.68	10.72
CP (%)	22.00	22.00	22.00	22.00
CFat (%)	2.09	2.00	2.03	2.01
CF (%)	6.34	6.73	6.56	6.48
Ca (%)	1.44	1.44	1.44	1.45
TP (%)	0.55	0.55	0.55	0.55
Na (%)	0.17	0.17	0.17	0.17
Met (%)	0.40	0.41	0.40	0.40
Met + Cys (%)	0.78	0.79	0.79	0.78
Lys (%)	0.89	0.90	0.90	0.89
Thr (%)	0.76	0.77	0.77	0.76
Trp (%)	0.25	0.25	0.25	0.25

ME - metabolizable energy; CP - crude protein; CFat - crude fat; CF - crude fiber; Ca - calcium; TP - total phosphorus; Na - sodium; Met - methionine; Met + Cys - methionine + cystine; Lys - lysine; Thr - threonine; Trp - tryptophan.

Table 3. Compound Feed Formulations - Grower 2 Phase (12 – 15 weeks)

Raw materials	Control (Diet 5)	Group 1 (Diet 6)	Group 2 (Diet 7)	Group 3 (Diet 8)
	%	%	%	%
Wheat	37.61	30.62	34.09	33.59
Maize	25.00	25.00	25.00	25.00
Sunflower seeds	21.55	23.05	19.07	21.47
Soybean meal	7.00	8.00	10.00	9.10
Peas	5.00	5.00	5.00	5.00
Calcium carbonate	3.44	3.43	3.45	3.44
Salt	0.39	0.40	0.40	0.40
Nettle	0.00	3.00	1.50	0.50
Garlic	0.00	1.00	1.00	1.00
Calendula	0.00	0.50	0.50	0.50
Total	100	100	100	100
ME (MJ/kg)	11.37	10.86	11.13	11.04
CP (%)	20.00	20.00	20.00	20.00
CFat (%)	2.13	2.03	2.06	2.08
CF (%)	5.62	5.76	5.24	5.38
Ca (%)	1.44	1.44	1.44	1.44
TP (%)	0.51	0.50	0.49	0.50
Na (%)	0.17	0.17	0.17	0.17
Met (%)	0.35	0.36	0.35	0.35
Met + Cys (%)	0.70	0.71	0.69	0.69
Lys (%)	0.77	0.80	0.82	0.79
Thr (%)	0.67	0.68	0.68	0.67
Trp (%)	0.22	0.22	0.22	0.22

ME - metabolizable energy; CP - crude protein; CFat - crude fat; CF - crude fiber; Ca - calcium; TP - total phosphorus; Na - sodium; Met - methionine; Met + Cys - methionine + cystine; Lys - lysine; Thr - threonine; Trp - tryptophan.

Table 4. Compound Feed Formulations - Finisher Phase (16-20 weeks)

Raw materials	Control (Diet 9)	Group 1 (Diet 10)	Group 2 (Diet 11)	Group 3 (Diet 12)
	%	%	%	%
Wheat	17.38	10.00	12.39	13.66
Maize	50.00	50.00	50.00	50.00
Sunflower seeds	18.72	21.68	20.72	20.45
Soybean meal	5.00	5.00	5.00	5.00
Peas	5.00	5.00	5.00	5.00
Calcium carbonate	3.50	3.42	3.49	3.49
Salt	0.40	0.40	0.40	0.40
Nettle	0.00	3.00	1.50	0.50
Garlic	0.00	1.00	1.00	1.00
Calendula	0.00	0.50	0.50	0.50
Total	100	100	100	100
ME (MJ/kg)	11.76	11.22	11.40	11.33
CP (%)	17.00	17.00	17.00	17.00
CFat (%)	2.63	2.54	2.57	2.55
CF (%)	5.12	5.45	5.35	5.39
Ca (%)	1.44	1.42	1.44	1.44
TP (%)	0.46	0.47	0.47	0.47
Na (%)	0.17	0.17	0.17	0.17
Met (%)	0.32	0.33	0.32	0.32
Met + Cys (%)	0.63	0.64	0.63	0.63
Lys (%)	0.65	0.66	0.66	0.65
Thr (%)	0.59	0.61	0.60	0.59
Trp (%)	0.18	0.18	0.18	0.18

ME - metabolizable energy; CP - crude protein; CFat - crude fat; CF - crude fiber; Ca - calcium; TP - total phosphorus; Na - sodium; Met - methionine; Met + Cys - methionine + cystine; Lys - lysine; Thr - threonine; Trp - tryptophan.

Analysis of productive performance

The results showed a slight decrease in final body weight in the groups supplemented with medicinal plants compared with the control (4.335 kg in M vs. 4.060-4.206 kg in G1-G3 variants), although these differences were not statistically significant ($p > 0.05$). This trend suggests that the inclusion of phytogenic additives within the range of 2-4.5% did not substantially affect the growth potential of the hybrid, but may be associated with the concurrent nutritional adjustments of the diets, particularly the slight reduction in energy density and the increase in the structural fraction.

The scientific literature indicates that the effects of medicinal plants on productive performance are dose-dependent and influenced by the composition of the basal diet. Studies conducted on meat-type poultry have reported either moderate improvements in growth at low inclusion levels or the absence of significant differences at higher doses when the dietary energy density is reduced (Windisch et al., 2008; Toghyani et al., 2011). Similarly, Ognik et al. (2021) showed that supplementation with plant extracts may modulate physiological parameters without inducing significant increases in body mass.

Therefore, in the case of the hybrid analyzed, the absence of statistically significant differences suggests that phytotherapeutic supplementation does not compromise growth performance, but neither does it generate a direct increase in body weight under conditions of a slight reduction in metabolizable energy. From a practical perspective, this result indicates that the use of medicinal plants can be implemented without significant losses in final body weight; however, their benefits should be evaluated in relation to feed efficiency and the associated production costs.

Table 5. Final production performance

Specification	Treatment							
	Control		Group 1		Group 2		Group 3	
	X	S_X	X	S_X	X	S_X	X	S_X
Live body weight (kg)	4.335	0.1548	4.206	0.1681	4.156	0.1621	4.060	0.1732
Average daily gain (kg)	0.031	0.0011	0.030	0.0012	0.029	0.0012	0.029	0.0012
Feed conversion ratio (kg)	4.448	0.1612	4.797	0.1881	5.123	0.1972	5.532	0.2320
Viability (%)	100		100		95.00		95.00	

Table 6. Testing the statistical significance of the observed differences in final body weight

Specification		Treatment			
		C	G1	G2	G3
Treatment	C	-	0.5652	0.7989	1.1844
	G1	NS	-	0.2138	0.6048
	G2	NS	NS	-	0.4049
	G3	NS	NS	NS	-

NS - statistically non-significant differences ($p > 0.05$)

Table 7. Testing the statistical significance of the observed differences in feed conversion ratio

Specification		Treatment			
		C	G1	G2	G3
Treatment	C	-	1.4083	2.6518	3.8407
	G1	NS	-	1.1981	2.4646
	G2	*	NS	-	1.3453
	G3	**	*	NS	-

NS - statistically non-significant differences ($p > 0.05$)
* - significant differences ($p < 0.05$)
** - significant differences ($p < 0.01$)

With regard to feed conversion, the results revealed a progressive increase in FCR values with the inclusion of medicinal plants, from 4.448 kg feed/kg gain in the control group to 4.797 kg in G1, 5.123 kg in G2, and 5.532 kg in G3. The differences compared with the control were statistically significant for groups G2 ($p < 0.05$) and G3 ($p < 0.01$), indicating a reduction in feed conversion efficiency at inclusion levels of 2–3% medicinal plants.

This trend may be explained by the slight reduction in dietary energy density and the increase in the structural fraction, which may lead to compensatory feed intake in order to meet the birds' energy requirements. The literature indicates that phytogenic additives may improve feed conversion when used at moderate levels and under conditions of constant dietary energy density; however, their effects may become neutral or even negative when their inclusion alters the energy balance of the diet (Windisch et al., 2008; Zhai et al., 2018). Furthermore, studies conducted on meat-type poultry indicate that plant-based additives rich in fiber may influence intestinal transit and nutrient digestibility, with a direct impact on FCR (Choct, 2006).

In the present case, the results suggest that the tested inclusion levels, particularly 2-3%, resulted in reduced feed efficiency without generating a significant advantage in weight gain. From a practical perspective, this aspect is essential, since feed conversion represents the main determinant of the cost per kilogram of meat produced. Therefore, the use of medicinal plants should be calibrated so that potential functional benefits are not offset by a reduction in nutritional efficiency.

The viability of the flock remained high across all experimental groups, reaching 100% in the control and G1 groups and 95% in G2 and G3. The absence of major differences suggests that the inclusion of medicinal plants at levels of up to 4.5% did not have adverse effects on the overall health status or on the birds' capacity to adapt to the experimental conditions.

The literature indicates that phytogenic additives based on plants with antioxidant and antimicrobial properties may contribute to maintaining physiological balance and reducing oxidative stress without necessarily affecting growth parameters (Windisch et al., 2008; Surai et al., 2019). In agreement with these observations, the results obtained in the present study suggest that the tested supplements are biologically safe; however, their effects on productive performance remain dependent on their interaction with the nutritional structure of the diet.

Analysis of the costs of the compound feeds used

The analysis of the costs associated with the compound feeds used highlights clear differences between the control group (M) and the experimental groups supplemented with phytotherapeutic additives (G1-G3), both in

terms of the average cost and the phase-specific costs across the growth stages. For the control group, the weighted average unit cost of the compound feed, calculated over the entire growth cycle, was 0.279 €/kg, representing the lowest value among the variants analyzed.

From the perspective of phase-specific costs, a decreasing trend can be observed with advancing age: from 0.694 €/kg in the starter phase to 0.282 €/kg (Grower 1), 0.264 €/kg (Grower 2), and 0.244 €/kg (Finisher). This profile is characteristic of poultry feed formulations, in which nutrient density (particularly protein supply, amino acids, and premixes) is higher at early ages, generating higher costs during the starter and early growth phases, followed by a gradual reduction as the formulation becomes simpler and the proportion of cereals increases in the later stages. This evolution is consistent with the principles of phase feeding described by Leeson & Summers (2001) and Rostagno et al. (2017), who emphasize that protein requirements and digestible amino acid levels are higher during early growth, resulting in higher starter feed costs, while finisher diets allow a greater inclusion of cereals and less expensive energy ingredients.

In the groups receiving medicinal plants (G1-G3), the average unit cost of compound feed increased substantially compared with the control, reaching 0.375 €/kg (G1), 0.375 €/kg (G2), and 0.375 €/kg (G3). The difference compared with the control is approximately +34%, indicating a substantial economic impact associated with the inclusion of phytotherapeutic components and the reformulation of the diets. The percentage increase reflects the difference relative to the weighted average cost across the entire production cycle, rather than the phase-specific costs.

The literature indicates that the introduction of unconventional ingredients or natural additives can modify the economic structure of feed formulations, even when included at moderate levels, due to their higher unit cost and the adjustments required to maintain nutritional balance (Donohue & Cunningham, 2009; Pesti & Choct, 2023). In intensive poultry production systems, any change in the cost per kilogram of feed is directly reflected in the total budget of the production cycle, given that feeding accounts for the largest share of overall production costs (Zampiga et al., 2021).

It is important to note that although the inclusion levels of the medicinal plant mixture differ among G1-G3, the resulting average costs are practically identical. This suggests that the increase in cost is not strictly proportional to the percentage of plant additives, but is mainly determined by the overall structure of the formulation and by the way in which the phytotherapeutic ingredients replace or complement other components of the diet.

At the level of phase-specific costs, the same decreasing trend is maintained in the phytotherapeutic groups; however, the values remain higher than in the control group, particularly during the growth phases, where costs reach 0.383 €/kg (Grower 1) and 0.366 €/kg (Grower 2), respectively 0.345 €/kg in the Finisher phase. Therefore, in phases characterized by high feed intake (Grower 2 and Finisher), even relatively small differences in cost per kilogram have the potential to significantly increase the total feeding expenditure, since these stages account for the largest share of cumulative feed consumption.

From this perspective, the economic relevance of the unit cost of feed should be interpreted not only through its absolute value, but also through the distribution of feed intake across growth phases. An increase in feed cost during periods of maximum consumption has a disproportionately large impact on the total feed budget of the production cycle. In modern poultry production, economic efficiency is largely determined by the relationship between feed cost and feed conversion performance, as feeding accounts for approximately 60-70% of total production expenses, and fluctuations in the prices of energy and protein ingredients directly influence the farm's profit margin (Donohue & Cunningham, 2009; Mottet & Tempio, 2017; OECD-FAO, 2023).

Although the starter phase records the highest specific cost per kilogram (0.694 €/kg in all variants), its impact on the total cost of the production cycle is limited by the relatively low feed intake (0.975-1.150 kg/bird). In contrast, the Grower 2 and Finisher phases account for the largest volume of feed consumption (6.45-6.70 kg and 8.75-11.00 kg/bird, respectively),

thereby becoming the most decisive stages in determining the overall structure of feeding costs.

In the control group, the majority of feed consumption is distributed across the Grower 2 and Finisher phases, where the specific feed costs are lower (0.264 €/kg and 0.244 €/kg), thus favoring a balanced economic relationship between nutrient density and feed cost. In the phytotherapeutic variants, however, the specific costs during these phases increase to 0.366 €/kg (Grower 2) and 0.345 €/kg (Finisher), and this difference becomes economically significant due to the volume effect.

For example, a difference of 0.101 €/kg in the Finisher phase, applied to a feed intake of 8.75-11.00 kg/bird, generates an additional cost of approximately 0.880-1.120 €/bird in this stage alone. The same principle applies to the Grower 2 phase, where cost differences are amplified by the high level of feed consumption. Consequently, even though the percentage differences in cost per kilogram may appear moderate, their cumulative economic impact becomes substantial, particularly during stages characterized by maximum feed intake.

This phenomenon is explained by the concept of "consumption-weighted cumulative cost", commonly used in economic evaluations of compound feeds, according to which the financial impact of a price difference is proportional not only to the unit value of the feed but also to the total volume consumed during the respective production phases (Donohue & Cunningham, 2009; OECD-FAO, 2023).

Overall, the analysis of the unit cost of compound feed confirms that the inclusion of medicinal plants modifies the economic structure of the formulations not only through the intrinsic cost of the phytotherapeutic ingredients themselves, but also through the adjustments required to maintain a constant crude protein level.

The specialized literature emphasizes that the economic evaluation of feed formulations should be carried out in an integrated manner, by correlating the unit feed cost with productive performance and with the distribution of feed intake across the different growth phases (Pesti & Choct, 2023; Zampiga et al., 2021). This approach is essential because seemingly moderate differences in cost per kilogram may generate significant cumulative effects at the level of the entire production cycle.

Table 8. Average unit cost of the compound feed

Specification		Duration (weeks)	Compound feed intake (g)	Production cost (€/kg)	Average unit cost (€/kg)
C	Starter	0-7	0.975	0.694	0.279
	Grower 1	8-11	2.950	0.282	
	Grower 2	12-15	6.450	0.264	
	Finisher	16-20	8.750	0.244	
G 1	Starter	0-7	0.975	0.694	0.375
	Grower 1	8-11	2.900	0.383	
	Grower 2	12-15	6.450	0.366	
	Finisher	16-20	9.250	0.345	
G 2	Starter	0-7	1.035	0.694	0.375
	Grower 1	8-11	3.200	0.383	
	Grower 2	12-15	6.700	0.366	
	Finisher	16-20	9.750	0.345	
G 3	Starter	0-7	1.150	0.694	0.375
	Grower 1	8-11	2.850	0.383	
	Grower 2	12-15	6.700	0.366	
	Finisher	16-20	11.000	0.345	

Analysis of the unit cost of the live weight meat (€/ton)

The unit cost of the product "live meat" increased progressively across the experimental treatments, reaching a maximum in G3, from 2638.659 €/ton in the control group (M) to 3209.752 €/ton (G1), 3389.219 €/ton (G2), and 3565.177 €/ton (G3). The absolute differences compared with the control group were +571.093 €/ton for G1, +750.560 €/ton for G2, and +926.518 €/ton for G3, corresponding to relative increases of approximately +21.6%, +28.4%, and +35.1%, respectively. This profile indicates a cumulative economic pressure associated with the experimental treatments, with the highest impact observed in G3.

Structure of the total cost and determining factors

The breakdown of total cost by components shows that the main determinant of the differences between treatments is the expenditure on feed, while labor costs remained constant (97.366 €/ton) and therefore cannot explain the observed variations. In the control group, feed costs amounted to 1242.682 €/ton, whereas in the groups supplemented with medicinal plants these costs increased substantially: 1797.696 €/ton (G1), 1920.895 €/ton (G2), and 2073.203 €/ton (G3). Consequently, the differences compared with the control group were +555.014 €/ton (G1), +678.213 €/ton (G2), and +830.521 €/ton (G3).

Analysis of the cost difference structure confirms the dominant role of feed expenditure: 97.184% of the total increase observed in G1 is attributed to feed costs, while in G2 and G3 the contribution of feed remains predominant (90.361% and 89.639%, respectively). Increases in the cost of biological material contribute only secondarily (approximately 4-10% of the total difference), whereas "other expenses" have a marginal contribution (including

slightly negative values compared with the control, which are negligible in magnitude). These findings are consistent with the literature emphasizing that, in poultry production systems, feeding represents the decisive component of production costs, and any changes in feed cost and/or feed conversion efficiency are immediately reflected in the cost per unit of product (Donohue & Cunningham, 2009; Pesti & Choct, 2023).

Economic interpretation integrated with biological performance

From an economic standpoint, the increase in feeding costs reflects a combined effect: a higher unit cost of compound feed in the phytotherapeutic groups and a reduced feed efficiency (higher FCR), both of which amplify the cost per unit of product. In intensive poultry production systems, even relatively small variations in the feed conversion ratio can generate significant economic differences at the level of the final product, given the dominant share of feeding in the overall cost structure (Donohue & Cunningham, 2009; Zampiga et al., 2021).

In practical terms, although differences in feed cost per kilogram may appear moderate, they are multiplied by the total feed intake and by the feed conversion ratio, becoming the main mechanism explaining the differences in the cost of the product "live meat." From this perspective, evaluating the economic feasibility of using medicinal plants cannot be based solely on the cost of the feed formulation, but requires the strict integration of feed cost per kilogram with FCR and with the cost per ton of final product.

Table 9. Comparative unit cost of the live weight meat (€/ton)

Specification		Biological material	Feed	Labor costs	Other expenses	Total production costs
Treatment	C	780.254	1242.682	97.366	518.357	2638.659
	G1	804.204	1797.696	97.366	510.486	3209.752
	G2	856.694	1920.895	97.366	514.263	3389.219
	G3	876.951	2073.203	97.366	517.657	3565.177

Table 10. Structure of the unit cost difference for live meat

Specification		Total difference	Biological material	Other expenses	Feed
G1	Value - €	571.093	23.950	-7.87	555.014
	Structure - %	100	4.194	-1.378	97.184
G2	Value - €	750.560	76.440	-4.094	678.213
	Structure - %	100	10.184	-0.545	90.361
G3	Value - €	926.518	96.697	-0.700	830.521
	Structure - %	100.000	10.437	-0.076	89.639

CONCLUSIONS

The study conducted on the interline hybrid Diana × White Large demonstrated that the use of locally available feed resources allows the formulation of nutritionally balanced diets with adequate crude protein levels, adapted to the specific requirements of each technological growth phase, in accordance with current nutritional recommendations (NRC, 1994; Rostagno et al., 2017).

The inclusion of medicinal plants (*Urtica dioica*, *Allium sativum*, *Calendula officinalis*) at levels of 2-4.5% did not produce significant changes in final body weight and did not affect flock viability, confirming the biological safety of these supplements, in agreement with findings reported in the scientific literature (Windisch et al., 2008; Surai et al., 2019).

However, the tested inclusion levels were associated with a progressive increase in the feed conversion ratio, statistically significant at inclusion rates of 2-3%, which ultimately led to an increase in the cost per unit of product. The economic analysis demonstrated that more than 89-97% of the cost difference observed in the product "live meat" is explained by feed expenditure, confirming the decisive role of feeding within the cost structure of poultry production systems (Donohue & Cunningham, 2009; Pesti & Choct, 2023).

The results suggest that the use of medicinal plants at moderate levels can be integrated into production systems based on local feed resources without negative effects on flock viability. Nevertheless, optimizing the balance between potential functional benefits and economic efficiency requires maintaining an

adequate dietary energy density. Under the experimental conditions analyzed, the tested supplements did not generate a direct economic advantage for the Diana × White Large hybrid, highlighting the importance of calibrating the inclusion level according to the nutritional structure of the diet and the economic objectives of the farm.

Overall, the use of locally available feed resources and medicinal plants may represent a sustainable nutritional strategy for turkey production systems, contributing to the reduction of dependence on imported feed ingredients and increasing resilience to market price fluctuations.

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IMPORTANCE OF RELATIONSHIP BETWEEN FERTILITY AND LACTATION FOR SUSTAINABILITY OF DAIRY FARMING

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Abstract

Data from 192 dairy cows with the mean lactation number of 2.51 (1 to 10) were subjected to descriptive statistics to evaluate lactation and fertility parameters. The mean days in milk (DIM, lactation stage) was 170 for lactating cows (N=172), excluding dry cows (N=20, 10.42%). Cows were housed in early fresh (N = 21, DIM = 20), late fresh (N = 17, DIM = 48), peak (N = 50, DIM = 107), mid lactation (N = 44, DIM = 246), and late lactation (N = 40, DIM = 294) paddocks. Dry cows were housed in far-off (N = 13) and close-up (N = 7) paddocks. The average milk yield was 33.18 L/head/day. The percentage pregnant and inseminated cows were 46.97 and 26.77%. Herd program required to synchronize cows within 70 days postpartum. Avoiding hypocalcemia and ketosis during early postpartum, while strictly providing comfort, cows were expected to achieve pregnancy at peak milk production paddocks. In conclusion, achieving greater than 50% pregnant cows within 180-200 DIM ascertains sustainability of dairy farming.

Key words: *fertility, lactation, sustainability, days in milk, pregnant cow percentage.*

INTRODUCTION

Milk and meat are two of the indispensable foods for mankind. As global warming and drought become major issues, ruminant farming faces limitation challenges. Strategies are setup to perform environmentally friendly practices while maximizing productivity (Wang et al., 2025). The sustainability of dairy farming relies on lactation-fertility synchronization within economically reasonable calving intervals.

With improvements in genomic selection, health, nutrition, and mechanization as well as data management, lactation and fertility considering longevity are prominent parameters. Briefly, calving interval of < 14 months, daily milk yield average of > 40 L, and longevity of > 2.7 are targeted in breeders and age at first calving < 22 months in replacement heifers (Hayirli and Colak, 2011). Peripartum period nutrition pacing with endocrinal adaptation is key point to achieve maximal lactation and efficient fertility, as reflected by rapid increase in milk production and uterine involution. Dry period management and nutrition are often neglected. In opposite, it is an investment period for subsequent lactation and fertility.

Rapid growth of fetus and production of colostrum as well as neurohormonal changes take place, particularly, during the close-up period (Overton & Waldron, 2004). Switching from high roughage-based diets fed during late prepartum to high concentrate-based diet fed during early postpartum is critical for ruminal health, thereby for dramatic increase in milk yield and for accelerated uterine involution.

The objective of this study was to evaluate the relationship between fertility and lactation parameters, which is crucial for sustainability of dairy farms.

MATERIALS AND METHODS

Lactation and fertility data were obtained from 192 (20 dry-10.42% and 172-89.58% lactating) Holstein cows with an average of parity of 2.51 (1 to 10). Cows were housed in 2 row-free stall barns at different paddocks according to DIM

and lactation status for easing reproduction and nutrition management (Table 1). Of 20 dry cows, 13 were far-off dry and 7 were close-up dry. There were 5 paddocks for lactating cows: fresh group, cyclic group, peak lactation group, mid-lactation group, and late-lactation group.

All cows were fed to meet nutrient requirements for dairy cows (NASEM, 2021). On a dry matter basis, ration for lactating cows contained 1.87% straw, 11.65% alfalfa hay, 4.32% barley silage, 29.87% corn silage, 24.16% protein concentrate (RasyoLife, Mugla Türkiye), 23.12% corn flake, 3.12% premix of vitamins, minerals, buffering agents, yeast-sodium bicarbonate-magnesium oxide, toxin binder, salt, limestone, and dicalcium phosphate (RasyoLife) [roughage:concentrate (R:C), 47.68:52.32; crude protein (CP), 16.73%; neutral detergent fiber (NDF), 32.56%; starch, 25.62%, ether extract (EE), 5.23%; and ash, 8.31%].

On a dry matter basis, ration for dry cows contained 21.95% straw, 15.21% alfalfa hay, 30.46% corn silage, 19.52% protein concentrate (RasyoLife, Mugla Türkiye), 11.68% corn flake, 1.19% premix of vitamins, minerals, buffering agents, yeast-sodium bicarbonate-magnesium oxide, toxin binder, salt, limestone, and dicalcium phosphate (RasyoLife) [R:C, 67.61:32.39; CP, 14,10%; NDF, 44.37%; starch, 18.28%, EE, 2.88%; and ash, 7.43%). Close-up cow ration was added with 250 g/h/d anionic salt to avoid hypocalcemia and its consequences.

Farm was equipped with herringbone milking parlor (HB50 with DelProTM, DeLaval, Sweden). Cows were milked thrice daily. After 45-d voluntary waiting period cows were subjected to reproductive synchronization protocol depending on their ovarian status.

A commercial statistics software was used in data analyses (MedCalc Software Version 9.6.2.0, Mariakerke, Belgium). Data (parity, paddock, days in milk, milk yield, and fertility status) from breeders (N = 192) as well as data (age in month and fertility status from replacement heifers (N = 228) were harvested from dairy management system. After sorting by the paddocks and parity, data were subjected to the descriptive statistics for determination of mean and variation and Q-Q plotting for determining concordance correlation between days in milk and fertility status. The effect of paddock (stage of lactation) and parity on response variables (days in milk, milk yield, and days in pregnancy) were established using one-way ANOVA. Differences among paddocks and parities were attained using the LSD option.

RESULTS AND DISCUSSIONS

The mean for DIM and milk yield was 170 and 33.18 L for lactating cow (N = 172), respectively.

In dairy herd, percentages of lactating and dry cows are 84 and 16%. In this herd lower percentage of dry cows (10.42%) is related to voluntarily skipped insemination in cyclic periods occurring during summer due to heat stress. In early summer, dry cow percentage can be as high as 35%. As can be seen in Table 1, cows reached peak production (37.9 L) by DIM 107 and remained in paddock 3 until DIM 246.

Group 1 constitutes early postpartum cows, Groups 2 and 3 constitute peak period cows. Breeding started by DIM 45 if there was natural estrus, otherwise cows were subjected to synchronization. Establishment of the peak period grouping is mostly based on parity pace with social hierarchy.

Group 4 includes mid lactating cows. Their milk yield was considerably lower than Group 3. One can notice a dilemma that persistency of lactation was sharp (46.81 ml/day).

The number of fresh, pregnant, inseminated, open, and to be culled were 48 (25.00%), 87 (45.31%), 53 (27.60%), 2 (1.04%), and 2 (1.04%), respectively. Overall pregnant cow and inseminated cow percentages were 45.31 and 27.60% (Table 1; Figure 1). Considering 40% of insemination efficiency, pregnant cow percentage reach 56.35% with an insemination efficiency of 2.53. This indicates that this farm is about 7 L milk per cow and 3.65% pregnancy below the target level. However, data obtained at the end of September 2025, after 4 months of extreme summer condition. Likewise milk yield, in general, increases in early winter. The agreement between stage of lactation and fertility status, considering either cows pregnant-inseminated or open-fresh was r = 0.89 (Figure 1).

Table 1. Lactation and fertility characteristics of paddocks[1]

	Lactation Status			Cow Distribution by Fertility Status				
Group	N	DIM	Milk, L	F	P	I	O	C
1	21	20±3^{e}	33.0±1.6^{b}	21	0	0	0	0
2	17	48±9^{d}	28.6±1.8^{c}	15	0	2	0	0
3	50	107±8^{c}	37.9±1.1^{a}	12	4	34	1	0
4	44	246±18^{b}	31.3±0.9^{bc}	0	29	14	1	0
5	40	294±23^{a}	31.4±1.2^{b}	0	34	3	0	2
6	13	355±30^{a}	-	0	13	0	0	0
7	7	303±19^{a}	-	0	7	0	0	0

[1]Data are mean±SEM and N. DIM = days in milk; F = fresh, P = pregnant; I = inseminated; O = open; C = to be culled. Different superscripts within the same columns differ (P<0.05).

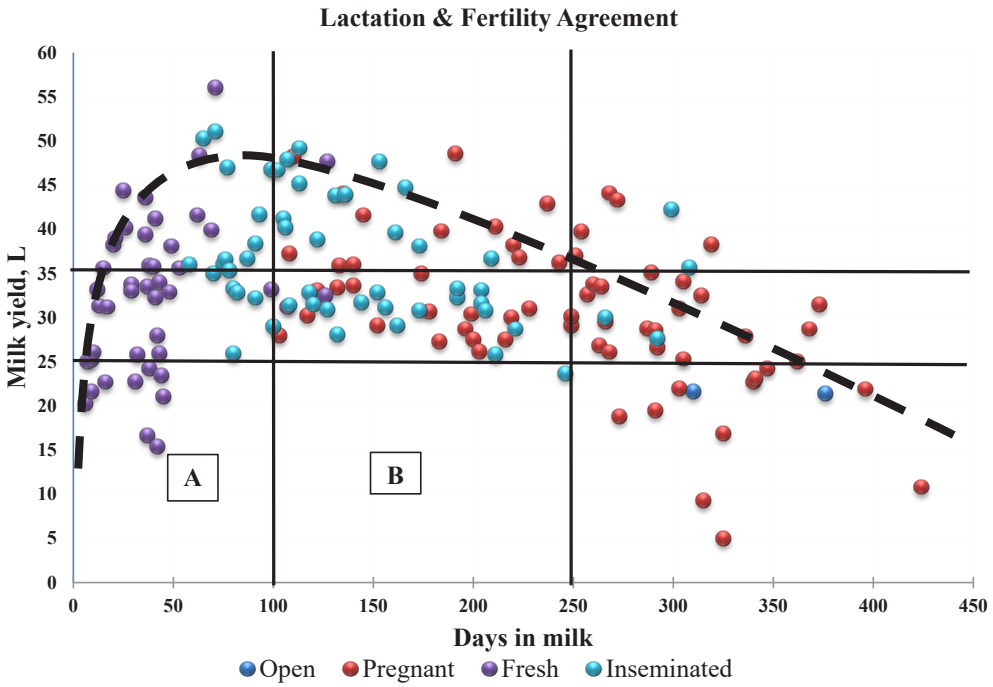


Figure 1. Distribution of cows according to milk yield and fertility status. Concordance correlation value was 0.89

Expectedly milk production continuously increased as the parity advanced up to 5th lactation and then decreased (Table 2).

Parity distribution provides information about longevity and/or culling rate of the breeder cows. 95.84% of the cows were up to 4th lactation (Table 2). This ratio may change depending upon selection for higher productivity accompanied by application of sex-semen. Longevity may be considered an indication of health and productivity. The mean parity was 2.51.

Figure 1 shows all cows based on their lactation performance and fertility status. One can establish upper and lower standard level (horizontal lines on y-axis) in milk yield that is economically feasible. This illustration ease visualization of ill and/or low producing cows considering DIM (Region A and B). One also can establish upper and lower standard level (vertical lines on x- axis) in fertility status that is approximate calving interval, consequently feed cost on milk yield as well as herd size. All cows must be inseminated at least once by DIM 100. Cows are still not pregnant after DIM of 250 have prolonged calving interval.

Table 2. Lactation and fertility characteristics of paddocks[1]

	Lactation Status				Pregnant Cow Distribution		
Parity	N	%	DIM	Milk, L	P, N	P, %	DIP
1	54	28.13	187±19	29.92±0.8[b]	23	26.44	101±11[b]
2	46	23.96	155±24	34.45±1.2[a]	16	18.39	65±11[c]
3	53	27.60	208±18	34.64±1.2[a]	37	42.53	147±11[a]
4	31	16.15	104±24	35.91±1.4[a]	7	8.05	43±11[c]
5	4	2.08	215±60	31.03±4.2[ab]	2	2.30	134±52[a]
7	2	1.04	120±91	29.75±4.0[ab]	0	0	0
8	1	0.52	206±na	31.12±na	1	1.15	177±na
10	1	0.52	341±na	23.10±na	1	1.15	208±na

[1]Data are mean±SEM, N, %. DIM = days in milk; P = pregnant cow as a count and a percentage; DIP = days in pregnancy, a mean number of inseminations for pregnancy was 2.53. Different superscripts within the same columns differ (P<0.05).

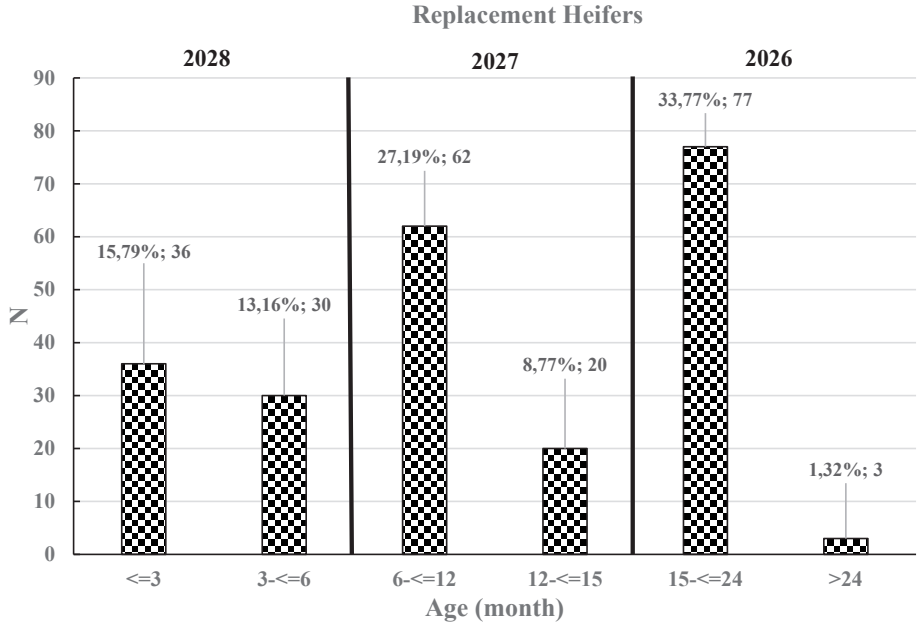


Figure 2. Age distribution of replacement heifers

Under normal conditions, herd complete capacity through replacement heifers (Figure 2). Thus, survival, growth and development of heifer calves are critical in addition to the breeder longevity. Based on culling rate of breeders, one either can expand herd size or sell heifers. Therefore, reproduction in heifers is as important as breeders (Table 3). If conventional sperm is used for insemination, while reaching first calving at age of 24 months, mother would have two lactations. Namely, one lactation yield is about to afford the cost of replacement heifer to make a cow. Therefore, parity average of equal to or greater than three is highly profitable and sustainable. Replacement heifers older than 15 months and 6 months and those aged up to 6 months are potential replacement of current year and following two years.

Achieving fertility parameters (Table 3) along with high lactation yield is directly related to nutrition management (Table 4). As compared to suggestions of National Research Council (NRC, 2001) of nutrient requirements for dairy cows, NASEM (2021) suggest single ration type of recommendation as reflected by elimination of time "t" variable from dry matter intake (DMI) equation. Cows are still grouped based on stage of lactation, lactation yield, or reproductive status in practice.

Table 3. Targets in reproductive parameters (adopted from Hayirli et al., 2011)

Parameter		Value
Age at the first estrus (month)		< 12
Age at the first insemination (month)		13-14
Age at the first calving (month)		24
Weight at the first insemination and after first calving (kg)		380-540
Onset of the first cyclist postpartum (day)		<40
Estrus detection rate (%)		85
Voluntary waiting period (day)		45-75
Conception rate at the first service (%)		35-50
Insemination per pregnancy		< 2.7
Estrus rate within two months postpartum (%)		> 90
Percentage of cows to be pregnant upon > 2 insemination (%)		< 20
Days open		85-110
Percentage of cows with days open >120 (%)		< 15
Calving interval (month)		13-14
Culled cows due to infertility (%)		< 10
Aborts (%)		< 4
Retained placenta (%)		< 8
Uterine infections and cystic ovary (%)		< 10

The grouping of cows is important to keep up with process that should be enforced and affirmed (Table 5). As can be seen in both Figure 1 and Table 5, primary focus should be on maintaining normocalcemia and normoketonemia. These are two major factors endogenously affecting feed intake. Normocalcemic cows are also less likely to have retained placenta and displaced abomasum. Cows consuming dry matter more than 3% of body weight are less likely to experience ketosis while having increased milk production. Metabolic profiling test is essential to maintain health and/or effective treatment for metabolic disorders and reproductive disorders (Emmam et al., 2024; Hayirli et al., 2016). Obese cows and undernourished cows are prone to ketosis and abomasum displacement and have suppressed immunity (Hayirli et al., 2012). Thus, they cannot reach lactation peak and experience silent estrus and frequent embryonic loss.

The periparturient period management, health, and nutrition have been focus of numerous research and review articles. A comprehensive analysis of factors affecting sustainability of dairy farms is well documented in serial studies by Kerwin et al (2021a; 2021b; 2023). Strong management of peripartum period management and nutrition (Bruinje and LeBlanc, 2025; Mezzetti et al., 2021) is the key factor for maximizing milk production and fertility success (Cook et al., 2024; Leroy and Kruif, 2006; Lucy, 2003; Opsomer, 2006). In addition to provision welfare, eliminating incidence of hypocalcemia (Goff, 2006) and ketosis (Kerwin et al., 2021a) allow cows to reflect their genetic capacity.

CONCLUSIONS

Milking cows with higher longevity produces about 40 L/d milk and calves before 14 months are economically profitable. Thus, pregnant cow ratio should be above 60%.

As lactation yield increases reproduction regresses is not correct phenomenon. If high producing cows fail in reproduction metabolic profile testing is necessary and nutrition should be reevaluated.

Undernourished cows would fail in lactation yield. It may result in excess culling rate (>30%).

Sustainability of dairy herds rely on reproductive efficiency while receiving maximal milk yield. This is the result of successful peripartum period management and nutrition protocols.

Table 4. Suggested nutritive values of rations offered by stages of lactation (adopted from Hayirli et al., 2011)

Parameter[1]	Far-off Dry	Close-up Dry*	Fresh**	Peak	Mid Lact.	Late Lact.
BW, kg	650	680	600	580	610	640
DMI, % BW	2.1	1.8	3.00	3.90	3.80	3.70
F:C***	80:20	70:30	50:50	45:55	55:45	60:40
NE_L, Mcal/kg	1.39	1.52	1.72	1.80	1.75	1.65
CP, %	12	13	18	17	16	15
RDP, %CP	70	60	55	60	55	55
EE, %	2	3	4	5.5	5	4
ADF, %	30	24	21	19	23	25
NDF, %	40	35	30	28	32	35
NSC, %	30	34	35	38	36	34
Ca, %	0.60	0.70	1.00	0.90	0.85	0.80
P, %	0.26	0.30	045	0.40	0.38	0.36
Mg, %	0.16	0.30	0.33	0.30	0.28	0.26
K, %	0.65	0.65	1.00	1.00	0.90	0.90
Na, %	0.10	0.05	0.33	0.30	0.28	0.26
Cl, %	0.15	0.70	0.30	0.25	0.24	0.23
S, %	0.16	0.40	0.25	0.25	0.25	0.25
Fe, mg/kg	150	150	150	150	150	150
Co, mg/kg	0.10	0.10	0.10	0.10	0.10	0.10
Mn, mg/kg	60	60	60	60	60	30
Cu, mg/kg	15	15	15	15	15	15
Zn, mg/kg	60	60	60	60	60	30
I, mg/kg	0.60	0.60	0.60	0.60	0.60	0.60
Se, mg/kg	0.30	0.35	0.35	0.35	0.30	0.30
Vitamin A, IU/d	100.000	125.000	125.000	125.000	100.000	100.000
Vitamin D, IU/d	25.000	30.000	30.000	30.000	25.000	25.000
Vitamin E, IU/d	1.000	2.000	2.000	2.000	1.000	800

[1]BW =body weight; DMI = dry matter intake; F:C = forage:concentrate; NEL = net energy for lactation; CP = crude protein; RDP = ruminally degradable protein; EE = ether extract; ADF = acid detergent fiber; NDF = neutral detergent fiber; NSC = non-structural carbohydrate.
*Anionic salt feeding. Antioxidant and vitamin-mineral premixes.
**Lipogenic agents and glucogenic precursors as well as ruminally protected amino acids. Antioxidant and vitamin-mineral premixes.
***Values vary by roughage quality and fecal consistency (+/-5-10%).

Table 5. Summary of expectations in cows during the lactation period

Stage	Days in Lactation	Expectation
Parturition	1-7	Placental membranes are removed, no vaginal bleeding and udder edema, normocalcemic with good appetite
First 3 weeks	8-21	No mastitis, normal locomotion and vaginal discharge, DMI >3% of BW, ketone z < 1.2 mmol, calcium >9.0 mg/dL, sharp increase in milk with good appetite
Early postpartum	22-60	Completed uterine involution, spontaneous estrus, synchronized for cyclicity, body condition score (BCS) loss < 0.5, DMI >3.5% of BW with reaching peak yield
Peak Period	61-200	DMI >3.8% of BW, reached peak yield, cows are either inseminated or >50% pregnant, maintaining BCS >2.75
Mid Lactation	201-300	DMI >3.8%, gradual decline in milk yield, pregnant cows >70%, gaining condition
Late Lactation	301- till dry (~360)	DMI >3.8%, gradual decline milk yield (40% of peak yield), pregnant cows >90%, gaining condition
Far-off Dry	First 40 d	Reached ideal BCS (~3.25), DMI about 2.2% of BW, nonmastitic, normal locomotion, all pregnant
Close-up Dry	Last 21 d	BCS (~3.50), DMI >1.5%, nonmastitic, normal condition, onset of mammogenesis, all pregnant

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THE INFLUENCE OF POLYPHENOLS FROM NETTLE EXTRACT ON IRON METABOLISM AND SOME MICROELEMENTS THAT PARTICIPATE IN MAINTAINING IRON HOMEOSTASIS

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Abstract

Iron is integrated into metabolic processes, cell growth and proliferation, while also being an essential cofactor of the mitochondrial respiratory chain, the Krebs cycle and DNA synthesis. Maintaining iron homeostasis in the body has always been in the sights of researchers at different levels of organization and composition of living organisms, its role can be determined by the biological functions of some proteins that contain this biometal, such as hemoglobin and myoglobin, enzymes involved in biological oxidation processes, enzymes that neutralize ROS and maintain redox balance in the body. At the same time, the excess of iron is associated with cytotoxic effects, which are caused by the peculiarities of iron, as a metal with variable valence, to trigger chain reactions of free radical formation, which lead to lipid peroxidation (LPO) of biological membranes, toxic damage to proteins and nucleic acids. Therefore, the content of this microelement is strictly regulated, which allows us to talk about iron homeostasis. The purpose of this paper is to familiarize with new scientific results of iron homeostasis under the influence of polyphenols extract from nettle.

Key words*: antioxidants, iron homeostasis, iron overload, oxidative stress, plant extracts.*

INTRODUCTION

Free radicals can be produced due to the influence of environmental pollutants, metal ions, radiation, etc. (Alkadi, 2020). As a result of ROS production, oxidative damage to biomacromolecules occurs, which can represent a suitable environment for disrupting the metabolism of energetic and detoxifying substances at the membrane and cell level (Gasmi et al., 2021). Endogenous defense mechanisms of living organisms cannot ensure complete prevention of damage caused by ROS (Gasmi et al., 2021). Natural antioxidants, as effective removers of free radicals, are indispensable in the prevention and treatment of many disorders (Alkadi, 2020). The concept of homeostasis currently plays an important role in the analysis of vital processes at different levels of biological systems. The homeostatic properties of the whole organism are the result of the simultaneous action of many complexly organized regulatory mechanisms, among which one of the important places is occupied by autonomic regulation, which ensures the constancy of metabolic and energy levels in the body, individual organs and tissues (Baevskii et al., 1984).

Iron is essential for oxygen transport, erythropoiesis, and plays an important role in many biological processes, in particular, such as the synthesis of deoxyribonucleic acids (DNA, RNA), amino acids, collagen, steroids, various neurotransmitters and proteins involved in electrolyte transport for mitochondrial respiration, cell proliferation, differentiation and regulation of gene expression, as well as in the functioning of the immune system, detoxification processes and metabolism of pharmacological remedies (Yiannikourides & Latunde-Dada, 2019). Iron is not synthesized in the human and animal organism, and its homeostasis is generally controlled by dietary

iron intake, intestinal absorption and its recycling mechanisms (Moustarah & Mohiuddin, 2022).

Iron deficiency can be caused by physiological, pathological, environmental, drug, genetic or erythropoietic reasons (WHO, 2020).

Excessive amounts of iron can cause toxicity and cell death through free radical formation and lipid peroxidation; therefore, iron homeostasis is strictly regulated (Bresgen & Eckl, 2015). Iron is mainly used as a component of heme in erythrocytes for oxygen transport, while relatively smaller amounts are present in muscle in the form of heme myoglobin and in the liver in the form of ferritin (Kohgo et al., 2008). In most other tissues in the body, iron serves as a component of protein cofactors in the form of iron-sulfur (Fe-S) clusters and heme, which are involved in the electron transport chain to produce ATP (Beinert et al., 1997). In this process, Fe^{2+} and Fe^{3+} serve as electron donors and acceptors, respectively. Basically, iron plays an essential role in maintaining adequate ATP reserves during oxidative phosphorylation in the mitochondrial internal membrane (Oexle et al., 1999).

The regulation of iron homeostasis is a complex multicomponent process involving a cascade of regulatory proteins. Due to the significant oxidative activity of iron and the ability to form chelate complexes with many compounds, its amount in the body is strictly limited and there are no physiological ways to naturally remove excess iron from the body. Iron levels can directly influence the activity of iron regulatory proteins in addition, with modulatory stimuli being nitric oxide, hypoxia, oxidative stress and erythropoiesis activity (Rumeantzev & Tokareva, 2004).

Disturbance of iron homeostasis is associated with various pathologies. Iron deficiency, resulting from impaired absorption or bioavailability of the metal, causes anemia. Excess iron leads to its deposition in tissues and becomes the pathophysiological basis of many pathologies (Yiannikourides & Latunde-Dada, 2019). High iron levels lead to the formation of free iron (II), which can cause oxidative stress and cell death through lipid peroxidation, known as ferroptosis (Rumeantzev & Tokareva, 2004).

Thus, both iron deficiency and iron overload have catastrophic consequences for the body, and the content of this microelement is strictly regulated.

The purpose of this research was to determine the influence of polyphenols from nettle on the metabolism of iron and other microelements, which participate in this process, namely in maintaining iron homeostasis. The study of iron homeostasis is of great interest due to the need to find new approaches to correcting the severe consequences of iron deficiency and excess in the body by including biologically active substances in the food ration.

MATERIALS AND METHODS

The purpose of this research conducted in our laboratory was to determine the influence of polyphenols from nettle extract on the iron homeostasis in the blood serum of roosters. Ten roosters were used for the research, which were divided into two groups: one control and the other experimental. The roosters in the control group were administered 1 ml of saline per os to each animal using the special dispenser for administering medicinal remedies to animals. The experimental group was administered polyphenol extract from nettle (urtica dioica), in a volume of 1 ml, with a total polyphenol content of 33.2 g/100 ml gallic acid equivalent (GAE).

Calorimetric (photometric) methods were used to determine chemical elements. The amount of iron was determined by the calorimetric method. The principle of the method consists in releasing iron from iron-binding proteins in blood serum and reducing it by the action of an acidic working solution (pH 1.6-1.7), which contains the reducing agent hydroxylamine and detergents. Then, after the addition of a chromogenic ligand (ferrozine) and a buffer, a stable, colored iron-ferrozine complex is formed, the optical density of which is measured at 570 nm.

The determination of zinc was based on the principle of the formation of a colored zinc complex with a special reagent (for example, Br-PPAS), the color intensity of which is proportional to the concentration of zinc, which is measured photometrically (endpoint).

The calorimetric (photocolorimetric) method for determining ceruloplasmin (CP) in blood serum is based on measuring its enzymatic activity (oxidase function). Ceruloplasmin is an enzyme that contains copper and catalyzes the oxidation of various polyamines, most commonly p-phenylenediamine. The principle of the method consists in the oxidation of the substrate, where, in the presence of serum ceruloplasmin, p-phenylenediamine is oxidized to form a colored compound (usually pink or brown). The color intensity of the solution formed during the reaction is directly proportional to the concentration and activity of ceruloplasmin. The optical density of the solution was measured using a photoelectrocolorimeter or a spectrophotometer.

Magnesium was also determined by the calorimetric (photometric) method. This method is based on measuring the intensity of the color formed during the reaction of magnesium ions with specific reagents; the color intensity of a solution is directly proportional to the concentration of magnesium in it (Beer-Lambert law).

The colorimetric method was used to determine phosphorus in blood serum (phosphomolybdate reaction). This method is based on the reaction of phosphates with ammonium molybdate in an acidic medium. The principle is that phosphates react with ammonium molybdate to form phosphomolybdic acid. This acid is reduced (usually with ascorbic acid or other reducing agents) to a blue compound, the intensity of which is directly proportional to the concentration of phosphorus.

The duration of administration of the preparations was for a period of 60 days.

The animals were maintained in the vivarium of the Institute of Physiology and Sanocreatology of the Moldova State University. The conditions of animal maintenance correspond to the sanitary and veterinary requirements for the maintenance of laboratory animals.

Biochemical investigations were carried out in the scientific-practical biochemistry laboratory of the "Nicolae Testemitanu" State University of Medicine and Pharmacy of the Republic of Moldova.

Blood samples from birds were collected from the ulnar cutaneous vein (wing vein), in compliance with all sanitary and veterinary requirements for collecting blood samples from laboratory animals.

The statistical analysis of the obtained data was carried out using the Student's t-test (Novikov, 1995).

RESULTS AND DISCUSSIONS

In these studies, the state of hematological indices in the general analysis of blood, which are dependent on iron, was evaluated after the administration of hydroalcoholic extract from nettle. The research results are presented in Table 1.

Table 1. Hematological indices of reproductive roosters included in experiments to study the action of polyphenols from nettle (*Urtica dioica* L.) on the reproductive system and the organism as a whole

Groups	Hgb (g/dl)	RBC, $10^{12/l}$	MCV (fl)	MCHC (g/dl)	MCH (pg)
Control	138.6±3.51	3.22±0.33	132.4±0.69	30.7±0.35	38.9±0.65
Experimental	151.0±2.00*	3.64±0.32	130.0±1.12	30.5±0.30	39.6±0.16

*The differences are statistically true between the experimental and control group ($P<0.05$)

Note: Hgb – hemoglobin concentration; RBC – absolute number of erythrocytes in the blood (red blood cells); MCV – mean corpuscular volume; MCHC – mean corpuscular hemoglobin concentration; MCH – mean corpuscular hemoglobin; fl – femtoliter; pg – pictograms.

From the results obtained, we observe that the amount of hemoglobin in the experimental group indicates a value of 151.0±2.0, compared to the control group with a value of 138.6±3.51 g/dl, which proves us about a stimulating effect of the polyphenol extract from nettle (*Urtica dioica* L.) and its influence on iron metabolism in living organisms.

As a result of the analysis of the specialized literature, it turns out that hemoglobin (Hb) is a molecule that is functionally linked to aerobic organisms and participates in the use of oxygen from the atmosphere, which it delivers to cells

and tissues. It is thanks to hemoglobin, the cardiovascular system is supplied with oxygen, through which are maintained cellular homeostasis and signaling the protective function of the cell. These roles are particularly highlighted in the binding of Hb to the gaseous molecules of carbon dioxide (CO_2), nitric oxide (NO) and carbon monoxide (CO), also serving indirectly or directly as sources of these signaling molecules.

In erythrocytes, $NO^{\bullet}$ will react with HbO_2 to form MetHb and nitrate, as well as with $O^{2\bullet}$- to form the strong oxidizing agent $ONOO^-$ (Stuehr & Haque, 2019).

The end result of oxidative damage to erythrocytes is hemolysis, loss of membrane integrity and release of hemoglobin and other intracellular proteins. Free hemoglobin is particularly toxic, and this is obvious that it can cause damage of erythrocytes (Schaer et al., 2013).

Polyunsaturated fatty acids (PUFAs), 18% of the total fatty acids in RBCs (Pacetti et al., 2016), are the lipid components that are more sensitive to oxidation in a series of reactions that trigger lipoperoxidation.

The antioxidant defense allows normal erythrocytes to survive 120 days in circulation. The main protection against oxidative damage is provided by various enzyme systems, such as superoxide dismutase, catalase, glutathione, etc.

MCV is a value that measures the average size of red blood cells. In states of excessive oxidative stress (EOS) in different individuals, the antioxidant capacity of erythrocytes is decreased (Maritim et al., 2003). From the results of the conducted researches we observe a difference in the MCV values, where for the control group a value of 132.4±0.69 fl is indicated, and for the experimental group it represents a value of 130.0±1.12 fl.

The next study of this experiment was devoted to the determination of the mineral antioxidant status in the blood serum of breeding roosters after administration of the polyphenol extract of nettle (*Urtica dioica* L.). The results obtained are presented in Table 2.

Table 2. Mineral antioxidant status in the blood serum of roosters after administration of polyphenol extract of nettle (*Urtica dioica* L.)

Groups	Iron, μM/L	Zinc, μM/L	CP, mg/L	Magnesium, mM/L	Phosphorus, mM/L
Control	12.15±0.31	31.32±0.45	118.17±0.35	0.67±0.03	1.45±0.11
Experimental	14.95±0.73*	39.73±0.89*	131.42±1.62*	0.97±0.06*	1.60±0.11

*The differences are statistically true between the experimental and control group ($P<0.05$)

Approximately 25% of all proteins included in the Protein Data Bank contain metals. Zinc ions are the most prevalent, followed by iron, calcium, manganese and magnesium. The role of transition metals in protein biochemistry is varied. They are part of the active site and actively participate in the formation of protein complexes, such as lipoproteins and nucleoproteins, and also stabilize the structure of proteins with several subunits (Dutta & Bahar, 2010). At the same time, many proteins, under stress conditions, form additional metal-binding centers, including iron.

The most important metal-ligand forms of iron are transferrin, albumin, ferritin, citrate and carnosine.

In the human body, iron metabolism is a strictly regulated process: iron enters the bloodstream from the intestine in the form of a divalent cation Fe(II), which is easily oxidized to Fe(III) and bound to transferrin (80 kDa) (Soldin et al., 2004). Plasma transferrin is the most important physiological source of iron and is synthesized in the liver. Together with ferritin, it binds virtually all circulating iron in the plasma. Under physiological conditions, this chelation maintains low levels of free iron in the bloodstream to prevent the formation of reactive oxygen species and promotes iron transport into cells (Soldin et al., 2004).

Iron-bound transferrin distributes iron to other cells in the body by binding to receptors on cell surfaces and then importing it into the cell through endocytosis. Once in the cytoplasm, iron is transported to various intracellular sites, including mitochondria for the biosynthesis of

heme and ferritin, which in turn serves as an intracellular depot of iron (Ren & Walczyk, 2014). Metabolically inactive iron is stored in ferritin and is in equilibrium with metabolic iron bound to carrier molecules (Ren & Walczyk, 2014).

The ability of albumin to bind iron ions has been repeatedly observed in experiments, although it is believed that this protein does not have specific binding sites for this metal. Serum albumin, known as a low-affinity iron-binding protein, has been proposed as a ligand for the reserve of iron unbound to transferrin, usually present where surplus iron is identified (Rabbani & Ahn, 2019). Albumin carries a net negative charge, with a large number of carboxylic acids on the surface of the molecule, which can form potential iron binding sites (Rabbani & Ahn, 2019).

The involvement of the carboxyl groups of aspartic and glutamic acid residues, as well as the imidazole group of histidine, in the coordination of iron ions was observed. The absence of specific binding sites of iron with albumin is due to ferritin, which is contained in the blood and has a role in the transport and deposition of this metal (Thompson, 2022).

Ferritin is a protein that forms as a complex between ferrous hydroxide and another protein called apoferritin. Ferritin represents the storage form of iron in the body, its concentration highlighting the iron deposits present in the organism of animals, including humans. Ferritin represents a 450 kDa complex that binds iron oxyhydroxide particles (up to 4500 iron atoms). Most ferritins are found inside cells and serve to store iron (Orino & Watanabe, 2019).

Research conducted on the chemical specification of elements in blood plasma suggested the presence of only the trivalent form of iron and indicated that among low molecular weight ligands, iron is present exclusively in the form of a hydroxycitrate complex (Dziuba et al., 2018). However, it has now been established that blood plasma and serum contain two to six types of low molecular weight iron complexes. Citrate, acetate, pyruvate and phosphates are potential low molecular weight ligands of the metal. Considering the affinity of each ligand, researchers have suggested that citrate is the most dominant ligand for unbound iron to transferrin (Dziuba et al., 2018).

Carnosine is a good chelator of iron, copper and zinc ions, eliminates reactive oxygen and nitrogen species and forms complexes with toxic electrophilic α, β-unsaturated aldehydes (Abdelkader et al., 2016). In living organisms, carnosine performs antioxidant and detoxifying functions, neutralizing many potentially harmful substances. It has been proven that it can exert both direct and indirect antioxidant effects. The direct antioxidant action of carnosine is mediated by binding reactive carbonyl compounds, harmful products of fat and carbohydrate oxidation, while its indirect action is mediated by activating the transcription factor Nrf2, which is essential for protecting cells from oxidative stress, metabolic byproducts and toxins.

Thus, the evaluation of the content of iron complexes with high and low molecular weight ligands in vivo is important for a surveillance of metabolism and maintenance at an optimal level the homeostasis of this microelement. In our research, we obtained the following iron values, 12.15±0.31 μM/L for the control group and 14.95±0.73 μM/L for the experimental group.

Zinc is a key modulator of iron absorption and its distribution in tissues. Reducing the level of zinc directly leads to decreased zinc content in the pancreas, which in turn reduces the intestinal absorption of iron by reducing the expression of divalent metal transport and ferroportin. Mechanically, the overall impact of zinc deficiency is associated with the development of iron deficiency caused by a combination of reduced intestinal absorption and reduced mobilization of iron from deposits (Kondaiah et al., 2019). In our research we obtained the following values of zinc, for the control group 31.32±0.45 μM/L, and for the experimental group 39.73±0.89 μM/L.

Ceruloplasmin (CP) also participates in the regulation of iron homeostasis, being an enzyme with an increased copper content, manifesting an increased ferroxidase activity, which is detected under the soluble isoform in plasma or as a membrane-associated isoform in different cell types. The ceruloplasmin-ferroportin system represents the main cellular iron exit pathway in vertebrates and is

responsible for the physiological regulation of cellular iron levels. This enzyme possesses several functions, from copper transport to the oxidation of ferrous iron and biological amines, as well as antioxidant activity by preventing the formation of free radicals in the serum (Floris et al., 2000). In our research, we obtained the following values of ceruloplasmin, for the control group 118.17±0.35 mg/l, and for the experimental group 131.42±1.62 mg/l, which demonstrates about the influence of polyphenols extracted from nettle on the amount of ceruloplasmin, which in turn, through various mechanisms, participates in maintaining iron homeostasis.

Magnesium plays a key role in the regulation of iron homeostasis, influencing its bioavailability, absorption and metabolic processes. The interaction between magnesium and iron is a complex electrolyte balance, in which magnesium can act as a regulator, as it is essential for the proper functioning of hundreds of enzymes, which indirectly support metabolic processes, including iron use.

As discussed in this paper iron can maintain the balance of the normal functioning of the body, but it can also trigger reactions that cause oxidative stress.

CONCLUSIONS

So as can be seen, Iron (Fe) is an essential element for all living organisms, since it is a component of the functional groups of proteins and enzymes that transport oxygen and catalyze energy-generating reactions and metabolic processes. Excess free iron leads to local tissue damage due to increased formation of free radicals. Therefore, the safe range of iron levels in the body is quite narrow and strictly controlled in order to avoid both iron deficiency and iron overload. The research results demonstrated a change in iron values under the influence of polyphenols extracted from *Urtica dioica*, namely 12.15±0.31 μM/L for the control group and 14.95±0.73 μM/L for the experimental group. Changes are also observed in other biochemical indices, which have been studied and are directly at the base of iron metabolism, which contributes to maintaining the homeostasis of this element.

Through the transport proteins of metal ions, such as albumin, ferritin and carnosine, and through the administration of polyphenol extracts with antioxidant effect it is possible to optimize iron metabolism, which will favor the establishment of iron homeostasis, the normal functioning and detoxification of the body's cells.

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BETWEEN TASTE AND WELFARE: METABOLIC PROFILE AND HISTOLOGICAL ASPECTS OF COMMON DUCKS (*Anas platyrhynchos*) FATTENED FOR CONSUMPTION

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Abstract

Forced fattening of ducks (Anas platyrhynchos) is a zootechnical practice used to obtain fatty liver (commercially called foie gras) and duck fat. Forced fattening raises important issues regarding animal welfare, being considered by many specialists to be an intensely stressful and invasive method. In this study, metabolic and histological changes were analyzed in a population of forced-fattened common ducks. Serum protein (TP, ALB, GG, BUN, CR), lipid (TL, TRY, CHOL), carbohydrate (BG), enzymatic (AST, ALT, ALP, GGT, TSB, DBI, LDH, ά-AMY, LIP, CK) and mineral (P, Mg) profiles were analyzed. The final results showed very significant differences between the initial and final values of the metabolic profiles, sometimes with the blood-brain barrier being overcome. Histological analyses also demonstrated pronounced, even degenerative, tissue structural changes.

Key words*: blood-brain barrier, ducks, forced fattening, stress, welfare.*

INTRODUCTION

Animal fattening represents an essential stage in meat production, with the primary objective of achieving rapid weight gain of the organism or, in some cases, of specific organs or tissues (liver, fat) (Michał Gornowicz et al., 2023; Kutay et al., 2024). Depending on the species (Marin et al., 2015; Bordei et al., 2020), fattening technologies differ in terms of digestive particularities (Marin et al., 2017), nutritional requirements, production systems, and performance parameters (Rehman & Zulfiqar, 2024; Gado et al., 2025). Optimizing the fattening process requires the correlation of genetics (Kremez et al., 2025; Răducuță et al., 2025), nutrition (Ungureanu et al., 2024), housing conditions, and sanitary-veterinary management in order to obtain high average daily gains and a low feed conversion ratio (Custură et al., 2021; Tudorache et al., 2021). Intensive fattening leads to high productivity but involves substantial feed costs. Extensive systems, sometimes traditional ones, are more environmentally sustainable (Mihai et al., 2022), but are less economically efficient (Syrůček et al., 2017). Animal fattening is not merely a technological process aimed at increasing body mass, but also an expression of the interaction between humans, the environment, and animals, reflecting the level of agricultural development and adaptation to local conditions (Adamczyk, 2018; Nica & Vidu, 2023).

Force-feeding is a procedure whereby animals are fed beyond their natural nutritional requirements, either to accelerate growth or to obtain certain culinary products (Skippon, 2013; Mohammed et al., 2014; Huber, 2024). Animal welfare organizations and some public health authorities consider force-feeding to be one of the most severe forms of suffering imposed on animals and reject gastronomic justifications

which, in their view, undermine animal welfare (Nollkaemper, 2023). In the European Union, which has some of the most advanced legislative standards concerning animal welfare, there are directives stipulating that animals must be fed in accordance with their physiological needs and must not be subjected to procedures that cause unnecessary suffering or injury (including psychological trauma) (Guémené & Guy, 2004; Nicolaisen et al., 2023). These aspects also derive from the level of education of the population in the member states (Ilovan et al., 2019), as well as from cultural-historical and socio-economic factors (Gligor et al., 2026). Nevertheless, there are several EU member states - such as France, Hungary, Bulgaria, and Spain -where force-feeding of birds and mammals is still practiced, despite public opposition and the recommendations of animal welfare institutions (Temple et al., 2012; Toth, 2012; Rochlitz & Broom, 2017). Other member states prohibit, or at least restrict the sale of animal products obtained through force-feeding. The most well-known form of force-feeding is the gavage of ducks and geese (Molnár et al., 2006; Wen et al., 2016), although similar practices are also encountered in other poultry species, such as turkeys (Vlad et al., 2025). Gavage results in the production of fatty liver (foie gras), defined under the legislation of France and the European Union as the liver of a duck or goose fattened through force-feeding.

The traditional fattening of palmipeds for the production of fatty liver represents one of the oldest agricultural practices in which biology, culinary tradition, and social history visibly intersect (Duhart, 2011; Saber, 2022). Originating from the ancestral history of hunting, humans observed the behavior of wild ducks and geese during the autumn period when, prior to migration, they consumed larger quantities of food, accumulating fat in their bodies and particularly in the liver. These fat reserves provided the energy required by the birds during migration (Johnson et al., 2014). Based on these behavioral observations, and later following the domestication of birds (Sheta et al., 2025), force-feeding became an important step toward obtaining high-quality animal products. Over the centuries, such techniques have been encountered in communities across Central and Eastern Europe, with Jewish communities playing a particularly significant role in their development and dissemination (Kuropatnicki, 2017). Approximately 100-150 years ago, rural areas where ducks and geese were raised - and consequently fattened - were mainly household farms located within the Pannonian Plain region, including Hungary, Slovakia, and Transylvania. Nevertheless, from a historical perspective, duck domestication originated in Asia (Chen et al., 2021). Fattening was a seasonal activity, typically practiced before the winter holidays, when corn - an important source of starch - had already been harvested and stored in barns. The birds were generally kept in confined spaces, which limited their freedom of movement and consequently reduced energy expenditure. Feed was introduced manually into the oral cavity, and within two to three weeks the ducks became visibly heavier. The outcomes of this process consisted of fatty liver, lard, and meat intended for winter consumption. These products represented an important source of calories during long and harsh winters.

During that period, the traditional fattening technique was practiced on a small scale, primarily for household needs (Bercu et al., 2025). Subsequently, with the development of large livestock and poultry production complexes, fattening practices began to be applied on an industrial scale. These techniques are undoubtedly unpleasant for ducks and geese, with evident psycho-affective and pathological impacts (Tremolada et al., 2020). Nevertheless, the outcomes were considered important in terms of taste and the nutritional needs of human populations. Consequently, the evaluation of such practices raises an important question regarding the balance between gastronomic preferences and animal welfare. In this context, the present study evaluated the physiological and histological impact of duck fattening performed in a traditional system within a family household located in Transylvania, Romania.

MATERIALS AND METHODS

The ducks and drakes included in the investigation originated from a family farm located in Cluj County, Romania. They were traditionally fattened by the owners for

household consumption over a period of two weeks, using a diet consisting of corn kernels soaked in water for approximately 30 minutes. For administration and lubrication of the pharynx and esophagus, sunflower oil (10 mL) was added. No tubes or beak-opening devices were used for gavage. The ducks were immobilized under the breeder's left arm; the beak was opened manually with the left hand, while the feed was administered with the right palm in small, successive portions. During the gavage procedure, after the feed was introduced between the two valves of the beak, the esophagus was gently massaged first in an oro-aboral direction and subsequently in a cranio-caudal direction, in order to facilitate efficient swallowing of the feed. The daily quantity of feed administered represented approximately 5% of the ducks' body weight. Thus, for ducks with an average body weight of approximately 2.5 kg, the daily feed ration was 125 g, whereas for drakes with an average body weight of approximately 3 kg, the daily ration was 150 g. This amount was administered in two daily meals, in the morning and in the evening. Drinking water was provided *ad libitum*. The stocking density for the birds subjected to fattening was 0.50 m² per duck.

For the blood analyses, a total of 12 ducks and 4 drakes were included in the study. Blood samples were collected by puncture of the brachial artery at the beginning of the fattening period (day 0) and at the end of the fattening period (day 12).

The collected samples were transferred into vacutainer tubes containing a clot activator to accelerate blood coagulation and facilitate serum separation. Under refrigerated conditions (2-4°C), the samples were transported to the Hematology and Biochemistry Laboratory of the University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca. Biochemical determinations were performed using a UV-VIS Screen Master Touch spectro-photometer. The wavelengths used for the biochemical analyses are presented in Table 1.

Table 1. Wavelengths (λ) and analytical methods used for the determination of biochemical constituents in the blood plasma of ducks

Blood parameter	λ (nm)	Usual method
TP - Total Protein	540-560 nm	Biuret
ALB - Albumin	628 nm	Bromocresol Green (BCG)
γ-GLOB - Gamma globulin	540-560 nm	electroforeză / calcul din TP-ALB
BUN - Blood Urea Nitrogen	340 nm	Urease-GLDH
Cr - Creatinin	492-520 nm	Jaffé
TL - Total Lipids	520-550 nm	sulfo-phospho-vanillin
TRY - Triglycerides	500-546 nm	GPO-PAP
CHOL - Cholesterol	500-520 nm	CHOD-PAP
BG - Blood Glucose	500-546 nm	GOD-POD
TC - Total Calcium	570-580 nm	Arsenazo III
P - Phosphorus	340 nm	UV phosphomolybdate
Mg - Magnesium	520-550 nm	Xylidyl blue
ASAT - Aspartate aminotransferase	340 nm	IFCC kinetic
ALAT- Alanine aminotransferase	340 nm	IFCC kinetic
ALP - Alkaline phosphatase	405 nm	p-nitrophenyl phosphate
GGT - Gamma-Glutamyl Transferase	405 nm	γ-glutamyl-p-nitroanilide
TB - Total bilirubin	540 nm	Jendrassik-Grof
DB - Direct bilirubin	540 nm	Jendrassik-Grof
LDH - Lactate dehydrogenase	340 nm	NADH kinetic
α-Amylase - Alpha amylase	405 nm	CNPG3
LIP - Lipase	405 nm	DGGR
CK - Creatine kinase	340 nm	IFCC kinetic

For the histological investigation, tissue samples (e.g., liver, breast muscle, and leg muscle) were collected and fixed in 10% buffered formalin for three days, followed by progressive dehydration in ethyl alcohol solutions of 70%, 96%, and absolute concentration, for one hour at each stage. Clarification was performed through three successive 1-hour baths of 1-butanol, after

which the samples were embedded in paraffin. Tissue sections of 5 µm thickness were obtained and subsequently stained using Goldner's trichrome method.

All statistical analyses were performed using GraphPad Prism (version 8.0.1; GraphPad Software Inc., San Diego, CA, USA). The collected data were first organized and inspected in order to identify potential errors or missing values prior to analysis. Descriptive statistics were calculated for all measured parameters and are presented as mean ± standard deviation (mean ± SD). To evaluate potential differences between sexes, the measured parameters from male and female individuals were compared using an independent-samples Student's t-test. This test was selected because it allows the comparison of mean values between two independent groups. The level of statistical significance (α) was set at 0.05 for all analyses. Differences were considered statistically significant when the calculated p-value was lower than the established significance threshold ($p < 0.05$). All statistical tests were two-tailed. The results of the statistical analyses were subsequently used to interpret possible sex-related differences in the studied parameters.

RESULTS AND DISCUSSIONS

Force-feeding in birds represents an experimental and economic practice used to investigate the metabolic, physiological, and biochemical adaptations of the organism to excessive energy intake. In the present study, blood changes were analyzed in two groups of birds, drakes (males) and ducks (females), by comparing parameters measured before and after the fattening period (MBF vs. MAF and FBF vs. FAF). The analyses focused on five metabolic profiles, protein, lipid, carbohydrate, mineral, and enzymatic, in order to identify the biological adaptations and metabolic stress associated with force-feeding.

In drakes, total protein (TP) showed a slight increase after fattening (2.78→2.97 g/dL; d=0.19±0.57), without statistical significance, while creatinine (Cr) decreased significantly (0.52→0.26 mg/dL; d=0.26± 0.08), suggesting a redistribution of muscle mass in favor of fat deposition. Albumin (ALB) and gamma globulins (γ-GLOB) showed minor, non-significant changes, indicating maintenance of the overall protein status, but with a slight decrease in components involved in immune function.

In females, protein-related changes were more pronounced and statistically significant. Albumin (ALB) increased considerably (0.99→1.43 g/dL; d=0.44±0.178), while gamma globulins (γ-GLOB) decreased (0.67→0.53 g/dL; d=0.14±0.069). Blood urea nitrogen (BUN) showed a marked decrease (57.67→22.72 mg/dL; d=34.95± 6.084), indicating a reduction in protein and carbohydrate catabolism (Massimino et al., 2020). Creatinine increased moderately, suggesting maintenance of muscle mass. Comparatively, females exhibited more pronounced and statistically significant protein-related changes, highlighting a stronger adaptation of protein metabolism to fattening, accompanied by a redistribution of plasma proteins (Table 2).

Thus, it can be observed that females exhibit more pronounced protein-related changes than drakes, with significant increases in albumin (ALB) and decreases in gamma globulins (γ-GLOB) and blood urea nitrogen (BUN), reflecting a more pronounced hepatic adaptation to excess energy intake.

In both drakes and ducks, force-feeding produced marked increases in plasma lipids (Davail et al., 2003; Gheorghe et al., 2022). In drakes, total lipids (TL) doubled (656.18 → 1348.03 mg/dL; d = 691.9 ± 88.32), and cholesterol (CHOL) increased significantly (183.2 → 372.83 mg/dL; d = 189.6 ± 34.53) (Table 3). In females, the increases were even more pronounced: total lipids (TL) rose markedly (725.08 → 1487 mg/dL; d = 761.9 ± 158.8), cholesterol (CHOL) also increased (276.03 → 404.53 mg/dL; d = 128.5 ± 32.37), and triglycerides (TRY) nearly doubled (286.56 → 586.61 mg/dL; d = 300.05 ± 50.74). These changes indicate rapid fat accumulation and an intensification of hepatic lipid metabolism (Lo et al., 2022), associated with increased adipose tissue mass and enhanced energy mobilization (Chartrin et al., 2006; Lungu & Moise, 2025).

Table 2. Mean values, differences, and statistical significance of the differences for metabolic constituents in ducks (females), before and after force-feeding

Metabolic profile	FBF	FAF	Mean of diff.	P value
Proteic profile				
TP (g/dL)	5.45±0.045	5.06±0.133	0.39±1.246	p=0.2946, ns
ALB (g/dL)	0.99±0.014	1.43±0.012	0.44±0.178	p<0.0001, ****
γ-GLOB (g/dL)	0.67±0.006	0.53±0.008	0.14±0.069	p<0.0001, ****
BUN (mg/dL)	57.67±0.64	22.72±0.513	34.95±6.084	p<0.0001, ****
Cr (mg/dL)	0.27±0.007	0.53±0.018	0.26±0.217	p=0.0014, **
Lipidic profile				
TL (mg/dL)	725.08±5.226	1487±12.969	761.9±158.8	p<0.0001, ****
TRY (mg/dL)	286.56±2.259	586.61±4.288	300.05±50.74	p<0.0001, ****
CHOL (mg/dL)	276.03±2.447	404.53±2.44	128.5±32.37	p<0.0001, ****
Carbohydrate profile				
BG (mg/dL)	211.72±1.533	335.16±5.836	123.44±64.24	p<0.0001, ****
Mineral profile				
TC (mg/dL)	10.99±0.058	8.43±0.055	2.56±0.686	p<0.0001, ****
P (mg/dL)	3.14±0.03	5.94±0.059	2.80±0.747	p<0.0001, ****
Mg (mg/dL)	2.12±0.021	2.48±0.028	0.36±0.291	p=0.0012, **
Enzyme profile				
ASAT (U/L)	77.92±0.281	40.17±0.243	37.75±4.207	p<0.0001, ****
ALAT (U/L)	121.42±0.461	164.78±1.277	43.36±15.05	p<0.0001, ****
ALP (U/L)	670.81±3.488	327.48±6.322	343.33±71.78	p<0.0001, ****
GGT (U/L)	18.47±0.098	79.63±0.366	61.16±4.277	p<0.0001, ****
TB (mg/dL)	0.96±0.009	1.14±0.015	0.18±0.191	p=0.0065, **
DB (mg/dL)	0.72±0.01	0.89±0.023	0.17±0.289	p=0.0711, ns
LDH (U/L)	375.13±4.17	1352.09±6.788	976.96±86.01	p<0.0001, ****
α – AMY (U/L)	5805.63±38.128	13799.5±42.165	7993.37±552.3	p<0.0001, ****
LIP (U/L)	39.84±0.345	171.62±2.513	131.78±24.0	p<0.0001, ****
CK (U/L)	2057.33±17.707	1631.27±6.238	426.06±202.8	p<0.0001, ****

FBF – females before fattening; FAF – females after fattening; TP – Total Protein; ALB – Albumin; γ-GLOB – Gamma Globulin; BUN – Blood Ureea Nitrogen; Cr – Creatinine; TL – Total Lipids; TRY – Triglycerides; CHOL – Cholesterol; BG – Blood Glucose; TC – Total Calcium; P – Phosphorus; Mg – Magnesium; ASAT - Aspartate aminotransferase; ALAT - Alanine aminotransferase; ALP - Alkaline phosphatase; GGT – Gamma-Glutamyl transferase; TB – Total bilirubin; DB – Direct bilirubin; LDH - Lactate dehydrogenase; α-amylase – Alpha-amylase; LIP – lipase; CK - Creatine kinase.

Table 3. Mean values, differences, and statistical significance of the differences for metabolic constituents in drakes (males), before and after force-feeding

Metabolic profile	MBF	MAF	Mean of diff.	p-value
Proteic profile				
TP (g/dL)	2.78 ± 0.021	2.97 ± 0.037	0.19 ± 0.57	p=0.5623, ns
ALB (g/dL)	1.28 ± 0.023	1.18±0.017	0.10 ± 0.25	p=0.4549, ns
γ-GLOB (g/dL)	0.2±0.005	0.17±0.004	0.03 ± 0.08	p=0.5117, ns
BUN (mg/dL)	13.65±0.186	15.8±0.08	2.15 ± 2.55	p=0.1907, ns
Cr (mg/dL)	0.52±0.009	0.26±0.005	0.26 ± 0.08	p=0.0075, **
Lipidic profile				
TL (mg/dL)	656.18±6.256	1348.03±11.492	691.9 ± 88.32	p=0.0006, ***
TRY (mg/dL)	255.33±3.707	355.68±4.395	100.4 ± 70.9	p=0.0062, ns
CHOL (mg/dL)	183.2±0.705	372.83±3.368	189.6 ± 34.53	p=0.0016, **
Carbohydrate profile				
BG (mg/dL)	237.13±4.28	289.38±2.591	52.25 ± 28.82	p=0.0361, *
Mineral profile				
TC (mg/dL)	10.79±0.05	8.96±0.025	1.83 ± 0.701	p=0.0136, *
P (mg/dL)	7.83±0.082	4.52±0.046	3.31 ± 0.414	p=0.0005, ***
Mg (mg/dL)	2.55±0.037	2.35±0.032	0.2 ± 0.654	p=0.584, ns
Enzyme profile				
ASAT (U/L)	24±0.339	28.75±0.24	4.75 ± 4.735	p=0.1385, ns
ALAT (U/L)	139.98±1.001	168.8±0.756	28.82 ± 15.54	p=0.0341, *
ALP (U/L)	221.23±2.466	382.98±1.833	161.75 ± 33.27	p=0.0023, **
GGT (U/L)	28.52±0.436	80.35±0.539	51.83 ± 8.674	p=0.0013, **
TB (mg/dL)	1.23±0.008	1.39±0.006	0.16 ± 0.131	p=0.0965, ns
DB (mg/dL)	1.1±0.014	1.08±0.01	0.02 ± 0.188	p=0.8684, ns
LDH (U/L)	408.88±4.449	1048.35±6.059	639.5 ± 46.23	p=0.0001, ***
α – AMY (U/L)	6189.25±23.041	14055.5±53.659	7866.25 ± 485.7	p<0.0001, ****
LIP (U/L)	42.3±0.508	388.35±3.523	346.05 ± 32.36	p=0.0002, ***
CK (U/L)	960.38±8.272	1217.28±13.781	256.9 ± 200.8	p=0.0833, ns

MBF – males before fattening; MAF – males after fattening; TP – Total Protein; ALB – Albumin; γ-GLOB – Gamma Globulin; BUN – Blood Ureea Nitrogen; Cr – Creatinine; TL – Total Lipids; TRY – Triglycerides; CHOL – Cholesterol; BG – Blood Glucose; TC – Total Calcium; P – Phosphorus; Mg – Magnesium; ASAT - Aspartate aminotransferase; ALAT - Alanine aminotransferase; ALP - Alkaline phosphatase; GGT - Gamma-Glutamyl transferase; TB – Total bilirubin; DB – Direct bilirubin; LDH - Lactate dehydrogenase; α-amylase - Alpha-amylase; LIP – lipase; CK - Creatine kinase.

Comparatively, lipid-related changes were more pronounced in females, with higher values of total lipids (TL) and triglycerides (TRY) and greater percentage increases than those observed in drakes (Atallah et al., 2024). This suggests a faster and more efficient metabolic adaptation of lipid metabolism in females, likely associated with sex-related hormonal differences.

Blood glucose increased significantly in both groups, reflecting the elevated energy intake. In drakes, blood glucose (BG) increased (237.13 → 289.38 mg/dL; d = 52.25 ± 28.82), while in females the increase was much more pronounced (211.72 → 335.16 mg/dL; d = 123.44 ± 64.24).

Thus, females exhibited more marked hyperglycemia, highlighting a stronger energetic adaptation for lipid synthesis and carbohydrate utilization (Sweazea, 2022).

Changes in mineral profiles showed a different pattern between the sexes. In drakes, calcium (TC) and especially phosphorus (P) decreased markedly (7.83 → 4.52 mg/dL; d = 3.31 ± 0.414), while magnesium (Mg) did not change significantly (2.55 → 2.35 mg/dL; d = 0.2 ± 0.654), indicating metabolic utilization associated with energy synthesis and hepatic adaptations. In females, calcium (TC) decreased (10.99 → 8.43 mg/dL; d = 2.56 ± 0.686), but phosphorus (P) increased significantly (3.14 → 5.94 mg/dL; d = 2.80 ± 0.747), and magnesium (Mg) showed a moderate increase (2.12 → 2.48 mg/dL; d = 0.36 ± 0.291). This pattern suggests a more complex mineral mobilization in females, associated with adaptations in energy and bone metabolism, possibly related to ATP synthesis and lipid storage. Therefore, females exhibited more complex and pronounced changes than drakes, with decreases in calcium (TC) and increases in phosphorus (P) and magnesium (Mg), indicating stronger metabolic and hormonal adaptations (Negm & Mohammed, 2023).

Enzymatic changes were pronounced in both sexes, although they followed different patterns. These alterations were primarily induced by force-feeding, although similar changes may also occur with advancing age in ducks (Hmangaihzuali et al., 2023). In drakes, very large post-fattening increases were observed for alpha-amylase (α-AMY) (6189.25 → 14055.5 U/L; d = 7866.25 ± 485.7), indicating intense pancreatic activity. Significant increases were also recorded for lactate dehydrogenase (LDH) (408.88 → 1048.35 U/L; d = 639.5 ± 46.23), gamma-glutamyl transferase (GGT) (28.52 → 80.35 U/L; d = 51.83 ± 8.674), alkaline phosphatase (ALP) (221.23 → 382.98 U/L; d = 161.75 ± 33.27), alanine aminotransferase (ALAT) (139.98 → 168.8 U/L; d = 28.82 ± 15.54), and lipase (LIP) (42.3 → 388.35 U/L; d = 346.05 ± 32.36). In contrast, the increases observed for aspartate aminotransferase (ASAT), direct bilirubin (DB), total bilirubin (TB), and creatine kinase (CK) were not statistically significant.

Dramatic increases in several enzymatic constituents were also observed in ducks, particularly in the case of alpha-amylase (α-AMY) (5805.63 → 13,799.5 U/L; d = 7,993.37 ± 552.3), alanine aminotransferase (ALAT) (121.42 → 164.78 U/L; d = 43.36 ± 15.05), gamma-glutamyl transferase (GGT) (18.47 → 79.63 U/L; d = 61.16 ± 4.277), lactate dehydrogenase (LDH) (375.13 → 1,352.09 U/L; d = 976.96 ± 86.01), and lipase (LIP) (39.84 → 171.62 U/L; d = 131.78 ± 24.0). Very significant differences were also observed in the opposite direction, with decreases after fattening for several enzymatic constituents in ducks: aspartate aminotransferase (ASAT) (77.92 → 40.17 U/L; d = 37.75 ± 4.207), alkaline phosphatase (ALP) (670.81 → 327.48 U/L; d = 343.33 ± 71.78), and creatine kinase (CK) (2057.33 → 1631.27 U/L; d = 426.06 ± 202.8). These findings suggest complex metabolic adaptations, involving intense hepatic and pancreatic redistribution, differentiated from the pattern observed in drakes by the decreases in ALP and ASAT.

Enzymatic–metabolic changes were pronounced in both sexes, with alpha-amylase (α-AMY) and lactate dehydrogenase (LDH) showing substantial increases. However, females exhibited a more complex redistribution between hepatic and bone-related enzymes, which may reflect hormonal and sex-related differences in metabolic adaptation.

The comparison of metabolic responses between female and male ducks highlights a markedly greater intensity of biochemical changes in females following force-feeding (Farhat & Chavez, 2000; Chia et al., 2022). Although both sexes exhibited increases in lipid parameters and

alterations in carbohydrate metabolism, the magnitude of metabolic reorganization was significantly more pronounced in females, suggesting a superior hepatic lipogenic capacity (Jerabek, 2018). In birds, the liver represents the primary site of fatty acid synthesis (Mancinelli et al., 2022), and the high carbohydrate intake derived from a corn-based diet promotes the conversion of glucose into triglycerides and other circulating lipids (Zaefarian et al., 2019; Cao et al., 2025). Females show a more intense activation of these processes, a phenomenon associated with physiological particularities of lipid metabolism influenced by estrogenic hormones, which stimulate lipid synthesis and transport (Yang et al., 2024). In contrast, males display smaller changes in serum proteins and nitrogen-related markers, suggesting a relative stability of protein metabolism and a more efficient metabolic adaptation to excess energy intake. Moreover, the hepatic enzymatic response is more pronounced in females, indicating greater hepatic metabolic activity and a higher sensitivity of the liver to nutritional overload. The increase in digestive and hepatic enzymes (Zeng et al., 2022) observed in both sexes reflects the adaptation of the digestive system to accelerated feed intake (Awde et al., 2013); however, its association with more extensive systemic metabolic changes in females suggests a metabolic redirection predominantly toward lipogenesis. Overall, these results indicate that females respond to force-feeding through a deeper metabolic reprogramming characterized by intensified hepatic lipid and carbohydrate metabolism, whereas males exhibit a more moderate and balanced metabolic adaptation.

Histologically, the liver lesions observed were represented by vacuolation of the hepatic parenchyma cells in the lobules. In both male and female ducks, the hepatocytes with lipidic inclusions were identified in all three zones of hepatic lobules, suggesting a diffuse hepatic lipidosis (Figures 1 and 2). Regarding the histological appearance of the affected hepatocytes, they had a multilocular appearance due to the presence of several uneven lipidic inclusions in the cytoplasm. The nucleus of these hepatocytes is usually eccentrically located but still with a spherical appearance and with a prominent central or paracentral nucleolus. As a consequence of a significant lipidic accumulation in hepatocytes, the cords of Remak were hypertrophied (Figure 2), a fact that induced the compression atrophy of the sinusoid capillaries (Figure 1). However, another aspect detected both in male and female ducks is the discrete fibrosis of the centrilobular venule (Figures 1 and 2). No inflammatory cells were identified in the specimens assessed.

The microscopic features detected in the muscles assessed were represented by the multifocal accumulation of lipids in the stromal connective tissue of the pectoral and leg muscle (Figures 3-6). The muscular lipomatosis was identified in both female and male ducks and induced multifocal hypertrophy of the perimysium, which is presenting large aggregates of monovacuolated or plurivacuolated adipocytes (Figures 3-6). In some microscopic areas, isolated or small groups of adipocytes were identified in between the myofibers, inducing the hypertrophy of the endomysium (Figures 4 and 6).

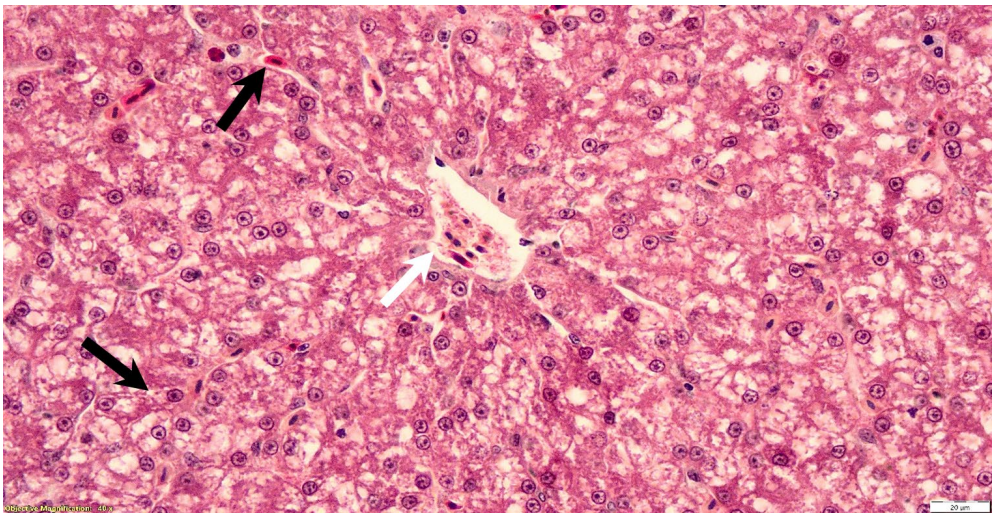


Figure 1. Diffuse hepatic lipidosis in a male duck: hypertrophied hepatocytes with a foamy aspect of the cytoplasm (arrowhead) due to accumulation of triglycerides and compression atrophy of the sinusoid capillaries (black arrow); discrete fibrosis of the centrilobular venule (blank arrow); GT stain (Original photo)

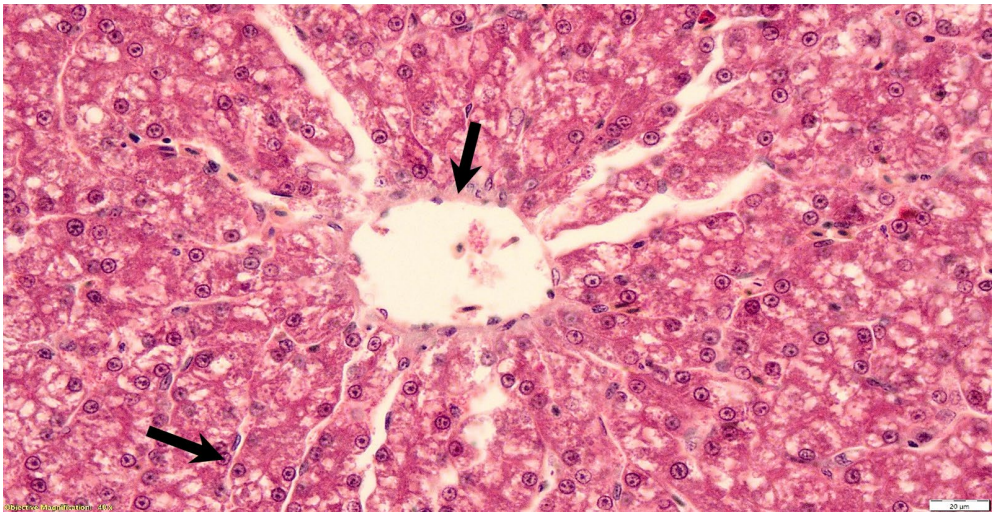


Figure 2. Diffuse hepatic lipidosis in a female duck: fibrosis of the centrilobular venule (black arrow) along with hypertrophy of the Remak's cords (arrowhead) due to accumulation of fatty material in the cytoplasm of hepatocytes; GT stain (Original photo)

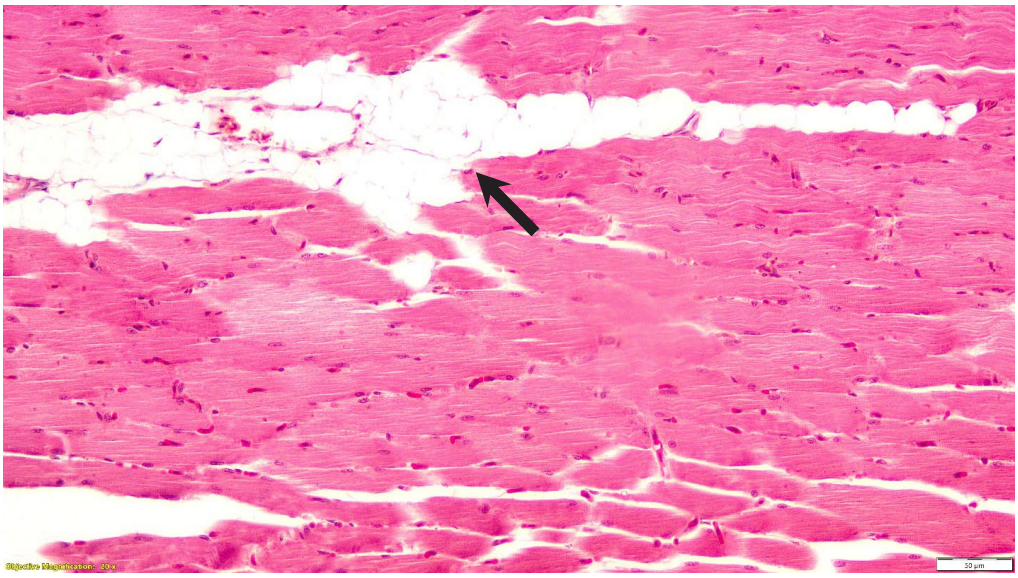


Figure 3. Muscular lipomatosis (pectoral muscle *Pectoralis major*, male duck): focal hypertrophy of the stromal connective tissue from the perimysium due to the presence of large aggregates of white adipocytes (arrow); GT stain (Original photo)

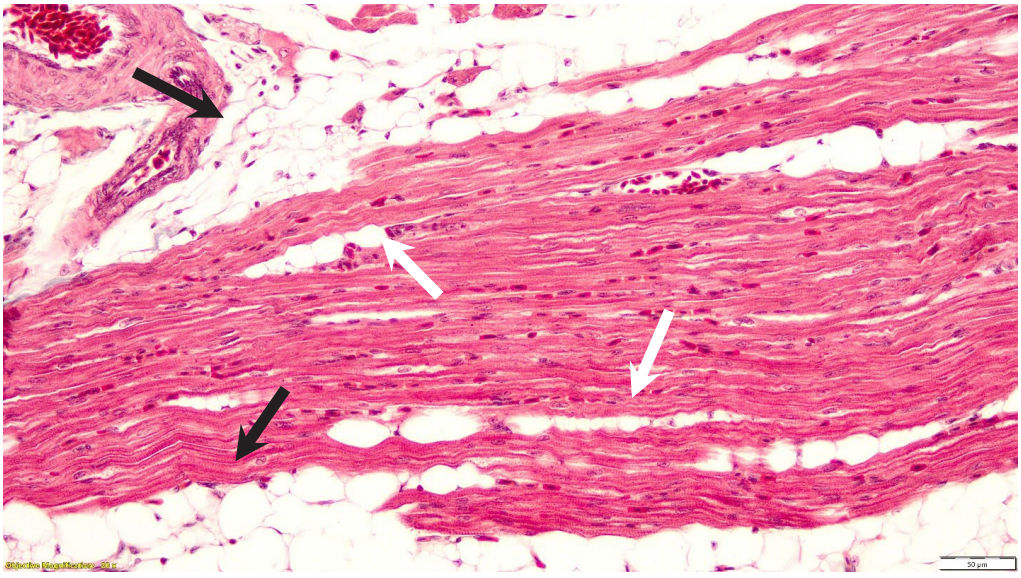


Figure 4. Muscular lipomatosis (pectoral muscle *Pectoralis major*, female duck): the presence of large groups of adipocytes in both perimysium (black arrows) and endomysium (blank arrows); GT stain (Original photo)

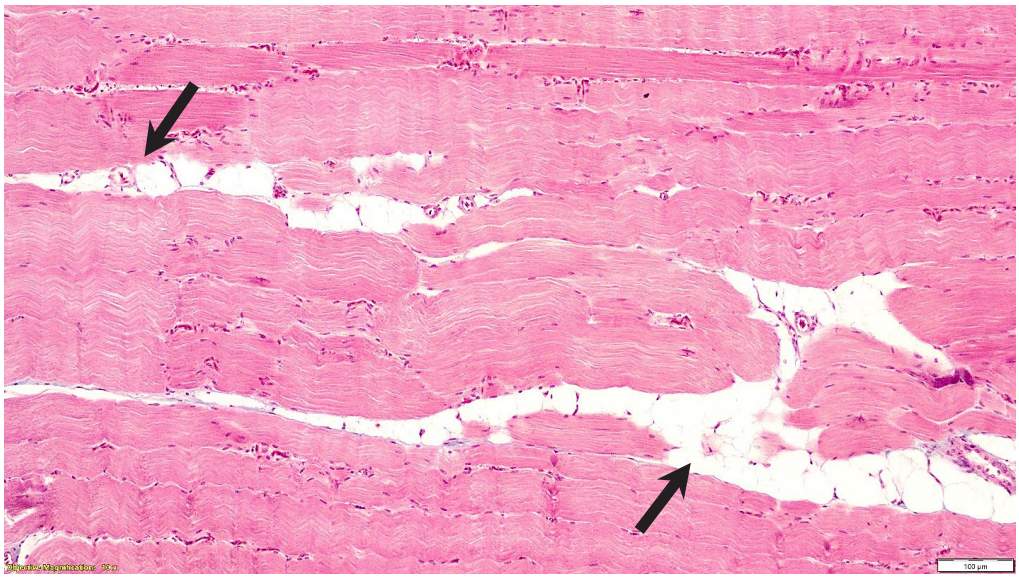


Figure 5. Muscular lipomatosis (leg muscle *Gluteus profundus*, male duck): groups of adipocytes in the perimysium (black arrows); GT stain (Original photo)

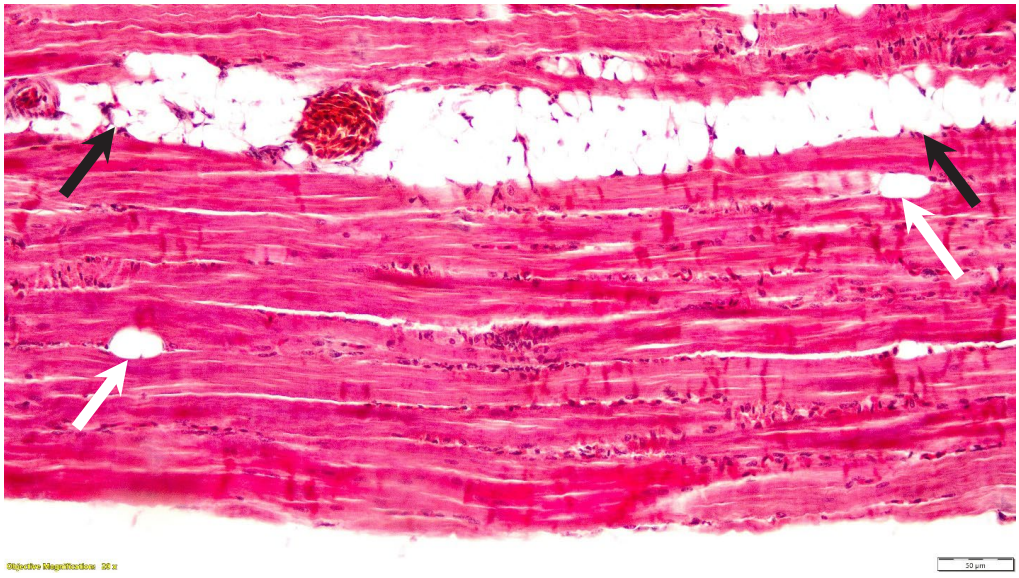


Figure 6. Muscular lipomatosis (leg muscle *Gluteus profundus*, female duck): large aggregates of adipocytes in perimysium (black arrows) and scattered adipocytes in between myofibers (blank arrows); GT stain (Original photo)

CONCLUSIONS

The metabolic study of ducks and drakes subjected to force-feeding highlights sex-related differences in physiological adaptation. Females exhibited pronounced changes in the protein compartment, with increases in albumin and decreases in globulins, suggesting an intense hepatic response and a redistribution of transport capacity and immune defense. These changes were accompanied by massive accumulations of lipids, triglycerides, and cholesterol, together

with marked increases in glucose, indicating a substantial metabolic transformation and significant energetic stress. Markers of hepatic and muscular enzymes signaled metabolic adaptations and evident micro-lesions at the tissue level, while the mineral balance remained actively regulated. In contrast, males displayed much more moderate protein-related changes: total proteins and albumin remained relatively stable, and urea and globulins suggested a balanced protein metabolism. Nevertheless, the lipid system also responded in males, with significant increases in total lipids and cholesterol, while hepatic and digestive enzymes indicated activation of energy metabolism and signs of moderate tissue stress. Glucose and mineral parameters showed less dramatic adjustments compared with females. Thus, females appear to be more vulnerable to metabolic imbalances and severe energetic stress, responding through marked hepatic and enzymatic adaptations, whereas males adopt a more conservative strategy, maintaining protein stability and regulating energy storage with a lower impact on overall metabolic balance. This difference highlights a clear sexual metabolic dimorphism, with females being more reactive to nutritional excess and males more resistant to protein-related disturbances, although both sexes exhibit adjustments in lipid metabolism and enzymatic activity. Ducks subjected to force-feeding showed clear signs of metabolic stress and blood biochemical imbalance, indicating a considerable level of physiological strain. Histological results revealed the presence of diffuse hepatic lipidosis in ducks, characterized by lipid accumulation within hepatocytes and structural alterations of the hepatic lobules, without associated inflammatory reaction. At the muscular level, multifocal lipomatosis was also observed, manifested by adipocyte accumulation within the stromal connective tissue, leading to hypertrophy of the perimysium and endomysium. These findings indicate significant lipid infiltration at both the hepatic and muscular levels. Despite these physiological effects, the practice continues to be employed for the production of high-quality traditional products, particularly fatty liver (foie gras), highlighting an ongoing compromise between animal welfare and gastronomic demand.

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LACTATION PERFORMANCE OF FIRST- AND SECOND-PARITY HOLSTEINS IN ROBOTIC MILKING SYSTEM

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Abstract

This experiment was conducted to evaluate performance parameters of first- and second-parity Holstein cows in the robotic milking system. Data from the first lactating cows (n = 124) and the second lactating cows (n = 57) were subjected to descriptive statistics and regression analysis. The mean DIM, milk yield, entrance to the robotic parlor, and compound feed consumption in the parlor were 222 days, 28.0 L, 2.89 times, and 2.97 kg for first-parity cows and 121 days, 36.7 L, 2.89 times, and 3.99 kg for second-parity cows, respectively. Entrance to robotic milking unit did not change as lactation advanced. Each entry was associated with increases in milk yield by 8.1 L and 1.00 kg compound feed consumption within the unit. In summary, the more entrance to the robotic milking unit, thereby the more feed consumption, increases milk yield in the robotic milking system, independent from the parity of cows.

Key words*: lactation, parity, robotic milking system.*

INTRODUCTION

Population in urban increases, whereas population in rural decreases. In relationship to this fact, the mean age of dairy farmers and workers is advanced, consequently farm sizes in rural decrease in global dairy operations. Thank to advancements in mechanization and electronics, robotic milking system (RMS), also known as automated milking system, is becoming common. Since the first RMS installation on a commercial farm in the Netherlands in 1992, switching to this system has dramatically increased to 35,000 units worldwide in 2022 (Geiger, 2022).

Robotic milking system technology allows voluntary milking, which is that each cow has ability to set its own milking schedule. Moreover, it provides data on volume, milking frequency, and milk quality that makes more accurate decisions on management, nutrition, and health (Urbanz & Cardaso, 2023; Marin et al., 2025). The advantages (Soffar, 2019) of this system include reducing number and cost of labor, obtaining more reliable data and experiencing less competition for feed banks, and holding area that create stress-free environment. It is reported that adoption of RMS caused reduction in labor cost by 86% and in number of employees by 69% and improvements in welfare by 77% and in herd performance by 73% (Malacco, 2022). The disadvantages (Soffar, 2019) of this system include high installation cost and initial adaptation.

Utilization of RMS changed feeding system. However, nutritional management in farms with RMS has not been fully elucidated yet. It is obvious that feeding management is key to eventual success. Not only content and physical form of concentrate offered in RMS, but also nutritive value and roughage: concentrate ratio in partial mixed ration (PMR) is important.

Cows were fed concentrate (averaging 3-5 kg) in RMS and given PMR at feed bunk (Tremblay et al., 2016). Data on palatability and amount of concentrate in RMS are not fully established. Bach et al. (2007) projected cows to consume either 3 or 8 kg concentrate in RMS and reported actual intakes were 2.6 and 6.9 kg. Similarly, Halachmi et al. (2005) projected either 1.2 kg per visit or 7 kg per day and reported an average actual intake of 3.85 kg. Menajovsky et al. (2018) set up groups in a

factorial arrangement of two levels of roughage : concentrate (54: 46 vs. 64: 36) in PMR at feed bunk and concentrate levels (2 vs. 6 kg/d) in RMR. They (Menajovsky et al., 2018) reported that feeding high level of concentrate increased milk yield while increasing day-to-day variability in concentrate consumption in RMS. Likewise feeding low roughage: concentrate ratio in PMR at feed bunk increased with yield without causing variation in concentrate consumption in RMS. However, low forage: concentrate in PMR at feed bunk induced ruminal acidosis as well increased time spent in holding area. Composition of PMR and responses to ruminal profiles may reciprocally influence performance in RMS. It is therefore inconclusive to claim that the more entry to consumption in RMS increases milk yield (Halachmi et al., 2005).

The objective of this study was to determine concentrate consumption in RMS and milk yield in the first and second lactating Holstein cows while *ad libitum* consumption of single type of PMR.

MATERIALS AND METHODS

Herd was generated from pregnant heifers purchased from Poland at different times, which caused parity difference.

Cows were housed in 2-row free stall farm equipped with 4 RMS units (VMS V300) and farm management software (DeLaval DelPro™) (DeLaval International, Tumba, Sweeden).

Days in milk (DIM), milk yield, entry to RMS, and concentrate consumption in RMS data from Holstein cows (N=124 1st lactation and N=57 2nd lactation).

Table 1. Summary of database[1]

RMS Unit	Cow (N)	DIM	Milk (L)	Entry to RMS	Concentrate intake in RMS (kg)
1st	30	197	37.3	3.08	4.33
2nd	51	391	28.8	2.72	2.90
3rd	56	33	27.8	2.88	3.50
4th	44	148	32.2	2.89	3.29
Mean ➔		189	30.7	2.89	3.29

[1]RMS = robotic milking system; DIM = days in milk

Cows were fed to meet nutrient requirements for dairy cows (NASEM, 2021). On a dry matter basis, PMR ration contained 1.55% straw, 13.15% alfalfa hay, 41.57% corn silage, 20.99% protein concentrate (RasyoLife, Mugla Türkiye), 17.14% corn flake, 2.05% palm oil, and 3.55% premix of vitamins, minerals, buffering agents, yeast-sodium bicarbonate-magnesium oxide, toxin binder, salt, limestone, and dicalcium phosphate (RasyoLife) [Roughage : Concentrate, 56.27 : 43.73; crude protein, 15.43%; neutral detergent fiber, 33.75; starch, 25.82%, ether extract, 5.25; and ash, 8.36%].

Cows were projected to consume 2 to 7 kg (crude protein, 22.27%, starch, 64.64%, ether extract, 4.59%, and ash 6.96%), 0.8 kg increment for 5 L extra milk after 20 L yield.

Data (Table 1) were subjected to one-way ANOVA and regression analyses.

RESULTS AND DISCUSSIONS

There was no difference in entry to RMS but concentrate consumption in RMS was higher in older cows than younger cows (Table 1). This could also be related to increased frame size and milk yield.

Table 2. Parity effect on performance of dairy cows housed with robotic milking system[1]

Parity	DIM	Milk, L	Concent. intake in RMS, kg	Entry to RMS
1 (N=124)	222	27.98	2.97	2.89
2 (N=57)	121	36.73	3.99	2.89

[1]RMS = robotic milking system unit; DIM = days in milk

As lactation advanced or DIM prolonged, entry to RMS decreased (Figure 1), due to lower milk yield associated with lower concentrate consumption in RMS (Figure 2). The linear regression equations revealed that these decreases started from 3.05 entry to RMS and 3.57 kg concentrate consumption in RMS.

Entry to RMS was associated with 8.1 L increase in milk production (Figure 3). Each kg concentrate consumption in RMS was associated with 4.7 L increase in milk production (Figure 4).

The study questioned if concentrate consumption increased the number of entries to RMS. Concentration consumption in RMS slightly encouraged voluntary entry to RMS (Figure 5).

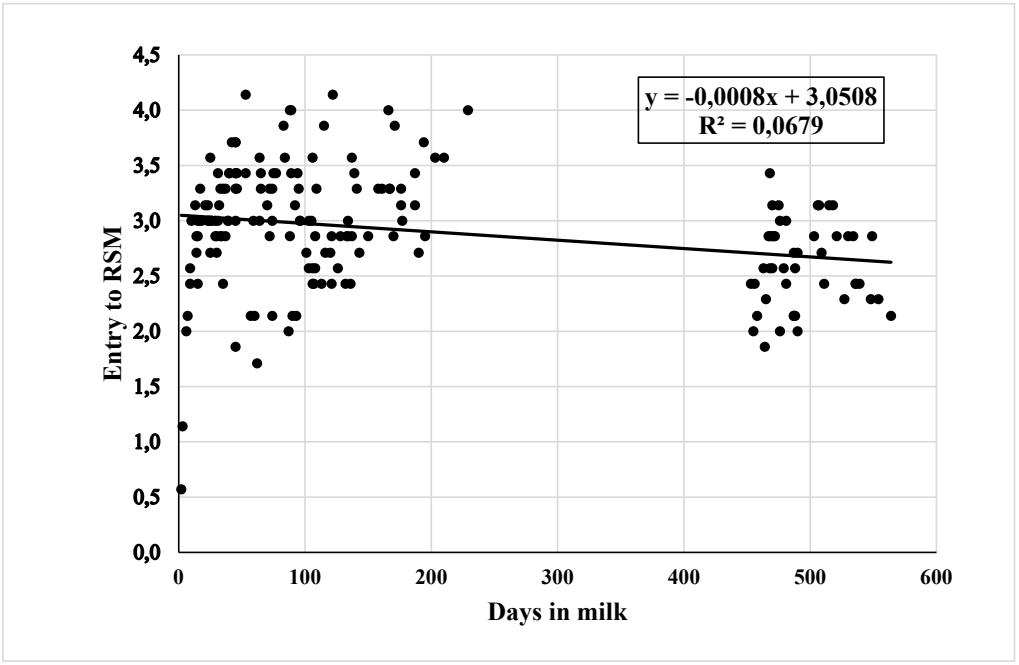


Figure 1. Relationship between entry to robotic milking system (RMS) and days in milk

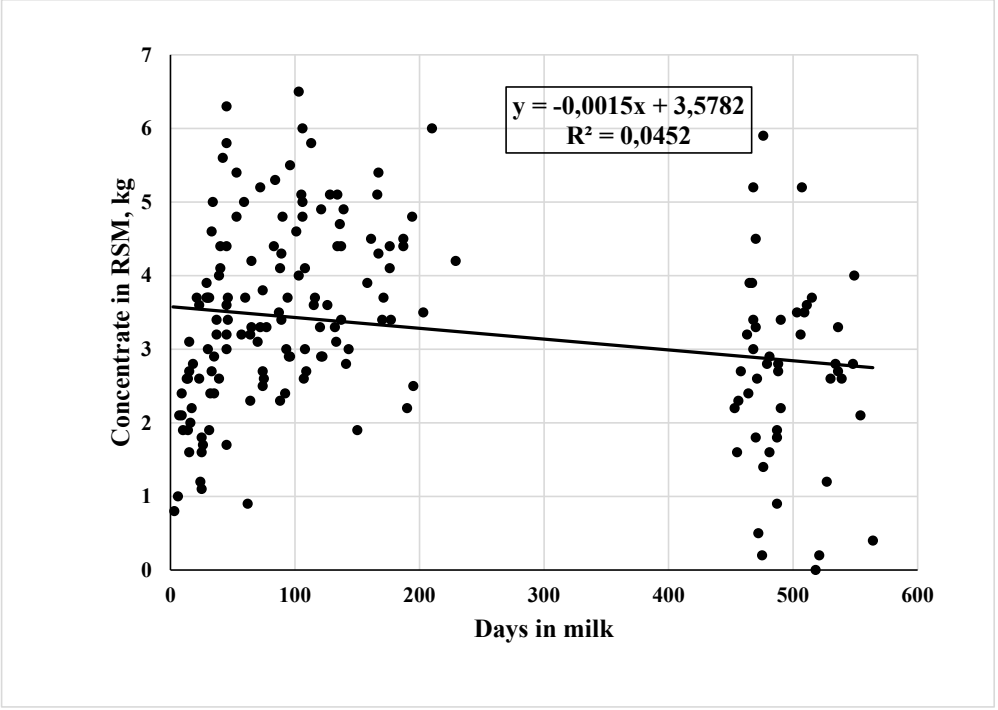


Figure 2. Relationship between concentrate consumption in robotic milking system (RMS) and days in milk

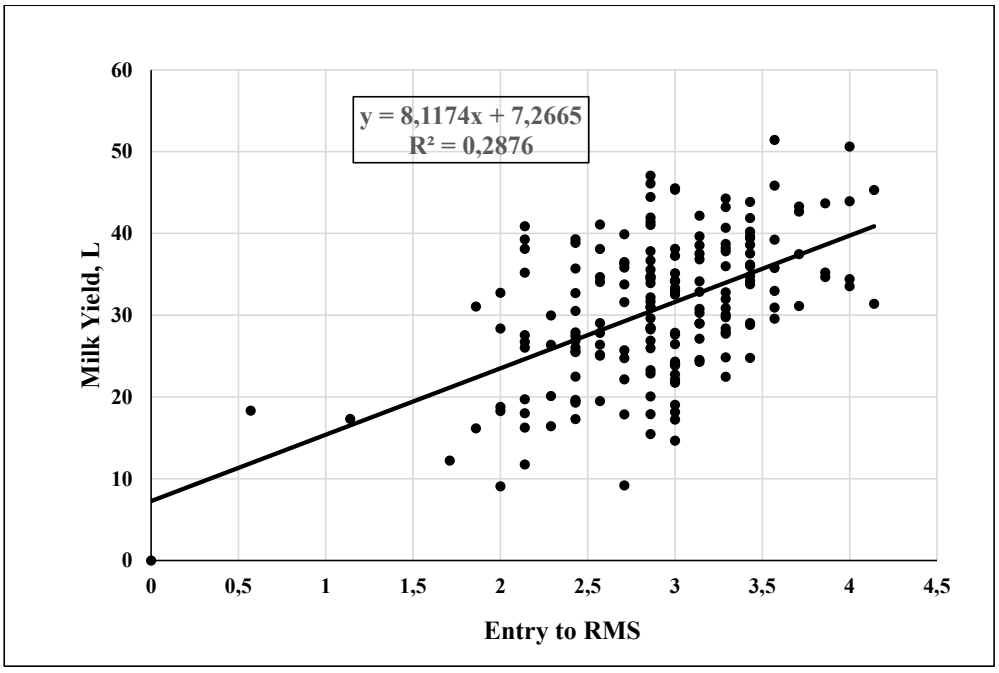


Figure 3. Relationship between entry to robotic milking system (RMS) and milk production

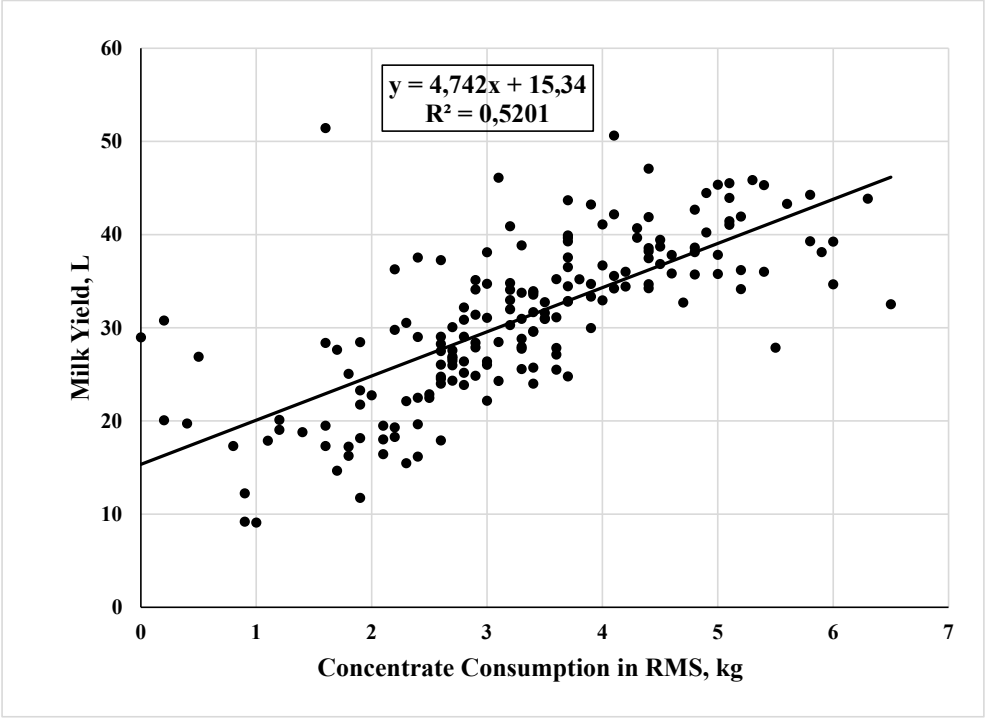


Figure 4. Relationship between concentrate consumption in robotic milking system (RMS) and milk production

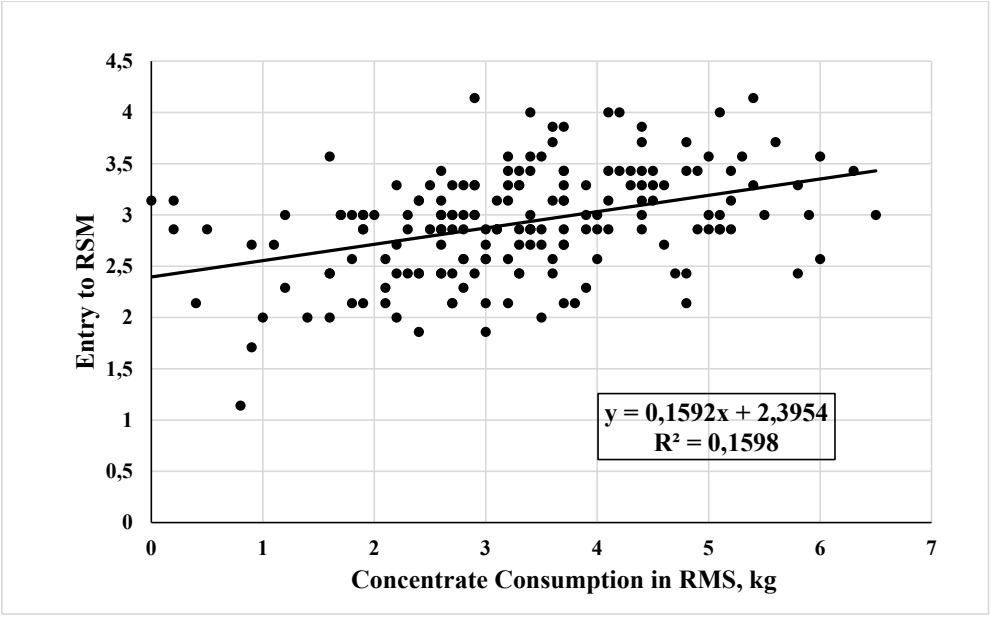


Figure 5. Relationship between concentrate consumption in robotic milking system (RMS) and entry to RMS

A palatable feed offered in the RMS unit is one of the motivating factors for cows to visit the RMS, with the other one being the availability and quality of the PMR in the feed bunk. Pellet feeding *vs*. steam-flaked barley was compared (2 kg/d) in terms of dry matter intake, entry to RMS, milk yield, and milk composition, and PMR consumption (Johnson et al., 2021). Cows receiving the steam-flaked barley fewer entry to RMS (2.71 *vs*. 2.90), tended to have a longer interval between milkings (541.7 *vs*. 505.8 min), spent more time in the holding pen before entering the RMS (139.9 *vs*. 81.2 min/d), and had lower total box time (19.7 *vs*. 21.4 min/d) than cows fed pellet feed. However, their milk yield (44.0 kg/d) and milk composition [fat (1.62 kg/d) and protein (1.47 kg/d)] were similar.

Paddick et al. (2019) offered from 0.5 to 5 kg concentrate in RMS reported no changes in milk yield. This response was attributed to compensation of total intake through PMR at bunk formulated to be *isocaloric*. Namely, cows projected to consume more concentrate in RMS consumed less amount of PMR at feed bunk. Therefore, daily visit to RMS remained unchanged. Despite single type concentrate formulation in this study, concentrate pellet in the RMS unit differing in nutrient density based on production level or lactation stage can be formulated.

In agreement with the present study, Siewert et al. (2019) reported that primiparous cows in free-flow systems produced less milk and had lower entry to RMS than multiparous cows until the late lactation stage. That concluded that there was a potential lagging performance for primiparous cows in early lactation as compared with multiparous cows.

Castro et al. (2012) evaluated efficiency of 34 RMS efficiency and reported that a RMS unit milked 52.7±9.0 cows daily at 2.69±0.28 milkings per cow, with a total milking downtime of 1,947±978 h/yr and a milk yield of 549,734±126,432 kg/yr. The predictor variables cow and milk flow rate had a greater level of influence on the milk yield per AMS than milkings per cow and rejections, which explained the 87% of the variation.

It is expected that health status of cows may influence performance in PMS. In this study, cows were not categorized and evaluated based on their health status. This is important for nutrient enriched concentrate option for cows with negative energy balance due to decreased number of entries to RMS, especially for ketotic cows having sluggish appetite (King et al., 2018). Cows with acidosis, as ascertained

by claw lesions, fecal inconsistency, and reduced ruminal intensity had an averaged entry to RMS was 2.2 milkings per day. After limiting concentrate dispensing in RMS and increasing roughage in PMR, entry to RMS reached an average of 2.6 milkings per day (Wichern et al., 2015). This can infer that amount of concentrate allowance in RMS and its starch level are important to maintain projected entry to RMS, thereby to achieve robotic efficiency and milk yield

CONCLUSIONS

Milk production level and entry to RMS as well as concentrate consumption in RMS are positively correlated.

It appears that genetic capacity drives entry to RMS and concentrate consumption in RMS.

The more entry to RMS and more concentrate consumption in RMS increased milk production.

Literature suggests that nutritive value and palatability of concentrate in RMR may attract more visits to increase milk yield.

Further studies should investigate PMR effect on performance in RMS. More studies are also needed for ingredients and nutritive value of concentrate offered in RMS.

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STUDY OF THE CORRELATION BETWEEN MORPHOLOGICAL INDICATORS OF EGGS AND EXTERIOR PARAMETERS IN LAYING HENS OF TWO BREEDING LINES AND THEIR SURVIVABILITY

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Abstract

The relationship between productive and exterior features and the survival of two breeding lines of laying hens population was studied. A direct correlation was established between two groups of features: 1 - exterior (density, color and shine of feathers; shape, size, color of the comb; height of the legs); 2 - morphological parameters of eggs (shape, size, condition of the cuticle, color of the shell and yolk, condition of the protein). Hens with dense black-pearl and zoz-like plumage, an elongated rose-shaped comb and high legs laid teardrop-shaped eggs of medium size, with a glossy shell, a large bright yolk, and a transparent dense protein. Their survival was 100%. Hens with red and white-black plumage (preserved 95%) had a leaf-shaped or rose-shaped comb, shorter legs and laid larger eggs (including round ones, with weak polar differentiation), dull and poorly mineralized shells, with smaller yolk and a larger zone of thinned protein. Hens with leaf-shaped combs laid fewer number of eggs that had defects in the shell texture in hot conditions.

Key words: *correlation, exterior indicators, laying hens, morphological parameters of egg, survivability.*

INTRODUCTION

The poultry industry plays a key role in meeting the global demand of the human population for substrates valuable for its health in the composition of chicken eggs. Chicken eggs are a valuable protein component of people's food chain all over the world (Khailov, 2018; Hliebova & Vertiichuk, 2014). It is known that, in addition to gastronomic uses, chicken eggs have a powerful biomedical potential. Their components (shell, subshell membrane, albumen and yolk) are used in bone transplantation, tissue engineering, wound healing, drug delivery and biosensors (Mohseni & Zhong, 2025; Usturoi et al., 2025).

Chicken eggs from private mini-farms and organic farms, where environmentally friendly poultry farming technologies are used, are extremely popular with modern consumers (Rahmani et al., 2019; Wägeli et al., 2016; Hidalgo et al., 2008). This technology of production minimizes the harmful impact on the environment. Optimal chicken hybrids are obtained for growing in such conditions because of breeding (Hamzău et al., 2025). Chickens with a meat-egg productivity profile are commercially profitable for mini-farms in rural areas. They are in steady demand among farmers. At the same time, chickens from amateur breeding lines are often used on rural poultry farms (Bondarenko & Khvostyk, 2020; Katerynych et al., 2015; Antos et al., 2013). Such chickens, as a rule, are distinguished by their resistance and high adaptation to environmental conditions. They are an optimal resource of potentially useful genetic parameters for industrial crossbred chickens (Preisinger, 2018; Katerynych et al., 2017; Hocking et al., 2003; Tudorache et al., 2022). Researchers are paying attention to studying the morphological features of local birds, despite the decreasing share of such breeds in the poultry industry of many countries, (Dudusola et al., 2020; Dana et al., 2010; Eganhi et al., 2010).

It is known that laying hens used in commercial poultry farming, as a rule, have solid or monochromatic plumage color. Local breeds of chickens often have a wide range of plumage colors and patterns (Chebo et al., 2023). This is explained by the presence or, conversely, the absence of pigments on the feathers, which is under genetic control (Davoodi et al., 2022). The unique spectrum of plumage pigmentation in local breeds of chickens is a significant genetic resource. Often, due to the uniqueness of the

plumage color, local breeds of chickens, despite the tendency of their population to decrease as a result of the introduction of a large number of commercial crosses, are properly appreciated by farmers and breeders and remain in the poultry sector (Chebo & Nigussie, 2016). Breeders consider the color of the plumage in chickens as a social signal and a tool for identifying breeding. Scientists emphasize the connection of periodic and seasonal fluctuations in the shape, size, texture and pigmentation of feathers in birds with the hormonal background and adaptation of the bird to changing environmental conditions (Widelitz et al., 2018). Investigation on morphological parameters of eggs of amateur breeding hens, depending on exterior characteristics, and their competitiveness in the spectrum of relevant products on the food market, deserve the attention of researchers. Highlighting these indicators and a model of specific experience of specialists and farmers working on optimizing the quality characteristics of chicken eggs will contribute to the development of the poultry industry as a whole. It is possible to optimize the production cycle, objectively investigate the state of egg productivity and morphological parameters of eggs in each breeding line of poultry in the conditions of private poultry farms.

MATERIALS AND METHODS

The relationship between morphological indicators of eggs and exterior characteristics in amateur breeding chickens and the survival of their population in two mini-farms was studied.

1. Chickens (n = 40) of the 1st mini-farm are the result of 18-year natural selection and selection of local chickens (Ukraine). The owner of the poultry farm is engaged in breeding chickens to form a collection of an ecological poultry farm. He raises chickens for a dual purpose (for decorative use - individuals with colorful feather color); to obtain: meat, eggs, fuzz and feathers. Chickens are obtained from incubation of eggs by hens and are grown in a mini-farm with free range. According to anamnestic data, chickens from this poultry farm are distinguished by endurance and a high level of viability. The owner uses combined individual-family selection based on indicators of families and individuals. The chickens breeders were evaluated based on the quality of the offspring if the line specificity was saved.

The chickens of the first mini-farm are descendants of local chickens (Ukraine): black-emerald, zozulyasty, with lacy, motley and striped feather patterns. The farm owner selected the chickens based on the following parameters: colorfulness and decorativeness of the plumage (shine and lacy and motley patterns of feathers; its density and splendor); resistance of the chickens to the influence of negative factors; high shins (the owner considers long legs in chickens to be a sign of both elegance and endurance, which is important in free-range conditions with wetlands); egg production, average egg size (this parameter, as established during monitoring of chickens of two generations, was inherited by laying hens from the paternal line - from the grandmother through the rooster line) and high yolk content.

The chickens of the first mini-farm are representatives of a breeding line, as they are a group of birds with common genetic traits that have been bred and fixed through selection to achieve certain qualities.

In the context of the modern concept of longevity of laying hens, the owner of the first mini-farm, on the advice of experts, uses a comprehensive approach to optimizing their life cycle: from day zero to the end of egg laying. This contributes to the sustainable and profitable production of high-quality food eggs. Observation of the chicken population lasted from the start of egg laying (at the age of 6 months, 100% of the chickens in the studied group laid eggs) to 70 weeks of age.

2. Chickens (n = 40) of the 2nd mini-farm were obtained as a result of amateur selection carried out by the owner of the farm for seven years. They were obtained from incubation of eggs by hens and grown in a free-range mini-farm. These chickens are descendants of local chickens with red and variegated plumage (Ukraine). The farm owner selected them based on the following characteristics: live weight (selection for live weight was carried out to reduce it to a level at which maximum egg production was achieved); at the same time, the owner selected the chickens based on the indicator of large eggs. Observation of the chicken population lasted from the start of egg laying (at the age of 5.5 months) to 70 weeks of age.

Both farms are located in the same biotope with the same type of relief and soil and plant cover. Chickens are kept in equipped poultry houses with optimal sanitary and hygienic conditions at night and in the cold season. Chickens are free-range during the day (in favorable weather). Nests in the poultry houses are equipped with video cameras to record the egg production of chickens, which makes it possible to carry out its individual accounting. The research was carried out in stages: the first stage included an individual assessment of productivity and morphological indicators of eggs; the second stage included an assessment of the exterior features of each laying hen and their comparison with the macromorphological features of eggs obtained from them. The ration of the studied chickens had significant seasonal differences in the set of feed components and their proportions. According to the analysis, the chickens' diet was dominated by wet mixtures of grain feeds (usually crushed and whole grains of wheat, barley, rolled oats, crushed corn) and crushed root crops (grown by farm owners), crushed mass of boiled beans, peas; periodically the chickens were fed dairy waste from their own farm. The seasonal feeding scheme for chickens was developed taking into account their age and operational characteristics, the corresponding needs of the body and the availability of specific feed sources at different times of the year. The most optimal period is the months from April to mid-November (at this time there are significant resources of grass in the grazing areas). The ambient air temperature, with the exception of hot summer and frosty winter days, allowed the chickens to roam during the day in search of plant and other feed components. The farm owners use a rotational system for keeping chickens: in certain areas, the chickens do not graze for 30 days and during this period the grass grows there. The chickens were fed with cultivated and wild plant species: aegopodium, chenopodium, wheatgrass; chopped radish, cabbage, spinach leaves. In autumn and winter, the chickens were fed wheat microgreens, hay flour, chopped hay of legumes (clover, sainfoin) and wild cereal crops, dandelion leaves, nettle, boiled root vegetables, potatoes, raw pumpkins.

The research was made in accordance with the topics of scientific activity of the Department of Morphology of the State Biotechnology University (Kharkiv, Ukraine).

The following methods were used: 1 – daily recording of individual egg productivity of chickens; 2 – method of selective assessment of morphological parameters of eggs (egg mass, thickness, strength and brightness of the shell; condition of the cuticle; proportion and condition of yolk and albumen); 3 – assessment of exterior features of chickens (density and color of plumage, shape, color and size of combs; height of thigh and shin); 4 – statistical analysis of the results obtained; 5 – method of regular chicken counting with calculation of survival rate; 6 – method of autopsy of dead chickens; 7 – method of photography.

For the purpose of selective assessment of morphological parameters of eggs, 5 eggs were selected from each laying hen of both farms aged 52-54 weeks and the egg weight and shell thickness were determined at three points (middle part, sharp end, blunt end), obtaining the average value per individual. 200 eggs were examined from each group of laying hens (total 400 eggs). 1. The weight of the egg and/or its components (g) was determined by weighing on an *Adventurer* laboratory electronic balance. 2. The thickness of the egg shell in the middle part / at the sharp end / at the blunt end (mm) was measured using an ultrasonic thickness gauge (Echometer 1061 device; *Karl Deutsch*; Co. Ltd., Wuppertal, Germany). 3. The strength of the shell was determined by a puncture resistance test (the ability of the shell to withstand a point mechanical impact/pressure of a dissecting metal needle in a certain area was assessed by two indicators: 1 - strong; 2 - fragile). 4. The degree of brightness of the egg shell was determined visually in daylight (in relative units) on the following scale: 1 - the shell is bright, the cuticle is uniform and smooth, reflects light rays in a specular direction; 2 - the shell is moderately shiny, the cuticle has separate matte areas; 3 - the shell is matte, without signs of shine. 5. The condition of the yolk and albumen was determined visually after breaking the shell; the integrity of the yolk and its pigmentation, the degree of protein density were noted. 6. The mass of the albumen and yolk was determined on the Adventurer laboratory electronic balance with the determination of their proportion.

The exterior features of the chickens were evaluated (the color of the plumage was visually determined; the brightness of the plumage was determined visually, in daylight, according to the following scale: 1 - bright plumage, with the presence of shiny black-tar, black-green, purple-pearl feathers; 2 - moderately bright plumage; with individual areas of dull feathers; 3 - dull plumage);
feather density was determined visually and palpated; comb shape (1 – rose-shaped; 2 – leaf-shaped) was determined visually; comb size was determined visually (by comparison in different chickens) and using a flexible laboratory ruler; thigh and shin height (cm) was measured using a laboratory ruler.
The survival rate of chickens on each farm was determined by regularly counting them, calculating the percentage of survival, and analyzing the reasons for their loss (death, culling). The survival rate of chickens was calculated as the ratio between the number of live chickens at the end of the study and their number at the beginning of the study.
Statistical analysis was performed using standard biometric methods using Microsoft *Excel* 2019 (Microsoft Corp., Redmond, WA, USA). Data are presented as mean ± standard deviation (M ± SD). The difference between the performance of chickens from two farms was assessed using Student's t-test for independent samples. The difference was considered statistically significant at $p < 0.05$. M ± SD (mean ± standard deviation; comparison of groups (chickens from two farms) – Student's t-test for independent samples; significance level $p < 0.05$). Correlation analysis was performed within each farm to assess the relationships between egg characteristics and exterior traits. For continuous variables (egg weight; shell thickness; thigh length; shin height), the Pearson correlation coefficient (r) was used. For ordinal brightness scores, the Spearman rank correlation coefficient (ρ) was used.

RESULTS AND DISCUSSIONS

Research on egg productivity, morphological parameters of eggs, exterior features and safety of laying hens with black-pearl and lace-striped plumage of farm №. 1

Parameters of egg productivity of the first mini-farm hens: age of laying the first egg – 175-180 days; age of reaching peak egg production – 29-31 weeks; egg production for 52 weeks of productivity (for one calendar year) – 245-260 eggs; egg weight at 52 weeks – 57-64 g; safety of hens – 100%. Live weight of adult laying hens – 2.1-2.3 kg. With daily monitoring and visual examination of all eggs laid by hens on the mini-farm, a connection between their morphological parameters and exterior features of the bird was established. Laying hens that had thick, lush black-pearl and black-emerald plumage stable and rhythmic laid eggs (except during the molting period) (Figure 1).



Figure 1. Appearance of part of the plumage of a domestic chicken of local selection: dense shiny black-emerald-pearl plumage in the area of the right wing (own source)

It is known that in nature the color of plumage in birds undergoes seasonal changes. In the studied chickens, the pigmentation of feathers acquired a particularly colorful appearance (to attract the male). Variations in the color of plumage in the studied chickens depended on primary and secondary color patterns and in some individuals were unique. The primary pattern depended on the distribution of colored feathers in different parts of the chicken's body, and the secondary pattern - on the distribution of pigment on individual feathers.
In the studied hens with dense lacy-zolaceous plumage, the eggs were also distinguished by 100% condition. It is known that in birds, feathers and skin are the first line of defense and play an important role in maintaining a stable internal temperature. Lush dense plumage helps maintain normal body temperature, regardless of the season (protects against hypothermia in the cold season and provides cooling in the heat). Elongated bright red rose-shaped combs, wide pectoral part, high shins were no less important exterior features that distinguished the studied hens with stable egg production (Figure 2).

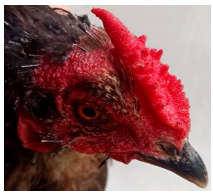


Figure 2. Appearance of a chicken's head with an elongated, bright rose-shaped comb (own source)

It is known, combs in birds play an important role in the prevention of heat stress (Hester et al., 2015; Hamzău et al., 2025). This depends on their shape and volume. The rose-shaped comb, in conditions of overheating of the bird, due to its specific volumetric structure, contributes to its faster cooling, dissipating heat from its surface, than the leaf-shaped one. The exterior features of the 1st farm chickens ensured and supported the adaptation of their organism to different conditions. The result of this was rhythmic egg laying, which indicates a high reproductive status, because it is a component of the reproductive cycle.

Results of visual assessment of the condition of the egg cuticle. The quality of the cuticle in individual egg specimens was consistently distinguished. Eggs from hens with dense colorful plumage (black-blue-pearl, with a metallic sheen and lacy-zulaceous) had a perfectly smooth cuticle. The best appearance was given to eggs from hens with the largest body and high legs. Their shells were shiny (Figure 3).



Figure 3. Appearance of an egg with a glossy (shiny) shell from a laying hen of local amateur breeding (own source)

It is known that gloss is conventionally defined as the specular component of light reflected from an object (Rasmussen & Dyck, 2000). Light is mostly reflected from a smooth glossy surface in a specular direction.

The appearance of a glossy, uniform eggshell was a successful sensory and marketing indicator: they were highly valued by buyers and consumers. It is known that the gloss of eggshells depends on the nanostructure. The shade and level of gloss of the eggshells of individual hens changed over time. But in general, eggs from hens from the first poultry farm had a uniform thickness of glossy golden-cream and pink-beige shells. (Figure 4).



Figure 4. Appearance of eggs from a hen with black-tarnish (on the left) and a hen with zozolish (on the right) plumage (own source)

In addition to marketing value, the uniform thickness of the eggshell ensured their high quality and long-term preservation.

Researchers note the nanostructural basis of the shine of the shell of chicken eggs and indicate the dependence of this trait on the breed and age of the laying hens (Li et al., 2020; Li et al., 2019; Yan et al., 2014). Researchers Lichovníková & Zeman (2008) note the influence of the housing system on the calcium needs of laying hens and the quality of eggshells. At the same time, scientists believe that the shine of the eggshell can potentially be integrated into the selection index as a moderate hereditary characteristic to achieve higher egg attractiveness.

Therefore, we consider the trait of the shine of the eggshell in chickens of the first farm to be extremely valuable and recommend that the owner use it as a breeding trait in chicken breeding programs. Researchers note that the hatchability of eggs depends on the quality of bioceramic calcite layers and the surface glycoprotein film of the egg (cuticle) (Bordunova et al., 2024).

Scientists Johnsson et al. (2012) indicate that calcium is more easily mobilized into the eggshell when hens have larger combs. At the same time, the heterogeneity of the thickness of the egg shell is a diagnostic criterion for a probable deficiency of minerals (calcium, phosphorus, etc.), vitamins of group *D* in the poultry diet and the influence of stressors

(Yenice, et al., 2025; Liakhovich et al., 2024; Liulin et al., 2023; Liakhovich et al., 2023).
Among the useful and husbandry traits in the chickens of the farm №1, it is important to note the indicator of long legs. The owner selected chickens, in particular, according to this trait, which is justified in free-range conditions.
Chickens with long legs move freely and quickly around the territory of the enclosure, compared to chickens with short legs. Long legs and distinctive camouflage plumage color variations (Figure 5) helped protect free-range chickens from predators.



Figure 5. Appearance of a chicken with camouflage lace plumage of beige-gray-brown shades (own source)

The survival rate of chickens on farm №. 1 was 100%.

Research on egg productivity, morphological parameters of eggs, exterior features and survival of hens with red-white and white-black plumage of amateur breeding of farm №. 2.
Parameters of egg productivity of hens of the second mini-farm: age of laying the first egg – 160-165 days; age of reaching peak egg production – 28-30 weeks; egg production for 52 weeks of productivity – 265-290 eggs; egg weight at 52 weeks – 57-67 g; survival rate of hens – 95.6%. The live weight of adult laying hens was 2.3-2.7 kg.
Hens with lush white and black plumage, a rose-shaped comb, an elongated body on medium-length legs, laid eggs with a white-cream shell. The shape of these eggs was distinguished by an elongated upper part and a smooth transition from the equatorial part to the rounded lower pole (Figure 6).



Figure 6. Appearance of an egg from a hen with white and black plumage with a rose-shaped comb, an elongated body on medium-length legs (own source)

Hens with lush red-and-white plumage, a short and thickened rose-like comb (Figure 7), and short legs laid relatively large eggs. These hens had a longer delay in feather regrowth after moulting compared to hens from the first farm. Hens from the farm №. 2 also had paler combs than hens from the farm №. 1.



Figure 7. Appearance of the head of a hen with red-and-white plumage and a short thickened light pink rose-like comb (own source)

The large eggs had rounded poles without clear differentiation, and their shells were often depigmented and dull (Figure 8).



Figure 8. Appearance of a large egg from a hen with red and white fluffy plumage: depigmentation and dullness of the shell, weak differentiation of the upper and lower poles (own source)

It was difficult to establish the basic color of the shell of such eggs, which is characteristic of chickens of certain crosses and breeds. Due to the large size of the egg and the squat body

structure, painful egg laying was observed in these chickens.

Daily recording of egg production, systematic visual assessment of the texture and pigmentation of the egg shell made it possible to establish the influence of specific factors that can change the indicated indicators. In chickens with a leaf-shaped comb (Figure 9), under conditions of comfortable summer temperatures, the color and texture of the egg shell were conditioned. And, at temperatures above 30°C, regardless of the color of the plumage of these chickens, their egg production decreased sharply (by 50-65%).



Figure 9. Appearance of hen head with a leaf-like comb that, under heat stress, laid eggs with defective shell texture (own source)

At the same time, eggs from hens with a leaf-shaped comb (in those individuals that maintained egg production in hot weather conditions) had a dull, depigmented and defective shell texture due to rough calcareous tubercles on the surface (Figure 10).



Figure 10. Appearance of an egg with a rough and depigmented shell from a hen with a leaf-shaped comb of amateur selection, laid at the background of heat stress (own source)

Researchers have found variability in some physical characteristics of chicken eggs and their shell quality throughout the entire laying cycle (Sirri, et al., 2018; Johnston & Gous, 2007). Regarding changes in internal and external egg quality parameters, scientists point out, first of all, their connection with the housing system, environmental temperature, age and genotype of the hens (Tumova & Gous, 2012; Tumova, 2011).

Data on the average egg weight from hens from both farms are given in Table 1.

Table 1. Egg mass of hens from two farms

Indicator	Farm 1 (n = 40)	Farm 2 (n = 40)	p
Egg mass (g)	59.88 ± 1.88	60.78 ± 2.69	0.087

As can be seen from the data in Table 1, the egg mass of chickens from the two farms is not statistically different.

Data on the thickness of the eggshells from chickens from both farms are given in Table 2.

Table 2. Eggshell thickness of chickens

Shell thickness (mm)	Farm 1 (M ± SD)	Farm 2 (M ± SD)	p
in the middle of the egg	0.332 ± 0.004	0.325 ± 0.012	0.001
at the sharp end	0.335 ± 0.004	0.328 ± 0.011	0.0004
at the blunt end	0.323 ± 0.005	0.317 ± 0.011	0.0013

The data in Table 2 show that the thickness of the eggshell at all three measurement points is significantly higher in chickens from the farm № 1; the differences are highly significant ($p < 0.01$).

Data on morphological and phenotypic indicators of chickens from the two farms are given in Table 3.

Table 3. Morphological and phenotypic indicators of chickens

Indicator	Farm 1 (M ± SD)	Farm 2 (M ± SD)	p
Brightness of the shell (relative units)	2.75 ± 0.44	1.65 ± 0.48	<0.001
Length of the thigh (cm)	14.97 ± 0.08	14.06 ± 0.15	<0.001
Height of the shin (cm)	8.14 ± 0.08	7.13 ± 0.14	<0.001
Brightness of the plumage (relative units)	2.70 ± 0.46	1.48 ± 0.51	<0.001

As can be seen from the data in Table 3, the brightness of the shell in chickens from the farm №1 is significantly higher than in chickens from the second farm; the length of the thigh and the height of the shin are also significantly higher in chickens from the farm №1; the brightness of the plumage is statistically higher in chickens from the first farm. In all cases, the differences are highly significant ($p < 0.001$).

The data of the correlation analysis within each farm to assess the relationships between egg characteristics and exterior traits of chickens are given in Table 4.

Table 4. Key correlations of indicators in chickens of farm 1 (n = 40)

Pair of indicators	Method / Coefficient (Pearson r; Spearman ρ)/	p
Egg mass ↔ Shell thickness (middle of egg)	r / −0.803	4.6×10^{-10}
Egg mass ↔ Shell thickness (sharp end of egg)	r / −0.703	4.1×10^{-7}
Egg mass ↔ Shell thickness (blunt end of egg)	r / −0.820	9.4×10^{-11}
Shell thickness middle of egg ↔ sharp end of egg	r / +0.851	3.5×10^{-12}
Shell thickness middle of egg ↔ blunt end	r / +0.667	2.6×10^{-6}
Shell thickness sharp end of egg ↔ blunt end	r / +0.768	7.3×10^{-9}
Thigh length ↔ Tibia height	r / +0.338	0.0327
Shell brightness ↔ Plumage brightness	ρ / +0.756	1.7×10^{-8}
Shell brightness ↔ Egg mass	ρ / −0.753	2.0×10^{-8}
Plumage brightness ↔ Egg mass	ρ / −0.764	9.4×10^{-9}

According to the results presented in Table 4, there is a strong negative relationship between the indicators “egg weight / shell thickness” in chickens of farm №. 1 (at all 3 measurement points).

To assess the relationships between morphometric parameters (thigh length, shin height, plumage brightness) and egg quality indicators (egg weight, shell thickness at three different points and its brightness), a correlation analysis was conducted. It showed the presence of a number of statistically significant relationships between morphometric characteristics of birds and egg quality indicators in hens of the farm №. 1.

The most pronounced relationships were found between egg mass and shell thickness, where a strong negative correlation was observed. Thus, the correlation coefficient between egg mass and shell thickness in the middle part was $r = -0.80$ ($p < 0.01$), at the sharp end of the egg $r = -0.70$ ($p < 0.01$), and at the blunt end $r = -0.82$ ($p < 0.01$). This indicates that with increasing egg mass there is a tendency for shell thickness to decrease. Shell thickness indicators in different parts of the egg demonstrate a high positive correlation with each other ($r = 0.67–0.85$; $p < 0.01$), which indicates the consistency of shell formation processes over the entire surface of the egg.

A strong positive correlation was also found between the brightness of the shell and the brightness of the plumage ($r = 0.76$; $p < 0.01$). A moderate positive relationship was found between the length of the thigh and the height of the shin ($r = 0.34$; $p < 0.05$), reflecting the overall proportionality of the development of the limbs in the studied chickens.

Correlation analysis of productivity indicators and morphometric characteristics of chickens of the farm № 2 revealed a number of statistically significant relationships between the studied traits. The most pronounced correlations were found between egg mass and shell thickness. A strong negative correlation was established between egg mass and shell thickness in the middle part of the egg ($r = -0.77$; $p < 0.01$), at the sharp end ($r = -0.73$; $p < 0.01$) and at the blunt end ($r = -0.79$; $p < 0.01$).

The results obtained indicate that with increasing egg mass there is a tendency for the shell thickness to decrease. Such a dependence may be due to the redistribution of mineral substances during the formation of larger eggs, which leads to a relative decrease in shell thickness. Strong positive correlations were found between shell thickness values measured in different parts of the egg. The correlation coefficient between shell thickness in the middle part of the egg and at the sharp end was $r = 0.88$ ($p < 0.01$), between the middle part and the blunt end - $r = 0.74$ ($p < 0.01$), and between the sharp

and blunt ends - r = 0. These results indicate a high consistency of shell formation processes across the entire surface of the egg and confirm the integrity of the shell mineralization mechanism.

A strong positive correlation was found between the brightness of the shell and the brightness of the plumage ($r = 0.73$; $p < 0.01$).

This may indicate common physiological or genetic mechanisms of pigmentation formation in birds, which are manifested both in the coloring of the plumage and the eggshell. Morphometric parameters in chickens of farm № 2 demonstrated a moderate positive relationship between the length of the thigh and the height of the shin ($r = 0.31$; $p < 0.05$), which reflects the proportionality of the development of the skeleton of the limbs in the studied birds.

At the same time, in chickens of farm № 2, the relationship between morphometric characteristics (thigh length, shin height) and egg quality indicators turned out to be weak or statistically unreliable, which indicates a relatively smaller influence of the bird's body size on the formation of egg quality characteristics in this sample. In general, the results of the correlation analysis show that close relationships are observed between the indicators of the structure of the egg shell, and even between the signs of pigmentation. This confirms the existence of functional and physiological relationships between individual components of egg formation and phenotypic characteristics of birds.

Comparative analysis of the results of the correlation study. Comparison of the results of the correlation analysis showed that in chickens on both farms similar patterns of relationships between egg quality indicators and morphometric characteristics of chickens are observed. In both groups, a strong negative correlation was established between egg weight and shell thickness in different parts of the egg, which indicates a tendency to decrease shell thickness with increasing egg weight.

Also, in chickens from both farms, close positive correlations were found between the shell thickness indicators measured in different parts of the egg, which confirms the consistency of the shell formation processes. In addition, in both groups, a positive relationship was observed between the brightness of the shell and the brightness of the plumage, which may indicate common mechanisms of their pigmentation. In general, the correlations between the studied indicators in chickens from the first and second farms are of a similar nature, which confirms the stability of the identified biological patterns.

Research on the internal content of eggs from chickens from two farms.

The subshell membrane in eggs from chickens from the first mini-farm was distinguished by its strength and elasticity. In eggs from chickens from the farm №2, the thickness of the subshell membrane was smaller.

The study of the internal contents of eggs from chickens from both farms showed a difference in the proportion and condition of the protein part. A larger proportion of protein was in the eggs of chickens from the farm №2, but the protein was watery and easily spread. Due to the diluted state of the protein part, the central position of the yolk was not preserved (Figure 11).

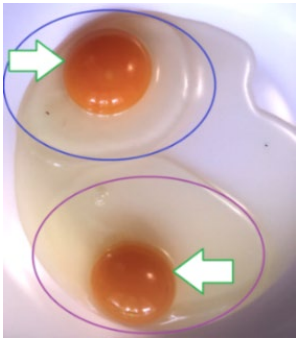


Figure 11. Appearance of the internal contents of eggs from experimental chickens (at the top – bright centrally located yolk and dense egg white from a chicken with black-tarry plumage; at the bottom – dull yolk and watery egg white from a chicken with red plumage) (own source)

The influence of breed and age of hens on the yolk-to-protein ratio in eggs and its differences in different genetic lines of poultry are known (Suk & Park, 2001). The mentioned traits are also influenced by the way the hens are kept, the length of the egg-laying cycle and many other factors (Sokołowicz et al., 2018; Ketta & Tumova, 2016).

The yolks in the eggs of the farm №1 chickens were consistently richer and brighter. At the

same time, the yolks in the eggs from chickens with glossy shells were the most pigmented.
Eggs from chickens with red-white and white-black plumage were heavier due to the albumen part and shell. And eggs from chickens with black-tarry and sable plumage had the most massive yolks. (Figure 12).



Figure 12 . Appearance of egg yolks on the left – a smaller yolk of an egg from a chicken with red-white plumage; on the right – a large yolk of an egg from a chicken with black-pearl plumage (own source)

At the same time, the proportion of yolk was the highest in eggs from hens with rich pitch-black plumage and reached 35% of the egg mass.
The feasibility of keeping hens with rose-shaped combs and long legs in free range is being investigated. A rose-shaped comb provides faster cooling of the hen's body than a leaf-shaped oneIn conditions of overheating. Accordingly, hens with a rose-shaped comb have a lower risk of heat stress. This contributes to the well-being of hens and their stable productivity.
Of the 40 chickens on farm №. 2, two chickens (5% of the initial population) died during the study period. This mortality rate is not statistically significant. It was determined that the deaths of two chickens were due to critical injuries sustained while free-range (one chicken was attacked by a hawk; the other chicken was attacked by a ferret). These incidents were recorded by surveillance cameras.
During the autopsy of one of the dead chickens, a formed egg was found in the oviduct. Hyperemia of the mucous membrane of the oviduct and stretching of its wall were detected (Figure 13), as well as a clear gradation of follicles in the ovary, which indicates the sudden death of the chicken at the moment of readiness for laying.
The difference in the survival rate of chickens on both farms (100% - farm № 1 and 95% - farm №. 2) is statistically insignificant.

Given that the two farms of local breeding laying hens where the study was conducted differ in a number of indicators (selection goal, live weight, age of first clutch), it is worth noting its limitations.



Figure 13. Appearance of the ovary and oviduct (with the presence of an egg in the lumen) in a dead hen (own source)

Given the differences in the directions of selection on both farms, it is advisable, first of all, to consider the impact of its consequences on the exterior and productive indicators of chickens. The number of studied chickens is also objectively limited, which accordingly reduces the possibility of wide application of the results obtained. It is advisable to continue research on the exterior and productive indicators of local breeding chickens on a larger population in the long term.

CONCLUSIONS

Laying hens with bright black-greenish-resinous and pearl plumage laid eggs with thicker and brighter shells, larger yolks and denser whites. They had longer thighs and legs. The weight of eggs from hens from the two farms was not statistically different. In hens with white-motley and red plumage, the white was larger and watery, and the yolk was smaller. In hens from both farms, a strong positive correlation was found between the brightness of the shell and the brightness of the feathers. This should be taken into account when selecting hens for breeding. The difference in survival of hens from both farms is statistically insignificant.

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LIVE ESTROUS FEMALE VERSUS PHEROMONAL STIMULATION: A COMPARATIVE ASSESSMENT OF CANINE SEMEN COLLECTION OUTCOMES

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Abstract

This pilot descriptive study compared manual semen collection outcomes in sexually experienced adult male dogs collected either in the presence of a live intact female in estrus (n = 3) or using isolated estrous pheromones sampled from an estrous female (n = 3). Outcome measures included ejaculation latency (time from stimulus exposure to ejaculation), ejaculate volume, semen pH, and macroscopic appearance (color, odor). Inferential statistical analysis was not performed because of the small sample size; results are presented descriptively. Median (range) ejaculation latency was shorter in the live - female group at 3:00 min (2:52–3:37) than in the pheromone group at 4:35 min (4:18–5:02). Mean ejaculate volumes were similar between groups - 3.93 mL (SD 2.70) for live - female stimulation and 4.03 mL (SD 2.85) for pheromone - only stimulation. Semen pH values were broadly comparable between groups, and all ejaculates were milky - white and odorless. These findings indicate that pheromone - only stimulation can induce ejaculation and produce grossly comparable ejaculates under low - stress conditions, although ejaculation latency was longer. Given the small parallel - group design, use of a single pheromone donor, and absence of objective sperm - quality parameters, results are preliminary. Further research using larger crossover designs and comprehensive fertility endpoints is warranted.

Key words*: dogs, ejaculate, pheromones, semen collection.*

INTRODUCTION

Assisted reproductive techniques (ART), including artificial insemination and semen cryopreservation, are increasingly important in canine breeding, particularly when natural mating is impractical because of anatomical, behavioral, geographic, or management constraints (Concannon, 2011; Luvoni, 2013). Reliable, low-risk semen collection methods are therefore essential for preserving genetic resources and supporting fertility management in domestic dogs. Manual semen collection is commonly facilitated by the presence of an estrous female, which provides multisensory cues: visual, behavioral, tactile, and olfactory, that promote sexual arousal and ejaculation. However, the use of live estrous females presents logistical, welfare, and safety challenges, including the need for suitable donors, coordinated timing, and potential stress or injury to animals and handlers (Micșa et al., 2024).

An alternative approach is the use of estrous pheromones sampled from females in estrus, providing primarily olfactory stimulation while avoiding direct contact. Pheromones are chemical signals that influence conspecific physiology and behavior and contribute to mating attraction and reproductive readiness in many mammalian species (Robertson & Martin, 2022; Patra, Barman & Kumar, 2026). Semio-chemical signaling is among the oldest forms of communication across taxa and plays a key role in initiating and modulating sexual behavior (Dzięcioł et al., 2016). In dogs, studies of female-derived cues have examined behavioral and endocrine responses, yet evidence supporting pheromone-only stimulation for reliable semen collection remains limited (Woszczyło et al., 2023).

Evaluating whether isolated pheromonal cues can produce ejaculates comparable, on gross characteristics, to those obtained in the presence of live-females may help simplify semen collection protocols and reduce risks

associated with direct male-female interaction. Semen evaluation is central to assisted reproduction, as ejaculate volume alone does not predict fertility; standardized assessment methods, including concentration and motility analysis, are essential for interpreting suitability for artificial insemination and cryopreservation (Johnson & Johnston, 2018). Semen analysis in dogs also allows repeated assessment across time and supports evaluation of functional impacts on male reproduction (Dostal, Juneau & Rothwell, 2001).

This study presents a pilot, descriptive comparison of manual semen collection outcomes in sexually experienced male dogs collected with either a live estrous female or pheromone-only olfactory stimulation. Ejaculation latency, ejaculate volume, semen pH, and macroscopic appearance (color, odor) were assessed. Owing to the small sample size and parallel-group design, results are presented descriptively without inferential statistics. Animals were washed and sanitized prior to collection to minimize bacterial contamination during sampling (Darie et al., 2020).

MATERIALS AND METHODS

This was a pilot, parallel-group, descriptive study. Six sexually experienced adult male dogs were enrolled and assigned to one of two groups (n = 3 per group) matched approximately by body size and weight to reduce breed/size-related confounding. Each dog contributed a single semen collection under the assigned condition.

Animals and grouping (see Table 1): Group 1 (live-female stimulus): Rottweiler (6 y 2 m; 49 kg), Boston Terrier (8 y 4 m; 11 kg), Wire-haired Dachshund (4 y 9 m; 9 kg). Group 2 (pheromone stimulus): Rottweiler (6 y 2 m; 52 kg), French Bulldog (7 y 7 m; 14 kg), Wire-haired Dachshund (3 y 1 m; 10 kg). Breed names are standardized.

Table 1. Breed, age and body weight of male dogs allocated to the two experimental groups

Group 1			Group 2		
Breed	**Age**	**Weight**	**Breed**	**Age**	**Weight**
Rottweiler	6y 2m	49 kg	Rottweiler	6y 2m	52 kg
Boston Terrier	8y 4m	11 kg	French Bulldog	7y 7m	14 kg
Wire haired Dachshund	4y 9m	9 kg	Wire haired Dachshund	3y 1m	10 kg

All males were sexually experienced and previously used for manual semen collection and natural mating. Dog semen is typically collected manually from conscious animals (Christensen et al., 2011).

Collections were performed in familiar, low-stress environments with owners present. Animals were washed and sanitized prior to handling to reduce contamination risk (Darie et al., 2020). Owner consent was obtained for participation and for use of photographs.

Female estrous status was confirmed by serum progesterone measurement performed by an external diagnostic laboratory (Figure 1), and only females with progesterone concentrations consistent with the peri-ovulatory/estrus period were used. For live stimuli, a clinically healthy intact female in estrus was positioned near the male during collection.

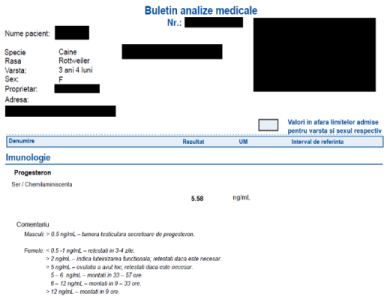

Buletin analize medicale
Nr.:
Nume pacient:
Specie Caine
Rasa Rottweiler
Varsta: 3 ani 4 luni
Sex: F
Proprietar:
Adresa:
Valori in afara limitelor admise pentru varsta si sexul respectiv
Denumire | Rezultat | UM | Interval de referinta
Imunologie
Progesteron
Ser / Chemiluminiscenta
5.58 ng/mL
Comentariu
Masculi: < 0.5 ng/mL – tumora testiculara secretoare de progesteron.
Femele: < 0.5 - 1 ng/mL – reticutati in 3-4 zile.
> 2 ng/mL – indica fatwizarea functionala, reteotati daca este necesar.
> 5 ng/mL – ovulatia a avut loc; reteotati daca este necesar.
5 - 6 ng/mL – montati in 33 – 57 ore.
6 – 12 ng/mL – montati in 9 – 33 ore.
> 12 ng/mL – montati in 9 ore.

Figure 1. Positive test bulletin for ovulation (own source)

For pheromone stimuli, a sterile cotton pad was gently applied to the vulvar region of an estrous donor female (progesterone 5.58 ng/mL for the donor used), then placed in a sterile container; during collection the container was opened and the pad positioned near the male's muzzle to provide olfactory stimulation only (See Figure 2).



Figure 2. Cotton pad previously applied to the vulvar region of an estrous female (own source)

Semen collection and evaluation

Manual semen collection was carried out by experienced personnel using standard manual stimulation techniques (Figure 3). Collections were performed once per male under assigned conditions.



Figure 3. Manual collection of the seminal material (own source)

Semen was evaluated immediately in fresh state. Parameters recorded were:

- Ejaculation latency: time from onset of exposure to stimulus (live-female present or pheromone container opened) to ejaculation (minutes: seconds).
- Ejaculate volume (mL).
- Semen pH (measured on fresh ejaculate).
- Macroscopic appearance: color and odor (descriptive). Erection latency was not measured and is not reported.

Rationale for not cryopreserving: the study focused on immediate ejaculatory and gross semen characteristics measured on fresh semen. While cryopreservation is important for long-term storage and ART, freeze-thaw processes can alter cellular structures and motility; therefore, cryopreservation was not performed for this pilot comparison (Bălan et al., 2020; Bradu et al., 2025).

RESULTS AND DISCUSSIONS

Because of small sample sizes, no inferential statistical testing was performed. Results are presented descriptively as individual values and as group summaries (means ± standard deviation, medians, and ranges). All original numeric values from the study dataset are preserved in this report (Tables 2 and 3).

Table 2. Ejaculation latency and macroscopic semen characteristics in Group 1 (live female stimuli)

Dog	Interval (min: s)	Volume (ml)	pH	Color	Smell
R1	3:37	7.1	6.4	milky-white	odorless
B1	2:52	2.3	6.2	milky-white	odorless
D1	2:59	2.4	6.3	milky-white	odorless

R1 - Rottweiler,
B1 - Boston Terrier
D1 - re-haired Dachshund

Table 3. Ejaculation latency and macroscopic semen characteristics in Group 2 (Pheromone stimuli)

Dog	Interval (min: s)	Volume (ml)	pH	Color	Smell
R2	4:18	7.3	6.4	milky-white	odorless
F2	5:02	2.2	6.9	milky-white	odorless
D2	4:35	2.6	6.4	milky-white	odorless

R2 - Rottweiler,
F2 - French Bulldog
D2 - Wire-haired Dachshund

Group descriptive summaries Ejaculation latency (converted to seconds)

- Group 1 (n = 3): Mean 185 s (3:05), SD 27 s; median 179 s (2:59); range 172-217 s (2:52-3:37).
- Group 2 (n = 3): Mean 277 s (4:37), SD 35 s; median 275 s (4:35); range 258-302 s (4:18-5:02).

Ejaculate volume

- Group 1: Mean 3.93 mL, SD 2.70; median 2.4 mL; range 2.3-7.1 mL.
- Group 2: Mean 4.03 mL, SD 2.85; median 2.6 mL; range 2.2-7.3 mL.

Semen pH

- Group 1: Mean 6.3, SD 0.1; median 6.3; range 6.2-6.4.
- Group 2: Mean 6.57, SD 0.35; median 6.4; range 6.4-6.9.

Macroscopic appearance: all ejaculates were described as milky-white and odorless in both groups; no gross discoloration, hemato-spermia, or malodor suggestive of contamination or infection was observed.

Discussion: summary of findings In this small pilot series, manual semen collection was successful under both stimulatory conditions:

live estrous females and isolated estrous pheromones. Ejaculation latency was consistently shorter when a live female was present (median 2:59) than when only pheromonal cues were used (median 4:35). Despite the latency difference, pH, and macroscopic characteristics (ejaculate volumes, color, odor) were broadly similar between groups.

Longer ejaculation latency during pheromone-only stimulation is plausibly explained by the reduced multisensory input: live females provide visual and behavioral cues (e.g., lordosis-like behaviors, attention-seeking) and potential tactile stimulation that may accelerate arousal and ejaculation (Robertson & Martin, 2022).

Olfactory cues alone may be sufficient to elicit sexual behavior and ejaculation in many males but may require more time or higher stimulus concentration to achieve equivalent arousal levels. Woszczyło et al. (2023) observed hormonal changes associated with female presence during manual collection, demonstrating that multisensory exposure can alter endocrine markers (cortisol, oxytocin, prolactin, testosterone) and potentially influence sexual behavior and semen parameters. Our descriptive results align with the idea that male arousal dynamics differ between full multisensory stimulation and olfactory-only stimulation, but do not provide data on endocrine correlates or objective sperm function. The similarity in ejaculate volumes between groups suggests that once ejaculation occurs, the quantitative output may not be markedly affected by the presence versus absence of a live female - at least in sexually experienced males under low-stress conditions. However, ejaculate volume alone is an incomplete indicator of semen quality: concentration and progressive motility are critical to fertility outcomes (Concannon, 2011; Luvoni, 2013). Standardized semen collection and evaluation methods - including careful manual collection techniques, objective motility assessment (preferably by CASA), and morphology and viability staining - are necessary to assess reproductive potential accurately (Ainsworth & Linde-Forsberg, 2009; Johnson & Johnston, 2018). The absence of motility, morphology, concentration, and viability data in the present study is a major limitation for interpreting reproductive utility.

Strengths and limitations Strengths of the study include standardized handling in familiar environments, use of progesterone-confirmed estrous females for pheromone sampling, and preservation of original numeric data for transparent descriptive reporting. The study also addresses a practical question of interest to clinicians and breeders: whether pheromone-only stimulation can provide a lower-contact alternative for collecting ejaculates.

Limitations are substantial: the small sample size (n = 6 total, 3 per group) and parallel-group design prevent separation of individual- or breed-related effects from treatment effects. Each male was collected once, so repeatability and intra-individual variability were not assessed. The pheromone stimulus was derived from a single donor female; pheromone composition and concentration likely vary among females and across estrous stages, so generalizability is limited. Importantly, objective sperm-quality parameters and fertility outcomes were not measured; without concentration and motility data, it is not possible to infer semen suitability for artificial insemination or cryopreservation. Finally, because no inferential statistics were performed, the results should be interpreted as descriptive observations rather than evidence of equivalence or superiority of either method.

Practical implications and recommendations For practitioners and breeders, pheromone-based stimulation may be a feasible lower-contact option to obtain ejaculates from sexually experienced males when live females are unavailable or pose logistic/welfare/safety concerns. However, given current evidence, pheromone-assisted collection should be used cautiously and validated on a case-by-case basis—for example by performing paired collections comparing methods in a subset of males and by assessing critical semen quality metrics (concentration, motility, morphology) before adopting pheromone-only protocols for artificial insemination or cryopreservation. For researchers, we recommend future studies that:

- Employ larger, adequately powered cohorts across multiple breeds.
- Use crossover designs (each male exposed to both conditions in randomized order with washout) to control for individual variation.

- Standardize and quantify pheromone sampling and presentation (e.g., consistent pad mass, solvent extraction and GC-MS profiling, or pooled/synthetic pheromone preparations).
- Include comprehensive semen analyses (sperm concentration, CASA-motility parameters, morphology, viability, membrane and acrosome integrity).
- Measure behavioral and endocrine correlates (arousal scoring, cortisol, testosterone, oxytocin) and safety/welfare metrics.
- Assess repeatability across multiple collections per male and across pheromone donors.

CONCLUSIONS

This pilot descriptive study showed that pheromone-only stimulation can elicit ejaculation in sexually experienced male dogs and yield ejaculates with macroscopic similar characteristics (volume, pH, color, odor) to those obtained in the presence of a live estrous female, while ejaculation latency was longer with pheromone-only stimulation. These observations suggest potential practical utility for pheromone-based semen collection as a lower-contact alternative in certain settings. However, due to the study's small sample size, parallel-group design, single pheromone donor, and absence of objective sperm-quality and fertility endpoints, the results are preliminary and insufficient to support routine adoption of pheromone-only collection for assisted reproduction. Rigorous follow-up studies incorporating the design and measurement recommendations above are required to establish whether pheromone-based protocols can reliably replace or complement live-female stimulation in clinical and breeding practice.

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EFFICIENCY OF USING ESTRUS SYNCHRONIZATION PROTOCOLS IN DAIRY COWS

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Abstract

As well as estrus synchronization programs have the role of increasing fecundity at the farm level, our experimental study was carried out during 2024-2025, on a herd of 111 dairy cows, with the aim of testing the efficiency of these protocols. Four groups were formed: group A consisting of 31 cows synchronized with OvSynch; group B consisting of 35 heads synchronized with OvSynch+PRID; group C consisting of 24 cows synchronized with d-cloprostenol and group D consisting of 21 cows inseminated on a spontaneous estrus cycle. The fecundity index, the services per conception rate, the calving to first services interval and the service period was monitored. Gestation control was performed with a portable ultrasound device, 30 days after insemination. The obtained results showed that the percentage of pregnancies was +8.61% in group A; +9.53% in group B; +16.67% in group C compared to group D. The analysis calving to first service interval shows that significant differences were recorded between groups of cows A - D ($p<0.05$; $p=0.03866$). Our findings support the implementation of hormone synchronization protocols to improve reproductive management in dairy herds.

Key words: *conception rate, dairy cows, OvSynch, reproductive performance.*

INTRODUCTION

The use of hormonal programs for estrus synchronization aims to improve reproductive performance in dairy cows (Strickland et al., 2024). Knowledge of how the endocrine system works has allowed the development of hormonal therapies for estrus synchronization. Currently, the use of these programs is a common practice (Macmillan, 2010). There are several estrus synchronization schemes, which are applied following a gynecological examination to establish the individual treatment/cow type.

The objectives of this study were to investigate the effectiveness of using hormonal synchronization programs in dairy cows in stimulating ovarian activity, their influence on twin pregnancies and abortions, in shortening the calving - first insemination interval, the service period and in improving herd fertility rates

Hormonal estrus synchronization protocols are developed to address ovarian pathology, stimulate ovarian hormonal activity, trigger ovulation, and enable timed artificial insemination, with the aim of improving fertility rates on farms (Simoes et al., 2024).

The use of hormonal therapy to synchronize the estrus phase may represent the solution to combat infertility in dairy cows in a context where more and more farms are faced with a decrease in the percentage of fecundity, an increase in the number of services/gestation, an increase in the open days period, and the calving interval in cows inseminated on a spontaneous heat cycle.

MATERIALS AND METHODS

This study was carried out during 2024-2025, in the Experimental Zootechnical Laboratory of the Research and Development Institute for Bovine Balotești, on a herd of 111 dairy cows from the Romanian Black Spotted breed owned by the Institute. The study followed the efficiency of estrus synchronization protocols in dairy cows.

The Experimental Zootechnical Laboratory is a medium-sized farm that had a herd of approximately 100 dairy cows during the experiment. The cows included in the research were selected based on several criteria: milk production in the first hundred postpartum days greater than 2500 kg, body condition score between 2.25 and 3.25 and the age of the animals to be no more than 3 lactations. The ovarian diagnosis was established by gynecological examination performed using an ultrasound device, by transrectal exploration. Based on the diagnosis, the group composition and hormonal treatment for estrus synchronization were determined.

Thus, the 111 cows were divided into four groups, namely: group A consisted of 31 cows diagnosed with follicular cysts and 'lazy' ovaries. These were synchronized through the OvSynch program. Group B, consisting of 35 cows diagnosed with ovarian hypotrophy and ovarian cysts, was synchronized through the OvSynch+PRID protocol. Group C, consisting of 24 cows diagnosed with persistent corpus luteum and luteal cysts, was synchronized with a synthetic analogue of prostaglandin Pg_2alpha called d-cloprostenol. Group D, consisting of 21 cows, was inseminated on a spontaneous heat cycle. The insemination of the cows was performed with conventional semen.

The OvSynch synchronization protocol consisted of administering 2.5 ml of gonadotropin-releasing hormone (Gn-RH) (Receptal®, MSD Animal Health, USA), injectable, intramuscular, on day 0. On day 7, 2 ml of d-cloprostenol (Sincrovall®, Mevet S.A.U., Spain), injectable, intramuscular, were administered, on day 9, 2.5 ml of Gn-RH, injectable, intramuscular, were administered and 16 hours after the last administration, insemination was performed.

The OvSynch+PRID protocol consisted of administering 2.5 ml of Gn-RH (Receptal®, MSD Animal Health, USA) intramuscularly on day 0 and inserting a progesterone (PRID Delta®, Ceva, France) device, containing 1.55 g into the vaginal cavity. On day 7, the PRID device was removed, and 2 ml of d-cloprostenol was administered intramuscularly. On day 9, 2.5 ml of Gn-RH was administered, and insemination was performed 16 hours after the last administration.

The synchronization protocol with the synthetic prostaglandin analogue $PgF_{2\alpha}$ (d-cloprostenol) consisted of administering 2 ml of, injectable, intramuscularly on day 0, and after 72 hours, the cows that responded to the treatment were inseminated.

Insemination on a spontaneous heat cycle was performed by monitoring the cows using the reproduction calendar, and those detected in estrus were inseminated 12 hours after the first signs of heat were triggered.

The efficiency of estrus synchronization protocols was interpreted using reproductive indicators. Thus, the fecundity index, number of services per conception (NSC), interval between calving and first insemination, service period, number of twin pregnancies, and abortion rate were analyzed. Gestation control was performed with a portable ultrasound device 30 days after insemination, and the re-control was performed 40 days after the diagnosis of gestation

The statistical analysis was performed using Microsoft Excel. The mean (X), standard deviation (SD) and coefficient of variability (CV%) were calculated for the 4 analyzed groups and significance tests of the F-test and CHITEST differences were performed. These were used to analyze and interpret the differences between the groups for each reproduction indicator. Thus, with the F-test, the significance differences were interpreted for the reproduction indicators the interval from calving to the first insemination, the service period and the number of services per conception, and with the CHITEST test, the differences between the results obtained and those expected for the reproduction indicators fecundity and number of services per conception were calculated. The differences were considered statistically significant at $p < 0.05$ and extremely significant at $p < 0.01$.

The sample size (n = 111 cows) was considered adequate for detecting biologically relevant differences in reproductive parameters between groups, based on similar studies reported in the literature

RESULTS AND DISCUSSIONS

Estrus synchronization programs in dairy cows aim to increase farm profitability by controlling ovarian pathology and improving reproductive

performance. They trigger the luteal phase (Gn-RH, progesterone), corpus luteum regression, progesterone reduction, follicular maturation ($PgF_{2\alpha}$), ovulation induction and artificial insemination at a fixed time (Gn-RH).

From Figure 1 it can be seen that group B was the most numerous. It was 12.9% larger than group A, 45.83% larger than group C and 66.66% larger than group D.

The analysis shows that group B recorded the best results among the three groups of cows diagnosed in the estrus phase and synchronized with different hormonal protocols. In this group, all cows were diagnosed with heat and inseminated. The percentage of cows inseminated in this group was +6.46% higher than that recorded in group A and +12.5% higher than the number of cows inseminated in group C. Similar results were recorded by other authors when detecting cows in the estrus phase following hormonal heat synchronization protocols (Mohammed et al., 2023)

The analysis of the fecundity index shows that the highest fecundity percentage, 50%, was obtained in group C. The result obtained is comparable to those presented in the specialized literature which reports a percentage of pregnancies between 40 and 65% in cows synchronized with a single injection of d-cloprostenol (Aregaw et al., 2024; Mohammed et al., 2021; Sharew et al., 2023). The second-best performance was recorded in group B, of 42.86%. The result obtained is comparable to that recorded by other authors, who reported a fecundity percentage in cows synchronized with OvSynch +PRID of up to 40% (Hölper et al., 2023). In group A, the fecundity percentage was 41.94%. The percentage of pregnancies obtained in this group is comparable to the results reported by other authors (Berean et al., 2025). The lowest fecundity percentage, 33.33%, was recorded in group D. This score is comparable to results reported in the literature for cows inseminated during a spontaneous heat cycle (Tippenhauer et al., 2023). This score is equivalent to poor fecundity.

From the mathematical analysis it is found that in groups synchronized by hormonal therapy the percentage of pregnancies obtained was higher than in cows inseminated on a spontaneous heat cycle as follows: in group C there were 16.67% more pregnancies recorded than in group D; in group B the percentage of fecundity was 9.53% higher than that recorded in group D and in group A the percentage of pregnancies obtained was 8.61% higher than that recorded in group D. Similar results were reported by other authors who found better fecundity in synchronous cows compared to those inseminated on a spontaneous heat cycle (Santos et al., 2017).

Figure 2 shows the relationship between the fecundity index, the percentage of twin pregnancies obtained and the percentage of abortions recorded in the 4 analyzed groups.

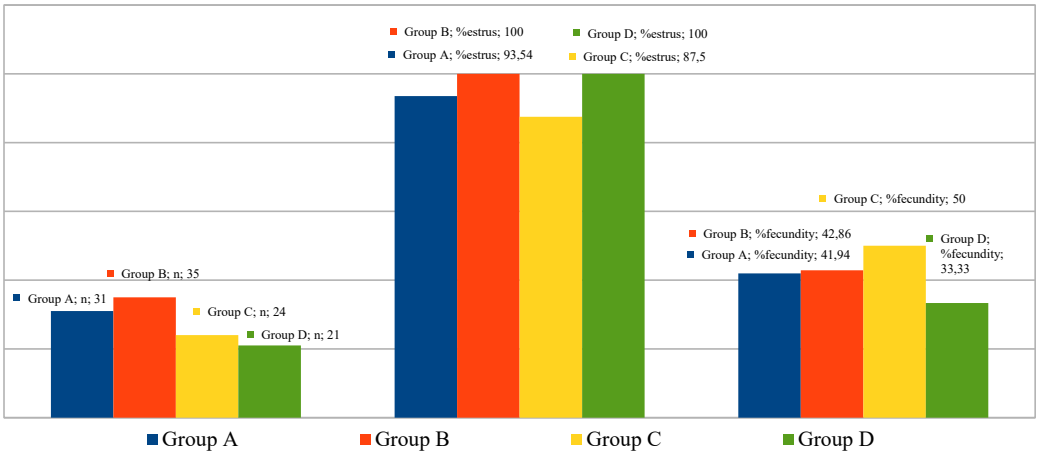


Figure 1. Distribution of cows from the 4 groups analyzed in relation to the fecundity index

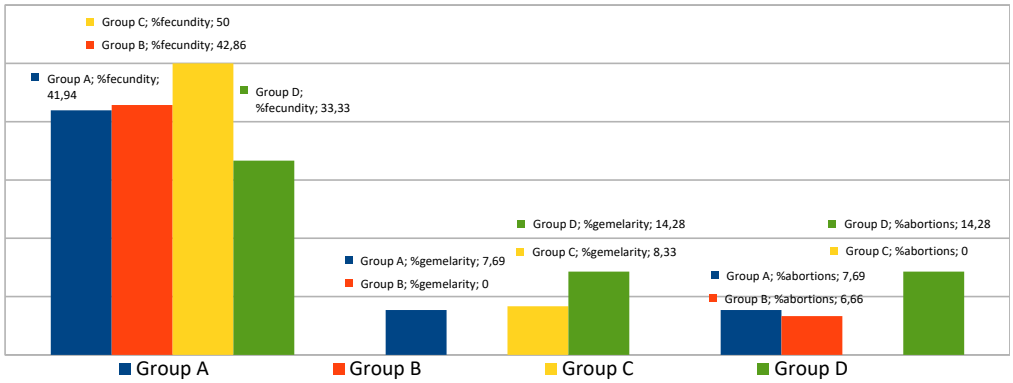


Figure 2. Analysis of the relationship between the fertility index, the percentage of twin pregnancies, and the number of abortions in cows from the 4 analyzed groups

The analysis of the number of cows that were diagnosed with twin pregnancies is justified, especially in groups of cows that were synchronized by hormonal therapy. Numerous authors have found that synchronization of cows with the help of hormonal treatments led to obtaining a higher number of twin pregnancies (Vazquez et al., 2012; Gatius et al., 2023; Arciniega et al., 2025;)

From Figure 2, it can be seen that the lowest percentages of twin pregnancies were recorded in groups of cows synchronized by hormonal treatments and the highest percentage of twin pregnancies was diagnosed in group D. The data obtained show that the use of synchronization programs in dairy farms does not influence the occurrence of twin calving, which is most often unwanted. The higher percentage of twin pregnancies recorded in group D is due to chance. Similar results were recorded by other authors who reported higher percentages of twin pregnancies obtained in cows inseminated on a spontaneous heat cycle compared to cows synchronized by hormonal treatments (Gatius et al., 2020).

The analysis of the incidence of abortions in early gestation (period 45-90 days of gestation) was performed because in the specialized literature, this interval is presented as a critical one with an incidence between 8% and 12% of the abortion rate (Pohler, 2020; Albaaj et al., 2022).

The analysis of embryonic mortality in the 4 groups shows that in groups A, B and C the percentage of abortions was between 0% and 7.69%. The highest percentage of abortions, 14.28%, was recorded in group D. The results obtained show that the incidence of abortions in groups A, B and C was lower compared to that reported by Pohler in 2020 and by Albaaj et al. in 2022 and higher in group D. This indicates that the use of hormonal therapy for estrus synchronization in dairy farms decreases the abortion rate by a percentage between 6.59% and 14.28%. The results recorded are similar to those presented by other authors who found that the use of hormonal therapy for estrus synchronization in dairy cows decreased the incidence of abortions (Madureira et al., 2019).

Fecundity is the ability of a cow to remain pregnant over a given period of time. It is interpreted using statistical and reproductive indicators and thus determines the economic efficiency of the herd. It is influenced by a number of factors such as milk production, energy balance and body condition score (Furnaris & Constantin, 2024).

The specialized literature estimates that fecundity in dairy farms is satisfactory when 50% of pregnant cows in the herd are diagnosed and a maximum of two services per conception are performed to obtain a pregnancy (Hutu, 2019).

Table 1. Analysis of cow fecundity in relation to the number of services per conception

Statistical indicators	Group A	Group B	Group C	Group D
n	31	35	24	21
Pregnant cows	13	15	12	7
% fecundity	41.94	42.86	50	33.33
Number of services per conception (NSC)	2.38	2.33	2	3
Comparative analysis of the significance of differences between group A vs. D				0.83282
Comparative analysis of the significance of differences between group B vs. D				0.81797
Comparative analysis of the significance of differences between group C vs. D				0.71751
Analysis of the significance of the differences between the obtained and expected fecundity percentage in group A (chitest)				0.20454
Analysis of the significance of the differences between the obtained and expected fecundity percentage in group B (chitest)				0.266210
Analysis of the significance of the differences between the obtained and expected fecundity percentage in group C (chitest)				1
Analysis of the significance of the differences between the obtained and expected fecundity percentage in group D (chitest)				0.003230

From the data analysis, it is found that in the groups of cows synchronized, by hormonal therapy the fecundity obtained was higher and the NSC was lower compared to the group inseminated by spontaneous heat cycle. This is justified by the fact that by using hormonal programs, the optimal moment of insemination of cows is identified by monitoring progesterone (P4) in milk and blood plasma. In dairy cows, the onset of the luteal phase is represented by the increase in the concentration of progesterone in milk from <5 ng/mL to values > 5 ng/mL, and the luteal phase lasts as long as the concentration of P4 is >5 ng/m. The determination of the moment of ovulation is deduced both by the decrease in the progesterone value and by transrectal ultrasound examination (Constantin et al., 2024). Thus in group C 1 (one) less insemination was performed to obtain a pregnancy than in group D (50%, 2 NSC vs. 33.33%, 3 NSC), in group A 0.62 fewer services per conception were performed compared to group D (41.94%, 2.38 NSC vs. 33.33%, 3 NSC) and in group B 0.67 fewer services per conception were performed compared to group D (42.86%, 2.33 NSC vs. 33.33%, 3 NSC).

The comparative analysis of significance of the FTEST differences showed that there were no significant differences between the hormonally synchronized groups and the one inseminated on a spontaneous heat cycle. Thus, between groups A vs D p=0.83285; p>0.05; B vs D p=0.81797, p>0.05; C vs D p=0.71751, p>0.05. Similar results have been recorded by other authors (Valenza & Bach, 2025).

The analysis of the CHITEST test shows that in the groups of cows A, B and C there were no significant differences between the results obtained and the expected ones. The performance threshold was chosen to achieved a fecundity rate of 50% with a maximum consumption of 2 services per conception. This threshold was chosen because it coincides with a good fecundity. Thus in group A p=0.20454, p>0.05; in group B p= 0.266210, p>0.05; in group C p=1, p>0.05. Distinctly significant differences were recorded in group D, with p=0.003230 (p<0.01), indicating that the results obtained in this group differ substantially from the expected ones, leading to economic losses.

Table 2 presents the analysis of the calving-first insemination indicator. This indicator is crucial because it influences farm management. In cows, the puerperal period lasts an average of 42 days. Once uterine involution has ended, the cow can be inseminated in an attempt to obtain a new pregnancy. Most studies indicate that during this heat cycle, the chances of becoming pregnant are low. The optimal insemination period is between 60 and 90 days from calving (Stangafero et al., 2018). The longer the calving-first insemination interval, the greater the economic losses.

Table 2. Index analysis, calving - first insemination in cows from the four analyzed groups

Statistical Indicators	Group A	Group B	Group C	Group D
n	31	35	24	21
X ± SD	85.6±18.03	91.48±19.47	90.04±17.62	104.1±27.72
CV%	21.05	21.28	19.57	26.63
Comparative analysis of significance of differences between group A vs. D				0.03227
Comparative analysis of significance of differences between group B vs. D				0.06771
Comparative analysis of significance of differences between group C vs. D				0.03868

From Table 2 it is found that the longest anestrus period was recorded in group D, 104.1 days. In this group, the calving-first insemination period was + 18.5 days longer than in group A, + 12.62 days longer than in group B, and + 14.06 days longer than in group C. The data show that, on dairy farms, the use of hormonal therapy for heat synchronization is indicated because these methods shorten the interval between first insemination and calving. Similar results showing that the use of estrus phase synchronization protocols shortens the calving-first insemination interval were reported by other authors (Fricke & Wiltbank, 2022). Recent research shows that monitoring hormones involved in ante and postpartum reproductive activity can influence the reduction of time intervals and improve pregnancy rates (Constantin et al., 2023)

This is to the farmer's advantage because it reduces the open days period.

The standard deviation analysis indicates that the greatest dispersion was observed in group D. In this group, the deviation from the mean was 27.72 days, indicating that some cows experienced a very long anestrus period due to various factors, such as high milk production, ovarian pathology, absent heats, and farmer's inattention. In groups A, B and C, the deviation from the mean was lower (between 17.62 and 19.47 days), which shows a greater homogeneity of the groups due to the use of hormonal therapy to synchronize the estrous phase. The result obtained is comparable to those reported by other authors, who found a smaller variation in cows synchronized with hormone therapy (Mekonnen et al., 2023).

From the analysis of the coefficient of variability (CV %) it is found that the highest dispersion was recorded in group D, 26.63%. In groups A, B and C the coefficient of variability was between 19.57% and 21.28%. This shows that the use of hormonal treatments for synchronizing heat in dairy cows leads to combating endogenous and exogenous factors and helps to improve reproduction indicators. Other authors (Carvalho et al., 2014) also found reduced variability in groups of cows synchronized by hormonal therapy.

The analysis of the F-test of significance of the differences found that there were insignificant differences between groups B vs D where $p=0.06771$, $p>0.05$. Other authors have also reported no significant differences in the calving-first insemination interval between cows synchronized with hormonal therapy and those inseminated during spontaneous heat cycles (Belandria et al., 2023). Significant differences were recorded between groups A vs D $p=0.03227$, $p<0.05$; respectively, C vs D where $p= 0.03866$, $p<0.05$. Significant differences in the first postpartum insemination between cows synchronized with hormonal therapy and those inseminated during a spontaneous heat cycle have also been reported by other authors (Valenza & Bach, 2026). The differences recorded between synchronized groups A and C vs D show the efficiency of these therapies in shortening the calving period, first insemination, and in improving farm management.

Table 3 presents the analysis of the open days period in cows from the 4 analyzed groups. Open days represent the mobile component of the calving interval (Gheorghe-Irimia et al., 2023). Ideally, this should last approximately 80-90 days (Vidu et al., 2025). The longer the interval, the lower the farm's managerial efficiency.

Table 3. Analysis of the open days period in the four groups

Statistical indicators	Group A	Group B	Group C	Group D
n	13	15	12	7
X ± SD (zile)	90.79±9.15	98.13±15.16	96.54±10.32	113.25±20.32
CV%	10.08	15.45	10.7	17.95
Comparative analysis of significance of differences between group A vs. D				0.013023
Comparative analysis of significance of differences between group B vs. D				0.322367
Comparative analysis of significance of differences between group C vs. D				0.038918

Table 3 reveals that group D recorded the longest period of open days, with 113.25 days. In this group the duration of the service period was higher by 22.46 days than in group A, by 15.12 days than in group B, and by 16.71 days than in group C. The analyzed data show that the use of heat synchronization programs in dairy cows shortens the duration of open days and promotes economic growth on the farm. The results obtained are comparable to those reported by other authors who found that the use of estrus synchronization hormone therapy shortens the service period in dairy cows (Santos et al., 2016).

The standard deviation analysis indicates that the greatest dispersion from the mean was observed in group D, with a standard deviation of ±20.32 days. In addition, these open days to the average service period of 113.25 days indicate that some cows in this group had open days approximately 53 days longer than the optimal 80-day period. This increase leads to a decrease in their performance and can decisively influence the farmer's decision to reform the cows. The results obtained are similar to those reported by other authors, who found, in the open days analysis, that dispersion is higher in cows inseminated during a spontaneous heat cycle than in cows synchronized through hormonal protocols (Santos et al., 2016).

The analysis of variance shows that group D had the highest variability, 17.95%, of the 4 groups analyzed. The high variability within this group indicates that inseminating cows during spontaneous heat cycles increases the open days period.

This variability in the service period is consistent with findings in the specialized literature, which indicates that it differs depending on the insemination strategy used (Sitko et al., 2023)

CONCLUSIONS

The use of estrus synchronization programs in dairy cows led to an increase in fecundity by 8.61% -16.67%

The use of estrus synchronization programs in dairy cows does not favor an increase in the percentage of twin pregnancies.

The use of heat synchronization programs tends to reduce the incidence of abortions in early pregnancy by up to 6.59% -14.28%.

The use of heat synchronization programs can reduce the number of services per conception in dairy cows by 0.62 - 1 services per conception.

The use of estrus synchronization programs can help shorten the interval between calving and first insemination by 12.62 days - 18.5 days

The use of estrus synchronization programs can shorten the service period by 15.12 days - 22.45 days.

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EVALUATION OF *Rosa canina* AS A NATURAL SOURCE OF VITAMIN C: IMPACT OF THE FORM OF ADMINISTRATION ON ERYTHROPOIESIS AND RENAL FUNCTION IN ROSS 308 BROILER CHICKENS

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Abstract

This study evaluated the effect of Rosa canina (rosehip) as a phytogenic supplement on the haematological profile of Ross 308 broiler chickens raised at the university experimental farm between July and August 2025. The experiment included a control group and two treatment groups, which received Rosa canina aqueous infusion (G1), and Rosa canina extract (G2) respectively, both administered via drinking water. Haematological parameters, specifically haemoglobin and haematocrit, were monitored and recorded at 7, 14, 21, 28 and 35 days of age. Erythropoietic stimulation was consistently observed in the treated groups compared to the control group. At 14 days of age, G1 showed a marked attenuation of anaemia, with individual haemoglobin values reaching 14.7 g/dL, increased compared to the 10.2 g/dL value obtained from the control group. At 35 days, G2 exhibited a pronounced haematological response attributed to enhanced vitamin C intake. The results also indicate variability in physiological responses, highlighting the need for further research to optimise dosage and forms of administration.

Key words*: haematological indicators, phytotherapy, Rosa canina.*

INTRODUCTION

When the haematocrit value falls below the lower physiological limit, anaemia may be diagnosed, whereas values exceeding the upper reference limit are indicative of polycythaemia (Goodwin, 1992). In general, marginally subnormal or supranormal values reflect an underlying pathophysiological state. Dehydration is a recognised cause of elevated haemoglobin concentration, due to haemoconcentration rather than true erythrocytosis (Barde et al., 2022). In cases of haemoglobin deficiency, the blood's ability to transport oxygen is reduced and this condition must be distinguished from hypoxaemia, which refers specifically to a decrease in the partial pressure of oxygen in the blood. In avian species, an erythrocyte volume exceeding the upper physiological reference limit may indicate the presence of toxic factors disrupting haematopoietic processes (Ogunola et al., 2025). Early-onset physiological anaemia has been documented in young broiler chickens during the first weeks of life (Goodwin et al., 1992; Legroux et al., 2025). An elevated haematocrit (HCT) value, secondary to dehydration, is characteristic of relative polycythaemia, which reflects haemoconcentration rather than true erythrocytosis, whereas decreased HCT values are indicative of the oligocythemic form of anaemia. In the latter condition, the underlying aetiology is most commonly attributable to impaired erythropoiesis within the haematopoietic bone marrow (HBM) (de Oliveira Santana et al., 2025). Of particular relevance in poultry production is the observation that housing facilities characterised by inadequate ventilation and prolonged exposure to elevated ammonia concentrations may overwhelm the physiological compensatory mechanisms, ultimately resulting in hypoxaemia (Barbosa et al., 2024). The ensuing tissue hypoxia triggers a compensatory erythropoiesis response, manifested as an increase in circulating erythrocyte mass. Furthermore, mast cell degranulation occurring under hypoxic conditions results in the local release of histamine which, through its vasoactive properties, initiates and sustains the

inflammatory cascade (Steiner et al., 2003; Nagamine et al., 2024). The subsequent vasodilatation and increased vascular permeability lead to vascular stasis, progressive elevation of blood viscosity, and consequent haemodynamic alterations. The aim of the present study was to investigate the erythropoietic and nephroprotective effects of *Rosa canina*, assessing two forms of administration, aqueous infusion and concentrated extract, in Ross 308 broiler chickens under intensive production conditions.

MATERIALS AND METHODS

A total of 128 Ross 308 hybrid broiler chickens of both sexes were experimentally reared at the experimental farm. All birds received primary vaccination against Newcastle disease (ND) and Infectious Bursal Disease (IBD or Gumboro disease) on day 1 post-hatch, within the hatchery. As part of the study, a repeat dose of the vaccine was administered on days 10 and 21, via ocular and intranasal instillation, consisting of one drop applied to each eye and each nasal cavity per individual. The lyophilised vaccine administered was Avipestisota (Romvac Company, Romania). All experimental procedures were performed in full compliance with the bioethical standards approved under registration number 839/21.10.2024.

In order to minimise physiological stress and maintain the stability of the intestinal microbiome, the same feed brand supplied by the hatchery of origin was used throughout the transition period.

The feed was sourced from a certified manufacturer, ensuring rigorous microbiological quality control. This precaution was considered essential in the local context, as long-term studies have demonstrated that certain raw materials, particularly grains received at feed mills in Romania, may be contaminated with mycotoxigenic fungi (Lăpușneanu et al., 2025). By eliminating the risk of mycotoxin exposure, renal and hepatic integrity was preserved, allowing for an accurate assessment of the effects of *Rosa canina* supplementation on the erythropoietic and excretory systems. The birds were fed a three-phase complete compound feed produced by a local manufacturer in northeastern Romania. The dietary phases consisted of a starter (0-10 days), a grower (11-24 days) and a finisher (25 days to slaughter). The specific ingredient inclusion levels for each phase are detailed in Table 1. Feed and water were provided *ad libitum* throughout the experimental period. The feeding regimen was designed to meet the nutritional requirements of the birds at each growth stage.

Table 1. Ingredient composition of the complete compound feed used across dietary phases

Ingredient (%)	Starter (0-10 days)	Grower (11-24 days)	Finisher (25-35 days)
Corn	50.50	60.5	65.50
Soybean Meal	35.50	27	24.00
Soybean Oil	5.50	5	3.50
Fodder yeast	5.00	4	4.00
Minerals/Premix	4.00	3.5	3.00
Total	100	100	100
Kcal ME/kg	3000 - 3200		

ME - Metabolizable Energy.

Birds were randomly allocated into three experimental groups: a control group (GC) and two treatment groups (G1 and G2). The treatment groups received *Rosa canina* via the drinking water, a route of administration previously documented in broiler supplementation studies (Iqbal et al., 2015). The control group (GC) received unsupplemented drinking water throughout the experimental period. Birds assigned to Group G1 received an aqueous *Rosa canina* infusion prepared by steeping dried rosehip fruits in drinking water, whereas birds in Group G2 received commercial *Rosa canina* extract administered according to the same protocol. Supplementation in both treatment groups was initiated 3 – 5 days prior to blood sampling performed at 21 days of age, at minimum doses of 10 g of dried *Rosa canina* fruits per 5 L of drinking water for G1 and 0.25 mL concentrated extract per 5 L of drinking water for G2. Thereafter, the administered doses were progressively increased throughout the experimental period, reaching a maximum concentration of 50 g dried fruits per 5 L of drinking water in G1 and 2.25 mL extract per 5 L of drinking water in G2. Between supplementation periods, birds had *ad libitum* access to unsupplemented drinking water until all supplemented solution had been consumed. The dried rosehip fruits used in the study were obtained from wild mountain rosehips collected from the Brașov region, Romania.

The blood samples were collected from three randomly selected birds per experimental group (n = 3 per group) at 7, 14, 21, 28 and 35 days of age. At 35 days of age, a larger number of samples was obtained (n = 10 per group), as blood collection was performed at the time of slaughter, eliminating the need for additional live bird restraint. Blood samples were obtained prior to slaughter, from the metatarsal vein of the left limb of the bird positioned on its right side. The puncture site was aseptically prepared using cotton wool soaked in 70% ethanol, and venipuncture was performed under sterile conditions using a 2.5 mL syringe for younger birds and a 5 mL syringe for adult birds aged 5 weeks. The blood was transferred into 500µL microtubes containing K_3EDTA (tripotassium ethylenediaminetetraacetic acid) and homogenised by gentle inversion. Haematocrit (HCT) values were determined by standard microhaematocrit method, using heparinised capillary tubes (75 mm) centrifuged in a HAEMATOKRIT 210 centrifuge (Hettich Zentrifugen). Haemoglobin concentration was determined using the Sahli acid-haematin method. Briefly, three drops of 0.1% hydrochloric acid (HCl) were placed into the central graduated tube of the Sahli haemoglobinometer. Subsequently, the blood sample was aspirated into the pipette up to the marked reference line and carefully discharged into the acid-containing tube. The mixture was allowed to react for 1-2 minutes to ensure complete conversion of haemoglobin to acid haematin. Once the colour changed to brown, distilled water was added dropwise with continuous homogenisation until the resulting solution matched that of the permanent brown glass comparator standards. Haemoglobin concentration was read directly from the graduated scale of the haemoglobinometer and expressed in g/dL.

Statistical comparisons were performed using one-way ANOVA (Single Factor) in Microsoft Excel. For each sampling time point, independent pairwise comparisons were conducted between each treatment group and the control (G1 vs. GC and G2 vs. GC), as well as between the two treatment groups (G1 vs. G2). Data are presented as mean ± standard error of the mean (SEM). Differences were considered statistically significant at $p < 0.05$. Different superscript letters (a, b, c) indicate statistically significant differences between groups within the same sampling age.

RESULTS AND DISCUSSIONS

The reference ranges for haematocrit in broiler chickens according to age, are presented in Table 2.

Table 2. Reference for packed cell volume in young broiler chickens, by age

Age (days)	PCV (%)
3	26-42
7	29-43
14	33-41
21	32-40
27*	27-36
28**	33-42
35	33-42
42	31-41
49	28-40

PCV - Packed cell volume; *Goodwin (1992); **Legroux et al. (2025).

The erythrocyte volume in broiler chickens, as shown in Table 2, remains relatively stable throughout the growing period, ranging from 26-43% during the first week to 28-41% at slaughter age, which is consistent with the physiological maturation of the erythropoietic system and the high metabolic demands of meat-type hybrids (Goodwin, 1992; Meluzzi et al., 1992; Al-Nedawi, 2018; Kongpechr et al., 2020; Scanes and Dridi, 2022; Legroux et al., 2025). Furthermore, previous studies report a transient increase during the second and third weeks of life (33-42%), followed by a gradual decline until slaughter age, reflecting physiological adaptation to increased oxygen demand during fast muscle growth and relative haemodilution associated with increased plasma volume in maturing birds (Sturkie, 1986; Zálešáková et al., 2025). Majekodunmi et al. (2022) reported reference values applicable to this study of PCV: 31.67 ± 0.45% and HGB: 10.77 ± 0.15 g/dL. Additionally, Abdel-Rahman et al. (2022) specified relatively stable values of haemoglobin (HGB) of approximately 10.5 g/dL for 28 days of age. Table 3 presents the results of haematological analyses. Haemoglobin concentration (HGB) was used as the primary indicator of erythrocyte functional status, given the high precision of the analytical method and the strong physiological correlation

between haemoglobin and haematocrit documented in avian species. Statistically significant differences in HGB were observed among experimental groups ($p < 0.05$), reflecting treatment-induced changes in oxygen transport capacity. The haematocrit values exhibited a trend consistent with that of haemoglobin, further supporting the effect of the experimental treatment on the overall haematological profile. According to Ike et al. (2025), a haemoglobin value of 9.98 g/dL ± 1.10 g/dL was reported in Ross 308, in the context of treatment with plant extract (*Phyllanthus amarus* leaf extract).

Table 3. Haematocrit (HCT %) values in Ross 308 broiler chickens by age and experimental group

Age (days)	G1	GC	G2	Reference interval*
7	20	14	10	29-43
14	40	20	30	33-41
21	15	10-26	25	33-41
28	19	20	17	27-42
35	25	23	20	33-42

Values represent individual measurements. *Goodwin (1992) and Legroux et al. (2025).

The haematological profile of G1 is consistent with that of clinically healthy birds, with values within the reference range for the species. The reduced HGB and HCT (20%) values observed in individual birds may be indicative of anaemia, probably secondary to elevated uric acid and glucose concentrations associated with metabolic stress and renal dysfunction in avian species. These findings are consistent with a previous study conducted on the same hybrid under identical phytotherapeutic conditions, in which *Rosa canina* administration via drinking water was associated with preserved renal biochemical markers during the final growth stage, supporting the hypothesis that haematological alterations observed in the current study are not secondary to nephrotoxic impairment (Stătescu et al., 2025).

The low haemoglobin (6.4 g/dL) and haematocrit (20%) values recorded in individual birds from GC at 7 days may reflect the influence of persistently elevated blood glucose concentrations on erythropoietic activity. Hyperglycaemia is a defining physiological feature of avian metabolism, characterised by the predominance of glucagon-mediated regulation over insulin-dependent pathways, which sustains persistently elevated glycaemia relative to mammals. The physiological response to metabolic stress in birds involves compensatory neuroendocrine adaptation aimed at maintaining systemic glucose homeostasis, during which transient hypoglycaemic episodes may secondarily occur. Furthermore, low HCT and HGB values may be associated with vascular hypoxia and, consequently, compromised tissue oxygenation.

Table 4. Results of haematological analyses in 7-day-old broiler chickens

Parameter	G1	GC	G2
HGB (g/dL)	16.4	9.4	8.2
	14.8	6.4	8.9
	15.2	8.8	9.1
Mean ± SEM	15.46 ± 0.48^{a}	8.20 ± 0.92^{b}	8.73 ± 0.27^{b}
P-value	0.00216		0.60677

HGB – Haemoglobin. Values are expressed as mean ± SEM (n = 3). Different superscript letters (a, b) indicate statistically significant differences among groups ($p < 0.05$).

At 7 days of age, birds receiving the *Rosa canina* aqueous infusion (G1) exhibited a marked increase in haemoglobin concentration (15.46 g/dL) compared with the control group (8.2 g/dL; $p = 0.002$), confirming an early antianaemic response. No significant difference was observed between the extract group and the control ($p = 0.60$). A low HGB result (6.4 g/dL) in the control group at 7 days may be associated with reduced blood glucose concentrations, suggesting a possible relationship between transient hypoglycaemia and impaired erythropoietic activity, which warrants further investigation. Hyperuricaemia may develop through two distinct pathophysiological mechanisms: impaired renal excretion secondary to nephropathy or accelerated proteolytic catabolism, which drives enhanced purine nucleotide turnover and a consequent increase in endogenous uric acid synthesis. It is normal for blood uric acid fluctuations to occur depending on biological and circadian rhythms, as well as age and nutritional conditions, thus influencing haematological parameters. Physiological compensatory and adaptive mechanisms are predominantly activated and subjected to maximal functional demand during the correction and homeostatic regulation of alterations in blood parameters. Previous research conducted by Egbuniwe et al. (2018), Johnson et al. (2023) and Al-Khalaifah (2025) highlighted the supportive effect of vitamin C on

haematological parameters, particularly haemoglobin concentration, by enhancing iron absorption (conversion of Fe^{3+} to Fe^{2+}). Furthermore, the protective role of vitamin C is also evident at the metabolic level: while stress (such as heat stress) tends to increase lipaemia, vitamin C intake counteracts this effect by reducing total cholesterol and triglyceride levels (Erfani et al., 2021). In research conducted by Unigwe et al. (2022), no substantial changes in haematological parameters were identified using a dose of 100 mg/kg Vitamin C. The results align with reports by Hassan & Asim (2020), supporting the efficacy of higher doses. In our study, according to Table 3 and Table 5, the administration of rosehip infusion (G1) generated an increase in haemoglobin at 14 days (individual maximum values of 14.7 g/dL vs. 9.7 g/dL in the control group) and haematocrit (40% vs. 20%), demonstrating that the phytochemical constituents from *Rosa canina* provided a sufficient concentration of active compounds to stimulate erythropoiesis, overcoming the limitations observed in studies with low doses.

Haematological analysis of 14-day-old Ross 308 broiler chickens revealed a significant erythropoietic response in the supplemented groups. According to Table 5, both treated groups demonstrated statistically significant elevations in haemoglobin concentration relative to control. The extract group (G2) recorded a mean haemoglobin of 16.46 g/dL, exceeding the upper physiological reference limit (8.70-11.80 g/dL), indicating a stronger erythropoietic stimulation and enhanced iron utilisation. The health status of the birds was considered satisfactory at this age, as no clinical signs of disease or systemic pathology were observed in either treatment group prior to the commencement of *Rosa canina* supplementation.

The superior haematological parameters, specifically elevated HCT and HGB values observed exclusively in the *Rosa canina*-supplemented groups, compared to the lower values recorded in the control group, may be attributed to the synergistic action of the bioactive compounds inherent to *Rosa canina* fruit, particularly ascorbic acid (vitamin C) and glutathione-activating polyphenols (Peña et al., 2023). Ascorbic acid is a well-established cofactor in haem biosynthesis, facilitating the reduction of ferric iron (Fe^{3+}) to the absorbable ferrous form (Fe^{2+}), thereby enhancing intestinal iron bioavailability and supporting the activity of ferrochelatase, which is the final enzymatic step in haem biosynthesis pathway, responsible for incorporating ferrous iron into protoporphyrin IX (Obi et al., 2022; Feijo et al., 2023). Concurrently, the polyphenolic compounds present in *Rosa canina*, including flavonols and catechins, are known to upregulate endogenous glutathione synthesis, which in turn protects erythroid progenitors from oxidative stress during active erythropoiesis in the HBM, preserving erythrocyte membrane integrity and extending erythrocyte lifespan (de Oliveira Santana et al., 2025). The cumulative effect of these mechanisms is reflected in the statistically superior HCT and HGB values recorded in the treated groups, suggesting that *Rosa canina* supplementation promotes erythropoietic efficiency via dual mechanisms: improved iron utilisation and antioxidant-mediated protection of erythropoietic precursor cells within the haematopoietic bone marrow (Aldemir et al., 2021).

Table 5. Haemoglobin concentration in 14-day-old broiler chickens

Parameter	G1	GC	G2
HGB (g/dL)	14.7	9.7	17.2
	13.5	9.1	16.4
	14.1	10.2	15.8
Mean ± SEM	14.10 ± 0.35^{b}	9.66 ± 0.32^{c}	16.46 ± 0.41^{a}
P-value	0.00070		0.00019

HGB – Haemoglobin; Values are expressed as mean ± SEM (n = 3). Different superscript letters (a, b, c) indicate statistically significant differences among groups ($p < 0.05$).

According to Table 6, no statistically significant differences in haemoglobin concentration were observed among experimental groups. These results likely reflect physiological haemodilution due to increased plasma volume during rapid growth, rather than suppression of erythropoiesis (Sitmo et al., 2023). The reduced haematocrit values recorded at this age are consistent with physiological haemodilution secondary to plasma volume expansion, rather than a primary haematological pathology.

The results presented in Tables 3 and 6 suggest that the botanical additive may have induced haemodilution with a consequent reduction in haematocrit, potentially attributable to an

increase in circulating plasma volume. Furthermore, *Rosa canina* contains not only ascorbic acid but also polyphenols and tannins, which are known to chelate non-haem iron and may thereby inhibit intestinal iron absorption, potentially contributing to the development of iron deficiency anaemia in broiler chickens.

Table 6. Results of haematological analysis at 21 days of age

Parameter	HGB (g/dL)		
	G1	GC	G2
Mean ± SEM	11.50 ± 1.88[a]	11.03 ± 0.12[a]	9.26 ± 0.81[a]
P-value[*]	0.81677		0.09746

HGB – Haemoglobin; Values are expressed as mean ± SEM (n = 3); [*]Not significant ($p > 0.05$)

According to Table 7, a modest but statistically significant improvement in haemoglobin concentration was observed in G1, whilst G2 showed no significant difference from GC. This phase may coincide with the onset of oxidative stress associated with accelerated metabolic activity, suggesting that the antioxidant compounds in the infusion provided partial protection to erythrocytes (Fathi et al., 2025).

Table 7. Haemoglobin concentration in 28-day-old broiler chickens

Parameter	HGB (g/dL)		
	G1	GC	G2
Mean ± SEM	9.80 ± 0.17[a]	8.16 ± 0.43[b]	8.23 ± 0.35[b]
P-value	0.02489		0.91030

HGB – Haemoglobin; Values are expressed as mean ± SEM (n = 3). Different superscript letters (a, b) indicate statistically significant differences among groups ($p < 0.05$).

Table 8. Mean haemoglobin concentration (g/dL) between G1 and G2 at selected time points

Age (days)	G1 Mean ± SEM	G2 Mean ± SEM	*P – value*	Significance
21	11.50 ± 1.88	9.26 ± 0.81	0.33713	NS
28	9.80 ± 0.17	8.23 ± 0.35	**0.01573**	*
35	9.03 ± 0.20	8.86 ± 0.20	0.59225	NS

Values are expressed as mean ± SEM (n = 3); [*]Differences were considered significant at $p < 0.05$; NS – not significant.

Haemoglobin represents the principal carrier of oxygen in the bloodstream and serves as an indicator of tissue oxygenation capacity. The relationship between HGB, HCT, and erythrocyte count reflects the metabolic and nutritional status of the organism. Reduced iron bioavailability contributes to impaired haemoglobin synthesis. Under conditions of haemodilution associated with a rosehip-based supplementation, erythropoiesis may be disrupted. Additionally, under the oxidative stress conditions evident at 28 days of age, mild haemolysis may occur as a consequence of reduced erythrocyte lifespan. The lowest haemoglobin and haematocrit values were recorded in GC and G2, consistent with this proposed mechanism.

The pairwise comparison of haemoglobin concentration between treatment groups, presented in Table 8, reveals a stage-dependent divergence in erythropoietic response. At 21 days, no significant difference was detected between G1 and G2, a result likely influenced by the high individual variability observed in G1, consistent with physiological haemodilution secondary to plasma volume expansion. However, at 28 days, G1 recorded a significantly higher mean haemoglobin concentration compared to G2 (9.80 ± 0.17 g/dL vs. 8.23 ± 0.35 g/dL), a finding that may be partially attributable to the nephroprotective properties of *Rosa canina* aqueous infusion. In avian species, impaired renal tubular excretion of uric acid leads to hyperuricaemia, which suppresses erythropoietin synthesis and consequently reduces erythropoietic activity within the haematopoietic bone marrow (Stătescu et al., 2025). The ascorbic acid and polyphenolic constituents present in the infusion may attenuate this mechanism by reducing oxidative renal tubular injury, thereby preserving erythropoietin-mediated stimulation of erythropoiesis. By 35 days, no significant difference had been observed between groups, suggesting a progressive convergence of haematological response as birds approached slaughter age.

Table 9. Haemoglobin concentrations in broiler chickens at 35 days of age

Parameter	G1	GC	G2
Mean ± SEM	9.03 ± 0.20[a]	8.70 ± 0.26[a]	8.86 ± 0.20[a]
P-value[*]	0.37390		0.64333

Values are expressed as mean ± SEM (n = 3). [*]Not significant ($p > 0.05$).

According to the results presented in Tables 9 and 10, although the birds in G1 received additional vitamin C through rosehip tea, a substantial proportion of individuals recorded haemoglobin values below the lower physiological limit. This suggests that the administered dose of ascorbic acid, or the

bioavailable concentration of active phytochemical constituents in the infusion, was insufficient to fully counteract physiological growth-related anaemia across all individuals. At 35 days, although no statistically significant differences were detected ($p > 0.05$), the maximum haemoglobin values observed in the G2 (up to 13.3 g/dL) exceeded the upper physiological limit (11.8 g/dL). This finding indicates a sustained erythropoietic response driven by the higher bioavailability of ascorbic acid found in the extract form.

Table 10. Descriptive statistics of the minimum haemoglobin concentration in treatment groups at age of 35 days

Group	Results Mean ± SEM	Interval for HGB (g/dL)	Reference interval
G1	7.96 ± 0.10	7.0 – 8.6	8.70 – 11.80
G2	8.24 ± 0.14	7.4 – 9.3	

HGB – Haemoglobin; Values are expressed as mean ± SEM (n = 10).

Table 11. Descriptive statistics of the maximum haemoglobin concentration in treatment groups at age of 35 days

Group	Mean ± SEM	Interval for HGB (g/dL)	Reference interval
G1	9.68 ± 0.09	9.0 – 10.5	8.70 – 11.80
G2	11.40 ± 0.25	9.3 – 13.3	

HGB – Haemoglobin; Values are expressed as mean ± SEM (n = 10).

The data presented in Table 11 reveal that the majority of haemoglobin values fell within the physiological reference range. However, a peak value of 13.3 g/dL was recorded in G2, which exceeded the upper reference limit of 11.8 g/dL. This compensation indicates a higher bioavailability of iron, mediated by the elevated ascorbic acid concentration present in the extract form. The study conducted by Hieu et al. (2022) confirmed that antioxidant supplementation, particularly with vitamin C, increases haemoglobin concentration by facilitating the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) in the duodenum. A decrease in Fe^{2+} availability represents a disturbance in the balance between the erythrocyte production and destruction, frequently associated with gastrointestinal disorders and impaired enteral absorption.

In contrast to the aqueous infusion group (G1), in which haemoglobin concentrations generally remained below the physiological threshold of 8.70 g/dL, indicating a tendency toward anaemia, the extract group (G2) showed a stimulation of erythropoiesis, with maximum values reaching 13.3 g/dL (Table 11). These results are consistent with those of Hussain and Al-Salhie (2022), who reported that supplementation with 100 mg/L of vitamin C in drinking water significantly improved haematological parameters, including erythrocyte count, haemoglobin concentration, and packed cell volume or haematocrit, by protecting erythrocyte membrane integrity against oxidative stress and damages (Opoola et al., 2018). The differential response between G1 and G2 underscores the importance of achieving an optimal phytochemical concentration and appropriate dosage to elicit a clinically meaningful haematological response (Gabriel et al., 2025). *Rosa canina* supplementation was associated with changes in haemoglobin concentration and significantly influenced erythropoietic activity in Ross 308 broiler chickens during the early and mid-growth stages. At 7 and 14 days of age, both treatments elicited significant increases in haemoglobin concentration ($p < 0.05$). No significant differences were recorded at 21 and 35 days ($p > 0.05$), although a modest but statistically significant improvement in HGB persisted in G1 at 28 days ($p = 0.025$), suggesting a progressive attenuation of the erythropoietic response as birds approached slaughter age. The transient reduction in haematocrit and haemoglobin at 21 and 28 days is consistent with physiological haemodilution associated with rapid somatic growth, rather than pathological anaemia. These results indicate that *Rosa canina*, as a natural source of vitamin C and antioxidant polyphenols, may effectively support erythropoiesis and maintain haematological balance in fast-growing broilers, representing a viable phytogenic alternative to synthetic ascorbic acid supplementation under intensive production conditions (Urban et al., 2025).

CONCLUSIONS

Rosa canina supplementation, administered as aqueous infusion or concentrated extract via drinking water, exerted a significant and stage-dependent erythropoietic effect in Ross 308 broiler chickens. The aqueous infusion (G1)

demonstrated superior efficacy in preventing physiological anaemia during the first weeks of life, whilst the concentrated extract (G2) elicited a more pronounced erythropoietic response at later growth stages (35 days). A modest but statistically significant improvement in haemoglobin concentration persisted in G1 at 28 days ($p = 0.025$), further supporting the sustained antianaemic potential of the aqueous infusion. The transient haematological decline observed at 21 days was consistent with physiological haemodilution rather than treatment-induced impairment. The preservation of renal integrity throughout the experimental period confirmed that the haematological findings were not confounded by nephrotoxic impairment.

Overall, *Rosa canina* supplementation demonstrated a dose- and stage-dependent erythropoietic effect, supporting its potential role as a natural source of vitamin C with antioxidant capacity in attenuating early post-hatch anaemia and enhancing the haematological status of broilers under intensive production conditions. Further studies are warranted to elucidate the optimal dosage, administration protocol and long-term effects on productive performance and carcass quality.

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RESEARCH ON THE INFLUENCE OF TECHNOLOGICAL FACTORS ON PRODUCTIVE PERFORMANCE IN A BROWN CATTLE HERD

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Abstract

This paper aims to highlight the influence of exploiting technology on productive performance in a dairy herd belonging to the Brown breed. The herd for which the study was carried out consisted of 48 head of cows in different lactations and which are exploited for semi-intensive milk production on a farm in NV Moldova, Romania. The data on milk production and quality for 2024 were obtained from records drawn up during the official control of the productions and those existing on the farm and were statistically processed using the software SAVC and SPSS 16.00 for WINDOWS. The average daily production was 20.03 kg of milk/head with 4.13% fat and 3.59% protein. The highest average production of 5,699.26 kg was achieved in the second lactation, when milk contained 3.83% fat and 3.42% protein. Analyzing the results obtained and comparing them with those characteristics of maternal ascendancy, we can conclude that, in the herd studied, milk is of good quality, but the animals do not externalize their genetic potential.

Key words: *Brown breed, milk, Romania.*

INTRODUCTION

Milk is a biological product synthesized by the mammary gland of mammals. In the case of taurines, its organoleptic properties are a white-yellowish color, a sweet taste, and a characteristic smell (Bosch et al., 2025). Of all foods of animal origin, milk is considered the most complete, easily assimilated by the body, and can be constituted as a basic food for human nutrition, especially during the period of growth and development of the body (Guzun, 2001).

Milk is a complete product and has a high biological value because its composition includes all essential amino acids and more, enzymes, over 10 fatty acids, lactose, 25 vitamins, and more than 45 mineral elements. (Mihai et al., 2020). Milk consumption benefits the body by increasing its resistance to infections and intoxications and by directly contributing to the improvement of the population's health status (Wirth & Duda, 2021).

The energy value of milk is 680 calories/kg and it is equivalent to the caloric energy contained in 600 g of beef, 500 g of fish, 400 g of pork, 7-8 eggs, 2.6 kg of cabbage, or 1.4 kg of apples. Due to its dietary properties, cow's milk is recommended for consumption by all age groups, especially children and the elderly (Maciuc et al., 2006; Fadhil & Zülkadir, 2020).

In Romania, several cattle breeds are raised under different production systems, including the Brown of Maramures (Brown Swiss strain), which represents an important genetic population in the country. The formation of the Brown of Maramures breed was not a random phenomenon, but the result of a breeding strategy initiated at the end of the 19th century, aimed at improving the productive performance of local rustic breeds from the country's mountainous areas, such as Mocanita and Grey steppe cattle. As a breed, it was developed through a directed process of grading-up crossbreeding using foreign genetic material, followed by intensive selection and adaptation to the specific environmental conditions of mountain regions (Maciuc et al., 2003).

The systematic importation of Schwyz-type (Brown Swiss) bovine genetic material was not restricted to a single source, but encompassed genetic lines originating from several regions of Central Europe. The continuous process of crossbreeding ("browning") led to the production of animals that progressively converged phenotypically toward the Brown breed standard (Roșu et al., 2024).

The final outcome was the development of a breed with superior combined productive traits, while maintaining high resilience and adaptation to mountainous environments - characteristics inherited from the local Mocanita and the Grey steppe cattle variety.

The resulting cattle populations were officially recognized in 1959 as a distinct population, being homologated as a breed and designated "Brown of Maramures" (Brown Association, 2024), the name refers to the region and to the area of origin and initial dissemination at the national level. Regarding population dynamics, the highest proportion of Brown of Maramures cows was recorded in 1980, when they accounted for more than 30% of the total national cow population, ranking as the second most important cattle breed; however, after 1990, the breed entered a period of continuous numerical decline (Maciuc et al., 2015).

Animals respond markedly to management and feeding conditions, and when nutritional and husbandry requirements are optimized, milk yields may reach up to 6,000 kg per lactation, with an average fat content exceeding 3.91% and a protein content of approximately 3.25% (ANARZ, 2024). Moreover, the milk is characterized by a high casein content, which makes it particularly suitable for cheese production.

The aim of the present study is to compare the productive performance of Brown of Maramures cattle under different physiological states, across successive lactations, and within varying age categories.

MATERIALS AND METHODS

The study was conducted on a herd of 48 dairy cows belonging to the breed Brown of Maramureș, exploited for milk production under the conditions of the SCDB Secuieni farm, located in Neamț County, in northeastern Romania. Research station was established on January 1, 1962, with the primary objective of improving the Brown of Maramures cattle population and developing modern technological solutions for their management.

The herd included in the present study comprises animals originating from the initial nucleus of 10 Brown of Maramures cows maintained at the time of the research station's establishment. The herd has been reared as a closed population and currently constitutes a conservation nucleus aimed at preserving the genetic resources of the Brown of Maramures breed. Productive performance data were collected and analyzed exclusively from animals belonging to this conservation herd.

The cows are maintained in permanent free-stall housing and are fed with stock fodder using a single-type ration, nutritionally balanced to meet the animals' requirements for basal metabolism and production, according to their age and physiological status. The animals are milked twice daily. Milking is performed using a pipeline system, and the milk is stored in cooling tanks until delivery.

The quantitative and qualitative parameters of milk production were obtained from analysis reports conducted during official production control and from farm records. Statistical processing focused on data from three normal lactations (LH1, LN2, LN3), including lactation length (DLN), milk yield (MY), fat yield (GY), protein yield (PY), and the percentage content of fat (G%) and protein (P%). Data from other lactations were excluded due to the very low number of animals with more than three lactations in the herd.

Statistical analyses were performed using the software packages SAVC (Statistical Analysis of Variance and Covariance) and SPSS 16.0 for Windows. The following statistical parameters were calculated: arithmetic mean ($\bar{X}$), standard error of the mean ($\pm s_{\bar{X}}$), standard deviation (s), and coefficient of variation (V%). Graphical representations included the regression line, Pearson correlation, Chi-Square test, ANOVA, significance test (p-value), and confidence intervals (C.I.).

RESULTS AND DISCUSSIONS

The aim of this study is to highlight the influence of external environmental factors, such as management and production technology, on the productive performance of Brown of Maramures dairy cows. Productive and qualitative performances are determined by the cumulative effect of internal factors, such as the genetic base, and external factors that are determined by the applied exploitation technology (Popovac, 2020; Yıldırır, 2024; Ma

et al., 2025; Mulianda et al., 2025; Rodriguez et al., 2025).

The statistical dynamics of the main parameters characterizing milk production, both quantitative and qualitative, were analyzed for the progeny across three normal lactations (LN1, LN2, LN3). Additionally, the study examines the productive performance of the dams (M) and maternal granddams (MM) of the cows, focusing on production traits and comparing the statistical values. All results of the statistical analyses were summarized in the following tables, with the statistics for the ancestors presented in Table 1 and those for the progeny in Table 2.

Table 1. Milk production statistics by ancestry of the studied cattle herd

Ascendence	Traits	n	$\bar{X}$	$\pm s_{\bar{x}}$	s	V%	Minim	Maxim
M	MY - kg	34	6,258.38	122.933	716.816	11.454	5,308.00	8,286.00
	G - %	34	3.99	0.046	0.271	6.773	3.56	4.63
	GY - kg	34	250.00	5.679	33.112	13.245	204.00	308.00
	P - %	33	3.49	0.026	0.150	4.289	3.21	3.91
	PY - kg	34	218.74	4.558	26.577	12.150	170.00	270.00
MM	MY - kg	9	6,471.00	178.614	535.843	8.281	5,704.00	6,993.00
	G - %	9	4.28	0.139	0.416	9.700	3.57	5.01
	GY - kg	9	277.67	12.301	36.902	13.290	204.00	324.00
	P - %	9	3.58	0.057	0.170	4.765	3.27	3.92
	PY - kg	9	231.67	7.853	23.558	10.169	198.00	254.00

M - mother; MM - mother's mother

For the herd of cows representing the progeny, the statistical values of milk production for the first three normal lactations are presented in Table 2.

Table 2. Milk production statistics for the normal lactations in the case of descendance from the studied herd

Parity (Lactation)	Traits	n	$\bar{X}$	$\pm s_{\bar{x}}$	s	V%	Minimum	Maximum
LN1	DLN	38	303.71	1.237	7.623	2.510	258.00	305.00
	MY - kg	38	5,683.26	90.244	556.304	9.788	4,797.00	6722.00
	G - %	38	4.04	0.049	0.304	7.519	3.21	4.44
	GY - kg	38	227.95	3.924	24.188	10.611	172.00	270.00
	P - %	38	3.47	0.022	0.138	3.959	3.28	3.77
	PY - kg	38	200.16	3.018	18.602	9.294	168.00	245.00
LN2	DLN	38	295.92	4.572	28.184	9.524	169.00	305.00
	MY - kg	38	5,699.26	128.728	793.531	13.923	3,642.00	8091.00
	G - %	38	3.83	0.066	0.409	10.680	3.14	4.50
	GY - kg	38	217.37	4.546	28.024	12.892	160.00	289.00
	P - %	37	3.42	0.021	0.130	3.819	3.20	3.75
	PY - kg	38	195.58	4.212	25.963	13.275	120.00	265.00
LN3	DLN	13	296.15	6.828	24.620	8.313	216.00	305.00
	MY - kg	13	5,089.92	186.791	673.486	13.232	3,193.00	5924.00
	G - %	13	3.88	0.091	0.329	8.475	3.40	4.40
	GY - kg	13	197.00	7.353	26.511	13.457	132.00	230.00
	P - %	13	3.48	0.025	0.088	2.546	3.31	3.64
	PY - kg	13	176.85	6.741	24.307	13.744	110.00	213.00

As shown in Table 2, for the studied herd of descendants, the highest milk yield (MY) of 5,699.26 kg was recorded in the second lactation, with milk containing 3.83% fat and

3.42% protein. The range of milk production varied between a minimum of 3,642 kg and a maximum of 8,091 kg, which represents the highest production record in the herd across all lactations.

In the first lactation, cows produced over 4,797 kg of milk, with an average of 5,683.26 kg, which is close to the maximum yield. The milk contained 4.04% fat and 3.47% protein. The coefficient of variation for the normal lactation length was 2.51%, and for milk yield it was 9.788%, the lowest values observed in the first lactation. Consequently, fat and protein yields were the highest, at 227.95 kg and 200.16 kg, respectively.

The variability of fat content was low in the first and third lactations, at 7.52% and 8.475% in the third lactation, respectively, and showed medium homogeneity in the second lactation. In LN3, although milk yield would be expected to increase, the average production of 5,089.92 kg was the lowest, with a minimum of 3,193 kg and a maximum of 5,924 kg, representing the lowest range. Although the variability of the trait was lower than in LN2, the production levels were still reduced, indicating that the animals did not fully express their genetic potential due to external environmental factors related to management and production technology.

Fat and protein yields were also the lowest; however, compared to LN2, the average lactation length of 296.15 days was similar, and the percentage content of fat (3.88%) and protein (3.48%) was higher.

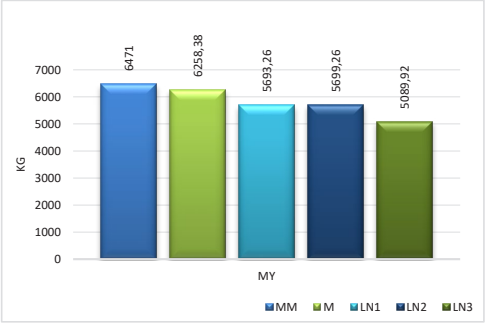


Figure1. Milk yield in the studied herd and its ancestry

Figure 1 provides a comparative graphical representation of milk production values between maternal ancestors and descendants. Maternal granddams (MM) had an average milk yield of 6,471 kg per lactation, while the dams (M) had an average milk yield of 6,258.38 kg, higher than the maximum mean of 5,699.26 kg recorded in LN2 among the ancestors. Compared to the maternal granddams (MM), the variability of the trait in the dams was higher, at 11.454%, with a production range from a minimum of 5,308 kg to a maximum of 8,286 kg of milk. While milk production in the dams was 3.28% lower than that of the MM, the highest mean production in LN2 of the progeny was 8.93% lower than that of the dams. Compared to the maternal granddams (MM), the variability of milk yield in the dams (M) was higher at 11.454%, with a production range from a minimum of 5,308 kg to a maximum of 8,286 kg of milk. While the average milk yield of the dams was 3.28% lower than that of the MM, the highest mean production in LN2 of the progeny was 8.93% lower than that of the dams.

Analyzing the values from Tables 1 and 2, it can be observed that the milk of maternal granddams (MM) contained 4.28% fat and 3.58% protein. In the case of the dams (M), the milk had comparatively lower percentages, with 3.99% fat and 3.49% protein. The average values for the dams were comparable to those of the progeny, where in LN1 the milk contained 4.04% fat and 3.47% protein, and in LN3 the averages were 3.88% fat and 3.48% protein.

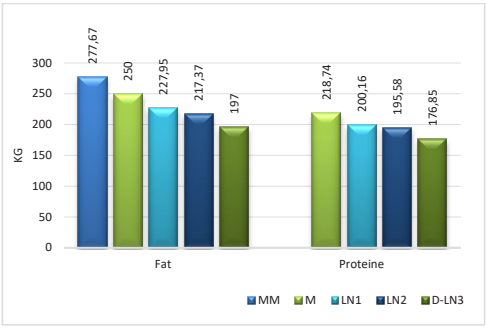


Figure 2. Fat and protein yield in the study herd and its ancestry

Figure 2 illustrates the average fat and protein yields in both the maternal ancestors and the progeny; these traits exhibited medium homogeneity in all cases.

In the case of the maternal granddams (MM), the highest average yields were recorded, with 277.67 kg of fat and 231.67 kg of protein per

lactation, reflecting the correlations between production traits. The dams of the progeny produced on average 250 kg of fat and 218.74 kg of protein, representing 9.96% less than the average performance of the granddams.
In the descendancy, these yields decreased from one lactation to the next, with the highest values recorded in LN1, where 227.95 kg of fat and 200.16 kg of protein were produced. From an analytical perspective, compared with the averages of the dams, these values were 8.82% lower for fat yield and 8.49% lower for protein yield.
Although the cows in the studied herd possess genetic potential, their performance does not fully reflect it. The average milk production in LN1 represents 99.72% of LN2 and is 10.44% higher than the average production in LN3. The standard deviations for the analyzed productive traits were also higher in LN2 and LN3 compared with LN1.
The high variability in milk, fat, and protein yields in LN2 and LN3 compared with LN1, along with the other observations, highlights that external environmental factors strongly influence the phenotypic expression of these traits in the studied herd, with animal nutrition being the most important factor.

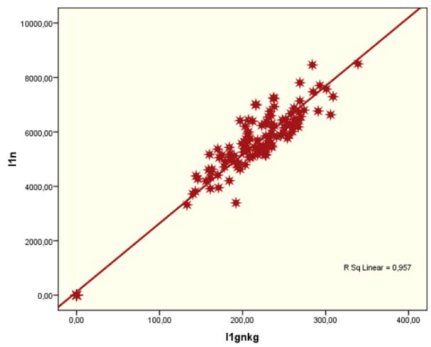


Figure 3. Regression line for milk yield (kg) and fat yield (kg)

Figure 3 presents the regression line for fat yield as a function of milk yield. The orientation of the line and the clustering of points close to it indicate the existence of a strong positive correlation (0,957) between milk yield (kg) and fat yield (kg). Any increase in milk production is accompanied by a corresponding increase in fat yield.

CONCLUSIONS

The study conducted on a herd of 48 cows belonging to Brown of Maramures breed, reared under a semi-intensive system, highlights that management practices and feeding conditions significantly influence productive performance and milk quality.
Although the average milk yields and qualitative parameters fall within the breed-specific range, they are lower than those of the ascendants, indicating that the animals' genetic potential is not fully expressed. The dams and maternal granddams exhibited higher production than the descendants, with differences of approximately 8-10% in favor of the ancestors for the main productive traits.
The analysis of the three normal lactations shows that maximum production was achieved in the second lactation, with an average of 5,699.26 kg; however, this remains lower than the values reported for the ancestors, confirming the unfavorable influence of environmental factors. In contrast, the first lactation exhibited the highest data homogeneity, the lowest variability, and the highest fat and protein yields relative to lactation length.
The third lactation most clearly reflects the negative impact of environmental conditions, with lower production levels despite lactation length and milk component percentages that would allow higher yields. This decline suggests limitations in management technology, particularly regarding nutrition, milking management, and animal comfort.
The very strong correlation (r = 0.957) between milk yield and fat yield confirms the direct relationship between total milk production and the output of its components. Overall, the results demonstrate that the herd's performance does not fully reflect the cows' genetic potential of the cows, with technological and environmental factors being the main limitations to its expression.
In these conditions, it can be made the following recommendations:
1. Optimize feeding rations by adapting nutritional intake to the physiological stage and production level of each cow.
2. Improve housing conditions (space, microclimate, bedding) to reduce stress and enhance productive efficiency.

3. Evaluate the milking technology and protocol to reduce losses and stimulate milk production.
4. Apply genetic selection focused on productive traits and milk quality, considering the performance of ascendants.
5. Continuously monitor performance through regular official controls and annual comparative analyses.

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RESEARCH ON THE GENETIC VALUE OF A FLECKVIEH BREED HERD AND ITS INFLUENCE ON THE PRODUCTIVE LEVEL

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Abstract

The present study aims to highlight the influence of the genetic merit of a Fleckvieh cattle herd on milk and beef production. The primary data were collected from 82 cows with completed lactations and 51 of their offspring, managed under a semi-intensive system on a farm located in Iași County, Romania. Using SAVC (Statistics, Analysis of Variance, and Covariance) software, the data were statistically processed to calculate the mean, variance, standard deviation, standard error of the mean, and coefficient of variation. For milk production, parameters included milk yield (MY), fat content (% F), and protein content (% P), as well as somatic cell count (SCC). For beef production, data regarding birth weight (BW) and average daily gain at 200 days (ADG200) were analyzed. Within the herd, the highest mean for milk yield was 6,643.80 kg/lactation, while the highest means for fat and protein percentages were 4.04% and 2.93%, respectively. Regarding beef production traits, the progeny group [family] of sire 20215 recorded the highest means, with a BW of 32.67 kg and a ADG200 of 973.33 g/day. The performances recorded by these animals fall within the specific standards of the breed, demonstrating that the herd possesses significant genetic potential.

Key words*: Fleckvieh, milk, meat production.*

INTRODUCTION

The Fleckvieh is a dual-purpose cattle breed exhibiting high performance in both milk and beef production, representing approximately 41 million head of cattle worldwide. Young cattle achieve an average average daily gain of 900 to 1,200 g up to 200 days of age. The average 305-day milk yield of cows ranges from 6,000 to 8,500 kg, with a content of 4.10 % fat and 3.40% protein. The average adult body weight ranges between 650 kg and 850 kg (Piccand et al., 2014).

In Romania, the breed's genetic improvement program aims to enhance milk production while simultaneously accounting for beef production and functional traits (Gociman et al., 2020). The breeding goals combine functional traits (with a weighting of 44%), milk production (38%), and beef performance (18%). The objective is to produce healthy, long-lived, productive, and efficient animals for both milk and beef outputs. The economic benefits of raising dual-purpose cattle are further reflected in the efficient utilization of increasingly limited resources, as high-quality forage is utilized in a balanced manner (Maciuc, 2006).

While the performance of the Fleckvieh breed is extensively documented in Central Europe, its phenotypic expression is influenced by genotype-by-environment interactions (Filipčík et al., 2020).

Fewer studies precisely demonstrate how Fleckvieh populations perform under the influence of management systems specific to Northeastern Romania.

The novelty of this study lies in its comprehensive analysis of growth performance in young bulls alongside the performance of dams from the same population, thereby providing an overview of dual-purpose production efficiency under the environmental conditions of the Moldavian Plateau.

The objective of the present study was to evaluate the influence of genetic factors on the zootechnical performance of a Fleckvieh cattle herd (Bucher et al., 2024). The main quantitative and qualitative indicators of milk production in adult cows were analyzed, as well as the determining parameters of beef production in young fattening bulls. Although microclimate factors and nutritional management modulate phenotypic expression, the genetic structure of the analyzed population remains the major

determinant of productive performance and carcass quality (Acatincăi, 2004).

MATERIALS AND METHODS

The research was conducted from January 2023 to December 2025 within a cattle cattle-breeding facility located in Iași County (Northeastern Region of Romania). The biological material for this study consisted of a herd of 82 adult cows belonging to the Fleckvieh breed (Figure 1) and 51 of their offspring. The animals were housed and managed under a semi-intensive system, benefiting from homogenous microclimate and herd management conditions. Nutritional management was provided through the administration of a Total Mixed Ration (TMR), computer-formulated to fully meet the energy, protein, and mineral requirements of the lactating cows.



Figure 1. Fleckvieh cows on the farm (original source)

The weighted daily ration structure per head of animal comprised: 4.00 kg alfalfa hay, 28.00 kg corn silage, 5.30 kg ground corn grain, 3.10 kg sunflower meal, along with a mineral intake consisting of 0.034 kg dicalcium phosphate and 0.06 kg feed-grade limestone [forage chalk]. The primary data regarding the quantitative and qualitative performance of milk production were extracted from the official analysis reports issued following official milk recording (OMR). The phenotypic indicators monitored were: milk yield per standard 305-day lactation (MY, kg), fat content (F, %), protein content (P, %), and somatic cell count (SCC X 10^3/mL). To ensure the accuracy of the transgenerational genetic potential evaluation, the statistical processing focused on three consecutive completed standard lactations, namely the 2nd, 3rd, and 4th lactations (SL2, SL3, SL4). Data pertaining to the first lactation (SL1) were excluded from the experimental model, considering that primiparous cows do not fully express their maximum productive ceiling due to concurrent processes of somatic growth and development. Regarding beef production traits, the analysis was based on raw data recorded in the technical logs of the farm, targeting calf birth weight (BW, kg) and average average daily gain from birth to 200 days of age (ADG200, g/day). The primary processing of the data and the calculation of measures of central tendency and dispersion (arithmetic mean, standard error of the mean, standard deviation, and coefficient of variation) were performed using the specialized SAVC (Statistics, Analysis of Variance, and Covariance) software package. This software was used to determine the degree of homogeneity of the experimental groups for the traits under study. To test the statistical significance of differences between means and to analyze the correlations and complex interdependencies between milk and beef indicators, the SPSS v.20.00 software package for Windows was utilized. The statistical significance thresholds considered for interpreting the results according to zootechnical methodology were set at $p < 0.05$ (significant), $p < 0.01$ (distinctly significant), and $p < 0.001$ highly significant).

RESULTS AND DISCUSSIONS

Within the study, the progeny structure and productive performances of several sire families were analyzed to evaluate the genetic potential and the stability of traits transmission for milk and beef production to the offspring. Regarding milk production, data concerning the productive performance and the values of the main quality indicators were processed for the second, third, and fourth standard lactations (SL2, SL3, SL4) across the entire analyzed cow herd.

Table 1 presents the mean values of milk production for the sire families within the studied herd. Thus, it can be observed that the productive performance of the cows ranges between 4,955 and 6,977 kg of milk per lactation.

Table 1. Values of means ($\bar{X}$) and coefficient of variability (V%) for milk yieald (MY) per lactation

Bull	Number of descendances	MY (kg)/ SL2 ($\bar{X}$)	V %	MY (kg)/ SL3 ($\bar{X}$)	V %	MY (kg)/ SL4 ($\bar{X}$)	V %
53756	18	6628.00	5.00	6642.60	9.71	6660.20	12.00
53435	13	6122.25	17.13	5782.00	15.32	6134.25	20.10
54464	11	6358.00	10.13	5847.00	16.31	6019.25	20.48
13272	6	5438.33	7.30	5666.00	19.34	6351.67	12.51
53050	7	5685.00	14.89	4955.00	20.43	6246.33	16.69
53433	6	5745.00	9.13	6199.00	12.67	5850.00	6.54
37218	6	6862.50	31.56	6780.50	4.29	6278.00	12.28
70264	9	6157.50	11.84	5685.00	27.59	5904.50	13.93
52033	6	5791.00	2.83	6977.00	4.26	5103.50	10.00

For milk production traits, sire 53756 exhibits the largest progeny group, with 5 daughters and 13 granddaughters within the herd, followed by sire 53435 with 4 daughters and 9 granddaughters, and sire 54464 with 4 daughters and 7 granddaughters recorded. The progeny of sire 53050 comprises 3 daughters and 4 granddaughters, while sire 59672 has 3 daughters and 6 granddaughters. Sires 53433, 37218, and 52033 each have 2 daughters and 4 granddaughters, and sire 70264 presents a progeny group consisting of 2 daughters and 3 granddaughters. The analysis of lactation performance across the three consecutive completed lactations (SL2, SL3, and SL4) highlights pronounced inter-line variability, driven by the genetic potential of the sires. Within the second lactation (SL2), the daughters of sire 37218 recorded the highest mean yield of 6,862.50 kg of milk per lactation, a performance that deviates positively from the general breed average reported in Eastern European semi-intensive operations (Maciuc, 2006).

However, this group was characterized by heterogeneity, with the coefficient of variation reaching a value of V % = 31.56%, indicating non-uniformity in individual performance and increased genotype instability in response to farm management factors. In contrast, during the same lactation stage (SL2), the daughters of sire 53756 achieved an average yield of 6,628.00 kg of milk per lactation, demonstrating homogeneity for this trait. This phenotypic uniformity denotes high stability in trait transmission from the paternal ancestor. At the opposite spectrum, the progeny group of sire 13272 recorded the lowest productive level (5,438.33 kg of milk per lactation), a value falling below current breeding standards for the breed, suggesting an inferior breeding value of this sire for milk production. In the third lactation (SL3), the group belonging to sire 37218 consolidated its top position in quantitative terms, recording 6,780.50 kg of milk per lactation. Unlike the previous stage, the trait showed evident genetic stabilization, materialized by an extremely low coefficient of variation (V % = 4.29%), which confirms that the expression of productive potential becomes more predictable as the herd reaches biological maturity.

Performance consistency is also firmly supported by the line of sire 53756, whose daughters achieved 6,642.60 kg of milk per lactation. The poorest results in SL3 were attributed to the progeny of sire 53050 (4,955.00 kg of milk per lactation), a performance with marked heterogeneity, translating into genetic instability and a non-uniform adaptive response to farm management conditions. In the advanced stage of exploitation, represented by the fourth lactation (SL4), the progeny group of sire 53756 ranked first, with a mean production value of 6,660.20 kg of milk per lactation.

Maintaining high phenotypic uniformity throughout the entire production dynamic (SL2-SL4) validates the genetic superiority of this sire relative to the rest of the analyzed nucleus. During the same period, the lines of sires 53435 and 54464 exhibited moderate mean yields and heterogeneous profiles, reflecting less conserved hereditary transmission mechanisms. The minimum yield in SL4 was reached by the progeny of sire 52033 (5,103.50 kg of milk per lactation), confirming the placement of this line within the category of genetic resources with a deficient dual-purpose potential for the lactation component.

The overall comparative evaluation of the three lactations indicates that while the line of sire 37218 excels punctually in the early stages, the line of sire 53756 offers the most robust productive consistency and metabolic resilience along the lactation curve.

The average production level obtained by the high-performing lines in this herd (6,600-6,800 kg) aligns with results obtained in similar Fleckvieh populations (Ducháček et al., 2020), demonstrating that a well-optimized semi-intensive management system can efficiently exploit the local genetic pool despite limitations induced by regional climate stress (Filipčík et al., 2020). From the perspective of future selection, sire 53756 is recommended as a proven bull for the milk yield per standard lactation trait within this nucleus.

The significance of productive differences among the studied paternal lines was established by statistical testing of the null hypothesis, with the interpretation of variability based on the following relevance thresholds:

- $p < 0.05$ (significant): validates the existence of real differences between the performance of daughters from different sires at the same lactation stage, such as the productive superiority of sire line 37218 compared to line 13272 within SL2;
- $p < 0.01$ (distinctly significant): confirms a pronounced genetic determination of the quantitative trait for lines that conserve their hierarchical position when transitioning from one lactation to another, despite microclimate changes during the experimental years;
- $p < 0.001$ (highly significant): demonstrates that extreme differences in production (the yield difference of 1,873.5 kg of milk in SL4 between the daughters of high-performing sire 53756 and those of sire 52033) are dictated by differing breeding values.

Table 2. Values of mean ($\bar{X}$) and coefficient of variability (V %) for fat (F %), protein (P %) and number of somatic cells (x 10^3 NSC/mL)

Bull	Number of descendances (n)	Lactation rank	Fat content (F %)		Protein content (P %)		NSC (10^3/mL)	
			($\bar{X}$)	V%	($\bar{X}$)	V%	($\bar{X}$)	V%
53756	18	LN2	4.29	17.99	3.03	5.67	117.08	25.47
		LN3	3.86	15.92	2.88	11.61	537.32	41.38
		LN4	3.49	8.14	2.72	12.09	421	56.41
53435	13	LN2	4.11	11.25	3	11.30	115.10	12.35
		LN3	3.88	10.32	2.77	14.26	129	14.48
		LN4	4.13	14.32	2.71	11.88	210	51.28
54464	11	LN2	3.93	14.11	2.57	9.07	58	12.00
		LN3	3.71	11.26	2.76	10.38	396	18.93
		LN4	3.87	11.63	2.57	12.21	222	25.59
13272	6	LN2	3.81	4.62	3.19	10.52	231	33.14
		LN3	4.50	11.91	2.80	16.50	93	31.00
		LN4	3.82	14.92	2.71	17.07	145	18.21
53050	7	LN2	3.57	13.25	2.93	25.59	91.90	18.50
		LN3	3.62	8.35	2.57	11.66	99	14.21
		LN4	3.83	10.54	2.84	8.86	60	12.61
53433	6	LN2	3.83	2.77	3.00	8.00	121	22.01
		LN3	3.97	1.25	2.00	4.18	470.50	20.21
		LN4	4.00	11.21	3.00	4.23	223	27.92
37218	6	LN2	3.66	8.11	2.55	19.41	186	19.90
		LN3	4.20	23.57	3.35	6.33	235.85	32.22
		LN4	4.07	20.65	2.39	3.25	376	17.00
70264	9	LN2	3.58	5.14	3.95	10.88	300	34.85
		LN3	3.15	2.24	2.63	13.93	336.30	24.90
		LN4	3.31	15.71	2.23	6.98	465	15.31
52033	6	LN2	3.55	9.96	2.84	5.72	210	32.01
		LN3	4.04	5.07	2.77	22.17	110	28.76
		LN4	3.67	4.43	2.92	0.97	532	19.89

The analysis of milk quality indicators (Table 2) highlights pronounced inter-line variability, reflecting the manner in which paternal selection influences the synthesis of useful milk components. Within the second lactation (SL2), the progeny group of sire 53756 stood out due to superior performances, recording a mean value of 4.29% fat and 3.03% protein.

The uniformity of this group (characterized by high-to-moderate homogeneity) indicates a high capacity for hereditary transmission of traits to the offspring. This was maintained during subsequent lactations (SL3 and SL4), where the daughters of sire 53756 exhibited stable profiles (3.86-4.20% fat; 2.72-2.88% protein).

The productive behavior of this line confirms findings in the literature, where certain Fleckvieh lines manifest a remarkable stability of the quality curve throughout their productive life (Filipčík et al., 2020). In contrast, the progeny of sire 37218 exhibited oscillating dynamics and a high degree of heterogeneity in SL2 (with V % for fat and protein exceeding stability thresholds), although individual punctual means were relatively high (4.20% fat in SL3). This association between high means and marked heterogeneity suggests an increased sensitivity of the genotype to environmental factors and a reduced capacity for genetic fixation of the trait during the early stages of exploitation.

A biological phenomenon of interest was observed in the progeny of sire 70264 in SL2, which recorded a maximum protein content (3.95%) but a sharp minimum for fat content in SL3 (3.15%). This phenotypic dissociation reflects the partial independence of genetic determinism for the two major milk components and confirms the complexity of breeding the Fleckvieh breed for quality traits.

The inferior lines, represented by sires 52033 and 53343 (which reached minimums of 3.55% fat and 2.00% protein, respectively), indicate the presence within the herd of genotypes with a deficient breeding value for milk quality, requiring selective culling.

From a quantitative genetics perspective, these inter-line differences are justified by the moderate-to-high heritability ($h^2 = 0.25$-0.40) of fat and protein percentages reported for the Fleckvieh breed (Maciuc, 2006). The results indicate that, under unified nutritional management (a single TMR ration), selection based on high-performing paternal lines (such as the line of sire 53756) represents an efficient strategy for consolidating milk quality while limiting the uncontrolled dispersion of performance in animal exploitation.

The evolution of somatic cell count (SCC) across the three lactations represents a fundamental indirect indicator of mastitis resistance and the immune status of the mammary gland. Unlike the quality parameters, the SCC analysis reveals generalized structural heterogeneity across all analyzed lines, with no single family falling into the category of homogeneous groups. In SL2, mean values oscillated within wide limits, from an optimal minimum of 58 x 10^3 cells/mL (line 54464) up to values of 300 x 10^3 cells/mL. Even from this early stage, lines such as those of sires 53756 and 53435 demonstrated frequent exceedances of the ideal physiological threshold (100 - 150 x 10^3 cells/mL), suggesting a latent genetic susceptibility to subclinical udder inflammation.

As the animals advanced in age, in SL3 and SL4, an exacerbation of both mean values and dispersion indices was observed. This phenomenon is best illustrated by the line of sire 53756, whose daughters recorded means of 537.32 x 10^3 cells/mL in SL3 and 421 x 10^3 cells/mL in SL4.

Substantial parallel increases were also identified in lines 54464 and 52033, whose extremes migrated toward the range of 210- 532 x 10^3 cells/mL. From a biological standpoint, this upward dynamic of SCC correlated with lactation number reflects the cumulative effect of teat sphincter microtraumas induced by mechanical milking and the physiological wear of the secretory glandular tissue (Filipčík et al., 2020).

In addition, the low heritability of this trait (h^2~ 0.15) explains why phenotypic variation is dominated by physiological factors (milking management, housing hygiene, and individual mammary pathology) (Mihai et al., 2019). However, maintaining constant moderate homogeneity and relatively low SCC values throughout the study in the lines of sires 54464, 53050, and 13272 suggests the existence of exploitable genetic variability for infection resistance. In modern Fleckvieh breeding programs, where functional and health traits carry a major selection weight (44%),

identifying and promoting these lines with controlled SCC represents a necessity to ensure productive longevity and reduce veterinary treatment costs on Romanian farms.

The results of the main statistical estimators for birth weight and average daily gain are included in the data in Table 3.

Table 3. Principal estimates for calving weight (BW) and average daily gain (ADG200)

Bull	Number of descendances (n)	Traits	$\bar{X}$	s_x	V %	MIN.	MAX.
54463	10	BW (kg)	31.20	0.59	15.39	29	34
		ADG200 (g/day)	914	28.06	8.63	800	1050
20191	18	BW (kg)	31.11	0.91	7.71	27	35
		ADG200 (g/day)	891.11	21.40	7.98	810	1100
54547	15	BW (kg)	31.80	0.86	5.88	28	35
		ADG200 (g/day)	903.33	25.61	4.93	820	990
20192	2	BW (kg)	30.50	0.68	2.32	30	31
		ADG200 (g/day)	955	16.52	8.14	900	1010
54461	3	BW (kg)	31	1	3.23	30	32
		ADG200 (g/day)	916.67	80	25.73	890	940
20215	3	BW (kg)	32.67	0.45	7.70	30	35
		ADG200 (g/day)	973.33	11.91	5.84	910	1020

The genetic structure of the analyzed herd for beef production traits highlights a contrasting numerical distribution among male offspring (sires' sons). The primary families were dominated by the lines of sires 20191 (n = 18 sons) and 54547 (n = 15 sons), followed by the sons of sire 54463 (n = 10 sons).

Lines 54461, 20125 (20215), and 20192 exhibit a limited representation, pooling between 2 and 3 offspring per sire - a scenario frequently encountered in commercial operations where bloodline rotation is practiced. Regarding birth weight (BW), the calculated mean values ranged from 30.50 kg (line 20192) to 32.67 kg (line 20215). From a biological standpoint, this pronounced uniformity in birth weights (with groups classified as homogeneous) reveals a rigorous control of the maternal factor and

gestation management, but also an intrinsic characteristic of the Fleckvieh breed, which tends to maintain moderate birth weights to prevent dystocia (Bucher et al., 2024).

The sole exception was represented by the line of sire 54463, which fell into the moderate homogeneity class, a fact correlated with a variation range of 5 kg (29.00-34.00 kg). The total absence of heterogeneous lines for the BW trait demonstrates remarkable stability in the hereditary transmission of this trait across the entire breeding nucleus.

The analysis of growth dynamics through average average daily gain from birth to weaning (ADG200) reveals an outstanding biological potential specific to this dual-purpose breed, with means ranging from 891.11 g/day (line 20191) to a maximum of 973.33 g/day (line 20215). A particularly strong performance was also achieved by the progeny of sire 20192 (955.00 g/day), with both lines highlighting sound genetic potential for early muscle accretion rates.

From a genetic perspective, a statistical paradox is observed in the line of sire 20191: although the group is classified as homogeneous (V % = 7.98%), it exhibits the largest absolute variation range (290 g/day, with values ranging between 810 and 1,100 g/day). Biologically, this phenomenon is explained by the large sample size (n = 18), which allowed the full spectrum of polygenic genetic recombinations to manifest under environmental influences without destabilizing the overall group mean.

At the opposite spectrum, the line of sire 54461 was defined as heterogeneous, recording a wide dispersion in individual growth rates among its offspring, despite a narrow absolute range (890-940 g/day). This pronounced heterogeneity within a limited sample suggests strong instability in the transmission of beef breeding value by the paternal genitor, with the offspring being highly reactive to fluctuations in the barn's structural microclimate.

When comparing these results with recent scientific literature focused on Fleckvieh performance under the pedoclimatic conditions of Romania, the values obtained for ADG200 (approaching or exceeding the 900-950 g/day threshold in 5 out of the 6 lines) confirm an excellent feed conversion efficiency of TMR rations into muscle tissue (Kosińska-Selbi et al., 2020). The results consolidate the status of the Fleckvieh breed as a viable economic alternative in the region, demonstrating that targeted selection utilizing the lines of sires 20215 or 20192 can maximize young beef production without inducing major risks associated with dystocic calvings on the farm.

CONCLUSIONS

The genetic structure of the analyzed herd denotes a pronounced inter-line variability for both quantitative and qualitative milk traits. The absence of a single paternal line that aggregates peak performances across all traits reflects the antagonistic genetic correlations between milk yield and the concentration of useful dry matter components (fat, protein). This scenario underscores the necessity of implementing weighted selection indexes rather than unidirectional selection.

For commercial farms focused on maximizing delivery volume, the line of sire 53756 represents an important genetic resource, recording a remarkable mean of 6,643.80 kg of milk per standard lactation. Conversely, for milk processing operations where coagulation yield is dictated by solids-not-fat (SNF), the use of lines 13272 and 53435 (with a maximum of 4.04% fat) or line 70264 (with a peak of 2.93% protein) is recommended to compensate for their lower quantitative potential.

Somatic cell count (SCC) proved to be the most unstable trait, being heavily influenced by genotype-by-environment interactions. In breeding programs aimed at increasing mastitis resistance and improving the functional longevity of cows, the line of sire 53050 presents major applicative interest, demonstrating the most stable and lowest SCC level (83.56 x 10^3 cells/mL), in contrast to line 53756.

Regarding young cattle, the line of sire 20215 positioned itself as the most valuable dual-purpose genetic resource within the herd, excelling in both optimal birth weight (32.67 kg, which reduces the risk of maternal dystocia) and the highest growth rate (ADG200 = 973.33 g/day). This line is strongly recommended for producing offspring intended for intensive fattening.

All identified mean values fall within the biological standards of the Fleckvieh breed; however, the dispersion of performances confirms the need for targeted corrective mating strategies.

It is proposed to utilize sire 53756 to improve quantitative milk traits and sire 20215 to consolidate the growth rate of young cattle, thereby ensuring the sustainable and balanced exploitation of this dual-purpose breed within the breeding area of Iași County.

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PRECISION TECHNOLOGIES FOR DAIRY COW MANAGEMENT: A REVIEW OF WELFARE, HEALTH, AND SUSTAINABILITY OUTCOMES

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Abstract

Dairy cow farming is a strategic sector of agriculture, with a major impact on food production, the rural economy, and food security. In the context of increased production and increasing demands on animal welfare and sustainability, modern dairy farms are increasingly integrating advanced management technologies. This review synthesizes and analyses the main modern technologies used in dairy cow maintenance, including health and behaviour monitoring systems, precision nutrition technologies, automated milking systems, environmental management solutions, and digitally assisted reproductive technologies. The effects of these systems on cows' productivity, milk quality, health, and longevity of cows, as well as on the economic and environmental sustainability of farms, are assessed. Through the integrated approach to modern maintenance systems, this review contributes to clarifying the relationship between technology, animal welfare, and production efficiency, addressing gaps in the literature, where these issues are often treated separately.

Key words*: animal health monitoring, animal welfare, automatic milking systems (AMS), dairy cattle management, precision livestock farming (PLF).*

INTRODUCTION

In recent decades, dairy cattle production has undergone profound transformations: herd sizes have increased, input costs have escalated, and standards regarding milk quality and animal welfare have become increasingly stringent. In this context, traditional herd management methods based on the stockman's direct observation and empirical knowledge can no longer sustain an efficient and profitable dairy operation on their own. Given these considerations, it is understandable that a growing number of dairy farmers are turning to modern technologies that enable both the continuous collection and analysis of individual animal data and the timely adoption of evidence-based management decisions (Neethirajan, 2023).

Precision livestock farming (PLF) has evolved from simple automation systems primarily aimed at reducing labour requirements (Kelemen et al., 2016), towards complex solutions centred on the continuous monitoring of animal needs, particularly with regard to health and welfare. Data collected at farm level are intended to support the farmer's decision-making process, facilitating timely interventions adapted both to the animals' physiological status and to the operational efficiency objectives of the holding.

A wide range of systems are currently available that can be attached to cows or installed on the farm (motion sensor collars, ear tags, ingested boluses for ruminal temperature measurement, thermal cameras, and others). Precision livestock farming relies on individual animal monitoring through internet-connected sensors, with data transmitted directly to the farmer to support better management decisions for each cow (Tangorra et al., 2024). One of the most significant advantages of these systems lies in their ability to detect health disorders before they become clinically apparent. The onset of disease in dairy cows is accompanied by characteristic behavioural changes - reduced rumination time, decreased dry matter intake, diminished locomotor activity, and prolonged recumbency - changes that sensors can signal several days before a conventional clinical

diagnosis (Tsai et al., 2021). When milk production data and physiological indicators are combined with thermal imaging, the accuracy of early disease detection increases considerably compared with the use of any single data source in isolation (Michelena et al., 2025; Paulauskaite-Taraseviciene et al., 2026).

An equally important consideration is the housing environment in which cows are kept. Barn temperature, air quality, and resting area comfort directly influence animal health and milk yield. The temperature-humidity index (THI) is a widely used indicator of heat stress risk in dairy cattle. When THI exceeds the threshold of 68, automated systems activate fans and water sprinklers to cool the animals, without requiring any intervention from the stockman (Tangorra et al., 2024; Neculai-Văleanu et al., 2025). As herd sizes have grown, individual monitoring of each cow has become increasingly challenging, with real consequences for animal health and milk production. Precision livestock farming offers a practical solution for managing large herds without losing sight of the individual animal, while simultaneously improving the economic efficiency of the farm and reducing its environmental footprint (Kaur et al., 2023; Tangorra et al., 2024).

Although the scientific literature contains a growing number of studies on individual technologies - automatic milking systems, wearable sensors, artificial intelligence algorithms, and individually tailored nutrition - studies examining all of these together as an integrated system remain scarce. Despite this growing body of research, integrated assessments that simultaneously link outcomes in terms of animal welfare, productivity, and farm sustainability remain limited in the scientific literature. The aim of this review is to critically evaluate the main precision livestock farming technologies applied in dairy cattle operations, assessing their combined effects on animal welfare, health, productivity, and farm sustainability. By analyzing and synthesizing the available evidence in the scientific literature, this study contributes to filling an existing gap in the field, highlighting the importance of a holistic approach in which data on health, production, housing environment, and reproduction are correlated to support long-term economic efficiency and animal welfare (Stygar et al., 2021; Papakonstantinou et al., 2024; Defta et al., 2025).

MATERIALS AND METHODS

To achieve the objectives of this review, the following scientific databases were consulted: Web of Science, Scopus, PubMed, and ScienceDirect (Elsevier), which host the most relevant peer-reviewed studies on precision livestock farming applied in dairy cattle operations. Articles published primarily between 2015 and 2026 were selected to reflect the most recent technological developments in the field. A total of 312 articles were identified through database searches, of which 68 were included in the final analysis based on relevance and compliance with the inclusion criteria. Search strings included combinations of terms such as: “precision livestock farming” and “dairy cow”, “automated milking system” and “welfare”, “sensor” and “dairy cattle” and “health”, “machine learning” and “dairy”, and “smart farming” and “sustainability”. An extensive review of the scientific literature was conducted on the application of Precision Livestock Farming (PLF) technologies in dairy cow farms, with an emphasis on their impact on animal health, welfare, and sustainability.

The keywords used in the literature search included: precision livestock farming, dairy cow monitoring, automated milking systems, sensor technologies for dairy, animal welfare in dairy cows, smart farming in dairy, feeding automation for dairy cattle, and reproductive monitoring in dairy cows.

Following the construction of the reference database, a set of inclusion and exclusion criteria was applied to the selected articles.

Inclusion criteria:

- original research articles and review papers published in ISI-indexed journals;
- studies addressing technologies applied directly in dairy cow farming operations;
- research evaluating the impact on animal health, welfare, milk production, or farm sustainability;
- studies employing sensors, artificial intelligence, automated systems, or digital management platforms.

Exclusion criteria:

- publications with restricted access to full text;
- studies without direct relevance to the dairy cattle sector;
- articles of a purely theoretical nature, lacking practical applicability;
- non-scientific or unvalidated sources (reports not subject to peer review).

The selected articles were managed and organised using the Mendeley reference management software and classified according to the technological domain addressed.

Automatic milking technologies, housing and environment management, and reproductive management technologies. The schematic representation of these technologies consisted in the development of conceptual diagrams using the IPE software, facilitating the visualisation of the relationships between the different components of precision livestock farming systems.

RESULTS AND DISCUSSIONS

A. Health and welfare monitoring technologies in dairy cows

The monitoring of health and welfare in dairy cows has become a priority in modern farming operations. Until recently, stockmen relied almost exclusively on daily visual observation to identify potential health disorders in individual animals. Today, digital sensors, cameras, and artificial intelligence algorithms perform this task automatically, around the clock, for each cow individually (Neculai-Văleanu et al., 2025). Farms adopting these technologies are able to detect disease more rapidly, reduce milk yield losses, and lower treatment costs (Silva et al., 2021), which explains the growing uptake of such solutions in dairy cattle operations. Figure 1 illustrates the main categories of health and welfare monitoring instruments currently deployed in dairy cow operations, encompassing sensor-based, imaging, and AI-driven systems.

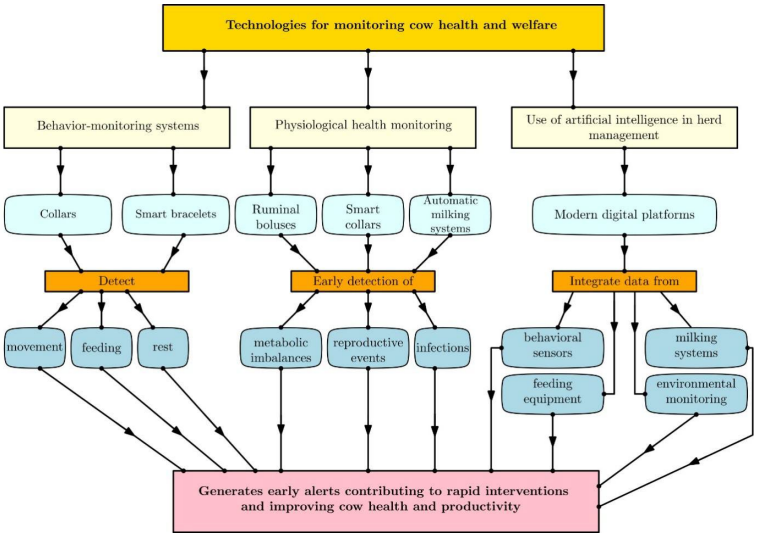


Figure 1. Schematic representation of health and welfare monitoring instruments in dairy cows
Own source (processed using IPE software)

a. Behaviour monitoring systems

The simplest and most widely used approach to monitoring the health status of dairy cows involves sensors attached directly to the animal's body. Smart collars and ear tags equipped with accelerometers continuously record the animal's activity - time spent feeding, ruminating, and lying down - automatically transmitting data to the farmer and generating alerts whenever an individual cow's behavioural patterns deviate from its established baseline (Lovarelli et al., 2022). Collars fitted with microphones add an additional layer of precision by acoustically differentiating rumination from primary ingestion (Alipio & Villena, 2023), while ear tags with integrated temperature sensors allow

fever detection without any physical contact with the animal. The limbs are monitored through pedometers or plantar pressure sensors, which identify gait abnormalities that may indicate pain or early-stage lameness (Lee & Seo, 2021).

The relevance of these devices increases substantially when they operate in parallel, with their data aggregated and analyzed on a shared digital platform. A decline in rumination time correlated with extended recumbency periods and reduced locomotor activity can thus signal the onset of disease several days before the appearance of visible clinical signs (Tsai et al., 2021).

Animal behaviour is a reliable indicator of health status, and deviations from normal behavioural patterns may signal underlying health disorders (Silva et al., 2021). For instance, a reduction in water intake may indicate heat stress or disease; increased locomotor activity is characteristic of oestrus, and a decline in rumination time may precede certain metabolic conditions (Tsai et al., 2021). Automated monitoring systems are capable of detecting these behavioural changes in real time, making them particularly valuable in large-scale dairy farming operations (de Oliveira et al., 2024).

b. Physiological health monitoring

The monitoring of physiological parameters through modern technologies - including ruminal boluses, smart collars, and automatic milking systems - enables the early detection of metabolic imbalances, reproductive events, and infectious conditions, facilitating timely interventions and contributing to improvements in animal health, milk yield, and milk quality (Lee & Seo, 2021; Michelena et al., 2025).

The most economically significant conditions in dairy farming - mastitis, lameness, and postpartum metabolic disorders - can be identified at an early stage through automated systems, before the onset of visible clinical signs. Mastitis is detected through an increase in milk electrical conductivity and a reduction in milk yield from the affected quarters (Michelena et al., 2025), and the integration of these data with behavioural changes reduces the rate of false alarms (Post et al., 2020; Mottram, 1997). Lameness is identified through changes in locomotion patterns and lying time (Swartz et al., 2024), and it can also be monitored through video-based systems with automated gait analysis (Sohan et al., 2025). Concurrently, postpartum metabolic disorders can be anticipated through declines in rumination time, locomotor activity, and milk yield, several days before clinical manifestation (Silva et al., 2021).

c. Application of artificial intelligence in herd management

Modern digital platforms integrate data from behavioural sensors, milking systems, feeding equipment, and environmental monitoring units, constructing a comprehensive profile for each individual cow. These profiles are analysed through machine learning algorithms capable of identifying patterns imperceptible to human observation and supporting the concept of a "digital twin" for the simulation of management decisions (Kaur et al., 2023; Cabrera, 2025; Rao et al., 2026). Such systems generate early health and reproductive alerts, including estimates of the optimal time for artificial insemination and the risk of culling, with high accuracy in mastitis prediction (Dharejo et al., 2025). Computer vision technologies further enable individual animal monitoring without the need for wearable devices (Mahato et al., 2025).

The integration of these technologies contributes substantially to animal welfare and farm sustainability through the early detection of mastitis and lameness, the reduction of antibiotic use and premature culling, and the anticipation of postpartum metabolic disorders based on behavioural and physiological changes (Silva et al., 2021; Stygar et al., 2021; Cabrera, 2025). Overall, the application of artificial intelligence transforms individual animal data into preventive management decisions, optimising veterinary interventions and extending the productive longevity of animals within the herd (Neculai-Văleanu et al., 2025).

All of the technologies described - behavioural sensors, ruminal boluses, and artificial intelligence platforms - do not operate in isolation. Their true value emerges only when they are connected within an integrated system that consolidates data from multiple sources and analyses them collectively.

A critical assessment of health and welfare monitoring technologies reveals both considerable advantages and significant limitations that must be acknowledged. On the

positive side, wearable sensors and AI-based platforms enable continuous, individual-level monitoring that substantially outperforms traditional visual observation, with reported sensitivities for oestrus detection ranging from 83% to 93% and mastitis detection accuracies exceeding 85% in well-validated systems (Stygar et al., 2021; Schilkowsky et al., 2021). Early disease detection reduces treatment costs, lowers antibiotic use, and decreases milk yield losses, generating measurable economic returns (Silva et al., 2021). However, several limitations constrain their practical impact. False positive rates remain non-negligible: alert systems based on a single parameter generate substantially more false alarms than multi-parameter platforms, imposing additional labour costs on farm staff who must investigate each alert (Post et al., 2020). Sensor accuracy is also affected by individual animal variability, breed differences, and parity, meaning that algorithms trained on one population may underperform when applied to another (Tsai et al., 2021). The economic viability of these systems depends heavily on herd size: on farms with fewer than 100 cows, the cost per animal of sensor infrastructure and data management software frequently exceeds the financial benefit from improved disease detection, limiting uptake to larger operations (Kaur et al., 2023). Furthermore, the lack of standardised communication protocols between sensor manufacturers prevents seamless data integration, requiring costly middleware solutions or manual data reconciliation (Tangorra et al., 2024).

B. Feeding and nutrition technologies

Nutrition is one of the most important factors influencing milk yield, metabolic health, and the welfare of dairy cows. Adequate feeding does not simply mean ensuring a sufficient supply of feedstuffs, but also the precise delivery of the appropriate nutrients, at the right time, to each production group or even to each individual animal (Neculai-Valeanu et al., 2025). Modern dairy operations that have adopted automated feed delivery systems report increases in milk yield, a reduction in the incidence of metabolic disorders, and lower feed costs (Tangorra et al., 2024). These outcomes make feeding automation one of the investments with the highest return on investment in modern dairy cattle production. Figure 2 illustrates the precision instruments used for feeding and nutrition management, including automated TMR systems, NIRS sensors, and feed monitoring platforms.

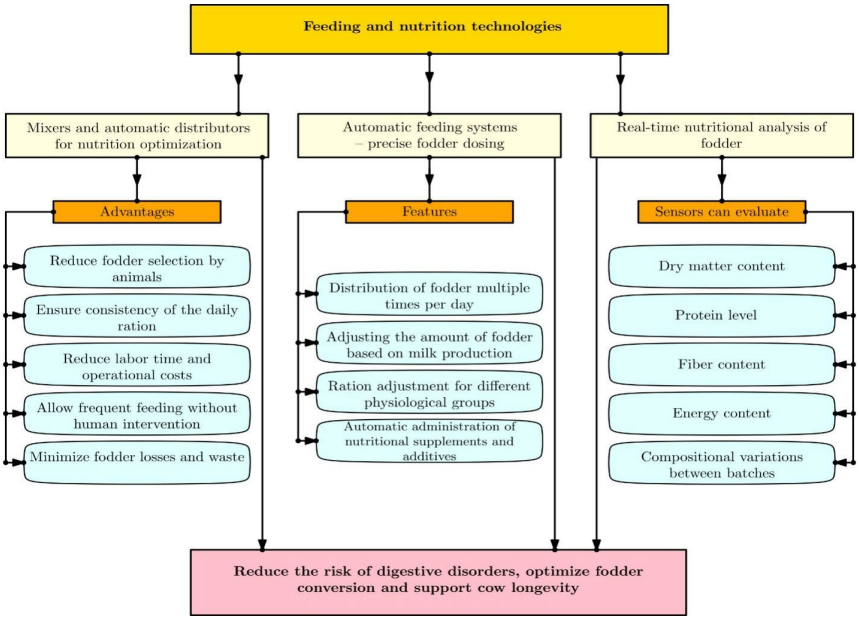


Figure 2. Schematic representation of precision instruments for feeding and nutrition technology
Own source (processed using IPE software)

a. Automatic feeding systems - precision feed delivery

Feeding dairy cows using a total mixed ration (TMR) ensures a uniform nutrient intake, stabilising ruminal fermentation, reducing the risk of acidosis, and stimulating voluntary dry matter intake (Romano et al., 2023). Automatic feeding systems (AFS) optimise this process through the automated preparation and delivery of feed several times per day, thereby increasing the frequency of feed access, reducing social competition at the feed bunk, and maintaining a stable ruminal pH (Mattachini et al., 2019; C Souza et al., 2025).

In addition, these technologies provide substantial operational benefits, considerably reducing labour requirements and energy consumption compared with conventional feeding systems, thus contributing to the efficiency and sustainability of modern dairy farming operations (Romano et al., 2023).

b. Automatic mixers and distributors for nutrition optimisation

The quality of a total mixed ration (TMR) depends not only on its composition but also on the homogeneity of the mix, as a non-uniform blend promotes feed sorting and increases the risk of digestive disorders (Chase & Fortina, 2023). In this context, feed pusher robots ensure continuous access to feed by redistributing it along the feed bunk, operating autonomously and completing multiple cycles per day (Tangorra et al., 2024).

Automated TMR preparation and delivery systems integrate mixing and feed distribution functions, enabling ration adjustments based on individual milk yield and the physiological status of the animals (Hun et al., 2023), drawing on data centralised within the farm's digital management platform (Mattachini et al., 2019; Hu et al., 2023). Furthermore, the monitoring of feeding behaviour through video-based systems and artificial intelligence provides detailed information on feed intake and feeding patterns, facilitating the early identification of health problems or adaptation difficulties (Yu et al., 2022).

c. Real-time nutritional analysis of feedstuffs

Variability in feedstuff composition represents a major challenge in ration formulation for dairy cows, as rations based on average values may lead to nutritional imbalances and milk yield losses (Evangelista et al., 2021). The use of near-infrared spectroscopy (NIRS) sensors enables rapid, real-time analysis of feedstuff composition and automatic adjustment of ingredient proportions to maintain optimal nutritive value (Evangelista et al., 2021; Yamada et al., 2024).

The integration of these technologies with in-line milk analysis provides detailed information on the metabolic status of individual cows, including the estimation of blood metabolites without invasive sampling methods, thereby facilitating continuous monitoring of nutritional balance (Giannuzzi et al., 2022). At a more advanced level, machine learning algorithms enable the formulation of individualised rations and nutritional grouping of animals, optimising milk production and reducing the environmental impact of dairy farming (Lee et al., 2025; Hu et al., 2023).

A comparative evaluation of precision feeding technologies highlights both their potential and their current constraints. Automated feeding systems (AFS) and TMR robots reduce labour requirements by an estimated 20-30% compared to conventional feeding and improve feed bunk access through multiple daily deliveries, stabilising ruminal pH and reducing the risk of subacute ruminal acidosis (Romano et al., 2023; Mattachini et al., 2019). NIRS-based real-time feedstuff analysis represents a significant advance over static ration formulation, accounting for natural variability in forage composition that conventional approaches ignore, with improvements in dry matter intake consistency reported in several studies (Evangelista et al., 2021). Nevertheless, the evidence base for fully individualised precision feeding remains limited. Souza et al. (2025) demonstrated that short-term historical production responses were insufficient to inform reliable supplementation decisions at the individual cow level, with neither of two tested precision feeding algorithms generating significant improvements in milk yield, feed efficiency, or income over feed cost compared to a conventional TMR. This finding challenges the assumption that individual cow data automatically translates into better feeding outcomes and underscores the need for more sophisticated data streams and longer training periods. The capital cost of fully integrated

precision feeding systems - including automated mixers, NIRS sensors, and individual cow identification infrastructure - represents a substantial financial barrier for many producers, particularly on small and medium-sized farms (Chase & Fortina, 2023).

C. Automatic milking technologies

Milking is the primary activity in any dairy farming operation and, at the same time, the most demanding in terms of time and labour. Whereas conventional milking was traditionally carried out two to three times per day at fixed hours with the direct involvement of farm staff, modern systems have fundamentally changed this approach (Gislon et al., 2024). The automation of the milking process represents one of the most significant technological transformations in modern dairy farming (Bull et al., 1996). Current systems aim not only to increase process efficiency, but also to improve animal comfort, reduce stress, and enable continuous monitoring of production and health parameters. Figure 3 summarises the main components of automatic milking technology, from animal identification and teat preparation to milk quality monitoring and data management.

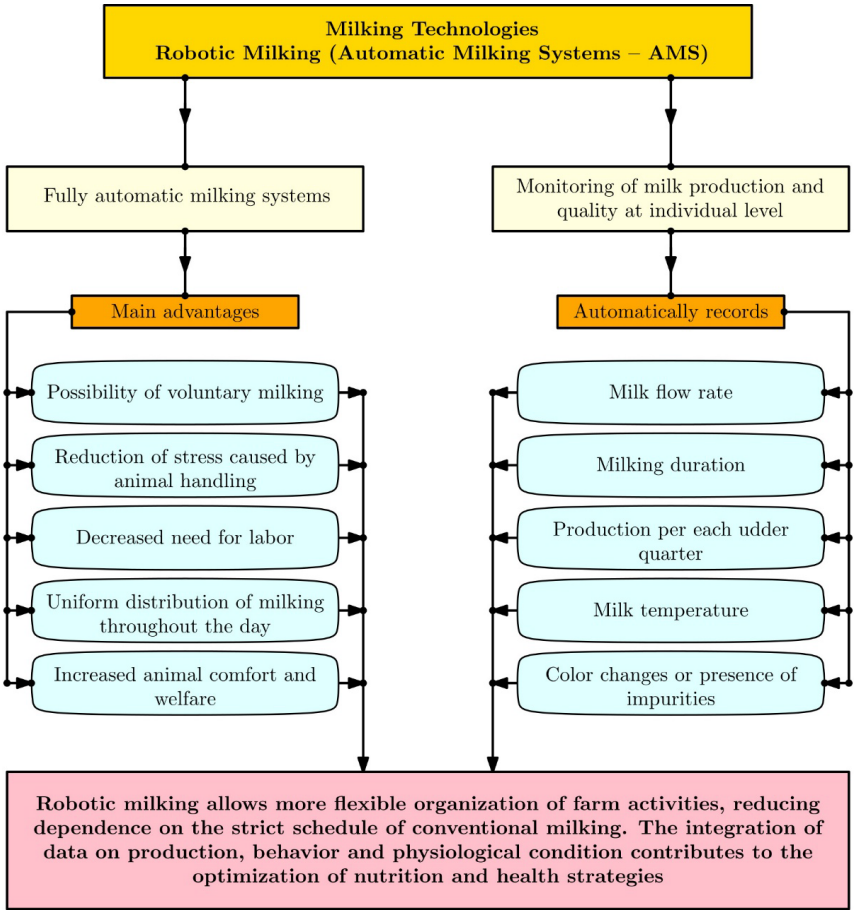


Figure 3. Schematic representation of precision instruments for milking technology
Own source (processed using IPE software)

Automatic milking systems (AMS) allow cows voluntary access to the milking unit, reducing stress and increasing milking frequency, with positive effects on milk yield and animal welfare (Lage et al., 2024). The entire process is fully automated - from animal identification and teat cleaning to teat cup attachment and milking monitoring - carried out without direct human

intervention (Aerts et al., 2022; Wang et al., 2023). Furthermore, cows spend less time waiting and more time resting and feeding, which contributes to reduced stress levels and improved behavioural expression (Lavrijsen-Kromwijk et al., 2024; Lage et al., 2024).

A major advantage of AMS is the continuous, individual monitoring of milk yield and milk quality through parameters such as milk quantity, flow rate, temperature, electrical conductivity, and visual changes in milk appearance (Cogato et al., 2021; Bausewein et al., 2022; Van Steenkiste et al., 2023). These data enable the early detection of mastitis through the correlation of multiple indicators, including a reduction in quarter milk yield and an increase in electrical conductivity, sometimes before the onset of clinical signs (Van Steenkiste et al., 2023; Zhang et al., 2023). Although detection accuracy varies between systems, these technologies significantly reduce the economic losses associated with mastitis (Bausewein et al., 2022; Van Steenkiste et al., 2023).

Furthermore, the integration of AMS data with other farm management systems enables reproductive monitoring, including oestrus detection and the analysis of hormones such as progesterone, contributing to the optimisation of artificial insemination timing and an improvement in conception rates (Jacobs & Siegford, 2012; Pan et al., 2025). In this way, AMS have become a central component of smart dairy farms, combining process automation with data analysis to improve overall herd performance and farm sustainability.

Automatic milking systems offer clear advantages in terms of labour savings, milking frequency, and individual cow monitoring, but a critical appraisal reveals important limitations. On the benefit side, AMS increase milking frequency from the conventional two to three times per day to four or more voluntary visits, which is associated with milk yield increases of 5-15% in cows that adapt readily to the system (Jacobs & Siegford, 2012; Cogato et al., 2021). The continuous recording of milk yield, electrical conductivity, and somatic cell count per quarter enables earlier mastitis detection than is achievable through conventional parlour milking (Bausewein et al., 2022). However, the sensitivity and specificity of AMS-based mastitis detection vary considerably across systems and farms, with reported sensitivity values for clinical mastitis ranging from 46% to 89%, indicating that AMS alone cannot replace structured herd health monitoring programmes (Bausewein et al., 2022). Cow traffic management in AMS systems - whether free, guided, or forced - significantly affects milking frequency, resting time, and social stress, and suboptimal traffic design can negate some of the welfare benefits associated with voluntary milking (Lavrijsen-Kromwijk et al., 2024). The capital investment for a single AMS unit ranges from approximately €100,000 to €180,000, with an additional requirement for adapted barn infrastructure, making the transition economically viable primarily for herds of 60 cows or more per robot under typical European milk pricing conditions (Lage et al., 2024). Furthermore, AMS dependency introduces operational risk: system downtime due to technical failure can rapidly compromise cow welfare, milk yield, and udder health in the absence of adequate contingency protocols.

D. Housing and environment management

Housing conditions have a direct impact on the health, welfare, and milk yield of dairy cows, as inadequate environments lead to reduced dry matter intake, decreased milk production, and an increased incidence of disease (Leliveld & Provolo, 2020). Consequently, modern livestock buildings are integrated into digital management systems and continuously optimised through technologies similar to those used for individual animal monitoring (Papakonstantinou et al., 2024).

Heat stress represents a major challenge in dairy cattle production, negatively affecting milk yield, fertility, and longevity, but can be mitigated through automated ventilation and cooling systems adapted to environmental conditions (Mihai et al., 2020). At the same time, the quality of flooring and resting areas influences the incidence of lameness, directly contributing to the maintenance of animal health and the reduction of premature culling from the herd (Losacco et al., 2025). Figure 4 presents the precision instruments deployed for housing management, covering climate control, environmental sensing, and resting area monitoring.

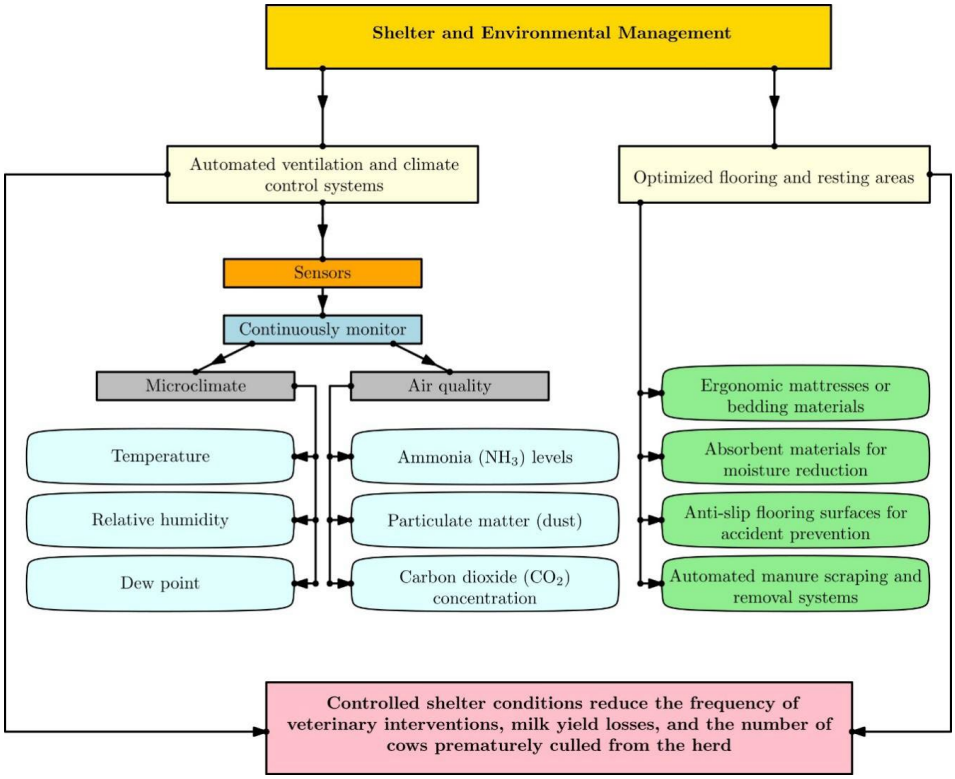


Figure 4. Schematic representation of precision instruments for housing management
Own source (processed using IPE software)

a. Automatic ventilation and climate control systems

Automatic ventilation and climate control systems are essential for maintaining animal health and milk quality, as poor air quality and elevated temperatures reduce dry matter intake and predispose animals to heat stress (Leliveld & Provolo, 2020). Heat stress risk is assessed using the temperature-humidity index (THI), where values above 72 indicate the onset of heat stress and values above 80 indicate severe effects on animal performance (Neves et al., 2022).

The integration of temperature and humidity sensors enables real-time monitoring and the automatic activation of ventilation and cooling systems when critical thresholds are exceeded (Provolo et al., 2025). Although natural ventilation is commonly used, modern solutions - such as high-volume low-speed (HVLS) fans and evaporative cooling systems - significantly reduce body temperature and physiological heat stress in dairy cows (Oliveira et al., 2025; Fournel et al., 2017).

Furthermore, the combination of microclimatic data with behavioural data from individual animals enables the precise identification of cows most affected by heat stress and the tailoring of cooling interventions accordingly, contributing to more efficient and individualised housing management (Fournel et al., 2017; Mylostyvyi & Izhboldina, 2025).

b. Optimised flooring and resting areas

The comfort of resting areas is essential, as dairy cows spend 10 to 14 hours per day in recumbency, a period that supports milk synthesis and limb health (Leliveld & Provolo, 2020). Inadequate surfaces or poorly dimensioned cubicles reduce lying time and increase the risk of integument injuries and lameness (EFSA AHAW et al., 2023).

Appropriate bedding materials, such as deep sand or rubber mats, improve resting comfort and reduce the incidence of limb disorders,

whereas slippery or abrasive flooring surfaces increase the risk of injuries, underscoring the importance of sound barn design (Andreasen & Forkman, 2012; Weerasinghe et al., 2021; B Sadiq et al., 2017).

c. Real-time environmental monitoring

Modern environmental monitoring systems in dairy farms employ IoT-based architectures in which sensors continuously collect data on microclimatic conditions and air quality and transmit them in real time to digital management platforms. These systems provide accurate and reliable long-term information, facilitating continuous surveillance of housing conditions (Leliveld et al., 2024).

The effectiveness of these technologies increases substantially when environmental data are correlated with individual behavioural indicators. For example, associating an increase in the temperature-humidity index (THI) with a reduction in feeding time or changes in resting patterns enables the rapid identification of heat stress and the automatic activation of corrective measures, reducing the rate of false alarms (Leliveld & Provolo, 2020; Leliveld et al., 2024).

The integration of environmental, behavioural, production, and health data within a single digital platform transforms monitoring from a simple measurement tool into an active decision-support system, contributing to the optimisation of farm management and the improvement of animal welfare (Tangorra et al., 2024).

A critical evaluation of housing and environmental management technologies reveals a clear gradient between the effectiveness of individual components and the complexity of fully integrated systems. Automated ventilation and cooling systems - particularly high-volume, low-speed (HVLS) fans combined with evaporative cooling - have demonstrated significant reductions in body temperature and physiological heat stress indicators, with reported improvements in milk yield of 4-8% under heat stress conditions when cooling is activated at THI thresholds above 68-72 (Oliveira et al., 2025; Fournel et al., 2017). Real-time THI monitoring through IoT sensor networks improves the timeliness and precision of cooling interventions relative to fixed timer-based systems, reducing unnecessary energy consumption and associated operating costs (Provolo et al., 2025). In terms of flooring and resting area design, deep sand bedding consistently outperforms rubber mats and concrete in reducing lameness prevalence and integument lesions, but its operational costs - in terms of sand procurement, manure separation, and maintenance - are substantially higher than those associated with alternative bedding materials (Andreasen & Forkman, 2012; Weerasinghe et al., 2021). A key limitation of current environmental monitoring systems is that most commercial solutions remain primarily descriptive: they report microclimatic conditions but lack validated decision rules that automatically translate environmental alerts into specific management actions. The integration of environmental data with individual animal behavioural data, while technically feasible, requires substantial investment in sensor infrastructure, data management platforms, and staff training, and its cost-effectiveness has not been rigorously evaluated across different farm types and climatic regions (Leliveld et al., 2024).

E. Reproductive management technologies

Reproductive performance is one of the most important economic drivers in dairy farming. Undetected oestrous events and extended calving to conception intervals generate significant economic losses and contribute to the premature culling of cows from the herd. In modern dairy cows, the oestrous period lasts on average 12 to 18 hours and occurs frequently at night, when farm staff are not present, resulting in a visual detection rate of only 34 to 40% under conventional observation (Das et al., 2023). Each missed oestrus represents a delay of 21 days until the next insemination opportunity (Adenuga et al., 2020). In modern dairy operations, digital technologies and automated systems enable precise monitoring of the reproductive cycle and the optimisation of artificial insemination programmes. Figure 5 illustrates the precision instruments used in reproductive management, from oestrus detection sensors to progesterone monitoring systems and reproductive decision-support platforms.

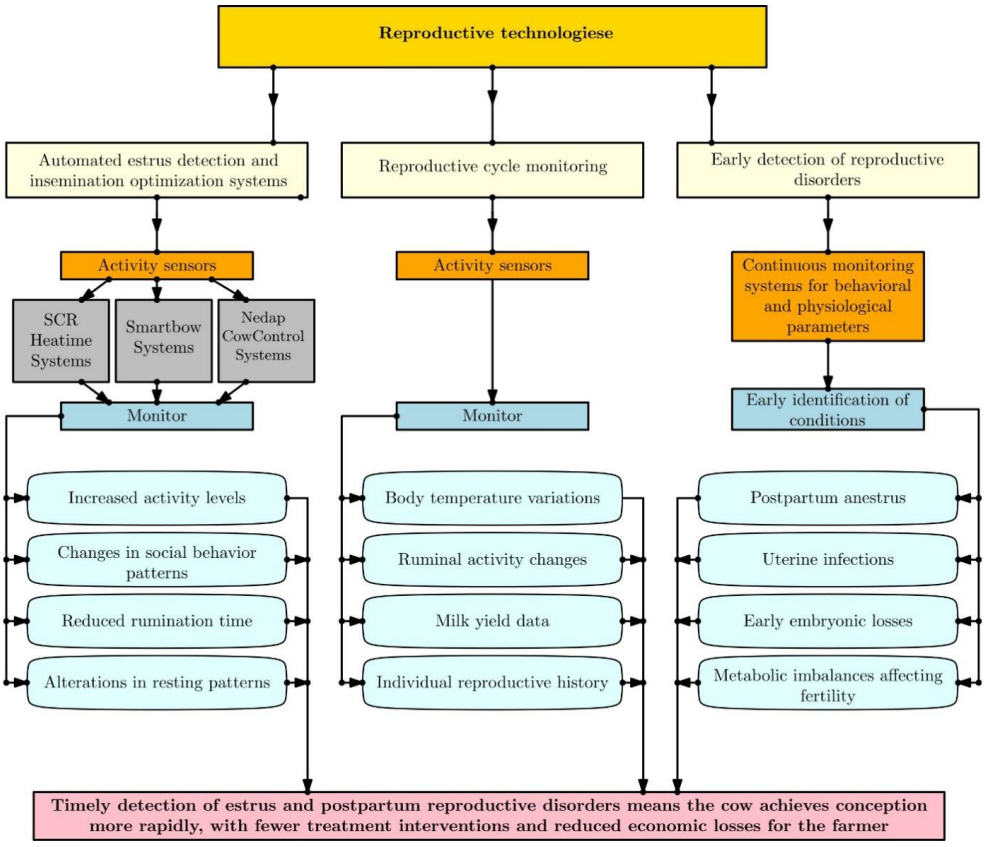


Figure 5. Schematic representation of precision instruments for reproductive management technology
Own source (processed using IPE software)

a. Automatic oestrus detection systems and insemination optimisation

Automatic oestrus detection systems use activity sensors - accelerometers mounted on collars or ear tags - to continuously monitor cow movement and compare it against each animal's individual baseline pattern. A sudden increase in locomotor activity, associated with a concurrent reduction in rumination time, automatically triggers an oestrus alert, regardless of the time of day (Antanaitis et al., 2020).

Systems such as SCR Heatime, Nedap CowControl, and Smartbow achieve a sensitivity of 83 to 93% and a specificity of 92 to 97%, contributing to an improvement in the pregnancy rate at 200 days in milk from 57% to 67-69% compared with visual observation (Schilkowsky et al., 2021).

For maximum reproductive efficiency, artificial insemination should be performed 11 to 16 hours after the onset of the detected oestrus, and these systems can automatically generate alerts indicating the optimal insemination window (Várhidi et al., 2024).

b. Reproductive cycle monitoring and early detection of reproductive disorders

Approximately 50% of dairy cows exhibit irregular ovarian cycles in the early postpartum period, and anovulatory anoestrus extending beyond 60 days significantly increases the calving-to-conception interval (Das et al., 2023). In this context, the Herd Navigator system (DeLaval) monitors milk progesterone concentration at each milking, estimating the resumption of ovarian cyclicity, the timing of oestrus, and gestational status, with an oestrus detection accuracy of 95 to 97% and conception rates of 42 to 50%, compared with 20 to 30% achieved through conventional methods (Adenuga et al., 2020; Antanaitis et al., 2020).

A further concern is represented by postpartum uterine diseases, which affect up to 40% of dairy

cows and reduce fertility by approximately 20% (Várhidi et al., 2024). Automated monitoring systems can detect metritis at an early stage through changes in rumination time, locomotor activity, and body temperature, occurring 1 to 3 days before the appearance of clinical signs (Zhou et al., 2022). Nguyen (2025), based on a study conducted on a herd of 3,747 cows equipped with SCR sensors, confirmed that automated alerts enable the early identification of genital tract infections and prompt veterinary intervention.

By integrating behavioural, hormonal, and reproductive data within a single digital platform, reproductive management becomes predominantly preventive in nature, reducing the interval to conception and improving the long-term reproductive sustainability of the herd (Das et al., 2023).

A critical appraisal of automated reproductive management technologies highlights both substantial performance gains and important implementation challenges. Activity-based oestrus detection systems achieve sensitivity values of 83-93% and specificity values of 92-97%, representing a marked improvement over visual observation rate of 34-40% (Schilkowsky et al., 2021; Das et al., 2023). When combined with progesterone-based cycle monitoring through systems such as Herd Navigator, conception rates of 42-50% have been reported, compared with 20-30% achieved through conventional reproductive management, with a corresponding reduction in the calving to conception interval and in the number of open days per cow (Adenuga et al., 2020; Antanaitis et al., 2020). These improvements translate directly into economic benefit: each additional open day costs a dairy farm an estimated €3-5 per cow, meaning that even modest reductions in calving-to-conception intervals generate measurable returns on investment (Adenuga et al., 2020). However, the cost of progesterone monitoring systems - which require dedicated milk sampling at each milking and specialised analytical equipment – is substantially higher than that of activity-based systems alone, and their cost-effectiveness depends on herd size and the baseline reproductive performance of the farm. A further limitation is that automated systems perform less reliably in cows with prolonged postpartum anoestrus, high body condition score losses, or concurrent subclinical health conditions, precisely the animals in which accurate reproductive monitoring is most needed (Das et al., 2023; Várhidi et al., 2024). Integration of reproductive data with health and nutritional platforms remains technically challenging and is not yet consistently achieved in commercial farm management software.

F. Limitations and challenges of PLF technologies

Despite the demonstrated benefits of PLF technologies, their widespread adoption is constrained by a set of persistent limitations. The initial investment costs for integrated sensor systems, automatic milking units, and digital management platforms remain high, limiting uptake particularly on small and medium sized farms. Furthermore, return on investment depends heavily on herd size, milk price, and the technical capacity of farm staff, making cost benefit assessments highly context specific (Adenuga et al., 2020; Romano et al., 2023).

A major research gap remains the lack of standardised data formats and measurement protocols across precision livestock farming technologies, limiting cross platform comparability and large-scale data integration. Limited interoperability between PLF systems from different manufacturers remains a key barrier to the effective integration of multi-source data for decision-making in dairy farming. The absence of universal communication standards means that data from milking systems, wearable sensors, and environmental monitors frequently cannot be consolidated without significant technical effort (Kaur et al., 2023; Tangorra et al., 2024).

Alert interpretation also presents challenges: false positive rates in disease detection remain non-negligible, and the effectiveness of automated alerts depends on the training and responsiveness of farm personnel. Machine learning algorithms developed on data from specific breeds or management systems may show reduced accuracy when transferred to different farm contexts, underscoring the need for broader validation studies (Dharejo et al., 2025; Stygar et al., 2021). Reliance on digital connectivity further introduces vulnerability in areas with limited network infrastructure, where

system downtime can result in data loss and delayed interventions

CONCLUSIONS

Precision livestock farming (PLF) technologies applied in dairy farming operations now cover all key aspects of dairy cattle management - animal health, nutrition, milking, housing conditions, and reproduction - delivering measurable outcomes in each of these domains. Their true value, however, emerges not when individual technologies are used in isolation, but when they are integrated within a shared platform that correlates data from multiple sources and supports better management decisions for each individual animal.

The adoption of these technologies contributes to improvements in milk yield and milk quality, a reduction in the incidence of stress-related conditions and disease, more efficient use of farm inputs, and a lower environmental impact. At the same time, their implementation presents challenges related to initial investment costs, staff training, equipment dependency, and the management of large volumes of data.

The main limitations of these technologies remain the high cost of implementation, the need for validation under diverse farm conditions, and reliance on digital connectivity. The integration of data from multiple sources also raises challenges related to platform compatibility and the training of personnel in alert interpretation. Future developments are expected to include the wider application of machine learning algorithms for individual-level disease prediction, the integration of genomic selection data with real-time physiological measurements, and the extension of the digital twin concept to the level of the dairy herd. The dairy farm of the future will not necessarily be larger or more automated - it will, above all, be better connected, with every management decision supported by objective, individual-animal data.

For farmers, the practical implication is that technology adoption should be approached incrementally and strategically: starting with systems that address the most economically significant pain points, mastitis detection, oestrus identification, heat stress management, before expanding towards fully integrated platforms. Staff training and change management are as critical as the technical infrastructure itself. For researchers, the priority should be the development of cross-platform validation studies, standardised welfare outcome metrics, and economic models that account for the diversity of farm sizes and management contexts across different regions.

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PRECISION LIVESTOCK FARMING IN CATTLE SYSTEMS: PRODUCTIVITY AND SUSTAINABILITY

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Abstract

Precision Livestock Farming (PLF) is an emerging field at the interface of animal science and agricultural engineering, enabling sensor-based monitoring and data-driven management in cattle production. This review synthesises current evidence on PLF applications in dairy and beef systems, focusing on welfare and behaviour, health surveillance (mastitis), reproduction, body condition assessment, productivity, extensive/pasture-based management, and environmental implications. A qualitative literature review was conducted using peer-reviewed papers retrieved from ScienceDirect, PubMed, Scopus, Web of Science, Frontiers, and Google Scholar. Overall, findings indicate that PLF enhances herd performance by enabling continuous monitoring of behavioural and physiological indicators, supporting earlier detection of subclinical disorders, improved reproductive event identification, and objective phenotyping through automated body weight and body condition scoring. Evidence also suggests improved milk and beef production through increased biological efficiency, optimised grazing allocation in extensive systems, and improved estimation of enteric methane emissions. These insights support the adoption of PLF decision-support tools to optimise feeding, health interventions, and pasture management, thereby increasing productivity and supporting sustainability targets.

Key words*: animal welfare, beef cattle, dairy cattle, precision livestock farming, sensor technologies.*

INTRODUCTION

The global livestock sector is undergoing profound transformations driven by population growth, increasing demand for animal-derived products, rising production costs, labor shortages, and intensified societal concerns regarding animal welfare and environmental sustainability. Within this context, cattle production systems, encompassing both dairy and beef sectors, remain fundamental to global food security and rural economies, while also representing a significant share of agricultural land use and greenhouse gas emissions (Banhazi et al., 2012; Lovarelli et al., 2020). Traditional livestock management approaches, largely based on visual observation and group-level decision-making, are increasingly inadequate in modern farming systems characterized by expanding herd sizes and reduced direct human-animal interaction (Caja et al., 2016; Halachmi et al., 2019). Moreover, evidence from other livestock sectors suggests that production conditions may influence product quality traits, including nutritional and sensory characteristics, highlighting the potential for value-added animal-derived products (Bonneau & Lebret, 2010; Lungu et al., 2024, Zinca et al., 2025).

Technological innovation has been identified as a key pathway for addressing these challenges and improving the resilience and efficiency of livestock production systems. In this regard, Precision Livestock Farming (PLF) has emerged as an innovative management paradigm that aims to support farmers through continuous, automated, and objective monitoring of individual animals (Banhazi et al., 2012; Caja et al., 2016; Kelemen et al., 2016). PLF relies on the integration of sensor

technologies, Internet of Things (IoT) devices, automated data acquisition systems, and advanced data processing techniques to generate real-time information on animal behavior, physiology, production, and environmental interactions (Halachmi et al., 2019; Lovarelli et al., 2020). By shifting management from a group-based to an individual-animal-centered approach, PLF enables earlier detection of deviations from normal biological patterns and facilitates timely, targeted interventions. One of the principal motivations for the development and implementation of PLF technologies is the need to enhance animal health and welfare while maintaining or increasing production efficiency. Continuous monitoring of behavioral and physiological indicators allows for the early identification of health disorders, often before the appearance of clinical signs, thereby reducing disease severity, treatment costs, and productivity losses (Neethirajan, 2017; Halachmi et al., 2019). Furthermore, PLF systems provide objective welfare indicators, supporting improved transparency and compliance with increasing societal and regulatory expectations related to animal well-being (Caja et al., 2016; Lovarelli et al., 2020). These capabilities are particularly relevant in cattle production, where subtle changes in activity, rumination, or social behavior may precede health or welfare impairments.

Beyond animal health and welfare, PLF has been widely recognized for its potential contribution to sustainable cattle management. Optimized feeding strategies, improved reproductive efficiency, and enhanced production monitoring can lead to more efficient use of resources and a reduction in the environmental footprint per unit of output (Halachmi et al., 2019; Lovarelli et al., 2020). The large volumes of data generated by PLF systems also enable evidence-based decision-making and support the evaluation of farm performance from both economic and environmental perspectives (Banhazi et al., 2012; Caja et al., 2016). Consequently, PLF is increasingly viewed as an enabling technology for achieving sustainability objectives within livestock production systems.

Most scientific research and commercial applications of PLF have focused on intensive dairy production systems, where indoor housing and automated milking technologies facilitate sensor deployment and data collection (Halachmi et al., 2019; Lovarelli et al., 2020). In contrast, the adoption of PLF in beef cattle and extensive or semi-extensive systems has progressed more slowly, largely due to challenges related to infrastructure availability, sensor robustness, data transmission, and economic feasibility (Caja et al., 2016; Neethirajan, 2020). Nevertheless, recent studies demonstrate that adapted PLF solutions can be successfully implemented in extensive environments, providing valuable insights into animal health, grazing behavior, and welfare status, and highlighting their relevance beyond intensive dairy systems (Caja et al., 2016; Neethirajan, 2020).

Despite the expanding body of literature, existing studies often address isolated PLF applications or specific production systems, resulting in a fragmented understanding of the overall potential and limitations of precision technologies in cattle farming (Banhazi et al., 2012; Halachmi et al., 2019). Moreover, the rapid pace of technological development necessitates continuous synthesis of current knowledge to identify research gaps, methodological challenges, and future research priorities. An integrated review encompassing both dairy and beef cattle systems, as well as intensive and extensive production contexts, is therefore essential to provide a comprehensive overview of the current state of PLF and its role in sustainable cattle management (Caja et al., 2016; Lovarelli et al., 2020). However, despite these advances, important challenges remain, including variability in system performance under commercial conditions, data integration issues, and limited adoption by farmers due to economic and practical constraints.

The objective of this review is to critically synthesize and evaluate recent scientific literature on Precision Livestock Farming in cattle production systems, including both dairy and beef sectors, with particular emphasis on technological approaches, applications in animal health, welfare, reproduction, and production, and their contribution to sustainable livestock management.

MATERIALS AND METHODS

In order to achieve the objectives of this review, a total of specialised scientific papers focusing on Precision Livestock Farming (PLF) in cattle systems were analysed. The selected literature covered the main PLF application domains in dairy and beef cattle, including welfare and behavioural monitoring, health management (with emphasis on mastitis), reproductive monitoring, body condition assessment, productivity-related outcomes, extensive/pasture-based systems, and environmental implications.

The publications were retrieved from major international scientific databases and platforms, namely ScienceDirect, PubMed, Scopus, Web of Science, Frontiers, and Google Scholar, using relevant keywords and combinations (e.g., precision livestock farming, dairy cattle, beef cattle, sensors, mastitis detection, oestrus detection, GPS monitoring, body condition score, and emissions). The literature search focused primarily on studies published in the last 10-15 years, with emphasis on recent advances in PLF technologies. Following the literature screening, the analysis was conducted through qualitative synthesis, based primarily on observation and thematic grouping. The results were subsequently structured into key sections reflecting the major biological and managerial functions targeted by PLF technologies, thereby providing an integrated overview of their role in improving productivity, welfare, and sustainability in cattle production systems.

RESULTS AND DISCUSSIONS

Welfare and Behaviour Monitoring

Welfare and behaviour monitoring represents one of the most mature and widely applied domains of Precision Livestock Farming (PLF), as behavioural changes frequently precede clinically detectable health disorders and declines in productivity. The analysed literature consistently shows that PLF enables continuous, objective, and individual-level assessment of cattle behaviour, overcoming the subjectivity and limited temporal resolution of conventional welfare evaluation based on periodic visual inspection (Mirzaei et al., 2013; Caja et al., 2016). Core behavioural indicators, including lying, resting, rumination, activity, and vocalisation, are repeatedly identified as reliable proxies of animal welfare across different production systems.

In dairy calves, lying and resting behaviour dominate early life stages and are closely associated with comfort and housing quality, while deviations from expected behavioural patterns are linked to stress and impaired welfare (Pesenti Rossi et al., 2024). In adult dairy cows, reductions in rumination time and alterations in activity patterns were consistently observed during periods of physiological challenge, particularly around parturition, enabling earlier identification of animals at risk compared with traditional monitoring approaches (Mirzaei et al., 2023). These findings underline the value of behavioural metrics as early-warning indicators of compromised welfare.

Wearable sensors, particularly accelerometer- and rumination-based systems, are widely reported as effective tools for behavioural monitoring, providing high temporal resolution and continuous data streams (Mirzaei et al., 2023; Pesenti Rossi et al., 2024). Complementary non-contact approaches further extend PLF capabilities. Acoustic monitoring based on vocalisation analysis demonstrated high accuracy in identifying behaviourally relevant events, suggesting potential applications beyond reproduction, including social behaviour and discomfort detection (Wang et al., 2023). Similarly, computer vision approaches using deep learning models enabled automated recognition of postures and activities without physical contact, offering clear welfare advantages by reducing animal disturbance and device-related constraints (Sim et al., 2024). However, despite these advantages, several studies highlight important limitations of behavioural monitoring systems. Sensor accuracy may be affected by environmental conditions, animal variability, and device placement, leading to inconsistencies in data quality under commercial farm conditions. In addition, wearable devices may require regular maintenance and calibration, which can limit their practical applicability, particularly in

large-scale or extensive systems (Berckmans, 2014).

Although implementation differs between intensive and extensive systems, the overall evidence indicates that PLF substantially improves welfare monitoring in both contexts. Controlled environments in intensive dairy systems facilitate higher accuracy, while in extensive systems PLF enables continuous assessment of activity, grazing behaviour, and spatial distribution that is otherwise difficult to achieve (Caja et al., 2016). Nevertheless, the effectiveness of PLF technologies is not uniform across studies. While some research reports high accuracy and reliability of sensor-based behavioural monitoring, other studies indicate moderate performance, particularly in complex or variable environments, suggesting that system performance is highly context-dependent (Banhazi et al., 2012; Berckmans, 2017). Collectively, the literature converges on the conclusion that PLF-based behavioural monitoring enhances welfare assessment through improved objectivity, earlier detection of welfare-related deviations, and greater capacity for proactive management interventions (Caja et al., 2016). From a practical perspective, these findings suggest that farmers can use PLF systems to monitor animal behaviour more efficiently, detect welfare issues at an early stage, and support timely management decisions, potentially reducing labour requirements and improving overall herd management (Lovarelli et al., 2020).

Health Monitoring: Mastitis Detection

Mastitis is one of the most important diseases affecting dairy cattle, generating substantial economic losses and negatively impacting milk quality, animal welfare and farm sustainability (Stanek et al., 2024). In addition, the microbiological quality of dairy products remains a relevant concern along the production chain, as contamination may affect both product safety and marketability (Şuler et al., 2021). Conventional diagnostic approaches, based on clinical observation and periodic somatic cell count (SCC) measurements, are often reactive and insufficient for early detection, particularly in subclinical cases. Precision Livestock Farming (PLF) technologies provide a data-driven alternative by enabling continuous, automated monitoring of udder health.

A comparative overview of conventional and PLF-based mastitis detection approaches is presented in Table 1.

Table 1. Comparison between conventional and PLF-based mastitis detection approaches in dairy cattle

Evaluated aspect	Conventional mastitis detection	PLF-based mastitis detection
Monitoring type	Periodic, reactive	Continuous, proactive
Data collection	Manual observation, periodic milk sampling	Automated, real-time sensor data
Main indicators	Clinical signs, laboratory SCC	Online SCC, electrical conductivity, milk yield, milk composition
Detection of subclinical mastitis	Limited	High
Response time	Delayed	Early
Diagnostic accuracy	Moderate	High (especially with multi-sensor integration)
Decision support	Farmer experience-based	Data-driven, algorithm-supported
Antibiotic use	Often empirical	Targeted, reduced
Labor requirement	High	Reduced
Impact on animal welfare	Limited	Positive
Suitability for large herds	Low	High

As shown in Table 1, PLF-based systems provide clear advantages over conventional methods, particularly in terms of continuous monitoring, earlier detection, and data-driven decision support. However, improvements in diagnostic accuracy are strongly dependent on the integration of multiple indicators, as single-parameter approaches may produce inconsistent results (Rutten et al., 2013).

The literature consistently identifies SCC as the most reliable indicator of intramammary inflammation, reflecting the immune response of the mammary gland (Pan et al., 2025). However, single-parameter monitoring systems frequently show limited diagnostic accuracy. Milk electrical conductivity, although widely used in automated milking systems, is influenced by several non-pathological factors, resulting in variable performance for mastitis detection (Rukmana et al., 2021). As a result, recent studies emphasize multi-sensor approaches that integrate SCC, milk yield deviations, electrical conductivity and milk

composition parameters. Nevertheless, despite the generally positive results, variability in diagnostic performance has been reported across studies. While several studies report high accuracy of multi-sensor systems for mastitis detection, others highlight an increased rate of false positives, particularly when relying on single indicators such as electrical conductivity. This inconsistency suggests that detection performance is influenced by herd-specific conditions, environmental factors, and sensor calibration, emphasizing the need for context-specific validation of PLF systems (Banhazi et al., 2012; Rutten et al., 2013).

The application of machine learning techniques has significantly improved mastitis detection performance. By modeling complex, nonlinear relationships among multiple indicators, machine learning-based systems consistently outperform traditional threshold-based methods, achieving higher sensitivity and specificity, particularly for subclinical mastitis (Pan et al., 2025). In parallel, fuzzy inference systems have demonstrated satisfactory detection accuracy using a limited number of milk parameters, offering a cost-effective solution for on-farm decision support (Rukmana et al., 2021).

Overall, the literature converges on the conclusion that PLF-based mastitis monitoring enables earlier detection, reduces unnecessary antibiotic use, supports targeted treatment strategies and improves udder health management. From a practical perspective, these findings suggest that farmers can use PLF systems to optimize health monitoring strategies, reduce veterinary costs through early disease detection, and improve treatment decision-making, ultimately enhancing farm profitability (Steeneveld et al., 2015; Lovarelli et al., 2020). Despite remaining challenges related to sensor accuracy, data integration and economic feasibility, PLF technologies represent a key advancement toward proactive, welfare-oriented and sustainable mastitis control in modern dairy farming (Stanek et al., 2024).

Reproduction Monitoring in Cattle Using Precision Livestock Farming

Efficient reproductive management is essential for productivity and economic sustainability in cattle systems, yet conventional monitoring based on visual observation is increasingly unreliable due to reduced estrus expression and limited labor availability (Mottram, 2015). Precision Livestock Farming (PLF) technologies provide an effective alternative by enabling continuous, automated monitoring of behavioural and physiological indicators associated with reproductive events (Neethirajan, 2017). Activity-based monitoring using accelerometers is the most widely adopted PLF approach for estrus detection, as estrus is typically accompanied by increased locomotor activity and restlessness (Mottram, 2015; Tullo et al., 2019). These systems consistently outperform traditional observation, particularly in intensive dairy systems, although false positives may occur due to non-reproductive activity changes (Mottram, 2015). The integration of accelerometer data with other behavioural indicators, such as rumination and lying behaviour, combined with advanced data processing and machine learning techniques, has been shown to improve detection robustness and adaptability across management contexts (Tullo et al., 2019).

PLF technologies are also increasingly applied to calving prediction, a critical aspect of reproductive management. Behavioural changes, including increased tail movement and altered activity patterns, intensify during the hours preceding parturition and can be detected using automated sensors (Szenci, 2022; Sedó et al., 2024). Although prediction accuracy varies due to individual and environmental factors, these systems enable earlier intervention compared with conventional monitoring, contributing to reduced calving-related risks. An overview of the main PLF technologies used for reproductive monitoring, including estrus detection and calving prediction, is illustrated in Figure 1.



Figure 1. Precision Livestock Farming (PLF) technologies for reproductive monitoring in dairy cattle: estrus detection and calving prediction (created with https://biorender.com)

As shown in Figure 1, PLF systems integrate multiple behavioural and physiological indicators to support the identification of reproductive events. This multi-sensor approach enhances detection reliability compared with single-indicator systems, although performance remains dependent on data quality and farm-specific conditions (Norton & Berckmans, 2017; Lovarelli et al., 2020) Evidence from the reviewed studies suggests that PLF-based reproductive monitoring is associated with improved accuracy in detecting reproductive events while reducing reliance on manual observation. The findings indicate that combining multiple physiological and behavioral indicators through data-driven approaches enables earlier and more reliable identification of reproductive status compared to conventional monitoring methods (Rutten et al., 2013). Nevertheless, the effectiveness of these systems is not consistent across all studies. While some research reports high detection accuracy for estrus and calving prediction, other studies highlight moderate performance and the occurrence of false positives, particularly under variable environmental conditions or in herds with high behavioural variability. This suggests that predictive performance is influenced by both animal-related and management-related factors, requiring further validation under commercial farming conditions (Wathes et al., 2008; Banhazi et al., 2012). At the same time, the literature highlights that individual variability constrains the predictive performance of any single indicator used in isolation, supporting the added value of multi-sensor integration for more proactive and welfare-oriented reproductive management (Mottram, 2015; Tullo et al., 2019; Szenci, 2022). From a practical perspective, these findings suggest that farmers can use PLF systems to improve estrus detection accuracy, optimize breeding timing, and better anticipate calving events, thereby reducing reproductive losses, improving herd fertility, and enhancing overall farm efficiency (Steeneveld et al., 2015).

Body Weight, Body Condition Score, and Conformation Assessment

Body weight, body condition score (BCS), and body conformation are key indicators of animal health, nutritional status, productivity, and welfare, playing a central role in management and breeding decisions (Wathes et al., 2008; Berckmans, 2017). Conventional assessment methods based on manual weighing and visual scoring are often labor-intensive, subjective, and may induce stress, particularly when

applied repeatedly or at large scale. These limitations have driven the adoption of automated approaches within Precision Livestock Farming (PLF) systems.

Recent evidence shows that computer vision–based technologies enable accurate and repeatable estimation of body weight and morphological traits using 2D imaging, depth sensors, and 3D reconstruction techniques (Dohmen et al., 2022; Wang Y. et al., 2023). The extraction of body dimensions and shape-related features allows continuous phenotypic monitoring under commercial conditions, reducing animal handling and increasing the temporal resolution of growth and condition assessment compared with conventional methods. Automated body condition scoring has similarly benefited from PLF-based imaging systems. An overview of PLF-based approaches for body weight, body condition score, and conformation assessment is presented in Figure 2. This is particularly relevant in extensive or large-scale production systems where routine manual evaluation is impractical.

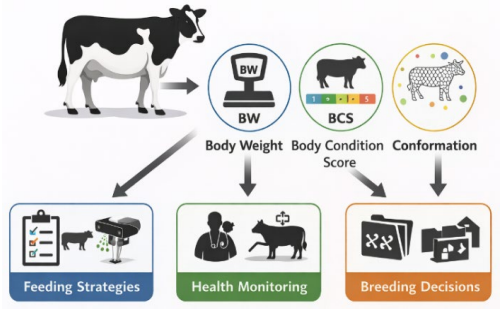


Figure 2. Precision phenotyping in cattle using body weight, body condition score and conformation traits to support feeding, health monitoring and breeding decisions (created with https://biorender.com)

As shown in Figure 2, geometric and surface features derived from dorsal or lateral views can be linked to BCS using machine learning and deep learning models, helping reduce observer-related variability and enabling earlier detection of suboptimal nutritional or metabolic conditions (Bewley et al., 2008; Halachmi et al., 2008; Song et al., 2019). However, the accuracy of these systems depends on image quality, animal positioning, and algorithm robustness, which may limit their performance under commercial farm conditions. At herd level, the integration of body weight and conformation data into PLF platforms supports dynamic monitoring of growth patterns, body reserve mobilization, and phenotypic variability across production stages (Martins et al., 2020; Wang Y. et al., 2023). At the same time, variability in system performance has been reported across studies. While some research demonstrates high accuracy and repeatability of computer vision–based methods, other studies highlight limitations related to model generalization and sensitivity to environmental conditions, suggesting that further validation is required before large-scale implementation (Norton & Berckmans, 2017; Dohmen et al., 2022). Overall, the synthesized results indicate a shift from sporadic, manual evaluation toward continuous and objective phenotyping, supporting more precise, welfare-oriented livestock management, despite ongoing challenges related to sensor calibration and model generalization (Norton & Berckmans, 2017). From a practical perspective, these technologies enable farmers to monitor animal condition more frequently and objectively, improve feeding management, and support timely interventions, thereby enhancing productivity and animal welfare.

Productivity Outcomes in Dairy and Beef Systems

The reviewed evidence indicates that the implementation of Precision Livestock Farming (PLF) technologies is associated with improved productive performance in both dairy and beef cattle systems, as illustrated in Figure 3, which highlights the main pathways through which continuous monitoring and data-driven decision-making contribute to enhanced productivity. Sensor-based, individual-level monitoring enabled more precise and timely management interventions, resulting in reduced performance variability and enhanced production consistency across herds (Lokhorst et al., 2019; Lovarelli et al., 2020).

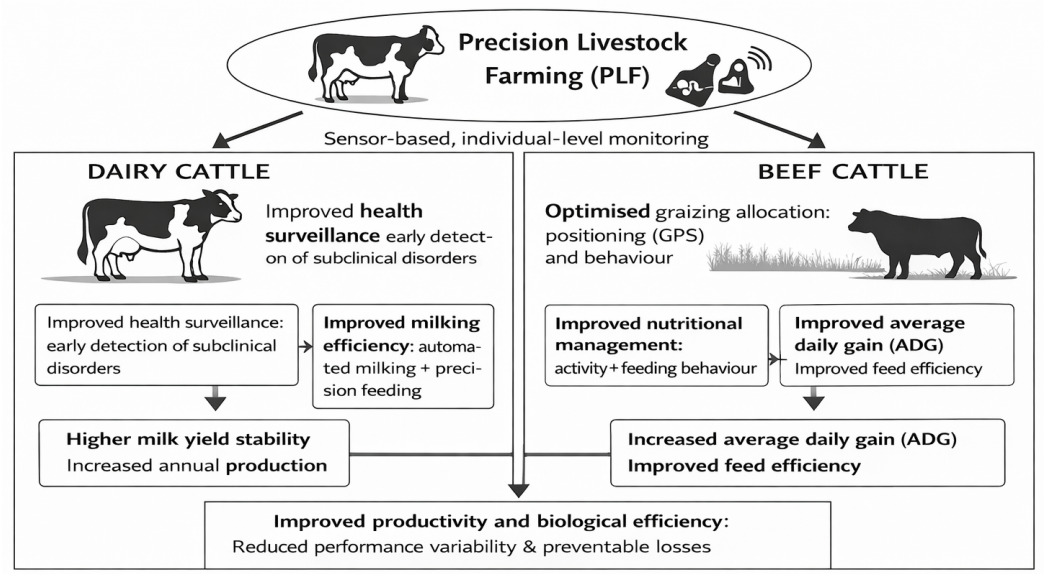


Figure 3. Precision Livestock Farming (PLF) pathways to improved productivity in dairy and beef cattle (created with https://biorender.com)

As shown in Figure 3, productivity improvements are primarily driven by the integration of continuous monitoring with timely, data-based management interventions at individual animal level. In dairy cattle, PLF adoption was primarily linked to increased milk yield stability and annual production per cow, largely through improved health management and milking efficiency. Continuous monitoring of rumination, activity, and milking behavior facilitated early detection of subclinical disorders, thereby limiting milk yield losses and improving lactation persistence. Automated milking and precision feeding systems further contributed to higher cumulative milk output under PLF-supported management strategies (Rutten et al., 2013; Steeneveld et al., 2015).

In beef production systems, PLF technologies were associated with improvements in growth performance, particularly through increased average daily gain and improved feed efficiency. Behavioral and positional monitoring supported optimized grazing and nutritional management, leading to more uniform growth and enhanced meat production. These effects suggest that PLF contributes to improved biological efficiency and resource utilization in beef cattle systems (González et al., 2015; Greenwood et al., 2017). At the same time, the magnitude of productivity gains varies across studies. While some research reports substantial improvements in milk yield and growth performance, other studies indicate more moderate benefits, particularly in systems where baseline management is already optimized. This variability suggests that the effectiveness of PLF technologies is influenced by farm-specific conditions, management practices, and the level of technology integration (Banhazi et al., 2012; Lovarelli et al., 2020).

Collectively, the findings support the interpretation that PLF enhances milk and beef production by improving biological efficiency and preventing avoidable performance losses. Productivity gains appear to result from the integration of continuous monitoring with targeted, animal-specific management responses, underscoring the role of PLF as an effective strategy for increasing cattle productivity while supporting animal welfare and production sustainability (Berckmans, 2014). From a practical perspective, these results indicate that farmers can use PLF systems to optimize feeding strategies, improve herd health management, and enhance reproductive efficiency, ultimately increasing

farm profitability and production sustainability (Steeneveld et al., 2015).

PLF in Extensive/Pasture-Based Systems

In extensive cattle production systems, Precision Livestock Farming (PLF) technologies improve management efficiency by enabling continuous monitoring of animal location, activity, and grazing behavior under conditions of limited direct supervision. GNSS-based tracking, accelerometers, and density-based data processing reduce information gaps inherent to pasture-based systems and support informed, animal-specific management decisions while preserving the low-input nature of extensive production, as depicted in Figure 4 (Aquilani et al., 2022).

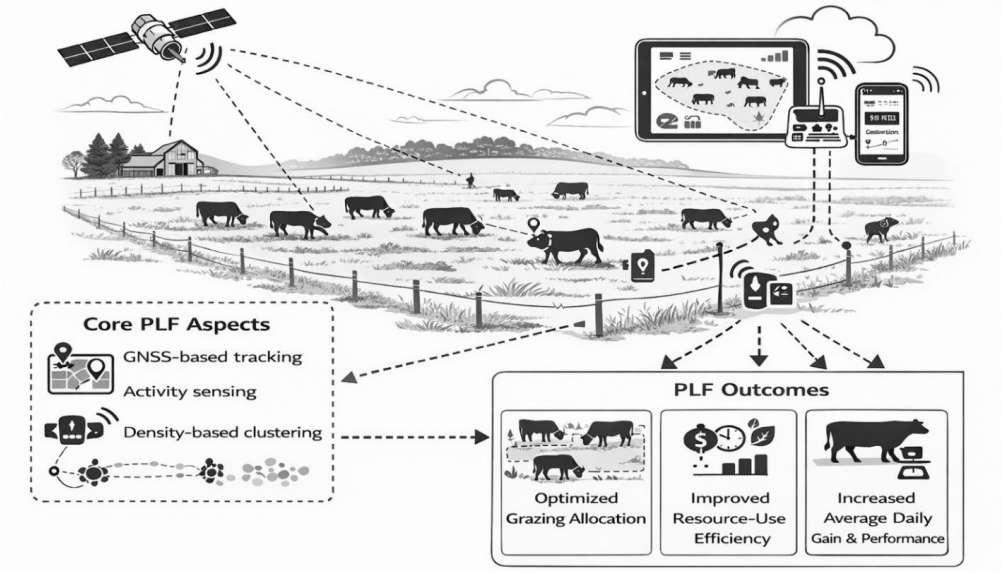


Figure 4. Precision Livestock Farming (PLF) in extensive and pasture-based cattle production systems: Global Navigation Satellite System (GNSS)-based monitoring, activity sensing and management outcomes (created with https://biorender.com)

As shown in Figure 4, PLF technologies enable the integration of spatial and behavioural data to support more precise grazing management and improved understanding of animal distribution and activity patterns in pasture-based systems (González et al., 2015; Williams et al., 2016). Evidence from grazing-based studies indicates that PLF applications allow objective quantification of pasture use and grazing time, which are closely associated with productive performance in extensive dairy and beef systems. The integration of GPS data with clustering algorithms enables accurate estimation of time spent grazing under variable pasture conditions, supporting improved assessment of feed intake opportunities and energy expenditure. This information facilitates more effective grazing management and stocking rate adjustments in highly dynamic forage environments (Williams et al., 2016). Even so, the adoption of PLF technologies in extensive systems remains constrained by several practical limitations. Data transmission challenges, sensor durability, and infrastructure requirements may reduce system reliability in remote or large grazing areas. In addition, economic considerations and the need for technical expertise may limit the willingness of farmers to adopt these technologies under extensive production conditions (Banhazi et al., 2012; Aquilani et al., 2022). From a production standpoint, PLF adoption in extensive systems has been linked to improved growth performance and resource-use efficiency, particularly in beef cattle. Optimized grazing allocation, early detection of behavioral or health deviations, and reduced handling-related stress contribute to increased average daily gain

and more uniform animal performance (Greenwood et al., 2017). At the same time, the magnitude of these benefits varies across studies, as environmental conditions, pasture availability, and herd management practices can influence system performance and limit the consistency of results. Although adoption remains limited, current evidence highlights the substantial potential of PLF to enhance productivity, animal welfare, and environmental sustainability in pasture-based cattle systems (Greenwood et al., 2017; Aquilani et al., 2022). These findings indicate that PLF technologies can support more efficient grazing management, better resource use, and enhanced decision-making in extensive cattle systems.

Environmental Implications: Emissions and Mitigation Potential

As illustrated in Figure 5, PLF technologies enable the continuous capture of animal-level data, including feed intake, feeding behaviour, rumination, and activity, all of which are closely associated with enteric methane production mechanisms (Wathes et al., 2008; Mottram, 2015; Berckmans, 2017).

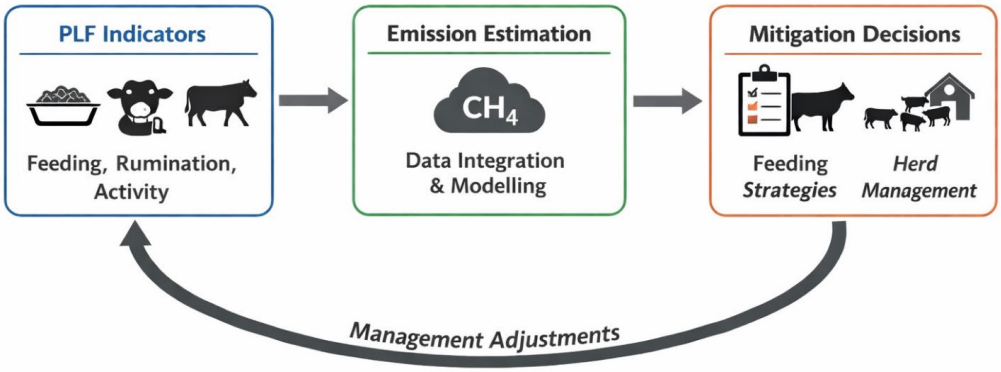


Figure 5. PLF-based framework for enteric methane (CH_4) emission estimation and mitigation through data-driven management decisions (created with https://biorender.com)

Figure 5 further highlights how the integration of behavioural, physiological, and production parameters supports more refined estimation of methane emissions and facilitates targeted mitigation strategies at both individual and herd level. In this context, PLF enables a more dynamic and animal-specific characterization of emission patterns compared with conventional assessment methods. Compared to conventional emission assessment methods, PLF enables a more dynamic and animal-specific characterization of emission patterns.

Evidence indicates that PLF-derived behavioural and physiological indicators can be used as indirect proxies for enteric methane emissions, supporting near real-time estimation under practical farming conditions (Tullo et al., 2019; O'Connor et al., 2024). Differences in feeding behaviour and feed efficiency between animals have been associated with substantial variation in methane emissions, reinforcing the value of PLF tools for identifying high-emitting individuals and supporting targeted mitigation strategies (Beauchemin et al., 2020). At herd scale, the integration of PLF data into dynamic modelling frameworks improves the representation of herd structure, productivity, and turnover, leading to more accurate estimates of total emissions over time (Rotz, 2018; Henn et al., 2023). These approaches allow emissions to be assessed in relation to production stages and management changes, thereby reducing uncertainty compared with static emission factor methods. Despite these advances, reliance on indirect indicators introduces important sources of uncertainty. Factors such as diet composition, environmental variability, and individual metabolic differences can influence methane production, potentially limiting the precision of proxy-based estimation under commercial farming conditions (Rotz, 2018).

Feeding management represents a key application area for PLF in emission mitigation. Precision feeding strategies, supported by continuous monitoring of intake and animal performance, enable the evaluation of dietary interventions aimed at reducing methane emissions while maintaining productivity (Beauchemin et al., 2020; Ouatahar et al., 2021). Available evidence indicates that mitigation efficiency varies considerably between systems, reflecting the influence of interacting biological and management factors such as diet formulation, animal variability, and production conditions.
Overall, the literature points toward a transition from retrospective emission assessment to more proactive, system-oriented emission management in livestock production systems (Wongpiyabovorn et al., 2025). These findings emphasize the role of PLF in supporting more informed management of feeding and production systems, facilitating a better balance between environmental impact and productive performance (Lovarelli et al., 2020; Ouatahar et al., 2021).

CONCLUSIONS

Precision Livestock Farming (PLF) provides a robust framework for improving cattle management by enabling continuous, objective monitoring and facilitating earlier, data-driven interventions at the individual animal level.
In both dairy and beef systems, PLF contributes to improved performance through integrated applications in welfare assessment, health surveillance (notably mastitis detection), reproductive monitoring, and automated phenotyping, thereby reducing preventable losses and improving production efficiency. The magnitude of these benefits varies across studies, as system performance is influenced by farm-specific conditions, data quality, and the level of technological integration. PLF also supports sustainability goals by improving resource-use efficiency, enabling better management of extensive pasture-based systems, and facilitating enteric methane emission estimation and mitigation, although wider adoption will require further validation and cost-effective implementation. At the same time, important challenges remain, including variability in sensor accuracy, limitations of proxy-based estimations, and economic and practical constraints affecting large-scale adoption. Overall, PLF represents a promising tool for enhancing productivity and sustainability in cattle systems, but its effective implementation requires context-specific validation, improved data integration, and greater accessibility for farmers.

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THE ROLE OF STIMULATIVE FEEDING OF NURSE BEES IN QUEEN REARING TECHNOLOGY

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Abstract

The aim of this study was to evaluate the role of stimulative feeding of nurse bees in the technological process of queen rearing. Under conditions of absent maintenance nectar flow, nurse colonies were fed 50% sugar syrup supplemented with the biostimulator Cloramicob. Supplemental feeding improved the physiological condition of the bees and stimulated royal jelly secretion for grafted larvae, which increased larval acceptance for rearing. It also promoted queen cell development, particularly length and diameter, and increased the body mass of both unmated and mated queens. The best overall response was generally obtained at the dose of 2.0 ml/L, while all stimulated groups outperformed the unstimulated control.

Key words: *biostimulants, queen cells, queen-rearing colonies, queens, sugar syrup.*

INTRODUCTION

The success of beekeeping largely depends on the organization and development level of the breeding base, queen-rearing technology, and colony management. Valuable breeding material can be rapidly multiplied through queen rearing. Queen quality is influenced by rearing methods, the condition of nurse colonies (cell builders), the number of grafted larvae, and the forage base, among other factors (Eremia, 2020).

Queen rearing by larval grafting involves transferring larvae from worker cells to artificial queen cups obtained by repeatedly dipping a template (2-3 times) in melted wax and then in cold water (Bura & Pătruică, 2004).

The production and breeding value of bee colonies is determined by hereditary traits and by the husbandry conditions provided for the queen. For breeder queens, rearing conditions are crucial: the main traits defining queen quality are formed during the larval stage. The development of the queen reproductive system depends on body size and overall body mass, which largely determine potential egg-laying capacity. Queen fecundity drives colony growth and development during the season and the expression of biological and productive traits (Borodachev & Savushkina, 2014).

Queen quality is also influenced by queen cell volume, which depends on colony strength and condition, nectar flow, bee race, etc. (Naumkin, 2000).

To obtain well-developed queens early in the season, special attention should be paid to nurse colonies and favourable conditions should be ensured, primarily warmth and sufficient feed (Belin, 1981).

In queen rearing, the influence of larval age was evaluated by comparing larvae aged 0-12 h, 24-36 h, and 48-60 h, as well as diets containing different proportions of pollen. It was found that the youngest larvae produced pupae with greater body mass, whereas the absence of pollen severely compromised rearing success, with acceptance falling to zero under complete pollen deprivation and being significantly higher at elevated protein levels (Kama & Shpigler, 2025).

Studies have shown that supplemental feeding significantly increased the acceptance rate of grafted larvae. Thus, 2-day-old larvae reached a mean acceptance rate of 82.3%, compared with 73.4% for 1-day-old larvae. At the same time, however, queens reared from younger larvae (1-day-old) exhibited a higher body weight (166.6 mg) than those developed from 2-day-old larvae (160.8 mg). These results suggest that, from a biological perspective, larval acceptance and the final quality of queens do not always coincide

and should therefore be interpreted together (Gençer et al., 2000).

Likewise, 1-day-old larvae produced queens with the best morphological traits, including a mean weight of 158.83 mg and a larger spermatheca volume, and supplemental feeding improved most traits except some more stable dimensions such as wing length (Mahbobi et al., 2012).

Among four pollen substitutes, the best-performing recipe for queen rearing consisted of brewer's yeast 30% + defatted soy flour 30% + skim milk 10% + cotton honey 20% + powdered sugar 10%. Orphaned colonies fed with this diet showed higher acceptance, more emerged queens, and better morphometric and reproductive traits than unfed controls (Taha et al., 2025). Pollen source strongly influences both queen-rearing success and queen morphophysiological quality (Abdel-Sattar et al., 2025).

Previous studies showed that feeding nurse colonies with stimulators during queen rearing under the absence of nectar flow increases graft acceptance and improves queen quality (Eremia & Zagareanu, 2012; Eremia et al., 2014; Eremia et al., 2015).

Queen-rearing success also depends on grafting technique and larval age. Testing different substrates placed in artificial cups showed that apple nectar gave the highest acceptance (81.06%), clearly above the control without substrate (47.04%), highlighting the importance of the larval microenvironment during the first 72 h after transfer (Contreras-Martinez et al., 2017). Similarly, queens reared from 1-day-old larvae were markedly superior to those from 2- or 3-day-old larvae (Zhong et al., 2024).

When queens originating from orphaned colonies fed with Apistimulin BM or Nanos-timul were introduced into swarms during the season, honey yields increased by 17% and 52% versus control, suggesting that improving queen quality during rearing has direct economic effects at colony level (Vedmed et al., 2016).

Overall, studies from different countries confirm that, when correctly applied and combined with efficient rearing methods, stimulative feeding improves graft acceptance and the morphological and reproductive quality of queens obtained (Gençer et al., 2000; Cengiz et al., 2019; Mahbobi et al., 2012; Taha et al., 2025). Thus, the analysed sources demonstrate that stimulative feeding is not only an energy-support measure, but a biotechnological tool capable of substantially shaping the response of cell-builder colonies and the quality of queens produced.

Based on the above, the aim of this study was to evaluate the role of stimulative feeding of nurse bees in the technological process of queen rearing.

MATERIALS AND METHODS

The research material consisted of bee colonies from a specialized and authorized queen-rearing apiary located in Dănceni village, Ialoveni district, maintaining a stock of 100 colonies and a nucleus yard with a capacity of 500 queen mating units. Based on the evaluation of morphoproductive traits (honey yield, early development, queen prolificacy, and overwintering resistance) and morphometric parameters (proboscis length; length and width of the 3rd tergite and 3rd sternite; wax glands; right forewing; tarsus; cubital index; and discoidal shift), the breeding stock of maternal and paternal colonies for queen reproduction was established. The experiment was conducted during May 2024.

Five groups of cell-builder colonies were formed by full queen removal; the queen and 2-3 frames with open brood were removed. The nest retained frames with honey, bee bread, and sealed brood. Open brood was removed to prevent the bees from building queen cells on larvae from those frames.

In the experimental colonies, the following morphoproductive traits were studied: the number of combs in the nest, colony strength (bee spaces), the amount of capped brood, and honey reserves.

Under the absence of maintenance nectar flow, each cell-builder colony received 1 L of 50% sugar syrup supplemented with the Cloramicob stimulator as follows: Group I - 1.0 ml/L; Group II - 2.0 ml/L; Group III - 3.0 ml/L; Group IV (Control I) - plain sugar syrup; Group V (Control II) - no stimulation.

In the first trial, conducted on 30 April 2024, a frame containing 36 grafted larvae was introduced between the frames with capped brood. From the first day after the introduction

of the grafted larval frame, nurse bees were fed 1 L of sugar syrup supplemented with the biostimulator, and feeding was continued daily until the queen cells were capped. Two days before queen emergence, the frame containing the capped queen cells was removed, and the diameter, length, and weight of the queen cells were measured, after which they were individually placed in cages.

The cages containing the queen cells were then introduced into incubator colonies and maintained there until queen emergence. The body weight of the unmated queens was determined using a torsion balance (measuring range: 500 mg), after which the queens were introduced into mating nuclei. After mating, the queens were marked and their body weight was reassessed.

In the second trial, the same procedures were followed. The number of grafted larvae introduced and accepted for rearing, the diameter and length of the queen cells, the weight of empty queen cells and queen cells containing queens, as well as the body weight of unmated and mated queens, were recorded.

Biometric processing of the data obtained was performed using the method of statistical variation, with determination of the standard value of Student's t-test to assess the significance of differences, according to the ANOVA method, using Microsoft Office.

RESULTS AND DISCUSSIONS

At the start of the experiment, nurse colonies had on average 9 frames in the nest, including 4 frames with sealed brood; colony strength was 8 bee spaces and honey reserves were 7.0 kg. Each colony received 36 grafted larvae. From the total number of larvae (36), acceptance was: Group I - 20 larvae (56%); Group II - 24 (67%); Group III - 20 (56%); Group IV (Control I) - 17 (47%); Group V (Control II) - 15 (42%) (Table 1).

Thus, colonies in Group II accepted 19% more larvae than Control IV and 25% more than Control V.

Table 1. Productive indicators of cell-builder colonies and number of grafted and accepted larvae for queen rearing, 10 May 2024 (I grafting)

Group	No. of frames, pcs	Colony strength, bee spaces	Honey stores, kg	No. of brood frames, pcs	No. of grafted larvae, pcs	No. of accepted larvae, pcs	No. of accepted larvae, %
I - Sugar syrup + Cloramicob, 1.0 ml/L	9	8	7.0	4	36	20	56
II - Sugar syrup + Cloramicob, 2.0 ml/L	9	8	7.0	4	36	24	67
III - Sugar syrup + Cloramicob, 3.0 ml/L	9	8	7.0	4	36	20	56
IV - Plain sugar syrup (Control I)	9	8	7.0	5	36	17	47
V - No stimulation (Control II)	9	8	7.0	4	36	15	42

Feeding nurse bees during larval rearing until queen cell capping increased queen cell length and the mass of capped queen cells, queen cells containing queens, and the body mass of unmated and mated queens.

The mean queen cell diameter in Group I was 12.81 mm, i.e., 0.54 mm larger than Group V; the difference was significant (*P1 >= 0.95). The largest queen cells were produced in Group II, with a length of 32.71 mm, which was 5.90 mm (22%) longer than Control IV and 4.7 mm (20%) longer than Control V (***P3 >= 0.999) (Table 2).

Table 2. Diameter, length and mass of empty queen cells (I grafting), 22 May 2024

Group	Queen cell diameter, mm	Queen cell length, mm	Mass of empty queen cells, mg
I - Sugar syrup + Cloramicob, 1.0 ml/L	12.81±0.14*	27.89±0.47	120.37±4.53
II - Sugar syrup + Cloramicob, 2.0 ml/L	12.36±0.13	32.71±0.56***	143.19±2.73**
III - Sugar syrup + Cloramicob, 3.0 ml/L	12.73±0.41	30.17±0.61**	169.35±7.21**
IV - Plain sugar syrup (Control I)	12.79±0.24	26.81±0.38	134.27±2.83
V - No stimulation (Control II)	12.27±0.20	27.93±0.68	137.0±0.05

Queen cell diameter, Group I / Group V - *P1 >= 0.95; Queen cell length, Group II / Group V - ***P3 >= 0.999; Queen cell length, Group II / Group IV - ***P3 >= 0.999; Queen cell length, Group III / Group IV - **P3 >= 0.999; Queen cell length, Group III / Group V - **P2 >= 0.99; Mass of empty queen cells, Group II / Group IV - **P2 >= 0.99; Mass of empty queen cells, Group II / Group V - *P1 >= 0.95; Mass of empty queen cells, Group III / Group IV - **P2 >= 0.99; Mass of empty queen cells, Group III / Group V - **P2 >= 0.99.

The heaviest empty queen cells after queen emergence were obtained in Group II (143.19 mg) and Group III (169.35 mg), which were 8.92 mg and 32.35 mg heavier than Control IV and Control V (**P2 >= 0.99).

The mass of queen cells containing queens averaged 1.066 g in Group II and 1.301 g in Group III, exceeding both controls.

Queen quality is primarily assessed by body mass. The mass of unmated queens in experimental groups ranged from 175.82 to 179.93 mg, whereas in Control I and Control II it ranged from 168.33 to 174.80 mg (Table 3).

The highest body mass of mated queens was recorded in Group I: 267.14 mg, which was 37.39 mg (16%) higher than Control IV (***P3 > = 0.999) and 44.33 mg (22%) higher than Control V (***P3 > = 0.999). Thus, feeding nurse bees from the introduction of grafted larvae until queen cell capping for 5 days with 1 L of sugar syrup supplemented with 1.0 ml/L Cloramicob ensured queens with increased body mass, which was 7% higher than previously reported results (Eremia & Zagareanu, 2012; Eremia et al., 2015).

Table 3. Mass of queen cells and body mass of unmated and mated queens (I grafting)

Group	Mass of queen cells containing queens, g	Mass of unmated queens, mg	Mass of mated queens, mg
I - Sugar syrup + Cloramicob, 1.0 ml/L	0.987±0.09	175.82±3.57	267.14±1.96***
II - Sugar syrup + Cloramicob, 2.0 ml/L	1.066±0.02*	179.93±5.07	264.00±7.57**
III - Sugar syrup + Cloramicob, 3.0 ml/L	1.301±0.04**	179.80±5.46	261.00±4.93**
IV - Plain sugar syrup (Control I)	1.128±0.06	174.80±4.65	229.75±2.95
V - No stimulation (Control II)	0.932±0.05	168.33±3.71	219.67±0.88

Mass of queen cells containing queens, Group II / Group V - *P3 >= 0.95; Mass of queen cells containing queens, Group III / Group IV - *P3 >= 0.95; Mass of queen cells containing queens, Group III / Group V - **P3 >= 0.95; Mass of mated queens, Group I / Group IV - ***P3 >= 0.999; Mass of mated queens, Group I / Group V - ***P3 >= 0.999; Mass of mated queens, Group II / Group IV - **P3 >= 0.999; Mass of mated queens, Group II / Group V - **P3 >= 0.999; Mass of mated queens, Group III / Group IV - **P3 >= 0.999; Mass of mated queens, Group III / Group V - **P3 >= 0.999.

In the second trial, a second frame containing grafted larvae was introduced into the nurse colonies for rearing.

From the 36 larvae, the highest acceptance was recorded in Group II - 23 larvae (64%). With an increased dose, acceptance decreased to 53% (Table 4).

Table 4. Number of grafted and accepted larvae, 13 May 2024 (II grafting)

Group	No. of grafted larvae, pcs	No. of accepted larvae, pcs	No. of accepted larvae, %
I - Sugar syrup + Cloramicob, 1.0 ml/L	36	21	58
II - Sugar syrup + Cloramicob, 2.0 ml/L	36	23	64
III - Sugar syrup + Cloramicob, 3.0 ml/L	36	19	53
IV - Plain sugar syrup (Control I)	36	20	56
V - No stimulation (Control II)	36	19	53

The number of accepted larvae in Group II was 8% higher than Control IV and 11% higher than Control V.

Queen cell diameter in experimental groups varied slightly, from 12.23 mm to 12.93 mm. By queen cell length, Group II was superior: 34.06 mm, which was 6.3 mm (23%) longer than Control IV and 6.13 mm (22%) longer than Control V (***P3 >= 0.999) (Table 5).

Compared with the data reported by Canverdi et al. (2023), queen cell length in Caucasian bees was 23.19 mm at low altitude and 22.75 mm at high altitude. In our case, particularly in Group II, queen cell length was greater, exceeding 32 mm after the first grafting and 34 mm after the second grafting. Queen cell dimensions and queen performance depend on the entire technological chain, which includes the type of cell cup, the rearing method, environmental conditions, process intensity, and supplemental feeding (Abou-shaara, 2023; Ahmat et al., 2024; Derafsh et al., 2025; Lashari et al., 2022; Mattiello et al., 2022; Poudel et al., 2024).

Table 5. Diameter, length and mass of queen cells containing queens (II grafting)

Group	Queen cell diameter, mm	Queen cell length, mm	Mass of queen cells containing queens, g
I - Sugar syrup + Cloramicob, 1.0 ml/L	12.96±0.23	28.09±0.51	1.25±0.47
II - Sugar syrup + Cloramicob, 2.0 ml/L	12.23±0.17	34.06±0.54***	1.47±0.05
III - Sugar syrup + Cloramicob, 3.0 ml/L	12.55±0.26	23.97±0.27	1.17±0.04
IV - Plain sugar syrup (Control I)	12.84±0.14	27.76±0.29	1.35±0.04
V - No stimulation (Control II)	12.27±0.19	27.93±0.67	1.37±0.05

Queen cell length, Group II / Group IV - ***P3 >= 0.999; Queen cell length, Group II / Group V - ***P3 >= 0.999.

After emergence, at the time of introducing queens into nuclei for maturation and mating, body mass in experimental groups was 182.19-186.50 mg, while in Control I and Control II it was 173.67-179.50 mg (Table 6).

Feeding nurse bees from the introduction of grafted larvae until queen cell capping with 1 L of 50% sugar syrup supplemented with Cloramicob at doses of 1.0-3.0 ml/L increased the body mass of mated queens that started laying; differences versus both controls were significant (*P1 >= 0.95).

The best results were obtained in Group II, where mated queens had an average body mass of 250.0 mg, which was 22.50 mg (10%) higher than Control IV and 24.5 mg (11%) higher than Control V.

The obtained data confirm the view of several authors that supplemental feeding of nurse colonies results in improved indicators of queen quality (Kama & Shpigler, 2025; Sakla & El-Shafeiy, 2022).

Table 6. Body mass of unmated and mated queens (II grafting)

Group	Mass of unmated queens, mg	Mass of mated queens, mg
I - Sugar syrup + Cloramicob, 1.0 ml/L	182.61±3.28	243.60±2.97*
II - Sugar syrup + Cloramicob, 2.0 ml/L	186.50±5.25	250.0±5.02*
III - Sugar syrup + Cloramicob, 3.0 ml/L	182.19±2.50	246.40±5.55*
IV - Plain sugar syrup (Control I)	179.50±4.42	227.50±3.50
V - No stimulation (Control II)	173.67±1.98	225.5±5.50

Mass of mated queens, Group I / Control IV - *P1 >= 0.95; Mass of mated queens, Group I / Control V - *P1 >= 0.95; Mass of mated queens, Group II / Control IV - *P1 >= 0.95; Mass of mated queens, Group II / Control V - *P1 >= 0.95; Mass of mated queens, Group III / Control IV - *P1 >= 0.95; Mass of mated queens, Group III / Control V - *P1 >= 0.95.

Therefore, stimulative feeding of nurse bees during queen rearing ensured, in the first grafting, an increase in larval acceptance by 19-25%, queen cell length by 20-22%, and mated queen body mass by 16-22%; in the second grafting, larval acceptance increased by 8-11%, queen cell length by 22-23%, and mated queen body mass by 10-11%.

CONCLUSIONS

Based on the results obtained, a queen-rearing method was developed and patented, which involves feeding nurse bees from the first day after the introduction of the frame containing grafted larvae with 1 L of sugar syrup supplemented with a biostimulator, and subsequently on a daily basis until the queen cells are capped.

It was established that, in the absence of a maintenance nectar flow, feeding nurse bees involved in the technological process of queen rearing with 50% sugar syrup supplemented with the biostimulator Cloramicob improves the physiological condition of the bees and stimulates royal jelly secretion for the rearing of grafted larvae.

This, in turn, increases the number of grafted larvae accepted for rearing and promotes the development of queen cells (length and diameter), as well as increasing the body weight of both unmated and mated queens.

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ADVANCES AND INNOVATIONS IN SERICULTURE - A REVIEW

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Abstract

This review aimed to emphasize recent discoveries and advances in sericulture. Current trends in silkworms rearing include technological progress and a diversification of products. Innovations like genetically modified silkworms, automation, and precision farming techniques including drone technology and monitoring systems are increasing efficiency by further optimized mulberry cultivation and silkworm rearing. There`s a growing effort to use silkworm pupae and sericin for high-value applications in medical, cosmetic, and nutraceutical industries. Genetic engineering has enabled the manipulation of silkworm genomes. Through genetic techniques, scientists can precisely edit the genes responsible for the synthesis of silk proteins. The production of disease-resistant silkworm breeds is part of an innovative line. Automated systems, drive units and artificial intelligence algorithms have offering new opportunities in the field. New opportunities for enhancing silk production have been made possible by biotechnology. Advances in molecular genetics, biotechnology, and engineering are leaving their mark on the evolution of sericulture, making paradigm shifts vital.

Key words*: innovations, sericulture, silkworms, trends.*

INTRODUCTION

Sericulture, the science of silk production, has experienced a remarkable and unprecedented transformation over the past several decades. Sericulture has gradually developed into a complex, technologically advanced field from its beginnings as a traditional agricultural practice passed down through the centuries. These days, it incorporates developments from a wide range of scientific and engineering domains (Kiruba et al., 2024; Thrilekha et al., 2024). Such as, historically, silkworm rearing and silk processing depended largely on manual work, close observation, and practical, experience-based techniques. In contrast, the modern sericulture industry now operates at the crossroads of biotechnology, genetic engineering, materials science, informatics, and sustainable agricultural systems. This transformation mirrors global movements toward precision agriculture, increased automation, environmental stewardship, and the development of high-value, bio-based (Fatima et al., 2024; Kiruba et al., 2024).

The rapid advancement of sericulture is further propelled by a growing worldwide demand for specialized silk-derived products such as medical-grade fibers, sustainable textiles, and bifunctional proteins. This rising interest has pushed scientists and industry professionals to explore new, innovative strategies across the entire production chain, from silkworm rearing and mulberry cultivation to improved disease control, silk processing methods, and the value-added use of materials that were once considered waste (Adikesavan et al., 2025). As a result, sericulture has evolved into a vibrant and multidisciplinary domain that seamlessly integrates long-standing traditional practices with the latest advancements in science and technology. This convergence has transformed the field from a primarily agricultural activity into a sophisticated bioindustry that draws upon innovations in molecular biology, engineering, data science, and sustainable resource management (Savu et al., 2024; Bora et al., 2025).

The growing volume of literature reflects this transformation, documenting significant progress in diverse areas such as silkworm genome engineering, the development of highly automated and AI-assisted rearing systems, and the expansion of silk and silkworm-derived materials into new commercial and biomedical applications. Additionally, precision agriculture techniques, such as sensor-guided mulberry cultivation, environmental monitoring, and

data-driven farming, have further strengthened the efficiency and productivity of sericulture (Yu, 2025). Together, these advancements underscore the field's shift toward a more technologically advanced, economically valuable, and ecologically sustainable industry.

Although technological innovations show promising results their large-scale implementation may be constrained by high initial investment costs, the need for specialized technical expertise, data reliability issues, and limited accessibility in developing countries.

Drawing from contemporary research, industrial advancements, and global market trends, this work provides a comprehensive overview of the changing landscape of sericulture and its far-reaching implications for silk production worldwide (Arya and Sharma, 2025).

Traditionally rooted in agricultural craftsmanship, it has steadily evolved into a multidisciplinary field incorporating biotechnology, engineering, materials science, and sustainable agriculture. This review highlights recent discoveries and technological advances shaping modern sericulture, placing particular emphasis on innovations in silkworm genetics, automated rearing systems, diversified product utilization, and precision farming. Drawing from contemporary research and industry developments, the following pages provide a detailed overview of the new directions in sericulture and their implications for global silk production (Kiruba et al., 2024; Lazar et al., 2025).

The objective of this review is to examine and synthesize recent scientific and technological advancements that are reshaping modern sericulture. Specifically, it aims to analyses innovations in silkworm genetics, automated rearing technologies, precision mulberry cultivation, and the emerging high-value applications of silk-derived biomaterials. By integrating insights from contemporary research, industrial developments, and global market trends, this review seeks to provide a comprehensive understanding of how sericulture is transitioning from a traditional agricultural practice into a multidisciplinary, technology-driven bioindustry with expanding economic, scientific, and environmental significance.

MATERIALS AND METHODS

Current findings in the field of sericulture, the review of 120 publications highlights a shift from traditional rearing to a high-tech, bio-based industry. High-impact journals (PubMed, Google Scholar, Research Gate, MDPI, etc.) from 2016 to 2025 were used to compile the data, with an emphasis on the last two years.

Keywords such as "silkworms", "sericulture", "biodiversity" and "trends", "innovation in sericulture", "advancement" were used. Selection criteria for the publications included subject relevance and the language used (English).

RESULTS AND DISCUSSIONS

INTEGRATED SERICULTURE SYSTEMS

Integrated sericulture systems have transitioned from linear silk production to circular bioeconomy models that prioritize total biomass utilization. Specialized literature has centred on silk production (Altman & Farell, 2022; Fatima et al., 2024) and byproduct valorization (Kishore et al., 2024a). There is a growing need for a holistic perspective on the entire sericulture value chain.

This comprehensive review integrates every stage of the process, from the fundamental importance of mulberry cultivation as the primary nutrient source to the latest technological innovations in silkworm rearing and the systematic conversion of all process byproducts into high-value waste valorization streams. In this study, our intention consists on connecting the upstream (farming and reeling), midstream (textile weaving and dyeing), and downstream (recycling) segments.

Briefly, sericulture is no longer viewed as a linear production process but as a circular bioeconomy. A holistic perspective integrates the following critical stages (Figure 1).

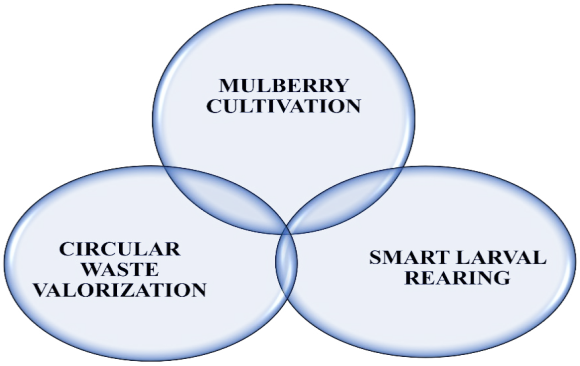


Figure 1. The integrated sericulture circular bioeconomy model (original)

1. Mulberry cultivation: moving beyond simple farming, modern practices emphasize sustainable, nutrient-dense cultivation of *Morus* spp. as the foundation for high-quality silkworm development. Mulberry (*Morus* spp.) is the foundational nutrient source for silkworms (*Bombyx mori*). Modern strategies focus on eco-friendly cultivation, utilizing organic fertilizers derived from recycled sericulture waste to improve soil fertility and leaf quality (Ayman, 2024; Hassan et al., 2025; Rajendran et al., 2025).

Highlighting the discrepancies in reported results is crucial. In practice, the performance of irrigation and fertilization systems varies based on local soil chemistry, water standards, and specific mulberry varieties. Therefore, theoretical findings often diverge from the inconsistent yields reported by farmers operating in different environments (Garai, 2025)

2. Smart silkworm larval rearing: has transitioned into a highly automated, data-driven discipline. Modern sericulture now exploits the "Precision Rearing" model, integrating Internet of Things (IoT), artificial intelligence, and advanced genetics to stabilize silk production against climate volatility while maximizing cocoon quality. Current advancements focus on automated monitoring, climate-controlled environments, and genetic improvements to enhance larvae health and cocoon quality (Dhanapriya, 2025; Veenita et al., 2025).

While Integrated Sericulture Systems offer clear benefits, switching to a high-tech model is difficult due to high costs. For small farmers, such as those in Romania, the price of IoT and AI tools is often too high without government help. This limits these advanced methods to wealthy research centers and large farms, making it hard to grow the industry globally (Manju & Binson, 2024; Garai, 2025). Additionally, high humidity can damage sensors, causing data errors. Finally, these systems require expert workers; without proper training, automation may actually cause more problems than it solves (Reddy et al., 2025a; 2025b)

3. Circular waste valorization: the transition of sericulture toward a circular bioeconomy has fundamentally redefined waste as a primary economic driver. By systematically valorising byproducts, the industry now generates multiple high-value revenue streams while significantly reducing its environmental footprint (Shravanilakshmi et al., 2025a). The industry is transitioning from a traditional linear model to a multidisciplinary field where sericulture supports diverse sectors like biotechnology and pharmaceuticals.

1. MULBERRY CULTIVATION ADVANCEMENT

Modern mulberry cultivation has transitioned from traditional agrarian methods to a highly sophisticated, data-driven science. The focus of *Morus* spp. cultivation has shifted toward maximizing leaf quality through precision technology and sustainable soil management, as the nutritional profile of the leaf directly

determines the quality of silk produced by the silkworm (Garai & Sadhu, 2025).

Among the benefits of organic mulberry cultivation, we can highlight: 1) enhances soil health and long-term productivity**:** organic fertilizers and composting enrich the soil with essential nutrients and organic matter, fostering a robust foundation for sustained agricultural yield; 2) improves soil structure and nutrient content: composting and green manure applications increase water retention and aeration, leading to healthier plant growth and reduced need for external inputs; 3) provides a sustainable source of nutrients for plants**:** these natural practices create a closed-loop system where waste is recycled into valuable fertilizer, ensuring continuous nutrient availability; 4) reduces dependency on chemical pesticides: biological pest control lessens costs and health risks associated with synthetic chemicals, leading to a more resilient farming system (Ahammed et al., 2025).

The modern practice in mulberry cultivation takes into account the following:

- Integrated Nutrient Management (INM): Modern practices combine organic manures like compost and green manure with scientifically calibrated doses of chemical fertilizers. INM has been shown to increase leaf yield by up to 22% while improving microbial activity and nutrient cycling, leading to foliage that is richer in protein and soluble sugars, key components for larval growth (Giora et al., 2022; Hăbeanu et al., 2024).
- Precision Soil Health Monitoring: Farmers can utilize IoT-based soil sensors to track pH, moisture, and specific nutrient levels such as nitrogen, potassium, and zinc in real-time. This allows for "prescriptive fertilization", where nutrients are applied only where and when needed, preventing over-fertilization and ensuring the optimal soil pH of 6.2 to 6.8 is maintained (Reza et al., 2025; Chen et al., 2025).
- Nano-Fertilizers and Foliar Spray: Nano-fertilizers deliver nutrients slowly and precisely to plants, preventing waste and protecting the environment. Multi-nutrient foliar sprays are also increasingly used to bypass soil-related limitations, directly boosting the chlorophyll content and moisture retention of the leaves (Kekeli et al., 2025; Goyal et al., 2025).
- Smart Irrigation Systems: Traditional flood irrigation is being replaced by smart drip irrigation, which delivers water directly to the root zone. These systems have improved water-use efficiency by approximately 35%, ensuring consistent moisture and reducing plant stress, especially in drought-prone regions (Rahman, 2025; Ali et al., 2025).
- Biotechnological Advancements: Genomic editing tools are being applied to develop mulberry varieties with enhanced photosynthetic efficiency and resistance to environmental stressors like soil salinity and extreme temperatures. These genetic improvements ensure that the leaves remain nutrient-dense even under challenging climatic conditions (Mangammal et al., 2025; Liu et al., 2025).
- Integrated Pest Management (IPM): in sericulture has evolved into a sophisticated, multi-layered framework that prioritizes the health of both the mulberry ecosystem and the silkworms (*B. mori*). This transition is increasingly supported by digital traceability and eco-friendly interventions (Kishore et al., 2024b).
- Drone-Assisted Field Management: Drones equipped with multispectral cameras are deployed to monitor large mulberry plantations. These drones can identify early signs of nutrient deficiency or disease outbreaks that are invisible to the naked eye, allowing for early, targeted interventions that save up to 28% in pesticide costs (Ahmed & Hossain, 2024).
- Soil-Less Cultivation Technologies: Innovations in hydroponics and aeroponics are enabling the year-round production of mulberry leaves in controlled environments. These soil-less systems are particularly valued for their ability to produce highly palatable leaves with increased medicinal components like 1-deoxynojirimycin (Baciu et al., 2025; Tricase et al., 2025).
- Sustainable Waste Recycling: in sericulture involves transforming byproducts like silkworm litter, leftover leaves, and pupae into valuable soil amendments such as biochar and nutrient-rich compost. This approach is central to a circular economy model, which improves soil health and enhances the long-term resilience of mulberry plantations ([a]Kaushik et al., 2025).

- Climate-Resilient Agronomy: has become the primary defence for the silk industry against environmental volatility. By integrating specialized genetics with advanced soil management, sericulture achieves stable leaf production despite increasing global temperatures and erratic rainfall (Kruthika et al., 2025).

2. LARVAL REARING INNOVATIONS

Larval rearing has fundamentally transitioned from a traditional agricultural practice to a sophisticated, technology-driven bio-industry (Reddy et al., 2025a; Arya & Sharma, 2025). This evolution is characterized by three pillars: i) integration of cutting-edge biotechnology; ii) intelligence automation; iii) circular economy principles (Kotsou et al., 2024; Reddy et al., 2025a). These advancements have significantly enhanced the productivity, efficiency, and sustainability of silk production, driven by a convergence of genetic engineering, automated environmental controls, and zero-waste models (Reddy et al., 2025a). The goal is no longer just producing silk, but creating a resilient and highly efficient value chain that can withstand modern environmental and economic pressures (Kaza, 2024; Reddy et al., 2025a).

The application of biotechnology in sericulture has undergone a profound shift, moving beyond passive data collection to proactive, intelligent decision-making systems. This transformation is particularly evident in the development of "super-industrial" silkworms, a term used to describe genetically optimized strains. The ability to monitor processes in real-time and apply genomic precision allows for unparalleled optimization of silk production. This new paradigm ensures maximum yield and quality while minimizing resource input and environmental impact (Thanga et al., 2025).

A key aspect of these biotechnological advancements is the widespread adoption of genome editing tools like CRISPR/Cas9, a revolutionary gene-editing technology, derived from a bacterial immune system (Chen et al., 2024). CRISPR-Cas9 driving significant advancements in medicine, and transforming agriculture with climate-resilient crops. This technology enables scientists to precisely modify silkworm genes, moving beyond traditional breeding's limitations. Specific modifications aim to enhance crucial industrial traits, such as increasing the raw fiber strength, improving tensile elasticity, and building resistance to pervasive environmental stressors and diseases. These engineered silkworms represent a new generation of robust, high-performance biological assets (Fan et al., 2025; Reddy et al., 2025b).

Scientists have also developed transgenic silkworms that produce specialized, high-value fibers. Furthermore, scientists have created strains that produce naturally fluorescent or colored silk, effectively eliminating the environmental need for chemical dyes and streamlining the production process. This innovation not only adds value but also aligns with the growing demand for eco-friendly manufacturing practices (Kiruba et al., 2024).

Modern rearing facilities utilize PCR and LAMP diagnostics to detect pathogens like pébrine at early stages, shifting disease management from reactive to proactive. These molecular tools safeguard high-value genetic investments by enabling rapid intervention, effectively preventing widespread crop losses and ensuring production stability (Fu et al., 2016).

The advent of precision sericulture utilizes the IoT and AI to create optimal, stable environments (Kaya, 2024). Modern facilities, often referred to as "smart rearing houses", use sophisticated IoT sensors to continuously monitor and automatically regulate key parameters such as temperature, humidity, and CO_2 levels in real-time. This data-driven approach ensures optimal growth conditions for the silkworms, leading to consistent, high-quality cocoon production and reducing the variability associated with manual management (Kaya, 2024).

Automation extends beyond environmental controls into the physical handling of the process. The introduction of automated silk reeling machines and robotic harvesters has significantly reduced manual labour requirements while ensuring high thread uniformity and minimizing waste. These automated systems increase efficiency, lower operational costs, and help maintain the strict hygienic standards necessary for disease prevention in large-scale operations (Saikia & Kaushiki, 2024). Sericulture adopts circular economy principles by transforming waste like

protein-rich pupae into high-value aquaculture feed, pharmaceuticals, and cosmetics. Furthermore, the industry repurposes sericin from wastewater into biocompatible materials for skincare and tissue engineering, turning former pollutants into sustainable, revenue-generating biomaterials (Hăbeanu et al., 2024; Lazăr & Pătruică, 2025). Finally, the sericulture industry has reached peak efficiency by integrating IoT-driven smart rearing houses and robotic management, reducing manual labour by over 80% while utilizing AI diagnostics to detect diseases with 99% accuracy (Reddy et al., 2025b). Despite the implementation of sophisticated models, such as Support Vector Machines combined with Histogram of Oriented Gradients and Kernel Principal Component Analysis (KPCA) proposed by Manju and Binson (2024) significant variability in outcomes remains a critical challenge. Improvements in cocoon yield and disease resistance are often dependent on climatic conditions, silkworm strains, and regional infrastructure; therefore, the high productivity increases reported in controlled studies may not be consistently reproducible across all production systems (Garai, 2025).

Environmental sustainability is prioritized through organic cultivation and green enzymatic processing, complemented by the development of climate-smart, heat-tolerant breeds that maintain high productivity in fluctuating temperatures (Reddy et al., 2025a).

3. CIRCULAR WASTE VALORIZATION

The sericulture industry has transitioned to a "*Pharma-Farming*" model that treats every byproduct as a high-value resource rather than waste. This circular approach is driven by the following key pillars:

- **Nutrient cycling and soil health**: silkworm litter (excreta and residual leaves) is processed into liquid organic manure or vermicompost and reintroduced into mulberry fields. This sustainable cycle improves soil structure and reduces reliance on chemical fertilizers while enhancing the nutritional quality of mulberry leaves (Shravanilakshmi et al., 2025b).
- **Functional byproduct valorization**:

Sericin Recovery: once a wastewater pollutant, sericin is now recovered for high-end biomedical and cosmetic applications, including use as a scaffold for tissue engineering and a moisture-retaining agent in anti-aging skincare.

Sericin used to be thrown away as waste from silk production, but today it is seen as a very valuable material for science and medicine (Chetia et al., 2025). This protein, which acts as the gummy binder holding silk fibroin fibers together, was traditionally chemically stripped and discarded, creating a significant environmental burden in the sericulture, however, advanced extraction methods and a shift toward circular economy principles have transformed sericin into one of the industry's most valuable functional byproducts (Darith, 2025). This paradigm shift not only solves a long-standing disposal problem but also unlocks new, high-margin revenue streams that align with global demands for sustainability and natural ingredients.

The sustainable recovery process fundamentally aligns with the industry's overall commitment to reducing its environmental footprint and maximizing resource efficiency, proving that industrial byproducts can become premium, sought-after biomaterials (Dragojlov et al., 2025).

The integrated approach not only minimizes waste and environmental impact but also significantly enhances the economic viability of silk production, driven by a convergence of technological innovation and circular economy principles (Dragojlov et al., 2025).

Pupae protein: silkworm pupae have transitioned from a low-value byproduct to a cornerstone of the circular bio-industry, providing a sustainable, high-protein alternative to traditional animal feeds. Generating roughly 6 kg of pupae for every 1 kg of raw silk produced, the industry now utilizes this vast biomass to reduce global dependency on fishmeal and soybean meal in aquaculture and poultry sectors (Hăbeanu et al., 2024; Naan et al., 2025; Xue et al., 2025). Pupae meal is not only nutrient-dense but also acts as a natural growth promoter, improving weight gain, specific growth rates, and feed conversion ratios while significantly lowering mortality rates. The "Pharma-Farming" model further elevates this byproduct by extracting high-value pharmaceutical proteins and health supplements ([b]Kaushik et al., 2025; Gokulakrishnaa et al., 2025).

Beyond bulk nutrition, biotechnological advancements allow for the precise extraction of bioactive peptides and omega-rich oils for specialized industrial use. Silkworm pupae oil, which can contain up to 44% alpha-linolenic acid (omega-3), is now a primary ingredient in anti-aging cosmetics and nutraceuticals designed to lower blood sugar and blood pressure. These oils and peptides also serve as functional food ingredients, acting as natural α-glucosidase inhibitors to manage post-meal glucose levels, thereby addressing the rising global demand for metabolic health solutions (Gokulakrishna, 2025).

Innovative applications also extend into regenerative medicine and biomanufacturing, where pupae-derived materials are used as pharmaceutical scaffolds for tissue engineering. Bioengineered hydrogels incorporating pupae extracts demonstrate potent antioxidant and anti-inflammatory properties, significantly accelerating wound closure in diabetic models by enhancing tissue re-epithelialization and vascular growth (Geng et al., 2025).

- **Resource and water efficiency**: advanced water management has become a pillar of large-scale sericulture, achieving up to a 35% increase in water-use efficiency through the systematic recycling of reeling effluents. Once considered a waste product, this nutrient-rich wastewater, packed with proteins like sericin and essential minerals, is now treated and redirected for the irrigation of mulberry fields. This "closed-loop" approach significantly reduces the industry's dependence on primary freshwater sources, such as borewells and surface water, while providing a reliable moisture supply that is resilient to climate-induced scarcity (Ashrith et al., 2025).

The adoption of these circular practices extends beyond simple conservation, acting as a "liquid biofertilizer" that enhances both soil fertility and crop productivity. Modern operations utilize real-time sensors and AI-driven predictive algorithms to optimize the delivery of this reclaimed water, ensuring that it meets the precise needs of different mulberry varieties while preventing salt accumulation. By transforming reeling effluents into a strategic resource, the sericulture industry not only mitigates its environmental footprint but also stabilizes the entire value chain, fostering a sustainable model for high-efficiency silk production (Ashrith et al., 2025).

ECONOMIC, SOCIAL AND ENVIRONMENT IMPACT

Advancements in sericulture have profound economic, social, and environmental effects.

Economic impact. Modern techniques, such as AI-driven precision farming and IoT monitoring, can increase productivity by 20-30% while significantly lowering operational costs.

The transition to integrated sericulture systems is significantly boosting rural economies by diversifying farmer income and tapping into high-growth biomedical markets. Integrated farming models allow farmers to maximize land use by cultivating secondary crops alongside mulberry trees.

Shifting from raw silk to diverse value-added products, such as silk fibroin for biomedical implants and biopharmaceuticals, is opening massive new revenue streams in non-textile sectors (Kairy, 2020; Mala et al., 2024). From a practical perspective, the initial expense for automated housing, IoT sensors, and climate control is often unaffordable for small-scale farmers without subsidies. However, economic feasibility depends on farm size, access to financial support, technical training, and market stability. Cost-benefit analyses are essential before adopting advanced automation technologies. For example, fluctuating of global silk prices can make it hard to predict the return on investment, creating financial risk for those with heavy debt from technology upgrades. Furthermore, high maintenance c**osts** increase long-term expenses compared to traditional methods.

Social impact. Sericulture is a primary vehicle for gender equity, with women making up approximately 58-60% of the workforce in major producing regions like India. Advancements provide women with flexible, home-based income, improving their financial independence and social status. The industry provides a stable livelihood for tribal and marginalized communities (Mala et al., 2024).

Environmental impact. Byproducts previously considered waste are now recycled: silkworm litter is used as high-protein animal feed or organic manure, and mulberry prunings are

composted into fertilizers, reducing reliance on chemical inputs. Innovations like drip irrigation and waterless dyeing technologies have drastically reduced the industry's water footprint. Perennial mulberry cultivation acts as a carbon sequestration and prevents soil erosion. Silk fibroin is increasingly replacing petrochemical-based plastics in various industrial applications due to its natural biodegradability and high mechanical strength (Popescu et al., 2025).

RECENT CHALENGES AND TRENDS IN SERICULTURE

Sericulture is currently confronted with a range of environmental, biological, and socio-economic challenges that threaten its sustainability and productivity. Climate change has emerged as a major environmental stressor, with increasing temperature extremes and unpredictable weather patterns adversely affecting mulberry cultivation, the primary food source for silkworms. Variations in temperature and humidity directly influence leaf quality, thereby impairing silkworm growth and cocoon yield. These climatic stresses are compounded by persistent disease outbreaks, notably pébrine and flacherie, which continue to pose a significant risk of large-scale crop losses. The transition toward mechanized rearing systems has further complicated disease management, as effective disinfection protocols remain technically challenging. Additionally, concerns over excessive water use and the environmental impact of chemical pesticides have intensified the need for sustainable waste management practices within the sericulture sector (Singh & Massey, 2025).

Beyond environmental constraints, sericulture faces pronounced socio-economic pressures. The labor-intensive nature of silk production has become increasingly difficult to sustain due to rural-to-urban migration, particularly among younger populations, resulting in a shortage of skilled labor. Market instability and fluctuating cocoon prices further exacerbate the vulnerability of small and marginal farmers. These economic challenges are intensified by competition from low-cost synthetic fibers, which continue to dominate global textile markets. Inadequate infrastructure, including unreliable electricity supply and limited access to high-quality, disease-free silkworm eggs, further undermines productivity and profitability. Consequently, there is a growing imperative for the industry to transition toward ethical, eco-friendly, and quality-driven production systems in order to align with evolving consumer expectations and global sustainability standards (Dhane et al., 2004; Singh & Massey, 2025). Despite these challenges, significant opportunities are emerging through the diversification of sericulture-based products. A biorefinery approach is gaining prominence, emphasizing the comprehensive utilization of sericultural by-products to enhance the overall value of the seri-economy. Silk-derived biomaterials and silkworm by-products have demonstrated considerable potential in high-value applications such as regenerative medicine, tissue engineering, medical textiles, drug delivery systems, and cosmetic formulations. In particular, silkworm pupae are increasingly recognized as valuable resources for therapeutic compounds, human nutrition, animal feed, and organic fertilizers. Moreover, residual sericulture waste is being explored as a renewable feedstock for sustainable biofuel production, contributing to circular economy models and environmental conservation (Jaiswal et al., 2020).

In summary, while sericulture faces mounting environmental and socio-economic challenges, technological innovation, sustainable management practices, and by-product valorization offer promising pathways for enhancing resilience, profitability, and long-term sustainability of the industry (Table 1).

Table 1. Constraints and technological trends shaping modern sericulture

Category	Key Issues / Trends	Impacts and Implications	References
Environmental & Biological Challenges	Climate change, unpredictable weather, temperature extremes	Degradation of mulberry leaf quality, reduced silkworm productivity	Singh & Massey, 2025
	Silkworm diseases (pébrine, flacherie)	High risk of disease outbreaks and potential total crop failure	
	Disinfection challenges in mechanized systems	Increased difficulty in disease control and hygiene management	
	Environmental pollution and resource use	Need for sustainable waste management due to pesticide use and high-water consumption	
Socio-Economic Challenges	Labor shortages and aging workforce	Reduced availability of skilled labor due to urban migration of youth	Singh & Massey, 2025
	Market instability and price volatility	Financial insecurity for small-scale farmers	Singh & Massey, 2025
	Competition from synthetic fibers	Decline in demand for natural silk products	Dhane et al., 2004
	Inadequate infrastructure	Unreliable electricity and limited access to disease-free silkworm eggs	Singh & Massey, 2025
	Changing consumer preferences	Need for ethical, eco-friendly, and sustainable silk production	Singh & Massey, 2025
Emerging Trends & Opportunities	Biorefinery approach in sericulture	Value addition through utilization of sericulture by-products	Jaiswal et al., 2020
	Use of silk by-products in healthcare	Applications in regenerative medicine, tissue engineering, medical textiles, drug delivery, and cosmetics	
	Utilization of silkworm pupae	Sources of therapeutics, nutrition, animal feed, and fertilizers	
	Sustainable energy solutions	Exploration of sericulture waste for biofuel generation	

CONCLUSIONS

Sericulture has transitioned from a traditional craft into a sophisticated bio-industry defined by "Precision Rearing". By integrating molecular biology, IoT-driven automation, and AI diagnostics, the industry achieves real-time environmental control and early disease detection.

Mulberry cultivation has evolved into a data-driven science using soil sensors, drones, and nano-fertilizers to optimize leaf quality. Precision irrigation and biotechnological breakthroughs, such as genomic editing and hydroponics, ensure a consistent supply of nutrient-dense, climate-resilient foliage.

Larval rearing now centers on "super-industrial" silkworms, genetically optimized via CRISPR/Cas9 for superior strength and natural fluorescence. Managed by robotic systems, these biological assets thrive in smart rearing houses featuring full environmental autonomy.

The industry has fully embraced a circular "Pharma-Farming" model, upcycling byproducts like sericin and pupae into high-value revenue streams. The zero-waste approach transforms potential pollutants into sought-after biomaterials, ensuring a sustainable and economically robust future for global silk production. Advancements in sericulture continue to reshape the industry into a sustainable, technology-driven bioeconomy with widespread global impact.

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PERFORMANCE, CARCASS TRAITS, AND PRODUCTIVE POTENTIAL OF SUFFOLK CROSSBRED LAMBS WITH ROMANIAN BREEDS: A LITERATURE REVIEW

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Abstract

This review compiles multi-decade results on crossbreeding Suffolk rams with major Romanian local sheep breeds - Tsigai, Tsurcana, Teleorman Black Head, Spanca, and Merino-type populations. Data extracted from previously published studies highlight significant improvements in pre-weaning growth, fattening efficiency, carcass classification, meat composition, and adult morpho-productive traits in F_1 crossbreds. Suffolk inheritance led to higher birth weights, increased weaning weights, and superior average daily gains compared to pure local breeds. Carcass evaluations using the EUROP system indicated substantial progress in muscularity (frequent U and R scores) and desirable fat cover classes. Meat quality assessments confirmed higher meat percentages and improved meat-to-bone ratios. Adult F_1 animals also demonstrated larger body frames and greater productive potential. Collectively, reviewed findings show that Suffolk crossbreeding enhances growth performance and carcass value while maintaining the adaptive resilience of local breeds, supporting its continued use for improving meat production in Romanian sheep farming.

Key words*: lamb performance, meat quality, Romanian breed, Suffolk.*

INTRODUCTION

The improvement of meat production in sheep farming remains a central objective of modern animal husbandry, especially in production systems where indigenous breeds possess remarkable adaptive qualities but show moderate performance in growth rate and carcass yield. Romania hosts several well-established local breeds - such as Tsigai, Tsurcana, Teleorman Black Head, and Merino-derived populations - each adapted to specific ecological zones and extensive grazing systems. These breeds are valued for their resilience, prolificacy, and ability to sustain production under low-input conditions; however, their meat production potential is limited by relatively low average daily gains and variable carcass conformation (Ilișiu et al., 2011; Zamfir et al., 2017). An empirical study conducted from Ilișiu et al. (2013) indicated that most sheep farmers in the traditional Tsigai region intend to enhance their flocks' genetic potential and herd size by crossing with Suffolk meat breed.

The Suffolk breed, internationally recognized for its superior meat production traits, has been widely used as a terminal sire in crossbreeding programs to enhance growth and carcass quality in lambs. Suffolk rams transmit rapid growth, excellent muscularity, early maturity, and desirable carcass characteristics that align with EUROP classification standards. Early Romanian research emphasized the significant improvement potential of Suffolk when crossed with local ewes, with some of the first evaluations showing notable increases in growth rate and slaughter performance (Ciolcă & Timariu, 1972; Pop et al., 1974, cited by Pop et al., 2007).

Subsequent studies carried out in different geographical regions confirmed that Suffolk × local breed F_1 lambs exhibit higher birth weights, improved suckling growth, and

superior weaning weights compared with purebred Tsigai or Tsurcana lambs (Vlaic et al., 2008; Popa et al., 2009; Popa et al., 2011; Popa et al., 2013). Additional evaluations demonstrated consistent improvements in post-weaning fattening ability and feed utilization efficiency, with lower Net Energy Meat (NEM) requirements per unit of gain in Suffolk crossbreds compared with local breeds (Ghiță et al., 2011; Zamfir et al., 2017).

Carcass quality studies further support the advantages of Suffolk inheritance. When assessed using the EUROP classification system, Suffolk × local breed lambs commonly achieved U and R conformation classes, in contrast with pure local lambs, which were more frequently classified in O or R classes (Ilișiu et al., 2011; Negre et al., 2020). Fat cover was also more favorable in crossbreds, with a higher proportion of animals falling within the commercially desirable 2 and 3 fat classes. Meat quality analyses revealed enhanced carcass composition, including higher meat percentages and improved meat-to-bone ratios in F_1 lambs relative to maternal breeds (Zamfir et al., 2017; Ilișiu et al., 2011).

Despite the abundance of individual studies, a comprehensive synthesis of Suffolk × Romanian local breed crossbreeding results has been lacking. This review aims to integrate multi-decade data to provide a clear and structured evaluation of how Suffolk genetics influence growth, fattening efficiency, carcass characteristics, and morpho-productive traits in Romanian sheep production systems.

MATERIALS AND METHODS

This review was carried out through a structured and systematic analysis of published research addressing the performance of Suffolk × Romanian local sheep crossbreeding. A comprehensive literature search was conducted in 2025 in several scientific databases, including Web of Science, Scopus, Google Scholar, CAB Abstracts, ScienceDirect, and the Romanian national academic repositories (e.g., USAMV institutional archives). The search strategy used combinations of keywords such as *"Suffolk crossbred lambs", "Romanian local sheep breeds", "Tsigai", "Tsurcana", "Teleorman Black Head", "Merino", "growth performance", "carcass quality",* and *"EUROP classification"*. Boolean operators (AND/OR), phrase searching, and breed-specific terms were applied to maximize relevance.

Inclusion criteria consisted of: (1) studies published up to and 2025; (2) research involving Suffolk rams crossed with Romanian local ewe breeds; (3) studies reporting quantitative data for at least one of the following categories: pre-weaning growth, fattening performance, carcass quality, meat composition, or adult morpho-productive indicators; (4) peer-reviewed articles, conference proceedings, doctoral theses, and institutional research reports.

Exclusion criteria included: (1) studies lacking numerical data; (2) purely genetic or molecular studies without productive traits; (3) crossbreeding trials not involving Suffolk as breed.

All data extracted for this review originated from studies listed in the provided tables. These tables served as the primary dataset, which was checked against the original publications when available. For each study, specific variables were recorded: birth weight, 30-day weight, weaning weight, average daily gain, slaughter percentage, EUROP carcass classification scores, tissue composition, wool traits, and adult body measurements.

Data extraction followed a standardized procedure:

- compilation of numerical values in comparative matrices;
- categorization into five trait groups: growth performance, fattening efficiency, carcass quality, meat/tissue composition, and adult morpho-productive indicators;
- cross-breed comparison, with emphasis on differences between F_1 crossbreds and the corresponding maternal pure breeds;
- qualitative interpretation;

Because the reviewed studies used different sample sizes, management systems, and regional conditions, no meta-analysis or statistical pooling was attempted. Instead, a narrative comparative synthesis was employed to highlight consistent trends across decades of research. This methodological approach ensures a reliable and integrative evaluation of

Suffolk crossbreeding outcomes in Romanian sheep production.

RESULTS AND DISCUSSIONS

Growth parameters during the suckling period

The data summarized in Table 1 demonstrate clear differences between local Romanian breeds and their F_1 crosses with Suffolk rams. F_1 lambs consistently exhibit higher birth weights, indicating a positive influence of Suffolk genetics and heterosis on prenatal development (Vlaic et al., 2008; Popa et al., 2009). For instance, F_1 S × TA lambs reached 4.3-4.5 kg at birth, compared with 3.3-3.6 kg in pure Tsurcana lambs. During the suckling period, F_1 lambs also show superior growth performance, measured both by weight at 30 days and weaning weight.

Table 1. Growth parameters during the suckling period in lambs from local breeds and F_1 crossbreds with the Suffolk breed

Genotype	Sex	Body weight - kg			WA (d)	Average daily gain - g			Author/ Location
		Birth	30 d	WW		Birth - 30 d	60 - W	Birth - W	
F_1 S x TI	M; F	3.35; 3.11	-	20.88; 20.88	89;88	-	-	195; 199	Vlaic et al. (2008), Jucu
TI	M; F	3.60; 3.40	-	17.26; 16.50	84;87	-	-	163; 151	
F_1 S x TA	M; F	4.33; 4.00	11.93; 11.57	25.03; 23.96	90	253; 252	199; 186	-	Popa et al. (2009), Sibiu
TA	M; F	3.63; 3.30	9.60; 8.40	18.70; 16.50	90	199; 170	138; 109	-	
F_1 S x TA	M; F	4.30; 3.90	-	20.95; 19.60	70	-	-	238; 224	Popa et al. (2011), Sibiu
TA	M/F	3.60/3.30	-	16.20/15.25	70	-	-	180/171	
F_1 S x TBH	M + F	5.36	15.03	24.92	60	-	-	322	Ghiță et al. (2011), INCDBNA Balotesti
TBH	M + F	4.77	13.40	18.31	60	-	-	253	
F_1 S x TA	M/F	4.52/3.87	-	25.52/23.87	90	-	-	-	Popa et al. (2013), Sibiu
TA	M/F	3.69/3.35	-	18.58/16.69	90	-	-	-	

TI – Tsigai breed; TA – Tsurcana breed; TBH – Teleorman Black Head breed; S – Suffolk breed; W – weaning; WA – weaning age; d – day; - = not reported.

Average daily gain (ADG) from birth to weaning was consistently higher in F_1 crosses, with increases of up to 30-35% compared to local breeds. This enhanced early growth is important for production systems targeting lambs for market at 90 days, as faster growth reduces time to slaughter and improves feed efficiency. The observed differences are consistent across studies and regions, confirming the robustness of Suffolk influence on pre-weaning performance.

Although the numerical differences between studies are evident, several critical aspects must be considered when interpreting these results:

i) Variability between studies and experimental conditions

The data originate from different studies (Vlaic et al., 2008; Popa et al., 2009; 2011; 2013; Ghiță et al., 2011), conducted in different years, locations, and management systems (Jucu, Sibiu, Balotești) introduces:

- variability in feeding systems;
- differences in environmental and climatic conditions;
- possible differences in flock health status;
- differences in maternal nutrition and milk production;

ii) Birth weight - genetic effect vs maternal effect

While higher birth weights of F1 lambs is attributed to Suffolk genetics and heterosis, birth weight is strongly influenced by:

- maternal nutrition during gestation;
- parity and body condition of ewes.

The lack of detailed information regarding parity and litter size makes it difficult to isolate the heterosis component from maternal environmental influences.

iii) Average daily gain (ADG) - consistency vs. biological interpretation

The reported 30-35% increase in ADG in F1 lambs is substantial and economically relevant,

but the following aspects should also be taken into consideration:

- in some cases, 30-day weights are missing, reducing the ability to evaluate growth dynamics;
- weaning ages vary (60, 70, 84-90 days), which complicates direct comparison of ADG values;
- some datasets combine males and females (M + F), while others separate them, potentially masking sex-related growth differences.

Without statistical parameters, it is not possible to evaluate whether the reported differences are statistically significant or only numerically superior.

Fattening efficiency

Table 2 provides insights into post-weaning growth performance and energy efficiency of F_1 crossbred lambs compared to pure local breeds. Net Energy Meat (NEM) represents the amount of energy required to produce 1 g of body weight gain. Lower NEM values indicate better feed conversion efficiency, which is crucial for reducing production costs and improving profitability. Suffolk crossbreds consistently demonstrated lower NEM requirements, suggesting that the genetic contribution of Suffolk rams enhances metabolic efficiency. For instance, S × TI lambs required 7938 kcal/gain, compared with 8243 kcal/gain for Transilvania Merino × Suffolk lambs (Ciolcă & Timariu, 1972). This difference illustrates how crossbreeding can improve energy utilization by promoting lean tissue deposits over fat accumulation.

F_1 lambs also exhibited higher average daily gains (ADG) during the fattening period, ranging from 203 to 256 g/day, compared with 170-198 g/day in pure local breeds. This enhanced growth rate is particularly advantageous for meat production systems targeting early market weights, as faster-growing lambs reach slaughter weight sooner, reducing feed and maintenance costs. Moreover, the observed slaughter percentages were consistently higher in F_1 crossbreds, sometimes reaching 57%, reflecting superior carcass composition with a higher proportion of muscle and lower fat deposition. These characteristics align with consumer preferences for leaner meat and with market requirements for uniform carcass quality.

Table 2. The results of fattening of F_1 crossbred lambs

Maternal breed	Paternal breed	Fattening and slaughter results			Author/Year
		Average daily gain - g -	NEM* kcal/gain	Slaughtering percentage - % -	(Ciolcă & Timariu, 1972)
Transilvania Merino	Suffolk	203	8243.20	53.8	
Spanca		256	7948.80	57.0	
Tsigai		198	7948.80	55.4	
Tsurcana		170	9273.60	50.3	
Suffolk	Tsigai	228	7938.15	-	Pop et al. (1974), cited by Pop et al. (2007)
Tsigai	Suffolk	174	7938.15	-	Pop et al. (1974), cited by Pop et al. (2007)
Suffolk	Tsigai	238	7520.54	51.9	Ilișiu et al. (2010)
Suffolk	Transilvania Meriono	209	-	-	Maier (1975), cited by Pascal (2007)
Suffolk	Merino	229	-	55.9	Zamfir et al. (2017)
Suffolk	Teleorman Black Head	-	-	54.5	Ghiță et al. (2011)

*The NU (nutrient unit) reported value were transformed in NEM according to Burlacu et al. (2002)

The improved fattening performance of crossbreds can be explained by heterosis, which enhances growth rate, feed efficiency, and muscular development. Suffolk sires contribute alleles associated with rapid postnatal growth, efficient protein deposits, and skeletal development, while local breeds provide adaptation traits, such as tolerance to local environmental conditions and pasture utilization efficiency. This combination allows

F_1 lambs to maximize growth without compromising adaptability or robustness.

From a practical standpoint, these findings suggest that Suffolk × local breed crossbreeding is an effective strategy for intensive and semi-intensive meat production systems. Producers can expect faster growth, improved feed efficiency, and higher slaughter yield, translating into greater economic returns. Additionally, the reduced energy requirement per gram of gain (lower NEM) implies a more sustainable system, with lower feed input per unit of meat produced.

However, variability between studies should be taken into consideration. The data originate from experiments conducted in different years, under diverse management and feeding systems, which may significantly influence fattening performance and slaughter parameters. Differences in housing conditions, feed quality, energy-protein balance, health status, and fattening duration can affect average daily gain and slaughter yield.

Moreover, some results may reflect optimized experimental conditions rather than commercial farm realities, potentially leading to an overestimation of performance parameters. The lack of uniform experimental design and incomplete reporting of statistical indicators further limits strict comparative interpretation across studies.

Carcass quality according to the EUROP system

F_1 crossbred lambs (Table 3) consistently achieved superior carcass conformation and fat cover compared with pure local breeds. Regarding carcass conformation, most F_1 lambs were classified in the U (very good) or R (good) categories, whereas local breeds were predominantly in the O (average) or R (good) categories (Ilișiu et al., 2011; Negre et al., 2020). This distribution indicates enhanced muscular development in crossbreds, particularly in the loin, back, and hindquarters, which are the most valuable parts of the carcass for meat production. The contribution of Suffolk genetics likely promotes faster muscle fiber proliferation and greater lean tissue deposition, resulting in improved carcass conformation.

Concerning fat cover, F_1 lambs were mostly classified in classes 2 (thin) and 3 (average), which represent the optimal range for commercial markets. This ensures adequate marbling and palatability without excessive fat deposition, improving consumer acceptance and market value. Local breeds showed more variable distribution, with some individuals in class 4 (thick), which may affect uniformity and marketability.

However, the relationship between growth rate and fat deposition must be interpreted carefully.

i) Market variability and commercial implications

Carcass value is highly market-dependent:

- Southern European and Mediterranean markets often prefer lighter carcasses with moderate fat cover.
- Western European markets may favor heavier carcasses with better conformation (U class).
- Export markets can impose strict fat limits.

Table 3. Carcass quality appreciation with the EUROP system at young ovine from different genotypes

Specification	**Ilișiu et al. (2011)**		**Negre et al. (2020)**	
	TI, %	F_1 S × TI	MP, %	F_1 S x MP
I. Class after conformation				
- E - excellent	-	-	-	-
- U - very good	-	60.0	-	100.0
- R - good	40.0	40.0	100.0	-
- O - average	60.0	-		-
- P weak	-	-	-	-
II. Class after the layer of fat				
- 1 - small	-	-	-	-
- 2 - thin	-	60.0	33.0	-
- 3 - average	60.0	40.0	67.00	100.0
- 4 - big	40.0	-	-	
- 5 - very big	-	-	-	-

Therefore, the superiority of F1 carcasses may vary depending on the target market. A genotype optimized for rapid growth and high conformation may not always align with consumer preferences for leaner meat or specific carcass weights.

ii) Potential trade-offs: growth vs. fat deposition

Crossbreeding with Suffolk improves growth rate and muscular conformation, but biological trade-offs must be considered:

Rapid growth vs. fat accumulation

Faster-growing lambs may deposit fat earlier if slaughter is delayed, potentially reducing carcass classification.

Muscle development vs. meat quality traits

Increased muscularity does not automatically imply improved meat quality (tenderness, intramuscular fat, flavor). Without compositional analysis (protein %, fat %, moisture), it is not possible to determine whether meat quality improved proportionally.

Efficiency vs. adaptability

While F1 lambs show superior carcass traits, local breeds may offer better adaptability and resilience under extensive systems. Under suboptimal feeding, genetic potential for superior conformation may not be fully expressed.

Tissue composition and meat quality indicators of carcasses

The tissue composition and meat quality indicators of carcasses from purebred and F_1 crossbred lambs are presented in Table 4. The data reveal significant differences between local breeds and F_1 crossbreds in meat, bone, and fat proportions, as well as in meat-to-bone and meat-to-fat ratios.

F_1 crossbreds consistently showed higher meat percentages and lower bone content compared with local breeds. For example, F_1 S × TI lambs had 62.53% meat and 18.84% bone, whereas pure TI lambs had 57.85% meat and 20.11% bone (Ilișiu et al., 2011). This represents a clear improvement in carcass yield and lean tissue deposition, which is crucial for commercial meat production. Similarly, the meat-to-bone ratio, an important indicator of carcass quality, was higher in crossbreds (3.32 in F_1 S × TI *vs*. 2.88 in TI), indicating more efficient allocation of body mass to edible muscle tissue.

Fat deposits also differed between genotypes. F_1 S × TI lambs had 18.63% fat compared with 22.04% in TI, reflecting a more balanced carcass composition with adequate energy reserves but not excessive fat, which aligns with market and consumer preferences for lean meat. The meat-to-fat ratio was also improved in crossbreds (3.35 *vs.* 2.62 in TI), further supporting the efficiency of Suffolk genetics in promoting lean growth while controlling fat accumulation.

Table 4. Tissue composition and meat quality indicators of carcasses

Genotyp	Meat (%)	Bone (%)	Fat (%)	Meat/ bone ratio	Meat/ fat ratio	Author and year
PM	60.72	23.32	15.96	2.60	-	Zamfir et al. (2017)
F1 S x PM	59.13	22.42	18.45	2.63	-	
TI	57.85	20.11	22.04	2.88	2.62	Ilișiu et al. (2011)
F1 S x TI	62.53	18.84	18.63	3.32	3.35	
TBH	-	-	-	2.19	-	Ghiță et al. (2011)
F1 S x TBH	-	-	-	2.68	-	

Similar trends were observed in other crossbreeds. F_1 S × PM (Palas Merino) lambs had slightly lower meat percentages than pure PM (59.13% *vs.* 60.72%), but a higher meat-to-bone ratio (2.63 *vs.* 2.60), indicating better carcass composition for processing purposes.

F_1 S × TBH crossbreds had a meat-to-bone ratio of 2.68 compared with 2.19 in TBH, highlighting the positive impact of crossbreeding on skeletal efficiency and edible yield (Ghiță et al., 2011).

Overall, these results confirm that Suffolk crossbreeding enhances carcass quality by increasing lean tissue proportion, improving meat-to-bone and meat-to-fat ratios, and reducing excessive fat deposition.

Morpho-productive indicators of adult F_1 crossbreds compared to maternal breeds

Table 5 summarizes the morpho-productive characteristics of adult F_1 crossbred sheep in comparison with their respective maternal breeds. The results reveal that F_1 crossbreds exhibited a consistent advantage over maternal breeds. For instance, F_1 S × MP rams reached 109.86 kg compared to 89.50 kg in pure MP rams, representing a 22.9% increase in adult body weight (Zamfir et al., 2012). Similarly, F_1 S × TA males and females were heavier than their TA counterparts (61.70 kg *vs*. 50.40 kg for males, and 47.15 kg *vs.* 40.10 kg for females). This demonstrates the effect of Suffolk genetics in enhancing growth potential, likely due to improved muscle development and skeletal frame, a clear manifestation of heterosis. In terms of wool production, F_1 S × MP rams produced 6.47 kg compared with 9.10 kg in MP rams. This reduction may reflect a trade-off between meat and wool traits, where selection for growth and muscularity in crossbreds slightly reduces wool yield. Wool length and fiber diameter also showed variations: F_1 S × MP rams had shorter and finer fibers than maternal MP, suggesting a modification of wool characteristics due to crossbreeding. For F_1 S × TA lambs, data are more limited, but ranges indicate a tendency for moderate wool production and fiber fineness, still within acceptable market ranges for Tsurcana wool.

Table 5. The morpho-productive indicators at the F1 crossbred adult animal comparatively to maternal breed

Genotyp	Sex	Body weight (kg)	Wool production (kg)	Wool length (cm)	Wool fiber diameter (µm)	Author and year
F_1S x MP	M	109.86	6.47	7.71	23.71	Zamfir et al. (2012)
MP	M	89.50	9.10	10.33	21.33	
F_1S x TA	M	61.70*	-	-	-	Popa et al. (2011)* Pădeanu et al. (2015)** Zamfir et al. (2022)*** Enciu et al. (2024)****
TA	M	50.40*	6.4 ≈ 8.2****	22 ≈ 30***	44.92 ≈ 59.79****	
F_1S x TA	F	47.15*	-	-	-	
TA	F	40.10*	2.3 ≈ 4.9****	30.89**	37.03 ≈ 46.67****	

≈ - interval

Overall, F_1 crossbreds combine superior growth and body size with wool traits that remain commercially viable. The increase in body weight and skeletal dimensions enhances meat yield, whereas wool characteristics are influenced by maternal genetics and are adequate for local textile production. This balance makes F_1 crossbreds suitable for dual-purpose systems in which both meat and wool are economically important.

The results highlight the practical benefits of crossbreeding Suffolk rams with local breeds, providing animals that outperform maternal breeds in growth and carcass yield while maintaining wool quality within acceptable limits. Such information is essential for breeders aiming to optimize profitability and efficiency in sheep production systems.

Main body dimensions of adult F_1 S × MP rams compared to maternal breed

Table 6 summarizes the morphometric measurements of adult F_1 S × MP rams in comparison with the maternal MP breed. All recorded parameters, indicate superior structural development in F_1 crossbreds.

These morphometric improvements demonstrate the positive influence of Suffolk genetics in enhancing frame size, structural robustness, and muscular potential, while maintaining balance and functional conformation in crossbred rams. The data confirm that F_1 S × MP rams are larger, more robust, and structurally superior compared with pure maternal MP rams, which translates into higher meat production potential and overall productivity for meat-oriented systems.

Table 6. Main body dimensions at the rams of F_1 S x MP comparatively to maternal breed (Zamfir et al., 2012)

Item	UM	Genotyp		Differences between F_1 S x M and MP	
		F_1 S x MP	MP	± cm	± %
Height at withers	Cm	76.00	70.11	+ 5.85	+ 8.40
Height at croup	Cm	75.86	71.11	+ 4.75	+ 6.68
Length of body	Cm	82.43	75.26	+ 7.17	+ 9.53
Length of croup	Cm	29.14	25.96	+ 3.18	+ 12.24
Width at shoulders	Cm	29.14	23.66	+ 5.48	+ 23.16
With at hip-femur articulations	Cm	30.14	24.59	+ 5.55	+ 22.57
Depth of thorax	Cm	36.57	34.15	+ 2.35	+ 6.88
Width of thorax	Cm	32.14	30.33	+ 1.81	+ 5.97
Thoracic perimeter	Cm	102.14	100.74	+ 1.40	+ 1.39
Perimeter of shinbone	Cm	11.50	11.30	+ 0.20	+ 1.77

Limitations of the crossbreeding strategy

While crossing local breeds with Suffolk improves growth rate, carcass conformation, and pre-weaning performance, several limitations and long-term considerations should be explicitly acknowledged:

Genetic dilution of local breeds

Repeated crossbreeding can reduce the genetic purity of local breeds, potentially eroding unique adaptive traits such as disease resistance, mothering ability, and resilience to extensive or harsh environments.

Long-term sustainability

Dependence on crossbreeding for production gains may compromise the long-term sustainability of local breeds. Maintaining purebred populations is essential to preserve biodiversity and ensure a genetic reservoir for future breeding programs.

Dependency on imported genetics

Using Suffolk rams requires continuous access to high-quality imported genetics, which can be costly and vulnerable to supply disruptions. This reliance may also limit local farmers' self-sufficiency and increase production risks.

CONCLUSIONS

The integrative analysis of the literature consistently highlights the superiority of F_1 hybrids produced using Suffolk rams as terminal sires, confirming the efficiency of this strategy for improving meat production in Romanian sheep farming. The consistency of results, regardless of maternal breed, study period, or rearing conditions, underscores the robustness of the Suffolk genetic contribution and supports the continuation and expansion of crossbreeding programs at the national level. Furthermore, the analyzed data indicate that this breeding approach aligns with current demands for carcass type and production efficiency, providing a clear genetic solution for optimizing economic performance. Overall, the systematic use of this crossbreeding scheme can significantly enhance the competitiveness of the Romanian sheep sector by producing more productive animals without compromising the adaptability of local breeds to specific rearing conditions.

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QUALITY ASSESSMENT OF *Longissimus dorsi* IN AUBRAC CATTLE: IMPLICATIONS FOR MEAT NUTRITION AND SUSTAINABILITY

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Abstract

The Aubrac cattle breed is renowned for its adaptability to mountainous environments, growth performance, and notably favorable nutritional properties of its meat. This study investigated the biochemical composition of the longissimus dorsi muscle in male (n = 28) and female (n = 10) Aubrac cattle, focusing on amino acid and fatty acid profiles. Males exhibited higher concentrations of essential amino acids (15.05% vs. 12.68% in females), suggesting superior protein quality, while fatty acid analysis revealed higher saturated fatty acids (48.96% vs. 46.82%) and polyunsaturated fatty acids (3.96% vs. 2.66%) in males, whereas females showed higher monounsaturated fatty acids (44.45% vs. 39.11%). These results indicate that sex significantly influences muscle composition, impacting both nutritional value and meat quality. The findings highlight the genetic potential of Aubrac cattle to produce protein- and lipid-rich beef with favorable health-related traits, making this breed particularly suitable for extensive and sustainable production systems. Such insights can guide breeders, meat producers, and researchers in implementing targeted management, selection, and breeding strategies to optimize beef quality and consumer benefits.

Key words*: Aubrac, meat, nutrition, quality, sustainability.*

INTRODUCTION

Cattle and beef production hold considerable importance for human society from nutritional, economic, and cultural perspectives. From a nutritional standpoint, beef represents a valuable source of high-quality proteins, essential for tissue development, maintenance of physiological functions, and proper functioning of the immune system. Regular consumption contributes to an adequate intake of essential amino acids required for maintaining a balanced diet (Poroșnicu et al., 2026). In addition, beef provides important micronutrients, including vitamin B12, iron, zinc, and selenium, which are critical for metabolic processes and overall human health. The presence of heme iron, a highly bioavailable form, further enhances its nutritional value by supporting hemoglobin synthesis and efficient oxygen transport within the body.

Beyond its dietary significance, the cattle and beef industry plays a vital role in the economy of many countries. It generates employment opportunities and contributes to income across multiple sectors, including farming, processing, and distribution. At the same time, beef occupies a central place in numerous culinary traditions, being a key ingredient in a wide variety of traditional dishes, appreciated for its distinctive flavor and versatility (Maysonnave et al., 2020). In this context, increasing attention has been directed toward specialized beef breeds, such as the Aubrac cattle breed (Bakharev et al., 2018).Originating from the Aubrac region in southern France, this breed is recognized for its long-standing history and its capacity to produce high-quality meat. Aubrac cattle are characterized by a robust and compact conformation, with medium to large body size, a well-developed frame, a broad and strong chest, and strong limbs (Mădescu et al., 2021). Their coat typically ranges from light grey to reddish tones, sometimes accompanied by darker pigmentation around the facial area. Despite their solid appearance, these animals exhibit notable agility.

A defining characteristic of the Aubrac breed is its remarkable adaptability to diverse environmental conditions (Matei et al., 2022). Traditionally raised in mountainous and hilly areas, these cattle demonstrate resilience to

harsh climates and the ability to efficiently utilize marginal grazing lands. This adaptability has facilitated their successful expansion into various regions, including lowland areas, where they continue to perform well under different production systems (Aviles et al., 2015).

The meat obtained from Aubrac cattle is widely appreciated for its superior sensory qualities, including fine texture, juiciness, and rich flavor profile. A key attribute is its excellent marbling, characterized by the uniform distribution of intramuscular fat, which enhances tenderness and palatability. As a result, Aubrac beef is highly valued on both local and international markets, meeting the growing consumer demand for premium-quality products (Mădescu et al., 2025).

Furthermore, beyond meat production, Aubrac cattle may also serve additional roles in certain regions, such as animal traction or participation in agricultural exhibitions, reflecting their multifunctional value within rural systems. The expansion of this breed in countries such as Romania illustrates a broader trend of agricultural diversification and alignment with evolving market requirements, offering benefits to both producers and consumers (Mădescu et al., 2025).

The chemical composition of beef is a key determinant of its nutritional value, with particular emphasis on lipid and protein fractions (Wu et al., 2016). Among these, fatty acids play a central role, as their profile directly influences both the technological properties of meat and its implications for human health (Bostami et al., 2023). Beef lipids consist of a complex mixture of saturated and unsaturated fatty acids, the relative proportions of which are influenced by genetic factors, feeding strategies, and production systems (Listrat et al., 2016). While saturated fatty acids are generally present in higher amounts, increasing attention has been directed toward the beneficial effects of unsaturated fatty acids, which are associated with improved lipid metabolism and a reduced risk of cardiovascular diseases (Davis et al., 2022; Davidescu et al., 2024). Consequently, the characterization of fatty acid composition has become an important criterion in the evaluation of meat quality (Mădescu et al., 2025).

In addition to lipids, the protein fraction of beef is of major nutritional importance, being defined by its amino acid composition (Ucar et al., 2024). Beef provides a complete profile of essential amino acids, which cannot be synthesized by the human body and must be obtained through dietary intake, thus playing a fundamental role in protein synthesis, tissue development, and metabolic regulation (Alekseeva et al., 2019). Furthermore, non-essential amino acids contribute to maintaining physiological balance and supporting various biochemical processes. The variability of amino acid composition, influenced by factors such as breed and feeding conditions, makes its assessment a valuable indicator of the biological quality of meat (Emery, 2013).

Within this context, the integrated evaluation of fatty acid and amino acid profiles offers a comprehensive approach to assessing beef quality (Lee et al., 2020). Such analyses are particularly relevant in the case of specialized beef breeds, including Aubrac cattle, where both genetic background and production conditions may contribute to the development of meat with enhanced nutritional and sensory attributes (O.M. Sheveleva et al., 2021).

MATERIALS AND METHODS

The study used 38 Aubrac beef cattle (28 males and 10 females), aged 15-18 months, raised in a semi-intensive system on a farm in southeastern Romania. The Aubrac cattle included in the study were slaughtered at 18 months of age in the farm's own slaughterhouse, which complies with the regulations set forth by Regulation (EC) No. 1099/2009 on the protection of animals at the time of slaughter, also adhering to hygiene and food safety standards. Meat samples were taken from the Longissimus dorsi muscle at the 13th rib (sirloin area), following ISO 3100-1:1991 guidelines for representative sampling. Each sample weighed 300-500 g and was prepared and stored for laboratory analysis.

The fatty acid profile of beef samples was determined using gas chromatography–mass spectrometry (GC-MS) following conversion to fatty acid methyl esters (FAME). Lipids were extracted with a chloroform-methanol mixture, after which transmethylation was performed using boron trifluoride (BF_3) in methanol at 60 °C for 30 min (AOAC, 1990).

FAME separation was carried out on a polar capillary column (30 m × 0.25 mm,

polyethylene glycol), using a temperature program from 100°C to 250°C. Helium was employed as the carrier gas (1.5 mL/min), with a split ratio of 40:1. Identification of compounds was achieved by mass spectrometry based on characteristic fragmentation patterns, while quantification was performed using a certified reference standard (FAME Supelco 37 Mix). Results were expressed as percentages of total identified fatty acids (FAO, 1991).

The analysis enabled the determination of major saturated, monounsaturated, and polyunsaturated fatty acids, including specific trans isomers. Based on these data, lipid quality indices were calculated, such as total saturated (ΣSFA), monounsaturated (ΣMUFA), polyunsaturated (ΣPUFA), and unsaturated fatty acids (ΣUFA), as well as omega-3 (Ω3), omega-6 (Ω6), and total trans fatty acids (TFA) (Simeanu et al., 2022; FAO, 1991).

The amino acid composition of beef samples was determined using an automatic amino acid analyzer (Mikrotechna AAA 881, Model 118/119 CL, CR). Prior to analysis, samples were subjected to acid hydrolysis with 6 M HCl at 110°C for 24 h to release free amino acids. Under these conditions, cysteine and methionine were converted into their oxidized forms (cysteic acid and methionine sulfone), allowing for their reliable quantification (Simeanu et al., 2022; FAO, 1991).

Tryptophan content was assessed separately following alkaline hydrolysis with NaOH at 110°C for 22 h, in accordance with standardized analytical procedures. Amino acid concentrations were expressed as g/16 g nitrogen (equivalent to g/100 g protein), enabling accurate evaluation of protein quality and nutritional value (Simeanu et al., 2022; FAO, 1991).

The collected data were first organized in a Microsoft Excel database for efficient handling and subsequently analyzed using SPSS (Statistical Package for the Social Sciences). Descriptive statistical analysis was applied, and the obtained results were expressed as percentages according to gender distribution. No inferential statistical tests were reported in the present study. The results are presented in tables as percentage distributions by gender.

RESULTS AND DISCUSSIONS

The fatty acid composition and the calculated lipid quality indices are summarized in Table 1. Analyses were performed on the M. Longissimus Dorsi, with samples collected from both male and female animals. The fatty acids in beef are an important aspect of the meat's chemical composition. We classify them into saturated and unsaturated fatty acids, and their proportion and type can influence the quality and nutritional characteristics of the meat.

Table 1.The content of saturated, monounsaturated, and polyunsaturated fatty acids in the *Longissimus dorsi* muscle

Specification	Gender	
	Male (n = 28)	**Female** (n = 10)
[1]SFA %	**48.96 %**	**46.82 %**
C10:0	0.40	0.10
C12:0	0.40	0.12
C14:0	3.28	3.36
C15:0	0.48	0.50
C16:0	23.43	28.23
C17:0	1.02	0.98
C18:0	19.86	13.45
C20:0	0.09	0.09
[2]MUFA %	**39.11 %**	**44.45 %**
C14:1	0.63	0.64
C16:1	2.92	4.38
C18:1	35.41	39.31
C20:1	0.16	0.12
[3]PUFA %	**3.96 %**	**2.66 %**
C18:2:cis	**3.49**	**2.10**
C18:3:cis:ω3	**0.47**	**0.56**

1 = saturated fatty acids (SFA); 2 = monounsaturated fatty acids (MUFA); 3 = polyunsaturated fatty acids (PUFA); n = number of samples.

Table 1 presents the fatty acid composition of the *Longissimus dorsi* muscle, highlighting differences between male and female Aubrac cattle. Saturated fatty acids (SFA) represented the predominant fraction in both sexes, accounting for 48.96% in males and 46.82% in females. Among individual SFAs, palmitic acid (C16:0) was notably higher in females (28.23%) compared to males (23.43%), whereas stearic acid (C18:0) was more abundant in males (19.86%) than in females (13.45%). Minor saturated fatty acids, including capric (C10:0), lauric (C12:0), myristic (C14:0), pentadecanoic (C15:0), heptadecanoic (C17:0), and arachidic acid (C20:0), were present in low concentrations with minimal sex-related variation.

Monounsaturated fatty acids (MUFA) were more prevalent in females (44.45%) than in males (39.11%). Oleic acid (C18:1) was the major MUFA, contributing 39.31% in females and 35.41% in males, while palmitoleic acid (C16:1) and myristoleic acid (C14:1) showed modest increases in females, reflecting an overall higher MUFA content in female beef. Eicosenoic acid (C20:1) remained minor in both sexes.

Polyunsaturated fatty acids (PUFA) constituted the smallest fraction, with males exhibiting 3.96% and females 2.66%. Linoleic acid (C18:2 cis) was predominant among PUFAs, particularly in males (3.49%), whereas alpha-linolenic acid (C18:3 cis, ω-3) was slightly higher in females (0.56% vs. 0.47% in males). Overall, male beef was characterized by higher total SFA and slightly elevated PUFA content, while female beef showed greater MUFA proportions, particularly oleic and palmitoleic acids, which may enhance nutritional quality and influence meat sensory attributes. These results underline sex-related differences in fatty acid composition that could inform breeding and dietary strategies aimed at optimizing meat quality.

Amino acids constitute the building blocks of proteins and their composition can be influenced by species, breed, and dietary factors (Albracht-Schulte et al., 2021). Assessing the amino acid profile provides valuable insights into the nutritional quality and biological value of meat. Table 2 summarizes the amino acid composition of the M. Longissimus dorsi muscle, including relevant indices, with samples obtained from both male and female animals.

Table 2. The content of essential and non-essential amino acids in the *Longissimus dorsi* muscle

Specification	Gender	
	Male (n = 28)	**Female** (n = 10)
[1]EAA %	**15.05 %**	**12.68 %**
Phenylalanine	1.12	0.93
Isoleucine	1.33	1.12
Histidine	1.17	0.95
Leucine	2.35	1.89
Lysine	2.60	2.15
Methionine	0.75	0.61
Threonine	1.30	1.09
Total tryptophan	3.06	2.85
Valine	1.38	1.11
[2]NEAA %	**15.56 %**	**13.28 %**
Aspartic acid	2.67	2.24
Glutamic acid	4.31	3.72
Alanine	1.61	1.42
Arginine	2.12	1.86
Cystine+Cysteine	0.29	0.27
Glycine	1.19	1.00
Hydroxyproline	0.01	0.01
Proline	1.10	0.87
Serine	1.21	1.02
Tyrosine	1.04	0.88

1 = essential amino acids (EAA); 2 = non-essential amino acids (NEAA);
n = number of samples.

The data presented in Table 2 highlight notable differences between male and female Longissimus dorsi muscles in terms of both essential (EAA) and non-essential amino acid (NEAA) content. Males consistently exhibit higher concentrations of all measured amino acids, with total EAA content reaching 15.05% compared to 12.68% in females, and total NEAA content of 15.56% versus 13.28% in females. Among essential amino acids, leucine and lysine were particularly abundant in males (2.35% and 2.60%, respectively), compared to females (1.89% and 2.15%). This trend is mirrored in non-essential amino acids, with glutamic acid and aspartic acid showing higher levels in males (4.31% and 2.67%) relative to females (3.72% and 2.24%).

These differences in amino acid composition may be linked to physiological and metabolic distinctions between genders. The higher EAA content in males suggests a greater capacity for protein synthesis and muscle growth, potentially reflecting the influence of anabolic hormones such as testosterone. Similarly, the elevated NEAA levels, particularly of glutamic acid and aspartic acid, indicate a higher availability of substrates for energy metabolism, nitrogen transport, and biosynthetic processes in male muscle tissue.

Moreover, the overall profile suggests that male *Longissimus dorsi* muscles may provide a more complete source of dietary protein in terms of both essential and non-essential amino acids. From a nutritional perspective, these findings underscore the importance of considering gender-specific differences when evaluating meat quality and protein value. The higher content of both EAA and NEAA in males could contribute to improved functional properties in processed meat products, such as enhanced texture and water-holding capacity, which are influenced by protein composition.

The observed differences between males and females in the *Longissimus dorsi* muscle amino acid profile reflect underlying biological variations in muscle metabolism, growth potential, and protein utilization.

CONCLUSIONS

The present study highlights clear sex-related differences in the fatty acid and amino acid composition of the *Longissimus dorsi* muscle in Aubrac cattle. Male muscles exhibited higher concentrations of both essential and non-essential amino acids, indicating greater protein quality and a higher potential for muscle growth. In contrast, female muscles were characterized by a higher content of monounsaturated fatty acids, particularly oleic and palmitoleic acids, which may contribute to improved meat flavor, tenderness, and nutritional value. Saturated fatty acids were predominant in both sexes, whereas polyunsaturated fatty acids were present in smaller proportions, with males showing slightly higher levels. These compositional differences have important implications for meat nutritional quality, sensory attributes, and processing characteristics, suggesting that sex should be considered when evaluating meat quality and developing breeding or feeding strategies aimed at optimizing both protein and lipid profiles. Overall, the findings provide valuable insights into the biological and nutritional distinctions between male and female Aubrac cattle, supporting targeted approaches to enhance meat quality and functional properties.

As a consequence of the reduced number of cows but an increased milk yield, milk production has continuously increased, except the year 1995 when it recorded the lowest level.

The North Eastern region is traditionally suitable for cow rearing, due to its pastures and meadows, the important number of cow livestock and possibilities to produce ecological milk.

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COMPARATIVE ANALYSIS OF MILK QUALITY PARAMATERS IN DAIRY FARMS FROM TRANSYLVANIA, ROMANIA

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Abstract

Milk quality, including physicochemical and microbiological parameters, is an essential element to increase the value of dairy products. Within the European Union, milk producers are incentivized to enhance these qualitative parameters by establishing solid standards and differentiated compensation systems. This study aims to present a comparative analysis of key milk quality parameters in dairy farms from Transylvania, Romania, between 2023 and 2025. For the scope of the study, four groups of farms, grouped by herd size, were assessed for milk physicochemical and microbiological indicators - fat, protein, density, total dry matter, lactose, somatic cell count, and total germ count. Data analysis included comparisons with Romanian national averages, EU averages, and top-performing EU countries (e.g., France, Germany), considering milk production volumes and predominant cattle breeds. The analysis revealed variations in milk quality parameters across farm groups, with differences influenced by herd size and breed composition. Comparative results highlighted areas where Transylvanian farms close in or diverge from national and EU benchmarks. The findings underscore opportunities for targeted improvements in milk quality among dairy farms in Transylvania. Implementing specific technical measures and adhering to sustainable milk quality standards could enhance raw milk valuation and increase processing yields on long-term, implicitly the farmers revenues.

Key words: *competitiveness, microbiology, measure, milk quality, physicochemical.*

INTRODUCTION

The physicochemical and microbiological quality of cow milk represent essential milk parameters with direct impact in the competitiveness of milk valorisation, especially considering the focus of European Union (EU) farmers to reduce the number of cows and to increase the production level and efficiency of milk production per cow, implicitly the level of revenues vs. costs, the resulting profitability per animal and overall exploitation.

The objective was to obtain representative conclusions concerning the dynamics of milk production level and milk quality parameters performance per group of farms pursuant to a detailed evaluation of the evolution for the period 2023-2025 of the most important technical indicators of cow milk production to the four groups of cow milk farms from Transylvania - Romania, grouped accordingly to the number of animals per farm; the results per group were compared among the evaluated groups and also with the performance level of similar indicators at Romanian (RO) and EU average and vs. top EU cow milk production performers, France (FR), Germany (DE) and Poland (PL). All the conclusions will support, as further steps of doctoral thesis in preparation, the development of specific technical and commercial farms management models per each group of farms and will be focused on the aspects mentioned above. Considering the importance of cow milk production performance, quantity and quality, the topic was addressed also in other countries, with focus on the impact of different factors of influence (e.g. breed, nutrition, physiology) in the milk production performance (Fiorillo & Amico, 2024; Duguma, 2021).

MATERIALS AND METHODS

Locations, period of research, main parameters evaluated

For the complete period 2023-2025 (at monthly and yearly level) the dynamics of core physics-chemical and microbiological milk quality indicators - fat, protein, density, total dry matter, lactose, somatic cells number, total number of germs - for four groups of cow milk farms from Transylvania (RO) was analysed. The evolution of milk quality parameters for all the groups was studied also in the relation to average milk production per cow for each group and in relation with the structure of cattle herds per each group for the same period 2023-2025. The groups were built considering the total number of cattle, out of which cows in lactations, cows in mammary rest and heifers; Group 50 - 100 cattle, Group 100 - 200 cattle, Group 200 - 300 cattle and Group +300 animals. Each group (lot) includes 8 farms. Simultaneously all the analysed indicators were evaluated in comparison with the first four groups for a fifth group which includes all 32 farms (Group Total). Concerning the breeds structure, at Group Total level, the predominant breed is Romanian Spotted Cattle (80% share), Holstein (15% share) and Montbeliard (5% share from total cumulated herd). Split per groups, Romanian Spotted Cattle has a minimal share of 73% (Group +300) and a maximal share of 99% (Group 50 - 100), Holstein with a share between 1% (Group 50 - 100) and 20% (Group +300) and Montbeliard from 0% to 7% (Group +300), with an increasing share for Holstein and Montbeliard as the group size is higher.

The laboratories used for determining all the analysed parameters of milk are the processor's certified factory laboratory and the Milk Quality Control Foundation from Cluj- Napoca.

Processing and statistical analysis of data

The experimental data collected were subjected to statistical analysis using Statistical Package SPSS software, version 20 (2011). To assess the influence of variation factors on milk production and quality parameters, Anova Multifactorial was applied. The main variation factors included in the model were group size and the year.

For the detection of significant differences among group averages, after achieving the overall effects through GLM (Multivariate general linear model), post-hoc multiple comparison tests were performed - Tukey test and LSD (Least Significant Difference) test. The Tukey test was performed for or the assessment of differences between the pairs, reducing the risk of errors. The LSD test allowed to underline more sensitive differences between groups when the main effect was significant. The results were stated as averages ± standard error, statistical differences considered significant at p 0.05. The dependent variables analysed included milk production level per cow per cow and the physics – chemical milk content: percentages of fat, protein, lactose, total dry matter (TDM), milk density.

As mentioned above, the evolution of each farm group focus on each criterion was studied for 2023, 2024, 2025 and as a comparison among the four groups; implicitly, for the fifth group which included the cattle from all 32 farms, the weighted average results per parameter were compared with the results of similar criteria from all 4 groups. For having representative conclusions, the group results were put in the balance with Romanian national average, European Union (28) average also vs. the national averages of the countries with high results on cow milk production (DE, FR, PL).

The main conclusions were presented, split per each evaluated criterion and focused on every group. Together with recommendations, they could consolidate the specific milk production quality performance of all studied 4 groups of cow milk farms from Transylvania. The primary data evaluated from all 32 cow milk farms (grouped in the mentioned 4 groups and Group total) are the results of own doctoral research, while the national and EU averages constitute public data.

The evaluated farms are independent specialised farms, organized as legal entities, with a mix of family farms and farms with permanent and temporary employees; the cow raising technology is based on free-range housing and the milking is carried out in milking parlors (with a higher degree of automation corresponding to a higher farm size). All the farms are sanitary -veterinary authorized and are included in all the national control programs.

RESULTS AND DISCUSSIONS

For each criterion and group of farms, a Grubbs Test was performed to identify eventual outliers that must be taken out from the group structure. The G critical value identified for n=8 (8 farms per group (lot)) and α equal with 0.05 was 2.126. All the G calculated values were below G critical value and implicitly all the farms from all the groups (for all evaluated criteria) were kept in the designed group structure (e.g. examples of G calculated value for the parameter of milk fat for each group and for 2023: 1.9211 for the Group +300 cattle, 1.4297 for the Group 200 - 300, 1.3395 for the Group 100 - 200, 1.4975 for the Group 50 - 100 cattle, all of them below the G critical value of 2.126 already mentioned). The results and conclusions of the statistical tests are presented within the article in relation to each criteria analysed.

2023-2025 average yearly milk production/ cow and dynamics of cattle herds

For 2023-2025 **the yearly average cow milk production** for all 32 farms (Group Total) registered a slow increase of 0.97% (2025 vs 2023) with a relative stagnation in 2024 vs 2023 of -0.07%; the total number of cattle increased with 5.23% in 2025 vs 2023 and with an intermediary increase of 2.82% in 2024 vs 2023. At the same time, analysing the dynamics of herds structure, it could be underlined that the share of total cows in lactation decreased in 2025 vs 2023 to 41% from 43% and the share of cows in lactation plus heifers decreased to 59% from 60% (as shares from total herd). As split per groups, the Group 200 - 300 registered an important increase of 9.38% in 2025 vs 2023, while the rest of the groups registered negative development (2025 vs 2023) of yearly milk production per cow: -0.77% for Group +300, -1.96% for Group 100 - 200 and -5.07% for Group 50 - 100 cattle. For all the groups (except Group 50 - 100 where the share of cows in lactation increased in 2025 to 47% from 45% in 2023) the shares of cows in lactation decreased with ~1 percentage point (pp). The share of cows in lactation plus heifers decreased in 2025 vs 2023 to 53% from 56% for Group +300, while for the rest of the groups the share is increasing with ~1 pp for Group 200 - 300 and Group 100 - 200 (to 63% from 62%) and with 2 pp at Group 50 - 100 (to 71% from 69%). As a preliminary statement, it could be pre-concluded that for Group Total the yearly milk production per cow registered an increase of 1% in 2025 vs 2023 (6,788 kg from 6,723 kg, with Romanian Spotted Cattle as majority breed - 80% share in the Group total) - even if the share of cows in lactation and cows in lactation plus heifers decreased for each category with ~ 1 pp for the same period. These yearly production levels were above the level of 5,907-5,868 kg per lactation every year, as registered for Romanian Spotted Cattle included in Official Production Control between 2013-2021 (Romanian Breeding Society ACVBR-SIM, Romanian Spotted Cattle Breed Breeding Program, 2025). We may underline the high focus of all evaluated Groups for a stable category share of cows in lactation and also combined with specific effective management practices for a stable or positive development of category share of cows in lactation and heifers– as an anchor to secure the stability of quantitative milk production per farm and the level of revenues, even if the value performance/cow is highly dependent by the received price per kilogram of milk sold for processing. The development of 2023-2025 yearly milk production/cow as a comparison among the groups is presented in the Figure 1.

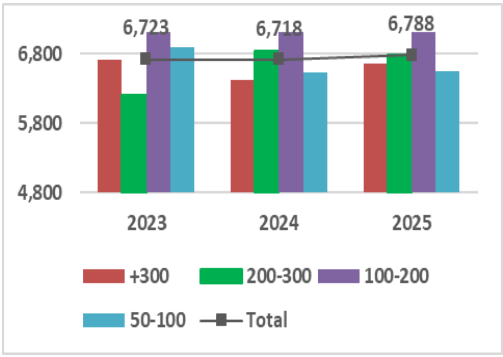


Figure 1. 2023-2025 yearly milk production/cow (kg)

For a much higher representativity, the above data were compared with the yearly average performance in RO and EU and compared to average national performance of DE, FR and PL. The performance of all groups is much higher than RO national average, in 2025 for Group Total with +79.58%, and with a

maximum of +91.74% for Group 100 - 200 and with a minimum of +73.21% for the Group 50 - 100 cattle.

At the same time, it's known that Romania owns the highest share in EU of agricultural exploitations below 2 cattle (~67%) and between 2 and 9 heads (~30%) (Maxim et al., 2025) - which means that more than 95% of RO agricultural exploitations number are just for subsistence and practically without chance to be economically feasible. Implicitly, all these very small exploitations are decreasing significantly RO average of yearly milk production per cow, national average being a very low one in the EU (National Institute of Statistics - Romania, 2025, Animal production. Eurostat, 2025, Milk and milk products, retrieved from https://ec.europa.eu/eurostat/ databrowser/ explore/all/alltheme; SectionAgriculture/ Agriculturalproduction; Dracea, 2020).

As effect, it is more relevant to compare the performance of all the groups with EU average and the countries mentioned above considering that in EU, in average, ~94% of total milk production is sold for processing - compared to RO where the share is below 35% (The National Institute of Statistics of Romania, 2025, Animal production. Eurostat, 2025, Milk and milk products, retrieved from https:/ec.europa.eu/ /eurostat/databrowser/explore/all/alltheme; SectionAgriculture/Agriculturalproduction).

For 2025, the yearly milk production of Group Total represented 82.36% (6,788 kg milk) from EU average (8,242 kg), in a decreasing trend, from 86.18% in 2023; the reason is the higher pace of yearly milk production/ cow increase in EU of 5.6 pp in 2025 vs 2023 (8,242 kg vs 7,800 kg) comparing just +0.97% in 2025 vs 2023 for the Group Total. Analysing per each group, the highest 2025 performance had the Group 100 - 200 cattle where the share of yearly milk production/cow from EU average was 87.93% and the lowest was at Group 50 - 100 cattle with a share of 79.44% from EU average (Eurostat, 2026, Milk and milk products statistics, retrieved from https:// ec.europa .eu/ euro stat/statistics-explained/index.php?title=Milk _and_milk_product_statistics#Data_sources).

Considering that from the analysed countries, only Poland has a performance below EU average - but showing significant efforts to increase the yearly rhythm to reduce the gap - it is obvious that the negative difference in performance vs all these countries is even higher. This evaluation was especially relevant in the context Romania is EU member and implicitly the Romanian farmers are part of the same EU milk market, implying raw material, fat and protein milk constituents, dairy products – therefore milk could travel without trade barriers within EU and the transport cost per unit of fat or per unit of protein is less and less a barrier if we discuss about transportation of concentrated milk or dairy products with a high content of dry matter, such as cheese, milk powder, butter or even cream. Additionally, these aspects underline not only the high importance of milk quantity performance/ cow but also the high contribution in revenues performance of a stronger fat and protein milk raw material content – issues that will be addressed further. Based on the GLM test, average milk production/cow was highly significantly influenced by farm size category (p<0.001). Although the peak of production was observed in 2024 within the 100-200 group, the temporal factor did not exert a statistically significant effect on overall yields. Specifically, LSD post-hoc analysis revealed significant differences between the 50-100 group and all other categories: 100-200 (p=0.012), 200-300 (p=0.001), and >300 (p<0.001). Furthermore, highly significant differences were identified between the 100-200 and >300 groups (p<0.0001), as well as between the 200-300 and >300 group (p<0.001). The 2023-2025 group performance comparison vs RO, EU and vs DE, FR and PL averages it's presented in Figure 2.



Figure 2. 2023-2025 yearly milk production/cow and group compared with RO, EU, DE, FR and PL (kg)

2023-2025 dynamics of physics - chemical milk quality parameters

The **fat and protein milk content** are considered essential milk parameters with a direct impact in the processing yields into dairy products; both milk farmers and processors, have a high focus to continuously improve the milk content in fat and protein; at EU level different standards exist concerning the minimal fat and protein milk content versus the basic milk price paid by the processors to the farms and also bonification and malus systems for these two parameters could be agreed between the two parts as part of their direct milk selling/purchasing contract. During 2023-2025, the **milk fat content** registered a continuous yearly increase for all 4 groups. Implicitly for the Group Total the fat content raised from a level of 3.84% in 2023 to 3.96% in 2025, with an intermediary increase to 3.88% in 2024; the weighted average fat content for the entire period is 3.89% and with a standard deviation of 0.043. The highest fat content of 3.94% (standard deviation of 0.077) had the Group 50 - 100 cattle, followed by Group +300 with 3.91% (standard deviation of 0.068) and Group 100 - 200 with 3.88% fat (standard deviation of 0.034); the lowest fat of 3.84% (standard deviation of 0.044) was registered at Group 200 - 300 - but which also had the highest yearly milk production/cow increase of 9.83% for the same period 2023- 2025. The highest rhythm of 0.19 pp to increase the fat percentage was revealed at Group 50 -100, from 3.85% in 2023 to 4.04% in 2025, while the lowest increasing rhythm of fat content of 0.02 pp had the Group 50 - 100, from 3.86% in 2023 to 3.88% in 2025. A Multivariate General Linear Model (GLM) revealed that size group exerted a significant influence on fat composition (p=0.045). Specifically, Fisher's Least Significant Difference (LSD) post-hoc analysis indicated significant variances between the 50-100 and 100-200 groups (p=0.022), as well as between the 50-100 and 200-300 groups (p=0.014). Conversely, the year of sampling did not yield a statistically significant effect on milk fat levels. The evolution of fat content/group/year and compared to performance of Group Total is described in Figure 3.

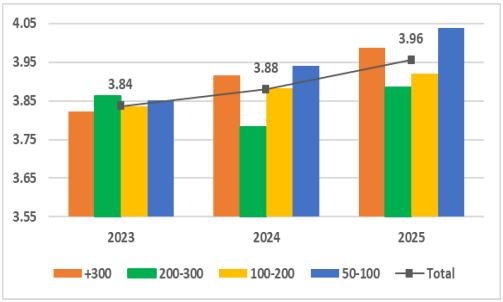


Figure 3. 2023-2025 yearly milk fat content dynamic compared among the four groups and Group Total (%)

Similarly to the criteria of yearly milk production/cow, for having a more relevant conclusion, the results concerning milk fat content dynamic were compared with the yearly average performance in Romania (RO) and European Union (EU) and with average national performance of DE, FR and PL. The performance of all groups is higher than RO national average during the period 2023-2025, except 2024 when the Group Total performance was below - at 3.88% vs 3.90% fat; implicitly, there were two groups with the fat content lower than RO average (Group 200 - 300 at 3.77% fat and Group 100 - 200 at 3.88% fat). Overall, all the groups had a fat content below EU average, even lower than Poland (which is still under EU average) and consistently below Germany and France which both performed still below EU average (CLAL, 2026, EU milk fat supply 2023-2025, retrieved from https://www.clal.it/en/?section=milkfat_supply_eu).

It is relevant that the pace of fat content rhythm of increase is higher between 2023 and 2025 for the analysed groups (e.g. 014 pp for the Group Total and the highest rhythm of 0.19 pp Group 50 - 100) vs EU total rhythm of 0.04 pp.

In absolute values, in 2025 The Group Total fat content was 3.96% with the highest fat content of 4.04 % for the Group 50-100 vs RO average of 3.8%, Poland 4.07%, France and Germany at 4.10% and EU average of 4.16%. Overall, these are encouraging results which underline the high investments the milk farms analyzed did during last years to enhance their milk quality performance to be more competitive vs the EU high milk quality performance already in place. The yearly evolution of fat content/ group and compared to yearly average performance of RO, EU, PL, DE and FR is presented in Figure 4.



Figure 4. 2023-2025 yearly milk fat content /group compared with RO, EU, DE, FR and PL (%)

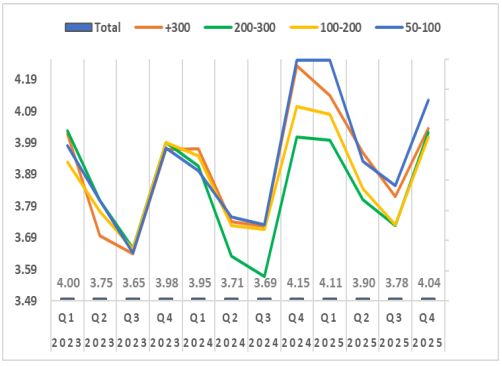


Figure 5. 2023-2025 quarterly milk fat content /group compared with RO, EU, DE, FR and PL (%)

Considering that the demand for fat is not constant during the year, traditionally a higher demand during winter and lower during summer, but still with a high unpredictability during last years at international and EU level due to changes in consumers behaviors trend and due to international political and trade regulation changes, the curve of fat content seasonality during the year was also studied for all 32 farms grouped in the mentioned 4 groups and Group Total. The trend of seasonality is the same for all the groups with higher fat content in winter and lower in summer and with a highest fat content of 4.25% and an amplitude of seasonality of 0.21 pp at the Group 50 - 100 cattle and lower seasonality for the rest of the groups - the lowest fat content and lowest deviations between seasons at Group 200 - 300 cattle. As Group Total, the amplitude of deviations vs the yearly average was for summer [(quarter) Q2 & Q3] between 0.17…0.19 pp and between 0.15…0.27 pp for winter (Q4 & Q1). This evolution could have a positive effect for both farmers and processors as the demand for fat, to be processed in dairy products such as cream, butter, other categories, is structurally higher for winter when also the peak of fat content was met to all four groups studied. The 2023-2025 evolution of fat content per quarter and group compared to quarterly average fat content of Group Total is presented in Figure 5.

During 2023-2025 the **milk protein content** registered a continuous yearly increase for all 4 groups. Implicitly for the Group Total the protein content raised from a level of 3.44% in 2023 to 3.51% in 2025, with an intermediary increase to 3.47% in 2024; the weighted average protein content for the entire period is 3.48% and with a standard deviation of 0.073.

The highest protein content of 3.53% (standard deviation of 0.034) had the Group +300 cattle, followed by Group 50 - 100 with 3.51% (standard deviation of 0.074) and the Group 200 - 300 with 3.45% protein (standard deviation of 0.040); the lowest protein of 3.37% (standard deviation of 0.026) was registered at Group 100 - 200 – but, as mentioned above, which also had the highest yearly milk production/cow increase of 9.83% for the same period 2023-2025. The highest rhythm of 0.14 pp to increase the protein percentage was revealed at Group 50-100, from 3.42% in 2023 to 3.56% in 2025, while the lowest increasing rhythm of protein content of 0.06 pp had the Group 100 - 200, from 3.34% in 2023 to 3.40% in 2025.

Although the highest protein values were recorded in 2025, the year of sampling did not exert a statistically significant influence on protein composition. In contrast, farm size was found to have a highly significant effect (p=0.001). Except for the comparison between the 50 - 100 and 200 - 300 groups, Fisher's Least Significant Difference (LSD) post-hoc tests revealed significant variances between the 100 - 200 and 200 - 300 groups (p=0.05). Furthermore, high levels of significance were observed between the 50 - 100 and 100 - 200 groups (p=0.001) and between the 100 - 200 and +300 groups (p<0.0001). The yearly evolution of protein content split per group and compared to the yearly average performance of Group Total is described below in Figure 6.

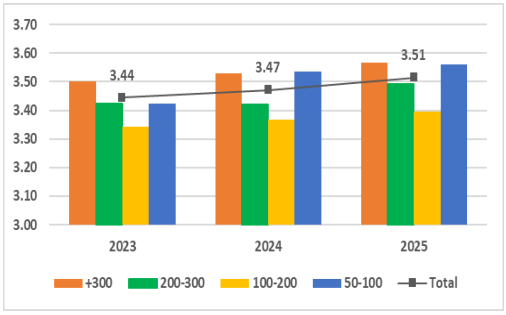


Figure 6. 2023-2025 yearly milk protein content dynamic among the four groups and Group Total (%)

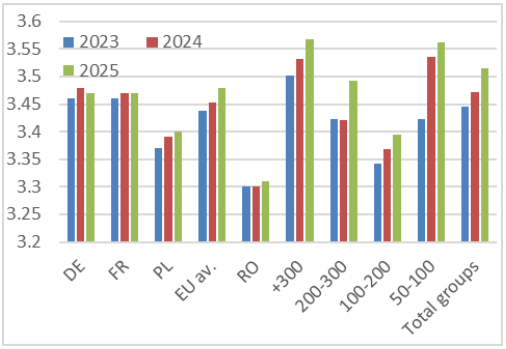


Figure 7. 2023-2025 yearly milk protein content /group compared with RO, EU, DE, FR and PL (%)

Similarly to the criteria of yearly milk production/cow, for more relevant conclusions, the milk protein dynamic was compared with the yearly average performance in Romania and European Union and vs the average national performance of DE, FR and PL.

The performance of all groups is significantly Above RO national average for the period 2023-2025. Overall, all groups had a yearly protein content above EU average, and even higher than Polish national average performance, which is still under EU average, and Germany and France national averages (which performed close to EU average) (CLAL, 2026, EU milk protein supply 2023-2025, retrieved from http://www.clal.it/en/?section=milkprotein_supply.eu).

The pace of protein content increasing rhythm is higher between 2023 and 2025 for the analysed groups, e.g. 0.07 pp for Group Total and the highest rhythm of 0.14 pp for Group 50 - 100), vs EU rhythm of 0.04 pp. In absolute values, in 2025 The Group Total protein content was 3.51% with the highest fat content of 3.56 % for the Group +300 vs RO average of 3.31%, Poland 3.40%, France and Germany at 3.47 and EU average of 3.48%.

Even if the comparison of group results was made vs national or EU averages, the groups protein content results, together with the fat milk content development, underline the high focus of all evaluated milk farms from all four groups to continuously increase their milk quality performance – as a strong need for to be able to compete with the milk quality available on the EU milk market.

The yearly evolution of protein content split per group and compared to the yearly average performance of RO, EU, PL, DE and FR is presented in Figure 7.

Similarly to fat, the curve of protein content seasonality was also studied for 2023 - 2025 for all 32 farms per the 4 groups and as Group Total. The trend of seasonality is the same for all the groups with higher protein content in winter and lower in summer. The highest protein content/ quarter of 3.72% and an amplitude of seasonality of 0.18 pp had the Group +300 cattle and with a lower seasonality and amplitude of quarterly protein content for the rest of the groups - the lowest fat content and lowest deviations between seasons at Group 100 - 200 cattle. As Group Total, the amplitude of deviations vs the yearly average was for summer season (Q2 & Q3) between 0.07…0.10 pp and between 0.10…0.18 pp for winter (Q4 & Q1). Similarly to fat, this development has a synergic effect for farmers (in terms of revenues per kg of milk) and for processors (concerning the production yields) as the demand for protein (to be transformed in dairy products – e.g. cottage, natural cheese) is structurally higher for winter when also the peak of protein content was registered to all four groups of farms analyzed. The variables of year, group size, and quarter exerted a highly significant influence on the observed outcomes, respective milk quantity, fat and protein level ($P<0.0001$). Regarding temporal variations, while milk yield and protein content exhibited highly significant fluctuations across years, fat percentage remained stable between 2023 and 2024. However, fat content differed highly significantly when comparing 2023-2025 and 2024-2025 ($P<0.0001$). Group size also significantly impacted yield, fat, and protein levels ($P<0.0001$). Specifically, LSD post-hoc analysis revealed highly significant differences

(P<0.01) between the following group size: 50 - 100 vs. 100 - 200, 50 - 100 vs. 200 - 300, and 200 - 300 vs. +300. Seasonality, analyzed by quarter (Q), showed that milk quantity in Q2 was significantly different from Q1, Q3, and Q4 (P<0.0001). Furthermore, the LSD test indicated highly significant variances in fat levels across all quarters, except for the Q1 vs. Q4 comparison. Similarly, protein levels differed significantly between all quarters, except for the comparison between Q2 and Q3.

The average fat content and protein content for Group Total (for fat from 3.84% in 2023 to 3.96% in 2025 and for protein from 3.44% in 2023 to 3.51% in 2025) are below the maximal level of 4.02% fat registered in 2013 and respectively significantly higher than the maximal milk protein content of 3.29% registered in 2020 by the Romanian Spotted Cattle included in the Official Production Control (OPC) between 2013 and 2021, (Romanian Breeding Society ACVBR- SIM, Romanian Spotted Cattle Breed Breeding Program, 2025). The data mentioned above (referring to the Official Production Control 2013-2021, Romanian Spotted Cattle) are superior vs the result of OPC from 1994 when the fat content was 3.85…3.95% and the average milk production per cow was between 4,345…5,388 kg (Dinescu & Tontsch, 2005). This development expressed the farmers' focus to increase the milk production per animal, even in the detriment of fat content, in order to increase the revenues per cow (based on an increased milk quantity per animal); this trend has the disadvantage of lower fat supply and availability in the context of an international higher demand for milk fat.

The evolution of protein content per quarter during 2023 and 2025 and split per group and compared to the quarterly average of Group Total is presented in Figure 8.

During 2023-2025 the **milk lactose content** evaluated to all 4 groups registered value in the normal range of cow milk content of 3.6%...5.5% and closer every year to the mean of ~5% (FAO, Gateway to dairy production and products, 2026, retrieved from https://www.fao.org/dairy-production-products/products/milk-composition/en). Except 2024, when lactose content had 0.1 pp decrease vs 2023 (from 4.65% to 4.64% at Group Total - a decrease which come from Group +300 (0.1 pp from 4.65 to 4.64%) and Group 200 - 300 (0.2 pp from 4.66 to 4.64%), the lactose content yearly raised for all the groups. Implicitly for the Group Total the lactose content grew overall (2025 vs 2023) from a level of 4.65% in 2023 to 4.69% in 2025; the weighted average lactose content for the entire period is 4.66% and with a standard deviation of 0.022.

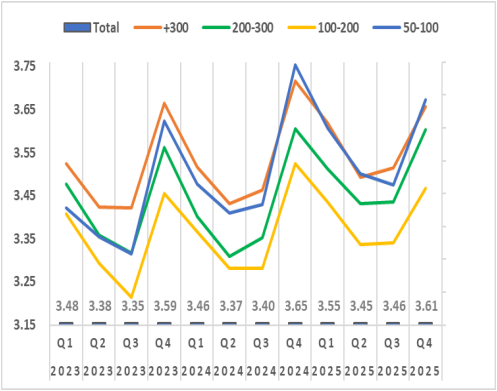


Figure 8. 2023-2025 quarterly milk protein content/ group compared with RO, EU, DE, FR and PL (%)

The peak of lactose content was reached in 2025 for all the groups, while the Group 50 - 100 cattle had the highest value of 4.74% (standard deviation of 0.044), followed by Group 200 -300 with 4.72% (standard deviation of 0.041) and the Group +300 with 4.68% lactose (standard deviation of 0.020); the lowest lactose level of 4.65% (standard deviation of 0.009) was registered at Group 100 - 200, which performed the best concerning yearly milk production/cow (an increase of 9.83% for the same period 2023-2025). The highest rhythm of 0.08 pp of increase of the lactose percentage was revealed at Group 50 - 100, from 4.66% in 2023 to 4.74% in 2025, while the lowest increasing rhythm of protein content of 0.02 pp had the Group 100 - 200, from 4.63% in 2023 to 4.65% in 2025. Relating the results of statistical tests, neither the sampling year nor the farm size category exerted a statistically significant influence on lactose concentration (p>0.05). As an overview, we may consider that all the groups secured positive trends for the dynamic of this indicator, as a positive effect of effective management practices of feed ratio (energy deficit avoided) and of the lactation curve end for their cows in

lactation. The yearly evolution of lactose content/group compared to average performance of Group Total is described in Figure 9.

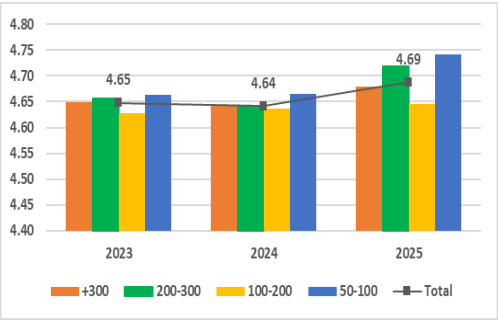


Figure 9. 2023-2025 yearly milk lactose content dynamic among the four groups and Group Total (%)

Between 2023-2025 the studied **milk total dry matter (TDM)** at Group Total and the 4 groups (except Group 200 - 300 in 2024 with a decrease of 0.07 pp from 12.54 to 12.47%) registered a consistent increasing trend. For Group Total the total dry matter content increased from 12.54% in 2023 to 12.75% in 2025 with a pace of 0.07 pp in 2024 and a double pace of 0.14 pp in 2025 (vs previous year); the weighted average TDM content for the entire period is 12.63% and with a standard deviation of 0.111. Considering the increasing trend for all the major milk components (with highest values in 2025) also the highest content of TDM was reached in 2025 to all four groups; highest value of 12.73% (standard deviation of 0.201) at Group 50 - 100 cattle, followed by Group +300 with 12.70% (standard deviation of 0.123) and the Group 200 - 300 with 12.57% TDM (standard deviation of 0.116); the lowest rate of 12.50% (standard deviation of 0.007) was registered at Group 100 - 200. As expected, the highest rhythm of 0.40 pp to increase TDM percentage was registered at Group 50 -100, from 12.53% in 2023 to 12.93% in 2025 - as they also registered the highest yearly increasing rhythm for fat and the second highest for protein. The lowest increasing rhythm of TDM of 0.14 pp had the Group 100 - 200, from 12.43% in 2023 to 12.57% in 2025 - figures which have to be analyzed in the relation to the fact that this group had the highest milk production per cow performance, at the average level 2023-2025 of 7,357 kg, +9.1% vs Group Total average of 6,743 kg/cow.

Relating the results of statistical tests, Total Dry Matter (TDM) was significantly influenced by both the sampling year (p=0.037) and farm size category (p=0.05). According to the LSD post-hoc test, significant differences regarding farm size were observed between the 50-100 and 100-200 groups (p=0.002), the 50-100 and 200-300 groups (P=0.02), and the 100-200 and +300 groups (p=0.008). Furthermore, regarding the year factor, a significant influence was identified between 2023-2025 (p=0.011).

We can pre-conclude that all the groups also expressed a consistent positive development for this indicator, as a positive consequence of the important growth for all main components part of TDM, focus on fat, protein also lactose. The yearly evolution of TDM per group and compared to the yearly average performance of Group Total is described below in Figure 10.

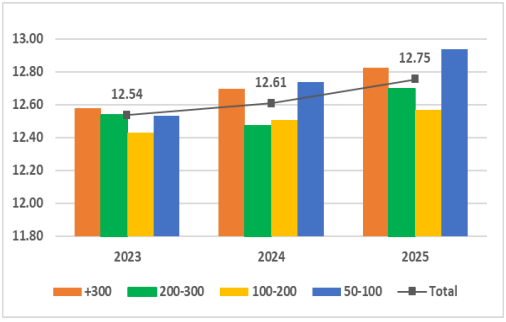


Figure 10. 2023-2025 yearly milk Total Dry Matter dynamic among the four groups and Group Total (%)

For the period of 2023-2025 the milk density showed values in the proximity of average mean for cow milk density of 1.030 g/ml (measured at 20 Celsius degrees) (Agriculture-Institute, 2023, Milk production and milk quality, retrieved from https://agriculture.institute/milk-production-and-quality/density-specific-gravity-of-milk). The Group Total and all 4 groups (except Group 200 - 300 in 2024 with a decrease from 1.0298 in 2023 to 1.0297 of 0.07) registered a stationary or increasing yearly results For Group Total the density from 1.0298 in 2023 to 1.0302 in 2025 with an unchanged 2024 performance vs 2023; the weighted average density for the entire period is 1.0299 and with a standard deviation of 0.0003.

Considering the important increasing fat content for the entire 2023-2025 maintaining and slowly increasing of the milk density it is an appreciated

result which emphasizes the stability and very well managed milk production process in the farms. The highest density (all in 2025) of 1.0304 g/ml (standard deviation of 0.0003) was recorded at Group 50 - 100 and Group 200 - 300 cattle, followed by Group +300 with 1.0303 g/ml (standard deviation of 0.0002); the lowest density of 1.0297 g/ml (standard deviation of 0.0002) was registered at Group 100 - 200.

The highest rhythm of ~0.0007 g/ml (for the entire period 2023-2025) to increase density was registered at Group 200 - 300, from 1.0298 g/ml in 2023 to 1.0304 g/ml in 2025 - as they also registered (together with Group 50 - 100 cattle) the highest density.

The lowest increasing rhythm of TDM of 0.0003 g/ml had the Group +300 and the Group 100 - 200 up to the 2025 level of 1.03033 and 1.0297 g/ml. Regarding the statistical test results, neither the sampling year nor the farm size category exerted a statistically significant influence on milk density (p>0.05).

The yearly evolution of density per group and compared to the yearly average performance of Group Total is described below in Figure 11.

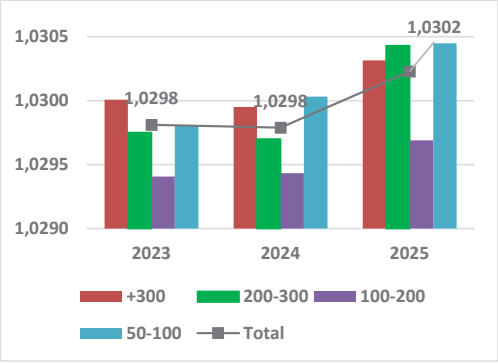


Figure 11. 2023 - 2025 yearly milk density dynamic among the four groups and Group Total (%)

The maximal limits concerning **total bacteria count (TBC) and somatic cells count (SCC)** in the cow milk are transparently expressed in the EU regulation – specifically in the Regulation (EC) No. 853/2004 of the European Parliament and of the European Council of 29 April 2004.

For TBC the limit is maximum 100,000 cfu/ml of milk and calculated as geometrical average of last two months with minimum 2 samples per month. For SCC the maximum limit is 400,000 cells/ml of milk and calculated as geometrical average of last three months with minimum 1sample per month. As the TBC parameter is an indicator of hygiene level of milking process and raw milk storage equipment and the SCC, it reflects the level of mammary gland health, both with direct effects on milk production quantity and quality. On top of being a legal duty according to Regulation (EC) No. 853/2004, all farmers already have in place established measures throughout the entire milk farm processes to reduce as much as possible these values, significantly lower than legal maximal limits.

For all evaluated four groups, both indicators (TBC and SCC) are at a very good level and consistently below the maximal limits, underlining the farmers strong efforts for a high efficiency also for these two parameters. The SCC started in 2023 from 196,032 cells/ml (standard deviation of 7,407) at Group Total and registered an increase of 16.97% until 2025, up to the level of 229,292 cells/ml (standard deviation of 4,160); at groups level the lowest increase (2025 vs 2023) of 10.36% (up to 214,477 cells/ml) was mentioned a Group 50 - 100 and the highest at Group 100 – 200 cattle, + 23.28% up to 212,151 cells/ml).

TBC stayed in a plateau during 2023 – 2025, at the level of ~42,000 ufc/ml as yearly average; as Group Total, 0.33% less TBC in 2025 vs 2023 (at 42,233 ufc/ml and a standard deviation of 205,76) with a maximal deviation of -5.06% TBC ufc/ml for Group 200 - 300 and +6.01% for Group 100 - 200 cattle.

The yearly evolution of TBC and SCC per group and compared to the yearly average performance of Group Total is described below in Figure 12 and Figure 13.

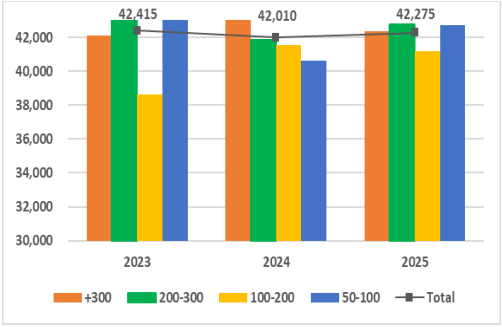


Figure 12. 2023-2025 milk TBC (total number of germs) dynamic among the groups and Group Total (%)

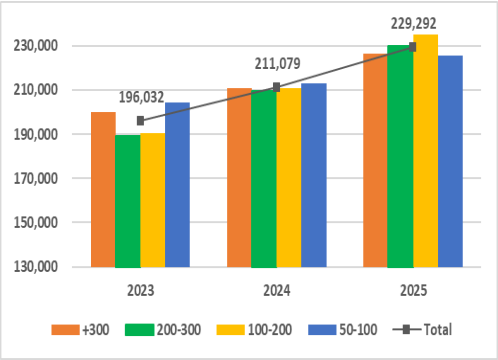


Figure 13. 2023-2025 milk SCC (somatic cells number) dynamic among the groups and Group Total (%)

Concerning the evolution of milk production per cow, the increase rate of EU rhythm of milk production per cow is stronger than the pace that the entire group achieved, leading to an increase in the gap vs. EU average, at ~82% from 86% in 2023 and is even higher compared to performers as France, Germany. Simultaneously, the fat content had a very encouraging dynamic, showing increasing pace of yearly fat content for all groups higher than EU average, with the effect of reducing yearly gap vs. EU average fat percent; for protein content, the performance of all groups is an excellent one, already above EU as well as FR, DE and PL averages. The performance for the rest of the parameters, both physicochemical and microbiological for all the groups, is in line with the EU performance averages.

CONCLUSIONS

Based on the main results and the discussions presented for each criterion during the article, the main conclusion is that for the period 2023-2025, all 32 analyzed cow milk farms from Transylvania – Romania, with Romanian Spotted Cattle as majority breed (~80% share from total herd and at group level from ~75% to ~99% share) performed significantly above Romanian average performance for all evaluated criteria – milk production/cow, fat, protein, density, lactose, total dry matter, density, somatic cells number and total number of germs. The level of influence of different factors such as farm size and yearly evolution for certain parameters such as fat and protein, for all the evaluated criteria and for every group was already presented in detailed *supra* in the article. A detailed analysis of the differences recorded for different parameters between the analyzed groups is already planned within the doctoral thesis in preparation; the focus will be to identify the causes (e.g. breed, growth technology, feeding) and implicitly the categories of improvements recommended especially for those parameters with higher practical and financial impact during the milk processing in dairy products.

Overall business conclusions about the relation between farm size and the milk production quantity and quality dynamics can be expressed only after the technical performance of milk production per group will be evaluated vs. milk production cost and profitability development per group. Such is necessary in order to assess to what extent the management of large vs. small/medium farms manages to reach a stable technical and economic performance, feasible on a long term; this integrated analysis will be a further step of the doctoral thesis in preparation.

Finally, the outlined development is an expression of the significant improvements, of the consistent and long-term investments, as well as of the effective management practices of all the evaluated farms in order to become more competitive on RO and EU milk markets.

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PARTICULARITIES OF CERTAIN BIOCHEMICAL INDICES IN SHEEP INFESTED WITH *Dicrocoelium dendriticum* (Rudolphi, 1819)

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Abstract

The obtained results highlight notable alterations in biochemical parameters in sheep infested with Dicrocoelium spp. Compared to the control group, total protein levels decreased by 11.22% ($p > 0.05$), primarily due to a 12.23% reduction in albumin concentration ($p > 0.05$). In contrast, globulin levels increased by 6.12% ($p > 0.05$), suggesting a compensatory response in protein fractions. Analysis of hepatic enzymes revealed elevated activities in infested sheep, indicating potential liver dysfunction. Specifically, alanine aminotransferase (ALT) activity was 24.63% higher ($p > 0.05$), while gamma-glutamyl transferase (GGT) increased by 21.31% ($p > 0.05$) relative to the control group. Additionally, aspartate aminotransferase (AST), an enzyme present in multiple tissues, showed an 11.77% increase ($p > 0.05$) in the infested animals. These biochemical alterations reflect disturbances in protein metabolism and hepatic function associated with dicroceliosis. Although the differences did not reach statistical significance, the observed trends suggest subclinical effects of the parasite on liver health and systemic metabolism in affected sheep.

Key words*: biochemical parameters, Dicrocoelium dendriticum, liver enzymes, protein metabolism, sheep.*

INTRODUCTION

In the animal world, parasitic organisms represent an essential and integral component of ecosystems, being present in a wide range of natural or anthropogenically modified biocenoses, both terrestrial and aquatic. Numerous investigations conducted on animal populations have demonstrated that parasitic diseases affecting animals and humans in the Republic of Moldova exhibit a wide distribution and are responsible for significant economic and social damage (Zgardan et al., 2001; Zgardan, 2008; Erhan at al., 2005; Erhan, 2010, 2020, 2024; Cercel et al., 2018; Nafornița, 2014, 2016, 2018; Nafornița et al., 2017).

In the study of parasitic diseases, a fundamental role in the control of parasitic agents is played by knowledge of their epidemiology, which depends on the technology of animal management, age, season, and, importantly, on the physiological, biochemical, and morphological processes that develop in the mono- or polyparasitized host organism.

Small ruminants, throughout their productive lifespan, are frequently subjected to parasitic invasions, including those localized in the liver and bile ducts, caused by trematodes such as *Fasciola hepatica* and *Dicrocoelium lanceolatum*, as well as by cestodes such as the larval stage of *Echinococcus granulosus* and *Cysticercus tenuicollis* (Blagburn & Dryden, 1999; Constantin, 2014).

In sheep, contamination with these parasitic agents occurs predominantly via the oral route, and the access of the parasites to the target organ depends on the species involved. The migration of immature forms toward the liver induces pathological changes of varying severity, from mild to severe lesions.

In sheep maintained under different technological systems of breeding and management, multiple parasite species are identified, including those localized in the liver, ultimately leading to increased morbidity and mortality, accompanied by substantial economic losses. The most important parasitic diseases diagnosed in sheep with hepatic localization are

trematodoses (fasciolosis and dicrocoeliosis) and cestodoses (echinococcosis/hydatidosis and cysticercosis) (Zgardan et al., 2001; Zgardan, 2008; Erhan et al., 2005; Erhan, 2010, 2020, 2024).

Within the animal organism, the liver is a metabolically vital organ, functioning both under physiological and pathological conditions, and is often referred to as the "central laboratory of the body". At the hepatic level, indispensable energetic and anabolic processes necessary for cellular life take place, along with catabolic degradation processes affecting multiple structural and functional components of various tissues and cells.

Among the principal functions of the liver are the uptake and distribution of nutrients introduced into the animal organism and the maintenance of their constant concentration in the bloodstream. At the same time, carbohydrate, lipid, and protein metabolism are carried out in the liver, highlighting its multifunctional role, while it also performs an essential excretory function (Dulceanu & Terinte, 2003; Treyer & Müsch, 2013).

The presence of immature or adult parasitic forms in the liver, bile ducts, or blood vessels constitutes the primary cause of pathological processes whose intensity, duration, and severity depend strictly on the parasite's life cycle, migration pathways, feeding type, localization within the organ, and parasite burden.

The Republic of Moldova benefits from favorable climatic and natural conditions for the development of the livestock sector. However, a major obstacle to profitable animal production is represented by a series of pathological conditions of diverse etiology, including those of parasitic origin.

Data reported by numerous researchers indicate that in large and small ruminants under the conditions of the Republic of Moldova, more than 70 species of parasitic etiological agents may be identified. Most of these infections have a subclinical course, yet they exert considerable harmful effects on the host organism, manifested by reduced functionality and productivity.

Each geographical area of the Republic of Moldova presents individual particularities related to distinct climatic and soil characteristics, which influence the composition of animal parasitofauna. According to economic estimates, annual losses generated by parasitic diseases, calculated only from the confiscation of edible organs and tissues, amount to approximately 70 tons of liver, 40 tons of lungs, and 15 tons of other tissues (Rusu, 2021).

A study conducted across the country established that between 2 and 20 species of parasitic organisms may parasitize a single sheep. The prevalence of hepatic trematode infections in sheep may reach 55.1%, cestode infections 19.6%, and nematode infections 100%. Trematode parasitoses are most frequently detected in the northern region of the Republic of Moldova (73.3%), followed by the central region (48.8%) and the southern region (17%). In the case of nematode infections, the prevalence across all geographical regions is 100% (Zgardan et al., 2001; Erhan, 2020, 2024).

Helminthological investigations performed on 1,528 sheep slaughtered in various abattoirs across the Republic of Moldova showed that up to 67.3% were infected with *Fasciola* spp. and *Dicrocoelium* spp. (Zgardan et al., 2001).

Within the host organism, a constant relationship of monoparasitism exists with each parasite species, while simultaneously a state of polyparasitism is present in relation to the total parasitic burden existing at a given time.

Extensive investigations carried out during 1960-1970 and 2000-2001, involving approximately 19,000 ruminants raised under various technological systems and located in different geographical regions of the Republic of Moldova, indicated that about 90-95% of adult ruminants were infected with *Fasciola*, *Dicrocoelium*, *Echinococcus* (hydatid disease), strongyles, *Eimeria*, and *Sarcocystis* species, occurring in variable associations. These studies also demonstrated that the overall level of infection in the two investigated periods did not undergo significant changes (Zgardan et al., 2001; Erhan, 2020, 2024).

In 2012-2013, a similar study was conducted on sheep of different age groups from various regions of the Republic of Moldova. Coproovoscopic examination outlined the presence of polyparasitism based on associations including *Cysticercus tenuicollis*, *Fasciola hepatica*, *Dicrocoelium lanceolatum*, larval *Echinococcus granulosus*, *Dictyocaulus*

filaria, and *Sarcocystis ovicanis*. Necropsy and sanitary-veterinary examination of carcasses also revealed polyinvasions in lambs, with 100% prevalence consisting of three species: *Cysticercus tenuicollis*, larval *Echinococcus granulosus*, and *Trichuris ovis*. In adult sheep, a 100% prevalence of polyparasitism was identified in associations formed by *Cysticercus tenuicollis*, larval *Echinococcus granulosus*, and *Trichuris ovis*, while associations including *Sarcocystis ovicanis* were recorded in 67% of cases, with *Fasciola hepatica* in 54%, and with *Dicrocoelium lanceolatum* in 27% of cases (Erhan et al., 2005; Erhan, 2020).

The pathogenic effects of parasitic forms on the animal organism vary depending on several factors, including the parasite species, migration route, parasite burden, physiological status of the host at the time of invasion, and degree of infestation (Rusu, 2021).

Compared with other trematodoses, the pathogenic impact of *Dicrocoelium dendriticum* is less pronounced than that produced by *Fasciola hepatica*. Among the pathogenic particularities underlying differences in virulence are the route of penetration into the host organism - via the common bile duct in the case of dicrocoelia, which is less destructive - the small size of the parasite, the absence of cuticular spines, and its mode of nutrition, which involves bile rather than biliary tissue (Theodoridis et al., 1991; Otranto & Traversa, 2003; Mandai, 2006; Arbabi et al., 2011).

Recent studies have shown that infestation with *Dicrocoelium dendriticum* leads to significant alterations in hepatic biochemical parameters and oxidative status, reflecting functional impairment of the liver and the host's immune response (Piegari et al., 2021; Hodžić et al., 2020).

During the evolution of these parasitic diseases, following parasite penetration into the liver, a local inflammatory reaction occurs, accompanied by destruction and desquamation of biliary epithelium. Excretory and metabolic products accumulate within the bile duct lumen, altering certain chemical parameters of bile. In chronic and massive infestations, biliary stasis, obstruction, and ductal hypertrophy develop, producing hepatotoxic and pyrogenic effects (Theodoridis et al., 1991).

During migration from the intestine of the definitive host, young dicrocoelia disseminate highly pathogenic bacteria such as *Escherichia coli*, *Staphylococcus albus*, *Streptococcus viridans*, and *Proteus vulgaris*, leading to a 5-18-fold reduction in the bactericidal and fermentative activity of bile and thereby favoring the proliferation of pathogenic bacteria.

Research has established a correlation between microbiological changes in bile and both parasite burden and time elapsed since infestation. At 30 days after infection of young sheep with 2,600 metacercariae of *Dicrocoelium lanceolatum*, the total bacterial count in bile reached 2.36 ± 0.33 million/ml, including: *E. coli* 2.32 ± 0.165; *Staphylococcus* spp. 0.033 ± 0.005; *Streptococcus* spp. 0.007 ± 0.001. At 60 days post-infection, total bacterial count was 1.42 ± 0.28 million/ml, including: *E. coli* 1.40 ± 0.29; *Staphylococcus* spp. 0.017 ± 0.003; *Streptococcus* spp. 0.003 ± 0.001 (Lalor R. et al., 2021).

At 30 days post-infection, total microbial count increased 6.8-fold compared with healthy sheep: *E. coli* increased 6.4-fold, *Clostridium* spp. 8.9-fold, aerobic bacteria 2-fold, *Staphylococcus* spp. 2.1-fold, while total fungi decreased by 22%, including yeasts by 21.4%. At 60 days post-infection, total microbial count increased slightly due to rises in *E. coli* (3-fold), *Staphylococcus* spp. (16.7%), *Clostridium* spp. (8.9%), and aerobic bacilli (32-fold) (Constantinescu et al., 2016; Lalor et al., 2021).

At 90 days post-infection, jejunal microflora in diseased sheep showed a total bacterial count seven times higher than in healthy sheep: *E. coli* 15.3-fold, *Clostridium* spp. 3.3-fold, aerobic bacilli 11.3-fold, *Staphylococcus* spp. 41.5-fold, while total fungi decreased 20-fold, including yeasts 20.2-fold.

In the large intestine of diseased animals, total microbial count increased 2.4-7.2-fold, including *E. coli* 2.4-7.2-fold, *Staphylococcus* spp. 2.6-5.3-fold, aerobic bacilli 3.1-77.6-fold, *Clostridium* spp. 16.3-fold, whereas total fungi decreased 5.2-fold, including yeasts 5.1-fold (Kublickene, 1976; Constantinescu et al., 2016; Lalor et al., 2021).

Simultaneously, at 55 and 65 days post-infection with dicrocoelia, a drastic decrease in hepatic glycogen content and a reduction in the degree

of fatty degeneration were observed. Lipid droplets in hepatocytes exceeded normal volume, causing cellular destruction and accumulation around necrotic foci or in adjacent connective tissue. A gradual decrease in hepatic phosphatase activity was also recorded.

Acid phosphatase activity in dicrocoeliosis manifested from the early stages of the pathological process, reaching maximum levels at 35 and 50 days post-infection (Irinca, 2008; Predescu, 2011; Constantinescu et al., 2016).

According to Bliugher (1964), increased acid phosphatase activity in organs and tissues has an adaptive character, reflecting adaptation to new pathological conditions. Hepatic phosphatase activity is more intense during the migration phase of dicrocoelia through hepatic tissue, reaching maximum values at 35-45 days post-infection, coinciding with morphological alterations and the onset of tissue repair processes (Manga-Gonzalez et al., 2004; Xhemollari et al., 2012; Irinca, 2008; Predescu, 2011).

Furthermore, current research highlights the importance of correlating parasite burden with biochemical and hematological changes, as well as the role of oxidative mechanisms in the pathogenesis of the disease, aspects also supported by recent analyses in the field (Kılıç Altun et al., 2023).

Many researchers reporting clinical signs of dicrocoeliosis have noted that affected animals exhibit visible signs of anemia to varying degrees, the primary cause being the spoliative syndrome induced by large numbers of parasites that deplete hepatic reserves of macro- and microelements (Manga-Gonzalez et al., 2004; Xhemollari et al., 2012; Irinca, 2008; Predescu, 2011).

In sheep affected by dicrocoeliosis, protein, carbohydrate, and mineral metabolism is disrupted, manifested by increased serum alanine and aspartate aminotransferase activity, accompanied by decreased concentrations of free amino acids - particularly essential ones - total protein, and albumins.

In the blood of sheep suffering from dicrocoeliosis, concentrations of Ca, P, Fe, Mg, and Cu decrease, alkaline phosphatase activity increases, and in hepatic tissue the concentrations of vitamins A, C, D, and especially B-complex vitamins are reduced (Otranto & Traversa, 2003; Manga-Gonzalez et al., 2004; Xhemollari et al., 2012; Irinca, 2008; Predescu, 2011).

MATERIALS AND METHODS

For the determination of biochemical changes in infested sheep, the first step consisted of identifying animals infected with dicrocoelia. Fresh fecal samples were collected from suspected sheep, representing 15-20% of the total number of animals in each flock.

Coproscopic examination was performed using standard coprological techniques: the qualitative Fulleborn flotation method, which involves the use of a hypertonic sodium chloride solution with a density of approximately 1.150-1.200, and the sedimentation method (successive washings, polyvalent), recommended for the detection of trematode eggs such as *Fasciola* spp., *Dicrocoelium* spp., etc., using ordinary tap water.

In the Fulleborn method, 5 g of fresh sheep feces were used for each analyzed sample, mixed with 20-30 ml of a supersaturated NaCl solution. The mixture was gently homogenized, filtered into a flotation glass, and allowed to stand for 20-30 minutes. Subsequently, using a bacteriological loop, material was collected from the surface film of the solution and placed on a glass slide. Several collections were made from different areas of the surface layer, covered with a coverslip, and examined under a microscope using the 10× objective. At least 10 microscopic fields were obligatorily examined. In parallel, the detected eggs were counted, the mean value was calculated, and the intensity of invasion was determined (Cozma et al., 2010; Modry et al., 2012).

The centrifugation sedimentation method (active sedimentation) involves concentrating parasitic elements into a reduced volume within a short period of time. A fecal sample of 3-5 g was homogenized with tap water and then filtered into 2-4 centrifuge tubes. The samples were centrifuged at low speed (1,500 rpm) for 2-3 minutes. After centrifugation, the supernatant was discarded, and several preparations were made from the sediment between slide and coverslip. These were examined microscopically using the 10× objective (Cozma et al., 2010; Modry et al., 2012).

Blood sampling for biochemical examination was performed using tubes intended for biochemical analysis containing EDTA. The volume of each blood sample was 10 ml, and each sample was strictly individualized. Blood collection was carried out from the jugular vein prior to slaughter, in order to minimize stress-related interference. In parallel, an additional blood sample without anticoagulant was collected for the determination of hepatic marker enzymes in serum. After collection, the samples were transported rapidly to the laboratory in fresh condition for serum and plasma separation. Hepatic marker enzymes (ALT, AST, GGT, ALP) and biochemical parameters (total bilirubin, total protein, albumin, globulins, Ca, P, Mg) were determined for each individual sample (n = 10) using a Spotchem biochemical analyzer, model SP-4410 (Medex, Biochemical Analysis System, SP-4410 Chemistry Analyzer, USA).

The percentage increase of biochemical parameters and hepatic marker enzyme values was calculated using the following formula:

$$\frac{(X_{\text{infected}} - X_{\text{control}}) \times 100}{X_{\text{control}}}$$

RESULTS AND DISCUSSIONS

The prevalence and particular characteristics of invasion in sheep infected with *Dicrocoelium lanceatum* were investigated over a one-year period, from spring 2017 to spring 2018, within a sheep farm operating under a traditional breeding and management system.

Fecal samples were collected directly from the rectum, individually from each animal, placed in polyethylene containers, strictly labeled, and transported to the Laboratory of Parasitology and Helminthology of the State University of Moldova (USM), as well as to the Faculty of Veterinary Medicine, Department of Animal Health and Public Safety (SASP) of the Technical University of Moldova (UTM). During sample collection, the presence of specific clinical signs was taken into consideration, including weight loss, decreased appetite, and reduced milk production.

In parallel, at slaughter, the livers of the sheep were thoroughly examined by inspection, palpation, and sectioning of the hepatic parenchyma. The macerated hepatic tissue was then subjected to successive washings in order to determine the presence of adult parasitic forms in infected livers (Skriabin, 1928).

Overall, during the study period, 250 fecal samples were collected from a flock consisting of 185 sheep, and liver examinations were performed on 15 slaughtered animals.

The prevalence rate of *Dicrocoelium dendriticum* (Rudolphi, 1819) infection in sheep is shown in Table 1, which indicates an infection rate in sheep of 71.9%, as determined by copro-oidoscopic examination, while the overall prevalence of dicrocelia in necropsy examination of the liver collected from slaughtered or dead animals was 93.3%.

Table 1. Prevalence of *Dicrocoelium dendriticum* (Rudolphi, 1819) infection in sheep

Host	Coproovoscopic examination					Necropsy examination of the liver				
	number animals examined	number animals infested.	number animals with eggs (%)			number animals examined.	number animals infested	number animals with adult parasitic forms (%)		
			<100	<500	<2000			<100	<500	<2000
Sheep	185	133	49	58	26	15	14	4	5	5
% infestation	-	71.9	36.9	43.6	19.5		93.3	26.6	33.3	33.3

At the same time, it was established that the number of parasite eggs detected in fecal samples subjected to coproscopic examination, expressed as eggs per gram of feces (EPG), ranged from 50.0 to 1089.9 in the sheep examined.

Depending on the quantity/number of parasite eggs identified, the severity of the parasitic disease was classified as mild (light), moderate, and severe invasions, according to the data presented in Table 2.

Table 2. Number of *Dicrocoelium dendriticum* (Rudolphi, 1819) eggs in coproovoscopic examination of fecal samples in sheep

Level of infestation	Sheep	
	No. of infected animals	EPG (average number of eggs)
Mild <100	49.0	50
Moderate <500	58	248.6
Severe <2000	26	1089.9
Total	133	-

As previously stated, the primary objective of the present study was to determine the changes occurring in blood biochemical and enzymatic parameters in sheep infected with *Dicrocoelium dendriticum*.

In order to establish the severity of alterations in blood biochemical and enzymatic indices, sheep were selected following coproscopic examination. Two groups of five-year-old sheep, each consisting of 10 animals, were formed. In the control group, coproscopic examination did not reveal infection with dicrocoelia, whereas in the second group, infection with *Dicrocoelium* spp. was confirmed, with a moderate degree of infestation.

The results regarding the modification of biochemical parameters in sheep infected with dicrocoelia are presented in Table 3. Analysis of the data revealed minor changes in plasma protein concentrations in the infected sheep compared with the control group, reflected by a decrease in albumin synthesis. This alteration is observed in the acute phase of the disease and represents a consequence of the migration of larval forms of dicrocoelia through the hepatic parenchyma toward the periphery of the hepatic lobes. Migration occurs via the medium and small bile duct branches, and due to parenchymal destruction, the concentration of certain hepatic enzymes in the blood increases.

Analysis of the data in Table 3 indicates that major importance in interpreting the physiological differences between the control and infected groups is attributed to the levels of enzymes originating from hepatocytes or endothelial cells of the bile ducts, namely ALT, GGT, and AST. Lesser importance is attributed to ALP concentration; however, its determination remains necessary for the differential diagnosis of hepatic lesions compared with other types of tissue damage.

Table 3, concerning the particularities of certain biochemical indices in sheep infested with *Dicrocoelium dendriticum* (Rudolphi, 1819), comprehensively highlights the impact of this helminthiasis on liver function, protein metabolism, and mineral balance. The comparative analysis of the parameters determined in the infested group versus the control group (n = 10 animals/group), expressed as mean ± standard deviation, allows the outline of a biochemical profile characteristic of moderate dicroceliosis.

A central element of the biochemical profile is the alteration of the serum protein pattern. The decrease in total protein concentration by 11.22% in infested sheep reflects the progressive impairment of hepatic synthetic function. The liver is the primary organ responsible for plasma protein synthesis, and the localization of the parasite within the bile ducts leads over time to structural changes in the hepatic parenchyma, including chronic inflammatory and fibrotic processes.

The reduction in albumin by 12.23% represents a sensitive indicator of hepatocellular dysfunction. Albumin, having a relatively long half-life and being synthesized exclusively by hepatocytes, is a reliable marker of protein synthesis capacity. Its decrease suggests chronic hepatic parenchymal damage, even in the absence of evident clinical signs. This alteration contributes to reduced plasma oncotic pressure and may influence the transport of various metabolites and drugs.

In contrast, the 6.12% increase in globulin concentration highlights activation of the humoral immune response. During infestation with *D. dendriticum*, the host organism develops a persistent immune response, leading to an increase in the globulin fraction, particularly immunoglobulins. This redistribution of protein fractions-decreased albumin and increased globulins-constitutes a biochemical feature suggestive of chronic hepatobiliary inflammatory processes.

Hepatic enzyme activity represents one of the most relevant indicators of functional liver impairment in dicroceliosis.

The 24.63% increase in ALT indicates direct hepatocellular damage. ALT is a cytoplasmic enzyme involved in amino acid metabolism, and its release into the bloodstream occurs following alteration of hepatocyte membrane integrity. In infestation with *D. dendriticum*, migration and localization of the parasite within the biliary system generate chronic inflammation, ductal compression, and secondary degenerative changes in adjacent hepatocytes.

GGT showed a 21.31% increase, which is particularly significant from a pathogenetic perspective. This enzyme is primarily localized in the epithelium of the bile ducts and is considered a sensitive marker of cholestasis and biliary tree involvement. The increase in GGT reflects irritation and inflammation of the bile ducts caused by the presence of adult parasites. Therefore, the alteration of GGT represents a specific biochemical feature of dicroceliosis with important diagnostic value.

AST increased by 11.77%, indicating moderate involvement. Since this enzyme is present in multiple tissues (liver, myocardium, skeletal muscle, kidneys, pancreas), its elevation may suggest both hepatic injury and a general state of systemic metabolic stress. In the context of dicroceliosis, increased AST values should be interpreted in correlation with ALT and GGT to confirm a predominantly hepatic origin.

ALP showed a slight increase, compatible with mild cholestatic changes. In the absence of severe bile duct obstruction, this enzyme does not reach markedly elevated values; however, its variation supports the presence of chronic hepatobiliary distress. Slightly elevated total bilirubin values in the infested group suggest moderate impairment of biliary excretory function. Although this alteration does not indicate severe cholestasis, it reflects difficulties in bile pigment elimination, possibly due to thickening and inflammation of the ductal walls. This finding complements the cholestatic profile suggested by increased GGT and ALP activities.

Changes in serum Ca, Mg, and P concentrations indicate metabolic disturbances secondary to hepatic dysfunction. The slight increase in calcium (+3.03%) and magnesium (+8.30%) may be associated with alterations in protein balance and plasma transport mechanisms, considering that part of these elements circulates bound to serum proteins, particularly albumin.

The decrease in phosphorus (−5.22%) may reflect alterations in calcium-phosphorus metabolism and possible influences on cellular energy metabolism. Even discrete mineral imbalances support the presence of systemic metabolic disturbances associated with chronic parasitism. Although the observed differences did not reach statistical significance ($p > 0.05$), the trends identified are biologically relevant. They suggest the development of progressive, predominantly subclinical hepatic dysfunction in sheep infested with *Dicrocoelium dendriticum*. The slow and insidious evolution of dicroceliosis explains the moderate but consistent changes observed in biochemical indices.

Overall, the identified biochemical particularrities - decreased total protein and albumin levels, increased globulins, elevated hepatic enzyme activities (especially ALT and GGT), and discrete mineral disturbances - outline a characteristic profile of chronic hepatobiliary impairment in infested sheep. These findings confirm that dicroceliosis produces functional hepatic alterations even in the absence of severe clinical manifestations, emphasizing the importance of biochemical investigations in assessing the health status of infested flocks.

Table 3. Some biochemical parameters determined in the control group and in sheep with *Dicrocoelium dendriticum* (Rudolphi, 1819)

Parameters	Number head	Animal groups studied	
		Control	*D. dendriticum*
Total bilirubin (mg/dl)	10	0.50 ± 0.06	0.56 ± 0.07
Total protein (g/dl)	10	7.40 ± 0.35	6.57 ± 0.40
Albumin (g/dl)	10	3.60 ± 0.20	3.16 ± 0.18
Globulin (g/dl)	10	3.80 ± 0.28	4.03 ± 0.30
AST (U/L)	10	90.00 ± 8.50	100.60 ± 9.20
ALT (U/L)	10	25.00 ± 3.10	31.16 ± 3.80
ALP (IU/l)	10	110.00 ± 12.00	118.00 ± 14.00
GGT (U/L)	10	35.00 ± 4.20	42.46 ± 5.10
Ca (mg/dl)	10	9.90 ± 0.45	10.20 ± 0.50
P (mg/dl)	10	5.75 ± 0.40	5.45 ± 0.38
Mg (mg/dl)	10	2.40 ± 0.18	2.60 ± 0.20

CONCLUSIONS

Natural infestation of sheep with *Dicrocoelium dendriticum* (Rudolphi, 1819) leads to measurable alterations in biochemical blood parameters, indicating functional impairment of the liver even in cases of moderate parasitic invasion.

Total serum protein concentration decreased by 11.22% ($p > 0.05$) in infested sheep compared to the control group, reflecting a reduction in hepatic synthetic capacity associated with chronic parasitic involvement of the liver.

Albumin levels showed a reduction of 12.23% ($p > 0.05$), confirming impaired protein synthesis at the hepatocellular level and highlighting albumin as a sensitive indicator of hepatic dysfunction in dicrocoeliosis.

In contrast, globulin concentration increased by 6.12% ($p > 0.05$) in the infested group, suggesting activation of the humoral immune response and the chronic inflammatory nature of the infection.

Alanine aminotransferase (ALT) activity increased by 24.63% ($p > 0.05$) in infested sheep, indicating hepatocellular membrane damage and cytoplasmic enzyme leakage into circulation.

Gamma-glutamyl transferase (GGT) activity was elevated by 21.31% ($p > 0.05$), reflecting biliary epithelial involvement and confirming the localization of adult parasites within the bile ducts.

Aspartate aminotransferase (AST) levels increased by 11.77% ($p > 0.05$) in infested animals, indicating not only hepatic involvement but also possible systemic metabolic disturbances due to the multisystemic distribution of this enzyme.

Alkaline phosphatase (ALP) and total bilirubin showed mild increases in the infested group, supporting the presence of moderate cholestatic processes without evidence of severe bile duct obstruction.

Mineral metabolism was also affected, with serum calcium increasing by 3.03% ($p > 0.05$) and magnesium by 8.30% ($p > 0.05$), while serum phosphorus decreased by 5.22% ($p > 0.05$) compared to the control group, indicating subtle metabolic imbalances.

Although the differences between groups did not reach statistical significance ($p > 0.05$), the consistent trends observed across protein, enzymatic, and mineral parameters demonstrate that dicroceliosis induces progressive subclinical hepatic dysfunction and systemic metabolic alterations in sheep.

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NATURAL ALTERNATIVES TO MITIGATE THE EFFECTS OF HEAT STRESS IN BROILER CHICKENS

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Abstract

Heat stress is one of the main challenges facing modern broiler production, having a significant impact on it, thus leading to reduced growth performance, the onset of physiological dysfunctions, and disruption of the oxidative balance. In recent years, numerous studies have been conducted on the use of natural feed additives as a sustainable alternative to mitigate the adverse effects of heat stress. This review article provides a comparative analysis of the main natural bioactive compounds used in the modern poultry industry (antioxidant molecules, plant-derived phytochemicals, and essential oils), highlighting their role in increasing heat tolerance, maintaining intestinal integrity, and supporting physiological homeostasis in broiler chickens. The results presented in the literature, highlight the fact that these natural alternatives can reduce oxidative stress, stimulate feed conversion performance, and partially restore productive performance without the disadvantages associated with the use of antibiotics. Summarizing the analysed information, it can be concluded that the rational integration of natural bioactive compounds into effective and sustainable nutritional programs supports both the resilience and productivity of broiler chickens exposed to heat stress conditions.

Key words*: broiler chicken, heat stress, nutrition, bioactive compounds.*

INTRODUCTION

Heat stress (HS) is defined as a physiological state in which animals, especially birds, cannot maintain a balance between internal heat production and the inability to dissipate it into the environment (Akbarian et al., 2016; Ahmad et al., 2022; Apalowo et al., 2024).

Birds are particularly sensitive to heat stress due to specific physiological and anatomical characteristics, such as full body feather coverage, which acts as a thermal insulator, reducing the efficiency of heat loss through convection and radiation, favouring the accumulation of body heat (Vandana et al., 2021; Emami et al., 2021; Nawaz et al., 2021; Ahmad et al., 2022; Apalowo et al., 2024). Another peculiarity is the lack of sweat glands, which severely limits one of the most effective physiological mechanisms of thermoregulation, namely sweat evaporation at skin level, thus relying on alternative mechanisms such as behavioural changes, peripheral vasodilation, and polypnea (Vandana et al., 2021; Emami et al., 2021; Apalowo et al., 2024; Insawake et al., 2025).

Heat stress is the result of the interaction of several environmental factors, such as high ambient temperature, humidity (which hinders cooling through evaporation), solar radiation, and air movement (Lara et al., 2013; Akbarian et al., 2016; Ahmad et al., 2022; Olayiwola & Adedokun, 2025).

Physiologically, birds are homeothermic organisms capable of maintaining a constant body temperature through a complex thermoregulatory system, but its efficiency critically depends on the ambient environment in which birds can maintain an average internal temperature of 41-42°C without the need for additional energy expenditure for thermoregulation, which is between 18-24°C (Apalowo et al., 2024; Oke et al., 2024). When the ambient temperature exceeds the critical threshold of 25-28°C, physiological stress reactions are triggered, leading to a decrease in feed intake and an increase in water consumption, with a negative effect on body weight and feed conversion rate (Wasti et al., 2020; Ahmad et al., 2022; Li & Wang, 2024).

The scientific literature classifies heat stress according to the temporal profile of exposure into acute heat stress and chronic heat stress, each type triggering different physiological and molecular responses (Oluwagbenga & Fraley, 2023; Moise et al., 2024).

Acute heat stress is defined by short-term exposure, from 1 to 24 hours to a sudden and rapid increase in ambient temperature (27°-38°C) (Ahmad et al., 2022; Oluwagbenga & Fraley, 2023). The initial phase of acute stress is characterized by an acceleration of mitochondrial substrate oxidation and electron transport chain activity, thus generating an exaggerated production of reactive oxygen species (ROS). At this stage, the antioxidant enzymes (superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase GPx), which form the cellular defence mechanism, are often rapidly activated to neutralize free radicals (Akbarian et al., 2016).

Chronic heat stress is prolonged exposure to high temperatures for 7 days or more (Vandana et al., 2021; Ahmad et al., 2022; Oluwagbenga & Fraley, 2023) and can be subdivided into two types: cyclic and constant. Cyclical heat stress is characterized by limited exposure to high temperatures during the day, followed by periods of comfortable temperatures, allowing for partial recovery, while constant heat stress involves exposing birds to constant heat without periods of cooling. The chronic form of exposure to high temperatures is characterized by a decrease in mitochondrial oxidative capacity and the depletion of antioxidant reserves in the body (vitamins A, C, E, and minerals such as Zn, Se), (Akbarian et al., 2016), which can influence overall enzyme activity and hormone levels, such as the T3 hormone (Gheorghe et al., 2022).

Phytogenic (phytobiotic or botanical) additives have gained particular importance in recent years, being integrated into feed rations for broiler chickens, identified as a natural, safe, and sustainable alternative to growth-promoting antibiotics, supporting consumers' growing interest and preference for poultry products obtained without drug residues.

The main advantages identified in the literature are: they do not cause the development of antibiotic resistance among pathogens (Rachwał & Gustaw, 2025); no residues in food products (Aminullah et al., 2025); multifunctional biological action, offering a wide range of benefits due to antioxidant, anti-inflammatory, antimicrobial, and immunomodulatory properties that can improve intestinal barrier integrity, modulate immune response, and reduce oxidative stress (Obianwuna et al., 2024; Urban et al., 2024; Rachwał & Gustaw, 2025; Khatun et al., 2023), safety and reduced toxicity, being considered non-toxic, biodegradable, and environmentally friendly (Mandey & Sompie, 2021; Ivanova et al., 2024). Other benefits of introducing feed additives into the diet of heat-stressed broilers include stimulating digestibility (certain feed additives can improve the aroma and taste of feed by stimulating appetite and digestive enzyme secretion) (Custură et al., 2019; Abdelli et al 2021; Urban et al., 2024), as well as improving the quality of the final product, with the use of phytobiotics being associated with higher meat quality (better retention capacity, favourable amino acid and ketone profile) (Custură et al., 2012; Ruff et al., 2021; Michalczuk et al., 2024).

The purpose of this article is to provide an overview of the main classes of natural bioactive compounds used in broiler nutrition, with a focus on their chemical structure, classification, mechanisms of action, and relevance of their use in the context of heat stress.

MATERIALS AND METHODS

A systematic review of the literature was conducted using the main international scientific databases, Google Scholar, Web of Science, Scopus, ScienceDirect, ResearchGate, and PubMed. The search strategy used predefined keywords such as broiler, heat stress, oxidative stress, phytobiotics, bioactive compounds, natural feed additives, and was structured according to the year and type of publication. Following the application of predefined inclusion and exclusion criteria, 90 studies were identified as relevant and included in the final synthesis.

RESULTS AND DISCUSSIONS

Natural bioactive compounds, also known as phytogenics or phytobiotics, are a class of secondary metabolites synthesized by plants to protect themselves against pathogens and environmental stress. To date, over 5,000 such compounds have been identified in fruits, vegetables, cereals, legumes, nuts, and medicinal plants (Urban et al., 2024). The effectiveness of these compounds is due to their

multiple biological actions, which not only inhibit the proliferation of pathogens by destroying bacterial cell membranes, but also optimize gastrointestinal health by stimulating the secretion of digestive enzymes (Obianwuna et al., 2024). Phytobiotics are also essential in reducing the effects of heat stress, helping to reduce oxidative stress and regulate inflammatory responses, thus contributing to maintaining growth performance and meat quality (Greene et al., 2021; Iliopoulou et al., 2025).

Phytobiotics are derived from a variety of botanical sources and can be classified according to their origin into herbs, spices, essential oils, and oleoresins (Pashtetsky et al., 2020; Abdelli et al., 2021; Mandey et al., 2021). The classification of feed additives into these four categories reflects both their botanical origin, the part of the plant used, and the method of processing (Abdelli et al., 2021; Mahasneh, 2024; Aminullah et al., 2025; Papatsiros et al., 2022).

From a chemical point of view, phytobiotics are an extremely heterogeneous class, including major groups such as phenolic compounds and polyphenols, terpenes and terpenoids, alkaloids, saponins, glycosides, and polysaccharides (Panaite et al., 2019; Pashtetsky et al., 2020; Abdelli et al., 2021; Obianwuna et al., 2024; Urban et al., 2024; Rachwał & Gustaw, 2025).

Phenolic compounds, considered the main bioactive components of feed additives, represent one of the most important groups of secondary metabolites, defined by the presence of at least one hydroxyl (-OH) group directly attached to an aromatic ring (Rachwał & Gustaw, 2025). They are generally divided into two main categories, the first being simple phenolic compounds containing a single phenol unit, such as hydroxyphenols and dihydroxybenzenes, phenolic acids such as hydroxybenzoic acids (gallic acid), hydroxycinnamic acids (ferulic acid, caffeic acid, cinnamic acid) and coumarins (umbelliferone and scopoletin). The second main category is polyphenols, defined by the presence of two or more hydroxyl groups attached to an aromatic ring, classified into four major groups, flavonoids, stilbenes, lignans, and tannins, found in various parts of plants, such as leaves, bark, stem, roots, fruits, and flowers (Goel et al., 2025).

Mechanisms of action and biological activity

In poultry farming, phenolic compounds exert a wide range of beneficial effects on broiler chickens, especially under heat stress conditions, such as mitigating oxidative stress through molecular mechanisms, activating the nuclear factor erythroid 2-related factor 2 (Nrf2/ARE) molecular pathway (stimulation of the Nrf2 transcription factor, which migrates to the nucleus activating the genes responsible for the synthesis of endogenous antioxidant enzymes (superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase GPx), facilitating the neutralization of free radicals before the initiation of lipid, protein, and DNA degradation processes) (Abd El-Hack et al., 2025; Madkour et al., 2025; Aminullah et al., 2025; Zhao et al., 2021) and inhibition of pro-oxidants by blocking pro-oxidant enzymes such as xanthine oxidase and by chelating transition metal ions (Fe^{2+}, Cu^{2+}), limiting the formation of extremely toxic hydroxyl radicals (Basiouni et al., 2023).

It also has anti-inflammatory and immune-regulating effects by suppressing nuclear factor kappa-b (NF-kB) activity and a decrease in pro-inflammatory cytokine levels, including IL-1β, IL-6, and tumour necrosis factor alpha (TNF-α), thereby reducing tissue damage induced by chronic inflammation (Madkour et al., 2025).

Studies conducted on broiler chickens subjected to heat stress (Table 1), have shown that the use of phenolic compounds in the diet acts on heat stress regulation mechanisms by:

- improving the physiological response to stress by reducing body temperature (Ruff et al., 2021), decreasing stress hormones (Oni et al., 2024), and improving biological indicators (Mohiti-Asli & Ghanaatparast-Rashti, 2017; Griela et al., 2021; Jimoh et al., 2022);
- protecting the intestinal barrier and modulating the microflora, intervening in reducing intestinal permeability (Ruff et al., 2021), balancing the microbiome (Saracila et al., 2021), improving morphology by increasing intestinal villi, optimizing nutrient absorption (Oni et al., 2024; Olayiwola & Adedokun, 2025);
- improving meat quality and slaughter parameters, directly influencing physicochemical properties, water retention

capacity, texture and composition, increasing aroma units (aromatic profile), as well as reducing defects (white streaks) in the breast (Chodkowska et al., 2022; Michalczuk et al., 2024);
- stimulating antibody production, increasing the ability to defend against infections (Mohiti-Asli & Ghanaatparast-Rashti, 2017) and reducing the concentration of pro-inflammatory cytokines, limiting heat-induced systemic inflammation (Ruff et al., 2021; Michalczuk et al., 2024; Obianwuna et al., 2024);
- have a hypolipidemic effect, helping to reduce triglyceride and cholesterol levels in meat and blood (Oni et al., 2024, Saracila et al., 2021).

Table 1. Phenolic compounds used in poultry farming

Phytogenic formulation	Hybrid HS (°C)	Dosage	Outcome	Source
Bioactive lipid compounds (oregano & mint)	Ross 308 38.2 ± 2.6°C (max 40.2°C)	150 mg/kg	- improvement in BW, BWG, FCR; - ↓ abdominal fat; - ↓ cholesterol, triglycerides, AST, ALT, urea creatinine; - ↓ water loss through cooking and dripping.	Abdel-Wareth & Lohakare, 2023
Encapsulated EOs (cinnamon, thyme, cloves)	Cobb 500 (THI = 30.52)	100 mg/kg	- ↑ BW, BWG, FCR; - ↑ TAC serum; - ↓hepatic expression of stress genes HSP70, HSP60.	Madkour et al., 2025
Polyphenols (resveratrol)	Yellow - feather 37 ± 2°C (8 h/day)	350-500 mg/kg	- improvement in SMZ; - ↓ rectal temperature; - ↓ corticosterone, ACTH; - ↑ SOD, CAT, GPx.	He et al., 2018
EO (*Lippia origanoides*) + vegetable betaine	Cobb 500 35°C (8 h/day)	350 mg/ton	- improvement in BW, BWG; - ↓ intestinal permeability; - ↑ intestinal villi.	Señas-Cuesta et al., 2023
Plant extracts (olive)	Arbor Acres (chronic)	15 ml/L (water)	- ↑ T3 plasmatic; - ↑ SOD; - ↓ MDA.	Pang et al., 2021
Powder (*Lippia citriodora*)	Ross 308 35°C	0.5% - 1%	- improved BW, FI, FCR; - ↓ cholesterol, LDL, triglycerides; - ↑ leukocytes; - improved meat quality.	Mehrparvar et al., 2016
Powder *(Salix alba)*	Cobb 500 32°C	50 g/100 kg (diet)	- ↓ cholesterol, triglycerides; - ↓ MDA in the liver; - ↓ E. coli, ↑ lactobacilli in the cecum.	Soica et al., 2019
Powder (*Mentha piperita)*	Ross 308 34°C 8 h/day (days 35 - 42)	1%, 2% (diet)	- ↓ cholesterol, triglycerides; - ↓ MDA in the liver; - ↓ *E. coli*, ↑ lactobacilli in the cecum.	Ameri et al., 2016
Dried oregano	Cobb 500 32°C, constant (days 14 - 35)	2%	- balancing the intestinal flora; - ↓ *Enterobacteriaceae* in the intestine.	Criste et al., 2017
Powder *(Rosa canina)*	Cobb 500 32°C, constant (days 14 - 35)	2%	- ↓ BW than in the control group; - colonization of the digestive tract with beneficial lactic acid bacteria.	Criste et al., 2017
Extract (grape seeds)	Cobb 500 (males) 34 ± 1°C, 5 h/day (days 29 - 42)	150, 300, 450 mg/kg	- 300 mg/kg dose: ↑ BW, EPEF; - 450 mg/kg dose: ↓ cholesterol, triglycerides, HSP70 gene expression in the heart and liver.	Hajati et al. 2015
Powder *(Artemisia annua)*	Cobb 500 32°C, constant (days 14 - 35)	2%	- limiting colonization with *Enterobacteriaceae* in the intestine; - ↑ concentration of lactobacilli; - did not improve growth performance.	Panaite et al., 2018
PSPP (steam-exploded pine particles)	Indian river 31°C (6 h/day)	0.5% - 1%	- improved oxidative status; - ↓ rectal temperature;	Goel et al, 2025

Phytogenic formulation	Hybrid HS (°C)	Dosage	Outcome	Source
			- suppression of stress marker genes HSP70, HSP90, NOX4; - ↑ number of beneficial intestinal ba.teria.	
Resveratrol	Crossbred 37 ± 2°C 8 h/day (14 days)	500 mg/kg	- improved ADG; - ↓ FCR; - ↓ corticosterone and ACTH; - ↓ splenic apoptosis and inflammation.	Meng et al., 2022
Curcumin (turmeric)	Ross 34°C (8 h/day)	100 mg/kg	- ↑ carcass yield; - ↓ abdominal fat; - ↓ MDA; - ↓ MUFA, PUFA fatty acid profile; - equilibrates hepatic ATP levels.	Salah et al., 2021
EGCG (green tea)	Huainan partridge 33 ± 1°C	400 mg/kg	- ↑ antioxidant enzyme activity; - ↓ muscle lactic acid; - activates the Nrf2 signalling pathway; - ↓ drip losses.	Zao et al., 2021
Tannic acid	Cobb500 35°C	1%	- ↓ weight gain and feed consumption; - ↑ FCR; - improvement of the lipid profile in muscles.	Ebrahim et al., 2015
ECGC (green tea)	Arbor Acres (males) 35°C (12 h/day)	300, 600 mg/kg	- ↑ BW, FI; ↓ FCR; - ↓ CK, LDH, AST; - improvement in SOD, CAT, GPx.	Luo et al., 2018
Ellagic acid	Arbor Acres 35 ± 2°C	75, 150, 300, 600 mg/kg	- improvement in BWG; - ↓ corticosterone, LPS, DAO; - ↑ antioxidant enzyme activity; - modulation of cecal microbiota.	Yang et al., 2022
Ginestein and hesperidin	Arbor Acres 34 - 37.3°C	5. 20 mg/kg + mix	- ↓ heterophil/ lymphocyte ratio; - improved growth performance BW, FI, CFR; - ↓ CK, LDH; - ↓ HSP70 in the breast muscle.	Kamboh et al., 2013
Liposomal resveratrol (nanoparticles)	Ross 308 36 ± 2°C (6 h/day)	50, 100, 150 mg/kg	- improvement in BWG, FCR; - restoration of renal and hepatic functions; - ↑ expression of SIRT1, SIRT3, SIRT7; - ↓ restoration of HSP70, HSP90 to normal levels.	Kishawy et al., 2023
Resveratrol	Arbor Acres 33 ± 1°C (10 h/day)	400 mg/kg	- ↑ final weight and average daily gain; - activation of the Nrf2 pathway in the intestinal mucosa; - increase in the height of intestinal villi.	Wang et al., 2021

[1]ACTH - adrenocorticotropic hormone; [2]ALT - alanine aminotransferase; [3]AP-1 - activator protein-1; [4]AST - aspartate aminotransferase; [5]ATP - adenosine triphosphate; [6]ADG - average daily gain; [7]BW - body weight; [8]BWG - body weight gain; [9]CAT - catalase; [10]CK - creatin kinase; [11]CKMB - creatine kinase MB; [12]CRYAB - alpha-crystallin B chain; [13]DAO - diamine oxidase; [14]EO - essential oil; [15]EPEF - European production efficacy factor; [16]FCR - feed conversion ratio; [17]FI - feed intake; [18]GPx - glutathione peroxidase; [19]H/L - heterophil/lymphocyte ratio; [20]HSP - heat shock protein; [21]Ig - immunoglobulin; [22]IL - interleukin; [23]LDL - low density lipoprotein; [24]LPS - lipopolysaccharide; [25]MDA - malondialdehyde; [26]MUFA - monounsaturated Fatty Acid; [27]NF-Kb - nuclear factor kappa-b; [28]Nrf2 - nuclear factor erythroid 2-related factor 2; [29]PUFA - polyunsaturated fatty acid; [30]SIRT - sirtuins; [31]SOD - superoxide dismutase; [32]T3 - triiodothyronine; [33]TAC - total antioxidant capacity; [34]THI - temperature - humidity index.

Terpenes/terpenoids are another significant class of secondary metabolites synthesized by plants, major components of essential oils, the largest group of natural products, with over 35000 known components (Dewick, 2009). Structurally, terpenes are hydrocarbons formed by the repetition of isoprene units, while terpenoids are natural organic compounds derived from terpenes by the introduction of oxygen units or the removal of methyl groups, modifications that confer diversity in structure and chemical functionality (Rachwał & Gustaw, 2025).

Terpenes are classified according to the number of isoprene units in their structure, as follows: hemiterpenes (C5) contain a single isoprene unit; monoterpenes (C10) consisting of two units (limonene, γ-terpinene, α-phellandrene, and oxygenated monoterpenes such as carvacrol, thymol, 1.8-cineole); sesquiterpenes (C15) consisting of three units, such as β-caryophyllene and α-humulene; diterpenes

(C20), four units (carnosic acid, carnosol, rosmanol); triterpenes (C30), ginsenosides, consisting of six units, often precursors of steroids; tetraterpenes (C40) represented mainly by carotenoids (astaxanthin, canthaxanthin, lutein, lycopene) and polyterpenes with high molecular weight, such as natural rubber (Dewick, 2009; Tang et al., 2018; Rammuni et al., 2019; Sandner et al., 2020; Jiang et al., 2021; Señas-Cuesta et al., 2023; Urban et al., 2024; Rachwał & Gustaw, 2025; Abeeb, 2025; Zhou et al., 2025).

In poultry farming, terpenes and terpenoids, especially in the form of essential oils (Table 2), exert multifunctional biological effects. The results published by Attia et al. (2017), demonstrate that the administration of thyme essential oil, rich in thymol and carvacrol, has a significant beneficial effect in mitigating heat stress, with direct effects on growth performance by improving feed conversion ratio (FCR); exercises a significant protective role by reducing malondialdehyde (MDA) levels, while also standing out through its high antioxidant activity, manifested by its ability to neutralize free radicals; improved immune protection by achieving higher antibody titers (infectious bursitis), as well as liver protection by lowering the level of liver stress enzymes (AST).

Table 2. Terpenic compounds used in poultry farming

Phytogenic formulation	Hybrid HS (°C)	Dosage	Outcome	Source
Thyme oil	Arbor Acre 33°C	1.0 g/ kg	- improvement in FCR, EPEI; - ↑ WBCs; - ↓ plasma AST.	Attia et al., 2017
Oregano leaf extract	Indian River (males) 32 ± 2°C	50 mg/kg	- ↑ BW, ↓FCR; - ↓ hepatic expression of stress genes HSP70, HSP60, HSP90A.	Madkour et al., 2024
Rosemary extract	Indian River (males) 32 ± 2°C	100 mg/kg	- improved TAC; - ↓ LDL cholesterol, AST enzymes; - ↑ T3 vs. control;	Madkour et al., 2024
EO (*Lippia origanoides*)	Cobb 500 35°C (12 h/day)	37 ppm	- improvement in BW, BWG, CFR; - ↑ SOD; - ↓ IgA and IFN; - ↑ tibia resistance to fracture.	Ruff et al., 2021
EO (*Lippia origanoides)* + EO (*Rosmarinus officinalis)* + Beetroot extract	Cobb 500 35°C (12 h/day)	45 ppm LO + 45 ppm RO + 300 ppm extract	- improvement in BW; - ↓ internal body temperature; - ↓ inflammatory biomarkers; - ↓ intestinal permeability; - ↑ bone mineralization.	Ruff et al., 2021
EO (*Lippia origanoides*) + EO (*Rosmarinus officinalis*) + Natural betaine	Cobb 500 35°C (12 h/day)	45 ppm LO + 45 ppm RO + 300 ppm betaine	- optimal improvement of FCR; - optimal improvement of carcass components (breast, legs); - ↓ intestinal permeability; - ↑ SOD; - improvement of bone mineralization.	Ruff et al., 2021
Thymol	Ross 308 35°C (8 h/day)	250 mg/kg (diet)	- ↑ BWG by 6%, - ↓ FCR by 4%; - ↓ heterophil/lymphocyte ratio (H/L), indicating ↓ stress levels; - ↑ % carcass, breast.	Shad et al., 2015
Carvacrol	Ross 308 35°C (8 h/day)	300 / 500 mg/kg (diet)	- = BWG, FCR; - ↓ cholesterol and H/L ratio; - ↑ GPx activity.	Shad et al., 2015

Phytogenic formulation	Hybrid HS (°C)	Dosage	Outcome	Source
1.8-Cineol / Eucalyptol (microencapsulated)	Ebayka broiler 30 ± 2°C (chronic)	1mg/kg (microcapsules)	- ↑ BWG; - ↓ inflammation (NF-κB, IL-1β, IL-6, TNF-α); - ↑ IL-10; - balancing the gut microbiota (↑ Lactobacillus, ↓ Salmonella).	Jiang et al., 2020
Eucalyptus oil	Cobb 500 32°C (chronic)	1 L oil/ 4000 Lwater / 1 L oil/ 200 L water (spray)	- no effect on growth performance, haematological parameters, leukocytes; -↓ haematocrit (indicator of physiological adaptation); - spray: ↓ Gram-negative bacteria in the respiratory tract.	Petrolli et al., 2019
EO blend (*Eucalyptus globulus labill, Tymus vulgaris, Cymbopogon nardus, Syzgium aromaticum*)	Ross 308 (males) 36°C	250, 500, 750 ml/ 1000 L (water)	- 250 ml/1000l water: - ↓ liver weight; - ↑ abdominal fat; - improved meat colour parameters (a* and L*).	Tekce et al., 2020
Oregano (BLCO)	Ross 308 29.5-38.3°C	150 mg/kg	- improvement in BW; BWG, FCR; - ↑ slaughter yield; - ↓ cholesterol, triglycerides, and liver enzymes (ALT, AST); - ↓ water loss in meat.	Abdel-Wareth & Lohakare, 2023
Mint (BLCP)	Ross 308 29.5-38.3°C	150 mg/kg	- improved growth performance; - ↓ creatinine and urea - ↓ abdominal fat; - improved meat quality.	Abdel-Wareth & Lohakare, 2023
Mint (powder)	Ross 308, 34°C, 8 h/day (days 35 - 42)	1%, 2% (diet)	- ↑ IgG, IgM; - ↑ leukocytes and lymphocytes; - ↓ LDL cholesterol, VLDL.	Ameri et al., 2016
Mint (powder)	Cobb 32-40°C	0.2% (diet)	- optimisation of comfort; - ↑ slaughter yield; - ↓ mortality; - ↑ erythrocytes and haemoglobin.	Elbaz et al., 2022
Mint (extract)	Arbor Acres 31°C (constant)	300 mg/kg	- reduction of splenic lesions; - suppression of inflammatory cytokines (IL-1β, IL-6, TNF-α) by regulating NF-kB and AP-1 signaling pathways.	Ma et al., 2024
Astaxanthin	Cobb 500 32-35°C (days 21-42)	1.33 mg/kg	- ↑ expression of cytoprotective genes / epithelial integrity of the ileum; - ↓ hepatic oxidative stress; - ↓ inflammation.	Kuehu et al., 2024
(*Yucca schidigera*) (powder)	Yellow featherd 34 ± 2°C (days 29-56)	100 mg/kg	- no changes in SOD, CAT, GPx; - ↓ rectal temperature; - ↓MDA.	Luo et al., 2022
Rosemary (purified extract)	SPF broiler 42 ± 1°C	3% nanoemulsion concentration 40 mg/day (water)	- ↓ LDH, CK, CKMB; - more pronounced oxidative stress; - ↑ CRYAB, HSP70.	Tang et al., 2018

[1]ACTH - adrenocorticotropic hormone; [2]ALT - alanine aminotransferase; [3]AP-1 - activator protein-1; [4]AST - aspartate aminotransferase; [5]ATP - adenosine triphosphate; [6]BLCO - bioactive lipid compounds of orindex; [7]BLCP - bioactive lipid compounds of peppermint; [8]BW - body weight; [9]BWG - body weight gain, [10]CAT - catalase; [11]CK - creatin kinase; [12]CKMB - creatine kinase MB; [13]CRYAB - alpha-crystallin B chain; [14]DAO - diamine oxidase; [15]EO - essential oil; [18]EPEI - enzyme protein efficiency index; [19]FCR - feed conversion ratio; [20]FI - feed intake; [21]GPx - glutathione peroxidase; [22]H/L - heterophil/lymphocyte ratio; [23]HSP - heat shock protein; [24]Ig - immunoglobulin; [25]IL - interleukin; [26]LDL - low density lipoprotein; [27]LO - *Lippia origanoides*; [28]LPS - lipopolysaccharide; [29]MDA - malondialdehyde; [30]NF-Kb - nuclear factor kappa-b; [31]RO - *Rosmarinus officinalis*; [32]SIRT - sirtuins; [33]SOD - superoxide dismutase; [34]T3 - triiodothyronine; [35]TAC - total antioxidant capacity; [36]THI - temperature - humidity index; [37]TNF - α-tumour necrosis factor alpha; [38]VLDL - very low density lipoprotein.

Alkaloids are a class of nitrogenous secondary metabolites with a low molecular weight, identified in over 27,000 structures, of which approximately 21,000 come from plants, demonstrating pronounced pharmacological effects (Dewick, 2009), They are used in poultry nutrition both for their digestive stimulation properties and to improve growth performance and the ability of birds to adapt to stress factors. The main alkaloids used in poultry farming are piperine, capsaicin, and isoquinoline alkaloids.

Experimental data presented by Ahammad & Kim (2025) show that piperine acts as a bio-improver, improving nutrient absorption by modifying the dynamics of lipids in the intestinal membrane. Research conducted on a flock of 640 Ross 308 chickens, without heat stress, showed that supplementing the diet with piperine in the form of micelles helps stimulate digestive enzymes (lipase, amylase, protease) and linear body weight gain. Another study conducted on 360 Ross 308 chickens subjected to heat stress revealed that supplementing the diet with capsaicin reduces oxidative stress by decreasing MDA and increasing the GPx enzyme, increases the number of red blood cells, hemoglobin, and total proteins, and decreases cholesterol, uric acid, and glucose levels. In the same experimental design, two groups were supplemented with chili pepper powder (*Capsicum anuum*), an intervention that stimulated GPx (antioxidant protection) and significantly improved erythrocyte count and haematocrit value (Abed & Al-Shukri, 2024). Kikusato et al., 2021, demonstrate that the administration of isoquinoline alkaloids (sanguinarine and chelerythrine) can mitigate growth retardation induced by heat stress (Ross 308, 33°C constant) by protecting the barrier function and inhibiting inflammatory factors, as well as suppressing the secretion of uric acid and corticosterone in plasma.

Saponins, glycosides, and polysaccharides

Experimental studies have shown that the use of saponins as heat stress modulators, especially steroidal and triterpenic saponins derived from *Yucca schidigera* and *Gynostemma pentaphyllum,* have beneficial effects in reducing heat stress in broiler chickens, leading to a decrease in plasma corticosterone levels, and indicating a favourable modulation of the hypothalamic-pituitary-adrenal axis (Luo et al., 2022). Research conducted by Yu et al., 2025, highlighted that, at the molecular level, saponins exert antioxidant and anti-inflammatory effects by improving the antioxidant activity of superoxide dismutase, (SOD). catalase (CAT), glutathione peroxidase (GPx) enzymes and inhibiting the activation of the nuclear factor kappa-b (NF-kB) pathway.

Glycosides also play a role in supporting the immune response and maintaining redox balance, Saracila et al., 2021, providing evidence of the ability of salicin, extracted from *Salix alba*, to increase the total antioxidant capacity (CAT) and reduce (MDA) malondialdehyde concentrations, indicating a decrease in lipid peroxidation. In the case of the Ross 308 hybrid, subjected to a constant temperature of 32 ± 2°C, the combination of stevia flour (20 g/kg) and xylanase (100 FXU/kg) significantly increased carotenoid concentrations in the liver (stevia), while xylanase improved nitrogen retention, metabolizable energy, and hepatic vitamin E, as well as butyric acid concentration in the cecum (Pirgozliev et al., 2021). Improvements in economic performance and structural integrity of the intestine were reported by Sadner et al., 2020, who provided evidence that the administration of ginsenosides (90 mg/kg) to Cobb 500 hybrids subjected to cyclic heat stress of 34°C, in the form of ginseng extract, has a positive effect on feed conversion ratio and body weight, as well as increasing the expression of tight junction proteins, occludin and claudin-1. Similarly, a relevant example are polysaccharides derived from the marine algae *Enteromorpha prolifera* and the microalgae *Euglena gracilis*, which have been shown to improve intestinal antioxidant status (increased total antioxidant capacity and decreased malondialdehyde), significant improvement in intestinal barrier function by decreasing tumor necrosis factor alpha TNF-α, interleukin IL-1β; increase in nuclear factor beta IκBα alpha inhibitor) (Liu et al., 2021), reduction in cell apoptosis and histological lesions, associated with negative regulation of the nuclear factor kappa-b (NF-kB) pathway in Fabricius' bursa (Liu et al., 2021), improvement of feed conversion rate, increase in energy digestibility, and reduction of ileal *E. coli* population (Zhang et al., 2020).

In an experimental model of heat stress in broiler chickens (37 ± 1°C), dietary supplementation with 200 mg/kg *Atractylodes macrocephala* proved to be beneficial, both in terms of oxidative stress (increased antioxidant parameters and decreased pro-oxidant markers) and in terms of splenic immune modulation, along with reduced apoptosis (Xu et al., 2017).

CONCLUSIONS

For modern poultry production, heat stress is one of the most important challenges due to its negative effects on growth performance, immunity, oxidative balance, and intestinal function, generating nonspecific physiological reactions such as oxidative stress, intestinal barrier dysfunction, and systemic inflammation. A review of the literature highlights that the integration of phytogenic bioactive compounds into the broiler chicken diet is a viable and effective nutritional strategy for mitigating the physiological and metabolic effects induced by high temperatures, being the most sustainable alternative to antibiotic growth promoters, without the risk of residues or microbial resistance.

It is unanimously accepted that bioactive compounds exert complementary and often synergistic effects; for example, phenols and terpenes act pleiotropically by activating the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, stimulating antioxidant defense and modulating the inflammatory response, saponins and polysaccharides play an important role in maintaining the integrity of the intestinal mucosa, while glycosides and alkaloids can influence energy metabolism and the neuroendocrine response to stress.

The use of additives, especially in microencapsulated form, improves zootechnical performance and the quality of the finished product, while contributing to improved feed conversion ratio (FCR), body weight gain (BWG) and increased oxidative stability of meat. Although the beneficial effect of phytobiotics is recognized, their effectiveness depends on the optimization of doses and synergistic combinations, botanical source, chemical structure, and form of administration, elements that must be adapted to the stressor (intensity and duration of heat stress).

Therefore, further research is needed to elucidate the molecular mechanisms of action, structure, and optimal dosage, but especially to validate the effectiveness of these interventions under production conditions.

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APPLICATION OF STEVIA AS AN ALTERNATIVE THERAPY IN BUFFALOES IN MODERN VETERINARY MEDICINE

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Abstract

Under conditions of increasing restrictions on the use of antimicrobial agents in animal husbandry, growing interest is being directed toward natural bioactive substances as alternatives for maintaining health status and metabolic balance in young animals. Among these, Stevia rebaudiana Bertoni extract stands out due to its proven antioxidant, hepatoprotective, and metabolism-regulating properties. The aim of the present study was to evaluate the effect of stevia extract on hematological and biochemical blood parameters in newborn buffalo calves. In the experimental design, newborn calves received a 5% extract of Stevia rebaudiana Bertoni at a dose of 20 mL per day, administered with milk over a 30-day period. Hematological and biochemical blood parameters were monitored at the beginning of the experiment and on day 31. A statistically significant increase in hemoglobin (HGB) concentration by 21.8% ($p \leq 0.01$) was observed, along with a tendency toward increased values of white blood cells (WBC - 17.8%), lymphocytes (LYM - 10.1%; LYM%), monocytes (MON - 2.43%), granulocytes (GRA - 8.45%), red blood cells (RBC - 15.7%), and platelets (PLT - 34.4%). A statistically significant decrease in alanine aminotransferase (ALAT) activity by 52.11% ($p \leq 0.001$) was also recorded. Blood glucose concentration decreased significantly at the end of the experiment ($p \leq 0.01$). Total cholesterol and LDL-cholesterol levels were significantly reduced ($p \leq 0.01$ and $p \leq 0.001$, respectively). Statistically significant differences in triglyceride levels were observed on day 31 ($p \leq 0.01$), as well as a significant decrease in urea concentration at the end of the experiment ($p \leq 0.01$). The obtained results indicate a pronounced hematopoietic and hepatoprotective effect of stevia and confirm its potential as a natural agent for supporting metabolism and overall health status in animals.

Key words*: antibiotic resistance, calves, hematological indicators, stevia.*

INTRODUCTION

Guided selection towards high growth intensity leads to the creation of precocious breeds and hybrids. Such physiological asynchrony often results in an attenuated immune system and suboptimal homeostatic regulation, predisposing the organisms to a spectrum of idiopathic pathologies and systemic metabolic dysregulation. They lead to both direct losses and impaired technologies, reproductive performance and susceptibility of the animal body to infectious and parasitic diseases. Neonatal hypoglycemia, anemia, enteritis, parasitosis, septicemia, respiratory diseases and omphalitis are common pathological conditions that can lead to serious health complications in newborn animals and are critical for their survival in the first 1 to 3 days. They have low glycogen reserves with which to initiate glycolysis and gluconeogenesis, as well as an underdeveloped liver. These conditions lead to neurological damage, reduced growth, high mortality and a weakened immune system. Oxidative stress has been shown to play an important role in the pathophysiology of diseases, suggesting that antioxidant-rich plants may offer therapeutic benefits (Nenova et al., 2024). *Stevia rebaudiana* Bertoni, often called the sweet herb of Paraguay, is a perennial shrub of the Asteraceae family, native to South America, specifically Paraguay and Brazil (Pirgozliev et al., 2022; Enchev and Kikindonov, 2023). The bioactive components derived from stevia possess a wide range of therapeutic potential for various diseases. Among its medicinal benefits are antihypertensive, antitumor, antidiabetic, and antihyperlipidemic. In recent years, there has been a significant paradigm shift toward the investigation of biogenic therapeutic agents derived from botanical resources. Among these, *Stevia rebaudiana Bertoni* has gained substantial recognition as a multifaceted natural matrix possessing diverse secondary metabolites with documented physiological

benefits (Takahashi et al., 2001; Shivanna et al., 2013; Bender et al., 2015; Lemus-Mondaca et al., 2018; Ntalli et al., 2020; Enchev et al., 2025).
The presented study demonstrates a way to limit the use of antibiotics in newborn calves, alternative strategies for improving animal health, the use of natural bioactive substances of plant origin, stevia and its positive impact on hematological parameters and general health.

MATERIALS AND METHODS

Animals, breeding and feeding

The experimental population of buffaloes was raised at the buffalo farm of the Agricultural Institute, Shumen (Northeastern Bulgaria, latitude: 43.28N, longitude: 26.93E). The feed ration included - whole milk 6 l, distributed in three rations per day. The study included 30 buffalo calves (n), matched by age and origin. 20 ml of 5% stevia extract were added to the milk of animals from the experimental group (n-15), for 30 days.

Blood samples

Blood samples for determining the indicators of hematological status were obtained from the *v. jugularis* using a vacuum container on the day of the beginning of the experiment and on the 31st day of the experiment. Subsequently, they were centrifuged and the serum was stored at -20°C until biochemical analysis was performed, hematological analysis was performed immediately.

Research Methods

Analytical procedures for blood tests were performed with an automatic biochemical and hematological analyzer EXICO C200 and EXICO H400 (Sweden) in accordance with the manufacturer's instructions. We monitored changes in the following hematological parameters - ASAT /aminotransferase/, ALAT (alanine aminotransferase), GLUCOSE, UREA, CHOLESTEROL, TRIGLYCERIDES, LDL hol (high-density lipoproteins), LDL cal (cholesterol concentration in low-density lipoproteins), WBC (leukocytes), LYM (lymphocytes), LYM% (lymphocytes), MON (monocytes), MON% (monocytes), GRA (granulocytes), GRA% (granulocytes), HGB (hemoglobin), MCH (mean hemoglobin concentration in one erythrocyte), MCHC (mean hemoglobin concentration in erythrocytes), RBC (erythrocytes), MCV (measures the average volume of red blood cells (erythrocytes) in the blood), HCT (hematocrit), RDWa (red blood cell distribution width), RDW%, PLT (platelets), MPV (mean platelet volume).
The stevia extract was obtained by the green extraction method with water + membranes (López-Carbón et al., 2019). The leaves were ground, the extraction was performed with pure water (without organic solvents). This was then followed by membrane filtration (to remove unwanted substances and concentrate glycosides). The concentration of the extract we worked with was 5%.

Statistical analysis

The primary data were analyzed using Statistica version 7.0 (StatSoft Inc., USA). To compare values between Day 0 and Day 31, a one-way repeated measures ANOVA was applied. Biochemical and hematological parameters quantified in the study are presented in detail in the accompanying tables. Statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSIONS

A statistically significant increase in the concentration of HGB (Hemoglobin) 21.8% was found on the 31th day of the experiment and a tendency to increase the values of WBC (17.8%), LYM (10.1%), MON (2.43%), GRA (8.45%), RBC (15.7%) and PLT (34.4%).
A statistically significant decrease in the values of ALAT (52.11%) ($p \leq 0.001$) was found. A statistically significant decrease was reported in the concentration of GLUCOSE at the end of the experiment ($p \leq 0.01$). In the values of CHOLESTEROL and LDL, statistically high reliability is observed, as they were significantly reduced with a level of significance of $p \leq 0.01$ and $p \leq 0.001$, respectively. In the concentration of TRIGLYCERIDES, statistically significant differences were reported on the 31 day, respectively day 0 - 0.137 and day 31 - 0.620 ($p \leq 0.01$). Statistically significant differences are observed in relation to Urea ($p \leq 0.01$), as the

results demonstrate a decrease in its values at the end of the experiment (Tables 1, 2)

In the results of the control group of animals, no statistically significant differences or trends in blood parameters were found.

A complex interplay exists between mitochondrial bioenergetics, oxidative stress, and metabolic adaptation during nutritional transitions, often manifesting as a functional decline in mitochondrial respiratory chain complexes within hepatic and intestinal tissues. The current investigation demonstrates that the administered extract exerts a systemic restorative effect on these metabolic pathways. Specifically, the data indicate that *Stevia rebaudiana* facilitates erythropoietic modulation, evidenced by a physiological increase in erythrocyte count, and exhibits potent anti-anemic properties by elevating hemoglobin concentrations. Furthermore, the extract appears to enhance systemic respiratory efficiency and cellular oxygen utilization in the experimental animals.

Table 1. Complete blood count of buffalo calves in the presence of stevia

Indicators	Day 0			Day 31		
	$\bar{x}$	$S\bar{x}$	C	$\bar{x}$	$S\bar{x}$	C
WBC g/L	7.774	0.749	22.12	9.160	0.702	24.23
LYM *x10^12/L*	5.740	0.796	44.84	6.320	0.740	37.04
LYM%	53.210	5.799	34.46	53.130	4.878	29.04
MON *x10^12/L*	0.820	0.042	16.06	0.840	0.092	34.69
MON%	8.390	0.496	18.69	7.600	0.397	16.52
GRA *x10^12/L*	4.140	0.676	51.67	4.490	0.653	46.01
GRA%	38.330	4.771	47.61	39.000	4.778	38.74
HGB g/L	125.800**	4.791	12.04	153.300**	6.893	14.20
MCH pg	14.710	0.455	9.78	15.160	0.616	12.85
MCHC g/L	393.900	19.006	15.26	426.400	23.098	17.13
RBC *x10^12/L*	9.026	0.796	27.87	10.451	0.786	23.79
MCV fL	37.970	1.880	15.66	36.720	1.850	15.93
HCT l/l	0.365	0.026	22.25	0.346	0.018	16.12
PLT g/L	424.300	80.539	60.03	570.600	48.189	26.71
MPV fL	9.750	0.971	45.48	6.730	0.903	42.44

** – $p \leq 0.01$

Table 2. Biochemical parameters in buffalo calves in the presence of stevia

Indicators	Day 0			Day 31		
	$\bar{x}$	$S\bar{x}$	C	$\bar{x}$	$S\bar{x}$	C
ALAT	10.800***	0.573	16.79	7.100***	0.674	30.02
ASAT	61.100	2.681	13.88	61.400	4.238	21.83
GLUCOSE mmol/L	2.880**	0.275	30.17	1.770**	0.177	31.63
UREA mmol/L	3.550**	0.463	41.23	1.570**	0.304	61.24
LDL mmol/L	1.234***	0.078	19.91	0.685***	0.089	41.11
CHOLESTEROL mmol/L	1.614***	0.131	25.70	0.593***	0.064	34.39
HDL mmol/L	0.863	0.065	23.88	0.828	0.196	74.86
TRIGLYCERIDE mmol/L	0.137**	0.024	54.30	0.620**	0.161	82.20

*** – $p \leq 0.001$, ** – $p \leq 0.01$

The regulatory influence of *S. rebaudiana* on the hematological profile of newborns may be due to the pleiotropic effects of its various phytochemical constituents. This physiological modulation is mediated by a complex of bioactive secondary metabolites, including steviol glycosides, iminosugars, and a potent polyphenolic fraction consisting of tannins and flavonoids, as well as volatile essential oils (Debnath, 2008; Abou-Arab & Abu-Salem, 2010; Lemus-Mondaca et al., 2012; Mali et al., 2015; Abdel-Rahman et al., 2015). These

directly or indirectly affect the oxygen-transporting function of hemoglobin. Stevia may increase hemoglobin levels in some experimental models, although this effect is not fully understood, but has been linked to several potential mechanisms of action based on the biochemical properties of the plant. Antioxidant activity - Stevia contains flavonoids, phenolic compounds, vitamin C, carotenoids, and other antioxidants that reduce oxidative stress in bone marrow and erythropoietic tissue, which supports erythropoiesis (red blood cell formation). Our results are consistent with a study by Kurek et al. (2021), which found that in rats with induced diabetes, high doses of stevioside (~2500 mg/kg body weight) significantly increased hemoglobin (HGB) and hematocrit (HCT). These results are consistent with our previous studies on the effect of stevia in sows (Nenova and Enchev, 2022). Some biologically active components in stevia may act as mitogens, substances that stimulate cell division. This enhances the proliferation and differentiation of erythroid cells in the bone marrow and leads to increased hemoglobin and erythrocyte production. In some studies, stevia improves liver function, intestinal absorption and microbiome, which may lead to better absorption of iron, vitamin B12, folic acid - essential cofactors for hemoglobin synthesis. Better iron absorption leads to more hemoglobin. Some studies have found an indirect effect of stevia on hematopoiesis through glucose regulation. In animals with insulin resistance or diabetes, anemia associated with oxidative stress and inflammation is observed. Stevia improves glycemic control and reduces metabolic stress, which indirectly improves hematopoiesis. In a study in which albino rats were given an aqueous extract of stevia (at doses of 200, 300, 400 and 500 ppm/kg body weight for 8 weeks), various hematological parameters increased significantly - including RBC, HGB, HCT, MCV, MCH and MCHC. This indicates that under these conditions, the number of erythrocytes and their associated parameters in the blood increases (Ahmad et al., 2020). In comparison, there are also studies that show the opposite effect. For example, in albino mice, prolonged use of stevia leads to a decrease in RBC, HGB and HCT. This highlights that the effect may be variable and dependent on the dose, form of stevia, animal species and duration (Farid et al., 2020). In the experimental buffalo calves, a quantitative increase in total leukocyte count was observed, increasing from 7.774 g/L on day 0 to 9.160 g/L by day 31. Although these values remain within the established physiological reference range, this upward trend suggests a potential immunomodulatory effect of the *S. rebaudiana* extract. This bioactivity is likely mediated by the presence of specific flavonols, namely quercetin, kaempferol and isorhamnetin, which are known to interact with immune signalling pathways and cellular defense mechanisms. Some authors note (Lillehoj et al., 2018) that these compounds promote the rapid proliferation of bone marrow cells and the release of young blood cells into the bloodstream. Stevia has an immunomodulatory (stimulating) effect on cells of the immune system. Stevioside stimulates both humoral and cellular immune responses. In studies on mice, it increased phagocytic activity, hemaglutination titer, and proliferation of B and T cells (Sehar et al., 2008). Kizhina et al. (2023) found in mice treated with stevia (10 mg/g body weight), an increased content of glycogen in leukocytes - an important substrate for phagocytosis. At the same time, the neutrophil/lymphocyte ratio (NLR) increased, indicating activation of phagocytic cells (granulocytes) (Kizhina et al., 2023). The authors found that chronic use of stevia (in the diet of mice) led to an increase in the number of $CD3^+$ T cells, $CD19^+$ B cells, and IgA^+ plasma cells in the walls of intestinal lymphoid structures (Peyer's patches and lamina propria), as well as changes in the levels of cytokines such as IL4, IL10, and TNFα. These effects promote local and systemic immune activity, which is reflected in blood parameters. (Rosales-Gómez et al., 2018; Noosud et al., 2017; Escoto et al., 2021).

When analyzing the results, an upward trend in platelet counts was observed. This is usually a reactive thrombocytosis related to the immune response, antioxidant effects and effects on the bone marrow. Stevia has anti-inflammatory and antioxidant properties that can alter the production of cytokines such as IL-6, which

stimulates thrombopoiesis by increasing thrombopoietin (TPO). Stevia reduces oxidative stress and damage to hematopoietic cells. This can lead to increased platelet production, especially in animals with previous hematological damage. Jeppesen et al. (2003) found in an experiment with rats with induced diabetes that high doses of stevioside (2500 mg/kg) were associated with an increase in platelets (PLT) in the blood, along with an increase in Hemoglobin and Hematocrit.

Shivanna et al. (2013) observed in albino rats with a diabetic model a moderate increase in PLT, probably related to the antioxidant effect of stevia and the improvement of the bone marrow. Analysis of the biochemical results revealed a statistically significant decrease in ALAT values (52.11%) ($p \leq 0.001$). The blood glucose concentration recorded at the end of the experimental period (1.77 mmol/L) requires careful interpretation with respect to the physiological range for neonatal calves. According to published reference values, normal blood glucose concentrations in healthy calves generally range between approximately 2.5 and 6.0 mmol/L, with mean values around 5.0-5.6 mmol/L (Knowles et al., 2000; Constable et al., 2013). In hospitalized neonatal calves, plasma glucose concentrations below 2.0 mmol/L have been defined as severe hypoglycaemia and have been associated with adverse clinical outcomes and increased mortality risk (Trefz et al., 2016).

The glucose value observed in the present study therefore falls below the commonly reported physiological range and approaches the threshold used to define hypoglycaemia. However, it is important to note that considerable physiological variability exists in glucose metabolism during the early postnatal period in ruminants, influenced by age, feeding regime, and metabolic adaptation after birth (Constable et al., 2013). In the current experiment, the reduced glucose concentration was not accompanied by clinical signs of hypoglycaemia or deterioration in general health status. On the contrary, improvements in hematological parameters and a marked reduction in hepatic enzyme activity were observed, suggesting a favorable metabolic adaptation rather than a pathological condition. Nevertheless, the obtained glucose values warrant cautious interpretation and should be discussed in the context of age-specific reference ranges and clinical observations, highlighting the need for further studies to better define physiological glucose limits in buffalo buffalo calves. Statistically high reliability was observed for cholesterol and LDL values, as they were significantly reduced, respectively, with a reliability level of $p \leq 0.01$ and $p \leq 0.001$. Statistically significant differences were observed for triglyceride concentrations at day 31, day 0 - 0.137 and day 31 - 0.620, respectively ($p \leq 0.01$). Statistically significant differences were observed for urea ($p \leq 0.01$), as the results showed a decrease in its values at the end of the experiment.

The addition of stevia (*Stevia rebaudiana* Bertoni) to the milk of buffalo calves has a positive effect on the biochemical parameters of the blood. The changes that occurred within 30 days in the experimental group indicate a hepatoprotective and antianemic effect of the plant, which is consistent with the findings of Ramos-Tovar et al. (2018) and Chang (1976). A decrease in ALAT values ($p \leq 0.01$) was observed in the animals of the experimental group. This is due to the antioxidant and anti-inflammatory effect through the activation of Nrf2 and the suppression of NFκB. In models of chronic liver injury, stevia prevents the increase in ALT by increasing the activity of Nrf2, which regulates antioxidant enzymes and provides protection against oxidative stress. It preserves the levels of glutathione (GSH) and GPx, thus reducing lipid peroxidation (4-HNE, MDA). Inhibits NFκB, resulting in reduced production of inflammatory cytokines (TNFα, IL1β, IL6). This is reflected in a significant decrease in serum ALAT and preservation of liver tissue (Ramos-Tovar et al., 2018). Latha et al., (2017) found that in acute liver injury in rats, both stevia aqueous extract and stevioside significantly restored elevated serum ALAT and ASAT levels, while simultaneously alleviating oxidative and inflammatory damage in the liver. In a study on diabetic rats, stevia significantly reduced ALAT (by about 62%) and other liver markers, while also reducing MDA levels. It increased GSH and improved the histological structure of liver tissue, including by increasing antioxidant and apoptosis regulators (Abdelwahab et al., 2017).

In the present study, a change in lipid status was found. In the values of Cholesterol and LDL, statistically high reliability was observed, as they were significantly reduced, respectively, with a level of reliability $p \leq 0.01$ and $p \leq 0.001$. In the concentration of Triglycerides, statistically significant differences were reported on the 31 day, respectively day 0 - 0.137 and day 31 - 0.620 ($p \leq 0.01$). An increase in triglyceride values has been found, which is due to a change in lipid metabolism: Steviol glycosides (mainly stevioside and rebaudioside A) are metabolized by the intestinal microflora to steviol, which can affect hepatic enzymes responsible for lipid synthesis and degradation (Chatsudthipong & Muanprasat, 2009). Improved insulin sensitivity has been observed in animals receiving stevia. Insulin stimulates lipogenesis in the liver and adipose tissue, which can lead to a transient increase in triglycerides (Jeppesen et al., 2003). In some experiments, low doses of stevia have been shown to reduce lipid levels, while at higher doses or with prolonged use, an increase in triglycerides may be observed due to adaptive mechanisms of hepatic metabolism (Melis, 1999). The effect depends on the animal species: in rodents, changes in serum lipids are often observed, while in ruminants (cows, sheep) the effect is more complex due to microbial fermentation in the rumen. The rumen microbiota can metabolize stevia components in a way that increases the production of volatile fatty acids, especially acetate – a major precursor for lipogenesis in ruminants (Bergman, 1990; Bauman & Griinari, 2003). In some conditions, stevia can reduce glucose utilization and increase lipid catabolism, which in short-term manifests as increased circulating triglycerides. In most cases, it is considered a hypoglycemic and hypolipidemic agent, but under certain conditions it can lead to reduced glucose utilization and compensatory increased lipid catabolism, which in the short-term manifests as increased circulating triglycerides (Melis, 1999; Kurek et al., 2020). This increase can be considered a positive effect, as higher triglyceride concentrations in the blood of buffaloes/cows reflect enhanced energy availability and improved metabolic function, which is particularly important for optimizing lactation and maintaining physiological balance. It is important to emphasize that the reported increase in triglycerides remains within the physiological reference values for the species, which excludes a pathological interpretation and supports its consideration as a functional metabolic adaptation.

Dietary supplementation with *S. rebaudiana* appears to enhance the hepatic biochemical profile and positively modulate lipid and carbohydrate metabolism, thereby mitigating hepatic steatosis. The liver and kidneys exhibit the highest gluconeogenic capacity within the organism. In accordance with the Cori cycle, lactate is translocated from skeletal muscle to the liver, where it serves as a primary substrate for gluconeogenesis. Through the catalytic activity of lactate oxidase, lactate is converted into pyruvate, which, along with glycogenic amino acids, facilitates the formation of alpha-keto acids for entry into systemic metabolic pathways. They are transformed into oxaloacetate, which is converted into glucose or glycogen in the reverse pathway of the glycolytic chain. With protected liver function, as in the present study, a reduced synthesis of glucose is observed.

Stevia and its main compounds (stevioside, rebaudioside A, etc.) lower blood glucose through several interrelated mechanisms - on the pancreas, liver, muscles and intestines. Stevioside and rebaudioside A act directly on β-cells in the pancreas. They activate TRPM5 calcium channels, which increases intracellular Ca^{2+} and stimulates insulin release. The effect is glucose-dependent - i.e. it increases secretion only at high glucose levels, which reduces the risk of hypoglycemia (Jeppesen et al., 2003; Philippaert et al., 2017) Stevioside enhances signaling along the PI3K/Akt pathway in muscle and adipose tissue. This leads to increased expression and transport of GLUT4 glucose transporters to the cell membrane and more glucose is taken up by the tissues. (Chatsudthipong & Muanprasat, 2009). Stevia inhibits key enzymes such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase. This limits the production of new glucose by the liver and leads to lower levels in the blood. (Shivanna et al., 2013). Stevia can partially inhibit Na^{+}-dependent glucose transporters (SGLT1) in the

small intestine and leads to a slower and lower rise in blood glucose. (Abudula et al., 2004). Oxidative stress and chronic inflammation damage β-cells and cause insulin resistance. Stevioside reduces the production of TNF-α, IL-6 and improves antioxidant defenses, better preserved pancreatic function and leads to better glucose homeostasis. (Lemus-Mondaca et al., 2012).

Jeppesen et al. (2003) found that stevioside directly stimulates pancreatic β-cells to secrete insulin in diabetic rats. This leads to a significant decrease in glucose. Shivanna et al. (2013) found that treatment with an extract of stevia reduces the expression of liver enzymes and glucose-6-phosphatase, which leads to lower production of endogenous glucose and a decrease in glycemia. Urea is a nitrogenous product, and its content in the blood depends on the protein supply of the body. A decrease in its value within the normal range was observed, which indicates that the carbamidosynthesis function of the liver of the animals is optimized, as also proven in the experiments of Borisova et al. (2018). Our results also confirm the conclusions of the study by Algendy et al. (2023) that metformin, as well as stevia as monotherapy or combination significantly improved the glycemic profile along with balancing the redox status and alleviating the inflammatory response in male albino rats with diabetes. The effect of stevia on urea is less discussed compared to glucose and lipids, but there is evidence that it lowers serum urea in animal models and in some clinical settings. This is explained by its effect on renal function, protein metabolism and antioxidant protection. Stevioside and extracts of *Stevia rebaudiana* reduce oxidative stress and inflammation in the kidneys. This improves glomerular filtration and reduces the accumulation of nitrogenous metabolites (urea, creatinine). Our results are in line with the studies of Shivanna et al. (2013) who explain the decrease in urea and creatinine in diabetic rats with antioxidant and nephroprotective effects similar to Sharma et al. (2018), Pari & Satheesh (2004).

Statistically significant differences in the values of CHOLESTEROL and LDL under the influence of the supplement are observed, which correlates with the hypocholesterolemic effect of the herb, proven in the studies of Akhtar et al. (2018), related to the reduction of hepatic cholesterol and this function is mainly due to the improved bioavailability mediated by fats and/or by inhibiting the absorption of dietary cholesterol. The diterpene glycosides of stevia, in addition to helping to normalize the concentration of blood sugar, also have the property of supporting the function of the pancreas and pancreatic juice. This leads to lower values of lipid indicators in the blood, which could also affect the biochemical analysis of meat and should be further studied. Clinical studies show that stevia can be used as a means of lowering cholesterol (Nassuato et al., 1991; Tang et al., 2010). By suppressing hepatic cholesterol biosynthesis and attenuating systemic levels through the inhibition of intestinal absorption, *S. rebaudiana* may effectively modulate lipid metabolism and the subsequent fatty acid composition of meat and milk. This reduction in circulating lipids is likely driven by the interference of steviol glycosides with micellar formation within the gastrointestinal tract, thereby influencing the partitioning of adipose tissue and the lipid profile of bovine secretory products. When comparing the changes in the biochemical parameters of experimental animals, the hematopoietic and hepatoprotective function of Stevia (*Stevia rebaudiana* Bertoni) was established. The results are consistent with those established by Borisova et al. (2018), Algendy et al. (2023), Schonfeld et al. (1997), Tang et al. (2010) Shivanna et al. (2013), Sharma et al. (2018), Pari & Satheesh (2004).

CONCLUSIONS

The addition of 5% extract of *Stevia rebaudiana* Bertoni in a dose of 20 ml for a period of 30 days in the milk of newborn buffalo calves has a beneficial effect on their metabolism and general health. This is confirmed by the statistically significant changes in the main biochemical parameters of the blood (HGB, ALAT, GLUCOSE, CHOLESTEROL, LDL, TRIGLYCERIDES, UREA), which are consistent with the results published in previous scientific studies. The results established demonstrate the hepatoprotective and hematopoietic function of Stevia.

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COMPARATIVE STUDY OF BIOLOGICAL AND TECHNOLOGICAL TRAITS IN *Bombyx mori* L. FED ARTIFICIAL FOOD AND ADDED PLANT EXTRACTS

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Abstract

Bombyx mori cultivation and cocoon production is an old and traditional industry in the country. With the creation and application of artificial nutrition, it is possible to grow them in any season of the year, regardless of the external climatic conditions and the stage of development of the mulberry. The purpose of this research is to add thyme and peppermint extracts to artificial food as plant stimulants. To follow the absorption of food and the most important biological and technological signs of the silkworm and the resulting cocoons. Larvae accept artificial food and add extracts with great willingness. In the experimental groups, exceptionally satisfactory results were observed in the larvae fed with artificial food and added thyme extract, and significantly poorer results in those with meat.

Key words: *artificial feeding, blueberry silkworm, mentha, plant extracts, Thymus vulgaris L.*

INTRODUCTION

Today, silk is considered the most valuable and healthy natural fiber (Bhattacharyya et al., 2016) Authors such as Panthee et al. (2017) and Aznar-Cervante et al. (2021) recommend the use of silkworms as laboratory animals due to their short biological development and not so expensive to raise.

The temperate continental climate in Bulgaria does not allow the cultivation of silkworms throughout the year and the production of mulberry leaves is in certain seasons. Therefore, the use of a substitute for mulberry leaves is necessary. In such cases, artificial food is a good alternative.

Researchers in Japan and China have focused on developing technologies for cultivating silkworms based on artificial food (Cui et al., 2016; Yanagawa et al., 2010; Dong et al., 2017). According to Hamamura (2001), with the introduction of artificial feeding into practice in countries such as Japan in the 1960s, the breeding of the species can be carried out at any time of the year without being dependent on the development of the mulberry. This way of feeding can solve many issues in the breeding of *Bombyx mori* L., such as maintaining a large number of mulberry trees for feeding, can reduce labor costs and those in growing and maintaining mulberry trees, collecting leaves, etc.

In Bulgaria, scientists such as Tsenov & Georgiev (Avramova et al., 2020) designed in 2010 an artificial food that could be used during the entire larval period and for all seasons.

Authors such as Sudhakar et al. (2000), Hiware (2006), Nguku et al. (2007), Kamel et al. (2016), Bhattacharyya et al. (2016) studied the effect of various plant extracts or other substances (Shankar & Rangaswamy, 1999; Rahmathulla et al., 2007) added to the food of *Bombyx mori* L., followed the growth and development of the larvae. One of the alternative ways to improve the growth of larvae and cocoon production is to enrich the food with additional nutrients.

Different breeds and hybrids of *Bombyx mori* L. have different requirements for artificial food, it must be adapted to them. Plant extracts reveal the factors that can be influenced positively or negatively in favor of silkworms (Nair & Kumar, 2004)

According to authors such as Etebari & Matindoost (2005), Etebari et al., (2004), Singh et al. (2005) and Amala Rani (2011) nutritional supplements such as vitamins, proteins, amino acids and probiotics added to the larval diet tend to enhance the nutritional adaptability and economic performance of silkworms. The way of rearing and feeding influences the growth and

development of larvae and cocoon production (Thapa & Ghimire, 2005). Enriching the diet with additional nutrients is a basic prerequisite for improving growth and development, and therefore on the yield and quality of cocoons (Buhroo et al., 2018; Jaiswal et al., 2020).

Authors such as Kumar & Balasubramanian (2014), Kumar et al. (2009), Ganeshprabu et al. (2011) and Thangapandiya & Dharanipriya (2019) added silver nanoparticles and spirulina (*Spirulina maxima*) to mulberry leaves and monitored their effect on silkworm larvae. The authors concluded that nanoparticles and spirulina had a good effect and positively affected the energetic and economic parameters of *B. mori* and could be used in commercial cocoon production.

Soliman (2021) investigated the effects of different concentrations of baker's yeast S. cerevisiae and spirulina Spirulina platensis on some biological parameters of *B. mori,* such as larval weight, pupal weight, cocoon mass, cocoon shell mass, and silk ratio. The addition of yeast and spirulina to the diet had a beneficial and significant effect on larval development and increased the yield and quality of cocoons. According to the author, the application of these two ingredients to the diet can significantly increase yields in the silkworm industry.

It has been shown that feeding without ascorbic acid has a beneficial effect on cocoon production and on slowing down the development cycle (Cappellozza et al., 2005). Ascorbic acid in the diet affects cocoon yield when feeding larvae in their first and last instars.

According to the same author (Cappellozza et al., 2011), preparations such as Methoprene can be added to artificial foods, which have a stimulating effect on the larvae, and an increase in protein accumulation in the body and cocoons is observed.

The proportion of defatted soybean meal in today's widely used artificial feed mixtures is similar to that of mulberry leaf meal and ranges between 25% and 35% (Ito, 1980; Shinbo & Yanagaw, 1994; Cappellozza et al., 2005; Dong et al., 2017, 2018; Qin et al., 2020). Rich sources of protein are known to improve larval growth and economic performance.

For thousands of years, various herbs and spices have been used to preserve food or to protect it from microbial contamination.

According to authors such as Yanishlieva et al. (2006), Al-Turki (2007), Solomakos et al. (2008), Hoffman-Pennesi & Wu, (2010), Thyme *(Thymus vulgaris* L.) and peppermint *(Mentha piperita* L.) are medicinal and aromatic plants belonging to the Lamiaceae family and have therapeutic and pharmaceutical effects.

Thyme has strong antimicrobial and antioxidant activity due to its high content of thymol (40%), carvacrol (15%), cymene, eugenol and tetra-allylphenol.

Plant extracts are extremely valuable in traditional medicine and herbal products. They contain concentrated active substances from herbs. They are absorbed significantly faster by the body. Extracts are concentrated mixtures, with organic solvents such as water, ethanol or other solvents, of various plant parts, such as leaves, roots, stems and flowers. The final product, compared to the raw plant material, has more potent forms of the active chemicals of the plant.

The components in the volatile oil of thyme have antimicrobial activity against a wide variety of bacteria and fungi. 100 g of dried thyme contains vitamin A - 50 mg, vitamin C - 1890 mg, calcium - 124 mg, iron - 220 mg and magnesium.

All above-ground parts of *Mentha piperita* contain essential oil with menthol as a component (45-65%). The herb has a rich biochemical composition, which includes: vitamins such as C, A, B-complex, macroelements and microelements such as calcium, iron, magnesium, potassium, zinc and plant compounds: menthol, rosmarinic acid and eucalyptol. Acetic and isovaleric acid, carotene, bitter substances, tannins and mint oil. Mint oil is an indispensable raw material in the perfumery, alcohol and sugar industries (Landzhev, 2010).

These two plants have special functions such as antiseptic, antimicrobial and antioxidant effects (Sengul, 2008).

MATERIALS AND METHODS

The study was conducted at the educational and experimental base of the Faculty of Agronomy at the University of Forestry, Sofia, in March 2023. The eggs were laid for incubation on March 20, 2023. The tetrahybrid Vr35 x VB1 x

XB2 x M2 created at the Scientific Center for Sericulture - Vratsa was used.

When conducting the experiment for feeding silkworm larvae, an artificial food containing 100% dried mulberry leaf flour was used, provided by the Scientific Center for Sericulture - Vratsa. The food was purchased from the same place. The artificial food was prepared according to the method recommended by the manufacturer. Five days after winding, the cocoons were collected and weighed using an electronic balance and caliper.

Plant extracts

Extract of thyme (*Thymus vulgaris* L.) and mint (*Mentha piperita* L.) In 1 liter of water, 10 g of the herb are placed in the experimental groups. The extract is boiled for 1 hour, distilled water is added to the decoction to a liter, after which it is left to stand for 24 hours. It is filtered through filter paper, and stored at low temperature.

Method of preparing artificial food

Distilled water or herb extract is added to the dry substance. 250 g of dry substance + 700 ml, extracts of both plants. It is homogenized using a mixer and placed in a box with a layer thickness of 2 cm. The resulting mixture undergoes heat treatment and is cooled. The finished food is stored in a closed container at a temperature of 2-5˚C until the moment of feeding. The food can be stored for up to 40 days without losing its nutritional qualities. The control larvae are fed artificial food without additives, their food mixture is prepared only with distilled water.

Incubation and rearing of the larvae was carried out according to the generally accepted methods in our country (Petkov, 1982; Petkov, 1995). The eggs were stored at a temperature of 2 to 5°C, a temperature shock was carried out (increasing t to 10-12°C), which aimed to accelerate the development of the embryo in the egg. After reaching the IV stage, the eggs were placed again in storage at 2-5°C. The silkworm eggs were set for incubation under controlled parameters in an incubator. Hatching began 11 days after their setting for incubation. The experiment was conducted with one control and two experimental groups of 50 worms. The rearing and feeding of the larvae was carried out in boxes in a specialized room up to the fifth instar inclusive, at an established temperature and humidity according to Petkov, 1980, which is a universal rearing regime.

RESULTS AND DISCUSSIONS

The results of the experiment show much better results for the larvae fed with artificial food and added thyme extract than those in the control and the variant with added mint extract. They develop better, spin larger and heavier cocoons than those in variant 2. The thyme extract has a stimulating effect on the silkworm larvae, they are more lively and take their food with a big appetite, unlike those fed with added mint extract.

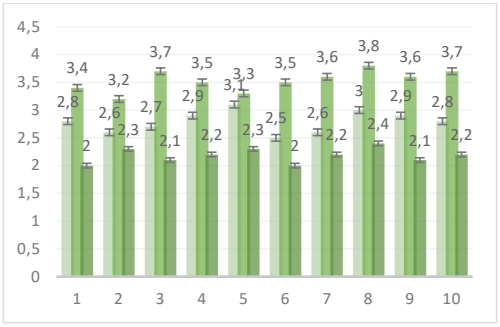
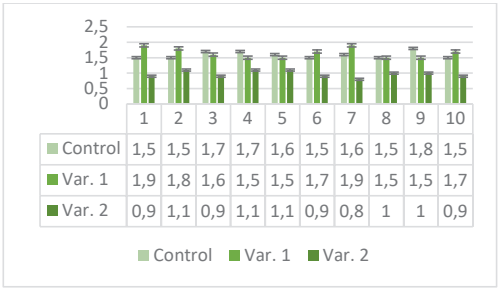


Figure 1. Larval mass in 5th instar

The results in Figure 1 show that much higher results (3.2 -3.7 g) were obtained in var.1 in larvae fed with artificial food and added thyme extract. In the control and var.2 the results obtained were significantly lower (2.0 -2.4 g).



	1	2	3	4	5	6	7	8	9	10
Control	1,5	1,5	1,7	1,7	1,6	1,5	1,6	1,5	1,8	1,5
Var. 1	1,9	1,8	1,6	1,5	1,5	1,7	1,9	1,5	1,5	1,7
Var. 2	0,9	1,1	0,9	1,1	1,1	0,9	0,8	1	1	0,9

Figure 2. Raw cocoon mass

In terms of raw cocoon mass, higher results were reported for the variant with added thyme extract (1.7 -1.9 mg). In those with added mint extract, they were low (0.9 -1.1 mg), the

resulting cocoons were small, petite and did not meet the hybrid's performance.

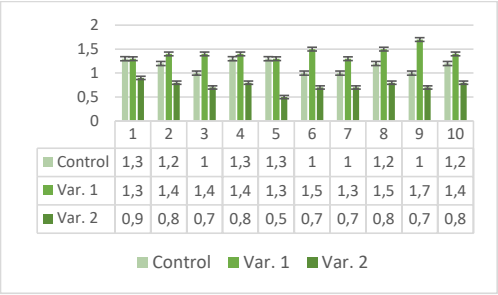


Figure 3. Dry cocoon weight

In the results of the dry cocoon mass trait, the same dependence is observed: higher results in var.1 (1.3-1.7 mg) and lower results in those fed with artificial food and added mint extract (0.5 - 0.8 mg).

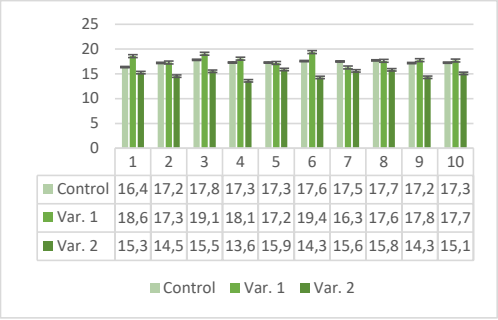


Figure 4. Cocoon width

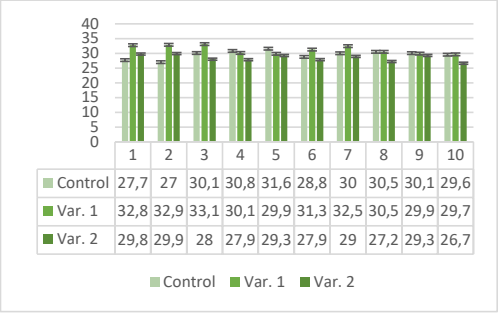


Figure 5. Cocoon height /mm

The results of Fig. 4 and Fig. 5 show higher values of the studied traits in the cocoons of variant 1, they are close to those of the control and significantly lower in variant 2. Mint has an adverse effect on silkworm larvae.

CONCLUSIONS

Thyme, as a plant extract added to the food of silkworm larvae, shows good results in all tested traits. The larvae fed with artificial food and thyme extract are significantly larger and spin larger and heavier cocoons.

Thyme is an alternative way to improve larval growth and cocoon production. It enriches the artificial food with additional nutrients.

Larvae fed with artificial food and mint extract are relatively smaller than the control and lime.

Mint does not give good results as a nutritional stimulant, and has a negative effect on the development of silkworm larvae and on the resulting cocoons. More in-depth studies are needed to establish this.

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EVALUATION OF QUANTITATIVE MEAT PRODUCTION IN TURKEY BROILERS RAISED IN DIFFERENT SYSTEMS

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Abstract

This study evaluated quantitative meat production in turkey broilers reared under different systems during the finishing period (105-136 days): intensive (controlled environment, compound feed) and extensive (access to paddock, cereal-based diet). At slaughter, birds from the intensive system showed higher live weight (+5.70%) and superior slaughter yields (hot: +2.78%; cold: +2.41%). Carcass dissection revealed higher proportions of breast (+1.54%), thighs (+1.14%), and wings (+1.05%) in intensively reared broilers, whereas extensively reared birds exhibited a higher proportion of the back portion (+3.74%). The proportion of liver and gizzard was greater in the extensive system (+0.14% and +0.80%, respectively), while heart yield was higher in the intensive system (+0.13%). Overall, the intensive system improved quantitative production parameters, whereas the extensive system mainly influenced organ development and carcass distribution, with potential benefits for meat quality.

Key words: *anatomical portions, edible organs, rearing system, turkey broiler, slaughter yield.*

INTRODUCTION

Turkey meat is appreciated for its low lipid content (lean meat), as well as for its important nutritional benefits and its positive effects on consumers' health (Costache et al., 2019; Üçtepe, 2025). Turkey meat production follows an upward trend due to the increasing demand, but also because of its higher profitability compared to chicken meat (Custură et al., 2012; Santos et al., 2005). Consequently, in addition to large commercial intensive turkey production complexes, an increasing number of backyard-type household farms have emerged in many regions of the world (Mottet & Tempio, 2017; Ghosh & Saha, 2023; Salgado-Pardo et al., 2025; Alemu et al., 2025).

Although the main concern of turkey breeders is to achieve high productivity and superior meat quality under conditions of the lowest possible production costs (Adhikari et al., 2024; Batkowska, 2026), they face complex and diverse challenges (disease prevention, biosecurity requirements, consumer concerns regarding food safety and animal welfare, environmental protection constraints, and rising feed costs), which significantly influence management practices, technological development, and the sustainability of the production chain (Bennett & Classen, 2003; Stadig et al., 2016; Hafez & Shehata, 2021).

One of the most important technological factors affecting productive performance in turkeys is the rearing system (Lungu et al., 2024). From this perspective, it is well known that intensive systems provide perfectly controlled environmental conditions and optimized nutrition, resulting in high body weight gains and superior carcass yield (Moise et al., 2024; Kaya et al., 2024), while extensive systems allow the expression of natural behaviors and improve welfare and meat quality, but are generally associated with lower performance and higher production costs (Vlad et al., 2025; Castellini et al., 2002; Castellini et al., 2006).

Experimental studies evaluating the effect of production systems on growth performance in turkeys have demonstrated that live weight gain, feed utilization efficiency, and production parameters differ significantly depending on management conditions (Gheorghe et al., 2021). Analyses based on repeated measurements have highlighted a strong statistical effect of the rearing system, age, and their interaction on growth dynamics, confirming the substantial influence of the

production environment on turkey development (Inci et al., 2020; Erener et al., 2006; Fanatico et al., 2007).

Studies conducted on a meat line belonging to the American Bronze breed, under different rearing systems (intensive, semi-intensive, and extensive), highlighted significant differences in live body weight and growth performance, especially in the later stages of life, emphasizing the importance of feeding level, access to pasture, and management conditions in determining productive outcomes (Arslan & Cetin, 2023).

In addition to growth performance, the production system directly influences carcass structure, the development of edible organs, and the qualitative characteristics of meat. This can be explained by differences in feeding regimes, birds' physical activity, and their exposure to natural environmental influences, technological factors that modify nutrient utilization, tissue deposition, and physiological development. These aspects determine variations in slaughter yield, the proportion of the main anatomical parts, and the weight of edible organs, which are considered essential indicators in evaluating the quantitative efficiency of poultry meat production systems (Ghosh & Saha, 2023; Vlad et al., 2025; Macovei et al., 2022).

Under these circumstances, the comparative evaluation of production systems has become essential for identifying optimal management strategies that ensure a balance between productivity, economic efficiency, animal welfare, and meat quality (Petracci & Cavani, 2012), especially in the context of the sustainable development of poultry production, where technological decisions must be supported by objective quantitative indicators related to live weight (Murawska, 2017; Inci et al., 2020; Vlad et al., 2025).

The present study aimed to evaluate the quantitative meat production in turkey broilers raised in two different production systems, intensive and extensive, in order to obtain scientifically substantiated data that would allow turkey breeders to optimize their production strategies so as to satisfy consumer demands while also improving the profitability of their activity.

MATERIALS AND METHODS

Experimental design

The study was conducted under production conditions in a unit specialized in turkey broiler rearing, using as biological material the commercial hybrid *Hybrid Converter*.

Day-old chicks were raised on permanent litter in a controlled-environment hall, with environmental factors (temperature, humidity, ventilation, and lighting program) and the quality conditions of compound feeds ensured at the levels recommended by the hybrid producing company; this was considered the "intensive system" of rearing.

At the beginning of the experimental period, a biological lot was formed consisting of a total stock of 150 turkey broilers. The birds were monitored throughout the experimental period, with weekly records of productive performance parameters, including body weight, feed consumption, water intake, feed conversion ratio, and mortality.

At the age of 105 days, a part of the birds was transferred to a free-range type hall with free access to an outdoor paddock, thus benefiting from natural environmental fluctuations as well as increased locomotor activity; this was the "extensive system" of rearing.

Until the moment of slaughter (136 days of age), the birds raised in the intensive system received a finishing compound feed in pellet form containing 18.5% C.P. and 3170 kcal M.E./kg, while the birds from the extensive system were fed a mixture of ground cereals containing 14.9% C.P. and 3220 kcal M.E./kg.

At the end of the rearing period (day 136), from both rearing systems (intensive and extensive), 6 (six) males and 6 (six) females, respectively, were selected, whose body weight was close to the average of the respective system/sex.

The selected birds were subjected to a 12-hour feed withdrawal to ensure carcass hygiene, after which they were slaughtered according to a standard procedure that included electrical stunning, bleeding for 10 minutes, scalding in water at +68°C, mechanical defeathering, and manual evisceration, thus resulting in the carcasses that constituted the subject of our study.

Working methods

Immediately after slaughter and evisceration, the carcasses (with head, neck, and claws) were individually weighed, and the obtained values were used to determine the hot carcass yield.

Subsequently, the carcasses were refrigerated (for 24 hours at +3.5°C), weighed again, and the cold carcass yield was determined.

The slaughter yield (hot and cold) was calculated as the percentage ratio between carcass weight (hot/cold) and live body weight.

The refrigerated carcasses were subjected to a uniform cutting procedure (performed by a single operator) into the four main regions (breast, thighs, wings, and back portion) (Figure 1).



Figure 1. Anatomical cutting of the carcasses (original source)

To determine the proportion in the carcass structure, each anatomical part was weighed individually, and the obtained value was related to the carcass weight.

After removal from the carcass, the edible organs (liver, heart, and gizzard) were cleaned of adhering tissues and then individually weighed; finally, the obtained weights were related to the weight of the carcass from which they originated, and the obtained data were used as quantitative indicators of physiological development under different rearing conditions (Figure 2).

Statistical analysis

The experimental data regarding live body weight, slaughter yield, carcass composition, and edible organ proportions were statistically processed using analysis of variance (ANOVA) to evaluate the differences between the intensive and extensive rearing systems.

The analysis was performed separately for males, females, and for both sexes combined. For each trait, the arithmetic mean (X), standard error of the mean (s_x), coefficient of variation (V%), as well as minimum and maximum values were calculated.

The significance of the differences between the compared groups was assessed based on probability values (p), and classified as follows: $p < 0.05$ (significant), $p < 0.01$ (distinctly significant), and $p < 0.001$ (highly significant), according to Field (2013).

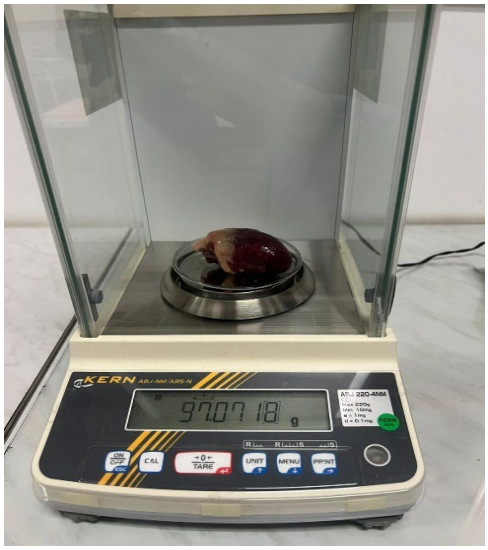


Figure 2. Determination of edible organ weight (original source)

RESULTS AND DISCUSSIONS

The quantitative evaluation of meat production in turkey broilers was based on the analysis of body weight at slaughter, slaughter yield (hot and cold), the anatomical structure of carcasses (the proportion of anatomical regions in carcass composition), and the weights of edible organs (heart, liver, and gizzard).

Body weight at slaughter

The comparative analysis of body weights at the time of slaughter highlighted differences both between the two sexes and between the tested rearing systems (Table 1).

Thus, males raised under intensive conditions reached an average body weight of 16.360±0.22 kg, compared to only 15.610±0.08

kg for those in the extensive system, the difference being statistically distinctly significant (p = 0.0053). In the case of females, the differences between groups were very statistically significant (p = 0.0004), against the background of average weights of 15.720±0.12 kg in the intensive system and only 14.641±0.12 kg in the extensive system. In both cases, the studied trait showed very good homogeneity, with coefficient of variation values ranging from 1.34-0.55% in males and 0.79-0.88% in females.

Table 1. Body weight at slaughter

Specification	Intensive rearing (n=6)		Extensive rearing (n=6)	
	$X \pm s_x$ (kg)	V%	$X \pm s_x$ (kg)	V%
Males	16.360±0.22	1.34	15.610±0.08	0.55
	** MI vs ME: p= 0.0053			
Females	15.720± 0.12	0.79	14.641±0,12	0.88
	*** FI vs. FE: p= 0.0004			
Both sexes	16.040± 0.49	3.10	15.126±0,54	0.57
	**I vs E: p= 0.0089			

Note: NS – non-significant; *significant differences (0.01 < p < 0.05); ** distinctly significant differences (0.001 < p < 0.01); *** very significant differences (p < 0.001)

From the comparative analysis of body weight (males + females) carried out within the two production systems, average values of 16.040±0.49 kg were obtained for the birds raised in the intensive system and 15.126±0.54 kg for those in the extensive system, leading to a statistically distinctly significant difference (p = 0.0089) between the two groups.

The studied trait was very homogeneous at the group level, an aspect validated by the very low values of the coefficient of variation (intensive system = 3.1%; extensive system = 0.57%).

The obtained results indicate a clear superiority of the intensive rearing system in terms of body weight at slaughter (16.04 kg vs. 15.13 kg). Similar findings were reported by Arslan & Cetin (2023), where higher final body weights were recorded in turkeys raised under intensive conditions compared to extensive systems (up to 7.79 kg vs. 6.71 kg in males). Ince et al. (2020) demonstrated that the rearing system has a significant effect on growth performance and body weight dynamics, confirming the strong influence of environmental and nutritional conditions.

Slaughter yield

The obtained data confirmed the same trend observed for body weight, namely that both carcass weights and slaughter yield were higher in birds raised under intensive conditions and, consistently, higher in males than in females.

For example, immediately after slaughter, the average weight of hot carcasses was 13.072±0.11 kg in males raised in the intensive system, compared to 12.046±0.05 kg in those raised in the extensive system, the difference being statistically distinctly significant (p = 0.004).

In the case of females, the average carcass weight was 12.197±0.16 kg in the intensive system and 10.946±0.04 kg in the extensive system, the difference between the two systems being very statistically significant (p = 0.0003).

The overall analysis of both sexes showed an average carcass weight of 12.635±0.21 kg in birds raised in the intensive system and 11.496±0.23 kg in those raised in the extensive system, the difference between systems being statistically distinctly significant (p = 0.006).

Regarding the hot slaughter yield, the values were also higher in birds raised in the intensive system. Thus, in males an average yield of 79.90±0.18% was recorded, compared to 77.15±0.14% in the extensive system, with distinctly significant differences (p = 0.003).

In females, the average yield was 77.59±0.90% in the intensive system and 74.76±0.13% in the extensive system, the difference being statistically distinctly significant (p = 0.001).

For the two sexes combined, the slaughter yield was 78.74±0.54% in the intensive system and 75.96±0.34% in the extensive system, the difference between systems being statistically distinctly significant (p = 0.002) (Table 2).

Table 2. Hot slaughter yield

Specification	Intensive rearing (n=6)		Extensive rearing (n=6)	
	$X \pm s_x$ (kg)	V%	$X \pm s_x$ (kg)	V%
Hot carcass weight				
Males	13.072 ± 0.11	1.50	12.046 ± 0,05	1.02
	**MI vs ME: p = 0.004			
Females	12.197 ± 0.16	3.27	10.946 ± 0.04	0.62
	***FI vs FE: p = 0.0003			
Both sexes	12.635 ± 0.21	2.54	11.496 ± 0.23	2.77
	**I vs E: p = 0.006			
Hot slaughter yield (%)				
Males	79.90 ± 0.18	0.40	77.15 ± 0.14	0.52
	**MI vs ME: p = 0.003			
Females	77.59 ± 0.90	2.02	74.76 ± 0.13	0.30
	**FI vs FE: p = 0.001			
Both sexes	78.74 ± 0.54	1.37	75.96 ± 0.34	0.89
	**I vs E: p = 0.002			

Note: NS – non-significant; *significant differences (0.01 < p < 0.05); ** distinctly significant differences (0.001 < p < 0.01); *** very significant differences (p < 0.001)

The higher slaughter yield recorded in intensively reared birds (78.74% vs. 75.96%) confirms the superior efficiency of nutrient utilization under controlled conditions. These results are in agreement with those reported by Adhikari et al. (2024), who observed improved growth performance and feed efficiency in turkeys raised under intensive systems, which are directly associated with higher carcass yield.

After a 24-hour refrigeration period, the carcass weight was 12.854±0.11 kg in males raised in the intensive system, compared to 11.899±0.05 kg in those raised in the extensive system, the difference being statistically distinctly significant ($p = 0.004$).

In the case of females, the average cold carcass weight was 11.994±0.16 kg in the intensive system and 10.810±0.04 kg in the extensive system, the difference being very statistically significant ($p = 0.0003$).

The overall analysis of both sexes showed an average cold carcass weight of 12.424±0.21 kg in birds raised in the intensive system and 11.355±0.23 kg in those raised in the extensive system, the difference being statistically distinctly significant ($p = 0.006$) (Table 3).

Table 3. Cold slaughter yield

Specification	Intensive rearing (n=6)		Extensive rearing (n=6)	
	$X \pm s_x$ (kg)	V%	$X \pm s_x$ (kg)	V%
Cold carcass weight				
Males	12.854 ± 0.11	1.20	11.899 ± 0.05	0.94
	**MI vs ME: p = 0,004			
Females	11.994 ± 0.16	3.27	10.810 ± 0.04	0.69
	***FI vs FE: p = 0,0003			
Both sexes	12.424 ± 0.21	2.54	11.355 ± 0.23	2.77
	**I vs E: p = 0,006			
Cold slaughter yield (%)				
Males	78.57 ± 0.16	0.44	76.21 ± 0.14	0.56
	**MI vs ME: p = 0.003			
Females	76.30 ± 0.88	2.04	73.83 ± 0.14	0.37
	**FI vs FE: p = 0.001			
Both sexes	77.43 ± 0.52	1.38	75.02 ± 0.33	0.91
	**I vs E: p = 0.002			

Note: NS – non-significant; *significant differences ($0.01<p<0.05$); ** distinctly significant differences ($0.001<p<0.01$); *** very significant differences ($p < 0.001$)

Proportion of anatomical parts

The distribution of the anatomical parts obtained from carcass cutting highlighted differences due to the experimental factors considered.

The proportion of the breast recorded the highest values among all carcass components and was higher in males raised under intensive conditions (33.62±0.18%), compared to those from the extensive system (32.07±0.24%), the statistical differences between the two groups being distinctly significant ($p < 0.001$).

A similar situation was also observed in females, where the breast proportion was 33.08±0.10% in the intensive system and 32.40±0.17% in the extensive system, the differences between systems also being statistically distinctly significant ($p < 0.001$).

Calculated as an average for the two sexes (males + females), the breast proportion was 33.35±0.11% in birds from the intensive system and only 31.81±0.17% in those from the extensive system, thus the statistical difference between groups was very significant (Table 4).

Table 4. Breast proportion in the carcass structure

Specification	Intensive rearing (n=6)		Extensive rearing (n=6)	
	$X \pm s_x$ (%)	V%	$X \pm s_x$ (%)	V%
Males	33.62 ± 0.18	0.55	32.07 ± 0.24	0.75
	**MI vs ME: p < 0.0010			
Females	33.08 ± 0.10	0.30	32.40 ± 0.17	0.52
	**FI vs FE: p < 0.0010			
Both sexes	33.35 ± 0.11	0.79	31.81 ± 0.17	1.31
	*** I vs E: p = 0.0002			

Note: NS – non-significant; *significant differences ($0.01 < p < 0.05$); ** distinctly significant differences ($0.001 < p < 0.01$); *** very significant differences ($p < 0.001$)

The determinations carried out to establish the proportion of thighs in the carcass structure highlighted higher values in birds raised in the intensive system compared to those raised in the extensive system (Table 5).

Thus, in males, the proportion of thighs was 32.83±0.18% in the intensive system and 31.65±0.19% in the extensive system, the differences between the two systems being statistically distinctly significant ($p < 0.001$).

A similar situation was also observed in the case of females, where the proportion of thighs was 32.46±0.12% in the intensive system and 31.54±0.16% in the extensive system, the difference between groups also being statistically distinctly significant ($p < 0.001$).

Calculated as an average for both sexes (males + females), the proportion of thighs in the carcass structure was 32.59±0.05% in birds raised in the intensive system and 31.45±0.09% in those from the extensive system, the statistical difference between the two systems being very significant ($p < 0.0001$).

Table 5. Proportion of thighs in the carcass structure

Specification	Intensive rearing (n=6)		Extensive rearing (n=6)	
	$X \pm s_x$ (%)	V%	$X \pm s_x$ (%)	V%
Males	32.83 ± 0.18	0.55	31.65 ± 0.19	0.60
	**MI vs ME: p < 0.001			
Females	32.46 ± 0.12	0.37	31.54 ± 0.16	0.52
	**FI vs FE: p < 0.001			
Both sexes	32.59 ± 0.05	0.37	31.45 ± 0.09	0.69
	*** I vs E: p < 0.0001			

Note: NS – non-significant; *significant differences (0.01 < p < 0.05); ** distinctly significant differences (0.001 < p < 0.01); *** very significant differences (p < 0.001)

The determinations carried out to establish the proportion of wings in the carcass structure revealed higher values in birds raised in the intensive system compared to those raised in the extensive system (Table 6).

Thus, in males, the proportion of wings was 12.20±0.18% in the intensive system and 11.25±0.54% in the extensive system, while in females the proportion of wings was 11.99±0.16% in the intensive system and 10.17±0.32% in the extensive system.

Calculated as an average for both sexes (males + females), the proportion of wings in the carcass structure was 12.13±0.08% in birds raised in the intensive system and 11.08±0.16% in those from the extensive system.

In all three situations presented, the statistical differences between the groups were distinctly significant.

Table 6. Proportion of wings in the carcass structure

Specification	Intensive rearing (n=6)		Extensive rearing (n=6)	
	$X \pm s_x$ (%)	V%	$X \pm s_x$ (%)	V%
Males	12.20 ± 0.18	1.47	11.25 ± 0.54	4.80
	**MI vs ME: p < 0.01			
Females	11.99 ± 0.16	1.33	10.17 ± 0.32	3.14
	**FI vs FE: p < 0.01			
Both sexes	12.13 ± 0.08	1.63	11.08 ± 0.16	3.59
	** I vs E: p = 0.0029			

Note: NS – non-significant; *significant differences (0.01 < p < 0.05); ** distinctly significant differences (0.001 < p < 0.01); *** very significant differences (p < 0.001)

The analysis of the anatomical structure of the carcass revealed that the proportion of the back portion was higher in birds raised in the extensive system compared to those raised in the intensive system (Table 7).

In the case of males, the back portion represented 21.34±0.34% of the carcass structure in birds raised intensively, while in birds raised in the extensive system the proportion was 25.03±0.26%, the difference between the two systems being very statistically significant (p < 0.001).

In females, the same trend was observed, the back-portion accounting for 22.47±0.18% in the intensive system and 25.89±0.44% in the extensive system, the difference between the groups also being very statistically significant (p < 0.001).

Considering the average values for both sexes (males + females), the back portion represented 21.92±0.20% of the carcass structure in birds raised in the intensive system and 25.66±0.23% in those raised in the extensive system, the difference between the two systems being very statistically significant (p < 0.0001).

Table 7. Proportion of the back portion in the carcass structure

Specification	Intensive rearing (n=6)		Extensive rearing (n=6)	
	$X \pm s_x$ (%)	V%	$X \pm s_x$ (%)	V%
Males	21.34 ± 0.34	1.59	25.03 ± 0.26	1.05
	*** MI vs ME: p < 0.0001			
Females	22.47 ± 0.18	0.80	25.89 ± 0.44	1.70
	*** FI vs FE: p < 0.0001			
Both sexes	21.92 ± 0.20	2.24	25.66 ± 0.23	2.17
	*** I vs E: p < 0.0001			

Note: NS – non-significant; *significant differences (0.01 < p < 0.05); ** distinctly significant differences (0.001 < p < 0.01); *** very significant differences (p < 0.001)

The higher proportion of breast (33.35% vs. 31.81%), thighs (32.59% vs. 31.45%), and wings (12.13% vs. 11.08%) in intensively reared birds indicates enhanced muscle development under optimized feeding and management conditions. A similar influence of feeding system and nutrient availability on carcass composition was reported by Erener et al. (2006), who highlighted that feeding strategy significantly affects tissue deposition and carcass traits.

In contrast, the higher proportion of the back portion in extensively reared birds (25.66% vs. 21.92%) may be associated with increased locomotor activity and different patterns of energy utilization. This observation is consistent with the findings of Ghosh & Saha (2023), who reported that birds raised in extensive systems exhibit different growth patterns and lower muscle deposition in commercially valuable cuts.

Yield of edible organs

The evaluation of the yield of edible organs highlighted distinct variations between turkey broilers raised in different systems, reflecting both metabolic adaptation and the physiological responses associated with

differences in rearing conditions, activity level, and feed utilization efficiency.

The liver yield showed moderate but statistically significant differences between the two production systems. Thus, males raised in the extensive system recorded a liver proportion of 1.44±0.06%, compared to 1.28±0.06% in those from the intensive system, the difference between the two groups being statistically significant ($p < 0.05$).

The same phenomenon was also observed in the case of females, in which the liver yield was 1.55±0.04% in the extensive system and 1.52±0.10% in the intensive system, the difference between systems also being statistically significant ($p < 0.05$).

The average liver proportion (males + females) was 1.50±0.04% in broilers raised in the extensive system, compared to 1.36±0.05% in birds raised in the intensive system, the difference being statistically significant ($p < 0.05$). This trend may be attributed to the increased metabolic activity associated with more intense locomotion and variable feed consumption patterns characteristic of extensive systems, which may stimulate hepatic development due to higher energy expenditure and physiological adaptation to fluctuating environmental conditions (Table 8).

Table 8. Liver proportion relative to carcass weight

Specification	Intensive rearing (n=6)		Extensive rearing (n=6)	
	$X \pm s_x$ (%)	V%	$X \pm s_x$ (%)	V%
Males	1.28 ± 0.06	8.60	1.44 ± 0.06	7.16
	*MI vs ME: $p < 0.05$			
Females	1.52 ± 0.10	6.71	1.55 ± 0.04	2.38
	*FI vs FE: $p < 0.05$			
Both sexes	1.36 ± 0.05	9.05	1.50 ± 0.04	6.72
	* I vs E: $p < 0.041$			

Note: NS – non-significant; *significant differences ($0.01 < p < 0.05$); ** distinctly significant differences ($0.001 < p < 0.01$); *** very significant differences ($p < 0.001$)

The analysis of the proportion of the gizzard in the carcass structure revealed clear differences between the rearing systems, with higher values recorded in birds from the extensive system (Table 9).

In males, the gizzard represented 1.66±0.02% of the carcass weight in the intensive system, while in birds raised extensively its proportion was 2.44±0.06%, the difference between the two systems being statistically distinctly significant ($p < 0.001$).

A similar trend was observed in females, where the gizzard proportion was 1.72±0.02% in the intensive system and 2.57±0.06% in the extensive system, the difference between the groups being distinctly significant ($p < 0.001$).

Considering the average values for both sexes (males + females), the gizzard proportion was 1.69±0.02% in birds raised in the intensive system and 2.49±0.03% in those from the extensive system, the statistical difference between the two systems being very significant ($p < 0.0001$).

Table 9. Gizzard proportion relative to carcass weight

Specification	Intensive rearing (n=6)		Extensive rearing (n=6)	
	$X \pm s_x$ (%)	V%	$X \pm s_x$ (%)	V%
Males	1.66 ± 0.02	1.70	2.44 ± 0.06	2.45
	**MI vs ME: $p < 0.001$			
Females	1.72 ± 0.02	1.40	2.57 ± 0.06	2.30
	**FI vs FE: $p < 0.001$			
Both sexes	1.69 ± 0.02	2.61	2.49 ± 0.03	3.03
	*** I vs E: $p < 0.0001$			

Note: NS – non-significant; *significant differences ($0.01 < p < 0.05$); ** distinctly significant differences ($0.001 < p < 0.01$); *** very significant differences ($p < 0.001$)

The evaluation of the proportion of the heart in the carcass structure revealed differences between the two rearing systems, with higher values recorded in birds raised in the intensive system (Table 10).

In the case of males, the heart proportion was 0.57±0.02% in the intensive system and 0.53±0.01% in birds raised in the extensive system, the difference between the two systems being statistically distinctly significant ($p < 0.01$).

In females, a similar trend was observed, the heart proportion being 0.67±0.02% in birds raised in the intensive system and 0.50±0.01% in those raised in the extensive system, the difference between groups also being statistically distinctly significant ($p < 0.01$).

Table 10. Heart proportion relative to carcass weight

Specification	Intensive rearing (n=6)		Extensive rearing (n=6)	
	$X \pm s_x$ (%)	V%	$X \pm s_x$ (%)	V%
Males	0.57 ± 0.02	3.51	0.53 ± 0.01	1.89
	**MI vs ME: $p < 0.001$			
Females	0.67 ± 0.02	2.99	0.50 ± 0.01	2.00
	**FI vs FE: $p < 0.001$			
Both sexes	0.62 ± 0.02	7.49	0.49 ± 0.02	10.64
	** I vs E: $p < 0.003$			

Note: NS – non-significant; *significant differences ($0.01 < p < 0.05$); ** distinctly significant differences ($0.001 < p < 0.01$); *** very significant differences ($p < 0.001$)

Calculated as an average for both sexes (males + females), the heart proportion in the carcass structure was 0.62±0.02% in birds from the intensive system and 0.49±0.02% in those raised in the extensive system, the statistical difference between the two systems being distinctly significant ($p < 0.003$).

The higher proportions of liver (1.50% vs. 1.36%) and gizzard (2.49% vs. 1.69%) in the extensive system reflect increased digestive activity and adaptation to less processed diets. Similar trends were reported by Macovei et al. (2022), who showed that alternative production systems influence metabolic activity and physiological development. Conversely, the higher heart proportion in intensively reared birds (0.62% vs. 0.49%) may be associated with accelerated growth rate and increased metabolic demands.

CONCLUSIONS

The obtained results demonstrate that the rearing system exerts a significant influence on the quantitative meat production in turkey broilers, with direct repercussions on body weight at slaughter, slaughter yield, carcass anatomical structure, and the development of internal organs.

The performed analysis highlighted that birds raised under intensive conditions reached significantly higher body weights at slaughter, both in males (by 4.58%) and in females (by 6.86%), confirming the superior efficiency of controlled rearing conditions, balanced feeding regimes, and optimized technological management.

The data obtained at slaughter followed the same trend, intensively raised broilers recording higher carcass weights, both hot (by 9.01%) and cold (by 8.60%), as well as higher slaughter yield values (by 2.78% – hot yield and by 2.41% – cold yield) compared to birds raised in the extensive system. These results highlight the direct relationship between growth rate, nutrient utilization efficiency, and muscle mass development under intensive management conditions.

The evaluation of the anatomical structure of the carcass also highlighted the influence of the rearing system on the distribution of carcass components. The intensive system favored the development of components with high commercial value, especially the breast (by 1.54%) and the thighs (by 1.14%). In contrast, birds raised in the extensive system showed higher proportions of the back portion (by 3.74%), an aspect associated with more intense locomotor activity and different utilization of energy resources.

The analysis of the yield of edible organs revealed additional differences between the two rearing systems. The extensive system was associated with a more pronounced development of the gizzard, suggesting more intense digestive activity, while birds raised in the intensive system showed a higher proportion of the heart, possibly as a result of the accelerated growth rate. The liver yield showed moderate but statistically significant variations, indicating metabolic adaptations to different rearing conditions and energy consumption.

The results of the study confirm that the rearing system significantly influences the productivity of turkey broilers. Thus, the intensive system maximizes productive performance and yield in meat production, while the extensive system leads to certain physiological adaptations reflected in the development of internal organs and less in carcass structure, but which will certainly be reflected in the quality of the obtained meat.

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PRODUCTIVE PERFORMANCE OF CHAROLAISE CATTLE EXPLOITED IN EXTENSIVE AND SEMI-INTENSIVE SYSTEMS ON FARMS IN SUCEAVA COUNTY, ROMANIA

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Abstract

The present study aims to analyze the productive performance of Charolaise beef cattle exploited in extensive and semi-intensive systems on farms in Suceava county. The biological material consists of animals under official production control through which body weight is monitored at birth (BW), at 200 days (W200) and at 365 days (W365) and based on the results obtained through measurements, it was calculated the average of daily weight gain (DWG). The primary data were statistically processed using specific computer programs, calculating the values of the estimators and testing the differences in means. For BW, the average of animals in the semi-intensive system was insignificantly lower by 0.54 kg ($p<0.05$). The differences are significant in favor of this system only for the W365 and DWG365 environments which are higher by 32.93 kg and 138.18 g/day respectively, the performances at one year being influenced by the management applied

Key words*: Charolaise, meat, production performance.*

INTRODUCTION

Meat and milk are the animal food for which animal husbandry technologies have been developed to increase their performance. Beef is a animal food with high biological value and special organoleptic qualities, containing 18.7% protein and 15.7% fat, the energy value of one kilogram of meat being 2270 kcal. The biological value of meat proteins is 74%, the net utilization coefficient is 74% and the digestibility is 97% (Gociman et al., 2019).Through the digestion and absorption of proteins following meat consumption, the entire spectrum of essential amino acids is made available to the human body (Maciuc, 2006). The weight gain of animals and the development of tissues is influenced by the neuro – endocrine system through the hormones produced, among which we mention somatotropic hormone, thyroxine, insulin, adrenal medullary hormones, etc. (Hozáková et al., 2020; Maciuc et al., 2015).

A particular influence on meat production has the management of technology and external environmental factors, the most important being feeding and ration structure, sanitation and water temperature, but also the thermal stress present during the summer months, especially in the case of animals raised on pastures (Gociman et al., 2020; Grossi et al., 2021; Amariții et al., 2026)

Among the meat breeds raised in Romania is the Charolaise breed, which was formed in France by improving local breeds and which were later crossed with the Simmental and Shorthorn breeds. As a result of the applied improvement programs, animals belonging to this breed have more abundant muscle mass and reduced fat content. In the case of intensively fattened calves, are recorded average daily weight gain (DWG) up to 1.4 kg.

The Charolaise breed is recognized as having a good capacity for body development, the improvement of productive performances being dependent on the correct use of exploitation technologies and the application of adequate management, especially nutritional. Some studies highlight that the bulls of this breed have a very good feed conversion and the achievement of the best average daily gains of 1.313 kg for the fattening period of 12-18

months which are represented by higher intramuscular fat deposits (Ünal et al., 2025). The quality of the meat in the carcass is different compared to other meat breeds, because Charolaise cattle and their crossbreeds gain weight during the fattening period due to the development of muscle mass in the front part of the trunk (pectoral muscles and neck region) and the accumulation of intramuscular fat (Robelin et al., 1990; Młynek et al., 2006). The slaughter age of the animal is very important for obtaining quality carcasses and superior meat and the target weights established for each breed are based mainly on consumer preferences and the economic efficiency of production (Kostusiak et al., 2023).

The nutritional management of these animals remains the determining factor of the productive performance, the level of concentrates in the ration significantly influencing the amount of meat in the carcass (Serrano et al., 2007). The productive performance of calves is influenced by the management of milk feeding. Corn silo is considered a very good source of food for fattening cattle due to the energy and fiber intake (Álvarez-Rodríguez et al., 2010; Cozzi et al., 2008).

Growth performance is influenced by the level of animal welfare through environmental factors and housing conditions that can reduce stress of any kind and thus influence health status (Cozzi et al., 2005; Álvarez-Rodríguez et al., 2010; Skaperda et al., 2021).

In Romania, the beef cattle herd is growing, this sector being supported by subsidies that stimulate farmers. The animals are registered in the Official Production Control (OPC) and based on the standardized methodology, the main indicators of animal growth are monitored until the age of one year. At the national level, in 2023 there were 207 farms that raised Charolaise animals, registered in the Genealogical Register, with the following herd structure: 5887 lactating cows, 1838 calves, 1920 heifers and 224 bulls used for breeding.

This paper work was carried out with the aim of contribution to the study of the performances achieved in Romanian farms by Charolaise cattle.

MATERIALS AND METHODS

The study is based on the analysis of data on the productive performance of animals born from January 2022 to December 2024 and belonging to the Charolaise breed, raised on farms in Suceava County, of which 7 apply semi-intensive breeding technologies and 24 raise animals in an extensive system. For these animals, the OPC is carried out, which is the basis for the development of the national breeding program for this breed.

For the evaluation of meat production, the most important parameter is the live weight of the animals. The animals whose performances were studied are raised and exploited both in extensive and semi-intensive systems. After birth, the calves are suckled by mother cows that are not milked, their weaning taking place naturally, 6-7 months after calving. The animals are raised on pastures during most of the year and the ration is supplemented with concentrates. The calves graze alongside the base herd and after weaning they are fattened for slaughter or to be used for reproduction if they are valuable. During the winter, the animals are kept in stables, being tied in the extensive system or free in the case of the semi-intensive system. The analyzed characters were the birth weight (BW), the weight at 200 days (W200) and at 365 days (W365), and based on these, the daily weight gain achieved is calculated (DWG).

In the extensive system, 24 farms and a total of 432 heads were taken into account, of which 182 males and 250 females. In the 7 farms analyzed that raise animals in a semi-intensive system, 164 males and 205 females were born during the reference period.

The primary data are those included in the OPC bulletins and were statistically processed using the PrismaGraph9 software program to perform descriptive statistics, apply the Fisher and Tukey tests, determine the significance of differences and coefficient of Pearson correlations. For the indicators describing the animal performances, the differences in the means of the achieved performances were tested and the correlations were calculated: arithmetic mean ($\bar{X}$), arithmetic mean error ($\pm s_{\bar{X}}$), standard deviation (s), coefficient of variation (V%), Fisher test and Tukey test.

The test result *p*, given as a number between 0 and 1, represents the probability of making an error if we reject the hypothesis H_0. If *p* is less than the chosen significance threshold α – usually α = 0.05 – we reject the hypothesis H_0 and accept the hypothesis H_1 as true.
The interpretation of p-values in most tests is as follows:
- $p < 0.05$, the difference is significant (S, 95% confidence interval, marked *);
- $p < 0.01$, the difference is significant (S, 99% confidence interval, marked **);
- $p < 0.001$, the difference is highly significant (HS, 99.9% confidence interval, marked ***);
- $p < 0.0001$, the difference is extremely highly significant (HS, 99.99% confidence interval, marked ****);
- $p > 0.05$, the difference is not significant (NS).

The Pearson correlation coefficient (r) is a measure of the linear association between two variables and has values between –1 and +1, with the significance of positive/negative association depending on the sign of the coefficient.
If the value $r_{XY} = 0$, the lack of association between the variables is signified.
The statistical significance of the values is as follows:
- $0 < r < 0.19$ – very weak association;
- $0.20 < r < 0.25$ – weak association;
- $0.26 < r < 0.40$ – moderate association;
- $0.41 < r < 0.69$ – strong association;
- $0.70 < r < 1$ – very strong association.

RESULTS AND DISCUSSIONS

The evolution of daily weight gain is an indicator of the degree of adaptation of a breed to the conditions of husbandry and animal handling (Hozáková et al., 2020; Maciuc, 2006). Animals of this breed have the highest body weights at maturity, bulls reaching 1000-1650 kg and cows between 700-1200 kg. (www.okstate.edu)
Table 1 presents the statistical values for the data from the period 2022-2024 for animals raised on farms in Suceava County, values presented separately depending on the maintenance system. It can be seen that there are differences in animal performance between the two raising systems, as expected as a result of management measures regarding technological factors.

Table 1. Statistics of productive performances achieved in young Charolaise cattle raised on farms in Suceava County during 2022-2024

Livestock farming system	Parameters	n	$\bar{X}$	S	$s_{\bar{x}}$	V %	Minimum	Maximum
Extensive	BW (kg)	*432*	42.59	7.1	0.34	16.68	30	66
	W200 (kg)	*413*	223.69	35.94	1.77	16.07	126.23	354.99
	DWG200 (g/day)	*413*	905.52	181.64	8.94	20.06	416.67	1589.93
	W365 (kg)	*237*	337.54	57.41	3.73	17.01	153.31	487.14
	DWG365 (g/day)	*237*	743.05	289.37	18.8	38.94	56.82	1452.38
Semi-intensive	BW (kg)	*369*	42.05	4.05	0.21	9.63	34	61
	W200 (kg)	*352*	228.17	40.87	2.18	17.91	104.97	410.31
	DWG200 (g/day)	*352*	930.83	204.17	10.88	21.93	339.84	1861.54
	W365 (kg)	*256*	370.48	50.6	3.16	13.66	204.3	534.51
	DWG365 (g/day)	*256*	881.23	261.39	16.34	29.66	109.89	1835.16

As can be seen, the average weight of calves born to cows raised in the extensive system was 42.59 kg, 0.54 kg heavier than calves obtained in the semi-intensive system whose average weight was 42.05 kg.
The fact that in the extensive system the animals are on average somewhat heavier at calving draws attention to the management of the last part of the gestation in the case of cows in the semi-intensive system in terms of exercise regime, feeding or other internal factors. Monitoring body condition score (BCS) in cows is a good management measure.
However, in the semi-intensive system the lowest calving weight was 34 kg, higher by 11.76% (Table 1) and that the batch of calves is homogeneous for this character, compared to

the extensive one which is medium homogeneous.

The calves in the extensive system had an average W200 of 223.69 kg and W365 of 337.54 kg with corresponding average daily weight gains (DWG) of 0.905 kg and 0.743 kg respectively. For DWG200, the maximum value recorded was 1.59 kg/day and at one year the daily weight gains were a maximum of 1452.38 kg/day. The variability is high because the values of the V% coefficient are 20.06 and 38.94 respectively, due to the large number of extensive farms analyzed (24).



Figure 1. Production performances achieved in extensive and semi-intensive systems

The performance of animals raised in semi-intensive system was better, as shown in the data in Table 1 and the graphic representation in Figure 1. Calves had a better growth rate because the average weights were W200 of 228.17 kg and for W365 of 370.48 kg, values higher by 2.19% and 8.89% respectively compared to the average body weights achieved by calves raised extensively.

The DWG200 and DWG365 were on average 0.931 kg/day and 0.881 kg/day, which means a daily gain of 26 g/day and 138 g/day respectively compared to the extensive system. The upper limit of DWG200 for variation range was for 1.861 kg/day and for DWG365 was 1.835 kg, the variability of the performances being high in this case, with V % values of 21.9 and 29.66.

Up to 200 days the rearing system does not influence W200 because the differences are insignificant, both systems ensuring relatively similar conditions for this period, being feeding based on maternal milk consumption in both cases to ensure nutrients. Discrepancies appear for the period that occurs, up to one year, with semi – intensively raised animals being significantly heavier, which means that feeding management differentiates the two systems.

Table 2 details the values of the statistics that describe the performances obtained by calves and heifers raised in extensive and intensive systems.

Table 2. Statistics of performance indicators achieved by Charolais calves and heifers in both rearing systems

Livestock farming system		Extensive					Semi-intensive				
Parameters		BW (kg)	W200 (kg)	DWG200 (g/day)	W365 (kg)	DWG365 (g/day)	BW (kg)	W200 (kg)	DWG200 (g/day)	W365 (kg)	DWG365 (g/day)
CALVES	n	*182*	*180*	*180*	*60*	*60*	*211*	*152*	*152*	*101*	*101*
	$\bar{X}$	43.78	233.44	948.26	354.99	820.42	42.81	238.22	974.51	386.03	917.23
	s	7.257	33.99	173	63.4	298.78	3.691	44.13	220.73	59.51	29.53
	$s_{\bar{x}}$	0.538	2.533	12.89	8.185	38.57	0.254	3.58	17.9	5.921	296.74
	V%	16.58	14.56	18.24	17.86	36.42	8.623	18.53	22.65	15.42	32.35
	Minimum	31	147.52	461.54	153.31	162.01	35	105	339.84	204.30	109.89
	Maximum	66	355	1590	487.14	1452.38	61.00	410.31	1861.54	534.51	1835.16
HEIFERS	n	*250*	*233*	*233*	*177*	*177*	*205*	*200*	*200*	*155*	*155*
	$\bar{X}$	41.72	216.15	872.50	331.63	716.83	40.97	220.54	897.63	360.34	857.78
	s	6.871	35.66	181.6	54.15	282.18	3.938	36.5	184.37	40.97	233.56
	$s_{\bar{x}}$	0.435	2.336	11.9	4.07	21.21	0.275	2.581	13.04	3.291	18.76
	V%	16.47	16.50	20.82	16.33	39.36	9.61	16.55	20.54	11.37	27.23
	Minimum	30.00	126.23	416.67	154.57	56.82	34.00	114.52	377.62	231.38	163.84
	Maximum	65.00	350.14	1535.71	449.32	1345.24	57.00	333.37	1455.36	454.08	1477.71

For birth weight, males have higher weights than females, in the extensive system they are on average 2.06 kg heavier and in the semi-intensive the difference is 2.42 kg. Comparing the rearing systems, the average birth weights are higher in the case of calves and heifers born in the extensive system which also presents greater character variability, being a homogeneous environment. From the data presented and represented in figures 2 and 3, it can be observed that the animals have different body development depending on sex in both exploitation systems, sexual dimorphism being a recognized factor of influence of body development.

Compared in the two rearing systems, calves have different weight gains. In the intensive system, males reach W200 of 238.22 kg and W365 of 386.03 kg, values that are 2% and respectively 8.04% higher compared to the extensive system where calves reach 233.44 kg at 200 days and about 355 kg at one year. In both groups analyzed, the variability of the character is moderately homogeneous and the differences become significant ($p < 0.05$) for the weight achieved at one year of age.

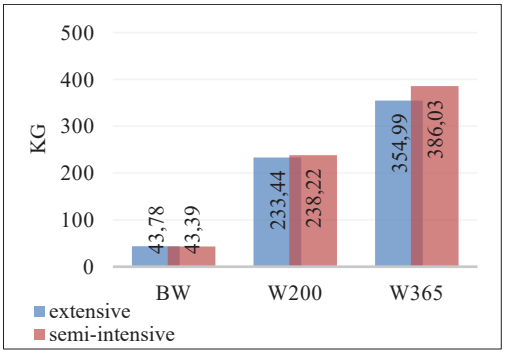


Figure 2. Body weight (kg) for calves in both rearing systems

Regarding the average daily weight gains achieved by males, they are different because in the extensive system the value of DWG200 is 0.948 kg/day and DWG365 is 0.820 kg/day in the latter case the variability being very high in the herd for V% of 36.42. In the semi-intensive system, calves achieve better DWG, because up to 200 days they grow by 0.975 kg/day and DWG365 is 0.917 kg/day values that are higher by 2.77% and 10.58% respectively compared to the extensive system, the differences being significant ($p < 0.05$) for DWG365.

If we analyze the evolution of the weight of calves in the both systems, it can be seen from table 2 and figure 3 that they are superior in the semi-intensive one. Although the calving weight is on average 40.97 kg, 0.8% lower than that of calves in the extensive system, subsequent performances are better. Thus, semi-intensively raised calves achieve a weight of 220.54 kg at 200 days with a DWG200 of 0.897 kg/day and at one year old the weight is on average 360.34 kg by achieving a DWG365 of 0.858 kg/day. These performances are highly significant ($p < 0.0001$) for the weight achieved by calves at one year old and for dwg365 compared to those achieved in the extensive system where calves reach an average W365 value of 331.63 kg based on a DWG365 of 0.717 kg/day.

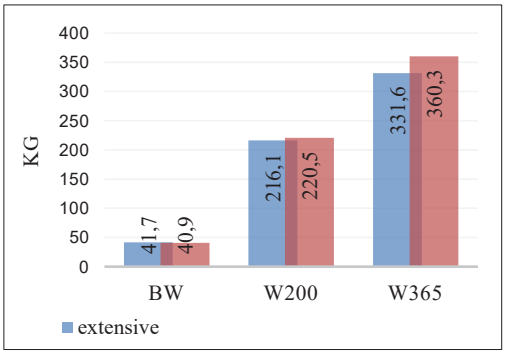
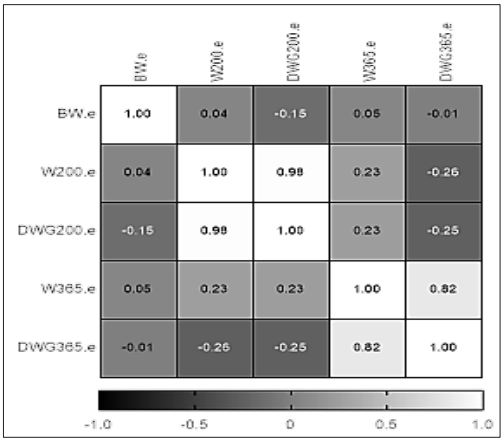


Figure 3. Body weight (kg) for heifers in both rearing systems

It is worth noting the variability of these traits for which the batches of calves are on average homogeneous for W200 and DWG200 and remain on average homogeneous for W365 with the lower value in the intensive system where V% is 11.37 compared to 16.33. The variability increases when we analyze DWG365 because in the case of calves raised extensively for this trait the batch is more heterogeneous (V % = 39.36) compared to the case of calves in the semi-intensive system (V % = 27.23). This means that animal feeding management is a major factor influencing performance which, by adjustment, can reduce variability in both situations. By testing the differences between the average performances by sex and between the two rearing systems,

they are statistically insignificant between farms for BW, W200 and DWG200 regardless of the group. This highlights the fact that the rearing system does not significantly influence BW of the animals and their growth performance in the first 200 days of life.

For W365 and DWG365 there are highly statistically significant differences for W365 and DWG365 between the exploitation systems but also between groups of animals of the same sex raised in this two different systems. Semi-intensive farms record final weights (W365) and DWG up to one year higher than extensive one as a result of better growth management that primarily targets animal nutrition, which is reflected in higher weight gains of animals after the age of 7 months.



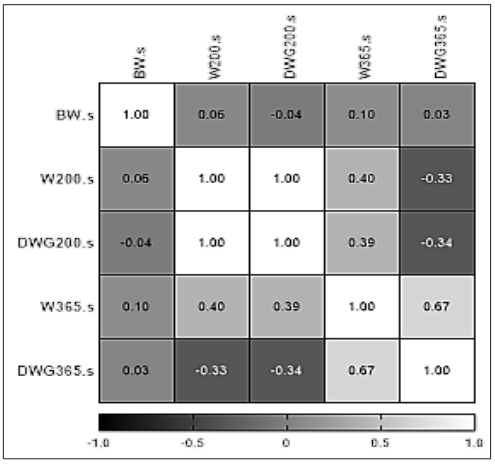
e – extensive

Figure 4. Pearson correlation coefficient values between BW, W200 and W365 in extensive systems

The statistical analysis of the data also included the calculation of Pearson correlation coefficients (r) between the characters for both rearing systems (Figures 4 and 5), to explain the linear relationships between the variables.

Even though they have negative values, the correlations between BW on the one hand and W200 and W365 are weak and very weak, which means that this indicator is not a predictor of subsequent weight gain, which depends mainly on the management of the animals. Animals with lower birth weights tend to have lower DWG in the extensive system but at the end of the control period, in the semi-intensive system, these animals could have slightly higher W365 (r = 0.03)

Positive correlations exist between W200 and W365 of the animals, being moderate also in extensive (0.23) and in semi-intensive (0.40) which means that the weight reached by the animals at the age of 200 days (at weaning) influences the weight they will have at the age of one year. The DWG of each period is very important in achieving the performances we want, a statement supported by the high values of the correlation coefficients which in extensive system is +0.82 and +0.98 and in semi-intensive system it is perfect (r = +1) at 200 days and strong (r = +0.67) for 365 days.



s – semi-intensive

Figure 5. Pearson correlation coefficient values between BW, W200 and W365 in semi-intensive systems

Animals in the extensive system have a lower growth rate up to 200 days compared to those in the semi-intensive system, due to fluctuations in animal feed, but increase their growth up to 365 days, because the correlation coefficient between DWG365 and W365 in the extensive system is higher. The way animals are fed in the post-weaning period is very important because those in the extensive system can also have similar performances up to 365 days. The values of the DWG200 and DWG365 correlation coefficients draw attention to the fact that the weaning weight of animals is more strongly influenced and supported by the average daily weight gain than the weight reached at the age of one year, for which the correlations remain strong but are lower in value. Also, because there are weak-moderate negative correlations between

DWG200 and DWG365, it means that animals with a higher growth rate until weaning will have a lower growth gain after this period. In an extensive system the calves can recover W365.

CONCLUSIONS

The influence of the semi-intensive rearing system is reflected in the better performances achieved by the animals, especially after the age of 200 days, regardless of the sex of the animal. Thus, even if the differences are insignificant between the exploitation systems in terms of BW, W200 and DWG200 days, they become evident and very significant when we analyze the performances between calves and heifers raised in the two systems.

In semi-intensive farms, the animals are heavier and have a higher average daily gain, especially in the period 200-365 days, which confirms the fact that this system brings benefits through the management applied, especially in terms of nutrition, which translates into better growth rates of the animals.

Other conclusions that can be drawn from the analysis performed:

- although there are differences in birth weight and animal growth up to 200 days of age between the two systems, these are not significant;
- the birth weight of the animals is not a limiting factor of subsequent performances which were, however, superior in the case of males compared to females;
- raising animals in a semi-intensive system leads to obtaining superior results for the W365 of the animals, the differences being highly significant ($p < 0.0001$) compared to the extensive system;
- comparing the performances achieved separately by calves and heifers in the two systems, there are differences for W365, significant in the case of calves ($p < 0.05$) and highly significant ($p < 0.0001$) in the case of heifers, which reveals the fact that the phenotypic expression is conditioned primarily by the exploitation system;
- for calving weight, the batches are homogeneous, being moderately homogeneous in the case of the W200 and W365;
- given the correlations, the strong relationship between W365 and W200 must be taken into account;
- there are negative correlations between DWG200 and DWG365, after 200 days the tendency is for the growth rate of the animals to decrease;
- in the intensive system, W200 moderately predicts W365, the controlled feeding of the animals supporting the predictability of their growth;
- animals with a lower DWG200 can recover the weight difference for up to one year if they are provided with optimal conditions after weaning.

The recommendations refer in particular to the management of the breeding system applied on farms:

- improving animal nutrition, especially in the extensive system, to increase the productive performance of the animals and the quality of the milk consumed by the young until weaning;
- evaluating both breeding systems to identify the causes that determine the very high variability of the batches in terms of their performance, which suggests the non-uniformity of technological factors, the most important being nutrition (quality of feed, unequal access to pastures, etc.);
- management measures regarding nutrition of cows in the last part of gestation in the intensive system;
- improving environmental conditions during the housing period to ensure the welfare of the animals and maintain their health;
- W365 should be the evaluation criterion for the animals due to the compensatory growth of the animals that occurs during this period.

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ANALYSIS OF CAROTENOID CONTENT AT VARIOUS PLANT SOURCES USED TO INTENSIFY MEAT COLOR IN CHICKEN BROILER

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Abstract

The increasing demands for the `yellow chicken` have led poultry farmers to adopt different technological strategies, including the administration of carotene-rich cereals, in quantities that ensure the desired coloration. In this context, we set out to determine the carotenoid content of the main color source used in poultry farming (six maize hybrids) and several plant species (marigold, red pepper, turmeric and sea buckthorn); all samples were analyzed by spectrophotometric methods. In maize hybrids, the total carotenoid content was only 0.29-0.74 mg/g, while in the tested plants it was 0.81-8.31 mg/g. The dosage of β-carotene (responsible for the red-orange color) showed that the highest content was in turmeric (0.84-96.63% higher than the other samples), a situation also valid for lycopene (yellow-orange-red color) at which the turmeric was ahead of the other samples by 21.15-99.72%. The results obtained indicate that, unlike the classic corn dye, the tested plants can ensure a much more intense coloring of the meat of the chicken broiler.

Key words*: lycopene, plant sources, pigments, β-carotene, yellow chicken.*

INTRODUCTION

Skin and muscle pigmentation in broiler chickens is a quality parameter with a major impact on the acceptability of the product on the market, being closely correlated with consumer preferences and the commercial value of the meat. The yellow colour of the skin and adipose tissue is mainly determined by the accumulation of dietary carotenoids in tissue lipids, since birds cannot synthesize these isoprenoid compounds and depend exclusively on their dietary intake (Pérez-Vendrell et al., 2001; Surai et al., 2002; Rosati et al., 2009).

In poultry nutrition, maize (*Zea mays* L.) is the traditional source of xanthophylls (especially lutein and zeaxanthin), being used both as a major energy ingredient and as a natural pigmenting agent. However, the total carotenoid content of commercial maize hybrids shows considerable variability, influenced by genetic, pedoclimatic and technological factors (Breithaupt et al., 2007; Popa et al., 2025). Relatively low levels of carotenoids can limit the intensity of pigmentation, especially in intensive growth systems, where the length of the production cycle is short.

In this context, interest in alternative natural sources rich in carotenoids has increased significantly, both for technological reasons and due to consumer pressure to reduce the use of synthetic dyes. Plant species such as marigold (*Calendula officinalis* L.), red pepper (*Capsicum annuum* L.), turmeric (*Curcuma longa* L.) and sea buckthorn (*Hippophae rhamnoides* L.) are recognized for their high carotenoid content, including β-carotene and lycopene, as well as for the presence of other bioactive compounds with antioxidant and immunomodulatory potential (Rao & Rao, 2007; Surai et al., 2014).

β-carotene, a precursor to vitamin A, contributes to both pigmentation and support immune function and protection against oxidative stress, while lycopene is associated with high antioxidant capacity due to its multi-conjugated double bond structure (Rao & Rao, 2007; Tudorache et al., 2023).

In poultry farming, supplementing diets with natural sources rich in carotenoids has been correlated not only with increased skin coloration, but also with improved health and performance parameters (Custură et al., 2024; Moise et al., 2024; Bittencourt et al., 2025).

In view of these considerations, the objective of the present study was to compare the total carotenoid content and specific fractions (β-carotene and lycopene) in medicinal plants and

maize, by spectrophotometric determinations, to identify effective natural alternatives for the intensification of meat pigmentation in chicken broiler.

MATERIALS AND METHODS

The study aimed to evaluate the colour potential of some medicinal plants, compared to that of some corn hybrids. In this regard, four medicinal plants recognized for their high pigment content were analyzed (sea buckthorn - *Hippophae rhamnoides*; red pepper - *Capsicum annuum*; turmeric - *Curcuma longa* and marigolds - *Calendula officinalis*) (Figure 1), as well as six corn hybrids - *Zea mays* L.: (FABULO CX5039; P9415; P9398; P9944; ZPSC388 and P9590 (Figure 2).



Figure 1. Analyzed medicinal plants: A - *Capsicum annum,* B - *Hippophae rhamnoides,* C - *Curcuma Longa*, D - *Calendula officinalis* (own source)

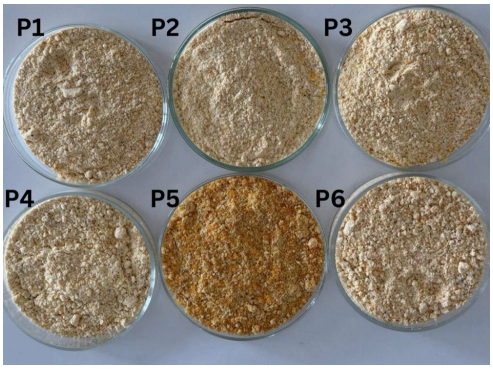


Figure 2. Corn hybrids analyzed:
P1- *P9398*, P2- *FABULO CX5039*, P3- *P9415,* P4- *P9944*, P5- *P9590*, P6- *ZPSC388* (own source)

The plant material was represented by powders obtained from parts specific to each plant. The samples (medicinal plants + maize hybrids) were dried and finely ground to the same grain to eliminate the influence of particle size on the extraction and spectrophotometric determination of carotenoids.

Solvent and ultrasonic extraction of biologically active compounds

The sample to be analyzed (1 g powder) was mixed, one at a time, with 10 mL hexane + acetone (3:1), then the mixture was homogenized at room temperature for 5-6 minutes (Figure 3).

The samples were kept in the ultrasonic bath for 45 minutes at 40 kHz and 40°C. Each sample was then centrifuged at 5000 rpm at 4°C for 10 minutes.

The resulting supernatants were analyzed from the point of view of phytochemical composition.



Figure 3. Solvent and ultrasonic extraction of compounds (own source)

Determination of the total content of carotenoids, β-carotene, lycopene

The quantitative determination of the total carotenoid content, including the compounds β-carotene and lycopene, was carried out by spectrophotometric methods, using a procedure adapted according to the literature (Butnariu, 2016). This method involves dissolving a defined amount of the extract obtained - i.e. 2 mL of a solution with a concentration of 10 mg/mL - in the solvent used in the extraction step (Figure 4), followed by measuring the spectrophotometric absorption at wavelengths specific to each compound of interest.

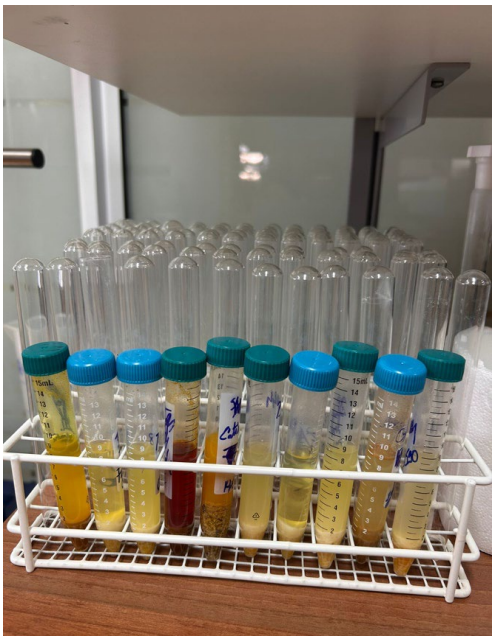


Figure 4. Quantitative determination of carotenoids, β-carotene and lycopene (own source)

More specifically, the absorption measurements were carried out at:
- 450 nm for the determination of total carotenoids;
- 470 nm for β-carotene quantification;
- 503 nm for lycopene content estimation.

With the help of the absorption values recorded at these wavelengths and based on the relationship proposed by Butnariu M. (2016) the concentration of carotenoid compounds in the analyzed samples was calculated, according to the following formula:

$$Carotenoid\ (mg/g\ s.u.) = \frac{A \times V \times DF}{m \times l \times \varepsilon}$$

where:

A = represents the absorption of the sample at the appropriate wavelength;

V = is the volume of solvent used to solubilize the concentrated extract (mL);

DF = is the dilution factor applied to the sample, where further dilution was required;

m = indicates the mass of the concentrated extract (g);

l = is the length of the optical path of the vessel used in spectrophotometry (1 cm in the case of quartz vessel);

ε = is the extinction coefficient specific to each compound analyzed:
- 2500 for total carotenoids,
- 2590 for β-carotene,
- 3450 for lycopene.

RESULTS AND DISCUSSIONS

Total carotenoid content

The comparative analysis of the total carotenes content highlights a clear demarcation between the metabolic profile of medicinal plants and maize hybrids (*Zea mays* L.). The results indicate a massive accumulation of carotenoids in the species *Hippophae rhamnoides* (sea buckthorn) and *Capsicum annum* (pepper), where concentrations of 8.312 mg/g and 7.827 mg/g, respectively, far exceed the biosynthetic potential of maize hybrids. This underlines the evolutionary specialization of medicinal plants in the storage of secondary metabolites with antioxidant role in chromoplasts. In contrast, although maize hybrids show significantly lower carotenoid values (with a maximum of 0.748 mg/g in sample P2), the significant variation observed within this group (over 150% between sample P2 and P5) suggests a different genetic potential despite the limitations imposed by the starchy matrix of the endosperm.

From the perspective of the total concentration of carotenoids, the comparison of the average of maize hybrids (0.546 mg/g) to the values recorded in medicinal plants highlights a major physiological contrast. Thus, the cereal matrix represents a massive accumulation deficit, the total carotenoid content being 93.35% lower compared to that of sea buckthorn (a reduction of 15.03 times) and 92.94% lower than that of red pepper (14.15 times lower). Even compared to turmeric, corn registers a 90.95% lower concentration of carotenoids (a decrease factor of 11.04 times).

A difference is also observed in the case of marigolds, which registers an average value of 0.81 mg/g, about 48.35% higher than the average for corn hybrids (0.546 mg/g).

The total carotenoid content determined in the six maize hybrids analysed showed significant difference, with values ranging from 0.296 to 0.748 mg/g (Figure 5).

This heterogeneity of the data can be attributed mainly to the genetic background of the hybrids, and it has been documented that the accumulation of these compounds in the endosperm is regulated by the differentiated expression of genes involved in the biosynthetic pathway (Yan et al., 2010; Gheorghe et al., 2021).

The values obtained converge with the previously reported data for conventional maize genotypes, confirming the influence of genetic and pedoclimatic factors on the metabolic profile (Chen et al., 2024). The higher levels identified in certain hybrids (P2) indicate outstanding antioxidant potential and additive nutritional value, essential in the context of biofortification and protection against oxidative stress (Rodriguez-Amaya et al., 2001; Bouis et al., 2010; Custură et al., 2019).

As for medicinal plants, there were marked differences, with concentrations ranging from 0.811 to 8.310 mg/g. The analysis highlighted Sample B (sea buckthorn) as the richest source (8.310 mg/g), while the minimum level was detected in Sample D (marigolds - 0.811 mg/g). The massive accumulation of carotenoids in sea buckthorn is consistent with the literature, which establishes this species as the primary source of b-carotene and zeaxanthin (Britton, 1995; Rodriguez-Amaya, 2010).

It should be noted that the minimum value recorded in the group of medicinal plants (0.811 mg/g) exceeds the maximum threshold determined in maize hybrids (0.748 mg/g). This value difference emphasizes the metabolic specialization of medicinal plants in the storage of secondary metabolites.

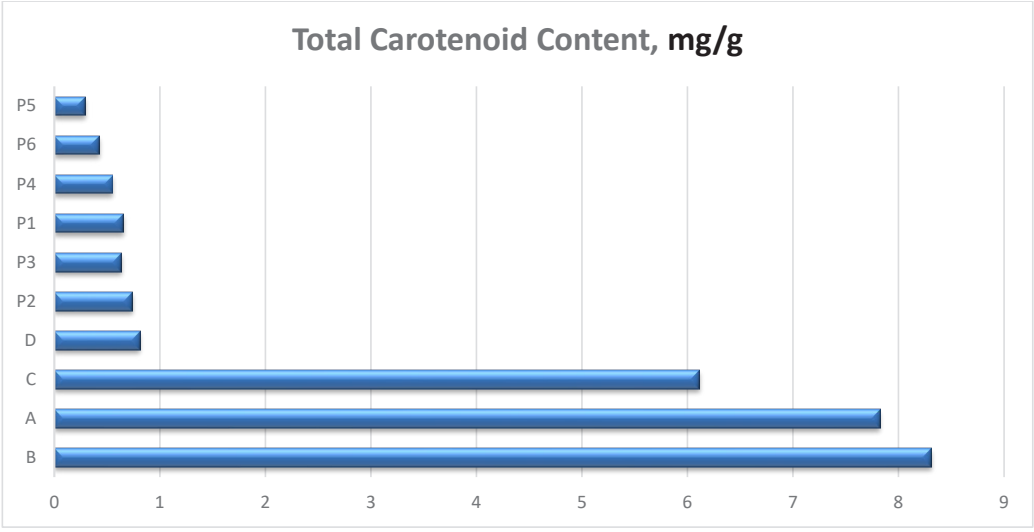


Figure 5. Variation in total carotenoid content by plant source

β-carotene content

As regards the fraction of b-carotene analysed, the disparity between the cereal matrix and medicinal species remains pronounced. The average of corn hybrids (0.410 mg/g) registers a critical deficit compared to red pepper, being 92.14% lower (a decrease factor of 12.73 times). Similar downward trends were identified in relation to sea buckthorn (-91.65%, factor 11.98) and turmeric (-90.83%, factor 10.90).

The β-carotene content determined in the six maize hybrids analyzed ranged from 0.207 to 0.598 mg/g produced, showing moderate variability between genotypes. The maximum level (0.598 mg/100 g) was recorded in sample P2, while the minimum concentration (0.207 mg/g) was observed in sample P5 (Figure 6). The values obtained fall within the ranges reported in the literature for conventional maize, where β-carotene is present in relatively small quantities compared to lutein, the predominant carotenoid in the endosperm (Ortiz-Monasterio et al., 2007; Baranowska et al., 2025). Inter-genotypic variability is associated with differences in the expression of genes involved in the carotenoid biosynthetic pathway, especially crtRB1 and lcyE, which influence the accumulation of β-carotene in the grain (Yan et al., 2010; Gupta et al., 2022)

In the case of the four medicinal plants analysed, the β-carotene content was considerably higher, ranging from 0.671 to 5.892 mg/100 g product.

The maximum level was found in red pepper (sample A) and indicates an increased capacity for carotenoid accumulation in photosynthetic tissues, a phenomenon explained by the structural and photoprotective role of these compounds in chloroplasts (Britton, 1995; Fraser & Bramley, 2004).
Compared to maize, medicinal plants showed concentrations up to about 10 times higher, which highlights major differences between the cereal matrix and green vegetative tissues in terms of biosynthesis and carotenoid storage.
From a nutritional point of view, β-carotene is the main precursor of vitamin A, and the high levels identified in plant species can contribute significantly to dietary intake of provitamin A (Stahl & Sies, 2003; Lungu & Moise, 2025; Kurilich, 1999). The differences observed between the two categories of samples underline the decisive influence of the type of plant matrix on the total and specific carotenoid content.

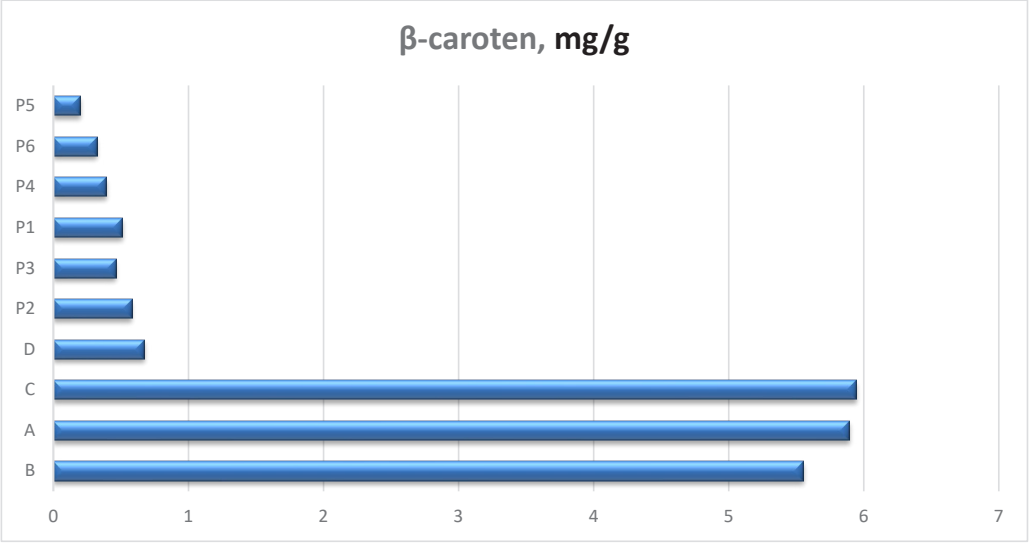


Figure 6. Variation in β-carotene content by plant source

Lycopene content
The assessment of lycopene content reflects the most pronounced discrepancy between medicinal plants and maize hybrids, highlighting the limited storage capacity of this carotenoid in the endosperm of *Zea mays* L. compared to the tissues of medicinal plants. The average of corn hybrids (0.066 mg/g) represents a drastic decrease compared to red pepper (4.681 mg/g), being 98.53% lower, which corresponds to a reduction factor of 67.84 times. A similar segregation trend is observed in relation to sea buckthorn (3.687 mg/g), where the average concentration in cereals is 53.49 times lower.
Although in the case of total carotenoid content the differences were more balanced, in terms of lycopene, even medicinal plants have moderate accumulation, such as turmeric and marigolds. Corn hybrids register deficiencies of 86.17% compared to turmeric and 85.53% compared to marigolds, being 7.23 and 6.91 times poorer in this bioactive compound, respectively.
The lycopene content determined in the six maize hybrids analyzed ranged from 0.014 to 0.125 mg/g produced, highlighting small but detectable quantitative differences between genotypes. The maximum value (0.125 mg/g) was recorded in sample P2, while the minimum concentration (0.014 mg/g) was observed in sample P5 (Figure 7). The value range obtained confirms that lycopene is a minor carotenoid in the cereal matrix of maize, compared to lycopene and β-carotene, which are the predominant compounds in the endosperm (Ortiz-Monasterio et al., 2007; Boileau, 2003).
Inter-genotypic variability may be correlated with differences in gene expression involved in the biosynthetic pathway of carotenoids,

especially the lycopene enzymes β-cyclase and ε-cyclase, which cause the conversion of lycopene to xanthophylls and cyclic carotenoids (Fraser & Bramley, 2004). In corn, the reduced accumulation of lycopene is explained by its rapid conversion into derived compounds, which limits its free concentration in grains (Yan et al., 2010).

The concentrations determined in the present study are within the limits previously reported for conventional maize, where lycopene is described as present in low or even undetectable amounts in certain genotypes (Rodriguez-Amaya et al., 2001; Cvetkovic et al., 2013). Although the levels identified are low, the contribution of lycopene to the total antioxidant capacity of grains cannot be neglected, given its high efficiency in neutralizing singlet oxygen and free radicals (Stahl & Sies, 2003; Hidayat et al., 2023; Buckler, 2008).

The lycopene content determined in the four analyzed medicinal plants showed considerable variations, the values being between 0.476 and 4.681 mg/g product. The highest concentration (4.681 mg/g) was recorded in sample A, while the minimum level (0.476 mg/g) was determined in sample D. The quantitative differences observed can be attributed both to the biochemical peculiarities specific to the raw materials and to the technological processing (drying, grinding, exposure to light), factors known to influence the stability of carotenoids.

According to the literature, lycopene is present in high concentrations in tomato and red pepper products, being one of the main compounds responsible for the intense red colour (Shi et al., 2000). Differences between plant species may reflect differences in the initial content of the raw material, but also the oxidative degradation of lycopene during processing and storage (Giovannucci et al., 1999; Rodriguez-Amaya et al., 2001; Martinez-Valvere et al., 2002).

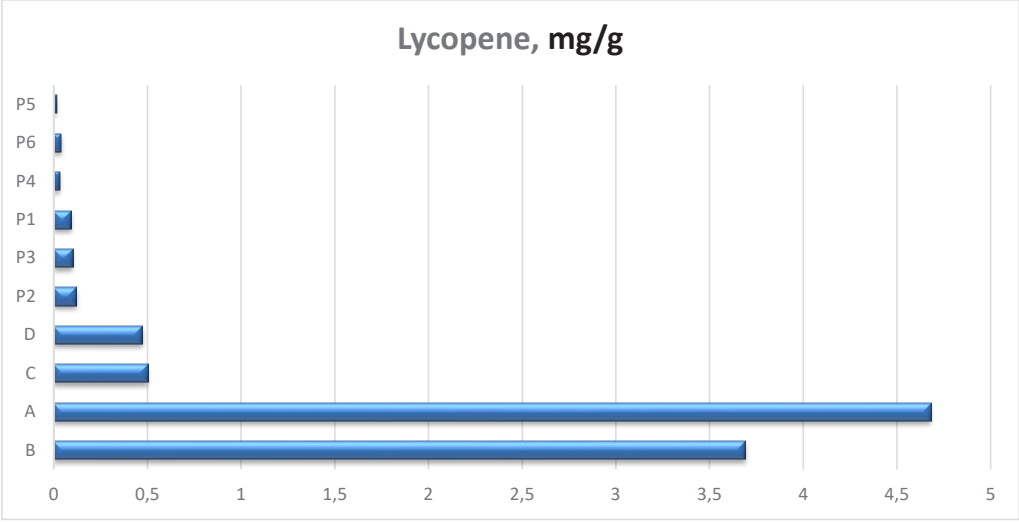


Figure 7. Variation in lycopene content by plant source

CONCLUSIONS

The results of the study highlight major differences in the pigmentation capacity of the analyzed raw materials, with direct implications on the correct formulation of feed rations for the chicken broiler

From the perspective of the hierarchy of pigmentation sources, medicinal plants, especially red pepper and sea buckthorn emerge as high-density feed additives.

The massive lycopene deficiency of maize compared to red pepper (67.84 times lower) suggests that the exclusive use of maize in the diet of the chicken broiler may be insufficient to achieve intense pigmentation, requiring supplementation with concentrated sources.

Red pepper and sea buckthorn are recommended for intensifying orange/red shades due to the extreme density of total carotenoids.

Lycopene, identified in higher concentrations in medicinal plants than corn (deficit of over 85% even for marigolds), is essential for the yellow base of pigmentation, highlighting the importance of these plants as chromatic "boosters".

Among the corn hybrids analyzed, the P2 sample represents the highest coloristic potential, having a β-carotene and lycopene content clearly higher than the other hybrids (P5 showed a pigment content 9.6 times lower than P2). The use of the P2 hybrid in feed recipes can reduce the dependence on synthetic pigments, providing a natural alternative for achieving a high colour score.

The conclusion of the study is that the inclusion of sea buckthorn powder or red pepper in compound feeds for the chicken broiler can represent an effective and sustainable strategy in obtaining high quality carcasses, but also to improve the health of the birds.

Although maize remains the basic energy source in poultry farming, the genetic variability identified between hybrids requires a rigorous selection of them based on pigment content criteria.

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RESEARCH REGARDING THE EFFECTS OF TECHNOLOGICAL MODERNIZATION AND PRECISION ENVIRONMENTAL CONTROL ON BROILER PRODUCTION PERFORMANCE IN A COMMERCIAL FARM

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Abstract

Technological modernization of broiler production systems has become an important strategy for improving productivity, animal welfare, and economic sustainability in commercial poultry farming. The present study evaluated the effects of structural and technological upgrades on broiler production performance by comparing conventional and modernized production systems in a commercial farm in Romania. Four matched production cycles were analyzed: summer and winter cycles before modernization (2023) and after modernization (2024). Each cycle involved approximately 160,000 broilers raised under intensive conditions. The modernization process included thermal insulation of poultry houses, installation of automated ventilation, heating and evaporative cooling systems, sensor-based environmental monitoring, and automated feeding and watering equipment integrated into a centralized management platform. Productive, environmental, and economic indicators were compared between systems. The modernized system significantly improved productive performance. Final body weight increased from 2.453 to 2.548 kg/bird ($p < 0.01$), while average daily gain increased from 58.4 to 60.7 g/bird/day ($p < 0.01$). Daily feed intake decreased from 99 to 97 g/bird/day, resulting in improved feed efficiency. Feed conversion ratio was reduced from 1.71 to 1.60 kg feed/kg gain ($p < 0.001$), and mortality declined from 4.5% to 3.2% ($p < 0.01$). Seasonal analysis showed that modernization reduced performance variability between summer and winter production cycles through improved environmental control. Economic evaluation demonstrated substantial increases in profitability, with net profit per cycle improving by 40.0% during summer and by 116.6% during winter compared with the conventional system. These findings demonstrate that technological modernization and precision environmental control can substantially enhance broiler production efficiency, improve bird welfare, and increase the economic sustainability of commercial poultry operations.

Key words*: broiler chickens, poultry housing modernization, precision livestock farming, production efficiency.*

INTRODUCTION

The global poultry sector has experienced rapid expansion over the past decades, driven by increasing demand for affordable, high-quality animal protein. Broiler meat, in particular, has become one of the most consumed animal products worldwide due to its relatively low production costs, high nutritional value, and reduced environmental footprint compared to other livestock systems (Han et al., 2013; Food and Agriculture Organization, 2020). This growth has intensified the need for more efficient, sustainable, and welfare-oriented production systems. Traditional broiler production systems are often characterized by limited automation and reliance on manual monitoring of environmental parameters such as temperature, humidity, and air quality. These conditions frequently lead to fluctuations in the microclimate, delayed corrective actions, and reduced consistency in production outcomes. As highlighted by Dawkins (2016), inadequate environmental control can significantly affect bird welfare, leading to increased stress, impaired growth performance, and higher mortality rates (Bessei, 2006). The concept of precision livestock farming has gained increasing attention as a means to optimize animal production systems through the integration of advanced technologies (Fontana et al., 2015). According to Banhazi et al.

(2012), modern broiler farms increasingly utilize automated control systems, environmental sensors, and data-driven decision-making tools to improve productivity and sustainability. These technologies enable continuous monitoring and real-time adjustment of critical parameters, thereby reducing variability and improving overall farm efficiency. Environmental control plays a crucial role in broiler production, as parameters such as temperature, humidity, ventilation rate, and air quality directly influence bird health and performance. High concentrations of ammonia (NH_3) and carbon dioxide (CO_2) have been associated with respiratory issues, reduced feed intake, and decreased growth rates (European Commission, 2007). Consequently, modern production systems incorporate ventilation and air quality monitoring technologies to maintain optimal environmental conditions (Blanes-Vidal et al., 2008). In addition to environmental improvements, technological modernization often includes structural upgrades, enhanced insulation, and the implementation of automated feeding and watering systems. These interventions contribute to improved feed efficiency, reduced energy consumption, and better overall economic performance. Studies have shown that optimizing feed utilization and environmental conditions can significantly improve production efficiency and reduce operational costs (Organisation for Economic Cooperation and Development, 2021). Despite the recognized benefits of modern technologies, many poultry farms still operate using outdated systems, limiting their production potential and economic competitiveness. Therefore, evaluating the impact of modernization at the farm level is essential for understanding its practical benefits and guiding future investments.

The objective of the present study is to assess the effects of technological modernization on broiler production performance by comparing key production indicators before and after the implementation of improved systems.

The study focuses on growth performance, feed efficiency, mortality rate, production capacity, and environmental control, providing a comprehensive evaluation of the benefits associated with transitioning from conventional to modernized broiler production systems.

MATERIALS AND METHODS

The study was conducted in a commercial broiler farm located in Romania, operating under intensive production conditions. The production system was based on raising broiler chickens on litter flooring, in environmentally controlled poultry houses, following standard commercial practices.

The objective of the study was to evaluate the impact of technological modernization on broiler production performance and economic efficiency by comparing key zootechnical and financial indicators recorded before and after the implementation of improved systems, across matched seasonal production cycles.

Animals and experimental design

The study was conducted at the commercial broiler farm Avicola Smirna SRL, located in Ialomița County, Romania, operating under intensive production conditions throughout the year. The production system was based on raising fast-growing broiler hybrids (Ross 308 and Cobb 500) sourced from a certified hatchery in the Czech Republic (MACH, Cehia), on litter flooring in environmentally controlled poultry houses. A total of four parallel production cycles were compared: two summer cycles (June-July 2023 in the conventional system vs. June-July 2024 in the modernized system) and two winter cycles (November-December 2023 in the conventional system vs. November-December 2024 in the modernized system). Each cycle began with 160,000 chicks, distributed across four production houses of approximately 2,000 m² each, at a stocking density of approximately 20 birds/m² during placement, respecting the provisions of Council Directive 2007/43/EC. Each production house accommodated 40,000 birds.

Description of the conventional system (before modernization)

Prior to modernization, the farm operated four poultry houses with a usable floor area of approximately 1,850 m² per building. The roofing consisted of conventional materials

without adequate thermal insulation, resulting in significant heat losses during winter and insufficient thermal protection during summer. Environmental parameters, temperature, humidity, and ventilation rate, were monitored periodically by farm staff, without automated, continuous data acquisition. Ventilation was achieved through manually adjusted natural and mechanical systems, without variable-speed regulation. Heating was provided by gas-powered heaters, but without real-time feedback from distributed sensors. The feeding system was based on manually supplied chain feeders, without automated dosing or overflow detection. The conventional system operated at a capacity of approximately 100,000 birds per cycle, completing five production cycles per year.

Description of the modernized system (after implementation)

The modernization project, co-financed through the European Union under the programme "Extension and Modernization of Avicola Smirna Farm", involved both structural and technological upgrades. The poultry houses were reconfigured and expanded to approximately 2,000 m² usable area per building. The roofs were thermally insulated using sandwich panels and polyurethane foam to reduce heat losses during winter and limit heat gain during summer, in line with recommendations for energy-efficient broiler housing (Oviedo-Rondon, 2007; Biswal et al., 2022). Additional structural modifications included the removal of internal partition walls, the creation of openings for ventilation and cooling equipment, and the construction of auxiliary spaces dedicated to climate control systems.

Each modernized house was equipped with a centralized, sensor-based climate management system. The ventilation system comprised 8 variable-speed ridge fans (12,000 m³/h each) and 12 exhaust fans mounted on reinforced concrete gable ends (40,000 m³/h each), providing a total ventilation capacity of approximately 500,000 m³/h per house. Air inlets with adjustable openings allowed precise control of airflow velocity and direction. During the first 10-14 days of life, only the ridge fans operated, maintaining low air velocities to prevent cold stress in chicks. Evaporative cooling was achieved via 70 polypropylene honeycomb cooling pad panels installed on dedicated extensions of each building. Heating was provided by 6 gas/LPG heaters per house (hot air throw: 40 m) with automatic regulation. Microclimate monitoring was performed continuously via 8 temperature sensors and 4 humidity sensors per house, together with CO_2 and NH_3 concentration sensors. The multi-point temperature measurements were averaged by the management computer to ensure uniform microclimate across the production area, a method shown to reduce spatial variability in broiler performance (Deng et al., 2024). The system enabled remote access via smartphone and PC software.

The automated feeding system comprised four external feed silos (30 m³/19.5 tons capacity) connected to 152 internal distribution sections, each serving 4 feeders (608 feeders per house), coordinated by a 175-litre hopper with overflow sensors. Water was supplied through nipple drinking systems (155 sections × 18 nipple drinkers per section) connected to a metered supply network maintaining a pressure of 0.3-6 bar. Additional investments included a front loader for manure management and bird transport, a backup generator, a concrete manure storage platform, and a deep-water well.

Production management and data collection

Production cycles lasted approximately 42 days, followed by a sanitary break for cleaning, disinfection, and facility preparation. Body weight was monitored weekly by weighing a representative sample of approximately 1% of the flock population per house (400 birds), with measurements taken at days 8, 15, 22, 29, 36, and at slaughter. Feed consumption was recorded daily via the automated feeding system. Mortality was registered daily by house staff and verified against slaughter records.

The primary zootechnical parameters analyzed were: average live weight at delivery (kg/bird), daily weight gain (g/bird/day), daily feed intake (g/bird/day), total feed consumption per cycle (kg/bird), feed conversion ratio (FCR, kg feed/kg gain), and mortality rate (%). Economic performance was evaluated using financial records extracted from the farm's management system, comprising total direct costs (lei/series), revenue from sales

(lei/series), net financial result per production cycle (lei), and cost of production per kilogram of live weight (lei/kg). The four analyzed series were: Series June-July 2023 (conventional, summer); Series November-December 2023 (conventional, winter); Series June-July 2024 (modernized, summer); and Series November-December 2024 (modernized, winter).

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Differences between the conventional and modernized systems were assessed using an independent samples t-test (for pairwise comparisons) and a two-way analysis of variance (ANOVA) with system (conventional vs. modern) and season (summer vs. winter) as fixed factors, to evaluate main effects and their interaction. Differences between groups were considered statistically significant at $p < 0.05$ (*), highly significant at $p < 0.01$ (**), and extremely significant at $p < 0.001$ (***). The level of statistical significance was set at $\alpha = 0.05$ for all analyses performed using IBM SPSS Statistics, version 25 (IBM Corp., Armonk, NY, USA).

RESULTS AND DISCUSSIONS

The implementation of modernization measures in the studied broiler farm led to significant improvements in production performance, environmental control, and overall operational efficiency. The comparative analysis between the conventional system and the modernized system highlights the impact of structural and technological upgrades on key production indicators.

Automated equipment and technologies implemented

Each modernized house was equipped with precision technologies managed through a centralized system:

- Storage and feeding system: Four feed silos with an auger system were installed (capacity of 30 cubic meters/19.5 tons, mechanical/pneumatic loading). The internal feeding system includes 152 sections with a transport pipe and 608 feeders per house, coordinated by a 175-liter hopper and overflow sensors;
- Watering system: Built using drip lines, totaling 155 watering sections with 18 nipple drinkers each per house. Water pressure is controlled (0.3-6 bar) and metered;
- Microclimate control (Ventilation, Cooling, Heating): Six variable-speed ridge fans were mounted on the roof, along with 12 wall fans on a rebuilt reinforced concrete gable end. For cooling, 70 polypropylene honeycomb panels (cooling pads) were implemented. Heating is supported by 6 gas/LPG heaters per house, with a hot air throw of 40 meters;
- Automated control and lighting: A management computer was installed in the main electrical panel, connected to a management network with smartphone access and a PC software license. The system collects data from 4 indoor temperature sensors, one outdoor sensor, humidity sensors, and pressure sensors. Lighting is provided by dimmable 36 W fluorescent tubes.

Recorded productive performances

One of the most notable outcomes of the modernization process was the increase in production capacity (Table 1).

Table 1. Production capacity before and after modernization

Parameter	Conventional system	Modern system	p-value
Final weight (kg/bird)	2.453±0.05	2.548±0.04**	<0.01
Daily weight gain (g/day/head)	58.4±1.23	60.7±1.17**	<0.01
Daily feed intake (g/bird/day)	99±3.16	97±2.76**	<0.01
Total feed consumption (kg/bird/cycle)	4.17±0.11	4.08±0.10**	<0.01
FCR (kg feed/kg gain)	1.71±0.03	1.60±0.02***	<0.001

p<0.01 highly significant differences; p<0.001 extremely significant differences.

The number of broilers per cycle increased from 100,000 to 160,000 birds, representing a 60% increase in stocking capacity. In addition, the number of production cycles per year increased from 5 to approximately 6–6.5 cycles, resulting in a substantial rise in annual output.

The modernization of the broiler production system resulted in significant improvements in growth performance, feed efficiency, and mortality rates. A comparative statistical analysis was performed between the conventional and modern systems, as well as between summer and winter production cycles.

The increase in final body weight and daily gain was statistically significant ($p < 0.01$), indicating improved growth conditions in the modernized system. The feed conversion ratio also showed a highly significant improvement ($p < 0.001$), reflecting better feed utilization.

The increase in average body weight can be attributed to improved microclimatic stability, particularly temperature and ventilation control. The presence of multiple sensors allowed more precise regulation of environmental conditions, reducing stress and promoting uniform growth.

Although the daily feed intake slightly decreased, the overall efficiency improved due to better environmental conditions and optimized feeding systems. This confirms that modernization leads to qualitative, not just quantitative improvements in production.

A reduction of 0.11 kg feed/kg gain in FCR represents a significant economic advantage, considering that feed costs account for the majority of production expenses. This improvement reflects better feed utilization due to optimized environmental conditions and improved feeding systems.

The mortality rate decreased significantly after modernization, indicating improved animal welfare and health status (Table 2).

Table 2. Mortality indicators

Parameter	Conventional system	Modern system	Reduction
Mortality (%)	4.5±0.41	3.2±0.35	-1.3%
Mortality (birds/cycle)	7200	5100	-2100

Statistical analysis showed a significant reduction in mortality ($p < 0.01$).

The reduction in mortality is closely linked to improved air quality, better ventilation, and more stable environmental conditions.

Lower ammonia and carbon dioxide levels contributed to better respiratory health and reduced disease incidence.

Environmental control and microclimate

The modernization of ventilation, heating, and cooling systems had a direct impact on environmental stability within the poultry houses.

The installation of cooling pads ensured optimal temperatures during summer, while thermal insulation significantly reduced heat loss during winter.

Humidity control improved due to the addition of dedicated sensors, leading to reduced litter moisture and lower ammonia emissions.

The monitoring of NH_3 and CO_2 concentrations allowed the maintenance of air quality within recommended limits, contributing to improved bird welfare and performance.

The use of multiple temperature sensors per poultry house enabled the calculation of average values, reducing temperature gradients and ensuring uniform conditions throughout the production space.

Environmental control systems had a strong impact on seasonal performance differences (Table 3).

Table 3. Seasonal comparison of performance indicators

Parameter	Summer (old system)	Summer (modern system)	Winter (old system)	Winter (modern system)
Final weight (kg/bird)	2.430±0.05	2.540±0.03	2.419±0.06	2.484±0.04
FCR (kg feed/kg gain)	1.72±0.01	1.61±0.04	1.70±0.03	1.60±0.06
Mortality (%)	5.2	3.5	4.3	3.0

Table 4. Economic performance by production series and system type

Parameter	Jun-Jul 2023 (conventional, summer)	Nov-Dec 2023 (conventional, winter)	Jun-Jul 2024 (modern, summer)	Nov-Dec 2024 (modern, winter)
Total direct costs (lei/series)	1,906,000	2,112,000	1,869,000	2,024,458
Feed costs (lei/series)	1,220,000	1,338,000	1,220,000	1,300,458
Feed cost as % of total costs	64.0%	63.4%	65.3%	64.2%
Average feed cost (lei/kg)	1.9365	2.1306	1.9837	2.0975
Total revenue (lei/series)	2,311,000	2,302,000	2,436,000	2,436,000
Net profit (lei/series)	**405,000**	**190,000**	**567,000**	**411,542**
Profit improvement vs. conventional (%)	—	—	+40.0% vs. summer 2023	+116.6% vs. winter 2023

Summer improvements were mainly due to cooling pad systems; improved ventilation; reduced heat stress.
Winter improvements were driven by thermal insulation; better humidity control; reduced energy losses.
Statistical comparison between seasons showed a significant interaction between system and season ($p < 0.05$) and reduced variability in the modern system.
This indicates that modernization not only improves average performance but also stabilizes production across seasons.

Economic performance analysis

The financial records extracted from the farm management system reveal that modernization generated substantial improvements in economic performance across all monitored production cycles (Table 4). Revenue per series remained constant (2,436,000 lei/series), as the number of birds delivered to slaughter and the selling price were stable across both systems. The economic gains were therefore driven exclusively by cost reductions achieved through improved feed efficiency, reduced mortality, and lower heating expenditures.
The most pronounced economic benefit was recorded during the winter cycles, where the net profit per series increased from 190,000 lei (conventional) to 411,542 lei (modernized), representing a gain of 116.6%. This disproportionate improvement during winter reflects the impact of thermal insulation on heating costs, which decreased despite the higher number of birds per series. In summer, profit increased by 40.0% (from 405,000 to 567,000 lei), driven mainly by FCR improvement and reduced mortality under optimized cooling conditions. Feed costs consistently represented the largest expense category (63-65% of total direct costs), confirming that FCR improvement has the highest leverage on the overall cost structure, as previously documented in commercial broiler production systems (Quintana-Ospina et al., 2023).
The results obtained in this study clearly demonstrate that technological modernization has a measurable and statistically significant positive impact on broiler production systems. Improvements in production capacity, growth performance, feed efficiency, and mortality rates confirm the effectiveness of integrating precision livestock farming technologies into commercial poultry operations.
The improvement in final body weight from 2.453 to 2.548 kg/bird (+3.9%; $p < 0.01$) is consistent with findings reported by Quintana-Ospina et al. (2023), who analysed over 2.7 million Ross 308 AP broiler flocks and found that tighter environmental control during weeks two through four of production was the primary driver of improved body weight at slaughter. Similarly, the study by Guerrero-Ginel et al. (2023), which analysed commercial flocks of Ross 95 and Cobb 500, confirmed that climate-controlled housing was among the strongest predictors of final body weight and production efficiency index, with a mean FCR of 1.65-1.72 reported in conventional houses. The FCR of 1.60 achieved in the present study after modernization therefore falls within the upper range of efficiency documented for well-managed commercial operations with automated environmental control, and is comparable to the target FCR of 1.6 considered achievable under tightly controlled conditions for these hybrids.
The reduction in FCR from 1.70 to 1.60 ($p < 0.001$) is of particular economic significance, as feed costs represented 63-65% of total direct production costs in this study. A reduction of 0.10 kg feed/kg gain in FCR at this scale (160,000 birds/cycle; ~395,000 kg live weight delivered) corresponds to a saving of approximately 39,500 kg of feed per cycle, or approximately 78,000-83,000 lei per cycle at current feed prices. This is consistent with industry modelling indicating that improving FCR from 1.8 to 1.6 can reduce feed costs by up to 12% (OECD, 2021). Brassó et al. (2025), in a comprehensive review of precision livestock farming technologies for broilers published in the journal Animals, confirmed that sensor-based environmental management systems are the single most impactful technology for improving FCR and reducing mortality in commercial broiler production.
The observed reduction in total cycle mortality from 4.5% to 3.2% ($p < 0.01$) places the modernized system within the range of 3.0-3.5% reported by Forseth et al. (2024) for commercial Ross 308 flocks under

contemporary management in Europe, and aligns with the benchmark of less than 5% considered indicative of effective disease control and an adequate cage environment (Kebede et al., 2020, as cited in Rahman et al., 2022). The decrease in mortality is attributable primarily to improved ammonia and CO_2 control: the sensor network maintained NH_3 concentrations below 20 ppm and CO_2 below 3,000 ppm throughout production cycles, in compliance with the minimum welfare thresholds established by European Commission Directive 2007/43/EC. These values are consistent with the NH_3 and CO_2 thresholds identified by the European Commission (2007) as critical for preventing respiratory disease and reducing mortality in commercial broiler flocks.

The stronger economic benefit observed in winter cycles (+116.6% profit improvement) compared to summer (+40.0%) highlights the critical role of thermal insulation in cold-season production. Deng et al. (2024), in a study on temperature distribution in commercial broiler houses, demonstrated that non-uniform temperature gradients driven by inadequate insulation significantly increase mortality and reduce performance uniformity during winter. The sandwich panel roof insulation installed in the present study directly addressed this vulnerability, reducing heating costs despite a 60% increase in bird numbers per cycle. Similar findings were reported by Ferreira et al. (2024) in a study on the thermal environment dynamics of climate-controlled broiler houses, who confirmed that well-insulated, sensor-managed houses maintained significantly smaller temperature differentials across the production area compared to conventionally built houses, directly translating into more uniform growth and reduced within-flock variability.

The integration of automated systems also reduced dependence on manual labour for environmental interventions. The shift from reactive to proactive management - enabled by real-time sensor monitoring and remote access via smartphone - represents a fundamental change in farm management philosophy, in alignment with the Precision Livestock Farming (PLF) framework described by Banhazi et al. (2012) and extended more recently by Brassó et al. (2025) and Morrone et al. (2022). The sensor data collected continuously in the present study provide a foundation for future implementation of machine learning algorithms to predict flock performance, as demonstrated by the IoT-XGBoost approach described by Abdou et al. (2025) in a study published in Computers and Electronics in Agriculture. Such predictive management systems represent the logical next step for farms at the modernization level described in this study.

CONCLUSIONS

The technological modernization of broiler production systems resulted in significant improvements across all evaluated performance indicators. The transition from conventional to AI-assisted, sensor-equipped management systems led to a statistically significant increase in final body weight (from 2.453 to 2.548 kg/bird; $p < 0.01$), a substantial improvement in feed conversion ratio (from 1.70 to 1.60 kg feed/kg gain; $p < 0.001$), and a reduction in mortality rate (from 4.5% to 3.2%; $p < 0.01$). Production capacity increased by 60%, from 100,000 to 160,000 birds per cycle, while the number of annual cycles increased from 5 to approximately 6-6.5.

Environmental control proved to be the primary driver of performance improvement, with automated ventilation, heating, and cooling systems ensuring microclimate stability throughout all seasons. Seasonal analysis confirmed that the modern system significantly reduced both heat stress in summer and cold stress in winter, resulting in more consistent production outcomes year-round ($p < 0.05$).

These findings confirm that investment in precision livestock farming technologies represents a viable and economically justified strategy for enhancing broiler production efficiency, improving animal welfare, and supporting the long-term sustainability of commercial poultry operations. Future research should focus on quantifying the return on investment of specific technological components and evaluating their applicability across diverse production scales and climatic conditions.

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EVALUATION OF QUANTITATIVE AND QUALITATIVE TRAITS OF MEAT PRODUCTION IN ROSS 308 BROILER CHICKENS

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Abstract

Poultry meat occupies an essential place in human nutrition due to its superior quality attributes. Compared to meat from other species, poultry meat offers major advantages: low in calories, rich in high-quality protein, characterized by a fine structure that facilitates mastication and digestion. Market demand shows a clear consumer preference for breast meat. The present study aimed to characterize this anatomical region in the Ross 308 hybrid. The analyses focused on several quantitative and qualitative parameters of the pectoral muscles (Pectoralis superficialis and Pectoralis profundus), comparing two slaughter ages: 35 and 42 days. Birds were reared under identical housing and feeding conditions, with a standardized diet. Differences in slaughter age resulted in significant variations in live weight (1985.11 ± 45.09 g at 35 days vs. 2788.63 ± 54.26 g at 42 days), which were reflected in breast weight with bone and skin, as well as in its proportion within the carcass. The study highlights notable differences between the two age groups and concludes that, from a quantitative standpoint, slaughtering Ross 308 broilers at 42 days is more economically efficient.

Key words*: food quality, meat quality, meat production, poultry, Ross 308.*

INTRODUCTION

The poultry sector represents an emblematic branch of animal husbandry, important due to the possibility of exploiting numerous domestic species (chickens, ducks, geese, turkeys, guinea fowl) and semi-domestic species (pheasants, partridges, ostriches), in accordance with each breeder's capacities (Usturoi et al., 2025).

Meat, the main product generated by poultry farming, holds an important place in human nutrition due to its outstanding qualities. Compared to other species, poultry has the advantage of providing always fresh meat, supported by the short time required to raise hybrids and the opportunities for advanced technological development of farms (Davidescu et al., 2025).

From a culinary point of view, it is a product that is quick and easy to prepare and possesses numerous remarkable organoleptic and nutritional properties (low calorie content, high protein content, and a fine structure that makes it easy to chew and digest). Its dietary qualities recommend it for all age groups, from children to the elderly and convalescents. In general, poultry meat is characterized by a low cholesterol content, contains all essential amino acids necessary for human nutrition, and, through its organs, provides a rich source of minerals and vitamins.

Assessing global consumption, poultry meat holds a very important position among foods of animal origin, mainly due to its nutritional qualities as well as the relatively low costs of production and processing (Tudorache et al., 2023).

Although the concept of poultry meat, in a broad sense, includes the entire skeletal musculature along with connective tissues, consumers' desire to include healthier products in their diets has made them increasingly selective, including with regard to the nutritional properties of each muscle consumed. For this reason, in the current context, an important role is played by "white meat," characteristic of the pectoral muscles. Based on this premise, the present paper aims to provide a quantitative and qualitative

characterization of the pectoral musculature from one of the most widely used commercial hybrids for meat production, namely Ross-308.

MATERIALS AND METHODS

The biological material studied consisted of the commercial hybrid Ross-308. A total of 80 specimens were selected randomly from the rearing hall after stocking and were divided into two equal groups, which were raised until 35 and 42 days of age, respectively. The birds were raised under industrial conditions, in a windowless house, on the floor with permanent litter. Throughout the research, efforts were made to comply with the microclimate conditions specified in the rearing guide, and the feeding regimen was standardized, as recommended by the genetic material producer. Slaughtering was carried out in a specialized facility, and the slaughter results were processed; laboratory analyses were performed in accordance with current standards.

Slaughter parameters - live weight, hot carcass weight, and cold carcass weight were recorded, followed by calculations to determine hot and cold dressing percentages.

Proportion of anatomical portions - the carcasses were cut into anatomical parts, and determinations focused on establishing both the weight and the proportion within the carcass for three components: breast with bone and skin, breast without bone and skin, and thighs.

Dimensional aspects of pectoral muscles - the pectoral musculature was measured by determining lengths, widths, and thicknesses of the regions, after which average values were calculated.

Chemical composition - aimed at the qualitative characterization of the pectoral muscles. Accordingly, analyses targeted the characteristics of the deep pectoral muscle and the superficial pectoral muscle.

Water content - determined by the oven-drying method to constant weight (SR ISO 936:2009; SR ISO 1442:2010).

Dry matter - determined by drying the samples in an oven at controlled temperature until constant weight was reached. This determination allows the evaluation of the total solid content of the analyzed tissue.

Protein content - determined using the Kjeldahl method (SR EN ISO 937:2007), which involves measuring the total nitrogen in the sample and converting it into protein content using a specific conversion factor.

Lipid content - determined by extraction with organic solvents using the Soxhlet method (SR ISO 1443:2008). This method enables the quantification of the total lipid fraction in the sample.

Mineral content - determined by incinerating the samples in a furnace at high temperatures (approximately 550°C) until ash is obtained, a method commonly used to estimate total mineral content.

Nitrogen-free extract substances - calculated indirectly by difference, by subtracting the protein, lipid, and mineral content from the dry matter. These represent mainly carbohydrates and other non-protein soluble components.

Data interpretation - the obtained results were statistically processed by calculating mean values and variability indicators. Comparative analysis between groups allowed the identification of significant differences and the formulation of conclusions regarding the influence of the studied factors.

RESULTS AND DISCUSSIONS

Slaughter Yield

The live weight of the birds recorded average values of 1985.1±75.1 g at 35 days of age, with variations ranging between 1910.2 g and 2060.1 g. The coefficient of variation was 16.7, indicating a medium homogeneity of the studied trait. At 42 days of age, the average body weight was 2788.6±84.3 g, with minimum values of 2701.4 g and maximum values of 2869.2 g. The calculated coefficient of variation (V = 13.4%) also indicated medium homogeneity (Table 1).

Hot carcass weight at 35 days averaged 1586.3±66.3 g, while at 42 days it reached 2267.8±76.2 g. The recorded values ranged from 1519.3 g (35 days) to 2190.6 g (42 days) for minimums, and from 1643.3 g (35 days) to 2344.2 g (42 days) for maximums. The trait showed medium homogeneity (V% = 15.2 and 11.6) (Table 1).

Cold carcass weight averaged 1555.2±54.1 g at 35 days, with values between 1502.1 g and 1609.4 g. In the second control period (42 days), the average value was 2233.1±46.2 g, with a minimum of 2186.3 g and a maximum of 2279.5 g (Table 1).

The hot dressing percentage averaged 79.91±1.9% at 35 days and increased to 81.3±0.8% at 42 days.

The cold dressing percentage recorded an average value of 78.34±1.3% for birds raised to 35 days, while for those slaughtered at 42 days it reached 80.1±1.1% (Table 1).

Weight and Proportion of Anatomical Regions

As the study focused on the pectoral musculature, measurements targeted this region, considering two commercially important cuts: breast with bone and skin, and boneless, skinless breast. At the same time, thigh weight and proportion were determined, as these represent the second most preferred portion and an important component of the carcass.

The average weight of breast with bone and skin at 35 days was 459.1±21.3 g, while at 42 days significantly higher values were obtained (641.1±20.1 g). The coefficients of variation indicated slightly higher homogeneity at 42 days (V % = 12.5 vs. 14.6) (Table 2).

These values corresponded to carcass proportions of 29.5±1.1% at 35 days and 29.7±0.9% at 42 days.

Boneless, skinless breast weight averaged 364.1±18.5 g at 35 days and increased to 538.2±18.1 g at 42 days (V% < 10 in both cases, indicating good homogeneity). The proportion was 23.4±0.9% at 35 days and 24.1±0.8% at 42 days.

Thigh weight averaged 384.1±16.2 g at 35 days, representing 24.7±0.5% of the carcass. At 42 days, the average weight increased to 562.8±15.8 g, with a proportion of 25.2±0.6%

Table 1. Live weight, carcass weight and slaughter yields recorded by the Ross-308 hybrid

Specification	Both sexes							
	35 days				42 days			
	$\bar{X} \pm s_{\bar{x}}$	Min.	Max.	V%	$\bar{X} \pm s_{\bar{x}}$	Min.	Max.	V%
Live weight (g)	1985.1±75.1	1910.2	2060.1	16.7	2788.6±84.3	2701.4	2869.2	13.4
Hot carcass weight (g)	1586.3±66.3	1519.3	1643.3	15.2	2267.8±76.2	2190.6	2344.2	11.6
Cold carcass weight (g)	1555.2±54.1	1502.1	1609.4	14.6	2233.1±46.2	2186.3	2279.5	9.2
Hot yield (%)	79.91±1.9	78.01	81.8	10.5	81.3±0.8	80.6	82.1	8.3
Cold yield (%)	78.34±1.3	77.04	79.6	10.4	80.1±1.1	78.9	81.2	8,2

Table 2. The share of the main cut portions in the composition of the carcasses of the Ross-308 hybrid

Specification	Both sexes							
	35 days				42 days			
	$\bar{X} \pm s_{\bar{x}}$	Min.	Max.	V%	$\bar{X} \pm s_{\bar{x}}$	Min.	Max.	V%
Breast weight with bone and skin	459.1±21.3	437	481	14.6	641.1±20.1	620	663	12.5
% of carcass	29.5±1.1	28.3	30.7	11.1	29.7±0.9	28.7	30.7	11.2
Breast weight without bone and skin	364.1±18.5	345	383	9.7	538.2±18.1	519	557	8.8
% of carcass	23.4±0.9	22.5	24.5	9.2	24.1±0.8	23.3	24.9	8.3
Thigh weight	384.1±16.2	367	410	7.7	562.8±15.8	547	578	7.1
% of carcass	24.7±0.5	24.1	25.3	5.6	25.2±0.6	24.6	24.9	6.7

Dimensional Characteristics of Pectoral Muscles

Given the increasing demand for marketing pectoral muscles separately (superficial and deep), several dimensional parameters were evaluated, including length, width, and thickness.

The average length of the superficial pectoral muscle was 179±5 mm at 35 days and 197±6 mm at 42 days, while the deep pectoral muscle measured 138±6 mm and 146±4 mm, respectively.

The average width of the deep pectoral muscle was 37±4 mm at 35 days and 39±3 mm at 42 days, while the superficial muscle measured 76±3 mm and 82±4 mm, respectively.

The average thickness of the deep pectoral muscle was 14±3 mm at both ages, while for the superficial muscle it was 29±4 mm at 35 days and 33±5 mm at 42 days (Table 3).

Table 3. Dimensional aspects of the pectoralis superficialis and pectoralis deepis muscles in the Ross-308 hybrid

Specification	Average length (mm)	Average width (mm)	Average thickness (mm)
35 days			
Superficial pectoral	179±5	76±3	29±4
Deep chest	138±6	37±4	14±3
42 days			
Superficial pectoral	197±6	82±4	33±5
Deep chest	146±4	39±3	19±3

Chemical Composition of Pectoral Musculature

The chemical composition analysis aimed to determine water, dry matter, lipids, proteins, nitrogen-free extract substances, and mineral content (Hudák et al., 2021).

In the first control period (35 days), for the superficial pectoral muscle, the average water content was 74.9±0.1%, with very narrow variation limits (74.7-75.0%), indicating very high homogeneity.

Correspondingly, dry matter averaged 25.1±0.1%, also showing very high homogeneity (V = 1.1).

The average lipid content was 0.92±0.01%, with values between 0.91 and 0.93%, indicating very high homogeneity.

Protein content averaged 22.22±0.02%, with minimal variation, resulting in a very low coefficient of variation.

Nitrogen-free extract substances averaged 0.94±0.01%, while mineral content was 1.02±0.03%.

In the second control period (42 days), for the superficial muscle, water content averaged 74.1±0.6%, and dry matter 25.9±0.5%.

Protein content reached 22.29±0.05%, while lipids showed a slight increase to 1.12±0.03%.

Mineral substances averaged 1.49±0.05%, and nitrogen-free extract substances reached 1.01±0.03%.

For the deep pectoral muscle at 35 days, water content was 75.3±0.3%, and dry matter 24.7±0.2%.

Protein content averaged 22.25±0.15%, while lipid content was 0.79±0.01%.

Mineral substances averaged 1.05±0.02%, and nitrogen-free extract substances 0.63±0.05%.

At 42 days, for the deep pectoral muscle, water content averaged 75.2±1.2%, while dry matter was 24.8±0.3%, both showing very good homogeneity (V = 1.2 and 0.7).

Protein content ranged between 22.05% and 22.25%, with an average of 22.15±0.10% and a very low coefficient of variation (V = 1.3).

Lipid content averaged 0.99±0.02%, with values between 0.97% and 1.01% (V = 2.9), indicating high homogeneity.

Mineral substances represented 0.98±0.01%, while nitrogen-free extract substances averaged 0.66±0.05% (Table 4).

Table 4. Dimensional aspects of the pectoralis superficialis and pectoralis deepis muscles in the Ross-308 hybrid

Age	Parameter	Superficial pectoralis muscle				Deep pectoralis muscle			
		$\overline{X} \pm S_{\overline{x}}$ (%)	V %	Min. (%)	Max. (%)	$\overline{X} \pm S_{\overline{x}}$ (%)	V %	Min. (%)	Max. (%)
35 days	Water	74.9±0.1	1.4	74.7	75.0	75.3±0.3	1.2	75	75.8
	Dry matter (SU)	25.1±0.1	1.1	25.0	25.3	24.7±0.2	0.8	24.5	25
	Mineral substances	1.02±0.03	4.1	0.99	1.05	1.05±0.02	3.2	1.03	1.07
	Fat	0.92±0.01	5.3	0.91	0.93	0.79±0.01	4.6	0.78	0.80
	Protein	22.22±0.02	1.2	22.20	22.24	22.25±0.15	1.1	22.10	22.40
	SEN	0.94±0.01	3.3	0.93	0.95	0.63±0.05	2.6	0.58	0.68
42 days	Water	74.1±0.6	1.3	73.5	74.7	75.2±1.2	1.2	74	76.4
	Dry matter (SU)	25.9±0.5	1.1	25.4	26.05	24.8±0.3	0.7	24.5	25.1
	Mineral substances	1.49±0.05	6.4	1.41	1.54	0.98±0.01	5.4	0.97	0.99
	Fat	1.12±0.03	3.2	1.09	1.15	0.99±0.02	2.9	0.97	1.01
	Protein	22.29±0.05	1.2	22.24	22.34	22.15±0.10	1.3	22.05	22.25
	SEN	1.01±0.03	3.6	0.98	1.04	0.66±0.05	3.2	0.61	0.71

Slaughter Yield

Regarding the body weight of the studied flocks, a favorable evolution was observed in relation to growth rate. Comparison with data from the literature revealed similar or even higher average values than those reported by other authors. Thus, at 35 days of age, Hassan et al. (2021) reported body weights of approximately

1756 g, while Ribeiro et al. (2021) obtained values of 1900-1950 g.

At 42 days of age, average body weights of 2636 g were reported by Gumowski (2025), while Rajcic et al. (2024) identified values ranging between 2600 and 2900 g.

Hot carcass weight showed values comparable to those reported in the literature. Namted et al. (2025) reported values of 1500-1600 g for commercial hybrids at 35 days of age, confirming their superiority over less improved breeds (Gheorghe et al., 2022).

Similarly, Dong et al. (2024) reported values between 2200 and 2350 g for Ross 308 at 42 days of age.

Comparable results were also reported by Curea et al. (2023), who observed that improvements in genetic and nutritional performance in broilers have led to significant increases in hot carcass weight.

Regarding cold carcass weight, Hudak et al. (2021) reported ranges of 1500–1580 g at 34–36 days of age. The Aviagen guide (2022) indicates that, under commercial conditions, Ross 308 carcass weights at 42 days range between 2150 and 2300 g, confirming that the value obtained in this study (2233.1 g) falls within the optimal performance range.

Liao et al. (2022) highlighted that weight loss during chilling varies between 1.5% and 3% of hot carcass weight, supporting the differences observed in the present study.

For hot dressing percentage, the genetic supplier reports values between 78% and 82% for Ross 308, while applied research (Custură et al., 2019) emphasizes the influence of nutrition, age, sex, and slaughterhouse processing conditions. Despite these multiple influencing factors, our results fall within the reference range.

Berri (2020) reported hot dressing percentages of approximately 78.0-80.0% at 35-42 days of age, values close but slightly lower than those obtained in this study. Similar findings were reported by Benahmed et al. (2023), who noted that dressing percentage tends to increase slightly with age and muscle development.

For cold dressing percentage, the obtained values are comparable to those reported by Olkowski et al. (2001), who found values of 77-79% at 35-42 days of age. Lilburn et al. (2015) reported values of approximately 75.5-80.0%, emphasizing the influence of rearing conditions and slaughtering technology. Martínez and Valdivié (2021) demonstrated that cold dressing percentage is generally 1.5-3% lower than hot dressing percentage, due to water loss through evaporation and post-slaughter drip.

Differences compared to other studies can generally be explained by technological and environmental factors such as stocking density, nutritional level, and microclimate conditions, all recognized as having a significant impact on bird growth (Usturoi et al., 2021).

Weight and Proportion of Anatomical Regions

The weight of breast with bone and skin was similar to the values reported by Wegner et al. (2024), who found values between 430 and 500 g at approximately 35 days of age. In the same context, Fletcher (2002) and Tudorache et al. (2022) showed that intensive genetic selection in meat-type hybrids, including Ross 308, has led to significant increases in pectoral muscle mass, with the breast accounting for over 30% of carcass weight at commercial ages.

At 42 days of age, Havenstein et al. (2003) and Nechitailo et al. (2024) reported breast weights (with bone and skin) ranging between 600 and 660 g.

The weight of boneless, skinless breast was consistent with the findings of Biesek et al. (2020), who demonstrated that genetic selection significantly increased the proportion of pectoral musculature, representing approximately 28-32% of carcass weight at commercial ages.

Novak et al. (2010) reported boneless breast weights of approximately 520-560 g at 42 days of age, values comparable to those obtained in the present study (538.2 g). Similarly, Lilburn. (2015) showed that pectoral muscle development accelerates toward the end of the growing period, explaining the differences observed between 35 and 42 days.

Dimensional Characteristics of Pectoral Muscles

Regarding the dimensional characteristics of pectoral muscles, Fletcher (2002) emphasized that intensive genetic selection has favored the accelerated development of the pectoralis superficialis (the main determinant of breast yield), while the pectoralis profundus remains a secondary functional muscle with slower

development. Hassan et al. (2021) demonstrated that the differences between the two muscles are due to their distinct biomechanical functions.
Similar results to those obtained in the present study were reported by Rajcic et al. (2024), who observed that in commercial hybrids, the superficial muscle shows a higher growth rate compared to the pectoralis profundus.

Chemical Composition of Pectoral Musculature

Regarding the chemical composition of the superficial pectoral muscle, Fletcher (2002) reported a water content ranging between 73% and 76%, values very close to those obtained in the present study (74.9% at 35 days and 74.1% at 42 days). Protein content reported in the literature is approximately 21-23% (Fletcher, 2002), corresponding to the values obtained (22.22% and 22.29%).
In the same context, Dalle et al. (2024), Dolis et al. (2024) reported intramuscular lipid contents ranging between 0.8% and 1.5%, which includes both values obtained in this study (0.92% at 35 days and 1.12% at 42 days). Other authors (Cojocariu et al., 2026) reported mineral contents of approximately 1.0-1.6%, confirming that the values obtained in this study (1.02-1.49%) fall within this range.
Nitrogen-free extract substances (0.94% and 1.01%) fall within the ranges reported for lean poultry meat, as Liao et al. (2022) indicated values below 1% for these compounds.
The chemical composition of the deep pectoral muscle showed that water content (75.2-75.3%) falls within the range reported by Liao et al. (2022), who identified values between 74% and 76% in broilers. Variations may be attributed to nutrition, rearing system, and slaughter age (Gheorghe et al., 2022, Martinez et al. 2021). Olkowski et al. (2001) also confirmed that deep pectoral muscle exhibits low variability in water content within the commercial slaughter range.
Dry matter values (24.7-24.8%) are similar to those reported by de Wegner et al. (2024), who found values between 23% and 26%.
Protein content (22.15-22.25%) is comparable to the results reported by Hudak et al. (2021), ranging between 21% and 23%.
Lipid content (0.79-0.99%) is consistent with the findings of Berri (2020), who reported values below 1.2% for deep pectoral muscle, highlighting its low lipid content.
Mineral content (0.98-1.05%) was similar to that reported by Dalle et al. (2024), who indicated a range of 1.0-1.4% without major differences between pectoral muscles.
Nitrogen-free extract substances (0.63-0.66%) fall within the low values reported by Havenstein et al. (2003), who showed that these components represent less than 1% in lean poultry meat, reflecting a low content of soluble non-protein compounds.

CONCLUSIONS

The results obtained highlight the extremely high productive performance of the Ross 308 hybrid, confirmed by rapid body weight gain and superior values of slaughter performance parameters recorded between 35 and 42 days of age.
Live weight and carcass weight were parameters that showed significant increases with advancing age, demonstrating efficient feed utilization and the expression of the hybrid's high genetic potential. Slaughter yields, both hot and cold, were high, with a slight increase observed at 42 days of age.
The overall analysis of carcass structure revealed a pronounced development of the breast region, especially of the pectoralis superficialis muscle, which recorded significant increases in both weight and size. This trend confirms the direction of genetic selection toward increasing the proportion of breast meat in carcass composition.
The chemical composition of the pectoral muscles showed a high and stable protein content, low lipid levels, and minimal variation in water and dry matter content, characteristics that confirm the pectoral muscle as being of superior quality

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BIoTa EcoToken: SUSTAINABLE DAIRY 4.0 THROUGH INTEGRATED BLOCKCHAIN AND ARTIFICIAL INTELLIGENCE

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Abstract

In high-volume dairy production, ensuring animal health and welfare across diverse environments is a logistical challenge that exceeds human capacity. Addressing the dual challenge of animal welfare and environmental sustainability requires next-generation monitoring. This paper introduces the BIoTa EcoToken system, a multi-layered architecture integrating Internet of Things, deep learning, and a hybrid blockchain to revolutionize Dairy 4.0 practices. The system leverages distributed sensors and visual data to provide continuous, objective monitoring of cattle behaviour, physiology, and barn microclimate. Central to its success is the Proof of Sustainability consensus mechanism, which tokenizes rewards and incentivizes proactive farmer behaviour based on verified eco-actions. Experimental results demonstrate a 40% reduction in anomaly detection response time and a 30% increase in farmer engagement due to the EcoToken structure. With a validated security performance of <1% false validation rate, BIoTa EcoToken establishes a secure and scalable framework that translates real-time farm data into verifiable sustainable outcomes.

Key words*: animal welfare, artificial intelligence, blockchain, dairy farming, Internet of Things.*

INTRODUCTION

Ensuring the welfare of dairy cows and reducing the ecological impact of farms represent two directions of modern agriculture, positioned at the core of European and international strategies for sustainable development (Pavanello et al., 2024). Cattle farms are under increasing pressure from consumers, authorities, and environmental organizations, who demand dairy products obtained under ethical conditions, with reduced emissions and high traceability (Linstädt et al., 2024). At the same time, the intensified growth of animal herds makes the continuous monitoring of each individual exceed traditional human capacities, increasing the risk of stress, disease, discomfort, or accidents (Maltz, 2020; Amaritii et al., 2024).

Animal welfare is now recognized as a fundamental pillar of milk production, being directly linked to health, fertility, and productive performance (Linstädt et al., 2024). Parameters such as natural behaviour, thermal comfort, movement, rumination, access to feed and water, as well as the prevention of metabolic and locomotor disorders are needed in assessing the well-being of cows (Amaritii et al., 2024).

Thermal stress, for example, represents one of the threats to welfare and sustainability, with direct implications for production, immunity, and feed intake (Polsky & von Keyserlingk, 2017). In the context of climate change, the frequency of high-temperature events has increased, which requires advanced monitoring and intervention strategies (Kadzere et al., 2002; Giannone et al., 2023). In parallel, the environmental impact generated by dairy farms, methane emissions, water and energy consumption, manure management, and soil degradation represents a sensitive topic for both the industry and the scientific community.

Modern farms are responsible for approximately 14% of global greenhouse gas emissions originating from the livestock sector, which makes it necessary to identify technologies capable of reducing the ecological footprint without compromising productivity (Gerber et al., 2013; Beauchemin et al., 2020). To address

these challenges, recent years have seen a transition toward smart farms, where digital technologies, especially the Internet of Things (IoT), data analytics, and artificial intelligence enable continuous monitoring of animal behaviour and physiology, as well as of the microclimate inside barns (Rutten et al., 2013; Norton et al., 2019).

The use of wearable sensors, accelerometers, intelligent video systems, and data analysis platforms facilitates early detection of anomalies, disease prevention, and optimization of environmental conditions. Numerous studies have shown that digitalized monitoring reduces stress, increases comfort, and improves the health status of dairy cows, thus directly contributing to their overall welfare (Soyeurt et al., 2019). Moreover, environmental sustainability cannot be separated from the smart management of resources.

Modern technologies can reduce water consumption through optimized watering systems, minimize waste through digital manure management, and reduce emissions by continuously analysing rumination behaviour, a key indicator of methane production (Tangorra et al., 2024; Wang et al., 2024). Integrating these processes into a digital architecture allows not only the identification of problems but also real-time adaptation of management strategies to reduce environmental impact. In this context, the digitalization of farms becomes more than a technological modernization: it represents a needed condition for the transition toward a sustainable, ethical, and high-performing dairy system. However, although IoT and AI can generate large volumes of valuable data regarding animal welfare and environmental parameters, the use of this information is often limited by the lack of transparent and secure mechanisms for validation and for incentivizing positive actions. This is where blockchain technology comes in, as it can transform such information into verifiable, aggregable, and usable assets that support environmentally friendly behaviours (Kampan et al., 2022). The BIoTa EcoToken platform offers a unified ecological monitoring and behaviour-incentivizing system. The current paper is structured to first introduce the dual challenges of animal welfare and environmental sustainability in dairy farming, justifying the need for next-generation monitoring. It details the Materials and Methods, outlining a multi-layered architecture utilizing IoT sensors (accelerometers, thermal probes) and deep learning for continuous monitoring, and introduces the Proof of Sustainability consensus mechanism to incentivize proactive farmer behaviour via EcoTokens. The Results and Discussions section presents experimental validation, highlighting the AI's peak performance (Full Ensemble + DTW) with 91.10% accuracy in behaviour recognition, the system's capacity for early anomaly detection (6-12 hours earlier with 87% accuracy) , and the positive behavioural impact of the EcoToken structure, which led to a 30% increase in farmer engagement. The paper concludes that the platform successfully merges animal health management, environmental sustainability, and economic incentives into a single, scalable framework.

MATERIALS AND METHODS

Recent advancements in Internet of Things technologies have facilitated extensive monitoring of cattle health, behaviour, and environmental conditions via integrated sensor networks and sophisticated processing systems. New research indicates progression towards multi-modal sensing architectures that integrate physiological, behavioural, and environmental data streams to deliver comprehensive farm management solutions.

Sensor configurations differ according to monitoring goals, although several recurring patterns emerge. Accelerometers and gyroscopes predominantly facilitate activity classification in behavioural monitoring (Alonso et al., 2020), frequently attached to collars or leg-worn devices to record standing, lying, walking, grazing, and rumination behaviours (Dutta et al., 2022).

Vision-based methodologies using RGB and infrared cameras were efficient in behaviour detection (Jeong et al., 2024) and individual identification via facial recognition (Chen et al., 2025), obtaining high accuracy.

Health monitoring systems generally use non-invasive sensors, such as infrared thermistors for measuring body temperature (Dutta et al., 2022; Arshad et al., 2022) pulse sensors, or

stethoscope-amplifier combinations for assessing heart rate (Arshad et al., 2022), and specialized gas sensors (MQ138, MQ-135) for identifying metabolic biomarkers like acetone, which indicates ketosis (Arshad et al., 2024).

Environmental monitoring combines ambient temperature and humidity sensors with hazardous gas detectors (CH_4, H_2S, NH_3) and agrometeorological stations that evaluate soil moisture and solar radiation (Alonso et al., 2020).

Communication architectures are balancing range, power consumption, and data throughput. Short-range protocols such as Wi-Fi and Bluetooth Low Energy (BLE) are commonly used in collar-based systems, necessitating frequent updates (Alonso et al., 2020; Arcidiacono et al., 2021), whereas LoRaWAN and ZigBee networks cater to applications requiring broader coverage with reduced power consumption (Alonso et al., 2020; Debdas et al., 2022). Cellular GSM/GPRS modules offer dependable connectivity for location tracking and alert notifications (Dutta et al., 2022; Debdas et al., 2022), although reliance on uninterrupted internet connectivity continues to be a constraint (Arshad et al., 2022). Multi-protocol gateways that integrate ZigBee, LoRa, Wi-Fi, and Ethernet/DSL show the industry's shift towards heterogeneous network integration (Alonso et al., 2020).

Computational task allocation between edge devices and cloud infrastructure differs between implementations. Edge-centric architectures executing comprehensive inference locally, such as YOLO-based object detection on the NVIDIA Jetson Nano (Jeong et al., 2024) and MobileNetV2 feature extraction on the Raspberry Pi (Chen et al., 2025) eliminate cloud dependencies and reduce latency, although they encounter limitations in memory and computational capacity (Chen et al., 2025). Hybrid methodologies use edge pre-processing for filtration and threshold-based notifications, while assigning intricate machine learning operations to cloud servers (Alonso et al., 2020; Dutta et al., 2022). This results in data traffic reductions surpassing 50% and notable enhancements in data reliability, evidenced by a 53% decrease in missing database values (Alonso et al., 2020). Federated learning architectures constitute a novel paradigm that facilitates collaborative model training across distributed edge nodes while maintaining data locality (Arshad et al., 2024).

Machine learning techniques range from basic threshold-based classification (Arcidiacono et al., 2021) to advanced ensemble methods and deep neural networks. Tree-based algorithms, such as XGBoost and Random Forest, show good multi-class activity recognition performance on short observation windows (Dutta et al., 2022). Deep learning methodologies using YOLO-v8 for the detection of cattle mounting behaviour show strong performance during both day and night (Jeong et al., 2024). In contrast, federated Gaussian Naïve Bayes classifiers obtained good precision for certain disease categories, but had a relatively low overall federated accuracy (Arshad et al., 2024).

Despite technological advancements, numerous constraints appear in the examined literature. Dataset sizes are limited, with standard implementations observing 3 to 53 animals over durations ranging from days to 17 months (Dutta et al., 2022; Chen et al., 2025; Arshad et al., 2022), which raises concerns regarding model generalizability. GPS signal attenuation within covered structures (Dutta et al., 2022), constraints on battery longevity (typically 11 hours or fewer per charge) (Dutta et al., 2022), and packet loss rates nearing 20% (Arcidiacono et al., 2021) indicate notable challenges for practical deployment. Measurement reliability can be compromised by inconsistent sensor placement and external interferences such wind, humidity, and occlusions (Jeong et al., 2024; Arshad et al., 2024).

The BIoTa EcoToken system was developed using a multi-layered architecture that integrates IoT sensors, deep learning models, and a hybrid blockchain framework to monitor dairy cows and barn environments in real time. Wearable sensors, accelerometers, thermal probes, and intelligent video systems were deployed to capture continuous data on cattle behaviour, physiology, and microclimate conditions. These data streams were processed using deep learning algorithms designed to detect anomalies, assess welfare indicators, and correlate environmental factors with animal responses. To ensure data security and to incentivize sustainable practices, all validated eco-actions were recorded through

a hybrid blockchain using the Proof of Sustainability consensus mechanism, which generated EcoTokens as rewards. The system is currently implemented as a pilot platform designed to evaluate how its integration can influence and improve animal welfare indicators, allowing researchers to analyze the changes produced following its deployment on dairy farms.

Distributed sensors collected multimodal data on physiological parameters (heart rate, body temperature), behavioural activity (walking, standing, drinking, licking, feeding), rumination patterns, and movement intensity, ambient environmental conditions (temperature, humidity, illumination), air pollutant and GHG levels (methane, nitrous oxide, ammonia). Wearable collars and pedometers further enabled dynamic monitoring of cattle welfare indicators, essential for early detection of stress or illness.

RESULTS AND DISCUSSIONS

A multi-classifier ensemble combining Random Forest, Support Vector Machines, and Convolutional Neural Networks was developed to recognize cattle behaviours with high accuracy. Time-windowed sensor matrices and the temporal alignment technique of Dynamic Time Warping (DTW) increased the behaviour recognition across individuals with different movement intensities, as in Table 1.

The performance comparison reveals a clear trend of increasing accuracy as the models move from simple, feature-engineered approaches to complex, deep learning, and ensemble techniques. The Random Forest model, utilizing raw sensor features, serves as the baseline, achieving an accuracy of 82.40% and an F1-score of 82.40%. This indicates good performance for static behaviours (lying, standing), suggesting the raw data is sufficient to distinguish non-movement states.

The Support Vector Machine model, leveraging statistical movement descriptors, slightly outperforms RF with an accuracy of 84.60% and an F1-score of 84.50%. The specific observations note its strength in the fine-grained distinction between feeding and drinking, highlighting the effectiveness of the statistical features in capturing the nuances of these dynamic behaviours. The introduction of the Convolutional Neural Network (CNN) marks a leap in performance. The standalone CNN, processing time-windowed sensor matrices, achieved an accuracy of 88.90% and an F1-score of 88.50%. This strong result confirms the CNN's ability to automatically learn complex temporal-spatial movement patterns directly from the raw time-series data, bypassing manual feature engineering.

Table 1. Comparison of classifier performance before and after ensemble integration

Model	Feature Type	Accuracy (%)	Precision (%)	Recall (%)	F1-Score (%)	Observations
Random Forest (RF)	Raw sensor features	82.40	83.10	81.70	82.40	Performs well for static behaviours (lying, standing)
Support Vector Machine (SVM)	Statistical movement descriptors	84.60	85.20	83.90	84.50	Strong separation for feeding vs drinking
CNN (Standalone)	Time-windowed sensor matrices	88.90	89.40	87.60	88.50	Learns complex temporal-spatial movement patterns
CNN + DTW Alignment	Time-warped motion sequences	89.80	90.10	88.90	89.50	Improved alignment across cows with different activity levels
RF + SVM (Hybrid Voting)	Combined feature sets	86.70	87.30	86.00	86.60	Reduces individual classifier biases
Full Ensemble (RF + SVM + CNN)	All sensor and temporal features	90.30	91.60	89.70	90.60	Best overall performance across behaviour types
Full Ensemble + DTW	Time-aligned temporal windows	91.10	92.00	90.50	91.20	Highest generalization and cross-animal consistency

Further refinement was achieved by incorporating dynamic time warping alignment. The CNN + DTW alignment model demonstrated improved metrics (accuracy of 89.80%, F1-

score of 89.50%). The DTW's contribution of creating time-warped motion sequences addresses natural variability in activity speed and duration across different subjects, leading to improved alignment across cows with different activity levels and boosting generalization.

Hybrid and full ensemble models consistently achieved the highest performance metrics, demonstrating the benefit of combining diverse learning strategies. The RF + SVM (hybrid voting) approach, which combines two distinct feature types and classifiers, elevated the performance over the individual RF and SVM models (accuracy of 86.70%, F1-score of 86.60%). This improvement is attributed to the mechanism of reducing individual classifier biases, making the hybrid prediction more reliable. The full ensemble (RF + SVM + CNN) model, leveraging all sensor and temporal features, surpassed all standalone and hybrid models (accuracy of 90.30%, F1-score of 90.60%). Its designation as the best overall performance across behaviour types highlights the power of combining deep temporal feature learning (CNN) with discriminative feature-based methods (RF, SVM). Finally, the full ensemble + DTW model represents peak performance, achieving the highest metrics across the board (accuracy of 91.10%, F1-score of 91.20%). By applying time alignment to the combined power of the full ensemble, this model achieved the highest generalization and cross-animal consistency, establishing it as the most effective solution for behaviour classification in this study.

To ensure secure, transparent, and verifiable data governance, all validated eco-actions and sensor-derived events were stored on a hybrid blockchain. This dual architecture, consisting of a public chain for transparency and a private chain for sensitive data, maintained immutability while allowing controlled access. Smart contracts enforced a Proof-of-Sustainability consensus mechanism that rewarded farmers with EcoTokens for verified improvements in welfare conditions, emission reductions, and environmentally responsible actions, such as proactive heat-stress mitigation and optimized manure management. The system was deployed for six months on a Romanian dairy farm, where it collected more than 1.2 million multimodal data points under variable climatic and operational conditions, as in Figure 2. Ground-truth annotations generated by human observers ensured accurate validation of the AI models.



Figure 1. Romanian dairy farm used for piloting (own source)

A key outcome of the system was its capacity to detect anomalies earlier than conventional observation methods. Predictive analytics identified deviations in locomotion, rumination time, or heart rate 6-12 hours before visible symptoms, with 87% accuracy in early anomaly detection (Figure 2). These predictions were particularly important in identifying heat-stress events, which were accompanied by decreased feeding behaviour, prolonged standing, and reduced lying time. By integrating wearable-based activity signatures with environmental inputs such as temperature and humidity, the platform provided real-time alerts and recommended mitigation strategies.

Environmental monitoring revealed clear relationships between microclimate variability, cattle behaviour, and pollutant emissions. The system's pollutant sensors captured fluctuations in methane (CH_4) linked with changes in rumination patterns, while ammonia (NH_3) exhibited marked increases during periods of high humidity or elevated temperature. Nitrous oxide (N_2O) concentrations rose in association with manure accumulation or suboptimal nitrogen balance in feed.

Table 2 summarizes the average pollutant concentrations recorded during typical barn routines. The integration of environmental and behavioural datasets enabled precise mapping of emission dynamics.

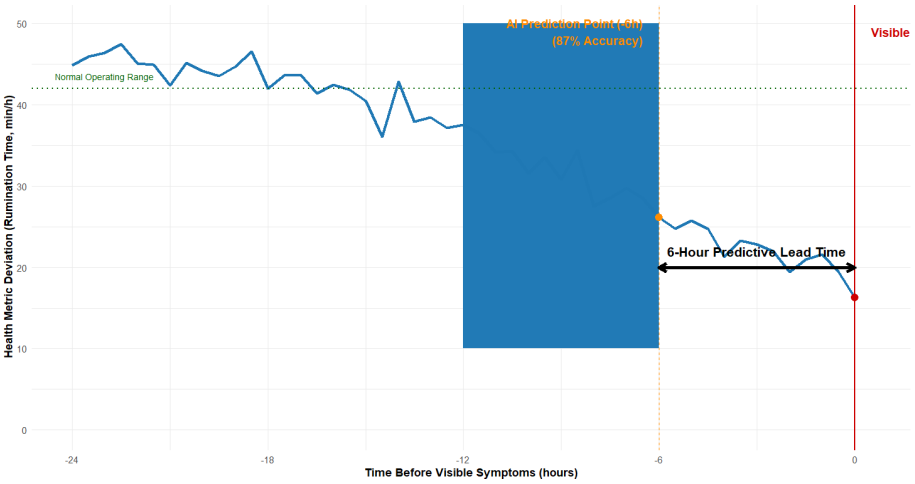


Figure 2. Early anomaly detection timeline for visible symptoms related to rumination

Table 2. Average pollutant concentrations during monitoring period

Pollutant	Average concentration	Observed peak conditions
Methane (CH_4)	Moderate baseline, variable peaks	Reduced rumination / heat stress
Ammonia (NH_3)	Elevated during high humidity	Warm, poorly ventilated periods
Nitrous oxide (N_2O)	Stable, with manure-related spikes	Manure accumulation events

Figure 3 shows a correlation between rumination levels and methane fluctuations during a heat-stress episode. Reduced rumination was associated with a measurable increase in methane variability, supporting the scientific consensus that heat stress alters digestion and contributes to emission instability.

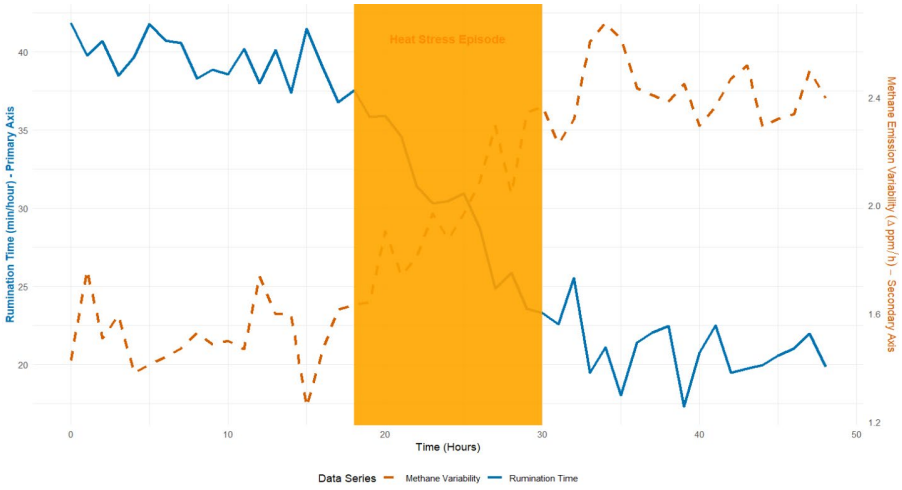


Figure 3. Correlation between rumination time and methane emission variability

Blockchain functionality played a central role in ensuring data integrity and traceability. The hybrid blockchain implementation achieved a transaction latency of 0.8 seconds and a throughput of 115 transactions per second, while reducing data redundancy by 35% compared to a single-chain structure. All environmental, welfare, and behavioural events were immutably recorded, enabling verifiable sustainability claims for regulatory audits and supply-chain

transparency. Additionally, the EcoToken mechanism enhanced stakeholder engagement. By automatically rewarding farmers for improved welfare metrics, heat-stress interventions, or emission reduction actions, the system generated a 30% increase in compliance and proactive behaviour adoption. User evaluation surveys revealed high acceptance rates, with a mean usefulness score of 4.7/5 and an ease-of-adoption score of 4.5/5 (Table 3).

The results from Table 4 highlight a multi-faceted evaluation spanning the physical infrastructure (IoT deployment), the underlying ledger technology (blockchain performance, deployment validation, and consensus integrity), and real-world utility (behavioural impact).

The enrichment highlights that the system's core technologies, IoT and consensus, are validated and ready for scaling, but its success hinges on addressing the user onboarding and training issue. The 63% knowledge gap is a hurdle that could negate the 30% increase in behavioural impact achieved through incentives if farmers cannot properly interact with the system. Future work must therefore prioritize interface simplification and dedicated educational programs to translate the system's technical success into broad user adoption and sustained farm-level benefits.

Table 3. User evaluation of the BIoTa EcoToken platform

Evaluation category	Mean score (1-5)
Perceived usefulness	4.7
Ease of adoption	4.5
Interface clarity	4.4
Overall satisfaction	4.6

Table 4. Experimental results and validation tests for Token-based IoT deployment

Category	Tested feature	Result	Significance	Future work
IoT Deployment	Smart Lighting Sensor Integration	-25% sensor installation cost, no loss in detection accuracy, -18% energy usage	Demonstrates cost-effective and energy-efficient deployment while maintaining sensor performance.	These efficiency gains justify rapid deployment across wider farming operations.
Deployment Validation	Block Reward Test	Reward = 5 Tokens/block, fixed during deployment	Validates the consistency of mining incentives across the blockchain lifecycle.	Use this fixed baseline to model the economic sustainability of variable reward schemes (dynamic rewards based on severity of sustainability event).
Consensus Integrity	Proof of Sustainability	<1% false validation rate across 5000+ sustainability events	Validates reliability of consensus in real-world sustainability reporting.	High reliability is suitable for seeking certification or integration with external regulatory platforms.
Behavioural Impact	Farmer Engagement Metrics	30% increase in proactive animal care interventions during token reward phases	Indicates behavioural change when eco-incentives are introduced.	Translate the +30% intervention rate into reduced animal health costs (or increased yield) to calculate the Return on Investment (ROI) of the token system.
Blockchain Performance	User Onboarding and Training	Identified knowledge gaps in 63% of participants during simulation trials	Points to the need for better UX design and educational materials.	Must allocate resources immediately to simplify the UI/UX and develop mandatory, interactive training modules to ensure platform utilization.

The future work for the BIoTa EcoToken system will focus on a strategic three-pronged approach: optimizing user experience, scaling deployment, and validating the economic model. The most immediate priority is addressing the knowledge gaps identified in 63% of participants by allocating resources to simplify the UI/UX and develop mandatory, interactive training modules to ensure confident platform utilization. Concurrently, the proven

cost-effectiveness of -25% installation cost and -18% energy usage justifies the rapid deployment across wider farming operations.

On the blockchain side, the established fixed reward baseline will be used to model the economic sustainability of variable reward schemes and, crucially, to calculate the Return on Investment (ROI) by translating the observed +30% increase in proactive interventions into quantifiable benefits such as reduced animal health costs or increased yield. Finally, the high reliability of the Proof of Sustainability mechanism, <1% false validation rate, positions the platform to pursue certification and integration with external regulatory platforms for verifiable sustainability reporting.

The results from Table 4 confirm that the combined IoT-AI-blockchain architecture not only improves data accuracy and welfare outcomes but also facilitates behavioural change among farmers. The system successfully merges animal health management, environmental sustainability, and economic incentives into a single framework.

CONCLUSIONS

The BIoTa EcoToken platform demonstrates that merging IoT, AI, and blockchain technologies yields a powerful and practical solution for sustainable dairy management. The system's high-resolution monitoring and advanced analytics enabled the early identification of welfare anomalies, detecting deviations up to 12 hours earlier than traditional observation methods, with 87% early-prediction success rate. The AI behaviour recognition model delivered 90.3% accuracy and a 90.6% F1-score, confirming its reliability for continuous livestock monitoring. Environmental sensing further enhanced decision-making by providing real-time insights into greenhouse gas dynamics, supporting interventions that reduce ammonia, methane, and nitrous oxide emissions and improve microclimate stability within the barn.

Blockchain integration ensured 100% traceability and tamper-proof verification of all sensor data and eco-actions, with system performance achieving 0.8-second latency and 115 transactions per second, proving its suitability for real-time agricultural environments. The hybrid blockchain architecture also reduced data redundancy by 35%, demonstrating strong efficiency benefits for large-scale sensor networks. The EcoToken mechanism significantly increased farmer participation by approximately 30%, illustrating how token-based incentives can successfully encourage timely interventions, promote welfare-supportive behaviour, and foster environmental responsibility. High user satisfaction (usefulness 4.7/5, adoption 4.5/5) confirms the platform's accessibility and practicality. Overall, the results show that BIoTa EcoToken achieves measurable improvements in farm sustainability, animal health, and environmental impact. Its modular architecture allows flexible deployment across farms of various sizes, making it well-suited for both industrial operations and small-scale producers facing modern regulatory and ecological pressures. By integrating welfare, emissions, and economic incentives into a single aligned framework, the system offers a replicable and scalable blueprint for ethical, climate-smart, and digitally transparent dairy farming.

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DEVELOPMENT OF A NEXT-GENERATION MEAT PRODUCT ENRICHED WITH *Astragalus membranaceus* AND EXPLORING ITS POTENTIAL

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Abstract

The food industry, and especially the meat industry, is in a constant search for new, innovative products that meet the increasingly demanding requirements of consumers who are looking for functional products, rich in benefits and without synthetic additives, in line with the trends of recent years. With this idea in mind, we developed this new type of beef product in the form of sticks, which would incorporate both the benefits of the meat and the added Astragalus membranaceus, an antioxidant superfood, and meet the demand for innovation that consumers are demanding. For this study, three batches of products were produced: a control batch without added Astrogalus membranaceus and two experimental batches with added Astrogalus in 1 and 3%. All three batches were evaluated for physicochemical characteristics, instrumental colour, texture, antioxidant activity, bioactive compound profile, and sensory properties, including hedonic assessment, QDA, and PCA. The results showed that Astragalus membranaceus powder significantly influenced most quality parameters, particularly antioxidant activity, phytochemical composition, colour, texture, and sensory descriptors, with the strongest effects generally observed at the 3% addition level.

Key words*: antioxidants, beef meat products, functional foods, innovative meat products, sensory evaluation.*

INTRODUCTION

The meat industry is one of the largest industries worldwide and, due to strong consumer demand and intense market competition, new products are continuously being developed (Boișteanu et al., 2025)

Having nutritional components of major importance, including essential amino acids, fatty acids, vitamins, and minerals that are crucial for regular physiological and biochemical activities, meat and meat products are valuable foods in the diet (Anchidin et al., 2024a, 2024b; Ciobanu et al., 2025; Manoliu et al., 2023). However, consumers frequently characterize meat in an unfavorable way, describing it as a food that promotes the occurrence of cancer and is rich in fat. Nevertheless, this perspective minimizes the vital role that meat plays in preserving human health (Anchidin et al., 2023, 2025b; Ciobotaru et al., 2025).

In recent years, the way in which consumers perceive meat products from a health perspective has changed (Sirini et al., 2022). The increased interest in the beneficial attributes of meat, which meet the nutritional needs of consumers, has been supported by recent studies on consumers' perceptions and attitudes toward meat (Boișteanu et al., 2024; Flocea et al., 2024). Also, because it combines flavor, tenderness, and nutritional value, meat quality is crucial for both producers and consumers (Mădescu et al., 2025).

Consumers are increasingly aware of the link between health and nutrition nowadays (Manoliu et al., 2024) and of the need to meet the growing consumer demand for healthier options (Ciobotaru et al., 2024). Therefore, the food industry conducts extensive research and develops new products in response to the growing consumer demand for these products (Gucianu et al., 2025). The reformulation of meat products that deliberately include animal proteins and valuable bioactive components obtained from plants has been driven by the global movement toward a sustainable and health-promoting diet (Manoliu et al., 2025b). In

addition, these functional additives may have two purposes: they can improve nutritional and energy intake, while at the same time providing some protection against the occurrence of chronic diseases (Gucianu et al., 2023; Manoliu et al., 2025a).

Beyond improving the nutritional profile, the reformulation of meat products must also respond to essential technological requirements, such as maintaining oxidative stability, extending shelf life, and preserving sensory characteristics, because these are significantly influenced by oxidative stability. Undesirable alterations in sensory qualities and nutritional value result from the oxidation of proteins and lipids (Torres-Martínez et al., 2026).

At the same time, these technological and nutritional concerns must be integrated into a broader perspective on sustainability and food security. Food preservation has represented and continues to represent one of the fundamental objectives of food security, considering the need to ensure physical and economic access to essential foods (Petcu et al., 2023). Due to its substantial effects on the economy, ecology, and society, food waste represents a key challenge for global food security (Gucianu et al., 2024), including the control of chemical contaminants that may originate from processing, packaging, or from food contact with different materials (Matei & Pop, 2023).

Antioxidants, vitamins, minerals, or fibers generated from plants and plant materials with a high content of bioactive components, such as phenolic compounds, are examples of natural substances that are increasingly used for preserving and improving meat quality (Anchidin et al., 2025). Since lipid and protein oxidation simultaneously impacts odor, taste, color, nutritional content, and sensory acceptability, it continues to be one of the most important causes of meat product deterioration from a technological point of view (Manessis et al., 2020; Pateiro et al., 2021).

Recent research on meat preservation supports this strategy, demonstrating that plant extracts, essential oils, and other bioactive substances derived from plants are investigated for their antimicrobial and antioxidant qualities, which can help prevent oxidation, prevent the development of microorganisms that cause spoilage, and extend the shelf life of meat products. In this food system, plant bioactive compounds include polyphenols, flavonoids, tannins, terpenes, alkaloids, saponins, and coumarin. These compounds can act as preservation agents and can have an impact on the physical and chemical properties of meat products when used under appropriate conditions and concentrations (Akacha et al., 2023; Campolina et al., 2023; Chang et al., 2022; Elgadir et al., 2025; Olvera-Aguirre et al., 2023; Shah et al., 2014).

Astragalus membranaceus is a plant species of interest for this type of use. This plant includes a variety of bioactive compounds, such as polysaccharides, saponins, flavonoids, amino acids, and trace elements. Astragalosides I-IV, formononetin, isorhamnetin, quercetin, kaempferol, and calycosin are mentioned in the specialized literature (Yao et al., 2024; Zhang et al., 2024). These elements have been associated with hepatoprotective, immunomodulatory, anti-inflammatory, and antioxidant properties (Guo et al., 2019). According to these points of view, *Astragalus membranaceus* is a viable source of bioactive components for functional foods, and research demonstrating its antioxidant effects and improvement of physiological status in a variety of biological and food systems supports its potential (Dong et al., 2023).

The capacity of *Astragalus membranaceus* to improve the oxidative stability and nutritional functionality of products justifies the interest in it from the perspective of food applications. Research on *Astragalus* supplementation in animal diets and in meat has shown improvements in their quality and antioxidant capacity, indicating that the components of this plant may also be useful in the creation of functional meat products (Luo et al., 2020; Qiao et al., 2022).

Simultaneously, research on meat snacks demonstrates that the addition of natural ingredients can considerably modify the physicochemical characteristics, oxidative stability, color, texture, and sensory acceptance (Aşkın et al., 2022; Aykın-Dinçer et al., 2026). Thus, the nutritional profile and shelf life of meat products can be considerably improved through the addition of plant bioingredients (Flocea et al., 2025). Plant-based ingredients have been efficiently incorporated into products

such as dried meat and meat sticks to improve their functional profile and to encourage the creation of more attractive substitutes for consumers seeking healthier options (Aşkın et al., 2022; Peulić et al., 2025).

In addition, meat snacks, such as dried meat and meat snacks, have become a significant category of products due to consumer demand for ready-to-eat foods with high protein content and long shelf life. However, the quality of the final product depends largely on the control of the technological process and oxidative stability (Aşkın et al., 2022; Peulić et al., 2025).

Natural additives from plant products can provide oxidative stability and can support consumer-friendly formulations, according to findings reported in the specialized literature for dried meat products and other meat products. However, the result ultimately depends on the dosage, the type of functional ingredient added, and the way in which it interacts with proteins and lipids in the meat matrix (Das et al., 2021; Domínguez et al., 2020; Gullón et al., 2020).

The addition of plant ingredients to meat snacks can have two different effects: on the one hand, it can increase the amount of phenolic compounds and antioxidant activity; on the other hand, it can modify texture parameters, such as elasticity, tenderness, and chewiness, requiring a careful balance between functional benefit and sensory quality (Aykın-Dinçer et al., 2026; Yiğit & Dinçer, 2025).

Based on these premises, the present study aims to develop an innovative beef-based snack product enriched with *Astragalus membranaceus* and to evaluate its technological and functional potential by determining color and texture, as well as physicochemical, sensory, antioxidant, and bioactive compound analyses. The main purpose is to determine whether the addition of this plant can support the development of an innovative meat product with improved properties and potential application in the category of meat products.

MATERIALS AND METHODS

Three different beef-based product variations were made for the experiment: two experimental formulations that were supplemented with 1% and 3% *Astragalus membranaceus* root powder, respectively, and one control formulation that did not contain the powder. The items were acquired in the IULS microproduction workshop for processing meat and meat products.

The meat raw materials used for product manufacture consisted of beef round and beef shoulder, purchased from Selgros Cash & Carry SRL, Iași, Romania. Semolina flour produced by La Molisana was purchased from a local retail store in Iași and was used as a technological ingredient in the formulation. The bioactive plant ingredient, represented by *Astragalus membranaceus* root powder, was purchased from Paradisul Verde, Brașov, Romania, under the commercial form of raw organic *Astragalus membranaceus* powder. The seasoning mixture and auxiliary ingredients were supplied by a local company from Iași County, Romania.

The experimental batches and the ingredients included in their formulation are presented in Table 1, expressed in percentage form.

Table 1. Experimental formulation of beef snack samples enriched with *Astragalus membranaceus* powder

Batch code	Composition of raw materials and ingredients										
	Beef round	Beef chuck	Semola flour	*Astragalus* root powder	Salt	Smoked paprika	Garlic powder	Ground pepper	Onion powder	Ground cumin	Chili flakes
SA0	51.00	35.50	11.00	0.00	1.30	0.25	0.28	0.18	0.18	0.16	0.10
SA1	50.40	34.90	11.20	1.00			0.27			0.17	
SA3	49.20	34.10	11.30	3.00		0.24	0.25			0.15	

SA0 – control sample without *Astragalus membranaceus* powder; SA1 – sample supplemented with 1% *Astragalus membranaceus* powder; SA3 – sample supplemented with 3% *Astragalus membranaceus* powder.

The beef snack samples were obtained through a series of steps in the technological process. The meat raw materials were first combined with the auxiliary ingredients, and then they were coarsely ground in a meat grinder. After that, the mixture was finely ground in a cutter to combine the spice mixture, semolina flour, and *Astragalus membranaceus* powder in

accordance with each batch's formulation. After the mixture was homogenised, it was moved to a dosing machine and formed into beef sticks using a filling nozzle that was about 20 cm long and had an 18 mm diameter. The created samples were put into the smoking and heat-treatment room after being arranged on stainless steel racks.

Table 2 presents the thermal processing parameters applied to all experimental batches.

Table 2. Thermal processing parameters applied to beef snack samples

Stage	Technological operation	Temperature	Time
I	Preliminary drying	60°C	30 min
II	Smoking	70°C	35 min
III	Heat treatment	-	Until reaching 72°C in the thermal center
IV	Final drying	75°C	90 min

Following the thermal treatment, the samples were vacuum-packed, chilled in a refrigerator, and kept at 2-4°C until additional laboratory testing.

The analytical evaluation of the beef snack samples included physicochemical, phytochemical, antioxidant, and sensory determinations. The physicochemical and instrumental analyses consisted of pH measurement, instrumental color assessment, texture profile analysis and Warner-Bratzler shear force test, and the determination of proximate composition, including moisture, protein, fat, carbohydrate, and ash contents. In addition, the phytochemical profile and antioxidant potential of the samples were evaluated by determining total phenolic content and radical-scavenging activity using DPPH and ABTS assays. A portable pH meter HI98163 (SC Hanna Instruments SRL) with a penetration electrode was used to measure the pH directly in the product, and the CIE L*a*b* method was used to determine colour parameters in accordance with the guidelines for evaluating meat colour (King et al., 2023). To better characterise the colour variance among the experimental samples, derived colour parameters such as Chroma (C*), hue angle (h°), and total colour difference (ΔE*) were computed in addition to the primary colour coordinates L*, a*, and b*.

Chroma (C*), hue angle (h°), and total color difference (ΔE*) were calculated according to Girolami et al. (2013), (Song et al., 2022), and (Tkacz et al., 2020), using the following equations:

$$\text{Chroma (C*)} = \sqrt{(a^*)^2 + (b*)^2} \quad (1)$$

$$\text{Hue angle (h*)} = tan^{-1} \times b^*/a^* \quad (2)$$

$$\Delta E^* = \sqrt{\Delta a^{*2} + \Delta b^{*2} + \Delta L^{*2}} \quad (3)$$

Moisture, protein, fat, and ash contents were determined using standard analytical procedures, namely the drying oven method, Kjeldahl method, Soxhlet extraction method, and muffle furnace method, respectively, according to AOAC Internațional (Horwitz & International, 2000). Carbohydrate content was calculated by difference (Vulić et al., 2024).

The antioxidant activity and bioactive compound content were determined according to (Echegaray et al., 2021), using the DPPH and ABTS assays for antioxidant activity and spectrophotometric methods for total polyphenols, flavonoids, total carotenoids, lycopene, and β-carotene. The absorbance was measured using a UV–Vis spectrophotometer, Specord Plus 210 (Analytik Jena, Germany).

In order to ensure that food products meet the necessary quality requirements, sensory testing is a crucial tool that helps identify defects, off-flavors, and other quality issues that could compromise the overall attractiveness of a product (Ciobanu et al., 2023). In the present study, sensory analysis was performed using hedonic evaluation, Quantitative Descriptive Analysis (QDA), and Principal Component Analysis (PCA), according to Boișteanu et al. (2025) with the participation of 20 semi-trained assessors.

The statistical processing of the experimental data was carried out using XLSTAT software.

One-way ANOVA was applied for the physicochemical, color, texture, and sensory parameters, while two-way ANOVA was used for antioxidant activity and bioactive compound content. The overall *p*-value was used to evaluate the significance of the tested factors. When significant differences were observed, Tukey's post hoc test was applied for multiple comparisons among samples, while Dunnett's test was used to compare each experimental sample with the control sample. Differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSIONS

Physicochemical characteristics of beef snack samples

The incorporation of *Astragalus membranaceus* powder significantly affected all physicochemical parameters of the beef snack samples (Table 3), as indicated by the global *p*-values ($p < 0.05$). Moisture, protein, fat, and pH decreased progressively with increasing levels of Astragalus, with both SA1 and SA3 differing significantly from the control according to Dunnett's test ($p < 0.05$). In contrast, dry matter and carbohydrate content increased significantly in the supplemented samples ($p < 0.05$), reflecting the contribution of the plant powder to the non-meat solid fraction of the formulation. Ash content showed a more moderate response: although the global effect was significant ($p = 0.014$), Dunnett's test indicated that only SA3 differed significantly from SA0, while SA1 remained statistically similar to the control. Similar effects have been reported in beef patties enriched with dried pumpkin pulp and seed mixture, where the addition of plant material decreased moisture and increased ash content, confirming that plant-based ingredients can modify the proximate composition of meat matrices (Serdaroğlu et al., 2018).

Table 3. Physicochemical characteristics of beef snack samples supplemented with *Astragalus membranaceus* powder

Parameter	Samples			Global	Dunnett	Dunnett
	SA0	SA1	SA3	*p*-value	SA0 vs. SA1	SA0 vs. SA3
Moisture (%)	57.51 ± 0.16^{a}	57.16 ± 0.07^{b}	56.90 ± 0.08^{c}	**<0.0001*** **	**0.001*** **	**<0.0001*** **
Dry matter (%)	42.49 ± 0.16^{c}	42.84 ± 0.07^{b}	43.10 ± 0.08^{a}	**<0.0001*** **	**0.001*** **	**<0.0001*** **
Protein (%)	18.26 ± 0.11^{a}	18.09 ± 0.05^{b}	17.85 ± 0.07^{c}	**<0.0001*** **	**0.015***	**<0.0001*** **
Fat (%)	22.82 ± 0.10^{a}	22.49 ± 0.06^{b}	22.17 ± 0.07^{c}	**<0.0001*** **	**<0.0001*** **	**<0.0001*** **
Carbohydrates	0.03 ± 0.05^{c}	0.81 ± 0.07^{b}	1.54 ± 0.17^{a}	**<0.0001*** **	**<0.0001*** **	**<0.0001*** **
Ash	1.37 ± 0.08^{b}	1.45 ± 0.06^{ab}	1.53 ± 0.06^{a}	**0.014***	0.188^{ns}	**0.008****
pH	6.42 ± 0.02^{a}	6.37 ± 0.02^{b}	6.32 ± 0.02^{c}	**<0.0001*** **	**0.002****	**<0.0001*** **

Data are presented as mean ± SEM (standard error of the mean). Different superscript letters in the same row indicate statistically significant differences among samples ($p < 0.05$). SA0, control sample; SA1, sample with 1% *Astragalus membranaceus* powder; SA3, sample with 3% *Astragalus membranaceus* powder. ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Results of colour analysis

The incorporation of *Astragalus membranaceus* powder produced a selective effect on the instrumental colour of the beef snack samples (Table 4). The lightness parameter L* was not significantly affected by formulation, as shown by both the global *p*-value (p = 0.784) and Dunnett comparisons for SA0 vs. SA1 and SA0 vs. SA3, indicating that the addition of *Astragalus* did not substantially modify sample brightness. In contrast, a* decreased significantly from SA0 to SA3, showing a reduction in redness, while b* showed significant differences among samples, with SA1 presenting the lowest value and SA3 partially recovering yellowness. These changes were also reflected in the derived colour parameters: Chroma (C*) decreased significantly with *Astragalus* addition, suggesting lower colour saturation, whereas hue angle (h°) increased progressively, indicating a shift in colour tone. The total colour difference ΔE increased from SA1 to SA3, confirming that the 3% addition produced a more pronounced visual deviation from the control.

The Dunnett test supports this interpretation: except for L*, both supplemented samples differed significantly ($p < 0.05$) from the control for a*, C* and h°, while for b* the difference

was significant but stronger in SA3 than in SA1. Similar effects have been reported in meat systems supplemented with plant-derived ingredients, where the incorporation of botanical powders or polyphenol-rich extracts modified redness, yellowness, Chroma and hue angle due to pigment dilution, matrix interactions and the intrinsic colour of the added plant material.

The colour changes observed in the present study are consistent with previous findings on beef products supplemented with plant-derived antioxidant ingredients. Zahid et al. (2018) reported that grape seed extract and clove extract influenced the evolution of L*, a*, b*, Chroma, and hue angle in beef patties during refrigerated storage, indicating that natural antioxidant extracts may contribute to colour stability by limiting oxidative discoloration. In a similar meat matrix, Fruet et al. (2020) showed that the addition of tannin extract from Quebracho Colorado wood reduced redness and Chroma values and increased hue angle, suggesting a shift toward a less intense red colour and greater browning tendency, depending on the concentration and intrinsic colour of the extract used.

Table 4. Instrumental color parameters of beef snack samples supplemented with *Astragalus membranaceus* powder

Colour parameter	Samples			Global *p*-value	Dunnett SA0 vs. SA1	Dunnett SA0 vs. SA3
	SA0	SA1	SA3			
L*	46.81 ± 0.65^{a}	44.42 ± 0.48^{a}	44.13 ± 0.24^{a}	0.784^{ns}	0.830^{ns}	0.981^{ns}
a*	18.03 ± 0.37^{a}	16.16 ± 0.10^{b}	15.26 ± 0.27^{c}	**<0.0001*****	**<0.0001*****	**<0.0001*****
b*	18.47 ± 0.48^{b}	17.42 ± 0.16^{b}	18.13 ± 0.14^{a}	**<0.0001*****	**0.042***	**<0.0001*****
Croma (C*)	25.96 ± 0.26^{a}	24.86 ± 0.23^{b}	24.27 ± 0.16^{c}	**<0.0001*****	**<0.0001*****	**<0.0001*****
Hue angle (h°)	0.80 ± 0.01^{c}	0.88 ± 0.01^{b}	0.97 ± 0.01^{a}	**<0.0001*****	**<0.0001*****	**<0.0001*****
ΔE	-	2.26 ± 0.40	4.59 ± 0.36	**<0.0001*****	-	-

Data are presented as mean ± SEM. Different superscript letters in the same row indicate statistically significant differences among samples ($p < 0.05$). SA0, control sample; SA1, sample with 1% *Astragalus membranaceus* powder; SA3, sample with 3% *Astragalus membranaceus* powder. ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Results of texture analysis

Texture analysis revealed marked changes in the mechanical behaviour of the beef snack samples supplemented with Astragalus membranaceus powder (Table 5). Warner–Bratzler shear force increased from SA0 to SA3, with a significant global effect ($p = 0.010$), although Dunnett's test indicated that only SA3 differed very significantly from the control ($p = 0.006$). Work of shear also increased significantly in both supplemented samples ($p = 0.000$), suggesting greater resistance of the product structure to cutting. This tendency can be associated with the physicochemical changes observed in the samples, particularly the reduction in moisture and the increase in dry matter and carbohydrate content, which may have contributed to a more compact meat matrix (Table 3).

The TPA parameters confirmed this trend. Hardness, cohesiveness, and gumminess increased significantly in SA1 and SA3 compared with SA0 ($p < 0.05$), indicating that the addition of *Astragalus powder* promoted a firmer and more cohesive structure. At the same time, springiness decreased significantly in the supplemented samples, suggesting a reduction in elasticity as the level of plant powder increased. In contrast, chewiness and adhesiveness were not significantly affected ($p > 0.05$), as shown by both the global *p*-values and Dunnett comparisons, indicating that the main structural effects were related to firmness, cohesion, and shear resistance rather than to all texture dimensions.

This behaviour is consistent with previous observations in meat products enriched with plant-derived ingredients, where fibre and carbohydrate-rich materials may modify water retention, increase the solid fraction of the formulation, and influence protein–matrix interactions, leading to changes in hardness, cohesiveness, gumminess, and shear force (Marczak & Mendes, 2024; Mehta et al., 2015).

Table 5.Warner-Bratzler and Texture Profile Analysis parameters of beef snack samples supplemented with *Astragalus membranaceus* powder

Texture test	Texture parameter	Samples			Global *p*-value	Dunnett SA0 vs. SA1	Dunnett SA0 vs. SA3
		SA0	SA1	SA3			
Warner-Bratzler	Shear force, N/cm^2	15.18 ± 0.97^{b}	16.71 ± 1.26^{ab}	17.49 ± 0.70^{a}	**0.010***	0.058^{ns}	**0.006****
	Work of shear, mJ	47.13 ± 4.67^{b}	55.72 ± 2.19^{a}	59.07 ± 1.34^{a}	**0.000*****	**0.002****	**0.000*****
Texture Profile analysis (TPA)	Hardness, N	33.36 ± 0.78^{b}	35.67 ± 1.16^{a}	36.72 ± 0.67^{a}	**0.000*****	**0.003****	**0.000*****
	Cohesiveness, -	0.46 ± 0.03^{b}	0.51 ± 0.02^{a}	0.52 ± 0.03^{a}	**0.002****	**0.006****	**0.001****
	Springiness, -	0.71 ± 0.07^{a}	0.59 ± 0.05^{b}	0.58 ± 0.06^{b}	**0.009****	**0.014***	**0.010***
	Gumminess, N	15.22 ± 1.13^{b}	18.20 ± 1.01^{a}	19.24 ± 0.94^{a}	**0.000*****	**0.001*****	**0.000*****
	Chewiness N	10.80 ± 1.56^{a}	10.71 ± 1.09^{a}	11.21 ± 1.46^{a}	0.833^{ns}	0.991^{ns}	0.858^{ns}
	Adhesiveness, mJ	0.52 ± 0.04^{a}	0.53 ± 0.02^{a}	0.56 ± 0.01^{a}	0.074^{ns}	0.987^{ns}	0.075^{ns}

Data are presented as mean ± SEM. Different superscript letters in the same row indicate statistically significant differences among samples ($p < 0.05$). SA0, control sample; SA1, sample with 1% *Astragalus membranaceus* powder; SA3, sample with 3% *Astragalus membranaceus* powder. ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Results of antioxidant and bioactive compounds

The bioactive profile of *Astragalus membranaceus* root powder is presented in Table 6. The powder showed high antioxidant activity, with DPPH and ABTS inhibition values of 74.59% and 76.94%, respectively, indicating a strong radical-scavenging potential.

Table 6. Bioactive compounds and antioxidant activity of *Astragalus membranaceus* root powder

Parameters	*Astragalus membranaceus* root powder
DPPH inhibition (%)	74.59 ± 0.34
ABTS inhibition (%)	76.94 ± 0.17
Total flavonoids (mg CE/g DW)	0.86 ± 0.01
Total polyphenols (mg GAE/g DW)	3.16 ± 0.03
Total carotenoids (mg CT/g DW)	0.50 ± 0.01
β-caroten (mg/g DW)	0.32 ± 0.00
Lycopene (mg/g DW)	0.13 ± 0.00

Values are expressed as mean ± standard error of the mean. DPPH and ABTS results are expressed as inhibition percentage. CE: catechin equivalents; GAE: gallic acid equivalents; CT: carotenoid equivalents; DW: dry weight.

Among the quantified bioactive compounds, total polyphenols had the highest value (3.16 mg GAE/g DW), followed by total flavonoids (0.86 mg CE/g DW) and total carotenoids (0.50 mg CT/g DW).

The presence of β-carotene and lycopene further supports the contribution of *Astragalus membranaceus* powder as a plant ingredient with antioxidant and bioactive potential, justifying its use in the formulation of the experimental beef snack samples.

The results presented in Table 7 show that both *Astragalus membranaceus* concentration and thermal treatment influenced the antioxidant activity and the bioactive compound profile of the beef snack samples, but the magnitude of these effects depended on the analysed parameter. The percentage of *Astragalus powder* had a significant effect on all parameters evaluated (*p*-value Concentration < 0.0001), confirming that the progressive enrichment from SA0 to SA3 was the main factor responsible for the increase in DPPH and ABTS inhibition, total flavonoids, total polyphenols, carotenoids, β-carotene and lycopene. This result is consistent with the composition of the plant powder itself, which showed high DPPH and ABTS inhibition values and relevant contents of total polyphenols, flavonoids and carotenoids Table 6. Therefore, the increase observed in SA1 and SA3 can be directly associated with the bioactive contribution of *Astragalus membranaceus* root powder.

Thermal treatment had a more selective influence. It significantly affected DPPH inhibition, total polyphenols, total carotenoids, β-carotene and lycopene ($p < 0.05$), while its effect was not significant for ABTS inhibition and total flavonoids ($p > 0.05$). This indicates that heating did not uniformly reduce the antioxidant potential of the samples; in some cases, heat-treated samples showed higher DPPH, ABTS or polyphenol values than untreated samples. Similar observations have been reported in studies on plant-derived antioxidants and meat systems, where phenolic-rich ingredients may increase antioxidant capacity and where processing can modify the availability and activity of bioactive compounds. Similar effects were reported by Xu

et al. (2007), who showed that heat treatment increased the free fraction of phenolic acids and the total antioxidant activity of citrus peel extracts. Călinoiu & Vodnar (2019) also reported that thermal processing improved total phenolic content in wheat and oat bran by 22.49% and 25.84%, respectively, supporting the idea that heating can promote the release of phenolic compounds from plant matrices. Therefore, in the present study, the higher DPPH, ABTS, and polyphenol values observed in some heat-treated samples may be associated with the thermal release of bioactive compounds from *Astragalus membranaceus* powder and their improved extractability within the meat matrix.

Table 7. Bioactive compounds and antioxidant activity of untreated and heat-treated beef snack samples supplemented with *Astragalus membranaceus* powder

Parameter	Thermal treatment	Samples			*p*-value Treatment	*p*-value Concentration	*p*-value % × Treatment
		SA0	SA1	SA3			
DPPH inhibition (%)	Untreated	15.76 ± 0.20	28.54 ± 0.15	32.65 ± 0.20	**<0.0001*** **	**<0.0001*** **	**<0.0001*** **
	Heat-treated	16.94 ± 0.12	30.54 ± 0.17	35.75 ± 0.12			
ABTS inhibition (%)	Untreated	16.74 ± 0.23	24.87 ± 13.47	34.41 ± 0.33	0.067^{ns}	**<0.0001*** **	0.455^{ns}
	Heat-treated	17.84 ± 0.23	32.15 ± 0.14	37.75 ± 0.12			
Total flavonoids (mg CE/g DW)	Untreated	0.20 ± 0.02	0.34 ± 0.16	0.76 ± 0.01	0.772^{ns}	**<0.0001*** **	**0.001*** **
	Heat-treated	0.24 ± 0.01	0.46 ± 0.01	0.62 ± 0.01			
Total polyphenols (mg GAE/g DW)	Untreated	0.18 ± 0.02	0.64 ± 0.02	0.90 ± 0.02	**0.003** *	**<0.0001*** **	**<0.0001*** **
	Heat-treated	0.22 ± 0.02	0.71 ± 0.03	0.72 ± 0.01			
Total carotenoids (mg CT/g DW)	Untreated	–	0.29 ± 0.01	0.41 ± 0.01	**<0.0001*** **	**<0.0001*** **	**<0.0001*** **
	Heat-treated	–	0.26 ± 0.01	0.32 ± 0.01			
β-caroten (mg/g DW)	Untreated	–	0.17 ± 0.01	0.27 ± 0.01	**0.022***	**<0.0001*** **	**<0.0001*** **
	Heat-treated	–	0.20 ± 0.01	0.22 ± 0.01			
Lycopene (mg/g DW)	Untreated	–	0.04 ± 0.01	0.07 ± 0.00	**<0.0001*** **	**<0.0001*** **	**<0.0001*** **
	Heat-treated	–	0.07 ± 0.01	0.08 ± 0.01			

Values are expressed as mean ± standard error of the mean. DPPH and ABTS results are expressed as inhibition percentage. CE: catechin equivalents; GAE: gallic acid equivalents; CT: carotenoid equivalents; DW: dry weight. SA0, control sample; SA1, sample with 1% *Astragalus membranaceus* powder; SA3, sample with 3% *Astragalus membranaceus* powder. ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The significant Concentration × Treatment interaction observed for DPPH inhibition, total flavonoids, total polyphenols, total carotenoids, β-carotene and lycopene indicates that the effect of thermal treatment depended on the concentration of *Astragalus* powder. For example, total polyphenols increased after heat treatment in SA0 and SA1, but decreased in SA3, suggesting that higher levels of plant powder may behave differently during thermal processing. Similarly, carotenoids and β-carotene showed concentration-dependent changes after heating, which may reflect both thermal degradation and enhanced release from the plant–meat matrix. In contrast, the interaction was not significant for ABTS inhibition ($p > 0.05$), indicating a more stable response of this assay across thermal conditions. Studies on *Astragalus membranaceus* also support its relevance as a source of phenolic and antioxidant compounds, with recent research reporting antioxidant activity and phenolic compounds in *A. membranaceus* extracts and

modified plant materials (Liu et al., 2024; Shahrivari-Baviloliaei et al., 2024).

Overall, the data indicate that *Astragalus membranaceus* root powder acted as an effective source of bioactive compounds in the beef snack formulation, with the 3% addition generally producing the highest antioxidant and phytochemical values. However, the significant interactions for several parameters show that the final bioactive profile was not determined only by concentration, but also by the way in which the plant powder responded to thermal processing within the meat matrix.

Hedonic analysis

The hedonic analysis of beef meat sticks supplemented with *Astragalus* showed a generally favourable sensory perception across all evaluated attributes (Figure 1). SA0 presented the highest scores, while SA1 remained very close to the control, indicating that the lower level of *Astragalus* incorporation maintained a balanced hedonic profile in terms of appearance, odour, taste, texture, and overall perception. Although SA3 showed slightly lower scores, the values remained within an acceptable sensory range, suggesting that *Astragalus* addition was compatible with the product matrix, with only moderate changes in consumer perception at the higher inclusion level. A similar trend was reported by Lee et al. (2021)who observed that the use of herbal medicinal extracts, including *Astragalus membranaceus*, in cooked meat products did not negatively affect key visual and eating-quality attributes, supporting the potential use of herbal ingredients in meat product development.

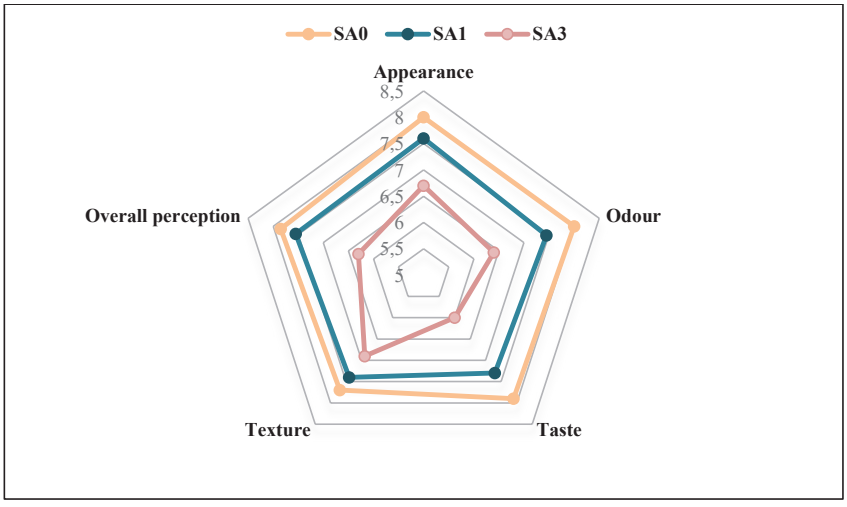


Figure 1. Hedonic analysis of samples

Quantitative Descriptive Analysis

The QDA results (Table 8) showed that the incorporation of *Astragalus membranaceus* powder induced a differentiated sensory profile in beef meat sticks, mainly by increasing the intensity of yellowish-brown colour, herbal odour, herbal taste, bitterness, astringency, and aftertaste. These changes were dose-dependent, with SA3 showing the most pronounced sensory differentiation from the control. However, the core meat-related attributes, including brown colour, cooked meat odour, specific beef odour, cooked meat aroma, texture, and juiciness, remained at relatively high intensity levels, indicating that the beef matrix was still sensorially recognizable after supplementation. The non-significant differences observed for spicy odour and texture suggest that *Astragalus* addition did not substantially alter these dimensions of the product. Similar findings were reported by Lee et al. (2021), who observed that herbal medicinal extracts, including *Astragalus membranaceus*, could be incorporated into cooked meat systems without impairing key quality traits, while producing specific changes in colour, flavour, and

palatability. In addition, Szymandera-Buszka et al. (2020) showed that plant-derived extracts can modify the aroma and taste profile of meat products, particularly through herbal, spicy, essential-oil-like or bitter notes, while the final acceptability depends on the balance between these added sensory notes and the characteristic meat flavour.

Table 8. Sensory attributes evaluated by Quantitative Descriptive Analysis (QDA) in beef snack samples supplemented with *Astragalus membranaceus* powder

Sensory attribute	Samples			*p*-value
	SA0	SA1	SA3	
Brown colour	8.10 ± 0.85^{a}	7.65 ± 1.23^{ab}	7.10 ± 1.12^{b}	**0.034***
Yellowish-brown colour	1.20 ± 0.41^{c}	2.35 ± 1.04^{b}	4.10 ± 0.97^{a}	**<0.0001****
Cooked meat odour	8.05 ± 0.76^{a}	7.60 ± 1.19^{ab}	6.90 ± 1.02^{b}	**0.004****
Specific beef odour	7.85 ± 0.99^{a}	7.45 ± 1.05^{ab}	6.75 ± 1.12^{b}	**0.009****
Herbal odour	1.10 ± 0.31^{c}	3.10 ± 1.07^{b}	5.70 ± 0.92^{a}	**<0.0001****
Spicy odour	3.80 ± 1.06^{a}	3.95 ± 1.23^{a}	4.10 ± 0.97^{a}	0.732^{ns}
Off-odour	1.05 ± 0.22^{b}	1.20 ± 0.41^{ab}	1.55 ± 0.60^{a}	**0.007****
Cooked meat aroma	8.00 ± 0.73^{a}	7.55 ± 1.28^{ab}	6.80 ± 1.01^{b}	**0.004****
Herbal taste	1.10 ± 0.31^{c}	3.05 ± 0.94^{b}	5.70 ± 0.92^{a}	**<0.0001****
Bitterness	1.25 ± 0.64^{c}	2.40 ± 0.99^{b}	4.75 ± 1.16^{a}	**<0.0001****
Astringency	1.15 ± 0.49^{c}	2.20 ± 1.01^{b}	4.40 ± 0.99^{a}	**<0.0001****
Texture	7.60 ± 1.19^{a}	7.35 ± 1.09^{a}	6.85 ± 0.88^{a}	0.070^{ns}
Juiciness	7.20 ± 1.06^{a}	7.00 ± 0.86^{ab}	6.45 ± 1.10^{b}	**0.050***
Aftertaste	1.40 ± 0.75^{c}	2.75 ± 1.21^{b}	5.10 ± 1.02^{a}	**<0.0001****
Overall acceptability	7.85 ± 0.99^{a}	7.65 ± 1.23^{a}	6.35 ± 1.14^{b}	**0.000****

Data are presented as mean ± SD. Different superscript letters in the same row indicate statistically significant differences among samples ($p < 0.05$). SA0, control sample; SA1, sample with 1% *Astragalus membranaceus* powder; SA3, sample with 3% *Astragalus membranaceus* powder. ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Principal Component Analysis

The PCA biplot (Figure 2) revealed a well-defined association between the beef meat stick formulations and the sensory descriptors generated by QDA. SA0 was mainly correlated with conventional meat-related attributes, including brown colour, cooked meat odour, cooked meat aroma, and specific beef odour, confirming the predominance of the typical beef sensory profile in the control sample. SA1 was closely associated with overall acceptability, juiciness, texture, and spicy odour, suggesting that the lower level of *Astragalus membranaceus* powder supported a balanced sensory configuration, in which the meat matrix and the added botanical ingredient were sensorially well integrated. SA3 was primarily correlated with descriptors reflecting the contribution of *Astragalus membranaceus*, such as herbal odour, herbal aroma, herbal taste, slightly green colour, aftertaste, bitterness, astringency, and off-odour. These associations indicate that increasing the addition level enhanced the perception of plant-derived sensory notes and contributed to a more differentiated sensory identity of the product. Overall, the PCA confirms that sample discrimination was governed mainly by the interaction between the beef matrix and the intensity of *Astragalus*-related sensory attributes.

The PCA partially supports the QDA results presented in Table 8, but also highlights some differences in the way sensory attributes contributed to sample discrimination. According to the QDA data, SA0 showed higher intensities for brown colour, cooked meat odour, specific beef odour, and cooked meat aroma, whereas SA3 presented significantly higher values for yellowish-brown colour, herbal odour, herbal taste, bitterness, astringency, aftertaste, and off-odour. These trends are generally reflected in the PCA, where SA0 was positioned in relation to meat-related descriptors, while SA3 was associated with attributes linked to the sensory contribution of *Astragalus membranaceus*.

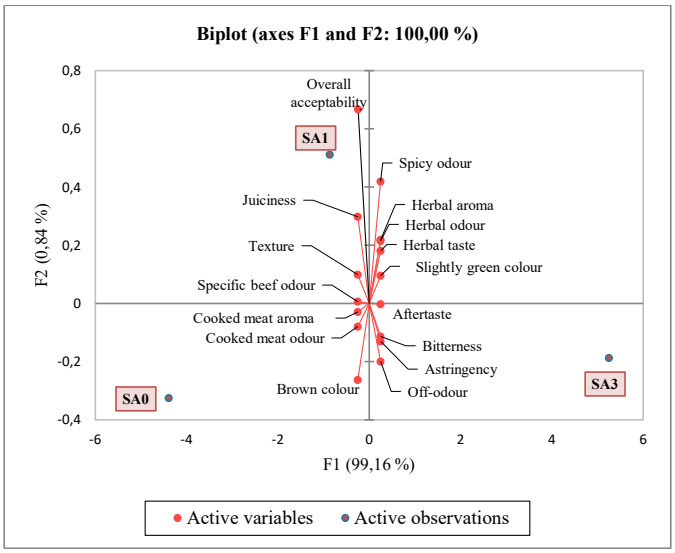


Figure 2. PCA biplot of QDA sensory attributes in beef meat sticks supplemented with *Astragalus membranaceus* powder

However, some attributes with significant differences in the QDA table, such as juiciness and off-odour, had a weaker contribution to the graphical separation in the PCA compared with herbal, bitter, astringent, and aftertaste descriptors. In contrast, texture and spicy odour, which were not significantly different among samples in the QDA analysis, appeared closer to SA1 in the PCA, suggesting that their role was more related to the overall sensory configuration of this sample rather than to strong statistical discrimination among formulations. Thus, PCA complements the univariate QDA results by showing how the attributes act together in defining the sensory identity of each beef snack formulation.

CONCLUSIONS

The incorporation of *Astragalus membranaceus* powder influenced the physicochemical, bioactive, textural, colour, and sensory characteristics of beef snack samples in a concentration-dependent manner. Increasing levels of *Astragalus* slightly reduced moisture, protein, fat, and pH, while increasing dry matter, carbohydrates, and ash content, indicating a clear contribution of the plant powder to the solid fraction of the formulation.

Instrumental colour and texture analyses showed that *Astragalus* addition modified redness, colour saturation, hue angle, shear resistance, hardness, cohesiveness, and gumminess, while lightness, chewiness, and adhesiveness were less affected. These changes suggest that the plant powder interacted with the meat matrix, contributing to a slightly firmer and more structured product.

From a bioactive perspective, *Astragalus membranaceus* root powder improved the antioxidant and phytochemical profile of beef snacks, mainly in a concentration-dependent manner. SA3 generally showed the highest values, confirming the contribution of the plant powder. Thermal treatment frequently increased DPPH and ABTS inhibition, suggesting a possible enhancement of antioxidant compound release or extractability within the meat matrix. Thus, the bioactive potential of the samples was influenced by both *Astragalus* concentration and thermal processing.

The sensory results confirmed that the supplemented samples maintained acceptable sensory characteristics. Hedonic analysis showed that SA1 remained close to the control sample, indicating good consumer perception at the lower inclusion level. QDA and PCA further demonstrated that SA0 was associated with typical beef-related attributes, SA1 with balanced sensory characteristics and overall acceptability, while SA3 was more strongly associated with plant-derived descriptors such

as herbal odour, herbal taste, bitterness, astringency, and aftertaste.

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BEYOND CONVENTIONAL CHICKEN MEATBALLS THROUGH OAT FIBER INCORPORATION AND ITS PHYSICOCHEMICAL, TEXTURAL, AND SENSORY EFFECTS

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Abstract

Growing interest in clean-label meat products has encouraged the use of dietary fibers that improve nutritional value and technological functionality. Oat fiber was incorporated at 3% and 6% into chicken meatballs from breast and thigh meat, and compared with a control formulation. The samples were evaluated for proximate composition, energy value, pH, water activity, color, Warner–Bratzler cutting properties, texture profile analysis, and sensory perception using hedonic evaluation, descriptive sensory analysis, and PCA. Oat fiber affected most quality parameters. Moisture decreased from 70.96% in the control to 70.05% and 69.50% in the 3% and 6% samples, while dry matter, ash, and fiber increased. Fiber increased from an undetectable level to 2.77% and 5.12%, respectively, whereas protein, fat, energy value, pH, and water activity decreased markedly ($p < 0.001$). Color analysis indicated significant changes in L, b*, and chroma, whereas a* and hue were not significantly affected. Oat fibre addition increased firmness-related parameters, with hardness, Warner-Bratzler shear force, and work of shear rising from 13.52 N, 9.31 N/cm², and 83.24 mJ to 19.29 N, 11.02 N/cm², and 122.55 mJ, respectively; however, springiness and cohesiveness decreased, indicating a firmer but less elastic and less cohesive structure.*

Key words*: chicken meatballs, dietary fiber, meat science, oat fiber, poultry meat products.*

INTRODUCTION

Nutrition is an essential activity that supports human survival. When it comes to resources, a diet based on animal proteins ranks first (Khillare & Kaushal, 2022). Meat and meat products represent a significant part of the human diet, as they contain important nutrients that are difficult to obtain from vegetables and their derivatives (Anchidin et al., 2024; Boișteanu et al., 2023; Boișteanu et al., 2024; Ciobanu et al., 2025; Ianiţchi et al., 2026).

Poultry meat is considered the most consumed meat of animal origin worldwide, recording high consumption rates, particularly in the case of chicken meat (Connolly & Campbell, 2023). Globally, poultry meat consumption has increased dramatically due to changes in trade, the economy, and dietary patterns (Sammani et al., 2025). In this context, it is estimated that by 2034, global poultry meat consumption will reach 173 million tons carcass weight equivalent, representing approximately 62% of the additional growth in global meat consumption (OECD/FAO, 2025). It can be inferred that these results are also due to the absence of cultural or religious restrictions regarding chicken meat, which is one of the most popular products among consumers (Coșarcă et al., 2025).

Demand for ready-to-eat foods, such as canned, frozen, and pre-prepared meals, has increased in the contemporary era due to changes in dietary patterns brought about by urbanization, smaller family units, higher incomes, and the growing number of working women (Hossan et al., 2024). Convenient meat products, which are easy to prepare and consume, are increasingly in demand as a result of rapid urbanization and lifestyle changes, factors that significantly influence consumption habits. Among these products, meatballs are among the most appreciated by consumers (Keerthi et al., 2023). One of the fastest-expanding segments of ready-to-eat meat products on the global market is represented by products such as schnitzels and

nuggets, in which the use of poultry meat accounts for a considerable share. Due to increasing consumption, various ready-to-eat chicken-based products, including chicken wings, marinated chicken breast, meatballs, nuggets, chicken popcorn, and chicken sausages, are increasingly in demand (Khillare & Kaushal, 2022). This orientation toward the use of chicken meat is due to certain characteristics, such as its neutral flavour, consistent and pleasant texture, and light colour. These characteristics make it more suitable for processing compared to other types of meat. They also allow producers to obtain the desired textures and flavour profiles according to market requirements and marketing strategy, as well as the targeted consumer category, such as adults or children (Petracci et al., 2013).

The consumption of meat and meat products consumption is increasing globally as a result of rising incomes and population growth (Flocea et al., 2024; Gucianu et al., 2024). Consumers are demanding healthier meat and meat products, with reduced fat, cholesterol, sodium chloride, and nitrite contents, as well as the addition of various natural ingredients that promote health (Anchidin et al., 2024; Ciobotaru et al., 2024; Manoliu et al., 2023). For this reason, the food industry is reformulating food products to enhance the physiological functionality of natural nutrients or by adding ingredients in order to meet this growing consumer demand (Anchidin et al., 2023; Ciobotaru et al., 2024; Manoliu et al., 2025; Gucianu et al., 2025).

Meat has several physiological functions in the human body and is usually devoid of dietary fibre, which is widely present in plants. According to epidemiological research, the consumption of foods deficient in dietary fibre is one of the main risk factors for the occurrence of numerous lifestyle-related disorders (Manoliu et al., 2024; Mishra et al., 2023). The industry is aware of the need to provide improved food products containing ingredients that support health and prevent nutrition-related disorders (Anchidin et al., 2025; Gucianu et al., 2023; Matei et al., 2024). The addition of substances not usually present in meat, such as dietary fibre, to meat products is a viable way to improve their quality, considering the increased demand for functional and healthy diets (Anchidin et al., 2025; Gucianu, et al., 2024; Ciobanu et al., 2025; Flocea et al., 2025; Manoliu et al., 2025).

The reformulation of meat products through the incorporation of dietary fibres is a well-established research direction in the scientific literature, due to the potential of these ingredients to improve the physicochemical characteristics, textural properties, and sensory acceptability of finished products. The use and incorporation of dietary fibres in meat products are becoming increasingly important. The suitability of dietary fibres for the reformulation of meat products is mainly associated with their functional properties, including water retention, lubrication, reduction of cooking losses, texture modification, and neutral flavour. Dietary fibres obtained from various plant sources, including dehydrated fruits, vegetables, and cereal-derived materials, have been widely used in the food industry and have shown promising technological results (Marczak & Mendes, 2024; Mehta et al., 2015; Mishra et al., 2023; Talukder & Sharma, 2010).

Oat is one of the oldest cereal crops cultivated and consumed worldwide. Its popularity has increased due to its valuable nutritional composition and the multifunctional benefits provided by selected bioactive compounds (Paudel et al., 2021). The technological relevance of oat fibres in meat products is determined by their hydrocolloidal properties, which allow them to function as texturizing agents, partial fat substitutes, and ingredients with a role in improving water- and fat-holding capacity during heat treatment (Piñero et al., 2008). Studies conducted on low-fat beef patties, low-fat burgers, chicken meat patties, and low-fat chicken nuggets have shown that oat fibre, bran, or flour can improve cooking yield, moisture retention, fat retention, water-holding capacity, and emulsion stability, while maintaining acceptable sensory characteristics in reformulated meat products (Piñero et al., 2008; Santhi & Kalaikannan, 2014; Summo et al., 2020; Talukder & Sharma, 2010).

Within meat product reformulation strategies, oat fibres represent a category of plant-based ingredients with significant technological and nutritional value. Interest in their use is mainly associated with the presence of β-glucan, an important component of oat dietary fibre, described as a hydrocolloidal polysaccharide

predominantly located in the soluble fibre fraction and composed of D-glucose monomers (Majumdar et al., 2024; Singla et al., 2024; Summo et al., 2020; Wang & Ellis, 2014).

Therefore, the present study aims to evaluate the effects of incorporating oat fibres on the physicochemical, textural, and sensory characteristics of chicken meatballs made from thigh and breast meat. The proposed approach focuses on obtaining a reformulated product that exploits the technological and nutritional potential of oat fibres by assessing their effects on the physicochemical composition, colour parameters, textural properties, and sensory acceptability of chicken meatballs. Thus, this research contributes to supporting the use of oat fibres as valuable ingredients in the development of chicken meat products with improved technological and nutritional value.

MATERIALS AND METHODS

For this study, three batches of chicken meatballs were manufactured: a control batch without oat fibre addition and two experimental batches formulated with 3% and 6% oat fibre, respectively. The products were prepared using refrigerated chicken meat purchased from METRO Cash & Carry SRL. The chicken meat used for meatball formulation consisted of a standardized mixture of 60% chicken thigh meat and 40% chicken breast meat, and this ratio was maintained constant across all experimental batches. Oat fibre powder was purchased from NO SUGAR SHOP SRL, while the seasoning ingredients, namely salt, garlic powder, sweet paprika, onion powder, dried parsley, black pepper, and ham flavour, were purchased from a local store in Iași, Romania.

The technological process consisted of trimming the chicken meat, mincing it through a 5 mm plate, mixing it with the seasoning ingredients and oat fibre, and manually shaping the composition into meatballs of approximately 40-50 g. Semolina flour was used during shaping to improve handling.

The heat treatment was identical for all batches and included preheating, thermal processing by boiling, drying, and baking, according to the parameters presented in Table 1. After thermal processing, the samples were cooled at 10-12°C for approximately 6h, vacuum-packed, labelled, and stored under refrigeration until laboratory analysis.

Table 1. Thermal treatment parameters applied to chicken meatballs

Technological stage	Time (min)	Chamber temperature (°C)	Core temperature (°C)	Relative humidity (%)
Preheating	10	60	52	80-85
Thermal processing by boiling	-	76	72	99
Drying	10	78	72	10
Baking	60	82	80	40-50

The physicochemical characterization of the chicken meatball samples included the determination of moisture, dry matter, protein, fat, ash, carbohydrates excluding fibre, fibre content, energy value, pH, and water activity. Moisture, protein, fat, and ash contents were determined according to the methods described by (Dinçer et al., 2018). Dietary fibre content was determined according to the method described by Bajcic et al. (2019), previously validated and applied for total dietary fibre determination in meat products. Carbohydrates excluding fibre and energy value were calculated by difference, according to the approach used for proximate composition assessment.

The pH values of the experimental batches were measured using a HI98163 pH meter equipped with an FC2323 electrode (SC Hanna Instruments SRL). Due to the blade-shaped tip of the electrode, measurements were performed by direct insertion into the meat samples.

Water activity (aw) was determined with an AquaLab 4TE instrument (Addium Inc., Washington, DC, USA) after sample homogenization by grinding.

The instrumental colour of the chicken meatball samples was determined using a Konica Minolta CR-410 colorimeter (Konica Minolta, Inc., Tokyo, Japan), under a D65 illuminant and a 10° standard observer. The results were expressed in the CIELAB colour space as lightness (L*), redness (a*), and yellowness (b*). Based on these coordinates, chroma (C*) and hue angle (h°) were calculated according to the equations

reported by Sen et al. (2014), while the total colour difference (ΔE) was calculated according to (Tkacz et al., 2020).

Texture analysis was performed using a TA1+1K Plus texturometer (Ametek, Inc., USA). Before analysis, the samples were cut into standardized shapes depending on the test performed. For Texture Profile Analysis (TPA), cylindrical samples measuring 2.5 cm in height and approximately 2.0 cm in diameter were used for the double-compression test. For Warner-Bratzler shear force determination, parallelepiped samples measuring approximately 5.0 × 1.5 cm were prepared and sheared using a Warner-Bratzler V-shaped notch blade.

$$\text{Chroma (C*)} = (a*^2 + b*^2)^{1/2} \quad (1)$$

$$\text{Hue angle (h*)} = tan^{-1} \times b^*/a^* \quad (2)$$

$$\Delta E^* = [(\Delta a^*)^2 + (\Delta b^*)^2 + (\Delta L^*)^2]^{0.5} \quad (3)$$

The sensory evaluation included hedonic analysis and quantitative descriptive analysis. Descriptive analysis was used because it allows the sensory profile of food products to be quantified through relevant attributes evaluated by trained assessors, while hedonic analysis provides information on product acceptability (Mongi & Gomezulu, 2022; Ruiz-Capillas et al., 2021). Principal component analysis (PCA) was subsequently applied to the descriptive sensory data, as this multivariate method is useful for reducing complex sensory datasets and identifying the main attributes responsible for differentiating the samples (Chapman et al., 2001).

Sensory analysis is an important method for assessing the perceived quality and acceptability of food products. Sensory characterization is used to obtain a complete qualitative and quantitative description of the sensory attributes of a product (Boișteanu et al., 2025). For centuries, this type of analysis has been used to approve or reject food products (Ciobanu et al., 2023).

In the present study, sensory evaluation was performed by a panel of 44 semi-trained assessors, consisting of 32 females and 12 males, aged between 20 and 22 years. Prior to the evaluation, the assessors were familiarized with the sensory descriptors and the evaluation procedure in order to ensure a common understanding of the attributes assessed. The descriptors “smoky odor” and “smoked taste” were included in the sensory profile to describe smoked/ham-like aromatic notes derived from the ham flavour used in the seasoning mixture, as the products were not subjected to a smoking process. Moreover, the baking stage applied during thermal treatment may have slightly enhanced the perception of these aroma notes, making them more detectable by the assessors. The sensory evaluation was performed in a specialized sensory analysis laboratory of IULS, under controlled environmental conditions. The samples were coded with random three-digit numbers and presented to the assessors in a randomized order to minimize order and expectation effects. A 5-minute break was provided between samples in order to reduce sensory fatigue and carry-over effects. During this interval, the assessors were provided with water and bread for palate cleansing and for neutralizing residual tastes before evaluating the next sample. The sensory session was conducted in accordance with the ethical principles of the Declaration of Helsinki. Participation was voluntary, and all assessors were informed about the purpose of the study and the nature of the samples before evaluation.

Statistical analysis was performed using XLSTAT software (Lumivero, Denver, CO, USA). Three experimental batches were analysed: CBO-0, CBO-3, and CBO-6. For each batch, all physicochemical determinations were performed in five analytical replicates (n = 5), except for colour measurements, which were carried out in ten analytical replicates (n = 10). The experimental data were processed to determine the mean values and standard deviations of the analysed parameters, and the results were expressed as mean ± standard deviation.

The significance of differences between batches was assessed using one-way analysis of variance (ANOVA), followed by Tukey’s post hoc test for multiple comparisons and Dunnett’s test for comparison of the experimental batches with the control batch, where appropriate. Statistical significance was considered at $p < 0.05$. Principal component analysis (PCA) was also

performed using XLSTAT to visualize the relationships between the experimental batches and the analysed variables.

RESULTS AND DISCUSSIONS

Effect of oat fibre addition on the physico-chemical properties of chicken meatballs

The nutritional composition of the oat fibre powder used in the formulation is presented in Table 2.

Table 2. Nutritional composition of oat fibre powder

Nutritional parameter of oat fiber powder	Value / 100 g
Fat	0.2 g
Carbohydrates	0.1 g
Dietary fibre	93.0 g
Protein	0.4 g
Salt	0.10 g

Values are expressed per 100 g of oat fibre powder

According to the product label, the ingredient was characterized by a very high dietary fibre content, representing 93.0 g/100 g, while the contents of carbohydrates, protein, fat and salt were very low, with values of 0.1, 0.4, 0.2 and 0.10 g/100 g, respectively. This composition indicates that the ingredient mainly contributed to the dietary fibre fraction of the chicken meatball formulations, with minimal direct contribution to available carbohydrates, protein or lipid content.

The physicochemical results in Table 3 show a clear reformulation effect induced by oat fibre addition. Moisture decreased significantly from 70.96 ± 0.09^{a} in CBO-0 to 70.05 ± 0.30^{b} in CBO-3 and 69.50 ± 0.30^{c} in CBO-6 (global $p < 0.0001$; Dunnett CBO-0 vs. CBO-3: $p = 0.000$; CBO-0 vs. CBO-6: $p < 0.0001$), while dry matter increased in the opposite direction from 29.04 ± 0.09^{c} to 29.95 ± 0.30^{b} and 30.50 ± 0.30^{a} under the same significance pattern. The decrease in moisture content observed in oat fibre-supplemented samples may be associated with the increased dry matter fraction introduced by the fibre ingredient. A similar trend was reported by Yılmaz (2005), who observed a progressive reduction in moisture content in low-fat meatballs as wheat bran concentration increased from 5% to 20%.

Protein and fat also decreased progressively, from 19.80 ± 0.02^{a} to 18.35 ± 0.08^{b} and 17.05 ± 0.08^{c} for protein, and from 7.56 ± 0.02^{a} to 7.00 ± 0.06^{b} and 6.35 ± 0.06^{c} for fat, with global $p < 0.0001$ and extremely significant Dunnett comparisons for both supplemented samples ($p < 0.001$). This trend is consistent with the low protein and fat contribution of the oat fibre powder and reflects the partial replacement of the meat matrix.

Table 3. Physicochemical characteristics of chicken meatball samples supplemented with oat fibre

Parameter	CBO-0	CBO-3	CBO-6	Global *p*-value	Dunnett CBO-0 vs. CBO-3	Dunnett CBO-0 vs. CBO-6
Moisture (%)	70.96 ± 0.09^{a}	70.05 ± 0.30^{b}	69.50 ± 0.30^{c}	**<0.0001*** **	**0.000*** **	**<0.0001*** **
Dry matter (%)	29.04 ± 0.09^{c}	29.95 ± 0.30^{b}	30.50 ± 0.30^{a}	**<0.0001*** **	**0.000*** **	**<0.0001*** **
Protein (%)	19.80 ± 0.02^{a}	18.35 ± 0.08^{b}	17.05 ± 0.08^{c}	**<0.0001*** **	**<0.0001*** **	**<0.0001*** **
Fat (%)	7.56 ± 0.02^{a}	7.00 ± 0.06^{b}	6.35 ± 0.06^{c}	**<0.0001*** **	**<0.0001*** **	**<0.0001*** **
Ash (%)	1.60 ± 0.02^{c}	1.68 ± 0.05^{b}	1.87 ± 0.05^{a}	**<0.0001*** **	**0.015***	**<0.0001*** **
Carbohydrates, excluding fibre **(%)**	0.08 ± 0.03^{b}	0.15 ± 0.03^{a}	0.11 ± 0.03^{ab}	**0.014***	**0.008****	0.266^{ns}
Fibre (%)	–	2.77 ± 0.08^{b}	5.12 ± 0.08^{a}	**<0.0001*** **	**<0.0001*** **	**<0.0001*** **
Energy value (kcal/100 g)	147.56 ± 0.36^{a}	142.54 ± 1.17^{b}	136.03 ± 1.17^{c}	**<0.0001*** **	**<0.0001*** **	**<0.0001*** **
Energy value (kJ/100 g)	617.35 ± 1.50^{a}	596.39 ± 4.89^{b}	569.15 ± 4.90^{c}	**<0.0001*** **	**<0.0001*** **	**<0.0001*** **
pH	6.52 ± 0.05^{a}	6.19 ± 0.01^{b}	6.12 ± 0.05^{c}	**<0.0001*** **	**<0.0001*** **	**<0.0001*** **
Water activity (a_w)	0.950 ± 0.001^{a}	0.936 ± 0.002^{b}	0.930 ± 0.001^{c}	**<0.0001*** **	**<0.0001*** **	**<0.0001*** **

Values are expressed as mean ± SD. Different superscript letters in the same row indicate significant differences among samples ($p < 0.05$). CBO-0, control; CBO-3, sample with 3% oat fibre; CBO-6, sample with 6% oat fibre. Carbohydrates were calculated by difference and exclude dietary fibre. Global *p*-value indicates the overall effect of oat fibre addition, while Dunnett comparisons show differences between the control and supplemented samples. ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Ash content increased from 1.60 ± 0.02^{c} to 1.68 ± 0.05^{b} and 1.87 ± 0.05^{a} (global $p < 0.0001$; Dunnett: $p = 0.015$ and $p < 0.0001$), while carbohydrates excluding fibre remained very low, as expected from the ingredient label: 0.08 ± 0.03^{b}, 0.15 ± 0.03^{a}, and 0.11 ± 0.03^{ab} (global *p*

= 0.014; Dunnett: p = 0.008 and p = 0.266). Fibre increased markedly from an undetectable level in CBO-0 to 2.77 ± 0.08^{b} in CBO-3 and 5.12 ± 0.08^{a} in CBO-6 (global p < 0.0001; Dunnett p < 0.0001 for both comparisons), confirming that oat fibre was the main driver of nutritional differentiation. Energy value decreased significantly from 147.56 ± 0.36^{a} kcal/100 g to 142.54 ± 1.17^{b} and 136.03 ± 1.17^{c} kcal/100 g (global p < 0.0001; Dunnett p < 0.0001), reflecting the replacement of energy-dense meat constituents by fibre. Similar decreasing patterns were observed for pH (6.52 ± 0.05^{a}, 6.19 ± 0.01^{b}, 6.12 ± 0.05^{c}) and water activity (0.950 ± 0.001^{a}, 0.936 ± 0.002^{b}, 0.930 ± 0.001^{c}), both with global p < 0.0001 and significant Dunnett comparisons.

These findings agree with previous studies on fibre-enriched poultry products. Santhi & Kalaikannan (2014) reported that oat flour addition in low-fat chicken nuggets significantly increased crude fibre, while reducing crude protein and fat, which supports the dilution pattern observed in the present study.

Overall, these results indicate that oat fibre incorporation effectively improved the fibre profile of chicken meatballs while inducing predictable compositional changes, mainly through reduced moisture, protein, fat, pH and water activity, and increased dry matter, ash and dietary fibre contents.

Table 4 displays the instrumental colour parameters of the chicken meatball samples that were treated with varying amounts of oat fibre. Significant changes were mostly observed in brightness, yellowness, and chroma when oat fibre was added (p < 0.05). From 59.93 ± 0.90^{b} in the control sample to 60.81 ± 0.76^{ab} in CBO-3 and 61.03 ± 1.22^{a} in CBO-6, the L* value increased slightly. Despite the modest numerical increase, the global p-value (p = 0.044) showed that the addition of oat fibre had a significant impact. Only the difference between CBO-0 and CBO-6 was significant, according to Dunnett's test (p = 0.034); CBO-3 did not differ substantially from the control (p = 0.100). This pattern was validated by Tukey's test, which classified CBO-3 as an intermediate group.

The redness parameter, a*, was not significantly affected by the formulation, with values ranging from 7.70 ± 1.67^{a} in CBO-0 to 8.00 ± 1.21^{a} in CBO-6. This was confirmed by the global p-value (p = 0.899), by the non-significant Dunnett comparisons for CBO-3 (p = 0.931) and CBO-6 (p = 0.864), and by the identical Tukey superscript letters assigned to all samples. In contrast, b* increased significantly from 17.51 ± 1.84^{b} in CBO-0 to 19.32 ± 0.95^{a} in CBO-3 and 19.57 ± 1.11^{a} in CBO-6. The global effect was significant (p = 0.004), and both Dunnett comparisons confirmed significant differences from the control sample (p = 0.011 for CBO-3 and p = 0.004 for CBO-6). A similar pattern was observed for chroma, which increased from 19.22 ± 1.45^{b} in CBO-0 to 20.93 ± 0.92^{a} in CBO-3 and 21.18 ± 0.88^{a} in CBO-6, with a significant global effect (p = 0.001) and significant Dunnett comparisons for both supplemented samples (p = 0.004 and p = 0.001, respectively).

Table 4. Instrumental colour characteristics of chicken meatball samples supplemented with different levels of oat fibre

Colour parameter	Samples			Global p-value	Dunnett CBO-0 vs. CBO-3	Dunnett CBO-0 vs. CBO-6
	CBO-0	CBO-3	CBO-6			
L*(D65)	59.93 ± 0.90^{b}	60.81 ± 0.76^{ab}	61.03 ± 1.22^{a}	**0.044***	0.100^{ns}	**0.034***
a*(D65)	7.70 ± 1.67^{a}	7.91 ± 1.58^{a}	8.00 ± 1.21^{a}	0.899^{ns}	0.931^{ns}	0.864^{ns}
b*(D65)	17.51 ± 1.84^{b}	19.32 ± 0.95^{a}	19.57 ± 1.11^{a}	**0.004****	**0.011***	**0.004****
Croma (C*)	19.22 ± 1.45^{b}	20.93 ± 0.92^{a}	21.18 ± 0.88^{a}	**0.001*****	**0.004****	**0.001*****
Hue angle (rad)	1.15 ± 0.11^{a}	1.18 ± 0.08^{a}	1.18 ± 0.07^{a}	0.691^{ns}	0.667^{ns}	0.686^{ns}
ΔE	—	3.47 ± 1.27^{a}	4.26 ± 1.34^{a}	0.191^{ns}	-	-

Values are expressed as mean ± standard deviation. CBO-0 = control sample without oat fibre; CBO-3 = sample supplemented with 3% oat fibre; CBO-6 = sample supplemented with 6% oat fibre. Different superscript letters within the same row indicate significant differences according to Tukey's HSD test (p < 0.05). Global p-value indicates the overall effect of oat fibre addition, while Dunnett comparisons show differences between the control and supplemented samples. ns = not significant; *, ** and *** indicate significance at p < 0.05, p < 0.01 and p < 0.001, respectively.

The addition of oat fibre did not significantly affect hue angle (rad) because the values for CBO-0, CBO-3, and CBO-6 were 1.15 ± 0.11^{a}, 1.18 ± 0.08^{a}, and 1.18 ± 0.07^{a}, respectively. The non-significant Dunnett comparisons, the global p-value (p = 0.691), and the identical Tukey

letter for every sample supported this finding. The total colour difference, ΔE, increased from 3.47 ± 1.27^{a} in CBO-3 to 4.26 ± 1.34^{a} in CBO-6. However, this difference was not statistically significant ($p = 0.191$), suggesting that the supplemented samples' overall colour variation was not significantly affected by the increase in oat fibre level from 3% to 6%.

Taken together, the findings show that while redness and hue angle remained unchanged, the inclusion of oat fibre mostly enhanced the chicken meatballs' lightness, yellowness, and colour intensity. These results are in line with earlier research demonstrating that adding dietary fibre to meat products might alter their instrumental colour characteristics. (Kilincceker & Yilmaz, 2019) found that the inclusion of fibre changed the L*, a*, and b* values in chicken meatballs, while Gore et al. (2022) found that the naturally yellowish tint of oat fibre boosted the yellowness of meat sausages.

Table 5 displays the chicken meatball samples' instrumental texture parameters. Almost every textural feature was significantly affected by the inclusion of oat fibre ($p < 0.05$). With a highly significant global effect ($p < 0.0001$), Warner-Bratzler shear force increased gradually from 9.31 ± 0.52^{c} in CBO-0 to 10.05 ± 0.27^{b} in CBO-3 and 11.02 ± 0.15^{a} in CBO-6. Tukey's test separated all samples into distinct groups, whereas Dunnett's test verified substantial differences between the control and both supplemented samples ($p = 0.010$ for CBO-3 and $p < 0.0001$ for CBO-6). Work of shear showed a similar increasing trend, rising from 83.24 ± 3.00^{c} to 101.03 ± 4.49^{b} and 122.55 ± 3.20^{a} ($p < 0.0001$), suggesting that the inclusion of oat fibre enhanced the product's mechanical resistance.

The TPA parameters showed the same tendency for hardness, which increased significantly from 13.52 ± 0.76^{c} in CBO-0 to 16.13 ± 1.18^{b} in CBO-3 and 19.29 ± 1.02^{a} in CBO-6 ($p < 0.0001$). Dunnett comparisons were significant for both fibre-supplemented samples ($p < 0.05$), and Tukey's test confirmed a clear separation among the three formulations. This increase may be related to the higher dry matter and fibre contents observed in the physicochemical results Table 3, together with the reduction in moisture and fat, which can contribute to a firmer and more compact meat matrix. A comparable effect was reported by Souza et al. (2019) in low-fat cooked sausages, where oat fibre increased hardness, especially at higher concentrations; similarly, Gore et al. (2022) reported that sausages with oat fibre showed good gel strength and a compact microstructure. Adhesiveness values became progressively more negative with oat fibre addition, changing from -4.11 ± 0.04 mJ in CBO-0 to -4.90 ± 0.12 mJ in CBO-3 and -5.21 ± 0.06 mJ in CBO-6, with extremely significant global and Dunnett effects ($p < 0.0001$). Since adhesiveness in TPA is expressed as the negative work required to detach the sample from the probe, the increase in the absolute value indicates higher adhesive work in the oat fibre-supplemented samples. A similar trend was reported by Dhanasekar & Kalaikannan (2014) in low-fat chicken nuggets supplemented with oat flour, where increasing oat flour levels significantly increased hardness and adhesiveness, while cohesiveness, springiness, and resilience decreased. Therefore, the present results are consistent with previous findings showing that oat-derived fibre ingredients can promote a firmer and more adhesive meat matrix, while reducing elasticity and internal cohesion.

Additionally, springiness markedly decreased from 0.70 ± 0.02^{a} to 0.59 ± 0.03^{b} and 0.50 ± 0.04^{c} ($p < 0.0001$), indicating a decrease in elasticity in the fibre-enriched samples. Cohesiveness decreased slightly but significantly from 0.40 ± 0.02^{a} in CBO-0 to 0.38 ± 0.02^{ab} in CBO-3 and 0.35 ± 0.02^{b} in CBO-6 ($p = 0.003$). Only CBO-6 showed a substantial decrease ($p = 0.001$), according to Dunnett's test, although CBO-3 continued to be statistically comparable to the control ($p = 0.081$).

Gumminess increased from 5.46 ± 0.23^{b} to 6.07 ± 0.52^{ab} and 6.70 ± 0.38^{a} ($p = 0.001$), with only CBO-6 showing a significant Dunnett difference ($p = 0.001$). On the other hand, chewiness dropped from 3.84 ± 0.23^{a} in CBO-0 to 3.56 ± 0.29^{ab} in CBO-3 and 3.37 ± 0.20^{b} in CBO-6 ($p = 0.029$). Dunnett's test only revealed a significant difference between CBO-0 and CBO-6 ($p = 0.018$). This decrease can be explained by the reduction in springiness and cohesiveness, since chewiness is calculated from hardness, cohesiveness and springiness. The findings indicate that oat fibre improved the firmness and shear resistance of chicken meatballs, but at greater concentrations, it decreased their cohesion, elasticity, and chewiness.

Table 5. Warner-Bratzler and texture profile analysis parameters of oat fibre-supplemented chicken meatball samples

Texture test	Texture parameter	Samples			Global *p*-value	Dunnett CBO-0 vs. CBO-3	Dunnett CBO-0 vs. CBO-6
		CBO-0	CBO-3	CBO-6			
Warner–Bratzler	Shear force, N/cm^2	9.31 ± 0.52^c	10.05 ± 0.27^b	11.02 ± 0.15^a	**<0.0001*** **	**0.010** **	**<0.0001*** **
	Work of shear, mJ	83.24 ± 3.00^c	101.03 ± 4.49^b	122.55 ± 3.20^a	**<0.0001*** **	**<0.0001*** **	**<0.0001*** **
Texture Profile Analysis (TPA)	Hardness, N	13.52 ± 0.76^c	16.13 ± 1.18^b	19.29 ± 1.02^a	**<0.0001*** **	**0.003** **	**<0.0001*** **
	Adhesiveness, mJ	-4.11 ± 0.04^a	-4.90 ± 0.12^b	-5.21 ± 0.06^c	**<0.0001*** **	**<0.0001*** **	**<0.0001*** **
	Springiness, -	0.70 ± 0.02^a	0.59 ± 0.03^b	0.50 ± 0.04^c	**<0.0001*** **	**0.000*** **	**<0.0001*** **
	Cohesiveness, -	0.40 ± 0.02^a	0.38 ± 0.02^{ab}	0.35 ± 0.02^b	**0.003** **	0.081^{ns}	**0.001*** **
	Gumminess, N	5.46 ± 0.23^b	6.07 ± 0.52^{ab}	6.70 ± 0.38^a	**0.001*** **	0.056^{ns}	**0.001*** **
	Chewiness, N	3.84 ± 0.23^a	3.56 ± 0.29^{ab}	3.37 ± 0.20^b	**0.029***	0.157^{ns}	**0.018***

Values are expressed as mean ± standard deviation. CBO-0 = control sample without oat fibre; CBO-3 = sample supplemented with 3% oat fibre; CBO-6 = sample supplemented with 6% oat fibre. Different superscript letters within the same row indicate significant differences according to Tukey's HSD test ($p < 0.05$). Global *p*-value indicates the overall effect of oat fibre addition, while Dunnett comparisons show differences between the control and supplemented samples. ns = not significant; *, ** and *** indicate significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Sensory profile and acceptability of chicken meatballs with oat fibre addition

Figure 1 shows the hedonic evaluation of chicken meatball samples that have been supplemented with oat fibre. Regardless of the amount of oat fibre, all samples obtained high scores overall, typically above 7.5, suggesting good consumer acceptability. The control sample, CBO-0, showed slightly higher scores for appearance, odour, texture and overall acceptability, suggesting that the traditional formulation was marginally preferred by the assessors.

The hedonic profile was slightly affected by the addition of oat fibre. Particularly in terms of look, aroma, and general acceptability, CBO-3 kept values extremely near to the control sample, suggesting that the addition of 3% oat fibre had no detrimental effects on consumer perception. CBO-6, on the other hand, had slightly lower ratings for appearance, aroma, texture, and overall acceptability. This could be related to the increased fibre content and how it affects the structure and sensory perception of the food.

The oat fibre-supplemented samples' taste was either slightly better than or equal to that of the control, suggesting that the addition of fibre did not negatively affect flavour perception. This finding is significant since adding dietary fibre to meat products might occasionally change their texture, taste intensity, or juiciness. Overall, the hedonic evaluation indicates that chicken meatballs supplemented with oat fibre were still well-liked, with CBO-3 emerging as the most balanced formulation because it improved the nutritional profile by adding fibre while maintaining a sensory profile similar to the control.

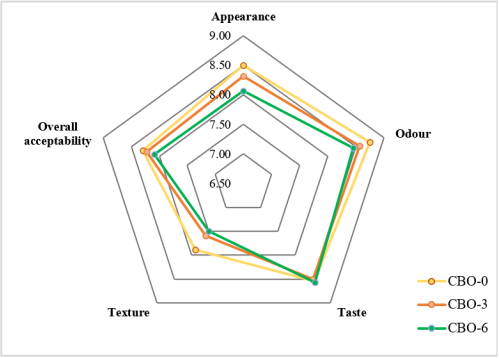


Figure 1. Hedonic evaluation of chicken meatball samples supplemented with different levels of oat fibre

Table 6 displays the descriptive sensory profile of oat fibre-supplemented chicken meatball samples. Most texture parameters were not significantly affected ($p > 0.05$), while significant differences were observed mainly in appearance-, odour-, and taste-related descriptors ($p < 0.05$). Compared to CBO-0 (7.59 ± 0.26^a) and CBO-3 (7.45 ± 0.28^a), the light pinkish-cream appearance score of CBO-6 (6.20 ± 0.39^b) was significantly lower ($p = 0.004$), suggesting that the highest oat fibre level altered the product's visual perception. Furthermore, the score for spherical shape with slight irregularities gradually decreased from 7.95 ± 0.15^a in CBO-0 to 6.84 ± 0.29^b in CBO-3 and 6.52 ± 0.38^c in CBO-6 ($p < 0.0001$). The homogeneous fat distribution decreased in the fibre-supplemented samples ($p = 0.000$), while the slightly dry, flaky aspect increased considerably in CBO-6 (2.95 ± 0.32^a) compared to the control (1.95 ± 0.22^b) ($p = 0.034$).

Table 6. Descriptive sensory profile of oat fibre-supplemented chicken meatball samples

Sensory characteristic	CBO-0	CBO-3	CBO-6	*p*-value
Ingredients evenly dispersed	7.12 ± 0.34^{a}	6.55 ± 0.35^{a}	6.18 ± 0.35^{a}	0.102^{ns}
Even color	6.36 ± 0.41^{a}	6.50 ± 0.36^{a}	6.48 ± 0.38^{a}	0.783^{ns}
Light pinkish-cream appearance	7.59 ± 0.26^{a}	7.45 ± 0.28^{a}	6.20 ± 0.39^{b}	**0.004****
Firm, dense structure	6.42 ± 0.37^{a}	6.61 ± 0.35^{a}	6.75 ± 0.35^{a}	0.943^{ns}
Perceptible connective tissue	3.15 ± 0.27^{a}	2.80 ± 0.32^{a}	3.02 ± 0.31^{a}	0.598^{ns}
Generally spherical with minor irregularities	7.95 ± 0.15^{a}	6.84 ± 0.29^{b}	6.52 ± 0.38^{c}	**<0.0001*****
Heterogeneous visual pattern	7.20 ± 0.31^{a}	7.05 ± 0.30^{a}	7.18 ± 0.30^{a}	0.889^{ns}
Slightly dry, flaky aspect	1.95 ± 0.22^{b}	2.73 ± 0.29^{b}	2.95 ± 0.32^{a}	**0.034***
Uniform fat distribution	7.11 ± 0.27^{a}	5.77 ± 0.41^{b}	6.23 ± 0.39^{b}	**0.000*****
Smoky odor	5.05 ± 0.42^{a}	3.84 ± 0.38^{b}	3.77 ± 0.37^{b}	**0.002****
Cooked meat odor	8.36 ± 0.29^{ab}	8.50 ± 0.19^{a}	7.23 ± 0.32^{b}	**0.004****
Odor typical of the product category	8.78 ± 0.06^{a}	8.25 ± 0.21^{a}	7.48 ± 0.31^{b}	**<0.0001*****
Faint odor intensity	1.86 ± 0.15^{b}	2.20 ± 0.26^{b}	4.07 ± 0.37^{a}	**<0.0001*****
Faint rancid odor	1.16 ± 0.07^{b}	1.39 ± 0.15^{ab}	1.66 ± 0.19^{a}	**0.042***
Aroma profile characteristic of commercial products	8.36 ± 0.19^{a}	8.11 ± 0.24^{a}	6.61 ± 0.31^{b}	**<0.0001*****
Taste of spices	5.16 ± 0.42^{a}	4.66 ± 0.35^{a}	5.02 ± 0.45^{a}	0.630^{ns}
Smoked taste	4.09 ± 0.39^{a}	3.95 ± 0.39^{a}	4.11 ± 0.33^{a}	0.955^{ns}
Well-balanced meat-seasoning taste	7.36 ± 0.31^{a}	6.48 ± 0.39^{a}	6.73 ± 0.36^{a}	0.148^{ns}
Pleasant flavor persistence	6.70 ± 0.37^{a}	6.55 ± 0.33^{a}	6.41 ± 0.39^{a}	0.888^{ns}
Perceptible aftertaste	6.14 ± 0.37^{a}	6.00 ± 0.35^{a}	5.84 ± 0.35^{a}	0.772^{ns}
Pronounced meat taste	8.42 ± 0.15^{a}	6.95 ± 0.39^{b}	6.66 ± 0.35^{b}	**0.000*****
Salty taste	4.80 ± 0.36^{a}	4.50 ± 0.34^{a}	4.45 ± 0.38^{a}	0.742^{ns}
Vegetal taste	2.32 ± 0.28^{b}	4.05 ± 0.38^{a}	4.25 ± 0.37^{a}	**<0.0001*****
Umami taste	4.74 ± 0.36^{a}	4.20 ± 0.38^{a}	4.05 ± 0.36^{a}	0.652^{ns}
Undesirable taste	1.13 ± 0.13^{a}	1.66 ± 0.19^{a}	1.73 ± 0.20^{a}	0.095^{ns}
Firm texture	5.98 ± 0.38^{a}	5.84 ± 0.40^{a}	6.20 ± 0.40^{a}	0.804^{ns}
Elastic texture	4.54 ± 0.37^{a}	4.84 ± 0.40^{a}	5.59 ± 0.37^{a}	0.141^{ns}
Adhesive texture	4.15 ± 0.36^{a}	3.61 ± 0.34^{a}	3.50 ± 0.37^{a}	0.790^{ns}
Well-bound texture	5.78 ± 0.32^{a}	6.02 ± 0.41^{a}	6.11 ± 0.37^{a}	0.974^{ns}
Friable texture	4.06 ± 0.30^{a}	3.41 ± 0.36^{a}	3.05 ± 0.33^{a}	0.465^{ns}
Structural integrity during mastication	6.02 ± 0.32^{a}	5.91 ± 0.36^{a}	6.20 ± 0.37^{a}	0.436^{ns}

Values are expressed as mean ± standard deviation. CBO-0 = control sample without oat fibre; CBO-3 = sample supplemented with 3% oat fibre; CBO-6 = sample supplemented with 6% oat fibre. Different superscript letters within the same row indicate significant differences according to Tukey's HSD test ($p < 0.05$). ns = not significant; *, ** and *** indicate significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

In terms of odour descriptors, the addition of oat fibre decreased the smoky odour from 5.05 ± 0.42^{a} in CBO-0 to 3.84 ± 0.38^{b} in CBO-3 and 3.77 ± 0.37^{b} in CBO-6 ($p = 0.002$). The odour profile typical of commercial products and the odour typical of the product category were also lower in CBO-6; however, the intensity of the faint odour increased significantly from 1.86 ± 0.15^{b} in CBO-0 to 4.07 ± 0.37^{a} in CBO-6 ($p < 0.0001$). The faint rancid odour also showed a slight but significant increase ($p = 0.042$), even though the absolute values remained low.

The most significant changes in taste characteristics were observed for pronounced meat taste and vegetal taste. While vegetal taste increased from 2.32 ± 0.28^{b} in the control to 4.05 ± 0.38^{a} and 4.25 ± 0.37^{a} in the oat fibre-supplemented samples ($p < 0.0001$), pronounced meat taste decreased from 8.42 ± 0.15^{a} in CBO-0 to 6.95 ± 0.39^{b} in CBO-3 and 6.66 ± 0.35^{b} in CBO-6 ($p < 0.001$). These findings suggest that the addition of oat fibre shifted the sensory perception from a strongly meat-associated profile towards a slightly more vegetal profile.

The current findings are consistent with those of Verma & Banerjee (2010), who noted that oat bran can maintain the inherent aromatic constituents of meat, mimic fat, increase mouthfeel, and lack a strong cereal flavour. These characteristics could help to explain why, especially at the 3% addition level, the oat fiber-supplemented samples retained good overall sensory acceptance. However, the decrease in strong meat flavour and smokey odour and the increase in vegetable flavour at higher fibre levels suggest that adding too much oat fibre may slightly change the typical meat sensory profile, causing perception to shift towards more vegetal overtones.

In general, Tukey's test revealed that the 6% oat fibre formulation was generally associated with the most pronounced sensory changes, whereas CBO-3 often remained closest to the control. Similar effects have been documented in meat

products supplemented with fibre, where the type and concentration of dietary fibre added affected sensory attributes related to colour, flavour, and texture. While Szczepaniak et al. (2005) found that oat fibre altered colour uniformity and decreased fatty aroma and taste intensity in finely comminuted sausages, Kilincceker & Yilmaz (2019) reported that fibre concentration affected the sensory qualities of chicken meatballs, with the highest acceptable level depending on the fibre source.

The PCA biplot presented in Figure 2 explains 100% of the total variability, with F1 accounting for 73.66% and F2 for 26.34%. The angle between the vectors indicates the relationships among sensory descriptors: variables oriented in a similar direction are positively correlated, variables positioned in opposite directions are negatively correlated, while approximately perpendicular vectors indicate weak or no correlation.

The position of CBO-0 indicates a close association with descriptors such as pronounced meat taste, salty taste, umami taste, adhesive texture, friable texture, ingredients evenly dispersed, pleasant flavor persistence, odour typical of the product category and aroma profile characteristic of commercial products. The small angles between these vectors suggest that these descriptors were positively correlated and contributed similarly to the sensory characterization of the control sample.

CBO-6 was located closer to descriptors such as faint odour intensity, faint rancid odour, elastic texture, firm, dense structure, firm texture and structural integrity during mastication. The similar orientation of these vectors indicates a relationship among these attributes and shows that they contributed together to the differentiation of the CBO-6 formulation. CBO-3 was positioned closer to vegetal taste, undesirable taste and even color, suggesting a stronger association with these descriptors.

Descriptors oriented in similar directions, such as pronounced meat taste, salty taste and umami taste, indicate close correlations, while descriptors placed in opposite directions, such as pronounced meat taste and vegetal taste, suggest contrasting sensory tendencies. Overall, the PCA shows that each formulation was associated with a distinct group of sensory descriptors, with CBO-0 linked mainly to meat- and aroma-related attributes, CBO-6 to texture- and faint odour-related descriptors, while CBO-3 occupied an intermediate position, suggesting a transitional sensory profile between the control and the highest oat fibre level.

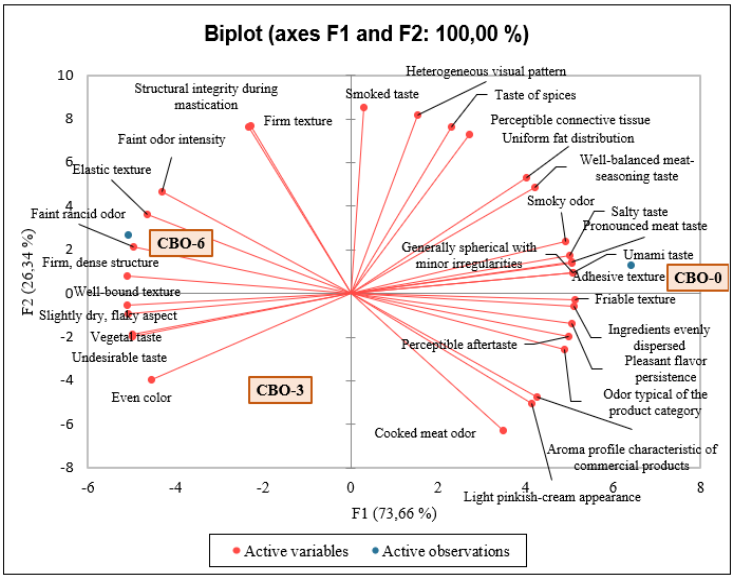


Figure 2. Principal component analysis (PCA) biplot of the sensory descriptors and chicken meatball samples supplemented with oat fibre

CONCLUSIONS

Chicken meatball samples showed distinct compositional, instrumental, and sensory alterations following the addition of oat fibre. As anticipated, the addition of oat fibre reduced moisture, protein, fat, energy value, pH, and water activity, while increasing dietary fibre content and dry matter. These modifications, primarily resulting from the substitution of a high-fibre, low-fat, and low-protein ingredient and the partial replacement of the meat matrix, confirm the reformulation effect of oat fibre.

The physicochemical changes were closely linked to the instrumental texture results. Higher shear force, work of shear, and hardness were associated with decreased moisture and fat contents, as well as increased dry matter and fibre content. This suggests the formation of a more robust and mechanically resistant product structure. Simultaneously, the decrease in chewiness, cohesiveness, and springiness, particularly in CBO-6, indicates that although firmness increased, a higher oat fibre content made the structure less cohesive and elastic.

The sensory evaluation largely mirrored the instrumental results. The increase in L*, b*, and chroma was in line with the changes seen in visual descriptors, particularly in CBO-6, where the greater dry/flaky aspect and lower score for light pinkish-cream look suggested that colour alterations were noticeable at the sensory level. Similarly, the PCA connection of CBO-6 with firm texture, solid and dense structure, and structural integrity during mastication was associated with increases in instrumental hardness, shear force, and work of shear. The reduction in cohesiveness and springiness, however, implies that the stiffer structure brought about by oat fibre was not always linked to greater elasticity or internal cohesiveness.

From a sensory standpoint, oat fibre mainly affected appearance-, taste-, and odour-related descriptors. While CBO-6 was more strongly associated with vegetal flavour, faint rotten odour, and faint odour intensity, CBO-0 was associated with meat-related and product-typical characteristics. CBO-3 exhibited an intermediate behaviour, retaining a profile more similar to the control. Overall, the findings suggest that oat fibre can enhance the nutritional profile of chicken meatballs; however, in terms of colour, texture, and sensory perception, the 3% level appears to be the most balanced formulation.

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PHYSICOCHEMICAL PROPERTIES OF DAIRY, FRUIT-BASED AND PLANT-BASED FROZEN DESSERTS: IMPLICATIONS FOR FUNCTIONAL PACKAGING DESIGN

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Abstract

This review compares dairy, fruit-based, and plant-based frozen desserts from a compositional and physicochemical perspective, with emphasis on their implications for functional packaging. The study is based on a literature review, focusing on fat, protein, pH, overrun, texture, viscosity, and melting behavior. Dairy ice cream demonstrated the highest structural stability due to its aerated fat-protein structure, resulting in slower melting. Fruit-based sorbets showed lower stability and faster melting, while plant-based products showed variable behavior depending on formulation. These differences directly influence packaging requirements. Products containing lipids require protection against oxygen and light, while structurally less stable systems demand improved thermal insulation and strict temperature control. Overall, adapted functional packaging solutions can represent an effective approach to maintaining quality and extending shelf life.

Key words*: ice cream, sorbet, plant-based frozen desserts, sensory properties, texture.*

INTRODUCTION

Ice cream is described as a multiphase system in which structural elements such as ice crystals, air cells, fat globules, and the unfrozen phase determine melting behavior and texture.
The relationship between these elements has been shown to directly influence hardness and melting rate, particularly through the interaction between air incorporation and fat structure (Muse & Hartel, 2004). In addition, the colloidal nature of ice cream systems highlights the importance of fat, proteins, and aqueous phases in defining stability and structural properties (Goff, 1997).
Milk is considered more than a basic dietary staple; it is a fundamental pillar of human nutrition that significantly supports overall health. As a complex food matrix, dairy products provide a vital balance of proteins, lipids, carbohydrates, and essential minerals necessary for physical development and well-being (Stefan et al., 2025).
The quality of finished dairy products is fundamentally determined by the integrity of the raw materials and ingredients used, making microbiological safety a critical factor in dairy product manufacturing (Şuler et al., 2024).
Furthermore, milk fat contributes significantly to both the physical manufacturing qualities and the nutritional profile of dairy products, as the balance of fatty acids is increasingly recognized for its impact on human health (Vasiliu et al., 2024).
This health-conscious trend is further driving the market toward non-dairy where novel formulations using plant-based milks not only aim to improve the biofunctional profile, such as antioxidant activity, but may also exhibit distinct technological properties, including modifications in overrun depending on the plant-based substrate and formulation strategy used (Acar et al., 2025).
Consequently, changes in formulation, particularly fat reduction and the use of fat replacers, influence viscosity, texture, and

melting behavior in ice cream by altering the properties of the unfrozen phase (Akbari et al., 2019). In dairy ice cream, fat globules form a partially coalesced network that stabilizes air cells and contributes to resistance to melting. The crystallization of lipids plays a key role in the development of this structure, influencing physical stability during freezing and storage (Adleman & Hartel, 2001).

The incorporation of fruit components leads to changes in physicochemical properties of frozen dairy systems. The addition of fruit and vegetable ingredients has been shown to influence acidity, texture, and overall stability, depending on their composition (Soodbar et al., 2025).

In systems where fruit juice is added, modifications in texture and melting behavior have been reported, indicating that fruit composition affects structural characteristics of frozen products (Marshall et al., 2003).

Studies on sorbet systems show that high water content and the absence of fat lead to differences in structural stability and melting behavior compared to dairy ice cream. Physicochemical analyses of sorbet matrices indicate lower overrun and faster melting rates, associated with higher water content and lack of fat structure (Petkova et al., 2022).

Understanding how composition influences physicochemical properties is essential for improving frozen dessert formulations. Research highlighting the role of fat content, stabilizers, and their impact on thermal behavior and ice crystallization (Akbari et al., 2019; Pintor-Jardines et al., 2018) provides a theoretical basis for comparing milk-based and fruit-based systems. Such comparisons help clarify how specific formulation differences dictate the final structure and melting characteristics of the product.

Therefore, this review aims to compare the compositional and physicochemical characteristics of dairy, fruit-based, and plant-based frozen desserts.

Differences in composition may lead to distinct sensitivities to temperature fluctuations, moisture migration, and oxygen exposure, which are key factors affecting shelf life and structural integrity. As a result, understanding these physicochemical variations provides a fundamental basis for developing functional packaging systems, such as moisture barriers, oxygen scavengers, and temperature-responsive materials, tailored to maintain the quality and stability of frozen desserts throughout storage and distribution.

MATERIALS AND METHODS

In order to compare the physicochemical properties of dairy, fruit-based, and plant-based frozen desserts, a literature review was conducted using scientific articles and review papers published up to 2025. The literature search was conducted between December 2025 and March 2026. A total of 78 articles were initially identified, of which 44 were selected based on their relevance to the physicochemical parameters investigated, including fat content, pH, overrun, melting behavior, texture, and thermal properties. Articles not directly related to ice cream or frozen desserts were excluded.

Scientific databases such as ScienceDirect, Wiley Online Library, MDPI, PubMed Central, and Taylor & Francis were used to identify relevant sources. Keywords included “ice cream”, “sorbet”, “fruit-based ice cream”, “plant-based ice cream”, “physicochemical properties”, “melting behavior”, and “texture”.

The selected studies were analysed comparatively, with particular emphasis on parameters that affect heat transfer, moisture migration, structural stability, and resistance to temperature fluctuations. The main variables considered included composition, fat content, water distribution, protein source, pH, overrun, and melting properties. The collected data were further interpreted to establish direct correlations between formulation-dependent physicochemical properties and packaging performance requirements. By identifying how structural differences influence heat sensitivity, melting resistance, and moisture loss, this analysis supports the development of targeted functional packaging solutions adapted to the specific stability challenges of each frozen dessert category.

Although efforts were made to include a wide range of relevant studies, the present review is limited by the variability in experimental conditions, formulations, and analytical methods reported in the literature, which may affect the direct comparability of results. In

addition, the correlations established between physicochemical properties and packaging requirements are based on secondary data interpretation and should be further validated through experimental studies.

RESULTS AND DISCUSSIONS

Dairy ice cream

Milk-based ice cream is formulated using milk, sugar, and stabilizing ingredients. A formulation reported in the literature includes sugar, dextrose, maltodextrin, stabilizer, milk powder, skim milk powder, water, fresh milk, and whipping cream, indicating the presence of fat, sugars, and milk solids (Limsuwan et al., 2014). Standard milk-based ice cream formulations are typically designed to achieve approximately 10% fat, 12% milk solids-not-fat (MSNF), and 12% sucrose in the mix, with a protein content of approximately 4.8% and total solids of approximately 34.26%. The dairy base may also include buttermilk, a by-product of butter production with a composition similar to skimmed milk, containing approximately 3.3% protein, 0.8% fat, 10.0% total solids, and a pH of 6.75 (Szkolnicka et al., 2020).

Buttermilk contains polar lipids derived from the milk fat globule membrane (MFGM), present at concentrations five times higher than in whole milk, which have been reported to confer emulsifying and stabilizing properties (Szkolnicka et al., 2020). Milk-based ice cream composition can be modified by replacing fat and sugar with alternative ingredients. The use of whey protein and maltodextrin as fat replacers has been reported to reduce fat content to less than 1%, while carbohydrate content increases (Limsuwan et al., 2014).

Proteins play a fundamental role in dairy ice cream, contributing to foam and lipid emulsion stabilization through partial coalescence of fat globules, while ice crystal sizes exceeding 50 µm have been associated with a rough texture and icy mouthfeel (Ng et al., 2024).

The physical properties of dairy ice cream are directly influenced by its formulation and processing conditions. Standard dairy ice cream mix exhibited an apparent viscosity of 112.33-132.83 mPas after aging, an overrun of 65.04%, and a melting rate of 0.29%/min, with a draw temperature of -5.42°C (Alvarez et al., 2005). The pH of dairy ice cream ranged between 6.47 and 6.80 (Beegum et al., 2022; Aydemir et al., 2025).

Differential scanning calorimetry analyses of dairy ice cream revealed an onset melting temperature of -7.12°C, an enthalpy of 170.3 J/g, and a freezable water content of 51.14% (Alvarez et al., 2005).

The hardness of dairy ice cream was reported at 7.08N, lower than the 19.99N recorded for plant-based ice cream formulated from cereal and legume ingredients (Pontonio et al., 2022). Milk protein concentrate ice cream exhibited the highest overall sensory acceptability score of 6.56 on a 9-point hedonic scale, demonstrating the closest resemblance to conventional dairy ice cream in terms of physical and sensory properties (Ng et al., 2024). The incorporation of probiotic cultures into dairy ice cream has also been investigated, with results indicating that propolis extract at concentrations up to 0.6% did not significantly affect color or texture, while higher concentrations resulted in decreased flavor and overall acceptability scores (Soodbar et al., 2025).

Fruit-based frozen desserts

Sorbets are non-dairy alternatives to ice cream, obtained by incorporating fruit pulp, juices, or pieces of fruit and other ingredients. Fruit-based frozen desserts may contain bioactive compounds derived from fruit, including phenolic compounds associated with antioxidant activity (Martins et al., 2025).

Fruit-based frozen desserts encompass several distinct product categories, each defined by specific compositional characteristics. While sorbets contain exclusively fruit-derived ingredients without any dairy components, sherbets represent an intermediate category incorporating both fruit and dairy ingredients such as cow's milk, and plant-based frozen desserts constitute a broader category produced using ingredients such as coconut, almond, oat, soy, cashew, or avocado as dairy substitutes (Sangteerakij et al., 2022; Taspinar et al., 2023).

The structural base of fruit-based frozen desserts differs fundamentally from that of dairy ice cream, being characterized by a high

water content and the absence of dairy fat. In mulberry sorbet, moisture content reaches 71.81 g/100 g, total fat is only 0.36 g/100 g, and pH is 3.32, reflecting the acidic nature of the organic acids naturally present in fruits (Sangteerakij et al., 2022). pH values in sorbet systems may range up to approximately 3.8 depending on formulation (Petkova et al., 2022). The sorbet exhibited higher acidity than sherbet ice cream, which was attributed to the absence of milk and the low pH of the fruit used in the formulation (Sangteerakij et al., 2022).

The absence of dairy components in fruit-based frozen desserts determines a fat and protein profile that differs fundamentally from that of conventional dairy ice cream.

Fruit-based sorbets are characterized by very low fat content, as reported in mango sorbet formulations where total fat reached only 0.50 g/100 g (Williams et al., 2022).

The protein content of fruit-based sorbets is equally low, as observed in peach sorbet matrices which presented reduced fat and protein contents (Petkova et al., 2022).

The sugar composition of fruit-based frozen desserts differs markedly from that of conventional dairy ice cream. While dairy ice cream contains sucrose as the primary added sweetener alongside lactose derived from milk, fruit-based sorbets are characterized by a predominance of fructose and glucose, as these are the dominant sugars naturally present in most fruits (Székelyhidi et al., 2025). Sorbets contain a minimum fruit content of 25%, with water and sugar as the other main components, and the fruit itself contributes substantially to the final sugar profile (Hipólito et al., 2016). In strawberry sorbet, fructose and glucose were each detected at approximately 41,663 mg/kg, while sucrose was present at only 7,932 mg/kg, the lowest concentration among the three sugars identified (Székelyhidi et al., 2025).

The total soluble solids content of peach sorbet matrices varied between 17.50 and 33.03%, depending on the formulation, reflecting the influence of both naturally occurring fruit sugars and added sweeteners on the overall sugar content of the product (Petkova et al., 2022).

In ice cream formulations enriched with increasing concentrations of persimmon puree, glucose and fructose contents increased progressively while lactose decreased from 7.30% to 3.76%, indicating that fruit incorporation modifies the sugar profile of frozen desserts (Karaman et al., 2014).

Beyond the naturally occurring sugars derived from fruit, the total sugar content of fruit-based frozen desserts is also influenced by the addition of sweetening agents during formulation.

In industrial sorbet production, added sugar and water content are typically higher than in artisanal products, where the fruit content is proportionally greater (Palka & Wilczyńska, 2023). Sugar addition increases viscosity, improves body and texture, and lowers the freezing point, thereby reducing the risk of large ice crystal formation during freezing and storage (Romulo et al., 2021). In frozen yogurt formulations, increasing sugar content from 18% to 22% resulted in a progressive increase in overrun from 22.15% to 31.63%, while complete melting time decreased, demonstrating the direct influence of sugar concentration on the structural and melting properties of frozen desserts (Güven & Karaca, 2002).

Sugar directly regulates the overall taste sensation of frozen desserts, and its removal or reduction requires the use of bulking agents and alternative sweeteners to compensate for its functional properties, including freezing point depression and body formation (Pinto & Dharaiya, 2014). The substitution of sucrose with alternative sweeteners and bulk fillers such as polydextrose, maltodextrin and sorbitol has been shown to achieve a 20% reduction in caloric value while maintaining acceptable physicochemical and sensory characteristics in the final product (Pinto & Dharaiya, 2014).

In mulberry sorbet, overrun was 44.72 and melting rate was 17.27 g/min, while in mulberry sherbet ice cream, overrun was 47.58% and melting rate was 13.81 g/min, with the higher overrun of sherbet attributed to the presence of cow's milk in its formulation (Sangteerakij et al., 2022). Sorbet melted faster than sherbet ice cream (Sangteerakij et al., 2022). In peach sorbet matrices, overrun ranged from 8.11 to 12.32%, with the control sample completely melted within 60 minutes, and low overrun values were attributed to the low

protein content of the sorbet matrices, with larger ice crystals detected at lower overrun (Petkova et al., 2022).

Fruit-based ice cream formulations incorporating dragon fruit and banana have also been investigated, with these tropical fruits contributing antioxidants, vitamin C, dietary fiber, and potassium to the nutritional profile of the final product (Van Thinh et al., 2025).

Novel frozen dessert formulations incorporating jujube fruit have been developed, with jujube contributing sweetening and coloring capacity, antioxidant activity, vitamin C content, and dietary fiber to the nutritional profile of the final product (Stan et al., 2025).

Persimmon sorbet mixes exhibited shear thinning behavior, with flow behavior index values between 0.29 and 0.37 and apparent viscosity between 1.14 and 1.40 Pa·s at a shear rate of 50 s^{-1}, while overrun ranged from 8.3 to 51% depending on the concentration of the foaming agent used, and higher overrun values were associated with slower melting rates due to the low thermal conductivity of air (Kilicli et al., 2025).

Plant-based frozen desserts

Plant-based frozen desserts are produced using ingredients such as coconut, almond, oat, or soy. An analysis of 358 commercial non-dairy frozen desserts reported that 90% of the products were classified as high in sugar and many contained saturated fat due to the widespread use of coconut oil (Craig & Brothers, 2022). Frozen desserts formulated with plant-based milk sources such as almond, hazelnut, or lupine differ in composition and physicochemical properties depending on the raw materials used (Akalın et al., 2024).

Among the plant-based alternatives to conventional dairy ice cream, coconut-based formulations represent the most extensively studied category. Non-dairy ice cream formulations produced from coconut milk, tender coconut pulp and water obtained overrun values ranging from 40.00% to 46.39%, significantly lower than the 86.00% recorded in the dairy control, and the incorporation of coconut products resulted in increased total phenolic content and mineral content compared to conventional dairy ice cream (Beegum et al., 2022). Cold-pressed coconut oil by-products have also been identified as a low-cost alternative source for plant-based ice cream production, with overrun values between 22.02% and 28.33% achieved without negatively impacting sensory qualities (Kasapoglu et al., 2023).

In non-dairy ice cream formulated from cashew milk and coconut cream, fat content varied from 6.04% to 10.46% and protein content from 3.71% to 4.60% as the proportion of coconut cream increased (Matabura, 2023).

Beyond coconut and fruit-based formulations, plant-based ice creams have also been developed using nuts, cereals, and legumes as dairy substitutes. Vegan ice cream produced from hazelnut cake contained 3.67% protein, 8.00% fat, 16.99% carbohydrate, and 3.22% dietary fiber per 100 g, with a caloric value of 133 kcal/100 g, representing an 18% reduction compared to conventional dairy ice cream.

The overrun of 4.01% compared to 15.39% in the dairy control (Aydemir et al., 2025).

Plant-based ice cream formulated from a fermented gluten-free and lactose-free base of rice, lentil and chickpea contained 3.52% protein, 4.65% fat, 25.69% carbohydrate and 5.20% dietary fiber, with an overrun of 19.3% (Pontonio et al., 2022).

Plant-based ice cream formulations produced from cereal-based milk sources such as oat, millet and spelt, combined with vegetable oleogels as fat sources, exhibited protein contents ranging from 1.32% to 3.40% and fat contents from 3.12% to 9.74% depending on the type of plant milk and oleogel concentration used (Ropciuc et al., 2024).

The physicochemical and rheological properties of plant-based ice creams vary with the type of plant milk and protein source used (Hidas et al., 2023; Leahu et al., 2022).

Hemp drink-based vegan ice creams produced with different plant proteins exhibited pH values ranging from 6.1 to 6.4 depending on the protein source used, with walnut protein yielding the lowest pH and hemp protein the highest (Hidas et al., 2023).

The rheological behavior of plant-based ice cream mixes was pseudoplastic, with apparent viscosity values between 0.016 and 0.426 Pa·s at a shear rate of 50 s^{-1} and flow behavior index values between 0.664 and 0.974, while

maximum meltdown rate decreased with increasing overrun (Hidas et al., 2023).
In almond milk and hemp milk ice creams, pH values of 6.2 and 5.3 were recorded for the control samples respectively, with fat content of 2.67% and protein content of 1.09% in almond milk formulations, and the addition of dietary fibers resulted in increased viscosity and hardness (Leahu et al., 2022).

Comparative analysis

The fundamental differences in raw materials and processing conditions significantly dictate the final technical and nutritional profile of the frozen desserts. Standard dairy ice cream mix exhibited an overrun of 65.04% (Alvarez et al., 2005) and a protein content of approximately 4.8% (Szkolnicka et al., 2020).
The pH of dairy ice cream was reported at 6.47 (Beegum et al., 2022) and 6.80 (Aydemir et al., 2025). Fruit-based sorbets exhibited a pH of 3.32 and a fat content of 0.36 g/100 g (Sangteerakij et al., 2022).
Plant-based formulations recorded overrun values as low as 4.01% (Aydemir et al., 2025), with fat content reaching values comparable to dairy ice cream depending on the fat source used (Matabura, 2023). Low overrun values in both fruit-based and plant-based categories were attributed to the low protein content of these matrices (Petkova et al., 2022).
Plant-based ice cream exhibited higher hardness than dairy ice cream (Pontonio et al., 2022). The primary physicochemical, textural, and nutritional parameters across the three categories are summarized in Table 1.

Table 1. Comparative Analysis of Frozen Matrices

Parameter	Dairy	Plant-Based	Sorbet	Source
pH	6.47–6.80	5.3–6.4	3.32–3.88	Beegum et al. (2022); Aydemir et al. (2025); Hidas et al. (2023); Leahu et al. (2022); Sangteerakij et al. (2022); Petkova et al. (2022)
Overrun (%)	65.04	4.01–19.3	8.11–44.72	Alvarez et al. (2005); Pontonio et al. (2022); Petkova et al. (2022); Sangteerakij et al. (2022)
Hardness (N)	7.08	19.99	-	Pontonio et al. (2022)
Fat (%)	~10	2.67–10.46	0.36–0.50	Szkolnicka et al. (2020); Leahu et al. (2022); Matabura (2023); Sangteerakij et al. (2022); Williams et al. (2022)

The melting rate of dairy ice cream was reported at 0.15 g/min (Ng et al., 2024).
The firmness of dairy ice cream was reported at 48.2 g, lower than the 350.8 g recorded for plant-based ice cream formulated with pea protein isolate (Ng et al., 2024). Vegan ice cream produced from hazelnut cake exhibited a melting time of 68 minutes, compared to 99 minutes recorded for dairy ice cream (Aydemir et al., 2025). Hemp drink-based vegan ice creams exhibited onset melting temperatures between -7.21 and -9.66°C, with unfreezable water content ranging from 25.27 to 48.10% (Hidas et al., 2023).

Table 2. Rheological, Textural and Thermal Properties of Dairy and Plant-Based Ice Cream

Parameter	Dairy	Plant-Based	Source
Melting rate (g/min)	0.15	-	Ng et al. (2024)
Firmness (g)	48.2	350.8	Ng et al. (2024)
Melting time (min)	99	68	Aydemir et al. (2025)
Onset temp (°C)	-7.12	-7.21 to -9.66	Alvarez et al. (2005); Hidas et al. (2023)
UFW (%)	51.14	25.27–48.10	Alvarez et al. (2005); Hidas et al. (2023)

Correlation between physicochemical properties and functional packaging requirements

The comparative analysis of dairy, fruit-based, and plant-based frozen desserts highlights significant differences in composition, structure, and stability, which directly translate into distinct packaging requirements.

Influence of fat content and structural network on oxidative and light sensitivity

Differences in lipid content and composition influence the susceptibility of frozen desserts to oxidative degradation, particularly under exposure to oxygen and light (Christophersen et al., 1992).
From a packaging perspective, products containing lipids require materials with effective oxygen barrier properties and protection against light in order to limit oxidation and preserve quality during storage (Christophersen et al., 1992).
Even in low-fat systems, packaging strategies should consider the protection of sensitive bioactive compounds, such as phenolics, which may also undergo oxidative degradation (Singh et al., 2022).

Influence of water content on moisture migration and recrystallization

Differences in water mobility and phase distribution influence the stability of frozen desserts during storage, particularly in relation to ice recrystallization and texture degradation.

From a packaging perspective, these variations require the use of materials with effective moisture barrier properties to prevent dehydration and freezer burn. Additionally, maintaining temperature stability throughout storage and distribution is essential to limit recrystallization and preserve product structure (Kummar et al., 2020).

Effect of overrun on thermal behavior and melting stability

Differences in overrun significantly influence heat transfer and melting behavior in frozen desserts. Products with lower overrun exhibit higher density and increased thermal conductivity, resulting in faster melting under temperature fluctuations (Kilicli et al., 2025; Hidas et al., 2023).

Consequently, these systems require enhanced thermal insulation and improved temperature buffering capacity during storage and distribution in order to maintain structural stability and delay melting (Kilicli et al., 2025; Hidas et al., 2023).

Impact of pH on material compatibility

Differences in pH influence the chemical stability of packaging materials and their interaction with frozen dessert matrices. Acidic systems may promote material degradation or increase the risk of compound migration.

Therefore, low-pH products require the use of acid-resistant materials with appropriate chemical stability, as well as polymers designed to ensure migration safety under acidic conditions (Gupta et al., 2024).

Role of sugar composition in freezing point depression and stability

Differences in sugar composition influence freezing behavior and sensitivity to temperature fluctuations in frozen desserts.

From a packaging perspective, systems with lower freezing stability require strict cold chain control and packaging solutions capable of minimizing temperature fluctuations in order to prevent partial melting and preserve product integrity during storage and distribution (Güven & Karaca, 2002; Kumar et al., 2020).

CONCLUSIONS

The three categories of frozen desserts show distinct physicochemical characteristics that directly influence their structural stability, melting behavior, and response to storage conditions. Differences in lipid composition, water mobility, air incorporation, pH, and sugar profile were identified as key factors governing thermal sensitivity, recrystallization, and overall product stability.

Dairy ice cream demonstrated greater structural stability due to its balanced multiphase system, while fruit-based desserts were more susceptible to temperature fluctuations and structural degradation. Plant-based systems showed high variability depending on the raw materials used, often presenting intermediate or less predictable stability profiles.

These compositional differences translate into specific packaging requirements. Systems with higher lipid content require effective oxygen and light barriers to prevent oxidation, while products with high water mobility demand strong moisture barriers and strict temperature control to limit recrystallization and dehydration. In addition, products with lower thermal stability or overrun require enhanced insulation and temperature buffering during storage and distribution.

Overall, the findings emphasize that frozen desserts should not be treated as a uniform category from a packaging perspective. Instead, the design of functional packaging systems must be tailored to the specific physicochemical characteristics of each product type in order to ensure quality preservation, extend shelf life, and improve product performance throughout the cold chain.

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FATTY ACIDS IN WILD BOAR MEAT

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Abstract

In the context of the increased interest in the nutritional quality of meat, this study aimed to evaluate the physicochemical composition and fatty acid profile of wild boar meat (Sus scrofa, Linnaeus, 1758) from three representative counties of the Transylvania region (Cluj, Sălaj and Bistrița-Năsăud, Romania). A total of 24 Longissimus dorsi muscle samples (including the control group) were collected from wild boars during hunting parties and analyzed from a compositional point of view. Physicochemical parameters were determined by FT-NIR spectroscopy, while lipid extraction and fatty acid profile were performed by solvent extraction, followed by chromatographic analysis. The results showed a protein content between 26.4–29.4%, a fat content of 1.1–1.2% and pH values between 6.2 and 6.6, with significant differences observed in Sălaj and Bistrița-Năsăud ($p < 0.05$). The total PUFA content ranged between 20.36% and 23.77%, the SFA content between 35.35% and 38.37%, and the MUFA content between 40.72% and 44.51%. The PUFA/SFA ratio ranged between 0.57 and 0.63, indicating a favorable lipid profile. Therefore, this study confirms that wild boar meat from Transylvania presents a lean composition and a favorable nutritional profile of fatty acids, with moderate regional variations, probably influenced by habitat and diet.

Key words: *fatty acids, meat, Transylvania, wild boar.*

INTRODUCTION

The wild boar (*Sus scrofa*, Linnaeus, 1758) is an omnivorous species with a high capacity for ecological adaptation, widely distributed across Europe, Asia and parts of North Africa, playing an important role in forest and agricultural ecosystems. Due to its behavioral flexibility and feeding adaptability, the species occupies a wide range of habitats, including deciduous and coniferous forests, wetlands, agricultural areas, semi-arid regions, and mountain ecosystems (Viganò et al., 2019; Kamieniarz et al., 2020; Dannenberger et al., 2013).

Therefore, this adaptability can determine significant variations in the composition and quality of the meat, especially in terms of lipid profile. Game meat, including wild boar meat, is considered a valuable source of protein with high biological value, characterized by a low lipid content and a superior mineral profile compared to meat from intensive farming systems (Sales & Kotrba, 2013; Niewiadomska et al., 2021). An additional advantage of wild boar meat is the increased nutritional interest of consumers, due to its high iron, zinc and phosphorus content and low sodium content compared to domestic pork (Sales & Kotrba, 2013).

Regarding meat quality, it can be influenced by biological and environmental factors, including sex, age, physical activity and type of habitat, these factors determining significant variations in chemical and lipid composition (Tesarova et al 2018; Morán et al., 2019; Hoffman et al., 2021; Quaresma et al., 2011).

It is worth noting that intramuscular fat influences the sensory properties of meat, including texture and flavor, being strongly dependent on the lipid metabolism of the animal. From a nutritional point of view, game meat is considered, according to the literature, a healthier alternative due to its favorable lipid profile, characterized by an optimal ratio between saturated and unsaturated fatty acids and an increased content of polyunsaturated fatty acids (Kelava Ugarković et al., 2021; Raba et al., 2016). Another important aspect is that the consumption of game meat is associated with

dietary diversification and the intake of essential nutrients and is recommended as part of a balanced diet (Raba et al., 2016).

According to the literature, the superiority of wild boar meat quality is primarily determined by living conditions in the natural environment, characterized by varied food resources and an unpolluted environment. These conditions can influence the chemical composition of the meat, including the content of macro- and microelements, the optimal ratio between polyunsaturated and saturated fatty acids, as well as the organoleptic characteristics of wild boar meat (Szmańko et al., 2007).

Regarding the fatty acid profile, it can be significantly influenced by diet, habitat, season and level of physical activity, with the predominance of palmitic (C16:0), stearic (C18:0), oleic (C18:1 n-9) and linoleic (C18:2 n-6) acids being reported in wild boar meat (Razmaite et al 2009; Raba et al., 2016; Niewiadomska et al., 2020). In this context, consumers show an increased interest in natural products with reduced fat content and, and game meat in cholesterol is an important alternative in this regard (Szmańko et al., 2007).

Compared to systems from intensive farming, wild boar meat has a lower lipid content, especially in the psoas major muscle, due to the high level of physical activity and species-specific metabolism (Viganò et al., 2019; Niewiadomska et al., 2020).

In the literature, the distribution of fatty acids is influenced by biological and ecological factors such as sex, age, season and food availability, which determines important variations between populations (Quaresma et al., 2011). These variations are nutritionally and technologically relevant, influencing both the oxidative stability and the sensory quality of the meat (Raba et al., 2016; Niewiadomska et al., 2021).

Recent studies confirm that not all types of game meat present an optimal lipid profile from a nutritional point of view, the differences being influenced by factors such as geographical area, diet, age and sex of the animal (Lešić et al., 2025). In addition, significant differences have been reported between game species and domestic pork, especially in terms of omega-3 fatty acid content (Sampels et al., 2023).

In a balanced diet, wild boar meat consumption is considered moderate, representing approximately 20% of the total food intake, and in terms of meat proteins, they have a high biological value due to the content of essential amino acids (Strazdiņa et al., 2013; Honikel, 2009). Therefore, meat represents an important source of essential amino acids such as lysine, leucine and isoleucine, which cannot be synthesized by the human body and must be provided through food (Strazdiņa et al., 2013). It is worth mentioning that in recent decades, game meat has become an important component of the diet, due to the increased interest in products from animals raised in natural conditions, being appreciated both for its sensory properties and for its high nutritional value (Soriano et al., 2006). However, the literature shows that the fatty acid profile can vary significantly depending on ecological and biological conditions, which requires detailed regional studies (Lešić et al., 2025). It is also worth noting that the composition of meat can be influenced by post-mortem and technological factors, including the handling and processing method, as well as by the enzymatic activity specific to the muscle tissue of wild animals, which influences meat maturation and protein degradation (Cioata et al., 2025).

MATERIALS AND METHODS

In this study, a total of 24 samples of wild boar meat (*Sus scrofa*, Linnaeus, 1758) were analyzed, divided into four experimental groups, with n = 6 samples each, and a reference group. The samples were collected following hunting parties in the Transylvania region (Cluj County, Sălaj County and Bistrița-Năsăud County).

All sampled specimens were classified as subadult animals aged 1-2 years, including both males and females.

The hunting was carried out authorized in accordance with the Hunting and Protection of Game Reserves Law no. 407/2006 from the Official Gazette, Part I no. 944 of 22 November 2006.

The samples in this study were taken from the *Longissimus dorsi* muscle considered a representative muscle for the analysis of the chemical and lipid composition of the meat**.**

The samples, after collection, were transported to the INCDO-INOE 2000 laboratory, Branch of the Research Institute for Analytical Instrumentation (ICIA), Cluj-Napoca, Romania, under refrigerated conditions (4 ± 1°C). The

samples were homogenized in the laboratory to obtain a uniform and representative sample before their analysis.

Wild boar samples were analyzed for dry matter, total lipid, NaCl content, protein and pH using a Bruker FT-NIR spectrometer in diffuse reflectance mode. The calibration used was internal, previously validated in the laboratory by reference methods, which include ash by calcination, lipid by solvent extraction, protein by Kjeldahl method, salt by titration or gravimetry, and pH by potentiometry.

Each wild boar meat sample was subjected to homogenization by comminution to uniform the particles and reduce the variation due to scattering effects. Spectra were collected in the range 12,000-4,000 cm^{-1} with a resolution of 8 cm^{-1} and 64 scans per analysis.

For the determination of lipids, we took a quantity of 0.5 g of the muscle tissue sample taken from the wild boar and mixed it with 20 ml of chloroform-methanol mixture (2:1) (v/v). For extraction, the mixture of biomass and solvents were extracted in an ultrasonic bath (Sonorex RK 512H), for 60 min.

After the extraction step, the mixture was filtered to separate the organic phase. The organic phase was treated with 10 ml of KCl (0.74%) for extraction and purification.

The chloroform phase was separated from the aqueous phase and filtered and dried using Na_2SO_4. As for the solvent, it was evaporated to dryness using a rotary evaporator, and the obtained oil was dried at 60°C, after which it was weighed. Regarding the analysis of the fatty acid profile of wild boar muscle tissue there may be changes, which may be associated with the type and quality of dietary lipids.

RESULTS AND DISCUSSIONS

The physicochemical composition of wild boar meat (*Sus scrofa*, Linnaeus, 1758) from Cluj, Sălaj and Bistrița-Năsăud counties is presented in Table 1. The results in the table indicate a relatively homogeneous profile between the studied batches. The sex of wild boar specimens was not included as a factor in the analysis due to the homogeneous age structure and limited batch size.

Regarding the protein content, it varied between 26.4% and 29.4%, slightly lower values being observed in Sălaj, while the highest values were recorded in Bistrița-Năsăud. The fat content was reduced (1.1-1.2%), confirming the lean meat character specific to game (Table 1).

Table 1. Physico-chemical composition of wild boar meat by area

Parameter	Wild boar reference group	Wild boar Cluj	Wild boar Sălaj	Wild boar Bistrița-Năsăud
Ash (%)	2.8±0.1	2.6±0.1^{ns}	2.9±0.1^{ns}	2.5±0.1^{ns}
Protein (%)	28.1±0.5	28.7±0.4^{ns}	26.4±0.6^{ns}	29.4±0.5^{ns}
Fat (%)	1.1±0.05	1.2±0.05^{ns}	1.1±0.05^{ns}	1.1±0.05^{ns}
Collagen (%)	2.2±0.1	2.4±0.1^{ns}	2.1±0.1^{ns}	2.4±0.1^{ns}
Water (%)	70.9±0.3	71.4±0.4^{ns}	70.6±0.3^{ns}	71.5±0.4^{ns}
pH	6.6±0.1	6.4±0.1^{ns}	6.3±0.1*	6.2±0.1*

(P-value; ns- insignificant; p >0.05 -ns; p < 0.05∗)

According to published studies, the results are in agreement with the data reported by Sales & Kotrba (2013), which highlighted that wild boar meat has a lower lipid content and a higher protein content compared to domestic pigs. Also, Honikel (2009) reported results regarding meat protein values between 13-23%, which confirms the high protein character of the analyzed samples. Regarding the significant differences in pH between some counties, they may be associated with post-mortem factors, such as harvesting stress, handling or cooling conditions of the meat.

For fatty acids, the results (Table 2) indicate higher levels of saturated fatty acids (SFA) in wild boar meat samples from Bistrița-Năsăud and Sălaj, compared to the control group. This increase is mainly associated with higher values of C14:0, C17:0 and C20:0. In contrast, total polyunsaturated fatty acids (PUFA) were higher in all investigated regions, compared to the control group, with the highest values observed in Sălaj and Bistrița-Năsăud, reaching the highest levels of significance. This pattern is largely related to the increased content of linoleic acid (C18:2 n-6) in these samples (Table 2). PUFAs showed higher values in all experimental groups compared to the control, with maximums in Sălaj (23.77%) and Bistrița-Năsăud (23.27%) (Table 2). The PUFA/SFA ratio ranged from 0.57 (control group) to 0.63 (experimental groups), with values ranging from 0.61 to 0.63 in wild boar meat from the studied regions. These values are considered nutritionally favorable, as a PUFA/SFA ratio

greater than 0.4 is generally associated with a lipid profile beneficial to human health. The results are similar to those reported by Quaresma et al. (2011), who demonstrated the influence of diet and habitat on the lipid profile in wild boar. Sampels et al. (2023) highlighted a higher content of polyunsaturated fatty acids in game meat compared to meat from intensive systems.

Table 2. SFA, MUFA and PUFA fatty acids in wild boar meat depending on the area

Fatty acids	Wild boar reference group	Wild boar Cluj vs reference group	Wild boar Sălaj vs reference group	Wild boar Bistrița-Năsăud vs reference group
∑ SFA	35.51	35.35 (ns)	37.50 (*)	38.37 (**)
∑ MUFA	41.01	42.20 (ns)	40.72 (ns)	44.51 (**)
∑ PUFA	20.36	21.79 (*)	23.77 (***)	23.27 (***)
PUFA/SFA	0.57	0.62 (*)	0.63 (*)	0.61 (*)
n-6/n-3	13.86	13.09 (ns)	16.68 (**)	15.14 (*)

ns (insignificant); * significant ($p < 0.05$); ** distinct significant ($p < 0.01$); *** high significant ($p < 0.001$)

The high protein values and favorable fatty acid profile observed in the present study support the claims of Soriano et al. (2006), according to which game meat represents a valuable and healthy food alternative due to its nutritional and sensory properties. The low fat content (1.1-1.2%) and high levels of polyunsaturated fatty acids identified in the analyzed samples contribute to the high biological value of wild boar meat.

Oleic acid (C18:1 n-9) was one of the main fatty acids identified in our study, with values between 37.25% and 40.28%, confirming its dominant role in the lipid structure of wild boar meat. Palmitic acid (C16:0) showed minimal variations between groups (20.9-21.9%), indicating a relative metabolic stability. Linoleic acid (C18:2 n-6) was significantly higher in Sălaj and Bistrița-Năsăud compared to the values in Cluj, suggesting the influence of habitat factors and available food resources, (Table 3). These results are in agreement with Quaresma et al. (2011), who concluded that the natural diet significantly influences the fatty acid composition of wild boar. Authors Razmaite et al. (2009) and Quaresma et al. (2011), who identified palmitic acid and stearic acid as the main saturated fatty acids in wild boar meat. Also, Sales & Kotrba (2013) highlighted the favorable lipid profile of wild boar meat compared to domestic pork.

Table 3. Average values for wild boar meat by area

Fatty Acids (%)	reference group	Wild boar Cluj	Wild boar Sălaj	Wild boar Bistrița-Năsăud	p-value	Semni-fication
C14:0	1.2	1.4	1.5	1.9	0.008	**
C15:0	0.4	0.9	0.4	0.7	0.042	*
C16:0	21	20.9	21.7	21.9	0.115	Ns
C16:1	2	2.2	2.3	2.6	0.004	**
C17:0	0.6	0.9	1.6	1.3	< 0.001	***
C17:1	0.45	0.52	0.61	0.93	< 0.001	***
C18:0	11.51	11.22	11.82	11.91	0.241	Ns
C18:1 n-9 (Oleic)	38.08	39.01	37.25	40.28	0.012	*
C18:2 n-6 (Linoleic)	16.02	17.13	19.22	18.42	< 0.001	***
C18:3 n-6	0.45	0.37	0.42	0.55	0.031	*
C18:3 n-3	0.88	0.99	0.91	1.02	0.045	*
C20:0	0.3	0.31	0.38	0.45	0.006	**
C20:1	0.37	0.33	0.4	0.51	0.018	*
C20:2 n-6	0.21	0.27	0.3	0.29	0.058	Ns
C20:4 n-6	2.34	2.49	2.51	2.54	0.382	Ns
C22:0	0.28	0.32	0.29	0.22	0.092	Ns
C22:1 n-9	0.11	0.13	0.16	0.19	0.009	**
C22:5 n-3	0.46	0.53	0.41	0.45	0.154	Ns
Others (C23:0, C24:0)	3.92	3.66	3.91	2.77	0.003	**

The differences observed between the counties analyzed by us can be explained by the particularities of the habitat, the diversity of natural food sources and the degree of physical activity of the wild boar. Cioată et al. (2025) highlighted the fact that the diet and living conditions of the wild boar directly influence the chemical composition and lipid profile of the meat. Also, the study conducted by Lešić et al. (2025) emphasized that not all wild boar populations present the same fatty acid profile, the differences being determined by factors such as season, sex, age and habitat conditions.

The high protein values and favorable fatty acid profile observed in the present study support the claims of Soriano et al. (2006), according to which game meat represents a valuable and healthy food alternative due to its nutritional and sensory properties. The low fat content (1.1-1.2%) and high levels of polyunsaturated fatty acids identified in the analyzed samples contribute to the high biological value of wild boar meat. According to the study conducted by Strazdiņa et al. (2013), among the Latvian population, special attention was paid to maintaining health by adopting a balanced diet. The authors mention that, in the food pyramid, meat is considered a product to be consumed in

moderation, representing approximately 20% of the total food.

Regarding the results obtained in this study, they are consistent with data reported in the specialized literature for wild boar meat from other regions, confirming the predominance of oleic, palmitic, stearic and linoleic acids in its lipid fraction (Skobrák et al., 2011; Quaresma et al., 2011; Tomljanović et al., 2014; Tesarova et al 2018; Blaška et al., 2023; Lešić et al. 2025).

CONCLUSIONS

This study highlighted that there may be some differences in the chemical composition and fatty acid profile of wild boar meat (*Sus scrofa* L., 1758) from different counties of the Transylvania region (Cluj, Sălaj and Bistrița-Năsăud). The results show, that from a physico-chemical point of view, most of the analyzed parameters (protein, fat, collagen, water and ash) did not present significant differences between the studied batches. The pH values were significantly different for the samples from Sălaj and Bistrița-Năsăud counties compared to the control batch ($p < 0.05$).

Regarding the lipid profile, the highest values of saturated fatty acids (SFA) were identified in the samples from Bistrița-Năsăud county (38.37%), followed by those from Sălaj county (37.50%). The total content of polyunsaturated fatty acids (PUFA) was higher in the samples from Sălaj County (23.77%), followed by those from Bistrița-Năsăud County (23.27%). Also, the highest content of oleic acid (C18:1 n-9) was recorded in Bistrița-Năsăud County (40.28%), and the highest content of linoleic acid (C18:2 n-6) in Sălaj County (19.22%).

The PUFA/SFA ratio ranged between 0.57 and 0.63, all values exceeding the threshold of 0.40 recommended for a balanced diet, which confirms the high nutritional value of the analyzed wild boar meat.

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HISTOPATHOLOGICAL EVALUATION OF SELECTED TISSUES FROM WILD BOARS IN TRANSYLVANIA, ROMANIA

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Abstract

The aim of this study was to evaluate histopathological changes in tissues from wild boars (Sus scrofa, L. 1758) from the Transylvania region, Romania. We collected tissue samples from lungs, heart, liver, kidneys and skeletal muscles from 5 wild boars. The samples were collected during post-mortem examinations. The samples were fixed in 10% buffered formalin, processed by paraffin embedding, sectioned at 2 µm, stained with hematoxylin and eosin and examined microscopically. Histopathological examination revealed severe parasitic and inflammatory lesions in the lungs, associated with cysts of Metastrongylus spp., Sarcocystis spp. in the myocardium, mild inflammatory lesions and occasional hepatic steatosis in the liver, as well as multifocal lymphohistiocytic interstitial nephritis in the kidneys. It should be noted that no histological changes were observed in skeletal muscle tissue. These observations provide essential information about the pathological state of wild boars in the studied region and emphasize the importance of histopathological monitoring for wildlife and meat quality assessment.

Key words*: histological analysis, organ lesions, wild boar.*

INTRODUCTION

The wild boar (*Sus scrofa,* L. 1758) is the most widespread species of wild and omnivorous mammal, in Europe, Asia and North Africa, which is part of the Suidae family and is the direct ancestor of the domestic pig. The wild boar is characterized by a high capacity to adapt to various environmental conditions and an opportunistic omnivorous diet. Due to its high mobility and variable feeding behavior, this wild species can be permanently exposed to numerous biological agents and environmental factors that can influence its health (Amici et al., 2012; Martin & Griswold, 2009). According to studies, game animals such as moose (*Alces alces*), roe deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), wild boar (*Sus scrofa*) and wild rabbit (*Lepus europaeus*) offer consumers opportunities to diversify their diet. (Rule et al., 2002; Falandysz et al., 2005). The use of wild species for monitoring environmental pollution is an essential tool, and according to the ethological characteristics of the wild boar, there is a probability of being exposed to heavy metal contamination (Martin & Griswold, 2009). Wild boar meat is an important source of protein with high biological value and is appreciated for its low fat content and favorable fatty acid profile compared to that of domestic animals (Quaresma et al., 2011; Kasprzyk et al., 2019). The consumption of wild boar meat has been steadily increasing, associated with consumer interests in organic products and extensive production systems (Kallas et al., 2021). Regarding wild animals, they can be frequently exposed to pathogens and parasites that can induce histopathological changes in internal organs. Infections with nematodes of the genus *Metastrongylus* are frequently found in wild boar and can cause bronchitis, bronchopneumonia and chronic pulmonary inflammatory reactions (García-González et al., 2013). Also, infections with protozoa of the genus *Sarcocystis* are often found in skeletal muscles and myocardium, where they can form tissue cysts (Dubey et al., 2015). Organs such as the liver and kidney are essential for metabolic processes including detoxification, due to their sensitivity to the action of infectious and environmental factors. Lesions such as lymphocytic hepatitis, hepatic steatosis and interstitial nephritis are described in the literature, changes associated with both

pathological processes and habitat conditions (Beiglböck et al., 2002; Falandysz et al., 2005).
Another study conducted on wild rodents, more specifically on cotton rats (*Sigmodon hispidus*) found in industrial habitats where there are petrochemical deposits; The authors aimed to identify structural lesions in liver and kidney tissue through histopathological evaluation (Kim et al., 2001).
Regarding histopathology, it is an essential method in assessing the effects of biological and environmental stress factors on living organisms, allowing the identification of structural changes at the cellular and tissue level. Histopathological biomarkers, such as inflammation, necrosis, fibrosis and cellular degeneration, are very often used in monitoring the health of wildlife and domestic animals (Au, 2004; Flora et al., 2008).

MATERIALS AND METHODS

This study included 8 wild boars, originating from authorized hunting activities in the Transylvania region, Romania. Regarding the study area, it is characterized by mixed habitat (forest and agricultural), with potential for exposure to biological and environmental factors specific to wildlife. The collection of samples from the wild boar was carried out during the 2023-2024 hunting season, in accordance with national legislation and regulations in force. Of the total of 8 wild boars that we included in the study, both male and female specimens were examined. The distribution by sex was as follows: 5 males and 3 females. A total of 40 tissue samples were collected from 8 wild boars, namely: lungs, heart, liver, kidneys and skeletal muscles. Histopathological tissue lesions were identified with variable frequencies in all examined samples, except for skeletal muscles, where no significant structural changes were observed. Thus, in the study we highlighted only the most relevant lesions. Regarding the tissue samples, they were processed and evaluated from a histological point of view at the Morphopathology Laboratory, Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania.
Tissue samples were collected during the postmortem examination and fixed in a 10% formalin solution for 24-48 hours. After fixation, they were processed and embedded in paraffin according to the standard paraffin-embedding protocol. The paraffin blocks were then sectioned using a Thermo Scientific HM325 manual microtome, producing 2 µm thick sections. These were mounted on histological slides, incubated at 60°C for 24 hours, stained with hematoxylin-eosin (HE), and examined using an Olympus BX40 microscope. Images were obtained with an Olympus SC180 camera and processed using the cellSens software.

RESULTS AND DISCUSSIONS

This study evaluated the histopathological changes in the main internal organs of wild boars from the Transylvania region, highlighting the presence of lesions of variable intensity in the lung, liver, kidney and heart, while skeletal muscle was unaffected.
Interest in the consumption of game meat, including wild boar, has become a concern at the level of Romania as well. Game meat diversifies the daily diet, being considered a high quality meat if it comes from unpolluted areas. Numerous studies in the specialized literature evaluate the nutritional quality and sustainability of wild boar meat, mentioning the high-quality proteins, the relatively low fat content, the vitamin and mineral content, and all the technological characteristics recommend it for inclusion in a diversified diet (Quaresma et al., 2011; Kasprzyk et al., 2019; Kallas et al., 2021).
We followed the results regarding the lesions at the tissue level, in order to confirm the quality of the boar meat, but also the environment in which it lives.
In the case of the lungs, the histological examination reveled severe inflammatory and necrotic changes, with intralesional presence of multiple parasitic forms, consisting of larvae and adults of *Metastrongylus* spp. They were found inside bronchi and bronchioles, in some cases obstructing the lumen, and leading to desquamation and sloughing of epithelial cells and, in some cases, to the hyperplasia of bronchiolar epithelium. These changes were

accompanied by hyperplasia of bronchial associated lymphoid tissue (BALT) and smooth muscle hyperplasia. Multifocal, we identified peribronchial granulomatous pneumonia, composed of numerous macrophages, lymphocytes, eosinophils and giant multinucleated cells, mixed with fibrosis and congestion. In some cases, the necrotic centre of granulomas presented multiple mineralised foci (Figure 1).

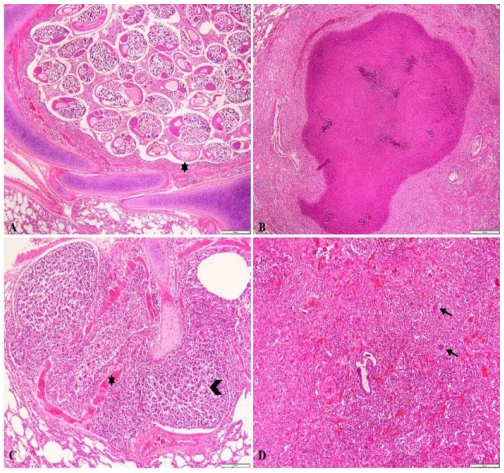


Figure 1. Histological aspect of the pulmonary lesions: A - bronchial obstruction by numerous parasitic forms of *Metastrongylus* spp., with epithelial hyperplasia (star), bar=500 µm; B - peribronchiolar granulomatous pneumonia, bar=500µm; C- hyperplasia of peribronchial associated lymphoid tissue (arrow head) and smooth muscle hyperplasia (star), bar=200µm; D - granulomatous and eosinophilic pneumonia, showing bronchiolar destruction and presence of multiple multinucleated giant macrophages (arrows), bar=200 µm; HE stain (original)

According to the literature, the lung lesions in this study are similar to those of *Metastrongylus* spp. infections in wild boar. These nematodes can colonize the bronchi and bronchioles, causing chronic inflammation, epithelial hyperplasia, eosinophil-rich inflammatory infiltrates and the formation of peribronchial granulomas. In more severe cases, airway obstruction can lead to major lung damage and of course to the appearance of foci of fibrosis and mineralization (Humbert & Henry, 1989; Cabaj et al., 1997).

Regarding the heart, there were no gross changes visible. Histologically, the only lesions was the presence of multiple protozoal cyst (*Sarcocystis* spp.) expanding the cardiomyocytes. These cysts consisted of a thin, hyalinized, acellular membrane that contained a myriad of bradyzoites. The muscle fibres were, occasionally swollen, with a hypereosinophilic sarcoplasm. There were no inflammatory infiltrates observed (Figure 2).

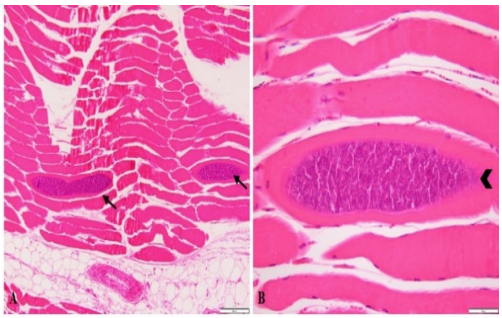


Figure 2. Histological aspect of the miocardial lesions: A - *Sarcocystis* cysts inside cardiomyocytes (arrows) containing numerous bradyzoites, bar=200µm; B - peribronchiolar granulomatous pneumonia, bar=50µm; HE stain (original)

Sarcocystis spp. cysts observed in the myocardium are associated with minimal degenerative changes in cardiomyocytes and the absence of inflammatory infiltrates. These observations are consistent with studies showing that cardiac sarcocystosis in wild boar is frequently a subclinical infection, characterized by the presence of intramuscular cysts without an essential inflammatory reaction from the host (Odening, 1998; Dubey et al., 2015).

The lesions present in the liver parenchyma were mild, and consisted of multiple foci of inflammatory infiltrate, with mononuclear cells, mainly lymphocytes. In some individuals we identified many intracytoplasmic clear vacuoles, of different sizes, causing cellular augmentation and multifocal necrosis (hepatic steatosis). When present, this lesion was diffuse and severe. The etiology of this degeneration is believed to be nutritional (influenced by the diet, feeding habits of wild boars, obesity), metabolic (hypothyroidism, adrenal pathologies) or circulatory (hypoxia related steatosis) (Figure 3).

In the case of the hepatic changes observed in the study, namely focal lymphocytic infiltrates and hepatic steatosis, these changes have also been reported in other wild boar populations. We also mention that the liver is an organ with

an important role in metabolic and detoxification processes, being frequently affected by all nutritional imbalances, metabolic changes or exposure to various environmental stress factors. Hepatocyte vacuolization and lipid accumulation may represent common changes described in wild animals under metabolic stress (Cullen & Stalker, 2016).

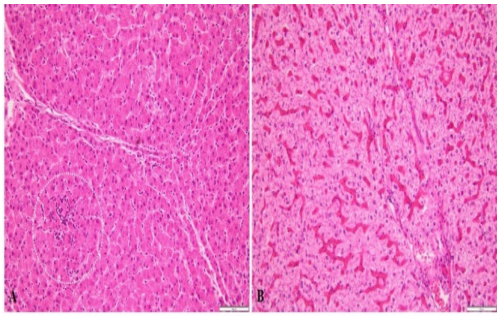


Figure 3. Histological aspect of the hepatic lesions: A - focal minimal lymphocytic hepatitis (circled area); B - diffuse severe hepatic steatosis; HE stain, bar=100 µm (original)

Multifocally, the renal parenchyma was distended by a moderate inflammatory infiltrate, composed of lymphocytes, histiocytes and a small number of plasma cells, located interstitially and perivascular. This change was present both in the cortex and the medulla, sparing the renal pelvis. Though no microbiology culture was performed in these cases, it is possible that these changes were brought on by chronic infectious diseases (Figure 4).

The lymphohistiocytic interstitial nephritis observed in our study is comparable to lesions described in cervids and other wildlife species exposed to various infectious agents or multiple external environmental factors. This lesion usually indicates a chronic but persistent inflammatory process, characterized by infiltration of the renal interstitium with mononuclear cells and long-term impairment of renal function (Beiglböck et al., 2002).

In the case of the muscle tissue in our study, the histopathological examination revealed no changes or cellular damage, suggesting that the muscle is safe for food consumption from this region.

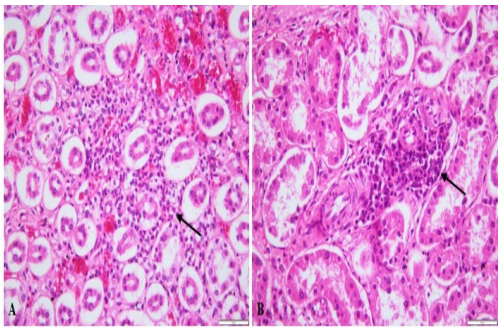


Figure 4. Histological aspect of the renal lesions: A, B - Moderate, multifocal, lympho-histiocytic interstitial nephritis; HE stain, bar=50µm (original)

Regarding the absence of muscle lesions, it is an essential result, especially from a food safety perspective. Therefore, according to studies in the specialized literature, skeletal muscle is usually less affected by chronic pathological processes or even by local accumulation of contaminants, being also the most consumed organ by the human population (Amici et al., 2012; Kallas et al., 2021).

CONCLUSIONS

The histopathological examination in this study revealed lesions with varying degrees of severity in the lungs, liver, kidneys and heart of the examined wild boars, while the skeletal muscles did not show significant changes. Pulmonary lesions associated with infestation with *Metastrongylus* spp. represented the most important pathological changes identified in the present study.

Sarcocystis spp. cysts were observed in the myocardium, and chronic inflammatory processes and degenerative changes were highlighted in the liver and kidneys. It is worth mentioning that the absence of significant lesions for the skeletal muscles indicates a good structural integrity of this tissue intended for human consumption. Our results emphasize the importance of constant monitoring of wild boar populations through histopathological examinations, as a useful tool in disease surveillance and environmental quality assessment.

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GAME MEAT AND CONVENTIONAL MEAT: A COMPARATIVE REVIEW OF NUTRITIONAL COMPOSITION AND TECHNOLOGICAL CHARACTERISTICS

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Abstract

This review analyzes the nutritional profile and technological properties of game meat in comparison with conventional meat, based on a structured search of scientific databases such as Scopus, Web of Science, and PubMed. Game meat is generally distinguished by a higher protein content, lower fat levels, and a more favorable fatty acid composition, particularly due to increased polyunsaturated fatty acids and omega-3 content. It is also an important source of essential micronutrients, including iron, zinc, and vitamin B12. From a technological standpoint, however, game meat is more prone to lipid oxidation, which can negatively impact shelf life and processing stability. In contrast, conventional meat exhibits greater oxidative stability but typically contains higher levels of saturated fat. These variations are influenced by species, diet, physical activity, and rearing conditions.

Key words*: conventional meat, game meat, nutritional profile.*

INTRODUCTION

Health-conscious consumers have driven the growing demand for meat with reduced fat and cholesterol content, which, in turn, has sparked renewed interest in suitable alternatives to meat products from traditional agricultural sources. Game meat could meet the needs of consumers today and in the coming years as an alternative to meat from domestic animals, as it is characterized by an excellent chemical composition, low fat content, an optimal ratio of unsaturated to saturated fatty acids, high protein content and a favorable protein composition, as well as a distinctive taste and aroma (Tomasevic et al., 2018). Growing consumer concern for health, food safety, and quality is driving an increasing demand for meat products with high nutritional value and optimal sensory properties (Anchidin et al., 2023; Boișteanu et al., 2025). However, consumer behavior is becoming increasingly complex and variable, influenced not only by preferences but also by product availability and past experiences. Game meat and conventional meat must be evaluated comparatively not only in terms of nutritional composition and technological characteristics but also in terms of how they align with consumer perceptions and expectations (Hoffman & Wiklund, 2006). Interest in hunted wild game meat (HWGM) is growing, but consumer perceptions and acceptance are strongly influenced by geographical and cultural contexts. Although this type of meat has characteristics that appeal to consumers (e.g., naturalness, nutritional value), market development is limited primarily by low availability and food safety concerns (Corradini et al., 2022). Game meat meets the demands of modern consumers due to its natural qualities and favorable nutritional profile; however, intensified production may compromise these advantages, particularly by altering the fatty acid composition, while gaps remain regarding the impact of production systems, post-mortem handling, and new consumption trends on the final quality (Hoffman & Wiklund, 2006). As for production and consumption strategies, the inclusion of natural antioxidants and fiber in processing methods is gaining increasing acceptance among consumers (Anchidin et al., 2024; Manoliu et al., 2024). In addition, the integration of biodegradable or active smart packaging aligns with the sustainability trends associated with the consumption of game meat, which is often considered a more natural alternative to conventional meat (Davidescu et al., 2025). In general, the available scientific evidence suggests that game meat products offer

a higher-protein, lower-fat, and lower-calorie alternative to conventional protein sources such as chicken, beef, and pork (Tomasevic et al., 2018; Ciobanu et al., 2020; Boișteanu et al., 2024). This article aims to highlight the differences between game meat, which has a superior nutritional profile compared to conventional meat (more protein, less fat, and a favorable fatty acid profile), but has technological limitations, particularly a higher susceptibility to lipid oxidation, which affects its stability and shelf life. The differences between the two types of meat are determined by factors such as species, diet, activity level, and rearing system.

MATERIALS AND METHODS

This study was designed as a narrative review, aiming to synthesize and critically discuss existing scientific literature regarding the nutritional composition and technological characteristics of game meat in comparison with conventional meat. A comprehensive literature search was conducted using major scientific databases, including Scopus, Web of Science, and PubMed. The search was performed using combinations of the following keywords:

1. *"game meat"*, *"venison", "wild meat"*;
2. *"conventional meat"*, *"red meat"*;
3. *"nutritional composition"*, *"fatty acid profile"*;
4. *"technological properties"*, *"meat quality"*, *"lipid oxidation"*.

Boolean operators (AND, OR) were applied to refine the search results.

Table 1. Inclusion and exclusion criteria applied in the literature selection process

Inclusion criteria	Exclusion criteria
Peer-reviewed articles published in English;	Non-scientific publications (e.g., reports without peer review);
Studies focusing on game meat and/or conventional meat;	Studies lacking relevant comparative or compositional data;
Research addressing nutritional composition and/or technological properties;	Duplicate records.
Publications from approximately the last 20-25 years, with emphasis on recent findings.	

The selection process involved screening titles, abstracts, and full texts to identify relevant studies. The retained articles were critically analyzed and grouped according to key themes as nutritional composition (proteins, lipids, micronutrients); fatty acid profile; technological characteristics; influencing factors (species, diet, rearing system, physical activity). As a narrative review, this study does not follow a fully systematic protocol and may be subject to selection bias. However, efforts were made to include representative and high-quality studies to ensure a balanced and comprehensive overview.

RESULTS AND DISCUSSIONS

Comparative Nutritional Composition of Game and Conventional Meat

Game meat is characterized by a very low fat content, typically below 3-4%, with minimal intramuscular fat deposition. However, from a nutritional perspective, the fatty acid composition, particularly the ratio of polyunsaturated to saturated fatty acids (PUFA/SFA) and the balance between omega-6 and omega-3 fatty acids, is more relevant than total fat content. A higher PUFA/SFA ratio and a lower n-6/n-3 ratio are associated with improved cardiovascular health outcomes. Meat from grazing animals, including most game species, tends to exhibit a more favorable fatty acid profile due to higher levels of omega-3 fatty acids, especially α-linolenic acid, contributing to a reduced risk of chronic diseases (Hoffman, L. C., & Wiklund, E., 2006).

Game meat is generally characterized by a superior nutritional profile compared to conventional meat, including higher protein content, lower fat levels, and a more favorable fatty acid composition (Boișteanu et al. 2024).

In particular, the PUFA/SFA and n-6/n-3 ratios are more aligned with dietary recommendations, mainly due to the natural feeding regimes and higher physical activity of wild animals (Hoffman & Wiklund, 2006; Wood et al., 2003).

In comparison with conventional meat, game meat generally contains lower intramuscular fat and higher levels of essential nutrients, which may influence both its technological behavior and consumer perception.

A comparative overview of these differences is presented in Table 2.

Table 2. Comparative nutritional composition of game meat and conventional meat

Parameter	Game meat	Conventional meat	Scientific interpretation	References
Protein content	High	Moderate to high	Slightly higher in game meat due to greater physical activity.	Hoffman & Wiklund (2006); Valencak et al. (2015); Wood et al. (1999); Wood et al. (2003); Cordain et al. (2002)
Intramuscular fat	Very low	Moderate to high	Game meat is leaner.	
Total fat content	Low	Higher	Influenced by feeding system.	
Fatty acid profile	More favorable	Less favorable	Higher PUFA in game meat.	
PUFA/SFA ratio	Higher	Lower	Closer to recommended values in game meat.	
n-6/n-3 ratio	Lower	Higher	More balanced in game meat.	
Cholesterol content	Similar	Similar	No major nutritional differences.	
Iron (Fe)	Higher	Moderate	Rich source of heme iron in game meat.	
Zinc (Zn)	Higher	Moderate	Important micronutrient from game meat.	
Vitamin B12	Higher	Moderate	Linked to muscle metabolism.	
Energy value	Lower	Higher	Due to lower fat content.	

Wild ruminant meat is characterized by extremely low intramuscular fat content, often below 1%, although notable interspecies differences exist. While cholesterol levels may be comparable to or slightly higher than those found in domestic animals, the fatty acid composition is generally more favorable from a nutritional perspective. Specifically, wild ruminant meat contains lower proportions of saturated fatty acids and higher levels of polyunsaturated fatty acids, along with a beneficial n-3/n-6 ratio and the presence of conjugated linoleic acid (CLA). These characteristics are primarily influenced by the animals' natural diet, whereas the use of concentrate-based feeding systems has been shown to increase total fat and saturated fatty acid content while reducing PUFA, particularly n-3 fatty acids. Additionally, feeding behavior (browsers vs. grazers) further contributes to variability in fatty acid profiles among species (Ramanzin et al., 2010).

Technological Properties of Game Meat in Comparison with Conventional Meat

Wild boar meat exhibits specific sensory characteristics, including the potential occurrence of undesirable off-odours such as boar taint, as well as an increased susceptibility to lipid oxidation compared to pork. Although differences in biogenic amine content between wild boar and domestic pig products are relatively minor, rancidity development tends to be more pronounced in wild boar-derived products. However, the application of appropriate processing technologies, particularly the use of starter cultures, can significantly reduce these negative effects. Consequently, it is possible to produce reduced-fat wild boar meat products with sensory attributes comparable to conventional counterparts in terms of taste, aroma, and texture (Bauer et al., 2014).

Processing technologies play an important role in determining the technological and sensory quality of meat products. For instance, the use of liquid smoke as an alternative to traditional smoking has been shown to influence oxidative stability, flavor development, and overall product quality depending on the structural characteristics of the meat matrix (Ciobotaru et al., 2024). Such effects are particularly relevant in the case of game meat, which, due to its lower fat content and higher susceptibility to oxidation, may respond differently to processing techniques compared to conventional meat.

The incorporation of additives significantly influences the physicochemical and technological properties of meat products, particularly in heterogeneous matrices, and is especially important for game meat, which, due to its low fat content and specific muscle structure, requires technological interventions to achieve quality attributes comparable to conventional meat (Flocea et al., 2024).

Another method, the incorporation of functional ingredients, represents an effective strategy to improve the sensory quality of meat products. For example, the addition of bone broth has been shown to enhance taste, texture, and overall acceptability in processed meat products with

heterogeneous structures (Ciobotaru et al., 2024). Such approaches may be particularly beneficial for game meat, which often exhibits firmer texture and variable sensory properties, thereby requiring technological interventions to increase consumer acceptance.

The incorporation of plant-based ingredients represents a promising strategy for enhancing both the technological and nutritional quality of meat products. For instance, the addition of apple and sugar beet fibers has been shown to improve structural properties and overall product stability in meat-based formulations (Manoliu et al., 2025). Sensory quality represents a key determinant of consumer acceptance for both game and conventional meat products. Studies on processed meat products have shown that attributes such as texture, flavor, and overall acceptability are strongly influenced by product formulation and processing conditions (Ciobanu et al., 2023). These aspects are particularly important in the case of game meat, which is often characterized by a firmer texture and a more intense flavor compared to conventional meat, requiring appropriate technological adjustments to meet consumer expectations.

Therefore, careful control of processing parameters is essential to maintain the technological quality of game meat and ensure its comparability with conventional meat products (Gucianu et al., 2023; Ayaz et al., 2024).

The valorization of animal by-products represents an important strategy for enhancing sustainability and adding value to meat production systems. For instance, bone broths derived from cattle have been shown to possess significant nutritional and functional properties, depending on factors such as animal age and processing conditions (Anchidin et al., 2024). Such approaches are highly relevant for both conventional and game meat sectors, particularly in the context of circular economy principles and the need to maximize resource utilization.

Factors Influencing Differences Between Game and Conventional Meat

Wild ungulate production systems vary significantly depending on their intended purpose, namely hunting, restocking or meat production, leading to distinct management and selection strategies. Systems aimed at restocking prioritize the preservation of natural behavior, low stocking densities, minimal human interaction, and traits such as agility and adaptability. In contrast, meat-oriented production systems focus on higher growth rates, increased body weight, and more intensive feeding regimes. These divergent approaches also extend to breeding objectives, where temperament and productivity are prioritized in venison production, whereas maintaining wild traits is essential in hunting systems. Consequently, these differences in management and selection criteria have important implications for animal performance, product quality, and overall production efficiency (Piasentier et al., 2005). The differences between game meat and conventional meat are influenced by multiple interacting factors, including diet, physical activity, environmental conditions, species-specific traits, and pre- and post-mortem handling, as well as processing technologies and consumer perception, all of which contribute to variations in nutritional composition and technological quality. Feed composition significantly influences meat quality, particularly in conventional systems where ingredients such as soybean can be replaced with alternatives like chickpea to modify nutritional and functional properties (Ciobanu et al., 2025). Feed-related contaminants, such as mineral oil hydrocarbons, may influence meat quality and safety, particularly in conventional production systems where animals are exposed to industrial feed sources (Matei & Pop, 2023).

CONCLUSIONS

Game meat represents a nutritionally valuable alternative to conventional meat, characterized by higher protein content, lower fat levels, and a more favorable fatty acid profile, particularly in terms of polyunsaturated and omega-3 fatty acids. However, its increased susceptibility to lipid oxidation poses technological challenges related to shelf life and processing stability. In contrast, conventional meat offers greater oxidative stability and production consistency but is generally associated with higher saturated fat content. Overall, the differences between these two meat types are primarily driven by

species, diet, physical activity, and rearing systems, highlighting the need for optimized processing strategies to fully exploit the nutritional advantages of game meat while ensuring product quality and consumer acceptability.

USE OF GENERATIVE ARTIFICIAL INTELLIGENCE (GenAI)

The authors state that ChatGPT was used exclusively for linguistic review and refinement of the text in the "Introduction" and "Conclusions" sections of this paper. All scientific content, data processing, analyses, and interpretations were developed independently and critically reviewed by the authors.

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DEVELOPMENT AND COMPREHENSIVE EVALUATION OF CHICKPEA-BASED VEGAN CHEESE ALTERNATIVES: PHYSICOCHEMICAL, NUTRITIONAL AND SENSORY PROFILES

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Abstract

In the light of increasing health, sustainability, and ethical concerns, plant-based diets are rapidly gaining traction. This study developed chickpea-based vegan cheese analogues, evaluating three formulations: a control (C), a variant enriched with turmeric, black pepper, and oregano (VC1), and one with dried tomatoes and basil (VC2). A multi-analytical approach was used to quantify total polyphenol content (TPC), antiradical activity (DPPH), and antioxidant capacity (CUPRAC), alongside proximate composition and sensory profiles. Results showed that enriched formulations (VC1 and VC2) possessed significantly higher phenolic concentrations and enhanced antioxidant potential than the control, with VC1 exhibiting the highest values. Moreover, these variants achieved superior hedonic ratings, suggesting that strategic integration of natural additives effectively optimizes both the functional architecture and consumer acceptability of plant-based dairy alternatives. .

Key words*: antioxidant activity, chickpea, polyphenols, proximate composition, vegan cheese alternatives.*

INTRODUCTION

Food systems exert a massive impact on the planet, accounting for up to 37% of net greenhouse gas emissions (Grasso et al., 2023). As a primary driver of the climate crisis and biodiversity loss, the animal-based sector necessitates a fundamental rethink of global dietary patterns (Benton et al., 2021). Driven by a growing global population and the urgent need to reduce our environmental footprint, the industry is increasingly focused on developing innovative plant-based alternatives to traditional staples like cheese. Consequently, the plant-based dairy sector is undergoing rapid expansion, challenging food producers to create vegan substitutes that balance innovation with nutritional density and affordability. This shift is further propelled by rising market demand from consumers adopting meat- and dairy-free diets for ethical, environmental, or health-related reasons (Mefleh et al., 2022). Currently, the nutritional profile of most commercial alternatives is deficient, as they primarily rely on starch and solid fats (such as coconut oil) as base ingredients. This results in a product with low protein content and high levels of saturated fats, making it significantly inferior to traditional cheese. Furthermore, discrepancies in taste and price prevent these products from meeting consumer demands, even though this category holds the highest untapped demand potential within the plant-based market (Grasso et al., 2023). With a view to developing plant-based dairy alternatives featuring optimized nutritional profiles and a reduced environmental footprint, various research collectives are currently evaluating the feasibility and

technological potential of plant-derived protein ingredients (Grasso et al., 2023; Grossmann & McClements, 2021; Mefleh et al., 2022).

Pulses are fundamental components of traditional diets and serve as a vital source of plant-based proteins. Through flours and isolates, they provide innovative solutions for meeting daily protein requirements (Boye et al., 2010). Among these, the chickpea (*Cicer arietinum* L.) ranks second in global consumption and is cultivated in over 50 countries. Beyond its therapeutic role in managing diabetes, it offers a dense matrix of essential micronutrients, including a broad spectrum of vitamins (A, C, E, K, and the B-complex) and critical minerals such as iron, zinc, magnesium, and calcium (Gaur et al., 2010). Chickpeas are particularly distinguished by a protein content of 20-25%, complemented by significant levels of dietary fiber, and bioactive compounds like phenolic acid (Hall et al., 2017). This nutritional versatility, coupled with superior functional properties, positions chickpea-derived ingredients as ideal candidates for plant-based cheese analogues. Specifically, chickpea proteins exhibit high efficiency in emulsification and gelling-traits largely attributed to the structural complexity of globulins and the hydrophobic interactions that drive their functionality (Kaur & Singh, 2007; Withana-Gamage et al., 2011).

Turmeric (*Curcuma longa* L.) is an ancient spice and traditional remedy used as a medicine, condiment, and dye, originating from the tropical regions of South Asia as a rhizomatous herbaceous perennial of the *Zingiberaceae* family (Prasath et al., 2024). Its yellow colour and medicinal properties are derived from curcuminoids, a group of polyphenolic compounds, among which curcumin stands out as the primary constituent evaluated for its health-promoting activities. Beyond these, turmeric contains sugars, proteins, resins, and volatile oils - such as turmerone, atlantone, and zingiberene - some of which may also exhibit bioactivity. Consequently, numerous preclinical investigations have identified a wide range of potential health benefits, including treatments for heart disease, arthritis, Alzheimer's, gastrointestinal disorders, and metabolic syndrome (Hay et al., 2019; Singletary, 2020).

Due to their highly perishable nature, tomatoes (*Solanum lycopersicum*) are frequently subjected to dehydration processes to extend shelf life and facilitate their use as versatile raw materials in the production of functional foods, nutraceuticals, and diverse processed commodities (Pravitha et al., 2024). These fruits are characterized by a dense micronutrient profile, notably providing an average of 20 mg/100 g of vitamin C, representing approximately 40% of the recommended daily intake - alongside a complex matrix of phenolic acids, flavonoids, and carotenoids that underpin their significant antioxidant capacity. Furthermore, the concentration of soluble sugars and organic acids within the dry matter fraction is a critical determinant of palatability, as these constituents govern the organoleptic balance between sweetness and acidity (Bakir et al., 2023). Empirical evidence suggests that thermal processing at a controlled temperature of 60°C optimizes the retention of these bioactive compounds, ensuring superior phytochemical stability both immediately post-dehydration and during subsequent storage periods (Kaur et al., 2020).

The aim of this work is to formulate and characterize three chickpea-based vegan cheese variants: a plain control and two enriched versions (turmeric/black pepper/oregano and sun-dried tomato/basil). The study assesses their nutritional and sensory attributes, alongside their antioxidant activity and total polyphenol content.

MATERIALS AND METHODS

Chickpea-based vegan cheese alternatives preparation

Three chickpea-based cheese alternatives (C, VC1 and VC2) were developed using raw materials sourced from the Romanian market (Figure 1), following the specific formulations detailed in Table 1.

In the preparation of the three chickpea-based cheese analogues, ingredients were precisely weighed in accordance with the established formulations. The mixture underwent homogenization until a uniform consistency was achieved, followed by thermal treatment (boiling) until the desired viscosity was

reached. The resulting hot matrix was transferred into molds pre-treated with a thin film of vegetable oil. Upon cooling, the samples were refrigerated for a duration of 2 hours, subsequently vacuum-sealed, and maintained under strictly controlled refrigeration conditions.

Table 1. Recipes for chickpea-based vegan cheese alternatives

Product / Materials (%)	C	VC1	VC2
Chickpea flour	30	29	29
Extra virgin olive oil	3	3	3
Lemon juice	3	3	3
Garlic powder	0.5	0.5	0.5
Onion flasks	0.5	0.5	0.5
Nutritional yeast flakes	1.5	2	2
Water	60	59	59
Turmeric powder		0.5	
Black pepper grounded		0.5	
Dried oregano		0.5	
Dried tomato flakes			1
Dried basil			0.5
Salt	1.5	1.5	1.5



C VC1 VC2

Figure 1. The three types of chickpea-based vegan cheese alternatives (own source)

Total polyphenol content evaluation

The total phenolic content (TPC) of the raw materials and the resulting chickpea-based cheese analogues was quantified via the Folin-Ciocalteu spectrophotometric assay. Samples were subjected to extraction using 70% (v/v) ethanol. The analytical protocol was executed according to the methodology described by Dumbrava et al. (2020). Quantitative results were calculated using a gallic acid calibration curve and expressed as milligrams of gallic acid equivalents per 100 grams (mg GAE/100g).

Antioxidant activity evaluation

The antioxidant capacity of both the raw materials and the finished chickpea-based cheese analogues was quantified via the Cupric Ion Reducing Antioxidant Capacity (CUPRAC) assay. To maintain methodological uniformity across all spectrophotometric measurements, the analysis was conducted on the previously prepared 70% (v/v) ethanolic extracts, strictly adhering to the protocol described by Dumbrava et al. (2020). Quantitative data were reported in milligrams of Trolox equivalents per gram of sample (mg TE/g).

Antiradical activity evaluation

The antiradical capacity of the raw materials and the resultant chickpea-based cheese analogues was quantified by determining their 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (RSA). The analytical procedure was conducted in accordance with the methodology previously presented by Poiana et al. (2022), ensuring technical consistency across the experimental framework.

Proximate composition and energy value determination

The proximate composition of the chickpea-based cheese analogues was determined in accordance with standardized methods established by the International Organization for Standardization (ISO). Specifically, the crude protein content was quantified according to SR ISO 937:2007, total lipids were extracted per SR ISO 1443:2008, and mineral (ash) content was determined via SR ISO 936:2009. Sugars and fiber concentrations were assessed following SR ISO 91-2007 and ISO 13906:2007, respectively. Moisture content was evaluated using a FOSS Fibertec 2010 M6 system (Sweden) following SR ISO 1442:2010. The total carbohydrate content was derived by difference, subtracting the combined percentages of protein, lipids, dietary fiber, minerals, and moisture from 100%. Finally, the energy value (kcal/100 g) was estimated by applying the Atwater conversion factors: 9 kcal/g for lipids and 4 kcal/g for both proteins and carbohydrates

Statistical data processing

Quantitative data, derived from triplicate determinations of total phenolic content (TPC), antioxidant capacity, antiradical activity, and proximate composition, were subjected to

statistical analysis. Data processing and descriptive statistics were performed using Microsoft Excel 2010 (Microsoft Corp., Redmond, WA, USA), with results expressed as the mean value ± standard deviation (SD) to ensure analytical reproducibility.

Sensory analysis

The organoleptic properties of the chickpea-based analogues were evaluated in accordance with the ISO 4121:2002 standard. The sensory panel comprised 20 semi-trained evaluators (balanced by gender, aged 21-60 years). Assessments were conducted using a 5-point hedonic scale, where scores ranged from 1 (dislike extremely) to 5 (like extremely), encompassing six key sensory descriptors: external appearance, appearance in section, smell, taste, flavour, texture, and overall acceptability. Ethical compliance was maintained through the acquisition of written informed consent from all participants, adhering to European Union research guidelines (Alfonsi et al., 2012). To ensure reproducibility, the evaluation was performed under controlled environmental conditions, with score interpretations and acceptability thresholds aligned with the methodology established by Lim (2011).

RESULTS AND DISCUSSIONS

Total polyphenol content

TPC data for the raw materials and chickpea-based vegan cheese analogues are presented in Table 2.

The assessment of the TPC within the raw ingredients reveals that turmeric powder yielded the most significant phenolic concentration (4147.25 ± 11.06 mg GAE/100 g), substantially surpassing dried oregano (1014.18 ± 9.28 mg GAE/100 g) and dried basil (741.82 ± 5.22 mg GAE/100 g). Conversely, the lowest concentration was identified in the chickpea flour (59.63 ± 1.06 mg GAE/100 g).

In a study of 40 chickpea genotypes, Gupta et al. (2017) observed lower TPC concentrations than those found in the present study, with values ranging from 4.70±0.10 to 35.40 ± 0.20 mg GAE/100 g. For commercial onion powder, a comparable value of 386.60±10.02 mg GAE/100 g DM was reported by Bamba et al. (2020). Bieżanowska-Kopeć et al. (2022) observed TPC levels in commercial dried thyme (632.09 ± 17.68 to 867.57 ± 18.16), basil (548.99 ± 12.90 to 707.43 ± 10.51), and black pepper (443.24 ± 18.16 to 610.14±0.96 mg GAE/100 g) that are consistent with our results. For dried tomatoes, the TPC values obtained in this study fall within the range reported by Toor și Savage (2006), which varied from 235 ± 23.6 to 387 ± 19.8 mg GAE/100 g.

Table 2. TPC in chickpea-based vegan cheese analogues and their constituent raw materials

Sample	TPC (mg GAE/100 g)
Chickpea flour	59.63 ± 1.06
Lemon juice	61.48 ± 0,81
Garlic powder	502.27 ± 4.21
Onion flasks	358.14 ± 3.12
Turmeric powder	4147.25 ± 11.06
Black pepper grounded	608.42 ± 4.18
Dried oregano	1014.18 ± 9.28
Dried tomato flakes	288.61 ± 3.11
Dried basil	741.82 ± 5.22
C	31.16 ± 0.62
VC1	48.61 ± 1.04
VC2	40.44 ± 0.83

For the chickpea-based vegan cheese analogues, the results highlighted that the lowest TPC was recorded for the control sample (C) (31.16±0.62 mg GAE/100 g). In the VC1 (48.61±1.04 mg GAE/100 g) and VC2 (40.44±0.83 mg GAE/100 g) formulations, the TPC values were higher than the control by 54.83% and 29.03%, respectively.

With respect to the total polyphenol content (TPC) in various dairy-based matrices, Sik et al. (2023) reported values of 19.01 mg GAE/100 g for Jersey ricotta, whereas Holstein ricotta exhibited the lowest concentration, at 10.55 mg GAE/100 g. Comparable TPC levels were documented for Simmental and Saanen goat ricotta samples, both quantified at 15.42 mg GAE/100 g. Notably, all of these dairy-derived values are substantially lower than the polyphenol concentrations determined in the present study for the chickpea-based vegan cheese analogues. Similarly, in the case of cottage cheese, Farrag et al. (2020) reported a TPC of 32.29 mg GAE/100 g, which also remains below the concentrations measured in the current investigation for the VC1 and VC2 vegan formulations. These findings underscore the superior antioxidant potential of the VC1 and VC2 formulations developed in the present

study. The elevated polyphenol concentrations observed in these chickpea-based matrices signify a robust capacity for free-radical scavenging, thereby positioning these vegan cheese analogues as functional dietary interventions with high biological value.

Antioxidant and antiradical activities

Antioxidant activity assessments of the raw materials and final products, conducted using the CUPRAC assay, resulted in the data summarized in Table 3.

The experimental data reveal a distinct hierarchy in antioxidant potency among the primary substrates: black pepper manifested the highest antioxidant and respectively DPPH free-radical scavenging capacity (60.11±0.90 mg TE/g, 96.42 ± 0.72%), followed by turmeric powder (57.25 ± 0.94 mg TE/g, 95.86 ± 0.81%), while chickpea flour (2.18 ± 0.14 mg TE/g, 34.28 ± 0.36%) yielded the lowest recorded values.

In comparison to the results reported by Bieżanowska-Kopeć et al. (2022) for commercial dehydrated basil and thyme (0.36 mg TE/g, ABTS assay), the present study yielded higher antioxidant values using the CUPRAC method. This discrepancy potentially reflects the distinct reaction mechanisms of these two analytical approaches. Specifically, the elevated results observed here may be attributed to the superior sensitivity of the CUPRAC assay toward the unique phenolic profile of these aromatic herbs compared to the ABTS radical scavenging method (Apak et al., 2006; Apak et al., 2007). This is particularly relevant as CUPRAC has been shown to correlate more strongly with total polyphenolic content in *Lamiaceae* species than the ABTS assay (Özyürek et al., 2008). Notably, the antioxidant capacities determined in the present study via the CUPRAC assay are in close agreement with the range (3.89-4.60 mg TE/g) reported by Toor and Savage (2006) for various tomato cultivars using the ABTS method. While these assays employ different reactive species, their mutual reliance on Single Electron Transfer (SET) mechanisms facilitates a meaningful comparison. These consistent findings suggest that the polyphenols and carotenoids, such as lycopene, within the dehydrated tomato matrix are equally proficient at reducing metallic ions and scavenging free radicals. Moreover, the capacity of the CUPRAC assay to simultaneously evaluate both hydrophilic and lipophilic antioxidants likely underpins the observed consistency with established literature. Furthermore, a comparative evaluation of multiple turmeric species and cultivars conducted by Akter et al. (2019) using the Oxygen Radical Absorbance Capacity (ORAC) assay revealed antioxidant capacities between 420.0 and 3522.5 mg TE/g - substantially exceeding those determined herein via the CUPRAC method. Regarding chickpeas, Jogihalli et al. (2017) reported a radical scavenging activity (RSA) of 22.85 ± 0.25%, a value lower than that observed in the present study.

For the three chickpea-based vegan cheese analogues, variants VC1 and VC2 demonstrated higher antioxidant activity and RSA values compared with the control sample. The incorporation of turmeric, black pepper, and dried thyme in variant VC1 resulted in an antioxidant activity of 1.68 ± 0.10 mg TE/g and an RSA of 32.16 ± 0.21%, corresponding to increases of 58.49% and 58.19%, respectively, relative to the control. Conversely, the addition of dried tomatoes and dried basil in variant VC2 produced an antioxidant activity of 1.14 ± 0.05 mg TE/g and an RSA of 25.28 ± 0.18%, representing increases of 7.55% and 24.23% compared with the control (1.06 ± 0.08 mg TE/g and 20.35 ± 0.12%, respectively).

Table 3. Antioxidant and antiradical activities of chickpea-based vegan cheese analogues and their constituent raw materials

Sample	Antioxidant activity (mg TE/g)	RSA (%)
Chickpea flour	2.18 ± 0.14	34.28 ± 0.36
Lemon juice	10.62 ± 0.24	90.74 ± 0.62
Garlic powder	12.62 ± 0.30	92.16 ± 0.53
Onion flasks	9.23 ± 0.28	83.86 ± 0.46
Turmeric powder	57.25 ± 0.94	95.86 ± 0.81
Black pepper grounded	60.11±0.90	96.42 ± 0.72
Dried oregano	11.33 ± 0.22	92.35 ± 0.48
Dried tomato flakes	4.34 ± 0.16	84.18 ± 0.38
Dried basil	7.53±0.61	83.68 ± 0.46
C	1.06 ± 0.08	20.35 ± 0.12
VC1	1.68 ± 0.10	32.16 ± 0.21
VC2	1.14 ± 0.05	25.28 ± 0.18

Proximate composition and energy value of chickpea-based vegan cheese analogues

The results concerning the proximate composition and energetic values of the three chickpea-based vegan cheese analogues are detailed in Table 4.

Table 4. Proximate composition and energy value of chickpea-based vegan analogues

Parameters	C	VC1	VC2
Proteins (g/100 g)	10.00±0.10	10.07±0.08	10.35±0.11
Lipids (g/100 g)	5.43±0.06	5.39±0,04	5.39±0,06
Saturated fatty acids (g/100 g)	0.57±0.02	0.57±0.01	0.57±0.01
Carbohydrates (g/100 g)	32.64	32.98	32.93
Sugar (g/100 g)	0.43±0.03	0.43±0.02	0.99±0.06
Dietary fiber (g/100 g)	7.71±0.12	8.37±0.13	8.09±0.11
Moisture (g/100 g)	42.86±0.31	41.84±0.28	41.84±0.32
Mineral substances (g/100 g)	1.36±0.04	1.35±0.05	1.40±0.03
Energy value (kcal/100 g)	221.07	220.71	221.63

As evidenced by the data, the proximate compositions of the three formulations exhibit a high degree of uniformity; nevertheless, the VC2 analogue demonstrates a marginal but statistically relevant superiority in protein content. Specifically, the protein concentration of VC2 (10.35 ± 0.11 g/100 g) exceeds that of VC1 (10.07 ± 0.08 g/100 g) and the control C (10.00 ± 0.10 g/100 g) by 2.78% and 3.5%, respectively. Furthermore, the sugar content, although low across all three formulations, was 2.30 times higher in VC2 (0.99 ± 0.06 g/100 g) compared to VC1 (0.43 ± 0.02 g/100 g) and C (0.43 ± 0.03 g/100 g). Additionally, the analysed formulations are distinguished by a significant dietary fiber profile; the highest concentration was identified in the VC1 analogue (8.37 ± 0.13 g/100 g), whereas the control sample (C) exhibited the lowest fiber content (7.71 ± 0.12 g/100 g).

Compared to the analogues presented in this work, the Romanian cow's milk cheeses analysed by Ibric et al. (2024) exhibited higher concentrations of moisture, crude protein, and total lipids, with maximum values reaching 57.71 ± 2.05 g/100 g (all proximate constituents were calculated based on the wet weight of the samples as analyzed, with values expressed in g/100 g of the total product weight), 17.50 ± 0.61 g/100 g, and 27.93 ± 0.95 g/100 g, respectively. Conversely, total carbohydrates showed a marked reduction (0.53 ± 0.01 to 1.44 ± 0.04 g/100 g), with no dietary fibre being reported.

In a study of vegan cheese analogues based on fava bean and respectively yellow pea flours, Ferawati et al. (2021) reported lower protein concentrations (4.2-4.4 g/100 g and 6.2-6.6 g/100 g, respectively) and total lipid contents (10.4-10.6 g/100 g and 9.9-10.8 g/100 g, respectively) compared to the formulations in the present work. Furthermore, while the total carbohydrate levels (3.3-8.3 g/100 g) were also reduced, the dietary fiber values (6.5-7.7 g/100 g) were found to be comparable to those observed in our current investigation.

Sensory analysis

The sensory evaluation of the three chickpea-based cheese analogues yielded the comparative data illustrated in Figure 2.

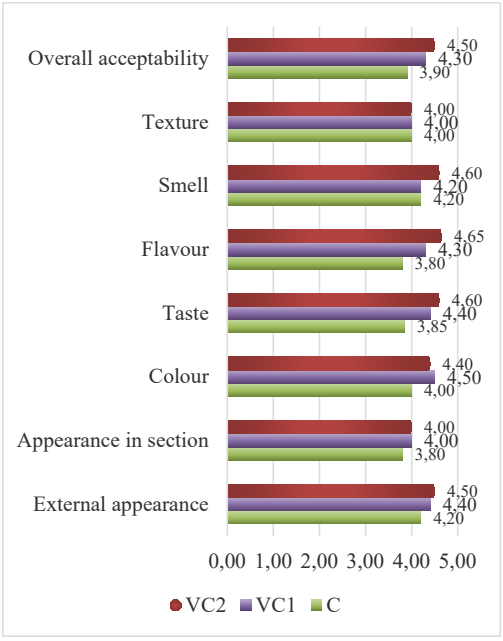


Figure 2. Comparative sensory profile and overall acceptability of chickpea-based cheese analogues

These results reflect the organoleptic performance of the formulations, assessed across various descriptors to determine consumer

acceptability and textural attributes. Although a uniform score of 4.00 was recorded for texture across all three samples, significant variations were observed in the remaining sensory attributes, with a distinct preference for the VC1 and, more prominently, VC2 variants. Specifically, VC2 exhibited superior performance, achieving "very good" ratings for external appearance (4.50), taste (4.60), flavour (4.65), smell (4.60), and overall acceptability (4.50), while VC1 followed with respective scores of 4.40, 4.40, 4.30, 4.20, and 4.30. In contrast, the control sample (C) yielded lower values for these parameters (4.20, 3.85, 3.80, 4.20, and 3.90, respectively); furthermore, while VC1 attained the highest acceptability for colour (4.50), followed by VC2 (4.40) and C (4.00), the cross-sectional appearance was rated equally for both VC1 and VC2 (4.00), surpassing the inferior score of 3.80 attributed to the control.

CONCLUSIONS

This research details the formulation and systematic evaluation of three distinct chickpea-based plant-derived cheese alternatives. These include a plain variant, a second variant fortified with turmeric, dried thyme, and black pepper, and a third variant incorporating dried tomatoes and dried basil.

The raw ingredients analysis identifies turmeric powder as the primary contributor to the total phenolic content, significantly outperforming the other botanical components and the leguminous base. The resulting chickpea-based analogues demonstrate a markedly superior antioxidant profile compared to the control sample, with the enriched formulations exhibiting a substantial increase in polyphenol concentration. Most notably, when benchmarked against traditional dairy matrices such as ricotta and cottage cheese, the developed vegan analogues consistently show higher phenolic levels. These findings highlight the formulations' robust capacity for free-radical scavenging and their potential as high-biological-value functional alternatives to conventional dairy products.

The research highlights a clear hierarchy in antioxidant potency among the utilized substrates, with black pepper and turmeric manifesting the most pronounced free-radical scavenging properties. Integrating these ingredients into the cheese analogue formulations optimized the antioxidant activity of the final products. Consequently, the addition of spices and herbs represents an effective strategy for improving the oxidative stability and nutritional quality of chickpea-based food matrices.

The developed formulations demonstrate a highly consistent proximate composition, with the VC2 analogue offering a marginal advantage in protein content. The primary distinguishing factor of these analogues is their robust dietary fiber profile, which significantly surpasses that of traditional dairy counterparts. While conventional Romanian cheeses and Gouda exhibit higher protein and lipid concentrations, the formulations presented in this work successfully address the nutritional limitations of traditional dairy products by providing a superior fibre-to-carbohydrate ratio. When compared to other pulse-based alternatives such as fava bean and yellow pea analogues, the chickpea formulations developed in this study exhibit a superior nutritional density, characterized by markedly higher protein and total lipid concentrations. While the dietary fibre profiles remain consistent across different leguminous sources, the elevated macronutrient levels in our formulations suggest a more robust nutritional profile than previously reported alternatives.

Sensory evaluation indicates that while texture remained consistent across all samples, the inclusion of functional ingredients positively influenced the remaining attributes. The enriched variants were markedly superior to the control, with the tomato and basil formulation being the most highly rated for taste, flavour, and overall acceptability. Simultaneously, the turmeric variant achieved the highest appreciation for colour, demonstrating that nutritional optimization through spice supplementation concurrently enhances the organoleptic properties of the products.

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PROCESSING OF CHICKPEAS FOR THE PRODUCTION OF MEAT-ALTERNATIVE PLANT-BASED FOODS

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Abstract

The growing demand for sustainable, nutritious, and ethical food systems has accelerated the development of plant-based meat alternatives. Chickpeas (Cicer arietinum L.) represent a promising raw material for such products due to their high protein content, favorable amino acid profile, functional starch, and widespread availability. Beyond nutritional and environmental considerations, consumer acceptance of meat alternatives is strongly driven by sensory attributes, including texture, juiciness, flavor, and appearance. This paper reviews and synthesizes current knowledge on the processing of chickpeas to obtain plant-based products with sensory, nutritional, and structural properties similar to meat. Particular emphasis is placed on how processing conditions and formulation strategies influence texture perception, flavor development, and overall sensory quality. Challenges and future research directions related to sensory optimization, digestibility, and consumer acceptance are also highlighted.

Key words*: chickpea, extrusion, meat alternatives, plant-based meat, protein extraction, sensory quality, texturization.*

INTRODUCTION

Global food systems face increasing pressure from population growth, climate change, and resource limitations (FAO, 2019). Livestock production contributes significantly to greenhouse gas emissions, land use, and water consumption (Poore & Nemecek, 2018). Consequently, plant-based meat analogs have emerged as viable alternatives to conventional meat products (Tso & Forde, 2021). Legumes are among the most studied protein sources for these applications, and chickpeas have attracted attention for their agronomic resilience, nutritional quality, and relatively neutral flavor profile (Boye, Zare, & Pletch, 2010). Chickpeas are traditionally consumed as whole seeds or in minimally processed products such as flour, hummus, and cooked seeds. However, advanced processing technologies now enable the transformation of chickpeas into structured, fibrous products that mimic the texture, appearance, and sensory attributes of meat (Dekkers et al., 2018). This paper focuses on the processing strategies required to convert chickpeas into meat-like plant-based foods.

MATERIALS AND METHODS

A systematic literature review was conducted to identify scientific studies addressing the processing of chickpeas (*Cicer arietinum* L.) for the development of plant-based meat alternatives. The literature search was performed using the Web of Science Core Collection database, which is widely used for indexing peer-reviewed publications in food science and technology. The search strategy included combinations of keywords related to chickpeas and plant-based meat analogues. The following search query was used:
(*"chickpea" OR "Cicer arietinum"*) *AND* (*"meat alternative" OR "meat analogue" OR "meat analog*" OR "plant-based meat").
The search was restricted to publications from 2016 to 2026 to capture the most recent technological developments in plant-based meat substitutes.
To ensure the relevance and quality of the reviewed literature, the following inclusion criteria were applied: peer-reviewed scientific articles and review papers; publications written in English; studies classified within the Food

Science and Technology research area; studies focusing on chickpea processing, chickpea protein extraction, functional properties, or applications in plant-based meat analogues.

The following exclusion criteria were applied: studies not related to food applications of chickpeas; publications focusing exclusively on agronomy, crop genetics, or animal feed; conference abstracts, editorials, and non-peer-reviewed sources; studies not addressing protein functionality, processing technologies, or meat analogue applications.

The initial search generated 14,949 records in the Web of Science Core Collection database. After applying filters for publication year (2016-2026), document type (article or review), research area (Food Science and Technology), and language (English), the dataset was reduced to 2,844 records. Further screening was performed by refining the search using Citation Topics Meso categories related to food science, resulting in 1,791 publications. To focus specifically on studies relevant to chickpea-based ingredients and their technological applications, an additional keyword filter ("chickpea flour") was applied, resulting in 1,325 references. Titles and abstracts were subsequently screened to identify publications directly related to: chickpea protein composition and functional properties, processing technologies (e.g., extrusion, fermentation, fractionation), and formulation of plant-based meat analogues using chickpea ingredients. Studies meeting these criteria were included in the qualitative synthesis presented in this review.

Relevant information was extracted from the selected publications, including chickpea composition and protein functionality; processing technologies used for the development of plant-based meat analogues; effects of processing on structural, nutritional, and sensory properties; and formulation strategies for chickpea-based meat substitutes.

The collected information was then organized into thematic categories covering processing technologies, functional properties, product formulation, sensory characteristics, and sustainability considerations. These categories were used to synthesize current knowledge on the potential of chickpeas as a raw material for plant-based meat alternatives.

RESULTS AND DISCUSSIONS

Composition and Functional Properties of Chickpeas

Chickpeas typically contain 18-24% protein, 40-50% carbohydrates (mainly starch), 5-7% lipids, and significant amounts of dietary fiber, vitamins, and minerals (Wood & Grusak, 2007; Jukanti et al., 2012). The protein fraction is rich in globulins (legumin and vicilin), which play a critical role in gelation, emulsification, and water-holding capacity, key properties for meat analog formulation (Kaur & Singh, 2007).

Functional properties of chickpea proteins include water and oil holding capacity, emulsifying ability, and gelation behavior (Grasso et al., 2022). These properties contribute to juiciness, fat stabilization, and texture formation in meat analogue systems. Native chickpea proteins, like most plant-derived proteins, are globulins that exist in a spherical (globular) shape rather than the elongated, fibrous structure found in animal muscle tissue. While they offer similar nutritional benefits and functional properties, such as emulsification and gelation, their natural structure lacks the anisotropy needed to mimic the chewy, fibrous texture of meat (Gulzar et al., 2025).

Processing of Chickpeas for Meat-Alternative Applications

Processing begins with cleaning, dehulling, and milling of chickpeas. Dehulling reduces fiber content and antinutritional factors while improving protein extractability (Khattab & Arntfield, 2009). Milling produces chickpea flour, which serves as the base material for further fractionation or direct texturization.

Based on the literature analysis, several key processing techniques are employed to convert chickpeas into meat-alternative structures: extrusion, fermentation, milling/fractionation, thermal processing, enzymatic hydrolysis, emulsion/gelation, and other emerging technologies.

Extrusion

High-Moisture Extrusion (Figure 1) is used to create "whole-muscle" meat analogues with a fibrous texture similar to chicken or beef. It typically uses moisture levels above 40%.

High-moisture extrusion is the most widely used technology for producing meat analogues (Cheftel et al., 1992). During extrusion, chickpea protein-rich material is subjected to heat, pressure, and shear, leading to protein denaturation, unfolding, and alignment (Akdogan, 1999). In high moisture extrusion, fibrous products are produced with moisture contents above 50 wt.%. The proteins are plasticized/molten inside the barrel by a combination of heating, hydration, and mechanical deformation. When this protein 'melt' flows into the die, it becomes aligned by the (inhomogeneous) laminar flow and is cooled to prevent expansion (Dekkers et al., 2018). Plant-based protein ingredients (such as flours, concentrates, or blends) are fed into the extruder barrel and are mixed with water. With help of high pressure and increasing temperature, friction and shear forces are achieved. Together with a cooling die, these conditions lead to the formation of fibrous structures in the plant proteins. The result is a meat-free product that mimic the texture and bite of meat.

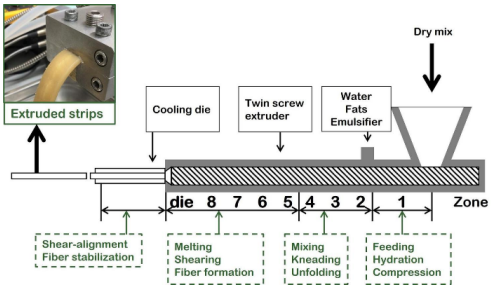


Figure 1. High moisture extrusion equipment (source: https://futurefoodsbv.com/high-moisture-extrusion)

Low-Moisture Extrusion (Figure 2) can also be used to produce texturized vegetable protein, which requires rehydration before incorporation into final products (Singh et al., 2007). The produced textured vegetable protein is often used as a meat extender or in "ground" meat products like burgers (Zahari et al., 2023; Yagci et al., 2020; Kaur et al., 2023). Plant protein powders (such as flours, concentrates, or blends) are loaded into the extruder barrel and are mixed with water. With increasing pressure and temperature, the material undergoes friction and shearing. In combination with a specific die, these conditions lead to fiber formation of the plant proteins. This technology yields a meat-free product with a texture and bite similar to meat. After extrusion, the products are dried to extend their shelf life to approximately 6-12 months.

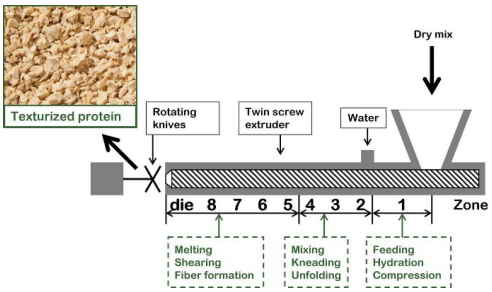


Figure 2. Low moisture extrusion equipment (source: https://futurefoodsbv.com/low-moisture-extrusion)

High-moisture extrusion is widely recognized as the most effective technology for producing fibrous meat analogues; however, significant variability exists across studies regarding its efficiency and product quality. For example, Dekkers et al. (2018) report that high-moisture extrusion (>50% water) enables strong anisotropic fiber formation due to phase separation and protein alignment. In contrast, Osen et al. (2014) observed that the formation of well-defined fibrous structures is highly dependent on protein source and composition, with legume proteins (including chickpea) often requiring blending with gluten or other structuring agents to achieve comparable textures.

This highlights a key limitation: chickpea proteins alone may not consistently produce optimal fibrous structures, due to their globular nature and lower elasticity compared to wheat gluten. Supporting this, Zahari et al. (2023) demonstrated improved texture only when chickpea proteins were co-extruded with wheat gluten, suggesting that protein-protein interactions are critical but not fully understood.

Moreover, while extrusion is industrially scalable, studies such as Akdogan (1999) emphasize its high energy consumption and process complexity, which may limit sustainability advantages when compared to less intensive structuring methods. Therefore,

although extrusion is considered the benchmark technology, its efficiency is formulation-dependent and may contradict claims of universal applicability across plant proteins.

Fermentation

Fermentation is increasingly applied to chickpea-based meat analogues to overcome the inherent limitations of plant proteins, such as "beany" off-flavors, low digestibility, and poor texture. Fermentation helps break down cell wall components and protein structures, creating more fibrous, meat-like textures. It increases the protein's water- and oil-holding capacity, enhancing juiciness. Fermentation reduces anti-nutritional factors (such as phytic acid) and increases the bioavailability of nutrients, amino acids, and phenolic compounds in chickpeas. It serves as a sustainable and natural processing method to enhance the nutritional value, sensory quality, and structural integrity of chickpea-based alternatives.

Fermentation, particularly with lactic acid bacteria like *Lactobacillus plantarum*, reduces green/beany notes by decreasing aldehydes (e.g., n-hexanal) and creating aromatic compounds (acids, alcohols, and esters) that mask off-flavors.

Homolactic fermentation (e.g., *Lactobacillus acidophilus*) can be used to improve protein digestibility, reduce antinutritional factors, and enhance flavor profiles (Hurtado-Murillo et al., 2025).

The use of fungi, such as *Rhizopus oryzae* or *Aspergillus oryzae*, can create a fibrous texture similar to muscle tissue, enhancing the savory, umami flavor of chickpea-based burgers.

Studies show that microbial fermentation (e.g., 24-hour lactic acid fermentation) causes profound structural changes in chickpea proteins. These include protein degradation, where complex, compact structures are broken down into smaller, more digestible peptides, and structural reconfiguration, characterized by decreased β-sheet content, increased α-helix content, and greater exposure of hydrophobic groups. These changes enhance protein solubility and improve its functionality for use in meat substitutes (Milcarz & Harasym, 2025).

Chiacchio et al. (2025) showed that submerged chickpea flour fermentation for 24 hours at 30-37°C with *Lacticaseibacillus casei*, *Lactiplantibacillus plantarum*, *Leuconostoc mesenteroides*, and *L. plantarum* reduced phytic acid.

Solid state fermentation of chickpea at 30-37°C, 48 hours with *A. oryzae*, *A. niger*, and *L. plantarum* determined a reduction of *in vitro* protein digestibility from 87% to 74-82% (Stone et al., 2024; Coronel-León et al., 2025).

Fermentation (Figure 3) enables the creation of "clean-label" products, minimizing the need for artificial additives to improve flavor and texture.

To mimic the fibrous structure of meat, fermented chickpeas are often combined with other technologies, such as high-moisture extrusion or 3D printing (Tao et al., 2022).



Figure 3. Converting chickpeas into meat-like plant-based foods based on fermentation (created with https://biorender.com)

Even if fermentation is frequently reported as an effective strategy to improve flavor and nutritional quality of chickpea-based products, findings across studies are not entirely consistent. For instance, Chiacchio et al. (2025) and Hurtado-Murillo et al. (2025) demonstrate that lactic acid fermentation significantly reduces antinutritional factors and enhances digestibility. Similarly, Tao et al. (2022)

highlight its role in reducing off-flavors through aldehyde degradation.

In contrast, Stone et al. (2024) reported a decrease in *in vitro* protein digestibility (from 87% to 74-82%) following solid-state fermentation, which contradicts the commonly reported improvement in digestibility. This discrepancy may be attributed to differences in microbial strains, fermentation conditions, and substrate composition, suggesting that fermentation outcomes are highly process-specific.

The long processing time and variability in microbial activity represent important limitations for industrial scalability of fermentation (Milcarz & Harasym, 2025). Therefore, although fermentation is promising, its effects are not universally beneficial and require precise optimization to avoid negative impacts on nutritional quality.

Milling/Fractionation

Wet and dry fractionation techniques present a clear trade-off between purity and sustainability. Wet-milling is a process used to obtain high-purity chickpea protein isolates by separating proteins from starch and fiber in a liquid medium.

Wet-milling is a high-purity liquid medium extraction process used to isolate chickpea protein (up to 94.2% purity) by separating it from starch and fiber. The process involves soaking chickpea seeds (often in a solution like sulphur dioxide to break down protein-starch interactions), grinding them into a slurry, and using centrifugal force to separate the components based on density.

Key aspects of the wet-milling process:

- Mechanism: The process utilizes high-speed grinding or homogenization to break down the tissue, followed by filtration, centrifugation, and drying to isolate the protein.
- Separation: It relies on the sedimentation rates of different components, where starch settles out, and protein remains in an aqueous suspension for further purification.
- Advantages: This method offers high protein purity (ranging from 49% to over 94%), high yields (up to 49%), and low fat content in the final product.
- Limitations: It is resource-intensive, requiring high amounts of water and energy, and can lead to the loss of certain functional properties or amino acids, such as sulfur-containing ones.
- Applications: The resulting protein isolates are used in various food and nutraceutical applications (Patil et al., 2024).

Wet-milling, compared with dry fractionation (Figure 4), produces higher-purity protein (usually> 70% pure), though it uses significantly more energy and water. While wet-milling can offer higher purity, it may yield lower yields than alkaline extraction followed by isoelectric precipitation (Patil et al., 2024).

Air Classification (Dry Fractionation) is a sustainable alternative to wet processing that uses air to separate protein-rich and starch-rich fractions based on particle size and density (Espinosa-Ramírez & Serna-Saldívar, 2019).

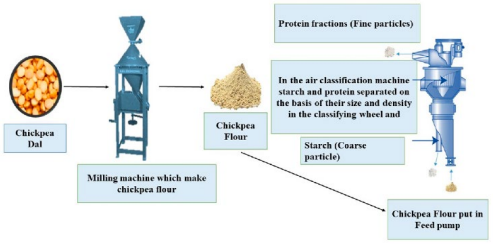


Figure 4. Chickpea protein extraction using the air classification method (source: Patil et al., 2024, https://doi.org/10.3390/foods13091398)

Comparing wet-milling processes and dry fractionation (air classification), we can conclude that dry fractionation is more sustainable (Espinosa-Ramírez & Serna-Saldívar, 2019) but it produces protein fractions with lower purity and higher starch content, which can negatively affect texturization efficiency during extrusion. On the other hand, wet milling is advantageous for controlled structuring applications. Both methods are resource-intensive, requiring large amounts of water and energy, and may lead to loss of functional properties, particularly of sulfur-containing amino acids. A notable contradiction in the literature concerns protein yield versus functionality: while wet extraction yields higher purity, some studies suggest that less refined protein fractions may retain better functional properties due to reduced denaturation (Lima et al., 2023). This indicates that higher purity does not necessarily translate into better performance in meat analog

applications, underscoring the need to balance processing intensity and functionality.

Thermal Processing

Thermal processing is widely used to improve protein functionality (Huang et al., 2023; Nosworthy et al., 2020; Xu et al., 2017; Avilés-Gaxiola et al., 2018); however, its effects depend heavily on processing conditions. Nosworthy et al. (2020) showed that excessive heat treatment can reduce protein quality, whereas moderate heating can enhance digestibility and functionality. This suggests a narrow optimal processing window, beyond which protein denaturation becomes detrimental.

Similarly, enzymatic hydrolysis is often reported to improve solubility and emulsification (Ghosh et al., 2025), yet excessive hydrolysis can lead to loss of gelation capacity and weakened texture, which is critical for meat analog structure. This creates a technological contradiction: improved interfacial properties *vs*. reduced structural integrity. Therefore, both approaches require careful control, as functional improvements in one property may compromise others, particularly texture.

Enzymatic Hydrolysis

Moderate proteolytic hydrolysis is used to create amphiphilic polypeptides that improve the emulsifying and foaming properties of chickpea proteins (Ghosh et al., 2025). Moderate proteolytic hydrolysis (typically 5-15% degree of hydrolysis) breaks down chickpea proteins into smaller, amphiphilic peptides, which improves their functional properties by increasing surface hydrophobicity and solubility. Enzymes like trypsin and alcalase expose hydrophobic groups, enabling better adsorption at oil-water interfaces and enhancing emulsion stability and foaming capacity.

Moderate hydrolysis improves protein solubility (especially at pH 4.0), increases surface hydrophobicity, and improves water/oil holding capacities. Trypsin and alcalase are effective in producing amphiphilic peptides, with trypsin specifically generating higher surface hydrophobicity and better functional improvements. The process reduces molecular weight, enabling smaller peptides to form more stable emulsions and foams than raw chickpea protein. Optimal results are often found at low degrees of hydrolysis (e.g., 6% to 15% DH) using controlled pH and temperature conditions (Ghosh et al., 2025). This enzymatic modification produces plant-based, functional ingredients suitable for food applications that require superior foaming and emulsification.

Emulsion/Gelation

Chickpeas can be used to produce thick pastes that are suitable for new spreads or pâte-like products. The chickpea pastes exhibit a lumpy texture (Durage et al., 2025; Viadel et al., 2024; Mazumder et al., 2023). While fat in animal tissue is embedded within the collagen scaffolds of adipose tissues, in meat analogues, it is typically present in a free, unbound form. Some of the negative textural sensory qualities of plant-based meat substitutes, such as their lack of hardness, chewiness, juiciness, and oil binding, are partly caused by the absence of adipose tissue. Chickpea flour minimizes oil loss without compromising texture, and an ideal gel consisting of 6% pea starch, 4% chickpea flour, and 40% oil (7:3 coconut: sunflower oil) gel was created (Czapalay et al., 2025).

Emerging Technologies

Emerging technologies such as shear cell processing, wet spinning, and 3D food printing have been explored for chickpea- and legume-based systems (Krintiras et al., 2015; Nieuwland et al., 2014). Shear cell processing of chickpeas (Figure 5) is an emerging thermomechanical method for creating fibrous, meat-like textures from plant proteins. Unlike traditional high-moisture extrusion, it uses a well-defined shear flow to align protein molecules into anisotropic structures under more moderate conditions. The process typically uses either a conical shear cell (cone-cone device) or a cylindrical Couette cell (concentric cylinders). Protein molecules are unfolded and aligned within the narrow gap between the rotating parts. This creates a layered, fibrous "meat-like" matrix. It requires lower energy input and lower shear forces than extrusion, enabling better control over the final texture and structure.

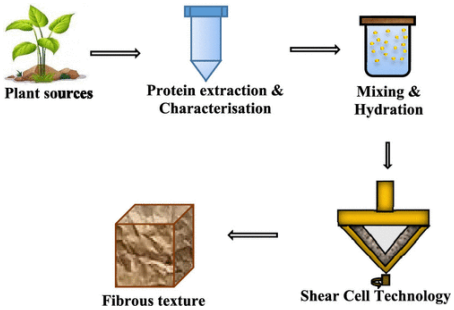


Figure 5. Shear Cell Technology on the production of plant-based meat analog (source: Ajay et al., 2025 https://pubs.acs.org/doi/10.1021/acsfoodscitech.5c00416)

The production of chickpea-based fibrous structures follows three main unit operations:

- Mixing and hydration: Combining chickpea protein concentrates or isolates with water and other ingredients (like hydrocolloids or lipids) to form a consistent dough;
- Thermo-mechanical treatment: Applying heat and shear forces simultaneously to promote protein-protein interactions and alignment;
- Cooling and Resting: The material is cooled and allowed to "rest" to stabilize the newly formed fibrous network before being removed from the cell (Cornet et al., 2022).

Chickpea is highly suitable for this technology because of its specific functional properties: high thermal stability: chickpea protein has high thermal expansion and stability, which helps maintain the integrity of the fibrous structure during heating; textural versatility: researchers use shear cell processing to develop chickpea-based meat analogs, such as emulsion gel meat substitutes, by aligning chickpea protein with oils like flaxseed. Cornet et al. (2022) suggest that shear cell technology provides better control over fiber alignment under milder conditions. However, its low throughput and limited scalability currently restrict commercial application.

3D food printing (Figure 6) is an emerging technology utilized to create sustainable, plant-based meat alternatives by using chickpea-based "inks" to mimic the texture, flavor, and nutritional profile of conventional meat. Chickpeas are particularly valued for this application due to their high protein content, digestibility, and rich mineral profile.


Figure 6. 3D food printing for creating plant-based meat alternatives (source: Dang Hui L 2024. https://www.foodnavigator-asia.com/Article/2024/02/07/3d-food-printing-vital-for-creating-plant-based-alternatives-with-buckwheat-being-most-promising-ingredient/)

The key aspects of 3D printed chickpea meat alternatives are:

- ink formulation**:** chickpea flour is blended with ingredients like water, oils, and binding agents to create a printable paste;
- textural optimization**:** research indicates that extruded chickpea flour has better printability at a water content of around 72%, while non-extruded flour is better at lower moisture levels (around 36%);
- process efficiency**:** extrusion-based 3D printers can create complex geometries, mimicking the fiber-like structure of meat. The process also allows for the integration of other ingredients to enhance texture and juiciness, such as mushrooms or omega-3-rich oils;
- aquafaba application**:** the liquid leftover from cooking chickpeas, known as aquafaba, is used in 3D food printing as a functional plant-based ingredient (like an egg substitute) due to its high viscosity and foaming capacity, which helps with structural retention.

This technology has many advantages: it is sustainable - chickpeas are an environmentally friendly crop, making them a sustainable source of protein for meat alternatives; it allows personalisation of products with tailored nutritional content to meet specific dietary requirements, such as high-fiber or low-fat diets; it enables the use of upcycled ingredients and precise ingredient portioning to minimize waste (Auyeskhan et al., 2024; Rodríguez-Herrera et al., 2024; Hena et al., 2024). The challenges of this technology are: the technical limitations- achieving the precise, fibrous

mouthfeel of meat remains difficult; cost and scalability- the technology is currently expensive and generally limited to, or focused on, high-end food service or specialized products; rheological properties -the "ink" must be viscous enough to hold its shape, yet fluid enough to be extruded, which requires precise control over temperature and formulation (Bhuiyan et al., 2025; Qiu et al., 2025). In contrast to extrusion, which is already industrialized, these technologies remain largely at the prototype or pilot scale, highlighting a gap between innovation and practical implementation.

Protein Extraction and Fractionation

Protein ingredients can be obtained through dry or wet fractionation methods (Pelgrom et al., 2013). Dry fractionation involves milling followed by air classification to separate protein-rich fractions and is considered more sustainable due to lower water and energy requirements. Wet fractionation typically includes alkaline solubilization, isoelectric precipitation, washing, and drying, yielding chickpea protein isolates with protein contents exceeding 80% (Lima et al., 2023). To enhance functionality, chickpea proteins may be subjected to physical, chemical, or enzymatic modifications (Nivala et al., 2021). Heat treatment, pH shifting, and enzymatic hydrolysis can improve solubility, reduce beany flavors, and promote protein-protein interactions during texturization (Osen et al., 2014).

Formulation of Meat-Analog Products

Processing technologies play a central role in developing meat-like texture in chickpea-based systems. Protein alignment and phase separation during processing contribute to the formation of fibrous structures that enhance chewiness and structural integrity. The incorporation of plant-based oils and structuring agents further improves mouthfeel by simulating intramuscular fat, which is critical for lubrication and flavor release during consumption. Chickpea-based meat analogs are commonly formulated with plant-based oils for fat mimicry, hydrocolloids such as methylcellulose for binding, and natural colorants to simulate meat appearance (Kyriakopoulou et al., 2021). Flavorings, salts, and umami compounds are carefully balanced to enhance overall palatability while maintaining clean-label considerations. Product formats include burgers, sausages, nuggets, and minced-meat analogs, each requiring tailored sensory and structural optimization. Table 1 presents examples of chickpea-based meat alternatives produced using various processing technologies.

Table 1. Chickpea-Based Meat Alternatives

Product Type	Processing Technology	Key Findings	Reference
Meat analogue	Freeze-alignment combined with emulsion/gelation	Chickpea protein–flaxseed oil emulsion gel created a fibrous structure with improved textural properties suitable for meat analogues	Durage et al., 2025
Sausage analogue	Emulsion gel formulation using chickpea and mung bean proteins	Chickpea incorporation improved rheological behavior, digestibility, and microstructural stability of plant-based sausages	Sivapragasam et al., 2025
Burger patty	Formulation with chickpea flour and textured vegetable protein	Higher chickpea concentrations improved protein content and influenced the texture and in vitro digestibility of plant-based burgers	Kea et al., 2025
Meat analogue	High-moisture extrusion with chickpea proteins and wheat gluten	Co-extrusion improved fibrous texture and sensory attributes in meat analogue products	Zahari et al., 2023
Sausage analogue	Emulsion-type gel system using chickpeas and grey oyster mushrooms	Chickpea flour contributed to emulsion stability and improved textural properties in plant-based sausages	Mazumder et al., 2023
Fermented burger	Solid-state fermentation with Aspergillus oryzae	Fermentation improved flavor profile, increased protein content, and reduced undesirable sensory notes	Mohamed & Ali, 2025

Flavor Development, Texture Perception, and Product Formulation

The success of chickpea-based meat analogues largely depends on their ability to replicate key meat sensory attributes, including fibrous texture, juiciness, cohesiveness, flavor intensity, and visual appearance (Tso & Forde, 2021). Texture is often identified as the most

critical factor, as consumers expect anisotropic, fibrous structures that resemble muscle tissue during chewing (Dekkers et al., 2018). Juiciness perception is influenced by water-holding capacity, fat distribution, and moisture release during mastication, all of which are affected by protein structure and formulation. Chickpeas contain volatile compounds associated with beany and grassy flavors derived mainly from lipid oxidation and enzymatic activity (Roland et al., 2017). These flavors can negatively impact consumer acceptance, particularly in products intended to mimic meat. Mitigation strategies include thermal processing, fermentation, enzymatic treatment, and the use of flavor-masking or flavor-generation systems, such as Maillard reaction precursors (Kyriakopoulou et al., 2019).

Nutritional and Sustainability Considerations

Chickpea-based meat analogs offer high protein content, dietary fiber, and cholesterol-free properties, while potentially reducing saturated fat intake (Loveday, 2020). From a sustainability perspective, chickpeas require less water and nitrogen fertilizer than many animal protein sources and contribute to soil fertility through biological nitrogen fixation (Merga & Haji, 2019). However, the environmental benefits may be influenced by processing intensity and ingredient complexity, underscoring the importance of life-cycle assessment in product development (Mari et al., 2025).

Challenges, Critical Synthesis and Future Perspectives

Despite technological advances, challenges remain in achieving full sensory equivalence to meat, particularly regarding juiciness, flavor release, and fibrousness (Sha & Xiong, 2020). The literature reveals that no single processing technology is sufficient to fully replicate the complex structure and sensory profile of meat. While extrusion provides the most advanced structuring capability, its dependence on formulation and high energy input represents a significant limitation. Fermentation and enzymatic treatments improve flavor and functionality but may introduce inconsistencies in digestibility and structural integrity. Similarly, fractionation techniques involve trade-offs between purity, sustainability, and functionality. These findings suggest a growing consensus toward hybrid processing strategies that combine multiple technologies to overcome individual limitations. However, the lack of standardized processing conditions across studies makes direct comparison difficult and highlights the need for systematic optimization and harmonized evaluation methods in future research. Future research should focus on improving protein functionality with minimal processing, enhancing digestibility, developing clean-label formulations, and scaling up novel structuring technologies.

CONCLUSIONS

Overall, the current literature demonstrates that while chickpeas are a promising raw material for plant-based meat analogues, their successful application depends on carefully balanced, multi-step processing strategies, and no single technology currently provides a complete solution, underscoring the need for integrated, interdisciplinary approaches in future research.

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EFFECT OF SALT CONCENTRATION ON MICROBIAL STABILITY AND QUALITY EVOLUTION OF CARP (*Cyprinus carpio*) ROE DURING MATURATION

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Abstract

Carp roe (Cyprinus carpio) is a rich, concentrated source of nutrients in the human diet, offering multiple health benefits. The maturation of Cyprinus carpio roe is a complex technological process characterized by a series of controlled biochemical transformations that contribute to the development of the final sensory profile. The aim of this study was to monitor the maturation process of Cyprinus carpio roe under controlled refrigeration conditions (0-4°C) for 20 days. Three experimental trials were conducted in which different amounts of NaCl (4%, 7%, and 9%) were added per 450 g of raw material. Controlling maturation parameters, including NaCl concentration, temperature, and duration of the process, is essential for obtaining a product with optimal consumer properties, quality, and safety. The experimental samples were tested microbiologically and physicochemically at 10 and 20 days to identify the optimal maturation time. According to the results, increasing the NaCl concentration led to a significant reduction (<0.005) in total microbial load and mold, with the effect being more pronounced at 9% NaCl. However, at 10 days of maturation, TVC values were slightly higher at 7% and 9% NaCl compared to 4%, suggesting a possible selection of halotolerant microbiota in the product. The physicochemical analyses were significantly influenced (<0.005) by both the salt concentration and the time period. Water activity is significantly influenced by time, salt concentration, and the interaction between time and salt concentration (<0.005). The L parameter is influenced by salt concentration (<0.005) and is not influenced by time. A maturation period of 20 days was identified as optimal for ensuring the safety and quality of the final product.*

Key words*: carp, roe, maturation, quality, safety*

INTRODUCTION

People consume fish roe products because of their pleasant aroma and taste, as well as their nutritional value. Value-added products made from fish roe are highly regarded and rank among the most widely consumed products worldwide. There are various methods for producing fish roe, depending on the fish species, which significantly influence their quality and marketability (Shirai et al., 2006).

In the current context of the food industry, there is growing interest in utilizing by-products and developing high-value-added products. The study conducted by Anchidin B. G. et al. (2024) highlights the potential for transforming secondary raw materials into functional foods, emphasizing the importance of qualitative characterization based on biological source and processing. Although fish roe is considered a high-value product, its integration into concepts of sustainable development and the efficient use of fishery resources aligns with modern trends in the food industry, as highlighted by recent studies (Gucianu I. et al., 2024).

The roe with the highest commercial value is caviar, which comes from the beluga sturgeon or the great sturgeon (*Huso huso*), but are also often harvested from other fish species such as cod (*Gadus morhua*), paddlefish (*Polyodon spathula*), lumpfish (*Cyclopterus lumpus*), carp (*Cyprinus carpio*), mullet (*Mugil cephalus*), etc. The roe from the fish species mentioned above can be considered substitutes for caviar at a much lower cost and are consumed as a spread (Křížek et al., 2011). Carp roe (*Cyprinus carpio*) is a rich source of nutrients, polyunsaturated fatty acids, essential amino acids, and various vitamins. However, its use is

currently limited to a few fish species, as most present various processing challenges (Binsi et al., 2019).

Roe is a product susceptible to oxidative processes, such that the concentration of NaCl significantly influences the microbiological stability and quality evolution of the roe during maturation. In the study by Mirsadeghi et al. (2022), the amount of salt influences the fatty acid composition during the production of salted rainbow trout roe. The results showed that, after salting, the fatty acid composition decreased in raw roe, but no changes were observed during storage. The impact of food additives on the structure and stability of food products depends on their nature and their interactions with the matrix components. The study conducted by Ciobotaru M.C. et al. (2024) highlights the fact that the use of alternative technologies, such as liquid smoke, can significantly influence the quality and sensory characteristics of food products. According to Flocea E.I. et al. (2024), these interactions can significantly influence physicochemical parameters, suggesting that the replacement of synthetic additives with natural alternatives must be analyzed in an integrated manner. The integration of plant-based functional ingredients, such as those rich in phenolic compounds and carotenoids, represents a promising direction, as demonstrated in studies by Anchidin B. G. et al. (2023), although further research is needed for their application in roe maturation. Improved product stability may be attributed to the presence of bioactive compounds in the plant-based ingredients used. In this regard, Manoliu Diana-Remina et al. (2024) highlight the role of fruit fibers in reducing lipid oxidation and improving the textural properties of food products. Sensory evaluation is an indispensable tool in assessing food quality, providing information that complements physicochemical analyses (Ciobanu M. M. et al., 2023). The sensory evaluation of matured roe is an essential parameter in determining product acceptability, as it is influenced by biochemical changes during maturation. Recent studies, such as the one conducted by Boișteanu P. C. et al. (2025), highlight the importance of detailed analysis of sensory attributes (color, aroma, texture, taste) in characterizing complex food products, emphasizing their role in consumer perception. The integration of plant-based bioingredients into fish-based products represents a promising strategy for the development of functional foods, as highlighted in recent literature (Flocea E. I. et al., 2025). The incorporation of smart packaging into products made from matured roe, combined with the use of natural bio-ingredients, represents an innovative approach to enhancing food safety and reducing post-processing losses (Davidescu et al., 2025).

The study aimed to monitor the maturation process of carp (*Cyprinus carpio*) roe under refrigerated conditions over 20 days. To this end, three salt concentrations were used: 4%, 7%, and 9% NaCl.

The samples underwent microbiological and physicochemical analyses (color, pH, and water activity) at 10- and 20-day intervals to determine the optimal maturation duration.

MATERIALS AND METHODS

The study began with the collection of carp roe from a local farm, followed by weighing the roe and subjecting it to a salting process using different salt concentrations (4%, 7%, and 9%) (Table 1). The samples were properly homogenized and then placed in the maturation chamber and stored at refrigeration temperatures (0-4°C). During maturation, the samples were protected from light to limit oxidative processes.

Table 1. Formulation of the experimental sample

	Sample Code	NaCl %	Raw Material (g)
1.	4SFR	4	450
2.	7SFR	7	459
3.	9SFR	9	453

Microbiological tests and physicochemical analyses were conducted under controlled conditions after 10 days and 20 days of maturation. Microbiological analyses were performed to evaluate the hygienic quality and safety of processed fish roe products (roe-based spreads), in accordance with internationally standardized methods. The following microbiological parameters were determined: total viable count (TVC), molds and yeasts, *Escherichia coli*, and coagulase-positive

staphylococci. For sample preparation, 10 g of product were aseptically weighed and homogenized with 90 mL of sterile peptone water (0.1%, w/v) using a stomacher (BagMixer, Interscience, France) to obtain the initial dilution (10^{-1}). Subsequent decimal dilutions were prepared in the same diluent as required. Appropriate aliquots were inoculated onto selective and non-selective culture media using standard pour-plate or surface-plating techniques. The total viable count (TVC) was determined on Plate Count Agar (PCA) following incubation at 30°C for 72 h, according to ISO 4833-1:2013. Molds and yeasts were enumerated on Dichloran Rose Bengal Chloramphenicol (DRBC) agar after incubation at 25°C for 3-5 days under aerobic conditions, in accordance with ISO 21527-1:2008. *Escherichia coli* was determined using Rapid *E. coli* agar after incubation at 37°C for 24 h, based on the principles described in ISO 16649-2:2001. Coagulase-positive staphylococci were enumerated using Rapid Staph agar following incubation at 37°C for 24–48 h, in agreement with ISO 6888-1:2021. Microbial counts were expressed as colony-forming units per gram (CFU/g). Results were log-transformed and reported as log CFU/g. Only plates containing 30-300 colonies were considered for enumeration. When counts were outside this range, results were reported as estimated values. All analyses were carried out in duplicate, and the mean values were used for statistical analysis (Lipșa et al., 2025; Anchidin et al., 2024). An integrated approach to food quality, which takes into account both physical and chemical parameters, is essential for evaluating complex products, as recent studies have shown (Manoliu Diana-Remina et al., 2023). The pH of the experimental batches was measured using the HI98163 pH meter, equipped with the FC2323 electrode, supplied by Hanna Instruments SRL (Gucianu et al., 2023; Ciobotaru et al., 2024). Colorimetric analyses were performed using a Konica Minolta CR-410 colorimeter (Konica Minolta, Inc.) to determine color parameters in the three-dimensional CIELAB color space (Manoliu et al., 2025). The moisture content of the samples was determined using a Humimeter RH2 (Schaller Messtechnik) in accordance with the manufacturer's instructions. Statistical analysis of the obtained data was performed in Microsoft Excel to verify the data's normality for two-way analysis of variance (ANOVA). The Chi-square (χ^2) test, with a significance level of $p < 0.001$, and PCA analysis were performed using the JASP software. These analyses were necessary to determine the influence of the NaCl percentage and the maturation time on the analyzed parameters.

RESULTS AND DISCUSSIONS

The microbiological parameters of the samples analyzed are presented in Table 2, showing the levels of the main groups of microorganisms monitored during the monitoring period. Increasing the NaCl concentration resulted in a significant reduction in total microbial load and mold populations, an effect attributed primarily to the decrease in water activity and the increase in osmotic pressure within the product matrix. These conditions become unfavorable for microbial growth, inhibiting metabolic processes and cell proliferation. The antimicrobial effect was more pronounced at a 9% NaCl concentration, suggesting a directly proportional relationship between salt concentration and the intensity of microbial inhibition, particularly in the case of filamentous fungi, which exhibit increased sensitivity to variations in water activity (Beuchat, 1981).

Table 2. Evolution of microbiological content in samples analyzed during the maturation process

Microorganism	Time (Days)	4SFR	7SFR	9SFR
E.coli	10	*nd*	*nd*	*nd*
	20	*nd*	*nd*	*nd*
S. aureus	10	*nd*	*nd*	*nd*
	20	*nd*	*nd*	*nd*
Molds*	10	2.6±0.00	2.48±0.03	2.18±0.00
	20	2.2±0.05	2±0.01	1.7±0.00
TVC*	10	3.65±0.00	3.86±0.05	3.84±0.15
	20	3.45±0.13	3.45±0.00	3.03±0.03

"*nd*" = not detected; *log CFU·g^{-1}; 4SFR- carp roe sample with 4% *NaCl*; 7SFR- carp roe sample with 7% NaCl; 9SFR-carp roe sample with 9% NaCl

Increasing the NaCl concentration resulted in a significant reduction in total microbial load and mold counts, with the effect being more pronounced at 9% NaCl. However, at 10 days of ripening, TVC values were slightly higher at 7% and 9% NaCl compared to 4%, suggesting a possible selection of halotolerant microbiota. According to Table 3, water activity is significantly influenced by time, the percentage of NaCl, and the interaction between NaCl and time in the carp roe samples studied. The significant effect of salt concentration on water activity can be attributed to NaCl's ability to bind free water through ionic interactions, thereby reducing water availability. Storage time also significantly influenced aw. Furthermore, the significant interaction between salt concentration and time indicates that the impact of NaCl on water activity is time-dependent, likely due to the gradual diffusion of salt and its progressive interaction with muscle proteins, which alters water-binding capacity over the course of storage (Tilapia et al., 2020; Shin & Rasco, 2007).

Table 3. Combined effect of salt concentration (S) and storage time (T) on physicochemical parameters of carp roe

Sample	T (days)	a_w	pH	L*(D65)	a*(D65)	b*(D65)
4SFR	10	0.99±0.00	5.86±0.02	33.98±1.29	1.59±0.04	7.01±0.45
	20	0.93±0.00	5.72±0.02	33.21±0.44	2.23±0.09	7.11±0.19
7SFR	10	0.96±0.00	5.85±0.01	34.29±1.24	2.31±0.08	7.40±0.46
	20	0.93±0.00	5.77±0.01	35.33±0.94	3.30±0.28	7.05±0.69
9SFR	10	0.96±0.00	6.41±1.35	37.34±2.18	2.29±0.35	8.31±0.92
	20	0.93±0.00	5.76±0.01	38.46±1.04	3.98±0.15	7.40±0.81
ANOVA-two-way-factor with replication (*p*-values)						
S		<0.005**	0.423^{ns}	<0.005**	<0.005**	<0.005**
T		<0.005**	0.162^{ns}	0.335^{ns}	<0.005**	<0.005**
S x T		<0.005**	0.448^{ns}	0.207^{ns}	<0.005**	0.019*

4SFR- carp roe sample with 4% NaCl; 7SFR- carp roe sample with 7% NaCl; 9SFR-carp roe sample with 9% NaCl

The influence of the higher salt concentration was observed in the experimental samples containing 7% NaCl and 9% NaCl. Therefore, time and salt concentration are key factors affecting the activity of water. In the case of pH, the p-values here are greater than 0.005, which means that pH is not influenced by the amount of salt or the maceration time. As can be seen during the maturation process, each sample showed a progressive decrease, which may contribute to the inhibition of pathogenic microorganisms, the development of the sensory profile, and increased product stability. With regard to color parameters, a significant influence of salt concentration on the lightness (L*) of the analyzed roe was observed. An increase in NaCl concentration can accelerate pigment oxidation processes, leading to an increase in L* values and, consequently, a reduction in color intensity. For the a* parameter, the results indicate that curing time has a more pronounced influence compared to salt concentration, although the latter also contributes to the observed variations. For the b* parameter, the results suggest a comparable influence of curing time and NaCl percentage on this chromatic component. The interaction between maturation time and salt concentration has a limited effect on color parameters, indicating a limited contribution of the combined effect of the two factors to the color variations in the roe.

Figure 2 shows a graphical representation of the main physicochemical parameters analyzed, namely water activity (aw), pH, and color parameters in the CIELAB system (L*, a*, b*), determined under D65 lighting conditions, highlighting their variations as a function of NaCl concentration and maturation period. (A) The evolution of water activity (a_w) shows a similar pattern during the first stage of ripening (after 10 days), indicating relatively uniform values regardless of the salt concentration used. After 20 days of maturation, a change in the slope of the curves is observed, a finding supported by the statistical significance of the differences (<0.005). In this context, salt concentrations ranging from 7-9% tend to stabilize water activity, reducing it and suggesting improved product stability from a food safety perspective. (B) pH values did not show significant variations under the influence of NaCl concentration, indicating relative stability of this parameter during maturation. (C) The lightness parameter (L*) showed an upward trend throughout the maturation

process, with higher values in the final stage, indicating an increase in the lightness of the samples. These results are consistent with the typical characteristics of carp roe. Salt concentration significantly influenced this parameter. (D) For the a* parameter (D65), the results show that an increase in salt concentration has a more pronounced effect on its dynamics, suggesting a gradual shift in the red-green chromatic component. (E) The values of the b* parameter also confirm the influence of salt concentration on the color characteristics of the analyzed roe.

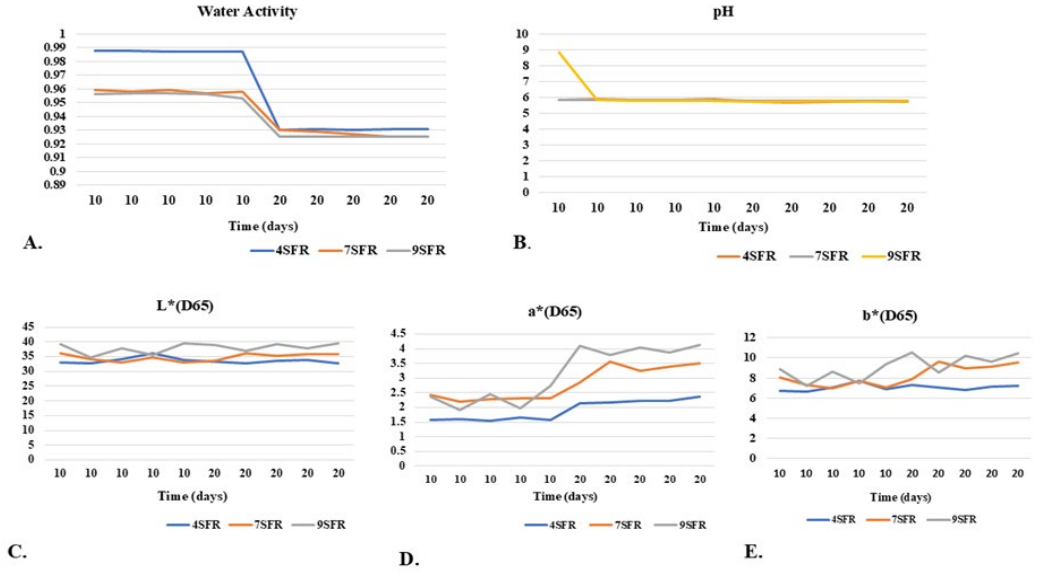


Figure 1. Graphical representation of the analyzed physicochemical parameters
[A. Water Activity; B. pH; C. L*(D65); D. a*(D65); E. b*(D65)]

According to Table 4, the results of the χ^2 (chi-squared) test showed a value of $p < 0.001$, indicating that both salt concentration and maturation time have a statistically significant effect on the parameters analyzed.

Table 4. Chi-square test of association between salt concentration, maceration time and analyzed parameters

Test	χ^2	Df	*p*-value
Chi-square test	31.486	1	< 0.001

The obtained *p*-value (< 0.001) demonstrates a highly significant statistical effect, indicating that the influence of salt concentration and maturation time on the analyzed parameters is not attributable to random variability, but reflects a real and consistent relationship within the studied system.

An interpretation of the loading coefficients (Table 5) reveals that RC1 is strongly correlated with color parameters (L*, a*, b*) and water activity (a_w), suggesting an interdependence between optical properties and product moisture content. In contrast, RC2 is dominated by pH, which exhibits a variation independent of the other parameters, a finding supported by the absence of significant correlations.

Table 5. Rotated component loadings obtained from Principal Component Analysis (PCA)

Variable	RC1	RC2
a*(D65)	0.975	
b*(D65)	0.951	
a_w	-0.781	
L*(D65)	0.775	0.476
pH		0.909

Principal component analysis (PCA) was applied to highlight the relationships among the analyzed physicochemical parameters. Based on the component characteristics (Table 6), two principal components were selected using the eigenvalue criterion (eigenvalue > 1), which explain a high cumulative percentage of the total variation in the data. After applying Varimax orthogonal rotation, the variance distribution was optimized, with component

RC1 explaining 61.5% of the variation and RC2 24.9%, indicating a good descriptive capacity of the analyzed system (Table 5).

Table 6. Total variance explained by principal components

Component	Eigenvalue	Variance	Cumulative
PC1	3.077	0.615	0.615
PC2	1.247	0.249	0.865

The graphical representation of the PCA scores (Figure 2) confirms these observations, highlighting a clear separation of the samples based on the parameters associated with each component. Thus, the samples are distributed along the RC1 axis based on color characteristics and water activity, while variations along the RC2 axis primarily reflect changes in pH. This distribution suggests that color parameters and water activity are the main factors differentiating the samples, while pH contributes secondarily to the system's variability.

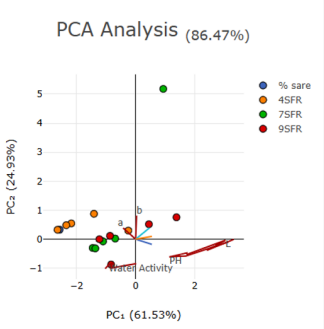


Figure 2. Principal component analysis (PCA), illustrating the distribution of the samples based on the analyzed physicochemical parameters

As for future research, although the present study did not focus on chemical contaminants, the literature highlights the importance of monitoring them in animal-derived products, including in the context of the safety of susceptible raw materials such as carp roe (Matei M. și Pop I. M., 2023; Matei M. et al., 2024).

CONCLUSIONS

The results highlight that NaCl plays a decisive role in both the microbiological stability and the physicochemical properties of the product. Although increasing the salt concentration effectively reduces the microbial load, high levels may likely favor the selection of halotolerant microorganisms during ripening; however, further studies are needed. The range of 7-9% NaCl, particularly at 20 days, appears optimal, ensuring a reduction in water activity and, consequently, improved stability from a food safety perspective, given that the ripening time did not have a statistically significant effect. At the same time, salt concentration significantly influences color parameters (L, a, b*), confirming its impact on the product's visual characteristics. Overall, the differentiation of samples is primarily determined by color parameters and water activity, while pH contributes secondarily to the system's variability.

USE OF GENERATIVE ARTIFICIAL INTELLIGENCE (GenAI)

The authors state that ChatGPT was used exclusively for linguistic review and refinement of the text in the "Introduction" and "Conclusions" sections of this paper. All scientific content, data processing, analyses, and interpretations were developed independently and critically reviewed by the authors.

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SUSTAINABLE USE OF SEA BUCKTHORN BY-PRODUCT AS FUNCTIONAL INGREDIENT IN FOOD APPLICATIONS: TRENDS AND CHALLENGES

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Abstract

Sea buckthorn juice processing generates substantial quantities of pomace, primarily consisting of seeds and peels, often discarded despite its potential as a valuable food ingredient. This work provides an overview of the literature on the conditioning, characterization, and valorization of sea buckthorn pomace (SBP) as a functional food ingredient. SBP is rich in valuable bioactive compounds and nutrients, allowing its transformation into a low-cost, highly functional food ingredient supporting circular economy principles and current food industry trends focused on sustainability and resource efficiency. Valorization of SBP for the development of value-added food products has attracted scientific interest due to its complex chemical composition, with high levels of phenolic compounds, flavonoids, proteins, and dietary fibers, enhancing both the nutritional and functional properties. This review addresses challenges related to SBP drying to preserve bioactive compounds and discusses applications in bakery, pastry, chocolate, dairy, and meat products, as well as its use as a natural antioxidant to improve the oxidative stability of edible oils. Overall, sustainable SBP exploitation provides environmental, economic, and nutritional benefits, supporting the development of foods with an enhanced functional profile.

Key words: *nutritional and functional benefits, sustainable use, upcycling of sea buckthorn pomace, value-added foods.*

INTRODUCTION

Sea buckthorn (*Hippophae rhamnoides* L.) is a small tree species belonging to the *Elaeagnaceae* family, recognized for its high tolerance to drought, strong winds, sandy environments, and saline soils (Tie et al., 2024). It is widely known under several common names, including vinegar tree, yellow thorn, sour willow, and black thorn (Teng et al., 2024). The classification of genus *Hippophae* has been modified over the years. Some studies found that originally, it was strong by only one species, namely *H. rhamnoides*, with 3 subspecies, namely *rhamnoides, salicifolia*, and *tibetana. Then,* the species was divided into nine subspecies: *carpatica, caucasica, fluviatilis, gyantsensis, mongolica, rhamnoides, sinensis, turkestanica, and yunnanensis* (Swenson et al., 2008).

Sea buckthorn (SB) is a highly resilient plant species, characterized by strong tolerance to drought, cold, salinity, and alkaline soils. Owing to its remarkable adaptability, it can survive in extreme environmental conditions, including temperatures as low as −40°C, as shown in Figure 1 (Chen et al., 2023).

In its natural distribution range, sea buckthorn occurs in North-Western Europe, Central Asia, China, Mongolia, the Caucasus, and Siberia (Zhu et al., 2025). In contrast, in North America, it has been cultivated experimentally for more than 20 years. Although efforts have been made to introduce it into commercial agriculture in the United States and Canada, it is still considered an exotic species in these regions. Its broader commercialization is limited by gaps in knowledge related to cultivation practices, the phytochemical

composition of the fruit, and potential market development (Danielski & Shahidi, 2025).

In the 1980s, sea buckthorn was included in the first group of medicinal and edible plant species recognized by the former Ministry of Health of China (Chen et al., 2023). Sea buckthorn (SB) berries are highly valued for their nutritional content and health-promoting properties, which have long been acknowledged in traditional medicine, including anti-inflammatory and detoxifying effects, as well as benefits for the regeneration and health of skin and hair. These properties are attributed to the presence of more than 200 nutritional and bioactive compounds. The wide environmental diversity across growing regions also contributes to variations in fruit quality and biochemical composition. As a result, sea buckthorn is already incorporated into a variety of food products.

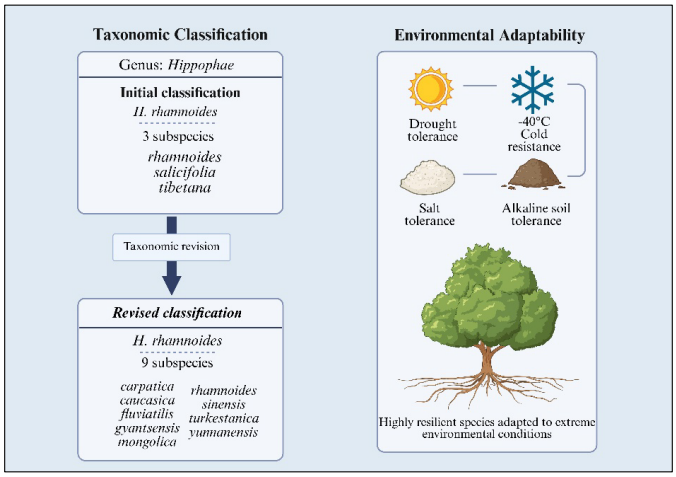


Figure 1. Taxonomic evolution and environmental adaptability of *Hippophae rhamnoides* L. (created in BioRender, https://BioRender.com, accessed in 25.03.2026)

The most common products derived from sea buckthorn include juices, jams, wines, pies, and liqueurs. Research has focused on identifying the compounds responsible for the fruit's characteristic and sometimes unpleasant taste. Among the organic acids present in sea buckthorn berries, malic and quinic acids are the predominant components and are largely associated with their pungent flavor (Jaśniewska & Diowksz, 2021).

The findings reported by Yang et al. (2011) and Jaśniewska & Diowksz (2021) indicate that the health-promoting effects of sea buckthorn (SB) have been acknowledged, mainly due to its high concentration of bioactive constituents such as antioxidants, phytosterols, essential fatty acids, amino acids, and vitamins C, K, and E. Moreover, the fruit is characterized by a relatively low sugar content and a complex profile of volatile compounds that shape its distinctive aroma. This particular sensory profile, together with its nutritional value and documented health benefits, supports the use of sea buckthorn as a valuable raw material in various cosmetic, pharmaceutical, and food applications (Jaśniewska & Diowksz, 2021).

A study conducted by Liu et al. (2021) classified sea buckthorn, alongside other fruits, as a "superfruit" due to its rich content of bioactive phytochemicals and associated health-promoting effects, particularly its high levels of antioxidant compounds. "Superfruit" is a term used to describe fruits that are believed to provide health benefits or therapeutic value, based on certain aspects of their nutritional composition or their overall nutrient density. Sea buckthorn contains a wide range of chemical constituents, including vitamins, carotenoids, and flavonoids (Wang et al., 2022). According to the same study, the juice exhibited elevated concentrations of vitamin E, vitamin C, and flavonoids, quantified at 13.3, 1.540, and 1.182 mg/L, respectively. More than 75% of the total

vitamin E content occurs as α-tocopherol, whereas isorhamnetin is recognized as one of the most biologically active flavonol aglycones present in sea buckthorn juice (Liu et al., 2021).

The global food processing industry produces large volumes of by-products, and their management is increasingly critical due to population growth and resource constraints (Metzner Ungureanu et al., 2020; Caponio et al., 2022; Chang et al., 2022). Fruit processing residues are rich in bioactive compounds with antioxidant activity, which may benefit human health. Valuable biological constituents can be recovered from plant-derived waste streams and converted into food ingredients, dietary supplements, or functional products (Wu et al., 2024).

Within this framework, recent research has increasingly focused on sea buckthorn by-products, recognizing their potential as valuable sources for supplements and functional ingredients. By incorporating sea buckthorn by-products as whole powders, functional fractions, or sources of extracted bioactive compounds, the food industry can enhance product quality and nutritional value while promoting waste valorization and reducing environmental impact. A continuous challenge in this field is the identification of processing methods that ensure minimal environmental impact and reduced risks to consumer health, while also lowering energy consumption and employing safe solvents (Stanciu & Popa, 2022).

Due to its delicate epicarp and high moisture content (80-90%), sea buckthorn fruit has limited post-harvest stability and is highly susceptible to microbial contamination, leading to rapid degradation and spoilage (Geng et al., 2023). Consequently, processing activities within the sea buckthorn industry are mainly directed toward beverage production and oil extraction. However, sea buckthorn pomace (SBP), the solid residue remaining after juice extraction, is frequently discarded, resulting in considerable resource losses (Zhou et al., 2024). Given its valuable nutritional and health-promoting properties, SBP represents a promising ingredient for the development of novel food products.

The recovery of processing by-products is closely aligned with the principles of the circular economy, aiming to reduce the environmental impact of production processes while increasing their economic value (Caponio et al., 2022). This perspective supports the expanded valorization of food industry residues by recovering and applying biologically active compounds across various sectors of the food industry (Luntraru et al., 2022).

The initial stage of by-product utilization involves conditioning immediately after processing to ensure biochemical and microbiological stabilization, particularly due to the high moisture content of most residues, followed by further transformation or extraction steps prior to final application in food products (Zhou et al., 2024).

Thermal treatments and drying methods applied to raw by-products may significantly influence quality attributes, particularly during dehydration processes. Drying can be performed using conventional techniques or advanced methods such as freeze-drying (lyophilization), infrared drying, and pulsed vacuum drying (Metzner Ungureanu et al., 2020; Jaśniewska & Diowksz, 2021; Zhou et al., 2024).

The main by-product generated during sea buckthorn juice processing is fruit pomace, consisting of seeds, peel, fibre, and residual pulp. Its high moisture content, approximately 67% (Zhou et al., 2024), contributes to its limited stability and susceptibility to microbial and biochemical degradation. Fruit peels are important sources of cellulose, hemicellulose, nutrients, and phenolic compounds (Kitrytė et al., 2017; Chang et al., 2022), while seeds represent valuable sources of proteins and lipids. Overall, fruit pomace contains approximately 50% fibre along with various bioactive compounds, highlighting its potential as a functional ingredient (Pedro et al., 2024).

The recovery of bioactive compounds from sea buckthorn pomace represents a critical stage, depending on the chosen extraction method and operational conditions, as these factors significantly affect extraction efficiency and compound stability. The study conducted by Luntraru et al. (2022) addresses the valorization of sea buckthorn juice by-products

through the extraction of bioactive compounds using two methods: maceration and ultrasound-assisted extraction (UAE). The findings indicate that UAE markedly increases the total polyphenol content, flavonoid concentration, and antioxidant activity compared to conventional maceration, highlighting the importance of optimizing extraction techniques from a technological perspective (Luntraru et al., 2022). From a broader perspective, Caponio et al. (2022) present a comprehensive view on the valorization of food industry by-products within the circular economy framework. Rather than focusing on a single raw material, it discusses multiple agro-industrial residues (including sea buckthorn flour, grape pomace, olive mill wastewater, and others) and emphasizes sustainable strategies, green extraction technologies, and functional food applications. While Luntraru et al. (2022) provide detailed analytical data on the composition and antioxidant activity of sea buckthorn pomace extracts, Caponio et al. (2022) extend the discussion to a broader sustainability framework. Consequently, Luntraru et al. (2022) contribute to the understanding of extraction efficiency and bioactive compound recovery, whereas Caponio et al. (2022) emphasize the environmental and economic significance of by-product valorization in the food sector.

Figure 2 provides a circular economy model in sea buckthorn processing, illustrating the transformation of berries into value-added products through by-product valorisation.

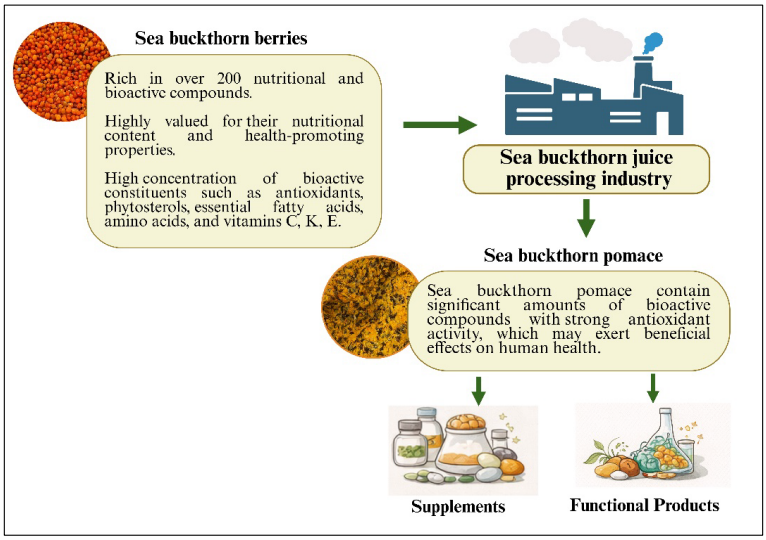


Figure 2. Circular economy model in sea buckthorn processing and by-product valorisation (created in BioRender, https://BioRender.com, accessed in 25.03.2026).

MATERIALS AND METHODS

This study was designed as a systematic literature review aimed at identifying and analyzing innovative strategies for the valorization of sea buckthorn (*Hippophae rhamnoides* L.) pomace and its applications in food systems.

The literature search was conducted using several major scientific databases, including Google Scholar, MDPI, ScienceDirect, SpringerLink, PubMed, Scopus, and Web of Science. The search strategy was based on combinations of relevant keywords such as "sea buckthorn pomace", "sea buckthorn by-products", "phytochemical composition", "functional foods", "food applications", "bioactive compounds", and "food enrichment".

Inclusion criteria comprised peer-reviewed articles published in English, focusing on the nutritional, phytochemical, and functional properties of sea buckthorn pomace, as well as its applications in food products. Review

papers and experimental studies were both considered, provided they contributed relevant data to the topic. Exclusion criteria included articles lacking full-text access, studies not directly related to sea buckthorn pomace, and publications outside the scope of food applications.

The selection process involved an initial screening of titles and abstracts, followed by full-text evaluation of potentially relevant studies, with priority given to recent publications. The collected literature was systematically analyzed and grouped into two main thematic sections: (1) nutritional and phytochemical properties of sea buckthorn pomace, and (2) sea buckthorn pomace as a functional food ingredient. The second category was further structured based on specific food applications, including: incorporation into jelly and candy formulations, chocolate products, beverages, bakery and pastry products, and edible oils for bioactive enrichment.

The methodology relied on critical analysis, comparison, and synthesis of the selected studies, allowing the identification of current research trends, technological approaches, and existing gaps in the valorization of sea buckthorn pomace.

RESULTS AND DISCUSSIONS

1. Nutritional and phytochemical properties of sea buckthorn pomace

Sea buckthorn pomace (SBP), consisting of seeds and residual solid fractions remaining after juice extraction, is subjected to drying, resulting in a nutritionally valuable by-product characterized by a high dry matter content of approximately 93-94%, confirming its low residual moisture and suitability for storage and further utilization (Nour et al., 2021).

Lipid content has been reported at approximately 12% (dry matter) (Kaminskytė et al., 2025) and up to approximately 20% (dry matter) (Peng et al., 2024). The lipid component is largely associated with residual seed material, as previously reported (Dienaitė et al., 2020; Sharma et al., 2022; Xiao et al., 2024). The fatty acid profile is nutritionally favourable, with monounsaturated fatty acids representing approximately 53% of total fatty acids and an n-6/n-3 ratio of approximately 1.4 (Peng et al., 2024).

The crude protein content of SBP ranges from approximately 15% (dry matter) (Nour et al., 2021) to approximately 21% (dry matter) (Kaminskytė et al., 2025). Essential amino acids account for approximately 38% of the total amino acid fraction, with leucine identified as a predominant component (Peng et al., 2024), highlighting the retained nutritional value of the pomace.

Dietary fiber represents the major fraction of SBP, reaching approximately 64 g/100 g dry matter (Kaminskytė et al., 2025). This fraction consists predominantly of insoluble dietary fiber (approximately 59 g/100 g dry matter), while soluble fibre contributes approximately 5 g/100 g dry matter (Kaminskytė et al., 2025). The high fibre content underlines its structural complexity and functional potential.

SBP also contains considerable amounts of ascorbic acid (approximately 100 mg/100 g dry matter) and high levels of total polyphenols, exceeding 2000 mg GAE/100 g dry matter depending on cultivar and drying conditions, along with notable antioxidant activity (Stanciu et al., 2023). Organic acids, including malic and quinic acids, have also been identified within the pomace matrix (Suo et al., 2025). Furthermore, the presence of carotenoids contributes to the characteristic colour and antioxidant functionality of the material (Hussain et al., 2021).

Sea buckthorn pomace (SBP) contains a wide range of bioactive compounds originating from the berries, such as phenolic acids (gallic, protocatechuic, *p*-coumaric, ferulic, and chlorogenic acids), flavonols (isorhamnetin, quercetin, and kaempferol), flavan-3-ols, and polymeric procyanidins (Singh et al., 2024). These compounds are largely responsible for the high antioxidant capacity reported for sea buckthorn derivatives, as demonstrated by various in vitro assays, including DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), FRAP (ferric reducing antioxidant power), and ORAC (oxygen radical absorbance capacity). In addition to polyphenols, pomace retains dietary fibre, carotenoids, tocopherols, essential fatty acids, vitamins, and minerals, which further enhance its nutritional value.

The biological activities associated with these bioactive constituents support the functional potential of sea buckthorn pomace. Therefore, sea buckthorn pomace should not be regarded merely as industrial waste but rather as a promising functional ingredient with considerable potential for application in functional foods, nutraceutical formulations, and natural antioxidant systems (Singh et al., 2024). In addition, the phytochemical profile of sea buckthorn pomace includes other bioactive classes such as carotenoids, triterpenes, unsaturated fatty acids, vitamins, and other isoprenoid-related compounds, further expanding its chemical complexity (Peng et al., 2024). These phytochemicals are linked to strong antioxidant and antimicrobial properties. In particular, flavonoids and other phenolic constituents contribute to the inhibition of undesirable microorganisms through mechanisms such as interference with nucleic acid synthesis, disruption of cell membrane integrity, and modulation of microbial energy metabolism. Moreover, polyphenolic compounds may interact with proteins to form complexes, which can reduce proteolytic activity and subsequently limit the formation of ammonium nitrogen during fermentation processes (Peng et al., 2024). The presence of these phytochemicals not only enhances the preservative potential of sea buckthorn pomace but also supports its application as a natural functional additive with protective effects against oxidative degradation and microbial spoilage (Peng et al., 2024). This diverse phytochemical composition contributes to the strong antioxidant potential of sea buckthorn pomace and supports its relevance as a functional ingredient in food and nutraceutical applications. These findings emphasize complementary aspects of the phytochemical profile of sea buckthorn by-products.

The study conducted by Sajfrtová et al. (2010) investigated the lipid fraction of sea buckthorn seeds, highlighting the presence of phytosterols, particularly β-sitosterol, which accounted for 57-76% of the total sterol content. Supercritical CO_2 extraction resulted in higher β-sitosterol concentrations compared to conventional Soxhlet extraction, emphasizing the seed oil fraction as a valuable source of bioactive sterols with potential hypocholesterolemic and anti-inflammatory effects (Sajfrtová et al., 2010).

In addition, Sławińska et al. (2023) analyzed the polar phytochemical profile of sea buckthorn seed extracts and reported a broad spectrum of flavonoids, mainly glycosides of isorhamnetin, kaempferol, and quercetin, as well as proanthocyanidins, catechin, and triterpenoid saponins. The total flavonoid content exceeded 150 mg/g in non-roasted seeds, confirming that the phenolic fraction represents a major group of bioactive compounds in the defatted seed matrix (Sławińska et al., 2023). These findings indicate that sea buckthorn seeds possess a dual phytochemical profile, consisting of a sterol-rich lipid fraction and a phenolic-dominant polar fraction, depending on the extraction method applied.

2. Sea buckthorn pomace as a functional food ingredient

The increasing interest in sustainable food systems has encouraged the use of fruit processing by-products as valuable ingredients in new product development. In this context, sea buckthorn pomace represents an important resource due to its significant content of bioactive compounds. Reintroducing sea buckthorn pomace into the production chain represents an effective strategy for reducing environmental burdens and improving the overall efficiency of resource utilization. The valorization of such fruit processing residues provides a sustainable alternative to conventional waste disposal methods, transforming secondary streams into valuable raw materials. Through this approach, agro-industrial by-products can be converted into functional ingredients and high-added-value products applicable in the food, cosmetic, and pharmaceutical sectors (Šalaševičienė et al., 2025).

Figure 3 provides an overview of the functional applications of sea buckthorn pomace across different food product categories.

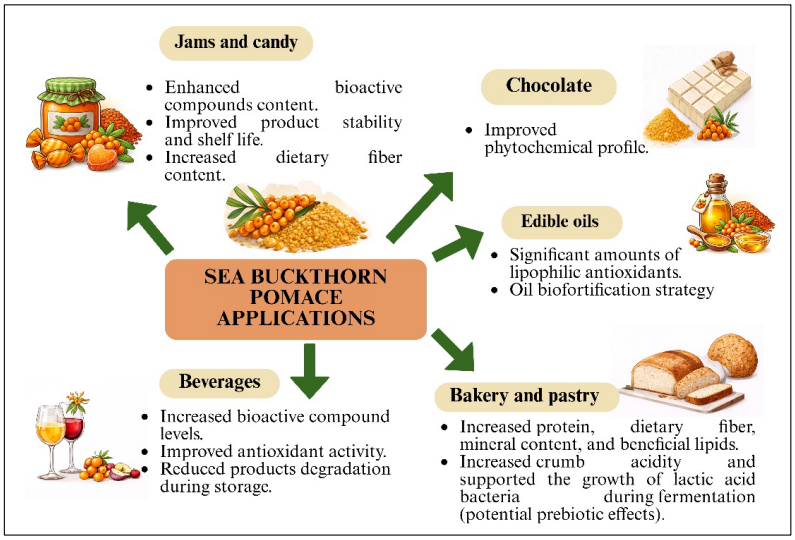


Figure 3. Functional applications of sea buckthorn pomace in food products (created in BioRender, https://BioRender.com, accessed in 25.03.2026)

2.1. Integration of sea buckthorn pomace in jelly and candy formulations

In the context of increasing consumer demand for clean-label products and natural ingredients, traditional preservation methods have gained renewed importance within the modern food industry (Urooj, 2021). Beyond preservation, producing fruit-based concentrates is a strategic way to maximize the use of fruit processing by-products. These products are valued for their sensory qualities and concentrate nutrients and beneficial compounds from the original fruit. The production of jellies and soft candies represents an important industrial application for fruit by-products. These products are developed based on the high-solids, high-acid principle, which ensures both stability and safety (Urooj, 2021).

According to Covaliov et al. (2025), the incorporation of dried SBP into quince-based jelly products provides both nutritional and functional advantages. From a nutritional perspective, the addition of pomace increased the content of carbohydrates, proteins, and minerals due to its intrinsic composition (approximately 13% protein and nearly 10% dietary fiber). The enrichment also contributed valuable bioactive compounds, particularly phenolics, resulting in enhanced antioxidant capacity of the jellies. The radical scavenging activity (DPPH assay) reached values above 90%, while hydrogen peroxide inhibition capacity also improved, demonstrating the significant antioxidant contribution of sea buckthorn pomace. From a functional standpoint, the high fiber content of the pomace positively influenced gel structure and texture. Its water-holding capacity contributed to increased firmness and chewiness of the jelly matrix while maintaining water activity below critical safety thresholds. Moreover, naturally occurring organic acids from the SBP enhanced titratable acidity and intensified the sensory profile. Formulations containing 1-2% SBP showed the most balanced combination of texture, taste, and overall acceptability (Covaliov et al., 2025).

Recent research shows that sea buckthorn juice and its by-products can be effectively incorporated into nutraceutical formulations. Lele et al. (2018) developed chewing candies enriched with sea buckthorn juice and by-products, demonstrating their antimicrobial and antioxidant potential. Both showed inhibitory activity against several pathogenic bacteria and significantly increased the total phenolic content and antioxidant capacity of the final product. The addition of by-products improved functional properties, confirming their suitability as value-added ingredients in sustainable food systems.

2.2. Integration of sea buckthorn pomace in chocolate formulations

Murariu et al. (2025) investigated the valorisation of sea buckthorn by-products obtained after juice extraction, with a particular focus on their incorporation into white chocolate as a strategy for developing a functional food product. The findings demonstrated that the addition of sea buckthorn fruit components, including pomace, to chocolate formulations provides significant nutritional and functional advantages, which may be extended to other confectionery systems enriched with sea buckthorn matrices. The incorporation of sea buckthorn by-products markedly increased the concentration of bioactive compounds in the final product. Higher inclusion levels were associated with elevated contents of total carotenoids, β-carotene, lycopene, vitamin C, flavonoids, and total phenolics. Antioxidant activity, including ABTS radical scavenging capacity, increased proportionally with the concentration of added material. In addition, mineral composition was enhanced, with enriched samples showing higher levels of calcium, magnesium, sodium, and phosphorus. Using by-product powder also increased protein and fat levels compared to formulations with only sea buckthorn juice.

From a technological perspective, the incorporation of sea buckthorn by-products influenced the textural and structural properties of the white chocolate matrix. Moderate inclusion levels improved cohesiveness and contributed to structural reinforcement, whereas higher concentrations reduced rigidity and hardness, resulting in softer products. Colour parameters were significantly affected by the presence of natural pigments, particularly carotenoids, leading to increased redness and distinct visual differentiation. Moreover, the enriched formulations remained stable throughout storage, indicating good functional integration of the pomace within the confectionery system (Murariu et al., 2025).

The incorporation of sea buckthorn pomace, either full-fat or defatted, into white chocolate provides significant nutritional and functional benefits (Murariu et al., 2024). The addition of pomace powder markedly increased the concentration of bioactive compounds, particularly carotenoids (β-carotene and lycopene), vitamin C, and total polyphenols. Antioxidant activity, including ABTS radical scavenging capacity, increased proportionally with pomace concentration, with the highest values recorded at a 15% inclusion level.

The full-fat pomace enhanced lipid content and fat-soluble bioactives such as carotenoids, whereas the defatted variant promoted higher polyphenol levels and stronger antioxidant capacity. Mineral composition was also improved, with enriched samples exhibiting increased calcium and magnesium levels, and in some formulations, elevated phosphorus content. Concerning technological properties, sea buckthorn pomace positively influenced rheological and textural parameters. At a 15% addition level, samples demonstrated greater firmness, shear force, cohesiveness, and gumminess compared to the control. The presence of natural pigments significantly altered colour characteristics, increasing brightness and redness and thereby enhancing visual appeal. A slight reduction in pH was observed with increasing pomace concentration, contributing to the distinct functional profile of the enriched products.

These findings demonstrate that sea buckthorn pomace can be effectively utilized as a multifunctional ingredient in chocolate formulations, improving antioxidant potential, mineral composition, and structural properties while supporting sustainable by-product valorisation (Murariu et al., 2024).

2.3. Integration of sea buckthorn pomace in beverage formulations

The upcycling of sea buckthorn pomace into beverage formulations represents an innovative strategy in sustainable product development. The utilization of this by-product through controlled fermentation enables the production of a low-alcohol (approximately 3% ethanol), effervescent beverage rich in phenolic antioxidants, thereby contributing to waste valorization and the development of functional products (Gatlan et al., 2020). However, current technological challenges, such as the tendency to sedimentation and astringency caused by tannins, require further research to optimize the stability and palatability of the finished product (Zhu et al., 2025).

The study conducted by Furulyas et al. (2024) highlights the valorization of sea buckthorn pomace (SBP) as a functional ingredient, emphasizing its potential to transform an agro-industrial by-product into a value-added resource. Although the bioactive properties of SBP are recognized, its antimicrobial potential remains insufficiently explored in the scientific literature. In this context, the authors aimed to investigate the antioxidant and antimicrobial characteristics of the pomace when incorporated into apple juice. Their findings, supported by the monitoring of total polyphenol content and microbial dynamics during storage, demonstrate that SBP contributes not only to improving the nutritional quality of the beverage but also to enhancing its microbiological stability, indicating a potential natural preservative effect. Another study evaluated the enrichment of sea buckthorn juice with different concentrations of dried sea buckthorn pomace (0.5-2%) and assessed physicochemical parameters, β-carotene content, total polyphenols, antioxidant capacity (FRAP), color stability, and storage behavior over 14 months. The results showed that adding pomace increased bioactive compounds and antioxidant activity while slowing their degradation during storage.

In addition, Rentsendavaa et al. (2021) investigated the stability of carotenoids and phenolic compounds in sea buckthorn (*Hippophae rhamnoides* L.) juice during storage, focusing specifically on the effect of sea buckthorn pomace addition. Juice samples were enriched with 0.5%, 1%, and 2% dried sea buckthorn pomace and stored at room temperature for 14 months. The study evaluated changes in total polyphenol content, antioxidant capacity (FRAP), β-carotene concentration, soluble solid content, pH, and colour parameters over the storage period.

The results showed that sea buckthorn pomace addition increased the initial β-carotene content and enhanced the antioxidant capacity of the juice in a dose-dependent manner. Although degradation of polyphenols and antioxidant activity occurred during storage, samples containing higher levels of pomace exhibited smaller reductions in bioactive compounds. Furthermore, a strong positive correlation between total polyphenols and antioxidant capacity confirmed the significant contribution of phenolic compounds to the overall antioxidant properties. The findings of the study demonstrate that sea buckthorn pomace addition improves the functional quality and storage stability of sea buckthorn juice.

2.4. Integration of sea buckthorn pomace in bakery and pastry formulations

The valorization of sea buckthorn pomace through its incorporation into bakery formulations represents a sustainable approach to converting this by-product into a functional ingredient with added nutritional value. Its inclusion in cereal-based products not only enhances the overall composition but also introduces technological considerations related to thermal processing. Baking is essential for developing desirable sensory attributes, ensuring microbial safety, and extending shelf life; however, it may also promote the formation of acrylamide through Maillard reaction pathways. Given the increasing regulatory attention and the need to comply with established benchmark levels, strategies to mitigate acrylamide formation are required (Ciesarová et al., 2023). In this context, the high L-asparagine content of sea buckthorn pomace makes it a potential contributor to acrylamide formation. To address this challenge, commercial asparaginase was applied to the pomace prior to drying and its incorporation into nutritionally enriched wholegrain cereal biscuits, thereby enabling its safe use while maintaining product quality and regulatory compliance (Ciesarová et al., 2023).

Recent research has explored incorporating sea buckthorn pomace into bakery products, showing that its technological and nutritional effects depend on the cereal matrix and processing conditions.

Wirkijowska et al. (2025) evaluated the use of sea buckthorn pomace in wheat bread fortification. Their findings showed that its inclusion improved the nutritional composition of bread by increasing protein, dietary fibre (particularly the insoluble fraction), mineral content, and beneficial lipids, while slightly lowering the caloric value. Although higher substitution levels negatively influenced dough stability, loaf volume, and crumb softness,

moderate additions (3–6%) achieved a suitable balance between enhanced nutritional value and acceptable technological and sensory properties. Overall, the study supports the sustainable valorization of sea buckthorn pomace as a clean-label ingredient aligned with circular economy principles, enabling the production of nutritionally improved bakery products without significantly compromising consumer acceptance at appropriate inclusion levels.

In contrast, Banach et al. (2024) investigated sea buckthorn pomace flour obtained as a by-product of juice and oil production and its application in rye bread formulated with sourdough fermentation. The incorporation of sea buckthorn pomace flour (SBPF) at moderate levels (approximately 10–15%) provided both nutritional and functional benefits. SBPF contributed substantial amounts of dietary fibre, lipids rich in bioactive fatty acids, protein, and phytochemicals, including polyphenols and carotenoids. Its addition increased crumb acidity and supported the growth of lactic acid bacteria during fermentation, suggesting potential prebiotic effects. The natural pigments intensified the crumb colour, reflecting the presence of carotenoid compounds associated with antioxidant activity. Furthermore, SBPF improved dough and bread yield, reduced baking losses, and enhanced water retention due to its high fibre content. At a 10% substitution level, loaf volume increased compared to the control, indicating improved gas retention and fermentation performance. However, higher inclusion levels (≥15-20%) adversely affected loaf volume and structure, demonstrating that excessive fibre can weaken the dough matrix. These studies confirm the significant potential of sea buckthorn pomace as a value-added ingredient in bakery formulations. Nevertheless, the optimal level of incorporation depends strongly on the type of flour, the technological process, and the structural characteristics of the final product. While wheat bread shows greater sensitivity to higher substitution levels, rye sourdough bread allows for more substantial incorporation, resulting in enhanced nutritional benefits and stronger alignment with sustainability and circular economy strategies.

According to Ciesarová et al. (2023), the incorporation of sea buckthorn pomace (SBP) into whole-grain biscuits offers significant nutritional benefits, as the pomace retains a considerable amount of bioactive compounds even after juice extraction. SBP contributes polyphenols, flavonoids, carotenoids, dietary fibre, vitamins, unsaturated fatty acids, essential amino acids, and minerals, thereby increasing the overall nutritional value of cereal-based products. The inclusion of 10% SBP enhances the concentration of health-promoting components while simultaneously supporting circular economy principles through by-product valorization. The study further demonstrates that enzymatic treatment of SBP effectively reduces acrylamide formation in biscuits. Given the naturally high free asparagine content of sea buckthorn, a major precursor of acrylamide, the application of asparaginase significantly decreases asparagine levels prior to baking. Consequently, biscuits formulated with treated SBP exhibit substantial reductions in acrylamide content, up to 64% in wheat biscuits and 35% in rye and triticale formulations, compared to those containing untreated pomace. Importantly, this mitigation approach does not compromise technological quality, as texture, spread ratio, and sensory-relevant attributes remain within acceptable ranges. Thus, sea buckthorn pomace serves as both a nutritional enhancer and, when appropriately processed, a safety-oriented ingredient that contributes to limiting the formation of heat-induced contaminants.

2.5. Integration of sea buckthorn pomace into edible oils for bioactive enrichment

In recent years, the use of certain synthetic antioxidants has been limited due to concerns regarding their potential toxic and carcinogenic effects; consequently, foods enriched with natural antioxidants have gained increasing global attention (Liu et al., 2021).

Vegetable oils are widely consumed and serve as efficient carriers for lipophilic bioactive compounds. However, many refined edible oils naturally contain low levels of carotenoids, limiting their intrinsic antioxidant capacity. Therefore, enrichment strategies aimed at enhancing oxidative stability and nutritional value have received increasing attention.

Integration of sea buckthorn pomace into edible oils for bioactive enrichment represents a promising strategy. The sea buckthorn pomace remaining after juice extraction retains significant amounts of lipophilic antioxidants, particularly carotenoids, representing an underutilized source of bioactive molecules that can be efficiently incorporated into lipid-based systems. These compounds are of considerable interest due to their antioxidant activity, coloring properties, and potential health-promoting effects, making sea buckthorn pomace a valuable raw material for functional ingredient development.

Chemat et al. (2012) investigated the direct enrichment of edible oils with carotenoids recovered from sea buckthorn pomace using ultrasound-assisted extraction. Sunflower, rapeseed, olive, and soybean oils were used both as final products and as extraction media, eliminating the need for organic solvents. Process optimization via response surface methodology showed that controlled sonication power and moderate temperatures significantly enhanced carotenoid transfer into the oil matrix. Compared to conventional maceration, ultrasound increased carotenoid concentration and drastically reduced extraction time, demonstrating the efficiency and feasibility of this solvent-free oil enrichment using sea buckthorn by-products and highlighting new perspectives for sustainable valorization in functional oils development**.**

An additional relevant contribution in the field of sea buckthorn by-product valorization was reported by Patra et al. (2022), who investigated microwave-assisted extraction (MAE) as an alternative to conventional Soxhlet extraction for obtaining bioactive compounds from sea buckthorn pomace and seeds. Both low-polarity (n-hexane) and medium-polarity (ethanol) solvents were evaluated, with the objective of maximizing extraction yield and antioxidant recovery. The study demonstrated that microwave processing significantly enhanced extraction efficiency compared to the traditional method, particularly when ethanol was used as solvent under optimized conditions, at 80°C and 150 W microwave power. The highest extraction yield was obtained for pomace using microwave-assisted ethanol extraction, substantially exceeding that achieved by Soxhlet extraction under similar solvent conditions. Moreover, the extracts obtained via MAE exhibited strong antioxidant properties, as confirmed by radical scavenging assays. In addition, when incorporated into edible oil systems, the sea buckthorn extracts demonstrated oxidative stability comparable to that of synthetic antioxidants, indicating their potential as natural alternatives for oil stabilization. These findings confirm that microwave-assisted extraction efficiently recovers antioxidant-rich fractions from sea buckthorn by-products, supporting their use in stabilizing and enhancing edible oils.

An additional application of sea buckthorn pomace valorization was reported by Bhimjiyan et al. (2021), who investigated the enrichment of cold-pressed flaxseed oil with carotenoids extracted from *Hippophae rhamnoides* pomace using ultrasound-assisted extraction. Flaxseed oil, although recognized for its high omega-3 fatty acid content and functional properties, naturally lacks carotenoids, which are important contributors to antioxidant activity and oxidative stability. Therefore, the incorporation of sea buckthorn-derived carotenoids represents a strategy to enhance both the nutritional and technological quality of the oil. The process was optimized using a statistical experimental design to maximize carotenoid transfer into the oil phase. Under optimized conditions, involving controlled sonication time, feed-to-oil ratio, and amplitude, a significant increase in carotenoid concentration was achieved in the enriched flaxseed oil. Compared to conventional extraction, ultrasound treatment resulted in substantially higher carotenoid incorporation while reducing processing time and eliminating the need for organic solvents. The enriched oil also exhibited improved radical scavenging activity and enhanced physicochemical stability. The study shows that ultrasound-assisted enrichment of flaxseed oil with sea buckthorn pomace carotenoids is an efficient, eco-friendly method, enhancing nutritional value and enabling applications in functional foods, nutraceuticals, and dietary supplements.

CONCLUSIONS

Sea buckthorn pomace is a valuable food industry by-product with significant nutritional and phytochemical potential. Rich in polyphenols, flavonoids, carotenoids, dietary fibre, vitamins, minerals, and bioactive lipids, it exhibits notable antioxidant and antimicrobial properties. Reviewed studies confirm that appropriate processing strategies, such as enzymatic treatments and controlled incorporation levels, can optimize its functional performance while preserving technological quality in final products. The integration of sea buckthorn pomace into different food systems such as beverages, bakery products, chocolate, jam and jelly, confectionery and edible oils, demonstrates its versatility as a functional ingredient. In addition to improving nutritional value, its incorporation may contribute to enhanced product stability, extended shelf life, and, in some applications, improved microbiological safety. However, the technological effects depend strongly on the formulation, processing conditions, and level of addition, highlighting the need for optimized product development strategies. Moreover, the valorization of sea buckthorn pomace supports sustainable production models by reducing food waste and promoting the efficient use of agro-industrial residues. This approach aligns with circular economy principles and contributes to the development of clean-label and value-added functional foods. Future research should further investigate bioavailability, consumer acceptance, scalability of processing technologies, and the long-term stability of bioactive compounds in complex food matrices to fully exploit the potential of sea buckthorn by-products.

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THE EFFECT OF ADDITION OF CRANBERRY SYRUP ON THE PHYSICOCHEMICAL AND SENSORY CHARACTERISTICS OF BEER

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Abstract

This study evaluates the effect of cranberry syrup addition on the quality of beer, focusing on physicochemical properties, sensory characteristics, and vitamin C content. Beer samples were produced with varying concentrations of cranberry syrup (5%, 10%, and 15%) and analyzed for pH, alcohol and vitamin C content, extract, color, and sensory profile. The results showed that increasing syrup concentration led to lower pH and alcohol content, while extract and color values increased. Sensory analysis indicated improved aroma and color, with the highest overall acceptability observed for the 10% sample. Additionally, vitamin C content increased significantly in enriched samples, confirming enhanced functional value. Although the 15% sample showed the highest vitamin C content, it presented slight sensory imbalance. Therefore, the 10% formulation was identified as optimal, offering a balance between improved quality and nutritional benefits.

Key words*: beer, cranberry, physicochemical properties, sensory analysis, vitamin C.*

INTRODUCTION

Beer is one of the oldest fermented beverages known to humanity, with a history spanning several millennia and a strong cultural, economic, and technological significance worldwide. In modern times, beer production has evolved from traditional practices into a highly controlled biotechnological process, involving precise manipulation of raw materials and fermentation conditions (Tirado-Kulieva et al., 2023). Today, beer remains one of the most widely consumed alcoholic beverages globally, reflecting both its accessibility and diversity of styles (Brewers of Europe, 2024).

From a compositional perspective, beer is a complex system consisting primarily of water, carbohydrates, alcohol, proteins, and a wide range of bioactive compounds. Among these, phenolic compounds derived from malt and hops have attracted increasing attention due to their antioxidant properties and potential health benefits when consumed in moderation (Piazzon et al., 2010; De Gaetano et al., 2016). These compounds contribute not only to the nutritional value of beer but also to its sensory characteristics, including color, flavor, and stability (Lentz, 2018).

In recent years, consumer demand has shifted toward innovative and functional beverages, leading to the development of specialty beers enriched with natural ingredients such as fruits, herbs, and plant extracts. Fruit beers, in particular, have gained popularity due to their enhanced sensory appeal and potential health-promoting properties (Aredes et al., 2023). The incorporation of fruits into beer formulations can increase the content of vitamins, organic acids, and polyphenols, thereby improving both the functional value and organoleptic profile of the final product (García-Guzmán et al., 2018).

Recent studies have highlighted that the incorporation of fruit-derived ingredients into beer not only enhances its nutritional profile but also contributes to product differentiation in an increasingly competitive market. Fruit beers are often perceived by consumers as more natural and healthier alternatives compared to conventional beer, mainly due to their higher content of bioactive compounds such as polyphenols, vitamins, and organic acids (Aredes et al., 2023). In addition, these ingredients can improve oxidative stability by limiting lipid oxidation and preserving flavor compounds during storage (Piazzon et al., 2010). As a result, fruit-enriched beers are

gaining attention not only from consumers but also from researchers and producers aiming to develop innovative functional beverages.

Cranberries (*Vaccinium macrocarpon*) are especially noteworthy among fruit ingredients due to their high content of polyphenols, flavonoids, and organic acids, which exhibit strong antioxidant and antimicrobial properties.

The use of cranberry-derived products in beverages has been associated with improved oxidative stability and enhanced sensory attributes, particularly in terms of color and aroma (De Gaetano et al., 2016). These characteristics make cranberry syrup a promising additive for the development of value-added beer products.

In addition to their antioxidant potential, cranberries contain significant amounts of anthocyanins, which are responsible for their characteristic red color and contribute to the visual appeal of food and beverage products. These pigments are known to be relatively stable under acidic conditions, making them particularly suitable for incorporation into beer, which typically exhibits a pH between 3.5 and 4.5 (Oszmiański & Wojdyło, 2005). Moreover, cranberry compounds may exert antimicrobial effects, potentially influencing microbial stability during storage and extending shelf life (De Gaetano et al., 2016). Therefore, the use of cranberry syrup may offer both technological and nutritional advantages in brewing applications.

Vitamin C (ascorbic acid) is an essential bioactive compound widely recognized for its strong antioxidant properties and its role in protecting biological systems against oxidative stress. In the context of beverages, vitamin C contributes not only to nutritional value but also to product stability by reducing oxidative degradation processes. Although conventional beer contains only trace amounts of vitamin C due to processing conditions and raw material composition, the incorporation of fruit-derived ingredients can significantly enhance its ascorbic acid content (Piazzon et al., 2010; De Gaetano et al., 2016).

Cranberries are particularly rich in vitamin C and other antioxidant compounds, making them a suitable candidate for the development of functional beer products with improved health-related attributes (Oszmiański & Wojdyło, 2005; Kylli et al., 2010). Therefore, the enrichment of beer with cranberry syrup may represent an effective approach to increase its antioxidant capacity and overall functional value.

At the same time, the brewing industry continues to adapt to changing consumer preferences, focusing on sustainability, natural ingredients, and product diversification. According to recent reports, the European beer market shows a growing interest in craft and specialty beers, including those with added functional ingredients (Brewers of Europe, 2024). In Romania, similar trends have been observed, with an increasing share of innovative and non-traditional beer products gaining market attention (Asociația Berarii României, 2024).

In this context, the present study aims to evaluate the influence of cranberry syrup addition on the quality characteristics of beer. The research focuses on analyzing the physicochemical and sensory properties of the final product, with the objective of identifying optimal formulations that enhance both functional value and consumer acceptability.

MATERIALS AND METHODS

The raw materials used for beer production were barley malt (Pilsner type), hops (pellet form, bittering and aroma varieties), brewing yeast (*Saccharomyces cerevisiae*) and potable water.

The experimental ingredient consisted of **cranberry syrup**, obtained from concentrated cranberry juice, standardized to a soluble solids content of approximately 65 °Brix.

All reagents used for physicochemical analyses were of analytical grade.

Four beer variants were produced, differing in the concentration of added cranberry syrup:

- **Control sample (C)** – without cranberry syrup;
- **Sample S1** – 5% cranberry syrup (v/v);
- **Sample S2** – 10% cranberry syrup (v/v);
- **Sample S3** – 15% cranberry syrup (v/v).

The cranberry syrup was added during the **secondary fermentation stage**, in order to preserve bioactive compounds and enhance aroma.

The beer was produced using a standard brewing process (Figure 1), consisting of the following steps:

– Milling of malt;
– Mashing – conducted at controlled temperatures (62-68°C) for enzymatic conversion;
– Filtration (lautering) – separation of wort from spent grains;
– Boiling with hops – for 60 minutes;
– Cooling of wort to fermentation temperature (~20°C);
– Primary fermentation – carried out for 5-7 days;
– Addition of cranberry syrup (according to experimental design);
– Secondary fermentation/maturation –10-14 days at 4°C;
– Filtration and bottling (Tirado-Kulieva et al., 2023).

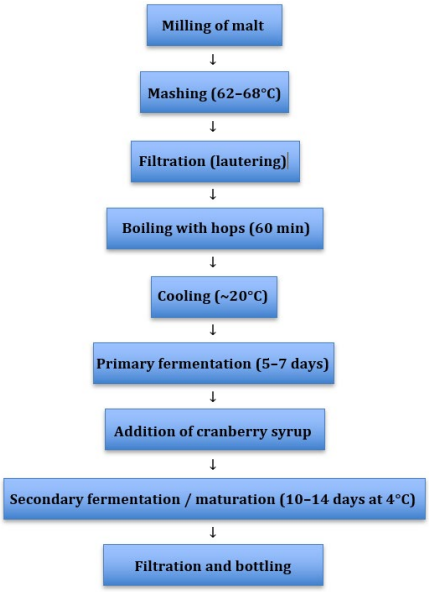


Figure 1. Technological process for obtaining beer with cranberry syrup

The obtained beer samples were analyzed for the determination of pH, alcohol and vitamin C content, extract, color and stability.

pH determination was conducted using a calibrated digital pH meter at 20°C. **Alcohol content (% v/v)** was determined using distillation followed by densimetric measurement. The **extract (Plato)** was measured using a refractometer. **Color** was determined spectrophotometrically and expressed in EBC units. **Stability** was evaluated through turbidity measurements and observation over a 14-day storage period at 4°C (Aredes et al., 2023).

The determination of vitamin C content in the beer samples was carried out using a spectrophotometric method based on the reduction of 2,6-dichlorophenolindophenol (DCPIP). Prior to analysis, the samples were filtered and appropriately diluted to ensure accurate measurement. Absorbance was recorded at a wavelength of 520 nm, and the vitamin C concentration was calculated using a calibration curve prepared with standard ascorbic acid solutions. All analyses were performed in triplicate, and results were expressed as mg/L of ascorbic acid. This method is widely used for the quantification of vitamin C in food and beverage matrices due to its reliability and sensitivity (Igual et al., 2010).

Sensory evaluation was conducted by a panel of **10 trained assessors** that evaluated the following attributes: aroma, taste, color and overall acceptability. A **9-point hedonic scale** was used (1 = extremely unpleasant, 9 = extremely pleasant) (Lentz, 2018).

All experimental determinations were carried out in triplicate, and the obtained results were expressed as mean values ± standard deviation. Statistical analysis was performed to evaluate the significance of differences between samples, using analysis of variance (ANOVA) followed by post hoc comparisons where appropriate. A significance level of $p < 0.05$ was considered statistically significant. This approach ensures the reliability and reproducibility of the results and allows for a more accurate interpretation of the influence of cranberry syrup addition on beer quality parameters (Aredes et al., 2023).

RESULTS AND DISCUSSIONS

The physicochemical parameters of the analyzed beer samples are presented in Table 1. The results highlight the influence of cranberry syrup addition on the main quality indicators of the finished product (Aredes et al., 2023).

Different superscript letters within the same column indicate significant differences between samples ($p < 0.05$).

Statistical analysis revealed significant differences ($p < 0.05$) between the control and cranberry-enriched beer samples for all analyzed

physicochemical parameters. Increasing cranberry syrup concentration significantly decreased pH and alcohol content, while extract and color values increased significantly.

Table 1. Physicochemical characteristics of beer samples enriched with cranberry syrup (mean ± SEM, n = 3)

Sample	pH	Alcohol (% v/v)	Extract (°P)	Color (EBC)
C	4.50 ± 0.03a	5.20 ± 0.05a	11.5 ± 0.1d	8.0 ± 0.2d
S1	4.30 ± 0.02b	5.00 ± 0.04b	11.8 ± 0.1c	12.0 ± 0.3c
S2	4.10 ± 0.04c	4.80 ± 0.03c	12.2 ± 0.2b	16.0 ± 0.4b
S3	3.90 ± 0.03d	4.60 ± 0.05d	12.8 ± 0.2a	21.0 ± 0.5a

The pH values of the samples decreased progressively with the increase in cranberry syrup concentration. The control sample recorded a pH of 4.5, while the sample with 15% syrup (S3) reached a pH of 3.9.

This decrease can be attributed to the presence of organic acids naturally found in cranberries, such as citric and malic acids. The reduction in pH contributes positively to microbial stability and freshness perception; however, excessively low pH values may negatively influence taste balance, leading to increased sourness (De Gaetano et al., 2016).

The decrease in pH may also influence the perception of freshness and crispness, which are desirable attributes in fruit beers. However, excessive acidity can lead to an imbalance between sweetness, bitterness, and sourness, negatively affecting overall palatability. Similar trends have been reported in other studies involving fruit-enriched beers, where optimal formulation requires careful control of acid content to maintain sensory harmony (Aredes et al., 2023).

The alcohol content showed a slight decreasing trend with increasing syrup concentration, from 5.2% in the control sample to 4.6% in sample S3. This variation can be explained by the dilution effect caused by syrup addition, the presence of non-fermentable sugars in cranberry syrup and by possible inhibition of yeast activity due to increased acidity. Although the decrease is moderate, it may influence the perception of body and warmth in the final product (Tirado-Kulieva et al., 2023).

The extract values increased proportionally with the amount of added cranberry syrup, reaching 12.8°P in sample S3. This increase is due to the contribution of soluble solids (mainly sugars and bioactive compounds) from the cranberry syrup. A higher extract content generally contributes to a fuller body and improved mouthfeel, although excessive extract may lead to a heavier sensory profile (Aredes et al., 2023).

Color intensity increased significantly with the addition of cranberry syrup, from 8 EBC in the control sample to 21 EBC in sample S3.

This change is directly related to the presence of anthocyanins and other pigments in cranberries. The enhanced color contributes positively to consumer appeal, especially for specialty or fruit beers, where visual characteristics are highly important (García-Guzmán et al., 2018).

The addition of cranberry syrup slightly influenced the **physical stability** of the beer. Samples with higher syrup concentrations showed a tendency toward increased turbidity during storage. This behavior may be associated with pectin content in cranberry syrup, interactions between polyphenols and proteins or incomplete clarification after fermentation.

However, no significant sedimentation or major stability defects were observed within the 14-day monitoring period (Piazzon et al., 2010).

The results of the sensory analysis are presented in Table 2.

Table 2. Sensory evaluation of beer samples enriched with cranberry syrup (mean ± SEM, n = 10)

Sample	Aroma	Taste	Color	Overall acceptability
C	6.5 ± 0.3c	6.8 ± 0.2c	6.0 ± 0.2d	6.6 ± 0.3c
S1	7.5 ± 0.2b	7.2 ± 0.3b	7.5 ± 0.3c	7.4 ± 0.2b
S2	8.2 ± 0.2a	7.8 ± 0.2a	8.3 ± 0.2b	8.0 ± 0.2a
S3	8.5 ± 0.3a	6.9 ± 0.3bc	9.0 ± 0.2a	7.2 ± 0.3b

Different superscript letters within the same column indicate significant differences between samples ($p < 0.05$).

The sensory evaluation results showed statistically significant differences ($p < 0.05$) among samples, particularly for aroma, color, and overall acceptability. Sample S2 recorded the highest overall acceptability score, indica-

ting the best balance between fruity characteristics and traditional beer flavor.

Aroma scores increased significantly with cranberry syrup addition, reaching the highest value (8.5) in sample S3. The improvement is due to volatile compounds and aromatic esters present in cranberries, which contribute fruity and fresh notes. This confirms that cranberry syrup enhances the aromatic complexity of beer (Lentz, 2018).

Taste evaluation showed a different trend. While samples S1 and S2 recorded higher scores than the control, sample S3 showed a slight decrease. This indicates that moderate addition of cranberry syrup improves taste balance, but excessive amounts may lead to increased acidity, slight astringency, or masking of the traditional beer flavor. Thus, taste acceptability is optimized at intermediate syrup concentrations (Lentz, 2018).

The perception of color improved significantly with increasing syrup concentration, with S3 receiving the highest score (9.0). Panelists appreciated the intense reddish hue, which is associated with fruit beers and considered visually attractive. Color is a critical quality parameter influencing consumer perception and purchase decisions, especially in the case of specialty and craft beers. The reddish hue imparted by cranberry syrup may also create expectations regarding flavor, often associated with fruity, fresh, and slightly acidic notes. Therefore, the enhancement of color not only improves visual appeal but also contributes to the overall sensory experience by shaping consumer expectations prior to tasting (García-Guzmán et al., 2018). The highest overall acceptability score was recorded for sample S2 (8.0), followed by S1 (7.4) and S3 (7.2). Although S3 had the best aroma and color, its lower taste score affected its overall perception. This demonstrates that consumer preference depends on a balance between sensory attributes rather than a single dominant characteristic (García-Guzmán et al., 2018).

Table 3: Vitamin C content of beer samples enriched with cranberry syrup (mean ± SEM, n = 3)

Sample	Vitamin C (mg/L)
C	3.2 ± 0.2d
S1	18.5 ± 0.5c
S2	45.0 ± 1.1b
S3	52.3 ± 1.4a

Different superscript letters indicate significant differences between samples ($p < 0.05$).

Vitamin C content increased significantly ($p < 0.05$) with increasing cranberry syrup concentration. The highest value was observed in sample S3, confirming the contribution of cranberry syrup to the antioxidant potential and functional value of the beer.

The results of the vitamin C analysis (Table 3) revealed a significant increase in ascorbic acid content with the addition of cranberry syrup.

The control sample showed a low vitamin C concentration (3.2 mg/L), which is typical for conventional beer, whereas the enriched samples exhibited markedly higher values, reaching up to 52.3 mg/L in sample S3. This increase can be attributed to the naturally high ascorbic acid content of cranberries, as well as the presence of phenolic compounds that are partially retained during the brewing process (Oszmiański & Wojdyło, 2005; Kylli et al., 2010).

Furthermore, these compounds contribute synergistically to the antioxidant potential of the final product, enhancing its functional properties and overall nutritional value (Piazzon et al., 2010). It is important to note that vitamin C is sensitive to processing conditions such as temperature, oxygen exposure, and light. Despite these factors, the addition of cranberry syrup during the secondary fermentation stage likely contributed to better preservation of ascorbic acid compared to earlier stages of processing. This technological approach may represent an effective strategy to retain heat-sensitive nutrients in functional beer formulations (Igual et al., 2010). However, although sample S3 recorded the highest vitamin C content, sample S2 (45.0 mg/L) appears to represent the optimal formulation when considering both nutritional benefits and sensory balance, as excessive addition may negatively influence organoleptic characteristics.

The radar chart presented in Figure 2 provides a comprehensive visualization of the overall quality characteristics of the analyzed beer samples, integrating physicochemical parameters, sensory attributes, and vitamin C content.

The graphical representation highlights the balanced profile of sample S2, which demonstrates optimal values across most

evaluated parameters, including aroma, taste, extract, and antioxidant potential. The increased values observed for color and vitamin C content in samples with higher cranberry syrup concentrations confirm the contribution of fruit-derived bioactive compounds, particularly phenolics and ascorbic acid (Piazzon et al., 2023; Oszmiański & Wojdyło, 2005).

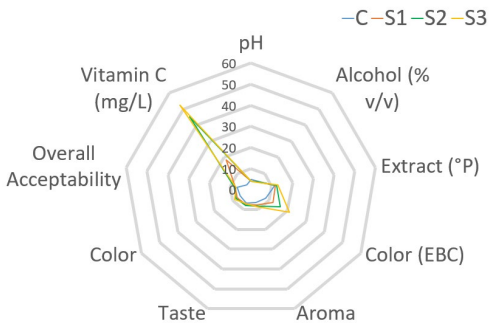


Figure 2. Radar Chart representing the overall quality characteristics of beer samples enriched with cranberry syrup

At the same time, the chart illustrates that excessive enrichment (S3) may lead to slight imbalances in sensory attributes, supporting the conclusion that moderate addition levels provide the best compromise between functional enhancement and consumer acceptability (Aredes et al., 2023). Such multivariate visualization tools are useful for identifying optimal formulations in functional beverage development.

The results confirm that the addition of cranberry syrup significantly influences both physicochemical and sensory characteristics of beer (Aredes et al., 2023; De Gaetano et al., 2016).

From a technological perspective, acidity increases with syrup addition, alcohol content slightly decreases and extract and color increase. From a sensory perspective, aroma and color are significantly improved and taste is optimized at moderate concentrations.

The optimal concentration identified in this study is 10% cranberry syrup (S2), which provides: balanced taste, enhanced aroma, attractive color and high overall acceptability. Higher concentrations (15%) lead to excessive acidity and slight sensory imbalance, despite improved visual and aromatic properties.

CONCLUSIONS

The use of cranberry syrup as an ingredient in beer production represents a promising approach for:

- developing **functional beers** enriched with antioxidants,
- diversifying product range,
- increasing consumer appeal through natural ingredients.

These findings support the potential of cranberry syrup as a value-added component in the brewing industry, especially in the segment of specialty and craft beers.

The incorporation of cranberry syrup into beer formulation proved to be an effective strategy for increasing vitamin C content and enhancing the functional value of the final product. The results demonstrated that enriched samples contained significantly higher levels of ascorbic acid compared to conventional beer, confirming the contribution of cranberry-derived compounds.

In addition to improving antioxidant potential, the presence of vitamin C may also contribute to product stability by limiting oxidative reactions (Piazzon et al., 2010; De Gaetano et al., 2016).

From an industrial perspective, the use of fruit-based ingredients such as cranberry syrup aligns with current trends in sustainable and innovative food production. The valorization of fruit-derived products can contribute to the development of high-value beverages while meeting consumer demand for natural and functional ingredients. Furthermore, the diversification of beer assortments through such formulations may provide competitive advantages for small-scale and craft breweries (Brewers of Europe, 2024; Asociația Berarii României, 2024).

Overall, the findings support the use of cranberry syrup as a natural ingredient for the development of innovative beer products with added nutritional benefits, provided that optimal concentrations are selected to maintain sensory acceptability.

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DEVELOPMENT AND NUTRITIONAL CHARACTERIZATION OF BEEF BONE MARROW-BASED PRODUCTS: PLAIN AND SOY-ENRICHED FORMULATIONS

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Abstract

The valorisation of animal by-products into nutrient-dense foods has received increasing attention in food science due to its potential to enhance resource use efficiency and reduce food waste within sustainable food systems. This study aimed to develop a beef marrow-based spread and to evaluate its nutritional value in both a plain formulation and a soy-enriched variant (soy at a 1:2 ratio with bone marrow on a w/w basis). Beef marrow is rich in lipids, proteins, and bioactive compounds; however, its application in paste-based food products remains limited. Both formulations were analysed for total fat content, fatty acid profile and protein content, oxidative stability, and shelf life. Results indicated a predominance of unsaturated fatty acids (UFA) in both products. The plain formulation exhibited higher levels of polyunsaturated fatty acids (PUFA) and more favourable lipid quality indices, whereas soy enrichment increased protein content and the proportion of monounsaturated fatty acids (MUFA), thereby improving oxidative stability, likely due to increased oleic acid content. Compared with conventional animal fat sources, beef bone marrow exhibited a favourable UFA profile, supporting its nutritional value and potential application in food formulations. Overall, bone marrow represents a valuable animal by-product with strong potential for incorporation into circular food systems.

Key words*: alternative protein sources, food valorisation, lipid quality, nutritional enhancement.*

INTRODUCTION

In the context of the circular economy and sustainable food production, increasing attention has been directed toward the valorisation of slaughterhouse by-products as value-added food ingredients (de Oliveira, Lago, & Dal'Magro, 2021; Mohan & Long, 2021). Beef bone marrow is a lipid-rich by-product containing essential fatty acids and bioactive compounds, representing a concentrated source of energy and nutrients (Adeyeye, 2014; Hoa et al., 2026). Compared with other animal fats, bone marrow has been reported to possess a distinct lipid composition and nutritional profile, which may provide functional and technological advantages in food applications. Although beef bone marrow has been consumed since ancient times (Blasco et al., 2019) in various cuisines worldwide (Adeyeye, 2014; Zahra & Cacciafoco, 2026), scientific literature remains limited regarding its use as a processed food ingredient and the development of marrow-based products. In particular, little information is available on the physicochemical properties of bone marrow pastes and on formulation strategies designed to modify lipid content and improve nutritional balance.

In this context, the present study aims to develop a beef bone marrow paste in both a simple formulation and an enriched version containing soy to adjust the lipid content. Furthermore, the study evaluates its properties through physicochemical composition analysis and other relevant characteristics to highlight its potential as a value-added food ingredient.

MATERIALS AND METHODS

Raw Materials, Auxiliary Ingredients, and Processing Flow

For the preparation of a beef bone marrow paste product and its soy-enriched formulation, the ingredients and quantities used are presented in Table 1.

Table 1. Ingredients used for Beef Bone Marrow Paste Products

Specification	Unit	Simple beef bone marrow	Soy-enriched beef bone marrow
Bone marrow	g	419	419
Lemon juice	ml	105	105
Soybean	g	-	210
Salt	g	4.5	4.5
Pepper	g	2	2

For the preparation of one batch of bone marrow paste, 7.236 kg of veal bones containing marrow were used. For each formulation (plain or soy-enriched), one independent production batch was prepared. From each batch, three subsamples were collected, and from each subsample, three analytical replicates were performed for each determination. The bones were roasted in an oven at 180°C for approximately 30 minutes, after which the marrow was extracted and homogenised using a blender. The resulting mass was subsequently passed through a fine sieve to remove any residual bone fragments that may have remained after cutting. The amount of unfiltered marrow obtained was 1081 g, corresponding to a yield of 14.9%, while 838 g remained after filtration, corresponding to a usable (filtered) yield of 11.6%. These yields may vary depending on the type of bones used for marrow extraction (Adeyeye, 2014).

In the soy-enriched bone marrow spread, 100 g of soybeans were combined with 200 g of beef bone marrow. The soybeans were first soaked for 8 hours, then boiled for 2 hours, after which they were blended and mixed with the bone marrow. Immediately after preparation, the beef bone marrow spread was stored under refrigerated conditions at 4°C. All analyses, except those related to freshness determination, were performed on day 1.

Physicochemical Analysis of Beef Marrow Paste

Moisture, protein, and fat contents were determined using standard analytical methods: moisture by oven drying, protein by the Kjeldahl method, and fat by Soxhlet extraction. Freshness of the marrow paste was evaluated through acidity determination, the Kreiss test, and the Nessler reaction (Răducu et al., 2025).

Determination of peroxide value

The peroxide value (PV) was determined in the lipid fraction extracted from marrow paste samples using an iodometric titration method adapted from ISO 3960 for fat-rich food matrices. Lipids were extracted from homogenized samples using a chloroform solvent system, followed by solvent evaporation under reduced pressure. Approximately 2.0-5.0 g of extracted fat were dissolved in a mixture of glacial acetic acid and chloroform (3:2, v/v). Subsequently, 0.5 mL of saturated potassium iodide solution was added, and the mixture was maintained in darkness for 1 min with continuous agitation. After the addition of 30 mL of distilled water, the liberated iodine was titrated with 0.01 N sodium thiosulfate solution using starch as an indicator near the endpoint. A blank sample was prepared and analyzed under identical conditions.

Analysis of fatty acids compositions

Extraction of lipids from samples

The lipid extraction procedure followed the method described by Senila, Kovacs & Senila (2025) with minor modifications. Briefly, 3 g of sample were extracted with 50 mL of a chloroform: methanol mixture (2:1, v/v) and subjected to ultrasonic treatment (ISOLAB, Germany) for 15 minutes. After extraction, the mixtures were filtered, and the resulting liquid phase was further treated with 20 mL of 0.74% KCl solution. The extracts were then centrifuged at 5000 rpm for 10 minutes to separate the organic layer, which was subsequently filtered through anhydrous Na_2SO_4 to remove residual water. The solvent was evaporated using a rotary evaporator (Laborota 4010, Heidolph), and the recovered oil was dried in an oven at 60 °C.

Fatty acid methyl esters (FAMEs)

The fatty acid composition of the samples was analyzed by gas chromatography coupled with a flame ionization detector (GC-FID), following transesterification of fatty acids into their corresponding fatty acid methyl esters (FAMEs) using potassium methoxide. For this purpose, 0.06 g of each sample was dissolved in

isooctane, after which 0.2 mL of a 2 mol L^{-1} methanolic potassium hydroxide solution (CH_5KO_2) was added, and the mixture was vigorously shaken for 30 seconds. The reaction mixture was then treated with 1 g of sodium hydrogen sulfate monohydrate ($NaHSO_4 \cdot H_2O$) to prevent saponification of the methyl esters and to neutralize the excess alkali. Each oil sample was subjected to trimethylation and analyzed in triplicate. A standard reference mixture - Supelco 37 Component FAME Mix (CRM47885, Sigma-Aldrich) was used for chromatographic identification and calibration. The fatty acid contents are expressed as the relative percentage of each component, calculated by internal normalization of the chromatographic peak areas.

Determination of fatty acid methyl esters (FAMEs) content using GC-FID

The fatty acid methyl esters (FAMEs) content in samples were determined using a gas chromatograph with flame ionization detector (GC-FID) (Agilent Technologies, 6890N), equipped with a ZB-WAX capillary column (30 m x 0.25 mm x 0.25 µm). The carrier gas was helium at a constant flow rate of 1 mL min-1. The split ratio was 1:20, and the injected volume was 1 µl. The GC oven temperature program consisted of three stages: 60°C for 1 min, from 60 to 200°C (rate 10°C min^{-1}, 2 min), and from 200 to 220°C (5°C min^{-1}, 20 min). Both injector and detector temperatures were at 250°C to ensure complete vaporization of the sample and detection sensitivity. The retention times of the sample FAMEs were compared with those of the Supelco FAME standard mixture.

Nutritional Lipid Indices Derived from Fatty Acid Composition

To calculate the nutritional indices of the fatty acid profile, the Atherogenic Index (AI) was determined according to the formula presented below (Dal Bosco et al., 2024; Ulbricht & Southgate, 1991). This index is widely used worldwide for the evaluation of food products, including meat, seeds, fish, dairy products, and related foods.

$$AI = [C12:0 + (4 \times C14:0) + C16:0]/(UFA)$$

UFA – unsaturated fatty acids.

The Thrombogenicity Index (TI), proposed by Ulbricht and Southgate (1991), is used to evaluate the potential of a food to contribute to blood clot formation and associated cardiovascular risk.

$$TI = (C14:0 + C16:0 + C18:0)/[(0.5 \times MUFA) + (0.5 \times n\text{-}6)\ n\text{-}3) + (n\text{-}3/n\text{-}6)]$$

MUFA – monounsaturated fatty acids.

The Healthy Fatty Index (HFI) is used to analyze which types of fats are predominant in food products. Beneficial fats are assigned higher values, while a score below 1 is associated with saturated fatty acids (SFA), which indicate an increased risk of cardiovascular diseases.

$$HFI = (MUFA \times 2) + (n-6 \times 4) + (n-3 \times 8) + \frac{n-3}{n-6} / (SFA \times 1) + (MUFA \times 0.5) + (n-6 \times 0.25) + (n-3 \times 0.125) + \frac{n-6}{n-3}$$

Microbiological analyses

Materials

Selective and non-selective culture media used in this study included Plate Count Agar, chromogenic coliform agar, chromogenic *Staphylococcus aureus* medium, *Salmonella–Shigella* Agar, chromogenic *Listeria* medium, Bile Esculin Azide agar, chromogenic ESBL agar and Columbia agar. All media were obtained from BioMaxima (Poland).

Total Aerobic Plate Count (TAPC) was determined using the standard plate count method (Datta et al., 2012), briefly, 10 g of sample were aseptically weighed and homogenised in 90 mL sterile saline solution to obtain the initial 10^{-1} dilution. Subsequent decimal serial dilutions were prepared up to 10^{-6}. For enumeration, 1.0 mL aliquots from appropriate dilutions were inoculated into sterile Petri dishes, followed by the addition of molten Plate Count Agar using the pour plate technique (Jay, 2002). After solidification, plates were incubated aerobically at 37°C for 48 h. Plates presenting countable colonies were selected, and results were expressed as colony forming units per gram (CFU/g). In addition, the presence of specific foodborne and indicator bacteria was investigated using selective culture media. Serial decimal dilutions of homogenised samples were inoculated onto chromogenic coliform agar for detection of *Escherichia coli*, chromogenic *Staphylococcus aureus* medium

for presumptive *Staphylococcus aureus*, *Salmonella-Shigella* Agar (SSA) for *Salmonella* spp. and *Shigella* spp., selective chromogenic *Listeria* medium for *Listeria monocytogenes,* Bile Esculin Azide agar for *Enterococcus* spp., and chromogenic ESBL agar for screening of ESBL producing *Enterobacteriaceae*. In parallel, dilutions were also inoculated onto Columbia agar, used as a non-selective nutritive medium to allow recovery of stressed or injured bacterial cells and to screen for overall microbial growth. Plates were incubated aerobically at 37 °C for 24–48 h, and results were recorded as the presence or absence of characteristic growth. Microbiological determinations were performed at days 1, 3, 7, and 14 during refrigerated storage of the product.

Statistical analyses

All data were statistically analysed using the GraphPad Prism version 8.0.1 (GraphPad Software, San Diego, California, USA), the results are presented as mean values ± standard deviation (mean ± SD).

RESULTS AND DISCUSSIONS

Physico-chemical composition

The physico-chemical composition of bone marrow varies depending on the species (Adeyeye, 2014; West & Shaw, 1975), the type of bone from which it is extracted (Abd-El-Aal & Mohamed, 1989), the maintenance system (Steiner-Bogdaszewska et al., 2022), the age (Field et al., 1974), and the physiological state of the animal (Murden et al., 2017). Following physico-chemical analyses of the two varieties of beef bone marrow paste, we obtained, as expected, a higher dry matter (DM) content in the plain beef bone marrow paste and a higher water content in the soy-enriched formulation (Figure 1). The moisture content of beef bone marrow is not constant and may vary depending on the anatomical origin of the bone (Calhoun, Schnell & Mandigo 1998). Literature reports that flat bones (e.g., pelvis, ribs, vertebrae) contain a higher proportion of red marrow and thus exhibit higher moisture levels, whereas long bones (e.g., femur, tibia, humerus) predominantly contain fatty marrow characterized by lower water content. The water content of raw beef bone marrow is approximately 11.51 ± 0.31 % as reported for unprocessed material (Fuerniss et al., 2024) In contrast, in the present study the sample consisted of roasted beef bone marrow that was subsequently blended, sieved, and filtered, leading to a reduction in moisture content due to the removal of liquid fractions. Lemon juice was then incorporated during formulation. The resulting final processed product of the plain beef bone marrow paste had a mean moisture content of 12.83%.

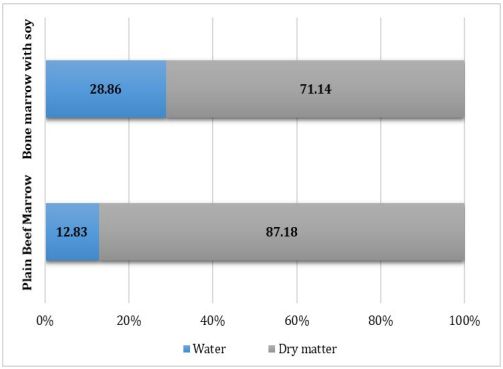


Figure 1. Water and dry matter content in plain and soy-enriched beef marrow pastes

In Figure 2, the chemical analyses of the two preparations are shown. Beef marrow is naturally highly lipid-rich, with the plain beef marrow paste exhibiting an average lipid content of 83.13 ± 1.78% (range 81.23-86.21%) on a wet basis. When expressed on a dry matter basis, this corresponds to approximately 95.4% (moisture 12.83%), confirming the strongly lipid-dominant nature of the product. Lipid content may vary depending on species, gender, and age of the animals. These findings are consistent with literature data reported by Adeyeye (2014), who described similarly high lipid levels in bovine marrow fat, with values of 98.7% in rams and 98.2% in bulls on a dry matter basis, highlighting natural biological variability between animal sources.

In the beef marrow paste formulated with soy, the average lipid content was lower, measuring 61.81 ± 4.49%. This was due to the addition of soy protein, with a minimum value of 54.30% and a maximum of 69.17%. In terms of protein content, it is generally quite low, given that marrow is primarily composed of lipids. For the plain beef marrow paste, the average protein content was 0.96 ± 0.05%, while in the

formulation containing soy, the average protein content increased to 6.06 ± 1.07%.

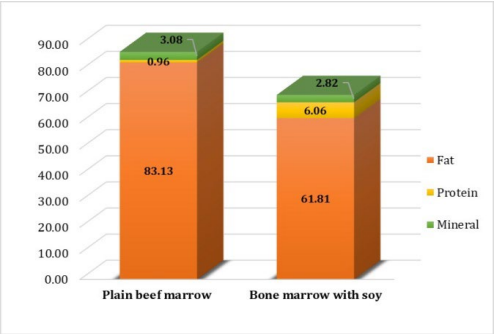


Figure 2. Chemical composition of plain and soy-enriched beef marrow pastes

The mineral content showed only small differences between the two beef marrow pastes, with values ranging from 2.36% to 3.87%. The plain beef marrow paste had an average mineral content of 3.08 ± 0.41%, whereas the beef marrow paste with soy showed a slightly lower average value of 2.82 ± 0.30%. The average salt content of the plain beef bone marrow paste was 2.26 ± 0.33 %, while the soy-enriched formulation exhibited a lower mean value of 1.62 ± 0.09%.

Fatty Acid Composition

Following the analysis of the two varieties of beef bone marrow paste (plain and soy-enriched), several differences can be observed regarding their nutritional value as well as the oxidative stability of the products (Table 2).

In both samples, oleic acid (C18:1 n-9) was identified as the predominant fatty acid. However, its concentration was considerably higher in the soy-enriched bone marrow paste (47.06%) compared with the plain beef bone marrow paste (31.47%). Scientific literature indicates that bovine bone marrow is naturally rich in oleic acid, with reported values ranging between 34.8% and 46.14% of the total fatty acid content, depending on factors such as the age of the animal, diet or bone type (Adeyeye, 2014; Küçük et al., 2018).

Oleic acid has been associated with reduced LDL cholesterol oxidation and moderate cardioprotective effects, being important from both nutritional (Xu, Xu, & Sohouli, 2026) and technological perspectives (Olmo-Cunillera et al., 2023).

Table 2. Fatty acid composition of beef bone marrow paste

Fatty acid	Soy-enriched	Simple
C8:0	-	-
C10:0	-	-
C11:0	-	-
C12:0	-	-
C13:0	-	-
C14:0	1.867	1.2799
C14:1 (n9)	-	-
C15:0	0.5533	0.4786
C15:1	-	-
C16:0	17.0526	19.1677
C16:1 (n7)	2.156	0.8487
C17:0	1.0707	2.4879
C17:1	0.1973	0.2353
C18:0	12.2186	7.3422
C18:1 (c+t) (n9)	47.062	31.4737
C18:2 (c+t) (n6)	13.0156	29.7971
C18:3 (n6)	0.3793	2.9385
C18:3 (n3)	1.3793	3.2013
C20:0	0.2781	-
C20:1 (n9)	0.5673	-
C20:2(n-6)	-	0.2156
C21:0	-	-
C20:4 (n6)	0.6726	0.5335
C20:3 (n3)	0.46253	-
C20:5 (n3)	0.40256	-
C22:0	0.66564	-
C22:1 (n9)	-	-
C22:2 (n6)	-	-
C23:0	-	-
C24:0	-	-
C22:6 (n3)+C24:1 (n9)	-	-
SFA	33.7	30.8
MUFA	50.0	32.6
PUFA	16.3	36.7
omega 6	14.1	33.5
omega 3	2.2	3.2
omega 9	47.6	31.5

SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA –polyunsaturated fatty acids.

Palmitic acid (C16:0), the principal SFA in animal lipids, was identified at levels of 17.05% in the soy-enriched bone marrow paste and 19.17% in the plain beef bone marrow paste. These values are lower than those commonly reported for bovine adipose tissue and are closer to the values described for human and bovine bone marrow, where palmitic acid accounts for approximately 23-31% of the total fatty acids (Adeyeye, 2014; Griffith et al., 2009). Lower levels of Palmitic acid (C16:0) are considered favorable.

Linoleic acid (C18:2 n-6) showed considerably higher levels in the plain bone marrow paste

(29.80%) compared with the soy-enriched bone marrow paste (13.02%). The elevated omega-6 content largely accounts for the increased PUFA value and the higher HFI score of the plain bone marrow sample. Thermal processing may additionally contribute to modifications in fatty acid composition, as PUFA are particularly susceptible to oxidative reactions and thermal degradation (Fogerty et al.,1990).

Alpha-linolenic acid (C18:3 n-3) was also higher in plain bone marrow sample (3.20%) compared with soy-enriched bone marrow paste (1.38%). Although the levels are moderate, the presence of omega-3 fatty acids is important due to their anti-inflammatory and cardioprotective effects. It should be noted that C18:3n-3 (α-linolenic acid ALA) was the only omega-3 fatty acid detected in the plain formulation, with no longer-chain omega-3 fatty acids identified. Furthermore, C18:3 n-6 (γ-linolenic acid, GLA) was included in the total omega-6 fraction in both formulations, and its contribution was explicitly accounted for in the omega-6 calculation (0.3793% in the soy-enriched sample and 2.9385% in the plain sample). The omega-6/omega-3 ratio was 6.41 in the soy-enriched sample and 10.47 in the plain beef bone marrow paste. From a nutritional perspective, lower ratios are considered more favourable, as an excess of omega-6 fatty acids may promote the synthesis of pro-inflammatory mediators. Dietary recommendations generally suggest ratios below 4:1. Thus, although the plain bone marrow paste exhibited a higher HFI, the soy-enriched sample showed a more balanced fatty acid profile in terms of inflammatory potential. This imbalance may also be attributed to the applied thermal processing.

The MUFA/SFA ratio was significantly higher in the soy-enriched beef bone marrow sample (1.48) compared with the plain bone marrow paste (1.06), confirming the predominance of MUFA in the soy-containing product. Higher MUFA/SFA ratios are associated with improved oxidative stability and beneficial metabolic effects (Mensink & WHO, 2016).

The PUFA/SFA ratio was higher in the plain bone marrow paste (1.19) compared with 0.48 in the soy-enriched paste. According to nutritional guidelines, PUFA/SFA ratios above 0.45 are considered beneficial for cardiovascular health. Thus, both products may be regarded as nutritionally adequate; however, the plain bone marrow paste exhibited a greater enrichment in PUFA (WHO, 2003).

Nutritional Lipid Quality Indices

The calculated lipid quality indices indicated a relatively low atherogenic potential for both formulations. Both samples were characterized by a reduced content of atherogenic fatty acids and a higher proportion of unsaturated fatty acids. The plain beef marrow paste exhibited an AI value of 0.35, while the beef marrow paste with soy showed a slightly higher AI value of 0.37. It should be noted that the contribution of C12:0 was zero in both samples, as this fatty acid was not detected, and therefore did not influence the calculated index. Regarding the Thrombogenicity Index (TI), it is lower in plain beef bone marrow paste (0.65 vs. 0.80) compared to the beef bone marrow with soy variant, indicating a reduced tendency for thrombus formation and a more cardioprotective lipid profile. Their values are below 1, which suggests that beef marrow paste has a lower thrombogenic potential than most processed meat products, much lower than high-fat dairy products, and similar to the values reported for certain marine foods considered cardioprotective (Rashidimehr et al., 2025).

The Healthy Fatty Index (HFI) exhibited a higher value in the plain beef bone marrow paste sample (3.39), indicating a superior lipid profile. The beef bone marrow paste supplemented with soy showed a slightly lower HFI value (2.53); however, it demonstrated improved oxidative stability and a predominantly MUFA profile.

Overall, the results indicate that the plain beef bone marrow paste exhibits a more PUFA-rich lipid profile and higher nutritional index values, whereas the soy-enriched formulation provides enhanced oxidative stability and a more balanced fatty acid composition, characterized by the predominance of oleic acid and other monounsaturated fatty acids.

Freshness and Storage Stability

To determine the freshness level and storage period under refrigeration conditions, acidity, Kreiss, and Nessler reactions were assessed. As shown in Table 3, due to the high fat content of the marrow, the plain marrow paste exhibited weakly positive changes in both Kreiss and Nessler reactions starting from day 3, while the

acidity was considerably higher compared to pork fat or beef tallow.

The peroxide value of the plain beef bone marrow paste showed an initial increase from day 3, exceeding 10 meq O_2/kg by day 7, in agreement with the other freshness indicators. In contrast, the soy-enriched paste remained relatively stable until day 7, with peroxide values exceeding 10 meq O_2/kg only from day 14 onwards.

Table 3. Assessment of freshness changes in beef bone marrow paste (plain vs. soy-enriched) during storage

Day	Peroxide value meq O_2/kg		Acidity g oleic acid %		Kreiss		Nessler	
	A	B	A	B	A	B	A	B
1	5.28 ± 0.05	4.74 ±0.04	1.53	1.12	-	-	-	-
3	7.60 ±0.05	5.45 ±0.07	1.86	1.25	±	-	±	-
7	> 10	7.75 ± 0.05	2.56	1.47	+	±	+	±
14		> 10	2.78	1.64	++	+	++	+

A – Plain beef marrow; B – Soy-enriched beef bone marrow paste; (–) negative (fresh); (±) weakly positive (incipient deterioration); (+) positive (spoiled); (++) strongly positive (advanced spoilage stage).

This increased susceptibility can be attributed to the high lipid content, as foods rich in fats, particularly unsaturated fatty acids, are prone to oxidation, which leads to the formation of free fatty acids and other degradation products that contribute to rancidity and reduced shelf life (Geng, Liu & Zhang, 2023). Throughout the storage period, Total Aerobic Plate Count remained below the detection limit, and no detectable growth of specific foodborne or indicator bacteria was observed on any of the selective culture media used. *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* spp., *Shigella* spp., *Listeria monocytogenes*, *Enterococcus* spp., and ESBL-producing *Enterobacteriaceae* were not detected at any sampling point. Similarly, no microbial growth was observed on the non-selective Columbia agar, indicating the absence of recoverable viable bacteria, including potentially stressed or injured cells.

CONCLUSIONS

This study demonstrates that beef bone marrow-based pastes, in both plain and soy-enriched formulations, possess distinct and tunable lipid profiles with potential relevance for the development of functional lipid-based foods. The plain formulation was characterised by a higher proportion of PUFA and more favourable nutritional lipid indices, whereas soy incorporation improved the MUFA's fraction and enhanced oxidative stability, likely associated with increased oleic acid content. From a valorisation perspective, beef bone marrow represents a high value animal by-product with strong potential for integration into circular food systems, contributing to resource efficiency through the transformation of a lipid-rich fraction into a functional food ingredient.

Compared with conventional animal fat sources, bone marrow exhibited a favourable fatty acid composition enriched in unsaturated lipids, supporting its nutritional relevance and potential application in reformulated fat-based products.

The study was conducted using a single production batch per formulation and a single thermal processing condition, which provides an initial controlled assessment of lipid composition. As such, the observed variability primarily reflects within-batch and analytical variation, while also indicating the need for future studies including multiple batches and a broader range of processing conditions further to strengthen the robustness and generalisability of the findings. In addition, sensory acceptability was not evaluated, representing an important direction for future work to better contextualise consumer relevance and application potential.

Furthermore, the omega-6/omega-3 ratio observed in the best-performing formulation (6.41) remains above the commonly recommended threshold of 4:1; however, this still reflects an improvement in fatty acid balance compared with conventional animal fat sources, highlighting the potential of formulation optimisation strategies to enhance the nutritional profile in future developments further.

Future research, including multiple independent production batches and optimised thermal treatments particularly milder and more controlled processing conditions may improve the robustness of the findings and allow a more comprehensive assessment of lipid stability and process-dependent changes, as well as sensory performance and consumer acceptability.

Overall, the findings highlight beef bone marrow as a promising sustainable raw material for the development of nutritionally enhanced lipid based products, with formulation dependent modulation of both nutritional quality and functional properties.

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SUSTAINABLE UTILIZATION OF ROSE PROCESSING BY-PRODUCT IN THE PRODUCTION OF WHITE BRINED CHEESE

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Abstract

This study examines the incorporation of distilled Rosa damascena extract into traditional Bulgarian white brined cheese as a potential natural preservative. Cheese was produced from cow's milk and ripened in brine containing three concentrations of rose extract (0, 33.3, and 66.6 mg/kg) to assess its influence on physicochemical stability, microbial safety, and sensory quality. Standard analytical methods - including pH and titratable acidity measurement, salt and moisture analysis, Kjeldahl nitrogen fractionation, microbiological enumeration, and instrumental color and texture profiling - were applied before and after a 30-day ripening period and throughout refrigerated storage at the 1st, 3rd, and 6th month. The rose extract demonstrated antimicrobial and antioxidant activity, supporting controlled microbial growth and inducing subtle structural changes likely related to interactions with casein. Sensory evaluation indicated acceptable consumer perception across all samples. Overall, the findings show that integrating distilled rose extract into the brining process can enhance cheese quality while promoting sustainable practices through the valorization of aromatic plant by-products.

Key words*: antioxidant, rose, waste, White Brined cheese.*

INTRODUCTION

The increasing demand for sustainable food production has intensified interest in the valorization of agro-industrial by-products (Silva et al., 2025). The rose processing industry, particularly in regions known for essential oil production, generates substantial quantities of by-products rich in bioactive compounds, including polyphenols, flavonoids, and natural antioxidants (Kolev et al., 2025; Khezerlou et al., 2025; Mileva et al., 2021). Despite their potential functional value, these by-products are often underutilized or discarded, posing environmental and economic challenges (McCarthy et al., 2019).

White brined cheese is one of the most widely consumed varieties of cheese worldwide, valued for its mild flavor, high moisture content, and unique textural properties (Hayaloglu, 2017). Incorporating natural functional ingredients derived from plant by-products offers a promising strategy to enhance the nutritional profile, oxidative stability, and shelf life of such cheeses (Ritota & Pamela Manzi, 2020). The sustainable utilization of rose processing by-products in white brined cheese production aligns with circular economy principles by reducing waste while adding value to traditional dairy products.

The aim of the present study was valorisation of rose distillation by-products for improving the quality of traditional White Brined cheese.

MATERIALS AND METHODS

Cow milk – with the following composition: fat content 3.38 ± 0.10%; protein 3.26 ± 0.10%; pH 6.56 ± 0.10; solid-non-fat 8.64 ± 0.20%; Titratable acidity 17 ± 2°T, density 1.029 ± 0.025 g/mL; freezing point -0.568 ± 0.020°C; no added water. Starter culture - a combination of DVS cultures was used: White classic 1-01 (with composition *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Streptococcus thermophilus*) and LB 144-12 (with composition *Streptococcus thermophilus*,

Lactobacillus delbrueckii subsp. *bulgaricus*) from the company Novonesis Group.

Distilled rose flower extract - the extract was analyzed by Kolev et al. (2025) and the used quantity was according to a previous experiment (Vlahova-Vangelova et al., 2025).

Calcium dichloride - with a concentration of 33-35%, purchased from Biokom Trendafilov EOOD, Bulgaria.

Rennet enzyme – chymosin CHY-MAX® Supreme from Novozymes A/S, part of Novonesis Group in amount 35 mL/100 L milk.

Sensory analysis - was done by 7 trained specialists taste and aroma (10 points), consistency at 18-20°C (10 points), cut surface, structure and color (10 points), brine (10 points), appearance of the pieces (10 points) and packaging and labelling (10 points) adapted from BDS 15612-83.

White Brined Cheese production process - A whole raw cow's milk was received by a local farmer and was checked to ensure it meets quality requirements. The milk must arrive between +4°C and <10°C. After acceptance, the milk was submitted to standardization, adjusting the casein-to-fat ratio to approximately 0.70 ± 0.20. The standardized milk was pasteurized at 72-74°C for 10 min, followed by cooling to 36 ± 2°C. At this stage, a direct-vat-set (DVS) starter culture was added, consisting of White Classic and LB 144-12, dosed at 1.5 g and 20 g per 100 L of milk, respectively. Then, calcium chloride (37%), previously diluted 1:10 in potable water, was added at a concentration of 50 mL per 100 L. Immediately afterward, chymosin (rennet enzyme) also diluted 1:10 was introduced at 35 mL per 500 L of milk. The milk was left to coagulate for 60 min at 34 ± 2°C. Once a firm curd has formed, it was cut vertically and horizontally into 2 cm cubes. The curd was allowed to rest for 15 min, followed by gentle turning to promote whey expulsion. This operation was repeated 5 times until reached the desired curd density. The curd was formed and a whey was separated. Then it was self-pressed and additionally drained over night at 20 ± 2°C. The drained curd was cut into blocks (approximately 123 × 123 mm) and placed into containers when active acidity reaches 5.2 ± 0.2 (or 170°T). Then it was submitted to pre-ripening process and dry salting with amount of salt 30 g/kg cheese at 20°C for 24 h. Cheese acidity reached pH 5.0 or 180-200°T. Next, the cheese was immersed in brine composed of water, salt, and lactic acid. The brine had a salt concentration of 5-6% and acidity of pH (4.0-4.5) reached by addition of lactic acid. Three different batches of white brined cheese were produced - with 0 mg/kg rose petals extract in brine (0WBC - control sample), with 33 mg/kg rose petals extract in brine (33WBC) and with 66 mg/kg rose petals extract in brine (66WBC). The cheese was submitted to ripening at 12°C for 30 days in brine. Finally, the matured cheese was packaged under vacuum, and stored at 2-4°C, for up to 6 months. The obtained cheese is presented on Figure 1.



Figure 1. White Brined Cheese matured in brine with rose petals extract (Source: author's own photograph)

Physicochemical analysis - fat content was measured using the Gerber volumetric technique (BDS ISO 19662:2019); dry matter - assessed with a KERN DBS moisture analyser; salt content - determined by the Mohr titration method following BDS 8274-82; ash content - evaluated according to BDS 6154:1974; active and titratable acidity - active acidity was quantified potentiometrically using a 7110 WTW (Germany) pH meter, while titratable acidity was established following the procedure described in BDS 1111-80.

Texture analysis - The texture parameters: hardness, cohesiveness, elasticity, gumminess, chewiness were obtained by analysis of cheese slices (15 mm thick and 60 mm in diameter) using a TX-700 analyzer equipped with a 25 kg strain gauge (Lamy Rheology, Champagne au Mont d'Or, France). A double compression method with a ½ spherical probe (30 mm in diameter) to compress 70% of the initial height at a compression rate of 1 mm/s and a 5-s delay between compressions was applied. The data

were processed using Rheotex software version 2.55 (Lamy Rheology, Champagne au Mont d'Or, France). The results from the measurements were calculated as described by Estevez et al. (2005).

Color - using the chroma meter cr-410, to evaluate the color of the surface and the cut surface;

Protein content and maturity level - Total nitrogen (TN), protein, non-casein nitrogen (NCN), non-protein nitrogen (NPN), and water-soluble nitrogen (WSN) were quantified following BDS EN ISO 8968-1:2014. Biogenic amines content was determined according to the following procedure: aqueous standard solutions of cadaverine, putrescine, tyramine, and histamine (Sigma, St. Louis, MO) were derivatized using the same procedure as for cheese samples. Biogenic amines were quantified according to Eerola et al. (1993) by HPLC-DAD using an ELITE LaChrom system (Hitachi) with a Purospher STAR C18 column (4.6 × 150 mm, 5 µm). Dansylated amines were detected at 254 nm, and quantification was performed using external standards.

Total phenolic content - Determined using a modified Folin-Ciocalteu assay (Pimentel-González et al., 2014). One milliliter of sample was combined with 5 ml diluted Folin-Ciocalteu reagent (1:10). After 6 min, 4 ml of 20% Na_2CO_3 was added, followed by 2 h incubation at room temperature (25 ± 0.5°C). Absorbance at 760 nm was read using a UV-Vis spectrophotometer. Results are expressed as mg gallic acid equivalents per gram of cheese.

Antioxidant activity - DPPH assay (Amirdivani et al., 2011): 250 µL cheese extract was vigorously mixed with 3 ml DPPH and stored in the dark at room temperature (25 ± 0.5°C) for 30 min. A control was prepared similarly, substituting the extract with 250 µL distilled water. Absorbance was measured at 517 nm, and activity expressed as mmol TE/100 ml. FRAP assay (Benzie and Strain, 1996): Supernatant was mixed with 50 ml acetate buffer, 5 ml TPTZ solution, and 5 ml $FeCl_3$, then incubated at 37 °C for 15 min. Absorbance of the coloured complex was measured at 593 nm. Results were expressed as mM TE/ml.

Microbiological analyses - The tested cheese samples are prepared according to ISO 6887-5:2020. Cheese samples were analyzed for the viable counts of different microbial groups: lactococci and lactobacilli were enumerated according to ISO 15214:1998, *Enterobacteriaceae* according to ISO 21528-2:2017, yeasts and molds were enumerated according to ISO 21527-1:2008, coagulase-positive *Staphylococcus aureus* according to ISO 6888-1:2021, and *E. coli* according to ISO 16649-2:2001/Amd 1:2017, while *Listeria monocytogenes* was detected according to ISO 11290-1:2017, and *Salmonella* spp. were detected by presence/ absence testing following ISO 6579-1:2017.

Statistical analysis - Data are presented as mean ± standard deviation (n = 4). Each experiment included four replicates, comprising two independent technological replicates and two analytical measurements per replicate. Statistical evaluation was performed using one-way analysis of variance (ANOVA). Differences between sample means were assessed using the least significant difference (LSD) post hoc test. Statistical significance was considered at $p < 0.05$.

RESULTS AND DISCUSSIONS

The results obtained from the physicochemical analysis are presented in Table 1.

Table 1. Physicochemical analysis of White Brined Cheese matured in brine with rose petals extract

Indicator	Sample	Ripening period		Storage period		
		1 day	60 days	1 month	3 months	6 months
Titratable acidity, °T	0WBC	224±2	223±2	264±2	238±2	218±1
	33WBC		235±2	240±	172±1	206±1
	66WBC		220±2	258±2	184±2	212±1
pH	0WBC	4.84±0.02	4.74± 0.05	4.80± 0.12	4.54± 0.40	4.57±0.12
	33WBC		4.70± 0.04	4.55± 0.12	4.72± 0.25	4.61±0.13
	66WBC		4.76± 0.05	4.60± 0.14	4.58± 0.14	4.64±0.22
Fat, %	0WBC	17.75±0.12	17.25± 0.75	19.50± 0.15	21.50± 1.22	19.50± 1.56
	33WBC		17.50± 0.69	18.50± 1.12	20.50± 2.00	22.00± 2.02
	66WBC		17.50± 0.45	19.00± 1.25	19.00± 1.48	19.75± 2.45
Dry matter, %	0WBC	46.62±2.22	42.49± 3.10	45.47± 2.02	49.30± 2.13	45.50± 2.12
	33WBC		45.96± 3.11	41.90± 2.55	50.15± 2.75	50.37± 2.08
	66WBC		47.36± 2.42	44.07± 2.46	48.07± 2.69	46.60± 2.33
Salt, %	0WBC	3.28±0.04	4.74± 0.05	3.51± 0.04	3.16± 0.10	3.51±0.05
	33WBC		4.39± 0.04	3.28± 0.06	3.63± 0.12	3.28±0.06
	66WBC		3.92± 0.05	3.29± 0.07	3.28± 0.07	3.16±0.08
Ash, %	0WBC	4.45±0.06	4.41± 0.05	3.87± 0.20	3.82± 0.07	3.86±0.06
	33WBC		4.73± 0.06	3.78± 0.11	3.93± 0.13	3.74±0.09
	66WBC		4.08± 0.07	3.93± 0.12	3.47± 0.14	3.88±0.14
Protein, %	0WBC	17.47±2.10	17.71± 1.23	18.79± 2.12	20.5± 1.12	18.46± 1.23
	33WBC		18.12± 2.22	18.98± 1.15	19.38± 1.05	19.05± 1.05
	66WBC		17.77± 1.05	18.97± 1.55	18.78± 2.00	20.11± 1.45

The physicochemical changes observed in Table 1 are typical for the ripening of white brined cheese and correlate with results reported in similar studies (Öner et al., 2006). The evolution of pH and titratable acidity follows normal trends associated with lactic fermentation and proteolysis, comparable to those described for Feta-type and white brined cheeses (Hayaloglu, 2017). Cheeses containing rose extract showed slightly higher acidity. This is in agreement which reports on cheeses enriched with plant extracts, where phenolic compounds were shown to influence acidification dynamics (Ritota & Manzi, 2020).
The increase in fat, protein, and dry matter content during ripening and storage is due to moisture loss, in line with findings reported by Pimentel-González et al. (2014) and other studies on brined cheeses. Similar data were found in cheeses supplemented with different herbal or antioxidant extracts, without negative influence on technological quality. Salt and ash contents remained within the typical ranges reported for white brined cheeses. This indicated that rose extract addition did not affect salt diffusion or mineral balance.
Table 2 represents the degree of ripeness and the presence of biogenic amine in the end of the storage period.

Table 2. Degree of ripeness and presence of biogenic amines in White Brined Cheese matured in brine with rose petals extract

Indicator	Sample	Ripening period		Storage period		
		1 day	60 days	1 month	3 months	6 months
NCN/TN, %	0WBC	5.07± 0.12	6.57± 0.12	5.22± 0.17	6.38± 0.14	6.18± 0.15
	33WBC		5.33± 0.14	4.08± 0.15	5.10± 0.14	5.32± 0.14
	66WBC		6.06± 0.15	4.46± 0.14	5.86± 0.15	6.15± 0.16
NPN/TN, %	0WBC	4.06± 0.14	4.82± 0.02	4.33± 0.15	4.94± 0.15	5.44± 0.17
	33WBC		3.83± 0.25	3.11± 0.16	3.87± 0.20	4.24± 0.21
	66WBC		4.53± 0.33	2.90± 0.15	4.46± 0.16	5.12± 0.09
WSN/TN, %	0WBC	4.24± 0.15	4.25± 0.25	3.33± 0.14	3.17± 0.16	3.39± 0.15
	33WBC		3.87± 0.26	2.15± 0.14	3.29± 0.19	3.22± 0.15
	66WBC		4.24± 0.26	2.22± 0.09	3.26± 0.14	3.68± 0.14
Putrescine, mg.kg^{-1}	0WBC	-				24.0±2.4
	33WBC	-				18.5±1.9
	66WBC	-				22.0±2.2
Cadaverine, mg.kg^{-1}	0WBC	-				19.0±1.9
	33WBC	-				14.5±1.5
	66WBC	-				17.0±1.7
Histamine, mg.kg^{-1}	0WBC	-				15.0±1.5
	33WBC	-				11.5±1.2
	66WBC	-				14.0±1.4
Tyramine, mg.kg^{-1}	0WBC	-				85.0±8.5
	33WBC	-				64.0±6.4
	66WBC	-				78.0±7.8

Values below 10 mg.kg^{-1} are below the limit of quantification (LOQ), i.e. they are outside the accuracy of the standard line.

The obtained results (NCN/TN, NPN/TN, and WSN/TN) show the extent of proteolysis occurring during ripening and storage of white brined cheese. The control sample (0WBC) presented showed progressive protein breakdown and accumulation of soluble nitrogen compounds demonstrated by the increase in NCN/TN and NPN/TN values. This indicates a slight evolution in cheese maturation. However, the results remain higher compared to those of other authors (Hayaloglu et al., 2002).
The relatively stable WSN/TN values observed during ripening may be attributed to the limited proteolysis typical for brined cheeses, where low pH, high salt concentration, and refrigerated storage conditions restrict enzymatic activity. Additionally, phenolic compounds from the rose extract may interact with casein or inhibit proteolytic enzymes, reducing the formation of water-soluble nitrogen fractions. This may be associated with interactions between phenolic compounds from the rose extract and casein fractions or microbial enzymes, as reported for cheeses supplemented with plant-derived bioactive compounds (Salik and Çakmakçi, 2024).
Biogenic amine accumulation in the studied white brined cheese after six months of storage remained low in all samples, with cheeses supplemented with rose petal extract generally showing lower concentrations compared to the control. The detected histamine levels (11.5-15.0 mg·kg^{-1}) were considerably below the commonly accepted safety threshold of 50-100 mg·kg^{-1} for fermented foods and below levels considered potentially hazardous by the EFSA Panel on Biological Hazards (2011), while regulatory limits established by the European Commission apply mainly to fish products. These values are lower than those reported for traditional and long-ripened cheeses, where concentrations may exceed 50 mg·kg^{-1} (Novella-Rodríguez et al., 2002; Natrella et al., 2024). Tyramine was the predominant amine (64.0-85.0 mg·kg^{-1}), which is typical for lactic acid fermentation, and the observed concentrations remained below those reported for long-ripened hard cheeses (>400 mg·kg^{-1}) (Spano et al., 2010; Ruiz-Capillas and Herrero, 2019) and under the safety threshold for healthy individuals (Linares et al., 2012). Putrescine (18.5-24.0 mg·kg^{-1}) and cadaverine

(14.5-19.0 mg·kg⁻¹) were also present at relatively low levels consistent with properly manufactured white brined cheeses (Andiç et al., 2010; Buňková, 2025), indicating limited proteolytic degradation and good hygienic practices (Alvarez & Moreno-Arribas, 2014; Doeun et al., 2017). The total biogenic amine content (108.5-143.0 mg·kg⁻¹) remained markedly below levels associated with defective fermentation (>500-1000 mg·kg⁻¹) (Ruiz-Capillas & Herrero, 2019), and the reduced accumulation observed in treated samples may be attributed to the antimicrobial activity of polyphenolic compounds present in plant extracts (Ritota & Manzi, 2020; Wang et al., 2021; Zhang et al., 2017).

The microbiological dynamics in the ripening and storage processes of the productc are presented in Figures 2 and 3.

From the results presented in Figure 2, it can be seen that in the initial stage of ripening of the White Brined Cheese, no significant difference was observed in the values of lactobacilli (8.2-8.3 log CFU/g) among all samples. These results are in accordance with the data presented by other studies, which show that rose petal extract does not have an inhibitory effect on the starter

culture in dairy products (Dimitrova et al., 2019). In the present study, at the end of ripening (60 days), an increase in the count of lactobacilli was observed in all samples (8.9 log CFU/g), which can be associated with the active phase of development of lactic acid microflora during ripening (Özer, 2014).

In the current investigation, all experimental variants showed a gradual decrease in the lactobacilli count during the storage period of 1, 3, and 6 months (Figure 2).

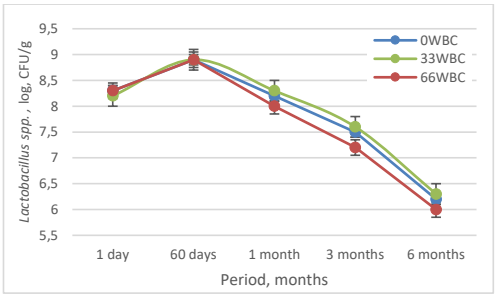


Figure 2. Dynamics of lactobacilli in White Brined Cheese matured in brine with rose petals extract

At the end of storage (6 months), the values reached 6.2 log CFU/g for the control sample (0WBC), 6.3 log CFU/g for 33WBC, and 6.0 log CFU/g for 66WBC, respectively.

The sample with a lower concentration of the extract (33WBC) followed the dynamics of the control sample (0WBC) without significant deviations. In the sample 66WBC, a less pronounced, but earlier occurring population reduction was observed during the storage period, which suggests a possible dose-dependent effect of the extract.

Figure 3 shows the dynamics of lactococci in studied samples (0WBC, 33WBC and 66WBC). According to the findings, there were no statistically significant changes between the control sample and the samples with added extract on the first day of ripening. The count of lactococci was relatively high and comparable among all samples ranging between 8.9-9.0 log CFU/g. This suggests that rose petal extract does not affect the initial microbial population in White Brined Cheeses.

After 60 days of ripening and the first month of storage, high levels of lactococci were also observed in all samples. The samples 33WBC and 66WBC were characterized by slightly lower values compared to the control sample (0WBC), but the differences were minimal and within the standard deviations.

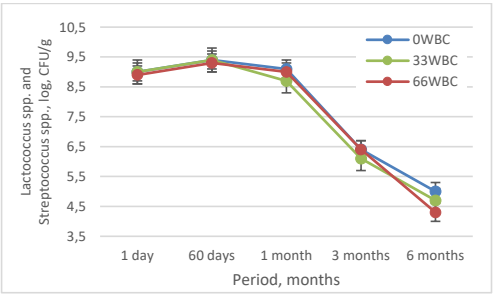


Figure 3. Dynamics of lactococci in White Brined Cheese matured in brine with rose petals extract

During prolonged storage (3 and 6 months), a significant decrease in the count of lactococci was observed in all samples. The samples matured in brine with rose petals extract (33WBC and 66WBC) showed a more pronounced decrease in lactococci count compared to the control (0WBC), with the values reaching about 4.3-4.7 log CFU/g at 6

months, while in the control they were slightly higher (about 5.0 log CFU/g). This is probably due to the antimicrobial effect of the rose petal extract during a longer storage period, which may be due to the presence of phenolic compounds and other bioactive substances in the plant extract. The results obtained indicate that the addition of rose petal extract in the studied concentrations does not significantly affect the development and survival of lactic acid microflora during ripening and storage of the White Brined Cheese.

The microbiological analysis of White Brined Cheese showed that all main sanitary-hygienic and pathogenic indicators remained within the acceptable limits or below the detection limits (Table 3). Regarding coliforms, *E. coli*, and *Staphylococcus aureus*, in all samples after 6 months of storage, the values remained below 10 CFU/g sample, indicating microbiological safety and a low level of secondary contamination. Similar low values have been reported in other studies of traditional White Brine cheeses (Makarijoski, 2023; Plessas et al., 2021).

Table 3. Microbiological analysis for basic sanitary and hygienic indicators of White Brined Cheese matured in brine with rose petals extract

Indicator	Sample	Ripening period		Storage period		
		1 day	60 days	1 month	3 months	6 months
Coliforms, log CFU/g	0WBC	-				Under 1
	33WBC	-				Under 1
	66WBC	-				Under 1
E. coli, log CFU/g	0WBC	-				Under 1
	33WBC	-				Under 1
	66WBC	-				Under 1
Salmonella subsp. presence/absence	0WBC	-				nd
	33WBC	-				nd
	66WBC	-				nd
L. monocytogenes, presence/absence	0WBC	-				nd
	33WBC	-				nd
	66WBC	-				nd
Staphylococcus aureus, log CFU/g	0WBC	-				Under 1
	33WBC	-				Under 1
	66WBC	-				Under 1
Molds/yeasts, log CFU/g	0WBC	-	3.5	-		2.4
	33WBC	-	4.5	-		2.4
	66WBC	-	4.0	-		2.3

"-" – not determined; nd – not detected

In the preset study, during ripening and storage periods in none of the examined variants of White Brined Cheeses was detected the presence of *Salmonella* subsp. and *Listeria monocytogenes* which is in accordance with the requirements of Commission Regulation (EC) No 2073/2005 (Commission Regulation, 2005).

A more significant difference was observed among the samples in the count of yeasts and molds. At 60 days of ripening, the control sample was characterized by yeast and mold values 3.5 log CFU/g, while the samples matured in brine with rose petals extract demonstrated significantly higher values (4.5 log CFU/g at 33WBC and 4.0 log CFU/g at 66WBC, respectively). However, at the end of the 6-month storage period, the count of yeasts and molds in the fortified samples decreased to levels similar to the control (2.4 log CFU/g for 33WBC and 2.3 log CFU/g for 66WBC vs. 2.4 log CFU/g for 0WBC). In a previous study on the antimicrobial activity of rose petal extract against some strains of molds and yeasts, it was found that the extract exhibits antifungal effects, making it suitable for application in food products (Kolev et al., 2025).

Table 4 represents texture analysis of White Brined Cheese matured in brine with rose petals extract

Table 4. Texture analysis of White Brined Cheese matured in brine with rose petals extract

Indicator	Sample	Ripening period		Storage period		
		1 day	60 days	1 month	3 months	6 months
Hardness, N	0WBC	11.87± 3.56	8.67± 1.65	20.35± 8.91	16.14± 2.43	11.65± 3.10
	33WBC		12.15± 2.19	14.64± 1.84	14.63± 2.29	15.31± 3.24
	66WBC		9.46± 1.89	10.23± 1.86	14.18± 4.25	10.81± 3.19
Cohesion	0WBC	0.26± 0.02	0.22± 0.02	0.23± 0.02	0.27± 0.02	0.33± 0.05
	33WBC		0.24± 0.04	0.28± 0.03	0.21± 0.03	0.26± 0.06
	66WBC		0.30± 0.07	0.34± 0.06	0.29± 0.12	0.26± 0.04
Elasticity	0WBC	0.35± 0.05	0.31± 0.06	0.35± 0.03	0.58± 0.08	3.57± 1.64
	33WBC		0.39± 0.10	0.55± 0.10	0.48± 0.06	1.81± 1.70
	66WBC		0.51± 0.11	0.63± 0.12	0.51± 0.10	3.44± 1.43
Gumminess, N	0WBC	3.05± 1.01	1.92± 0.44	4.76± 2.51	4.37± 0.60	3.75± 0.98
	33WBC		2.96± 0.72	4.17± 0.80	3.15± 0.72	3.86± 1.02
	66WBC		2.85± 0.66	3.48± 0.88	3.96± 1.12	2.71± 0.76
Chewiness, N	0WBC	1.10± 0.51	0.61± 0.26	1.64± 0.82	2.56± 0.66	11.45± 4.91
	33WBC		1.19± 0.55	2.33± 0.82	1.54± 0.51	8.24± 9.25
	66WBC		1.48± 0.61	2.27± 0.96	2.06± 0.74	8.86± 3.66

The obtained results demonstrated that the texture parameters of white brined cheese were influenced by ripening time and the addition of rose petals extract. Their represented a

combined influence of different chemical changes in cheeses. Some indices - hardness, gumminess, and chewiness increased during storage, indicating progressive structure strengthening, particularly after prolonged storage. Sample with rose extract showed comparable (66WBC) or moderated (33WBC) hardness and higher elasticity (66WBC) compared to the control. Fox and McSweeney (2017) suggested that protein matrix rearrangement and water loss led to increased firmness in brined cheeses. This was a possible explanation why the extract affected the structural stability and flexibility of the cheese matrix. It is in correlation with the results reported by (Yerlikaya et al., 2020). The demonstrated increase in moisture content (0WBC and 66WBC) decreased hardness, while an increase in fat content (33WBC) increased elasticity. Lower gumminess in extract-treated cheese with highest applied dose (66WBC) may result from reduced protein aggregation intensity. This is an indirect correlation with the values reported for hardness and cohesiveness of the sample (Alkhalaileh, 2024).

Table 5 represents the results for the determined antioxidant activity as well as the total phenolic compounds (TPC) values.

Table 5. Total phenolic content and antioxidant activity of White Brined Cheese matured in brine with rose petals extract

Indicator	Sample	Ripening period		Storage period		
		1 day	60 days	1 month	3 months	6 months
TPC (mg GAE/g)	0WBC	476.22 ±1.94	982.99 ±5.25	484.80 ±1.38	524.12± 2.71	542.28± 3.03
	33WBC	974.20 ±5.28	975.59 ±5.36	1004.66±5.72	1021.16 ±5.40	1026.70 ±5.91
	66WBC	975.42 ±5.24	985.42 ±5.68	966.91 ±5.59	1024.65 ±5.63	1021.37 ±5.62
DPPH, µmol TE/g	0WBC	8.94± 1.38	21.14 ±1.12	10.82± 0.93	6.54± 0.69	6.63±0. 70
	33WBC	117.33 ±1.97	103.05 ±1.72	126.69 ±1.17	118.97± 1.52	119.18± 1.88
	66WBC	120.26 ±1.99	115.87 ±1.56	125.13 ±2.07	124.93± 1.31	125.81± 1.93
FRAP, µmol TE/g	0WBC	13.06± 1.10	3.93 ±0.74	10.95± 0.68	13.41±0 .79	13.13± 0.71
	33WBC	8.92± 0.54	7.45 ±0.92	8.43± 0.62	9.22± 0.72	9.24± 0.51
	66WBC	4.66± 0.60	4.66± 1.07	7.80± 0.75	7.76± 0.82	6.73± 0.68

The statistical analysis revealed that both extract concentration and storage time influenced the total phenolic content of the cheese (Table 5). The effect of sample was high, while storage period also was important. Moreover, an interaction between sample and storage period was observed, indicating that changes in TPC during storage depended on the concentration of rose petal extract added to the brine.

Cheeses containing rose petal extract had considerably higher phenolic content compared with the control sample. The control cheese (0WBC) showed the lowest TPC value, while cheeses produced with 33 mg/kg and 66 mg/kg extract exhibited significantly higher values (Table 5). The analysis confirmed that both enriched samples differed from the control, whereas no difference was detected between the two extract concentrations.

This result suggests that the incorporation of rose petal extract effectively enriches the cheese with phenolic compounds, although increasing the concentration above 33 mg/kg does not result in a further significant increase.

The increase in TPC during the early stages of ripening can be attributed to several biochemical processes occurring within the cheese matrix. During cheese maturation, proteolysis and lipolysis modify the protein network and release phenolic compounds that may initially be bound to proteins or other macromolecules. In addition, diffusion of phenolic compounds from the brine into the cheese matrix continues during ripening, which can further increase the measurable phenolic content. These structural changes enhance the extractability and detectability of phenolic compounds in the cheese.

The antioxidant activity measured by the DPPH radical scavenging method showed a strong dependence on the extract concentration (Table 5). The analysis demonstrated a high effect of sample, indicating that the addition of rose petal extract markedly enhanced the radical scavenging capacity of the cheese. In contrast, the storage period did not influence DPPH activity, and the interaction between sample and storage was also not important.

These findings indicate that the antioxidant activity determined by the DPPH assay is mainly associated with the presence of phenolic compounds introduced through the rose petal extract rather than changes occurring during storage (Vlahova-Vangelova et al., 2025). The absence of significant storage effects suggests that the antioxidant compounds remain relatively stable within the cheese matrix during ripening and refrigerated storage.

Phenolic compounds are known to possess strong free radical scavenging properties due to their ability to donate hydrogen atoms or electrons to neutralize free radicals. Rose petals are particularly rich in flavonoids, phenolic acids, and anthocyanins, which are recognized for their antioxidant potential. Therefore, the higher DPPH activity observed in the extract-enriched cheeses can be directly attributed to the higher phenolic content provided by the rose petal extract (Kolev et al., 2025).

Similar trends were observed for FRAP values, which measure the reducing power of antio-xidant compounds. The FRAP assay reflects the ability of antioxidants to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}), indicating their electron-donating capacity. In general, samples enriched with rose petal extract exhibited higher FRAP values compared with the control cheese, confirming the enhanced antioxidant potential of the fortified cheeses (Table 5).

The relationship between FRAP values and phenolic content is well documented in food systems, as many phenolic compounds act as reducing agents (Vlahova-Vangelova et al., 2025). Therefore, the increased FRAP activity in cheeses containing rose petal extract is consistent with the higher levels of phenolic compounds observed in these samples. Furthermore, the relatively stable FRAP values during storage suggest that the antioxidant compounds remain stable in the cheese matrix throughout ripening and refrigerated storage.

Overall, the results demonstrate a strong relationship between total phenolic content and antioxidant activity measured by both DPPH and FRAP assays. Cheeses enriched with rose petal extract consistently exhibited higher phenolic content and stronger antioxidant activity than the control sample. This correlation indicates that phenolic compounds are the primary contributors to the antioxidant capacity of the enriched cheeses.

The stability of antioxidant activity during storage is an important technological advantage, as it suggests that functional dairy products enriched with plant extracts can maintain their bioactive properties throughout their shelf life. The cheese matrix may also provide protective effects against oxidation or degradation of phenolic compounds by limiting exposure to oxygen and light.

The results clearly demonstrate that the addition of rose petal extract to the brine significantly enhances the antioxidant properties of white brined cheese. The enriched cheeses showed higher phenolic content and improved antioxidant capacity compared with the control sample. Moreover, these bioactive compounds remained relatively stable during ripening and refrigerated storage.

Table 6. Color of the cut surface of White Brined Cheese matured in brine with rose petals extract

Indicator	Sample	Ripening period		Storage period		
		1 day	60 days	1 month	3 months	6 months
Color of the cut surface						
L*	0WBC	90.12± 0.19	90.16± 0.26	90.13± 0.14	90.86± 0.35	90.71± 0.12
	33WBC		91.16± 0.50	90.63± 0.22	90.68± 0.61	90.59± 0.16
	66WBC		90.84± 0.92	90.78± 0.25	91.61± 0.21	90.04± 0.31
a*	0WBC	-1.95± 0.11	-1.56± 0.07	-1.69± 0.15	-2.05± 0.09	-1.71± 0.07
	33WBC		-1.79± 0.10	-2.19± 0.66	-2.07± 0.16	-1.67± 0.11
	66WBC		-1.69± 0.12	-1.51± 0.14	-1.66± 0.03	-1.28± 0.08
b*	0WBC	18.09± 0.24	18.45± 0.10	18.04± 0.50	16.69± 0.63	16.70± 0.14
	33WBC		17.84± 0.24	18.60± 0.21	17.28± 0.83	16.66± 0.22
	66WBC		16.66± 0.52	16.04± 0.25	14.99± 0.28	16.89± 0.115
C*	0WBC	18.19± 0.25	18.52± 0.11	18.12± 0.51	16.82± 0.63	16.77± 0.20
	33WBC		18.03± 0.25	18.73± 0.22	17.41± 0.84	16.72± 0.24
	66WBC		16.92± 0.52	16.11± 0.25	15.08± 0.27	16.93± 0.14
h	0WBC	96.16± 0.30	94.83± 0.21	95.35± 0.42	97.00± 0.29	95.81± 0.20
	33WBC		95.68± 0.24	96.700.1 5	96.83± 0.36	95.76± 0.25
	66WBC		95.52± 0.26	95.540.2 7	96.32± 0.17	94.33± 0.19

The color parameters of the cut surface of white brined cheese matured in brine with rose petals extract showed high stability throughout ripening and storage. The addition of rose petals extract to the brining solution preserved the typical color characteristics of white brined cheese, maintaining high lightness and stable hue values throughout ripening and storage, while slightly reducing yellowness and color intensity at higher extract concentrations. The lightness values (L*) remained consistently high in all samples, indicating preservation of the typical bright white appearance of the cheese. Negative a* values in all samples confirmed the characteristic slight greenish tone of white brined cheese less pronounced in the sample with the highest dose of applied rose petals extract (66WBC). This indicated a reduction in green hues, which may be associated with the presence of plant pigments and phenolic compounds from the extract. The yellowness parameter (b*) and chroma (C*)

generally decreased during storage but were found similar for both three samples. Rose petals are rich in antioxidant compounds, including phenolic and flavonoids, which may limit lipid oxidation and pigment degradation, thereby contributing to the observed color stability (Felix da Silva et al., 2015). Hue angle values remained within typical range, confirming that the dominant yellowish color, typical of white brined cheese from cow milk, was maintained regardless of treatment (Kandyliari et al., 2023).

The results for the sensory analysis are presented on Figure 4.

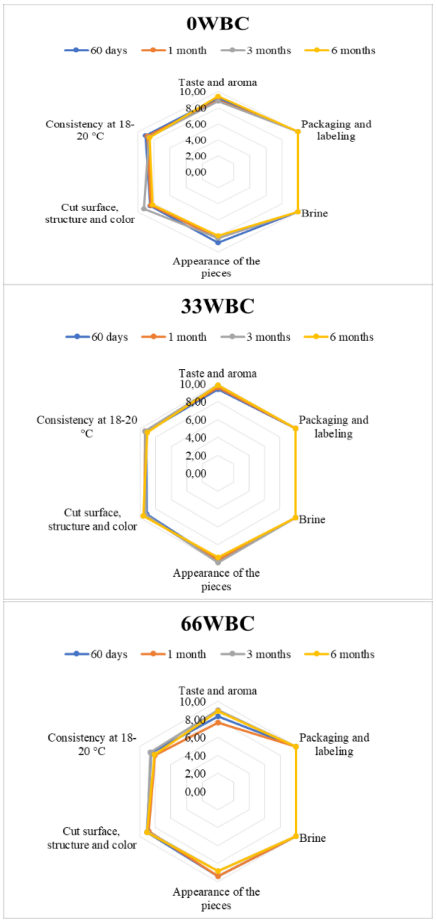


Figure 4. Sensory analysis of White Brined Cheese matured in brine with rose petals extract

It is well known that ripening significantly improves flavor development and texture due to biochemical processes such as proteolysis (Massouras et al., 2023). In white brined cheese the sensory changes are mainly due to pH decrease and protein breakdown. In the present study taste and aroma were both enhanced by the addition of rose petals extract. It is in correspondence with results of other authors who found that different herbs and plant extracts could contribute to excellent sensory scores (Hayaloglu & Farkye, 2011). According to Shahidi & Ambigaipalan (2015) some plant phenolic extracts can also improve overall sensory acceptability. The present findings are further supported by research on the incorporation of herbs and plant extracts in dairy products, which demonstrated that phenolic compounds enhance aroma and flavor while maintaining consumer acceptability (Setyawardani et al., 2023). It is evident that sample 66WBC had values closer to those of the control, while sample 33WBC has the highest score on all indicators, which indicates its preference by consumers.

CONCLUSIONS

The obtained results confirmed that the incorporation of rose petals extract preserved the physicochemical stability of white brined cheese. Low dose extract reduced proteolysis by partially inhabiting the activity of proteolytic enzymes. This resulted in a decrease in soluble nitrogen fractions and therefore a slowdown in cheese maturation. Incorporating rose could be a way to modulate the ripening speed, to adjust the texture and aroma development of the final product according to consumer preferences. The incorporation of rose petals extract did not affect the visual characteristics of white brined cheese and preserved the characteristic color profile during storage. The present study on plant-waste derived extracts in white brined cheese reported improved taste and aroma, appearance, and acceptable texture, confirming the potential of natural extracts as functional ingredients.

These findings suggest that rose petal extract can be successfully used as a natural functional ingredient in dairy products, contributing not only to improved antioxidant properties but also to the development of value-added cheeses with potential health-promoting benefits.

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COMPARATIVE EVALUATION OF PROXIMATE COMPOSITION AND PHYTOCHEMICAL PROPERTIES OF BANANA, MANGO, AND PAPAYA PULP AND PEEL

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Abstract

This study evaluated the proximate composition and phytochemical properties of the pulp and peel of banana (Musa spp.), mango (Mangifera indica L.), and papaya (Carica papaya L.). Proximate parameters, including moisture, ash, fat, protein, and carbohydrates, along with functional characteristics based on total phenolic content (TPC), total flavonoid content (TFC), and antioxidant capacity measured by ferric reducing antioxidant power (FRAP), were analyzed in samples from local agri-food markets. Results showed significant differences between peel and pulp in both composition and functional properties, highlighting their nutritional and health potential. Antioxidant activity was consistently higher in peels than in pulps, with mango peel exhibiting the highest value (166.89 μM Fe^{2+}/g d.w.). TPC and TFC followed a similar trend, with peels showing higher concentrations than edible fractions. These findings suggest that fruit peels are rich sources of natural antioxidants and have potential as functional ingredients, supporting the sustainable valorization of fruit-processing by-products for food and nutraceutical applications.

Key words*: banana, mango and papaya, by-products, functional properties, peel and pulp, proximate composition.*

INTRODUCTION

Fruits play a fundamental role in human nutrition due to their rich content of essential nutrients and bioactive compounds, contributing significantly to the prevention of chronic diseases and the promotion of overall health. Among tropical fruits, banana (*Musa spp.*), mango (*Mangifera indica* L.), and papaya (*Carica papaya* L.) are widely cultivated and consumed worldwide, owing to their sensory attributes, nutritional value, and economic importance (FAO, 2022; Slavin et al., 2012). These fruits are commonly consumed fresh or processed into juices, purees, dried products, jams, and other value-added foods, generating substantial quantities of by-products, particularly peels, which are often discarded as waste.

Banana is one of the most consumed fruits globally and represents a major staple food in many developing countries. It is a valuable source of carbohydrates, dietary fiber, potassium, vitamins, and bioactive compounds that support digestive health, cardiovascular function, and energy metabolism (Anyasi et al., 2013; Wall, 2006).

Mango, commonly referred to as the "king of fruits," is appreciated for its pleasant flavor and aroma and is recognized for its high content of vitamins A and C, sugars, minerals, and phenolic compounds with antioxidant and anti-inflammatory activities (Masibo et al., 2009). Papaya is valued not only for its nutritional quality but also for its medicinal properties, being rich in vitamin C, carotenoids, dietary fiber, and proteolytic enzymes such as papain, which contribute to digestive health and anti-inflammatory effects (Aravind et al., 2013; Rivera-Pastrana et al., 2010).

The nutritional and functional properties of fruits are mainly attributed to their proximate composition (moisture, proteins, fats, carbohydrates, ash, and fiber) as well as their phytochemical constituents, including phenolic

acids, flavonoids, carotenoids, and other antioxidant compounds. Numerous studies have demonstrated that regular consumption of fruits rich in antioxidants is associated with a reduced risk of oxidative stress-related diseases, such as cardiovascular disorders, diabetes, cancer, and neurodegenerative conditions (Liu, 2013; Shahidi et al., 2015). As a result, there is growing scientific and industrial interest in identifying new natural sources of bioactive compounds that can be used in functional foods, nutraceuticals, and pharmaceutical products.

In recent years, attention has increasingly shifted toward fruit-processing by-products, particularly peels, which are produced in large quantities during industrial processing and household consumption. Fruit peels are often considered waste and disposed of, leading to environmental concerns and economic losses. However, emerging evidence suggests that these by-products contain significant amounts of valuable nutrients and phytochemicals, often in higher concentrations than the edible pulp (Ayala-Zavala et al., 2011; Ajila et al., 2010). The sustainable valorization of fruit peels aligns with the principles of circular economy and waste reduction, promoting the efficient use of natural resources while generating added-value products.

Banana peel, which accounts for approximately 30-40% of the total fruit weight, has been reported to contain appreciable levels of dietary fiber, starch, proteins, essential minerals, and phenolic compounds with strong antioxidant activity (Emaga et al., 2008; Emaga et al., 2011). Studies have shown that banana peel extracts exhibit antioxidant, antimicrobial, and anti-inflammatory properties, supporting their potential use as functional ingredients in food formulations and animal feed (Vu et al., 2018; Pereira et al., 2015).

Similarly, mango peel represents about 15–20% of the fruit weight and is considered a rich source of polyphenols, flavonoids, carotenoids, and dietary fiber, often surpassing the pulp in antioxidant capacity (Ajila et al., 2010; Berardini et al., 2005). Mango peel extracts have demonstrated strong free radical scavenging activity and have been proposed for use as natural antioxidants and colorants in food and cosmetic industries (Macedo et al., 2022).

Papaya peel, though less extensively studied than banana and mango peels, has also been identified as a promising source of bioactive compounds. Research indicates that papaya peel contains phenolic compounds, flavonoids, carotenoids, and dietary fiber, contributing to its antioxidant and antimicrobial properties (Calvache et al., 2016; Nwofia et al., 2012). The presence of these compounds suggests that papaya peel may be exploited as a functional ingredient or as a raw material for the extraction of nutraceutical compounds.

The chemical composition and bioactive potential of fruit pulps and peels can vary significantly depending on species, cultivar, maturity stage, climatic conditions, soil type, agricultural practices, and post-harvest handling (Kader, 2008). Moreover, differences between pulp and peel composition highlight the importance of comparative evaluations to fully understand the nutritional and functional potential of each fruit fraction.

Several studies have reported that fruit peels generally contain higher concentrations of total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity compared to pulps, emphasizing their underutilized value (Ayala-Zavala et al., 2011; Shahidi et al., 2015). Despite increasing evidence on the nutritional richness of fruit peels, their utilization in food systems remains limited, mainly due to a lack of comprehensive compositional data, consumer acceptance issues, and technological challenges. Comparative studies that simultaneously assess proximate composition and phytochemical properties of both pulp and peel across different fruit species are essential to support their effective valorization. Such studies provide scientific justification for incorporating fruit by-products into functional foods, dietary supplements, and natural antioxidant formulations.

In this context, the present study aims to comparatively evaluate the proximate composition and phytochemical properties of the pulp and peel of banana (*Musa* spp.), mango (*Mangifera indica* L.), and papaya (*Carica papaya* L.) collected from local agri-food markets. The analysis focuses on key nutritional parameters, including moisture, ash, fat, protein, and carbohydrate content, as well as functional characteristics based on total phenolic content, total flavonoid content, and antioxidant capacity assessed by ferric reducing antioxidant power (FRAP).

By highlighting the differences between edible and non-edible fractions, this study seeks to contribute to the growing body of knowledge supporting the sustainable utilization of fruit-processing by-products as valuable sources of natural antioxidants and functional ingredients for food and nutraceutical applications.

MATERIALS AND METHODS

Three types of tropical fruit: banana (*Musa* spp.), mango (*Mangifera indica* L.), and papaya (*Carica papaya* L.) were collected from various local agri-food markets (Timisoara, Romania), in three batches, two weeks apart between collections. For further analysis, banana, mango, and papaya were coded as B, M, and P, respectively.

The ripe fruits were washed in tap water, dried with paper towel, and subsequently separated into peels, pulps, and seeds. The pulp and raw peel of each fruit variety were vacuum-sealed in separate polyethylene bags and stored at -18°C pending analysis. These samples were used for proximate analysis (moisture, ash content, fat, protein, and carbohydrates) and for determining phytochemical properties (total phenolic content – TPC, expressed as gallic acid equivalents, total flavonoid content – TFC, expressed as quercetin equivalents, and antioxidant capacity assessed by ferric reducing antioxidant power, FRAP).

All reagents used for the chemical analyses were of analytical grade and were purchased from three primary suppliers: Merck (Darmstadt, Germany) for the Folin-Ciocalteu reagent and sodium carbonate; Sigma-Aldrich (Taufkirchen, Germany) for antioxidant standards (gallic acid, quercitin, trolox), and inorganic reagents; and Amex-Lab (Bucharest, Romania) for 70% ethanol. To ensure statistical reliability, all measurements were performed in triplicate for every sampling interval.

Proximate analysis

Proximate composition was determined in accordance with the protocols established by the Association of Official Analytical Chemists (AOAC, 2000). Thus, samples were dried at 103±2°C to a constant weight to evaluate moisture content, while ash content was determined by incinerating samples in a muffle furnace at 550°C for about 4-hour duration.

Additionally, crude protein and fat content were measured using the Kjeldahl and Soxhlet extraction (using petroleum ether) methods, respectively.

The total carbohydrate content was derived by difference using the following formula:

Carbohydrate (%) = 100 – (% moisture + % crude protein + % ash + % crude fat)

The energy value was computed using the methodology of Das et al. (2019), considering that 1 g of fat provides 9 calories, 1 g of carbohydrates provides 4 calories and 1 g of protein provides 4 calories.

Energy value (kcal/100 g) = carbohydrates (%) x 4 + lipids (%) x 9 + proteins (%) x 4

Preparation of the antioxidant extracts

2 g of ground sample were treated with 20 mL 70% (v/v) ethanol for 120 minutes in a constant temperature chamber (25°C) under continuous stirring using a Heidolph Promax 1020 (Germany) shaker. After extraction, the samples were filtered and the extract was used to determine the total phenolic content – TPC (expressed as gallic acid equivalents), total flavonoid content – TFC (expressed as quercetin equivalents), and antioxidant capacity assessed by ferric reducing antioxidant power, FRAP.

Evaluation of total phenolic content (TPC)

Total phenolic content – TPC was quantified via the Folin-Ciocalteu assay. Extracts were first diluted (1:10 v/v) in distilled water; subsequently, 0.5 mL of the sample was reacted with 2.5 mL of Folin-Ciocalteu reagent (1:10 v/v) and 2 mL of 7.5% sodium carbonate. After incubating for 30 minutes at 50°C, the absorbance at 750 nm was measured. A gallic acid standard curve (prepared using standard solutions with concentrations ranging from 0.1 to 1.0 µM GAE/mL) was used to calculate TPC, which was reported as mg GAE/100 g d.w. for both pulp and peel samples.

Evaluation of total flavonoid content (TFC)

Total flavonoids were measured using the aluminum nitrate colorimetric method. The reaction involved sequential additions of 0.3% sodium nitrite, 10% aluminum nitrate, and 4%

sodium hydroxide to the sample extract. Following volume adjustment to 25.0 mL and a 15-minute stabilized reaction period, the absorbance was read at 510 nm (UV-VIS spectrophotometer). Results were normalized to dry weight (mg QE/100 g d.w.) based on a quercetin calibration curve prepared using standard solutions with concentrations ranging from 0.5 to 50 µg/mL.

Assessment of antioxidant activity by ferric-reducing antioxidant power (FRAP) assay

Antioxidant capacity was determined by the FRAP method, based on the electron-donating ability of the extracts to reduce Fe^{3+} to Fe^{2+}. The working reagent consisted of acetate buffer (300 mM, pH 3.6), 10 mM tripyridyltriazine (TPTZ), and 20 mM ferric chloride in a 10:1:1 (v/v/v) ratio. Aliquots of the 1:10 (v/v) diluted extracts (0.5 mL) were mixed with 2.5 mL of the FRAP solution and incubated at 37°C for 30 minutes. The chromogenic reaction was monitored at 593 nm using UV-VIS spectrophotometer. Antioxidant activity was quantified using a ferrous sulfate standard curve and normalized to the dry weight of the pulp and peel samples. Results were calculated as µM Fe^{2+}/g d.w.

Statistical analysis

Data were collected from three independent batches, each performed in triplicate. Results are presented as the mean ± standard deviation (SD). Statistical evaluation was performed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons and Levene's test to assess variance equality. Variations in the evaluated parameters among the three fruit types were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSIONS

Proximate composition of banana, mango and papaya pulp and peel

Table 1 presents the proximate composition and calculated energy value of banana (B), mango (M) and papaya (P) pulp and peel. Significant differences ($p < 0.05$) were observed among fruits within each group (pulp or peel), indicating distinct compositional characteristics between species and anatomical fractions.

Table 1. Proximate composition and energy value of the investigated fruit pulp and peel samples

Sample	Moisture (%)	Ash (%)	Crude protein (%)	Crude fat (%)	Carbohydrates (%)	Energy value (kcal/100 g)
B pulp	73.212 ± 0.011 ^{c}	1.980 ± 0.010 ^{a}	1.172 ± 0.013 ^{b}	0.440 ± 0.011 ^{a}	23.196±0.012^{a}	101.432 ± 0.030 ^{a}
M pulp	87.090 ± 0.023 ^{b}	0.330 ± 0.011 ^{c}	1.255 ± 0.015 ^{a}	0.074 ± 0.012 ^{c}	11.251±0.019^{b}	50.690 ± 0.095 ^{b}
P pulp	88.693 ± 0.044 ^{a}	0.430 ± 0.028 ^{b}	0.736 ± 0.011 ^{c}	0.162 ± 0.010 ^{b}	9.979±0.065^{c}	44.318 ± 0.032 ^{c}
B peel	88.156 ± 0.013 ^{a}	1.700 ± 0.012 ^{a}	1.127 ± 0.010 ^{c}	0.643 ± 0.011 ^{b}	8.374±0.013^{c}	43.791 ± 0.096 ^{c}
M peel	81.151 ± 0.041 ^{b}	0.901 ± 0.018 ^{c}	1.634 ± 0.019 ^{b}	2.466 ± 0.012 ^{a}	13.848±0.115 ^{b}	84.122 ± 0.013 ^{b}
P peel	77.260 ± 0.059 ^{c}	1.493 ± 0.012 ^{b}	4.592 ± 0.055 ^{a}	0.518 ± 0.010 ^{c}	16.237 ± 0.103 ^{a}	87.578 ± 0.044 ^{a}

B: banana; M: mango; P: papaya. Data are expressed as mean ± standard deviation of three independent analyses (n = 3). Within each column group (pulp or peel), values followed by different letters are significantly different among fruits (one-way ANOVA, $p < 0.05$)

Among pulps, papaya showed the highest moisture content (88.693%), followed by mango (87.090%) and banana (73.212%).

The lower moisture content of banana pulp may be associated with its higher carbohydrate accumulation during ripening, which contributes to a denser matrix and greater dry matter content.

The pulp moisture values are consistent with those reported in food composition databases such as TACO, which indicate moisture contents of approximately 73-75% for banana, 85-88% for mango and 88-90% for papaya pulps. Similar values were also reported by Morais et al. (2017) and Forster et al. (2003) for banana pulp (73.6%), respectively (71.94%). Papaya pulp humidity values determined in this experiment are comparable to those determined by Morais et al. (2017) (87.8%), confirming the reliability of the present results. For peels, banana peel exhibited the highest moisture content (88.156%), followed by mango (81.151%) and papaya (77.260%). These findings agree with Emaga et al. (2008), who reported high moisture content levels in banana peel (~85-90%). High moisture levels in peels indicate susceptibility to microbial spoilage,

reinforcing the need for drying or stabilization processes before industrial application.

Ash values, which reflect total mineral content, were significantly different among samples. In pulps, banana presented the highest ash content (1.980%), while mango (0.330%) and papaya (0.430%) showed lower values.

These values are comparable to those reported in the literature for banana pulp (1.18-3.89%) (Nadeeshani et al., 2021), mango pulp (0.31-0.66%) (Ubwa et al., 2014), and papaya pulp (0.31-0.61%) (Nwofia et al., 2012), where banana pulp typically contains higher mineral concentrations than mango and papaya pulps.

In peels, banana (1.7%) and papaya (1.493%) exhibited higher ash contents compared to mango peel (0.901%). Similar observations have been reported by Emaga et al. (2008) for banana peel and by other researchers evaluating mango and papaya processing residues. The relatively elevated ash content in peels suggests their potential as mineral-rich ingredients for food fortification or animal feed applications.

Protein levels in pulps were generally low, as expected for fruits. Mango pulp showed the highest protein content (1.255%), followed by banana (1.172%) and papaya (0.736%). These values are consistent with previously reported ranges: 0.5-1.4% for banana, 0.5-1.0% for papaya, and approximately 0.8-1.3% for mango pulp.

In contrast, peels, particularly papaya peel, presented higher protein levels. Papaya peel contained 4.592% protein, significantly higher than mango (1.634%) and banana (1.127%) peels.

Comparable findings have been reported for papaya peel by several authors, who described protein values ranging between 3-11%, namely 3.5% (Akintunde et al., 2022), 6.1% (Maisarah et al., 2014), and 10.51% (Mukta et al., 2025).

This indicates that papaya peel may represent a more promising protein source among the evaluated by-products.

Fat content in pulps was low, ranging from 0.074% (mango) to 0.440% (banana), consistent with literature data describing these fruits as low-lipid foods. Similar values have been documented for banana pulp, with contents ranging from 0.1-1.7% (Morais et al., 2017), mango pulp (0.06-0.16%) (Ubwa et al., 2014), and papaya pulp (0.14-0.53%) (Nwofia et al., 2012).

Peels showed more variability. Mango peel exhibited the highest fat content (2.466%), followed by banana (0.643%) and papaya (0.518%). Previous studies on mango peel have also reported lipid values between 2.0-2.56% (Tokas et al., 2020), which may be attributed to the presence of cuticular waxes and bioactive lipid fractions. The higher content of mango peel contributes to its increased caloric value relative to the other peels.

Carbohydrates were calculated by difference and represented the major macronutrient fraction in all pulps. Banana pulp showed the highest carbohydrate content (23.196%), significantly higher than mango (11.251%) and papaya (9.979%). This aligns with existing data indicating banana as a carbohydrate-rich fruit, particularly due to starch-to-sugar conversion during ripening.

In peels, papaya peel presented the highest carbohydrate content (16.237%), followed by mango (13.848%) and banana (8.374%). Literature reports similarly indicate that fruit peels can contain substantial amounts of structural carbohydrates, including dietary fibre and non-starch polysaccharides (Ismail et al., 2024).

The energy value reflected the macronutrient composition. Banana pulp presented the highest caloric value (101.432 kcal/100 g), approximately double that of mango (50.690 kcal/100 g) and papaya (44.318 kcal/100 g). These values are in line with commonly reported energy values for banana (approximately 89-105 kcal/100 g), mango (56-60 kcal/100 g), and papaya (40-45 kcal/100 g), according to the USDA FoodData Central database.

For peels, papaya (87.578 kcal/100 g) and mango (84.122 kcal/100 g) showed higher energy values than banana peel (43.791 kcal/100 g). The higher caloric values of mango and papaya peels are associated with their higher protein, lipid and carbohydrate contents. These findings support previous studies highlighting the nutritional potential of fruit by-products as alternative ingredients in functional foods or feed formulations (Zou et al., 2022; Tewari et al., 2025; Nitisuk et al., 2025).

In general, pulps were characterized by higher moisture and lower ash, protein and fat contents, while peels exhibited greater concentrations of minerals, proteins (notably papaya peel), and in

some cases lipids (mango peel). Banana pulp stood out for its higher carbohydrate and energy values, whereas papaya peel demonstrated superior protein and carbohydrate levels among the by-products.

These results are consistent with previously published data on banana, mango and papaya fractions, confirming that fruit processing residues, especially peels, retain considerable nutritional value. The compositional differences observed highlight the potential for valorisation of these by-products in food, nutraceutical or animal feed applications, contributing to waste reduction and sustainable agro-industrial practices.

Phytochemical properties of banana, mango and papaya pulp and peel

The phytochemical characteristics of B, M, and P (pulps and peels) are presented in Figures 1–3. Significant differences ($p < 0.05$) were observed among fruits within each anatomical fraction (pulp and peel), indicating that both species and fruit part strongly influence bioactive compound content and antioxidant capacity.

Among pulps, mango exhibited the highest TPC (1,363.091 mg GAE/100 g d.w.), followed by papaya (1,061.433 mg GAE/100 g d.w.) and banana (698.994 mg GAE/100 g d.w.). The elevated TPC of mango pulp is consistent with previous findings. Kim et al. (2010) reported TPC values for mango pulp ranging between 900 and 1,500 mg GAE/100 g d.w., depending on cultivar and ripening stage. Similarly, Maisuthisakul et al. (2007) observed that mango pulp possesses higher phenolic concentrations compared to several other tropical fruits. Banana pulp TPC values reported in the literature (Maisuthisakul et al., 2007) typically range from 500 to 900 mg GAE/100 g d.w., which aligns with those obtained in the present study.

Papaya pulp phenolic content has been reported between 800 and 1,200 mg GAE/100 g d.w. (Oloyede, 2005), which is consistent with the obtained data.

For peels, significantly higher TPC values were observed compared to pulps. Mango peel showed the highest value (2,442.065 mg GAE/100 g d.w.), followed by banana (1,605.081 mg GAE/100 g d.w.) and papaya (1,264.272 mg GAR/100 g d.w.) (Figure 1). These findings agree with Ajila et al. (2007), who reported mango peel TPC values ranging from 2,000 to 4,500 mg GAE/100 g d.w., emphasizing the richness of mango peel in phenolic acids and flavonoids. Emaga et al. (2008) also reported high phenolic levels in banana peel (approximately 1,500–2,500 mg GAE/100 g d.w.), which are similar to the current results.

The higher phenolic concentration in peels compared to pulps reflects the protective function of phenolic compounds in fruit outer tissues, where they act as defence molecules against environmental stress.

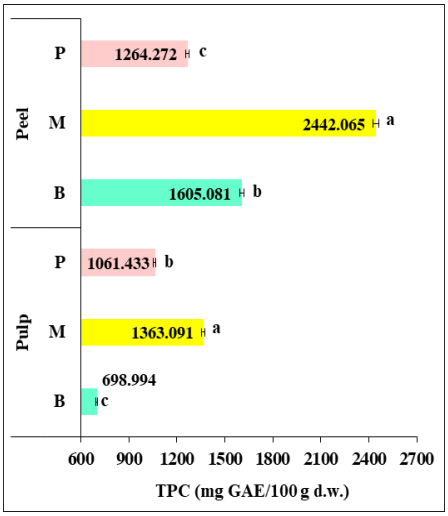


Figure 1. Total phenolic content (TPC) in the investigated fruit samples (pulp and peel). B: banana; M: mango; P: papaya. Data are expressed as mean ± standard deviation of three independent analyses (n = 3). Different letters above the columns indicate significant differences within the same group (pulp or peel) according to one-way ANOVA ($p < 0.05$)

A similar trend was observed for TFC. Mango pulp presented the highest value (631.767 mg QE/100 g d.w.), followed by papaya (497.471 mg QE/100 g d.w.) and banana (321.226 mg QE/100 g d.w.). These results are consistent with reports by Maisuthisakul et al. (2007), who found mango pulp flavonoid content significantly higher than banana and papaya.

In peel samples, mango peel again exhibited the highest TFC (1,058.748 mg QE/100 g d.w.), followed by banana (762.231 mg QE/100 g d.w.) and papaya (669.251 mg QE/100 g d.w.) (Figure 2). Ajila et al. (2007) and Larrauri et al.

(1996) also reported elevated flavonoid concentrations in mango peel due to the presence of quercetin, kaempferol and catechin derivates.
The consistently higher TFC values in peels reinforce their potential as sources of natural antioxidants for functional food formulations.

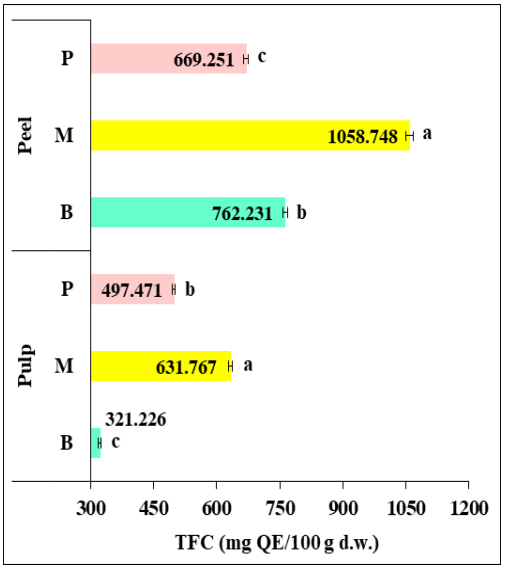


Figure 2. Total flavonoid content (TFC) in the investigated fruit samples (pulp and peel). B: banana; M: mango; P: papaya. Data are presented as mean ± standard deviation of three independent analyses (n = 3). Different letters above the columns indicate significant differences within each group (pulp or peel) according to one-way ANOVA ($p < 0.05$)

The antioxidant capacity measured by FRAP followed the same general pattern as TPC and TFC. Mango pulp showed the highest FRAP value (95.882 µM Fe^{2+}/g d.w.), followed by banana (59.574 µM Fe^{2+}/g d.w.) and papaya (41.635 µM Fe^{2+}/g d.w.). These results are comparable with those reported by Thaipong et al. (2006), who observed FRAP values in mango pulp between 50 and 120 µM Fe^{2+}/g d.w., depending on cultivar.
For peels, mango (166.894 µM Fe^{2+}/g d.w.) and banana (150.569 µM Fe^{2+}/g d.w.) exhibited significantly higher antioxidant capacity than papaya peel (48.788 µM Fe^{2+}/g d.w.). Similar trends were reported by Emaga et al. (2008) and Ajila et al. (2007), who demonstrated strong antioxidant activity in mango and banana peels due to their elevated phenolic and flavonoid concentrations (Figure 3).

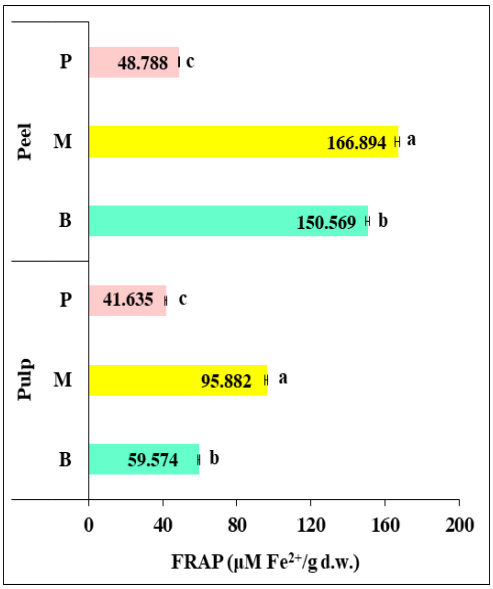


Figure 3. Antioxidant activity (FRAP assay) in the investigated fruit samples (pulp and peel). B: banana; M: mango; P: papaya. Data are presented as mean ± standard deviation (n = 3). Different letters above the columns indicate significant differences within each group (pulp or peel) according to one-way ANOVA ($p < 0.05$)

The similar trends observed between TPC and FRAP, as well as between TFC and FRAP, indicate that both total phenolics and flavonoids may contribute to antioxidant capacity, as previously reported in other plant-origin matrices (Metzner Ungureanu et al., 2020). The increased TPC and TFC contents in the fruit fractions are closely associated with their improved FRAP antioxidant capacity. This pattern further supports the role of phenolic compounds as major contributors to the overall reducing power of plant materials, in agreement with the findings of Poiana et al. (2023), who also reported a strong relationship between total phenolic content and antioxidant activity.
In all investigated fruits, peel samples exhibited significantly higher TPC, TFC, and FRAP values compared to pulp samples ($p < 0.05$). Mango peel was identified as the richest source of bioactive compounds, followed by banana peel and papaya peel. Among pulps, mango pulp exhibited superior phytochemical and antioxidant properties.
These findings are in agreement with previous literature demonstrating that fruit processing by-products, particularly peels, contain higher

concentrations of phenolic compounds than edible pulp fractions (Islam et al., 2021; Rodrigues-Rodrigues et al., 2026; Ismail et al., 2024). The results highlight the potential of banana, mango and papaya peels as low-cost sources of natural antioxidants for nutraceutical, pharmaceutical and food industry applications.
The valorisation of these by-products may contribute to waste reduction and to the development of functional ingredients with enhanced health-promoting properties.

Potential applications of banana, mango and papaya pulp and peel

The compositional and phytochemical profiles obtained in this study highlight the significant potential of both pulp and peel fractions for various industrial and nutritional applications. The observed differences between fruit parts suggest that each fraction may be better suited for specific valorization pathway.
The high carbohydrate content and energy value of banana pulp indicate its suitability as an energy-rich ingredient in food formulations such as purees, beverages, and bakery products. In contrast, mango and papaya pulps, characterized by higher moisture and lower caloric content, may be more appropriate for low-energy or refreshing food products, including juices and functional beverages.
Fruit peels, often considered agro-industrial waste, demonstrated higher concentrations of ash, protein (particular in papaya peel), and bioactive compounds (phenolics and flavonoids), as well as stronger antioxidant activity. These characteristics make them promising candidates for incorporation into functional foods as natural sources of dietary fiber and antioxidants. For instance, mango peel, which exhibited the highest total phenolic and flavonoid contents, could be used as a natural antioxidant extract to enhance the shelf life and nutritional quality of food products.
Also, banana peel, with its appreciable fiber and phenolic content, may be utilized in the development of fiber-enriched flours or as an ingredient in bakery products. Previous studies (Ahmed et al., 2021; Ismail et al., 2024; Weshah, 2026) have demonstrated successful incorporation of banana peel flour into bread and biscuits, improving both nutritional value and capacity.
Papaya peel, characterized by its relatively high protein and carbohydrate content, also presents potential for use in animal feed formulations, contributing to both energy and nutrient supply. In addition, its bioactive profile suggests possible applications in nutraceutical formulations.
The high antioxidant activity observed in peel fractions, particularly mango and banana, supports their use as natural alternatives to synthetic antioxidants in food preservation systems. This is increasingly important given consumer demand for clean-label products.
The results emphasize that fruit peels should not be regarded as waste but rather as valuable raw materials for the development of functional ingredients, nutraceuticals, and sustainable food products. These potential applications place our analytical findings into a broader applied context and highlights the relevance of fruit by-products in sustainable food systems and waste valorization strategies. The valorization of these by-products aligns with current trends in circular economy and sustainable agro-industrial practices, contributing to waste reduction and added economic value.

CONCLUSIONS

The results of the present study demonstrate that both pulp and peel fraction of banana, mango and papaya possess distinct and valuable nutritional and phytochemical characteristics. Significant differences ($p < 0.05$) were observed among fruit species and anatomical fractions, confirming that both botanical origin and fruit part strongly influence proximate composition and antioxidant potential. The findings confirm that fruit processing by-products, particularly peels, retain considerable nutritional and functional value. Their elevated mineral, protein (notably in papaya peel), lipid (in mango peel), and phenolic contents highlight their potential for valorization as functional food ingredients, natural antioxidants, nutraceutical raw materials, or components in animal feed formulations. In this context, the present research addresses the broader challenge of managing fruit processing residues, emphasizing their potential integration into sustainable production systems. The recovery and utilization of banana, mango and papaya peels represent a sustainable strategy for waste reduction and the develop-

ment of value-added products, supporting resource efficiency and the advancement of circular economy principles within the agro-industrial sector.

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ANIMAL FAT CONSUMPTION VERSUS SUGAR INTAKE: IMPLICATIONS FOR OBESITY IN THE POPULATION OF THE REPUBLIC OF MOLDOVA

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Abstract

This study examines the relationship between obesity prevalence and dietary patterns in the Republic of Moldova, with a comparative focus on animal fat and sugar consumption. The analysis uses national-level data from FAOSTAT, the World Health Organization, and the World Bank for the period 2010–2022. Obesity prevalence among adults and children was analysed alongside indicators of animal fat and sugar supply. The results indicate a closer descriptive association between obesity prevalence and sugar availability than with animal fat intake; however, these findings are based on seven biennial co-trending observations and should be interpreted only as population-level trend indicators rather than evidence of causation. Both dietary components increased during the study period, although animal fat consumption showed a weaker and more variable association with obesity trends. The study also includes a policy-oriented discussion regarding HFSS food advertising and public health implications in the Republic of Moldova.

Key words*: animal fat consumption, population obesity, Republic of Moldova, sugar intake.*

INTRODUCTION

Obesity has become one of the most pressing public health challenges of the 21st century, affecting populations across all income levels and geographic regions.

According to the World Health Organization (WHO, 2022), the global prevalence of obesity has nearly tripled since 1975, with more than 650 million adults classified as obese worldwide.

In low- and middle-income countries, the epidemiological transition from undernutrition to overnutrition has accelerated in recent decades, driven by changes in dietary habits, reduced physical activity, and urbanization (FAO, 2023).

The Republic of Moldova, a small, landlocked country in Eastern Europe, represents a relevant case for analysing obesity-related dietary patterns. As a former Soviet republic undergoing economic and social transition, Moldova has experienced significant changes in food consumption behaviour, characterized by increased access to processed and energy-dense foods.

At the same time, the country continues to struggle with poverty, food insecurity, and limited public health resources, creating a complex nutritional environment (Stratan et al., 2024).

The Republic of Moldova has a population of approximately 2.5 million inhabitants and is classified as an upper-middle-income economy with a large share of the population living in rural areas. The country has undergone substantial economic and social transitions since the 1990s, including changes in dietary patterns, urbanization, and food availability.

Despite recent economic growth, Moldova continues to face important public health challenges associated with nutrition, poverty, and unequal access to healthcare services.

According to the World Health Organization (WHO, 2022), the prevalence of adult obesity in Moldova has risen steadily over the past two decades, reaching approximately 23.4% in

2022, according to WHO estimates, and placing Moldova above the European average.

The relative contribution of macronutrients, dietary fat versus sugar, in driving obesity has been widely debated in the nutritional science literature (Siminiuc et al, 2025).

The traditional lipid hypothesis, prominent from the 1960s through the 1980s, postulated that dietary fat, particularly saturated fat derived from animal products, was the primary driver of excess caloric intake and weight gain (Keys, 1953; Ancel & Margaret, 1975).

Subsequent decades saw a partial paradigm shift, as researchers and public health bodies began to emphasize the role of added sugars and refined carbohydrates in metabolic dysfunction and obesity (Lustig et al., 2012; Stanhope, 2016).

More recent epidemiological evidence has supported the sugar hypothesis more strongly. Studies have demonstrated that high consumption of free sugars, particularly in the form of sugar-sweetened beverages, confectionery, and ultra-processed foods, is associated with excess caloric intake, insulin resistance, hepatic fat accumulation, and ultimately increased obesity risk (Te Morenga et al., 2013; Malik et al., 2013; WHO, 2015).

In contrast, the relationship between total animal fat intake and obesity appears more nuanced, with some research suggesting that the food matrix and overall dietary context may be more relevant than fat content alone (Astrup et al., 2019).

In addition to dietary patterns, the role of mass media advertising in shaping food consumption behaviours has received increasing scholarly attention. The promotion of foods and beverages high in fat, sugar, and salt (HFSS) through television, digital platforms, and social media has been associated with increased consumption of such products, particularly among children and adolescents (WHO, 2023; Boyland et al., 2016).

Because nationally representative monitoring data regarding HFSS food advertising are currently unavailable for the Republic of Moldova, this section is intended as a policy-oriented contextual discussion based on international literature and regional regulatory comparisons.

In the Republic of Moldova, the regulatory framework governing food advertising remains limited compared to Western European standards, which may contribute to higher population exposure to persuasive food marketing practices.

In contrast, several European countries have introduced restrictions on HFSS food advertising directed at children, including limitations on television advertising during children's programming and regulations targeting digital marketing exposure (WHO, 2023).

The World Health Organization has also recommended comprehensive monitoring systems and stronger regulatory frameworks to reduce the influence of unhealthy food marketing on dietary behaviour among vulnerable population groups.

This study aims to analyse the temporal association between animal fat consumption, sugar intake, and obesity prevalence using nationally representative data for the period 2010-2022, with specific attention to obesity trends between 2020 and 2025.

Additionally, a dedicated section examines the role of mass media in promoting HFSS food and beverage products and discusses the implications for public health in Moldova.

MATERIALS AND METHODS

This study employs a quantitative, longitudinal approach to analyse the association between dietary macronutrient availability and obesity prevalence in the Republic of Moldova over the period 2010-2022. The research design is based on the analysis of secondary statistical data from internationally recognized sources, following a methodology consistent with similar population-level dietary and health studies (Rojco et al., 2020; Munteanu & Stanciu, 2019).

Data on food supply were retrieved from the FAOSTAT Food Balance Sheets database (FAO, 2025), which provides annual estimates of the per capita availability of major food commodities and nutrients, expressed in grams per capita per day. It should be noted that these data reflect food availability rather than actual individual consumption. For this study, two key dietary indicators were extracted: animal fat

supply (g/capita/day) and sugar supply (g/capita/day).

Obesity prevalence data were obtained from the WHO Global Health Observatory and the World Bank Health Nutrition and Population Database, using the prevalence of obesity among adults (BMI ≥ 30) and the prevalence of overweight and obesity among children (ages 5-19).

GDP per capita in purchasing power parity (PPP, constant 2017 international USD) from the World Bank was included as a contextual economic indicator.

For the analysis of obesity trends during the period 2020-2025, data from the WHO Global Health Observatory, the National Bureau of Statistics of the Republic of Moldova, and published estimates from the Global Burden of Disease Study (GBD, 2024) were used. Where 2024 and 2025 data are not yet finalized, projected values based on linear trend extrapolation from confirmed series are indicated with an asterisk (*).

The section on mass media food advertising is based on a review of academic literature, WHO and Pan American Health Organization (PAHO) policy reports, and documented advertising monitoring studies from Eastern Europe and Central Asia.

Although this section is based primarily on international literature and regional policy comparisons rather than Moldova-specific empirical data, it is included to contextualize the observed obesity trends within broader public health and food environment frameworks associated with HFSS food marketing and media exposure.

Data were statistically processed, systematized in tables, and graphically represented. Pearson correlation coefficients were calculated to explore descriptive relationships between dietary indicators and obesity prevalence, with statistical significance evaluated at $p < 0.05$. It should be noted that the correlation analysis is based on seven biennial observations (2010-2022), all of which display co-trending upward trajectories. Under these conditions, high r values are mathematically expected and carry limited inferential weight. Accordingly, all reported associations should be interpreted strictly as descriptive population-level trend indicators and must not be construed as evidence of differential dietary effect or causation. To further contextualise the dietary–obesity relationships, GDP per capita (PPP, constant 2017 international USD), already present in the dataset, was considered as a proxy for the broader socioeconomic trends that co-evolve with both dietary patterns and obesity prevalence over the study period.

During the drafting and revision phase, language editing tools were used to improve the clarity and accuracy of the English text. All results were interpreted and validated by the authors through comparison with the specialized literature.

RESULTS AND DISCUSSIONS

The selected indicators and their analytical relevance are presented in Table 1.

Table 1. Indicators selected for analysis

Dimension	Indicator	Relevance for obesity
Diet Quality	Animal fat supply (g/capita/day)	Excess fat intake linked to caloric surplus and weight gain
Diet Quality	Sugar supply (g/capita/day)	High sugar consumption strongly associated with obesity
Health Outcome	Obesity prevalence – adults (%)	WHO-reported BMI ≥ 30 among adults
Health Outcome	Obesity prevalence – children (%)	WHO-reported overweight/ obesity among children
Economic context	GDP per capita, PPP (USD)	Proxy for dietary transition and lifestyle changes

Source: Authors, based on FAO (2025), WHO (2022), World Bank (2024).

The selection of these indicators reflects a multidimensional approach, capturing both dietary drivers and health outcomes of obesity, while also accounting for the broader economic context influencing nutrition transitions.

The broader policy discussion regarding HFSS food marketing is intended to complement these descriptive findings by situating obesity trends within the wider food environment and media exposure context.

Trends in Animal Fat and Sugar Consumption and Obesity Prevalence (2010-2022)

The data presented in Table 2 summarize the evolution of animal fat supply, sugar supply, adult obesity prevalence, and child obesity prevalence in the Republic of Moldova over the period 2010-2022.

Table 2. Animal fat supply, sugar supply, and obesity prevalence in the Republic of Moldova (2010-2022)

Year	Animal fat supply (g/capita/day)	Sugar supply (g/capita/day)	Adult Obesity (%)	Child Obesity (%)
2010	32.5	88.2	19.8	4.2
2012	33.8	91.5	20.3	4.5
2014	34.2	93.8	20.9	4.8
2016	35.1	96.4	21.5	5.1
2018	36.0	99.1	22.2	5.4
2020	36.8	100.3	22.7	5.6
2022	37.5	103.2	23.4	5.9

Source: Authors, based on FAO (2025), WHO (2022), World Bank (2024).

Both dietary components exhibit upward trends over the analysis period, consistent with broader patterns of nutritional transition observed in lower- and middle-income countries undergoing economic development (FAO, 2023).

These trends provide a basis for examining the statistical relationships between dietary indicators and obesity outcomes. Animal fat supply increased from approximately 32.5 g/capita/day in 2010 to 37.5 g/capita/day in 2022, representing a growth of approximately 15.4%. Sugar supply increased more rapidly, from 88.2 g/capita/day to 103.2 g/capita/day, representing a growth of 17.0%. Adult obesity prevalence increased from 19.8% in 2010 to 23.4% in 2022, an absolute increase of 3.6 percentage points, while child obesity rose from 4.2% to 5.9%.

GDP per capita (PPP) increased steadily in the Republic of Moldova during the study period, reflecting broader socioeconomic transitions that may have influenced both dietary patterns and obesity prevalence. This contextual trend suggests that the observed co-evolution between dietary indicators and obesity should be interpreted within a wider framework of economic and lifestyle changes.

The Pearson correlation analysis suggests that sugar supply co-trended more closely with obesity prevalence indicators than animal fat supply over the study period (adult obesity: $r = 0.96$ versus $r = 0.87$; child obesity: $r = 0.94$ versus $r = 0.82$).

It should be noted that these associations may partly reflect common upward trends over time (co-trending effect) and therefore should not be interpreted as evidence of causality.

These findings are broadly consistent with the international literature on the role of added sugars in driving population-level obesity (Te Morenga et al., 2013; Malik et al., 2013).

Obesity Evolution in the Republic of Moldova (2020-2025)

The period 2020-2025 is characterized by the convergence of multiple stressors that have affected population health and dietary behaviour in the Republic of Moldova.

The COVID-19 pandemic (2020-2022) induced significant lifestyle changes, including reduced physical activity, increased sedentary behaviour, and heightened psychological stress, all of which are recognized risk factors for weight gain and obesity (WHO, 2022, Stanciu, 2022).

The economic disruptions associated with the pandemic, combined with the effects of the war in Ukraine (2022-present) on food prices and household incomes, have further complicated the nutritional landscape in Moldova.

Table 3 presents the estimated evolution of obesity indicators in the Republic of Moldova for the period 2020-2025, based on WHO Global Health Observatory data, national statistics, and trend projections.

Table 3. Obesity prevalence in the Republic of Moldova (2020-2025)

Year	Adult Obesity (%)	Adult Overweight (%)	Child Obesity (%)	Est. Obese Population	Trend
2020	22.7	41.3	5.6	approximately 197,000	↑
2021	23.0	41.8	5.7	approximately 200,000	↑
2022	23.4	42.5	5.9	approximately 203,000	↑
2023	23.8	43.1	6.1	approximately 207,000	↑
2024*	24.1	43.6	6.3	approximately 210,000	↑
2025*	24.4	44.0	6.5	approximately 212,000	↑

*Projected values based on linear trend extrapolation from confirmed WHO/GBD data series.

Source: Authors, based on WHO Global Health Observatory (2022), GBD 2023 Obesity Collaborators (2024), National Bureau of Statistics of the Republic of Moldova.

The data indicate a continuous increase in adult obesity prevalence, from 22.7% in 2020 to an estimated 24.4% in 2025, corresponding to an increase of approximately 1.7 percentage points over five years. Adult overweight (BMI ≥ 25) is estimated to affect approximately 44% of the adult population by 2025, placing Moldova above the regional average for Eastern Europe.

Child obesity is also rising, from 5.6% in 2020 to an estimated 6.5% in 2025.

In absolute terms, the estimated number of obese individuals in Moldova has increased from approximately 197,000 in 2020 to over 210,000 in 2025, based on WHO population estimates and obesity prevalence rates. These figures

underscore the scale of the public health challenge facing the country and the urgency of implementing effective prevention and management strategies.

The COVID-19 pandemic has likely acted as an accelerating factor for obesity trends in Moldova. National surveys conducted during the pandemic period reported increased consumption of comfort foods, higher-calorie snacks, and sugar-sweetened beverages, alongside significant reductions in physical activity levels (National Bureau of Statistics, 2022).

These behavioural changes, if persistent, may contribute to elevated obesity rates beyond the immediate pandemic period.

Mass Media Promotion of Foods and Beverages High in Fat and Sugar

Mass media advertising plays a significant role in shaping food consumption patterns at the population level. The World Health Organization has repeatedly identified the marketing of foods and beverages high in fat, sugar, and salt (HFSS products) as a major modifiable risk factor for unhealthy dietary behaviour and obesity, particularly among children and adolescents (WHO, 2023; WHO, 2010).

In the Republic of Moldova, as in many other transitioning economies, food advertising through television, digital platforms, and out-of-home channels remains largely self-regulated and insufficiently aligned with international best practice standards.

Table 4 provides a systematic overview of the main media channels used to promote HFSS food and beverage products in Moldova, along with the frequency, product categories, and key characteristics of advertising impact across each channel.

Television remains one of the most influential advertising channels for HFSS food products in Moldova. Studies conducted across Eastern Europe have documented that a significant proportion of food advertising on national television channels is dedicated to snack foods, fast food, sugar-sweetened beverages, and processed meats - product categories characterized by high caloric density and low nutritional value (Boyland et al., 2016). Children are disproportionately exposed to such advertising, as food commercials are frequently aired during or adjacent to programming designed for younger audiences.

Table 4. Mass media channels for promotion of high-fat and high-sugar food products in the Republic of Moldova

Media Channel	Frequency of Food Advertising	Most Promoted Product Categories	Key Impact Characteristics
Television	High	Chips, fast food, sweet beverages, processed meats	Reaches all age groups; highest impact on children 6–12
Online / social media	Very High	Energy drinks, sweets, ultra-processed snacks	Targeted algorithms; influencer marketing; 24/7 exposure
Outdoor Advertising	Medium	Fast food chains, soft drinks, confectionery	Urban concentration; high frequency near schools
Radio	Low–Medium	Processed foods, dairy with added sugar	Limited visual appeal; mainly adult audience
Print Media	Low	Cooking fats, dairy products, cured meats	Declining reach; older demographic
In-store Promotions	High	Sugar-sweetened beverages, snacks, fatty processed foods	Point-of-sale nudge; price promotions; prominent placement

Source: Authors, based on WHO (2023), Boyland et al. (2016), PAHO (2021), and national media monitoring reports.

The rapid expansion of digital and social media platforms has created new and more targeted pathways for HFSS food marketing. Online advertising algorithms enable food manufacturers to reach specific demographic segments with personalized content, while influencer marketing - involving popular social media personalities who promote food and beverage products - has become increasingly prevalent among younger Moldovan consumers. The WHO (2023) notes that the digital marketing environment poses challenges for regulatory oversight, as content is often produced and disseminated outside the jurisdictions of national advertising standards bodies.

In-store promotions represent another high-impact marketing channel that operates largely outside the scope of traditional advertising regulation. Point-of-sale displays, price promotions, and prominent shelf placement of HFSS products have been shown to significantly influence consumer purchasing behaviour, including impulse purchases of energy-dense foods (PAHO, 2021).

In Moldova, supermarket and hypermarket formats have expanded rapidly over the past

decade, increasing consumer exposure to commercially promoted HFSS products daily.

The cumulative effect of multi-channel food marketing exposure on dietary behaviour and obesity risk has been well-documented in the international literature. A systematic review by Boyland et al. (2016) found that food advertising consistently influences children's food preferences, purchase requests, and actual consumption, with effects that are independent of parental influence. In adult populations, repeated exposure to food advertising has been associated with increased consumption of promoted products and greater caloric intake, particularly in the context of emotional or stress-related eating (Sadeghirad et al., 2016).

In the Republic of Moldova, the absence of a comprehensive regulatory framework for HFSS food advertising constitutes a significant gap in the national public health architecture. At present, food advertising in Moldova is governed primarily by the Law on Advertising (No. 1227/1997) and its subsequent amendments, which contain limited provisions specifically addressing the marketing of unhealthy food products or the protection of children from food advertising. In contrast, several EU Member States and WHO European Region countries have implemented or are in the process of implementing statutory restrictions on HFSS food advertising across television, digital, and outdoor media channels (WHO, 2023).

The alignment of Moldova's food advertising regulatory framework with WHO recommendations and EU standards would represent an important step toward reducing population-level exposure to commercial HFSS food marketing.

Recommended measures include the restriction of HFSS food advertising during children's broadcasting hours, the implementation of nutrient profiling systems to define which products qualify as HFSS, mandatory disclosure of nutritional information in food advertising, and the regulation of influencer marketing practices involving food and beverage products. Such measures, if implemented as part of a comprehensive national obesity prevention strategy, could contribute meaningfully to reducing the dietary risk factors associated with the ongoing rise in obesity rates observed in the Republic of Moldova.

These findings underscore the need to integrate regulatory, educational, and public health interventions to effectively mitigate the impact of HFSS food marketing on dietary behaviour.

CONCLUSIONS

This study analysed the temporal association between animal fat supply, sugar supply, and obesity prevalence in the Republic of Moldova over the period 2010-2022, with extended attention to obesity trends during 2020-2025 and the role of mass media in promoting HFSS food and beverage products.

This study contributes to the existing literature by providing a country-level, longitudinal assessment of dietary drivers of obesity in a transitioning Eastern European context.

The results indicate that both dietary components increased over the study period alongside rising obesity rates, while sugar supply appeared to co-trend more closely with obesity indicators ($r = 0.96$ for adult obesity; $r = 0.94$ for child obesity) than animal fat supply ($r = 0.87$ and $r = 0.82$, respectively). It must be emphasised, however, that these results are derived from seven biennial co-trending time series, a condition under which high Pearson r values are mathematically expected even in the absence of a demonstrable causal relationship. The reported associations therefore reflect descriptive population-level trends only and should not be interpreted as evidence of differential dietary effect or causation.

The analysis of obesity trends during 2020-2025 reveals a continuous upward trajectory, with adult obesity estimated to reach approximately 24.4% by 2025. The COVID-19 pandemic and the economic effects of the regional geopolitical situation may have contributed to a further deterioration in dietary behaviours, reinforcing pre-existing trends toward higher consumption of energy-dense, nutrient-poor food products.

The examination of mass media food advertising highlights the significant role of commercial food marketing in shaping dietary behaviour and contributing to population-level obesity in Moldova. However, this section should be interpreted as a contextual policy discussion based on international evidence and regional

policy comparisons rather than as original Moldova-specific empirical research. The absence of nationally representative HFSS advertising monitoring data remains an important research gap for future studies.

Based on the findings of this analysis, the following policy recommendations are proposed: (1) prioritize sugar reduction strategies in national dietary guidelines and public health interventions; (2) implement statutory restrictions on HFSS food advertising across television, digital, and outdoor media; (3) introduce mandatory front-of-pack nutritional labelling to enable informed consumer choices; (4) invest in national dietary monitoring systems to generate representative data on actual food consumption; and (5) develop integrated obesity prevention programs that address both dietary risk factors and physical activity levels across all age groups.

Future research should pursue at least three directions to build upon the findings of this study. First, nationally representative dietary consumption surveys should be conducted in the Republic of Moldova to replace the food supply proxies derived from FAOSTAT Food Balance Sheets; actual individual-level intake data would allow more precise estimation of the dietary determinants of obesity. Second, longitudinal multivariate modelling studies controlling for income levels, urbanisation, and physical activity are needed to disentangle the relative contributions of dietary and non-dietary factors to obesity trends, thereby moving beyond the descriptive trend associations characterising the present analysis. Third, dedicated HFSS media monitoring studies specific to the Republic of Moldova should be undertaken to generate an empirical national evidence base for the regulatory recommendations proposed in this paper. Such studies could assess the volume, reach, and composition of HFSS food advertising across television, digital, and out-of-home channels, providing country-level data that are currently absent from the literature.

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COMPARATIVE ASSESSMENT OF SPICE MIXTURES ON THE OXIDATIVE STABILITY OF WALNUT OIL DURING STORAGE

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Abstract

This study investigated the impact of supplementing walnut oil with spice mixtures on its oxidative stability during six months of storage at 20°C. Three spice mixtures were incorporated into the oil: (1) garlic (Allium sativum) combined with black pepper (Piper nigrum); (2) pistachio (Pistacia vera), pine seed (Pinus pinea), and black pepper; and (3) cinnamon (Cinnamomum zeylanicum) together with vanilla (Vanilla planifolia). Oxidative stability was assessed using peroxide value (PV) for primary oxidation and para-anisidine value (p-AV) for secondary oxidation. In addition, the TOTOX value provides an overall measure of oxidation, reflecting cumulative formation of primary and secondary products, calculated by combining PV and pAV. The enhancement of walnut oil oxidative stability by spice mixtures was directly related to their antioxidant content, with higher levels providing greater protection. Among the mixtures tested, the pistachio, pine seed, and black pepper combination showed the strongest protective effect, delaying lipid oxidation and improving oil stability. These findings demonstrate the potential of spice mixtures as functional additives with antioxidant properties to extend the shelf life and quality of walnut oil.

Key words*: antioxidant compounds, oxidative stability, primary and secondary oxidation, spice mixtures, walnut oil.*

INTRODUCTION

The walnut (*Juglans regia* L.) is the seed of a drupe and is commonly consumed as a nut in various culinary applications. Oil produced by cold pressing walnut kernels at approximately 60°C is appreciated both for its distinctive walnut aroma and for its valuable nutraceutical profile (Anwar et al., 2020).

Because it contains a large proportion of unsaturated fatty acids, walnut oil is highly vulnerable to oxidative processes. Linoleic acid is the dominant component of its fatty acid profile, followed by oleic and linolenic acids. The oil also provides considerable amounts of vitamin E (tocopherols), which contributes to slowing oxidative deterioration (Leahu et al., 2016). In addition to enhancing oxidative stability, naturally occurring antioxidants and their derivatives offer notable health benefits to consumers of vegetable oils (Mishra et al., 2021).

Even with the presence of antioxidants such as polyphenols, vitamin E, and sterols, walnut oil continues to face significant limitations in effectively resisting oxidation.

There are three main categories of oil oxidation: enzymatic oxidation, auto-oxidation and photo-oxidation.

The process of auto-oxidation can be delayed by the addition of antioxidants. Today's, the edible oil and oil-containing products industry primarily utilizes two types of antioxidants, natural and synthetic, to ensure product freshness and stability. The following synthetic antioxidants are well-known: BHA (butylhydroxyanisole), BHT (dibutylhydroxytoluene) and TB-HQ (tert-butylhydroquinone) (Jin et al., 2023).

Natural antioxidants occurring in fruits, vegetables, peels, seeds and various spices have demonstrated strong effectiveness in limiting hydrolysis, polymerization and the development of secondary oxidation compounds (Yildiz et al., 2025). At the same time, the growing consumer preference for "natural" ingredients has encouraged the edible oil industry to increasingly rely on plant-derived antioxidant sources. Over the past two decades, numerous studies have examined the incurporation of pure antioxidants, antioxidant blends and plant extracts into bulk edible oils. These compounds, including tocopherols, flavonoids, phenolic acids, polyphenols, carotenoids, lignans and ascorbate derivatives, have been associated with anti-inflammatory, anti-carcinogenic and anti-atherosclerotic activities (Mishra et al., 2021).

Spices also contain high levels of phenolic compounds and are used in food products to enhance their flavour profile, as well as to provide beneficial bioactive components, thereby increasing their value as functional foods (Somogyi et al., 2015). Except for pine seed (*Pinus pinea*), all spices included in this study are well known to the consumers in our country: garlic (*Allium sativum*), black pepper (*Piper nigrum*), pistachio (*Pistacia vera*), cinnamon (*Cinnamomum zeylanicum*) and vanilla (*Vanilla planifolia*). The genus *Pinus L.* represents the largest and most diverse group within the Pinaceae family, primarily grows throughout the Northern hemisphere. While pine-based products are common globally, they are rarely used as food ingredients. However, research highlights the significant therapeutic benefits of pine preparations for human health (Dziedzinski et al., 2021).

This study examined the improvement of oxidative stability in cold-pressed walnut oil enriched with different dried spices (garlic, black pepper, pistachio, pine seed, cinnamon, and vanilla) during six months of storage at ambient temperature. The progression of lipid oxidation was assessed by determining the peroxide value, the p-anisidine value and the total oxidation value, referred to respectively as PV, p-AV and TOTOX.

MATERIALS AND METHODS

Preparation of walnut oil samples

Walnut oil produced in Romania is commercially available in supermarkets across Timisoara, and this product served as the base material for preparing the spice-enriched walnut oil samples. Three mixtures were added to oil: garlic and black pepper, noted WOS1; pistachio, pine seed and black pepper - WOS2; cinnamon and vanilla - WOS3. Walnut oil was the control sample (C) used for this study. The dry spice mixtures were incorporated into the oil at a proportion of 5:100 (w/v), and the samples were monitored monthly over a six-month storage period at 20°C. Oxidative stability was assessed by determining the peroxide value, the p-anisidine value and the total oxidation value, which together reflect the progression of primary and secondary lipid oxidation.

Chemical analysis

The extraction of bioactive compounds from the oil was carried out by mixing 2 mL of each walnut oil sample with 20 mL of a 70% (v/v) ethanol solution, followed by a 30-minutes rest at room temperature. The mixture was then centrifuged at 5000 rpm for 10 minutes using a Hettich EBA 200 centrifuge, after which the resulting supernatant was collected and used for determining the total antioxidant capacity (TAC), which reflects the sample's overall reducing power, and the total phenolic content (TPC), representing the concentration of phenolic compounds responsible for antioxidant activity.

The total antioxidant capacity was assessed using the FRAP method, which measures the ability of the sample to convert ferric ions into their ferrous form, following the procedure originally introduced by Benzie and Strain (1996). To prepare the working solution, three components were freshly combined: a 10 mM TPTZ solution prepared in 40 mM hydrochloric acid, a 20 mM ferric chloride solution and a 300 mM sodium acetate buffer adjusted to pH 3.6, mixed in a 1:1:10 proportion. Each supernatant sample was diluted tenfold with distilled water, after which 2.5 mL of the FRAP reagent was added.

The reaction mixture was left to stand for 30 minutes at room temperature to allow full color development. Absorbance was then recorded at 593 nm using a SPECORD 205 UV-VIS spectrophotometer (Analytik Jena). The antioxidant capacity was expressed as millimoles of Fe^{2+} per gram of sample.

The total phenolic content was determined using the Folin-Ciocalteu method described by Singleton et al. (1999).

For this analysis, 0.5 mL of each supernatant sample, previously diluted tenfold with distilled water, was combined with 2.5 mL of the diluted Folin-Ciocalteu reagent and 2.0 mL of a sodium carbonate solution at a concentration of 7.5 percent. The mixture was kept at room temperature for 120 minutes to allow the reaction to proceed. Absorbance was then recorded at 750 nm. Gallic acid was used to generate the calibration curve, and the results were expressed as milligrams of gallic acid equivalents per gram of sample, as reported by Gao et al. (2022).

The peroxide value was determined using the classical iodometric procedure commonly applied for evaluating the primary oxidation products formed in edible oils, as described by Ampofo et al. (2022). The results were expressed as milliequivalents of active oxygen per kilogram of oil.

The p-anisidine value was determined to evaluate the secondary oxidation compounds present in the oil. The method is based on the reaction between aldehydes formed during lipid degradation and the amine group of p-anisidine, which produces a colored Schiff base with maximum absorbance at 350 nm. Isooctane served as the reference solution. For the analysis, 2 g of each walnut oil sample were dissolved in 25 mL of isooctane, and the absorbance was recorded at 350 nm. A portion of this solution was then mixed with a p-anisidine reagent prepared in glacial acetic acid, and after a reaction time of 10 minutes, the absorbance was measured again at the same wavelength. The p-anisidine value was calculated following the approach described by Gharby et al. (2021).

The total oxidation value (TOTOX) was determined following the method described by Gharby et al. (2021).

This index was obtained by combining the contribution of primary and secondary oxidation products, being calculated as twice the peroxide value added to the p-anisidine value.

All analyses were performed in triplicate to ensure the reliability of the results. All chemicals and solvents used throughout the experiments were of analytical grade.

Statistical analysis

Origin 9.0 software was used to conduct simple linear regression, correlating storage time with the PV, p-AV and TOTOX values of walnut oils samples with spice mixtures.

Principal Components Analysis (PCA) is a technique for reducing data complexity while preserving its essential features and patterns, without losing significant statistical information (Zakaria, 2025).

RESULTS AND DISCUSSIONS

The evolution of the total antioxidant capacity during storage was monitored for all samples (C, WOS1, WOS2 and WOS3), and the corresponding results are illustrated in Figure 1. The figure includes both the experimental TAC values recorded at each sampling point and the linear regression lines fitted for each sample, which provide a clearer representation of the overall trend over time.

Figure 2 illustrates how the total phenolic content changed during the storage period for the four walnut oil variants (C, WOS1, WOS2 and WOS3).

In addition to the experimental values recorded at each sampling point, the figure also includes the fitted regression lines for each sample, which help visualize the overall trends and the way phenolic compounds evolved over time.

The regression analysis describing the temporal evolution of the WOS3 sample showed R^2 values of 0.9694 for TAC and 0.9304 for TPC. Adding dry spices to walnut oil has been shown to enrich its bioactive compound profile.

In contrast, the WOS2 sample showed lower correlations over time, with R^2 values of 0.3723 for TAC and 0.4432 for TPC.

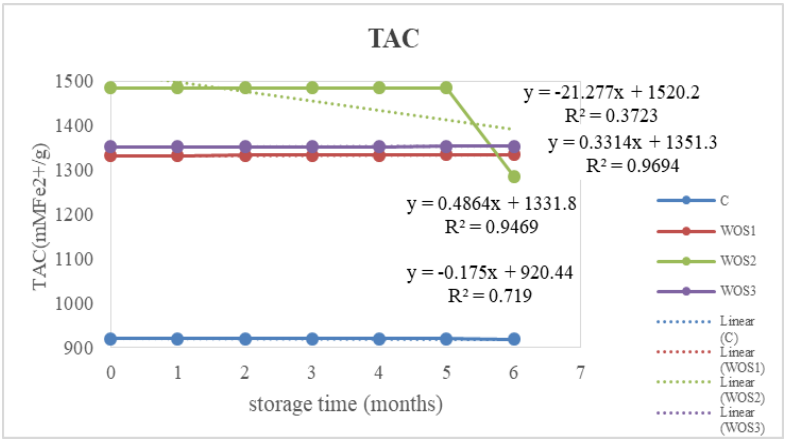


Figure 1. Evolution of the total antioxidant capacity of walnut oil samples during storage, together with the fitted linear regression lines

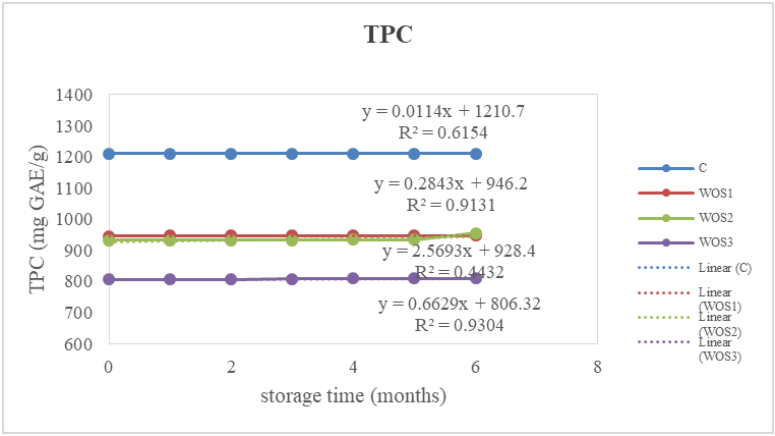


Figure 2. Evolution of the total phenolic content of walnut oil samples during storage, together with the fitted linear regression lines

The oxidative stability of oils is commonly used to estimate their shelf life, defined as the time required for the peroxide value to reach 10 mEq O_2/kg (Moslovac et al., 2023). Previous studies (Leahu et al., 2016; Masoodi et al., 2025) indicate that fresh walnut oil typically presents very low peroxide values, ranging from 0.4 to 0.83 mEq O_2/kg, which increase during storage, particularly over several months. According to the American Oil Chemists' Society standard methods (1997), the peroxide value of fresh oils should remain below 10 mEq O_2/kg. In contrast, oils with a p-anisidine value exceeding 30 mEq O_2/kg are considered rancid (Rebufa et al., 2022). Based on peroxide value measurements, Nosenko et al. (2019) reported that walnut oil maintains stability for approximately 56 days (induction period) and reaches the end of its shelf life after around 90 days.

In Figure 3, the evolution of the peroxide value of walnut oil samples during storage is shown together with the corresponding linear regression lines. After six months of storage (180 days), the walnut oil in our study reached a peroxide value of 2.5 mEq O_2/kg, indicating that the oil maintained a low level of primary oxidation throughout the storage period. This behavior is consistent with previous findings showing that the oxidative stability of walnut oil is influenced by its phenolic and tocopherol content, which varies among different walnut varieties (Rebufa et al., 2022; Moslovac et al., 2023).

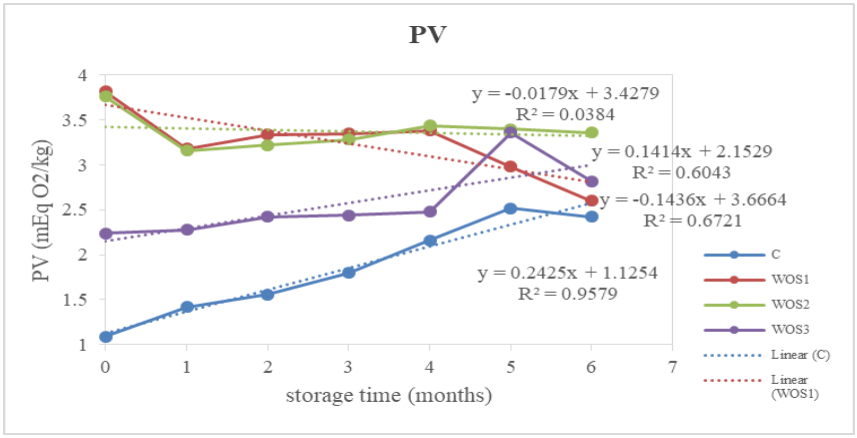


Figure 3. Evolution of the peroxide value of walnut oil samples during storage, together with the fitted linear regression lines

Figure 4 illustrates the evolution of the p-anisidine value of the walnut oil samples during storage, together with the fitted linear regression lines. With an R^2 value of 0.9632, the WOS2 sample shows that the linear model provides a good fit for the temporal evolution of p-AV. After six months of storage, the p-AV reached 1.01 for WOS1, a lower value compared to WOS3, which recorded 1.44

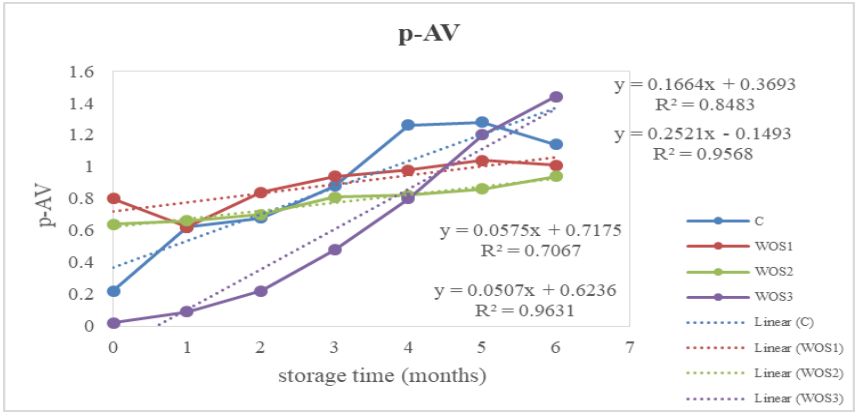


Figure 4. Evolution of the para-anisidine value of walnut oil samples during storage, together with the fitted linear regression lines

Figure 5 presents the evolution of the TOTOX value of the oil samples during storage, together with the fitted linear regression lines. The TOTOX value is an empirical indicator that reflects the overall oxidation state of an oil, obtained by combining two oxidation parameters: PV (specific to primary oxidation) and p-AV (specific to secondary oxidation). A lower TOTOX value is associated with better oil quality (Popovici & Deseatnicova, 2013).

The findings obtained from the oxidative stability indices showed strong consistency, indicating that all parameters related to lipid oxidation responded similarly to the oxidative changes occurring during storage.

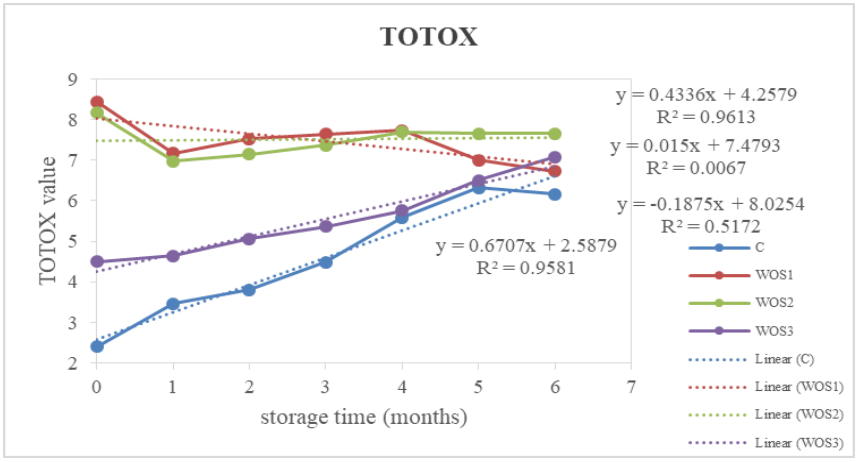


Figure 5. Evolution of the TOTOX value of walnut oil samples during storage, together with the fitted linear regression lines

This coherence highlights the protective effect of the spice mixtures incorporated into the walnut oils, which acted as natural antioxidants and contributed to slowing down both primary and secondary oxidation processes. Figure 6 shows the PCA biplot of antioxidant parameters (TAC, TPC) and oxidative markers (PV, p-AV, TOTOX).

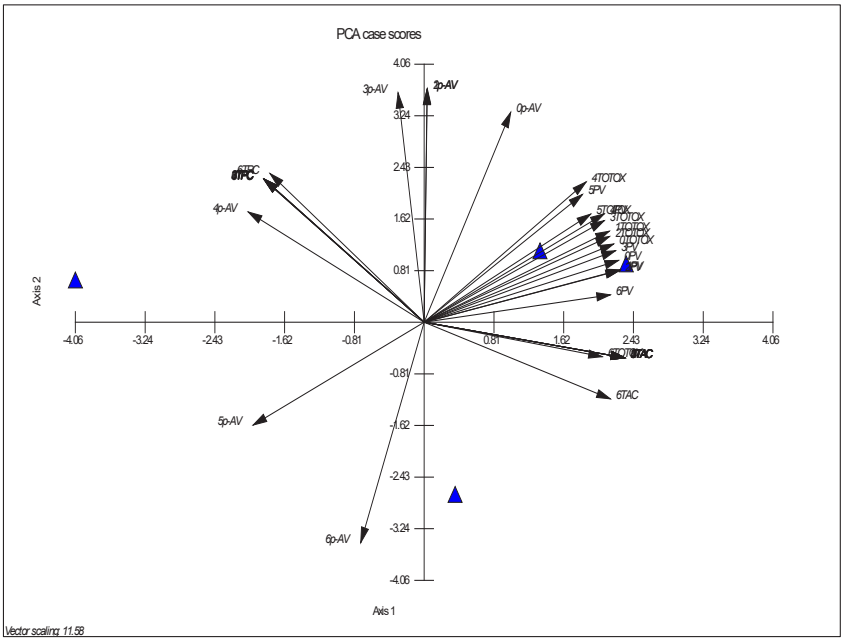


Figure 6. PCA biplot of antioxidant and oxidative markers

Legend: 0TAC- total antioxidant capacity at initial time, 1TAC- total antioxidant capacity after 1 month at storage, 2TAC- total antioxidant capacity after 2 months at storage, 3TAC- total antioxidant capacity after 3 month at storage, 4TAC- total antioxidant capacity after 4 month at storage, 5TAC- total antioxidant capacity after 5 month at storage, 6TAC- total antioxidant capacity after 6 month at storage; idem for TPC, PV, p-AV and TOTOX value.

Principal Components Analysis (PCA) applied to standardized data square-root transformed shows the following: the PCA biplot clearly separated antioxidant parameters (TAC, TPC) from oxidative markers (PV, p-AV, TOTOX),

confirming distinct biochemical responses during storage.

WOS2 demonstrated a delayed oxidative pattern, suggesting a protective effect on lipid stability, while WOS3 exhibited a rapid transition toward the oxidative zone, indicating limited storage suitability. p-AV and TOTOX were identified as the most sensitive predictors of oxidative deterioration (Figure 6).

The heatmap generated from standardized values (Figure 7) illustrates the associations between the oxidative and antioxidant parameters. Based on the heatmap with standardized data, we observe two clear clusters.

Cluster 1: TAC and TPC which show high intensity which is equivalent with strong antioxidant capacity.

Cluster 2: PV, p-AV, TOTOX, which show low intensity which is equivalent with oxidative degradation; WOS2 shows intermediate-high stability, but degradation begins after month 4-5; WOS3 shows the lowest phenolic stability and highest oxidation risk (late months). Control sample (C) remains chemically predictable; is ideal as a kinetic reference.

The heatmap clearly separates functional antioxidant behavior (TAC/TPC) from oxidative stress markers, confirming different metabolic pathways during storage.

The findings from the oxidative stability indices were in good correlation with each other, showcasing the impact of the spice mixtures that were present in the walnut oils about oxidative stability.

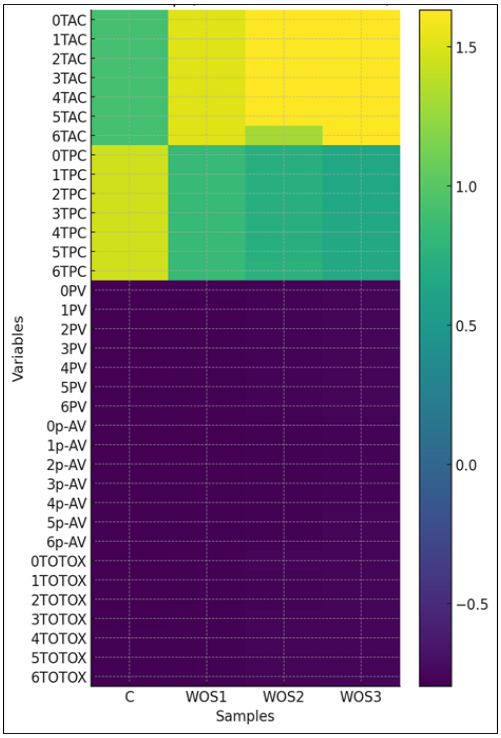


Figure 7. Heatmap based on standardized values

CONCLUSIONS

Our findings provide strong evidence that dried spice mixtures act as effective natural antioxidants in cold-pressed walnut oil. Pistachio, pine seed, and black pepper showed the strongest protective effect, significantly delaying lipid oxidation and improving overall stability. By enhancing resistance to oxidative damage during storage, these natural additives help maintain product quality and may offer additional health benefits. Multivariate analyses supported these results, distinguishing antioxidant parameters from oxidative markers

and identifying p-AV and TOTOX as the most sensitive indicators of deterioration. Overall, this study highlights the potential of spice mixtures as clean-label, functional ingredients capable of extending the shelf life and technological value of walnut oil.

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THE IMPACT OF EGGPLANT, ZUCCHINI, AND APPLE PEEL POWDER ADDITION ON THE PROXIMATE COMPOSITION, POROSITY AND SENSORY PROFILE OF SAVORY MUFFINS

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Abstract

Fruit and vegetable peels contain a variety of health-beneficial nutrients and serve as excellent nutritional sources. Their utilization contributes to the reduction of food waste, having a positive impact on both the environment and the economy. This article presents a study on the advanced valorization of apple, zucchini, and eggplant peels for the development of savory muffins. Four types of savory muffins were formulated using apple (MA), zucchini (MZ), eggplant (ME) peel flours, and control (MC). These were subjected to sensory evaluation and physicochemical analysis. The MA, MZ, and ME muffins demonstrated a high degree of acceptability. Proximate composition analysis revealed that the ME variant had the highest protein content. MA muffins recorded the highest levels of carbohydrates and dry matter, as well as the highest acidity. MZ and ME muffins had a higher salt content, while the ME variant exhibited the best porosity.

Key words*: acidity, antiradical activity, moisture, muffins, peel powder, porosity.*

INTRODUCTION

Globally, the massive generation and deficient management of waste resulting from the post-harvest processing of horticultural products exert considerable pressure on waste management systems, simultaneously generating negative environmental externalities and public health risks (Sharma et al., 2022). In this context, the valorization of residual fractions and by-products from the fruit and vegetable industry constitutes a fundamental pillar of sustainable agri-food systems, offering strategic opportunities for value-added generation. Implementing circular economy principles requires the adoption of integrated and collaborative strategies among value chain actors to reintegrate these resources into the productive circuit (Geissdoerfer et al., 2017; Kumar et al., 2024). The fruit and vegetable processing industry, a critical component of the agri-food sector, generates significant volumes of residual biomass throughout the technological flow, from cultivation and harvesting phases to conditioning and packaging. These flows include lignocellulosic plant remains (leaves, stems, roots), as well as specific processing by-products: peels, seeds, pits, exhausted pulp, and other organic materials. Although traditionally classified as waste, current scientific evidence (FAO, 2019) highlights their high bioconversion potential, transforming them into essential resources for the transition toward a sustainable food system. Optimizing the management of these flows not only mitigates the ecological footprint by reducing landfill disposal but also facilitates the procurement of high commercial value products, such as functional food ingredients, energy biomass, bio-fertilizers, and chemical

precursors (Kumar et al., 2021). Under the pressure of climate change and global demographic growth, the valorization of horticultural by-products becomes an imperative necessity, offering multidimensional benefits. Thus, food waste is minimized by reintegrating by-products into the consumption chain, optimizing the material balance and reducing net biomass losses, while generating added value and economic innovation by transforming residues into biocompounds with applications in the food, pharmaceutical, and cosmetic industries (Chemat et al., 2017), thereby stimulating economic growth. For example, the peels of certain fruits represent dense sources of dietary fiber and polyphenolic antioxidants with demonstrated benefits for human health. The integral valorization of biomass reduces dependence on virgin raw materials and optimizes resource consumption associated with primary production. Furthermore, substituting the linear model with a circular one diminishes greenhouse gas (GHG) emissions, prevents soil and water resource contamination, and reduces specific energy consumption related to waste management (D'Adamo et al., 2022).

Plant by-products are defined as morphological parts and fractions derived from primary agri-food production processes. As global fruit and vegetable production intensifies, the recovery of nutrients and bioactive compounds from these matrices has become a subject of maximum interest in biochemical and nutritional research (Tsegay et al., 2024). Valorization methodologies are diverse, including composting (Soltan et al., 2019), biogas production, extraction of bioactive compounds (Călinoiu & Vodnar, 2018), and the development of functional food ingredients (Mirabella et al., 2014).

Despite evident benefits, the valorization process faces critical barriers, such as material composition heterogeneity, technological costs, and infrastructure limitations. Nevertheless, the current context offers unprecedented opportunities through advances in biotechnology and the increasing demand for sustainable products. These initiatives align with the objectives of the European Green Deal and the Circular Economy Action Plan, which impose strict targets on member states for reducing landfilled biodegradable waste and promoting long-term sustainability. Given that the Green Deal places immense emphasis on transforming waste into resources, a strategic component of this endeavor is the valorization of peels, which represent excellent reservoirs of polyphenols, antioxidants, dietary fibers (pectins, cellulose), and essential micronutrients. The recovery of these compounds not only minimizes organic waste volume but also opens new horizons for innovative industrial applications. Thus, transforming peels from „residue" into „secondary raw material" becomes an essential case study for the resilience of the entire agri-food value chain. The valorization of fruit and vegetable peels represents a critical opportunity to recover essential nutrients that are traditionally eliminated from the food chain. These matrices are concentrated sources of fiber, vitamins, minerals, and phytochemicals, whose reintroduction into the consumption circuit has a positive multidimensional impact on the environment and the economy.

Apple peel (*Malus domestica*) constitutes an exceptional natural source of bioactive compounds, with concentrations of phenolic compounds, fiber, and minerals significantly higher than the fruit pulp (Henríquez et al., 2010a). A particularly relevant aspect is the presence of quercetin, a flavonoid with recognized anti-inflammatory and anti-allergic properties. Fundamental research by Wolfe and Liu (2003) demonstrated that waste from the apple puree industry can be transformed into a valuable food ingredient in powder form. The study highlighted the importance of preservation techniques; blanching treatments for 10 seconds proved most effective in preserving the phenolic profile. Through lyophilization and air drying, a powder with very strong antioxidant activity was obtained: 1 g of powder has an antioxidant potential equivalent to 220 mg of vitamin C. Physiologically, the soluble and insoluble fibers in apple peel regulate intestinal transit, prevent constipation, and favor a balanced probiotic environment. Additionally, the intake of B-complex vitamins (B_6) and vitamin C supports energy metabolism and protects cells against oxidative stress (Boyer & Liu, 2004).

Zucchini peel (*Cucurbita pepo*) is appreciated for its low caloric value; however, its peel, representing approximately 10% of the fruit's mass, concentrates the majority of nutrients. Compositional studies reveal major differences between tissues. Thus, the peel presents double the concentrations of soluble solids (Brix degrees) compared to the core. The level of total flavonoids is 59.06 mg CE/100 g in the peel, and antioxidant capacity is 159.13 µmol TEAC/100 g (Gomes et al., 2023). Nutritional profile (per 100 g fresh matter): 2.5% fiber, 1.41% protein, and a diversified vitamin intake (Vitamin C – 15.1 mg, Vitamin A, B_1, and B_2) (Li et al., 2021).

Eggplant peel (*Solanum melongena*) is recognized for its high anthocyanin content, particularly nasunin. This powerful antioxidant protects lipids in brain cell membranes and has demonstrated anti-cancer properties (Noda et al., 2000). The antioxidant activity is 250.94 µmol TEAC/100 g, with a polyphenol content between 51-63 mg tannic acid/g DM (Gomes et al., 2023). The presence of chlorogenic acid and vitamin K contributes to reducing inflammation and supports bone and vascular health.

To transform these by-products into high-quality secondary raw materials, such as functional flours, the contemporary industry implements state-of-the-art extraction techniques that maximize nutrient recovery. Among these, ultrasound- or microwave-assisted extraction plays a crucial role, allowing efficient isolation of polyphenols without causing thermal degradation, while encapsulation technologies are used to protect sensitive compounds and ensure their controlled release in finished products. Moreover, directed fermentation processes, carried out through the action of specific microorganisms, contribute significantly to enhancing the bioavailability of bioactive compounds, thus optimizing the nutritional profile of the resulting products.

Beyond technical benefits, the valorization of fruit and vegetable peels represents a strategic move for economic efficiency, surpassing simple ecological responsibility. By transforming waste into value-added products, production units manage to considerably reduce logistic costs associated with organic residue management and disposal. Simultaneously, this approach generates new revenue streams by accessing market niches dedicated to functional foods and „clean label" products. Ultimately, adopting these innovations strengthens the competitiveness of agri-food companies and reduces dependence on virgin raw materials, facilitating the transition toward a circular, sustainable, and resilient production cycle in the long term.

In this context, the present study aims to incorporate powders obtained from apple, zucchini, and eggplant peels into the recipe for savory muffins, evaluating their impact on proximate properties, porosity, acidity, scavenging capacity and the sensory profile.

MATERIALS AND METHODS

Raw materials and ingredients

The raw materials used in the manufacturing process were selected to ensure a high degree of reproducibility and food safety. The basic ingredients required for the preparation of the control sample (Control) and the fortified variants were purchased from the local commercial network: white wheat flour (type 000), fresh eggs, sunflower vegetable oil, milk (1.5% fat), telemea cheese (traditional Romanian brined cheese), green onions, fresh dill, and a leavening agent (baking powder). Particular attention was paid to the plant materials intended for fortification, represented by the peels of apple (*Malus domestica*), zucchini (*Cucurbita pepo*), and eggplant (*Solanum melongena*). The raw material was selected exclusively from the category of products originating from organic farming (BIO certified), a critical condition in this study to guarantee the absence of pesticide residues and other chemical contaminants that tend to accumulate predominantly in the outer layers of fruits and vegetables.

Production of functional flours from horticultural by-products

The transformation of horticultural by-products into secondary raw materials (functional flours) was carried out through a rigorous preliminary processing protocol, designed to maximize the preservation of bioactive compounds. The process was structured into several technological stages:

Sanitization and preparation. Apple, zucchini, and eggplant samples were subjected to rigorous washing with deionized water to ensure the complete removal of mechanical impurities and potential surface microorganisms.

Mechanical separation. The peeling of the samples was performed manually using stainless steel utensils to minimize the risk of enzymatic oxidation of phenolic compounds upon contact with air and metallic surfaces (Figure 1).



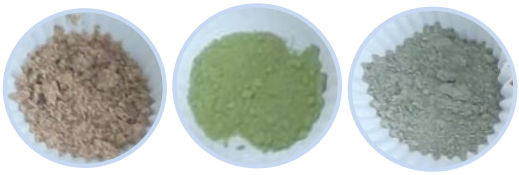
Figure 1. Cleaning of apple, zucchini, and eggplant peel (original source)

Controlled dehydration. The plant material was subjected to controlled dehydration in a professional device (Figure 2) at a constant temperature of 45°C. This thermal treatment lasted between 12 and 24 hours, depending on the specific moisture content of each matrix. Maintaining such moderate conditions was critical to prevent the degradation of thermolabile fractions and to preserve antioxidant stability. The process was rigorously monitored until reaching a residual moisture threshold of 15%, ensuring both microbiological and mechanical stability for subsequent processing.



Figure 2. Dehydration of apple, zucchini, and eggplant peel (original source)

Grinding and sorting. Subsequently, the dehydrated plant material underwent a fine grinding process using a laboratory mill, resulting in three types of homogeneous functional flours (Figure 3). To prevent the degradation of bioactive compounds, these were stored in hermetically sealed amber glass containers, thus ensuring protection against oxidative factors, light, and ambient humidity until their incorporation into the muffin matrix.



Apple peel flour (APF) | Zucchini peel flour (ZPF) | Eggplant peel flour (EPF)

Figure 3. Apple, zucchini, and eggplant peel flour (original source)

Manufacturing technology of the functional muffins

The manufacturing process followed a standardized technological flow, designed to ensure sample reproducibility and the stability of the food matrix. The experimental design included a control sample (Control) and three fortified (experimental) variants, developed by partially substituting wheat flour with functional flours derived from apple, zucchini, and eggplant peels at a ratio of 3% (w/w). The preparation protocol was structured into successive homogenization stages:

Preparation of the protein-lipid base. The process began by mixing the eggs and salt, followed by the incorporation of the liquid phase (milk and vegetable oil), ensuring the formation of a stable emulsion.

Formation of the structural matrix. The solid fraction, consisting of wheat flour, the leavening agent, and the specific functional flour for each sample, was gradually integrated under continuous mechanical stirring. This stage was monitored until a dough with specific rheological properties (dense-elastic texture) was obtained, ensuring a uniform distribution of fibers within the gluten network.

Incorporation of texture ingredients. In the final mixing phase, *telemea* cheese and aromatic ingredients (green onions and fresh

dill) were added, maintaining their structural integrity.

Thermal treatment (baking). The dough, portioned into standardized molds, was subjected to a baking process under controlled conditions at a temperature of 180°C for 25-30 minutes. This standardization was essential to ensure sample uniformity and to allow for an objective evaluation of physicochemical indicators, such as porosity, and sensory attributes.

Analysis methods for raw materials and finished products

Sensory evaluation

The sensory profile of the fortified muffins was determined using the intensity profile method, conducted by a panel of regular consumers (untrained tasters) to simulate actual market perception. The evaluation focused on several critical sensory attributes: general appearance, color, crumb consistency (texture), odor, taste, and flavor persistence (after-taste). A 5-point hedonic scale was employed for quantification, where: 1 represented minimum intensity/low acceptability and 5 indicated superior quality/ maximum acceptability. The data were aggregated to establish the comparative sensory profile of the samples. Overall acceptability represents the mean of all sensory attributes and constitutes the decisive indicator for the market potential of the fortified muffins.

Determination of crumb porosity

Crumb porosity was determined using the volumetric method, a parameter that indicates the ratio between the volume of interstitial spaces (pores) and the total volume of the crumb sample. The experimental procedure involved extracting three crumb cylinders from the central area of each sample using a punch of known geometry and dimensions. The porosity calculation was based on determining the specific mass of the compact crumb using the following mathematical relationships:

$$Porosity, \% = \frac{V_p - V_{cc}}{V_p} \cdot 100$$

$$V_{cc} = \frac{m}{\rho}$$

Where:

V_p - volume of the cylinder containing pores (cm^3); V_{cc} - volume of the compact crumb (pore-free, cm^3); m is the mass of the sample (g); ρ - density of the compact crumb, experimentally established at 1.26 g/cm^3.

This methodology allowed for the evaluation of how the addition of fruit and vegetable peel flours influenced the alveolar structure of the muffins, providing a quantitative basis for the structural changes observed in the fortified samples.

Determination of total acidity

The total acidity of the bakery products was considered a quality parameter, representing the sum of free acids and acid-reacting compounds formed during the fermentation and baking processes. The method was based on the neutralization of acids within the aqueous extract of the analyzed sample by titration with a 0.1 N sodium hydroxide solution. Phenolphthalein was used as a pH indicator. The endpoint of the titration was identified by the appearance of a faint pink coloration that persisted for at least 30-60 seconds. Acidity was expressed in acidity degrees, where one degree represented the acidity in 100 g of sample that was neutralized by 1ml 1 N NaOH solution. The following formula was used for the calculation:

$$Acidity\ (degrees) = \frac{V \cdot 0{,}1}{m} \cdot 100$$

Where:

V - volume of 0.1N NaOH solution used for titration (ml); m - mass of the sample taken for analysis (g); 0.1 - the normality factor of the sodium hydroxide solution; 100 - conversion factor to report the result per 100 g of product.

Proximate chemical analysis and nutritional evaluation

The proximate composition of the muffins was determined to establish their nutritional profile and to evaluate the impact of functional flour incorporation. This analysis included the determination of moisture, minerals (as salt content), proteins, lipids, and carbohydrates.

The crude chemical composition (proteins, lipids, and carbohydrates) and the total energy value were estimated using a theoretical calculation method. This involved integrating the nutritional data for each ingredient from standardized databases (nutritionvalue.org). Results were expressed per 100 g of finished

product, and the energy value was calculated using the Atwater conversion factors.

As a fundamental component of the proximate analysis, the water content was determined using the standard gravimetric method. Muffin samples were finely homogenized using a mortar and pestle. Approximately 5 g of each sample was weighed and dried in a thermostatic oven at 105 ± 2°C until a constant mass was achieved. The moisture content was calculated based on the weight loss during heating:

$$Moisture(\%) = \frac{m_1 - m_2}{m_1 - m_0} \cdot 100$$

Where:

m_0 represents the tare of the empty vessel; m_1 is the initial mass of the vessel containing the homogenized muffin sample; m_2 is the final mass recorded after oven-drying at 105 ± 2°C until a constant weight was achieved.

All determinations were performed in triplicate to ensure data precision.

The salt content (NaCl) was quantified through direct argentometric titration according to the Mohr method. Chloride ions were extracted into an aqueous phase and titrated with 0.1 N silver nitrate ($AgNO_3$) in the presence of potassium chromate (K_2CrO_4) as a pH - sensitive indicator. The process was conducted within a strictly controlled pH range (6.5 - 8.5). The final NaCl percentage was calculated by correcting the titrant volume with a blank sample and relating it to the initial sample mass. The sodium chloride content (NaCl, %) was calculated using the following formula:

$$NaCl, \% = \frac{(V_{AgNO3} - V_{blank}) \cdot N_{AgNO3} \cdot M_{NaCl} \cdot \frac{V_{extract}}{V_{aliquot}}}{m_{sample}} \cdot 100$$

Where:

V_{AgNO3} volume of silver nitrate solution used for sample titration (ml); V_{blank} volume of silver nitrate solution used for the blank titration (ml);

N_{AgNO3} normality of the silver nitrate solution (typically 0.1 N); M_{NaCl} milliequivalent weight of sodium chloride (0.05844 g/meq); $V_{extract}$ total volume of the initial aqueous extract (ml); $V_{aliquot}$ volume of the extract taken for titration (ml); m_{sample} mass of the muffin sample taken for analysis (g).

All determinations were performed in triplicate.

Methodology for determining antioxidant inhibition (DPPH Assay)

The antioxidant capacity of the functional flours (apple, zucchini, and eggplant) was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay. The principle of this method relies on the ability of bioactive compounds to reduce the stable DPPH radical, resulting in a colorimetric change from deep purple to yellow. Hydroalcoholic extracts were prepared from the functional flour samples. Due to the high concentration of bioactive constituents, the final extracts were subjected to a 1:100 (v/v) dilution in 70% ethanol to ensure that absorbance readings remained within the linear range of the spectrophotometer. The reaction system was set to a final volume of 3.2 ml. To ensure optimal interaction between the reactant species, the components were added into the reaction cuvettes according to the following specific sequence: 0.3 ml of diluted extract, 0.3 ml of DPPH radical solution (1 mM concentration) and 2.6 ml of 70% ethanol (solvent). Following the sequential addition, the mixture was thoroughly homogenized. The samples were then kept in the dark at room temperature for 30 minutes to allow the radical scavenging reaction to reach equilibrium. The absorbance of the samples (A_{sample}) was recorded at 517 nm using a UV-VIS spectrophotometer. A DPPH stock solution was used as a control (blank), prepared using the same addition sequence but replacing the extract with the solvent. The antioxidant activity was expressed as a percentage of inhibition (I%), calculated as follows:

$$I, \% = \frac{A_{blank} - A_{sample}}{V_{blank}} \cdot 100$$

All measurements were performed in triplicate, and the results were expressed as the mean value of the determinations.

RESULTS AND DISCUSSIONS

The sensory analysis of the fortified muffins

The sensory characteristics of the muffins were evaluated to determine the impact of the

functional ingredients on the final product's quality.

External appearance. The muffins exhibited a well-defined, rounded shape with a slightly domed top and a stable base. The crust was thin, crisp, and slightly firm to the touch, featuring a fine texture. The color profile ranged from light golden-green to golden-brown on the surface and edges, indicating uniform baking. Occasional darker spots were observed due to the caramelization or melting of the cheese on the surface. Fresh green dill specks and lightly browned cheese fragments were clearly visible on the exterior.

Cross-sectional appearance. The cross-section (Figure 4) reveals a slightly moist and aerated crumb with a uniform yellowish-cream hue, punctuated by subtle green inclusions.



Figure 4. Apple, zucchini, and eggplant peel muffins (original source)

The internal structure exhibits a well-developed medium-to-high porosity, indicating a soft and elastic matrix without dense or gummy zones. Cheese fragments are clearly visible and evenly distributed, contributing to the overall structural complexity, while the darker nuances in certain areas mark the optimal integration of the ingredients into the dough.

Color. Sample MA achieved the highest score for color (4.77), while the Control sample (C) obtained a lower score (4.57). These results are in agreement with those reported by Henríquez et al. (2010b), demonstrating that natural pigments from apples increase visual appeal.

Aroma. The aroma was of moderate to high intensity, characteristic of freshly baked pastry. The profile was dominated by the distinctive, sweet-herbal scent of fresh dill, which was well-balanced by the lactic, slightly acidic, and salty notes of the cheese. Subtle undertones of baked dough (cereal and egg) were also perceived, with no off-odors (such as rancid or burnt notes) detected.

Flavor. The taste was rich and savory, providing a harmonious balance between the key ingredients. It featured specific notes of salty, creamy, and slightly tangy cheese, complemented by the herbal and fresh anise-like flavor of the dill. The dough provided a neutral to slightly sweet base that integrated the aromatic notes perfectly. Salt levels were optimal, enhancing the overall flavor profile without being overpowering. The integration of apple peel powder resulted in a higher score for the fortified products compared to the control. Similar results were also reported by Alongi et al. (2019).

Texture and after-taste. The texture was described as crisp on the outside while remaining light and tender on the inside. The after-taste had a pleasant, medium persistence, leaving a fresh sensation of dill and a discrete salty-lactic note from the cheese.

The sensory evaluation, synthesized through the spider plot (Figure 5), provided a detailed perspectiveon the impact of partially replacing wheat flour with functional powders derived from vegetable by-products (apple, zucchini, and eggplant peels) on consumer perception. The control sample served as a benchmark for structural stability, achieving maximum scores for external appearance (4.8) and section appearance (4.8). These results confirm that the classic muffin meets the traditional visual standards of consumers. However, the control sample recorded the lowest score for the taste parameter (4.08), being perceived as having a less complex aromatic profile compared to the fortified variants.

The MA variant emerged as the favorite of the tasting panel, achieving an optimal harmony between innovation and acceptability. It obtained the highest score (4.77), suggesting that the natural pigments in the apple peel conferred an attractive, warm golden hue to the product. With a score of 4.57, the MA sample outperformed the control (4.44), demonstrating that the addition of apple by-products enhances the overall sensory profile through aromatic complexity. The high score for after-taste (4.5) indicated a pleasant balance between native sugars and the organic acidity of the apple. Muffins fortified with zucchini and eggplant

presented acceptability scores very close to the control, demonstrating the feasibility of integrating these by-products without negatively altering sensory perception.

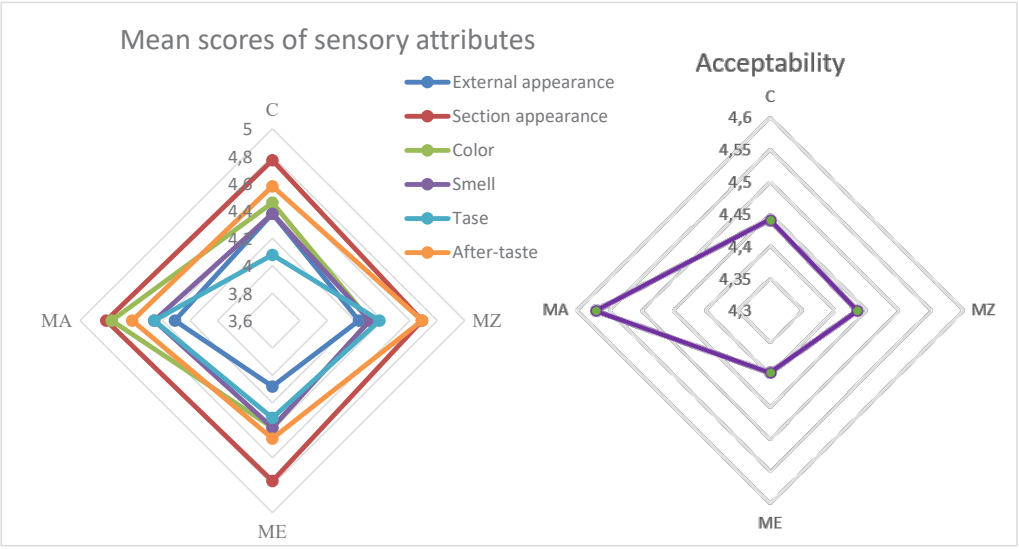


Figure 5. Sensory profile of the muffins

The MZ sample stood out for its balanced profile, with high scores for after-taste (4.5) and section appearance (4.5). The greenish nuances were perceived by consumers as indicators of "freshness" and a "natural product".

Although it recorded slightly lower scores for external appearance (4.3) and smell (4.3) due to darker pigments, the ME sample excelled in internal structure (4.6).

A key conclusion of the study is that all samples with added functional powders (MZ: 4.38; ME: 4.31; MA: 4.46) were rated superior to the control sample in terms of taste. This observation suggests that the fibers and compounds within the peels not only increase biological value but also appear to enhance the savory flavors (cheese and green onions) within the product matrix. Furthermore, the high scores for after-taste (above 4.46 for all samples) confirmed the absence of residual bitter or astringent tastes, which are often associated with the use of vegetable by-products in bakery applications.

Sample MA achieved the highest overall acceptability score (4.57), significantly outperforming the control sample (4.44). This suggests that the addition of apple peel is not only a method for increasing nutritional value but also enhances the global sensory profile, as the product was perceived to have a more complex aroma and superior aesthetics (due to the golden hues).

Samples MZ and ME recorded acceptability scores nearly identical to the control sample (approximately 4.45). This observation is crucial for the food industry: it demonstrates that by-products such as zucchini or eggplant peels can be integrated into muffins without negatively altering consumer perception, while maintaining the quality standards of the traditional product.

Our sensory results align with the findings of Henríquez et al. (2010a), Valková et al. (2022), who reported that apple peel inclusion significantly enhances the golden hue and overall appeal of bakery items. Furthermore, the high acceptability scores for zucchini and eggplant variants (MZ and ME) are consistent with the research of Lucera et al. (2012) and Mirani & Goli (2021), proving that these by-products can be successfully upcycled into savory matrices without compromising consumer perception. The absence of bitter notes in the after-taste (scores > 4.46) further validates the use of a gentle drying process at 45°C, as suggested by literature for preserving thermolabile aromatic compounds.

Muffin porosity results

The crumb porosity results reflect the direct impact of partially replacing wheat flour with fruit and vegetable peel powders on the alveolar architecture of the muffins. In the baking industry, porosity is a critical quality indicator, directly influencing texture and the consumer's perception of ”freshness”. The porosity values obtained for the analyzed muffins ranged between 52.82% and 53.14% (Figure 6).

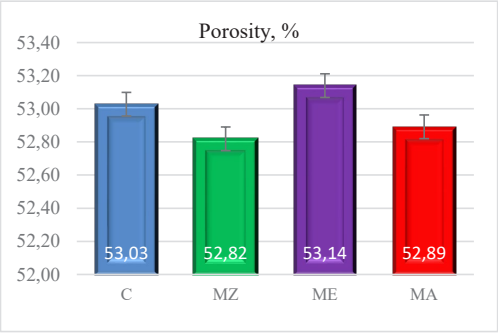


Figure 6. Muffin porosity

Although these values are lower than the porosity of a classic white bread (which frequently exceeds 62%), they fall perfectly within the standards for muffins or savory muffins, where the optimal range reported in the literature is 45-55%.

The control sample (C) exhibited a porosity of 53.03%, indicating an optimal gas-holding capacity and a well-developed gluten network. The ME sample recorded the highest value (53.14%). This phenomenon suggests that the fibers in the eggplant peel may act as stabilizers for the gas bubbles, preventing the network from collapsing during baking - an observation supported by the research of Lucera et al. (2012) and Mirani & Goli (2021). Samples MZ (52.82%) and MA (52.89%) showed minor decreases compared to the control. This trend is explained by the mechanical interference of the fibers within the protein matrix, partially reducing the dough's elasticity, as reported by Stojceska et al. (2009) in studies regarding fortification with vegetable by-products.

The minimal variations in porosity between samples (less than 0.3%) demonstrate an excellent integration of the functional powders into the muffin matrix. The uniform, yet relatively dense, alveolar structure is the result of two key factors: lipid intake and the presence of solid ingredients. The presence of oil and telemea cheese creates a ”shortening effect” on the gluten network, resulting in smaller alveoli and a more tender texture, according to studies by Alongi et al. (2019). Furthermore, the spring onion fragments and dill specks act as physical barriers to large alveolar expansion, favoring a fine and homogeneous porosity.

The crumb density, correlated with a porosity of approximately 53%, ensured high structural integrity, avoiding the formation of dense or gummy areas. This physical stability explains the superior sensory scores obtained for section appearance (4.6-4.8), confirming that the muffins maintained their aerated and ”soft and fluffy” character despite the addition of fibers.

Results regarding the titratable acidity of muffins

Titratable acidity is an essential physicochemical parameter that directly influences both the sensory properties (taste, freshness) and the technological characteristics (activation of leavening agents) of pastry products. The values obtained for the analyzed muffin batches ranged between 6.1 and 6.4 degrees of acidity (Figure 7).

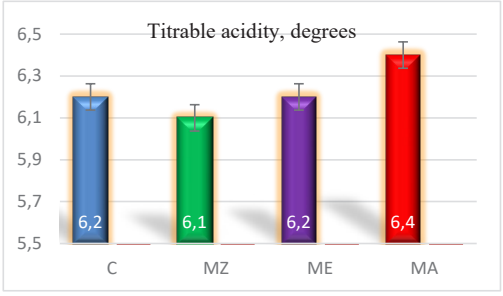


Figure 7. Titrable acidity

The comparative analysis of the samples reveals specific variations in acidity depending on the type of powder used; the MA (Apple) sample recorded the highest value of 6.4 degrees, a fact attributed to the presence of natural organic acids in the apple peel (primarily malic acid), which supplements the base acidity of the dough. In contrast, the Control sample (C) and the ME sample

presented identical values of 6.2 degrees, indicating that the addition of eggplant peel powder does not significantly alter the acid balance of the matrix compared to the standard recipe. At the opposite end, the MZ sample showed the lowest value of 6.1 degrees, suggesting either a slight acid-buffering capacity of the compounds in zucchini or a lower content of free acids in this specific by-product. A titratable acidity level within this range is considered optimal for savory pastry products utilizing fermented ingredients, such as telemea cheese. From a technological perspective, acidity plays a crucial role in activating sodium bicarbonate and baking powder. The resulting acid-base reaction releases carbon dioxide, the gas responsible for the superior porosity observed in our samples (52.82-53.14%).

According to the literature (Stojceska et al., 2009), an adequate level of acidity ensures an aerated alveolar structure, preventing the formation of a dense crumb. Furthermore, these acids enhance the flavor profile by potencying the lactic notes of the cheese and the freshness of the dill and green onions. In the case of the MA sample, the higher acidity aligns with reports by Alongi et al. (2019), who emphasize that fruit by-products can increase the concentration of organic acids, improving both the taste and the microbiological stability of the finished product. This optimal acidic balance explains the maximum sensory scores obtained for taste and overall acceptability, as it provides a necessary contrast to the richness of the lipids from the oil and cheese.

The moderate and constant range of acidity confirms that the partial replacement of flour with peel powders does not disturb the biochemical balance of the dough. On the contrary, the addition of apple peel powder (MA) optimizes acidity, contributing to an efficient release of fermentation gases and resulting in a fluffy muffin with a well-developed cellular structure, as confirmed by the porosity and moisture analyses.

Proximate chemical composition of muffins

The chemical composition analysis highlights the impact of fortification with vegetable by-product powders on the nutritional profile of the muffins. It is important to specify that while protein, carbohydrate, and lipid contents were calculated using specialized nutritional databases, the moisture and salt levels were both determined through experimental analysis. These experimental data were integrated to provide a comprehensive and verified overview of the products' functional value.

According to Figure 8, the moisture content determined by the oven-drying method ranged between 41.8% and 42.5%. The control sample (C) recorded the highest value (42.5%), followed by a progressive decline in the fortified samples: 42.1% (MZ), 41.9% (ME), and a minimum of 41.8% (MA).

This trend is generally attributed to the high water absorption capacity of the fibers within the peels, which alters the water release dynamics of the matrix during baking compared to pure wheat flour. In the food industry, savory muffins typically maintain a moisture level between 41-42% due to ingredients like telemea cheese and fresh vegetables. This level is considered ideal, as a content above 40% ensures the "moist" and succulent texture described in the sensory analysis, preventing premature drying.

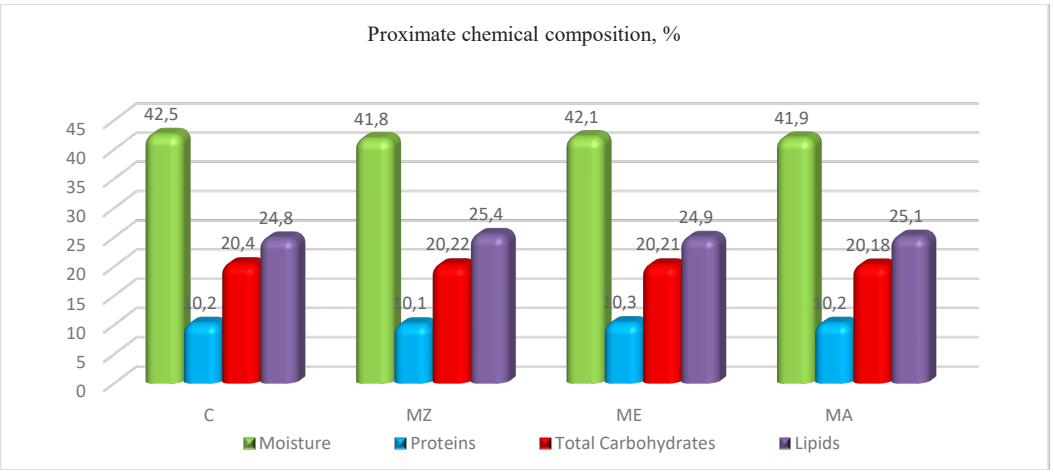


Figure 8. Proximate composition of the muffins

Our observations align with Alongi et al. (2019), who reported that apple-fortified muffins often exhibit lower final moisture than a control, likely because fibers disrupt the gluten network that typically "seals" moisture within the crumb. Furthermore, Henríquez et al. (2010b) demonstrated that apple peel powder reduces total moisture and water activity (a_w), providing a functional advantage by enhancing shelf-life and reducing microbial risk. Despite this decrease, the literature suggests that a lower moisture content is often compensated by a softer crumb, as fibers retain water in a bound form, providing a persistent sensation of freshness.

Regarding macronutrients, proteins (~10.2%), total carbohydrates (~20.3%), and lipids (~25%) remained relatively constant across samples. The significant lipid level is justified by the oil and telemea cheese, essential for the savory profile. However, a marked increase in fiber was observed: from 0.98% (C) to a peak of 1.38% (ME), confirming the success of the upcycling process. Henríquez et al. (2010a) noted that such increases in insoluble and soluble fibers have a major technological impact. For instance, Lucera et al. (2012) observed that certain vegetable fibers stabilize gas bubbles, explaining why our ME sample achieved the highest porosity (53.14%) despite its lower moisture, as the eggplant fibers supported superior alveolar expansion.

Salt values also rose slightly from 0.77% (C) to 0.947% (ME) and 0.936% (MZ), reflecting the natural mineral contribution of the peels. A salt content below 1% is considered moderate and balanced for savory bakery products, complementing the cheese and dill flavors without becoming overwhelming. This correlation is supported by Stojceska et al. (2009), who found that vegetable by-products are rich in mineral salts, contributing to the electrolytic profile of the finished product without the need for additional refined salt.

Antiradical activity: DPPH radical scavenging capacity of muffins

The comparative analysis of the antioxidant activity (Figure 9) demonstrates a significant increase in the radical scavenging capacity of the fortified muffins compared to the control sample (C - 5.01%). The highest inhibition was observed in the muffins enriched with apple peel flour (MA - 11.14%), followed by the zucchini variant (MZ - 10.04%) and the eggplant variant (ME - 8.76%). An increase in the antioxidant capacity of muffins supplemented with eggplant powder was also reported by Mirani & Goli (2021). Although the raw functional flours exhibited very high inhibition percentages - ranging from 76.86% to 82.09% at a 1:100 dilution - the values recorded for the finished products are lower.

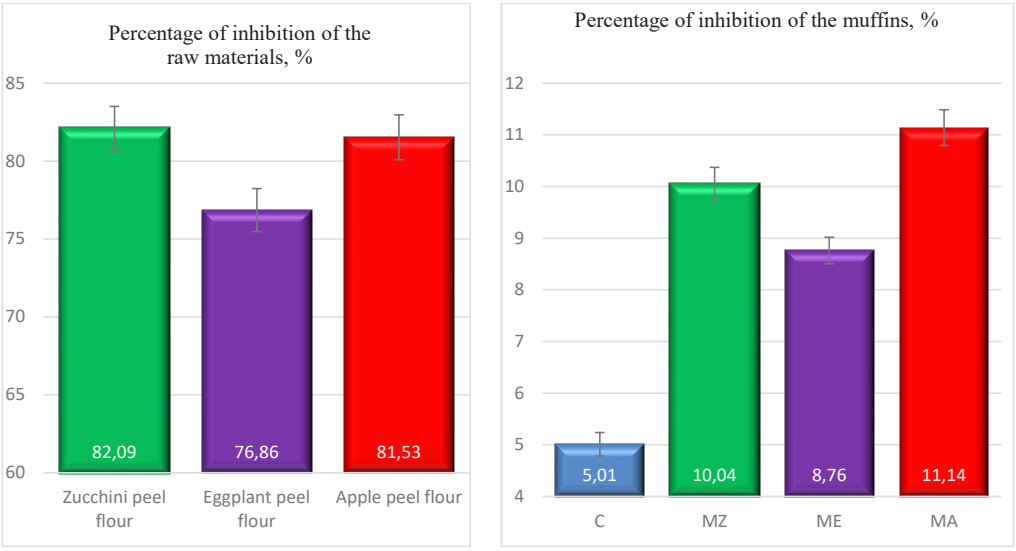


Figure 9. DPPH radical scavenging activity of muffins and raw materials

This trend is standard in bakery science, being attributed to the dilution of the functional ingredient within the complex food matrix and the thermal degradation of polyphenols during the baking process (typically at 180°C). Notably, the incorporation of these vegetable by-products successfully doubled the antioxidant potential of the final product.

The retention of approximately 10% inhibition in the final product, despite the harsh thermal treatment, is consistent with findings by Lucera et al. (2012), who noted that while heat decreases raw antioxidant power, the resulting functional bread or pastry remains significantly more protective than standard versions.

Furthermore, these results can be correlated with other physicochemical parameters determined in this study.

The MA sample, which exhibited the highest acidity (6.4 degrees), also showed the strongest antioxidant activity. This suggests that the acidic environment provided by the malic acid in the apple peel may have helped stabilize certain heat-sensitive antioxidants during baking.

Despite the slight decrease in moisture compared to the control (41.8-42.1% vs. 42.5%), the high fiber content in the MA and ME samples supported a superior alveolar structure. This indicates that the antioxidant-rich powders did not interfere with the leavening agents, allowing for a high porosity (over 52%) and an aerated volume.

The increased salt levels in the MZ and ME samples (0.936-0.947%) reflect the high mineral density of the peels, which typically accompanies the polyphenol complexes responsible for the observed DPPH inhibition. This aligns with Stojceska et al. (2009), who emphasized that vegetable by-products enhance the overall electrolytic and bioactive profile of baked goods.

CONCLUSIONS

The present study explored an innovative direction in food technology, responding to the current consumer trend of reducing the carbohydrate content of foods. In this context, the most effective strategy identified in the literature is the reduction of free sugar content, an aspect that formed the basis for the development of these savory muffins. Parallel to nutritional optimization, the research addressed major concerns regarding the valorization of food by-products within the framework of the circular economy, transforming often-ignored resources into high-value-added ingredients. The main achievements of the study include a successful nutritional reformulation where, by partially substituting wheat flour with powders obtained from zucchini, eggplant, and apple peels, the

goal of reducing carbohydrate density was achieved without compromising the product's structure. This integration effectively transformed the conventional muffin into a functional food.

Furthermore, the study highlighted a significant bioactive retention; although the baking process diminishes the raw antioxidant potential of the materials, the finished product offers a level of protective compounds and DPPH inhibition capacity significantly higher than the standard recipe, doubling the antioxidant potential in the case of the apple sample. From a technological standpoint, it was demonstrated that the use of by-products maintains an optimal physicochemical balance between the titratable acidity required for leavening, appropriate moisture levels between 41.8% and 42.5%, and a superior structural porosity of over 52%.

Beyond the technical parameters, the sensory acceptability of these muffins was remarkably high, proving that the use of these by-products results in a gourmet-quality product where expectations regarding health and taste are effectively satisfied simultaneously. In conclusion, the integration of apple, zucchini, and eggplant peel powders transforms the classic muffin into an innovative product capable of providing enhanced levels of protective compounds and fiber while maintaining an excellent sensory profile. The results confirm that the valorization of vegetable peels represents a sustainable solution for the modern food industry, offering products that do not compromise between nutritional benefits and the pleasure of taste.

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BIOTECHNOLOGICAL METHODS FOR IMPROVING QUALITY OF GLUTEN-FREE BREAD: A REVIEW

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Abstract

The manufacture of gluten-free bread remains a major technological and nutritional challenge due to the absence of the viscoelastic gluten network, which often results in products with reduced volume, weak crumb structure, rapid staling and limited sensory acceptance. In this review, recent biotechnological approaches applied to the improvement of gluten-free bread quality are critically analyzed, with emphasis on technological performance, nutritional enhancement and shelf-life extension. A systematic literature review was conducted in accordance with PRISMA guidelines in order to identify relevant scientific studies published within the last five years. The search focused on peer-reviewed articles addressing microbial fermentation strategies , enzyme-based technologies, and the incorporation of alternative protein sources and hydrocolloid systems. Additionally, the strategic combination of plant proteins and hydrocolloids was reported to compensate for the lack of gluten, especially when integrated with fermentation-based approaches, leading to synergistic improvements in technological and sensory quality. Overall, the findings support the potential of integrated biotechnological strategies to guide the development of gluten-free breads with improved quality and commercial viability.

Key words*: biotechnological methods, bread quality improvement, food biotechnology, gluten-free bread, gluten-free sourdough, selected microbial cultures, shelf-life extension, starter cultures, sourdough fermentation, texture enhancement.*

INTRODUCTION

The greatest challenge faced by those engaging in gluten-free (GF) baking is related to the absence of the bipartite gluten network, as this is what provides extensibility as well as elastic strength to regular baking products. Monomeric gliadins act as plasticizers that give viscosity and extensibility to wheat dough, thus, the dough matrix is able to expand continuously under the pressure of CO_2 generated continuously during the process of yeast fermentation (Šmídová & Rysová, 2022). At the same time, polymeric glutenins are able to supply the essential elasticity which is required to retain gas cells without any structural damage. This is only possible due to the robust, crosslinked macromolecular network they form via inter-chain disulphide bonds. This is the primary rheological hurdle faced in GF matrix design, extensibility without collapse (Calle et al., 2020). Furthermore, the shelf life of many GF products is compromised due to the heavy reliance on refined starches such as potato, rice, or corn. This leads to a plethora of effects such as moisture migration, rapid staling, and increased crumb firmness (Capriles et al., 2020). These starches are also known to undergo full gelatinization during the thermal baking process, however, there is a rapid re-association and crystallization process (retrogradation) due to the disruption of amylose and amylopectin chains, which happens during cooling. Gluten would provide a protein continuum which physically interrupts this recrystallization and permanently binds free water, but, due to its absence in GF products, this leads to a rapid transition of the crumb into a rigid, brittle structure within 24 to 48 hours of said GF products (Šmídová & Rysová, 2022). Xanthan and guar gums were initially used to mitigate these structural deficits, and while these polysaccharides successfully increase the viscosity of the batter and trap free water in order to simulate raw dough consistency, they are unable to form a hear-coagulable, continuous, three-dimensional network during baking. As a result, while it is possible for simple hydrocolloids to temporarily mimic the hydration properties of

gluten, they are unable to provide some of its benefits, such as, structural chewiness, mechanical resistance, or prolonged anti-staling properties characteristic of a true protein-based matrix, which leads to a necessity of using advanced biotechnological interventions (Manik & Nur, 2021). The use of certain enzyme treatments to cross-link proteins, the application of physical stressors like ultrasound to induce gelation, and the exploitation of microbial fermentation are among the most notable of said interventions (Azizi & Azizi, 2023). Sourdough technology is also such a tool, which has long since evolved from its rudimentary form of only being used as a traditional methods, as now selected lactic acid bacteria strains are used to degrade anti-nutritional factors and generate exopolysaccharides that function as natural texturizers (Ramos et al., 2021).

The purpose of this review is to critically analyze the latest biotechnological advancements that have been made in GF bread production. To be more specific, it tackles the efficacy of novel protein isolates, such as pea and zein, combined with the process of ultrasonic modification, the synergistic role of hydrocolloid-protein systems and the integration of sustainable, upcycled ingredients like apple pomace. As it is ultimately the acceptance of the consumers which determines the success of such a product, this paper aims to identify some of the most promising technological methods for producing high-quality, nutritionally balanced gluten-free bread by synthesizing data from the most recent peer-reviewed studies.

MATERIALS AND METHODS

A systematic literature review was conducted in accordance with PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines to identify relevant scientific studies. The search was deliberately restricted to articles published within the previous five years in order to capture the quick, recent advancements in precision food biotechnology, such as focused enzymatic cross-linking, ultrasonic cavitation, and specific lactic acid bacteria (LAB) fermentation. This specific time frame was chosen because it represents a significant technological paradigm change from more outdated, traditional hydrocolloid-substitution methods to active molecular modification of gluten-free matrices. The initial search yielded 850 records, which were systematically filtered down to 32 highly relevant, peer-reviewed studies focused on biotechnological improvements to the gluten-free matrix (Figure 1).

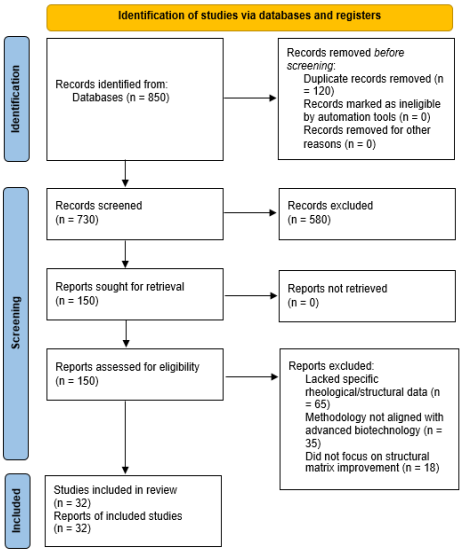


Figure 1. PRISMA flow diagram detailing the literature search, screening, and selection process for the systematic review

Major international databases such as Science Direct, Springer, PubMed and Web of Science were used in our search for relevant scientific articles. To ensure comprehensive coverage of the topic, the literature search was conducted using specific combinations of keywords and Boolean operators. The primary search strings utilized were: (“Gluten-free bread” or “GF bread” or “Gluten-free dough”) and (“Biotechnological methods” or “Sourdough fermentation” or “Enzymatic treatment” or “Alternative protein isolates” or “Hydrocolloid synergy” or "Ultrasound"). A secondary filter was applied in order to spare the peer-reviewed studies, and to further focus the current manuscript on methods such as microbial fermentation strategies, enzymatic treatments, or the integration of plant proteins in order to compensate for the lack of gluten.

References from the selected articles served as additional bibliographic sources for this paper as they ensured a thorough overview of the current methods being used.

RESULTS AND DISCUSSIONS

Diversity of additives and mechanisms of action

There are a number of practical issues in regards to the formulation of optimal gluten-free bread as it is considered that the technological requirements depend on the selected matrices (Calle et al., 2020). Generally speaking, the structure of a successful formulation is as follows: a major ingredient, an additive, and other ingredients (Djeukeu et al., 2024). The major ingredient commonly used is a source of carbohydrates, which helps in the formation of the basic structure of the bread. It is possible to simplify the formulation and potentially reduce production costs by using a whole matrix as the only major ingredient. The most frequently used matrix is rice flour, which has a usage percentage of 41. Following it are various combinations of corn starch and potato starch (15%), rice flour and cassava starch (6%), and corn starch and rice flour (4%) (Djeukeu et al., 2024).

It is necessary to use specific additives in order to satisfy the structural and sensory requirements of GF bread as optimal volume continues to be one of the biggest challenges in the field (Monteiro et al., 2021). It has been determined that consumers deem a bakery product to be satisfactory when it well-risen and regular in shape, as proper volume is a sign of quality to them. Proteins and protein sources (28.4%), enzymes (21.6%), fibers and fiber sources (12.7%), and hydrocolloids (11.8%) are some of the most commonly used additive groups to achieve this effect (Table 1; Djeukeu et al., 2024).

The role of enzymes in the development of high-volume gluten-free bread has been highlighted by a considerable number of researchers. Macromolecules are broken down into simpler compounds by amylases and proteases, this action provides yeast with carbon source to increase their activity and gas production, which, in turn, contributes to bread rising. Additionally, there is a possible improvement of the texture and volume of the bread due to the fact that transglutaminase can create bonds between proteins (Monteiro et al., 2021).

Furthermore, there is a correlation between the incorporation of fibers and bread volume due to mechanisms such as water absorption, gas retention, structural support, and starch gelatinization (Djordjević et al., 2021). Soluble fibers, such as inulin, lead to increased dough hydration and improved gas retention during fermentation because of their water absorption properties. An enhanced leavening and subsequent expansion of the dough is the result of this incorporation. On the opposite end of the spectrum, insoluble fibers, such as *Plantago psyllium* whole seed and oat fiber, may be used to prevent excessive collapse during baking as they provide structural support to the dough matrix, which results in them maintaining the shape, texture, and volume of the bread (Djeukeu et al., 2024). Researchers often try to find certain combinations of multiple mechanisms of action, such as a Protein and Enzyme Mix or a Protein and Fiber Mix in order to achieve a better synergistic volume effect (Parsamajd et al., 2025).

Table 1. Usage frequency of main matrices and additive groups in gluten-free formulations (Adapted from Djeukeu et al., 2024)

Category	Ingredient / Additive Type	Usage Frequency
Major Matrix	Rice flour	41%
	Corn starch and potato starch	15%
	Rice flour and cassava starch	6%
	Corn starch and rice flour	4%
Additive Group	Proteins and protein sources	28.4%
	Enzymes	21.6%
	Fibers and fiber sources	12.7%
	Hydrocolloids	11.8%

Functional Protein Isolates and Ultrasonic Modification

Plant-based protein isolates have recently been used in research in order to mimic the viscoelastic properties of gluten. Fan et al. (2026) studied these effects and has found out, via a comparison of the effects of pea protein, chickpea protein, and zein on rice dough, that zein exhibits a strong hydrophobicity which enhances the structural integrity of the dough through network formation, while due to the

highly hydrophilic nature of pea and chickpea proteins, their best use is in regards to water absorption. Furthermore, while there are universal improvements in the specific volume of gluten-free rice bread when plant-based proteins are used, their microstructural mechanisms of action diverge significantly depending on the profiles of their amino acids (Skendi et al., 2021). Pea and chickpea proteins, similarly to most legume proteins, are rich in polar amino acids and display strong hydrophilicity (Jiang et al., 2026). During the process of dough mixing, rice starch competes with them for available water, which ultimately results in a restriction of the swelling and gelatinization of starch granules. This, in turn, allows proteins to form a soft, hydrophilic gel network that significantly improves dough viscoelasticity. Whereas zein, which is rich in non-polar amino acids, forms a highly hydrophobic, film-like three-dimensional network during the process of heating as it undergoes self-assembly. Said structure is able to form a barrier which encapsulates the starch granules while at the same time inhibiting starch retrogradation far more effectively than legume proteins (Fan et al., 2026).

When taking the extension of shelf-life into account, another hurdle in GF bread-making, the addition of these proteins, which fundamentally alters water mobility within the dough matrix, must be paid close attention to (Capriles et al., 2020). Magnetic Resonance analysis has revealed that optimal amounts of pea protein, chickpea protein, and zein additions can shift free water into bound water. This enhanced hydration capacity prevents another problem that has been mentioned in this paper, namely moisture migration during storage, and there is a direct improvement of yeast fermentation rheology due to this stable viscoelastic structure (Haixi et al., 2025). When put in direct comparison to unfortified gluten-free dough, it has been determined that the addition of zein and chickpea protein at a 12% substitution level increases CO_2, resulting in a highly porous and aerated crumb (Fan et al., 2026).

Despite all of this, over-fortification has been identified as a potential issue in these studies, particularly the threshold of protein over-fortification (Tandazo et al., 2021). In an ideal scenario, exact additions of these proteins improve the matrix, however, excessive protein incorporation instead lead to reduced gas retention and collapsed loaf volumes. This results in a steady degradation of the structural integrity of the dough when protein additions exceed 6% for pea protein, 12% for chickpea protein, and 12% for zein. Ultimately, this causes the bread to become excessively hard and chewy (Fan et al., 2026).

On this note, the type of protein used has several effects on the final visual quality of the bread. Due to the fact that pea and chickpea proteins possess distinct natural pigments, an undesirable darkening may be noticed in the bread crumb when higher level of additions are implemented (Jiang et al., 2026). Zein would be a more suitable substitute in this regard, as it possesses high thermal stability and a pale-yellow hue that blends better with baking crusts than pea and chickpea. Thus, it results in the lowest total colour difference and is the best in terms of preserving the traditional appearance of the bread (Ozturk et al., 2025).

Furthermore, ultrasound treatment, among other physical modification techniques such, is used alongside protein fortification to enhance functionality (Haixi et al., 2025). Zhang et al. (2026) demonstrated how rice flour blended with Soy Protein Isolate (SPI) resulted in the oxidation of thiol groups to form disulfide bonds, constructing a gluten-like 3D network due to having undergone ultrasonic treatment. This synergistic treatment (optimized at 9% SPI with ultrasound) had favourable results compared to untreated samples, as the treatment helped in significantly reducing bread hardness by 6% and increasing specific volume by 10%.

Mechanistic and Microstructural Insights

The microstructure and rheology of the dough must be drastically altered for this synergistic method to be most effective. According to a plethora rheological studies, the majority of gluten-free doughs have significant elastic and loss moduli, but, when ultrasonic treatment is combined with SPI, these moduli are significantly reduced, making the dough behave much more like regular wheat flour (Aguiar et al., 2021; Ronda et al., 2023).

Amylose is liberated and attaches to the exposed amino and carboxyl groups of the SPI when the surface of starch granules is microscopically scratched by ultrasonic cavitation. Starch granules in untreated GF dough are mostly exposed, according to scanning electron microscopy (SEM), however, after ultrasonic treatment, these granules firmly wrap within the newly created protein network, greatly enhancing the structural stability of the dough (Zhang et al., 2026).

The Critical Role of Baking Parameters

Biotechnological changes can improve the raw dough matrix, however these benefits could be totally negated if heat processing is not precisely regulated (Table 2).

Table 2. Impact of thermal processing parameters on ultrasonic-SPI modified gluten-free dough (Adapted from Zhang et al., 2026)

Baking Temperature	Baking Time	Structural & Rheological Outcome
180°C	25 minutes	Increased specific volume and decreased baking loss to 9.19% due to enhanced water-holding capacity.
190°C	25 minutes	Excessive moisture evaporation, excessive starch gelatinization, and irreversible protein coagulation into a rigid network. Bread hardened and lost elasticity.
180°C	30 minutes	Excessive moisture evaporation, excessive starch gelatinization, and irreversible protein coagulation into a rigid network. Bread hardened and lost elasticity.

Zhang et al. (2026) claim that the structural advantages of the SPI-ultrasound synergy are significantly influenced by baking temperature and duration. Baking at 180°C for 25 minutes increased the specific volume of the loaf and decreased baking loss to 9.19% thanks to the modified protein network's enhanced capacity to hold water.

In reverse, due to excessive moisture evaporation, excessive starch gelatinization, as well as the irreversible protein coagulation into a rigid network brought on by raising the temperature to 190°C or extending the baking time by as little as 5 minutes, to 30 minutes, the bread eventually hardened and lost its elasticity.

These findings suggest that the disadvantages of inert starch matrices can be overcome by changing protein structure through cavitation effects if the subsequent heat processing is properly regulated (Nie et al., 2019).

Synergistic Hydrocolloid-Protein Systems

Even though individual functional additions provide partial improvements to gluten-free matrices, recent formulation techniques emphasize the necessity of multi-component systems to accurately replicate the complex viscoelasticity of gluten (Haixi et al., 2025). A recent study by Mensah et al. (2026) found that when a hydrocolloid, such as xanthan gum, is used with two distinct protein sources, like egg powder and gelatin, there are certain notable synergistic effects on dough rheology and final bread construction. Pure rice flour doughs are rather weak, incredibly sticky, and easily compressible due to the lack of structural support (Šmídová & Rysová, 2022). However, the employment of this ternary additive system significantly changes the mechanical profile of the dough, maximizing dough cohesiveness and firmness while reducing undesired adhesiveness, this effect has also been evidenced in other products, such as pasta (Culetu et al., 2021).

This particular combination works because of the complementary mechanisms of each individual ingredient during the stages of mixing, proofing, and baking. Xanthan gum primarily functions in the aqueous phase during proofing, forming an entangled network of polysaccharides that enhances dough viscosity, delays starch gelatinization, and inhibits the coalescence of gas bubbles (Mensah et al., 2026). Simultaneously, adding the thermoreversible protein gelatin creates a hydrated gel that expands to bind water and, when heated, changes into a denatured elastic network that allows the dough to stretch without rupturing. Finally, but perhaps just as importantly, egg powder provides vital structural proteins like albumin, which coagulate during baking to form a continuous, heat-set film around the expanding gas cells, increasing the volume of the loaf and stabilizing the crumb structure (Mensah et al., 2026).

Beyond the aspect of maintaining structural integrity, this hydrocolloid-protein synergy fixes two of the main sensory flaws in GF

bread, namely light crust hue and quick staling (Osei Tutu et al., 2024). The ideal bread formulation's final moisture content was raised to about 37% by the combined water-binding ability of xanthan, egg proteins, and gelatin (Mensah et al., 2026). This reduced water mobility and significantly enhanced both the shelf life and the crumb softness. Standard rice-based breads also have pale crusts because they often don't contain enough reducing sugars and reactive amino acids. During the process of thermal processing, the high amounts of glycine, proline, and other amino acids supplied by the egg powder and gelatin readily take part in Maillard browning reactions (Khan et al., 2025). Higher consumer acceptability scores are produced by this particular biochemical interaction, which successfully produces a rich, golden-brown crust and complex olfactory profile that more closely resembles conventional wheat bread (Capriles et al., 2020).

Advanced Fermentation and Sustainable Ingredients

The use of focused enzymatic biotechnology provided the largest increase in gluten-free bread volume, up to 122.79% when compared to control samples, according to a comprehensive review which detailed several formulation approaches (Djeukeu et al., 2024). This perfect formulation specifically used rice flour that was coated with a mold source, which cooperated with ordinary yeast and sugars. During the pretreatment and enzymatic action of mold preparations, including those derived from koji, very active proteases and amylases are released, cleaving native proteins into free amino acids and highly functional, and tastier peptides (Moradi et al., 2025). Additionally, the addition of these biological enzymes encourages the creation of complex molecular bonds between proteins, particularly when combined with cross-linking agents such as transglutaminase. In order to maximize carbon dioxide gas retention during proofing and produce improved structural aeration, this enzyme-driven structural alteration efficiently substitutes the missing gluten network (Šmídová & Rysová, 2022).

Furthermore, it has been shown that the addition of organic acids, such as lactic or acetic acid, can further optimize this process by controlling the dough's pH, which lowers the molecular weight of proteins and starches to enhance gas retention capacity. Sourdough fermentation remains one of the greatest microbiological techniques for improving the quality of gluten-free bread. Unlike ordinary baker's yeast (*Saccharomyces cerevisiae*), sourdough relies on a complex symbiotic culture of yeasts and lactic acid bacteria (LAB). The main mechanism that can lead to improvement is the dough's acidification; as LAB produces lactic and acetic acids, the pH drops, triggering natural cereal proteases and modifying the batter's viscosity in a positive manner (Seyedahmadi et al., 2025).

Recent studies indicate that this acidity solubilizes pentosans (arabinoxylans), increasing the water-holding capacity of rye and other similar GF flours (Zhang et al., 2026). Additionally, through their metabolic processes, some LAB strains, such as *L. plantarum* and *L. brevis*, can create exopolysaccharides (EPS), which serve as naturally occurring hydrocolloids (Parsamajd et al., 2025). By simulating the structural role of gluten, these microbial gums stabilize the gas bubbles during proving, producing a loaf with a higher specific volume and lower staling rates. Interestingly, a recent study indicates that through the act of concealing the "grassy" off-flavors that are frequently associated with novel plant proteins, like leaf protein concentrates, sourdough fermentation may potentially enhance the sensory profile of breads enriched with these proteins, potentially serving as a new synergistic system (Zhang et al., 2026).

The utilization of repurposed ingredients is also being driven by sustainability. Apple pomace and flaxseed were assessed by Cantero-Ruiz de Eguino et al. (2025) as potentially sustainable sources of fiber. Customers strongly favored compositions with up to 6% apple pomace and 12% flaxseed, according to the sensory analysis carried out by them, which assessed features like "crispy" crust and "artisan-like" appearance. However, an overabundance of 10% apple pomace in the mix resulted in unfavorable textural remarks like "rubbery" underscoring the necessity of optimizing fiber-rich residues in this field.

A sequential schematic of these integrated biotechnological solutions is visualized in Figure 2, while Table 3 provides a comprehensive overview of the novel protein sources driving these advancements.

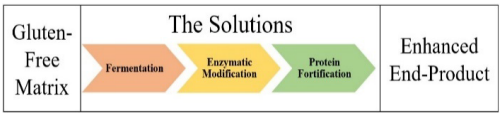


Figure 2. Schematic representation of several biotechnological strategies applied to improve the structural and nutritional quality of gluten-free bread

Table 3. Overview of novel biotechnological sources for gluten-free matrices

Additive/ Source	Processing Method/Intervention	Primary Rheological & Structural Functional Property	Reference
Zein (Corn Protein)	Physical blending (6–12% substitution)	Hydrophobic film-forming capability; significantly improves gas retention and specific volume by mimicking the gluten network.	Fan et al. (2026)
Soy Protein Isolate (SPI)	Ultrasound treatment (360 W, 40 kHz, 20 min)	Promotes thiol oxidation to form disulfide bonds; creates a "pseudo-gluten" 3D network that reduces crumb hardness.	Zhang et al. (2026)
Chickpea Protein	Sourdough fermentation (*L. plantarum* LB-1)	Formation of "weak gel" structures (G' > G"); significantly enhances batter viscosity and delays retrogradation (staling).	Jiang et al. (2026)
Egg Powder, Xanthan, & Gelatin	Synergistic composite blending	Distributes structural roles: increases viscosity, provides heat-coagulable proteins, and maximizes moisture retention and loaf volume.	Mensah et al. (2026)
Apple Pomace & Flaxseed	Dehydration and particle size reduction	High functional fiber content improves water-holding capacity; yields an "artisan-like" crispy crust at optimized inclusion levels (≤6%).	Cantero-Ruiz de Eguino et al. (2025)

Synergistic Fermentation of Legume-Based Matrices

Proteins derived from legumes, such as those found in peas, lentils, lupine, and chickpeas, are a novel method of giving GF doughs structural support (Imam et al., 2024). However, the fermentation step is where these legume matrices realize their full technological potential in regards to the aforementioned issues. The inclusion of these hydrophilic bean proteins significantly changes the dough's capacity to hold CO_2, in accordance with rheological fermentation tracking (Zeng et al., 2024). Because pure rice doughs lack a cohesive network, gas escapes rather quickly during proofing. On the other hand, robust molecular cross-linking is promoted by the targeted addition of pea protein, at 6% substitution, and chickpea protein, at 12% substitution, which changes the matrix into a viscoelastic structure with a high expansion potential. In addition to increasing the maximum dough height and overall gas retention coefficient by up to 1.15 times, this legume-protein network significantly prolongs the time at which carbon dioxide gas starts to escape the dough increasing it from 25 minutes in unfortified doughs to up to 50 minutes in chickpea-fortified formulations (Fan et al., 2026).

Modern biotechnological methods combine specialized sourdough fermentation with legume fortification to further optimize these structural advantages to their full potential. The addition of sourdough starters promotes the binding of starch granules by collaborating with the legume proteins in these matrices. As such, certain lactic acid bacteria, including *Lactobacillus plantarum*, alter the protein structure to create a "weak gel" network during this biological acidification process(Lin et al., 2022). In addition to maximizing the retention of CO_2 gas produced by yeast, this synergistic fermentation greatly delays staling and enhances the final gluten-free product's overall texture and volume, addressing some of GF bread's greatest hurdles (Fan et al., 2026).

Recent studies show that this synergistic application of legume flours and targeted sourdough fermentation significantly optimizes GF dough structure, whereas the sole application of alternative proteins and microbial fermentation can deliver quantifiable benefits. Recent research examined this interaction by carefully assessing the effects of *Lactobacillus plantarum* LB-1-fermented chickpea flour on a rice-based GF matrix (Jiang et al., 2026).

In terms of rheology, this chickpea sourdough greatly decreased the peak viscosity and setback values of the batter. The fermentation process improved the short-range order of starch molecules and actively helped in the stabilization of the conformations of protein molecules (Ramos et al., 2021). As a result, according to Jiang et al. (2026), the fermented batter presented remarkable viscoelastic behavior, maintaining a well-balanced loss tangent tan δ = 0.38-0.58 and reaching a

maximum storage modulus of G' = 11,347 Pa. These microscopic and rheological changes were strongly associated with improved macroscopic baking characteristics. The chickpea sourdough gluten-free (CSGF) bread had its baking moisture loss reduced to just 5.03% while achieving an amazing specific volume of 2.50 mL/g when compared to standard unfermented rice-based standards (Jiang et al., 2026). The crumb hardness was only 3.93 N due to the structural matrix that the fermented chickpea proteins and dietary fibers provided to the structure as a whole.

Additionally, a continuous, dense crumb porosity (AF = 29.94%) in macroscopic image analysis shown by SEM confirmed the creation of a very homogenous and compact structural matrix in the tested bread (Jiang et al., 2026).

Consumer Acceptance of Novel Ingredients

The *L. plantarum* fermentation greatly improved the physical rheology and antioxidant activity of the tested bread. These results demonstrate that using legume-based sourdoughs offers a dual-purpose technological approach that improves the nutritional and sensory qualities of GF bread, which improves its chances on the market, while simultaneously addressing its mechanical shortcomings (Seyedahmadi et al., 2025). The success of biotechnologically modified gluten-free products ultimately depends on consumer acceptance, even though technological functionality is crucial. Recent studies highlight the importance of sensory evaluation for products enriched with novel protein sources, as it is ultimately the acceptance of the consumers that will determine the success of any such experiment (Aguiar et al., 2023).

For example, Duta et al. (2025) stressed that in order to gain market acceptance, enhancing bread matrices, such as grissini, with protein by-products necessitates careful balance of nutritional benefits with sensory qualities.

Similarly, Scheibenzuber et al. (2023) showed that the presentation of "sustainability" and "nutritional" claims has a considerable impact on consumers' readiness to test foods containing industrial by-products, as markets change and these aspects are becoming more and more important.

CONCLUSIONS

The development of gluten-free bread quality has progressed far beyond the levels of simple starch substitution thanks to the application of sophisticated, cooperative biotechnological techniques. According to recent research, a multimodal strategy involving functional proteins, intricate physical alterations, and focused biological therapies is needed to replicate the viscoelastic characteristics of the absent gluten network.

Plant-based protein isolates, such as soy, pea, chickpea, and zein, have been shown to be highly successful in inducing molecular cross-linking and generating a functional, pseudo-gluten three-dimensional network when paired with techniques like ultrasonic cavitation. Additionally, by optimizing CO_2 gas retention during proofing, customized enzymatic treatments and especially those that combine organic acids with proteases and amylases derived from mold, have been able to produce record-breaking loaf volume increases of up to 122.79% in perfect environments. Further bridging the technological gap, there are formulation synergies, such as triple-additive systems made up of microbial hydrocolloids, heat-coagulable proteins, and thermoreversible gels. In addition to increasing specific volume and lowering crumb density, these intricate systems effectively restore the amino acids required for Maillard browning, so this is the best present method of overcoming the ongoing problem of pale crusts in gluten-free baking. In a similar vein, advanced sourdough fermentation combined with legume-based matrices demonstrates how biochemical acidification can modify protein structures to postpone staling and significantly increase gas retention, another longstanding issue.

Nevertheless, successful industrial application is still quite sensitive to processing conditions in spite of these important structural advancements. Over-fortification beyond essential ingredient limits (usually between 6% and 12% for new proteins) or even inadequate heat processing during the baking phase can completely offset the structural benefits of biotechnology. As such, the room for error is rather small and one must be careful when balancing these factors.

In the end, customer approval plays a major role in the commercial success of these innovative formulas, as they have the final word. Formulators must use focused sensory evaluations to remove important negative drivers, such as rubbery or too dense textures, before positive traits, such as crispiness and crumb lightness, can positively influence purchase intent in order to compete in the market. This is a tall task considering the competitors do not have to work around such inconveniences. A gluten-free bread that closely resembles the quality, appearance, and flavor profile of conventional wheat bread can be produced by striking a balance between these complementary biotechnological methods, exact thermal control, and stringent consumer validation.

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DETERMINANTS OF FOOD WASTE AMONG CONSUMERS: RESULTS FROM A CROSS-SECTIONAL STUDY

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Abstract

This study investigates consumer behavior in relation to food waste based on a questionnaire administered to 273 respondents from both urban and rural areas. The survey examined meal and shopping planning habits, food purchasing criteria, management of household food supplies, and practices related to preserving and reusing leftovers. It also assessed the frequency of discarding perishable foods, purchasing behavior during holiday periods, awareness of environmental impacts, and willingness to adopt preventive measures. The findings indicate that meal planning and regular grocery shopping are common practices, and food purchases are mainly driven by household needs. Many consumers report using food already available at home before buying new products. However, behaviors such as repurposing leftovers and consistently consuming foods close to their expiration date are less frequently practiced. Although most respondents consider reducing food waste important and express concern about the issue, preventive behaviors are applied inconsistently. The results highlight a gap between consumer attitudes and actual practices, emphasizing the need for educational initiatives and accessible information to promote sustainable consumption and reduce household food waste.

Key words: *environmental awareness, food preservation, food purchasing habits, household food management, sustainable consumption.*

INTRODUCTION

Food waste is a pressing global issue, leading to substantial economic losses while placing heavy demands on natural resources, food systems, and the environment. Globally, about one-third of all food produced for human consumption is lost or wasted, with households accounting for a significant share (FAO, 2013). The impacts of food waste extend beyond environmental consequences (e.g., greenhouse gas emissions, water use, and land degradation) to ethical and social concerns, including food insecurity and unequal access to resources (Papargyropoulou et al., 2014; Kibler et al., 2018).

In recent years, research has increasingly focused on food waste at the household level, where consumer behavior plays a critical role (Barnisca et al., 2024; Simões et al., 2022). Evidence indicates that food waste is not simply the result of poor storage or technical limitations, but arises from the complex interplay of planning habits, purchasing decisions, food management practices, and individual attitudes (Abdelradi, 2018; Aktas et al., 2018).

Factors such as meal planning, use of shopping lists, shopping frequency, and responses to promotions have been shown to influence the likelihood of over-purchasing and subsequent food disposal (Farr-Wharton et al., 2014; McCarthy & Liu, 2017; Ananda et al., 2021).

Household food management practices, particularly those related to leftover handling and food preservation, represent another important dimension of food waste generation. Previous studies highlight that ineffective leftover management and low levels of food

reutilization significantly contribute to avoidable waste (Aloysius et al., 2023; Dyen et al., 2021). Although consumers often express intentions to reduce waste, behaviors such as transforming leftovers into new meals or consuming foods close to their expiration date are inconsistently applied (Aydin & Yildirim, 2021; Farr-Wharton et al., 2014). This discrepancy points to a persistent attitude - behavior gap in household food waste reduction efforts.

Attitudes, moral norms, and awareness of environmental impacts play an important role in shaping food waste behaviors (Adams & Raisborough, 2010). Studies show that people who view food waste as morally wrong or harmful to the environment tend to generate less waste (Misiak et al., 2020; Ang et al., 2021). Yet, awareness and concern vary widely across populations, influenced by socio-demographic factors, cultural practices, and access to information (Hengi & House, 2022; Phasha et al., 2020). Social occasions, such as holidays and gatherings, are also linked to higher food waste, driven by social norms, hospitality expectations, and perceived moral obligations to provide abundant food (Aleshaiwi, 2023; Cavazza et al., 2011; Adams & Raisborough, 2010).

In Romania, recent research has highlighted the influence of socio-demographic characteristics (Defta et al., 2025), consumer perceptions, and communication strategies on food-related behaviors, including responses to promotions and sustainable consumption choices (Barbu et al., 2023; Defta et al., 2024). Despite this, there is still limited empirical evidence on how planning, purchasing, food management, and attitudinal factors interact to affect household food waste, especially from a consumer-focused perspective (Aiello et al., 2014).

Against this background, the present study provides new insights based on a cross-sectional survey of urban and rural consumers. It has two main objectives: (1) to analyze consumers' food planning, purchasing, and household management behaviors to identify practices that lead to food waste, and (2) to assess their awareness, attitudes, and preventive measures regarding food waste reduction. By examining both behavior and attitudes, this study aims to deepen our understanding of the drivers of household food waste and to inform targeted educational campaigns and policy measures that promote sustainable consumption

MATERIALS AND METHODS

This study employed a quantitative, cross-sectional research design to examine the determinants of food waste among consumers. This approach was selected to capture self-reported behaviors, attitudes, and practices related to food planning, purchasing, and household food management at a single point in time. The cross-sectional design enables the identification of prevailing patterns and associations between consumer behaviors and food waste-related outcomes, in line with the study objectives.

Data were collected using a structured questionnaire developed specifically for this research. The questionnaire was designed based on relevant literature on household food waste and consumer behavior and consisted of three main sections:

a. socio-demographic characteristics of respondents;

b. consumer behaviors related to food planning, purchasing, and household food management;

c. attitudes, awareness, and preventive measures regarding food waste. The instrument included both Likert-scale items and multiple-choice questions to ensure comprehensive coverage of the studied constructs.

The study sample comprised 273 respondents from both urban and rural areas. Participants were selected using a non-probabilistic sampling approach, and data were collected through voluntary participation. This sampling strategy allowed for the inclusion of diverse consumer profiles, facilitating the exploration of behavioral differences across socio-demographic contexts (Table 1).

Table 1. Distribution of respondents by socio-demographic characteristics

Characteristics	Share in the Sample	N	Ratio (%)
Gender	Female	175	64.1
	Male	98	35.9
Age (years)	18–30	105	38.5
	31–40	68	24.9
	41-50	46	16.8
	51-60	31	11.4
	Over 60	23	8.4
Education level	High school education (ongoing/completed)	16	5.9
	Post-secondary education (ongoing/completed)	5	1.8
	University education (ongoing)	134	49.1
	University education (completed)	97	35.5
	Postgraduate education (ongoing/completed)	21	7.7
Household monthly net income (RON)	Under 1500	82	30.1
	1501–3000	77	28.2
	3001–5000	58	21.2
	5001–5500	32	11.7
	5001–8000	16	5.9
	Over 8000	8	2.9
Residence	Urban	146	53.5
	Rural	127	46.5

Data Analysis

Collected data were coded and processed using Microsoft Excel. The analysis was conducted using descriptive statistical methods, including frequency distributions and percentage calculations. The results were subsequently presented in the form of tables and graphical representations to facilitate interpretation and to support the discussion of key findings in relation to the research objectives.

RESULTS AND DISCUSSIONS

Identifying the factors that drive food waste among consumers is essential given the substantial economic, social, and environmental implications of this phenomenon

Objective 1. To analyze consumers' food planning, purchasing, and household food management behaviors in order to identify practices that contribute to food waste.

Specific objectives and associated items:

O1.1. Planning and purchasing behaviors

Items:

a. How often do you plan what you are going to cook before going shopping?

The results show that approximately 45.4% of respondents always plan what they are going to cook before shopping, while 31.9% do so most of the time. In contrast, 19.4% report planning rarely and 3.3% never plan their meals in advance. These proportions indicate that although meal planning is common among consumers, a segment of the population still engages in limited planning, which may contribute to higher levels of food waste (Figure 1).

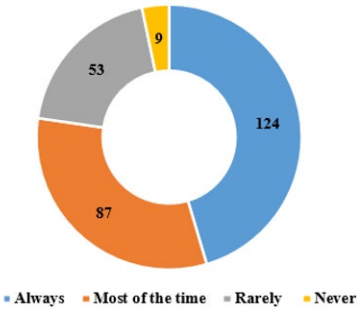


Figure 1. Frequency of meal planning prior to grocery shopping

b. When purchasing food, how often do you use a shopping list?

The results indicate that approximately 24.9% of respondents always use a shopping list when purchasing food, while 53.5% do so sometimes. In comparison, 12.5% report using a list rarely, and 9.2% never rely on one. These proportions suggest that although more than half of consumers use shopping lists occasionally, consistent use is less common, which may influence the likelihood of unplanned purchases and potential food waste (Figure 2).

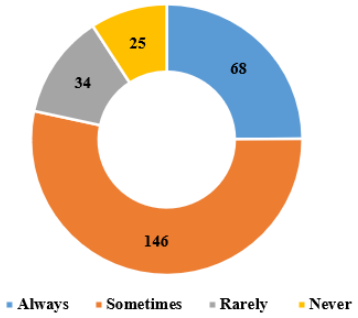


Figure 2. Frequency of shopping list use during food purchases

c. How do you decide which foods to buy?

The results show that 53.5% of respondents decide which foods to buy based on household needs, while 15.8% rely on discounts and promotions. Additionally, 21.6% make decisions according to the recipes they plan to prepare, and 9.2% follow other criteria. This indicates that practical household requirements are the primary factor guiding food purchase decisions (Figure 3).

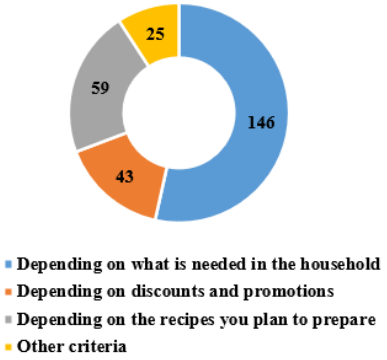


Figure 3. Distribution of respondents according to food purchase decision criteria

d. How often do you go grocery shopping?

The results indicate that grocery shopping is predominantly a frequent activity, with approximately 81% of respondents shopping once a week or more often. This high frequency suggests that consumers tend to restock their households regularly, which may help maintain food freshness but can also increase the risk of over-purchasing if shopping is not well planned (Figure 4).

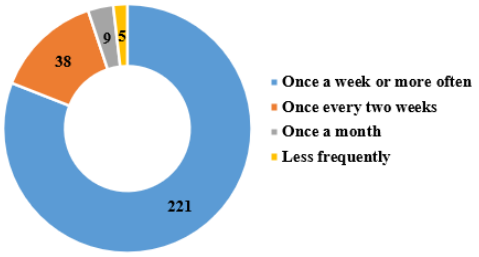


Figure 4. Distribution of respondents by grocery shopping frequency

e. How often do you purchase food products during holidays?

The findings indicate that 54.3% of respondents maintain their usual frequency of food purchases during holidays, while 45.3% report buying more than usual. A small minority, 5%, purchase less than usual. These results suggest that holiday periods are associated with either sustained or increased food purchasing, reflecting heightened consumption needs or festive behaviors (Figure 5).

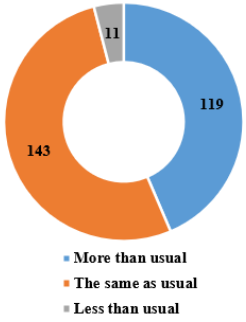


Figure 5. Frequency of food purchasing during holidays

O1.2. Household food management and handling of leftovers

a. How often do you use the food you already have at home before buying new items?

The results show that 55.3% of respondents use the food they already have at home before purchasing new items most of the time, while 35.9% always do so. Only a small proportion use existing food rarely (6.2%) or never (2.6%). This indicates that the majority of consumers make a conscious effort to utilize available food, which may help reduce household food waste (Figure 6).

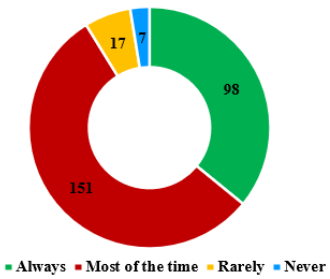


Figure 6. Distribution of respondents by frequency of using food already available at home prior to new purchases

b. What measures do you take to preserve fresh food?

The results indicate that 46.5% of respondents use airtight containers, 37.4% freeze food, 7% label and date items for proper rotation, and 9.1% apply other measures. This suggests that the majority prioritize practical methods to extend the shelf life of fresh food (Figure 7).

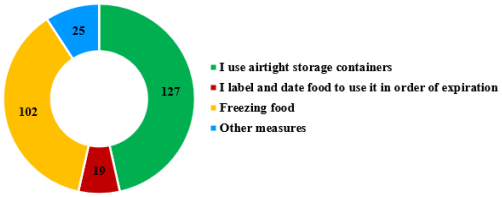


Figure 7. Distribution of respondents according to fresh food preservation practices

c. What do you do with the food after consuming the first portion?

The results show that most respondents keep leftovers for later meals (51.3% always, 34.9% often). Freezing is less common, with the majority doing it sometimes (55.3%). Sharing leftovers is done sometimes by most (49.4%), while discarding food is infrequent, with most respondents throwing it away sometimes (46.9%) or never (35.6%). This suggests a general tendency to preserve or reuse food rather than discard it (Figure 8).

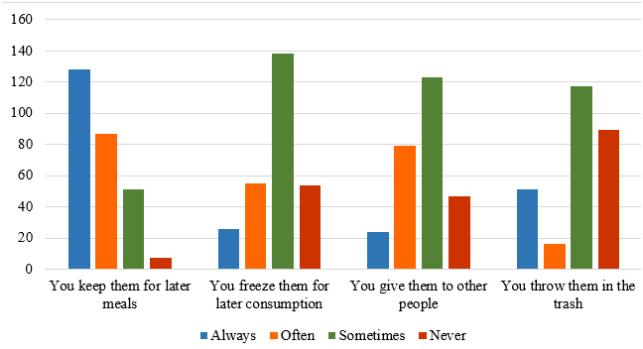


Figure 8. Distribution of respondents by practices related to leftover food management

d. How often do you try to transform leftovers into new dishes?

The results indicate that most respondents transform leftovers into new dishes infrequently, with 39.9% doing so rarely and 22.3% never.

Only a minority consistently repurpose leftovers, suggesting limited adoption of this food-saving practice (Figure 9).

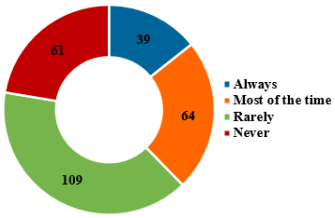


Figure 9. Frequency of transforming leftovers into new dishes

e. How often do you consume foods that are close to their expiration date?

The results show that the majority of respondents consume foods close to their expiration date at least occasionally, with 48.4% doing so sometimes and 22.3% always. A smaller proportion consumes such foods rarely (31.5%) or never (2.7%), indicating a moderate willingness among consumers to use products before they expire (Figure 10).

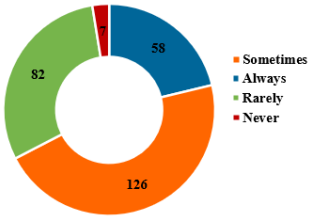


Figure 10. Frequency of consuming foods close to their expiration date

Objective 2. To assess consumers' level of awareness and attitudes toward food waste, as well as the preventive measures they adopt to reduce it.

Specific objectives and associated items: awareness, attitudes, and information sources

Items:

a. How important is it for you to reduce food waste?

The findings indicate that most respondents consider reducing food waste to be important, with 48.7% rating it as very important and 35.5% as moderately important. Only a small proportion view it as not very important (13.9%) or not important at all (1.8%), reflecting a generally high level of concern for minimizing food waste (Figure 11).

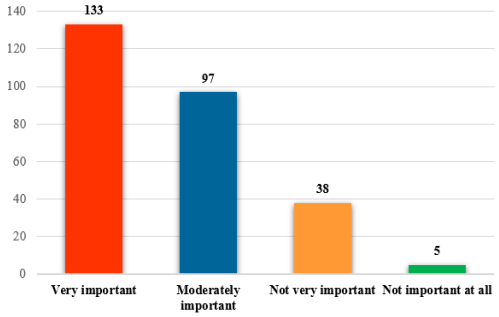


Figure 11. Importance of reducing food waste

b. How well-informed are you about the negative impact of food waste on the environment?

The data indicate that while a substantial proportion of respondents (50.4%) consider themselves moderately informed about the environmental impact of food waste, only a minority (24.2%) perceive themselves as very well-informed. Moreover, nearly one-quarter (25.2%) report being slightly or not informed at all. These findings suggest that, although awareness is present, there remains significant potential to enhance consumer knowledge regarding the ecological consequences of food waste, which could facilitate the adoption of more sustainable practices (Figure 12).

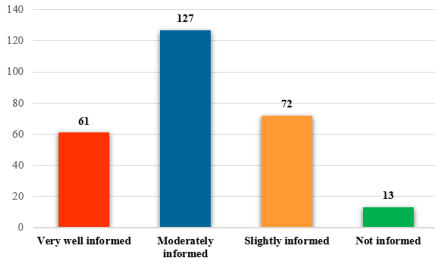


Figure 12. Distribution of respondents by level of awareness of the environmental impact of food waste

c. How concerned are you about food waste?

The findings indicate that the majority of respondents express concern about food waste, with 50.9% being moderately concerned and 23.3% very concerned. A smaller proportion are indifferent (9.0%) or have not considered this issue (4.2%). These results suggest that while most consumers recognize food waste as an important issue, there is variability in the intensity of concern, highlighting the need for targeted awareness initiatives to further engage less concerned individuals (Figure 13).

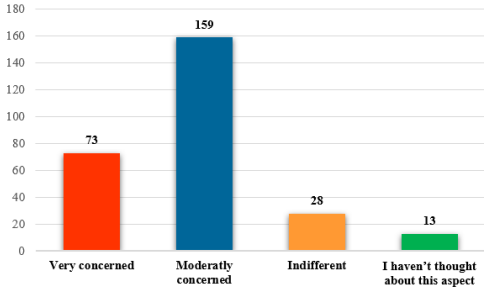


Figure 13. Distribution of respondents by level of concern about food waste.

The findings of this study reinforce the central role of consumer behavior in shaping household food waste outcomes (Silvennoinen et al. 2015). Consistent with previous research, the results indicate that planning practices, food management behaviors, and attitudes toward food waste are key determinants of waste generation at the household level (Abdelradi, 2018; Aktas et al., 2018, Vittuari et al. 2023). Although most respondents report a high level of concern regarding food waste and recognize the importance of reducing it, preventive behaviors - such as systematic meal planning, repurposing leftovers, or consuming near-expiration foods - are not uniformly adopted. This discrepancy confirms the persistence of the attitude - behavior gap identified in earlier studies, suggesting that awareness alone is insufficient to drive consistent waste-reducing practices (Aydin & Yildirim, 2021; Misiak et al., 2023).

From a behavioral perspective, the results highlight the importance of habitual actions embedded in everyday food - related routines (Silvennoinen et al., 2015). Frequent grocery shopping, occasional reliance on shopping lists, and limited transformation of leftovers into new meals may unintentionally increase the risk of over-purchasing and food disposal, especially during high - consumption periods such as holidays. Similar patterns have been documented in the literature, where social norms, convenience, and time constraints often outweigh intentions to reduce food waste (Aloysius et al., 2023; Aleshaiwi, 2023). These findings underscore the need for interventions that target not only individual attitudes but also practical skills and routines related to food planning and management (Chaboud, 2017).

In this context, marketing strategies can play a critical role in supporting food waste reduction by influencing consumer choices and behaviors at the point of purchase and beyond. Responsible marketing approaches - such as promoting realistic portion sizes, encouraging the purchase of "imperfect" or near-expiration products, and providing clear information on storage and leftover use - have been shown to positively influence waste-reducing behaviors (Ang et al., 2021; McCarthy & Liu, 2017; Chereji et al., 2023; Ögel et al., 2023). Furthermore, communication campaigns that emphasize both environmental and economic benefits, combined with nudges such as meal-planning tools or recipe suggestions for leftovers, may help bridge the gap between concern and action. Aligning marketing practices with sustainability goals can therefore contribute to fostering more responsible consumption patterns and reducing household food waste in the long term (Dyen et al., 2021; Papargyropoulou et al., 2014; Grapă et al., 2022).

CONCLUSIONS

Meal planning and use of shopping lists are common among respondents and help reduce food waste. Food purchases are primarily driven by household needs, reflecting a practical approach. Most participants store and reuse leftovers, though transforming or freezing food is less frequent. Consumers moderately use near-expiration products, showing some concern for waste reduction. While awareness and concern about food waste are generally high, preventive practices are only partially adopted, revealing a gap between attitudes and actual behaviors.

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COMPARATIVE ANALYSIS OF ANTIOXIDANT ACTIVITY AND PHENOLIC CONTENT IN FRESH AND DRIED EDIBLE GREEN PLANTS

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Abstract

The paper evaluates the antioxidant potential and polyphenol content of seven green plants of dietary and therapeutic importance, used in various diets: garden orache (Atriplex hortensis L.), sorrel (Rumex acetosa), patience dock (Rumex patientia) wild garlic (Allium ursinum), lovage (Levisticum officinale), tarragon (Artemisia dracunculus), and dill (Anethum graveolens). The importance of these determinations lies in the need to quantify the actual nutritional value of plants, beyond their traditional use, providing a scientific basis for dietary recommendations. The study focuses on quantifying the total polyphenol content and evaluating the antioxidant capacity, comparing fresh samples with those subjected to conventional drying. The total polyphenol content was determined using the Folin-Ciocalteu spectrophotometric method, while the antioxidant capacity was evaluated using the DPPH method (RSA %). Monitoring these parameters is important for the food industry to optimize preservation methods, and the research highlights the essential role of these green plants in nutrition, promoting their use as natural and accessible sources of antioxidants.

Key words*: antioxidant activity, fresh and dried edible green plants, phenolic content.*

INTRODUCTION

Phytochemical analysis of early plant species such as garden orache (*Atriplex hortensis* L.), sorrel (*Rumex acetosa*), and patience dock (*Rumex patientia*) reveals a complex nutritional profile with high levels of micronutrients and essential bioactive compounds (Zeipina et al., 2015; Dudaš et al., 2025; Helena Korpelainen & Maria Pietiläinen, 2020). The phenolic compounds present in *A. ursinum*, including p-coumaric and ferulic acids, are potent antioxidants that play a role in reducing oxidative stress. Research indicates that these molecules show considerable free radical scavenge activity when extracted with methanol or ethanol, which would be of interest for nutraceutical applications. Both antioxidants are represented in significant amounts, and their activities are necessary to neutralize oxidative stress (Popovschi & Ivanova, 2025). In addition to flavor-carrying organosulfur compounds and amino acids, other compounds, such as polyphenols, greatly contribute to the antioxidant properties of wild garlic. Benkeblia et al. (2007) and Pavlović et al. (2017) (cited by Kovačević et al., 2023) have reported high antioxidant and radical scavenging activity in *Allium ursinum* extracts. On the other hand, aromatic plants such as tarragon (*Artemisia dracunculus*), dill (*Anethum graveolens*), and lovage (*Levisticum officinale*) incorporate essential oils digestive and antimicrobial properties into their phytochemical complex (Chaleshtori et al., 2013; Chahal et al., 2017; Jannesar et al., 2024; Sahlabgi et al., 2025). Their integration into diet goes beyond their gastronomic value,

constituting an effective strategy for strengthening the immune system through the intake of phytonutrients. Thus, the study of their chemical composition remains an area of major interest for food biochemistry and contemporary nutrition sciences.

Atriplex hortensis (garden orache), an annual edible halophyte in the Chenopodioideae subfamily, is emerging as a high-potential nutrient source. Taxonomically related to spinach and quinoa, this climate-resilient species demonstrates superior tolerance to abiotic stressors, including drought, extreme temperatures, and nutrient-deficient soils. While its foliage exhibits diverse pigments from lime-green to deep purple. The green variety is particularly notable for its superior concentrations of protein, magnesium, vitamin C, and carotenoids compared to conventional spinach (*Spinacia oeracea*) (Ali et al., 2021; Kumorkiewicz et al., 2025).

Although plants of the genus *Rumex* (family Polygonaceae) are traditionally used in food and herbal medicine due to their beneficial effects, the specific biological properties of many of these species remain insufficiently documented in the scientific literature. Previous research has highlighted the properties of these plants, which are rich in bioactive phytochemicals, reporting anticancer, antioxidant, and anti-inflammatory effects, as well as antidiarrheal, analgesic, anthelmintic, and antimicrobial effects (Mishra et al., 2018; Lee et al., 2005; Vasas et al, 2015; Feduraev et al., 2022; Kurhe & Diwane, 2024. Patience dock (*Rumex patientia* L.), a perennial species of the Polygonaceae family, is widely cultivated in Eastern Europe. Its chemical composition includes anthraquinones, tannins, flavonoids, and phenolic acids, compounds that studies have attributed remarkable anti-inflammatory and antioxidant properties to (Jovin et al., 2011).

An analysis of the literature on the *Rumex* genus indicates that the species *R. acetosa, R. obtusifolius, R. crispus, R. acetosella, and R. dentatus* receive the most attention in scientific publications. However, comparative studies on the phytochemical composition of plants of this genus that coexist in the same geographical area are extremely rare (Feduraev et al., 2022). Sorrel (*Rumex acetosa* L.) is highly valued by consumers for its distinctive flavor and specific resistance, having high commercial potential. It can enrich the daily diet due to its significant macro- and micronutrient content, while also being an important source of natural antioxidants, such as phenolic compounds and carotenoids (Torres et al., 2019). Sorrel is an herbaceous plant rich in polyphenolic compounds with potential therapeutic benefits. These include flavan-3-ols, such as epicatechin and catechin, along with several essential phenolic acids: gallic, ferulic, protocatechuic, and p-coumaric acids (Kurhe & Diwane, 2024). Sorrel (*Rumex acetosa*) is the most widely used species of the genus, valued for its leaves rich in oxalic acid, (Bello et al., 2019) which give them a distinctive sour taste. However, the presence of high levels of the anti-nutritive factor, oxalic acid, reduces the bioavailability of some minerals, especially calcium, thus causing mineral deficiencies and other serious problems (calcium oxalate stone formation in kidneys, decreased iron absorption) (Tuazon-Nartea & Savage, 2013). With a tradition dating back thousands of years, it is used extensively in food, folk medicine, and as a source of natural dyes, offering rich and sustainable harvests (Korpelainen & Pietiläinen, 2020).

The distinct aromatic profile of the *Allium* genus, together with its nutritional and pharmacological potential, has sparked major interest in food and medical research. The properties of wild garlic are comparable to those of common garlic, with harvesting focusing mainly on its developed, intensely green leaves. Currently, there is an upward trend in the use of this species as a functional food and a basic ingredient in food supplements (Bernas, 2024). Wild garlic (*Allium ursinum* L.) has a centuries-old tradition as a food and remedy. Although it is a geophyte that blooms in spring in its natural environment - moist, shady beech forests - there is little documented information on how its phytochemical composition and antioxidant capacity are influenced by soil variety (Gordanić et al., 2022).

The therapeutic properties of *Allium ursinum* derive from its complex phytochemical profile, defined by the presence of sulfur compounds, phenolic acids, flavonoids, and essential oils

(Lenková et al., 2016). These constituents exhibit diverse bioactive activity, giving the plant high pharmaceutical potential. The strong aroma, specific to wild garlic, is generated by essential oils, while the characteristic smell is due to allicin. This sulfoxide compound is synthesized when plant tissues are mechanically destroyed (Popovschi & Ivanova, 2025). A wide range of studies have confirmed the antioxidant, cytostatic, antimicrobial, and antifungal properties of this species. (Sobolewska et al., 2006; Stajner et al., 2008; Pârvu et al., 2011; Bagiu et al., 2012; Shahrajabian, 2021; Barbu et al. 2023; Kovacević et al., 2023; Kalaba et al., 2024). The therapeutic effects of wild garlic are mainly related to the presence of organosulfur compounds. High concentrations of volatile substances, such as sulphides and disulfides, directly determine its therapeutic quality and value in the food industry as a spice (Boacă et al., 2025). In folk medicine, wild garlic (bear's garlic) is known for its ability to prevent disease and improve health. These benefits are linked to the presence of minerals (such as iron, phosphorus, sodium, and copper) and vitamins A and C, as well as being a rich source of fiber, phenolic compounds, and sulphur compounds (Filipčev et al., 2023).

A member of the *Asteraceae* family, tarragon (*Artemisia dracunculus* L.) is a perennial aromatic plant valued as a natural ingredient in food. With a distinct aroma and remarkable biological activity, its dried leaves are widely used to add flavor and aroma to culinary preparations (Kara & Çağlak, 2022). *Artemisia* species are known for their specific aroma, due to monoterpenes and sesquiterpenes, compounds that give them value in traditional medicine. This plant is used for its nutritional, stimulating, and disinfectant properties, while also having appetizing and vasodilatory effects (Behbahani et al., 2017; Mumivand et al., 2017).

The leaves of tarragon are popular in salads, soups, and grilled meats, whether used fresh or dried. Furthermore, essential oil is a key flavoring and aromatic ingredient in industrial food applications (Chaleshtori et al., 2013). While the stems and leaves of *A. dracunculus* L. are basic ingredients in international and traditional cuisine, the flowers and other parts of the plant are mainly used in alternative medicine (Cristea & Șandru, 2023).

Dill (*Anethum graveolens* L.) is an important source of essential oils, notable for its content of D-carvone and D-phellandrene. Although these compounds are defined for the plant profile, in the case of using the vegetative organs before flowering, carvone is not found among the major constituents of the essential oil (Jannesar et al., 2024).

Phytochemical investigations carried out on dill have confirmed the existence of various classes of bioactive compounds, such as terpenoids, saponins, tannins and flavonoids (including flavonoids), as well as alkaloids and steroids (Nguyen et al., 2020; Kaur et al., 2018; Basavegowda et al., 2022; Jadhav & Pawar, 2023; Thanuma et al., 2025). Tests have also indicated the absence of reducing sugars, glycosides, anthraquinones and paleobotanies in the plant composition (Chahal et al., 2017). Dill (*Anethum graveolens* L.) is a staple herb used to flavor a wide range of dishes, from salads, sauces, and soups to seafood and, especially, pickles (Jadhav & Pawar, 2023). In addition to its distinctive flavor, dill leaves are an important source of minerals, such as phosphorus, potassium, and magnesium and are used both fresh and dried (Isbilir & Sagiroglu, 2011). Phytochemical investigations have revealed the presence of diverse bioactive compounds in the leaves, stems, and roots of dill, such as tannins, terpenoids, cardiac glycosides, and flavonoid (Jana & Shekhawat, 2010) While previous studies have focused primarily on the seeds, the oil extracted from them has been shown to possess valuable antioxidant, antimicrobial, antiproliferative, and anticancer properties (Thanuma et al., 2025). Used since ancient times to alleviate gastrointestinal ailments, dill has a remarkable phytochemical profile. Approximately 90% of the composition of dill fruit extract is represented by D-carvone, D-limonene and alpha-phellandrene (Tizghadam et al., 2021).

Aromatic plants are widely used in the food and pharmaceutical sectors for their high antioxidant potential. This is generated by phenolic compounds (such as flavonoids, phenolic acids, stilbenes, lignans and tannins) found in various parts of the plant, from leaves and fruits to seeds and rhizomes (Kozłowska et

al., 2021). Aromatic plants used in food are valuable sources of essential nutrients, such as vitamins, minerals and antioxidant compounds. (Nour et al., 2017). Although *Levisticum officinale* (lovage) was once a staple herb in the food industry and in home kitchens, essential for seasoning soups and meat dishes, its popularity has declined drastically (Mohamadi et al., 2017). Today, its culinary utility remains a closely guarded secret among a few culinary experts, largely unknown to the public. Lovage is a relevant aromatic plant, but the literature contains only a limited number of studies on the volatile composition of lovage essential oil from various geographical regions (Spréa et al., 2020). All parts of lovage are intensely aromatic, presenting characteristic earthy notes, like those of celery (Moradalizadeh et al., 2012). Due to its compounds, lovage can act as a natural antioxidant, a property mainly attributed to the high content of polyphenols found in the plant material (Tomsone & Kruma, 2014). Another essential class of bioactive compounds are phenolic acids-such as chlorogenic, ferulic, and caffeic acids-recognized for their antioxidant and cardioprotective effects. Research indicates that lovage leaf extracts possess antioxidant capacity comparable to that of sage and rosemary, two of the medicinal plants with the most intense antioxidant activity (Sahlabgi et al., 2025).

MATERIALS AND METHODS

Extracts preparation

The plants analyzed included garden orache, sorrel, patience dock, wild garlic, lovage, tarragon, and dill. All were purchased at the local market in Timișoara, Romania.

The fresh leaves of these plants were cleaned of impurities, washed, and divided into two batches. One batch (fresh) was chopped and used immediately for extraction. The other batch of fresh plant material was dried at room temperature (21°C). The dried leaves were grounded using a mortar and pestle, packed, and stored in airtight containers until further use.

The extracts were obtained from fresh and dried material using 1 g of finely ground material in 20 mL of extraction solvent (70% ethanol). The extractions were performed in the dark at room temperature in a glass-stoppered Erlenmeyer flask, and the extraction time was 24 hours. The resulting extracts were filtered and then subjected to analysis.

Evaluation of the free radical scavenging activity

The free radical scavenging activity (RSA) was determined using the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) method (Dumbrava et al., 2023) for the obtained extracts. Briefly, 2 g of each sample was extracted with 20 mL of 70% ethanol for 2 hours at 25°C under continuous stirring. After filtration through Whatman No. 1 filter paper, 1 mL of each filtrate was mixed with 2.5 mL of 0.1 mM DPPH solution (in 70% ethanol). The mixture was homogenized and incubated in the dark at room temperature for 30 minutes. Absorbance was measured at 517 nm against a blank with 70% ethanol. A control sample was also prepared (1 mL of 70% ethanol and 2.5 mL of DPPH solution). RSA was calculated using the following equation:

$$RSA\ (\%) = \frac{\mathrm{Ac} - \mathrm{As}}{Ac}\ x\ 100$$

where: Ac is the absorbance of the control sample, As is the absorbance in the presence of the sample.

Evaluation of the total polyphenol content

Total polyphenols content was determined calorimetrically with the Folin-Ciocalteu reagent (Moldovan et al., 2023), the results being expressed in mg gallic acid equivalents/g.

Statistical analysis

All TPC and RSA determinations were performed in triplicate, with results expressed as mean ± standard deviation (SD). Data processing was conducted using Microsoft Excel 2010.

RESULTS AND DISCUSSIONS

DPPH free radical scavenging activity

The results of RSA are shown in Table 1.

Analysis of the free radical scavenging capacity by the DPPH method revealed significant differences between the seven fresh plant species studied. The values obtained reflect the immediate antioxidant potential of the plant

tissues, before any thermal degradation or drying process. According to experimental data, the DPPH radical scavenging capacity ranges from 55.71± 1.46% (lovage) to 72.65 ±2.27% (patience dock). The most remarkable results were recorded for patience dock (72.65%) and sorrel (70.38%).

Table 1. RSA (%) results in fresh and dried leaves

Sample	Fresh	Dried
Garden orache	61.53±1.47	84.34±0.79
Sorrel	70.38±1.33	90.02±1.13
Patience dock	72.65±2.27	91.86±0.27
Wild garlic	68.27±1.82	89.84±1.04
Lovage	55.71±1.46	80.77±0.40
Tarragon	60.45±1.00	92.45±1.18
Dill	66.82±1.02	88.73±1.30

All dried samples show higher RSA values than fresh ones, indicating a concentration of antioxidant compounds through the removal of water. The average increase in RSA is approximately 25-30%, confirming that drying enhances the free radical neutralization capacity. The largest increases are observed in tarragon from 60.45→92.45 (+32%), patience dock, from 72.65→91.86 (+19%) and lovage, from 55.71→80.77 (+25%).

Regarding fresh leaf samples, patience dock and sorrel have the best antioxidant activity in the fresh state, indicating a high content of polyphenols and flavonoids.

Drying causes a clear amplification of antioxidant activity in all samples, by increasing the concentration of active substances and reducing oxidative degradation.

In the fresh state, patience dock dominates, and in the dried state, tarragon shows the highest capacity to neutralize DPPH radicals.

The RSA values of the seven dried leaf samples show, overall, a very high antioxidant activity, but each plant stands out with a slightly different profile. Tarragon clearly stands out as having the highest antioxidant capacity, exceeding 92%, suggesting a particularly high concentration of active compounds that remain stable after drying. Patience dock is close behind, with an almost equally high value, indicating excellent efficiency in neutralizing free radicals. Sorrel and wild garlic form an upper intermediate group, both exceeding 89–90%, indicating that drying significantly enhances their antioxidant potential, without any notable loss of bioactive compounds.

Dill falls slightly below this group, but remains in the very high range, with almost 89%, confirming the presence of stable and effective antioxidants. Garden orache has good antioxidant activity, but noticeably lower than the samples, at around 84%. At the end of the range is lovage, which, although it has the lowest value among the dried samples, still exceeds 80%, which still places it in the category of plants with strong antioxidant activity.

Overall, all dried samples demonstrate considerable antioxidant capacity, the differences between them reflecting the peculiarities of the phytochemical composition rather than a loss of activity following drying.

Total phenolic compound

Polyphenols are one of the most important classes of plant secondary metabolites and are recognized for their antioxidant, anti-inflammatory, and antimicrobial properties. In this study, the total polyphenol content (TPC), expressed in mg GAE/g (gallic acid equivalent), is analyzed for seven species of green plants: garden orache, sorrel, patience dock, wild garlic, lovage, tarragon, and dill.

The results obtained from the analysis of total phenolic compounds (TPC) were presented in Tables 2 and 3.

Table 2. TPC in fresh leaves

Sample	mg GAE/g FW	mg GAE/g DW
Garden orache	0.71±0.02	6.45±0.04
Sorrel	0.63±0	5.80±0.43
Patience dock	1.94±0.03	15.08±1.45
Wild garlic	0.73 ±0.01	6.66±0.27
Lovage	0.44±0.01	2.93±0.04
Tarragon	0.55±0.01	2.75±0.06
Dill	1.03±0.00	5.72±0.04

Evaluation of fresh samples

In their fresh state but expressed on a dry - weight basis to eliminate moisture variability, the samples show a high polyphenol content. Patience dock stands out clearly, having the highest concentration (15.08 mg GAE/g DW), over five times richer than lovage or tarragon. The intermediate group - wild garlic (6.66), orache (6.45), sorrel (5.80), and dill (5.72) - shows similar average values, suggesting comparable antioxidant potential. Lovage and

tarragon record the lowest concentrations (< 3 mg GAE/g DW).

Evaluation of dried samples

After the drying process and correction for residual moisture, the profile changes significantly: garden orache (1.5) and dill (1.48) become the samples with the highest content of extracted polyphenols after drying. In terms of stability, the values are much more homogeneous in the dried samples (range 0.84-1.5) compared to the large variations observed in the fresh state. Although the residual moisture content varies between 8% (dill) and 13.5% (sorrel), it directly influences the final extraction yield but does not compensate for the substantial losses incurred. Since drying was carried out at room temperature (natural drying), the massive degradation of polyphenols (especially in the case of patience dock) cannot be attributed to thermal shock rather than to biochemical and physical processes occurring during slow drying.

Table 3. TPC in dry leaves

Sample	mg GAE/g DW	Residual water content (%)	mg GAE/g DW
Garden orache	1.32	12	1.5
Sorrel	1.07	13.5	1.24
Patience dock	0.81	12.5	0.93
Wild garlic	0.99	11.5	1.12
Lovage	0.83	10	0.92
Tarragon	0.76	9.5	0.84
Dill	1.37	8	1.48

Fresh vs. dried comparison

Comparative analysis highlights a severe degradation of polyphenols following the drying process.

Comparing the results between fresh and dried samples reveals a phenomenon of phytocompound degradation, suggesting that the drying process at room temperature was destructive to the integrity of polyphenols. Although one might intuit that the removal of water should lead to a "concentration" of the active substance, the data indicates exactly the opposite: the values expressed as dry matter (mg GAE/g DW) decrease drastically in the processed samples compared to their theoretical equivalent in the fresh state.

This major discrepancy, as in the case of patience dock (where the concentration decreases from approximately 15.08 to below 1 mg GAE/g DW), can be explained by the biochemical dynamics of slow drying. At room temperature, the dehydration process takes a long time, maintaining plant cells in a state in which endogenous enzymes, such as polyphenol oxidase, remain active and systematically oxidize phenolic compounds in the presence of atmospheric oxygen.

If in the fresh state the differences between species were very clearly marked (with a ratio of almost 5:1 between the richest and poorest sample), after drying, the values become relatively uniform and low for all samples. This demonstrates that the preservation method has standardized the antioxidant profile, transforming the plants from an excellent source of polyphenols into one with minimal residual biological value. Basically, natural drying facilitated a series of secondary degradation and polymerization reactions that made these compounds no longer detectable by the Folin-Ciocâlteu method.

Comparison with literature

The polyphenol content determined from fresh leaves of garden orache, (6.45 mg GAE/g DW) is higher than the range reported by Uslu et al. (2020), who identified values between 1.90 and 4.77 mg GAE/g DW. This indicates that the sample analyzed by you possesses a higher density of antioxidant compounds than the previously studied specimens.

Regarding the values obtained from dried leaves (1.5 mg GAE/gDW), they are relatively close to the values reported by Uslu et al. (2020).

The value of 15.08 mg GAE/g DW obtained in the case of dried samples of Patience dock (*Rumex patientia*) considerably exceeds the value of 2.45 mg GAE/g DW reported by Yuksel et al. (2024). This major difference suggests a high phytochemical potential of the sample analyzed.

In the case of sorrel, the value obtained of 5.80 mg GAE/g DW in fresh leaf samples is slightly lower than the 8.70 mg GAE/g DW identified by Torres et al. (2019), indicating a relative proximity of the results, despite the geographical variability of the plants. Greater differences are observed in dried leaf samples where the TPC is 1.24 mg GAE/gDW.

The results regarding the total polyphenol content of wild garlic (6.66 mg GAE/g DW) in

fresh leaves show an interesting variability compared to the literature: they are significantly higher than those reported by Barbu et al. (2023) - 0.354 mg GAE/g DW - and lower than the range of 1.41-2.14 g GAE/100g DW (equivalent to 14.1-21.4 mg/g) found by Bernas et al. (2023). At the same time, it is closest to the result reported by Boacă (2025) of 3.61 mg GAE/g DW. The TPC content in dried leaves (1.12 mg GAE/g DW) is comparable to that in the literature of 1.41-2.14 g GAE/100g DW (Bernas et al., 2023).

The value determined for fresh tarragon leaves of 2.75 mg GAE/g DW is comparable to the 3.75 mg GAE/g DW reported by Kara (2022). The value obtained for dried leaves is much lower than the value of 24.10 mg GAE/g DW mentioned by Behbahani (2017), which highlights the major impact that the plant variety and the extraction method can have on the result.

For dill (*Anethum graveolens*), the result of 5.72 mg GAE/g DW for fresh leaves is between the extremes of the literature: it is much higher than the range of 0.57-1.11 mg GAE/g DW reported by Leahu et al. (2023), but in dried leaves, the TPC value (1.48 mg GAE/g DW) is considerably lower than the value of 16.91 mg GAE/g DW found by Jadhav (2023).

The content of 2.93 mg GAE/g DW obtained in fresh lovage leaves is in full agreement with the value of 3.59 mg GAE/g DW reported by Tomsone (2014). In contrast, compared to the TPC values in dried lovage leaves (0.92 mg GAE/g DW), the value of 20.84 mg GAE/g DW published by Sahlabgi (2025) represents a much higher threshold, which probably reflects the use of optimized extraction techniques or a plant material with an unusual metabolic concentration.

Table 4. Correlation between RSA% and TPC in fresh leaves

Fresh samples	RSA %	TPC
Garden orache	61.53	6.45
Sorrel	70.38	5.8
Patience dock	72.65	15.08
Wild garlic	68.27	6.66
Lovage	55.71	2.93
Tarragon	60.45	2.75
Dill	66.82	5.72

0.744513

Fresh	RSA%	TPC
RSA%	1	0.744513
TPC	0.744513	1

As indicated in Table 4, RSA% exhibits a strong positive correlation with TPC (r = 0.74) in fresh leaf samples.

In the case of fresh plants, there is a close and direct link between polyphenols and antioxidant activity.

The higher the TPC (total polyphenols) content of the plant, the greater its ability to neutralize free radicals (RSA %).

Table 5. Correlation between RSA% and TPC in dried leaves

Dried samples	RSA %	TPC
Garden orache	84.34	1.5
Sorrel	90.02	1.24
Patience dock	91.86	0.93
Wild garlic	89.84	1.12
Lovage	80.77	0.92
Tarragon	92.45	0.84
Dill	88.73	1.48

-0.21452

Dried	RSA%	TPC
RSA%	1	-0.21452
TPC	-0.21452	1

As shown in Table 5, a negative correlation (-0.21) is observed between RSA and TPC in the dried samples.

After drying, although the RSA% values increased significantly (from ~60-70% to ~80-90%), the TPC values decreased drastically.

As a drying process seems to have degraded the polyphenols (low TPC), which could be attributed to the concentration or creation of other antioxidant substances that maintain the RSA % very high.

CONCLUSIONS

This research allowed the quantification and comparison of the polyphenolic profile for seven plants of food and phytotherapeutic importance: garden orache, sorrel, patience dock, leurda, lovage, tarragon and dill.

The comparative analysis of the seven green plants investigated reveals major differences between the fresh and dried states, both in terms of total polyphenol content (TPC) and antioxidant capacity (RSA %). In the fresh samples, TPC varies considerably among species, with high values in patience dock (15.08 mg GAE/g DW) and low levels in lovage and tarragon (<3 mg GAE/g DW), confirming the importance of these plants as natural sources of bioactive compounds. However, conventional room temperature

drying leads to a drastic reduction in TPC across all samples, with values converging into a narrow range (0.84-1.50 mg GAE/g DW), regardless of the initial content. This uniformity indicates massive polyphenol degradation, most likely caused by prolonged enzymatic oxidation during slow dehydration.

In contrast to the decline in TPC, drying results in a significant increase in RSA % in all samples. Values rise from 55-73% in fresh leaves to 80-92% in dried samples, with tarragon, sorrel, and patience dock reaching the highest levels (>90%). This dissociation between TPC and RSA suggests that post drying antioxidant activity is no longer determined exclusively by polyphenols but may be influenced by the formation of secondary compounds with increased reactivity in the DPPH system or by greater accessibility of non-phenolic antioxidants.

Overall, the results demonstrate that natural drying, although a traditional preservation method, does not maintain the integrity of phenolic compounds and significantly reduces the nutritional value associated with them. Nevertheless, the increase in RSA% indicates that dried plants retain, to some extent, their antioxidant potential, but through mechanisms different from those in fresh state. These findings highlight the need to optimize preservation methods to maintain the bioactive profile of green plants and provide a solid scientific basis for dietary recommendations and applications in the food industry.

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EFFECT OF KOMBUCHA-ENRICHED BRINE ON THE TENDERIZATION OF INJECTED BEEF: IMPLICATIONS FOR TEXTURE PROFILE, PHYSICOCHEMICAL PROPERTIES, AND SENSORY ACCEPTABILITY

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Abstract

This study evaluated the potential of kombucha – a fermented beverage produced through the symbiotic activity of acetic acid bacteria and yeasts – incorporated at three concentration levels (5%, 10%, and 15%) into the injection brine, as a natural source of organic acids with potential tenderizing and quality-modifying effects for smoked beef loin. Four batches were produced and assessed: one control (CB0%, with standard brine) and three formulations (KB5%, KB10%, KB15%), all injected at a rate of 10% (w/w). The products were evaluated for chemical composition, color parameters, texture profile, pH, water activity, and sensory acceptability. The addition of kombucha to the injection brine was associated with an improvement in meat tenderness, reflected by significantly lower Warner-Bratzler shear force and TPA hardness values, decreasing from 116.81 N and 57.60 N in the control sample to 71.66 N and 37.78 N in the sample containing 15% kombucha ($p < 0.05$). Sensory evaluation indicated good overall acceptability for all experimental formulations. Among the tested variants, the KB5% sample showed the highest scores for smoked aroma (7.86 ± 0.95), flavour (7.33 ± 0.66) and overall acceptability (7.58 ± 0.32), suggesting it as the most favorable formulation.

Key words*: injection brine, kombucha, meat tenderization, smoked beef, texture profile analysis.*

INTRODUCTION

The growing consumer demand for more natural and healthier meat products is posing new challenges for manufacturers in terms of the use of plant-based ingredients to improve the nutritional quality of products (Anchidin et al., 2024a; Manoliu et al., 2024). The meat industry, in particular, has been affected by this new movement owing to negative attitudes consumers associate with consuming heavily processed meat products (Inguglia et al., 2023). In this sense, nutritional enrichment with natural bioactive plant compounds including antioxidants, dietary fibers, probiotics, as well as the reduction of potentially harmful components such as salt, nitrate/nitrite, and artificial additives, are among the most commonly applied current strategies to improve the quality and safety of meat products (Ursachi et al., 2020; Flocea et al., 2024; Manoliu et al., 2025a).

At the same time, meat, and particularly beef, remains a nutritionally valuable food matrix, providing high-quality proteins with a complete amino acid profile, alongside essential lipids and micronutrients contributing to human health (Mădescu et al., 2025). Moreover, bovine resources can be further valorised through the superior utilisation of bones, which are used to obtain collagen-rich preparations such as bone broths, contributing additional nutritional and functional benefits (Anchidin et al., 2024b).

The use of fermented ingredients of natural origin, such as starter cultures (Baytukenova et al., 2025), plant extracts (Anchidin et al, 2025a; Ciobotaru et al., 2025a), vegetables (Bento de Carvalho et al., 2024; Boișteanu et al., 2023), or acid whey/vinegar (Sionek et al., 2024), as functional components in meat processing has gained considerable scientific interest, offering both technological advantages and improved consumer acceptability aligned with clean-label principles (Inguglia et al., 2023; Coșarcă et al., 2025).

The functional potential of plant-based bioingredients in food systems has been increasingly explored in recent years, with

numerous studies focusing on their incorporation into diverse food matrices. These investigations demonstrate significant antioxidant, antimicrobial, and nutritional enrichment effects, as well as improvements in sensory quality, across a wide range of products such as fish-based products (Flocea et al., 2025), beef snacks (Anchidin et al., 2025), chicken roulades (Boișteanu et al., 2025), or pork tenderloin (Anchidin et al., 2023).

Meat tenderness is considered one of the most decisive quality attributes influencing consumer acceptance of beef and processed beef products (Joo et al., 2023). The fundamental mechanism by which post-mortem aging and technological treatments improve the tenderness of meat involves the activation of endogenous proteolytic systems, particularly the calpain system, which causes the degradation of key myofibrillar and cytoskeletal proteins, disrupting the overall structural integrity of muscle cells (Inguglia et al., 2023; Joo et al., 2023). The optimal conditions for calpain activity, measured at the temperature of 25°C, have been estimated to occur around pH 7.5, with residual activity still detectable at pH 5, and tenderization in beef is known to occur at approximately pH 6.3 post-mortem, as μ-calpain is activated at low calcium concentrations (Kaur et al., 2021). Beyond the calpain system, lysosomal cathepsins – particularly cathepsins B, D, H, and L – are active under acidic conditions and contribute to tenderization during prolonged cold storage or under conditions of controlled pH reduction (Sentandreu et al., 2002; Huff-Lonergan & Lonergan, 2005).

In addition to technological and nutritional considerations, ensuring the safety of meat products remains essential, particularly in the context of processing operations that may introduce contaminants through interactions with equipment or food contact materials (Matei et al., 2024). In the context of increasingly complex food supply chains, systems such as the Rapid Alert System for Food and Feed (RASFF) highlight the persistent occurrence of contamination events and the need for continuous monitoring and risk management strategies (Postolache et al., 2020). These risks encompass a wide range of hazards, including microbiological contamination and chemical contaminants such as heavy metals (Ciobanu et al., 2020), which may accumulate in animal tissues and pose potential risks to human health, particularly due to their persistence and bioaccumulation along the food chain (Boișteanu et al., 2024), while contamination may also originate from upstream sources such as animal feed, where pollutants including mineral oil hydrocarbons have been identified (Matei et al., 2023).

Marination is a well-established meat processing technique that improves the natural rate of proteolysis by lowering the pH of the meat after slaughter, and stimulating enzymatic and proteolytic activities during muscle maturation. In some applications, the use of different acids helps break down the connective tissue, contributing to tenderness, while also exerting antimicrobial effects that extend shelf life (Rahman et al., 2023). A variety of ingredients have been incorporated into marinades over time, shifting the primary focus from preservation or pickling toward tenderization and flavor enhancement. Among these, biological marinades, including fermented dairy products such as kefir, yogurt, and buttermilk (Latoch, 2020; Rahman et al., 2023), fruit vinegars (Hafez et al., 2024), or juices (Zhang et al., 2024) – have demonstrated promising effects on the textural and sensory properties of meat. In marinated chilled chicken meat treated with papaya juice, an increase in protein solubility and a decrease in shear force was observed with the increase of papaya juice concentration (Zhang et al., 2024).

Injection marination, as opposed to surface marination, represents a particularly effective method for the uniform delivery of functional ingredients into the muscle matrix, enabling deeper penetration of bioactive compounds while improving water-holding capacity and overall technological yield (Sikes et al., 2009; Pietrasik & Shand, 2005; Kim et al., 2020). Brine injection has been reported to positively affect water retention and thereby the tenderness of meat, as salt and other dissolved compounds penetrate the protein-ion interactions within meat fibers, causing swelling of the matrix and solubilization of myofibrillar proteins (Rahman et al., 2023). The combination of injection with naturally acidic functional liquids thus provides a dual technological advantage: mechanical

distribution of the marinade throughout the muscle structure, and biochemical stimulation of proteolytic pathways (Chen et al., 2024; Ciobotaru et al., 2025b).

Kombucha is a fermented beverage produced by the fermentation of sweetened tea with a symbiotic culture of bacteria and yeasts (SCOBY), typically dominated by acetic acid bacteria of the genus *Komagataeibacter* and osmophilic yeast genera such as *Zygosaccharomyces* and *Saccharomyces* (Jayabalan et al., 2014). Through fermentation, this microbial consortium converts sucrose into a variety of metabolites, including ethanol, carbon dioxide, vitamins, polyphenolic derivatives, and organic acids such as acetic acid, gluconic acid, and glucuronic acid – bioactive compounds associated with antioxidant, antimicrobial, and anti-inflammatory effects (Tandhanskul et al., 2025). Several species of bacteria and yeasts involved in the fermentation process generate beneficial compounds, including polyphenols, organic acids, amino acids, vitamins, minerals, organic nitrogens, and hydrolytic enzymes, which confer significant antioxidant, anti-inflammatory, anticancer, and antimicrobial properties (Kitwetcharoen et al., 2023). The resulting low pH of kombucha (typically 2.5-3.5), combined with its content of organic acids and hydrolytic enzymes, positions it as a candidate for use as a natural tenderizing agent in meat processing applications, analogous to other acidic fermented substrates previously studied in this context (Rahman et al., 2023).

Despite the growing body of literature on the functional properties of kombucha as a beverage or as feed supplement (Adriani et al., 2007; Adriani et al., 2012; Ahmed et al., 2024), there are currently few studies regarding the application of kombucha in comminuted meat products (Bordea et al., 2017a; Bordea et al., 2017b), with no research regarding its use in meat marination or as a functional component of injection brine formulations.

This study aimed to investigate the effect of incorporating kombucha at different concentrations into the injection brine on the physicochemical properties, textural characteristics, color attributes, and sensory acceptability of smoked beef, with particular emphasis on tenderization as a key quality outcome.

MATERIALS AND METHODS

The study was conducted in two stages: the production of the experimental formulations and the qualitative analysis of the finished products, both carried out in the meat processing workshop and the specialized laboratories of the "Ion Ionescu de la Brad" Iași University of Life Sciences.

The beef loin and kombucha beverage were purchased from a local supplier in Iași County, kombucha having an initial pH of 4.1 Four experimental batches were produced, consisting of one control batch (CB 0%) injected with a standard brine solution, and three experimental batches (KB 5%, KB 10%, KB 15%) injected with brine solutions containing 5%, 10%, and 15% kombucha, respectively. Prior to incorporation into the brine, the kombucha was homogenized and used as a direct liquid replacement for a portion of the water in the brine solution, while the salt concentration in the brine was constant at 10% across all experimental batches. All batches were injected at a rate of 10% (w/w) relative to the meat weight, using an automatic injector (Inwestpol A-10), following the protocol described by Ciobotaru et al. (2025a). The brine uptake corresponding to the target injection level of 10% was verified gravimetrically by weighing the meat before and after injection. The brine composition for each formulation is presented in Table 1.

After production, the finished products were cooled and stored at refrigeration temperature (0-3°C) until the subsequent analysis stages.

All analyses were conducted in the Meat and Meat Products Technology Laboratory and the Sensory Analysis Laboratory of "Ion Ionescu de la Brad" Iași University of Life Sciences.

The chemical composition was determined using standard analytical methods: moisture by drying at 105°C to constant weight (ISO 1442:2023), ash by calcination at 550°C (ISO 936:1998), total fat by Soxhlet extraction (ISO 1443:1973) and total protein by the Kjeldahl method, with a conversion factor of 6.25 (ISO 937:2023).

The instrumental color parameters (L*, a*, b*) were assessed in the CIELAB color space on both the external surface and the cross-section of each sample, using the Chroma Meter CR-410 (Konica Minolta Inc., Japan). The instrument was calibrated using a standard white calibration plate under a D65 illuminant and a 2° standard observer. Based on the measured values for CIELAB color coordinates (L* – lightness, a* – green-red, b* – blue-yellow), the derived color parameters, hue angle (h*), Chroma (C*) and color difference (ΔE) were calculated according to the formulas (1), (2), and (3) described by Ciobanu et al. (2024).

Table 1. Formulations and heat treatment of experimental batches for beef loin injected with different concentrations of kombucha

Experimental batch	Injection level	Brine composition			Heat treatment		
		Water (%)	Kombucha (%)	Salt (%)	Air drying (°C/min)	Smoking (°C/min)	Hot air cooking (°C/min)
CB 0%	10%	90	0	10	65/30	72/30	86/50
KB 5%	10%	85	5		65/30	72/30	86/50
KB 10%	10%	80	10		65/30	72/30	86/50
KB 15%	10%	75	15		65/30	72/30	86/50

CB 0% – Control beef loin batch; KB 5%, KB 10%, KB 15% – Beef loin injected with brine containing 5%, 10% and 15% kombucha.

The pH value of the products was determined using a direct insertion pH meter (HI98163, Hanna Instruments Romania). The water activity (a_w) of the samples was determined using the RH2 humimeter instrument (Schaller Messtechnik, Austria).

$$h^* = tan^{-1} \times \frac{b^*}{a^*} \quad (1)$$

$$C^* = \sqrt{(a^*)^2 + (b^*)^2} \quad (2)$$

$$\Delta E = \sqrt{(L_0 - L_1)^2 + (a_0^* - a_1^*)^2 + (b_0^* - b_1^*)^2} \quad (3)$$

The textural parameters were determined using the TA1Plus texture analyzer (Lloyd Instruments, AMETEK, UK), equipped with a 500 N load cell. Two types of measurements were performed, as described by Manoliu et al. (2025b), with minor modifications regarding sample preparation: the Warner-Bratzler shear test, in which samples were prepared as strips (18 × 10 mm) aligned parallel to the muscle fibers, and shear force (N) and shear work (mJ) were recorded; and texture profile analysis (TPA), in which cylindrical samples of approximately 15 mm in height were subjected to double compression, and the following parameters were determined: hardness (N), cohesiveness, elasticity and gumminess (N).

The sensory analysis, an essential tool for ensuring that the products meet the required quality standards, helping to identify any unfavorable sensory characteristics (Ciobanu et al., 2023), was conducted under controlled temperature and lighting conditions in the Sensory Analysis Laboratory of University of Life Sciences, following procedures previously applied for similar meat products evaluated in the laboratory (Manoliu et al., 2023). A panel of 21 semi-trained evaluators, familiar with meat products, participated in a hedonic session, evaluating the following sensory attributes: appearance, section color, odour (smoked aroma), flavour, tenderness, juiciness, overall texture and overall acceptability. Each attribute was rated on a nine-point hedonic scale (1 – dislike extremely; 9 – like extremely). Samples were presented in uniform portions, coded with randomly assigned three-digit codes, and served in randomized order at room temperature.

All results for physicochemical evaluations were expressed as mean values (± standard deviation) from five analytical determinations performed on subsamples obtained from each experimental batch. Differences between the experimental batches and the control were assessed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test at a 5% significance level ($p < 0.05$), using IBM SPSS Statistics v. 21.

RESULTS AND DISCUSSIONS

The results for the physico-chemical analyses obtained for the beef loin injected with brine and smomed are shown in Table 2.

Moisture content increased significantly ($p < 0.0001$) with increasing kombucha concentration in the injection brine, ranging from 62.30 ± 0.69% (CB 0%) to 65.55 ± 0.27% (KB 15%), with corresponding decreases in dry matter from 37.70 ± 0.69% to 34.45 ± 0.27%. These values are consistent with the typical proximate composition of thermally processed whole-muscle beef products reported in the literature, ground beef samples analyzed showed moisture contents ranging from 59.6 to 72.9%, confirming the broad natural variability of this parameter across beef products (Isaksson et al., 1996).

The progressive increase in moisture with increasing kombucha concentration may be associated with the biochemical effects of the organic acids naturally present in kombucha beverage - primarily acetic, lactic, and gluconic acids (Jayabalan et al. 2014) - which can contribute to the local pH of the injected muscle tissue, shift the net charge of myofibrillar proteins away from their isoelectric point (~5.1), and thereby increase electrostatic repulsion between myofilaments and water-holding capacity of the protein matrix, , although this interpretation remains indicative due to the limited characterization of the kombucha used. However, a similar mechanism has been reported for acidic brine injection in beef, where pH reduction following organic acid treatment was associated with myofibrillar swelling and improved moisture retention within the muscle structure (Berge et al., 2001; Burke & Monahan, 2003).

Fat content decreased significantly ($p < 0.0001$) from 16.31 ± 1.06% (CB 0%) to 11.86 ± 0.42% (KB 15%). This decline is primarily attributable to a dilution effect: as moisture content increases through brine injection, the lipid fraction expressed as a percentage of total product mass decreases proportionally, even without actual lipolytic degradation of the lipid constituents. A similar inverse relationship between moisture and fat content has been consistently reported in the literature (Renou, 1995). One-point increase in intramuscular fat resulted in linear decreases in moisture and crude protein at an approximate rate of 0.7 and 0.3 percentage points, respectively, confirming the compositional interdependence between moisture, fat, and protein in muscle tissue (Matsumoto et al., 2024). An additional contribution to fat reduction at higher kombucha concentrations may arise from the organic acid content of kombucha (Cao & Qiu, 2024), which could promote limited lipolytic activity at the injection site; however, this mechanism requires further investigation to be confirmed in this specific matrix.

Table 2. Physico-chemical data of control and kombucha brine injected beef loin

Chemical compound	Sample				p-value
	CB 0%	KB 5%	KB 10%	KB 15%	
Moisture (%)	62.30 ± 0.69 ^{c}	62.97 ± 0.24 ^{bc}	63.20 ± 0.23 ^{b}	65.55 ± 0.27 ^{a}	<0.0001(***)
Dry Matter (%)	37.70 ± 0.69 ^{a}	37.03 ± 0.24 ^{ab}	36.80 ± 0.23 ^{b}	34.45 ± 0.27 ^{c}	<0.0001(***)
Fat (%)	16.31 ± 1.06 ^{a}	15.28 ± 0.46 ^{ab}	14.74 ± 0.33 ^{b}	11.86 ± 0.42 ^{c}	<0.0001(***)
Protein (%)	20.28 ± 0.37 ^{b}	20.53 ± 0.35 ^{b}	20.66 ± 0.21 ^{ab}	21.12 ± 0.22 ^{a}	0.003(**)
Ash (%)	1.09 ± 0.07 ^{b}	1.22 ± 0.05 ^{b}	1.40 ± 0.09 ^{a}	1.47 ± 0.09 ^{a}	<0.0001(***)
a_w	0.908 ± 0.00 ^{c}	0.935 ± 0.00 ^{ab}	0.937 ± 0.00 ^{a}	0.933 ± 0.00 ^{b}	<0.0001(***)
pH	6.32 ± 0.06 ^{a}	6.22 ± 0.02 ^{ab}	6.06 ± 0.06 ^{b}	6.34 ± 0.24 ^{a}	0.015(*)

Values are given as means ± standard deviation. Means with different superscripts in a row orientation indicate significant differences ($p < 0.05$) between samples determined by the Tukey test. Signification codes for the p-value: 0 < *** < 0.001 < ** < 0.01 < * < 0.05 < ns.

Crude protein content showed a moderate but statistically significant increase ($p = 0.003$) from 20.28 ± 0.37% (CB 0%) to 21.12 ± 0.22% (KB 15%), with significant differences identified between the control and the two highest kombucha concentrations. The observed protein values (20.28-21.12%) fall within the range of 18.1-20.7% reported for raw ground beef and above it for thermally processed products, reflecting the concentrating effect of the smoking and cooking process applied in the present study (Isaksson et al., 1996).

Ash content increased significantly ($p < 0.0001$) from 1.09 ± 0.07% (CB 0%) to 1.47 ± 0.09%

(KB 15%), consistent with the incorporation of mineral components from the injection brine. Bolognesi et al. (2020) reported that brine solutions containing 2.5% and 5.0% NaCl increased the mineral residue content from 1.04% (raw meat) to 3.1% at the higher salt concentration, demonstrating the direct and proportional relationship between NaCl concentration in the brine and ash content in the finished product. Moreover, different studies showed that the chemical composition of kombucha includes minerals, such as potassium, magnesium, calcium, copper, iron, zinc and manganese (Bauer-Petrovska & Petrushevska-Tozi, 2000; Bishop et al., 2022), which may contribute to the progressive increase in ash content from 1.09% to 1.47%.

In terms of pH, Neffe-Skocińska et al. (2017) state that the organic acids naturally present in kombucha beverage (acetic, lactic, and gluconic acids) are the main bioactive products of kombucha fermentation and are responsible for the characteristic acidic pH of the beverage. In the present study, pH values decreased from 6.32 ± 0.06 (CB 0%) to 6.06 ± 0.06 (KB 10%), followed by an increase at KB 15% (6.34 ± 0.24), as shown in Table 2. The initial pH decrease at intermediate concentrations may be associated with the presence of organic acids (Neffe-Skocińska et al., 2017). Moreover, Shi et al. (2021) show that changes in pH relative to the isoelectric point of myofibrillar proteins may influence their structural organization and water-holding capacity. When the pH departs from the isoelectric point of myosin (pI ≈ 5.4), the net charge of the proteins increases, which may lead to electrostatic repulsion between myofilaments and expansion of the interfibrillar space (Huff-Lonergan & Lonergan, 2005).

These structural changes may be associated with the increase in water activity (aw) observed in the kombucha-treated samples (0.908 for CB 0% vs. 0.935-0.937 for KB 5-10%), as a greater amount of water may be retained within the protein matrix.

In contrast to the trends observed at lower kombucha concentrations, the KB 15% sample showed an increase in pH accompanied by a slight decrease in water activity. This behavior indicates a non-linear response, suggesting the influence of additional factors beyond the effects typically attributed to organic acids reported in kombucha in the literature. This divergence may be associated with interactions between the brine constituents and the muscle matrix at higher incorporation levels, as well as with structural or compositional variability within the sample.

Table 3 shows the mean results registered for the colorimetric parameters (L*, a*, b*, C*, h*, and ΔE) for the injected smoked beef loin samples, measured both on the external surface and at the cross-section. These parameters are key indicators of product visual quality, serving as determinants of consumer acceptance before any tasting occurs.

In terms of surface color, lightness (L*) values on the product surface did not differ significantly between CB 0%, KB 5%, and KB 10% (27.73, 28.82, and 28.80, respectively), while KB 15% recorded a significantly higher value of 34.06 ± 0.98 ($p = 0.000$). This increase in surface lightness at the highest kombucha concentration can be attributed to the higher moisture content (65.55%) that alters the reflection of light at the surface by increasing the light reflectance at the product surface through modification of the surface microstructure during smoking and thermal treatment (Karamucki et al., 2013).

Redness (a*) showed a slight but significant decrease with increasing kombucha concentration ($p = 0.020$), from 9.45 ± 1.02 (CB 0%) to 7.57 ± 0.76 (KB 10%), with a partial recovery at KB 15% (8.93 ± 0.82). This reduction in surface redness is consistent with the dilution of myoglobin-derived pigments caused by the higher moisture content retained in the injected samples. Redness (a*) depends primarily on the forms of myoglobin present in the tissue and on the chromatic absorbance at 525 nm, and is directly influenced by the relative proportions of oxymyoglobin, metmyoglobin, and deoxymyoglobin in the muscle matrix (Karamucki et al., 2013). The introduction of organic acids from kombucha may additionally promote the formation of metmyoglobin at the product surface, further reducing the perceived redness.

The blue-yellow (b*) values on the surface showed significant differences among samples ($p = 0.008$), with KB 15% recording the highest value (9.68 ± 1.32) compared to KB 10% (5.56 ± 0.67), which was the lowest. Yellowness (b*)

increases with a decrease in the relative amount of deoxymyoglobin and an increase in oxymyoglobin and metmyoglobin, indicating that the oxidative state of myoglobin directly drives the yellow tone of the product (Karamucki et al., 2013). The elevated b* at KB 15% is consistent with the greater oxidative challenge posed by the higher concentration of organic acids introduced through kombucha, which may accelerate lipid oxidation secondary products contributing to yellow hue development.

Table 3. Colorimetric parameters of injected smoked beef (surface and section measurements)

Sample	L* (D65)	a* (D65)	b* (D65)	C*	h*	ΔE
			Surface color			
CB 0%	27.73 ± 0.60 ^{b}	9.45 ± 1.02 ^{a}	6.35 ± 1.06 ^{b}	11.39 ± 1.42 ^{ab}	33.74 ± 1.95 ^{b}	–
KB 5%	28.82 ± 3.35 ^{b}	8.77 ± 0.78 ^{ab}	7.23 ± 2.82 ^{ab}	11.48 ± 2.26 ^{ab}	38.12 ± 9.38 ^{ab}	4.55 ± 3.09 ^{ab}
KB 10%	28.80 ± 0.51 ^{b}	7.57 ± 0.76 ^{b}	5.56 ± 0.67 ^{b}	9.39 ± 1.01 ^{b}	36.25 ± 0.66 ^{b}	2.61 ± 0.93 ^{b}
KB 15%	34.06 ± 0.98 ^{a}	8.93 ± 0.82 ^{ab}	9.68 ± 1.32 ^{a}	13.19 ± 1.23 ^{a}	47.15 ± 4.05 ^{a}	7.39 ± 2.22 ^{a}
p-value	0.000 (***)	0.020(*)	0.008 (**)	0.012(*)	0.005(**)	0.000 (***)
			Section color			
CB 0%	49.33 ± 0.71 ^{d}	14.22 ± 0.49 ^{ab}	10.99 ± 0.20 ^{b}	17.97 ± 0.48 ^{ab}	37.71 ± 0.74 ^{bc}	–
KB 5%	54.19 ± 1.21 ^{b}	11.78 ± 0.35 ^{c}	11.62 ± 0.33 ^{a}	16.55 ± 0.34 ^{c}	44.61 ± 1.20 ^{a}	5.52 ± 1.83 ^{b}
KB 10%	59.22 ± 1.21 ^{a}	13.24 ± 0.41 ^{bc}	11.38 ± 0.15 ^{ab}	17.47 ± 0.27 ^{bc}	40.69 ± 1.16 ^{b}	9.97 ± 1.89 ^{a}
KB 15%	52.39 ± 0.49 ^{c}	15.17 ± 1.58 ^{a}	11.05 ± 0.21 ^{b}	18.80 ± 1.13 ^{a}	36.23 ± 3.51 ^{c}	3.56 ± 1.10 ^{b}
p-value	<0.0001(***)	0.000 (***)	0.002(**)	0.000 (***)	<0.0001(***)	0.019 (*)

L* = 0 (black); L* = 100 (white); –a * = green color; +a* = red color; –b* = blue color; +b* = yellow color; h* (hue angle, describes the dominant color tone), values closer to zero = more pure red, while increasing values indicate a shift toward yellow hues. Chroma (C*, color saturation or vividness) = larger values indicating more intense and saturated color. Results are given as means ± standard deviation from five repeated determinations. Means with different superscripts in a column orientation indicate significant differences ($p < 0.05$) between samples determined by the Tukey test. Signification codes for the p value: 0 < *** < 0.001 < ** < 0.01 < * < 0.05 < ns.

The significant differences in surface colorimetric parameters observed among the experimental batches are consistent with findings from studies conducted on smoked meat products, which confirm that both the intensity of the thermal treatment and the type of smoking method applied are key determinants of the colorimetric profile of thermally processed products (Gucianu et al., 2023; Ciobotaru et al., 2024a).

Color saturation (Chroma, C*) showed significant differences ($p = 0.012$), with KB 15% recording the highest value (13.19 ± 1.23) and KB 10% the lowest (9.39 ± 1.01). The hue angle (h*) increased significantly with kombucha concentration ($p = 0.005$), from 33.74° ± 1.95 (CB 0%) to 47.15° ± 4.05 (KB 15%), indicating a progressive shift of the dominant surface color away from red tones toward yellow-orange hues. This trend is directly interpretable in the context of the simultaneous decrease in a* and increase in b* described above, and is consistent with the increased moisture content and modified myoglobin redox state of the KB15% sample.

At the cross-section level, all four colorimetric parameters (L*, a*, b*, and C*) showed highly significant differences among samples ($p < 0.0001$ to $p = 0.002$), reflecting a more systematic influence of kombucha on the internal protein matrix than on the smoked surface, where Maillard reaction products and smoke deposits partially mask the treatment effects (Gucianu et al., 2024).

Lightness (L*) increased progressively from 49.33 ± 0.71 (CB 0%) to a maximum of 59.22 ± 1.21 (KB 10%), then decreased to 52.39 ± 0.49 (KB 15%), consistent with the non-linear pH pattern observed in Table 2, where KB 10% recorded the lowest pH (6.06 ± 0.06) and KB 15% showed a partial recovery (6.34 ± 0.24). This relationship is explained by a well-established structural mechanism: as muscle pH increases, the water-holding capacity of proteins increases and the higher intracellular water content causes swelling of fibers and shrinkage of the interfibrillar space, which decreases light scattering and increases light absorption by myoglobin, making the muscle appear darker (Wu et al., 2020).

Redness (a*) at the cross-section showed a distinct pattern from the surface, with KB 15% recording the highest value (15.17 ± 1.58), suggesting greater myoglobin preservation in the internal layers away from oxidative surface effects. Heat-induced denaturation of myoglobin in cooked meats is influenced by multiple endogenous factors including pH,

muscle source, and redox state, as well as exogenous factors such as ingredients incorporated during processing (Suman et al., 2016).

Hue angle (h*) showed significant differences ($p < 0.0001$), with CB 0% (37.71) and KB 15% (36.23) presenting lower values indicating a more red-dominant internal color, while KB 5% (44.61) and KB 10% (40.69) registered higher angles. Hue angle values closer to zero represent more pure red, while increasing values indicate a progressive shift toward yellow hues; chroma quantifies color saturation, with larger values indicating greater color intensity at a given hue (Claus, 2006).

The total color difference (ΔE) quantifies the overall chromatic distance between each kombucha-treated formulation and the control batch. Regarding the color difference at the external surface, ΔE values ranged from 2.61 ± 0.93 (KB 10%) to 7.39 ± 2.22 (KB 15%) on the surface. Altmann et al. (2022) state that a total color difference (ΔE) of 1 is discriminable by consumers under normal observation conditions, indicating that even modest ΔE values in the range observed for KB 10% represent perceptible color changes relative to the control. The ΔE of KB 15% (7.39) substantially exceeds this threshold, confirming that the color modification induced by 15% kombucha incorporation is clearly perceptible to consumers on the product surface.

At the section surface, the ΔE values ranged from 3.56 ± 1.10 (KB 15%) to 9.97 ± 1.89 (KB 10%), confirming that the color differences induced by kombucha incorporation are perceptible to consumers at the internal level and are most pronounced at the 10% incorporation level, consistent with the most pronounced pH reduction observed at this concentration.

In terms of textural attributes, this study evaluated the effect of kombucha beverage incorporation at varying concentrations (5%, 10%, and 15%) in the injection brine by Warner-Bratzler shear force and texture profile analysis (TPA) parameters including hardness, cohesiveness, gumminess, and elasticity. The data in Table 4 shows a gradual and significant reduction ($p < 0.0001$) of shear force with increasing kombucha concentration – from 116.81 ± 3.93 N in the control sample to 71.66 ± 2.15 N in the KB 15% formulation. The work of shear followed the same pattern, decreasing significantly from 1349.20 ± 9.61 mJ (CB 0%) to 948.57 ± 11.68 mJ (KB 15%), with all batches differing significantly from each other.

These results demonstrate that kombucha incorporation contributes to a progressive improvement in tenderness of the injected smoked beef loin, consistent with the tenderizing mechanisms of organic acids described in the literature. The tenderizing effects of organic acid injection in beef have been attributed to two concurrent mechanisms: (1) direct weakening of the perimysial connective tissue; and (2) acceleration of post-mortem proteolysis by cathepsins, whose activity is optimal at low pH values (Berge et al., 2001; Shi et al., 2021). In the present study, the organic acids naturally present in kombucha - primarily acetic, lactic, and gluconic acids - delivered via injection into the muscle matrix, reduced the local pH and thereby activated these mechanisms in a dose-dependent manner.

Hardness results followed a similar pattern to shear force, decreasing significantly ($p < 0.0001$) from 57.60 ± 5.03 N (CB 0%) to 37.78 ± 3.53 N (KB 15%). This may reflect the water-holding and myofibrillar swelling behavior of the organic acids within the protein matrix, leading to increased matrix density reduction and a softer internal consistency. Moisture enhancement with salt and phosphate solutions has been demonstrated to significantly reduce Warner-Bratzler shear force and to increase sensory scores for juiciness and tenderness in beef, confirming the structural softening effect of brine injection on the meat protein matrix (Pietrasik & Shand, 2011). In the present study, the additional biochemical contribution of kombucha's organic acids amplifies this effect beyond what would be expected from the NaCl component of the brine alone.

Table 4. Texture profile of beef loin injected with different concentrations of kombucha in brine

Analyzed parameter	CB 0%	KB 5%	KB 10%	KB 15%	p-value
Shear force (N)	116.81 ± 3.93 [a]	106.80 ± 5.65 [b]	74.39 ± 4.02 [c]	71.66 ± 2.15 [c]	<0.0001(***)
Work of shear (mJ)	1349.20 ± 9.61 [a]	1248.13 ± 8.89 [b]	980.52 ± 12.56 [c]	948.57 ± 11.68 [d]	<0.0001(***)
Hardness (N)	57.60 ± 5.03 [a]	45.63 ± 2.96 [b]	44.47 ± 4.61 [bc]	37.78 ± 3.53 [c]	<0.0001(***)
Cohesiveness (N)	0.03 ± 0.01 [b]	0.06 ± 0.01 [a]	0.06 ± 0.01 [a]	0.05 ± 0.02 [ab]	0.002(**)
Gumminess (N)	1.75 ± 0.19 [c]	2.87 ± 0.29 [a]	2.50 ± 0.38 [ab]	1.83 ± 0.65 [bc]	0.001(**)
Elasticity	1.01 ± 0.02 [a]	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.01	0.871(ns)

Values are given as means ± standard deviation from five repeted determinations. Means with different superscripts in a row orientation indicate significant differences ($p < 0.05$) between samples determined by the Tukey test. Signification codes for the p value: 0 < *** < 0.001 < ** < 0.01 < * < 0.05 < ns.

In terms of cohesiveness, the samples showed significant differences ($p = 0.002$), with KB 5% and KB 10% recording the highest values (0.06 ± 0.01), significantly greater than CB 0% (0.03 ± 0.01), while KB 15% returned to intermediate values (0.05 ± 0.02). This pattern suggests that at low and intermediate kombucha concentrations, the organic acids enhance the integrity of the internal protein network by promoting myofibrillar swelling and water retention within the gel matrix, creating a more cohesive structure. At the highest concentration (KB 15%), the more extensive proteolysis and connective tissue weakening may partially disrupt this internal cohesion, reducing the cohesiveness value relative to KB 5% and KB 10%.

Gumminess decreased significantly ($p = 0.001$) from 2.87 ± 0.29 N (KB 5%) to 1.83 ± 0.65 N (KB 15%), with the control recording the lowest value (1.75 ± 0.19 N). The superior value at KB 5% is consistent with the increase in cohesiveness observed at this concentration, as gumminess is is a parameter derived from the results obtained for hardness and cohesiveness. The decrease at KB 15% reflects the dominant effect of hardness reduction at the highest kombucha concentration, suggesting that the viscoelastic matrix was partially disrupted at this level, facilitating easier breakdown during mastication.

Elasticity did not differ significantly among samples ($p = 0.871$), with all batches recording values of 1.00 ± 0.00 to 1.01 ± 0.02. This result indicates that kombucha incorporation, regardless of concentration, did not alter the elastic recovery properties of the protein matrix under the compression conditions applied, suggesting that the structural modifications induced by organic acid injection affect primarily the resistance to shear and the firmness of the matrix, rather than its capacity for elastic recovery after deformation.

The sensory evaluation of the four experimental batches, performed by a panel of 21 semi-trained assessors on a 9-point hedonic scale, revealed that all samples scored above 6.0 for all evaluated attributes, indicating an acceptable sensory quality across all kombucha concentrations tested (Table 5).

For the attributes appearance, odour, and flavour, the sensory perception did not differ significantly among samples ($p = 0.614$, $p = 0.176$, and $p = 0.691$, respectively), confirming that kombucha incorporation into the injection brine did not alter the visual exterior or the characteristic smoked aroma and flavor profile of the product. The organic acids naturally present in kombucha, such as acetic, lactic, and gluconic acids, did not introduce perceptible off-notes at the concentrations used in the finished product after thermal treatment, a finding consistent with results reported by Braïek & Smaoui (2021), who demonstrated that lactic acid and sodium lactate incorporation into meat products improved sensory attributes including odour, flavour, and texture, with the highest overall acceptability score recorded at 1% lactic acid, without introduction of undesirable acidic off-notes perceptible to consumers.

Significant differences among samples were observed for section color, tenderness, juiciness, and overall texture ($p = 0.003$, $p = 0.016$, $p = 0.007$, and $p = 0.004$, respectively), with scores generally improving with increasing kombucha concentration. KB15% received the highest scores for tenderness (7.71 ± 0.81), juiciness (8.19 ± 0.88), and overall texture (7.86 ± 0.62).

Table 5. Sensory evaluation for control and kombucha brine injected smoked beef

Sensory attribute	Sample				p-value
	CB0%	KB 5%	KB 10%	KB 15%	
Appearance	7.33 ± 1.17 [a]	7.52 ± 0.89 [a]	7.57 ± 0.89 [a]	7.24 ± 0.77 [a]	0.614(ns)
Section color	7.05 ± 0.76 [ab]	7.29 ± 1.27 [a]	7.67 ± 0.94 [a]	6.38 ± 1.36 [b]	0.003(**)
Odour (smoked aroma)	7.33 ± 0.68 [a]	7.86 ± 0.95 [a]	7.48 ± 1.23 [a]	7.29 ± 0.55 [a]	0.176(ns)
Flavour	7.14 ± 0.88 [a]	7.33 ± 0.66 [a]	7.24 ± 0.89 [a]	7.05 ± 0.83 [a]	0.691(ns)
Tenderness	6.95 ± 0.86 [b]	7.57 ± 0.89 [ab]	7.05 ± 1.00 [ab]	7.71 ± 0.81 [a]	0.016(*)
Juiciness	7.19 ± 0.85 [b]	7.90 ± 1.12 [ab]	7.62 ± 0.89 [ab]	8.19 ± 0.88 [a]	0.007(**)
Overall texture	7.10 ± 0.67 [b]	7.57 ± 0.68 [ab]	7.40 ± 0.60 [ab]	7.86 ± 0.62 [a]	0.004(**)
Overall acceptability	7.16 ± 0.40 [b]	7.58 ± 0.32 [a]	7.43 ± 0.39 [ab]	7.39 ± 0.33 [ab]	0.006(**)

Values are given as means ± standard deviation (n=21). Means with different superscripts in a row orientation indicate significant differences ($p < 0.05$) between samples determined by the Tukey test. Signification codes for the p value: 0 < *** < 0.001 < ** < 0.01 < * < 0.05 < ns.

These results are consistent with the established tenderizing and moisture-retaining effects of organic acid injection reported in the literature. Berge et al. (2001) demonstrated that lactic acid injection significantly improved the textural traits of beef, including shear force values and sensory scores, through a combination of myofibrillar swelling, lysosomal enzyme release, and collagen weakening. Similarly, marination of beef with acid whey, a matrix rich in lactic acid, increased tenderness and juiciness ratings without decreasing hedonic liking scores for overall flavor or aftertaste (Kim, 2018).

Overall acceptability differed significantly among samples ($p = 0.006$), with KB 5% recording the highest score (7.58 ± 0.32) and CB 0% the lowest (7.16 ± 0.40), suggesting that, as overall perception is shaped by the combined contribution of all sensory attributes, moderate kombucha concentrations achieve an optimal sensory balance from the consumer's perspective. The slight decrease in section color score at KB 15% (6.38 ± 1.36), combined with greater inter-assessor variability for this attribute, may have partially offset the textural advantages of this batch in the overall hedonic evaluation. These findings indicate that KB 5% represents the optimal kombucha concentration for brine injection in smoked beef loin, achieving the most favorable balance between physicochemical improvement and overall consumer acceptability This observation aligns with the broader principle that functional additives incorporated into meat products at moderate concentrations tend to optimize sensory acceptance, whereas excessive levels risk compromising texture and overall sensory perception (Ciobotaru et al., 2024b).

The PCA biplot presented in Figure 1 covers 91.68% of the total variability of the dataset, with F1 accounting for 46.25% and F2 for 45.42%, indicating that the two principal components together provide a highly representative projection of the relationships among the analyzed parameters and experimental batches.

The control batch (CB 0%) is positioned in the lower-left quadrant of the biplot, strongly correlated with the parameters associated with textural resistance, shear force, work of shear, hardness, and dry matter and fat content, reflecting the firmer structure of smoked beef loin. These results confirm that the control sample presents the highest mechanical resistance among the analyzed batches, as showed by the instrumental texture data.

KB 5% is located in the upper-left area of the biplot and is associated primarily with gumminess, hue angle (h*), and b* values, suggesting that this batch is characterized by a moderate tenderizing effect combined with a slight shift toward yellow hues at the product surface.

Batch KB 10% is positioned in the upper-central area and correlates positively with cohesiveness, lightness, and water activity, reflecting the combined effect of pH reduction at this concentration on myofibrillar structure, promoting greater light scattering at the cross-section (higher L*) and improved internal cohesion, as well as the increased moisture retention.

Sample KB 15% occupies the lower-right quadrant and is strongly associated with moisture, protein, ash, chroma (C*), and redness (a*), as well as with a lower pH. This positioning confirms that the highest kombucha concentration produces the most pronounced

modification of the internal protein matrix and mineral profile, driving the highest redness and color saturation at the cross-section while simultaneously achieving the greatest moisture and protein retention. The pH vector points downward in the same general direction as KB 15%, consistent with the non-linear pH trend, where KB 15% records a partial pH recovery relative to KB 10%.

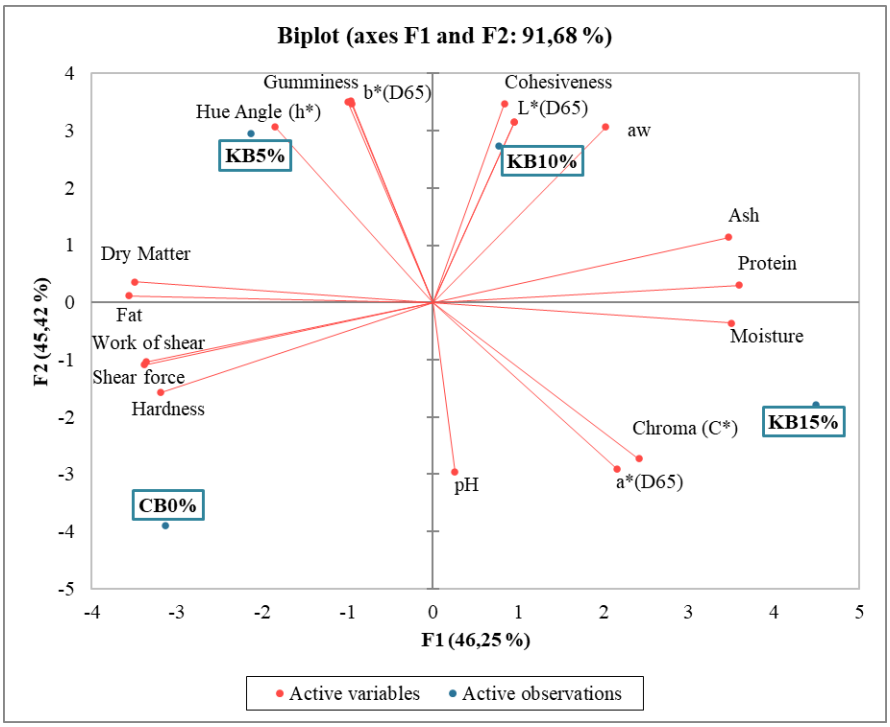


Figure 1. Principal component analysis (PCA) biplot illustrating the relationships between physicochemical, colorimetric, and textural parameters of control and kombucha brine-injected smoked beef loin (CB 0%, KB 5%, KB 10%, KB 15%).

CONCLUSIONS

The incorporation of kombucha beverage into the injection brine at concentrations of 5%, 10%, and 15%, from a textural perspective, resulted in a dose-dependent reduction in meat toughness, reflected by decreases in Warner–Bratzler shear force and hardness values.

In addition, kombucha incorporation was associated with significant modifications in the physicochemical profile of smoked beef loin, including progressive increases in moisture content, protein, and ash, alongside a corresponding decrease in fat content. Colorimetric analysis revealed perceptible color differences ($\Delta E > 1$) in all kombucha-treated samples relative to the control, both at the surface and cross-section, with the most pronounced internal lightness recorded at KB 10% and the highest surface redness and chroma at KB 15%, effects attributable to the pH-driven structural modifications of the myofibrillar matrix.

In terms of sensory evaluation, all four experimental batches maintained acceptable hedonic scores above 6.0 for all evaluated attributes. Although improvements were observed in tenderness, juiciness, overall texture, and section color, with KB 15% achieving the highest scores for textural attributes, the KB 5% sample showed a more balanced sensory profile in terms of appearance, odour, and flavour, resulting in the highest overall acceptability.

The mechanisms involved remain hypothesized based on literature data rather than directly

demonstrated in the present study; therefore, further research should aim to characterize the chemical composition of the kombucha used and to further explore its effects on product quality and stability.

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IMPACT OF MILKING SEQUENCE AND LACTATION NUMBER ON THE FATTY ACID COMPOSITION OF SHEEP MILK

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Abstract

The fatty acid profile of ewe milk is a critical indicator of its nutritional value and technological quality, being influenced by various physiological factors, including the stage of lactation and the order of milking. The aim of this study was to evaluate the evolution of the fatty acid composition in ewe milk across three consecutive lactations (L1, L2, and L3) and to determine the impact of milking sequence on these parameters. A total of 45 samples were collected over three milking sequences, from family farm of 15 heads of the Tsurcana (Țurcană) sheep breed from Șieuț locality, Bistrița Năsăud county. The findings revealed a significant improvement in the nutritional quality of the milk as the lactation rank advanced. A favorable decrease in the n-6/n-3 polyunsaturated fatty acid ratio was observed, shifting from 3.21 in the first lactation (L1) to 2.88 in the third lactation (L3). The analysis confirmed that the biochemical composition of ewe milk undergoes progressive modifications, with the lipid profile becoming more nutritionally balanced.

Key words*: ewe milk, fatty acid profile, lactation, lipid quality, n-6/n-3 ratio.*

INTRODUCTION

Consumption of dairy products has benefits in lowering blood pressure and cholesterol, helps in muscle development, prevents cancer (colorectal and bladder cancer), diabetes, obesity, has protective effects on coronary and cardiovascular diseases, stroke, dementia (Kliem & Givens, 2011; Huth & Park, 2012; Tunick & Van Hekken, 2014; Kanekanian, 2014). Milk is a complete and complex food that contains a variety of essential nutrients to ensure a healthy and balanced diet, being rich in valuable amino acids, lipids, carbohydrates, vitamins, minerals, enzymes, immunoglobulins (Kumar et al., 2017). It is also rich in functional ingredients such as immunoglobulins, α-lactalbumin, β-lactoglobulin, lactoferrin, lactoperoxidase, caseins and calcium (Anastasova et al., 2019). Bone health is ensured by the consumption of dairy products due to the valuable intake of calcium, vitamin D, protein and potassium (Rizzoli, 2014). The main factors influencing the fatty acid profile in milk are biological (breed, lactation, individuality, lactation: parity and stage) and external: animal nutrition (presence of concentrates, fat content and oil supplementation) and management (maintenance system, season, milking) (Hanuš et al., 2018). Fatty acids in milk are the result of the feed administered to the animals and the microbial activity of the rumen. Of all natural fats, the most structurally complex is milk fat because it is made up of approximately 400 different fatty acids (Lindmark, 2008). Sheep and goat milk is rich in medium-chain fatty acids: caproic (C6:0), caprylic (C8:0) and capric (C10:0), which is why its flavor is different compared to other species. Adding fresh grass to the animal diet increases the proportion of unsaturated fatty acids in milk fat (Djordjevic et al., 2019). Grazing has the effect of increasing milk production by 30% compared to feeding sheep in shelter (de Renobales et al., 2014). The largest seasonal variations were observed within the season, namely polyunsaturated fatty acids

with maximum values were identified in spring (44%) and summer and minimum values were observed in winter (De La Fuente et al., 2009). The main sources of fatty acids are found in oleaginous feeds, fish oil and fat supplements (Mansbridge & Blake, 1997). Grazing and the use of supplements rich in vegetable oils have an influence on the biohydrogenation process of fatty acids in the rumen, therefore improving the nutritional value of the fatty acid profile (Nudda et al., 2014). Other categories of vegetable lipids are flaxseed, soybean, safflower and sunflower which have positive effects on the content of conjugated linoleic acid and unsaturated fatty acids in milk fat (Luna et al., 2005; Bodas et al., 2010). The objective of this work was to investigate the dynamics of the fatty acid profile in sheep milk, with a focus on the sequential impact of milking and the evolution of these parameters during lactations I, II and III.

MATERIALS AND METHODS

The study was conducted on a flock of sheep belonging to a private family farm. As milk sampling was performed during the usual milking technological flow, without subjecting the animals to invasive experimental procedures, dietary changes or additional stress, specific approval from an ethics committee was not required (according to Directive 2010/63/EU on the protection of animals used for scientific purposes). All animal handling procedures complied with national farm animal welfare standards.

Animals

The research was conducted on a family sheep farm located in the Transylvania region: Șieuț locality, Bistrița-Năsăud county. The biological material was represented by Tsurcana sheep, Brează variety. The geographical coordinates of the pasture are: 46º58'37.50"N and 24º39'00.10"E. Lactating females are fed in shelter during the winter and in the summer they graze in pastures. The feed received by the animals during the winter is diversified, consisting of good quality hay, vetch, alfalfa and corn in a quantity of 0.5 kg/head. The meadow consists of grasses and spontaneous legumes (wild clover). Drinking water is provided at discretion since the pastures have natural springs. Milking is done manually, twice a day, starting with April and until October. The biological material is represented by 15 lactating female heads grouped into 3 categories: 5 primiparous females, 5 multiparous females (lactation 2) and 5 multiparous females (lactation 3).



Figure 1. Tsurcana sheep (original source)

Sampling

Milk samples collected for fatty acid analysis were collected individually on 3 milking sequences (sequence 1 - beginning of milking; sequence 2 - middle milking; sequence 3 - end of milking). Milking sequences were determined based on the milking duration that were previously recorded by a milking test, then they were divided equally. Milk was collected from the two quarters as a single sample. Subsequently, the samples were taken in sterile tubes and frozen until the analysis was determined. The samples were collected in June 2025.



Figure 2. Milk samples (original source)

Gas chromatographic analysis of fatty acids

Fatty acid composition was analyzed using a gas chromatograph system (GC-FID) from Agilent Technologies, model 6890N, located in Wilmington, DE, USA. This system included a DB-WAX capillary column with dimensions 30 meters long, 0.25 mm in diameter, and a 0.25 micrometer thick layer of polyethylene glycol as the stationary phase, along with a flame ionization detector. Initially, lipids were extracted from the samples. A volume of 50 mL of the sample was mixed with 50 mL of a chloroform:methanol solution in a 2:1 ratio by volume. The mixture underwent ultrasonic extraction for 30 minutes at room temperature using an ultrasonic bath, model Sonorex RK 512H, made by BANDELIN electronic GmbH & Co. KG, based in Berlin, Germany. The bath had internal dimensions of 150 × 138 × 65 mm and a working volume of 1.3 liters. It operated at a frequency of 40 kHz and a power of 60 W. After extraction, the mixture was filtered using Whatman No. 40 filter paper, sourced from Sigma-Aldrich, located in St. Louis, MO, USA. The organic layer was then collected. To purify the lipid extract, the sample was washed with 20 mL of a 0.74% solution of potassium chloride. The mixture was then centrifuged at a speed of 5000 revolutions per minute for 10 minutes to aid in the separation of phases. The organic layer was passed through anhydrous sodium sulfate to absorb any residual water. The solvent was evaporated using a rotary evaporator, model Laborota 4010, from Heidolph. The resulting oil was dried at a temperature of 60°C in an oven. To prepare fatty acid methyl esters (FAME), about 30 ± 0.1 mg of the extracted oil was dissolved in 2 mL of isooctane. Transesterification was then conducted by adding 200 microliters of a 2 mol/L methanolic potassium hydroxide solution while vigorously stirring. The organic layer was then treated with sodium hydrogen sulfate. Following phase separation, the supernatant was collected and used directly for gas chromatographic analysis.

Chromatographic analysis was performed by injecting 1 microliter of the prepared solution in split mode (1:20). The temperatures of the injector and detector were kept constant at 250°C. The oven temperature program started at 60°C and was held for 1 minute, then it increased from 60 to 200°C at a rate of 10°C per minute, with a 2-minute hold. The temperature was then raised from 200 to 220°C at a rate of 5°C per minute and held for 20 minutes. The detector gases used were hydrogen at 40 mL/min, air at 450 mL/min, and helium at 30 mL/min. Fatty acids were identified by comparing the retention times of the peaks from the sample with those from a certified reference standard mixture, Supelco 37-component FAME mix, CRM47885, from Sigma-Aldrich. The relative composition of each fatty acid was determined by normalizing the chromatographic peak areas internally and expressed as a percentage of the total identified fatty acids.

Statistical Methods

The data were analyzed using a General Linear Model (GLM) approach. A Two-Way Analysis of Variance (ANOVA) was conducted to assess the effects of lactation stage (L1, L2, L3) and milking sequence (S1, S2, S3), as well as their interaction (L × S). Since multiple samples were obtained from the same animals, 'Sheep ID' was included as a random effect in a mixed model to account for individual variation. The normality of the data distribution and homogeneity of variance were checked using the Shapiro-Wilk and Levene tests, respectively. If significant effects were found ($P < 0.05$), means were compared using Tukey's Honestly Significant Difference (HSD) post-hoc test. All results obtained were reported as mean and standard error of the mean (SEM). Statistical significance was set at $P < 0.05$. Calculations for Health Indices (Atherogenicity Index - AI and Thrombogenicity Index - TI) were based on the formulas given by Ulbricht & Southgate in 1991.

RESULTS AND DISCUSSIONS

The results indicate that the number of lactations exerts a direct influence on the quality of the lipid profile of sheep milk. ANOVA analysis confirmed significant differences ($p < 0.05$) in the case of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA). Table 1 presents the distribution of fatty acids in sheep milk according to lactation stage (L1, L2, L3). Data are expressed as mean ± SEM, and the statistical significance of variations between

physiological stages is highlighted by the calculated p-values.

Table 1. Fatty acid profile according to lactation (Means, Standard Error of the Mean)

Indicator (g/100 g)	Lactation 1 (Mean ± SEM)	Lactation 2 (Mean ± SEM)	Lactation 3 (Mean ± SEM)	p-value (ANOVA)
SFA (Saturated Fatty Acids)	71.42± 0.85	69.85± 0.72	68.12± 0.94	p<0.05
MUFA (Monounsaturated Fatty Acids)	22.15± 0.45	23.60± 0.51	24.85± 0.62	p<0.05
PUFA (Polyunsaturated Fatty Acids)	6.43± 0.22	6.55± 0.18	7.03± 0.31	p>0.05 (NS)
Report n-6/n-3	3.21± 0.12	3.05± 0.10	2.88± 0.14	p<0.05

Legend: *** - highly significant (P < 0.001); * - significant (P < 0.05); ns - not significant (P > 0.05)

An improvement in nutritional value was found as lactation progressed, highlighted by the reduction of the n-6/n-3 ratio (from 3.21 in L1 to 2.88 in L3). The favorable fatty acid profile observed in our study, especially in the third lactation (L3), can be attributed to the synergistic effect of animal maturity and the diet exclusively with green forage during the summer season. Grazing is an advantageous option for obtaining a higher concentration of phenolic compounds and antioxidant capacity of sheep milk (Ruiz de Gordoa et al., 2011; Chávez-Servín et al., 2018). The grazing-based agricultural system produces milk rich in fat and microcomponents beneficial to human health (fatty acids and vitamins) and volatile components (aroma, terpenes) (Morand-Fehr et al., 2007). Sheep farmed extensively on pasture have a higher content of cis-9, trans-11 and the milk fat profile is more beneficial for human health due to the high content of PUFA (Tsiplakou et al., 2006; Mierlita & Vicas, 2015). Natural pastures are a primary source of polyunsaturated fatty acids (PUFA), especially alpha-linolenic acid (C18:3 n-3), which acts as a precursor for the synthesis of beneficial lipids in milk (Mierlița, 2016). Sheep kept on pasture generally produce higher values of milk fat such as C18:3 linolenic acid, and unsaturated fatty acids MUFA and PUFA while feedlot sheep have a higher protein content (Atti et al., 2006; Ptáček et al., 2019). Standard Error (SEM) values confirm the precision of the measurements and the stability of the composition at the group level. Statistical analysis demonstrates that the maturity of the animal (expressed by the number of lactations) improves the quality of sheep milk by reducing the share of saturated fatty acids and optimizing the n-6/n-3 ratio. The differences observed between L1 and L3 are statistically significant ($p < 0.05$), confirming the hypothesis that the lipid profile evolves with the physiological age of the animal. The distribution of the main fatty acid classes (SFA, MUFA and PUFA) according to the milking sequence is presented in Table 2. The data (mean ± SEM) reflect the dynamics of lipid fractions during milking, the statistical significance of the variations being indicated by p-values.

Table 2. Impact of milking sequence on fatty acid profile (Mean ± SEM)

Indicator (g/100 g)	S1 (Initial Milking)	S2 (Middle Milking)	S3 (Final Milking)	p-value (ANOVA)
Total Fat (estimated)	Low	Average	High	p<0.01
SFA (Saturated Fatty Acids)	70.25± 0.90	69.90± 0.85	69.25± 0.95	p>0.05 (NS)
MUFA (Monounsaturated Fatty Acids)	23.10± 0.55	23.45± 0.50	23.95± 0.60	p>0.05 (NS)
PUFA (Polyunsaturated Fatty Acids)	6.65± 0.25	6.65± 0.22	6.80± 0.28	p>0.05 (NS)

Legend: *** - highly significant (P < 0.001); * - significant (P < 0.05); ns - not significant (P > 0.05)

Analysis of the milking sequences (S1-S3) indicates a remarkable stability of the fatty acid profile. Although the literature confirms an increase in total lipid content towards the end of milking, the percentage distribution of the SFA, MUFA and PUFA groups did not undergo statistically significant changes ($p > 0.05$). This result suggests that the milking process influences the amount of fat extracted, but does not alter the intrinsic quality of the lipid profile established at the level of mammary alveolar metabolism. To assess the nutritional quality of the lipid fraction of sheep milk and its potential impact on human health, atherogenicity (AI) and thrombogenicity (TI) indices were calculated, along with the n-6/n-3 fatty acid ratio. The mean values of these health parameters, recorded according to lactation rank, are summarized in Table 3. The results presented highlight a progressive improvement in the bioactive

properties of milk, suggesting a direct correlation between the physiological maturity of the animal and the reduction of the atherogenic and thrombogenic potential of milk fat.

Table 3. Lipid health indices according to lactation

Parameter	L1 (Media ± SEM)	L2 (Media ± SEM)	L3 (Media ± SEM)	p-value
n-6/n-3 ratio	3.21±0.04	3.05±0.05	2.88±0.04	<0.001
AI (Atherogenicity Index)	2.85±0.05	2.62±0.04	2.41±0.06	<0.001
TI (Thrombogenicity Index)	2.58±0.03	2.34±0.04	2.21±0.05	<0.01

Legend: *** - highly significant ($P < 0.001$); * - significant ($P < 0.05$); ns - not significant ($P > 0.05$)

Cardiovascular health indicators (AI and TI) showed a favorable evolution correlated with the number of lactations. The atherogenic index decreased from 2.85 (L1) to 2.41 (L3), indicating an improvement in lipid quality as the animals reach productive maturity. This decrease is determined by the rebalancing of the ratio between atherogenic fatty acids (C12:0-C16:0) and the unsaturated fraction. The stability of AI and TI indices across milking sequences (S1-S3) suggests that although fat content increases towards the end of milking due to the displacement of large fat globules from the alveolar lumen (Ontsouka et al., 2003), the health-promoting properties of milk remain constant throughout the milking process. The results demonstrate that although the milking sequence (S1-S3) modifies the lipid density, the AI and TI indices remain constant, the health profile of the milk being an intrinsic feature of the physiological stage of the sheep. The study confirms that lactation rank is a determining factor in establishing the nutritional value of sheep milk fat. The third lactation (L3) offers the most balanced lipid profile, characterized by the lowest values of the atherogenic and thrombogenic indices, as well as the most optimal n-6/n-3 ratio. The graphical analysis highlights a restructuring of the lipid composition, illustrating in detail the evolution of the major classes: SFA in Figure 1, MUFA in Figure 2 and PUFA in Figure 3, highlighting the trends in the modification of the fatty acid profile throughout the lactation cycles. The results of the ANOVA analysis revealed a significant effect of the lactation number on the main groups of fatty acids ($p < 0.05$). A progressive decrease in saturated fatty acids was observed along with an optimization of the n-6/n-3 ratio towards the end of the productive cycle of the animals (L3), a fact supported by the low values of the standard error (SEM), which indicates a stability of the lipid profile in mature animals.

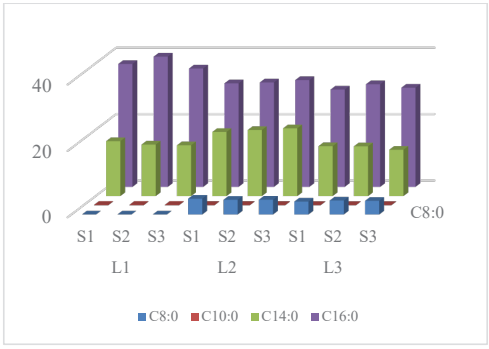


Figure 1. SFA milking sequence
Lactation stage (L1, L2, L3) and milking sequence (S1, S2, S3)

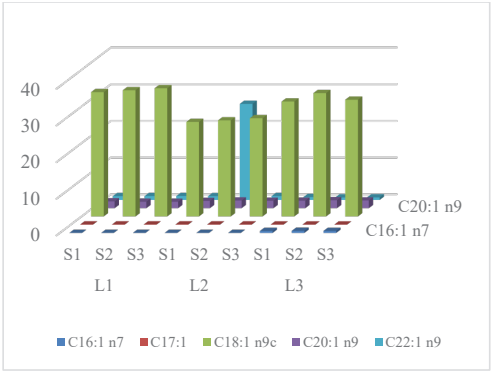


Figure 2. MUFA milking sequence

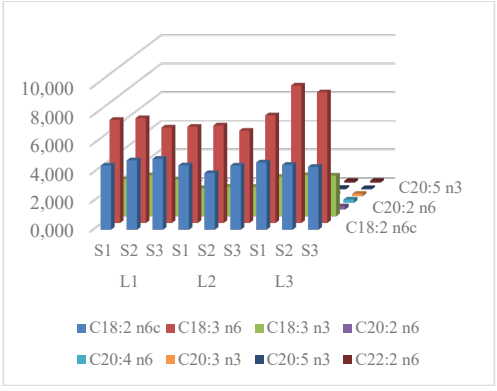


Figure 3. PUFA milking sequence

The fatty acid profile differs depending on the season, for example during the summer there are higher concentrations of α-linolenic acid, cis-9, trans 11-CLA, trans 11, C18:1 and PUFA and a low content of saturated fatty acids C12:0, C14:0 and C16:0 (Papaloukas et al., 2016). Altomonte et al. (2019) consider the milking period of sheep as a factor influencing milk characteristics and find that in the spring season there is a higher level of trans-11 C18:1, cis-9, cis-12, cis-15 C18:3 compared to the winter season. Mierliță et al. (2011a) observed that during the lactation period, milk production decreases while fat and protein content increases. With advancing lactation, the concentration of fatty acids C10:0, C14:0, C16:0, C16:1, C18:0, C18:3n3 and PUFA increases, while a decrease in fatty acids C4:0, C6:0, C12:0, C13:0, C15:0, C18:2n6 is observed. In the early lactation period, milk recorded the highest oleic acid content, which decreased during advancing lactation (Inostroza et al., 2020). Tsurcana sheep have a healthy fatty acid profile due to the low concentration of SFA (60.9%) and the high concentration of MUFA (31.0%) and PUFA (8.06%), as well as the C18:1, trans 11 C18:1, cis 9, trans 11 - CLA isomers (Mierlita & Dărăban, 2011, Mierliță et al., 2011b). Hemp seeds improve milk production, fat content by increasing polyunsaturated fatty acids and α-linolenic acid, while reducing unsaturated fats (Mierlita & Vicas, 2015). Adding soy to sheep diets increases the values of C18:0 and C18:1 isomers threefold compared to the control sample (Gómez-Cortés et al., 2008). At the same time, flaxseed supplementation increases milk production by 8%, increases C18:3 and CLA, decreases the proportion of unsaturated fatty acids without affecting the fat and protein components of milk, while the addition of sunflower seeds had no positive effects on milk production (Zhang et al., 2006).

To assess the contribution of experimental factors to lipid profile variability, two-way ANOVA was applied. Table 4 summarizes the statistical indicators of this model, including sums of squares (SS), degrees of freedom (df), and F-test values. This approach allows the identification of statistical significance for the main effects - lactation rank (L) and milking sequence (S) - as well as for the interaction between them (L x S). In this context, parameters such as sums of squares (SS) and F-test value become predictive indicators for the stability of the lipid profile under the given experimental conditions.

Table 4. Statistical indicators of lipid fraction variability according to experimental factors (Lactation and Milking Sequence)

Source of Variation	SS (Sum of Squares)	df (Deg. of Freedom)	MS (Mean Square)	F-value	p-value	Meaning
Lactation (L)	82.45	2	41.22	12.45	p<0.01	***
Sequence (S)	6.12	2	3.06	0.92	p>0.05	NS
Interaction (LxS)	2.34	4	0.58	0.17	p>0.05	NS
Error (Residual)	119.2	36	3.31	-	-	-
Total	210.11	44	-	-	-	-

Legend: *** - highly significant (P < 0.001); * - significant (P < 0.05); ns - not significant (P > 0.05)

The results of the analysis of variance (Table 4) confirm that lactation stage is the main source of variation in the lipid profile ($p < 0.001$), exerting a determining influence on the proportion of fatty acid classes. In contrast, milking sequence and the interaction between the two factors (L x S) did not generate statistically significant differences ($p > 0.05$). This indicates that, although the composition of milk changes as the animal matures, the distribution of fatty acids remains remarkably stable throughout the milk ejection process from the mammary gland, regardless of lactation stage.

CONCLUSIONS

The third lactation (L3) offered the most favorable nutritional profile, characterized by the lowest levels of saturated fatty acids (SFA) and the most beneficial values for the Atherogenicity (2.41) and Thrombogenicity (2.21) indices. This suggests that mature ewes exhibit superior metabolic efficiency in the synthesis of unsaturated lipids when maintained on exclusive summer pasture. Although fat concentration increases significantly towards the end of the milking process (S3), the quality of the lipid profile (AI, TI and n-6/n-3 ratio) does not very significantly between the cisternal and alveolar fractions. It is confirmed that the sanogenic properties of sheep milk are constant throughout the milking process. The exclusive

summer grazing system acts as a determining factor for the high concentration of polyunsaturated fatty acids, resulting in an optimal n-6/n-3 ratio (<3.0) in mature animals. The results demonstrate that, although the milking sequence (S1-S3) modifies lipid density, the AI and TI indices remain constant, the health profile of the milk being an intrinsic feature of the physiological stage of the ewe.

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IMPACT OF PARTIAL SUBSTITUTION OF WHEAT FLOUR WITH CHESTNUT FLOUR AND CHOKEBERRY (*Aronia melanocarpa*) POWDER ON THE NUTRITIONAL, FUNCTIONAL AND COOKING QUALITY OF PASTA

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Abstract

This study aimed to evaluate the potential of chestnut flour (CF) and chokeberry powder (CP) as alternative sources of bioactive compounds for the development of pasta formulations with improved nutritional and functional properties. Four pasta formulations were obtained by partially replacing wheat flour (WF) with CF at levels of 0%, 10%, 20%, and 30%. In addition, three formulations were produced by further substituting wheat flour in the 20% CF sample with CP at levels of 2%, 4%, and 6%. The samples were analyzed for proximate composition, total phenolic content (TPC), antioxidant activity (DPPH assay), and cooking quality. Substitution of WF with CF at 10–30% resulted in significant improvements in nutritional and phytochemical profiles of pasta while maintaining acceptable cooking quality. Further substitution of wheat flour in the 20% CF formulation with CP up to 4% led to a marked increase in bioactive compounds and is therefore recommended for enhancing pasta functionality without compromising cooking quality. These findings support the use of CF and CP as functional ingredients for the development of innovative flour-based products.

Key words*: chestnut flour, chokeberry powder, cooking quality, functional pasta, nutritional profile.*

INTRODUCTION

Pasta is one of the most widely consumed cereal-based foods, valued for its versatility, technological stability, and sensory acceptability. Its quality largely depends on the formation of a compact starch–protein network during dough processing and cooking, which determines texture and cooking behaviour (Dziki, 2021; Brochard et al., 2021). Despite these advantages, conventional pasta is often regarded as nutritionally limited, as it is typically produced from refined wheat flour with reduced fibre and bioactive compound content (Dziki, 2021). For this reason, increasing research attention has been directed toward enriching pasta with plant-derived ingredients that improve nutritional value while maintaining technological performance. Owing to its structural stability and formulation flexibility, pasta represents a suitable carrier for functional ingredients (Littardi et al., 2020).

Among alternative raw materials, chestnut flour has received growing attention due to its distinctive nutritional profile and functional properties. It provides complex carbohydrates and resistant starch, moderate levels of dietary fiber (commonly around 4-10 g/100 g), and relatively low lipid content dominated by unsaturated fatty acids. In addition, chestnut flour supplies vitamins (B-group, C, and E), essential minerals such as potassium and magnesium, and phenolic compounds associated with antioxidant activity (Demirkesen et al., 2010; Littardi et al., 2020; Ciucure et al., 2022; Alinovi et al., 2023; Stoin et al., 2025). These characteristics support its use as a fortifying ingredient that can enhance fibre, mineral, and phytochemical content in cereal-based foods (Dall'Asta et al., 2013; Oniszczuk et al., 2019; Pop et al., 2025; Stoin et al., 2025). Its application has been explored in a wide range of products, including bread (Raczyk et al., 2021), cookies (Sharma & Devi, 2021),

muffins (Choi et al., 2024) and pasta (Littardi et al., 2020), where moderate substitution levels generally improve nutritional quality and antioxidant potential. However, because chestnut flour is naturally gluten-free, its incorporation can alter dough rheology and structural integrity, highlighting the need for careful formulation to balance functional benefits and technological performance (Altıner & Mete, 2020; Raczyk et al., 2021; Pop et al., 2025).

Alongside alternative flours, fruit-derived ingredients rich in polyphenols have gained interest as natural fortifying agents. Chokeberry (*Aronia melanocarpa*) is widely recognized for its exceptionally high polyphenol content, particularly anthocyanins and related phenolic compounds, as well as for its contribution of vitamins, minerals, and dietary fibre. The incorporation of chokeberry-derived ingredients into cereal matrices has been associated with increased total phenolic content and enhanced antioxidant capacity, commonly evaluated using assays such as DPPH and FRAP (Adamczyk et al., 2025; Stoin et al., 2025). Importantly, relatively low inclusion levels can provide meaningful phytochemical enrichment while maintaining acceptable technological and sensory properties, whereas excessive addition may negatively affect colour and flavour (Stoin et al., 2025). Taken together, these findings support the potential of combining chestnut flour with chokeberry powder as a strategy for developing value-added cereal products with improved nutritional density and bioactive profiles. This approach allows simultaneous enhancement of fibre and mineral content through chestnut flour and polyphenol enrichment through chokeberry-derived compounds, while still requiring careful formulation to preserve product quality (Demirkesen et al., 2010; Pop et al., 2025; Stoin et al., 2025).

In this context, the present study investigated the combined use of chestnut flour and chokeberry powder as functional ingredients for the development of nutritionally enhanced pasta. Wheat flour was partially substituted with chestnut flour to improve fibre content and micronutrient density, followed by the incorporation of chokeberry powder as a natural source of polyphenols and antioxidant compounds (Borczak et al., 2023; Krajewska & Dziki, 2023). The resulting formulations were evaluated in terms of nutritional composition, antioxidant capacity, and key cooking quality parameters to better understand the relationship between functional enrichment and technological performance. By integrating nutrient-dense flour substitution with polyphenol-rich fruit fortification, this study provides insights into the formulation of value-added pasta with improved functional attributes while maintaining quality characteristics relevant to consumer acceptance and processing applicability.

MATERIALS AND METHODS

Raw materials and formulation design

For pasta production, the following raw materials were used: wheat flour type 000 (WF) with a moisture content of 13.72% (Boromir, Romania), chestnut flour (CF) produced by Targroch, and chokeberry powder (CP) produced by Dragon Superfoods purchased from a local natural products store in Timișoara, Romania. Wheat flour type 000 was selected instead of durum wheat semolina due to its fine particle size, comparable to that of CF and CP. Prior to use, CF and CP were sieved through an 80-mesh sieve to standardize particle size. Salt and potable water from local commercial sources were also included in the dough formulation. Two sets of composite flours were prepared through partial substitution of WF. In the first set, WF was replaced with CF at levels of 0% (control), 10%, 20%, and 30%, resulting in the CF0-CF30 blends. In the second set, a portion of WF in the 20% CF formulation (CF20) was further replaced with CP at levels of 2%, 4%, and 6%, yielding the CF20CP2-CF20CP6 blends. Pasta samples prepared from these blends were coded as PCF0 (control), PCF10, PCF20, and PCF30 for Group I, and PCF20 (control), PCF20CP2, PCF20CP4, and PCF20CP6 for Group II.

Pasta production process

Pasta samples were prepared following a conventional technological process adapted from the procedures described by Brochard et al. (2021) and Stoin et al. (2024). The dry ingredients (WF, CF, CP, and salt) were weighed and blended according to each experimental formulation to obtain a homogeneous mixture. Salt was incorporated at a level of 2 g per 100 g of flour blend. Tap water

(55 mL per 100 g flour) was added gradually, and the mixture was kneaded by hand for 10 min until a firm, uniform dough was obtained (Table 1). The dough was then allowed to rest for 1 h at room temperature (approximately 25°C), covered with plastic wrap to prevent surface drying. After resting, the dough was sheeted using a pasta machine (Marcato Ampia 150, Italy) to a uniform thickness of 2 mm. Drying was performed in a cabinet dryer (Italgi S.R.L., Italy) at 40 °C for approximately 12 h, until a final moisture content of about 11% was reached. The dried pasta was portioned (300 g), sealed in polypropylene bags, and stored at room temperature (25°C) for 60 days prior to analysis. Each formulation was prepared in triplicate (n = 3).

Table 1. Pasta formulations: control sample and variants fortified with CF or with CF and CP

Pasta formulations	Ingredients (%)				
	WF	CF	CP	S	W
	Pasta formulations (Set I)				
PCF0	100	0	0	2	55
PCF10	90	10	0	2	55
PCF20	80	20	0	2	55
PCF30	70	30	0	2	55
	Pasta formulations (Set II)				
PCF20	80	20	0	2	55
PCF20CP2	78	20	2	2	55
PCF20CP4	76	20	4	2	55
PCF20CP6	74	20	6	2	55

WF - Wheat flour; CF - chestnut flour; CP: chokeberry powder**;** PCF0 – Control pasta (100% wheat flour (WF): 0% chestnut flour (CF); PCF10 – Pasta sample with 90% wheat flour (WF): 10% chestnut flour (CF); PCF20 – Pasta sample with 80% wheat flour (WF): 20% chestnut flour (CF); PCF30 – Pasta sample with 70% wheat flour (WF): 30% chestnut flour (CF); PCF20CP2 – Pasta sample with 78% wheat flour (WF): 20% chestnut flour (CF): 2% chokeberry powder (CP); PCF20CP4 – Pasta sample with 76% wheat flour (WF): 20% chestnut flour (CF): 4% chokeberry powder (CP); PCF20CP6 – Pasta sample with 74% wheat flour (WF): 20% chestnut flour (CF): 6% chokeberry powder (CP).

Proximate composition of raw materials and pasta samples

The proximate composition of both raw materials and pasta samples was determined following standard analytical procedures of the Association of Official Analytical Chemists (AOAC, 2005). Moisture content was determined gravimetrically by oven-drying samples at 105°C until constant weight was achieved (AOAC 925.10). Protein content was quantified by the Kjeldahl method (AOAC 990.03), using a nitrogen-to-protein conversion factor of 5.70, as recommended for wheat-based products. Fat content was determined by Soxhlet extraction using petroleum ether as solvent (AOAC 920.39). Total dietary fibre was quantified by an enzymatic-gravimetric method according to AOAC 985.29. Ash content was obtained by incineration at 550°C for 5 h (AOAC 923.03). Total carbohydrate content was calculated by difference according to Equation (1), following the approach reported by Borșa et al. (2025):

$$\text{Total carbohydrates (\%)} = 100 - [\text{lipids (\%)} + \text{proteins (\%)} + \text{ash (\%)} + \text{moisture (\%)}] \quad (1)$$

The energy value was estimated using Atwater conversion factors (9 kcal/g for fat, 4 kcal/g for protein, and 4 kcal/g for carbohydrates), as shown in Equation (2):

$$\text{Energy value (kcal/100 g)} = 9 \times \text{fat (\%)} + 4 \times \text{carbohydrates (\%)} + 4 \times \text{proteins (\%)} \quad (2)$$

All analyses were performed in triplicate on both flour blends and finished pasta samples.

Cooking quality of pasta

Pasta cooking quality was evaluated based on established methods reported in previous studies (Chetrariu & Dabija, 2021; Schettino et al., 2021; Littardi et al., 2020). The optimum cooking time (OCT) was determined by cooking 25 g of pasta in 300 mL of boiling water without salt addition. At 30 s intervals, a pasta strand was pressed between two glass plates, and the OCT was defined as the complete disappearance of the starchy central core (Schettino et al., 2021). Determinations were performed in duplicate.

Cooking loss (CL) was evaluated gravimetrically by boiling 10 g of dried pasta in 200 mL of deionised water for the duration of the OCT. The cooking water was collected and evaporated to dryness at 105°C, and the residue was expressed as a percentage of the initial dry pasta weight, following gravimetric procedures adapted from Littardi et al. (2020).

Water absorption (WA) was calculated from the weight of pasta before and after cooking at the optimal cooking time and expressed as g per 100 g of dry pasta (Chetrariu & Dabija, 2021), according to Equation (3):

$$WA = [(w - w_0) / w_0] \times 100 \quad (3)$$

where w and w_0 represent the weights of cooked and raw pasta, respectively.

The swelling index (SI) was calculated as the ratio between the weight of cooked and

uncooked pasta. All measurements were performed in triplicate.

Preparation of Alcoholic Extracts

All analyses were conducted on dried pasta samples, with results reported on a dry-weight basis. Alcoholic extracts were prepared for the assessment of total phenolic content and antioxidant activity, based on the procedure described by Stoin et al. (2025), with minor adaptations for dried pasta matrices. Approximately 1.0 g of finely ground dried pasta was combined with 10 mL of 70% ethanol (*v/v*) and stirred for 2 h at room temperature using a magnetic stirrer. The suspension was then centrifuged at 10.000 rpm for 10 min, and the supernatant was carefully collected. The remaining residue underwent a second extraction with 70% ethanol for an additional 1 h under continuous stirring, followed by centrifugation under identical conditions. The pooled supernatants were stored at −20°C in the dark until further analysis. All samples were prepared and analysed independently in triplicate.

Total Phenolic Content (TPC)

Total phenolic content was determined using the Folin–Ciocalteu method as described by Stoin et al. (2025). Before analysis, alcoholic extracts were diluted 1:5 (v/v) with distilled water. A 0.5 mL aliquot of the diluted extract was mixed with 2.5 mL of Folin–Ciocalteu reagent and left to react for 5 min at room temperature. Subsequently, 2 mL of 7.5% Na_2CO_3 solution was added, and the mixture was incubated at 50 °C for 30 min. Absorbance was recorded at 750 nm using a UV–Vis spectrophotometer, using a reagent blank as reference. Results were expressed as milligrams of gallic acid equivalents per 100 g dry weight (mg GAE/100 g d.w.), calculated from a gallic acid calibration curve ($R^2 > 0.999$). All determinations were performed in triplicate.

Antioxidant Activity (DPPH Assay)

The free radical scavenging activity of the samples was determined using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, following the protocol described by Pop et al. (2025). A 0.1 mM DPPH working solution was prepared in 70% (*v/v*) ethanol. Before analysis, extracts obtained from wheat flour (WF), chestnut flour (CF), and pasta samples were diluted with 70% (v/v) ethanol at a ratio of 1:5 (*v/v*), while the chokeberry powder (CP) extract was diluted at a ratio of 1:100 (*v/v*).

A 1.0 mL aliquot of each diluted extract was mixed with 2.5 mL of the DPPH solution and incubated in the dark at 20°C for 30 min. Absorbance was measured at 517 nm using 70% ethanol as the blank. A control sample was prepared under identical conditions by replacing the extract with 1.0 mL of 70% ethanol.

DPPH scavenging activity (%) was calculated using the following equation (4):

$$\text{DPPH scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} x100 \quad (4)$$

where *A_control* represents the absorbance of the control and *A_sample* represents the absorbance of the sample.

To quantify antioxidant activity, DPPH scavenging activity values were plotted against Trolox standard concentrations (1.0–25 µg/mL) to generate a calibration curve. Results were expressed as µmol Trolox equivalents per gram of dry weight (µmol TE/g d.w.). All determinations were performed in triplicate.

Statistical analysis

Experimental data were collected from three independent replicates, each performed in triplicate, and are expressed as mean ± standard deviation (SD). Prior to statistical analysis, the assumptions of normality, homogeneity of variances (assessed via Levene's test), and independence of observations were verified and confirmed. Differences among formulations were evaluated by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons, with statistical significance set at $p < 0.05$.

RESULTS AND DISCUSSIONS

Proximate composition of raw materials and pasta formulations

The proximate composition of the raw materials (WF, CF, and CP) and pasta formulations (Set I and Set II) is presented in Table 2. Moisture content differed significantly ($p < 0.05$) among samples, with WF showing the highest value (12.72%), followed by CF (10.59%) and CP (7.62%). The lower moisture content of CP may contribute to improved shelf-life.

Table 2. Proximate composition and energy value of raw materials and pasta formulations

Samples	Chemical Parameters						Energy value (kcal/ 100 g)
	Moisture (%)	Fat (%)	Protein (%)	Ash (%)	*CRB (%)	Fiber (%)	
Raw materials							
WF	12.72±0.14^{a}	1.47±0.03^{c}	12.74±0.04^{a}	0.58±0.08^{c}	72.49±0.02^{c}	3.39±0.03^{c}	354.15±0.06^{c}
CF	10.59±0.15^{b}	3.88±0.09^{b}	7.67±0.02^{b}	3.07±0.09^{b}	74.79±0.08^{b}	7.12±0.09^{b}	364.76±0.04^{b}
CP	7.62±0.11^{c}	4.55±0.04^{a}	6.46±0.05^{c}	4.19±0.11^{a}	77.18±0.04^{a}	16.69±0.11^{a}	375.51±0.03^{a}
Pasta formulations (Set I)							
PCF0	12.24±0.02^{a}	1.22±0.04^{d}	9.68±0.02^{a}	0.55±0.04^{d}	76.31±0.03^{a}	2.58±0.05^{d}	354.94±0.05^{d}
PCF10	12.08±0.03^{b}	1.49±0.03^{c}	9.46±0.04^{b}	0.80±0.03^{c}	76.16±0.03^{b}	3.03±0.02^{c}	355.92±0.06^{c}
PCF20	11.91±0.02^{c}	1.75±0.05^{b}	9.28±0.05^{c}	1.05±0.07^{b}	76.01±0.04^{c}	3.49±0.02^{b}	356.89±0.04^{b}
PCF30	11.75±0.03^{d}	2.02±0.07^{a}	9.09±0.07^{d}	1.31±0.02^{a}	75.85±0.02^{d}	3.94±0.03^{a}	357.87±0.05^{a}
Pasta formulations (Set II)							
PCF20	11.91±0.02^{a}	1.75±0.05^{a}	9.28±0.05^{a}	1.05±0.07^{a}	76.01±0.04^{a}	3.49±0.02^{d}	356.89±0.04^{d}
PCF20CP2	11.82±0.03^{b}	1.81±0.02^{a}	9.22±0.02^{a}	1.11±0.05^{a}	76.03±0.12^{a}	3.75±0.06^{c}	357.28±0.22^{c}
PCF20CP4	11.74±0.03^{c}	1.86±0.06^{a}	9.17±0.02^{b}	1.18±0.05^{a}	76.06±0.11^{a}	4.02±0.04^{b}	357.65±0.12^{b}
PCF20CP6	11.65±0.04^{d}	1.92±0.04^{a}	9.11±0.03^{c}	1.24±0.02^{a}	76.08±0.10^{a}	4.28±0.02^{a}	358.03±0.19^{a}

WF - Wheat flour; CF - chestnut flour; CP: chokeberry powder**;** PCF0 – Control pasta (100% wheat flour (WF): 0% chestnut flour (CF); PCF10 – Pasta sample with 90% wheat flour (WF): 10% chestnut flour (CF); PCF20 – Pasta sample with 80% wheat flour (WF): 20% chestnut flour (CF); PCF30 – Pasta sample with 70% wheat flour (WF): 30% chestnut flour (CF); PCF20CP2 – Pasta sample with 78% wheat flour (WF): 20% chestnut flour (CF): 2% chokeberry powder (CP); PCF20CP4 – Pasta sample with 76% wheat flour (WF): 20% chestnut flour (CF): 4% chokeberry powder (CP); PCF20CP6 – Pasta sample with 74% wheat flour (WF): 20% chestnut flour (CF): 6% chokeberry powder (CP). *CRB – carbohydrates. Data are presented as mean ± standard deviation of three independent analyses. Values with different letters within the same column are significantly different (one-way ANOVA, $p < 0.05$), while those with the same letter are not significantly different ($p > 0.05$).

Similar moisture ranges were reported by Stoin et al. (2025) for functional cereal raw materials. Most compositional parameters differed significantly ($p < 0.05$) among samples, as indicated by different superscript letters in Table 2, with the exception of fat, ash, and carbohydrate contents in Set II formulations, which showed no significant differences upon chokeberry powder addition. The three raw materials presented strikingly different nutritional profiles, each contributing distinct functional properties to the final formulations. WF had the highest protein content (12.74%) alongside the lowest ash (0.58%) and fat (1.47%) levels, reflecting the typical composition of a refined cereal dominated by starch and gluten-forming proteins (Dziki, 2021). CF brought a richer mineral and fiber profile, with notably higher fat (3.88%), ash (3.07%), and dietary fiber (7.12%) contents and lower protein levels (7.67%), in agreement with reported ranges for chestnut-derived ingredients (Altıner & Mete, 2020; Torra et al., 2021; Ciucure et al., 2022). CP stood out for its exceptional fiber content (16.69%), the highest among all raw materials, alongside elevated fat (4.55%) and ash (4.19%) values, reflecting the concentrated nature of dried berry-derived powders. This high fiber fraction underlines the potential of CP as a functional ingredient for nutritional enrichment of cereal products (Sójka et al., 2013; Adamczyk et al., 2025). The presence of dietary fiber in both CF and CP may also influence dough properties through competition with gluten proteins for available water (Littardi et al., 2020).

Energy value increased progressively from WF (354.15 kcal/100 g) to CF (364.76 kcal/100 g) and CP (375.51 kcal/100 g), driven by the higher lipid and carbohydrate contents of the alternative ingredients, a trend previously reported in plant-enriched cereal systems (Brochard et al., 2021). Replacing WF with increasing levels of CF drove clear and consistent compositional shifts across Set I formulations.

Moisture decreased gradually from 12.24% (PCF0) to 11.75% (PCF30), likely reflecting the lower water retention capacity of non-gluten materials (Altıner & Mete, 2020). Protein content dropped from 9.68% to 9.09% as gluten-forming proteins were progressively diluted, a reduction of 0.5-1.5% typical of chestnut-enriched cereal products (Dziki, 2021; Alinovi et al., 2023), which may weaken the gluten network and affect dough structure (Littardi et al., 2020). Fat rose from 1.22% to 2.02%, ash increased markedly from 0.55% to 1.31%, and dietary fiber climbed from 2.58% to 3.94%, together reflecting the mineral- and fiber-rich character of CF (Ciucure et al., 2022; Alinovi et al., 2023). Energy value increased slightly (354.94-357.87 kcal/100 g), mainly driven by the higher lipid fraction of CF (Brochard et al., 2021). CF incorporation produced a gradual dilution of structural proteins accompanied by

meaningful enrichment in fiber, lipids, and minerals, supporting its use as a functional cereal ingredient (Altıner & Mete, 2020; Pop et al., 2025). Adding CP to the PCF20 base matrix brought more targeted changes. Moisture decreased slightly (11.91-11.65%) and protein showed only minor reductions (9.28-9.11%), indicating that chokeberry fortification has limited impact on the macronutrient backbone of the formulation, a pattern typical of berry-enriched cereal products (Dziki, 2021; Adamczyk et al., 2025). The most pronounced effect was on dietary fiber, which rose from 3.49% to 4.28%, and ash, which increased from 1.05% to 1.24%, reflecting the concentrated fiber and mineral content of CP (Sójka et al., 2013; Zbikowska et al., 2024). Fat increased slightly (1.75-1.92%), consistent with the lipid fraction of berry seeds (Brochard et al., 2021), and energy value remained nearly stable (356.89-358.03 kcal/100 g). CP delivered stronger fiber and mineral enrichment at lower substitution levels, without the protein dilution effect associated with CF. This complementary behavior makes the combined use of chestnut flour and chokeberry powder a balanced strategy for developing multifunctional cereal products with enhanced nutritional profiles (Sady & Sielicka-Różyńska, 2019; Zbikowska et al., 2024; Zeng et al., 2024; Adamczyk et al., 2025; Stoin et al., 2025).

Cooking quality of pasta formulations

The cooking quality parameters of the pasta samples are presented in Table 3. All cooking quality parameters differed significantly ($p < 0.05$) among Set I formulations, as indicated by different superscript letters in Table 3, while in Set II, the swelling index showed no significant differences among samples. Replacing WF with CF had a clear and progressive impact on all four cooking parameters. OCT dropped from 9.03 min (PCF0) to 7.10 min (PCF30), pointing to faster hydration and starch gelatinization as the gluten network became increasingly disrupted, a well-documented effect of non-gluten flour incorporation in pasta systems (Littardi et al., 2020; Alinovi et al., 2023). The reduction in cooking time may also be partly attributed to the lower protein content of CF, which reduces the resistance of the pasta structure to water penetration during thermal treatment. CL climbed from 3.54% to 4.27%, reflecting the reduced ability of the weakened protein matrix to retain soluble components during cooking (Dziki, 2021; Brochard et al., 2021). Although the CL values recorded remained within the acceptable range typically reported for enriched pasta products, the progressive increase with CF substitution level confirms that gluten dilution is the primary factor compromising structural integrity. WA rose sharply from 170.08 to 218.09 g/100 g dry pasta, driven by the higher fiber content of CF, since dietary fibers bind water and promote swelling during thermal processing (Altıner & Mete, 2020). The marked increase in WA also suggests that the composite pasta matrix became more porous and accessible to water as CF content increased, which is consistent with the simultaneous decrease in OCT. SI followed the same upward trend, increasing from 2.70 to 3.18, further supporting enhanced water uptake and structural expansion in chestnut-containing formulations (Alinovi et al., 2023).

The addition of CP to the PCF20 base produced more modest but consistent changes. OCT decreased slightly from 8.00 min (PCF20) to 7.04 min (PCF20CP6), suggesting that berry-derived particles may facilitate water diffusion within the pasta matrix (Sójka et al., 2013). This effect, though less pronounced than that of CF, indicates that even small amounts of chokeberry powder can influence the kinetics of water absorption during cooking. CL increased moderately from 4.05% to 4.48%, likely due to additional disruption of protein continuity by insoluble berry fibers (Mildner-Szkudlarz et al., 2009). WA continued to rise, reaching 216.88 g/100 g dry pasta at the highest CP level, while SI remained statistically stable across all Set II samples (3.08-3.17), indicating that CP enhances hydration capacity without substantially altering structural expansion, a behavior attributed to the water-binding properties of berry cell wall polysaccharides (Adamczyk et al., 2025). The stability of SI in Set II, despite increasing WA values, suggests that the berry-derived components promote uniform water distribution within the matrix rather than driving overall structural loosening.

Considering both sets together, protein dilution emerged as the primary driver of structural changes and cooking loss, while fiber enrichment governed hydration behavior.

Table 3. Cooking properties of pasta

Samples	Cooking properties of pasta			
	OCT (min)	CL (%)	WA (g/100 g dry pasta)	SI
Pasta formulations (Set I)				
PCF0	9.03±0.20^{a}	3.54±0.04^{d}	170.08±0.22^{d}	2.70±0.04^{d}
PCF10	8.08±0.11^{b}	3.88±0.03^{c}	192.46±0.24^{c}	2.92±0.03^{c}
PCF20	8.00±0.12^{b}	4.05±0.05^{b}	208.28±0.21^{b}	3.08±0.07^{b}
PCF30	7.10±0.13^{c}	4.27±0.07^{a}	218.09±0.25^{a}	3.18±0.02^{a}
Pasta formulations (Set II)				
PCF20	8.00±0.12^{a}	4.05±0.05^{d}	208.28±0.21^{d}	3.08±0.07^{a}
PCF20CP2	7.13±0.10^{b}	4.18±0.04^{c}	211.25±0.23^{c}	3.11±0.06^{a}
PCF20CP4	7.08±0.12^{b}	4.32±0.05^{b}	214.66±0.19^{b}	3.15±0.05^{a}
PCF20CP6	7.04±0.14^{b}	4.48±0.06^{a}	216.88±0.26^{a}	3.17±0.02^{a}

WF - Wheat flour; CF - chestnut flour; CP: chokeberry powder**;** PCF0 - Control pasta (100% wheat flour (WF): 0% chestnut flour (CF); PCF10 - Pasta sample with 90% wheat flour (WF): 10% chestnut flour (CF); PCF20 - Pasta sample with 80% wheat flour (WF): 20% chestnut flour (CF); PCF30 - Pasta sample with 70% wheat flour (WF): 30% chestnut flour (CF); PCF20CP2 - Pasta sample with 78% wheat flour (WF): 20% chestnut flour (CF): 2% chokeberry powder (CP); PCF20CP4 - Pasta sample with 76% wheat flour (WF): 20% chestnut flour (CF): 4% chokeberry powder (CP); PCF20CP6 - Pasta sample with 74% wheat flour (WF): 20% chestnut flour (CF): 6% chokeberry powder (CP). *CRB - carbohydrates. Data are presented as mean ± standard deviation of three independent analyses. Values with different letters within the same column are significantly different (one-way ANOVA, $p < 0.05$), while those with the same letter are not significantly different ($p > 0.05$).

Considering both sets together, protein dilution emerged as the primary driver of structural changes and cooking loss, while fiber enrichment governed hydration behavior. CF, acting through gluten dilution, produced stronger shifts in cooking parameters, whereas CP contributed additional hydration and minor structural effects. The magnitude of change was directly proportional to the compositional modifications described in the proximate analysis, supporting the view that technological performance in composite pasta is largely determined by the balance between gluten-forming proteins and non-gluten components (Dziki, 2021; Brochard et al., 2021).

Phytochemical profile of pasta formulations

The phytochemical profile of the raw materials and pasta formulations, expressed as total phenolic content (TPC) and antioxidant activity (DPPH, µmol TE/g d.w.), is presented in Table 4. All phytochemical parameters differed significantly ($p < 0.05$) among samples, as indicated by different superscript letters in Table 4. The three raw materials differed substantially in their phytochemical profiles, highlighting the central role of the enrichment ingredients in driving the functional potential of the final products. WF showed low TPC (52.64 mg GAE/100 g d.w.) and antioxidant activity (2.96 µmol TE/g d.w.), which is expected for refined wheat matrices, where phenolic depletion during milling substantially reduces radical-scavenging capacity (Shahidi & Ambigaipalan, 2015; Dziki, 2021). CF reached moderately higher phytochemical levels (186.36 mg GAE/100 g d.w.; 28.82 µmol TE/g d.w.), consistent with reported ranges of 150-300 mg GAE/100 g for chestnut-derived ingredients, with variation attributed to cultivar and processing conditions (Ciucure et al., 2022; Alinovi et al., 2023).

Table 4. Phytochemical profile of pasta formulations

Samples	Phytochemical Parameters	
	Total phenolic compounds (mg GAE / 100g d.w.)	Antioxidant activity (µmol TE/g d.w.)
Raw materials		
WF	52.64±0.36^{e}	2.96±0.12^{e}
CF	186.36±1.22^{b}	28.82±0.13^{b}
CP	5390.66±0.24^{a}	860.44±0.16^{a}
Pasta formulations (Set I)		
PCF0	26.84±0.20^{d}	1.68±0.14^{d}
PCF10	34.78±0.33^{c}	3.04±0.12^{c}
PCF20	45.02±0.44^{b}	4.36±0.11^{b}
PCF30	52.89±0.26^{a}	5.89±0.15^{a}
Pasta formulations (Set II)		
PCF20	45.02±0.44^{d}	4.36±0.11^{d}
PCF20CP2	145.86±0.32^{c}	20.87±0.16^{c}
PCF20CP4	178.66±0.26^{b}	35.66±0.18^{b}
PCF20CP6	198.44±0.11^{a}	52.44±0.20^{a}

WF - Wheat flour; CF - chestnut flour; CP: chokeberry powder**;** PCF0 - Control pasta (100% wheat flour (WF): 0% chestnut flour (CF); PCF10 – Pasta sample with 90% wheat flour (WF): 10% chestnut flour (CF); PCF20 - Pasta sample with 80% wheat flour (WF): 20% chestnut flour (CF); PCF30 - Pasta sample with 70% wheat flour (WF): 30% chestnut flour (CF); PCF20CP2 - Pasta sample with 78% wheat flour (WF): 20% chestnut flour (CF): 2% chokeberry powder (CP); PCF20CP4 - Pasta sample with 76% wheat flour (WF): 20% chestnut flour (CF): 4% chokeberry powder (CP); PCF20CP6 - Pasta sample with 74% wheat flour (WF): 20% chestnut flour (CF): 6% chokeberry powder (CP). *CRB - carbohydrates. Data are presented as mean ± standard deviation of three independent analyses. Values with different letters within the same column are significantly different (one-way ANOVA, $p < 0.05$), while those with the same letter are not significantly different ($p > 0.05$).

CP stood out with exceptionally high phytochemical density (5390.66 mg GAE/100 g d.w.; 860.44 µmol TE/g d.w.), placing it within the wide range documented for aronia-derived products (2000-8000 mg GAE/100 g d.w.) and

reinforcing its status as one of the most antioxidant-rich berry ingredients available (Kulling & Rawel, 2008; Sójka et al., 2013; Jakobek et al., 2025). A notable observation concerns the control pasta (PCF0: 26.84 mg GAE/100 g d.w.), whose TPC was considerably lower than that of WF (52.64 mg GAE/100 g d.w.). This reduction suggests that the pasta manufacturing process, including extrusion and drying, induces partial degradation of phenolic compounds, a phenomenon previously documented in functional pasta systems (Melini et al., 2020; Bresciani et al., 2022). In Set I formulations, replacing WF with increasing levels of CF led to a steady rise in both TPC and antioxidant activity, with TPC growing from 26.84 mg GAE/100 g d.w. (PCF0) to 52.89 mg GAE/100 g d.w. (PCF30) and antioxidant activity climbing from 1.68 to 5.89 µmol TE/g d.w. This roughly two-fold gain in TPC agrees with the 1.5-2.5-fold increases reported in chestnut-enriched cereal products (Littardi et al., 2020; Alinovi et al., 2023). While the absolute values remain modest relative to berry-enriched systems, the trend is fully consistent with the moderate but reliable phytochemical contribution of chestnut flour in composite matrices (Dziki, 2021). In Set II, even small additions of CP triggered a steep rise in both parameters. TPC climbed from 45.02 mg GAE/100 g d.w. (PCF20) to 198.44 mg GAE/100 g d.w. (PCF20CP6), while antioxidant activity jumped from 4.36 to 52.44 µmol TE/g d.w., a more than ten-fold increase. This response reflects the extraordinary phenolic richness of chokeberry and is well supported by studies showing that berry powders can elevate the phenolic content of cereal matrices by 2–5 times depending on inclusion level (Mildner-Szkudlarz et al., 2009; Jakobek et al., 2025; Stoin et al., 2025). Comparable gains in antioxidant capacity have been reported in berry-fortified pasta and bakery products, where anthocyanin-rich ingredients play a dominant role in enhancing radical-scavenging activity (Shahidi & Ambigaipalan, 2015). Throughout all formulations, TPC and DPPH values increased in parallel, reflecting the well-known link between phenolic concentration and antioxidant capacity in plant-based foods, with correlations above $r > 0.8$ consistently reported in cereal-berry composite systems (Shahidi & Ambigaipalan, 2015; Brochard et al., 2021). These results position CF as a moderate phytochemical enhancer and CP as a high-impact functional ingredient capable of substantially elevating the antioxidant profile of pasta formulations, a pattern consistent with recent findings on cereal products fortified with berry-derived bioactive ingredients (Mildner-Szkudlarz et al., 2009; Pop et al., 2025; Stoin et al., 2025).

Changes in the cooking time of pasta formulations

As shown in Table 5, optimal cooking time (OCT) differed significantly ($p < 0.05$) among pasta formulations at each storage interval, indicating that ingredient composition influenced the cooking behavior of the samples. Optimal cooking time is defined as the time required for complete starch gelatinization and disappearance of the uncooked core and is commonly used as an indicator of pasta cooking quality (Littardi et al., 2020).

Table 5. Changes in the cooking time of pasta formulations

Samples	Storage days		
	0	30	60
Pasta formulations (Set I)			
PCF0	9.03±0.20^{a}	8.90±0.10^{a}	8.40±0.21^{a}
PCF10	8.08±0.11^{b}	8.00±0.14^{b}	7.50±0.24^{b}
PCF20	8.00±0.12^{b}	7.90±0.11^{c}	7.40±0.21^{c}
PCF30	7.10±0.13^{c}	7.00±0.15^{d}	6.50±0.25^{d}
Pasta formulations (Set II)			
PCF20	8.00±0.12^{a}	7.90±0.11^{a}	7.40±0.21^{a}
PCF20CP2	7.13±0.10^{b}	7.00±0.12^{b}	6.40±0.12^{b}
PCF20CP4	7.08±0.12^{b}	6.95±0.15^{b}	6.25±0.15^{b}
PCF20CP6	7.04±0.14^{b}	6·90±0.14^{b}	6·10±0.14^{b}

WF - Wheat flour; CF - chestnut flour; CP: chokeberry powder; PCF0 - Control pasta (100% wheat flour (WF): 0% chestnut flour (CF); PCF10 - Pasta sample with 90% wheat flour (WF): 10% chestnut flour (CF); PCF20 - Pasta sample with 80% wheat flour (WF): 20% chestnut flour (CF); PCF30 - Pasta sample with 70% wheat flour (WF): 30% chestnut flour (CF); PCF20CP2 - Pasta sample with 78% wheat flour (WF): 20% chestnut flour (CF): 2% chokeberry powder (CP); PCF20CP4 - Pasta sample with 76% wheat flour (WF): 20% chestnut flour (CF): 4% chokeberry powder (CP); PCF20CP6 - Pasta sample with 74% wheat flour (WF): 20% chestnut flour (CF): 6% chokeberry powder (CP). *CRB - carbohydrates. Data are presented as mean ± standard deviation of three independent analyses. Values with different letters within the same column are significantly different (one-way ANOVA, $p < 0.05$), while those with the same letter

At day 0, the control sample (PCF0) showed the longest cooking time (9.03 min), significantly higher ($p < 0.05$) than all composite formulations. Progressive substitution of wheat

flour (WF) with chestnut flour (CF) reduced OCT to 8.08 min (PCF10), 8.00 min (PCF20), and 7.10 min (PCF30). This reduction suggests that the presence of chestnut flour facilitates water penetration and heat transfer during cooking, likely due to partial weakening of the gluten network (Alinovi et al., 2023). In Set II, the incorporation of chokeberry powder (CP) into the PCF20 formulation further reduced OCT, with values ranging from 7.13 min (PCF20CP2) to 7.04 min (PCF20CP6). Although differences among chokeberry-enriched samples were not statistically significant, OCT values remained lower than those of the PCF20 reference. This suggests that berry-derived fiber may facilitate water diffusion within the pasta matrix (Mildner-Szkudlarz et al., 2009; Foschia et al., 2013; Biernacka et al., 2019). During storage, a slight decrease in OCT was observed across all formulations while maintaining the same ranking among samples. After 60 days, the control sample decreased to 8.40 min, whereas CF-enriched samples reached values as low as 6.50 min (PCF30). In Set II, the lowest OCT was recorded for PCF20CP6 (6.10 min). Similar reductions in cooking time during storage have been reported for pasta formulated with alternative flours, where structural relaxation and moisture redistribution may occur over time (Vimercati et al., 2020; Dziki, 2021; Bresciani et al., 2022; Santos et al., 2025).

CONCLUSIONS

This study confirms that the combined use of CF and CP is an effective approach to improve the nutritional and functional profile of enriched pasta while maintaining acceptable technological quality. CF addition mainly increased dietary fiber and mineral content and induced moderate changes in cooking behavior associated with partial weakening of the gluten matrix. In contrast, CP exerted a stronger functional effect, significantly enhancing phenolic content and antioxidant capacity even at low inclusion levels. Despite these compositional changes, the enriched formulations retained satisfactory cooking performance, indicating that functional fortification can be achieved without major structural deterioration. The complementary roles of CF and CP enabled the development of pasta with improved nutritional value, enhanced bioactive potential, and balanced technological characteristics. Among the evaluated samples, PCF20CP4 showed the most favorable overall profile, providing the best compromise between improved nutritional value, elevated antioxidant activity, and acceptable cooking properties. These findings support the use of CF and CP as synergistic ingredients in the formulation of value-added cereal-based products. Overall, the results contribute to a better understanding of how ingredient interactions influence quality attributes in composite pasta systems and provide a solid basis for the development of functional cereal products with enhanced health-related potential.

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EFFICACY OF ANTIOXIDANT MARINATION IN THERMAL RISK MODULATION: COMPARATIVE PAH CONTAMINATION DYNAMICS IN BEEF BRISKET AND SHOULDER MUSCLES SUBJECTED TO GRILLING, FRYING, AND PRESSURE COOKING

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Abstract

The Polycyclic Aromatic Hydrocarbons (PAH) are carcinogenic and mutagenic contaminants generated by thermal processes mainly during high temperature cooking, via lipid degradation and pyrolytic reactions. In this study, the effect of thermal processing and marination on the occurrence of PAHs in beef brisket and shoulder muscles cooked by charcoal grilling (170°C), pan frying (114°C) and electric pot cooking (90 °C) was investigated. Significant differences between treatments were observed for Σ15 PAHs, PAH4, benzo[a]pyrene (BaP) and marination uptake values as a function of cooking method and muscle type. Samples of charcoal-grilled brisket (B-MC) had the highest concentration of Σ15 PAHs (22.50 ± 0.48 µg/kg) and PAH4 (0.86 ± 0.09 µg/kg). On the electric pot cooking at 90°C resulted in a lower concentration of PAH4 especially in B-ME (0.25 ± 0.01 µg/kg) and S-ME (0.22 ± 0.01 µg/kg), which reflects the mitigative effect of moderate thermal processing conditions.Two-way ANOVA showed significant interaction of muscle type and cooking treatment ($p < 0.001$). There was a significant negative correlation between the cooking loss and BaP concentration ($r = -0.714$, $p < 0.05$). Uptake of marination differed among treatments but the effect of marination on the occurrence of PAHs was treatment-dependent. In general, the main factors affecting the occurrence of PAHs in beef products were cooking temperature, smoke exposure and physicochemical alterations during cooking.

Key words*: beef brisket, beef shoulder, electric pressure cooking, marination, PAH.*

INTRODUCTION

Beef is a food with superior nutritional value (Zhang et al., 2025), but it requires cooking processes that can lead to the formation of potentially toxic compounds, such as polycyclic aromatic hydrocarbons (Adeyeye, 2020; Sahin et al., 2020). Thermal processes, such as frying in a pan, grilling, baking, and smoking, not only modify the sensory properties of the meat but also the levels of hydrocarbons generated. Consumers appreciate the particular taste of roasted beef acquired during thermal processing, for flavor creation at high temperatures (Kathuria et al., 2023). The Maillard reaction results from the interaction of amino compounds and reducing sugars, which provides food-rich flavors and sensory qualities (Chu et al., 2025). Different studies have shown that polycyclic aromatic hydrocarbons (PAH) (Chiang et al., 2020) are toxic chemicals formed during the incomplete combustion (Kalteh et al., 2024) of organic materials by smoking, roasting, grilling, barbecuing (Coroian et al., 2020; Coroian et al., 2022; Mastan et al., 2023; Șoimușan et al., 2024). Marinating, beside adding extra flavor to the prepared meat (Onopiuk et al., 2022; Oformata et al., 2024), also plays a role in inhibiting the formation of polycyclic aromatic compounds (Sampaio et al., 2021). Ciecierska et al. (2025) shown using electric grilling equipment or aluminium trays and marinating the meat, reduce the effects of PAH compounds.Research has demonstrated that choosing appropriate cooking methods by avoiding contact with flames, dripping fat into the heat source (Zahidah et al., 2024; Kwon et

al., 2025), and controlling the temperature substantially reduces the formation of PAHs (Duedahl-Olesen and Ionas, 2022). This study investigates the mitigation of 15 PAHs in beef meat (brisket and shoulder) through an antioxidant fruit-based marinade (sea buckthorn, cranberries, plums, sour cherries, and spices) across four thermal treatments.

MATERIALS AND METHODS

Certified organic beef from the Romanian cattle breed *Bălțată Românească*, sourced from the Silvania Bio farm (Sălaj County, Romania) was used as the raw material in this study.

Two muscle types with distinct anatomical and compositional characteristics were selected: brisket (*Musculus pectoralis)* and shoulder (*Musculus supraspinatus*). Prior to the experiment, visible connective tissue and excess surface fat were removed. Meat was portioned into samples weighing 60 g, with a uniform thickness of 1 cm to ensure comparable heat transfer during thermal processing. The experimental design consisted of two muscle types × four cooking treatments × five replicates, resulting in a total of 40 samples. The treatments were defined as follows:each treatment was performed in five independent replicates (n = 5). The sample codes presented in the Table 1 have been defined according to the experimental design. The first letter indicates the type of beef muscle (B = brisket, S = shoulder), the second letter indicates the marination state (C = control, M = marinated), and the last letter indicates the cooking method (C = charcoal grill, P = pan, E = electric pot).

Table 1. Experimental desing and sample coding

Sample code	Type Muscle	Treatment description
BC	Brisket control	Charcoal grill
BMC	Brisket marinated	Charcoal grill
BMP	Brisket marinated	Frying pan
BME	Brisket marinated	Electric pot
SC	Shoulder control	Charcoal grill
SCM	Shoulder marinated	Charcoal grill
SMP	Shoulder marinated	Frying pan
SME	Shoulder marinated	Electric pot

For treatments involving marination (MC, MP and ME), meat samples were subjected to an antioxidant fruit-based marinade designed to potentially reduce the formation of polycyclic aromatic hydrocarbons (PAHs) during thermal processing. The marinade formulation contained the following ingredients (g/1000 g of meat): 2.5% sea buckthorn (*Hippophae rhamnoides*), 2.5% cranberries (*Vaccinium macrocarpon*), 2.5% plums (*Prunus domestica*), 2.5% sour cherries (*Prunus cerasus*), 0.38% oregano (*Origanum vulgare*), 0.38% black pepper (*Piper nigrum*), 0.7% bay leaves (*Laurus nobilis*), salt 5%. The marination mixture was prepared using fruit-based ingredients with different processing conditions. Organic sea buckthorn berries and organic cranberries were used in frozen form ("quick frozen and optically selected"), supplied by Terarium Monte (Romania), no added sugar. According to the product specifications, the sea buckthorn berries contained 0.98% protein, 9.7% carbohydrates, 2.5% sugars, and 1.78% lipids per 100 g, with an energy value of 59 kcal. The cranberries contained 0.47% protein, 13.14% carbohydrates, 4.20% sugars, and 0.17% lipids per 100 g, with an energy value of 56 kcal. According to the manufacturer's specifications, the product was quick-frozen and stored at −18°C until use. Frozen sour cherries without pits supplied by FrescoVerde (Romania) were incorporated into the marinade formulation. According to the manufacturer's specifications, the product was quick-frozen and stored at −18°C until use. The nutritional composition per 100 g included 12.2 g carbohydrates, 8.5 g sugars, 1 g protein, and 0.3 g lipids, with an energy value of 50 kcal. The product originated from the European Union and was distributed by FrescoVerde SRL, Bihor County, Romania.Dried plums without pits (Prunus domestica) were used in dehydrated form and supplied by Fruit2Go Monicol (Republic of Moldova). According to the manufacturer's label, the dried plums contained protein 2.5 g, 49.0 g carbohydrates, 36.0 g sugars, 9.6 g fiber, and 0.9 g lipids per 100 g, with an energy value of 237 kcal. All fruit ingredients were commercially available food-grade products selected to reflect ingredients that are easily accessible to household consumers for domestic marination practices. The fruits were also chosen due to their natural content of organic acids, sugars, fibers, and bioactive

compounds potentially associated with physicochemical changes occurring during thermal processing. The ingredients were homogenized, by blender, to obtain a uniform mixture rich in natural antioxidant compounds such as polyphenols and organic acids. For each meat samples was aplicated 6 g of the marinade composition, with a brush and stored for 12 h at 4°C under refrigerated conditions. After the marination, 2 g of duck fat was applied with a brush to each sample prior to thermal processing. The fat layer was evenly distributed on the meat surface to simulate typical culinary conditions and to promote lipid interaction during cooking. Samples were subjected to three different cooking methods. Thermal processing was conducted using three cooking methods representing different heat transfer mechanisms and temperature regimes: charcoal grilling, frying pan cooking on a gas stove, and electric pot cooking. For charcoal grilling treatments (BC/SC and BMC/SMC), meat samples were cooked on a grill fueled with hexagonal coconut-shell bio charcoal briquettes, which provided a stable heat source and combustion environment. The samples were placed on the grill side away from the flame for indirect heat transfer at an average surface temperature of approximately 170 °C.For the frying pan treatment (BMP/SMP), samples were cooked in a ceramic frying pan placed on a gas-flame stove, with the pan surface temperature maintained at approximately 114°C.

The electric pot treatment (BME/SME) represented a lower-temperature cooking environment, where samples were prepared in an electric cooking pot, Tefal One Pot CY505E30, by setting the DIY Chef program that corresponds to level 3 at 90°C, without pressure and with the lid slightly open. During cooking, the internal temperature of each meat sample was continuously monitored using a calibrated digital thermocouple probe inserted into the geometric center of the sample. The endpoint temperature was standardized at 72°C, corresponding to the recommended safe internal temperature for well-done beef products. Temperature monitoring allowed accurate control of the cooking process and ensured comparability among the experimental treatments. For all cooking methods, the endpoint temperature was measured at the geometric center of each sample using a calibrated digital thermoprobe upon reaching the target temperature. Cooking was stopped immediately once this internal temperature was reached to ensure comparable thermal exposure across treatments, they were immediately taken from the grill, cooled to room temperature and re-weighed.To assess the weight loss, each sample was weighed before and after the respective treatment (grill, frying pan, electric pot). The formula for evaluating the cooking loss of the heat-treated meats is as follows:

Weight loss (%) = $M-M_1/M *100$

M - the weight of the unprocessed beef meat.

M_1 - the weight of the each treatment beef meat.

All chemicals and solvents used in the analysis were of HPLC grade and purchased from VWR International (Germany). Ultra-pure water was obtained using an ULTRACLEAR UV UF EVOQUA purification system (USA).

Antioxidant capacity was determined using the ACL assay kit (Analytik Jena, Germany). Analytical standards for polycyclic aromatic hydrocarbons and Trolox were obtained from Sigma-Aldrich (Darmstadt, Germany).

The analyzed samples consisted of beef brisket and beef shoulder muscles. Prior to analysis, the samples were homogenized to ensure sample uniformity. All measurements were performed in quintuplicate (n = 5). Polycyclic Aromatic Hydrocarbons (PAHs) Analysis were determined using a saponification followed by solid-phase purification procedure. Briefly, 10 g of homogenized beef brisket or shoulder sample were treated with 50 mL of 0.4 M KOH solution prepared in an ethanol-water mixture (9:1, v/v). The saponification process was performed in an ultrasonic bath at 60°C for 30 min. Following saponification, the mixture was filtered through filter paper, and the resulting extract was subjected to liquid-liquid extraction twice with 15 mL of cyclohexane. The combined organic phases were subsequently purified using a Florisil column. The purified extract was evaporated to dryness under a gentle stream of nitrogen and reconstituted in 1 mL of acetonitrile. Prior to chromatographic analysis, samples were filtered through 0.45 µm syringe filters. Analysis of PAHs by Chromatographic analysis was carried out

using a Waters Arc HPLC system (Waters Corporation, Milford, MA, USA) equipped with a fluorescence detector (FLD). Separation of 15 PAHs was achieved on an Inertsil ODS-P column (5 µm, 4.6 × 150 mm) maintained at 24°C. The injection volume was 10 µL. The mobile phase consisted of a water-acetonitrile gradient system. Detection was performed using a time-programmed fluorescence detection method optimized for each PAH compound.The PAHs analyzed were selected according to priority toxicological indicators defined by European food safety regulations. Benzo[a]pyrene (BaP) was evaluated separately as an important carcinogenic marker used in food safety assessment of thermally processed meat products. The analyzed PAHs compounds included naphthalene, acenaphthene, fluorene, pyrene, phenanthrene,anthracene,fluoranthene, benzo[a]anthracene(BaA),benzo[b]fluoranthen e (BbF) chrysene(CHR), benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene, indeno[1,2,3-cd]pyrene. PAH4 concentrations were calculated as the sum of BaA, CHR, BbF, and BaP, while Σ15 PAHs represented the sum of all quantified compounds. All concentrations were expressed as µg/kg of sample. According to Commission Regulation (EU) No. 915/2023 regulates the sets the maximum Benzo[a]pyrene (BaP) concentration at 2 µg /kg and the content of the four heavy PAHs at 12 µg/kg in food products. All statistical analysis was performed using RStudio software (R version 4.5.2). All experiments were carried out in five independent replicates (n = 5) for each treatment and results are shown as mean values ± standard deviation (SD). The effect of cooking treatments on the concentrations of polycyclic aromatic hydrocarbons (PAHs) was evaluated by one-way analysis of variance (ANOVA). For significant differences among treatments ($p < 0.05$), pairwise comparisons among treatment means were done by the Tukey's honestly significant difference (HSD) post hoc test. Statistical analyses were performed separately for each individual PAH compound as well as for the calculated indicators Σ15 PAHs, PAH4 and benzo[a]pyrene (BaP). Statistical significance was accepted at $p < 0.05$ for differences among treatments. Different lower case letters above bars in graphs indicate statistically significant differences based on Tukey's HSD test, and treatments with at least one common letter were not significantly different.

RESULTS AND DISCUSSIONS

The comparison of experimental values demonstrates that the cooking method is the determining factor in the formation of polycyclic aromatic hydrocarbons in beef.The physicochemical composition of raw beef muscles as shown in Table 2.

Table 2. Physicochemical properties

Parameter	Brisket	Shoulder
Moisture (%)	71.68 ± 0.42	72.18 ± 0.37
Protein (%)	26.72 ± 0.56	27.11 ± 0.97
Fat (%)	0.42 ± 0.54	0.51 ± 0.05
Minerals (%)	0.93 ± 0.08	0.93 ± 0.08

Values are expressed as mean ± standard deviation (n = 5). Moisture, protein, fat, and mineral contents were determined prior to thermal processing according to standard AOAC methods. The initial composition of brisket and shoulder was similar as indicated by the moisture, protein, lipid and mineral contents. The observed differences in PAH occurrence between treatments are likely related not only to compositional factors, but also to treatment specific thermal conditions, cooking loss, marination uptake and physicochemical changes during cooking. Lipids are recognized precursors in PAH formation through thermal degradation and pyrolytic reactions; the relatively small differences in lipid content observed between the two muscles suggest that other factors played a more important role. The differences in PAH concentrations between brisket and shoulder samples cannot be explained by chemical composition alone. Other factors such as muscle microstructure, connective tissue content, water holding capacity, cooking loss and heat and smoke exposure may also have contributed to the treatment dependent variability observed in this study.This would indicate that physicochemical changes taking place during cooking may be more important determinants of PAH occurrence than initial compositional differences found between the two muscle types.The limits set by the European Food Safety Authority (EFSA, 2008)

indicate that all analyzed samples have concentrations of benzo(a)pyrene and PAH4 significantly below the maximum permissible thresholds for meat products. The concentrations of benzo[a]pyrene varied between 0.01 and 0.23 µg/kg, depending on the marination treatment (Figure 1).

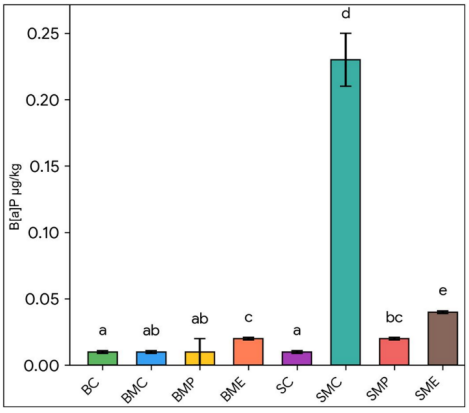


Figure 1. Benzo[a]pyrene (B[a]P) concentrations

The highest BaP value observed in the study (0.23 µg/kg, SMC treatment) remains nearly ten times lower than the European limit of 2 µg/kg. Different letters (a-e) above the bars indicate statistically significant differences ($p < 0.05$) according to Tukey's test.The linear scale emphasizes the critical accumulation of B[a]P in the SMC group compared to other treatments. Considered a carcinogenic marker, B[a]P is used as a classic indicator of PAH contamination. These results suggest that, under the evaluated experimental conditions, the preparation methods used do not generate PAH levels considered concerning from a toxicological standpoint. However, the differences observed between treatments indicate that cooking methods characterized by higher temperatures and direct contact with the heat source, such as charcoal grilling and gas frying, may promote a relatively higher accumulation of PAHs compared to methods based on indirect heat transfer.The formation of toxic compounds benzo[a]pyrene and other PAH4 compounds during thermal treatments is due to the pyrolysis of lipids in meat and incomplete combustion reactions at high temperatures. The dripping onto hot surfaces or charcoal can produce smoke rich in PAH precursors, which will condense on the surface of the meat (Singh et al., 2016). Using indirect heat transfer at lower temperatures plays an essential role in reducing the formation of compounds such as benzo[a]pyrene and PAHs (Sumer & Oz, 2023). The calculated 4 PAHs (Figure 2) values for all treatments (0.22–0.86 µg/kg) are well below the 12 µg/kg limit set for meat products subjected to thermal processing.

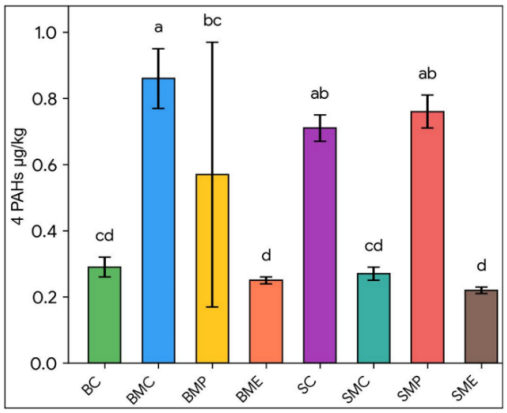


Figure 2. 4 PAHs concentrations

The maximum value was observed for BMC = 0.86 µg/kg followed by SMP = 0.76 µg/kg, SC = 0.71 µg/kg. Different letters above the bars indicate statistically significant differences between treatments ($p < 0.05$).

The toxicological indicator PAH4, which includes the compounds: Benzo(a)pyrene, Benzo(a)anthracene, Benzo(b)fluoranthene and Chrysene, is recommended by the European Food Safety Authority for contamination assessment (EFSA, 2008). All values are well below the European limit of 12 µg/kg, indicating a low toxicological risk for the analyzed samples. These results indicate that cooking on charcoal at 170°C promotes the formation of PAHs, especially in the case of brisket muscle, where the highest total value was recorded. The high temperature and contact with smoke generated by fat pyrolysis promote the formation of aromatic radicals (Lee et al., 2016). These proceses by condensation reactions, and the deposition of PAHs on the meat surface from direct contact with the heat source (Anjum et al., 2019). Abdel Monem et al., (2024) suggested that marinating beef steaks before charcoal grilling,

by using ingredients with antioxidant properties, to reduce PAHs formation and minimize risks to human health. In Figure 3, the highest total PAH concentration (Σ15 PAHs) was observed in the BMC samples (22.50 µg/kg), SMP (11.76 µg/kg), and SME (11.75 µg/kg).

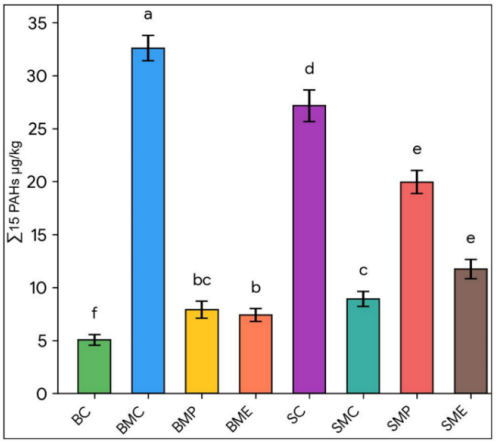


Figure 3. Total PAHs (Σ15 HAPs) concentrations

For the Brisket charcoal grill, the control sample BC = 5.05 µg/kg, and for the Shoulder, the results were for the control sample SC (17.76 µg/kg). The influence of marinating produced different effects depending on the cooking method. In the Brisket, the marinated sample BMC = 22.50 µg/kg, respectively the marinated Shoulder SMC = 8.91 µg/kg. Marination efficiency was strongly dependent on the meat cut. In the shoulder samples, total 15 PAHs levels were significantly reduced following marination, with reductions of 50% for SMC, 34% for SME, and 34% for SMP compared with the control. A similar trend was observed for total 4 PAHs, which decreased by 62% for SMC and 69% for SME and 14% BME. Marination of the brisket samples resulted in increased PAH concentrations relative to the control.Antioxidant compounds present in marinades can suppress PAH formation during cooking, by inhibition of lipid oxidation, but it can increase PAH if it contains sugars that promote Maillard reactions, due to marinading based on natural fruits (sea buckthorn, cranberries, plums, sour cherries).
Differences are observed between the types of muscle Brisket vs Shoulder, due to muscle structure, fat distribution, and water retention capacity. As shown in Table 3, both cooking temperature and marination uptake had a significant effect on the occurrence of PAHs in beef muscles.

Table 3. Effect of thermal processing and marination uptake on PAH levels in beef muscles

Sample code	∑15 HAPs	4 PAHs	B[a]P	Marinade uptake (%)
BMC	22.50 ± 0.48 ^{a}	0.86 ± 0.09 ^{a}	0.01 ± 0.00 ^{ab}	3,67 ± 1,39 ^{ab}
BMP	7.92 ± 0.48 ^{bc}	0.57 ± 0.40 ^{bc}	0.01 ± 0.01 ^{ab}	5,00 ± 1,67 ^{ab}
BME	7.40 ± 0.58 ^{b}	0.25 ± 0.01 ^{d}	0.02 ± 0.00 ^{c}	3,00 ± 13,9 ^{a}
SMC	8.91 ± 0.37 ^{c}	0.27 ± 0.02 ^{cd}	0.23 ± 0.02 ^{d}	6,67 ± 1,18 ^{b}
SMP	11.76 ± 0.40 ^{e}	0.76 ± 0.05 ^{ab}	0.02 ± 0.00 ^{bc}	4,33 ± 1,90 ^{ab}
SME	11.75 ± 0.43 ^{f}	0.22 ± 0.01 ^{d}	0.04 ± 0.00 ^{e}	2,67 ± 1,90 ^{a}

Two-way ANOVA analysis confirmed a significant interaction between muscle type and cooking treatment ($p < 0.001$), indicating that PAH occurrence is influenced simultaneously by muscle characteristics and thermal processing parameters. The different thermal treatments studied here, such as charcoal grilling (170°C), pan frying (114°C) and electric pot cooking (90°C), resulted in different contamination profiles for Σ15 PAHs, PAH4 and benzo[a]pyrene concentrations. Differences in the values for marinade uptake were also statistically significant. The highest marinade uptake was noted in SMC (6.67 ± 1.18) and the lowest value was in SME (2.67 ± 1.90). These differences may be due to differences in muscle structure, connective tissue content and water holding capacity between the brisket and shoulder muscles. The association between marinade uptake and PAH occurrence was not consistent across all treatments. Despite the high marinade uptake of SMC, high concentrations of benzo[a]pyrene (0.23 ± 0.02 µg/kg) were still detected. In contrast, BMC showed very high Σ15 PAHs concentrations despite comparatively low

values of marinade uptake. These results indicate that the formation of PAHs was mainly dependent on the intensity of the thermal exposure, physicochemical changes, smoke contact, cooking temperature, and during the thermal processing rather than the uptake of marinade alone. Analysis of the correlation between weight loss (%) and Benzo[a]pyrene concentration (µg/kg). The points represent the analyzed experimental groups, and the dotted line represents the linear regression trend in Figure 4.

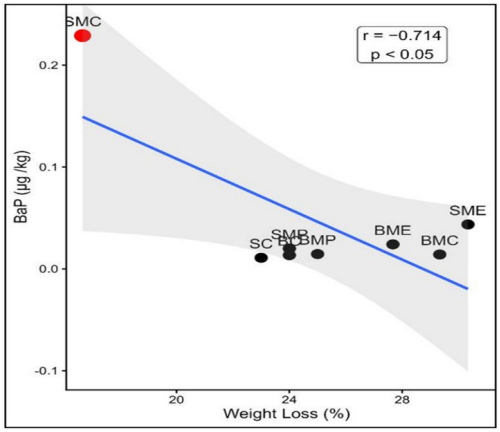


Figure 4. Linear regression analysis between weight loss (%) and Benzo[a]pyrene (B[a]P)concentration

A significant negative correlation ($r = -0.71$, $p < 0.05$) was observed between weight loss and Benzo[a]pyrene concentration, indicating that greater losses during cooking were associated with lower levels of BaP. Fat loss during cooking is another important factor that influences PAH formation. The weight loss values recorded in this study ranged from approximately 16% to 30%, depending on the treatment. Higher fat losses generally correspond to greater melting and release of lipids during cooking, may promote the formation of polycyclic aromatic hydrocarbons (PAH) as a result of pyrolytic reactions. In treatments where fat loss was high, such as BMC (29.33%) and SME (30.33%), high concentrations of several PAHs were observed. This relationship can be explained by the increased availability of lipid substrates undergoing thermal degradation. During thermal processing, meat loses moisture, leading to changes in texture and a reduction in cooking yield (Purslow et al., 2016). When the melted fat reaches hot surfaces or heating elements, it undergoes rapid pyrolysis, producing volatile compounds that participate in the formation of PAHs. In contrast, lower weight loss values can reduce the intensity of these reactions. For example, the SMC treatment, which showed the lowest fat loss (16.67%), generally exhibited reduced PAH concentrations compared to other treatments. This observation suggests, during cooking, that limiting fat dripping and subsequent pyrolysis could represent an effective strategy for reducing PAH contamination. Differences between brisket and shoulder samples may be partially related to differences in muscle structure, moisture retention, lipid distribution and thermal behavior during cooking, all of which can have an impact on PAH formation during thermal processing. However, the detailed physicochemical characterization of the muscle matrices studied was beyond the primary focus of the present study and will be reported separately in future work on marinade delivery efficiency and physicochemical properties of cooked beef products.

CONCLUSIONS

Results showed that the presence of PAHs in beef samples was significantly affected by the cooking conditions, thermal exposure and the type of muscles. The greatest concentrations of PAHs were found after the charcoal grilling (170°C), particularly in brisket. The lower cooking temperatures were associated with reduced PAH concentrations in the analysed samples, particularly during pan frying (114 °C) and electric pot cooking (90°C). Differences between brisket and shoulder muscles could be related to muscle structure, lipid distribution, water retention capacity and thermal behaviour during cooking. Shoulder samples showed higher values of marinade uptake and the effect of marination on the occurrence of PAHs was dependent on the treatment and not uniform in all cooking conditions. Correlation and regression analysis also indicated that the physicochemical changes in thermal processing could be a factor that contribute to the variability of PAHs in cooked beef products. The main factors

associated with the total occurrence of PAHs reported were cooking temperature, exposure to smoke and the intensity of heat. There were statistically significant differences among treatments. The concentrations of benzo[a]pyrene (BaP) and PAH4 were significantly lower than the maximum levels, 2 µg/kg for BaP and 12 µg/kg for PAH4, established by Commission Regulation (EU) No. 915/2023. It is recommended to minimize the amount of fat that drips onto the heat source, maintain controlled cooking temperatures and reduce exposure to smoke. The concentrations of PAHs in beef products can be reduced by these practices.

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FROM WASTE TO RESOURCE: MODERN APPROACHES TO WHEY UTILIZATION IN THE FOOD INDUSTRY

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Abstract

This review examines current approaches to whey valorization in the food industry, focusing on its transformation from a dairy by-product into a source of proteins, minerals, and bioactive compounds for functional foods and beverages. The paper summarizes contemporary scientific approaches to its use – from the development of functional drinks and protein concentrates to biotechnological and ecological solutions for managing waste streams, thereby closing the resource loop and supporting the circular economy. Emphasis is placed on the role of whey as a key element in the sustainable development of the dairy industry, as well as the opportunities for its effective utilization. Efficient whey valorization increases the profitability of enterprises, reduces environmental impacts, and leads to the creation of new health-promoting products that contribute to improved human health and quality of life.

Key words: *bioactive compounds, circular economy, dairy by-products, functional beverages, whey valorization.*

INTRODUCTION

Dairy products are indispensable foods that have an important place in human nutrition due to their rich composition, which includes a wide range of nutrients and biologically active substances. During cheese manufacture, whey is generated as the main liquid by-product remaining after milk coagulation and curd separation (Rocha-Mendoza et al., 2021; Tsermoula et al., 2021). During the production of one kilogram of cheese, 9 to 10 liters are separated as whey (Rocha-Mendoza et al., 2021).

According to Kim et al. (2022), global cheese production is expected to increase by up to 14% by 2029, which will lead to an increase in whey volumes. This relationship is direct because whey represents the major liquid fraction separated during cheese manufacture, accounting for approximately 85-90% of the initial milk volume depending on the type of cheese and processing conditions. The quantities are projected to reach approximately 203-241 million tons by 2030, with an anticipated annual growth of up to 2%. In 2020, Europe produced nearly 148 million tons of milk, and almost 50% of this milk was used for cheese production, where about 90% of the milk volume is separated as whey, resulting in approximately 55 million tons of liquid whey. This makes whey the largest-volume waste stream in the dairy industry (Švikruhová et al., 2022). Therefore, the increasing availability of whey is not only an environmental challenge but also a technological and economic opportunity for the dairy sector.

Of this amount, 13 million tons represent the annual surplus of cheese whey, while 40 million tons are generated solely within the European Union (Zotta et al., 2020; Barba, 2021).

The challenge associated with the effective utilization of whey is characteristic of all countries with developed dairy industries. With the advancement of modern technologies and the growing awareness of whey's beneficial properties, its application in the food industry is expanding, transforming it into a significant component of modern food production. In industrialized countries, between 70% and 90% of whey is already processed for food purposes. Nevertheless, the issue of its full and rational utilization has not yet been completely resolved at the global level. Therefore, research aimed at developing new products and technologies for whey utilization is becoming particularly relevant (Blais et al., 2022; Vera-Santander et al., 2024).

Unprocessed whey contains significant amounts of organic substances, mainly lactose and proteins, which, when discharged into water bodies, increase biochemical oxygen demand

and may lead to serious ecological imbalance (Mirzakulova et al., 2025).

It is considered one of the most polluting by-products of the food industry, as the oxidation of the organic compounds contained in 1 liter of whey requires between 34 and 39.2 grams of oxygen. Each ton of whey discharged into wastewater pollutes water bodies to a degree equivalent to approximately 100 m³ of domestic wastewater. Whey discharged into wastewater leads to reduced levels of dissolved oxygen and toxicity issues, which alter the physicochemical properties of aquatic ecosystems (Zandona et al., 2021). It is regarded as industrial waste by enterprises and is directed to wastewater treatment plants (Tsermoula et al., 2021). The separated whey, as a secondary product, must undergo appropriate treatment methods before being discharged into the ocean, which represents an additional cost for the dairy processing industry.

The main pathways of whey valorization, from its generation during cheese manufacture to its application in food systems and circular-economy processes, are schematically presented in Figure 1.

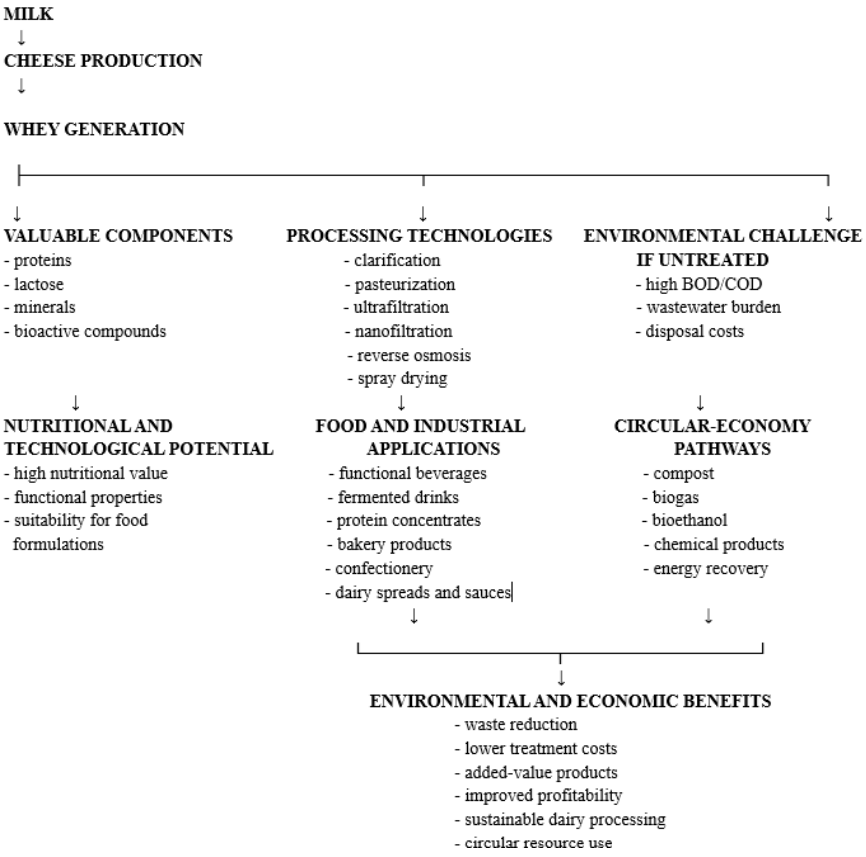


Figure 1. General pathways for whey valorization in the food industry and circular economy

Scientific research emphasizes the urgent need for sustainable solutions that would enable the processing of up to 90% of whey into valuable products (Mirzakulova et al., 2025).

The introduction of efficient technologies for whey valorization not only reduces pollutant emissions into the environment but also supports the development of sustainable, zero-waste production systems. This approach increases the efficiency and profitability of the dairy industry by transforming whey into a source of high value-added food products and functional beverages (Graikini et al., 2024).

New production systems must be sustainable and minimize the environmental impact of the dairy industry. In addition, the industry must

comply with standards for waste disposal, treatment, and sanitation in order to reduce environmental pressure. Management practices must remain sustainable, productive, and economically efficient (Roufou et al., 2021). In this context, whey treatment and recovery are of key importance for its valorization and for reducing environmental burden.

Despite the growing body of literature on whey processing, many studies focus separately on membrane treatment, beverage development, or environmental management. A more integrated perspective linking whey composition, purification technologies, food applications, and circular-economy pathways is still needed. Therefore, the aim of this review is to synthesize recent research on whey valorization in the food industry, with emphasis on functional beverages, product development opportunities, and sustainable strategies for reducing dairy waste.

COMPOSITION AND PROPERTIES OF WHEY

The use of whey as a secondary dairy product date back several millennia and is historically associated with traditional cheese-making practices. In appearance, it is a cloudy liquid with a sweet-sour taste and a mild characteristic aroma. The composition and properties of whey are influenced by the type of milk, the feeding regime of the animals, the milk processing technology used, and other environmental factors (Tsermoula et al., 2021; Sinđić & Pajic, 2025). Two types of whey are known - sweet and acid - depending on the production method and the coagulation process. Sweet whey is typically obtained during rennet coagulation in cheese production, whereas acid whey results from acid coagulation processes such as the manufacture of fresh cheeses or strained fermented dairy products. These two whey types differ in pH, mineral balance, and potential applications, which is important when selecting appropriate valorization strategies.

Fresh, unprocessed cow's milk whey contains approximately 93% water, and its main component is lactose, which accounts for up to 70-75% of the dry matter; about 14% consists of protein compounds, around 6% of milk fat, and the remainder is represented by minerals. The predominant minerals are calcium, phosphorus, magnesium, potassium, sodium, and zinc. The vitamins present are mainly from the B group. Bioactive peptides in whey are an important indicator for improving immune function and provide an antioxidant effect on human health (Pașca et al., 2024). These compositional characteristics determine the technological and nutritional value of whey and explain its suitability for incorporation into functional beverages, protein-enriched foods, fermented products, and bio-based ingredients.

The organoleptic characteristics of whey are defined by its appearance, color, odor, and taste (Larionov et al., 2020). In terms of appearance and color, it is a homogeneous liquid without foreign impurities, with a pale greenish hue. The presence of protein sediment may be observed. It has a characteristic whey taste and aroma. One of the most important and initial processes before whey is used in products is clarification by centrifugal force. The next step is pasteurization, which is the most important process from a microbiological standpoint.

PURIFICATION TECHNOLOGIES

More than 90% of whey is recycled into valuable products in countries with advanced processing systems that set the standard for sustainable practices in the dairy industry (Costa et al., 2021). The use of reverse osmosis and ultrafiltration membranes can remove up to 100% of the biological and chemical oxygen demand from whey wastewater (Mansor et al., 2021), while the combination of ultrafiltration and a membrane bioreactor achieves up to 99% removal (Kumari et al., 2021). Alawamleh et al. (2022) report that combining adsorption and microfiltration ensures up to 98% removal of biological and chemical oxygen demand. Through microfiltration and chromatographic separation, individual components such as glycomacropeptides and immunoglobulins can be isolated (Pires et al., 2024; Zheng & Meletharayil, 2025).

Pressure-driven membrane technologies such as ultrafiltration, reverse osmosis, nanofiltration, and electrodialysis are used for whey processing, improving quality while preserving the biological value of its components (Mirzakulova et al., 2025). Ultrafiltration

represents one of the key methods for obtaining whey protein isolates and concentrates that are used in functional foods (Mc Entee et al., 2025). Among these approaches, membrane technologies are particularly attractive for food applications because they allow component fractionation under relatively mild conditions, thereby preserving protein functionality and reducing thermal damage. Another method for raw material treatment is filtration, after which the obtained whey can be used in infant foods (Muuronen et al., 2021; Rosseto et al., 2024). High-temperature treatment, however, has been observed to negatively affect the quality of the proteins present. For this reason, the choice of treatment should depend on the intended end use of whey, whether for direct beverage production, protein concentration, lactose recovery, or waste minimization.

Whey powder is a dry dairy product produced by concentration followed by drying. Drying is typically carried out by spray drying, which converts liquid whey into powder, facilitating its storage and transportation (Burmistrov et al., 2021; Costa et al., 2025).

The treatment of whey waste before disposal encourages modern approaches to its utilization, particularly for the synthesis of whey protein (Khanam, 2026).

WHEY IN THE FOOD INDUSTRY

Whey has functional and nutritional properties and is therefore incorporated into various food formulations as a biofunctional component – for example, in milk-based products and refreshing beverages. Today, it is increasingly used in the production of environmentally friendly and healthy products, such as fermented drinks and protein snacks.

Whey is a widely used protein ingredient that has been shown to exhibit antimicrobial activity and immunomodulatory properties, to support muscle mass development and body composition optimization, and to contribute to the prevention of cardiovascular diseases and osteoporosis.

Protein-rich whey beverages are commonly associated with post-exercise recovery and may support muscle protein synthesis when consumed as part of an appropriate dietary and training regimen. The use of whey, obtained as a by-product of cheese production, as a beverage for human consumption demonstrates pronounced therapeutic potential with respect to emotional well-being (Korotkova et al., 2020). In the 18th century there were specialized treatment facilities, where it was used as a primary “medical remedy”, with treatments involving the consumption of approximately 4 liters of whey per day. In this context, the inclusion of whey in the daily diet, particularly in its natural (pure) form, may contribute to the optimal functioning of the body. A functional food or beverage is defined as a natural or processed product that, when consumed regularly, does not exhibit toxic effects (Granato et al., 2022) and provides health benefits beyond basic nutrition. In the case of whey, such benefits are associated not only with its protein quality, but also with its versatility as a carrier of probiotics, fruit-derived bioactives, plant extracts, vitamins, and minerals in beverage formulations.

To better illustrate the current diversity of whey utilization in food systems, its main applications can be grouped according to product type, technological role, nutritional relevance, and formulation challenges. Such a structured comparison makes it possible to evaluate whey not only as a dairy by-product, but also as a multifunctional ingredient with broad industrial potential. The principal directions of whey application in modern food production are summarized in Table 1. As shown in Table 1, beverage production remains one of the most promising and versatile directions for whey valorization. This is mainly due to the possibility of combining whey with fruits, probiotic cultures, plant extracts, and other functional ingredients in formulations that are both nutritionally enriched and technologically feasible. For this reason, recent studies have paid particular attention to the development of whey-based beverages with improved sensory properties, enhanced biological value, and greater commercial appeal.

Table 1. Main food applications of whey: products, representative studies, technological advantages, and potential health benefits

Application/ product type	Representative studies	Technological role of whey	Main advantages	Potential nutritional/ functional benefits	Limitations/ challenges
Fruit-based whey beverages	Kolotiy & Kovalenko (2021); Utebaeva et al. (2024)	Liquid base; source of lactose, proteins, and minerals; matrix for flavor incorporation	Improves utilization of liquid whey; enhances sensory appeal through fruit addition; supports product diversification	Hydration; intake of whey proteins and minerals; possible enrichment with fruit antioxidants	Sweet-sour whey flavor must be masked; shelf-life stability; risk of phase separation; consumer acceptance
Fermented and probiotic whey drinks	Kadyan et al. (2021); Özer & Evrendilek (2022)	Fermentation substrate for lactic acid bacteria and yeasts; carrier of probiotic cultures	Adds value to whey; enables production of functional dairy-type beverages; improves microbiological transformation of the substrate	Potential probiotic effect; antimicrobial activity; digestive support; added biofunctional value	Viability of probiotic cultures during storage; acidic taste; process standardization; refrigeration requirements
Protein-enriched sports and recovery beverages	Minj & Anand (2020)	Source of high-quality whey proteins with good digestibility and technological versatility	Easy incorporation into ready-to-drink products; suitable for protein fortification; high market relevance	May support post-exercise recovery and muscle protein synthesis as part of an appropriate diet	Sedimentation and instability in high-protein systems; flavor optimization; higher formulation cost
Whey beverages enriched with plant extracts, vitamins, or bioactive ingredients	Zhuravlev et al. (2021); Rosseto et al. (2024)	Carrier matrix for herbs, plant extracts, vitamins, and complementary proteins	Expands product functionality; increases biological value; allows development of innovative formulations	Antioxidant enrichment; diversified micronutrient profile; broader functional positioning	Possible interactions between whey proteins and plant compounds; taste balance; color and stability issues
Bakery and confectionery products	Minj & Anand (2020)	Emulsifying, foaming, gelling, and water-binding ingredient; partial replacer of other dairy solids	Improves texture, structure, and moisture retention; increases nutritional value; broad applicability in formulations	Higher protein content; improved technological quality of the final product	Thermal sensitivity of proteins; recipe reformulation needed; process adaptation at industrial scale
Dairy formulations, spreads, and sauces	Rocha-Mendoza et al. (2021); Özer & Evrendilek (2022)	Functional dairy ingredient contributing to emulsification, consistency, and solids content	Better resource efficiency within dairy processing; supports reformulation of soft dairy systems and emulsified products	Added proteins and minerals; improved product functionality	Flavor compatibility; labeling and regulatory compliance; need for standardized composition
Whey protein concentrates and whey powder used for food fortification	Blais et al. (2022); Muuronen et al. (2021); Burmistrov et al. (2021)	Concentrated protein ingredient for beverages, snacks, bakery, and specialized foods	Longer shelf life; easier transport and storage; wide industrial applicability	High protein density; convenient fortification of multiple food systems	Processing cost; denaturation risk during heat treatment and drying; reconstitution and solubility challenges

Today, processed whey is used for the production of various types of beverages that have been successfully developed on a small scale. These drinks may be fermented or non-fermented, with or without the addition of flavors, fruit juices or pulp, vegetable extracts, or carbonated components. A large portion of these products have a limited market due to the need for additional equipment for their production (Schulte et al., 2026). This limited market penetration is often related to sensory constraints, insufficient consumer familiarity, short shelf life, and the need for cost-effective stabilization technologies. Over the past decade, whey beverages with added fruit concentrates have been patented. Tropical fruit flavors and citrus tastes are recommended, as they effectively mask the sweet-sour flavor of whey. Positive results have been obtained in studies involving concentrates of mulberry, peach, cherry, and pear. Other flavoring agents used include vanilla, honey, chocolate, and even certain cereals such as bran and oats. Whey can also be used in the production of alcoholic beverages, where lactose-accounting for approximately 70% of the dry matter – plays a major role. Although not widely recognized in some countries, whey is applicable in the production of cream, yogurt, fermented milk, ice cream, or coffee-based beverages. To enhance nutritional and biological value, beverages may be enriched with herbal extracts from medicinal plants with high antioxidant properties, as well as vitamins and proteins (Lima et al., 2022; Rosseto et al., 2024). With modern technologies and developments, whey has become an integral part of the human diet.

The creation of functional beverages from whey mixed with fruit juices is one of the latest trends in its utilization. These beverages improve sensory qualities and extend shelf life (Anichkina et al., 2021; Kolotiy & Kovalenko, 2021; Kaya et al., 2024; Utebaeva et al., 2024). Furthermore, the natural sugars present in fruits can serve as a safe and natural alternative to artificial sweeteners in beverages. It has been demonstrated that high-protein whey may contribute to reducing glycemic response (Minj & Anand, 2020).

The preparation of functional beverages and foods is one of the most promising and important opportunities for managing whey (Kumar., 2023). Functional beverages provide specific health benefits and contribute to the hydration of the body (Navarrete-González et al., 2026). These products may be particularly attractive to younger consumer groups seeking alternatives to conventional soft drinks and protein beverages. Over the past decade, whey and its components have found wide application in the commercial market, particularly in the production of whey-based beverages containing probiotic or prebiotic ingredients (Özer & Evrendilek, 2022). In this context, Kadyan et al. (2021) developed a naturally carbonated probiotic whey-based beverage that demonstrated good antimicrobial activity against pathogenic strains.

An increasing variety of beverages containing whey protein is available on the market. The developed technological processes can be easily integrated into existing production lines, contributing to the minimization of wastewater volumes and reducing treatment-related costs (Kadyan et al., 2021). This creates opportunities for new product development and expansion of the market potential of whey beverages. The literature describes a significant diversity of whey-based beverages.

Beyond beverages, whey proteins are widely applied in bakery, confectionery, dairy, and sauce formulations because of their emulsifying, foaming, gelling, and water-binding properties. In desserts, cakes, and pastries they can improve texture and nutritional value, while in products such as mayonnaise, processed cheese spreads, and meat sauces they may contribute to emulsion stability and consistency. These multifunctional properties make whey ingredients suitable for the reformulation of foods with improved protein content and enhanced technological performance. The Sustainable Development Goals imply the need for dairy producers and processors to take targeted actions to limit the generation of by-products and waste streams, aiming to achieve a more efficient resource cycle through the development of nutrient-rich products by 2030 (Granato et al., 2022). Current challenges in whey-based product development include flavor masking, microbial stability, shelf-life extension, process standardization, consumer acceptance, and cost-effective scale-up. Addressing these issues is essential for the

broader industrial adoption of whey-derived foods and beverages.

CIRCULAR ECONOMY

Whey plays an important role in promoting the circular economy, as this by-product of dairy production can be utilized and transformed into a valuable resource in other sectors of the food industry (Tsermoula et al., 2021). Deproteinized whey can be effectively used for compost production in combination with grape pomace and pruning residues intended for organic farming. In this way, the environmental sustainability of production chains in both the dairy and wine industries is enhanced, while costs associated with by-product management are reduced (Alfonzo et al., 2022).

The by-product of cheese production can also be effectively used for electricity generation. This is mainly achieved through anaerobic digestion, in which the organic matter in whey is converted by microorganisms into biogas rich in methane. The resulting biogas can then be used in combined heat and power systems to generate electricity and thermal energy for dairy plants. It has significant potential to serve as a substitute for fossil fuels. In this way, whey valorization contributes not only to waste reduction but also to partial energy self-sufficiency in dairy processing facilities. Life-cycle assessments indicate that bioethanol production from whey may substantially reduce the environmental burden compared with conventional waste management and fossil-based alternatives (Gosalvitr et al., 2021). Other alternative approaches include the conversion of whey into chemical products.

As noted above, contemporary research focuses on processing methods for the production of biofuels, electricity, and chemical products. These processes are key to the efficient and economically viable utilization of whey. The combination of two or more of these processes enables the production of various high-value products without generating waste in the dairy industry (Asunis et al., 2020).

The valorization of whey is enhanced following initial spray drying, when both acid and sweet whey can be converted into value-added products for the food, cosmetic, and pharmaceutical industries (Soumati et al., 2023). Thanks to modern technologies, high-quality proteins, lactose, minerals, and bioactive compounds can be extracted in accordance with the criteria for sustainable and healthy products (Zhuravlev et al., 2021; Kashung & Karuthapandian, 2025). Russo et al. (2021; 2023) used whey as a substrate for processing algal biomass.

The complete utilization of whey is essential for achieving a full transition of the dairy sector toward a circular economy and for developing strategies designed to address unforeseen situations, such as the coronavirus pandemic of 2019 (Uvarova et al., 2020).

CONCLUSIONS

Whey should no longer be regarded merely as a dairy waste stream, but as a multifunctional raw material with significant nutritional, technological, environmental, and economic value. Its composition makes it suitable for the development of functional beverages, protein-enriched foods, fermented products, and other value-added formulations, while modern purification and biorefinery approaches expand the possibilities for its complete utilization. At the same time, successful whey valorization requires solutions to sensory, technological, logistical, and regulatory challenges. Future research should focus on scalable product development, consumer acceptance, and integrated processing systems that support both circular economy goals and the sustainability of the dairy industry.

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WILD LIFE MANAGEMENT, FISHERY AND AQUACULTURE

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FISH WELFARE RESPONSES TO HEAVY METAL EXPOSURE: A REVIEW

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Abstract

Heavy metals are persistent contaminants with a high potential for bioaccumulation and biomagnification in aquatic ecosystems, exerting toxic effects that impair physiological functions and compromise fish welfare. Although numerous studies have investigated oxidative stress, endocrine disruption, histopathological alterations, and sublethal effects, the integration of these processes within a comprehensive fish welfare framework remains limited.This review synthesizes current knowledge on the toxic effects of major heavy metals (Cd, Pb, Hg, As), focusing on their bioaccumulation patterns, tissue distribution, physiological and behavioral alterations, and their implications for fish welfare. By integrating ecotoxicological evidence with contemporary welfare concepts, this study highlights key mechanisms underlying welfare impairment and identifies major gaps in the current literature. The findings emphasize that sublethal and chronic exposure to heavy metals can compromise fish welfare even in the absence of immediate mortality. Overall, this review provides a functional perspective on heavy metal toxicity and outlines priority directions for future research and the development of monitoring and management strategies aimed at mitigating the impact of heavy metal contamination in aquatic ecosystems.

Key words*: aquatic ecotoxicology, bioaccumulation, biomagnification, oxidative stress, physiological responses.*

INTRODUCTION

Globally, the pollution of aquatic ecosystems with heavy metals represents a major concern due to the toxicity of these elements and their ability to accumulate within aquatic ecosystems (Ahmad et al., 2010; Aski et al., 2023). Heavy metals enter aquatic ecosystems through natural sources and, in particular, through anthropogenic activities, with anthropogenic inputs often exceeding natural fluxes and thereby increasing the risk of contamination in water bodies and aquatic organisms (Masindi & Muedi, 2018).

Unlike other pollutants, heavy metals are non-biodegradable and can undergo bioaccumulation and biomagnification throughout the food web, generating toxic effects even at locations distant from the original pollution source (Ahmad et al., 2010).

Sediment serves as a reservoir for heavy metals in aquatic ecosystems, gradually releasing these contaminants into the water and contributing to the chronic exposure of fish (Pelić et al., 2024). Fish can absorb heavy metals through multiple mechanisms, including ingestion of suspended particles, consumption of contaminated food, ionic exchange at the gill surface, and direct adsorption onto tissues (Islam et al., 2015). Fish may accumulate substantial quantities of heavy metals in their tissues (Łuczyńska et al., 2022). However, muscle tissue generally tends to accumulate the lowest concentrations of metals (Zeitoun & Mehana, 2014).

In contrast, accumulation occurs more frequently in organs such as the liver, kidneys, gills, and reproductive tissues, where metals can induce cellular alterations such as vacuolization, necrosis, or endocrine disruption, thereby affecting health and physiological function (Sharma et al., 2024).

Furthermore, heavy metals can directly impair fish welfare through complex toxicological mechanisms. Exposure to elevated concentrations can induce oxidative stress, genotoxicity, and endocrine disturbances, altering essential physiological functions, growth, and reproduction. At the morphological

and functional level, heavy metals are associated with vertebral deformities, fin abnormalities, and developmental impairments during embryonic and larval stages, which reduce swimming performance, feeding capacity, and survival. Fish exhibit heightened sensitivity to these effects, especially during early life stages, which represent critical periods for normal development and the maintaining of adequate welfare (Sfakianakis et al., 2015).

Exposure to heavy metals affects not only tissue structures but also behavioral patterns essential for survival and welfare. Several studies have reported alterations in vital functions such as swimming performance, avoidance responses, feeding behavior, and cognitive processes, even at sublethal metal concentrations. Such behavioral modifications represent sensitive indicators of toxic stress and can serve as early warning signs of declining welfare in aquatic organisms (Morales, 2022).

Additionally, fish are considered sensitive bioindicators of heavy metal pollution because they respond rapidly to changes in the aquatic environment and reflect the level of ecosystem contamination (Habib et al., 2023). In the European Union, Council Directive 98/58/EC establishes general welfare standards for animals kept for production purposes, explicitly including fish, which must be protected from suffering and inadequate environmental conditions (European Council, 1998).

However, the scientific literature still lacks an integrated synthesis connecting the toxicological mechanisms of heavy metals with their physiological, behavioral, and welfare-related impacts on fish. Most studies address these aspects in a fragmented manner, without integrating their consequences in accordance with modern welfare evaluation criteria.

In this context, the present review aims to provide a critical and comprehensive analysis of the impact of heavy metals on fish, bringing together evidence on bioaccumulation, tissue-level effects, physiological responses, behavioral alterations, and welfare implications. Such an integrative approach is essential for understanding the real risks at both individual and population levels and for informing future directions in monitoring and protecting fish within aquatic ecosystems.

MATERIALS AND METHODS

A comprehensive literature search was conducted to identify studies on heavy metal exposure and its implications for fish welfare, using Web of Science, Google Scholar, ResearchGate, and ScienceDirect.

The search strategy employed combinations of keywords such as “heavy metals”, “fish”, “fish welfare”, “bioaccumulation”, “oxidative stress”, “toxicity”, and “behavioral alterations”, applied individually and in combination to ensure broad coverage.

Studies published between 2015 and 2026 were primarily considered to reflect current knowledge, while earlier highly cited or relevant studies were also included.

Selection criteria focused on studies addressing aquatic organisms, particularly fish, with emphasis on peer-reviewed articles investigating physiological, biochemical, behavioral, or toxicological responses to heavy metal exposure. Studies lacking direct relevance or scientific robustness were excluded as far as possible.

The selected literature was analyzed and organized into key thematic areas, including toxicity mechanisms, bioaccumulation and tissue distribution, environmental behavior of heavy metals, and their implications for fish welfare, enabling an integrated synthesis of the available evidence.

RESULTS AND DISCUSSIONS

Linking Heavy Metal Toxicity to Fish Welfare: A Functional Perspective

Fish welfare is recognised as an integral component of aquatic animal health and is closely linked to the maintenance of normal biological functioning and adaptive capacity, with chemical agents acknowledged as potential hazards capable of adversely affecting aquatic animal health (OIE, 2019).

Physiological stress responses and associated biomarkers are widely used to assess fish welfare (EFSA, 2009). Prolonged or chronic stress has been shown to impair immune function, growth and reproduction in fish, providing strong indications of compromised welfare, reflecting disruptions of physiological homeostasis and tertiary stress responses

(Huntingford et al., 2006; Jerez-Cepa & Ruiz-Jarabo, 2021).

Fish welfare is increasingly recognised as an attribute of the individual, with individuals showing consistent behavioural and physiological differences in their responses to the same environmental stimulus (Torgerson-White & Sanchez-Suarez, 2022), and can therefore be functionally defined as the capacity of an individual to maintain health and appropriate biological functioning when exposed to environmental challenges (Huntingford et al., 2006).

Given their intimate contact with the aquatic environment through the gills, fish are particularly vulnerable to water-borne pollutants, including heavy metals, which may compromise welfare through chronic physiological stress and tissue damage (Huntingford et al., 2006).

Traditional fish welfare assessments have primarily focused on mitigating negative states such as chronic stress and pain; however, increasing evidence suggests that the absence of suffering does not equate to good welfare, highlighting the importance of evaluating both physiological and behavioural indicators within a broader welfare framework (Fife-Cook & Franks, 2019).

Within this context, exposure to both sublethal and lethal concentrations of heavy metals has been associated with the induction of physiological stress responses in fish, following accumulation in various tissues. Several metals promote redox reactions that contribute to the formation of reactive oxygen species (ROS), which play essential roles under controlled conditions. However, excessive ROS production may disrupt redox homeostasis and induce oxidative stress and subsequent impairment of cellular components such as lipids, proteins, and DNA (Shahjahan et al., 2022). Such cellular damage is particularly relevant for organs directly involved in essential physiological functions, as it can impair processes such as gas exchange, ion regulation, and energy metabolism.

To counteract these effects, oxidative balance in fish is maintained through antioxidant defence systems that include enzymatic antioxidants such as superoxide dismutase, catalase and glutathione peroxidase, together with non-enzymatic antioxidants like glutathione. In addition, metal-binding proteins such as metallothioneins play an important role in the detoxification of toxic metals and the regulation of essential metal homeostasis (Sevcikova et al., 2011).

Accordingly, physiological biomarkers are widely employed in fish welfare assessment. They provide measurable indicators of endocrine, oxidative and immune alterations associated with environmental stress. Cortisol is commonly used to evaluate stress responses and associated shifts in energy allocation. Likewise, changes in antioxidant defences and immune parameters reflect disturbances in homeostatic regulation, supporting their relevance as indicators of compromised welfare (Oliveira et al., 2024). Importantly, these biomarker responses reflect underlying physiological adjustments that may reduce the energy available for growth, reproduction, and immune defence.

Beyond oxidative imbalance, heavy metal exposure has been associated with alterations in antioxidant enzyme activities, including catalase, superoxide dismutase and glutathione-dependent systems. Such disturbances may contribute to oxidative damage at the cellular level, including genotoxic effects and histopathological alterations in target organs. Moreover, heavy metal accumulation has been linked to disruptions in reproductive organs, altered hormone production and impaired gamete development (Bhat et al., 2025).

Heavy metal exposure has also been associated with developmental abnormalities and morphological deformities in fish, which may impair survival, growth and overall fitness, thereby representing additional indicators of compromised welfare (Ali et al., 2019). Collectively, these physiological impairments may interfere with normal biological functioning by disrupting key processes such as respiration and osmoregulation (via gill impairment), as well as metabolism and reproduction. These functional alterations reduce the capacity of fish to maintain homeostasis and cope with environmental challenges, ultimately resulting in compromised welfare even in the absence of mortality. Thus, heavy metal exposure should not be interpreted solely as a toxicological stressor, but also as a direct welfare challenge that affects the functional integrity, adaptive capacity, and biological performance of fish.

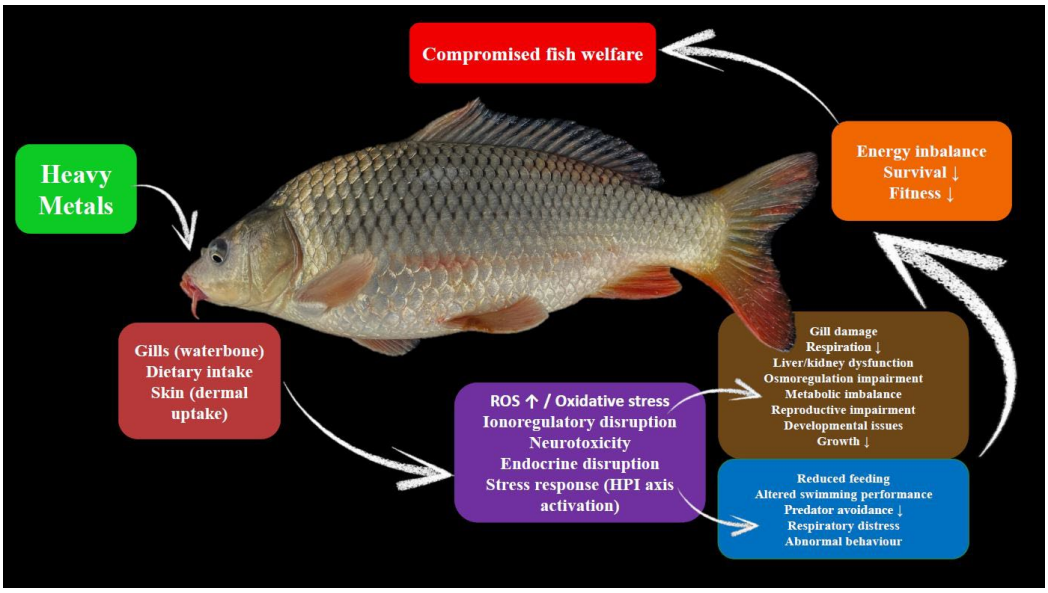


Figure 1. Conceptual framework linking heavy metal exposure pathways, physiological mechanisms and resulting impacts on fish welfare (generated using artificial intelligence tools)

Bioaccumulation and Tissue Distribution of Heavy Metals in Fish

The distribution and behavior of heavy metals in aquatic environments, including speciation, bioaccumulation, and toxicity, represent major concerns (Oziegbe et al., 2025).

Bioaccumulation represents the progressive increase in the concentration of a contaminant within an organism as a result of environmental exposure and trophic intake, whereas biomagnification describes the progressive increase in contaminant concentration across successive trophic levels of the food chain (Ali et al., 2025; Ali & Khan, 2018). Bioaccumulation and biomagnification reflect the limited capacity of organisms to detoxify heavy metals, which contributes to their persistence and toxicity in aquatic systems (Ray & Vashishth, 2024).

Heavy metals enter fish via waterborne and dietary exposure, and their accumulation is influenced by both biological and environmental factors. Age and lifespan represent important determinants of metal accumulation, as metal concentrations generally increase with prolonged exposure of fish to contaminated aquatic environments. Once introduced into aquatic systems, metals are distributed between water, sediments, and food webs, where their accumulation in benthivorous and predatory species largely depends on metal concentrations in water and habitat conditions. Environmental parameters such as water hardness, pH, and organic matter can further influence metal bioaccumulation in fish (Moiseenko & Gashkina, 2020).

Habitat type also plays a significant role in determining metal accumulation patterns. Differences in metal concentrations among fish species may reflect variations in ecological requirements, metabolism, and feeding behaviour. Several studies have reported that metal concentrations in fish largely depend on habitat characteristics. In aquatic systems, sediments are considered a major pathway for metal uptake and play an important role in the accumulation of heavy metals in fish. Fish inhabiting areas close to the sediments and feeding on benthic invertebrates or organic material may accumulate metals transferred from the sediment compartment into aquatic food webs. Consequently, benthic and benthopelagic fish generally exhibit higher metal concentrations than demersal species. Trophic level may further influence the accumulation of heavy metals. Previous studies have reported that piscivorous fish occupying higher trophic levels tend to accumulate greater concentrations of metals compared with omnivorous and herbivorous species, reflecting

trophic transfer processes within aquatic food chains (Hossain et al., 2022). However, this relationship is not always consistent, as some studies have reported higher metal accumulation in omnivorous species compared to carnivorous fish (Ali & Khan, 2019). These inconsistencies suggest that trophic transfer alone cannot fully explain metal accumulation patterns, highlighting the importance of species-specific ecology and exposure pathways.

In addition, uptake pathways may differ depending on metal form, with dissolved fractions being primarily absorbed through the gills, while particulate forms are mainly taken up via the alimentary tract (Ali & Khan, 2019). Contrasting patterns have also been reported. For example, Bawuro et al. (2018) observed higher metal concentrations in pelagic fish (omnivorous or herbivorous) compared with benthic carnivorous species, suggesting that accumulation patterns may vary depending on ecological conditions and species-specific feeding strategies.

Furthermore, ontogenetic changes may influence contaminant dynamics. Older fish may possess more developed enzymatic systems and enhanced elimination pathways, such as excretion or reproduction, compared with younger individuals. Changes in diet during growth may also influence contaminant accumulation, as dietary composition can affect the intake of heavy metals (Salaah et al., 2025).

Fish accumulate heavy metals through multiple pathways, including branchial absorption from the aquatic environment, dermal contact with contaminated sediments, and dietary ingestion via the gastrointestinal tract through ingestion of food or sediment particles, as well as across permeable body surfaces (Ali & Khan, 2018; Sonone et al., 2020; Azar & Vajargah, 2023; Oros, 2025). Following uptake, heavy metals are distributed through the bloodstream to different organs, where they interact with metal-binding proteins, leading to their accumulation in tissues (Pargi et al., 2024). The degree of accumulation is determined by the physicochemical properties of the metals as well as by the physiological characteristics of the species. Gills represent the primary site of absorption due to their extensive contact surface and ion-exchange functions, while direct interaction with sediments also promotes accumulation, as reflected by the higher concentrations observed in benthic species compared with pelagic ones. Trophic exposure also constitutes a major pathway for metal incorporation, particularly at higher trophic levels (Oros, 2025). Zhou & Wong (2000) demonstrated that mercury undergoes clear biomagnification along the aquatic food chain, with higher concentrations in predatory species, whereas other metals such as Pb and Cd did not show biomagnification within the same system, indicating lower trophic transfer potential.

Exposure of fish to sublethal or lethal concentrations induces stress responses and leads to the accumulation of metals in various tissues and organs, including gills, kidneys, liver, skin, and muscle (Naz et al., 2023).

Most metals accumulate preferentially in the liver, kidneys, and gills, while muscle tissue generally exhibits lower concentrations compared with the other organs (Sonone et al., 2020). Given the essential roles of these organs in respiration, detoxification, and osmoregulation, their preferential accumulation of heavy metals may directly impair key physiological functions, thereby contributing to reduced biological performance and compromised welfare.

Sediments act as accumulation compartments for heavy metals (Ali et al., 2019; El-Sharkawy et al., 2025), as well as for a wide range of contaminants originating from both natural processes and anthropogenic activities (El-Sharkawy et al., 2025). They are recognized as long-term sinks for heavy metals, capable of retaining contaminants and acting as secondary sources under certain conditions (Ali et al., 2019; El-Sharkawy et al., 2025).

Due to their high density, heavy metals tend to accumulate in bottom sediments, where they can be taken up by benthic organisms and reintroduced into aquatic food webs (Almashhadany et al., 2024).

Fish, being positioned at higher trophic levels in aquatic ecosystems, are considered suitable model organisms for toxicological studies. Their high sensitivity to environmental changes, as well as their capacity to metabolize, detoxify, and accumulate heavy metals, make them effective bioindicators for monitoring the condition of aquatic ecosystems (Ray & Vashishth, 2024).

Overall, these findings indicate that heavy metal bioaccumulation in fish is a highly context-dependent process, influenced by species-specific traits, habitat characteristics, and exposure pathways. In particular, benthic and higher-trophic-level species are more prone to chronic exposure due to their feeding strategies and environmental interactions. Consequently, such patterns may lead to sustained accumulation and increase the risk of sublethal physiological disturbances, even in the absence of acute toxicity.

Heavy metals in aquatic environments: sources, behavior, and relevance for fish welfare

The term heavy metals refers to metals and metalloids characterized by an atomic density greater than 4 g/cm³ or at least five times greater than the density of water (Masindi & Muedi, 2018; Piwowarska et al., 2024).

Depending on their toxic properties, heavy metals are classified into essential and non-essential metals. Essential metals, such as zinc (Zn), copper (Cu), iron (Fe), nickel (Ni), and molybdenum (Mo), are required in small amounts for fundamental biological processes in animal life, exhibiting low toxicity at low concentrations and playing an important role in maintaining physiological functions, including enzymatic activity and metabolic regulation. In contrast, non-essential metals are characterized by a high toxic potential; cadmium (Cd), lead (Pb), mercury (Hg), and arsenic (As) are frequently identified as the most hazardous elements in this category (Ali & Khan, 2018; Piwowarska et al., 2024; Mustapha et al., 2025). The behavior of these metals in the environment is controlled by specific physicochemical properties that determine their mobility, persistence, and bioavailability (Mustapha et al., 2025).

Heavy metals are commonly present in natural waters at low concentrations; however, many of them exhibit toxicity even at very low levels (Masindi & Muedi, 2018). The fate of heavy metals in aquatic systems is controlled by physicochemical factors such as pH, redox potential, solubility, and metal speciation, which determine their mobility, persistence, and toxicity (Mustapha et al., 2025). These physicochemical characteristics directly influence metal bioavailability, thereby determining the extent of uptake and potential toxic effects in aquatic organisms, including fish.

In general, decreasing pH increases the solubility and mobility of metals, promoting their release into the water column, while oxidation state and complexation processes influence the chemical form and toxic potential of metals (Mustapha et al., 2025).

Environmental contamination with heavy metals is primarily driven by anthropogenic pressure associated with population growth, industrialization, and intensive agriculture (Aziz et al., 2023), although both natural and anthropogenic activities may contribute to environmental contamination, often exceeding the assimilative capacity of ecosystems (Mustapha et al., 2025).

The introduction of heavy metals into aquatic environments occurs through both anthropogenic and natural sources, with anthropogenic inputs being dominant and largely associated with industrial and agricultural activities such as mining operations, metallurgical processes, irrigation with contaminated water, and various industrial discharges. In particular, modern industrial processes such as electroplating, electronic equipment manufacturing, mining, metal smelting, fertilizer production, and activities within the chemical and energy sectors generate significant volumes of wastewater containing heavy metals, which enter aquatic environments through direct discharge or surface runoff. Natural contributions, although generally secondary in magnitude, include processes such as rock weathering, volcanic activity, fires, and biogenic mechanisms, which also supply heavy metals to aquatic ecosystems (Sonone et al., 2020; Aziz et al., 2023; Singh & Sharma, 2024). Once introduced into the environment, heavy metals may occur in various chemical forms, including hydroxides, oxides, sulfides, sulfates, phosphates, silicates, and organic compounds (Sonone et al., 2020). These properties result in their non-biodegradability, capacity for bioaccumulation, environmental stability, long-term persistence, and biotoxicity (Aziz et al., 2023; Edo et al., 2024). As a result, fish inhabiting contaminated environments may be continuously exposed to elevated metal

concentrations, increasing the likelihood of chronic accumulation and sublethal physiological stress.

Among the highly toxic heavy metals and metalloids of environmental relevance are chromium (Cr), nickel (Ni), copper (Cu), zinc (Zn), cadmium (Cd), lead (Pb), mercury (Hg), and arsenic (As) (Edo et al., 2024). Unlike organic compounds, most metals cannot be readily metabolized into less toxic forms, reflecting their lack of biodegradability. Consequently, once introduced into aquatic environments, metals are redistributed within the water column, accumulate in sediments, or are incorporated into biomass through trophic processes (Sonone et al., 2020). As a result, the persistence of heavy metals in aquatic systems is largely determined by their chemical stability, low degradation rates, and high affinity for sediment particles (El-Sharkawy et al., 2025).

Overall, the persistence and bioavailability of heavy metals in aquatic environments highlight their potential to act as chronic stressors for fish. Continuous exposure, even at low concentrations, may facilitate bioaccumulation and increase the risk of sublethal physiological disturbances, with potential implications for fish welfare.

A synthesis of the main findings on heavy metal exposure, including exposure routes, target organs, and observed effects in fish, is presented in Table 1.

Table 1. Summary of heavy metal exposure routes, target organs and observed effects in fish

Heavy Metal	Species	Concentration	Exposure Route	Target Organ	Observed Effect	Reference
Cd^{2+}	*Cyprinus carpio*	60-100 mg/L	Waterborne	Gills, liver, kidney	Gill damage (hyperplasia, necrosis); liver degeneration; kidney lesions; behavioral alterations (erratic swimming, loss of equilibrium, increased breathing).	Ambiya et al., 2024
Cd^{2+}	*Paralichthys olivaceus*	2-8 mg/L	Waterborne	Liver, kidney, gills, muscle	Oxidative stress (altered antioxidant enzymes, increased lipid peroxidation); growth reduction; behavioral alterations (reduced feeding, sluggish swimming, decreased activity), (liver > kidney > gills > muscle).	Cao et al., 2012
Cd^{2+}	*Lates calcarifer*	10 mg/L	Waterborne	Gills, liver, kidney	Behavioral alterations (erratic swimming, excessive mucus production, hyperventilation); gill lesions (edema, aneurism, hyperplasia, lamellar fusion); liver swelling/vacuolation; kidney tubular damage and necrosis.	Thophon et al., 2003
Cd^{2+}	*Lates calcarifer*	0.8 mg/L	Waterborne	Gills, liver, kidney	Reduced food intake; gill epithelial edema, hypertrophy/hyperplasia and lamellar fusion; liver lipid droplet and dark granule accumulation; kidney tubular degeneration, vacuolation and necrosis.	Thophon et al., 2003
Cd^{2+}	*Terapon jarbua*	0.08-0.86 μg g^{-1} day^{-1}	Dietary	Digestive tract, liver, gills	Metallothionein induction; limited physiological effects (no significant growth reduction).	Dang & Wang, 2009
Pb^{2+}	*Oreochromis mossambicus*	0.5-5 mg/L	Waterborne	Gills, liver, kidney, intestine, brain	Oxidative stress (GSH alteration, lipid peroxidation); ionoregulatory disruption (Na^+/K^+-ATPase inhibition); reduced feeding; lethargy; abnormal swimming behavior; respiratory distress (excessive mucus production).	Kaya & Akbulut, 2015
Pb^{2+}	*Squalus acanthias*	4.14 mg/L	Waterborne	Skin, gills, kidney, gut	Pb accumulation mainly in skin and gills; moderate accumulation in kidney and gut; decreased plasma K+; transient increase in blood pH; no mortality.	Eyckmans et al., 2013
Pb^{2+}	*Squalus acanthias*	20.72 mg/L	Waterborne	Skin, gills, kidney, gut, liver, muscle	Pb accumulation in all tissues (highest in skin and gills); decreased plasma K+, Na+ and Cl−; reduced gill Na+/K+-ATPase activity (ionoregulatory disruption); increased urea and ammonia excretion; decreased liver protein and muscle glycogen; no mortality.	Eyckmans et al., 2013
Pb^{2+}	*Cyprinus carpio*	0.21 mg/L	Waterborne	Gills, liver, kidney	Histopathological alterations in gills (hyperplasia, epithelial lifting); liver degeneration; renal histopathological alterations (tubular degeneration, glomerular alterations).	Mustafa et al., 2017

Pb^{2+}	*Cyprinus carpio*	0.42 mg/L	Waterborne	Gills, liver, kidney	Severe histopathological alterations in gills (edema, lamellar fusion, necrosis); liver degeneration (vacuolation) and necrosis; renal histopathological alterations (tubular degeneration, vacuolation, necrosis).	Mustafa et al., 2017
Pb^{2+}	*Clarias gariepinus*	6.1 mg/L	Waterborne	Gills, liver, kidney, muscle	Mild histopathological alterations in gills (epithelial lifting, blood congestion); liver vacuolar degeneration; kidney glomerular alterations; muscle structural gaps.	Al-Balawi et al., 2013
Pb^{2+}	*Clarias gariepinus*	24.4 mg/L	Waterborne	Gills, liver, kidney, muscle	Severe histopathological alterations in gills (hypertrophy, lamellar degeneration, vacuolar degeneration); liver vacuolation and necrosis; kidney tubular necrosis and glomerular alterations; muscle fiber separation.	Al-Balawi et al., 2013
Pb^{2+}	*Oncorhynchus mykiss*	0.57-1.2 mg/L	Waterborne	Kidney	Renal Pb accumulation; decreased glomerular filtration rate (GFR); altered urine pH; increased ammonia and glucose excretion; disrupted Ca^{2+} and Mg^{2+} reabsorption; disruption of ion homeostasis.	Patel et al., 2006
Pb^{2+}	*Danio rerio*	0.0025-0.005 mg/L	Waterborne	Liver	Dose-dependent histopathological alterations, ranging from mild to severe, including bile duct degeneration and epithelial detachment, vessel/sinusoid congestion, cytoplasmic vacuolization, increased lipid droplets, macrophage proliferation, apoptotic and necrotic hepatocytes, and lysed areas; progressive increase in severity and earlier onset at higher concentration; upregulation of mtf1, cat and sod1.	Macirella et al., 2023
Pb^{2+}	*Oreochromis niloticus*	100-800 mg/kg diet	Dietary	Kidney, liver, gills, spleen (lower in muscle, brain)	Dose-dependent Pb accumulation (highest in kidney); oxidative stress (↑ MDA, ↓ GSH, SOD, GSH-Px, T-AOC); DNA damage in blood cells. Although dietary exposure implies gastrointestinal uptake, no direct measurements of Pb accumulation in intestinal tissues were reported.	Dai et al., 2012
Pb^{2+}	*Sebastes schlegelii*	30-240 mg/kg diet	Dietary	Liver, gills, brain, muscle	Increased SOD and GST activity, decreased GSH levels (oxidative stress); AChE inhibition in brain and muscle (neurotoxicity); increased MT expression in liver; effects varying with exposure concentration and duration.	Kim et al., 2017
iHg	*Brycon amazonicus*	0.15 mg/L	Waterborne	Gills, liver, white muscle, heart	Behavioral alterations (hyperactivity, aggressiveness, loss of equilibrium, abnormal swimming, dark skin pigmentation, loss of scales); Hg bioaccumulation (gills > liver > heart ≈ white muscle); antioxidant response activation; lipid peroxidation and protein oxidation; MT increase in liver, gills and heart.	Monteiro et al., 2010
iHg	*Gambusia affinis*	0.02 mg/L	Waterborne	Gills, viscera, head, body	Behavioral alterations (erratic swimming, loss of equilibrium, lethargy) and reduced swimming performance; severe gill damage (hyperplasia, hypertrophy, lamellar fusion, necrosis); Hg bioaccumulation (viscera > head > body).	Jakka et al., 2007
iHg	*Diplodus sargus*	0.002 mg/L	Waterborne	Brain (hypothalamus, optic tectum, cerebellum)	Brain cell loss (neuronal and glial), reduced cerebellum volume; impaired swimming performance (reduced distance and velocity); altered anxiety-like and mood-related behavior; persistent post-exposure effects.	Pereira et al., 2016
MeHg	*Perca flavescens (larvae)*	0.007-0.075 mg/L	Waterborne (embryonic exposure)	Not specified	Impaired locomotor activity, altered visual motor response, reduced foraging efficiency, and decreased survival at highest exposure concentration.	Mora-Zamorano et al., 2017

MeHg	*Danio rerio*	0.001-0.015 mg/L	Waterborne	Not specified	Significant behavioral alterations including reduced swimming performance, speed, and acceleration, impaired orientation, increased inactivity, decreased aggression, and memory deficits; increased oxidative stress biomarkers (SOD, MDA), particularly at higher concentration.	Strungaru et al., 2018
iHg	*Hoplias malabaricus*	Exposure via contaminated prey (0.15 mg L^{-1} $HgCl_2$; 8.11 mg kg^{-1} Hg in prey; ~0.45 mg Hg/fish/96 h)	Dietary	Liver, gills, white muscle, heart	Increased aggressiveness and altered feeding behavior; increased liver mass and HSI; decreased condition factor; induction of oxidative stress (↑ SOD, CAT, GPx, GST, GR); lipid and protein oxidation; glutathione imbalance (↓ GSH/GSSG ratio); increased metallothioneins; tissue-specific mercury bioaccumulation (gills > liver > muscle = heart).	Monteiro et al., 2013
As V	*Danio rerio*	0.001-0.1 mg/L	Waterborne	Brain (functional)	Impaired long-term memory (LTM); decreased avoidance latency; increased protein oxidation.	de Castro et al., 2009
As III	*Oreochromis mossambicus*	(3-56 mg/L; LC50 = 28.22 mg/L)	Waterborne	Gills, liver	Dose-dependent behavioral alterations (hyperactivity followed by lethargy and loss of equilibrium); respiratory impairment; mucus hypersecretion; dose-dependent mortality; gill damage (necrosis, epithelial lifting, hyperplasia, lamellar fusion, aneurism); liver damage (vacuolation, necrosis, macrophage infiltration, sinusoidal dilation).	Ahmed et al., 2013
As III	*Betta splendens*	0.01-0.1 mg/L	Waterborne	Not specified	Loss of mate preference at 0.1 mg/L (no effect at 0.01 mg/L); increased anxiety-like behavior (increased time in dark); no change in total association time; no change in cortisol levels.	Tudor et al., 2019
As III	*Sebastes schlegelii*	0.05-0.4 mg/L	Waterborne	Liver, kidney, spleen, gill, intestine, muscle	Dose- and time-dependent bioaccumulation (liver > kidney > spleen > gill > intestine > muscle); induction of antioxidant defense system (↑ SOD, GST, GSH in liver and gills); strong positive correlation between tissue accumulation and antioxidant enzyme activity.	Kim & Kang, 2015
As III & As V	*Carassius auratus*	50 mg/kg diet	Dietary	Liver	Time-dependent bioaccumulation in liver (peak at 30 d followed by decline); species-dependent accumulation (higher in AsV vs AsIII at specific time points); biotransformation to less toxic forms (increased AsB, MMA, DMA); time-dependent modulation of antioxidant responses (SOD, CAT, GSH); transient oxidative damage (elevated MDA at 15 d in AsIII group); subsequent recovery indicating adaptive physiological response.	Cui et al., 2020

Table 1 summarizes the main patterns of heavy metal toxicity in fish, showing consistent tissue-specific accumulation and multi-level biological effects. Oxidative stress, histopathological damage, and behavioral alterations are among the most commonly reported responses across different metals and exposure routes. These findings support the relevance of these endpoints in assessing fish welfare under metal exposure.

Cadmium

Cadmium is a highly toxic non-essential metal that can induce both acute and chronic effects in aquatic organisms even at low concentrations (Elbeshti et al., 2018). In aquatic environments, cadmium originates from both natural geological processes and anthropogenic sources, the latter being the dominant contributors, particularly through agricultural inputs (e.g., fertilizers), mining, metal smelting, and the use of pesticides and herbicides (Yang et al., 2025). Fish absorb dissolved cadmium mainly primarily through the gills, but also via the gastrointestinal tract and skin, while trophic transfer through a contaminated diet represents an additional route of exposure (Garai et al., 2021). The skin and, especially, the gills constitute the primary entry routes for waterborne cadmium, as indicated by the absence of digestive tract lesions commonly associated with dietary exposure (Annabi et al., 2013).

Following uptake, cadmium accumulates in fish tissues through binding to metallothioneins, which facilitate its intracellular sequestration. In addition, renal excretion of cadmium involves multidrug and toxic extrusion (MATE) transporters; however, increased intracellular Cd^{2+} levels can inhibit this pathway, potentially enhancing metal retention within the organism (Lee et al., 2024).

At the cellular level, cadmium toxicity is strongly associated with oxidative stress. It can impair mitochondrial electron transport and indirectly stimulate the formation of reactive oxygen species (ROS) by increasing the availability of redox-active iron, thereby promoting Fenton-related reactions (Garai et al., 2021; Zahran et al., 2025).

Verbost et al. (1987) demonstrated that cadmium inhibits Ca^{2+} uptake at the gill level in rainbow trout by impairing branchial calcium transport mechanisms, resulting in disturbances in calcium homeostasis and hypocalcemia. However, Franklin et al. (2005) reported no significant alterations in Ca^{2+} uptake or plasma Ca^{2+} levels under chronic exposure to lower cadmium concentrations, suggesting that these effects may be concentration-dependent. Such disruptions in calcium homeostasis may impair ion regulation and gill function, with direct consequences for respiratory efficiency and overall physiological stability.

Once absorbed, cadmium is unevenly distributed among fish tissues, with the highest accumulation typically observed in the liver and kidney, while gills, intestine, and muscle may also show elevated concentrations depending on exposure conditions and duration (Perera et al., 2015). Similarly, cadmium exposure has been associated with structural and functional impairment of major organs, particularly the gills, liver, and kidneys (Elbeshti et al., 2018; Garai et al., 2021; Emon et al., 2023). Cadmium exposure leads to physiological disturbances and reduced growth performance (Perera et al., 2015; Emon et al., 2023), and has also been associated with renal dysfunction and impaired reproductive capacity in fish, reflecting chronic toxic effects on key physiological systems (Afshan et al., 2014). These effects may reflect increased energetic costs associated with detoxification and stress responses, reducing the resources available for growth and reproduction. Collectively, these alterations impair key physiological functions, particularly those associated with gill and kidney function, including respiration, osmoregulation, and detoxification, thereby compromising fish welfare by reducing their capacity to cope with environmental stressors.

Lead

Lead is a non-biodegradable and bioaccumulative metal that readily binds to oxygen- and sulfur-containing groups in proteins, forming stable complexes that contribute to its persistence and toxicity in biological systems (Sarkar et al., 2022).

Lead occurs naturally in the Earth's crust; however, elevated concentrations in aquatic environments are primarily associated with anthropogenic activities such as mining, smelting, industrial discharges, and the use of lead-containing products, as well as atmospheric deposition and surface runoff (Ishaque et al., 2020; Simatupang et al., 2024).

In aquatic environments, lead bioavailability is strongly influenced by its speciation, as it rapidly binds to suspended particles and sediments, reducing its dissolved ionic fraction. However, sediment-associated lead and interstitial water can still represent important exposure routes, particularly for benthic organisms and fish interacting with bottom substrates (Prosi, 1989).

The meta-analysis conducted by Demir et al. (2025) showed that Pb accumulation in fish varies among tissues, with higher concentrations generally observed in the liver and gills, followed by muscle tissue. Mean Pb concentrations were reported as 0.41 μg/g in liver, 0.33 μg/g in gills, and 0.27 μg/g in muscle. Lead accumulation and its physiological effects in fish show considerable variability depending on exposure route and duration. For instance, Crespo et al. (1986) reported pronounced intestinal and systemic alterations following chronic dietary exposure, highlighting the importance of gastrointestinal uptake in mediating toxicity. Ciardullo et al. (2008) observed relatively low muscle accumulation under long-term exposure conditions, but distinct tissue-specific distribution patterns, with lead preferentially accumulating in skin, kidney, and gills. Similarly, Javed (2012)

demonstrated that sublethal exposure resulted in age-dependent differences in both accumulation and growth performance, suggesting that physiological responses to lead are strongly influenced by developmental stage. Together, these findings indicate that lead toxicity cannot be generalized across exposure scenarios, but rather reflects a complex interaction between exposure pathway, duration, and organismal characteristics.

Histopathological alterations such as lamellar shortening, epithelial disruption, and structural disorganization have also been reported in gill tissues of lead-exposed fish (Jan et al., 2022). Acute lead toxicity damages the gill epithelium, impairing respiration and potentially leading to suffocation, while also promoting reactive oxygen species (ROS) production, which induces oxidative stress and results in damage to lipids, proteins, and DNA (Authman et al., 2015). Given the central role of gills in gas exchange and ion regulation, such structural damage may directly impair respiratory efficiency and osmoregulatory balance, thereby affecting overall physiological stability in fish.

Lead exposure in fish induces neurotoxicity by disrupting cellular signaling and neurotransmission, leading to behavioral impairments, while also altering immune function and overall physiological performance, ultimately compromising long-term fitness (Lee et al., 2019; Das et al., 2022). These alterations may reduce the ability of fish to respond to environmental challenges, affecting feeding behavior, predator avoidance, and overall performance.

Lead can also impair early life stages in fish and is associated with delayed hatching, developmental retardation, and morphological abnormalities in embryos and larvae (Sneha et al., 2026). In addition, lead exposure alters hematological and biochemical parameters, including changes in blood profiles and enzyme activities in plasma and liver, indicating physiological stress and tissue damage (Saket et al., 2025). Fish acutely exposed to Pb may exhibit haemoglobin alterations, black tail disease, and spinal deformities (Das et al., 2022).

Collectively, these physiological and behavioral alterations indicate that lead exposure disrupts key functions such as respiration, ion regulation, and neurological control, linking tissue-level damage to reduced organismal performance and compromised fish welfare.

Mercury

Mercury occurs in various chemical forms in aquatic environments, and its toxicity largely depends on its speciation. In particular, microbial processes can transform inorganic mercury into methylmercury, a highly bioavailable form responsible for most toxic effects in aquatic organisms (Ullrich et al., 2001). This biomethylation process occurs predominantly under anaerobic conditions in sediments, where microbial activity facilitates the conversion of inorganic mercury into methylmercury (Gworek et al., 2016).

Methylmercury is efficiently absorbed in fish, with approximately 95% of ingested MeHg taken up through the gastrointestinal tract. Following absorption, it readily binds to thiol-containing molecules such as cysteine and glutathione, facilitating its distribution into red blood cells and critical target tissues, including the brain. Due to its limited excretion and long biological half-life, methylmercury is retained within the organism, contributing to its progressive accumulation and persistence in fish tissues (Rice et al., 2014). Methylmercury undergoes efficient trophic transfer in aquatic food webs, resulting in biomagnification and elevated mercury concentrations in predatory fish, while bioconcentration from the water column further contributes to its overall bioaccumulation in aquatic organisms (Lavoie et al., 2013; Wu et al., 2019). Wang et al. (2010) showed that trophic transfer plays a more important role than waterborne exposure in mercury accumulation in tilapia species. This highlights the importance of dietary exposure in higher trophic levels, where biomagnification may lead to sustained internal mercury burdens and increased risk of chronic toxicity.

Mercury contamination in aquatic systems arises from both natural and anthropogenic sources. Natural emissions are associated with geological and geochemical processes, including volcanic eruptions, geothermal activity, soil degassing, and forest fires, with their intensity depending on regional characteristics. However, anthropogenic inputs represent a major source of mercury release,

mainly driven by industrial activities such as mining and smelting, artisanal gold mining, coal combustion, oil refining, chlor-alkali processes, and cement production (Al-Sulaiti et al., 2022).

In fish tissues, mercury is predominantly present as methylmercury (≈70-95%), with inorganic forms representing only a minor fraction (≈5-15%) (Suedel et al., 1994). Mercury in fish tissues is typically highest in the liver, followed by the kidney, muscle, gonads, and brain, with the liver being a primary target organ for toxicity and histopathological assessment (Zulkipli et al., 2021). Mieiro et al. (2009) reported that in *Liza aurata*, mercury accumulation followed the hierarchy kidney > liver > muscle > brain > gills > blood, with the highest concentrations observed in kidney and liver. The authors also noted that tissue distribution patterns may vary among species, although most studies consistently identify liver and kidney as the main organs of mercury accumulation.

Vieira et al. (2009) showed that acute exposure (96 h) to environmentally relevant mercury concentrations (3.1-200 μg L^{-1}) in *Pomatoschistus microps* impaired swimming behavior and induced oxidative stress and neurotoxicity (AChE inhibition), leading to reduced functional performance and fitness. Such neurotoxic effects may impair essential behaviors such as feeding, predator avoidance, and spatial orientation, thereby reducing survival and ecological performance.

Jasim et al. (2016) reported that acute mercury exposure in *Oreochromis* sp. induced behavioral disturbances, including hyperactivity, aggressiveness, and respiratory distress, while also resulting in preferential accumulation in the liver and gills, accompanied by marked branchial and hepatic lesions. Kania & O'Hara (1974) reported that mercury exposure in mosquitofish (*Gambusia affinis*) impairs escape responses, increasing vulnerability to predation and reducing survival, with behavioral alterations observed at body burdens as low as 0.67 ppm (wet weight).

Mercury exposure has been associated with reproductive impairment in fish, including reduced gametogenesis, altered steroidogenesis, and decreased spawning success, likely mediated by disruption of the hypothalamic-pituitary-gonadal axis (Crump & Trudeanu, 2009). These effects are further exacerbated during early life stages, where exposure reduces hatching success, delays development, increases mortality, and induces morphological abnormalities, including skeletal deformities. Such alterations impair functional performance and survival, while additional effects on feeding behavior may further compromise growth and overall fitness (Zheng et al., 2019).

Collectively, these alterations indicate that mercury exposure disrupts key functions such as neurological control, reproduction, and behavior, linking tissue-level accumulation to reduced performance and compromised fish welfare.

Arsenic

Inorganic arsenic forms generally exhibit higher toxicity than organic species. In aquatic environments, arsenic occurs mainly as arsenite As(III) and arsenate As(V), with arsenite being the more toxic form. The relative distribution of these species is controlled by environmental redox conditions, which in turn influence arsenic bioavailability and toxicity in aquatic organisms (Sharma & Sohn, 2009; Kumari et al., 2017). In addition to redox conditions, arsenic speciation is influenced by environmental factors such as pH and interactions with other elements, contributing to its dynamic behavior in aquatic systems (Campbell & Nordstrom, 2014).

Arsenic enters aquatic environments from both natural and anthropogenic sources. Natural inputs mainly result from rock weathering and geothermal activity, while major anthropogenic sources include mining, smelting, industrial discharges, and agricultural practices. Atmospheric transport and deposition also contribute to arsenic input in aquatic systems. Once released, arsenic accumulates in water and sediments, which can act as both sinks and secondary sources of contamination (Wang et al., 2022; Patel et al., 2023).

In fish, dietary arsenic is absorbed through the gastrointestinal tract and subsequently distributed to various organs via the circulatory system (Zhang et al., 2022). In addition to dietary exposure, waterborne arsenic can be absorbed through the gills, skin, and digestive tract, with the skin representing a potentially important uptake route in smaller fish due to their higher surface area-to-volume ratio (Ghosh

et al., 2022). In higher trophic level organisms, food intake represents a major exposure pathway and can account for the majority of arsenic bioaccumulation (Zhang et al., 2022). Due to its structural similarity to phosphate, arsenate can also enter cells via phosphate transport pathways, representing a key mechanism of cellular uptake (Yang et al., 2012).

Following uptake, arsenic is unevenly distributed among fish tissues, with higher concentrations typically found in the liver, gills, and gastrointestinal tract, and lower levels in muscle. Both inorganic (As(III), As(V)) and organic species, such as arsenobetaine and DMA, are present across tissues, with their relative proportions varying depending on tissue type and species (Hoy et al., 2023). Arsenic does not consistently biomagnify across aquatic food webs and behaves differently from elements such as mercury, with trophic transfer often being variable or even characterized by biodilution (Córdoba-Tovar et al., 2022). The liver is consistently reported as the primary site of arsenic accumulation, reflecting its central role in metabolism and detoxification processes. In general, arsenic distribution follows a decreasing pattern from metabolically active organs such as the liver and kidney to less active tissues, including muscle (Jabeen & Javed, 2011).

Arsenic exposure has been shown to alter fish behavior, including increased aggression and reduced opercular movement, indicating potential respiratory impairment. Additionally, arsenic exposure can negatively affect growth and increase mortality, with both waterborne and dietary pathways contributing to these effects (Kanwal et al., 2019). Tang et al. (2024) further demonstrated that acute arsenic exposure induces pronounced behavioral alterations, respiratory distress, and visible gill damage, including loss of equilibrium, increased ventilation, and structural deterioration of gill tissues. At the tissue level, Ahmed et al. (2013) demonstrated that arsenic exposure induces histopathological alterations in fish, including gill lesions such as epithelial lifting, hyperplasia, and lamellar fusion, as well as degenerative changes in the liver. Such impairments in gill structure and function may directly reduce respiratory efficiency and limit the ability of fish to maintain adequate oxygen supply under environmental stress.

Consistent with these findings, arsenic exposure is associated with increased aminotransferase activity, indicating hepatotoxic effects in fish (Kanwal et al., 2019). In addition, arsenic toxicity is associated with the induction of oxidative stress through the generation of reactive oxygen species (ROS), which can lead to lipid peroxidation and protein damage. Antioxidant defenses, particularly glutathione (GSH), play a key role in mitigating these effects, while depletion of GSH is linked to increased susceptibility to arsenic-induced damage (Ventura-Lima et al., 2011). This imbalance between pro-oxidant and antioxidant processes may compromise cellular integrity and reduce the overall physiological resilience of fish.

Overall, the reviewed evidence indicates that the relative toxicity of heavy metals in fish depends on metal speciation, exposure route, tissue distribution, and the physiological system affected. Cadmium primarily targets the gills, liver, and kidneys, with toxicity strongly linked to oxidative stress, calcium homeostasis disruption, impaired ion regulation, and reduced growth or reproductive performance. Lead also affects gill and kidney function, but its toxicity is particularly relevant through ionoregulatory disruption, neurotoxicity, hematological alterations, and behavioral impairment. Mercury, especially in its methylated form, shows the highest concern for trophic transfer and biomagnification, with strong neurotoxic, behavioral, reproductive, and developmental effects, particularly in predatory fish. Arsenic toxicity is strongly influenced by speciation, with inorganic forms, especially As(III), being more toxic than organic forms; its effects are mainly associated with gill and liver damage, oxidative stress, impaired respiration, altered behavior, and variable trophic transfer. Therefore, mercury appears most critical in terms of biomagnification and neurobehavioral risk, cadmium and lead are highly relevant for organ-level physiological disruption, while arsenic toxicity is strongly dependent on chemical form and environmental conditions.

CONCLUSIONS

Heavy metals represent persistent aquatic contaminants of major concern due to their non-biodegradability, bioaccumulation potential, and capacity to impair fish health and welfare.

The reviewed literature indicates that heavy metal exposure affects fish through multiple toxicological pathways, including oxidative stress, endocrine disruption, tissue damage, and developmental impairment, with consequences for physiological functioning.

Bioaccumulation patterns are influenced by environmental conditions, trophic position, exposure route, and species-specific traits, while metal accumulation occurs preferentially in organs such as the liver, kidneys, and gills rather than in muscle tissue.

Beyond tissue-level and physiological alterations, heavy metals also induce behavioral disturbances that serve as sensitive indicators of toxic stress and declining welfare.

Evidence synthesized across cadmium, lead, mercury, and arsenic highlights that both acute and chronic exposure can compromise respiration, osmoregulation, growth, reproduction, behavior, and overall functional performance in fish.

Overall, this review supports the view that heavy metal exposure represents a significant welfare risk in aquatic environments, including under sublethal conditions where impaired biological functioning occurs in the absence of immediate mortality. Importantly, these findings demonstrate that traditional mortality-based assessments underestimate the real impact of heavy metals, as substantial welfare impairment can arise even at sublethal exposure levels.

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EFFECTS OF DIETARY SPIRULINA ON THE GROWTH AND HEALTH STATUS OF CARP (*Cyprinus carpio*, Linnaeus 1758)

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Abstract

The study aimed to investigate the influence of dietary supplementation with S. platensis on the growth performance of carp (Cyprinus carpio, Linnaeus, 1758), reared in a recirculating aquaculture system, and on its health status, assessed by primary hematological indicators and blood biochemical parameters. For this purpose, three experimental variants were used, differentiated by the concentration of spirulina added to the feed: V0 – control, without spirulina supplementation; V1 – diet supplemented with 4% spirulina powder; V2 – diet supplemented with 6% spirulina powder. At the end of the study, it was found that all biotechnological indicators analyzed showed higher values in the experimental variant in which the diet was supplemented with 4% spirulina. The main conclusion derived from the evaluation of hematological parameters is that supplementation with 4% spirulina enhances the process of erythropoiesis, as evidenced by increased hematocrit values and erythrocyte counts. The data obtained indicate that spirulina is a safe and effective protein additive, and that including 4% in the diet is the optimal option for stimulating growth, improving protein utilization, and increasing feed conversion efficiency without compromising fish health and while ensuring a favorable cost-benefit ratio.

Key words*: carp, growth, health status, RAS, spirulina.*

INTRODUCTION

The growing demand for aquaculture as a major source of high-quality animal protein, combined with fish's high protein requirements and the high cost or limited availability of fishmeal and soybean meal, is driving intensified research to identify affordable and sustainable alternative protein sources (Youssef et al., 2023).

Over the last few years, increased attention has been paid to dietary supplements such as probiotics, microalgae, and medicinal plants, to improve growth performance, nonspecific immune responses, and feed efficiency in aquatic animals, as these supplements are inexpensive and easy to prepare, are effective, have fewer side effects during disease treatment, and do not pose environmental or safety concerns (Watanuki et al., 2006; Milad et al., 2016). Spirulina is the dried biomass of microscopic filamentous cyanobacteria of the genus Arthrospira, particularly *A. platensis*, *A. fusiformis*, and *A. maxima*, which are widely cultivated for human and animal consumption. Since the cell wall of spirulina does not contain cellulose, 85–95% of the substance is absorbed by the body (Babadzhanov et al., 1999). *Spirulina platensis* is a microalga (filamentous cyanobacterium) recognized as an excellent dietary supplement because of its high content of protein, vitamins, minerals, and carbohydrates, including vitamins (B, C, D, A, E) and minerals (potassium, calcium, iron, magnesium, copper, etc.) (Milad et al., 2016; Hayashi et al., 1998). For animals - including dogs, cats, and fish - spirulina offers significant benefits: it supports immune, skin, and coat health (Iatrou et al., 2023), improves digestion, aids in detoxification, and enhances fish coloration. Spirulina is often given in powder or tablet form, mixed with the animals' regular feed (Selmi et al., 2011). Including spirulina in the diet has immunostimulatory effects on the

innate immune system of carp (Watanuki et al., 2006).

Previous studies have shown various beneficial effects of spirulina, especially on growth performance, immunity, and disease resistance (Hirahashi et al., 2002; Sayed & Fawzy, 2014), through dietary supplementation with *S. platensis* in different fish species, such as the Siberian sturgeon, *Acipenser baeri* (Milad et al., 2016); sturgeon *Huso huso* (Adel et al., 2016); common carp, *Cyprinus carpio* (Javeed et al., 2021); rainbow trout, *Oncorhynchus mykiss* (Yeganeh et al., 2015); and Nile tilapia, *Oreochromis niloticus* (Lu & Takeuchi, 2004).

The study focused on the common carp, which is considered one of the most economically valuable species due to its rapid growth rate, the superior organoleptic quality of its meat, its high natural resistance, its ability to adapt to cold water, and the ease with which it can be farmed in closed aquaculture systems (Javeed et al., 2021).

It is well known that fish have a close relationship with their aquatic environment; therefore, their blood can indicate health issues in their bodies long before any visible signs of disease appear (Musa & Omoregie, 1999; Okechukwu et al., 2007). Hematological and biochemical parameters serve as indicators of health and physiological responses that can be affected by various stressors (Sayed & Authman, 2018; Sayed et al., 2020, 2021). Hematological indices are strongly connected to an animal's response to its environment, suggesting that the environment where fish live may influence hematological characteristics (Gabriel et al., 2004).

The main goal of this study was, based on the well-known benefits of spirulina (*S. platensis*), to assess the effects of dietary supplementation with this microalga on the growth performance of carp (*Cyprinus carpio*, Linnaeus, 1758), as well as on their health status, indicated by key hematological and biochemical blood parameters.

MATERIALS AND METHODS

Experimental design

The study was performed in an intensive recirculating aquaculture system (RAS) achieved of side glass tanks, each with a capacity of 60 liters. To maintain water quality in the culture system, Tetratec EX 1500 external filters were used equipment effective at keeping optimal water parameters. These filters have technical features that enable efficient removal of solid particles and control of other parameters essential for the aquatic environment. A total of six such filters were used, with one for each tank. The biological material consisted of 5-month-old carp juveniles with an average weight of 89.5 grams per fish.

The experiment was conducted from September 22, 2025, to October 31, 2025, spanning 40 days. It was designed to assess the impact of spirulina powder supplementation on the biological and physiological parameters of the subjects. The main parameters were chosen to enable a clear comparison between a control group and two experimental groups, differentiated by the amount of spirulina added to their diet. Consequently, the following experimental groups were established:

- V0 – control group without added spirulina, used as a reference for evaluating effects, consisting of two rearing units, B1 and B2. Ten carp juveniles, averaging 87±14.3 g per fish, were placed in each rearing unit;
- V1 – the experimental treatment with 4% Spirulina powder added, designed to highlight the impact of a moderate level of supplementation, comprises two rearing units, B3 and B4. Ten carp juveniles, with an average weight of 89.9±21 g per fish, were introduced into each grow-out unit;
- V2 – the experimental treatment with 6% Spirulina powder added, designed to analyze the effect of a higher level of dietary supplement, comprises two rearing units, B5 and B6. Ten carp juveniles, with an average weight of 89.9±25.3 g per fish, were introduced into each rearing unit.

Throughout the experiment, all treatment groups experienced the same environmental conditions, basic diet, and care routine to ensure comparable results.

Diet preparation

The biochemical and granulometric characteristics of the feed administered are shown in Table 1. For all experimental groups, the feeding rate was 2.5% of body weight per day (2.5% BW/day), ensuring a consistent diet

and allowing for supplementation. The feeding schedule consisted of four meals per day, with pellets administered manually at 8:00 a.m., 11:00 a.m., 2:00 p.m., and 4:00 p.m., based on the subjects' daily needs and feeding behavior. The food was evenly distributed across meals to promote optimal digestion and efficient nutrient uptake.

Table 1. The biochemical composition of Aqua Start 1 mm basic feed and spirulina-enriched feed

Nutrients	Measurement unit	Aqua Classic 2.5 mm	Variant with 4% spirulina	Variant with 6% spirulina
Crude protein	%	34	35	35,5
Crude fat	%	9	8,92	8,88
Cellulose	%	4	4.12	4,17
Digestible energy	MJ/kg	16,8	16,9	17

For this experiment, spirulina powder from the "Pilot Research and Development Station for Algae Cultivation" was used; it was cultivated in modern photobioreactors operated in a fully controlled environment at the Research and Development Institute for Aquatic Ecology, Fisheries, and Aquaculture in Galați. Growing spirulina in photobioreactors guarantees a stable and nutritionally consistent biomass, which directly helps produce high-quality feed with superior biological value and positive effects on fish growth. The spirulina used in this experiment has the following composition: crude protein 60%, crude fat 7%, crude fiber 7%, and digestible energy 19 MJ/kg.

The protocol for adding spirulina into feed was designed to maintain its physicochemical properties and was carried out as follows: dissolving and homogenizing spirulina powder in a 2% gelatine solution; incorporating the resulting solution into the pelleted feed; drying the pellets in an oven at $T^0 = 25°C$ for 2 hours.

Throughout the experimental period (40 days), environmental conditions in the rearing units were monitored through physicochemical analysis of water samples taken from the carp juvenile rearing units.

Water temperature, dissolved oxygen (DO), and pH were recorded daily, while the other water quality parameters (concentrations of NH_4-N, NO_2-N, and NO_3-N) were monitored through sampling twice a week.

Growth parameters

At the beginning and end of the experiment, the juvenile carp were weighed, and the following parameters were calculated: actual growth rate [Sr], daily growth rate [GR], specific growth rate [SGR], feed conversion ratio [FCR], and protein conversion ratio [PER], using equations already established in the literature.

Hematological and biochemical blood parameters

To evaluate the effect of supplementing the feed with spirulina and to determine whether the health status of the biological material was influenced, blood samples (approx. 0.3 ml) were collected at the end of the experiment from 10 fish in each experimental group, selected at random. The sampling was performed on previously sedated carp juveniles with clove oil. After collecting blood samples, the main hematological parameters (hematocrit, hemoglobin, red blood cells, and erythrocyte constants) were determined using methods already established in the scientific literature and veterinary medicine (Blaxhall & Daisley, 1973; Ghergariu et al., 1985; Svobodova et al., 1991; Dima et al., 2022). Various blood biochemical parameters (TP, ALT, AST, ALB, GLO, A/G, glucose) were analyzed using a hematology analyzer (automated blood analyzer), specifically the Seamaty SMT-30V model, employing a reagent/test disc preloaded with reagents for biochemical analysis.

Hemoglobin was measured using the colorimetric method with Drabkin reagent on a SPECORD 210 spectrophotometer (Analytik Jena, Jena, Germany) at 540 nm. Hematocrit was determined by centrifuging blood in heparinized microhematocrit capillary tubes with the Haematokrit 210 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany) at 12,000 rpm for 5 minutes. The red blood cell count was performed using the classical method, with Vulpian's solution as the diluent (prepared in the laboratory from sodium citrate, potassium iodide, and metallic iodine, Sigma-Aldrich, St. Louis, MO, USA), a Neubauer counting chamber, and a Potain pipette for erythrocytes. Based on the results of hemoglobin, hematocrit, and red blood cell count measurements, erythrocyte constants such as MCV (mean corpuscular volume), MCH (mean corpuscular

hemoglobin), and MCHC (mean corpuscular hemoglobin concentration) were calculated. These are important parameters in characterizing the physiological state of fish.
The study was conducted in accordance with the guidelines of the EC Directive 86/609/EEC regarding the protection of animals used for experimental and other scientific purposes and the Romanian legislation (Low 43 of 11 April 2014), approved by the Ethical Commission of Academy of Agricultural and Forestry Sciences "Gheorghe Ionescu Sișești", Department of Veterinary Medicine (Decision No 2048 of 04 April 2023).

Statistical analysis
Statistical analysis of data was performed using Excel 2019 and SPSS Statistics 26 software. Single-factor ANOVA and t-test were used to compare the experimental groups. Significant differences between the experimental groups were assessed using paired post-hoc comparisons with the Tukey HSD test. Results were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSIONS

The physicochemical parameters of water
The fundamental biological principle of aquaculture is the principle of the unity of the organism with its environment. According to this principle, every organism and every species as a whole can exist and develop only under the conditions of a specific environment to which that species has adapted during its evolution. The primary technological objective in a recirculating aquaculture system is to ensure environmental conditions that closely correspond to the ecophysiological characteristics of the cultured species (Cristea et al., 2002).
By analyzing the values recorded for each water quality parameter during the experimental period, the following observations were made:
- Water temperature, which is closely connected to dissolved oxygen the key water characteristic influencing fish health averaged 24.5±0.90°C during the experiment. The water temperature patterns across different experimental groups are naturally similar due to the recirculating system, and they stayed within the species' optimal range. The juvenile carp displayed active feeding behavior, consuming all the feed provided.
- Concentration of dissolved oxygen, measured at the grow-out units, recorded average values of 6.55±0.58 mg/L, which is optimal for the cultured species. Some warm-water species, such as tilapia and carp, are better adapted to survive under low DO conditions, whereas cold-water species cannot survive (Svobodova et al., 1993).
- The pH range where carp can survive is between 5 and 10, but they grow best at a pH of 6 to 9. During the experiment, the pH stayed within the optimal range needed by the species' biological functions.
- Throughout the experiment, nitrogen compounds remained within the optimal range (<0.1 mg/L for nitrites and 15-23 mg/L for nitrates) by replacing 20-30% of the total water volume in the grow-out unit and leveraging the filters' ability to treat the process water. Assessing nitrogen compounds is especially important in aquaculture, as some decomposition products mainly ammonia and nitrites are toxic. To a lesser extent, nitrates also become toxic when they build up to high concentrations (Romano & Zeng, 2013; Zhang et al., 2016).

Growth parameters
From the analysis of the growth equations, plotted in Figures 1, 2, and 3 for each experimental treatment, it can be observed that the coefficient of determination (R^2) in the control group where the fish were fed without added spirulina was 0.8274 (Figure 1). Although this value indicates a strong correlation, it still highlights the presence of considerable biological variability, likely caused by factors such as individual differences among fish, uneven feed intake, or microenvironmental variations.
Adding 4% spirulina to the feed significantly raised the R^2 value to 0.948 (Figure 2). This indicates an extremely strong correlation between dietary supplementation and growth response, with the model explaining 94.8% of the variation observed. This notable improvement suggests that spirulina helps reduce individual variability and stabilize the metabolic processes involved in growth, resulting in much more consistent outcomes.

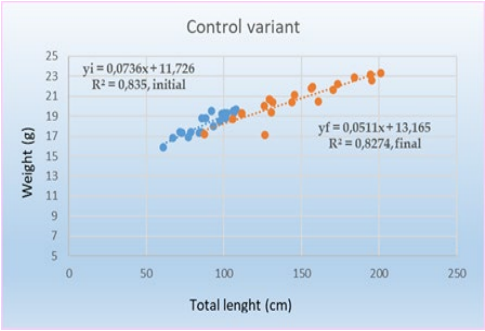


Figure 1. Length-weight regression (initial and final) for juvenile carp from the control variant

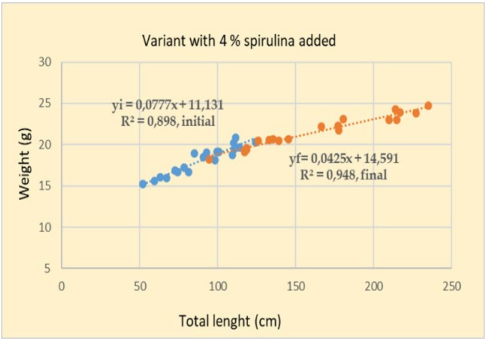


Figure 2. Length-weight regression (initial and final) for juvenile carp in the variant with 4% spirulina added

In the treatment with 6% spirulina supplementation, the R^2 value obtained was 0.9331 (Figure 3). Although it is slightly lower than the value recorded at the 4% spirulina level, it remains very high, indicating a strong correlation between spirulina supplementation in the diet and growth performance. The result suggests that higher levels of spirulina continue to support a consistent biological response, even though maximum uniformity was achieved with the 4% treatment.

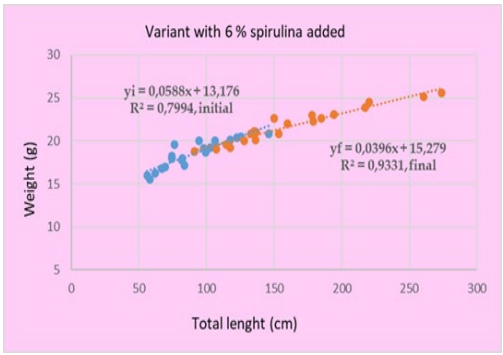


Figure 3. Length-weight regression (initial and final) for juvenile carp in the variant with 6% spirulina added

This upward trend in R^2 values shows that spirulina supplementation not only boosts growth but also lowers individual variability, with the 4% concentration being the most effective in producing a consistent and predictable growth response. The present study aimed to evaluate the effect of adding spirulina (4% and 6%) to fish feed on growth performance, feed conversion, and protein efficiency, compared to a control group without supplementation. The results emphasize the positive impact of spirulina on growth indicators, with variations depending on the dose, and it is evident that the experimental group with 4% spirulina achieved a higher growth rate than the other two experimental groups (Table 2).

Table 2. Biotechnological indicators for juvenile carp reared in a recirculating aquaculture system

Experimental Variant	**Control variant**	**The 4% spirulina variant**	**The 6% spirulina variant**
Tank indicator	**B1, B2**	**B3, B4**	**B5, B6**
Initial number of fish	20	20	20
Final number of fish	20	20	20
Survival (%)	100	100	100
Initial biomass (g)	1776.7	1798	1798.4
Initial biomass (kg/m³)	3.55	3.6	3.6
Final biomass (g)	2941.1	3267.4	3245.6
Final biomass (kg/m³)	5.88	6.53	6.49
Biomass gain (g)	1164.4	1469.4	1447.2
Biomass gain (kg/m³)	2.33	2.94	2.89
Initial average weight (g/fish)	88.84	89.9	89.92
Final average weight (g/fish)	147.06	163.37	162.28
Individual weight gain (g)	58.22	73.47	72.36

Experimental Variant	Control variant	The 4% spirulina variant	The 6% spirulina variant
Growth days	40	40	40
Crude protein in feed (CP %)	34	34	34
Daily ration (% biomass)	2.5	2.5	2.5
Total feed distributed (g)	1929.6	2045.7	2187.6
GR (Daily growth rate) (g/day)	29.11	36.74	36.18
SGR (specific growth rate)(%/day)	1.26	1.49	1.48
FCR (feed conversion ratio) (g feed/g biomass gain)	1.66	1.39	1.51
PER (protein efficiency ratio) (g/g)	1.77	2.11	1.95

Analysis of the data shows that survival was 100% across all experimental groups, indicating that spirulina has no toxic effects at the levels used and that stocking density and rearing conditions were optimal. This result confirms earlier research indicating that spirulina can serve as a protein supplement without harming fish health (Habib et al., 2008; Nuhu, 2013; Palmegiano et al., 2005).

The SGR and individual growth rates were significantly higher in the spirulina-treated groups, indicating superior metabolic efficiency and uniform fish growth. A similar observation was made using *Spirulina platensis* at different concentrations in *C. carpio* by Ramakrishnan et al. (2008). Although 6% spirulina yielded good results, the highest values were observed at 4%, suggesting that a higher dose does not necessarily yield maximum performance and that a ceiling effect may exist.

The lower FCR values seen in the spirulina-supplemented groups indicate more efficient feed utilization. PER increased significantly at 4% spirulina, confirming that the feed's protein is used more effectively for body growth. The results of this study on carp juveniles align with data from Al-Sulivany (2023). Similarly, Sayed et al. (2021), reported comparable results for catfish, emphasizing that growth parameters are optimal at a 2.5% spirulina concentration and decline at 5%. Unlike the results for carp juveniles, tilapia showed higher growth parameter values at a 10% spirulina concentration, which is above the optimal level for carp juveniles (Youssef et al., 2023).

Hematological and biochemical blood parameters

Knowledge of the hematological profile is an essential tool for monitoring physiological and pathological changes in fish (Kori-Siakpere et al., 2005).

Hematological indices, including hemoglobin concentration, hematocrit, and erythrocyte count, are particularly significant, reflecting the blood's oxygen-carrying capacity and overall physiological condition (Witeska, 2013; Youssef et al., 2023). By integrating hematological and biochemical data, scientists can gain a comprehensive understanding of fish physiology and the impact of nutritional strategies or environmental stressors on aquaculture species (Hrubec & Smith, 2010; Abdel-Tawwab et al., 2008). The values of the blood parameters and erythrocyte constants are presented analytically in Table 3.

Table 3. Hematological indices values

Experimental variant	RBC ($\times 10^6$cel/µl) (media±st. dev)	Hb (g/dl) (media±st. dev)	Ht (%) (media±st. dev)	MCV (µm^3) (media±st. dev)	MHC (pg) (media±st. dev)	MCHC (g/dl) (media±st. dev)
V0	1.58±0.20^a	10.82±0.35^a	27.1±2.44^a	173.14±13.05^a	69.77±9.82^a	40.25±9.82^a
V1	1.64±0.17^a	9.99±0.26^b	29.7±1.30^a	184.08±23.51^a	61.65±6.74^a	33.59±1.85^b
V2	1.55±0.19^a	10.5±0.88^a	25.6±3.96^b	166.6±30.08^a	68.68±9.64^a	41.92±7.14^a

Different letters (a,b) in the same column represent significant differences ($p<0.05$).
RBC- red blood cells; Hb - Hemoglobin; Ht - Hematocrit; MCV - Mean corpuscular volume; MCH - Mean corpuscular hemoglobin; MCHC - Mean corpuscular hemoglobin concentration.

In the experiment that studied the effects of spirulina supplementation on carp juveniles, the observed changes in erythrocytes reflect specific, dose-dependent physiological

responses. In variant V1, where the feed was supplemented with 4% spirulina, the erythrocyte count showed a slight, statistically insignificant increase ($p > 0.05$), reaching 1.64×10^6/µl, compared with the control group. This increase may suggest a moderate stimulation of erythropoiesis, possibly due to the bioactive compounds in spirulina, such as phycocyanin, chlorophyll, B-complex vitamins, and trace elements involved in heme synthesis. Therefore, a moderate supplemental intake of spirulina could enhance the efficiency of hematopoietic processes and the body's ability to adapt to increased metabolic demands. These results are also consistent with the data obtained by Abdulrahman et al. (2019), who observed no significant differences for added spirulina concentrations of 3% and 5%.

In contrast, in the 6% spirulina group, the red blood cell count decreased from not significantly ($p>0.05$) to 1.55×10^6/µl, indicating a change opposite to that seen at the lower concentration. This decrease may suggest metabolic overload caused by increased intake of bioactive nutrients, which could indirectly inhibit erythrocyte synthesis or redirect metabolic resources toward other physiological processes. Additionally, high concentrations of spirulina may impact osmotic balance, erythrocyte turnover, or alter endocrine parameters involved in regulating erythropoiesis (Abdel-Tawwab et al., 2008; Witeska, 2013; Hrubec & Smith, 2010; Promya & Chitmanat, 2011).

A decrease in red blood cell count at high spirulina levels may indicate a disturbance in physiological and metabolic balance, as numerous studies have shown that spirulina affects hematological parameters in a dose-dependent way, capable of both stimulating and modifying erythropoiesis depending on the level of inclusion in the diet (Rasheed et al., 2024). In experiments studying the effect of spirulina supplementation on the hematological parameters of carp juveniles, hemoglobin (Hb) concentration is a key indicator of physiological status, oxygen transport efficiency, and the organism's metabolic capacity (Javeed et al., 2021). A comparative analysis of Hb values between the control group and the experimental groups reveals subtle but biologically relevant changes.

In the experimental group where the feed was supplemented with 4% spirulina, the average hemoglobin level showed a statistically significant ($p<0.05$) decrease (9.99 g/dL) compared to the control group (10.82 g/dL). The same effect of spirulina concentration was also observed by Abdulrahman et al. (2019) in carp juveniles. In the variant where supplementation was increased to 6% spirulina, the hemoglobin level (10.5 g/dL) was considerably closer to that of the control group but still showed statistically significant differences compared to variant V1 ($p<0.05$). Overall, the results indicate that moderate spirulina supplementation (4%) can cause temporary changes in the hematological profile (Al-Sulivany, 2023), while a higher level (6%) appears to support the stabilization of hemoglobin, possibly through a gradual adaptation of fish metabolism to increased intake of functional nutrients. These findings highlight the importance of determining the optimal spirulina dose to maintain hematological balance and promote the overall health of carp juveniles.

Hematocrit (Ht) values increased in the experimental group treated with 4% spirulina (29.7%), but this was not statistically significant ($p>0.05$), compared to the control group (27.1%). The 6% spirulina group showed a statistically significant decrease ($p<0.05$) to 25.6%. This change may relate to variations in plasma volume or differences in erythrocyte size and volume, indicating a distinct adaptive physiological response based on the level of spirulina supplementation. Compared to the current study, Javeed et al. (2021) reported higher hematocrit values at a 7.5% spirulina concentration but observed decreases in hematological parameters at a 10% concentration in the feed.

The erythrocyte constants are vital hematological markers for evaluating red blood cell quality and function, offering additional insight into the physiological mechanisms behind changes in red blood cell count. Analysis of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) enables a comprehensive understanding of how spirulina supplementation affects erythrocyte maturation and efficiency in carp juveniles.

The mean corpuscular volume (MCV) showed a significant increase in the experimental group receiving 4% spirulina, reaching 184.08 µm³, compared with the control group. This increase suggests that erythrocytes formed in the presence of a moderate intake of spirulina are larger, possibly as a result of intensified erythropoiesis or accelerated erythrocyte maturation. At the same time, an increased erythrocyte volume may indicate increased availability of nutrients involved in red blood cell formation. In the 6% spirulina group, MCV decreased to 166.6 µm³, falling just below the control value, which may reflect the appearance of smaller erythrocytes, potentially due to incomplete cellular maturation, rapid nutrient utilization, or metabolic stress caused by excessive supplementation.

MCH (mean corpuscular hemoglobin) followed a different pattern, being lower at 4% spirulina (61.65 pg), suggesting that, although the red blood cells are larger in this treatment group, they contain less hemoglobin per cell. Similar data for carp were also obtained by Macoveiu et al. (2024). This combination (increased MCV, decreased MCH) may indicate a temporary imbalance in hemoglobin synthesis, possibly due to the redistribution of iron and other micronutrients toward other metabolic processes. At the 6% spirulina level, the MCH value increased to 68.68 pg, approaching control levels, suggesting a normalization of hemoglobin synthesis as the body adapts to higher supplementation.

The mean corpuscular hemoglobin concentration (MCHC) was highest in the 6% spirulina group, reaching 41.92 g/dL. This increase indicates a clear improvement in the hemoglobin content of erythrocytes, suggesting that, despite a slightly reduced erythrocyte volume in this variant, the hemoglobin density within the cells is significantly higher. Thus, the erythrocytes become more efficient at transporting oxygen, which may represent an important physiological advantage for fish under intensive rearing conditions.

Blood biochemical parameters are essential indicators of the physiological and metabolic status of fish, providing valuable information about their health, nutritional condition, and responses to environmental or experimental factors. These parameters include glucose, total proteins, albumin, liver enzymes (AST, ALT, ALP) (Hrubec & Smith, 2010; Abdel-Tawwab et al., 2008).

Supplementing the diet with spirulina resulted in significant changes in the analysed serum biochemical parameters, indicating a direct influence on protein metabolism, liver function, immunity, and physiological stress levels. The results highlight clear differences between the experimental groups, suggesting a dose-response relationship for some parameters.

Table 4. Blood biochemical parameters in the three experimental variants

Parameters	UM	Experimental variants		
		V0	V1	V2
Total serum protein (TP)	g/dl	3.08±0,01^{a}	3,02±0,01^{b}	3,61±0,02^{c}
Serum albumin (ALB)	g/dl	1.20±0,01^{a}	1,22±0,01^{b}	1,33±0,01^{c}
Globulin (GLO)	g/dl	1,87±0,01^{a}	1,80±0,01^{b}	2,29±0,01^{c}
ALB/GLO	-	0,64±0^{a}	0,68±0^{b}	0,58±0^{c}
Glucose (GLU)	mmol/l	13,36±0,40^{a}	8,06±0,09^{b}	7,98±0,08^{b}
Aspartate aminotransferase (AST)	U/l	133±2,46^{a}	106±2,57^{b}	210±3,02^{c}
Alanine aminotransferase (ALT)	U/l	46±2,21^{a}	47,5±0,48^{a}	34±2,22^{b}

Different letters (a,b,c) in the same column represent significant differences ($p<0.05$).

Total protein, albumin, and globulin levels showed consistent changes, particularly in the 6% spirulina variant, with statistically significant differences among the three variants. A significant increase in total protein and albumin content confirms that spirulina, because of its high crude protein, essential amino acid, and bioactive compound content, can stimulate protein synthesis and metabolic efficiency in fish. The ALB/GLO ratio decreased in the 6% spirulina variant, mainly due to a significant increase in globulins. A lower ALB/GLO ratio

is typical of organisms with heightened immune activity, indicating stimulation of defense mechanisms. This finding aligns with the literature emphasizing spirulina's role in activating macrophages, boosting antibody production, and modulating the nonspecific immune response (Hayashi et al., 1994). Serum glucose decreased significantly in both spirulina variants, indicating reduced metabolic stress and a potential improvement in stress tolerance (Macoveiu et al., 2024). Spirulina is known for its antioxidant properties. Analysis of liver enzymes reveals a clear and significant difference between variants. At 4% spirulina, the notable statistically significant decrease ($p < 0.05$) in AST levels indicates a hepatoprotective effect, likely due to the antioxidants and anti-inflammatory compounds in spirulina (Belay et al., 1993; Khan et al., 2005; Wu et al., 2016). Conversely, at 6% spirulina, AST levels increased significantly statistically ($p<0.05$), implying either metabolic overload or an adaptive response related to an increased overall metabolic rate (Ambroise et al., 2008; Holman & Malau-Aduli, 2013).

It is important to highlight that ALT levels, the more specific marker of liver health, decrease significantly ($p<0.05$) in the 6% group, indicating good liver function regardless of the increase in AST levels. Similar findings have been reported in common carp and other fish species, where spirulina supplementation reduced or maintained ALT levels even when AST levels varied, suggesting preserved liver health and potential metabolic adaptation (Abdalla et al., 2014). Furthermore, studies on fish like tilapia and catfish show that spirulina supplementation generally lowers ALT and AST under stress, highlighting its liver-protective and antioxidant effects (Sayed et al., 2021; Abdel-Daim et al., 2020). These findings support the idea that decreased ALT, even with elevated AST, may indicate that liver integrity remains intact but could also reflect increased metabolic activity or the release of enzymes outside the liver.

CONCLUSIONS

The experimental infrastructure, supported by advanced water quality control techniques, maintained optimal conditions for fish biomass growth across all three experimental variants, lending scientific credibility to the results.

Overall, the length-weight regression analysis shows that adding spirulina to feed enhances growth uniformity in fish. The 4% spirulina level is the most effective for producing a consistent and predictable response, while the 6% level continues to yield excellent results, confirming the beneficial role of spirulina in fish nutrition.

Analysis of key biotechnology indicators shows that spirulina is an effective and safe protein additive. A 4% spirulina dose is optimal for maximizing growth, protein utilization, and feed efficiency, without any negative effects on fish survival, thus making the feed cost-effective.

The findings align with existing scientific research, which emphasizes spirulina's role as a functional nutritional supplement with antioxidant, hepatoprotective, and immunostimulatory properties. The obtained results emphasize the importance of determining the optimal spirulina dose to maintain hematological, biochemical, and immune balance, as well as to promote the overall health of juvenile carp.

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ASSESSING BIODIVERSITY IN PĂDUREA CRÂNG (CODRII VLĂSIEI, BUZĂU, ROMANIA): A BASELINE STUDY FOR NATURA 2000 PROTECTED AREAS

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Abstract

Pădurea Crâng (Buzău County) is a valuable remnant of the former Codrii Vlăsiei forest, designated a county-level natural reserve in 1995. This study provides the first comprehensive assessment of its biodiversity and conservation value, establishing a scientific baseline for potential designation as a Natura 2000 site. Field surveys indicate high conservation importance, reflected by mature forest structures dominated by old-growth oak stands and the presence of rare, vulnerable, and protected species. The priority habitat 91I0 (Euro-Siberian steppic woods with Quercus spp.) was confirmed, underscoring the site's European significance. The flora includes notable species such as Tulipa sylvestris* subsp. *australis, Klasea bulgarica, Dianthus guttatus, Chaerophyllum nodosum, and Fraxinus pallisiae. Invertebrate fauna comprises Habitats Directive species (Cerambyx cerdo, Lucanus cervus) and nationally protected taxa, indicating structurally complex forests. Avifaunal surveys recorded several Birds Directive species, while the presence of Emys orbicularis confirms reptile conservation value. Overall, Pădurea Crâng represents a key biodiversity refuge in southeastern Romania and strongly merits designation as a Natura 2000 Site of Community Importance.*

Key words*: Codrii Vlăsiei, conservation value, Euro-Siberian steppic oak woods, forest biodiversity, Natura 2000.*

INTRODUCTION

Anthropogenic land use is the main cause of change in terrestrial ecosystems and has altered over half of the Earth's land surface (Vitousek et al., 1997; Foley et al., 2005). Forest ecosystems support a disproportionate amount of global biodiversity and provide essential services such as carbon sequestration, water regulation and nutrient cycling, that are vital for human welfare (Goodale et al., 2002; Millennium Ecosystem Assessment, 2005). In Europe, lowland deciduous forests have been particularly vulnerable to agricultural expansion, urbanisation, and drainage over the past two centuries. This has resulted in severe habitat fragmentation and the loss of old-growth structural attributes across much of the continent (Angelstam et al., 2004; Hanski, 2011).

In Romania, intensified land use from the early 19th century onwards has profoundly altered and fragmented the lowland forest ecosystems of the southern plains (Geacu et al., 2018). The Codrii Vlăsiei, a continuous deciduous forest complex that once covered much of the Wallachian Plain, was progressively cleared for agriculture and urban development. Only scattered fragments remain today, serving as ecological relics of the formerly extensive woodlands (Geacu et al., 2018). These remnant patches contain flora and fauna characteristic of the original forest, but their small size and isolation make them sensitive to edge effects, changes to the water cycle and recreational pressure (Laurance et al., 2002; Fischer & Lindenmayer, 2007).

Remnant lowland oak forests act as vital refuges for species that have disappeared from the surrounding agricultural landscape. These include flora specialized in old-growth forests, saproxylic invertebrates, cavity-nesting birds, and freshwater herpetofauna (Siitonen, 2001; Gutowski et al., 2005; Lindenmayer et al., 2012). Veteran trees (large, old individuals with structural features such as cavities, deadwood, and loose bark) are critical for biodiversity, supporting a disproportionately high number of species of conservation concern per unit area

(Ranius et al., 2009; Sebek et al., 2015). The conservation value of such fragments is further enhanced when mature stand structure coincides with rare or priority habitats, providing grounds for the formal protection of multiple taxa (Franc et al., 2007).

One of the most threatened forest types in Central and Eastern Europe is the Annex I priority habitat 91I0*, Euro-Siberian steppic woods with *Quercus* spp. (Habitats Directive 92/43/EEC). At the EU biogeographical level (2013-2018 assessment cycle), this habitat type has an unfavourable conservation status in both the continental and steppic biogeographical regions (EEA, 2020), primarily due to its rarity, ongoing hydrological impoverishment, and the progressive loss of veteran oak trees (EEA, 2024).

Saproxylic beetles, particularly those listed in Annex II of the Habitats Directive, have emerged as reliable indicators of the structural quality of forests and their management history (Siitonen, 2001). *Cerambyx cerdo* and *Lucanus cervus* are obligate associates of veteran oaks with decaying heartwood, and their presence indicates the persistence of old growth conditions that benefit a wide variety of co-occurring species (Buse et al., 2008; Thomaes et al., 2008). The occurrence of these saproxylic species, combined with Habitats Directive reptiles, such as *Emys orbicularis*, and Birds Directive Annex I taxa, provides particularly robust justification for site-level protection, an approach increasingly recognised as central to Natura 2000 site selection. The umbrella effect of such flagship invertebrates and reptiles thus strengthens the conservation case for habitats where they co-occur alongside rare or relict plant assemblages (Buse et al., 2007).

Despite the ecological importance of lowland forest remnants, the Natura 2000 network in Romania exhibits significant gaps at low elevations; protected areas represent less than 10% of the land surface at altitudes between 0 and 200 m a.s.l., making this the least represented elevation band in the national network (Iojă et al., 2010). The designation gap is particularly acute in the Wallachian Plain, where the few surviving forest fragments lack the formal European protection status that would trigger obligation-based management and long-term monitoring. This structural under-representation of lowland habitats in the Natura 2000 network has been identified as a key deficiency in biodiversity conservation policy in south-eastern Romania (Iojă et al., 2010).

Pădurea Crâng in Buzău County is one of the last surviving fragments of the Codrii Vlăsiei forest and has been recognised for its botanical significance since at least the late 19th century. In 1898, Grecescu recorded the presence of *Tulipa sylvestris* subsp. *australis* in the forest. The reserve was formally designated a county-level botanical nature reserve in 1995 (HCJ No. 13/1995) due to its wild tulip populations, veteran oak stands, and cultivated *Taxodium distichum* specimens. The site's floristic value has been further corroborated by the recent documentation of a genetically intact wild leek (*Allium scorodoprasum*) genotype within the forest (Vînătoru et al., 2025), attesting to the long-term ecological continuity of its herbaceous layer. Despite its recognised value, no systematic, multi-taxon biodiversity baseline had been compiled for the site prior to this study. Furthermore, Pădurea Crâng had not been evaluated for potential designation as a Natura 2000 Site of Community Importance.

This study aims to address this gap by providing the first comprehensive biodiversity assessment of Pădurea Crâng, integrating data on vascular plant flora, priority habitat occurrence, the dendrometric structure of veteran trees, and the herpetofauna, avifauna, and saproxylic Coleoptera. Specifically, the study aims to: confirm the presence of, and characterise the conservation status of, priority habitat 91I0*; document species of European conservation concern, as well as nationally rare taxa, across multiple taxonomic groups; provide the scientific basis for proposing 'Crângul Buzăului' as a Natura 2000 Site of Community Importance, thereby ensuring the long-term protection of its habitats and species.

MATERIALS AND METHODS

Study Area

The study was conducted in Pădurea Crâng, a county-level botanical nature reserve (HCJ no. 13/1995) situated within Buzău Municipality, southeastern Romania (45°08′42″N, 26°47′40″E). The reserve covers approximately 189 ha, of which some 178 ha constitute the

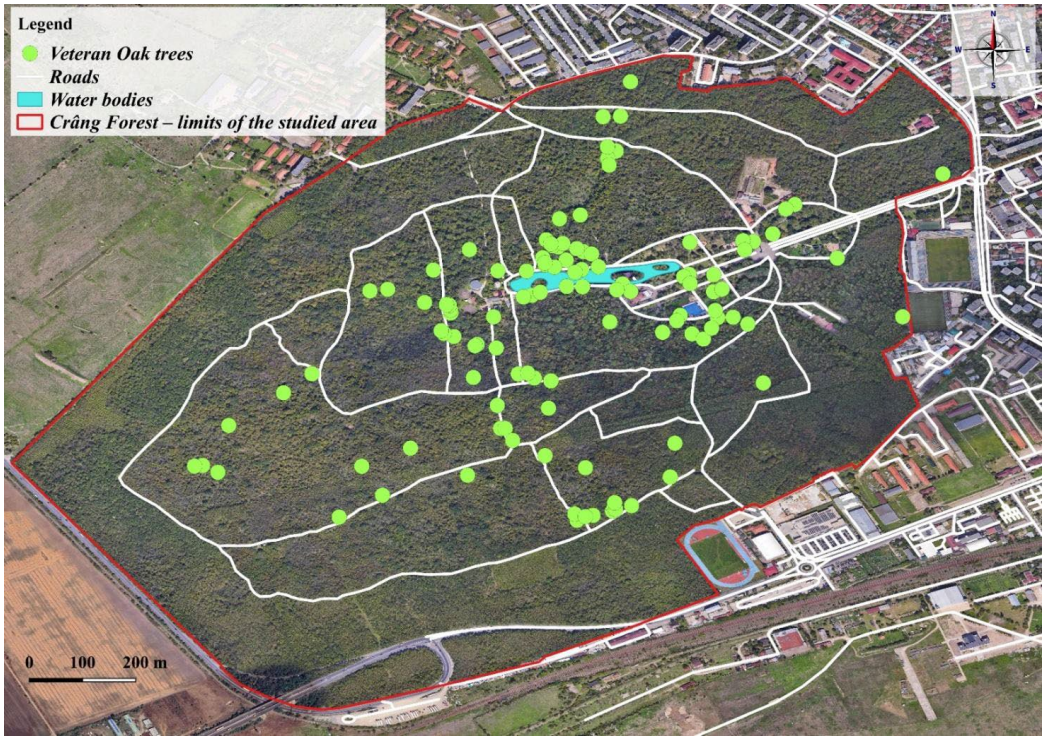


Figure 1. The spatial distribution of inventoried *Quercus robur* individuals within the Pădurea Crâng nature reserve in Buzău Municipality, Romania. Green circles indicate veteran oak trees (estimated age ≥100 years; n = 113). White lines show the internal road network and forest alleys. Blue polygon: pond (helești) and associated water bodies. The red boundary shows the limits of the studied area (approximately 189 ha)

actual forested area; the central-eastern sector is occupied by Parc Crâng, a managed recreational parkland. Elevation ranges from 89 to 107 m a.s.l. (Figure 1).

Floristic Surveys and Habitat Characterisation

All field and laboratory work was carried out between December 2024 and November 2025, encompassing bibliographic and herbarium analysis, repeated field campaigns across the principal phenological windows (March-November 2025), species distribution mapping and data processing. The floristic inventory relied on systematically distributed transects covering the full extent of the study area; species were identified in situ or, where necessary, in the taxonomy laboratory of the Institute of Biology Bucharest, using the determination keys of Săvulescu (1952-1976), Ciocârlan (2009) and Sârbu et al. (2013). Nomenclature was verified against the Euro+Med PlantBase and the Plants of the World Online database (POWO, Royal Botanic Gardens Kew). The inventory was complemented by a systematic review of published botanical literature, herbarium records and archival data, providing a long-term floristic baseline for the site. Taxa cited in the original reserve decree (HCJ 13/1995), but unconfirmed in the recent literature, were specifically targeted during seasonally appropriate surveys (March-May 2025), ensuring that non-detection reflects a deliberate, phenologically appropriate search effort.

Conservation status was evaluated against national Red Lists (Oltean et al., 1994; Boşcaiu et al., 1994; Dihoru & Dihoru, 1994; Dihoru & Negrean, 2009) and relevant legislative instruments (OUG 57/2007; HCJ 13/1995). Phytosociological surveys followed the two-stage analytical-synthetic methodology of Cristea et al. (2004), applying the Braun-Blanquet relevé method (Braun-Blanquet, 1951). Habitat identification and classification adhered to the *Interpretation Manual of Natura 2000 Romanian Habitats* (Gafta & Mountford,

2008) and the *Synthetic monitoring guide for habitats of Community interest* (Biriş et al., 2013).

Dendrometric Assessment of Veteran Trees
Tree age was estimated using two complementary methods. The indirect dendrometric method applied an age-diameter coefficient of 2.1, as established for silvosteppe oak stands (Netsvetov, 2016). For calibration, a subset of representative individuals was increment-cored using a 400 mm Pressler borer; ring counting and width measurement yielded a locally adjusted coefficient of 1.7. The discrepancy between the two estimates reflects natural variation in radial growth and enabled refinement of the age model to local site conditions. The combined approach provides a robust characterisation of the age structure of the mature and veteran oak population of Pădurea Crâng (Figure 1).

Herpetofauna
Surveys were conducted across eight field sessions between February and November 2025 (totalling 12 days, ~82 km walked), using active diurnal searching along transects, direct observation and long-range photography for subsequent taxonomic validation. A preliminary bibliographic review reconstructed the historical herpetofaunal baseline of the site. Species were identified from external morphological characters (Fuhn, 1960; Fuhn & Vancea, 1961); the *Pelophylax* spp. complex was assigned to complex level, as reliable in-field separation of *P. ridibundus*, *P. esculentus* and their hybrids requires acoustic or genetic analyses.

Avifauna
Birds were monitored over several field visits spanning February-November 2025, covering different phenological aspects (ranging from wintering to autumn). Bird surveys were conducted through visual observations with binoculars, in the morning (06:00-12:00) and in favourable meteorological conditions, following Bibby et al. (2000) and *Ghidul Standard de Monitorizare a Speciilor de Păsări de interes comunitar din România* (2020). A mix of line transects and point-counts was employed to ensure a comprehensive species inventory. Transects were drawn along the main park alleys while point counts were done in randomly selected locations (QGIS generated) inside the forested area. Counts were done with 10 x 50 Nikon binoculars, within a 150-200 m visual range. Acoustic recognition and song playback were used to confirm the presence of more cryptic species (e.g. the treecreeper - *Certhia familiaris*). Photographic documentation completed the ornithological assessment.

Saproxylic Coleoptera
Xylophagous beetle surveys were carried out between June and August 2025 along 25 transects distributed across the principal vegetation units of the reserve (oak stands of habitat 91I0*, urban park woodland with veteran oaks, and an ash plantation). Along each transect, favourable microhabitats for saproxylic species were characterised, and all encountered individuals of the four target taxa (*Cerambyx cerdo, Lucanus cervus, Oryctes nasicornis* and *Dorcus parallelipipedus*) were counted, distinguishing live adults from exuviae.

RESULTS AND DISCUSSIONS

This study provides the first systematic biodiversity baseline for the site, covering floristic composition, habitat characterization, dendrology, herpetofauna, avifauna, and saproxylic invertebrates.

Vegetation, habitat structure and flora
Pădurea Crâng is one of the last remnants of the former Codrii Vlăsiei forest complex, a lowland mixed deciduous woodland that once covered much of the Wallachian Plain. Field surveys revealed an overall mosaic vegetation structure, with natural and semi-natural forest communities alternating with planted stands, park areas, and open glades. This pattern is attributable to the site's complex land-use history and the ecological constraints of the subcontinental steppic zone.
The canopy is co-dominated by *Quercus robur* and *Q. robur* subsp. *pedunculiflora*, while the ash guild (*Fraxinus pallisiae, F. excelsior* and *F. angustifolia*) collectively accounts for around 29% of the composition of the stands, according to the forest management plan for U.P. I Pădurea Crâng (SC IRISILVA SRL, 2023). Pedunculate and sessile oak together contribute 24%, while

field elm (*Ulmus minor*) contributes a further 21%. Field maple (*Acer campestre*) and Tatar maple (*Acer tataricum*) are recurrent sub-canopy constituents, contributing 11% and 8% respectively. Deciduous broadleaves cover virtually the entire forested area (178 ha), while conifer plantations are confined to a mere 0.47 ha.

Priority Habitat 91I0* Euro-Siberian Steppic Woods with *Quercus* spp.

Field surveys confirmed the presence of Annex I priority habitat 91I0*, Euro-Siberian steppic woods with *Quercus* spp., within the reserve (Habitats Directive 92/43/EEC). According to the Romanian national forest typology (Doniţă et al., 2005), this community is classified as type R4159 - Danubian oak woodland of *Quercus pedunculiflora* and *Q. robur* with *Tulipa biebersteiniana,* and is assigned to the thermophytic steppe subzone of the Romanian Plain.

It is present in areas with a continental character, being recorded in 42 Natura 2000 sites distributed in the southern part of the country in the Danube Plain, Dobrogea, fragmented areas of Transylvania, but also in the Carei Plain in the Pannonian bioregion.

In the Crâng Forest, habitat 91I0* is confirmed by the composition of the stand, the vegetation structure and the presence of characteristic species. The defining elements of this habitat type are the pedunculate oak (*Quercus robur*) and species associated with the forest-steppe. The understory and shrub layer include *Acer tataricum*, *Ulmus minor*, *Crataegus pentagyna*, *Cornus mas*, Euonymus europaeus, *E. verrucosus*, *Pyrus communis*, *Viburnum lantana*.

The diagnostic species *Tulipa sylvestris* subsp. *australis* (syn. *T. biebersteiniana*) (Figure 2) was recorded at multiple locations during transect surveys (March-May 2025), with seven high-density population patches predominantly delineated in the central-eastern sector, near the principal access routes. This species was first reported in this area by Grecescu in 1898, attesting to over a century of floristic continuity. Given the documented anthropogenic pressures and the ongoing hydrological impoverishment of the site, the confirmed presence of this priority habitat is a strong case for strengthening existing conservation measures.



Figure 2. *Tulipa sylvestris* subsp. *australis*, flower detail showing hairy stamen filaments at the base (a diagnostic morphological character of the taxon) (left); leaf of a sterile shoot (right). Photographs by R. Nicoară (2025)

Plant Species of Conservation Concern

Floristic inventories identified several vascular plant taxa that warrant conservation attention at national and regional levels. The species were designated following an integrated assessment that incorporated national legislation (OUG 57/2007 and Law 49/2011), Romanian Red Lists, and ex situ conservation data. This approach is aligned with Romanian statutory frameworks, which evaluate rarity, endemism, relict status, and ecological function independently of IUCN global threat categories.

Klasea bulgarica (Figure 3) is the most significant floristic record of the present study. This sub-endemic taxon has a restricted range, with documented populations confined to a few localities in Buzău and Galaţi counties (Dihoru & Negrean, 2009). During the 2025 campaigns, two individuals were found in the south-southwestern part of the forest, growing on a slightly saline, light-exposed substrate alongside *Dianthus guttatus* and *Artemisia santonicum*. The species is categorized as Vulnerable across three national Red Lists (Dihoru & Negrean, 2009; Oltean et al., 1994; Dihoru & Dihoru, 1994) and is the focus of in vitro conservation programmes (Manole-Aiftimie et al., 2013; Ioniţă & Tofan-Dorofeev, 2025). It is also cultivated outside its natural habitat at the "Dimitrie Brandza" Botanical Garden in Bucharest.

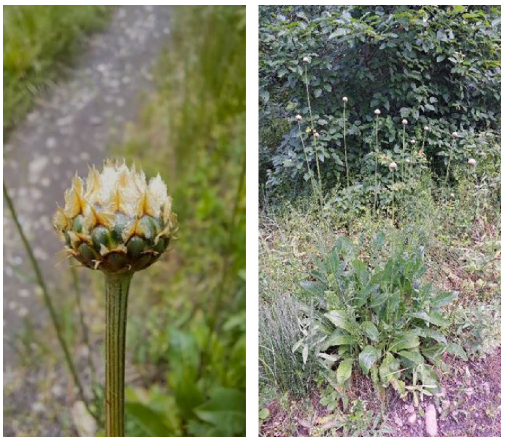


Figure 3. *Klasea bulgarica* in Pădurea Crâng: pre-anthesis stage (left); general habit of the species (right). Photographs by R. Nicoară (2025)

Dianthus guttatus was recorded at four locations in the southern and south-western parts of the reserve. It is assessed as Vulnerable (Oltean et al., 1994) and Endangered (Dihoru & Dihoru, 1994). *Chaerophyllum nodosum* (Figure 4), assessed as Vulnerable by Oltean et al. (1994), occurs consistently throughout zones of partially open canopy. *Fraxinus pallisiae*, a Pontic-Pannonian species with a limited natural distribution in Romania, plays a significant role in the structure of the canopy and is representative of the 91I0* habitat.



Figure 4. *Chaerophyllum nodosum* in Pădurea Crâng: detail of stem with inflated nodes (left); *C. nodosum* within the herb layer of the forest (upper right); detail of fruits (lower right). Photographs by R. Nicoară (2025)

The occurrence of *Fritillaria meleagris*, cited in the original nature reserve decree (HCJ 13/1995), could not be confirmed during the 2025 surveys. In ecological terms, however, its former presence is plausible given the historical hydrological continuity between Pădurea Crâng and the adjacent Pădurea Frasinu, where the species has been recorded (Zahariadi, 1962). Its apparent local extinction is most likely due to the progressive desiccation of the water table caused by deep borehole drilling and the construction of drainage canals for the Buzău municipal water supply, which eliminated the microhabitats essential for this species.

Old-Growth Trees and Stand Structure

A total of 139 veteran trees belonging to *Quercus robur* were inventoried within the reserve (Figure 1). The stem circumferences spanned a wide range, from 80 to 438 cm, corresponding to calculated diameters of 25-139 cm (mean: 75 cm). Age estimates derived from the relationship between diameter and growth rings range between 45 and 285 years, with an average for the stand of around 150 years. The individuals in the 200-250 year cohort are ecologically irreplaceable; they harbour cavity microhabitats and supply coarse deadwood for saproxylic assemblages. They also serve as refugia for arthropods and hole-nesting birds while bearing witness to the original structure of the Codrii Vlăsiei forest.

The spatial distribution of the oldest individuals is notably diffuse, with the largest specimens dispersed across the central and north-western sectors rather than concentrated in a single area. This pattern suggests a multi-generational relict origin rather than a single plantation event. A secondary cluster of very old trees around the pond and the central park area is most plausibly explained by selective retention during the conversion of part of the forest into a public park in the late 19th-early 20th century, when large-crowned oaks were deliberately preserved for their aesthetic qualities and ability to provide shade. Thus, the dendrometric dataset confirms that Pădurea Crâng is an important lowland refuge for veteran *Quercus robur*, with direct implications for the conservation of saproxylic invertebrate communities and cavity-dependent avifauna (Figure 5).



Figure 5. Veteran *Quercus robur* individuals inventoried in Pădurea Crâng nature reserve, Buzău, Romania. Photographs by R. Nicoară (2025)

Herpetofauna

A total of five taxa were identified in the surveys: three species of community interest, two reptiles and one amphibian, and two non-native aquatic species, which were most likely introduced through the intentional or accidental release of captive individuals. This phenomenon has been documented in urban parks throughout Romania (Iftime & Iftime, 2021).

The European pond turtle, *Emys orbicularis* (Linnaeus, 1758) is the herpetofaunal species of greatest conservation importance. It is assessed as Near Threatened by the IUCN (Cox & Temple, 2009) and as Vulnerable in the Red Book of Vertebrates of Romania (Botnariuc & Tatole, 2005). The species is strictly protected under OUG 57/2007 (Annexes 3 and 4A) and is listed in Annexes II and IV of the Habitats Directive. An individual was previously reported in the forest pond in 2020 (Iftime & Iftime, 2021) and was observed again near the pond island during the 2025 surveys, though the distance precluded definitive visual confirmation. The habitat requirements of this species, which include good water quality, adequate basking substrates and suitable oviposition sites on adjacent land, appear to be met at this location.

The green lizard, *Lacerta viridis* (Laurenti, 1768), which is protected under Annex IV of the Habitats Directive (IUCN: LC) and Annex 4A of OUG 57/2007, was the most consistently recorded terrestrial reptile and was present throughout glades, ecotones and areas of open canopy. The *Pelophylax* spp. complex (encompassing *P. ridibundus* and the hybrid *P. esculentus*) was detected in both the pond and its supply canal. However, specific identification in the field was hindered by the close morphological affinity between the different species.

Avifauna

Bird surveys recorded 67 taxa belonging to 29 families. The families *Muscicapidae* (8 taxa) and *Fringillidae* (6 taxa) displayed the greatest species richness; *Picidae* and *Corvidae* each accounted for five species.

Seven species are listed on Annex I of the Birds Directive (2009/147/EC): *Dryocopus martius, Picus canus* (Figure 6)*, Dendrocopos syriacus, Dendrocoptes medius, Ficedula parva, Ficedula albicollis* and *Lanius collurio*.

Confirmed seasonal occurrence of 3 woodpecker species (the grey headed woodpecker, the Syrian woodpecker, the middle spotted woodpecker), with the added occasional observation of *Dryocopus martius*, is a direct reflection of the abundance of large diameter trees, sensible amounts of decaying matter and ground branches that offer both shelter and foraging opportunities (Czeszczewik et al., 2013; Fröhlich & Ciach, 2020).

Two species are protected under the National Red List (OUG 2015/2022): the barn swallow (*Hirundo rustica*, Near Threatened nationally) and redwing (*Turdus iliacus* Near Threatened globally).

The dominance of insectivorous oscine taxa (Goldcrest, Eurasian wren, Blackcap, Red-breasted flycatcher - Figure 7) is an expression of habitat diversity and structural heterogeneity (Shaw et al., 2024).



Figure 6. *Picus canus* (grey headed woodpecker) male and female, Pădurea Crâng nature reserve, Buzău, Romania. Photographs by A. & M. Ciobotă (2025)



Figure 7. *Ficedula parva* (red-breasted flycatcher) recorded in Pădurea Crâng nature reserve, Buzău, Romania. Photograph by M. Ciobotă (2025)

Saproxylic Coleoptera

Four species of xylophagous beetle with recognized conservation value were recorded in the forest, including two taxa listed in the Habitats Directive: *Cerambyx cerdo* (Annexes II and IV) and *Lucanus cervus* (Annex II). Two further species of ecological significance, *Oryctes nasicornis* and *Dorcus parallelipipedus*, were also detected. All four species are obligate saproxylic beetles, meaning their life cycles depend on large-diameter oak trees with decaying wood or root systems. Consequently, their presence is closely linked to the availability of deadwood and veteran trees (De Groot et al., 2016). Surveys covered 25 transects, 22 of which contained favourable saproxylic microhabitats. A clear positive relationship was observed between mean tree circumference and beetle abundance. Transects with the highest coleopteran densities were characterised by mean stem circumferences of 206-320 cm compared to 92-184 cm in transects with low abundance.

Cerambyx cerdo (Figure 8) was recorded throughout the study area, yielding a total of 40 individuals, including live adults and exuviae. Comparisons with data from two Natura 2000 reference sites (ROSCI0359 Prigoria-Bengeşti and ROSCI0354 Platforma Cotmeana; Manu et al., 2017; Bărbuceanu & Truță, 2021) indicate that the Crâng population is numerically viable. All detections were made within the 91I0* habitat, which is consistent with the well-documented dependence of this species on old-growth oak. *Lucanus cervus* (Figure 8) was equally widespread, with 45 individuals recorded across the same proportion of surveyed transects.



Figure 8. *Lucanus cervus* (Linnaeus, 1758), male (left), and *Cerambyx cerdo* (Linnaeus, 1758), female (right), on the bark of *Quercus robur* in Pădurea Crâng nature reserve, Buzău, Romania. Photograph by M. Manu (2025)

Although the absolute number of individuals is lower than that recorded in the larger reference sites (228 and 314 individuals, respectively), the population density relative to the restricted area of Pădurea Crâng is considered satisfactory. The spatial co-occurrence of *C. cerdo* and *L. cervus* at forest ecotones and clearings around veteran trees (a pattern previously noted in Romanian oak forests (Panin & Săvulescu, 1961;

Bărbuceanu & Truţă, 2021) was also evident here. *Oryctes nasicornis* was widespread (44 individuals, principally exuviae), while *Dorcus parallelipipedus* was recorded as two isolated live individuals, reflecting its generally low density in inland oak forests.

Overall Conservation Assessment

Taken together, the results of this baseline study demonstrate that Pădurea Crâng supports a biologically diverse and structurally complex assemblage of flora and fauna of national and European conservation significance. The confirmed presence of priority habitat 91I0*, Habitats Directive species (*Cerambyx cerdo*, *Lucanus cervus* and *Emys orbicularis*), seven Birds Directive Annex I taxa and a variety of nationally rare or vulnerable plants, including the sub-endemic *Klasea bulgarica*, collectively provide robust scientific justification for designating this site as a Natura 2000 Site of Community Importance. Much of this biodiversity is underpinned by the old-growth *Quercus robur* stand, which has a mean age of around 150 years and includes specimens dating back 285 years. This stand functions as the principal structural and ecological foundation of the reserve. The long-term retention of veteran trees and coarse deadwood, combined with effective management of hydrological impoverishment, invasive species and recreational pressure, is essential for the sustained conservation of this forest remnant. Without such interventions, the ecological integrity of this irreplaceable lowland forest in south-eastern Romania cannot be guaranteed.

CONCLUSIONS

This baseline assessment confirms that Pădurea Crâng is home to biodiversity of national and European conservation importance across multiple taxonomic groups. Priority habitat 91I0*, Euro-Siberian steppic woods with *Quercus* spp., was identified and characterised. This is underpinned by a veteran *Quercus robur* stand with an estimated mean age of around 150 years, with some specimens reaching 285 years. The flora includes the sub-endemic *Klasea bulgarica*, which is assessed as Vulnerable in three national Red Lists and is the subject of active in vitro conservation programmes. Other species of regional conservation concern include *Dianthus guttatus*, *Chaerophyllum nodosum* and *Fraxinus pallisiae*. Habitat Directive species that were recorded include *Cerambyx cerdo* (Annexes II and IV) and *Lucanus cervus* (Annexes II), both of which have numerically viable populations, as well as *Emys orbicularis* (Annexes II and IV). Seven species listed on Annex I of the Birds Directive were documented, including four woodpecker species of European conservation concern. Their simultaneous occurrence within a single, small area can reflect advanced stand maturity. The presence of a priority habitat, Habitats Directive invertebrate and reptile species, Birds Directive Annex I taxa, and nationally rare plants collectively provides a robust, multi-taxon scientific basis for designating Pădurea Crâng as a Natura 2000 Site of Community Importance.
Given that fewer than 10% of the land surface at altitudes of 0-200 m a.s.l. in Romania is covered by protected areas (Iojă et al., 2010), the formal designation of this site would address a structural gap in the national Natura 2000 network while ensuring obligation-based management of one of the last remnants of the Codrii Vlăsiei. Long-term conservation will require strict retention of veteran trees and coarse deadwood, control of invasive alien species and regulation of recreational pressure. Without these measures, the ecological integrity of this irreplaceable lowland forest in south-eastern Romania cannot be guaranteed

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UTILISATION OF INSECTS IN ORNAMENTAL FISH NUTRITION: A REVIEW

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Abstract

Starting from the limitations surrounding fishmeal and the relevance of ornamental fish farming, this paper highlights the possibility of utilizing insects in their diet. Based on the scientific literature, specific nutritional requirements of ornamental fish and the valuable properties of certain insects were identified. Furthermore, experiments using insect protein biomass and artificial feeds formulated with varying percentages of insect meal for feeding ornamental fish were thoroughly analysed. It was demonstrated that insect meal can be included in the diets of goldfish and zebrafish at levels of 60% and 100% without adversely affecting growth or welfare, while for the Siamese fighting fish, the optimal level is 13%. In guppies and swordtails, testing inclusion levels of 50-75-100% yielded results indicating the potential for the complete replacement of fishmeal in their diet. The analysed experiments highlighted that ornamental fish are capable of assimilating nutrients and carotenoids from the composition of certain insects. A positive influence on fish health and coloration was observed, and significant performance was recorded regarding the survival and growth of fry in several freshwater and marine species.

Key words*: food, insects, ornamental fish.*

INTRODUCTION

It is estimated that a significant portion of the pet market revenue is derived from food production and distribution (Kowalska et al., 2021). Fishmeal and fish oil represent the most critical ingredients utilized in the formulation of nutritionally and energetically complete feeds (https://sealineexports.in/the-role-of-fish-meal-in-pet-food-formulations/; https://www.999.dk/en/products-and-segments/segments/pet-food/). The rising costs of fishmeal and fish oil, the depletion of oceanic fish stocks, and the imposed restrictions on their usage (FAO, 2024, 2025; OECD, 2025) are factors driving the identification and investigation of potential substitutes for these feed components (Alfiko et al., 2022; Sales & Janssens, 2003; Shepherd & Jackson, 2013; Turek et al., 2020). Recent studies and research consider insects to be a sustainable source of proteins with high biological value (Cullere et al., 2019; Fantatto et al., 2024; Motte et al., 2019; Surendra et al., 2016). Insect protein biomass is expected to command a market similar to that of fishmeal, with significant potential for use in aquaculture, livestock, and pet feed production (Freccia et al, 2020; Ragozzino-Paulino et al., 2025; Tomberlin et al., 2015; van Huis et al., 2013; Vrij, 2013). In this regard, since 2017, the European Union has approved the Regulation concerning the use of insect meal in aquaculture and pet food (EC 2017/893). Rearing and maintaining aquarium fish is emerging as one of the most popular hobbies (Maia et al., 2025; Miller-Morgan, 2010; Valdez & Mandrekar, 2019). The first public aquariums appeared as early as the 19th century, and it is estimated that there are more than 300 internationally (Karydis, 2011; Penning et al., 2009). Through its complexity and diversity, the aquarium industry has contributed and continues to contribute to the development of scientific research in various fields (Almunia et al., 2024; Safian et al., 2021; Novák et al., 2025).

The international trade in ornamental fish is a substantial industry, which has grown steadily (14%) since 1970 (Arapi & Cable, 2025; Novák et al., 2022; Valdez & Mandrekar, 2019). Its

market value is in the billions of dollars and encompasses over 5,300 freshwater species and 1,800 marine species (Evers et al., 2019; Marchio, 2018; Penning et al., 2009; Raghavan et al., 2013). The field's contribution to the global economy is therefore significant, and the captive breeding and rearing of an increasing number of species is of current interest to many countries (Lafi & Al-Shammari, 2025; Novák et al., 2022, 2025). Nutrition and feeding, along with ensuring water quality and organism welfare, are defining factors for the successful rearing of ornamental fish (Karydis, 2011; Sirimanna & Dissanayake, 2019). Within this general context, the present work highlights the possibility of utilizing insects for feeding ornamental fish.

MATERIALS AND METHODS

A thorough review of the scientific literature was performed to identify studies addressing the use of insects in ornamental fish food. Several major scientific databases were consulted, including PubMed, Google Scholar, Web of Science, Scopus, and ScienceDirect. The search approach relied on combinations of terms relevant to the subject area. Among the main search terms employed were "ornamental", "fish", "insects", "aquafeeds", "requirements", "nutrition" and "protein".

RESULTS AND DISCUSSIONS

NUTRITIONAL PARTICULARS OF ORNAMENTAL FISH

To ensure proper nutrient digestibility and support the metabolic requirements of aquarium organisms, a correct dietary intake is essential (Altaff et al., 2015). Both the feed and its ingredients must contain nutrients and energy sources vital for growth and development, depending on the species, life stage, and physiological state of the fish (NRC, 1993). There is limited research regarding the nutritional requirements of ornamental fish, and most information is based on results obtained from the aquaculture of food fish species (Pour et al., 2014; Sales & Janssens, 2003; Sicuro, 2018; Velasco-Santamaría & Corredor-Santamaría, 2011). However, it must be considered that certain differences exist. In ornamental fish farming, the primary objectives are colour enhancement and fish welfare; the feeding behaviour of each species is distinct, the quantitative feed requirement is lower, and the life cycle is longer (Altaff et al., 2015; Karydis, 2011; Sicuro, 2018). Nutritional requirements can be established for energy, proteins and amino acids, lipids, minerals, and vitamins (NRC, 1993). Specific nutritional values for some of the most well-known freshwater ornamental species are presented in Table 1. Protein is considered the most important nutrient for growth and development, and the requirement is dependent on the amino acid profile and digestibility (Miller & Mitchell, 2009; Nogales-Merida et al., 2019; Sirimanna & Dissanayake, 2019). These nutritional exigencies remain unchanged regardless of the source or type of feed. Under natural conditions, most fish rely on a protein-rich diet (Miller & Mitchell, 2009). In contrast to herbivorous and detritivorous species, omnivorous and carnivorous fish require a higher proportion of dietary protein (Henry et al., 2015; Nogales-Merida et al., 2019). Under aquarium conditions, it is crucial that fish utilize dietary proteins efficiently, as the resulting decomposition products have major implications for the living environment and, consequently, for the welfare of the organisms (Altaff et al., 2015; Sales & Janssens, 2003).

Table 1. Proximate composition of diets utilized for feeding aquarium fish*

Specification	Crude protein [%]	Crude fat [%]	Crude fiber [%]	Vitamin and Mineral [%]
Earlier stage	40-45	4-6	-	1-2
Adult	30-40	6-8	-	1-2
C. auratus	40-59	3.5-13.8	1-3	3
P. latipina	30-40	6-10	-	5
P.reticulata	15-52	3-9	6-8	5
P. sphenops	20-59	9.0-9.5	3.0-4.6	3
T.trichopterus	30-40	6-10	-	5
X. helleri	50	16	5	2-5
X. maculatus	18.3-26.3	4.9-5.9	2.2-8.8	-

*Data are compiled from published literature (Abdul Khader & Altaff, 2021; Fernando et al., 1991; Harsha Haridaet al., 2019; Mohanta & Subramanian, 2011; Pour et al., 2014; Sales & Janssens, 2003; Sirimanna & Dissanayake, 2019; Tamaru et al., 2001).

THE PRACTICE OF UTILIZING INSECTS IN FOOD FOR AQUATIC SPECIES

The exceptional properties of insects have been demonstrated through chemical assays. Insects are characterized by a high crude protein content and other nutrients (Table 2), comparable to those of fishmeal and soybean meal - the most

common ingredients used in aquafeed production (Barroso et al., 2014; Irungu et al., 2018). Their nutritional value is further characterized by a well-balanced essential amino acid profile, high lipid content, and also includes vitamins and minerals (Fantatto et al., 2024). The concentration of these nutritional components is strongly influenced by the species, the developmental stage, the nature of the growth substrate, and the extraction and processing methods (Makkar et al., 2014). Similarly, the lipid content and fatty acid profile are diet-dependent and can be adjusted by modifying the substrate (Georgescu et al., 2022). The presence of chitin in the insect exoskeleton may restrict its use in fish feed (Alfiko et al., 2022). On the other hand, positive influences have been reported by adding small amounts of chitin to aquatic feeds; furthermore, it has been proven that certain fish species can effectively utilize feed containing chitin (Georgescu et al., 2025; Tran et al., 2015).

Table 2. Nutritional value of some insect species

Specification	Crude protein [%]	Crude fat [%]	Crude fiber [%]	Ash [%]	Gross energy [MJ/ kg]
Fish meal Soy meal	70.6 51.8-55.2	9.9 1.7-2.0	- 4.4	- 7.3	- 20.0
Tenebrio molitor	52.8	36.1	2-5	3.1	26.8
Zophobas morio meal	49.3	33.6	5.7	2.9	26.8
Musca domestica maggot meal pupae meal larvae	 50.4 70.8 49.2	 18.9 15.5 17.5	 5.7 15.7 5.1	 10.1 7.7 10.2	 22.9 24.3 -
Hermetia illucens larvae meal	 42.1 41.8	 26.0 24.9	 7.0 8.8	 20.6 10.8	 22.1 -
Bombyx mori meal	 23.1-75.6	 4.7-14.2	 3.9-6.6	 1.2-6.8	 9.6
Galleria mellonella	38.8	51.4-58.6	8.9	2.2-3.3	-
Acheta domestica imago meal	 66.6 62.3	 22.1 13.3	 3.2 8.6	 3.6 8.4	 - -
Gryllodes sigillatus	45.0-70.0	16.8-18.2	3.6-11.8	4.7	19.0
Schidtocerca gregaria	76.0	13.0	2.5	3.3	18.0

*Data are compiled from published literature (Hawkey et al., 2021; Irungu et al., 2018; Kowalska et al., 2021; Makkar et al., 2014; Tran et al., 2015).

In accordance with EU legislation, eight insect species have been approved for use in aquaculture (Alfiko et al., 2022; EC 2017/893); among these, the black soldier fly (*H. illucens*), the housefly (*M. domestica*), the yellow mealworm (*T. molitor*), and crickets (*A. domestica, G. sigillatus*) are among the most extensively tested and utilized.

Preliminary studies and research on replacing fishmeal with insect meal have been conducted on various aquaculture species (Alfiko et al., 2022; Bake et al., 2021; Irungu et al., 2018; Magalhães et al., 2017; Motte et al., 2019; Oteri et al., 2021; Renna et al., 2017; Stamer et al., 2014; Tilami et al., 2020). According to these, it has been proven that many species efficiently utilize feed containing insects (Kowalska et al., 2021), and some experiments conducted on ornamental fish have reported that their diets can consist of insect protein biomass; furthermore, fishmeal in artificial feeds can be replaced with insect meal. In an attempt to determine the potential of insects as feed for ornamental fish, Das (2023) evaluates the growth, survival, and coloration of platy (*X. maculatus*) fed with three types of insects (grasshoppers, crickets, and mealworms) that were dried and crushed. At the end of the experiment, the results showed differences in favor of the groups fed with insects compared to the control group, which was fed with commercial feed. Some conclusions have also emerged indicating that a diet consisting of insects can be successfully administered, as the species is capable of assimilating the nutrients and carotenoids within their composition. The use of fishmeal has been tested on highly valued ornamental fish species (Table 3).

Table 3. Substitution of fishmeal with insect meal in the diet of some ornamental fish species

Insect species	Fish species	Replacement %	References
HI	*Amphiprion ocellaris*	25-50-75	Vargas-Abúndez et al., 2019
HI	*Betta splendens*	6.5-13-19.5-24.5	Kari et al., 2023
ZM	*Carassius auratus*	25-50-75-100	Das et al., 2024
HI	*Carassius auratus*	20-40-60-80-100	Kamalii et al., 2023
HI	*Cyprinus carpio* var. *koi*	25-50-75-100	Linh et al., 2024
HI	*Danio rerio*	25-50-75-100	Zarantoniello et al., 2021
AD GB	*Poecilia reticulate*	50-75-100	Perera & Bhujel, 2022
AD GB TM	*Xiphophorus helleri*	100	Perera et al., 2025.

AD - *Acheta domesticus*; GB - *Gryllus bimaculatus;* HI - *Hermetia illucens*; TM - *Teleogryllus mitratus*; ZM - *Zoophobas morio*.

In the Siamese fighting fish (*B. splendens*), in an experiment conducted by Kari et al. (2023), different inclusion percentages of insect meal in the feed were tested, and the results showed that at a level of 13%, growth and health status are improved. Research conducted by Das et al. (2024) highlights that in goldfish (*C. auratus*), it is possible to completely replace fishmeal with superworm meal (*Z. morio*). The research results indicate improved performance in terms of growth and survival, as well as fish coloration. The study by Kamalii et al. (2023) also focuses on the use of insect meal in goldfish. In an attempt to include different levels of black soldier fly larvae meal (*H. illucens*) in the diet of juveniles, the findings demonstrate better growth and feed utilization and specifically recommend 60% to ensure fish welfare. In another study conducted by Linh et al. (2024) on ornamental carp (*C. carpio* var. *koi*), results were obtained indicating the ability of *H. illucens* larvae meal to replace fishmeal and fish oil; furthermore, it is recommended as a possible way to increase disease resistance. The aquarium fish *Danio rerio* is validated as a model organism, so experiments are conducted, including on nutrition and feeding, which can also be extrapolated to aquaculture fish.

According to a detailed study conducted by Zarantoniello et al. (2021), it was highlighted that, through nutritional programming, fishmeal can be 100% substituted with *H. illucens* prepupae meal without negatively affecting fish growth and welfare. Other research also confirms the possibility of partial or total replacement of fishmeal with *H. illucens* meal in the diet of zebrafish, without affecting production parameters (Barca et al., 2023; Fronte et al., 2021). Given the need for sustainable ingredients for feed formulation, the objective of the study by Vargas-Abúndez et al. (2019) was to develop insect-based diets for clownfish (*A. ocellaris*), which is one of the most popular marine ornamental fish and is also considered a model organism. By using experimental diets with different levels of *H. illucens* meal, results with favorable perspectives were obtained, with no negative effects recorded on fish growth and survival. In the feed of viviparous fish, fishmeal was replaced with cricket meal. In the experiments conducted by Perera & Bhujel (2022) and Perera et al. (2025), it was proven that it can completely replace fishmeal without affecting the growth performance of guppy (*P. reticulata*) and swordtail (*X. helleri*). Additionally, the findings indicate the potential of using cricket meal in ornamental fish feed without the need for additives to enhance fish coloration.

CONCLUSIONS

Similar to food fish aquaculture, for production to be efficient, the feeding of aquarium organisms must be carried out in accordance with species requirements and depending on the ontogenetic stage and physiological state. The proven effect of insects on the growth and welfare of aquaculture organisms recommends their use in the production of feed for ornamental fish farming. The trend towards developing sustainable products is a current priority. The ability of some species to assimilate nutrients and carotenoids from certain insects can contribute to the elimination of taste attractants and color enhancers from ornamental fish feed. The development of insect-based artificial feeds can constitute an economic advantage for the ornamental fish industry.

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VALORIZATION OF BLACK SOLDIER FLY (*Hermetia illucens*) LARVAE MEAL IN GUPPY (*Poecilia reticulata*) FEED

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Abstract

The global trade in ornamental fish, feed, and aquarium equipment is an important component of the world economy. The majority of ornamental species are freshwater, fish of the Poeciliidae family are recognized as the most popular. Fishmeal is the most important ingredient in animal feed. The substantial reduction in oceanic fish stocks has imposed strategies to limit the use of fishmeal. Among these, the identification of alternative replacement sources is noteworthy. This study aimed to test a diet for guppy fish in which fishmeal was replaced by 60% insect meal. It also investigated whether the addition of enzymes promotes better availability and assimilation of this feed. The experiment lasted 75 days. The results showed no significant differences in growth between fish fed a commercial diet and those fed the insect-based diet. It was also found that feeding insect meal did not negatively influence the health of the fish and did not affect water quality parameters. It was also found that insect meal feed did not negatively affect the health of the fish or the water quality parameters.

Key words*: black soldier fly, guppy, protein larval meal.*

INTRODUCTION

The breeding of ornamental fish is in continuous evolution and is gaining increasing global interest (Olivier, 2001; Tolon, 2018). Due to current demand and environmental pressures, ornamental fish, particularly tropical live-bearing species, represent a true industry in certain countries (Sales & Janssens, 2003). Among the most popular species are the guppy (*Poecilia reticulata*), molly (*Poecilia sphenops*), swordtail (*Xiphophorus helleri*), and platy (*Xiphophorus maculatus*) (Alderton, 2012; Gavriloaie, 2023; Ghosh et al., 2007; Tolon, 2018). These species are also utilized in a wide range of biological and medical studies (Safian et al., 2021), serving as models for understanding invasive species, studying ecological consequences, and assessing the long-term effects of environmental pollutants (Boaru et al., 2021; Petrescu-Mag, 2023).

Native to South America, the guppy (*P. reticulata*) belongs to the family *Poeciliidae* (https://www.itis.gov/). It is a small fish with a broad tolerance for median environmental parameters (Gelsano & Demayo, 2022) and exhibits very pronounced sexual dimorphism (Petrescu-Mag & Bourne, 2008). Females give birth to 20-40 fry and have the capacity to store seminal material for subsequent fertilization (García & Giraldo-Gongora, 2023). Considered omnivorous like most poeciliids, the guppy's diet consists of zooplankton, insects, algae, and detritus (Arthington, 1989; Perera & Bhujel, 2022; Lawal et al., 2012; Tamaru et al., 2001; Yanong, 1999).

Fishmeal is one of the ingredients with the most complete properties utilized in feed formulations. In recent years, there has been a clear downward trend in fishmeal production due to the reduction of oceanic fish populations (FAO, 2016, 2018, 2020), coupled with an increased demand for feed production destined for aquaculture, livestock, and pets (Fontes et al., 2019; OCDE/FAO, 2021). Sustainability is now a mandatory requirement in all fields; therefore, the rearing and utilization of insects to substitute fishmeal in aquaculture and aquarium feeds is of high current relevance.

This study aimed to test a feed for guppies (*P. reticulata*) in which fishmeal was replaced by insect meal. Additionally, it investigated whether the addition of enzymes favors the availability and better assimilation of this feed.

MATERIALS AND METHODS

The experiment was conducted in 2023 at the Aquaculture Laboratory of the Faculty of Animal Science and Biotechnology, USAMV Cluj-Napoca. Sexually mature guppies (*P. reticulata* var. *Full Red Albino*), aged 2-3 months, were sourced from the Aquarium Complex of USAMV Cluj-Napoca. The insect meal feed was provided by the Laboratory of Aquaculture Rearing Technologies within the Faculty of Animal Science and Biotechnology.
Aquariums measuring 40 x 25 x 27 cm (L x l x h) with a useful water volume of 17 L were used. Each aquarium was equipped with a sponge filter (15 x 5 x 5 cm) powered by an air pump (Osaga MK-9510) at 10 W, with a maximum flow rate of 10 L/min. For lighting, a lamp (Resun DL-20R) with two neon tubes (OSRAM) at 18 W was used, and water temperature was maintained using heaters (Resun RH9000) at 25 W. Each aquarium was prepared with a maternity separator for the reproduction of live-bearing fish (Figure 1).
Tap water stored in containers was used; 35% of the water was replaced twice a week, and solid waste was vacuumed using a manual aquarium cleaner (Boyu model SC-002). Once every two weeks, filters were partially cleaned in water removed from the aquarium. The fish were brought into the laboratory and acclimated for 14 days, after which one male and one female (at first parturition) were released into the maternity separator of each aquarium (Figure 1), with the male being removed after 2-3 days. Following the expulsion of the fry, the female was also removed from the aquarium.

Fish feeding protocol

Differentiated feeding was initiated with the breeding stock, with two meals administered daily. The fry was fed *ad libitum* six days per week, with four daily feedings scheduled between 08:30 and 16:00. Based on the dietary treatments, the following experimental groups were established:

LGM: Consisted of a commercial control diet ("Hikari Fancy Guppy") specifically formulated for live-bearing fish. The nutritional profile, as specified by the manufacturer, included: Crude Protein (min. 50%), Fat (min. 8%), Fiber (max. 2%), Moisture (max. 10%), Ash (max. 18%), Phosphorus (min. 1%), Vitamin A (min. 19,000 IU/kg), Vitamin D3 (min. 2,800 IU/kg), Vitamin E (min. 850 IU/kg), and Ascorbic acid (min. 340 mg/kg).
LG60: Utilized a diet where 60% of the fishmeal was substituted with Black Soldier Fly (BSF) larvae meal (*Hermetia illucens*). This group was further divided into enzyme-supplemented (**60+**) and non-supplemented (**60-**) treatments. The calculated nutritional values for the BSF-based diet were: Crude Protein 44.12%, Crude Fat 3.26%, Cellulose 1.65%, Crude Ash 2.86%, Lysine 1.27%, Methionine 0.70%, and a Vitamin-Mineral Premix at 1.00%. For the supplemented group, the commercial preparation Hostazyme X (0.02%) was incorporated. Feed pellets were crushed into a coarse meal for adults, while particle size was calibrated according to the growth stage of the fry (Figure 2).

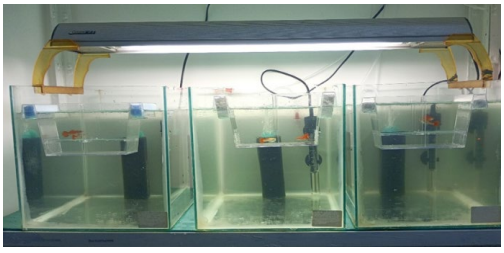


Figure 1. Experimental aquarium setup stocked with guppy (*Poecilia reticulata*) breeding pairs (♀ ♂) (original source)



Figure 2. Experimental diets used (from left to right: commercial control, LG60+, LG60-) (original source)

Water quality monitoring

Water temperature, electrical conductivity, and pH were recorded daily, while ammonia (NH_4) levels were determined on a weekly basis. The

monitoring was performed using a digital thermometer, a digital pH meter, a digital TDS meter, and a colorimetric titration kit (JBL ProAquaTest Combi Set Plus).

Growth parameters

To evaluate whether the substitution of fishmeal with insect meal influenced the growth performance and overall condition of the guppy fry, body weight (W), total length (Lt), and the condition factor (K) were assessed. The experimental period was divided into two evaluation stages: at 42 and 75 days post-parturition, respectively. At each interval, ten fish from each aquarium were weighed and measured. Body weight was recorded using an analytical balance (Ohaus Explorer Pro ep 114c) with a resolution of 0.1 mg. To determine the total length, the fish were photographed against a metric grid background; scale calibration was performed by measuring a standard 1 cm reference unit (Figure 3).



Figure 3. Biometric measurement and weighing of guppy fry (original source)

The following formula was used to calculate the condition factor (Gelsano & Demayo, 2022; Grozea, 2007):

$$K = \frac{(W \times 100)}{Lt^3}$$

where: K = condition factor/Fulton's index [%]; W = body weight [mg]; Lt = total length [cm].

The animal study protocol was approved by the Bioethics Committee of The University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca (decision no. 308 from 17 March 2022).

Statistical analysis

Raw data were statistically processed to determine mean values. For the monitored growth parameters, statistical significance was tested using one-way Analysis of Variance (ANOVA) with IBM SPSS Statistics 21 software.

RESULTS AND DISCUSSIONS

Water quality parameters

Mean water temperature values (Figure 4) were 26.28 ± 0.02°C (LGM), 26.06 ± 0.03°C (LG60+), and 26.10 ± 0.02°C (LG60-), falling within the ranges reported by other authors for guppy rearing (Perera & Bhujel, 2022; Shah et al., 2017).

Through daily pH monitoring (Figure 5), values of 7.76 ± 0.01 (LGM), 7.71 ± 0.01 (LG60+), and 7.77 ± 0.01 (LG60-) were recorded. These results indicate the alkaline nature of the water, which is specific to the growth requirements for this species (Bierbach et al., 2024; Serihollo et al., 2024). The consistency of the mean values obtained across all aquariums suggests that the administered feed did not influence water pH in any of the experimental groups.

Regarding electrical conductivity (Figure 6), the mean values obtained for each group were 165.25 ± 1.43 µS/cm (LGM), 154.11 ± 1.13 µS/cm (LG60+), and 158.94 ± 1.43 µS/cm (LG60-). These measurements fall within the recommended limits (100–4000 µS/cm) for guppy rearing (Bierbach et al., 2024).

Regarding ammonia (NH_4), recorded values were below the detection limit of the colorimetric assay (0.05 ppm) across all aquariums. Consequently, water quality relative to this parameter remained at an optimal level for the welfare of the ornamental fish (Bierbach et al., 2024; Shelton, 2010).

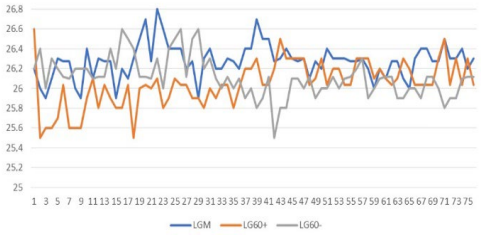


Figure 4. Water temperature monitoring results

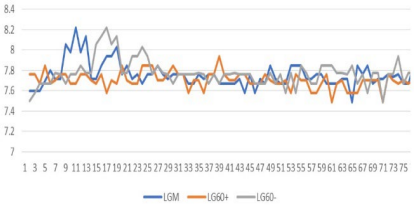


Figure 5. Water pH monitoring results

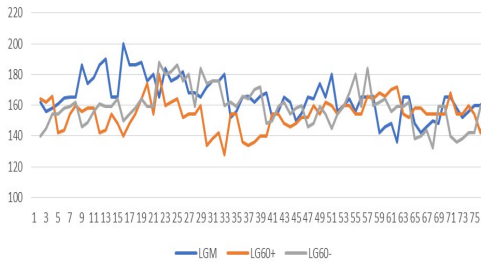


Figure 6. Water electrical conductivity monitoring results

Throughout the experimental period, fish behaviour was monitored, specifically focusing on feed acceptance or refusal. At the beginning of the experiment, females were less active; however, no differences in appetite were observed across any of the groups. During the first 2–3 days post-parturition, the fry exhibited low activity levels and remained concentrated in the central area of the aquarium floor. Subsequently, the fish began rising to the water surface to forage, with no feed refusal observed in any of the groups. A distinction was noted regarding the dispersion and physical stability of the feed in water. The commercial feed was drier and maintained its structural integrity in water longer, whereas the experimental feed dispersed significantly faster. Nevertheless, the water quality parameters indicated no significant differences between groups, confirming that the experimental diet did not adversely affect water quality.

Growth performance in relation to the experimental diets

The results obtained from the two evaluations are presented in tables, including the mean and standard error of the mean ($X \pm s_x$), the coefficient of variation (V %), and the variation limits (minimum-maximum) for each monitored trait.

The data obtained from the first evaluation are presented in Table 1.

Regarding body weight, the mean values were 21.51 mg (LGM), 20.94 mg (LG60+), and 20.75 mg (LG60-), with ranges between 9.05 mg (LG60-) and 49.95 mg (LG60+). High variability was observed for this parameter both within and between groups. From this perspective, the LGM group showed the lowest coefficient of variation (27.64%), followed by the LG60- group (39.38%), while the highest value was recorded for the LG60+ group (54.25%).

Regarding body length, the mean values were 1.35 cm (LGM), 1.33 cm (LG60+), and 1.40 cm (LG60-), with ranges between 1.12 cm (LG60+) and 1.69 cm (LG60-). For this parameter, the calculated coefficients of variation showed similar values (9.42% for LGM, 10.90% for LG60+, and 13.19% for LG60-).

Regarding the condition factor, mean values were similar for the LGM (0.863) and LG60+ (0.826) groups, while a slightly lower value was recorded for LG60- (0.713). The coefficients of variation were 11.43% (LGM), 13.21% (LG60-), and 19.61% (LG60+).

The data obtained at the second evaluation are listed in Table 2.

Regarding body weight, the mean values obtained were 98.09 mg (LGM), 79.58 mg (LG60+), and 95.54 mg (LG60-). The calculated coefficients of variation indicate the same high variability both within and between groups; however, the lowest value (44.17%) was recorded for LG60+, followed by LG60- (57.22%), while the highest value was determined for LGM (73.33%).

Regarding body length, mean values of 1.99 cm were obtained for both LGM and LG60+, while the LG60- group recorded a mean of 2.06 cm. The calculated coefficients of variation were 13.04% (LG60+), 13.41% (LG60-), and 19.58% (LGM).

Regarding the condition factor, mean values were 1.031 (LGM), 0.961 (LG60+), and 1.011 (LG60-), with coefficients of variation of 18.74% (LGM), 15.58% (LG60+), and 16.53% (LG60-).

Table 1. Mean values and dispersion indices at 42 days

Parameter		$X \pm s_x$	V%	Variation limits
LGM	W	21.51±1.88	27.64	13.85-32.80
	L_t	1.34±0.04	9.42	1.16-1.52
	K	0.863±0.03	11.43	0.74-1.02
LG60+	W	20.94±3.59	54.25	10.85-49.95
	Lt	1.33±0.04	10.90	1.12-1.58
	K	0.826±0.05	19.61	0.68-1.27
LG60-	W	20.75±2.58	39.38	9.05-32.10
	Lt	1.40±0.06	13.19	1.17-1.69
	K	0.713±0.03	13.21	0.56-0.89

Table 2. Mean values and dispersion indices at 75 days

Parameter		$X \pm s_x$	V%	Variation limits
LGM	W	98.09±25.43	73.33	31.75-207.95
	L_t	1.99±0.13	19.58	1.60-2.57
	K	1.031±0.07	18.74	0.77-1.32
LG60+	W	79.58±12.42	44.17	39.35-159.8
	Lt	1.99±0.09	13.04	1.56-2.51
	K	0.961±0.02	15.58	0.87-1.04
LG60-	W	95.54±22.32	57.22	43.95-198.75
	Lt	2.06±0.11	13.41	1.80-2.51
	K	1.011±0.07	16.53	0.75-1.26

In both evaluations, the recorded limits and coefficients of variation revealed moderate variability for body length and the condition factor (V<20%), while more pronounced variability was observed for body weight (V>20%). Based on the mean values and the observed variability across the analysed traits, the biological material in all groups exhibited appropriate growth and development. These results fall within the ranges reported by other authors for this species (Harpaz et al., 2005).

For each evaluation stage, an analysis of variance was performed, and the results are presented in Table 3.

Statistical data obtained from both weighings did not indicate significant differences, suggesting that there are no functional distinctions between the commercial and experimental feeds regarding weight gain. Similar to body weight, the results for total length proved to be statistically non-significant (NS) in both evaluations. Regarding the condition factor (K), although differences between groups were observed at 42 days, these were not maintained by the 75-day evaluation.

Table 3. F-values and the significance of differences obtained in the two evaluations

Parameter	F statistic	Critical F (0.05 level)	Effect
W 42 days	0.02	3.35	Ns
L_t 42 days	0.61	3.35	Ns
K 42 days	4.05	3.35	S
W 75 days	0.25	3.52	Ns
Lt 75 days	0.09	3.52	Ns
K 75 days	0.46	3.52	Ns

Ns = Non-significant; S = Significant.

The condition factor (K) is an indicator of the physiological state of fish and feed availability, with higher values reflecting a robust state of development (Grozea, 2007; Savin et al., 2022). From this perspective, the indices obtained in both evaluations reveal a good nutritional status for the fish in all groups, falling within optimal limits. Furthermore, at the end of the experiment, the fish condition factor values (1.031, 0.961, and 1.011) were consistent with those obtained by Abdul Khader & Altaff (2021) for guppy fry fed four different diets over 90 days (0.950, 0.962, 0.969, and 1.039). This highlights the healthy status of the fish and an isometric growth pattern. The analysis of variance reveals that no significant differences exist between the group fed with commercial feed-containing approximately 50% crude protein and based on fishmeal, and the groups fed with the experimental diet, where the crude protein content was approximately 44.12% and fishmeal was substituted at a 60% rate with BSF larvae meal. Furthermore, it can be stated that for this species, the addition of enzymes to BSF larvae meal-based feed is not necessary, as no significant differences were observed between the LG60+ and LG60- groups. This hypothesis is further supported by the fact that previous studies on *P. reticulata* have shown that insect meal, including Black Soldier Fly (BSF), acts as an attractant and increases feed palatability. Research conducted by Kowalska et al. (2021) demonstrated that guppies exhibit a significant interest in insects and even prefer diets based on insect meal over those containing fishmeal.

The effects of replacing fishmeal with insect meal derived from two species (*Acheta domesticus* and *Gryllus bimaculatus*) at varying inclusion levels (50%, 75%, and 100%) on the growth and reproduction of *P. reticulata* were also investigated by Perera & Bhujel (2022). No differences were recorded between the experimental groups and the control group regarding the growth performance of guppy fry. Furthermore, as the fry continued to grow, their reproductive performance was tested, revealing that insect meal had no influence on the number of offspring obtained or their dimensions. The conclusions and results of this study confirmed that meal from both insect species can replace fishmeal at a 100% substitution rate in guppy feed. In line with these findings, research conducted by other authors recommends the use of insects in aquarium fish diets to achieve optimal growth and reproductive performance (Adil et al., 2014; Ahmed et al., 2022). BSF larvae meal has been successfully utilized to replace fishmeal in the diets of other ornamental

species as well. Kamalii et al. (2023) reported that goldfish (*Carassius auratus*) fry fed with BSF larvae meal achieved superior growth performance and demonstrated enhanced feed utilization. The conclusions drawn indicate that a 60% substitution rate of fishmeal with BSF larvae meal is optimal for goldfish nutrition. Furthermore, the results of the study conducted by Linh et al. (2024) showed that replacing both fishmeal and fish oil with BSF larvae meal has significant implications for ornamental fish nutrition and may represent a novel and sustainable strategy for enhancing disease resistance. Through the improvement of captive breeding techniques, marine ornamental aquaculture continues to expand (King, 2019; Sinha et al., 2023), and addressing feeding-related challenges represents a current trend. Research is particularly focused on identifying high-value nutritional sources with minimal environmental impact. From this perspective, insects are being extensively studied, and the black soldier fly (*H. illucens*) shows significant potential. The partial inclusion of BSF meal in the diet of clownfish (*Amphiprion ocellaris*) fry was first reported by Vargas-Abúndez et al. in 2019. Experimental results demonstrated that no negative effects were recorded on fish growth, survival, or stress response; however, further studies are necessary.

CONCLUSIONS

By utilizing *P. reticulata*, a highly popular species in the aquarium hobby and a recognized model organism, and analysing the data obtained in this research, it was found that no differences in growth and development occurred between fish fed a fishmeal-based diet and those fed an insect meal-based diet. Furthermore, BSF larvae meal can be administered in the diet of these fish without adversely affecting water quality parameters. Given the unique anatomical and physiological specificity of each species, additional testing is recommended prior to the broad implementation of such diets. In the context of declining fishmeal production and the increasing trend of reserving marine fish strictly for human consumption, the use of insect meal in ornamental fish diets is highly opportune.

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CONSTITUTIONAL AND GROWTH INDICES, HEMATOLOGICAL AND METABOLIC PROFILES IN CARP (*Cyprinus carpio* Linnaeus, 1758), DUE TO THE ADMINISTRATION OF BREWERY YEAST (*Saccharomyces cerevisiae*)

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Abstract

This work aimed to determine the influence of brewer`s yeast (Saccharomyces cerevisiae) on the growth dynamics, health and physiological status of common carp (Cyprinus carpio Linnaeus, 1758). An experimental device was installed with four groups of carp in summer I. The control group was fed with classic feed. Groups II, III and IV are the experimental groups to which brewer`s yeast was added to the feed in a proportion of 3% (Group II), 5% (Group III) and 7% (Group IV). At the end of the experiment conducted over a period of 30 days, total weight gain (TWG) and average daily gain (ADG) as growth indices and the Fulton index (K) as a constitutional index were calculated, as well as the following physiological indices: hemogram and metabolic profiles. A 3% dietary supplementation of brewer's yeast produced the highest growth performance (TWG 69.58 g; ADG 2.32 g/day) and significantly improved Fulton's condition factor, while higher inclusion levels (5-7%) reduced growth performance and induced signs of metabolic stress. Hematological parameters remained stable across treatments, whereas moderate supplementation improved protein metabolism and physiological status without disrupting homeostasis. The addition of brewer`s yeast to the feed structure proved beneficial both in terms of growth dynamics and the health of the fish.

Key words*: yeast, physiological status, growth indices, constitutional indices, cyprinids.*

INTRODUCTION

Brewer's yeast (*Saccharomyces cerevisiae*) is a unicellular, eukaryotic yeast belonging to the phylum *Ascomycota* and the order *Saccharomycetales*. It is widely used in the baking, winemaking, and brewing industries (Walker & Stewart, 2016; Maicas, 2020). Due to its genetic and biochemical characteristics, it represents one of the main model organisms in molecular biology, possessing a genome organized into 16 linear chromosomes and functioning as a facultatively anaerobic chemoorganotrophic organism (Vanderwaeren et al., 2022). It has an active capacity to metabolize carbohydrates through glycolysis and alcoholic fermentation. As a result of these metabolic processes, ethanol and CO_2 are produced; these features are defined by the Crabtree effect, which involves the avoidance of complete oxidation via mitochondrial respiration and the inhibition of the activity of competing microorganisms (Pfeiffer & Morley, 2014). These particular characteristics have significant technological and industrial implications.

Biochemically, brewer's yeast synthesizes a wide range of biologically active compounds. It contains a high level of proteins with high biological value, B-complex vitamins (thiamine, riboflavin, pyridoxine, niacin, folic acid), nucleotides and nucleotide derivatives, and structural polysaccharides such as β-1,3/1,6-glucans and mannans (Badia et al., 2012; Bertolo et al., 2019; Grigore et al., 2025). The immunomodulatory effects attributed to

brewer's yeast are mainly due to the cell wall, which is largely composed of β-glucans and mannoproteins.

Nutritionally, brewer's yeast represents an important source of microbial protein, characterized by a favorable amino acid profile, with a high lysine content as well as a significant contribution of minerals and trace elements (Jach et al., 2022). Due to the presence of mannan oligosaccharides (MOS) as components of the cell wall, brewer's yeast reduces the adhesion of pathogenic bacteria such as *Escherichia coli* and *Salmonella* spp. to the intestinal mucosa (Alvarez et al., 2024). Although brewer's yeast in its inactive form is not considered a probiotic, its components can exert prebiotic and immunomodulatory functions. Following the administration of brewer's yeast, intestinal absorption functions are improved as intestinal villi are stimulated and increase in height (Jiang et al., 2015), the villus-to-crypt ratio is improved, and the activity of digestive enzymes is stimulated.

From an immunological and immunomodulatory perspective, β-glucans - recognized as ligands for receptors on phagocytic cells - stimulate the activity of macrophages and neutrophils, leading to increased phagocytic activity, enhanced lysozyme production, and improved resistance to bacterial infections (Ciszek-Lenda et al., 2024; Chattaraj et al., 2024).

Brewer's yeast has an increasingly wide range of applications in the field of farm animal nutrition (Ciurescu et al., 2021; Grigore et al., 2022) and in aquaculture (Mareš et al., 2023). The nutritional supplementation of brewer's yeast in ruminants leads to increased fiber digestibility, a reduced risk of acidosis, and improved milk production. In monogastric animals, studies have demonstrated a reduction in the incidence of diarrhea in piglets, improved growth rates, and optimization of feed conversion (Sampath et al., 2023).

In aquaculture, the inclusion of brewer's yeast plays a dual role: reducing the use of antibiotics and, more importantly, partially replacing fish meal, whose price has been steadily increasing and whose production volumes fluctuate on agricultural commodity markets. For this reason, various agricultural products as well as living organisms are being tested with the aim of replacing fish meal (Vrabec et al., 2015; Kulma et al., 2019). Due to its high protein content (40-50% of dry matter), brewer's yeast can partially substitute fish meal in the diets of carp, trout, salmon, or tilapia. However, it cannot fully replace fish meal because of its limited methionine content and the relatively low digestibility of its cell wall at high inclusion levels in feed. From the perspective of immunostimulatory effects in fish, the β-glucans and nucleotides present in yeast have been shown to increase phagocytic activity, elevate plasma lysozyme levels, and reduce mortality caused by bacterial infections (e.g., *Aeromonas hydrophila*).

Thus, brewer's yeast represents a valuable source of microbial protein and a functional ingredient with multiple applications in animal nutrition. Its metabolic properties, well characterized in numerous studies conducted over time, together with its content of bioactive compounds and its effects on intestinal health and immunity, justify its use in modern production systems. In aquaculture, the role of brewer's yeast extends beyond its strictly nutritional value, exerting immunomodulatory effects and promoting intestinal health, thereby contributing to the sustainability and efficiency of aquaculture production (Nicolae et al., 2012; Dobre et al., 2024).

In order to improve and increase the efficiency of cyprinid production, particularly that of common carp (*Cyprinus carpio*), new technologies for the exploitation of this species have been implemented (Filep et al., 2016; Bucur et al., 2016; Arcade et al., 2024), including genetic selection programs (Nicolae et al., 2013). The structural and functional methods underlying these technologies (Costache et al., 2021) are complemented by innovations in the field of nutrition. The common carp is an omnivorous species and, under intensive culture conditions and in various climatic environments (Dobrotă et al., 2024), readily accepts formulated feeds that include cereal-based ingredients, fish meal, plant protein sources, and vitamin-mineral premixes to ensure a balanced nutrient supply at all stages of development. In addition to the basic nutritional components, recent research emphasizes the use of functional additives with more complex roles, such as probiotics,

prebiotics, plant extracts (phytogenics), and other bioactive compounds that can stimulate digestion, improve nutrient utilization, strengthen the immune system, and reduce stress in fish (Cristea et al., 2012; Crețu et al., 2022). Plant extracts, essential oils, herbs, and certain bioactive substances are incorporated into diets at low inclusion levels in order to support both growth performance and overall health.

The aim of this study was to determine the influence of brewer's yeast (*Saccharomyces cerevisiae*) on growth performance indices and the physiological status of common carp (*Cyprinus carpio* Linnaeus, 1758).

MATERIALS AND METHODS

The experiment was conducted over a 30-day period, from May to June 2025, at the Mărtinești fish farm in Cluj County. Four cages were installed and four groups were formed, each consisting of 50 common carp specimens whose initial body weight (BW) and total length (TL) were recorded, at the start and at the end of the experiment. Group 1 (C) was considered the control group, while Group 2 (G2), Group 3 (G3) and Group 4 (G4) represented the experimental groups. Feeding was carried out according to an established schedule, with the specification that fish were not fed on Sundays, which were considered fasting days. Feed was administered once daily on a sliding feeding tray that could be immersed and lifted, allowing monitoring of feed consumption. Group 1 (control) was fed a conventional carp diet produced within the farm (Table 1). For Group 2, the feed was supplemented with 3% brewer's yeast, for Group 3 with 5% brewer's yeast and for Group 4 with 7% brewer's yeast. Each group received a daily ration equivalent to 1.5% of the total biomass of the group.

Growth performance indices were calculated, including total weight gain (TWG) and average daily gain (ADG), as well as Fulton's condition factor (K) (Lațiu et al., 2022). At the end of the experiment, blood samples were collected to assess the physiological status of the fish. Blood samples required for the analyses were obtained from the caudal vein, from five individuals in each experimental group. Sampling was performed using syringes with a usable volume of 3 mL. The collected blood was subsequently transferred into vacutainers containing anticoagulant (heparin) for hematological determinations, and into vacutainers with sodium citrate (Clot Activator) for serum separation required for biochemical analyses. Hematocrit (Hct) was determined by centrifugation at 12,000 rpm for 3 minutes. Hemoglobin concentration (Hb) and erythrocyte count (RBC) were determined using spectrophotometric techniques (UV-VIS Screen Master Touch). Hemoglobin determination was performed using the End-Point colorimetric reaction. Readings were carried out in the NIS spectral range using 0.4% ammonium hydroxide (NH_4OH) in distilled water as reagent. The erythrocyte count was determined using a turbidimetric and colorimetric EP-type reaction with visible reading, employing a sodium sulfate (Na_2SO_4) acetic solution in distilled water (Gowen reagent). The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated.

The biochemical profile was obtained through spectrophotometric techniques (UV-VIS Screen Master Touch) (Table 2).

All statistical analyses were performed using GraphPad Prism (version 8.0.1, GraphPad Software, USA). Data were initially tested for normality using the Shapiro-Wilk test. When the assumption of normal distribution was met, differences among groups were evaluated using analysis of variance (ANOVA), followed by Tukey's Honestly Significant Difference (HSD) test for post hoc multiple comparisons. For datasets that did not meet the assumption of normality, the non-parametric Kruskal-Wallis test was applied, followed by Dunn's test for pairwise comparisons. The level of statistical significance was set at $p < 0.05$. Results are presented as mean ± standard deviation (SD), and the number of replicates (n) for each parameter is reported in the corresponding tables or figures. Parameters that could not be determined using the available analytical methods or that were below the limit of detection (LOD) were recorded as below detection limit (BDL). In certain cases, the possibility of a Type II error cannot be

excluded due to limitations related to sample size or analytical sensitivity; such instances are acknowledged and discussed in the Results and Discussions section.

Table 1. Composition of the feed administered to common carp specimens

Ingredients	%	DM, %	OM, %	ME kcal, %	CP, %	CL, %	CDF, %	Ash, %	NFE, %	TP, %
FM	5	4.55	99.23	162.50	3.50	0.40	0.00	0.77	0.24	0.10
MM	7	6.30	98.45	213.50	4.58	1.83	0.00	1.55	0.35	0.28
SM-C1	15	13.20	99.10	510.00	6.93	0.24	1.83	0.90	4.56	0.10
SM-G2	12	10.56	99.28	408.00	5.54	0.19	0.66	0.72	3.65	0.08
SM-G3	10	8.80	99.40	340.00	4.62	0.16	0.55	0.60	3.04	0.07
SM-G4	8	7.04	99.52	272.00	3.70	0.13	0.44	0.48	2.43	0.05
SFM	5	4.49	99.70	127.50	1.75	0.06	1.09	0.30	1.22	0.06
BY-C	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
BY-E2	3	2.72	99.77	65.10	1.29	0.04	0.04	0.23	1.00	0.03
BY-E3	5	4.54	99.62	108.50	2.15	0.07	0.06	0.38	1.67	0.05
BY-E4	7	6.35	99.47	151.90	3.01	0.10	0.08	0.53	2.34	0.07
CF	30	25.98	99.58	1050.00	2.67	2.83	0.30	0.42	20.97	0.14
WF	30	26.28	99.49	975.00	3.39	2.83	0.30	0.51	20.97	0.08
BM	7	13.58	99.64	227.50	6.23	0.38	0.00	0.36	0.06	0.07
VMP	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01
Total-C	100	94.37	95.19	3266.00	29.05	8.56	2.51	4.81	48.36	0.83
Total-G2	100	91.74	95.15	3229.10	28.96	8.56	2.38	4.85	48.45	0.85
Total-G3	100	89.98	95.12	3204.50	28.89	8.55	2.30	4.88	48.51	0.85
Total-G4	100	88.22	95.09	3179.90	28.83	8.55	2.21	4.91	48.57	0.86

DM-dry matter; **OM**-organic matter; **ME**-metabolizable energy; **CP**-crude protein; **CL**-crude lipids; **CDF**-crude dietary fiber; **NFE**-nitrogen free-extract; **TP**-total phosphorus; **FM**-fish meal; **MM**-meat meal; **SM**-soybean meal; **SFM**-sunflower meal; **BY**-brewer's yeast; **CF**-corn flour; **WF**-wheat flour; **BM**-blood meal; **VMP**-vitamin-mineral premix; **C**-control group; **E**-experimental groups.

Table 2. Biochemical parameters of blood analyzed in this study

Blood parameter	λ (nm)	Usual method
TP - total proteins (g/dL)	546 nm	Burette
ALB - albumins (g/dL)	628 nm	BCG
ɣ-GLOB - gamma globulins (g/dL)	405 nm	Indirectly Computation
CHOL - cholesterol (mg/dL)	500 nm	CHOD-PAP (Enzymatic)
TRY - triglycerides (mg/dL)	550 nm	GPO-PAP (Enzymatic)
GLU - blood glucose (mg/dL)	500 nm	GOD-PAP (glucose oxidase-peroxidase)
TC - total calcium	575 nm	OCPC (o-cresolftalein complexon)
Fe^{3+} - serum iron (mg/L)	560 nm	Ferrozine
Na^{+} - sodium (mmol/L)	405 nm	ISE (ion selective electrode)
K^{+} - poatassium (mmol/L)	380 nm	ISE (ion selective electrode)
GGT - gamma-glutamyl transpeptidase (U/I)	405 nm	Enzymatic-Kinetic
ASAT - aspartate aminotransferase (U/I)	340 nm	Kinetic UV (IFCC)
ALAT - alanine aminotransferase (U/I)	340 nm	Kinetic UV (IFCC)
LDH - lactate dehydrogenase (U/I)	340 nm	Kinetic UV
DB - direct bilirubin (mg/dL)	546 nm	Diazo
TB - total bilirubin (mg/dL)	540 nm	Diazo with accelerator

RESULTS AND DISCUSSIONS

The mean body weight values of the specimens are presented in Table 3. The initial body weight of fish in the four groups was homogeneous, with values ranging from 123.00 ± 1.02 g (C) to 124.84 ± 0.96 g (G3). At the end of the experiment, the mean body weight values ranged between 185.88 ± 1.21 g (G4) and 193.14 ± 1.18 g (G2). The highest TWG (69.58 g) and ADG (2.32 g/day) were recorded in G2, which received a dietary supplementation of 3% brewer's yeast. Similar values to those in C were observed in G3, which

received a 5% supplementation of brewer's yeast. The lowest growth performance was recorded in G4, with 7% inclusion of brewer's yeast in the diet.

The results confirm those of other studies indicating that the optimal inclusion level of brewer's yeast ranges between 2.5 and 5% for optimal growth performance (Mihai et al., 2023). When this range is exceeded, inhibition of certain metabolic processes may occur (Mareš et al., 2023), resulting in reduced growth dynamics, potential nutritional imbalances, and alterations in the intestinal microbiota (Tao et al., 2025). This may appear paradoxical, considering the high protein content of brewer's yeast; however, its amino acid profile is not fully compatible with the nutritional requirements of fish. At high inclusion levels, brewer's yeast may "dilute" other more balanced protein sources in the feed, contribute to increased excretion of ammonia or uric acid (Jaeger et al., 2020), and may significantly reduce overall digestibility.

Regarding the constitutional indices, Fulton's condition factor (K) was calculated for all four groups, both at the beginning and at the end of the experiment (Figure 1). Fulton's condition factor represents an important indicator of the physiological status and the degree of somatic development in fish and is frequently used to evaluate feeding efficiency and growth conditions in common carp. The statistical analysis was performed using Dunn's test for multiple comparisons, with a significance threshold of $\alpha = 0.05$, comparing the initial and final values of Fulton's condition factor for each experimental group (Table 4).

Table 3. Initial and final mean body weight values of common carp specimens from the experimental groups and the growth performance indices of the four groups

GI	**C**	**G2**	**G3**	**G4**
IBW (g)	123.00 ± 1.02	123.56 ± 1.13	124.84 ± 0.96	123.42 ± 1.07
FBW (g)	189.43 ± 1.26	193.14 ± 1.18	190.15 ± 1.17	185.88 ± 1.21
TWG (g)	66.43	69.58	65.31	62.46
ADG (g/day)	2.21	2.32	2.18	2.08

GI-growth indices; **IBW**-initial body weight; **FBW**-final body weight; **TWG**-total weight gain; **ADG**-average daily gain; **C**-control group; **G2**-experimental group 2; **G3**-experimental group 3; **G4**-experimental group 4.

Table 4. Statistical differences in Fulton's condition factor (K) among the four experimental groups, comparing the initial and final values

Number of families	1			
Number of comparisons per family	4			
α	0.05			
Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary	Adjusted P Value
Group C Initial vs. Group C Final	-40.67	Yes	*	0.0318
Group G2 Initial vs. Group G2 Final	-75.73	Yes	***	0.0002
Group G3 Initial vs. Group G3 Final	-37.41	No	ns	0.1077
Group G4 Initial vs. Group G4 Final	-25.18	No	ns	0.4641

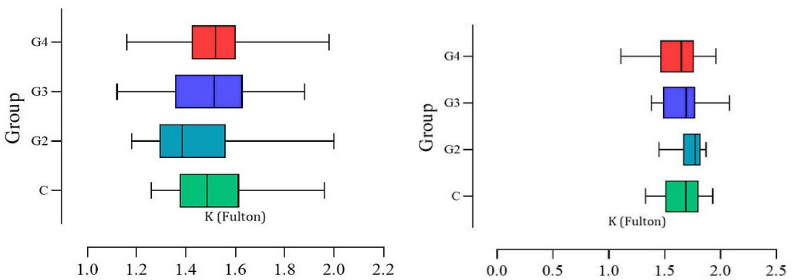


Figure 1. Fulton's condition factor (K) for the four experimental groups at the beginning of the experiment (left) and at the end of the experiment (right)

The control group showed a statistically significant difference between the initial and final values of Fulton's condition factor (Mean rank diff. = −40.67; $p = 0.0318$; $p < 0.05$). This result indicates a significant change in the body condition status of the fish during the experimental period. G2 (supplemented with 3% brewer's yeast) recorded the most pronounced statistical difference between the initial and final values (Mean rank diff. = −75.73; $p = 0.0002$; $p < 0.001$). This highly significant difference suggests a strong influence of brewer's yeast supplementation on the body condition of the carp specimens. G3 (supplemented with 5% brewer's yeast) did not show statistically significant differences between the initial and final values of Fulton's condition factor (Mean rank diff. = −37.41; $p = 0.1077$; $p > 0.05$), indicating that the observed changes were not sufficiently pronounced to be considered statistically significant. Similarly, G4 (supplemented with 7% brewer's yeast) showed no statistically significant differences between the beginning and the end of the experiment (Mean rank diff. = −25.18; $p = 0.4641$; $p > 0.05$), suggesting that the high level of brewer's yeast supplementation did not produce relevant changes in Fulton's condition factor.

Overall, the results indicate that moderate supplementation with brewer's yeast (particularly in G2) produced the greatest change in the condition index, whereas higher supplementation levels (5% and 7%) did not result in statistically significant differences during the experimental period. These observations may suggest the existence of an optimal inclusion level of brewer's yeast in the diet for improving the body condition of common carp.

Fulton's condition factor (K) is commonly used to assess the nutritional status, somatic development, and the relationship between weight and length in fish (Nash et al., 2006), being influenced by feed quality, nutrient conversion efficiency, and growth conditions. A higher Fulton's condition factor generally indicates a better body condition and a greater accumulation of body mass.

In the analyzed experiment, statistically significant differences were observed only in the control group and in the group supplemented with a moderate level of brewer's yeast, whereas the groups receiving higher yeast inclusion levels did not show significant differences. These results may be explained by several biological mechanisms (Zhou et al., 2024). The obtained results suggest that moderate supplementation of feed with brewer's yeast may positively influence the condition status (Fulton's condition factor K) of common carp (Mareš et al., 2023; Mihai et al., 2023), by improving nutrient digestibility and protein utilization efficiency. Brewer's yeast, particularly *S. cerevisiae*, represents a valuable source of proteins, B-complex vitamins, and bioactive compounds that can stimulate metabolism and the immune system of fish (Rhema, 2022; Sumbal et al., 2024). However, high inclusion levels do not necessarily lead to a further increase in Fulton's condition factor, as increases in length and weight may occur proportionally, thereby maintaining this body condition indicator relatively constant.

The physiological status and health condition of the common carp specimens from the four experimental groups are characterized by the hematological analyses and biochemical analyses performed at the end of the experiment (Tables 5, 6, 7, 8, 9).

Hematological parameters (Table 5) represent important indicators of the physiological and health status of fish, reflecting processes such as oxygen transport, energy metabolism, nutritional status, and immune response. In aquaculture, these parameters are frequently used to evaluate the effects of various dietary supplements on fish physiology. In the analyzed experiment, common carp were fed diets supplemented with brewer's yeast at different inclusion levels: 3, 5, and 7%, compared with the control group without supplementation. Statistical analysis of the hematological values determined at the end of the experiment showed that no significant differences were observed among the groups for any of the analyzed parameters. From a biological perspective, the absence of significant differences suggests that the administration of brewer's yeast at the tested inclusion levels did not produce major hematological changes, indicating that supplementation of the feed with this additive did not induce physiological stress or disrupt the homeostasis of the organism

(Boonanuntanasarn et al., 2019). This stability of hematological parameters may be interpreted as an indicator of the good tolerance of common carp to the inclusion of brewer's yeast in the diet, even at relatively high levels (Figure 2).

Table 5. Mean values of hematological parameters and erythrocyte indices in specimens from the four groups at the end of the experiment

Parameter	Group	Hb (g/dL)	Hct (%)	RBCx10^{12}	MCV (fL)	MCH (pG)	MCHC (g/dL)
Mean ± SEM	C	5.844 ± 1.726	37.200 ± 7.662	3.312 ± 0.532	111.7 ± 6.337	17.39 ± 2.752	8.977 ± 0.5223
	G2	7.200 ± 1.856	39.400 ± 11.800	3.500 ± 0.396	110.6 ± 22.20	20.35 ± 3.438	9.369 ± 2.029
	G3	6.712 ± 0.736	44.400 ± 1.140	3.532 ± 0.195	126.1 ± 8.643	19.03 ± 2.082	7.962 ± 0.5519
	G4	6.786 ± 1.038	41.200 ± 1.304	3.532 ± 0.128	116.7 ± 2.378	19.19 ± 2.644	8.573 ± 0.1714

Table 6. Mean values of plasma protein constituents in specimens from the four groups at the end of the experiment

Parameter	Group	TP (g/dL)	ALB (g/dL)	γ-GLOB (g/dL)
Mean ± SEM	C	3.592 ± 0.338	1.068 ± 0.105	0.138 ± 0.040
	G2	4.390 ± 0.157	1.324 ± 0.084	0.121 ± 0.015
	G3	4.310 ± 0.482	1.364 ± 0.125	0.120 ± 0.013
	G4	4.480 ± 0.376	1.252 ± 0.109	0.110 ± 0.013

Table 7. Mean values of lipid and carbohydrate constituents in the blood plasma of specimens from the four groups at the end of the experiment

Parameter	Group	TRY (mg/dL)	CHOL (mg/dL)	GLU (mg/dL)
Mean ± SEM	C	283.000 ± 21.360	213.300 ± 23.240	107.200 ± 15.180
	G2	305.700 ± 48.300	187.300 ± 24.410	106.500 ± 24.880
	G3	280.800 ± 14.550	185.200 ± 4.823	122.300 ± 3.984
	G4	241.700 ± 22.790	187.800 ± 11.730	184.700 ± 42.720

Table 8. Mean values of mineral constituents in the blood plasma of specimens from the four groups at the end of the experiment

Parameter	Group	Fe^{3+} (mg/L)	Na^{+} (mmol/L)	K^{+} (mmol/L)
Mean ± SEM	C	43.980 ± 12.570	154.200 ± 3.197	2.814 ± 0.249
	G2	40.400 ± 9.301	151.500 ± 2.205	3.216 ± 0.268
	G3	36.600 ± 7.570	160.800 ± 2.044	2.880 ± 0.212
	G4	38.860 ± 7.237	164.200 ± 4.257	2.706 ± 0.080

Table 9. Mean values of enzymatic constituents in the blood plasma of specimens from the four groups at the end of the experiment

Par.	Group	ASAT (U/l)	ALAT (U/l)	LDH (U/l)	GGT (U/l)	DB (mg/dL)	TB (mg/dL)
Mean± SEM	C	51.940 ± 7.89	25.300 ± 10.24	224.900 ± 28.61	3.340 ± 0.42	0.001 ± 0.01	0.054 ± 0.08
	G2	43.160 ± 13.18	17.400 ± 2.45	204.000 ± 12.80	8.000 ± 1.27	0.000 ± 0.00	0.006 ± 0.01
	G3	57.280 ± 4.24	14.320 ± 3.95	293.200 ± 8.25	3.676 ± 0.62	0.007 ± 0.01	0.231 ± 0.25
	G4	49.300 ± 1.49	21.620 ± 4.60	267.700 ± 4.77	3.144 ± 0.33	0.002 ± 0.01	0.019 ± 0.03

The increase in TP observed in all experimental groups compared with the control suggests stimulation of protein synthesis or a metabolic adaptation to the experimental conditions. G4 shows the highest increase, which may indicate a more pronounced physiological response of the organism to the treatment or to the experimental conditions. The significant increase in albumin in G2 and G3 suggests enhanced and efficient hepatic activity, since albumin is the principal marker of hepatic protein synthesis and plays an important role in maintaining colloid osmotic pressure and in the transport of various molecules. The absence of a significant difference in G4 may reflect either a physiological adaptation or stabilization of albumin concentration at the level of this experimental group (Gokulakrishnan et al., 2022).

The stability of γ-GLOB indicates that the humoral immune response was not significantly affected by the experimental factor, as γ-globulins represent the main component of plasma immunoglobulins (Table 6).

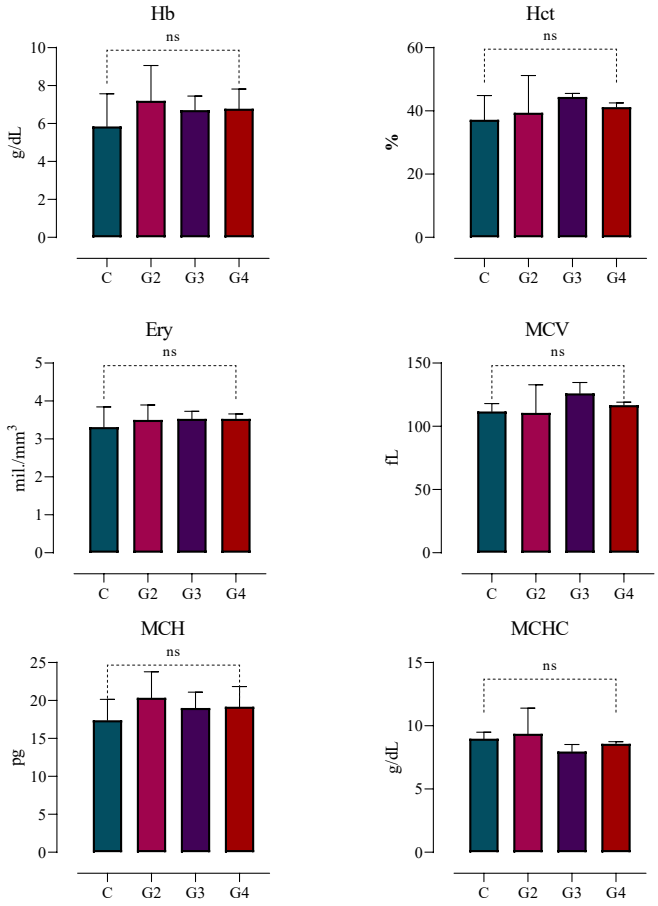


Figure 2. Significance of the differences in hematological parameters (Hb, Hct, RBC) and erythrocyte indices (MCV, MCH, and MCHC) among the four experimental groups

Triglyceride levels reflect lipid metabolism and energy storage (Table 7). The increase observed in G2 may indicate mobilization of energy reserves or metabolic adaptation, whereas the decrease in G4 suggests increased energy expenditure or inhibition of lipid synthesis. The decrease in cholesterol levels in all experimental groups indicates possible alterations in hepatic metabolism, reduced lipoprotein synthesis, or increased lipid catabolism (Hassaan et al., 2018).

The balance of blood glucose levels in G2 indicates the absence of a major effect (Goran et al., 2017), whereas the increases observed in G3 and especially G4 suggest metabolic stress and possible disturbances in glucose regulation (hyperglycemia), which may reflect effects on glycogenolysis, gluconeogenesis, or insulin sensitivity.

The decrease in Fe^{3+} values observed in all experimental groups suggests possible effects on iron metabolism, either through reduced absorption or through redistribution among different compartments (plasma, liver, tissue reserves) (Table 8). The lower values recorded in G3 and G4 may indicate increased utilization or mobilization for the synthesis of hemoproteins (e.g., hemoglobin, cytochromes) (Huyben et al., 2016).

The significant increases in Na^{+} levels in G3 and G4 suggest modifications in hydroelectrolytic balance, possibly through effects on renal or hormonal function (aldosterone, vasopressin). The slight decrease observed in

G2 may reflect an initial physiological adaptation to the treatment (Sutthi & Thaimuangphol, 2020). The stable or only slightly modified K^+ values indicate that the intracellular/extracellular potassium balance was largely maintained, although the slight decrease observed in G4 may signal minor renal loss or cellular redistribution (Figure 3).

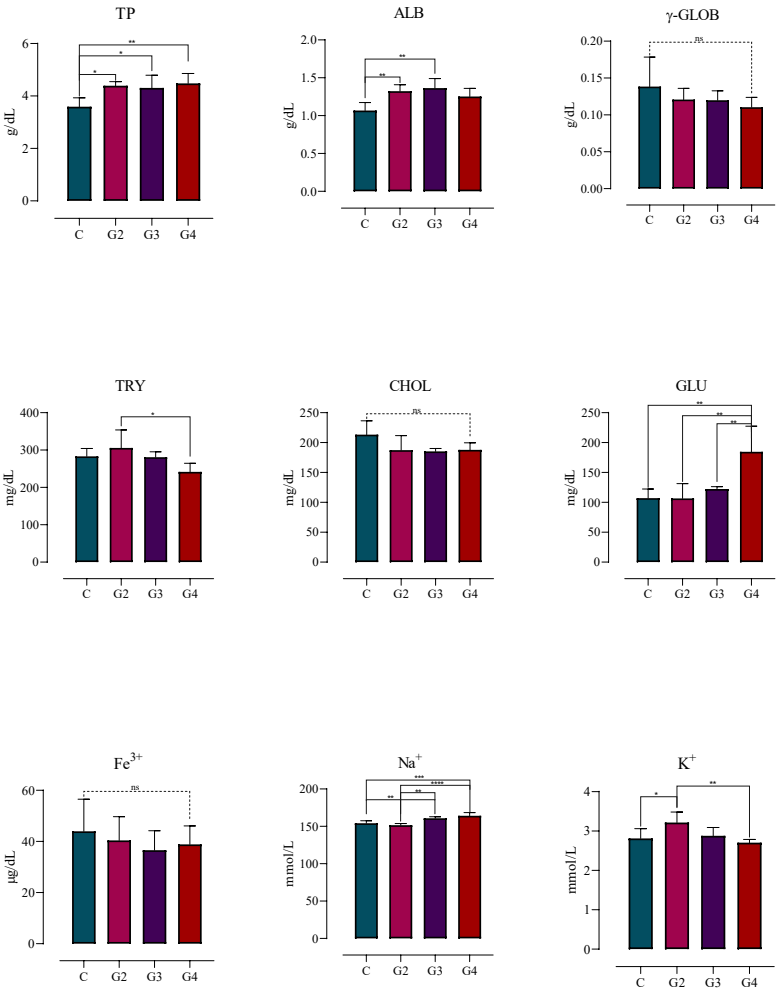


Figure 3. Significance of the differences in protein metabolic profiles (TP, ALB, γ-GLOB), lipid profiles (TRY, CHOL), carbohydrate profile (GLU), and mineral profiles (Fe^{3+}, Na^+, K^+) among the four experimental groups

ASAT and ALAT are markers of hepatic cytolysis. The decrease in ALAT observed in G2 and G3 may indicate metabolic adaptation or reduced hepatic enzymatic activity, whereas variations in ASAT may reflect influences on both muscular and hepatic metabolism (Table 9). The significant increase in LDH in G3 and G4 indicates tissue stress or cellular damage, as LDH is a nonspecific marker of cellular injury.

The marked increase in GGT in G2 suggests stimulation of the biliary pathways or cholestasis, while the normal values recorded in G3 and G4 indicate the absence of biliary canalicular impairment (Figure 4).

The nearly normal values of DB and TB indicate the maintenance of hepatic excretory function, with the exception of a slight increase observed in G3.

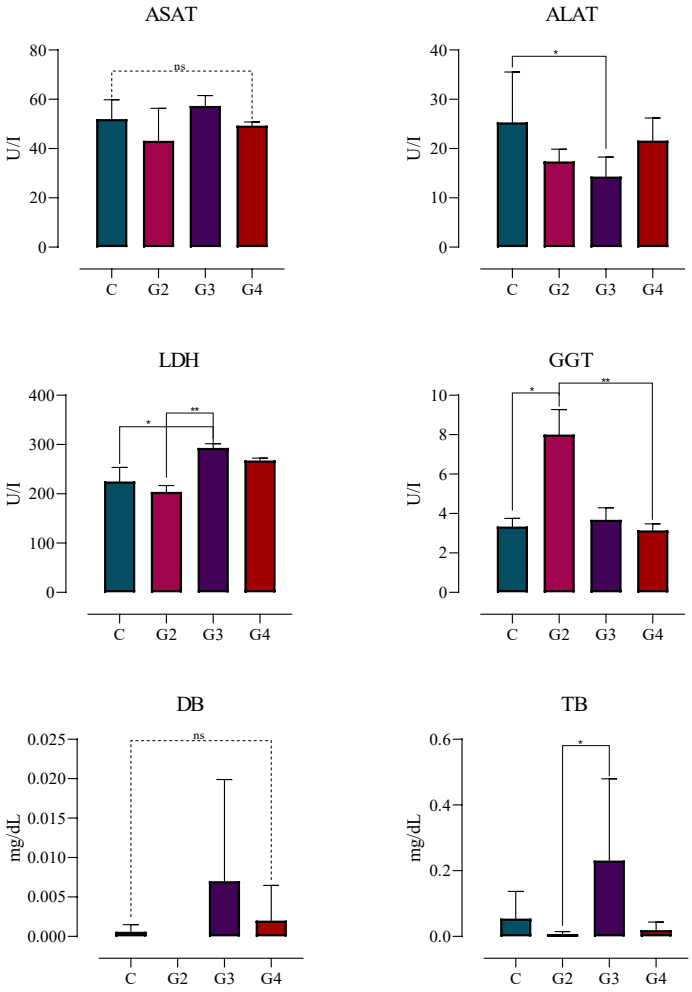


Figure 4. Significance of the differences in enzymatic metabolic profiles (ASAT, ALAT, LDH, GGT, DB, TB) among the four experimental groups

CONCLUSIONS

Brewer's yeast may be considered as an additive in common carp feeds, although only to a limited extent. Its high protein content recommends this product as an additive; however, the different amino acid profile may lead to inhibition of metabolic processes and, consequently, reduced growth of common carp when included at excessively high levels. Our results demonstrated that a 3% supplementation level is beneficial both in terms of growth performance and immunomodulatory effects. From a hematological perspective, no significant changes were observed in the experimental groups compared with the control group. Regarding metabolic profiles, an increase in biochemical protein indicators was observed, suggesting stimulation of protein synthesis and an adaptive response of metabolic processes in the experimental groups. However, at high inclusion levels, brewer's yeast may lead to an imbalance of the intestinal microbiota, generating digestive dysfunctions, specifically a reduction in digestibility. The increased level of lipid fractions observed in G2 (3% brewer's yeast supplementation) suggests mobilization of energy reserves, whereas at higher inclusion levels, as in G4 (7% brewer's yeast supplementation), inhibition of

lipid synthesis was observed. The levels of mineral fractions showed certain variations, particularly in the case of sodium, which produced alterations in the hydroelectrolytic balance and affected renal functions. Enzymatic fractions also exhibited some differences between the control group and the experimental groups, indicating tissue stress and stimulation of biliary function. Overall, brewer's yeast can be considered a suitable partial substitute for fish meal, but only when included in relatively low amounts in the diet.

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POPULATION DYNAMICS, PHYSIOLOGICAL ADAPTATIONS AND BEHAVIORAL RESPONSES OF GAME SPECIES IN SUBMONTANE ECOSYSTEMS: A MINI REVIEW

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Abstract

Understanding the interactions between physiological processes, population structure, and behavioral responses is essential for the sustainable management of game species in submontane ecosystems, especially under increasing environmental and anthropogenic pressures. This review aims to synthesize current knowledge on population dynamics and physiological adaptations of game species, with a particular focus on cervids and wild boar. The study is based on a comprehensive analysis of recent scientific literature, integrating data on population structure parameters (density, demographic composition) and key physiological mechanisms underlying behavior, including metabolism, thermoregulation, reproduction, and stress response. The results indicate that species-specific physiological adaptations influence survival, reproductive success, and spatial distribution, leading to distinct population patterns in submontane habitats. Overall, the results show that effective game management should take into account the ecological context, physiological constraints, and behavioral patterns of species.

Key words*: behavioral responses, game species, physiological adaptations, population dynamics.*

INTRODUCTION

Game species comprise a diverse group of terrestrial mammals and birds that live in the wild and are not classified as domestic animals. From a hunting perspective, these species are commonly categorized according to body size and management relevance, including large game species such as bear, deer, roe deer, and wild boar, as well as small game species such as hare, fox, lynx, marten, and various bird species, including pheasant, wild ducks, and quail. The composition and diversity of game species vary across geographical regions, being strongly influenced by ecological factors such as climate, habitat characteristics, and food availability, as well as by local traditions and wildlife management practices (Sălăjan, 2017).

Populations are subject to self-regulation mechanisms, such as migration, territorial behavior, and physiological responses to stress, which contribute to maintaining numerical equilibrium. In this context, population dynamics describe temporal variations in these parameters, typically manifested as seasonal, irregular, or cyclic fluctuations (Fryxell et al., 2014).

Research conducted over time on the behavior, ecology, ethology, and physiology of game species has an important role in supporting the development of effective conservation and management strategies (Matei et al., 2024; Vedel et al., 2024; Newman et al., 2024). Ethology, defined as the biological study of behavior and social organization, represents a central field within modern behavioral sciences. Ethological observation and analysis traditionally require considerable effort due to the inherent complexity and variability of behavioral patterns. Nonetheless, a range of methodological approaches has been developed to facilitate the identification, characterization, and classification of species-specific behavioral patterns (Datta et al., 2019; Goodwin et al., 2022).

Within natural ecosystems, wildlife resources have traditionally represented an essential component in meeting human food requirements, being among the most accessible sources of animal-derived food (Boișteanu et al., 2024; Ciobanu et al., 2020). Moreover, the quality and biological characteristics of game products are closely related to the physiological

status, feeding behavior, and environmental adaptability of wild species (Flocea et al., 2024; Gucianu et al., 2023). Beyond their primary role as a direct food source, game species are increasingly recognized for their potential in the development of value-added meat products (Anchidin et al., 2024; Manoliu et al., 2025).

The study of game species in submontane ecosystems represents a complex and interdisciplinary field at the interface of ecology, ethology, and physiology, with scientific and practical important. In the current context, characterized by accelerated climate change and increasing anthropogenic pressure on natural ecosystems, understanding the physiological mechanisms underlying animal behavior becomes essential for the development of effective and sustainable wildlife management strategies.

Game mammals, including especially cervids and suids, exhibit a range of specific physiological adaptations that enable survival and reproduction under the variable environmental conditions typical of submontane areas. These adaptations include seasonal modulation of energy metabolism, efficient thermoregulatory mechanisms, reproductive strategies synchronized with environmental conditions, and complex responses to stress factors. Such physiological processes are directly reflected in behavioral patterns, influencing population structure, spatial distribution, and overall population dynamics (Vilela ș.a., 2020). From an ethological perspective, the behavior of game species results from the dynamic interaction between intrinsic factors (physiological and endocrine) and extrinsic factors (climatic, trophic, and anthropogenic). Therefore, an integrated analysis of these components is essential for improving our understanding of how species respond to environmental changes and wildlife management interventions.

In this context, the present review aims to synthesize current knowledge on population dynamics, physiological adaptations, and behavioral responses of game species in submontane ecosystems, providing a conceptual framework to support sustainable management practices.

This paper aims to review the specialized literature on the physiological characterization of game mammals, with a particular emphasis on the relationships between bioecological, ethological, and physiological foundations and the behavioral manifestations observed under the specific conditions of submontane areas. To provide a clearer overview of the study direction and structure, the main objectives and their interconnections are illustrated in Figure 1.

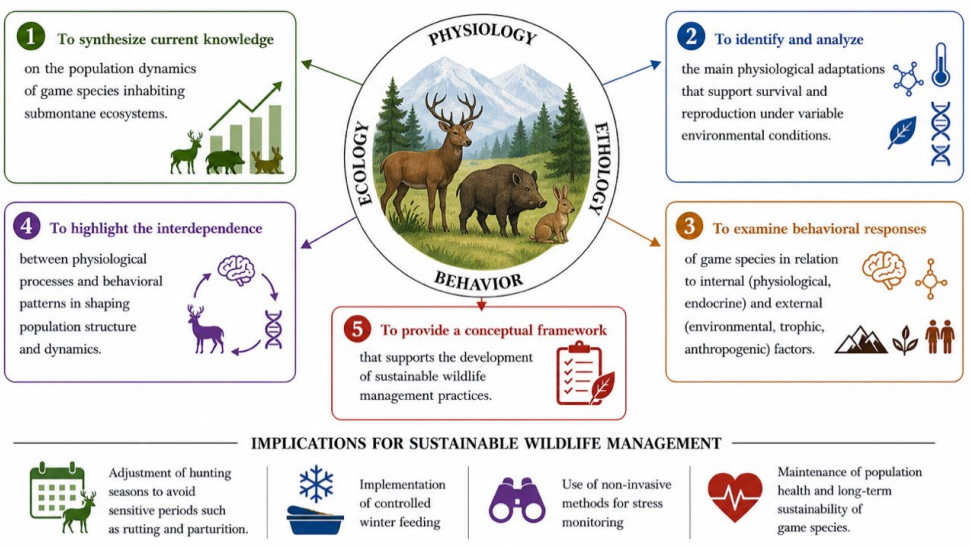


Figure 1. Representation of the study objectives (own elaboration, generated with AI support)

MATERIALS AND METHODS

The present study is based on a review of the scientific literature addressing population dynamics, physiological adaptations, and behavioral responses of game species, with a particular focus on submontane ecosystems. The literature search was conducted using major international scientific databases, including Web of Science, Scopus, PubMed, ScienceDirect, and Google Scholar. Additional sources were identified through institutional resources. The search process focused primarily on studies published between 2010 and 2025, while selected earlier references were included when considered essential for the scientific background of the topic.

The selection of sources was guided by their relevance to the topic, scientific quality, and recency, with priority given to seminal works in the field. Keywords such as "game species", "population dynamics", "physiological adaptations", "behavioral responses", and "submontane ecosystems" were used to identify these studies.

The inclusion criteria considered scientific reports and authoritative publications focusing on the ecological, physiological, ethological, and management-related aspects of European game species. In addition, because the evidence from the literature highlights the need for adaptive management strategies, this review is structured to synthesize the fundamental physiological mechanisms underlying behavior in European game species, within an ethological and wildlife management context.

Four major domains are addressed: energy metabolism, thermoregulation, reproductive physiology, and stress response. These are analyzed in relation to seasonal, age-specific, and sex-related behavioral patterns, as well as to the main ecological drivers influencing species adaptation.

RESULTS AND DISCUSSIONS

Behavioral determinants and ethological context

Understanding the determinants of animal behavior represents a key aspect in the study of game species, particularly in relation to their adaptation to dynamic environmental conditions. The available literature consistently indicates that animal behavior is fundamentally shaped by the interaction between genetic factors, specific to each individual, and environmental influences. Genetic inheritance has different roles in defining metabolic, hormonal, and neurosensory characteristics, thereby influencing how organisms respond to external stimuli and contributing to the development of species-specific behavioral patterns.

Innate behavior, resulting from these genetic determinants, tends to be relatively consistent among individuals of the same species, although individual variability may occur due to genetic differences. In parallel, acquired behavior develops through individual experience, complementing and refining innate responses. The relative contribution of innate and learned behavior varies depending on species and developmental stage, with learned components becoming more prominent in adult individuals and in more evolutionarily advanced species.

Environmental factors, both biotic and abiotic, exert a strong influence on behavioral expression by imposing adaptive pressures that shape species responses over time. In this context, the literature emphasizes that ethological studies cannot be dissociated from ecological approaches, as these disciplines are closely interconnected in understanding the relationships between organisms and their environment (Alanis-Hernandez et al., 2023).

Global anthropogenic changes are increasingly recognized as major threats to animal survival and reproduction, primarily through their effects on behavioral patterns. The literature highlights that migratory species are particularly vulnerable, as they depend on a well-coordinated spatio-temporal sequence of habitats and resources; consequently, even minor disruptions within this chain can lead to some consequences for their development and survival (Vickery et al., 2014; Mizrahi & Peters, 2009).

In addition, climate change has been shown to generate phenological mismatches between species and their trophic resources, with negative implications for population dynamics (Both et al., 2009; Visser & Gienapp, 2019). Several studies emphasize that such disruptions can alter feeding behavior, reproductive timing,

and overall fitness, ultimately affecting species persistence.

From an ethological perspective, research provides valuable insights into how global changes influence animal welfare, performance, and reproductive success. At the same time, the literature underscores the important role of ethology in identifying, understanding, and, in some cases, mitigating human-induced impacts. Within the context of game management, sustained hunting pressure that does not lead to population collapse may still act as a selective force, promoting evolutionary adaptations at both behavioral and morphological levels. The literature suggests that such adaptations arise as a result of natural selection, enabling species to adjust to persistent anthropogenic pressures (Matei et al., 2025). These changes can, in turn, generate cascading effects on interspecific interactions and overall ecosystem functioning (Potratz et al., 2024).

Human hunting activity has been widely reported to induce alterations in the activity patterns of wildlife with broader implications for ecological communities and ecosystem processes (Werner et al., 2003; Schmitz et al., 2004). These behavioral modifications may trigger behaviorally mediated trophic cascades, influencing predator-prey relationships as well as population structure and stability.

For instance, the reduction of age structure within populations due to human-induced mortality has been associated with increased vulnerability to additional environmental disturbances (Audzijonyte et al., 2013). Furthermore, the indirect effects on non-target species represent an often underestimated, yet important component in understanding ecosystem dynamics, highlighting the complex and far-reaching consequences of hunting practices.

Population dynamics of game species in submontane ecosystems

A population represents the fundamental organizational form of a species within a given habitat, functioning as a dynamic and self-regulating system. The principal parameters that characterize populations include population size, density, natality, mortality, and age and sex structure. Population structure is determined by the sex ratio and the distribution of individuals across age classes (juveniles, adults, and senescent individuals), these indicators reflecting both the growth potential and the overall stability of the population.

Individual variability contributes to the adaptability and evolutionary potential of populations under changing environmental conditions. Population size results from the balance between birth and death rates and is influenced by both intrinsic factors, such as longevity and genetic structure, and extrinsic factors, including environmental conditions, resource availability, competition, predation, and anthropogenic pressure. Population density expresses the number of individuals per unit area, with optimal values ensuring efficient resource use without generating ecological imbalances (Fryxell et al., 2014).

The analysis of population dynamics represents a key component in understanding the adaptive responses of game species to both environmental and anthropogenic pressures. The available literature indicates a general increasing trend in ungulate populations, particularly in wild boar and deer, over recent decades, largely attributed to their high ecological adaptability and the reduction of natural predator pressure (Abrantes & Vieira-Pinto, 2023).

Game populations in submontane areas are characterized by a dynamic and adaptive structure, reflected in the expansion of large ungulate populations, high variability among small game species, moderate genetic differentiation between populations, and a strong influence of both ecological and anthropogenic factors. These patterns are closely linked to the physiological foundations of behavior, particularly energy metabolism, reproductive strategies, and stress responses, which collectively shape population resilience and adaptability.

The analysis of population densities of the main game species in submontane ecosystems reveals important interspecific differences, primarily determined by their ecological and physiological traits (Table 1).

The analysis of population densities further highlights species-specific differences in adaptive strategies among game species. Roe deer (*Capreolus capreolus* L.) exhibit relatively high population densities (5–40

individuals/km²), reflecting a high adaptive capacity associated with efficient energy metabolism and considerable trophic plasticity. In contrast, red deer (*Cervus elaphus* L.) typically occur at lower densities (2-10 individuals/km²), which can be explained by their higher energetic requirements and pronounced territorial behavior.

Table 1. Average population densities of game species in temperate and submontane regions of Europe

Species	Density (individuals/km²)	Source	References
Roebuck (*Capreolus capreolus*)	**5-40**	ecological modeling studies (Germany)	Benjamin et al. (2022)
Deer (*Cervus elaphus*)	**2-10**	forestry studies Central Europe	Ionescu et al. (2025)
Wild boar (*Sus scrofa*)	**1-15**	European level estimates	Smith et al. (2026)
Fox (*Vulpes vulpes*)	**0.5-3**	general ecological studies	Benjamin et al. (2022)
Rabbit (*Lepus europaeus*)	**10-60**	European agro-ecosystem studies	

Wild boar (*Sus scrofa*) display a wide range of population densities (1-15 individuals/km²), a pattern closely linked to their high reproductive rate and remarkable ability to exploit diverse food resources. These traits make the species highly responsive to environmental changes. Small game species, such as the European hare (*Lepus europaeus*), generally show relatively high but unstable population densities, being strongly influenced by climatic variability and predation pressure.

At the European scale, population size estimates of major game species confirm the trends observed at local levels, particularly the marked increase in ungulate populations, with wild boar showing the most pronounced expansion (Table 2).

Table 2. Estimated population sizes of major game species in Europe

Species	Estimated population
Wild boar	13.5-19.6 million individuals
Deer	several million (wide distribution)
Roebuck	the most abundant ungulate species in Europe
Rabbit	declining in many regions

Smith et al., 2026; Abrantes & Vieira-Pinto, 2023

The available literature indicates that wild boar (*Sus scrofa*) populations in Europe are currently estimated at approximately 13.5-19.6 million individuals, reflecting a marked expansion of the species over recent decades. This trend has been widely associated with a combination of physiological and behavioral traits, including high fecundity, early sexual maturity, and strong metabolic adaptability, which collectively enhance the species' resilience to environmental variability.

Roe deer (*Capreolus capreolus* L.) are considered the most abundant ungulate species in Europe, a pattern that reflects their high ecological efficiency and superior ability to exploit fragmented habitats. In contrast, the European hare (*Lepus europaeus*) has experienced a decline in many regions, a phenomenon frequently linked to increased sensitivity to environmental stress, agricultural intensification, and elevated predation pressure.

Population structure analyses based on genetic studies reveal varying degrees of differentiation among game species, depending on their mobility and the presence of ecological barriers (Table 3). In the case of roe deer, the analysis of 920 individuals across 16 European sites indicates a relatively homogeneous structure at a large spatial scale, with regional differentiation patterns suggesting moderate dispersal capacity and relatively high ecological connectivity.

Table 3. Population structure of selected game species

Species	No. of individuals analyzed	Population structure
Roebuck	**920 individuals** (16 sites Europe)	relatively homogeneous, but regionally differentiated populations
Wild boar	**486 individuals** (Carpathian Basin)	2 distinct subpopulations (Fst = 0.03)

Niedziałkowska et al., 2024; Mihalik et al., 2020

For wild boar populations in the Carpathian Basin, studies have identified the presence of two distinct subpopulations (Fst = 0.03), indicating a low but important level of genetic differentiation. This structure is influenced by the species' high mobility, social behavior, and elevated reproductive rate. Overall, these findings highlight that population structure is the result of complex interactions between physiological, ethological, and environmental factors, with direct implications for wildlife management strategies.

From a game management perspective, large and productive populations of wild boar and cervids represent valuable resources, providing game meat that is increasingly appreciated in developed countries (Marescotti et al., 2019), as well as generating substantial economic benefits. The literature highlights that recreational hunting contributes positively to rural economies by generating income for landowners, conservation institutions, public authorities, and local communities (Di Minin et al., 2021; Delisle et al., 2024).

In addition, hunting-related services further support economic development at both local and national levels, reinforcing the multifunctional role of wildlife resources within socio-ecological systems (Di Minin et al., 2021; Martín-Delgado et al., 2020).

Physiological adaptations and associated behavioral responses in game species

Seasonal energy metabolism and behavioral adaptations

The literature highlights that seasonal energy metabolism represents a key adaptive mechanism in game species inhabiting temperate and submontane ecosystems. Large mammals typically exhibit pronounced annual metabolic cycles, characterized by increased energy allocation during periods of resource abundance (spring-summer) for growth, reproduction, and fat accumulation, and reduced metabolic activity during winter, when food availability is limited. For example, in red deer (*Cervus elaphus*), basal metabolic rate and heart rate decrease during winter, even in the absence of major changes in body mass or food intake, suggesting the involvement of endogenous biological rhythms and thermoregulatory adjustments (Turbill et al., 2010).

In contrast, wild boar (*Sus scrofa*) display a distinct metabolic pattern, with elevated energy expenditure in early spring (associated with gestation) and late autumn (linked to mating and fat accumulation), and lower metabolic activity during summer. This pattern reflects both the energetic costs of reproduction and the species' omnivorous diet, which supports metabolic flexibility under varying environmental conditions (Ruf et al., 2021).

Reproductive status and age further influence metabolic dynamics. Pregnant and lactating females exhibit increased energy demands, as observed in the European hare (*Lepus europaeus*), where peak energy expenditure occurs during advanced lactation. However, studies suggest that such metabolic limits may be self-regulated to prevent thermal stress, indicating adaptive trade-offs between reproduction and thermoregulation (Valencak et al., 2010). Similarly, in roe deer (*Capreolus capreolus*), metabolic rates increase during late gestation, with important nutritional implications (Mauget et al., 2003).

These physiological processes are closely reflected in behavioral adaptations. During winter, energy conservation strategies include reduced movement, selection of sheltered habitats, and crepuscular feeding patterns, while spring and summer are associated with increased activity levels, driven by reproductive and growth demands. From a management perspective, understanding these metabolic cycles is essential for optimizing hunting practices and habitat management, particularly by avoiding periods of peak physiological stress and ensuring adequate resource availability (Table 4).

Thermoregulation and behavioral adaptations

The literature indicates that thermoregulation in game species is achieved through a combination of physiological and behavioral mechanisms, which are essential for maintaining homeostasis under variable environmental conditions. Physiological adaptations include insulation through seasonal coat changes and peripheral heat exchange, while behavioral strategies are important in modulating thermal stress.

For instance, wild boar (*Sus scrofa*), lacking efficient sweat glands, rely on behavioral thermoregulation such as wallowing in mud and seeking shaded or humid areas to facilitate heat

dissipation. Similarly, cervids adjust their thermal balance through seasonal changes in fur density and by shifting activity patterns, with feeding behavior predominantly occurring during crepuscular periods to avoid thermal extremes. In winter, large mammals may reduce peripheral body temperatures and metabolic activity as part of energy conservation strategies (Turbill et al., 2010).

Table 4. Overview of physiological mechanisms in major game species

Physiological mechanisms	Red deer (*Cervus elaphus* L.)	Roebuck (*Capreolus capreolus* L.)	Wild boar (*Sus scrofa*)	Rabbit (*Lepus europaeus*)	Game birds (pheasants, quails)
Energy metabolism (seasonality)	Intense in spring–summer; reduced in winter through energy saving	Increased in gestation; reduced in winter, efficient use of resources	Increased during breeding periods; reduced at high temperatures	High, maximum in lactation; low in winter, use of reserves	High; increases in reproduction and raising offspring
Thermoregulation and adaptations	Seasonal fur; crepuscular activity; adaptation to thermal variations	Dense fur in winter; avoids exposure; adapted activity	Behavioral thermoregulation (mud, shade); no sweating	Fur insulation; heat preservation posts	Regulation by plumage; shelter and radiation avoidance
Reproduction (seasonality)	Autumn rut (Sept–Oct); calving in spring; intense hormonal activity in males	Summer mating; delayed implantation; spring calving	Breeds in autumn; gestation ~115 days; calving in spring, possibly 2 series/year	Mating in winter; short gestation; 3-4 litters/year under favorable conditions	Breeding in spring; seasonal egg laying; rapid development of young
Stress response (mechanisms)	HPA activation; increased cortisol; chronic stress reduces immunity	Similar HPA response; increased sensitivity in gestation	Intense HPA response; stress reduces resistance to disease	Rapid increases in cortisol; high sensitivity to manipulation	Increased corticosteron; sensitivity to capture and translocation
Associated behavior and ethological implications	Intense rutting behavior; formation of male groups; seasonal activity; under stress: agitation and dispersal	Solitary behavior; high alertness; avoidance reactions in disturbed conditions	Complex social structure; stable hierarchies; territorial behavior	Solitary behavior; rapid avoidance reactions; chicks use camouflage strategies	Rapid flight reactions; sheltering behavior; sensitivity to disturbances
Management and hunting implications	Avoiding hunting during the rut; protecting pregnant females; monitoring stress	Limiting hunting during the breeding season; trophic management during the cold season	Controlling hunting pressure; avoiding artificial selection; managing resources	Hunting during the breeding season; ensuring refuge habitats	Banning hunting during the nesting period; reducing stress through appropriate handling

Turbill et al. 2010; Mauget et al., 1997; Ruf et al., 2021; Valencak et al., 2010.

Behavioral adjustments also include habitat selection in response to thermal constraints. Species may move to higher altitudes or shaded areas during warm periods, reflecting trade-offs between thermoregulation and foraging efficiency (Mason et al., 2017). In birds of hunting interest, thermoregulation involves sun exposure for warming, feather maintenance behaviors, and group aggregation during colder periods to reduce heat loss.

Seasonal variation strongly influences these responses. During harsh winters, animals adopt strategies to conserve heat, such as reduced movement, use of sheltered microhabitats, and aggregation. Conversely, during heat stress, species seek shade and water sources, and shift activity to cooler periods of the day. These thermoregulatory behaviors directly influence spatial and temporal distribution patterns.

From a management perspective, the literature emphasizes the importance of habitat features that support thermoregulation, including the availability of shelter, vegetation cover, and water resources, particularly under changing climatic conditions.

Reproductive physiology and behavioral manifestations

The literature consistently shows that reproduction in European game species is strongly seasonal, primarily regulated by photoperiod and endocrine mechanisms. In cervids and wild boar, shorter day length during

autumn stimulates melatonin secretion and the activation of the hypothalamic–pituitary-gonadal axis, leading to mating periods typically occurring in early autumn, with births synchronized in spring. In roe deer (*Capreolus capreolus*), delayed implantation represents a key adaptive mechanism, allowing mating in summer while postponing embryo development until winter, thereby ensuring parturition under favorable spring conditions (Asher, 2011).

Reproductive cycles are closely associated with pronounced behavioral changes. During the rutting season, males exhibit increased activity, vocalization, and competitive interactions, driven by elevated testosterone levels, often accompanied by reduced feeding and increased energetic stress. After conception, females display more cryptic behavior, selecting sheltered areas for parturition and offspring rearing, while lactation imposes substantial energetic demands. These physiological and behavioral adjustments ensure reproductive success under variable environmental conditions.

Reproductive performance also varies with age and sex. Younger females may delay reproduction until reaching adequate body condition, while adult individuals with established energy reserves are better able to sustain the energetic costs of gestation and lactation. In males, dominance hierarchies during the rut influence reproductive success, and selective pressures, including hunting, may alter population structure and breeding dynamics.

From a management perspective, the literature emphasizes the importance of aligning hunting practices with reproductive cycles. Avoiding the harvest of pregnant females and limiting hunting pressure on dominant males during the rut are key measures for maintaining population stability. Additionally, ensuring adequate nutritional support during critical periods, such as late gestation and lactation, can contribute to improved reproductive outcomes and long-term population sustainability (Matei et al., 2026).

Stress response and associated behavioral manifestations

The literature highlights that the physiological response to stress in game species is primarily mediated by the hypothalamic-pituitary-adrenal (HPA) axis. Exposure to stressors such as predation risk, hunting activities, or extreme climatic conditions triggers the release of glucocorticoids (cortisol in mammals and corticosterone in birds), which mobilize energy reserves and redirect physiological resources toward immediate survival (Vilela et al., 2020; Güldenpfennig et al., 2021). While acute stress responses are adaptive, chronic stress has been associated with suppressed immune function, reduced reproductive performance, and altered population structure.

Behaviorally, stress is reflected in increased vigilance, flight responses, and avoidance strategies, often leading to changes in habitat use and activity patterns. Empirical studies demonstrate that repeated hunting pressure can induce long-term physiological stress. For example, elevated cortisol levels have been recorded in hunted deer populations during hunting seasons, indicating cumulative stress effects (Vilela et al., 2020). Similarly, hunting with dogs has been associated with higher stress responses compared to less intrusive hunting methods (Gentsch et al., 2018). In wild boar, sex-related differences in stress responses have been observed, with females showing higher cortisol levels under hunting pressure (Güldenpfennig et al., 2021).

In birds of hunting interest, repeated exposure to anthropogenic stressors, such as capture and translocation, has been shown to alter baseline corticosterone levels and negatively affect body condition (Dickens et al., 2009), suggesting long-term impacts on physiological regulation. These findings indicate that sustained exposure to stressors may disrupt the HPA axis and compromise species resilience.

From a behavioral and ecological perspective, stress responses can lead to habitat avoidance, increased energy expenditure, and shifts in spatial distribution, particularly in areas subjected to intense anthropogenic pressure. Consequently, the literature emphasizes the importance of monitoring stress levels using non-invasive methods and adopting management strategies that reduce chronic stress exposure. These include adjusting hunting intensity and methods, as well as avoiding hunting during extreme environmental conditions.

CONCLUSIONS

The present review highlights the complex interdependence between population dynamics, physiological adaptations, and behavioral responses in game species inhabiting submontane ecosystems. The available literature indicates that these components are closely interconnected, with physiological mechanisms such as energy metabolism, thermoregulation, reproductive processes, and stress responses has some roles in shaping behavioral patterns and population structure.

The findings emphasize that game species exhibit a high degree of adaptability to both environmental variability and anthropogenic pressures. However, ongoing global changes, including climate variability and intensified hunting activities, may disrupt these adaptive mechanisms, with potential consequences for population stability and ecosystem functioning.

In this context, the integration of physiological and ethological knowledge into wildlife management strategies is essential. Adaptive management approaches, including the alignment of hunting practices with biological cycles, the protection of critical reproductive periods, and the reduction of chronic stressors, are key elements in ensuring the sustainability of game populations.

Furthermore, the literature underscores the importance of continuous monitoring and the use of non-invasive methods to assess population health and stress levels. Such approaches can provide valuable insights for improving management decisions and mitigating the impact of human activities.

Overall, a comprehensive understanding of the interactions between physiological processes, behavior, and environmental factors provides a robust framework for the development of sustainable and scientifically grounded game management practices in submontane ecosystems.

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HEAT SHOCK PROTEINS (HSPs), THERMAL STRESS AND EPIGENETIC REGULATION IN WILD FISH POPULATIONS: A REVIEW

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Abstract

Rising water temperatures associated with climate change represent a major stressor for wild fish populations, affecting their physiology, distribution, and adaptive capacity. Heat shock proteins (HSPs), particularly HSP27, HSP70, and HSP90, are central components of the cellular response to thermal stress and are widely used as molecular indicators of sublethal stress. This review synthesizes current literature on the role of HSP expression in assessing thermal stress and temperature adaptation in wild fish populations, with a specific focus on epigenetic regulatory mechanisms. Available evidence indicates that epigenetic modifications, including DNA methylation and histone remodelling, can modulate HSP gene expression, thereby contributing to physiological plasticity and population-level differences in thermal responses. Integrating HSP-based biomarkers with epigenetic information provides a robust framework for understanding adaptive responses of wild fish populations to climate-driven thermal stress.

Key words*: DNA methylation, histone modifications, temperature adaptation, stress response, wild fish stocks.*

INTRODUCTION

The increasing frequency and intensity of thermal extremes represent one of the most significant selective pressures acting on aquatic organisms, particularly on wild fish populations (EPA, 2021; Almeida et al., 2024; Stroe et al., 2024). Climate change is reshaping thermal regimes in both freshwater and marine ecosystems, directly affecting key physiological processes such as metabolism (Munteanu & Bogatu, 2008; Jutfelt, 2020; Volkoff & Rønnestad, 2020), development and reproduction (Dobre et al., 2025a), and survival (Alborali, 2006; D'Abramo & Slater, 2019; Islam et al., 2022). As ectothermic organisms, fish are especially sensitive to temperature fluctuations, which directly regulate cellular function and overall biological performance (Aadland, 1993; Dobre et al., 2025b). Consequently, thermal stress represents a major driver of adaptation, natural selection, and population dynamics in aquatic environments (Dobre et al., 2025c). For example, marine heatwave events have been shown to cause acute physiological stress and population-level impacts in marine ecosystems, highlighting the biological relevance of thermal extremes beyond gradual warming trends (Ryu et al., 2020).

Against this background of climate-driven thermal instability, a critical synthesis is needed to evaluate how heat shock protein responses are modulated by epigenetic mechanisms and to assess their role in shaping physiological

plasticity and thermal resilience in wild fish populations.

One of the most conserved and well-characterized cellular responses to thermal stress is the induction of heat shock proteins (HSPs) (Lindquist & Craig, 1988; Arias et al., 2011; Folguera et al., 2011; Dawood et al., 2020; Somero, 2020; Elbahnaswy et al., 2024). These proteins function as molecular chaperones (Zarei et al., 2024), playing a central role in maintaining proteostasis by preventing protein denaturation, facilitating proper protein folding, and promoting the degradation of irreversibly damaged proteins (Hartl et al., 2011; Balchin et al., 2016). Increasing evidence indicates that cellular proteostasis is a key determinant of thermal sensitivity, integrating multiple molecular pathways that regulate protein stability and cellular function under thermal stress (Dorrity et al., 2023). In this context, HSPs represent central components of the proteostasis network, linking environmental temperature variability to cellular performance and survival (Somero, 2010).

HSP expression is rapidly upregulated in response to elevated or reduced temperatures, hypoxia, pollution, and osmotic stress (Steinberg, 2012; Ahmad et al., 2020; Chen et al., 2021; Zarei et al., 2024; Zhang et al., 2024), making these proteins reliable biomarkers of cellular stress (Iwama et al., 2004; Dawood et al., 2020; Somero, 2020; Elbahnaswy et al., 2024). Importantly, both the magnitude and duration of HSP responses vary among populations, reflecting differences in environmental history and physiological sensitivity. Such variability is shaped not only by genetic background but also by environmentally induced plasticity, enabling population-specific thermal tolerance and adaptive capacity (Basu et al., 2002; Somero, 2010; Lallias et al., 2021; Chapelle & Silvestre, 2022).

However, responses to thermal stress are not governed solely by fixed genetic mechanisms. Increasing evidence indicates that the regulation of HSP expression is strongly influenced by epigenetic processes that modulate gene activity without altering the underlying DNA sequence. Mechanisms such as DNA methylation, histone modifications, and regulation by non-coding RNAs enable rapid and reversible adjustments of HSP expression (Eid et al., 2016), thereby enhancing phenotypic plasticity under fluctuating environmental conditions (Lallias et al., 2021; Chapelle & Silvestre, 2022). These mechanisms may contribute not only to short-term acclimation but also to transgenerational thermal tolerance, a feature of particular relevance for wild populations exposed to ongoing climate change (Piferrer et al., 2024).

In this context, integrating thermal stress, heat shock protein expression, and epigenetic regulation provides a coherent framework for understanding adaptive capacity in wild fish populations. Although the HSP response to thermal stress is well documented and epigenetic mechanisms have been extensively studied independently, evidence integrating these two regulatory levels under natural environmental conditions remains limited. In particular, the extent to which epigenetic modulation of HSP expression contributes to thermal resilience in wild fish populations remains poorly understood. Addressing this gap is essential for understanding how natural populations cope with increasing thermal variability and for predicting their resilience under ongoing climate change.

MATERIALS AND METHODS

This review was conducted using a structured narrative approach with systematic elements to synthesize current knowledge on heat shock proteins (HSPs), thermal stress, and epigenetic regulation in wild fish populations. A comprehensive literature search was performed across major scientific databases, including Web of Science, Scopus, and PubMed, to ensure broad coverage of molecular, physiological, and ecological studies.

Search queries combined terms related to heat shock proteins, thermal stress, and epigenetic regulation, using Boolean operators. Key terms included “heat shock proteins”, “HSP70”, “thermal stress”, “epigenetic regulation”, “DNA methylation”, “histone modifications”, “non-coding RNAs”, “thermal plasticity”, “transgenerational effects”, and “fish” or “teleost”. Additional targeted searches were performed for specific processes (e.g., “thermal stress memory”, “trained immunity”, “epigenetic inheritance”) and for taxa frequently used as ecological or physiological models.

Studies were selected based on their relevance to (i) HSP expression under thermal stress, (ii) epigenetic mechanisms regulating stress responses, and (iii) experimental or observational evidence in fish, with emphasis on wild populations or environmentally realistic conditions. Laboratory studies from aquaculture or model species were included when they provided mechanistic insights directly applicable to natural systems.

Rather than applying strict quantitative selection criteria, studies were evaluated qualitatively for biological relevance, mechanistic clarity, and ecological interpretability. Priority was given to peer-reviewed articles presenting experimental evidence, field-based observations, or integrative frameworks linking molecular regulation to physiological performance under thermal variability.

The selected literature was synthesized thematically to integrate classical HSP regulation with emerging evidence on epigenetic plasticity, thermal stress memory, and transgenerational effects. Particular attention was given to studies that explicitly addressed epigenetic rewiring, environmentally induced regulatory persistence, and context-dependent plasticity, allowing comparison with related concepts such as trained immunity.

Table 1. Summary of PRISMA 2020 Checklist Compliance for This Systematic Review

PRISMA Section	Item No.	Requirement (Expanded Checklist)	How Applied in This Study
Title		Identify the report as a systematic review	The title explicitly identifies the manuscript as a systematic review addressing thermal stress, HSPs, and epigenetic mechanisms in fish
Abstract		Structured summary including background, objectives, methods, results, and conclusions	Abstract structured according to PRISMA 2020, summarizing scope, mechanisms (HSPs, epigenetics), and implications
Introduction	**Rationale**	Describe the rationale for the review	Justifies the review based on increasing thermal stress, phenotypic plasticity, and molecular stress responses in fish
	Objectives	Provide explicit objectives or questions	Objectives defined to synthesize evidence on HSPs, epigenetic regulation, and thermal tolerance across fish taxa
Methods	**Eligibility criteria**	Specify inclusion/exclusion criteria	Included peer-reviewed studies on fish (and selected aquatic ectotherms) reporting thermal stress, HSPs, epigenetic or transcriptomic responses; excluded non-relevant taxa and non-thermal stressors
	Information sources	Specify all databases and sources searched	Searches conducted in Web of Science, Scopus, PubMed, and Google Scholar; reference lists manually screened
	Search strategy	Present full search strategy	Keywords combined terms related to “heat shock proteins”, “thermal stress”, “epigenetics”, “fish”, “climate change”
	Selection process	Describe study selection process	Titles/abstracts screened, followed by full-text assessment according to eligibility criteria
	Data collection process	Describe how data were extracted	Data extracted on species, life stage, temperature regime, molecular endpoints (HSPs, miRNA, DNA methylation)
	Data items	List outcomes and variables	Outcomes included HSP expression (gene/protein), epigenetic marks, thermal tolerance metrics
	Risk of bias	Describe methods to assess bias	Formal meta-analytic bias assessment not applicable; study quality assessed narratively based on experimental design
	Effect measures	Specify effect measures used	Qualitative synthesis; no pooled quantitative effect sizes due to heterogeneity
Results	**Study selection**	Report numbers of records screened/included	PRISMA flow diagram reports identification, screening, eligibility, and inclusion stages
	Study characteristics	Cites and describes included studies	Studies summarized by taxa, stress regime, molecular level (gene, protein, epigenetic)

PRISMA Section	Item No.	Requirement (Expanded Checklist)	How Applied in This Study
	Risk of bias	Present risk of bias within studies	Discussed narratively, highlighting experimental vs observational approaches
	Results of individual studies	Present results for each study	Key findings on HSP70/Hsp90, miRNA regulation, and epigenetic memory summarized
	Synthesis of results	Synthesize results across studies	Evidence integrated to show conserved and population-specific thermal stress responses
Discussion	**Interpretation**	Interpret results in context	Results interpreted in relation to phenotypic plasticity, acclimation, and climate adaptation
	Limitations of evidence	Discuss limitations of included studies	Notes about heterogeneity of species, temperatures, and molecular endpoints
	Limitations of review	Discuss limitations of the review process	Acknowledges taxonomic and methodological bias in available literature
	Implications	Implications for research and management	Highlights relevance for aquaculture resilience and conservation under climate change
Other	**Registration and protocol**	Indicate registration/protocol	Review not registered; protocol developed internally

Reporting quality and methodological transparency were evaluated following the PRISMA 2020 guidelines, with detailed compliance summarized in Table 1.

This integrative approach enabled the identification of current knowledge gaps, methodological limitations, and future research directions relevant to predicting thermal resilience in wild fish populations under climate change.

Figures were created by the authors using https://www.biorender.com to illustrate conceptual and mechanistic frameworks discussed in the manuscript.

RESULTS AND DISCUSSIONS

Heat shock proteins (HSPs) represent a highly conserved family of molecular chaperones that play a central role in maintaining cellular proteostasis under both basal and stress conditions (Iwama et al., 1998; Kayhan & Susleyici Duman, 2010; Mohanty et al., 2018).

HSPs as a primary response to thermal stress

Initially described as proteins induced by elevated temperatures, HSPs are now recognized as key components of the cellular stress response activated by a wide range of environmental stressors, including thermal fluctuations, hypoxia, oxidative stress, pollutants, and osmotic challenges (Iwama et al., 1999; Tort & Teles, 2012; Jeyachandran et al., 2023), in order to maintain homeostasis (Kumar et al., 2022). Their primary function is to preserve protein integrity by assisting in proper protein folding, preventing aggregation of misfolded proteins, and facilitating the refolding or degradation of damaged polypeptides (Kayhan & Susleyici Duman, 2010; Franke et al., 2024).

HSPs are classified into several families according to their molecular weight and functional properties, including Hsp70, Hsp90, Hsp60, Hsp47, and small heat shock proteins (sHSPs) historically known as crystallins (Timofeyev et al., 2009; Kayhan & Susleyici Duman, 2010; Basha et al., 2012; Heikkilä, 2017).

The sHSP family corresponds to the HspB group and includes proteins such as Hsp27 (HspB1) and members of the α-crystallin family (e.g., αA/HspB4 and αB/HspB5). Small heat shock proteins (sHSPs), such as Hsp27 (HspB1), act as early responders to cellular stress by binding unfolded or partially denatured proteins, thereby preventing irreversible aggregation and maintaining proteostasis during acute stress conditions (Jakob et al., 1993; Halsbeck et al., 2005; Ryu et al., 2020). In addition, sHSPs contribute to cytoskeletal stabilization by interacting with actin and intermediate filaments, supporting cellular integrity under thermal stress (Basha et al., 2012).

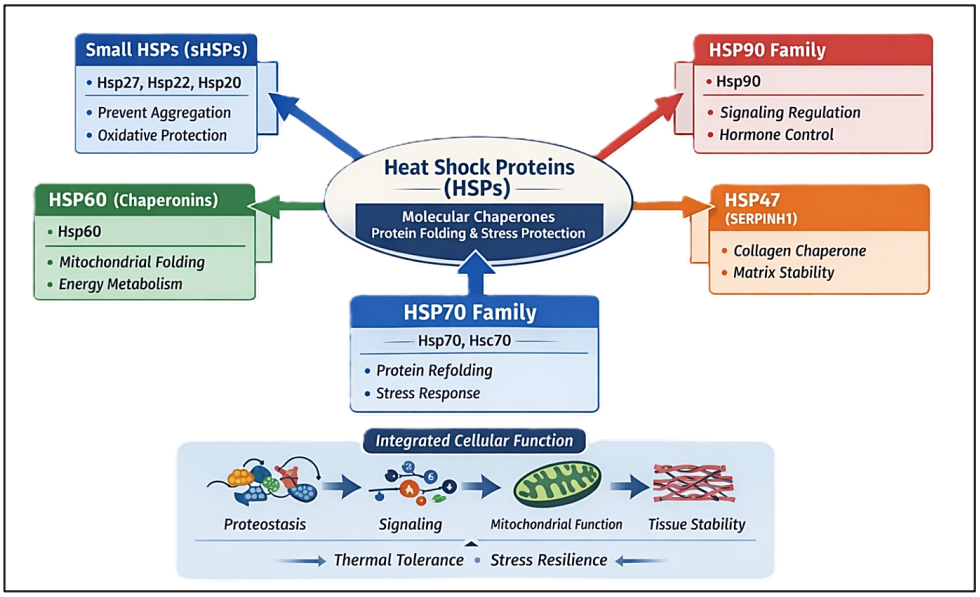


Figure 1. Classification and functional roles of heat shock proteins (HSPs) involved in cellular responses to thermal stress (Figure create with https://www.biorender.com)

Figure 1 illustrates the major families of heat shock proteins (HSPs) and their coordinated roles in maintaining cellular homeostasis under thermal stress. HSPs are grouped according to their molecular weight and functional specialization.

The HSP70 family represents a central hub of the cellular stress response, facilitating correct protein folding, refolding of denatured proteins, and targeting irreversibly damaged proteins for degradation, thereby preserving proteostasis. HSP90 further supports cellular adaptation by stabilizing a wide range of signalling proteins, transcription factors, and kinases involved in stress signalling pathways.

In parallel, HSP60, primarily localized in mitochondria, ensures proper protein folding and mitochondrial integrity, while HSP47 functions as a collagen-specific chaperone essential for maintaining extracellular matrix stability and tissue structure under stress. Together, these HSP families form an integrated chaperone network that safeguards cellular functionality and enhances stress tolerance under fluctuating environmental conditions (Heikkilä, 2017; Roberts et al., 2010).

In ectothermic organisms such as fish, HSPs play a particularly critical role, reactingto the direct influence of environmental temperature on cellular processes. The induction of HSPs allows cells to rapidly respond to thermal perturbations, thereby minimizing protein damage and sustaining metabolic performance (Iwama et al., 1998; Yamashita et al., 2010). Consequently, HSPs are widely regarded as sensitive and reliable biomarkers of cellular stress and physiological resilience in aquatic organisms (Iwama et al., 1999; Steinberg, 2012; Dawood et al., 2020; Elbahnaswy et al., 2024).

Importantly, although HSPs are evolutionarily conserved, their expression dynamics and functional relevance vary among species, populations, and environmental contexts. Such variability reflects differences in thermal history, habitat stability, and adaptive capacity, highlighting the importance of integrating molecular, physiological, and ecological perspectives when evaluating stress responses in natural populations (Basu et al., 2002; Oksala et al., 2014; Thia et al., 2021).

Empirical evidence from multiple fish species supports the central role of HSPs in mediating thermal tolerance under natural and experimental conditions. In common carp (*Cyprinus carpio*), thermal stress has been associated with elevated HSP expression, reflecting adaptive physiological responses to fluctuating temperature regimes (Iwama et al.,

1998; Yamashita et al., 2010). Similarly, in Nile tilapia (*Oreochromis niloticus*), exposure to elevated temperatures induces HSP expression and modulates immune and oxidative stress responses, highlighting the integrative role of HSPs in maintaining physiological resilience under thermal challenge (Hofmann et al., 2005; Girilal et al., 2015; Elbahnaswy et al., 2024).

In zebrafish (*Danio rerio*), differential cellular sensitivity to temperature has been linked to variation in proteostasis capacity, emphasizing the importance of HSP-mediated mechanisms in shaping thermal tolerance at the cellular level (Dorrity et al., 2023). Eid et al. (2016) demonstrated that snakehead fish (*Channa striata*) populations adapted to warmer environments exhibit distinct *hsp70* sequence and expression profiles, supporting the role of HSP70 as a key molecular component underlying interpopulation differences in thermal tolerance.

Collectively, these findings demonstrate that although HSPs represent a conserved stress-response system, their expression dynamics and functional relevance vary among species, populations, and environmental contexts. Such variability reflects differences in thermal history, habitat stability, and adaptive capacity, emphasizing the need to integrate molecular, physiological, and ecological perspectives when evaluating stress responses in natural populations (Basu et al., 2002; Oksala et al., 2014; Thia et al., 2021).

As a proof of concept, HSPs are typically not produced in organisms living in thermally stable environments. To identify how widespread the loss of the HSPs is within the Antarctic and cold-temperate non-Antarctic notothenioids, Hofmann et al. (2005) studied the thornfish, *Bovichtus variegatus*, and the black cod, *Notothenia angustata*. Interestingly, one of the two New Zealand notothenioids possessed a heat-shock response: *B. variegates* expressed HSPs in response to heat stress, whereas *N. angustata* did not display robust stress-inducible HSP synthesis at the protein-level. However, mRNA for a common hsp gene, *hsp70*, was present in cells of both New Zealand species following exposure to elevated temperatures. Consequently, the question arises, then the *hsp70* mRNA was unstable and, hence responsible for the lack of the heat-shock response in *N. angustata.* One may speculate that the expressed mRNAs are subject to an epigenetic mechanism; this could include cleavage by non-coding RNAs.

Epigenetic plasticity and variation in HSP responses

Epigenetic plasticity represents a key mechanism underlying variation in HSP responses among populations exposed to contrasting thermal environments. Differences in HSP expression patterns cannot be fully explained by genetic divergence alone but often reflect environmentally induced epigenetic modulation shaped by local thermal histories (Lallias et al., 2021; Chapelle & Silvestre, 2022).

Studies in teleost fish demonstrate that epigenetic mechanisms - including DNA methylation, histone modifications, and non-coding RNAs - contribute substantially to environmentally responsive gene regulation and phenotypic plasticity (Gavery & Roberts, 2017; Best et al., 2018). Ryu et al. (2020) identified temperature-dependent DNA methylation signatures associated with within-generational acclimation to ocean warming in a reef fish (*Acanthochromis polyacanthus*).

These mechanisms provide a molecular framework through which environmental cues can be translated into stable yet reversible changes in gene expression, allowing organisms to fine-tune physiological responses without altering their genetic code.

Populations inhabiting thermally variable or chronically warm environments frequently exhibit modified thresholds for HSP induction, altered response kinetics, or elevated baseline expression levels. Such adjustments contribute to maintaining cellular performance under thermal stress while minimizing energetic costs associated with prolonged stress responses (Oksala et al., 2014; Todgham et al., 2005; Ryu et al., 2020). Beyond their canonical chaperone function, heat shock proteins - particularly HSP90 - have been proposed to act as central modulators of genetic and epigenetic variation, buffering or releasing phenotypic diversity under environmental stress and thereby shaping stress-induced plasticity (Zabinsky et al., 2019).

Recent experimental studies support the role of epigenetic regulation in shaping thermal

responses in fish. For example, altered gene expression profiles and metabolic performance under elevated temperatures have been reported in clownfish larvae exposed to future ocean warming scenarios (Moore et al., 2023). In addition, transgenerational effects of environmentally induced epigenetic regulation have been demonstrated in fish, where parental thermal or environmental exposure influences offspring phenotypes (Pierron et al., 2021). The study by Ren et al. (2025), in western mosquitofish (*Gambusia affinis*), highlights directions for future research by showing that strong and immediate molecular responses to acute thermal stress in mosquitofish involve variation in gene expression, alternative splicing, and DNA methylation, but not miRNA expression. These individual molecular mechanisms regulate different sets of genes and play non-redundant roles in thermal stress responses. Interestingly, variation in gene expression in response to temperature stress does not appear to be directly regulated by epigenetic mechanisms, at least in this species.

Despite growing recognition of epigenetic regulation as a key modulator of HSP expression (Vihervaara et al., 2018; Lallias et al., 2021; Chapelle & Silvestre, 2022; Piferrer et al., 2024), its ecological relevance in wild fish populations remains insufficiently explored. Most available evidence derives from controlled laboratory studies, and the extent to which epigenetic modulation of HSP responses operates under natural thermal variability remains poorly understood.

Consequently, epigenetic regulation represents a critical mechanistic link between environmental heterogeneity and phenotypic variability, enabling rapid adjustment to changing thermal conditions without requiring genetic change. Clarifying how these mechanisms operate in natural populations is essential for predicting species responses to ongoing climate change and for understanding the processes underlying physiological plasticity in aquatic ecosystems.

Classical regulation of HSP expression

The classical response to thermal stress is rapid and transient, occurring within minutes to hours. It is primarily mediated by Heat Shock Factor 1 (HSF1), the main transcriptional regulator of *hsp* genes (Evans et al., 2007; Xiao et al., 2025). Under basal conditions, HSF1 is maintained in an inactive state through its association with molecular chaperones such as HSP70 and HSP90 (Figure 2, left side). Elevated temperatures promote the accumulation of misfolded proteins, leading to the dissociation of HSF1 from these inhibitory complexes, followed by its trimerization and activation (Figure 2, center).

Activated HSF1 binds to specific DNA sequences known as heat shock elements (HSEs) located in the promoter regions of *hsp* genes, such as *hsp70* and *hsp90*. This binding rapidly initiates transcription of genes involved in cellular protection.

Heat shock elements (HSEs) are conserved DNA motifs located in the promoter regions of heat shock genes and serve as binding sites for Heat Shock Factor 1 (HSF1). Upon thermal stress, HSF1 trimerizes and binds to HSEs, initiating rapid transcription of HSP genes (Pirkkala et al., 2001). The accessibility of HSEs is tightly regulated by epigenetic mechanisms, including DNA methylation and histone modifications, which modulate the magnitude and duration of the heat shock response. This regulatory architecture enables rapid, reversible, and environmentally responsive control of HSP expression (Jolly et al., 1999; Pirkkala et al., 2001; Yamashita et al., 2010; Lallias et al., 2021; Piferrer et al., 2024). This mechanism is essential for preventing protein aggregation and maintaining cellular functionality under acute stress conditions (Pirkkala et al., 2001; Yamashita et al., 2010).

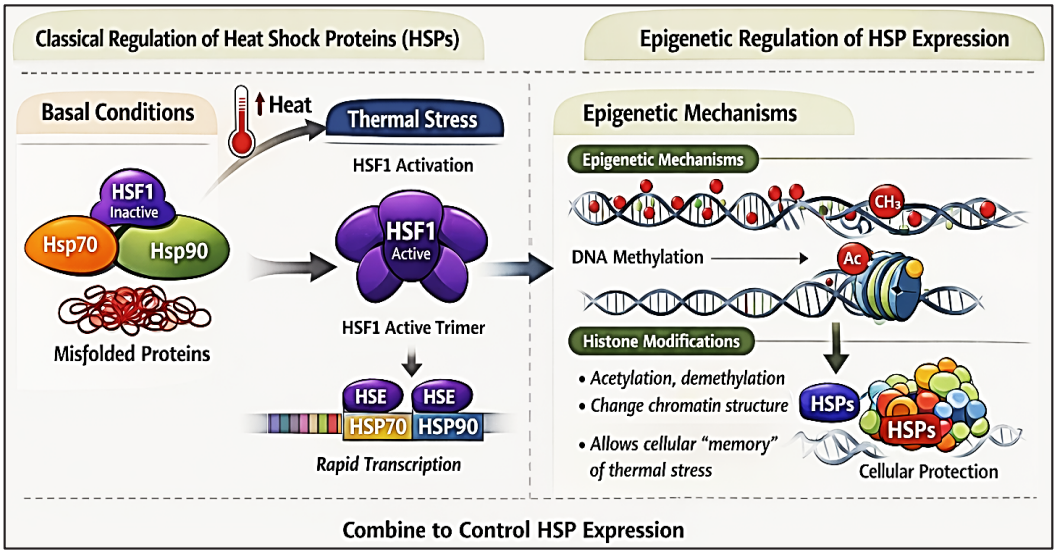


Figure 2. Schematic representation of the molecular mechanisms underlying heat shock protein (HSP) activation in response to thermal stress, integrating classical transcriptional regulation and epigenetic control (Figure created with https://www.biorender.com)

Epigenetic regulation as a mediator between environmental variability and physiological plasticity

Beyond classical genetic control, the expression of HSPs is strongly influenced by epigenetic mechanisms that modulate gene activity without altering the underlying DNA sequence.

Epigenetic regulation, including DNA methylation, histone modifications, and non-coding RNAs, modulates the magnitude, duration, and sensitivity of this response (Figure 2, right side).

DNA methylation within promoter regions of HSP genes plays a central role in regulating transcriptional accessibility, while histone modifications contribute to chromatin remodeling and fine-tuning of gene expression (Lallias et al., 2021; Piferrer et al., 2024). In addition, non-coding RNAs, including microRNAs, participate in post-transcriptional regulation by affecting mRNA stability and translation efficiency (Piferrer et al., 2024).

At the molecular level, Hsp90 has been proposed to function as a regulatory hub linking stress responses to both genetic and epigenetic control of gene expression. By interacting with chromatin regulators and transcriptional machinery, Hsp90 can influence the expression of stress-responsive genes and modulate the exposure of epigenetically regulated phenotypes under thermal stress (Zabinsky et al., 2019).

DNA methylation represents one of the primary epigenetic mechanisms influencing HSP regulation. It involves the enzymatic addition of a methyl group (–CH_3) to cytosine residues, primarily within CpG dinucleotides, catalyzed by DNA methyltransferases (DNMTs). This reaction uses *S-adenosylmethionine (SAM)* as the universal methyl group donor and results in the formation of 5-methylcytosine (5mC). In the context of *hsp* genes, methylation within promoter regions generally reduces chromatin accessibility and limits transcription factor binding, thereby repressing gene expression (Figure 3). Conversely, hypomethylation of HSP promoters, particularly under thermal stress, facilitates transcriptional activation by allowing binding of heat shock Factor 1 (HSF1) to heat shock elements (HSEs). This mechanism enables rapid induction of *hsp* expression during acute thermal challenges and may contribute to stress memory when maintained over time (Steinberg, 2012; Lallias et al., 2021; Chapelle & Silvestre, 2022).

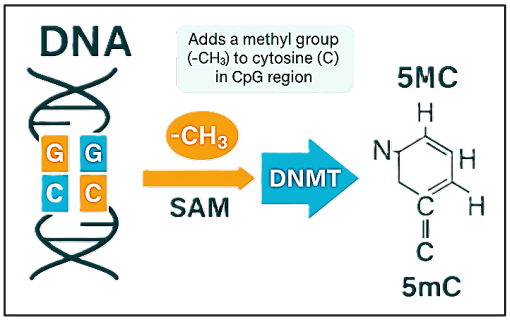


Figure 3. DNA methylation as an epigenetic mechanism regulating heat shock protein (HSP) expression (Figure created with https://www.biorender.com)

Figure 3 illustrates the enzymatic addition of a methyl group (-CH_3) from *S-adenosylmethionine* (*SAM*) to cytosine residues within CpG dinucleotides, catalyzed by DNA methyltransferases (DNMTs), resulting in the formation of 5-methylcytosine (5mC). DNA methylation in promoter regions of *hsp* genes can modulate chromatin accessibility and transcriptional activity, thereby influencing *hsp* expression.

Histone modifications play a central role in regulating HSP gene expression by modulating chromatin structure and transcriptional accessibility.

Acetylation of histone H4 or histone H3 at lysine 9 (H3K9ac) promotes an open chromatin configuration, facilitating transcription factor access to promoter regions and activating stress-responsive genes such as *hsp70* (Pirkkala et al., 2001; Pfluger & Wagner, 2007; Vihervaara et al., 2018).

In parallel, histone methylation contributes to both activation and repression of HSP loci depending on the specific residue and methylation state. For example, the removal of repressive histone methylation marks, such as di- or tri-methylation at specific lysine residues (e.g., H3K27me3), contributes to derepression of stress-responsive genes (Pfluger & Wagner, 2007; Chapelle & Silvestre, 2022; Piferrer et al., 2024), whereas activating methylation marks (e.g., H3K4me3) are associated with transcriptionally permissive chromatin states (Vihervaara et al., 2018; Zabinsky et al., 2019; Thorstensen et al., 2022).

Together, the dynamic interplay between histone acetylation and methylation enables fine, reversible regulation of HSP expression according to the intensity and duration of thermal stress (Pfluger & Wagner, 2007; Lin et al., 2022; Piferrer et al., 2024).

However, while the role of histone modifications in heat stress responses has been extensively documented in plants and terrestrial models, direct evidence linking histone-based regulation to heat shock protein expression under thermal stress in fish remains scarce. Most available insights into histone-mediated plasticity in fish derive from general stress or developmental contexts rather than HSP-specific regulation. This represents a critical knowledge gap that needs to be addressed to fully understand epigenetic regulation of thermal tolerance in aquatic ectotherms (Abdelnour et al., 2024; Piferrer et al., 2024).

In the context of heat stress, **non-coding RNAs** (ncRNAs) play a critical role in the post-transcriptional regulation of heat shock protein (HSP) expression, fine-tuning cellular responses to environmental challenges (Figure 4), as proven by Liu et al. (2024) in spotted sea bass (*Lateolabrax maculatus*).

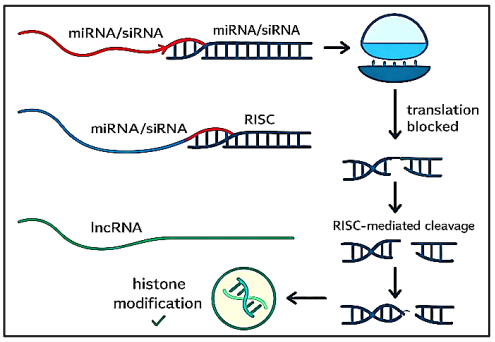


Figure 4. Post - transcriptional regulation of gene expression mediated by non-coding RNAs (Figure created with https://www.biorender.com)

Among these, microRNAs (miRNAs) are very sensitive to environmental changes, therefore the most extensively studied. They act primarily by binding to complementary sequences within the 3′ untranslated regions (UTRs) of HSP mRNAs. This interaction can either inhibit translation or promote mRNA degradation, thereby modulating the abundance of HSP proteins in a rapid and reversible manner.

In cases of high sequence complementarity, miRNA–mRNA interaction can trigger

endonucleolytic cleavage of HSP transcripts, mediated by the RNA-induced silencing complex (RISC), leading to irreversible degradation of the target mRNA and complete suppression of HSP translation. Such miRNA-mediated mRNA cleavage provides an efficient mechanism to rapidly downregulate HSP expression after stress relief, preventing excessive chaperone synthesis and reducing energetic costs associated with prolonged stress responses (Hutvágner & Zamore, 2002; Bartel, 2004; Meister & Tuschl, 2004).

Under thermal stress, changes in miRNA expression profiles can dynamically regulate HSP synthesis (Liu et al., 2024; Lin & Meegaskumbura, 2025), like HSP47, HSP70, and HSP90α.

Recent experimental evidence further supports the role of post-transcriptional epigenetic regulation in shaping thermal stress responses in fish. In zebrafish (*Danio rerio*), developmental exposure to constant elevated temperatures and diel thermal variation significantly altered microRNA expression profiles, with concomitant effects on physiological performance (Gavarikar & Craig, 2025). These findings indicate that thermal regimes experienced during early life-stages can program miRNA-mediated regulatory networks, potentially influencing the expression of stress-responsive genes, including heat shock proteins.

Long non-coding RNAs (lncRNAs) further contribute to HSP regulation by interacting with chromatin-modifying complexes or transcriptional machinery, indirectly influencing HSP gene accessibility and expression. Some lncRNAs act as molecular scaffolds that recruit histone-modifying enzymes to HSP loci, while others function as molecular sponges that sequester miRNAs, thereby modulating their regulatory activity (Piferrer et al., 2024). Evidence from rainbow trout indicates that thermal stress can reshape lncRNA-miRNA-mRNA regulatory networks, influencing the expression of stress-related genes, including those involved in protein folding and cellular protection (Zhou et al., 2022). These regulatory effects may operate in **cis**, meaning that lncRNAs or epigenetic marks act locally to modulate chromatin accessibility and transcription of adjacent *hsp* genes, or in **trans**, whereby regulatory molecules influence the expression of distantly located genes through interactions with transcriptional or epigenetic regulatory networks (Huo et al., 2019; Zhou et al., 2022; Liu et al., 2024).

The interaction between classical transcriptional control and epigenetic regulation can lead to a process of ***epigenetic rewiring***, whereby stress-induced chromatin modifications persist beyond the initial stimulus and alter the responsiveness of target genes. In the context of thermal stress, this rewiring underpins the phenomenon of ***thermal stress memory***, in which prior heat exposure primes HSP loci for enhanced activation upon subsequent stress. As a result, *hsp* genes are transcribed more rapidly and robustly during re-exposure, without any change in the underlying DNA sequence.

This form of epigenetic plasticity represents a major adaptive advantage for species exposed to climate variability, particularly for wild fish populations inhabiting dynamic ecosystems (Chapelle & Silvestre, 2022; Piferrer et al., 2024) and may be compared with trained immunity, also known as immune memory (Gourbal et al., 2018).

In trained immunity, initial gene activation is accompanied by the accumulation of specific epigenetic marks (e.g., H3K4me3 on gene promoters). Some of these epigenetic marks persist in the absence of the initial stimulus (e.g., H3K4me1 and H3K4me3, etc.). As a consequence of epigenetic rewiring, upon re-challenge with a secondary stimulus, the pro-inflammatory genes are more robustly transcribed. **TF** transcription factor (You et al., 2024).

In thermal stress memory, initial heat exposure induces rapid activation of heat shock genes, accompanied by epigenetic rewiring at HSP loci (Pirkkala et al., 2001; Jolly et al., 1999; Vihervaara et al., 2018). This process involves stress-induced chromatin remodeling and the deposition of activating epigenetic marks at promoters and regulatory regions of HSP genes, including permissive histone modifications and temperature-dependent changes in DNA methylation status (Lallias et al., 2021; Chapelle & Silvestre, 2022; Piferrer et al., 2024). Importantly, a subset of these epigenetic marks can be maintained after the thermal stressor is removed, preserving HSP loci in a transcriptionally primed chromatin state that

facilitates rapid reactivation upon subsequent stress exposure (Thorstensen et al., 2022).

Because of this epigenetic rewiring, subsequent thermal challenges elicit faster and more robust transcription of *hsp* genes, characterized by lower activation thresholds and reduced dependence on de novo chromatin remodeling (Vihervaara et al., 2018; Zheng et al., 2017). This primed transcriptional landscape enhances cellular thermotolerance, limits proteotoxic damage, and reduces the energetic costs associated with repeated stress responses, thereby supporting sustained physiological performance under fluctuating thermal conditions (Norouzitallab et al., 2014; Chapelle & Silvestre, 2022). Importantly, these effects occur without alterations to the underlying DNA sequence, reflecting a reversible and environmentally responsive form of epigenetic regulation rather than genetic adaptation (Skinner, 2014; Burggren, 2016).

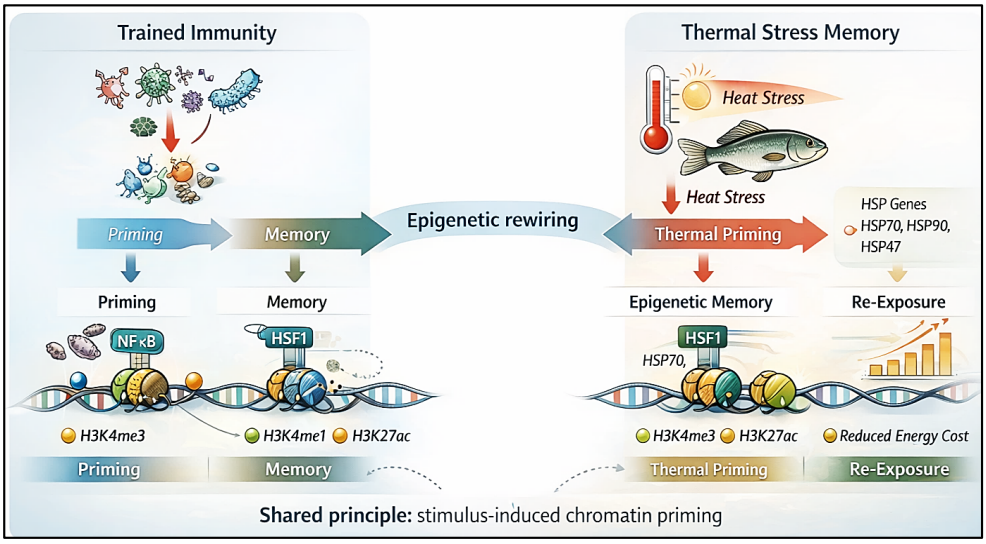


Figure 5. Conceptual comparison between trained immunity and thermal stress memory (Figure created with https://www.biorender.com)

Figure 5 describes how both phenomena rely on stimulus-induced epigenetic rewiring, whereby initial gene activation is accompanied by the deposition of activating histone marks. A subset of these epigenetic modifications persists after stimulus removal, mantaining target loci in a primed chromatin state. Upon re-exposure, this epigenetic memory enables faster and stronger transcriptional responses. In trained immunity, this mechanism enhances inflammatory gene expression, whereas in thermal stress memory it facilitates rapid induction of heat shock proteins. Current evidence suggests that trained immunity and thermal stress memory may involve similar classes of epigenetic regulators, including histone methyltransferases (e.g., SET1/MLL complexes; Sands et al., 2025), histone acetyltransferases such as p300/CBP and GCN5 that generate activating marks (Weinert et al., 2018) like H3K27ac, chromatin-remodeling complexes of the SWI/SNF family, and DNA methylation–associated enzymes (DNMTs and TETs), which contribute to the establishment of transcriptionally permissive chromatin states (Pirkkala et al., 2001; Creyghton et al., 2010; Shilatifard, 2012; Norouzitallab et al., 2014; Zheng et al., 2017; Best et al., 2018; Vihervaara et al., 2018; Gong et al., 2020; Lallias et al., 2021; Abdelnour et al., 2024; dos Santos David et al., 2026). In fish, temperature-dependent epigenetic regulation is associated with activating histone marks such as H3K4me3 and H3K4me1, characteristic of trained immunity-like responses and enhanced transcription upon re-exposure (Krishnan et al., 2022). However, direct evidence that the same specific enzymes operate in both processes in fish remains limited.

Thus far, most studies have examined methyltransferases in histone modulation and *HSP* methylation independently. However, emerging evidence suggests that other chromatin-associated enzymes, such as PARP1, a poly(ADP-ribose) polymerase involved in DNA repair, chromatin structure, and transcription, has been implicated in chromatin-based regulation under stress conditions and may also contribute to both processes (Thomas et al., 2019; Bamgbose et al., 2024). Two decades ago, Ouararhni et al. (2006) demonstrated that heat shock released PARP-1 from the *HSP70.1* promoter, thereby activating PARP-1 automodification activity, therefore regulating HSP gene expression under heat shock conditions This raises the possibility that PARP1 represents a functionally shared regulatory component, although direct evidence linking its role across both thermal stress memory and trained immunity in fish remains limited.

Experimental evidence indicates that exposure to thermal stress during early life stages can induce long-lasting changes in stress responsiveness. For example, in rainbow trout (*Oncorhynchus mykiss*), thermal conditioning during early development has been shown to shape later-life Hsp70 expression patterns, supporting increased tolerance upon re-exposure and suggesting persistent modulation of stress sensitivity potentially mediated by epigenetic mechanisms (McFarling et al., 2025). At the molecular level, thermal stress induces marked regulatory changes in stress-responsive genes. Lallias et al. (2021) demonstrated that early developmental temperature significantly influences DNA methylation patterns, with both the magnitude and direction of these epigenetic modifications depending on genetic background. Consistent with this, resistance to acute hyperthermia exhibits a heritable component and significant genetic correlations with performance-related traits, indicating that genetic architecture contributes to thermal tolerance (Lagarde et al., 2023).

At the functional level, exposure to elevated temperatures (25°C) or thermal acclimation significantly increases *hsp70* and *hsp30* transcript abundance in rainbow trout, highlighting the sensitivity of HSP expression to thermal conditions (Currie et al., 2000). However, genetic architecture alone cannot fully account for the rapid, reversible, and context-dependent nature of thermal acclimation observed in natural populations. This discrepancy highlights the importance of regulatory mechanisms beyond DNA sequence variation, particularly epigenetic modulation, which may enable flexible and reversible tuning of HSP expression in response to fluctuating thermal environments. This issue merits further investigation.

Similarly, in zebrafish (*Danio rerio*), early-life thermal preconditioning ("*thermal priming*") has been shown to modify DNA methylation patterns within the promoter region of *HSP70*, resulting in altered transcriptional responsiveness under subsequent stress exposure (Zheng et al., 2017). Under cadmium-induced stress conditions, this epigenetic modulation was associated with enhanced cellular tolerance. This indicates that early thermal experience can shape stress resilience by causing persistent regulatory changes in HSP expression, even in response to a different stressor than the priming one.

Complementary evidence from the brine shrimp *Artemia* demonstrates that thermal stress induces epigenetic modifications, including histone remodelling, which are associated with sustained changes in *Hsp70* expression and enhanced stress tolerance later in life (Norouzitallab et al., 2014). Although derived from invertebrate models, these findings are relevant for fish biology because core components of the cellular stress response, including heat shock proteins and their regulatory pathways, are highly conserved across metazoans. Moreover, both crustaceans and teleost fish are ectothermic organisms whose physiological performance is tightly coupled to environmental temperature.

These epigenetic mechanisms enable rapid, reversible, and context-dependent adjustments of HSP expression, facilitating physiological flexibility under fluctuating thermal conditions.

Increasing evidence indicates that exposure to elevated temperatures can induce persistent epigenetic modifications, that shape subsequent stress responsiveness, supporting the concept of *heat stress memory* and long-term acclimation (Thorstensen et al., 2022; Chapelle & Silvestre, 2022; Strand et al., 2023; Bisanti et al., 2025).

Such regulatory plasticity enhances the capacity of organisms to cope with environmental variability and supports functional resilience at the cellular level.

Transgenerational implications

Growing evidence suggests that epigenetic modifications induced by thermal stress can persist beyond the directly exposed generation, contributing to transgenerational plasticity (Skinner et al., 2014; 2015; Burggren, 2016), often referred to as epimutations (Piferrer et al., 2024) - stable changes in the epigenetic status. While epigenetic plasticity operates primarily within an individual's lifespan, transgenerational effects involve the persistence of epigenetic marks across generations.

This phenomenon refers to the capacity of parental environments to influence offspring phenotypes through non-genetic mechanisms (Figure 6), without changes to the underlying DNA sequence (Steinberg, 2012; Roy et al., 2023), and may be mediated by environmentally sensitive epigenetic reprogramming of germ cells, including temperature-dependent DNA methylation dynamics (Brionne et al., 2023), as well as broader environmentally induced epigenetic effects with transgenerational phenotypic consequences documented in fish (Pierron et al., 2021).

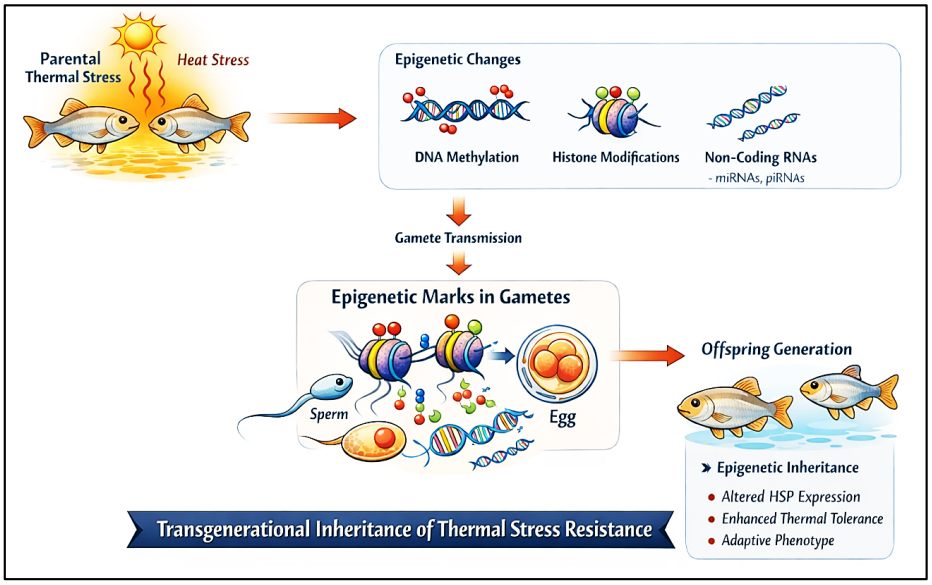


Figure 6. Epigenetic mechanisms mediating transgenerational responses to thermal stress (Figure created with https://www.biorender.com)

In the context of thermal stress, epimutations affecting heat shock protein (HSP) genes may contribute to adaptive phenotypic plasticity by altering transcriptional sensitivity and cellular stress tolerance. However, these epigenetic effects are often reversible and strongly dependent on the ecological context, including environmental variability and exposure history. Moreover, epimutations are often maintained only as long as the environmental stressor persists, as their stability frequently depends on sustained or repeated exposure to the inducing conditions (Piferrer et al., 2024). Consistent with this, experimental evidence in rainbow trout demonstrates that DNA methylation patterns in spermatozoa are highly sensitive to water temperature, indicating that paternal thermal environment can directly shape the gametic epigenome and potentially influence offspring phenotypes (Brionne et al., 2023).

At the molecular level, thermal stress can induce changes in DNA methylation patterns, particularly within promoter and regulatory regions of stress-responsive genes such as HSPs. Alterations in CpG methylation can modulate chromatin accessibility, thereby influencing transcription factor binding and transcriptional activation. For example, hypomethylation of

HSP gene promoters has been associated with enhanced transcriptional responsiveness during subsequent stress exposure, enabling faster and stronger induction of protective molecular pathways.

In parallel, histone modifications play a crucial role in shaping chromatin structure and transcriptional competence. Thermal stress can promote activating marks such as histone H3 lysine 9 acetylation (H3K9ac) or H4 acetylation, which relax chromatin structure and facilitate transcriptional activation of stress-response genes, including HSPs (Pfluger & Wagner, 2007; Liu et al., 2022). Conversely, the removal or redistribution of repressive marks such as H3K27me3 can further enhance transcriptional plasticity (Piferrer et al., 2024). These chromatin-based modifications are particularly important because they are potentially reversible yet sufficiently stable to influence gene expression beyond the initial stress exposure (Chapelle & Silvestre, 2022; Abdelnour et al., 2024).

Non-coding RNAs, including microRNAs (miRNAs) and other small regulatory RNAs, add an additional layer of post-transcriptional regulation. These molecules can fine-tune HSP expression by modulating mRNA stability, translation efficiency, or indirectly regulating transcription factors such as HSF1. Importantly, small RNAs can be transmitted through gametes, providing a mechanistic link between parental environmental experience and offspring phenotype (Skinner, 2014; Piferrer et al., 2024; Lin & Meegaskumbura, 2025).

Experimental studies indicate that parental exposure to elevated temperatures can enhance offspring thermal tolerance, even in the absence of direct exposure to the initial stressor. This suggests that epigenetic memory mechanisms may prime offspring physiology for anticipated environmental conditions, thereby increasing survival and performance under thermal challenge (Piferrer et al., 2024). Such responses may involve persistent modulation of stress-related pathways, including altered regulation of heat shock proteins (HSPs), which play a central role in cellular protection and proteostasis.

For instance, in three-spined stickleback (*Gasterosteus aculeatus*), exposure to recurring marine heatwaves not only impacted reproductive output and offspring performance but also resulted in enhanced fecundity and growth in subsequent generations, indicating the presence of heat stress memory and transgenerational plasticity (Spence-Jones et al., 2025)

From an ecological perspective, transgenerational plasticity may provide a rapid buffering mechanism against environmental change, particularly under scenarios of increasing thermal variability driven by climate change. By enabling offspring to better match anticipated environmental conditions, epigenetic inheritance can enhance population persistence in the short term, even in the absence of genetic adaptation. However, the stability, duration, and ecological relevance of these epigenetic effects remain incompletely understood, particularly in wild populations where environmental pressures are complex and multifactorial.

However, the persistence and ecological relevance of these epigenetic marks in natural populations remain incompletely understood. While laboratory studies provide strong evidence for epigenetically mediated plasticity, the stability of these modifications under fluctuating environmental conditions, their interaction with genetic background, and their contribution to long-term adaptation in the wild are still under active investigation. Understanding how epigenetic regulation of HSPs operates across generations in natural populations is therefore critical for predicting organismal resilience under ongoing climate change.

Limitations and uncertainties

Despite growing evidence supporting the role of epigenetic regulation in shaping thermal stress responses, significant knowledge gaps remain regarding the ecological relevance and long-term stability of these mechanisms in wild fish populations. Much of the current understanding of HSP regulation and epigenetic modulation is derived from controlled laboratory experiments, often conducted under simplified and stable conditions. While these studies provide critical mechanistic insights, they may not fully capture the complexity of natural environments, where organisms are simultaneously exposed to fluctuating temperatures, variable food availability, biotic interactions, and additional stressors.

Consequently, the extent to which epigenetic modifications of HSP expression persist, interact with genetic background, and contribute to adaptive capacity under natural conditions remains insufficiently resolved. Disentangling genetic effects from epigenetic influences is particularly challenging in wild populations characterized by complex demographic histories and heterogeneous selective pressures. Moreover, thermal stress rarely acts in isolation; interactions with other environmental stressors such as hypoxia, pollution, or nutritional limitation can profoundly modulate physiological responses and complicate interpretation of stress-related molecular patterns (Alborali, 2006; Nakano et al., 2014).

Importantly, separating environmentally induced epigenetic variation from genetic adaptation remains a major methodological challenge in natural populations.

Despite these limitations, converging evidence from experimental models, aquaculture studies, and emerging field-based observations suggests that epigenetic modulation of HSP expression represents a biologically plausible mechanism contributing to thermal resilience. Such plasticity may provide short-term adaptive benefits, allowing organisms to cope with rapidly changing thermal regimes while longer-term genetic adaptation unfolds. Addressing these knowledge gaps will require integrative approaches that combine molecular, physiological, and ecological perspectives, ideally within natural or semi-natural systems. This integrative framework is essential for accurately predicting organismal responses to climate change and for understanding the role of epigenetic regulation in shaping resilience and persistence of wild fish populations.

Beyond these general constraints, several HSP-specific uncertainties further limit current interpretations.

Another major uncertainty concerns the degree to which epigenetic modifications observed at the genome-wide level specifically target heat shock protein loci. While global DNA methylation or chromatin changes are frequently reported under thermal stress, direct evidence linking locus-specific epigenetic regulation of HSP genes to measurable thermal tolerance phenotypes in wild fish remains limited.

In addition, the temporal stability of epigenetic rewiring remains poorly constrained. It is often unclear whether observed modifications represent transient stress memory, short-term carry-over effects, or stable multigenerational inheritance. This uncertainty complicates attempts to link epigenetic regulation of HSPs to long-term adaptive trajectories.

Moreover, most epigenetic studies rely on single tissues, despite strong tissue-specific variation in both HSP expression and epigenetic regulation. This raises uncertainty regarding the extent to which observed molecular patterns reflect organism-wide stress responses or tissue-restricted regulatory processes.

Finally, current knowledge is biased toward a limited number of model or aquaculture species, potentially underrepresenting the diversity of epigenetic strategies present in wild fish populations inhabiting thermally complex and unstable ecosystems.

CONCLUSIONS

Heat shock proteins (HSPs) represent a central component of the cellular stress response in fish, acting as key molecular mediators that link environmental variability to physiological performance. This review highlights that HSP regulation is not solely governed by classical transcriptional mechanisms but is strongly influenced by epigenetic processes that modulate gene expression dynamics in response to thermal stress. DNA methylation, histone modifications, and non-coding RNAs collectively shape the magnitude, timing, and persistence of HSP expression, enabling organisms to respond rapidly and efficiently to fluctuating environmental conditions. DNA methylation is the most extensively studied epigenetic mechanism. However, histone modifications and the action of non-coding RNAs may be equally important and warrant further research.

Accumulating evidence indicates that thermal stress experienced during early life stages can induce lasting epigenetic modifications that influence stress responsiveness later in life. Such effects have been documented across multiple aquatic taxa, including teleost fish, and appear to contribute to phenotypic plasticity and thermal tolerance. Importantly, these

mechanisms may extend beyond individual lifespans, supporting transgenerational plasticity and providing a potential buffering capacity against rapid environmental change.

Despite significant progress, major knowledge gaps remain. Most current evidence derives from controlled laboratory studies, while the ecological relevance and stability of epigenetic modifications in wild populations remain poorly understood. Disentangling the relative contributions of genetic background, epigenetic regulation, and environmental variability continues to represent a major challenge, particularly under complex and fluctuating natural conditions.

Understanding how epigenetic regulation of HSPs operates in natural populations is therefore essential for predicting organismal resilience under ongoing climate change. Integrating molecular, physiological, and ecological approaches will be critical for advancing our understanding of how thermal stress shapes adaptive potential in wild fish populations. Such integrative frameworks will not only refine our understanding of stress biology but also support more accurate assessments of population vulnerability and adaptive capacity in a rapidly warming world.

This synthesis supports using HSP–epigenetic signatures as candidate indicators of thermal resilience, but emphasizes the need for field-validated, locus-specific, multi-tissue approaches.

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INTERANNUAL VARIATION IN LENGTH STRUCTURE AND REPRODUCTIVE CONTRIBUTION OF *Alosa immaculata* IN THE BLACK SEA-DANUBE SYSTEM (2020-2025)

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Abstract

An anadromous keystone species in the Black Sea-Danube system, Alosa immaculata plays an important ecological role by linking marine feeding habitats with riverine spawning grounds. Alterations in size structure can directly affect its reproductive output and long-term population stability. This work evaluates the interannual evolution (2020-2024) of length-frequency structure in Black Sea and Danube catches to assess changes in population composition, size-selective migration, and reproductive contribution. Marine data describe the feeding population, while riverine catches represent the spawning stock. Temporal comparisons focus on shifts in size spectra, variation in the proportion of sexually mature individuals, and persistence of the biological filter separating juvenile marine components from migrating adults. Results highlight consistent dominance of smaller size classes in the marine environment and the prevalence of mature individuals in riverine catches, with interannual variability in the representation of large size classes. These patterns indicate a stable but potentially vulnerable reproductive bottleneck, amplified by selective fishing during migration. Integrating multi-year marine and riverine length-based data supports assessment of population resilience and protection of mature individuals during reproduction.

Key words*: anadromous fish, interannual variability, length-frequency structure, pontic shad, size-selective migration, spawning stock dynamics.*

INTRODUCTION

The Pontic shad (*Alosa immaculata*) is an anadromous species of major ecological importance within the Black Sea–Danube system, contributing to the transfer of energy and nutrients between marine and freshwater ecosystems (Navodaru, 2001; Višnjić-Jeftić et al., 2013; Raikova-Petrova, 2013; Rozdina et al., 2015; Lenhardt et al., 2016; Grecu et al., 2020). Through its seasonal migration, downstream for juveniles and upstream for spawners, the species supports trophic connectivity and ecosystem functioning along the river-sea continuum (Țiganov et al., 2016; Leonov et al., 2022; 2023; Kirin et al., 2023).
Because individuals of different sizes use marine and riverine habitats unevenly throughout their life cycle, comparisons of length–frequency structure between habitats can provide insight into population connectivity across the Black Sea-Danube continuum. In anadromous fishes, size-dependent habitat use and migration timing are widely recognized mechanisms linking marine feeding areas to freshwater reproductive

habitats (Gross et al., 1988; Quinn & Myers, 2004; Secor, 2015). Accordingly, analysing size structure across habitats allows the evaluation of how demographic components are spatially distributed within this migratory system.

Connectivity between the Black Sea and the Danube system is a defining feature of the Pontic shad life cycle. Early juveniles drift seaward through the Danube Delta under the influence of river hydrology (Navodaru, 2001), subadults and adults exploit marine feeding habitats for growth, and sexually mature individuals migrate upstream to spawn. In many anadromous fishes, migration timing and river entry are influenced by size, age, and physiological condition (Quinn & Myers, 2004; Secor, 2015). In this context, analysing size structure across habitats provides a framework for exploring how different demographic components are distributed along the marine–freshwater continuum.

Owing to its complex life cycle, the Pontic shad is particularly vulnerable to anthropogenic pressures, including overfishing, habitat fragmentation, and hydrological alterations (Navodaru, 1996; Navodaru & Waldman, 2003; Višnjić-Jeftić et al., 2010; Smederevac-Lalić et al., 2018, Balik, 2019; Mocanu et al., 2020).

Body size is a key indicator of sexual maturity and reproductive contribution at the population level (Murua et al., 2003; Raikova-Petrova et al., 2013; Dobre et al., 2025) Length-frequency structure reflects both natural biological processes and the effects of external pressures and is therefore widely used to assess stock status. In Pontic shad, and differences between marine and riverine stock structures provide critical insight into biological selectivity and fishing impacts (Stroe et al., 2024).

Although previous studies have addressed interannual variation in length-frequency structure as central components of demographic assessments, with significant variation in cohort composition and sex ratio observed during reproductive migration in the Danube River (Nastase et al., 2018; Leonov et al., 2022; 2023), integrated comparisons between marine and riverine components remain limited. In particular, few studies have linked interannual changes in size structure to reproductive contribution within a unified analytical framework across habitats.

The present analysis focuses specifically on the marine feeding component (including juveniles, subadults, and adults present at sea prior to spawning migration) and on the upstream spawning component in the Danube River. It does not address downstream-drifting larvae or early juveniles within the riverine system. Therefore, the comparison targets size structure between the marine feeding assemblage and the riverine spawning assemblage, rather than across all life stages occurring within the river.

Interannual changes (2020-2025) in the length-frequency structure of Pontic shad are evaluated by integrating data from the Black Sea feeding grounds and the Danube River spawning migration. By jointly analysing marine and riverine components, the analysis aims to clarify population dynamics, assess the stability of the size-selective migration process, and explore the potential of marine length structure as an early structural indicator of subsequent spawning run composition in the Danube.

MATERIALS AND METHODS

Study area. The study was conducted within the Black Sea-Danube system, which represents a key ecological continuum for the life cycle of the Pontic shad (*Alosa immaculata*).

The Black Sea represents the main feeding and growth habitat for subadult and adult Pontic shad, where individuals accumulate energetic reserves prior to reproductive migration. The Danube River functions as the seasonal spawning migration corridor and reproductive habitat for mature individuals. This spatial structuring of life-history stages enables comparative assessment of size structure across feeding and spawning habitats within the migratory system. The study area represents a critical habitat for the species, functioning both as a spawning ground and a key migratory corridor.

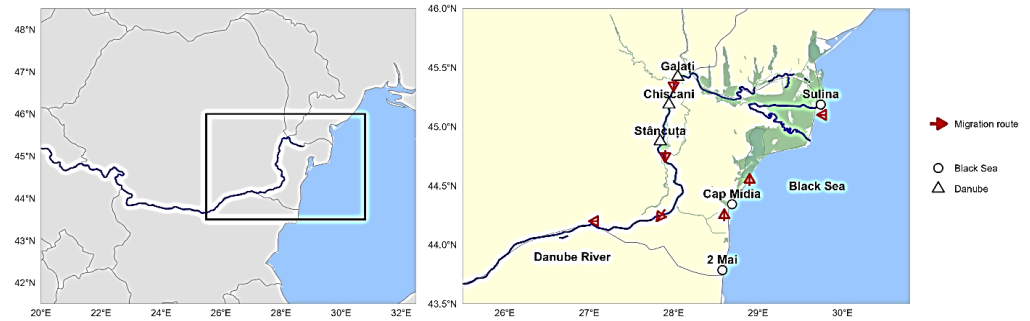


Figure 1. Study area (Source: original)

Data collection. Data were collected over a six-year period (2020-2025). Pontic shad specimens from the Danube River were obtained during scientific fishing surveys conducted by researchers from ICDEAPA Galați during the reproductive migration period, in the lower Danube sector, specifically in the areas of Galați, Chiscani, and Stâncuța. Marine data were collected by researchers from INCDM Constanța along the Romanian Black Sea coast, in the areas of 2 Mai, Cap Midia, and Sulina, corresponding to key coastal and nearshore habitats used by the species prior to spawning migration.

The Black Sea individuals were captured using fixed gillnets with a mesh size of 32-40 mm, a maximum net length of 100 m, a maximum net height of 8 m, and a twine diameter ranging from 0.2 to 0.3 mm, while the Danube River samples were captured using drift gillnets with a mesh size of 30-35 mm, as stated in Order 59/2021. The individuals were randomly selected to ensure the representation of individuals across all length classes. Total length (TL, cm) was measured with an ichthyometer, with an accuracy of 0.1 cm and sex was determined by gonadal inspection.

Length-frequency distributions were constructed using 1-cm class intervals. Class-based percentage frequencies were calculated separately for each year and habitat prior to statistical analyses.

Individuals in this study were classified as mature at a threshold length of 22 cm in the Black Sea. Pontic shad typically reaches sexual maturity at 2-3 years of age (Balik, 2019), corresponding to body lengths of approximately 20-30 cm (Stroe et al., 2021). The minimum legal retention size under current fisheries regulations is 22 cm (Order no. 304/2023). This threshold of 22 cm ensure consistency between biological interpretation and management practice, although actual maturity may vary within this size interval.

Statistical analyses. Length-frequency data were available as percentage distributions by 1-cm size classes for each year and habitat. Because individual-level observations were not available, proportional distributions were transformed into pseudo-individual datasets by replicating each class midpoint according to its reported percentage frequency. This reconstruction preserves the relative structure of the empirical distributions and allows application of distribution-based non-parametric tests that require individual-level input. The procedure represents an analytical transformation of proportional data and does not introduce additional biological information. Consequently, statistical inference focuses primarily on distributional divergence (K-S Dstatistic) rather than on absolute frequency-based significance levels.

Interannual variation in size structure was analysed separately for the marine and riverine components. Interannual and sex-specific length-frequency distributions were compared using the Kolmogorov-Smirnov test, a non-parametric method suitable for aggregated length-class data that evaluates differences in distributional shape and position and is widely applied in fisheries and population ecology when assumptions of normality and independence are not met (Sokal & Rohlf, 1995; Anderson & Neumann, 1996; Quinn & Deriso, 1999; Zar, 2010).

To account for multiple pairwise comparisons, p-values were adjusted using both false discovery rate (FDR) and Bonferroni corrections.

Interannual differences in the proportions of juvenile and mature individuals were assessed using the chi-square test, appropriate for categorical, frequency-based data with independent yearly groups (Sokal & Rohlf, 1995; Anderson & Neumann, 1996; Zar, 2010)

All statistical analysis were made using RStudio/2025.09.2+418.

To facilitate visual interpretation of modal structure in the length-frequency distributions, primary and secondary peaks were identified for each year based on the relative frequency of length classes. Using proportional length-frequency data, the two length classes with the highest percentages within each annual distribution were extracted. The class with the highest frequency was defined as the primary modal class, while the class with the second-highest frequency was considered the secondary modal class. These modal classes were used solely as descriptive indicators of distributional structure and were illustrated in the figures using dashed vertical lines. Because modal identification was based on aggregated proportional data, the resulting peaks represent the most prominent frequency classes rather than statistically validated local maxima.

Figures and maps. All figures were created using RStudio/2026.01.1+403 version.

RESULTS AND DISCUSSIONS

The size composition of Pontic shad catches differed between the Black Sea and the Danube River and varied among years between 2020 and 2025. In the Black Sea, captured individuals covered a wide range of total lengths, from 17 to 38 cm, with most of the catch consistently concentrated between 26 and 33 cm across years. The highest relative frequencies were generally observed in the 28-31 cm length classes, particularly in 2021-2023. Similar mean body sizes were reported for Pontic shad in the Black Sea by Tiganov et al. (2016), who observed an average total length of approximately 30.7 cm in commercial catches, while a comparable length range (18-36 cm) was previously documented in 2013 (Tiganov et al., 2013). For the Turkish part of the Black Sea, the maximum length in catches was 34.2 cm for *A. immaculata* (Erguden et al., 2011).

In the Danube River, catches were more restricted to larger size classes, with individuals ranging between 25 and 36 cm. Length classes below 23 cm were absent or rare in most years. The modal length classes were typically between 28 and 32 cm, with a pronounced contribution of larger individuals in several years, especially from 2020 onwards. For instance, Grecu et al. (2020) reported length classes ranging from 29 to 34.5 cm in 2020, whereas Ibănescu et al. (2017) documented a broader size spectrum, spanning 24-39 cm. For the Bulgarian sector of the Danube River, Kirin et al. (2023) found a maximum length in captures of 30 cm.

Sex composition of Pontic shad catches showed interannual variability in both study areas between 2020 and 2025 (Table 1). In the Black Sea, females generally represented a higher proportion of the annual samples, accounting for 49.4-64.2% of individuals in most years, except for 2022, when males predominated (56.0%). A female-dominated sex structure in Romanian Black Sea catches was also reported by Tiganov et al. (2016). In the Danube River, females also tended to be more abundant, representing between 50.9% and 64.2% of the catches, while males accounted for 35.8-49.1%. Same female predominance was reported also by Leonov et al. (2022) in 2022

Table 1. Sex composition (%) of Pontic shad catches by study area (Black Sea and Danube River) and year (2020-2025)

Area	Black Sea	Black Sea	Black Sea	Black Sea	Black Sea	Black Sea	Danube	Danube	Danube	Danube	Danube	Danube
Year	**2020**	**2021**	**2022**	**2023**	**2024**	**2025**	**2020**	**2021**	**2022**	**2023**	**2024**	**2025**
F	54.76	56.18	44.00	49.42	58.15	64.18	53.73	52.48	58.82	50.89	55.54	64.24
M	45.24	43.82	56.00	50.58	41.85	35.82	46.27	47.52	41.18	49.11	44.46	35.76
	100	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**

The predominance of females observed in both marine and riverine catches likely reflects a combination of biological and methodological factors. Sexual size dimorphism in Pontic shad, with females typically attaining larger body sizes (Navodaru, 2001; Năstase, 2018; Leonov et al., 2022), increases their probability of retention in gillnets targeting commercial size classes (Hamley, 1975; Millar & Mainguy, 2025). In marine habitats, sex-specific spatial distribution or differences in feeding intensity may further influence capture rates. During spawning migration in the Danube River, the female-biased structure may additionally reflect differential reproductive investment, as fecundity in clupeid fishes increases with body size and larger females contribute disproportionately to reproductive output (Dobre et al., 2025).

Size-dependent participation in migration has been widely described in anadromous fishes (Quinn & Myers, 2004; Secor, 2015). Together, these biological characteristics, combined with gear selectivity, likely explain the recurrent female dominance across habitats. Across both study areas, the relative contribution of sexes fluctuated among years.

Size structure of the Black Sea component

The Black Sea represents the primary feeding and growth habitat of Pontic shad, where population structure reflects the combined effects of recruitment variability, growth processes, and migratory dynamics. Accordingly, the size structure of the marine component provides insight into interannual changes in cohort composition prior to spawning migration.

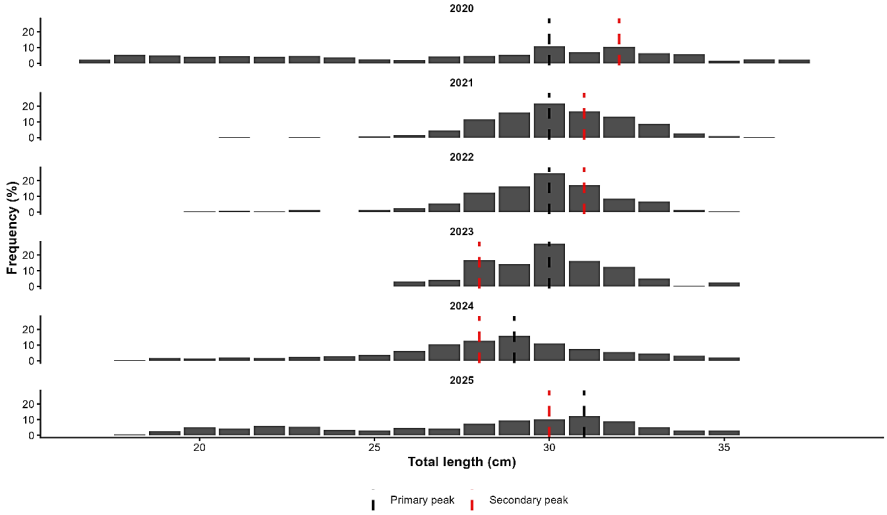


Figure 2. Length-frequency structure of Pontic shad in the Black Sea by year (Source: original)

Figure 2 illustrates pronounced interannual variation in the length-frequency distributions of Pontic shad within the Black Sea feeding component between 2020 and 2025. The vertical arrangement facilitates direct comparison of modal displacement across consecutive years, allowing visual inspection of potential cohort progression.

The 2020 distribution is relatively broad and includes a higher contribution of smaller size classes, as evidenced by the extended left tail of the histogram and the presence of a secondary peak below the dominant modal class around 29-31 cm. In that year, length classes below approximately 26-27 cm contribute noticeably higher proportions compared to subsequent years, resulting in a wider overall spread of the distribution. In contrast, distributions observed during 2021-2023 are more concentrated and dominated by intermediate to larger individuals, suggesting a phase of relative structural stability and modal consolidation around 29-31 cm.

The distributions recorded in 2024 and particularly in 2025 appear more dispersed and partially shifted toward smaller size classes,

indicating renewed variability in cohort representation in the most recent years.

Interannual variability is also evident in the relative prominence and position of secondary peaks, indicating fluctuations in cohort representation. In the figure, dashed vertical lines indicate the primary and secondary modal classes, defined as the length classes with the highest and second-highest frequencies, respectively; their relative position along the length axis may vary among years depending on cohort composition**.** Although these modal shifts may suggest cohort progression, confirmation would require age-based data.

These visual patterns are strongly supported by the Kolmogorov-Smirnov analysis, which detected highly significant differences in length-frequency distributions across all interannual comparisons. Biological interpretation should therefore focus primarily on the magnitude and temporal pattern of K-S Dstatistic values rather than on p-values alone, given the large pseudo-sample sizes used to represent proportional data.

Large effect sizes between 2020 and 2021-2023, as well as between 2025 and all preceding years, point to major structural shifts in population composition, while comparatively lower D values among 2021-2023 indicate a phase of relative stability in the Black Sea stock. Consistent with these results, the chi-square analysis revealed significant interannual variation in the relative proportions of juvenile and mature individuals, further supporting the presence of marked temporal variability in size structure rather than a static demographic pattern. Taken together, the concordant outcomes of distribution-based (K-S) and proportion-based (χ^2) analyses indicate that the Black Sea feeding component exhibits year-specific variability in cohort composition. These differences are best interpreted as interannual fluctuations in size structure rather than as evidence of long-term demographic change.

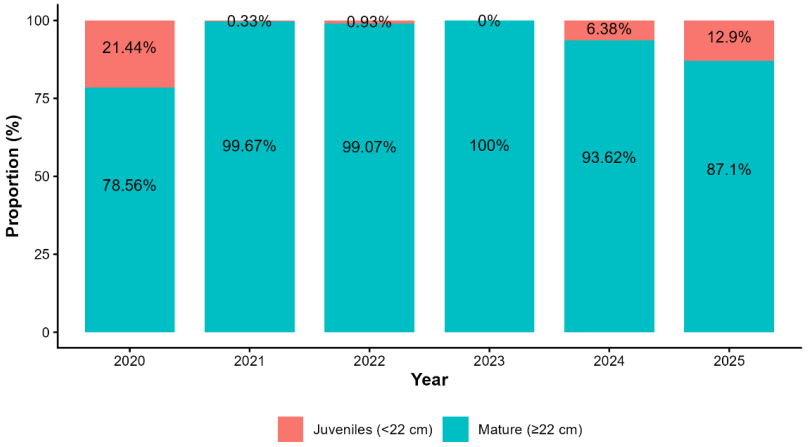


Figure 3. Interannual variation in the proportion of juvenile and mature Pontic shad in the Black Sea (Source: original)

In addition to distributional shape and central tendency, interannual changes in size structure within the Black Sea feeding component are further illustrated by the relative contribution of juvenile and mature individuals (Figure 3). The stacked bar chart shows pronounced interannual variability in the proportion of juveniles (<22 cm) and mature individuals (≥22 cm), a pattern that is statistically supported by the chi-square test, which detected highly significant differences among years (χ^2 = 5748.2, df = 5, $p < 0.001$). Standardized residuals indicate a higher-than-expected contribution of juveniles in 2020 and 2025, whereas the period 2021–2023 was characterized by an overwhelming predominance of mature individuals.

Years dominated by mature individuals (2021-2023) coincide with relatively concentrated length-frequency distributions, suggesting a phase of cohort progression and dominance of larger size classes within the marine feeding

assemblage. In contrast, the increased juvenile contribution observed in 2020 and especially in 2025 corresponds to broader distributions, indicating a greater relative presence of smaller size classes during these years.

Although the Black Sea represents the primary feeding and growth area for Pontic shad, persistent juvenile dominance is not expected, as early life stages primarily occupy coastal and nearshore habitats and are typically underrepresented in offshore samples. Additionally, gear selectivity may further reduce the capture probability of smaller individuals, as gillnets retain fish within specific size ranges determined by mesh size (Tiganov et al., 2022). Consequently, the low proportion of juveniles in marine samples likely reflects both ontogenetic habitat segregation and methodological constraints. Consequently, the observed interannual fluctuations in juvenile contribution likely reflect variability in recruitment strength, timing of offshore habitat use, and migration dynamics, rather than a fixed ecological pattern. Collectively, the concordant outcomes of the chi-square analysis, distribution-based comparisons (K-S), reinforce the interpretation that the marine feeding component exhibits dynamic, year-specific cohort composition, emphasizing the importance of interannual perspectives when evaluating population structure and demographic processes in the Black Sea-Danube system.

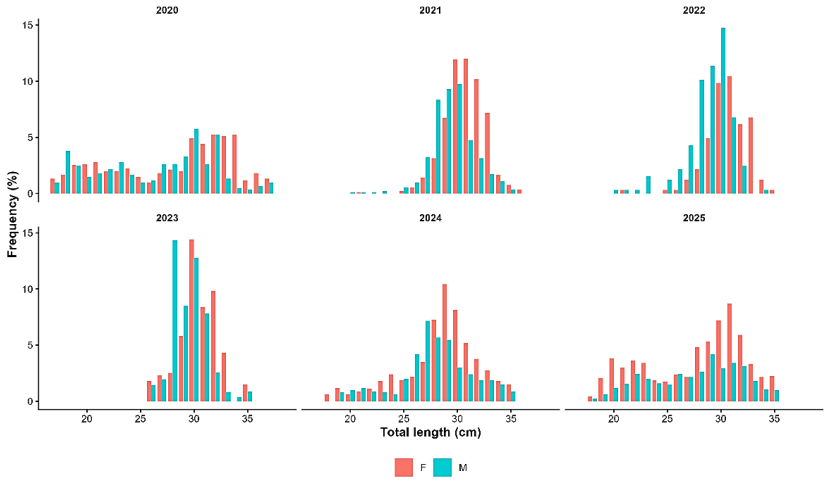


Figure 4. Sex-specific length-frequency distributions of Pontic shad by year in the Black Sea (Source: original)

Sex-specific patterns in size structure within the Black Sea feeding component are illustrated in Figure 4, which shows female and male length-frequency distributions for each year between 2020 and 2025. Visual inspection of the histograms indicates consistent differences in distributional shape, modal length, and spread between sexes, although the degree of overlap varies among years. In several years (notably 2021-2023), females and males exhibit partially distinct modal classes, whereas in other years (e.g., 2025) their distributions appear more similar, suggesting higher overlap in size composition.

Overall, these results indicate persistent but temporally variable sex-related differences in size structure within the marine feeding assemblage. Such differences may reflect sex-specific growth trajectories, differential habitat use, or variation in migration timing between females and males, although these interpretations remain indicative given the aggregated nature of the data. The consistently strong statistical significance across years highlights the sensitivity of distribution-based methods applied to large pseudo-samples derived from length-class frequencies.

Interannual variability in size structure within the Black Sea feeding component highlights the role of migratory selectivity in shaping marine assemblages. The predominance of larger individuals suggests that the Black Sea

functions primarily as a feeding and growth habitat for subadult and adult Pontic shad, whereas early juveniles are more frequently associated with shallow coastal and delta-influenced habitats, leading to their reduced representation in offshore samples. Consequently, the marine size structure does not represent a random cross-section of the population but reflects selective habitat use linked to ontogenetic stage and migration timing.

The reproductive structure inferred from the marine component suggests that temporal shifts in size composition are more likely driven by interannual variation in cohort representation and migration timing than by abrupt changes in the overall abundance of spawning individuals. Across all years, a consistent modal peak around 29-31 cm is observed, indicating the persistent presence of a dominant subadult/adult cohort in the marine feeding component. Periods characterized by higher relative juvenile contribution reflect the additional presence of smaller size classes superimposed on this stable modal structure, rather than a replacement of the larger cohort. These fluctuations therefore suggest variability in cohort representation rather than shifts in overall size dominance.

These patterns underscore the importance of interpreting marine size distributions within the context of the Black Sea-Danube migratory continuum, rather than as standalone indicators of reproductive potential.

Size structure of the Danube River component

In contrast to the marine feeding component, the Danube River represents the reproductive migration corridor of Pontic shad, where size structure is shaped by maturation selectivity and spawning-related processes rather than by recruitment or feeding dynamics. The ecological importance of the lower Danube and its distributaries as selective reproductive habitats have been extensively documented for anadromous fishes (Lenhardt et al., 2006; Danalache et al., 2017).

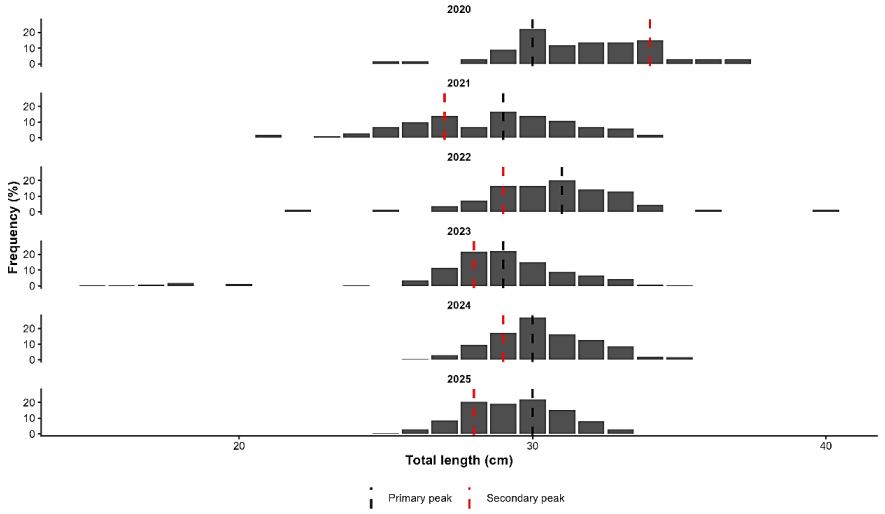


Figure 5. Length-frequency structure of Pontic shad in the Danube River by year (Source: original)

The annual length-frequency histograms for the Danube component (Figure 5) reveal clear interannual variation in modal structure and dispersion. For example, spawning seasons 2022 and 2024 are visually dominated by intermediate size classes (~28-31 cm), where the primary modal peak is sharply defined and frequencies are concentrated within a relatively narrow range. In contrast, 2020, 2021, and 2025 display broader distributions with increased representation of both smaller and larger individuals, resulting in a wider spread of the histogram and the appearance of secondary peaks. These features indicate the

simultaneous presence of multiple size cohorts within the spawning assemblage during those years. In the figure, dashed vertical lines mark the primary and secondary modal classes, corresponding to the length classes with the highest and second-highest frequencies within each annual distribution.

These visual differences are quantitatively supported by Kolmogorov-Smirnov comparesons, which detected significant distributional divergence between all year pairs (adjusted $p < 0.001$). The largest effect sizes (K-S D statistic) were observed between structurally contrasting years, such as 2020-2023 (D = 0.488) and 2020-2025 (D = 0.393), reflecting pronounced shifts in distributional shape. Conversely, lower D values between more similar years (e.g., 2024-2025: D = 0.196) indicate partial structural overlap. Together, the concordance between histogram patterns and D statistics demonstrates substantial interannual variability in the size structure of the Danube spawning population.

Across all analyzed years (2020-2025), the Kolmogorov-Smirnov test revealed large and highly significant differences between female and male length distributions (D = 0.41-0.62, $p_adj < 0.001$), confirming persistent sexual size dimorphism within the spawning population. Females consistently dominated larger size classes (~29-33 cm), whereas males were more frequently concentrated in slightly smaller ranges (~26-30 cm), with limited overlap.

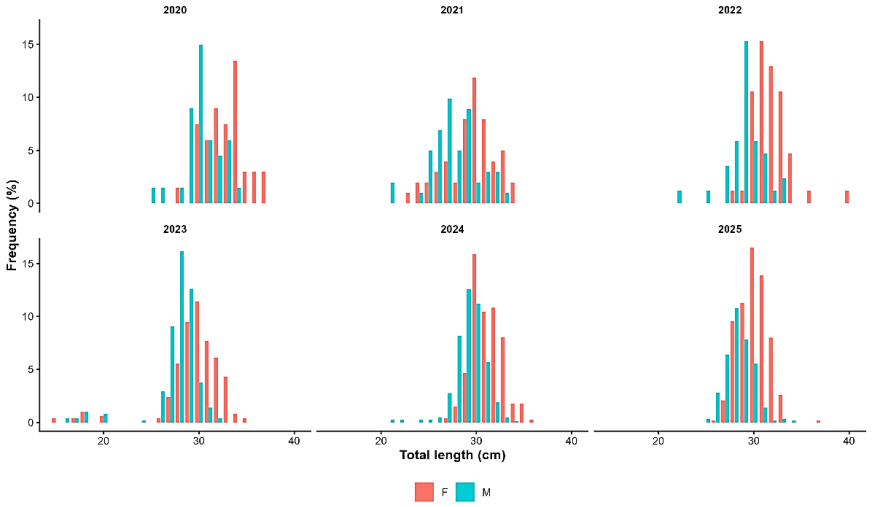


Figure 6. Sex-specific length-frequency distributions of Pontic shad by year in the Danube River (Source: original)

Importantly, interannual shifts in modal structure are evident within both sexes (Figure 6), indicating that year-to-year variability is not merely a consequence of sex aggregation but reflects genuine changes in cohort representation. Thus, the combined analysis captures overall demographic structure, while sex-specific patterns confirm a stable size hierarchy characteristic of the reproductive component.

Together, these results may indicate that the Danube River component reflects a biologically coherent reproductive assemblage, in which females attain larger sizes than males, consistent with differential energetic allocation to gonadal development and reproductive output. The concordance between statistical tests, and graphical patterns may underscore a robust and temporally stable biological signal, rather than sampling or methodological artifacts.

Conceptual framework linking marine feeding and riverine reproductive components of Pontic shad through size-selective migration in the Black Sea-Danube system

Figure 7 synthesizes this relationship conceptually, illustrating how size structure emerging from the marine feeding component is selectively transferred to the riverine reproductive habitat through spawning migration.

This framework is consistent with ecological models describing anadromous fishes as vectors linking marine feeding habitats to freshwater reproduction, thereby coupling energy flow and demographic processes across ecosystems (Gross et al., 1988; MacAvoy et al., 2009; Dodson et al., 2013).

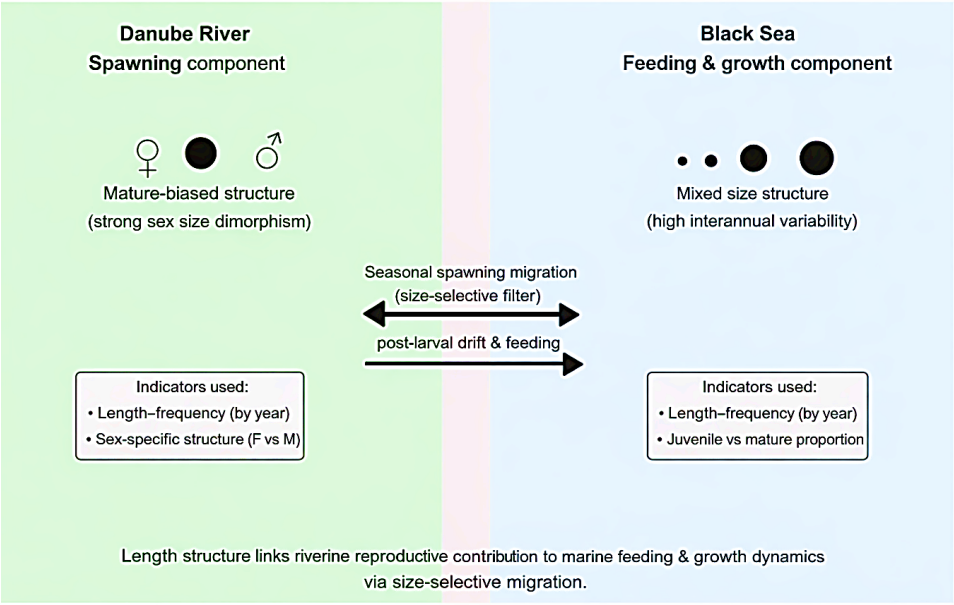


Figure 7. Length structure integrating marine feeding dynamics with riverine reproductive contribution through size-selective migration (Source: original)

Anadromous migration is widely interpreted as an adaptive strategy coupling productive marine feeding habitats with freshwater reproductive environments, mediated by size- and condition-dependent migratory decisions (Dodson et al., 2013; Schindler et al., 2015; Secor, 2015).

Within this continuum, participation in reproductive migration functions as a biologically selective process, whereby larger and sexually mature individuals exhibit a higher probability of upstream movement, particularly females. This size- and sex-dependent participation pattern has been widely documented for Pontic shad and other anadromous clupeids (Navodaru, 2001; Raikova-Petrova, 2013; Lenhardt et al., 2016; Năstase et al., 2018; Leonov et al., 2022; Quinn & Myers, 2004; Secor, 2015).

In this context, the size structure observed in the Black Sea may function as an upstream demographic signal that, after a maturation lag and size-selective migration, is progressively translated into the structure of the spawning population in the Danube. Pontic shad typically reaches sexual maturity at approximately 2-3 years of age (Navodaru, 2001; Raikova-Petrova, 2013; Lenhardt et al., 2016), implying that size cohorts observed in the marine feeding grounds may contribute to the spawning migration after a temporal lag. Consequently, fluctuations in marine length structure may contain predictive information regarding the composition of subsequent spawning runs. However, confirming such anticipatory capacity would require quantitative indicators of abundance (e.g., standardized CPUE) in both marine and riverine components, which were beyond the scope of the present study (Anderson & Neumann, 1996; Quinn & Deriso, 1999; Shin et al., 2005).

Research limitations

Several limitations should be acknowledged. The analyses were based on aggregated length–frequency data rather than individual-level measurements, and interpretations therefore reflect population-level structure rather than

individual growth trajectories or reproductive status. In addition, sampling reflects the inherent size selectivity of fishing gear and monitoring programs and may underrepresent early life stages or nearshore components of the population. However, this limitation primarily affects the representation of smaller size classes within each habitat and does not account for the consistent differences observed between the marine and riverine components, which are more plausibly explained by biological processes related to maturation and migration.

Finally, environmental drivers and fishing effort were not explicitly modelled, limiting inference on causal mechanisms underlying the observed variability.

The study focused on structural indicators and did not include standardized abundance indices (e.g., CPUE or biomass estimates), limiting inference regarding absolute changes in spawning stock size or recruitment magnitude. Age determination was not conducted; consequently, cohort progression is inferred from modal displacement rather than validated through age-structured analysis. The six-year time series may also be insufficient to detect longer-term demographic cycles. Additionally, hydrological drivers and fishing effort were not explicitly modelled, although they likely contribute to observed variability. Finally, the reconstruction of pseudo-individual datasets from proportional data may influence nominal statistical power; therefore, interpretation emphasizes effect sizes and distributional patterns rather than p-values alone.

CONCLUSIONS

This study provides an integrated assessment of interannual variability in the size structure of Pontic shad (*Alosa immaculata*) across the Black Sea-Danube system, highlighting the value of size-based metrics for linking marine feeding dynamics with riverine reproductive contribution. Analyses of length-frequency distributions and juvenile-mature proportions, consistently revealed pronounced year-specific variability in the marine feeding component, rather than a monotonic trend over the 2020-2025 period.

The Black Sea component was characterized by dynamic cohort representation, with significant interannual shifts in distributional shape and size composition. In contrast, the Danube River component displayed a more selectively filtered structure dominated by larger, sexually mature individuals, particularly females, supporting the interpretation of the river as a size-selective reproductive habitat. These patterns are consistent with the role of spawning migration as a size-selective biologicalfilter that transfers variability generated in marine feeding habitats into freshwater reproductive output.

Despite these constraints, this study contributes novel, system-level insight into how interannual variability in size structure propagates across the Black Sea-Danube continuum. By integrating marine and riverine components within a unified analytical framework, the results underscore the importance of considering habitat connectivity and size-selective migration when assessing anadromous fish populations.

Future research should combine size-based indicators with individual-based data, such as age, condition, reproductive status, and tagging or telemetry information, to better resolve the mechanisms driving cohort variability and migration timing. Integrating environmental covariates and fishing pressure into dynamic models would further strengthen understanding of how climatic and anthropogenic factors influence population structure and reproductive contribution in the Black Sea-Danube system.

Beyond its structural insights, this study highlights the potential management value of marine length–frequency information as an early indicator of spawning run composition in the Danube. If supported by future integration of standardized abundance indices (e.g., CPUE) and hydrological covariates, fluctuations in size structure at sea could contribute to anticipatory management of the riverine fishery by providing advance signals of spawning population structure. Such an approach would strengthen adaptive management within the Black Sea–Danube system by linking marine monitoring to riverine fishery regulation.

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Research on the influence of hydroclimatic changes on the stocks and migration of Danube shad (Alosa immaculata) schools, from the mouth of the Danube to the Iron Gates 2 Dam.

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LENGTH–WEIGHT RELATIONSHIP AND GROWTH OF ARTIFICIALLY REARED DANUBE SALMON (*Hucho hucho*, Linnaeus 1758)

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Abstract

The Danube salmon, Hucho hucho (Linnaeus, 1758), is a species of high economic and conservation interest, for which aquaculture plays an essential role in stock maintenance and restocking programs. This study presents biometric and growth-related information on H. hucho specimens reared under controlled farming conditions at the Valea Bistrişorii Trout Farm, Alba County, Romania. Fish were captured using routine aquaculture handling methods and measured to assess the length-weight relationship and relative condition factor. The results indicated a positive allometric growth pattern, suggesting suitable rearing conditions. The relative condition factor showed variation among individuals, reflecting differences in growth performance within the stock. These findings provide useful data on the growth characteristics of farm-reared Danube salmon and support the importance of aquaculture facilities in research and management of this species.

Key words*: biometric assessment, captive rearing, growth performance, salmonid culture, stock management.*

INTRODUCTION

The Danube Salmon (*Hucho hucho)* is regarded as one of the most distinctive and aesthetically remarkable species within the Salmonidae family, owing to its morphological features and significant ecological role. The species exhibits an impressive and elegant appearance, with adult individuals frequently exceeding 1 meter in total length in natural environments (Holčík et al.,1988; Ihuț et al., 2014; Kottelat & Freyhof, 2007). Its morphology is characterized by a streamlined, fusiform body adapted to fast-flowing waters, a large head, and a silvery coloration with brownish-red reflections, all of which contribute to its aesthetic and biological value (Holčík et al.,1988; Ihuț et al., 2014).

From an ecological perspective, *Hucho hucho* functions as an apex predator in montane river ecosystems, playing a crucial role in maintaining trophic balance. The species is endemic to the Danube River basin and is currently undergoing a pronounced population decline (Ford, 2024). The Danube Salmon inhabits cold, well-oxygenated, and highly natural river sections, which makes it a reliable indicator of minimally disturbed freshwater ecosystems. It inhabits cold, well-oxygenated, natural river sections, making it a reliable indicator of minimally disturbed freshwater ecosystems. Its conservation depends on preserving river habitats in the Danube basin; therefore, it is widely recognised as a flagship species for conservation strategies (Ihuț et al., 2014; Pinter et al., 2024). The European huchen *(Hucho hucho)* is an endangered salmonid flagship species, yet detailed biological parameters for populations under intensive

culture remain disproportionately scarce compared to their wild counterparts. The present study establishes an important reference point that complements existing literature, enabling the comparison of growth patterns across different environments. Integrating these data with previous research provides the necessary framework to quantify how habitat degradation impacts the species' physiological fitness.

This study aims to evaluate the growth patterns and physiological status of *Hucho hucho* over three years (2013-2015) using length-weight relationships (LWR) and condition indices. By comparing Fulton's (*K*) and relative (*K*n) condition factors, the research seeks to identify inter-annual variations in fish well-being and determine the specific type of allometric growth. These findings provide essential baseline data for the monitoring and sustainable management of this species in its natural habitat.

MATERIALS AND METHODS

Fish Rearing Conditions and Feeding Regime

Huchen *(Hucho hucho)* specimens were reared in a conventional concrete tank (2.2 m × 2.0 m) under a continuous flow-through water system. The experimental stock consisted of 150 individuals belonging to the same age cohort, reared under identical conditions in a single concrete flow-through tank. Each month, 50 fish were randomly sampled from the tank and individually weighed. As fish were not individually marked, sampling was conducted at the population level and did not involve tracking of specific individuals over time. The mean initial body weight (BW) was 22.45 ± 0.79 g, and the mean total length (TL) was 14.25 ± 0.167 cm. Fish were fed commercially formulated diets produced by Aqua Garant, with pellet size adjusted according to age class and developmental stage. The feeding program included Aqua Start 1.5 mm pellets for fry weighing 5-15 g and measuring 8-13 cm TL; Aqua Dynamic 2 mm pellets for juveniles weighing 15-50 g and measuring 13-18 cm TL; Aqua Dynamic 3 mm pellets for fish weighing 50-150 g and measuring 18-25 cm TL; and Aqua Dynamic 4 mm pellets for huchen weighing 150-350 g and measuring 25-33 cm TL. Feed was provided ad libitum using an automatic feeder throughout the study period. The water temperature in this salmonid rearing unit fell within the optimal range for salmonid culture, ranging from a minimum of 2.6°C in December to a maximum of 19.3°C in August.

Ethical approval

This study was approved by the Animal Ethics Committee of the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca (Approval No. 117/2018).

Fish body measurements

The morphometric data collection followed the general principles for fish analysis. For each specimen, two primary parameters were recorded: total length (TL) and body weight (BW). Total length was measured from the tip of the snout to the end of the caudal fin (to the nearest 0.1 cm), while body weight was determined using a high-precision digital balance (to the nearest 0.01 g) (Lațiu, 2025).

Length-weight relationship

The length-weight relationship (LWR) was estimated using the power function $BW = aTL^b$. To linearize the relationship, the data were log-transformed: log BW = log a + b log TL, where BW is the wet body weight of fish, TL is the total length, a is the intercept of the regression and b is the slope of the regression. To assess the growth type, the calculated allometry coefficient (b) was compared against the theoretical isometric value ($b = 3$) using the Student's t-test: $ts = (b - 3)/Sb$. Growth was categorized as isometric ($b = 3$), positive allometric ($b > 3$), or negative allometric ($b < 3$) (Le Cren, 1951; Froese, 2006; Simonović et al., 2011).

Condition factors

The physiological status of the specimens was evaluated using two complementary indices:

Fulton's Condition Factor (*K*): Calculated as $K = 100 * (BW/TL^3)$, where BW is the body weight (g) and TL is the total length (cm). In this isometric model, the power of 3 reflects the cube law, assuming that fish shape and density remain constant as the fish grows.

Relative Condition Factor (*K*n): Calculated as $Kn = BW_o/BW_p$, where BW_o is the observed weight and BW_p is the predicted weight derived from the specific LWR of the population ($BW = aTL^b$). Fulton's condition factor was used to compare the general well-being assuming a cubic growth, while the relative condition factor was included to compensate for the allometric

growth observed in specific years (Froese, 2006; Lațiu et al., 2023).

Fish sampling

All analyzed specimens of *Hucho hucho* originated from the same population. The variation in the number of individuals across the sampling years (2013, 2014, and 2015) resulted from a non-standardized, opportunistic sampling strategy. Data collection was performed across different months within each year, and the sample sizes reflect the natural availability and accessibility of the specimens during those specific periods. Consequently, the fluctuating number of individuals per year represents a series of seasonal snapshots of the same population rather than a pre-determined experimental design.

Statistical Analysis

Condition indices (*K* and *K*n) were compared across the sampling years (2013, 2014, and 2015) and for the pooled dataset. Data normality was assessed using the Shapiro-Wilk test. Since the data deviated significantly from a normal distribution, non-parametric methods were employed. The Kruskal-Wallis *H* test was used to identify overall differences between groups, followed by Dunn's post-hoc test for pairwise multiple comparisons to determine specific inter-annual variations.

All statistical analyses and graphical representations were performed using GraphPad Prism version 8.0.1 (GraphPad Software, San Diego, California, USA). The threshold for statistical significance was set at alpha = 0.05.

RESULTS AND DISCUSSIONS

Fulton condition factor K

Descriptive statistics for Fulton's condition factor (*K*) showed an overall mean of 0.8485 ± 0.1004. The lowest mean condition was recorded in 2013 (0.7663 ± 0.049), while the highest values were observed in 2014 (0.8695 ± 0.111) and 2015 (0.8614 ± 0.068). The coefficient of variation was notably higher in 2014 (12.87%), indicating a wider disparity in fish physiological status during that period compared to the more homogenous group from 2013 (6.43%). Regardless of the year, the low standard error of the mean (SEM < 0.005) indicates high reliability of the estimated indices.

The Kruskal-Wallis test revealed highly significant differences in the condition factor (*K*) across the studied periods ($H = 246.4$, $p < 0.0001$). Subsequent pairwise comparisons using Dunn's post-hoc test showed that the condition of fish in 2013 was significantly lower than in all other groups, including the pooled dataset ($p < 0.0001$ for all comparisons involving 2013).

In contrast, no significant difference was observed between the years 2014 and 2015 ($p > 0.9999$, ns). Additionally, both 2014 and 2015 showed significantly higher condition indices compared to the overall pooled average ($p < 0.0001$ and $p = 0.0089$, respectively), suggesting that the physiological status of the population improved notably after the first study year.

Relative condition factor Kn

Descriptive statistics for the relative condition factor (*K*n) showed mean values very close to unity across all analyzed periods: 1.002 ± 0.064 in 2013, 1.008 ± 0.121 in 2014, and 1.003 ± 0.073 in 2015, with an overall pooled mean of 1.006 ± 0.118 (Figure 1). The Kruskal-Wallis test indicated no significant differences in *K*n among the studied years ($H = 5.206$, $p = 0.1573$). This lack of significance was further confirmed by Dunn's post-hoc test, which showed no significant pairwise differences between any of the analyzed years or the pooled dataset ($p > 0.05$ for all comparisons). These results suggest that when the specific allometric growth pattern of the population is taken into account, the relative physiological status of the fish remained remarkably stable throughout the study period, regardless of the variations observed in Fulton's condition factor.

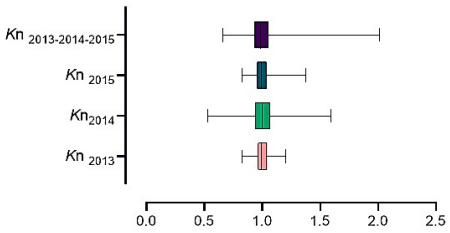


Figure 1. Relative condition factor *K*n

Length-weight relationship

All fish belonged to the same cohort, and the observed differences among years reflect

growth progression of the individuals over time. The length-weight relationship (LWR) parameters and growth patterns for *Hucho hucho* across the study periods are summarized in the table. For the years 2013 and 2014, the allometry coefficient (*b*) values were 2.964 and 2.971, respectively (Figure 2,3). The Student's t-test confirmed that these values did not differ significantly from the theoretical value of 3 ($t_{calculated} < t_{critical}$, $p > 0.05$), indicating an isometric growth (ISO) for these cohort.

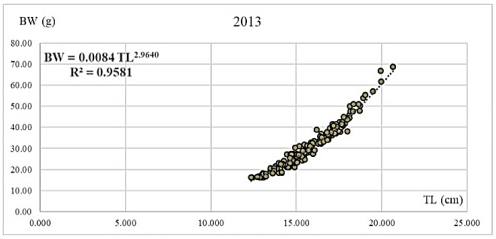


Figure 2. LWR of *H. hucho* in 2013

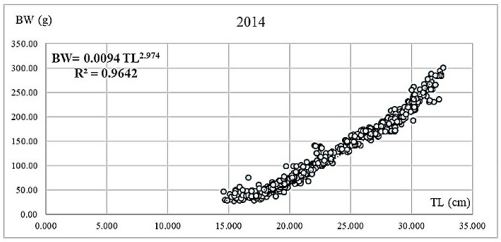


Figure 3. LWR of *H. hucho* in 2014

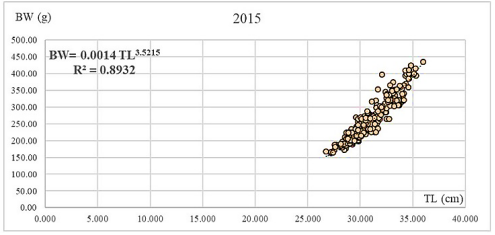


Figure 4. LWR of *H. hucho* in 2015

In contrast, the year 2015 showed a significantly higher allometry coefficient (*b* = 3.5215) (Figure 4) with the t-test revealing a highly significant difference ($t_{calculated} = 7.3824$, $p < 0.05$). This indicates a positive allometric growth (ALLO+), suggesting that the fish gained weight more rapidly relative to their length during this period. For the pooled dataset (2013-2015, n = 1100), the overall growth was also significantly positive allometric (b = 3.1020, $t_{calculated} = 8.6808$, $p < 0.05$). High coefficients of determination ($R^2 > 0.89$) were recorded for all models, demonstrating a strong correlation between length and weight across all sampled years.

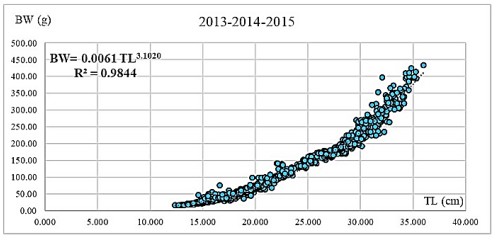


Figure 5. LWR of *H. hucho* in the 3 year analyzed period

Comparable studies on wild populations of *Hucho hucho* have reported b-values ranging from 3.25 to 3.39 (Andreji & Stráňai, 2013; Lațiu et al., 2022), indicating positive allometric growth under natural riverine conditions. For example, individuals from the Ruscova River (Romania) exhibited a b-value of 3.3879, which is consistent with the results obtained in the present study (Lațiu et al., 2022). Similarly, cultured populations have also shown positive allometric or near-isometric growth, with reported b-values ranging from 3.256 to 3.774, depending on rearing conditions and broodstock origin (Andreji & Stráňai, 2013). In contrast, Savić (2022), studying huchen fingerlings reared under cultivated conditions, reported a lower regression coefficient (b = 2.822), indicating negative allometric growth, together with a mean condition factor of 0.82. In addition, Simonović et al. (2011) emphasized that huchen growth is strongly influenced by both age and environmental conditions, with notable differences in growth performance between natural river systems and aquaculture settings. In the present study, huchen sampled during 2013 exhibited isometric growth. These results suggest a more balanced increase in length and weight during the juvenile stage,

Table 1. The length-weight relationship parameters and growth patterns for *Hucho hucho*

Year	N	Total length (mm)			Body weight (g)			Parameters of LWRs					Test-$t_{0.05}$		Growth type
		TL min.	TL. max	TL mean+-SD	BW min.	BW max.	BW mean+-SD	*a*	*b*	*a* CI95%	*b* CI95%	R^2	$t_{calculated}$	$t_{critical}$	
2013	200	12.35	20.66	15.74±1.623	16.15	68.73	30.84±9.916	0.00844	2.96404	0.00664-0.01073	2.87695-3.05113	0.95810	0.8142	1.972	*b*=3 (ISO)
2014	600	14.59	32.57	23.38±4.901	27.5	300.4	124.7±70.95	0.00945	2.97116	0.00818-0.01091	2.92515-3.01718	0.96420	1.2308	1.964	*b*=3 (ISO)
2015	300	26.75	35.96	30.92±1.825	163.3	434.4	258.8±58.83	0.001144	3.52154	0.00089-0.00231	3.38251-3.66057	0.89323	7.3824	1.968	*b*>3 (ALLO+)
2013/ 2014/ 2015	1100	12.35	35.96	24.05±6.338	16.15	434.4	144.2±99.16	0.00612	3.10205	0.00569-0.00658	3.07899-3.12512	0.98449	8.6808	1.9621	*b*>3 (ALLO+)

TL - total length; BW - body weight; SD - standard deviation;

a - the intercept of the regression; b - the slope of the regression.

representing an intermediate growth pattern between the negative allometric growth reported by Savić (2022) and the positive allometric growth observed in both wild and older cultured huchen populations.

Overall, these findings confirm that length-weight relationships in *Hucho hucho* vary considerably depending on age class and environmental conditions, with juvenile stages often showing more balanced growth patterns compared to later developmental stages or differently managed populations.

CONCLUSIONS

The study revealed significant inter-annual variations in the growth patterns and physiological status of *Hucho hucho*. The allometric growth shifted from isometric in 2013 and 2014 to positive allometric in 2015, indicating an increase in body robustness over time. While Fulton's condition factor (*K*) showed significant differences between years, suggesting a lower condition in 2013, the relative condition factor (*K*n) remained stable and close to unity throughout the study. This demonstrates that when accounting for the population's specific allometry, the overall well-being of the fish was consistently maintained across all sampling periods.

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PARASITES AND PARASITE COMMUNITIES OF FRESHWATER FISH FROM THE LUDA YANA RIVER

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Abstract

The aim of the study is to investigate and track 1) differences in parasite communities between mining-influenced biotopes versus wastewater impacts, and 2) expected relationships between parasite load and Fulton condition factor. The species composition and dominant structure of the fish complexes in the two biotopes were compared. The dominant species in both biotopes is Alburnus alburnus (Linnaeus, 1758), followed by Barbus cyclolepis Heckel, 1837. All specimens of the identified fish species were examined for parasites using standard methods. The dominant structure of the parasite communities is discussed at the level of component communities and infracommunities. A common parasite species for both biotopes is Acanthocephalus anquillae (Müller, 1780) Lühe, 1911. The similarity of the fish and parasite communities in both biotopes is presented. The factors influencing their formation are analysed. Alb. aburnus from the Luda Yana River is a new host for four parasite species: Rutilus rutilus (Linnaeus, 1758), B. cyclolepis and Gobio gobio (Linnaeus, 1758), for one parasite species each. Biotope Svoboda is a new habitat for five parasite species, and biotope Pazardzhik – for three species.

Key words*: component parasite communities, dominant structure, fish species, parasite infracommunities, similarity.*

INTRODUCTION

The Luda Yana River is 74 km long, which ranks it among the largest tributaries of the Maritsa River. The Luda Yana River springs from Sashtinska Sredna Gora, crosses the Pazardzhik-Plovdiv field and flows to the left of the Maritsa River, in the area of the village of Sinitovo, Pazardzhik municipality (Kiradzhiev, 2013; Kolev, 2020). Kolev (2013; 2020) reports on the ichthyofauna of the river eight native fish species (*Squalius orpheus* Kottelat & Economidis, 2006; *Barbus cyclolepis* Heckel, 1837; *Salmo* sp.; *Rutilus rutilus* (Linnaeus, 1758); *Gobio bulgaricus* Drensky, 1926; *Perca fluviatilis* Linnaeus, 1758; *Alburnus alburnus* (Linnaeus, 1758); *Cobitis strumicae* Karaman, 1955) and three alien fish species (*Carassius gibelio* (Bloch, 1782); *Lepomis gibbosus* (Linnaeus, 1758); *Pseudorasbora parva* (Temminck & Schlegel, 1846). Fish are of great importance to ecosystem biodiversity (Kumar et al., 2025). Parasites use a variety of fish species as intermediate and definitive hosts in their life cycles (Lehun et al., 2023). Parasites are organisms that live in or on other organisms from which they derive benefits, such as protection, food, and a place to live. On the one hand, they cause diseases. On the other hand, they contribute to biodiversity in ecosystems and have an important role in them, as they influence the dynamics of their host populations, the structure of host communities and trophic relationships (de Lima et al., 2025; Hasegawa & Poulin, 2025). Studies on the parasite fauna of fish from the Luda Yana River have been conducted by a few authors (Kirin, 2002; Kirin et al., 2019; Zaharieva, 2023; Zaharieva et al., 2024).

The aim of the study is to investigate and track 1) differences in parasite communities between mining-influenced biotopes versus wastewater impacts, and 2) expected relationships between parasite load and Fulton condition factor.

MATERIALS AND METHODS

In the spring of 2025, two places along the Luda Yana River were visited – Svoboda and Pazardzhik (designated as biotopes). The Svoboda biotope (42°23'59.1"N 24°17'31.2"E) is located in the middle reaches of the river near the Radka copper ore deposit (part of the Panagyurishte ore region). The mine's operations were suspended in 1995. The Pazardzhik biotope (42°09'38.8"N 24°22'52.0"E) is located in the lower reaches of the Luda Yana River, before its confluence with the Maritsa River. The selected biotope is located near the wastewater treatment plant (WWTP) of the town of Pazardzhik (Figures 1-2).

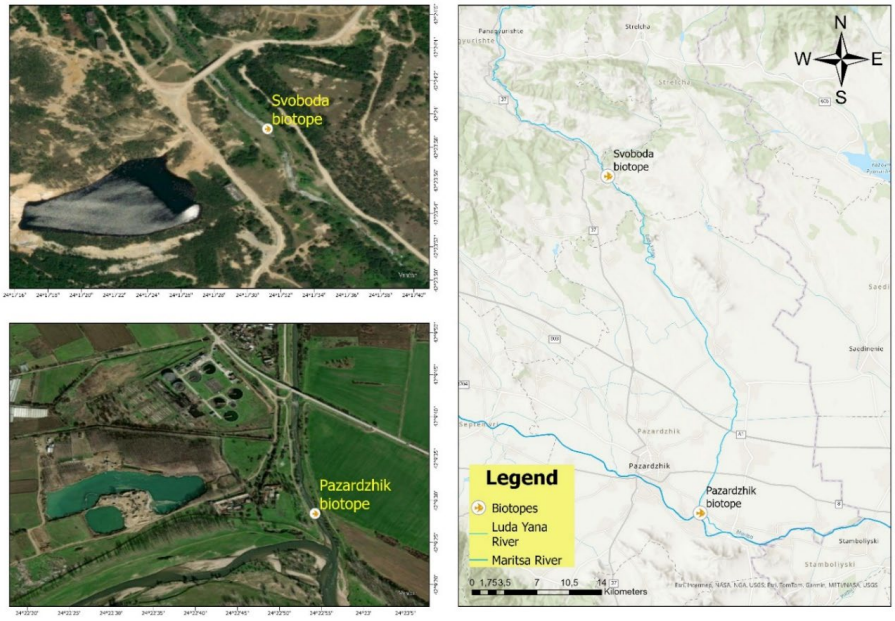


Figure 1. Map of the studied biotopes from the Luda Yana River (own source)



Figure 2. Photographs of the studied biotopes from the Luda Yana River (Svoboda and Pazardzhik – mouth; from left to right) (own source)

A total of 95 specimens of 6 fish species were caught (BDS EN 14757:2015) - Bleak *Alb. alburnus*, Round-scaled barbell *B. cyclolepis*, Gudgeon *Gobio gobio* (Linnaeus, 1758), Roach *R. rutilus*, Orpheus dace *Sq. orpheus* и European bitterling *Rhodeus amarus* (Bloch, 1782). Dominant species in both biotopes is *Alb. alburnus*. Fish species were identified according to Froese & Pauly (2025). The number of specimens of each species caught (N) is reflected; sex (male specimens - ♂; female specimens - ♀) and basic metric data (total body length (TL) in centimeters and body weight (BW) in grams) (Table 1).

Table 1. Metric data and numbers of the studied fish from the Luda Yana River

Biotope	Fish species	N	Sex	TL Min.-Max. (Mean±SD)	BW Min.-Max. (Mean±SD)
Svoboda	*Alburnus alburnus*	31	26 ♂; 5 ♀	7-12.2 (9.797±1.024)	4-12 (7.61±2.186)
	Barbus cyclolepis	25	24 ♂; 1 ♀	8.2-16 (9.608±1.479)	5-35 (9±5.694)
	Gobio gobio	5	2 ♂; 3 ♀	7.3-8.3 (7.7±0.4)	4-5 (4.6±0.548)
	Rutilus rutilus	1	1 ♂	8	4
	Squalius orpheus	1	1 ♂	12	15
Pazardzhik	*Alburnus alburnus*	30	19 ♂; 11 ♀	9-12 (10.423±0.722)	5-12 (7.53±2.113)
	Barbus cyclolepis	1	1 ♂	8.7	5
	Rhodeus amarus	1	1 ♂	5.3	3

A visual inspection of the fish body surface, abdominal cavity and all organs was performed. Organs were prepared and examined under a Micros Austria MZ 1240 stereomicroscope according to standard methodology (Zashev & Margaritov, 1966; Moravec, 2013). Temporary microscope slides with glycerin and permanent microscope slides with Canada balsam were prepared from the found parasites (Dubinina, 1948; Scholz & Hanzelová, 1998). With the XS-213 microscope, species determination of the established parasites was carried out according to Kakacheva-Avramova (1983), Moravec (2013), and others. The parasite communities of the dominant fish species in the two biotopes are presented at two levels: component community and infracommunity. Basic ecological indices (mean intensity (MI), mean abundance (MA) and prevalence (P%)) and diversity indices (Pielou's evenness index (J'), Simpson's index (D), and Simpson's index of diversity (1-D) according to McDonald, 2010; Okpiliya, 2012) were calculated and discussed. Fulton's condition factor (K) is presented. Fish with a Fulton's condition factor value above one are in good condition (Zencir Tanir, 2020). The relationship between sex and infection of *Alb. alburnus* from the Svoboda and Pazardzhik biotopes was studied (χ^2 test). The relationship between Fulton's condition factor and the number of parasites in the studied *Alb. alburnus* specimens from both biotopes was also studied (regression and correlation analysis). The statistical analysis of the data was carried out using IBM SPSS Statistics (Version 19).

RESULTS AND DISCUSSIONS

Species diversity of fish parasites from the Luda Yana River

In the present ecoparasitological study of 63 specimens of five fish species (Bleak, Round-scaled barbell, Gudgeon, Roach, Orpheus dace) from the Svoboda biotope and 32 specimens of three fish species (Bleak, Round-scaled barbell, European bitterling) from the Pazardzhik biotope, infection was found with 6 and 3 parasite species, respectively (Table 2).

Table 2. Species diversity of the isolated parasites from the Luda Yana River

Class/Phylum	Parasite species
TREMATODA	**Allocreadium isoporum* (Looss, 1894) Looss, 1902
CESTODA	**Caryophyllaeus brachycollis* Janiszewska, 1953 **Caryophyllaeides fennica* (Schneider, 1902) Nybelin, 1922
ACANTHOCEPHALA	****Acanthocephalus anguillae* (Müller, 1780) Lühe, 1911 ***Acanthocephalus tenuirostris* (Achmerov & Dombrovskaja-Achmerova, 1941) Yamaguti, 1963 ****Pomphorhynchus laevis* (Zoega in Müller, 1776) Porta, 1908
NEMATODA	**Rhabdochona denudata* (Dujardin, 1845) Railliet, 1916

Legend: * - a parasite found in Svoboda biotope; ** - a parasite found in the Pazardzhik biotope; *** - a parasite found in both biotopes.

In the study of *Alb. alburnus* from the Luda Yana River (Svoboda), 3 parasite species were found: *Caryophyllaeus brachycollis* Janiszewska, 1953; *Acanthocephalus anguillae* (Müller, 1780) Lühe, 1911; and *Rhabdochona denudata* (Dujardin, 1845) Railliet, 1916. In *B. cyclolepis* from Svoboda, 3 parasite species were established: *Allocreadium isoporum* (Looss, 1894) Looss, 1902; *Caryophyllaeides fennica* (Schneider, 1902) Nybelin, 1922 and *Ac. anguillae*. In *G. gobio* and *R. rutilus*, one parasite species was found: *Pomphorhynchus laevis* (Zoega in Müller, 1776) Porta, 1908 and *Ac. anguillae*, respectively. *Sq. orpheus* from the Svoboda biotope is free of parasites.

In the study of *Alb. alburnus* from the Luda Yana River (Pazardzhik), two parasite species were found: *Ac. anguillae* and *P. laevis*. One parasite species was found in *B. cyclolepis* – *Acanthocephalus tenuirostris* (Achmerov & Dombrovskaja-Achmerova, 1941) Yamaguti, 1963. *Rh. amarus* from the Pazardzhik biotope is free of parasites.

In the present study of 25 *B. cyclolepis* specimens from the Svoboda biotope, infection was observed in 44%. In 10 specimens of Round-scaled barbell, infection by a single parasite species was found, and in one specimen of fish, two parasite species were found. A total of 85 specimens of the three parasite species were studied. The number of *All. isoporum* in one specimen of *B. cyclolepis* varies from 2 to 22, and of *C. fennica* – from 2 to 34. Three specimens of *B. cyclolepis* were infected with one *Ac. anguillae* specimen each. Pielou's evenness index (J) indicates a high evenness in the distribution of parasites in the community. Simpson's dominance index (D) and Simpson's index of diversity (1-D) indicate moderate community diversity (Table 3).

Table 3. Indices of diversity of parasites of *Barbus cyclolepis* from Svoboda biotope

Indices	
Total number of species	3
Total number of specimens	85
Pielou's evenness index $J' = \frac{H'}{H_{max}} = \frac{H'}{\ln S}$	0.75
Simpson's index $D = \sum \left(\frac{n}{N}\right)^2$	0.47
Simpson's index of diversity (1-D)	0.54

In one specimen of *B. cyclolepis* from the Pazardzhik biotope, one *Ac. tenuirostris* specimen was found. During parasitological examination of five *G. gobio* specimens from the Svoboda biotope, infection was found in only one specimen of *G. gobio* (20%) with one specimen of *P. laevis*. In one *R. rutilus* specimen from the same biotope, infection with one *Ac. anguillae* was found.

Component communities of parasites of Alburnus alburnus from the Luda Yana River

In the component communities of Bleak from the Svoboda and Pazardzhik biotopes, the largest numbers of specimens are acanthocephalans: 12 of one species and 73 of two species, respectively. In the Pazardzhik biotope, no infections of Cestoda and Nematoda were found. In the two studied biotopes, no infection of Trematoda was found. In the parasite community of *Alb. alburnus* from the Svoboda biotope, one core parasite species (P%>20), one component (10<P%<20), and one accidental parasite species (P%<10) were found (according to Kennedy, 1993). Both parasite species found in the Pazardzhik biotope are the core species in the parasite community of *Alb. alburnus*. The highest mean intensity in the Svoboda biotope has the cestode species *C. brachycollis*, and in the Pazardzhik biotope, the acanthocephalan species *P. laevis* (Table 4).

Table 4. Component communities of *Alburnus alburnus* from the Luda Yana River

Biotope	**Svoboda**						**Pazardzhik**					
Fish species	*Alburnus alburnus* (N = 31)						*Alburnus alburnus* (N = 30)					
Parasite species	**n**	**p**	**MI**	**MA**	**P%**	**R**	**n**	**p**	**MI**	**MA**	**P%**	**R**
Caryophyllaeus brachycollis	3	8	2.67	0.26	9.68	1-5	-	-	-	-	-	
Acanthocephalus anguillae	7	12	1.71	0.39	22.58	1-5	20	55	2.75	1.83	66.67	1-7
Pomphorhynchus laevis	-	-	-	-	-	-	6	18	3.00	0.60	20.00	2-5
Rhabdochona denudata	4	5	1.25	0.16	12.90	1-2	-	-	-	-	-	

Legend: N - number of examined fish, n - number of infected fish, p - number of fish parasites, MI - mean intensity, MA - mean abundance, P% - prevalence, R – range

Infracommunities of parasites of Alburnus alburnus from the Luda Yana River

In the present ecoparasitological study of 31 specimens of Bleak from the Svoboda biotope, parasites were found in 12 specimens of fish. Of these, 10 specimens were infected with one parasite species, and two specimens with two parasite species. Three Bleak specimens were infected with 1, 2, and 5 *C. brachycollis* specimens, respectively. Five specimens of Bleak were infected with one *Ac. anguillae* specimen. Two specimens of Bleak were infected with two and five *Ac. anguillae* specimens, respectively. Three and one Bleak specimens were infected with one and two *Rh. denudata* specimens, respectively. In *Alb. alburnus* from the Svoboda biotope, infection with three parasite species (25 specimens) was found, and their number in one Bleak specimen varied from 1 to 5 specimens. Simpson's index (D) indicates moderate diversity in the parasite community of *Alb. alburnus* from the Svoboda biotope, with dominance of two parasite species. The value of Pielou's evenness index (J') indicates a very high evenness in the distribution.

During the examination of 30 specimens of Bleak from the Pazardzhik biotope, infection was found in 24 specimens. Twenty-two specimens of Bleak were infected with one parasite species, and two specimens were infected with two parasite species. In 8, 3, 3, 2, 1, 2, and 1 Bleak specimen, 1, 2, 3, 4, 5, 6, and 7 *Ac. anguillae* specimens were found, respectively. Two, three, and one Bleak specimens were infected with 2, 3, and 5 *P. laevis* specimens, respectively. In *Alb. alburnus* from the Pazardzhik biotope, two parasite species were found with a total of 73 specimens. The number of parasite specimens in one Bleak specimen varies from 1 to 7. Simpson's index (D) shows low diversity in the parasite community of *Alb. alburnus* from the Pazardzhik biotope, with the dominance of one species (*Ac. anguillae*).

Pielou's evenness index (J') reports a high evenness in the distribution of specimens in the parasite community (Table 5).

Table 5. Infracommunities of *Alburnus alburnus* from the Luda Yana River

	Svoboda			**Pazardzhik**		
	Number of parasite species			**Number of parasite species**		
	0	1	2	0	1	2
Number of specimens *Alb. alburnus*	19	10	2	6	22	2
Total number of species (Mean number of species ± SD)	3 (0.45±0.65)			2 (0.87±0.49)		
Total number of specimens (Mean number of specimens ± SD)	25 (0.81±1.35)			73 (2.43±2.32)		
Range	1-5			1-7		
Pielou's evenness index (J')	0.95			0.81		
Simpson's index (D)	0.37			0.63		

A moderate relationship was found between the sex of Bleak specimens (Svoboda and Pazardzhik biotopes) and infection (Sig<0.05; Cramer's $V_{Svoboda}$=0.372, Cramer's $V_{Pazardzhik}$=0.380). In both biotopes, higher infection was found in female Bleak specimens. All female Bleak specimens from the Pazardzhik biotope were infected. Male Bleak specimens from the Pazardzhik biotope are more infected than those from the Svoboda biotope (Figure 3).

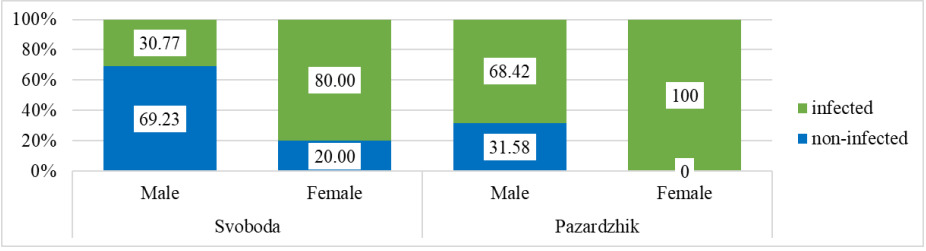


Figure 3. Infected male and female *Alburnus alburnus* specimens (in %) from Svoboda and Pazardzhik biotopes

According to the obtained mean values for Fulton's condition factor, *G. gobio* from Svoboda is in the best condition (K=1.01±0.12). In close to good condition are *B. cyclolepis* and *R. rutilus* again from the Svoboda biotope (Table 6).

Table 6. Fulton's condition factor (K)

Biotope	Fish species	K Min.-Max. (Mean±SD)
Svoboda	*Alburnus alburnus*	0.55-1.17 (0.80±0.14)
	Barbus cyclolepis	0.79-1.14 (0.95±0.08)
	Gobio gobio	0.87-1.19 (1.01±0.12)
	Rutilus rutilus	0.87
	Squalius orpheus	0.78
Pazardzhik	*Alburnus alburnus*	0.38-1.10 (0.66±0.14)
	Barbus cyclolepis	0.76
	Rhodeus amarus	2.02

The regression (Linear model; Figure 4) and correlation analysis (Pearson's correlation coefficient) report the presence of a moderate inverse relationship between the values of Fulton's condition factor and Number of Bleak parasites from the Svoboda biotope (Sig.=0.014<0.05; R=0.435; Pearson Correlation=-0.435). The analysis shows that 18.9% of the differences in the Number of fish parasites in the Svoboda biotope are explained by Fulton's condition factor values. For the Pazardzhik biotope, no relationship was established between Fulton's condition factor and the Number of fish parasites (Sig.=0.860>0.05; R=0.034).

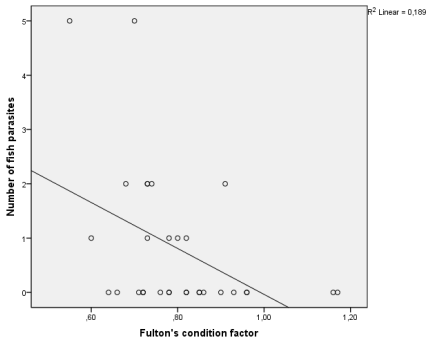


Figure 4. Linear model of the relationship between Fulton's condition factor and Number of *Alburnus alburnus* parasites from Svoboda biotope

Discussions

Of the 7 parasite species identified in the present study, six species (*All. isoporum*, *C. fennica*, *Ac. anguillae*, *C. brachycollis*, *P. laevis* and *Rh. denudata*) have been reported for different fish species from the Luda Yana River. While one species (*Ac. tenuirostris*) has not been reported as a parasite of fish from the Luda Yana River (Table 7).

Table 7. Parasite species in fish from the Luda Yana River from the study and according to other authors

Present study	Previous studies	
Parasite (Fish host / Biotope)	**Fish host / Biotope**	**Author**
TREMATODA		
All. isoporum (*B. cyclolepis* / Svoboda)	**** B. cyclolepis*** / Panagyurishte	Kirin (2002)
CESTODA		
C. fennica (*B. cyclolepis* / Svoboda)	*R. rutilus* / Popintsi	Kirin et al. (2019)
	Sq. orpehus / Popintsi	Zaharieva (2023)
	Sq. orpehus / Popintsi **** B. cyclolepis*** / ****Svoboda**	Zaharieva et al. (2024)
C. brachycollis (*Alb. alburnus* / Svoboda)	*B. cyclolepis* / Panagyurishte	Kirin (2002)
ACANTHOCEPHALA		
Ac. anguillae (*Alb. alburnus* / Svoboda, Pazardzhik; *B. cyclolepis* / Svoboda; *R. rutilus* / Svoboda)	**** B. cyclolepis*** / Panagyurishte	Kirin (2002)
Ac. tenuirostris (*B. cyclolepis* / Pazardzhik)	-	-
P. laevis (*Alb. alburnus* / Pazardzhik; *G. gobio* / Svoboda)	*Sq. orpehus* / Chernogorovo	Zaharieva et al. (2024)

Present study	Previous studies	
Parasite (Fish host / Biotope)	**Fish host / Biotope**	**Author**
NEMATODA		
Rh. denudata (*Alb. alburnus* / Svoboda)	*R. rutilus* / Popintsi	Kirin et al. (2019)
	Sq. orpehus / Popintsi	Zaharieva (2023)
	Sq. orpehus / Popintsi	Zaharieva et al. (2024)

Legend: * - host match; ** - biotope match

In this study, single specimens of two fish species from the Svoboda biotope (Roach, Orpheus dace) and two fish species from the Pazardzhik biotope (Round-scaled barbell, European bitterling) were studied; the results for these species are purely descriptive, and conclusions for them cannot be extrapolated.

CONCLUSIONS

During the parasitological examination of 31 specimens of Bleak, 25 specimens of Round-scaled barbell, 5 specimens of Gudgeon, one specimen of Roach and one specimen of Orpheus dace from the Svoboda biotope, and 30 specimens of Bleak, one specimen of Round-scaled barbell and one specimen of European bitterling from the Pazardzhik biotope, infection was found with a total of 7 parasite species with 186 specimens. *Sq. orpheus* and *Rh. amarus* are free of parasites. In the parasite community of *Alb. alburnus* from the Svoboda biotope, one core, one component and one accidental parasite species were found. In the Pazardzhik biotope, both parasites found in Bleak are core species. In both biotopes, higher infection was found in female Bleak specimens. Svoboda and Pazardzhik biotopes are new habitats for five and three parasite species, respectively, in the present study. *Alb. aburnus* from the Luda Yana River is a new host record for the parasite species *C. brachycollis*, *Ac. anguillae*, *P. laevis* and *Rh. denudata*; *R. rutilus* – for *Ac. anguillae*; *B. cyclolepis* – for *Ac. tenuirostris,* and *G. gobio* – for *P. laevis*.

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IMPACT OF HABITAT FRAGMENTATION ON SOIL MITE COMMUNITIES (Acari-Mesostigmata) FROM TWO SMALL PROTECTED AREAS FROM BRAŞOV COUNTY, ROMANIA

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Abstract

One of the main threats of terrestrial ecosystems is habitat fragmentation. This threat has impact even on soil fauna, as predator mites. The present study wants to demonstrate that the mite community structures and species diversity are strongly correlated with the type of fragmented habitats, from two fragmented Natura 2000 site: Forest and Eutrophic Marshes from Prejmer (ROSCI0170) and Lempeş Fortress Hill-Hărman Marsh (ROSCI0055), characterized by the presence of different ecosystems: calcareous fens, alluvial, oak-hornbeam and beech forests. In 2018, eighty soil samples were investigated. In total, 29 species of Mesostigmata were identified. Considering the numerical abundance and species richness of Mesostigmata, we observed that the highest value was obtained in Prejmer protected area. If we make this comparison, considering the four fragments from each investigated area, we observed that in the fragments characterized by wet ecosystems (calcareous fens with Cladium mariscus (L.) Pohl), soil mites found the most favorable environmental conditions for their development. Each investigated fragments from the two protected areas were defined by the characteristically pattern of the soil mite structures, according to the applied statistical analysis.

Key words*: habitat, mite, predator, soil*, *structure.*

INTRODUCTION

One of the most threats on terrestrial ecosystems is habitat fragmentation. If we consider the soil fauna this threat significantly impacts it through many mechanisms: isolating populations, decreasing population parameters (as species richness and abundance), altering community composition, and increasing species/population vulnerability to extinction (Rantalainen et al., 2008). The habitat fragmentation, which is often caused by anthropic activities (as forest cutting, urban extinction, intensive agriculture), creates disconnected, smaller patches, disrupting the ability of soil organisms to disperse and access resources, which can lead to ecosystem decay and instability in soil food webs (Rantalainen et al., 2008; Lopezosa et al., 2023; Winfrey et al., 2025; Yan et al., 2025). In this situation, what soil fauna should do? One of the possible answers is to respond. But the response of the on many soil processes (pedogenesis, nutrient cycle, decomposition of organic matter, etc.) (Krantz & Walter, 2009). Mesostigmata mites soil fauna to the habitat fragmentation depends on one hand, by organism's body size, trophic level, mobility and generation time. On the other hand some features of habitat fragments (as shape, size, isolation, as well as the presence and connectivity of habitat corridors, influence the survival and dispersal of soil fauna (Rantalainen et al., 2008; Lopezosa et al., 2023; Winfrey et al., 2025). Environmental microclimate (soil water content, soil acidity, precipitations) and the vegetation coverage constitute local drivers that could influence the impact of habitat fragmentation on soil biodiversity, which is a vital biological component for ecosystem functions like nutrient cycling and soil building. Mites (Acari) are one of the most abundant taxonomic groups from soil fauna, occupying diverse trophic niches (from decomposers to predators), participating directly or indirectly are those soil invertebrates that are mainly predators, free-living, very mobile species,

which participate indirectly on decomposing process of organic matter (Potapov et al., 2022). In Europe, studies regarding the influence of habitat fragmentation on soil mesostigmatids revealed that the communities' composition was changed, the species diversity and abundance were decreased (Rantalainen et al., 2008; Kamczyc et al., 2023). In Romania, studies regarding the influence of fragmented habitats on soil fauna communities are scarce (Manu et al., 2021).

In this context presented above, the main objectives of this study are: 1) to identify the community structures of Mesostigmata mites two protected areas, characterized by different types of ecosystems influenced by habitat fragmentation; 2) to measure some population parameters of Mesostigmata communities (Shannon diversity, dominance, evenness and equitability indices) and 3) to highlighting the correspondence between investigated habitats and Mesostigmata species.

MATERIALS AND METHODS

Study area

The research on soil mite fauna was made in November 2018, in two fragmented Natura 2000 sites from Braşov County, Romania: Forest and Eutrophic Marshes from Prejmer (ROSCI0170) and Lempeş Fortress Hill-Hărman Marsh (ROSCI0055).

The nature reserve Forest and Eutrophic Marshes from Prejmer has an area of 345 hectares. If we consider the type of habitats it is characterized first of all by eutrophic marshes and then by meadows, forests and shrubs.

The second protected area Lempeş Fortress Hill-Hărman Marsh has an area of 374 hectares, characterized by alluvial forests, calcareous fens, beech forests and oak-hornbeam forests (Figure 1; Table 1).

The classification of habitats was made according to Doniţă et al., 2005; Gafta & Mountford, 2008 (Table 1).

Table 1. Geographical and habitat description of investigated fragments from Forest and Eutrophic Marshes from Prejmer (ROSCI0170) and Lempeş Fortress Hill-Hărman Marsh (ROSCI0055) ecosystems, Romania

Investigated area	Short name	N	E	Altitude	Habitat
Prejmer - first fragment	PF1	45^{0}43'46.04"	25^{0}44'09.03"	514 m	7210* calcareous fens with *Cladium mariscus*
Prejmer - second fragment	PF2	45^{0}44'52.22"	25^{0}43'48.66"	503 m	91 EO alluvial forests with *Alnus glutinosa* and *Fraxinus excelsior (Alno-Padion, Alnion incanae, Salicion albae*).
Prejmer - third fragment	PF3	45^{0}44'56.90"	25^{0}42'08.94"	508 m	91 EO alluvial forests with *Alnus glutinosa* and *Fraxinus excelsior (Alno-Padion, Alnion incanae, Salicion albae)*.
Prejmer - fourth fragment	PF4	45^{0}44'55.46"	25^{0}41'15.64"	501 m	91 EO alluvial forests with *Alnus glutinosa* and *Fraxinus excelsior (Alno-Padion, Alnion incanae, Salicion albae*).
Hărman - first fragment	HF1	45^{0}43'03.63"	25^{0}40'03.61"	514 m	7210* calcareous fens with *Cladium mariscus*
Hărman - second fragment	HF2	45^{0}43'07.30"	25^{0}40'00.08"	498 m	9170 Galio-Carpinetum oak-hornbeam forests
Hărman - third fragment	HF3	45^{0}44'12.63"	25^{0}40'27.07"	511 m	9170 Galio-Carpinetum oak-hornbeam forests
Hărman - fourth fragment	HF4	45^{0}43'07.30"	25^{0}40'00.08"	498 m	9130 Asperulo-Fagetum beech forests

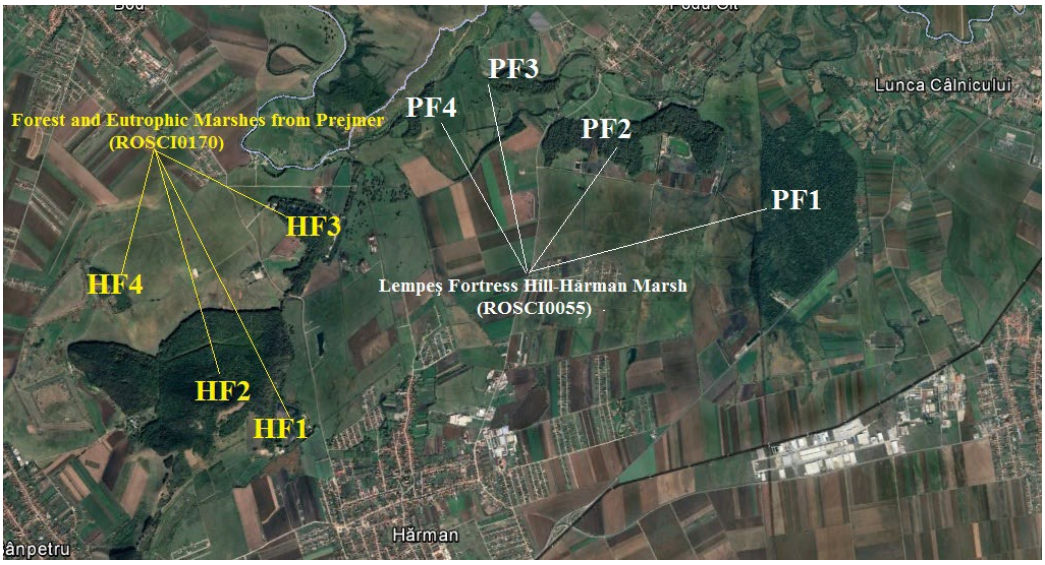


Figure 1. Localization of the investigated fragments from Forest and Eutrophic Marshes from Prejmer (ROSCI0170) (yellow color) and Lempeș Fortress Hill-Hărman Marsh (ROSCI0055) (white color) ecosystems, Romania (original source)

Soil samples

In each protected areas 40 soil samples were collected, with Macfadyen corer (to a depth of 10 cm with a, by 5 cm diameter) (Macfadyen, 1961). In total 80 soil samples were analyzed. The samples were taken randomly. Each soil sample was put in plastic bags, labeled and transported in the lab for extraction. The fauna were extracted for ten days, using natural light and heat, with a modified Berlese-Tullgren funnels, in ethylic alcohol (Berlese, 1905; Tullgren, 1917; Krantz & Walter, 2009). For taxonomical identification the most specialized keys were used (Ghilyarov & Bregetova 1977; Hyatt, 1980; Karg, 1993; Mašán & Fendá, 2004; Mašán, 2007; Mašán et al., 2008; Mašán & Halliday, 2010, 2013; Özbek & Halliday, 2015; Mašán, 2018).

Data analysis

The individual rarefaction and the correspondence analysis (CA) between identified Mesostigmata species and analyzed fragments from the two protected areas were calculated using the BioDiversity Pro 2.0 software, PAST (Hammer et al., 2001). The same software was used to calculate some population parameters for mites as Shannon diversity index (H), dominance (D), evenness (H/S) and equitability (J).

RESULTS AND DISCUSSIONS

The individual rarefaction shows us that the sampling effort was not sufficient, although in Prejmer area the results are better (Figure 2). In total 29 species of Mesostigmata was identified. Considering the numerical abundance of Mesostigmata communities, we observed that the highest value was obtained in Prejmer protected area (Table 2). If we make this comparison, considering the four fragments from each investigated area, we observed that fragments characterized by wet ecosystems (calcareous fens with *Cladium mariscus*) the soil mites found the most favorable environmental conditions for their development (PF1 and HF1). The same trend was observed for species richness (Table 2).

Table 2. Numerical abundance of the identified Mesostigmata species from Forest and Eutrophic Marshes from Prejmer (ROSCI0170) and Lempeş Fortress Hill-Hărman Marsh (ROSCI0055), Romania (± standard error)

Species	PF1	PF2	PF3	PF4	Total P	HF1	HF2	HF3	HF4	Total H
Amblyseius sp. (Am sp)						1 ± 0.1				1± 0.25
Cilliba sp. (Cill sp)	1 ± 0.1				1 ± 0.25					
Discourella sp. (Dis sp)								2 ± 0.13		2± 0.50
Eviphis ostrius (Ev os)	5 ± 0.17				5 ± 1.25	1 ± 0.1				1± 0.25
Gaeolaelaps praesternalis (Ga pr)						1 ± 0.1				1± 0.25
Gamasellodes bicolor (Ga bi)						1 ± 0.1		1 ± 0.1		2± 0.28
Geholaspis mandibularis (Ge ma)		2 ± 0.13			2 ± 0.50	1 ± 0.1				1± 0.25
Holoparasitus sp. (Ho sp)						1 ± 0.1	1 ± 0.1			2± 0.28
Laelaspis astronomicus (La as)						2 ± 0.13				2± 0.5
Leioseius lawrencei (Le la)						1 ± 0.1				1± 0.25
Lysigamasus misellus (Ly mi)	1 ± 0.1	1 ± 0.1			2 ± 0.29					
Lysigamasus tectegynellus (Ly te)	1 ± 0.1				1 ± 0.25					
Macrocheles recki (Ma re)						1 ± 0.1				1± 0.25
Olopachys suecicus (Ol su)		1 ± 0.1			1 ± 0.25					
Oodinychus sp. (Oo sp)	6 ± 0.43			13 ± 1.19	19 ± 3.09					
Pachydellus furcifer (Pa fu)						1 ± 0.1				1± 0.25
Pachyseius humeralis (Pa hu)	1 ± 0.1	2 ± 0.13			3 ± 0.48				1 ± 0.1	1± 0.25
Pachyseius subhumeralis (Pa sub)		1 ± 0.1			1 ± 0.25					
Pachyseius wideventris (Pa wi)					1 ± 0.25					
Paragamasus similis (Pa si)		1 ± 0.1			1 ± 0.25	1 ± 0.1				1± 0.25
Pergamasus sp. (Pe sp)				1 ± 0.1	1 ± 0.25					
Prozercon traegardhi (Pr tr)	2 ± 0.13	3 ± 0.21			5 ± 0.75			1 ± 0.1	1 ± 0.1	2± 0.28
Pseudouropoda sp. (Ps sp)		1 ± 0.1			1 ± 0.25					
Trachytes aegrota (Tr ae)	2 ± 0.13				2 ± 0.50					
Trachytes sp (Tr sp)	3± 0.21				3 ± 0.48					
Veigaia exigua (Ve ex)	2 ± 0.20	2 ± 0.1		1 ± 0.1	5 ± 0.75					
Veigaia nemorensis (Ve ne)	4 ± 0.22	2 ± 0.1			6 ± 1					
Vulgarogamasus kraepelini (Vu kr)	1± 0.1				1 ± 0.25					
Vulgarogamasus remberti (Vu re)						2 ± 0.13				2± 0.28
Total number individuals	29	16	0	16	**61**	14	1	4	2	**21**
Total number species	12	10	0	4	**19**	12	1	3	2	**15**

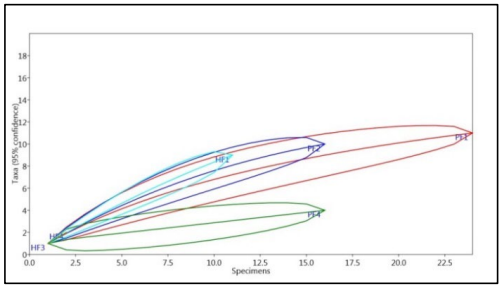


Figure 2. The individual rarefaction of the Mesostigmata communities from Forest and Eutrophic Marshes from Prejmer (ROSCI0170) and Lempeş Fortress Hill-Hărman Marsh (ROSCI0055) ecosystems, Romania

Comparing with other studies from other pedoclimatic regions from Europe, the species diversity from our study is lower with studied made in forests from continental area (20 species) or Atlantic zone (14 species), but in peat bogs this parameter recorded comparable or even higher value that in other countries (12 species in Romania, comparing with 3 species in Ireland or 14 species in Poland (Arroyo et al., 2013; Diriligen et al., 2016; Graczyk et al., 2025).

At national level, the numerical abundance and species richness is much lower in studied fragments, comparing with deciduous forests from other regions of Romania (13-22 Mesostigmata species) (Manu et al., 2013).

If we consider the population parameters, we observed that the highest value of dominance was obtained in Prejmer area (0.14), in comparison with Hărman (0.04). In Prejmer

area, fragment PF4 recorded the highest dominance (0.67), PF1 the highest Shannon index of diversity (2.27), PF2 the highest value of evenness (0.92) and the highest value of equitability (0.96) (Figure 3). In Hărman area the HF2 and HF3 recorded the highest values of dominance (0.50), of evenness and equitability indices (1 for each) and fragment HF1 the highest Shannon index of diversity (1.71) (Figure 3).

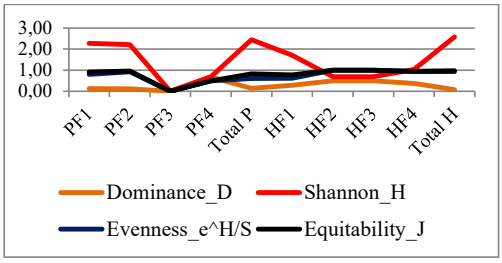


Figure 3. The statistical parameter of the Mesostigmata communities from Forest and Eutrophic Marshes from Prejmer (ROSCI0170) and Lempeş Fortress Hill-Hărman Marsh (ROSCI0055), Romania

In Prejmer area, the high dominance in PF4 was due the presence in high numerical abundance of Oodinychus species. On the whole ecosystem the dominant species were *Eviphis ostrinus, Prozercon traegardhi, Veigaia exigua* and *Veigaia nemorensis*, species which equitable distributed especially in fragments PF2 and PF3 (Table 2). The most humid soil from fen fragment (PF1) possible to create more favorable conditions for a high species richness, but the characteristic edaphic conditions from alluvial forest fragments (especially more organic matter, soil water content, lower temperature, etc) are preferred habitat for the dominant species. *Veigaia nemorensis* prefers habitats with moderate to low humidity, such as certain forest and grassland areas. It is a surface dweller and is commonly found near the top of the soil layers, often in association with organic matter and humus (Manu et al., 2017; Graczyk et al., 2025). *Veigaia exigua* prefers temperate forest habitats, particularly the upper soil layers like moss or the litter and organic humus layers (Manu et al., 2017). *Eviphis ostrinus* is recognized as generalist species, being found in a variety of environments, including fens, forests, grasslands, and other ecosystems (Salmane & Brumelis, 2010; Madej et al., 2011). Forests are habitats where *Prozercon traegardhi* had been frequently identified as a dominant species, showing high numerical densities in the litter and fermentation layer and the humus layer (Salmane & Brumelis, 2010).

In Hărman area, in the species were mostly equitable distributed (especially in HF2 and HF3), due to the similar habitat conditions (oak-hornbeam forests) (Table 2). If we consider the numerical abundance, besides *Prozercon traegardh,* there are few species more dominant, as: *Gamasellodes bicolor, Laelaspis astronomicus* and *Vulgarogamasus remberti* (Table 2). All species are predators, preferring upper layers of soil and leaf litter, especially in forest ecosystems like pine, beech and deciduous forests, where can find other small invertebrates, like nematodes-their preferred food. These species are associated with other animal taxa, as *Laelaspis astronomicus* which is often found in association with other arthropods, such as ants or *Vulgarogamasus remberti* which is associated with mice and voles (Hyatt, 1980; De Moraes et al., 2022).

Using correspondence analysis we highlighted the correlation between the investigated habitats/fragments and soil mite species. This analysis revealed the presence of four groups: 1) HF1 and HF2, characterized by the presence of *Vulgarogamasus remberti, Pachydellus furcifer, Laelaspis astronomicus, Leioseius lawrencei* and *Gaeolaelaps praesternalis*; 2) HF3, HF4, PF1, PF2 favorable for species *Veigaia exigua, Veigaia nemorensis, Vulgarogamasus kraepelini, Lysigamasus misellus, Prozercon traegardhi, Pachyseius subhumeralis, Pachyseius humeralis*; 3) characteristics species only for PF4, as *Pachyseius wideventris;* 4) species which have any habitat preference as *Paragamasus similis* and *Geholaspis mandibularis*, which are opportunistic predators, always searching for prey (Figure 4). These results demonstrated that even from the vegetation point of view the investigated fragments are different, it is possible that the similar environmental conditions (as presence of moss layer, high soil water content, presence of organic matter from marsh and forests soils) to be the cause of the characteristic spatial dynamics and species grouping.

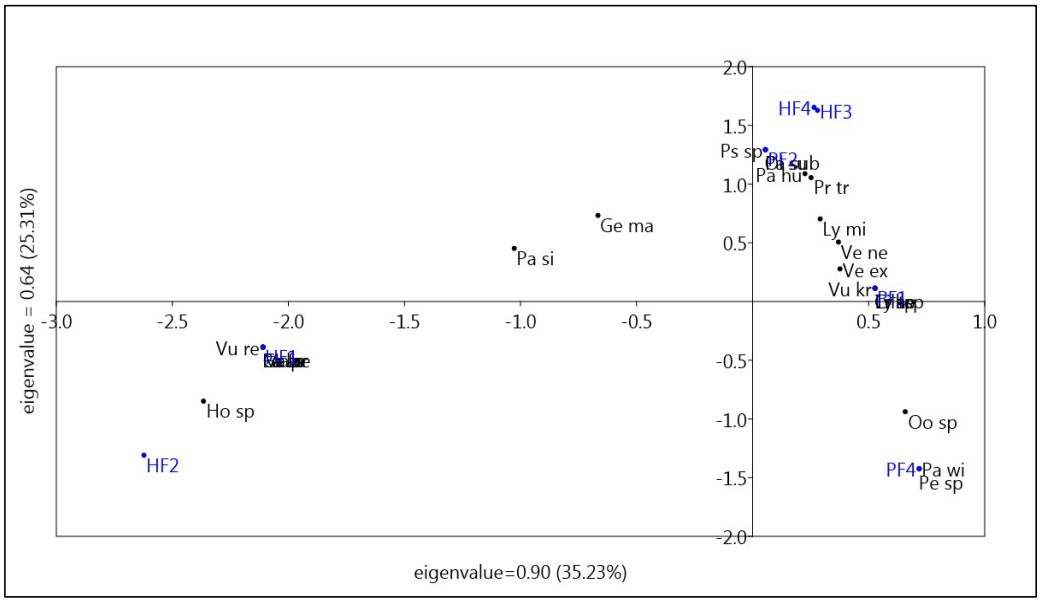


Figure 4. Correspondence analysis (CA) between identified Mesostigmata species and analysed fragments from Forest and Eutrophic Marshes from Prejmer (ROSCI0170) and Lempeş Fortress Hill-Hărman Marsh (ROSCI0055), Romania (the short names of the Mesostigmata species are mentioned in Table 1)

CONCLUSIONS

In 2018, an ecological study was made on soil predator mite fauna (Acari-Mesostigmata) from two Natura 2000 sites (Forest and Eutrophic Marshes from Prejmer - ROSCI0170 and Lempeş Fortress Hill-Hărman Marsh - ROSCI0055), which are characterized by the fragmented habitats, as: 7210* calcareous fens with *Cladium mariscus*; 91 EO alluvial forests with *Alnus glutinosa* and *Fraxinus excelsior*; 9170 Galio-Carpinetum oak-hornbeam forests and 9130 Asperulo-Fagetum beech forests. In total, 80 soil samples were analyzed, revealing the presence of 29 Mesostigmata species, which characteristic community structure for each fragment/ecosystem. This feature was highlined by some population parameters as: numerical abundance, specis richness, dominance, Shannon index of diversity, evenness and equitability. The highest values of numerical abundance, species richness and dominance were obtained in Prejmer protected area. On the other hand the highest values of evenness and equitability indices were obtained in Hărman protected area. Considering the investigated fragments, we observed that in the calcareous fens the numerical abundance and species richness recorded the highest values, in comparison with the other fragments, represented by forests. Canonical analysis revealed the presence of four groups of mites, revealing the influence of habitat characteristics of invertebrate's spatial distribution.

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DISTRIBUTION AND CONSERVATIVE ISSUES OF *Lucanus cervus* (Linnaeus, 1758) AND *Cerambyx cerdo* (Linnaeus, 1758) FROM CRÂNG FOREST, BUZĂU - ROMANIA

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Abstract

According to the European legislation invertebrate species Lucanus cervus (Linnaeus, 1758) and Cerambyx cerdo Linnaeus, 1758 are two species with conservative value, being included in the European Union Habitats Directive 92/43/EEC. They are considered indicator species for mature deciduous forest ecosystems, characterized by old oak trees and dead wood. Crâng forest located near Buzău city provided favorable habitats for these invertebrates. The Crâng forest is located in the forest-steppe zone in the southeast of Romania, has an area of 189 ha. The distributions of Lucanus cervus and Cerambyx cerdo were established, in this location. If we consider the numerical abundance, 40 individuals of Cerambyx cerdo and 45 individuals of Lucanus cervus were counted, on 25 studied transects. On the other hand another two species were investigated: Oryctes nasicornis (Linnaeus, 1758) and Dorcus parallelipipedus Linnaeus 1758. In order to protect these invertebrates, the pressures, threats and conservation measures were established. The present study demonstrated that a mature, old deciduous forest dominated by Quercur robur tree species, even if it is located near an urban area, could constitute a favorable habitat for two Natura 2000 invertebrates' species.

Key words*: abundance, forest, habitat, protection, threat.*

INTRODUCTION

Romania contains five major European bioregions: Alpine, Continental, Pannonian, Steppic and Pontic (Black Sea). Our study, which wants to establish the distribution and conservative issues for *Lucanus cervus* (Linnaeus, 1758) and *Cerambyx cerdo* Linnaeus, 1758 from Crâng forest area, which is located in Buzău County. According to the above classification, the Buzău city is located in the Steppic bioregion. From the conservative point of view, *Cerambyx cerdo* is protected at European and national level through many legislative frames, as: Article 17 of the Habitats Directive (GEO no. 57/2007 -annexes 2, 3, 4 A; HD 92/43 EEC -annexes II; IV), IUCN (being considered vulnerable species) and Bern Convention (annex II). The similar conservation status could define the species *Lucanus cervus*. It is protected by national and European legislation (GEO no. 57/2007 - annexes 2, 3; HD 92/43 EEC – annexes II); IUCN (near threated species) and Bern Convention, which entered into force on June 1, 1982 (annex III).

Considering the conservative value of these two invertebrates species and the Steppic bioregion (where this studied was made), in the last monitoring report to EU (2013-2018) regarding the overall assessment of the conservation status (considering the population, habitat and future prospects) for *Cerambyx cerdo* and *Lucanus cervus* are unfavorable-inadequate (U1) (https://nature-art17.eionet.europa.eu). The national distribution of these two protected species is quite well known, being signaled in many protected areas from Romania (Serafim & Chimişliu, 2010; Prunar et al., 2014; Iorgu et al., 2015; Bărbuceanu, 2017; Manu et al., 2017; 2025; Bărbuceanu & Truță, 2021). Saproxylic species *Cerambyx cerdo* lives mainly in the deciduous forests, especially in oak forests (*Quercus* spp.), preferring older/mature or dying trees (over 100 years old with a diameter of more than 40 cm), even in the seminatural or unnatural (anthropic) forest ecosystems

(Carpaneto et al., 2010; Iorgu et al., 2015; Redolfi De Zan et al., 2017; Mannu et al., 2021). *Lucanus cervus* is another protected species which preferred Quercus spp. forests. It is considered a polyphagous species, growing in the rotten wood of many deciduous species. It can be found in deciduous forests as well as in open areas with isolated trees or hedges, in urban and suburban gardens, parks, wooded pastures, wherever there is a sufficient source of dead wood (Iorgu et al., 2015; Bardiani et al., 2017). Even if these species were recorded especially in protected areas, however we identified them in a small forest area, near to Buzău city. On the other hand the Habitats Directive 92/43/EEC contains no explicit information concerning conservation measures or management of threatened species in artificial habitats like urban/peri-urban areas. According to Carpaneto et al., 2010, this is a critical issue because parks, gardens, avenues, roadsides and other 'unnatural' places can have old and sun-exposed trees, which are important hosts for saproxylic species. The present paper wants to highlight the presence and the numerical abundance of these two protected species in such small peri-urban forest area, to indicate their distribution and their pressures and threat connected with conservation measures. In the same time the present study demonstrated that even a small size forest could provide favorable habitats for the protected invertebrate species. Any information brought from the field observations about *Cerambyx cerdo* and *Lucanus cervus* is very important in order to evaluate their habitat requirements, their distribution and conservation measures, especially in urban forest ecosystem.

MATERIALS AND METHODS

The study was accomplished in June-September 2025, in Crâng forest- Buzău Plain, located near Buzău city (N: 45°08'37.86"; E: 026°47'51.56", altitude by 95 m) (Figure 1). This study area is located in Buzău county Romania, on an area of 189 hectares. The Crâng Forest is part of the medium plain area, bordering the Municipality of Buzău, in the Buzău Plain. For the beetle species, in the filed we used visual searching for adults along transects. The length of transect was by 500 m and 20 m wide. Transects were carried out in a zigzag pattern to cover all researched areas as best as possible. The fauna observations were done directly on the substrate: soil, rocks, logs, herbaceous vegetation, shrubs and bark of trees. For saproxylic Coleoptera species, in areas with dead wood (fallen or standing trees, wood stacks), there were investigated the adequate micro-refuges, through removing the bark on the trunks of dry trees and on logs (Iorgu et al., 2015; Lotrean et al., 2015; Redolfi De Zan et al., 2017). For taxonomical identification of species, we used an actual key (Panin, 1955; Panin & Săvulescu, 1961). In total 25 transects were established (Figure 1). Maps were generated using QGIS 3.42 software (project coordinate reference system: WGS 84). Google Satellite was the base layer, with other layers of interest being sourced from the following databases: (1) Romanian borders and administrative regions (eea.europa.eu/data-and-maps); (2) regional water bodies and (3) roads (data.humdata.org/dataset). Multipoint vector layers for species distributions were generated after the gathered data was compiled into CSV files. From the vegetation point of view those 25 investigated transects were described as following: T1 – forest clearing; T8, T9, T10, T22, T25 – urban forest area with secular oaks (park); T1-T7 and T11-T21, T24 -Habitat 91I0* Euro-Siberian steppic woods with Quercus spp; T23 -ash plantation with diverse shrub layer (Doniţa et al., 1990; 2005). Analyzing the microhabitats we observed that invertebrates' species with conservative value were observed on: dry log (T1), soil (T17), dead *Quercus robur* (T16, T24) and live *Quercus robur* (T2-T15; T18-T25). The tree circumferences were quantified with a flexible tape measure at breast height (Elias, 2000).For both protected species, threats and pressures were evaluated (in degree of intensity). In order to differentiate the pressure from threat, we used "the standard lexicon for biodiversity conservation: unified classifications of threats and actions" (Salafsky et al., 2008).

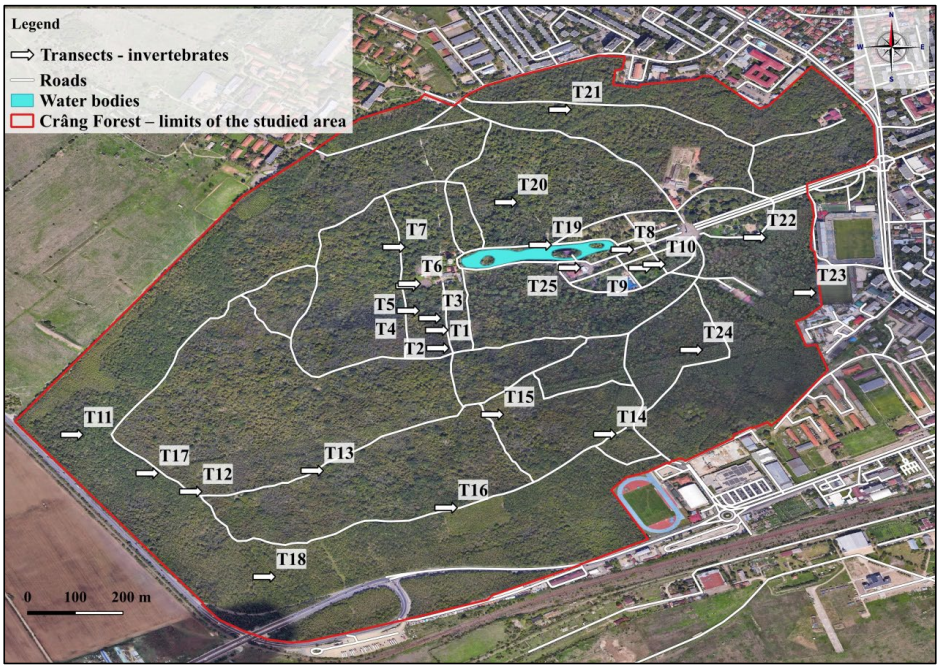


Figure 1. The geographical location of the Crâng forest and investigated transects, Buzău County, Romania, 2025 (original)

RESULTS AND DISCUSSIONS

In the Crâng forest two Natura 2000 invertebrate species were identified: *Cerambyx cerdo* and *Lucanus cervus*. In the same time, we observed another important xylophage coleopterans species: *Oryctes nasicornis* (Linnaeus, 1758) and *Dorcus parallelipipedus* Linnaeus 1758. Even if in Romania *Oryctes nasicornis* is not a legal protected species, the specialists consider that due to the decreasing of the forests with old trees we must to consider this species from the conservative point of view. It has been included in conservation efforts and recommended for the Red List of the Prut River (Popescu & Davideanu, 2009). According to the IUCN criteria, *Dorcus parallelipipedus* is listed as least concern species (IUCN, 2024).

All these xylophages species have an important role in forest ecosystem, participating in the decomposition proces, and representing a food source for other animal species (birds, micromammals, etc.). Their ecologycal role, their diverse habitat requirements, ecological niches and, in particular, their rapid responses to environmental changes, could constitue orguments to considered these invertebrates excellent bioindicators of management and, consequently, of the conservation status of forests. The strict relationship between saproxylic insects and the quantity and type of dead wood implies a very strong sensitivity to forest management practices (De Groot et al., 2016).

From 25 investigated transects, in 22 of them we identified favorable habitats and microhabitats for xylophagous coleopterans. The highest number of individuals were recorded in T7, T14, T16, T19, T24 and T25. On the opposite the lowest nmerical abundance were obtained in T, T2, T9, T12 and T17. With only tow exceptions (T9 and T25 from urban park) all those transects were integrated in Natura 2000 habitat: 91I0* Euro-Siberian steppic woods with Quercus spp. On the other hand, the transects where the high coleopterans abundaces were recorded, are characterized by the most increased average trees cicumferences (between 206 and 320 cm), despite the lowest reprezented transects (92-184 cm). According to Doniţă et al., 2005, habitat 91I0* Euro-Siberian steppic woods with Quercus spp. has a high conservative value. The type of soil is phaeozem (cambic chernozem), rich in humus, slightly acidic, eubasic, water

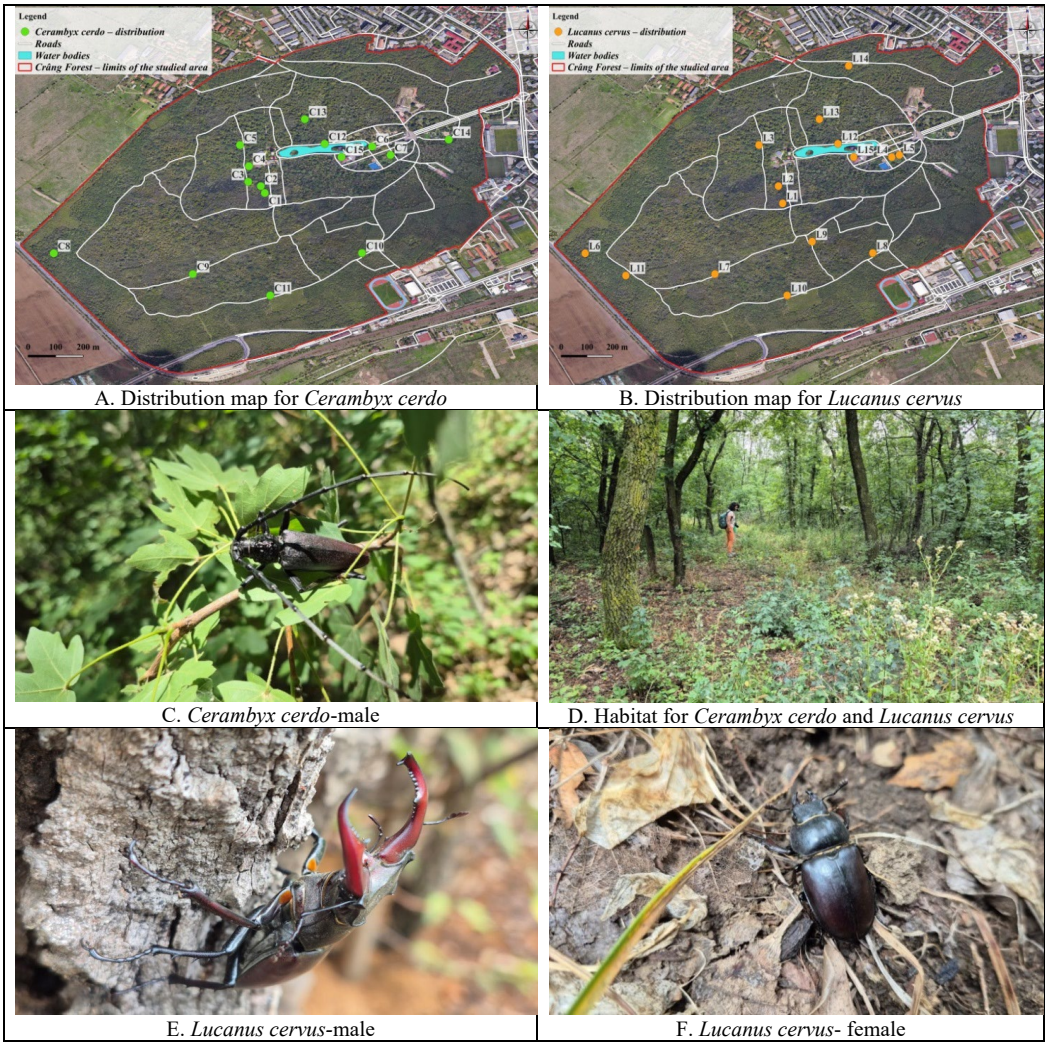
deficient during the summer, eutrophic. The tree layer is composed by: *Quercus pedunculiflora, Q. pubescens, Acer tataricum, Acer campestre, Ulmus minor, Ulmus procera, Pyrus pyraste*, etc. The shrub layer is well developed, composed by: *Crataegus monogyna, Prunus spinosa, Viburnum lantana, Rhamnus cathartica, Ligustrum vulgare, Evonymus verrucosus, E. europaeus, Rosa canina, Sambucus nigra,* etc. The herbaceous layer is dominated by the *Asperula taurina, Polygonatum latifolium, Geum urbanum* or *Glechoma hirsuta*.

Table 1: The abundance of investigated invertebrate species with conservative value in Crâng forest, Buzău county, Romania, 2025 (Cc=*Cerambyx cerdo*, Lc=*Lucanus cervus*, On=*Oryctes nasicornis*, Dp= *Dorcus parallelipipedus*).

T	Period	Altitude (m)	Cc	Lc	On	Dp	Tree circumference (cm)
T1	June 2025	98		1			289
T2	June 2025	98	1				184
T3	June 2025	97	2	3			280, 180,122
T4	June 2025	98	5				190, 233
T5	June 2025	98					172
T6	June 2025	105	3				170, 260, 186, 175
T7	June 2025	107	1	1	9		164
T8	June 2025	99	4				376
T9	June 2025	99		2			438, 378, 87, 268
T10	June 2025	100	2	1	2		229, 72, 203, 266, 255, 371
T11	June 2025	72	5	3			160, 121, 109, 98, 119, 137
T12	June 2025	97				1	92
T13	June 2025	97	2	4			183, 188
T14	July 2025	102	4	10			243, 254, 120, 208, 187, 201, 252, 215, 130
T15	July 2025	99		4			263, 183, 187
T16	July 2025	98	2	5	3		347, 248, 182, 178
T17	July 2025	99		1			
T18	July 2025	93					
T19	July 2025	89	3	1	8		265, 252, 249, 311, 347, 246, 149
T20	July 2025	92	3	2		1	411, 399, 259, 302, 254, 346
T21	July 2025	95		2	1		233, 226, 211, 127, 226
T22	August 2025	102	2				260
T23	August 2025	97					221, 231, 145
T24	August 2025	98			12		280
T25	August 2025	99	1	5	9		373, 285, 232, 238, 282

The protected species *Cerambyx cerdo* was identified in 15 transects, which represent 60% from all investigated ones (Figures 1 A, C, D). The total number of individuals was 40 individuals (13 females and 27 males), with a higher abundance in T4 and T11. The lowest numerical densities were obtained in T2, T7, T25 (Table 1). All investigated specimens were identified in 91I0* Euro-Siberian steppic woods with *Quercus* spp. If we consider the sex ratio, the highest number of females were recorded in T14 (3 specimens), on opposite being transects T2, T3, T4, T6, T7, T10 and T16 (any females). Considering the males, the highest abundances were obtained in T4 and T11 (5 specimens and respectively 4 ones). On the other hand, considering the physiological stage, we identified 20 live specimens and 20 exuvia. But, what about the *Cerambyx cerdo* population? It is viable population or not, considering the number of identified individuals? To answer at this question, we made some comparisons with other studies, from two protected areas where the conservation status of their populations is in

good condition. In ROSCI0359 Prigoria-Bengeşti, in 9 investigated transects were identified 22 individuals and in ROSCI0354 Platforma Cotmeana 41 individuals (Manu et al., 2027; Bărbuceanu & Truţă, 2021). So, having these data we can say that population of *Cerambyx cerdo* from Crâng forest is a viable one, well numerically represented.



A. Distribution map for *Cerambyx cerdo*

B. Distribution map for *Lucanus cervus*

C. *Cerambyx cerdo*-male

D. Habitat for *Cerambyx cerdo* and *Lucanus cervus*

E. *Lucanus cervus*-male

F. *Lucanus cervus*- female

Figure 1. Distribution and habitat of species *Cerambyx cerdo* and *Lucanus cervus* in Crâng forest, Buzău, 2025 (original)

Specialists observed that *Cerambyx cerdo* has often been mentioned in association with *Lucanus cervus*, a situation noted in the forest clearings or at the edges with secular trees (Panin & Săvulescu, 1961; Bărbuceanu & Truţă, 2021). This phenomen was observed in Crâng forest as well.

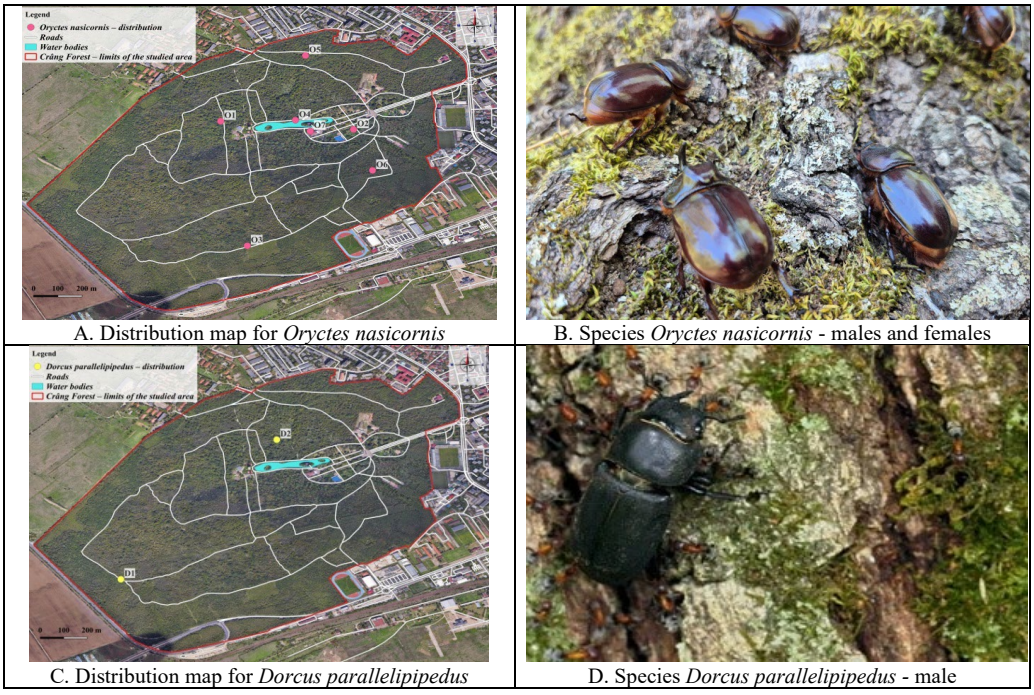
Species *Lucanus cervus* was identified in 15 transects, which represent 60% from all investigated ones (Figure 1 B, D, E, F). The total number of individuals was 45 individuals (22 females and 23 males), with a higher abundance in T14, T16 and T25. The lowest numerical densities were obtained in T1, T7, T10, T17 and T19 (Table 1). The majority of specimens were identified in 91I0* Euro-Siberian steppic woods with *Quercus* spp. If we consider the sex ratio, the highest number of females were recorded in

T14 (8 specimens) and T 25 (5 specimens), on opposite being transects T3, T7, T10, T11, T13, T15 and T19, with any females. Transects were the males were dominant were: T3, T11, and T15. On the other hand, considering the physiological stage, we identified 16 live specimens and 29 exuvia.

Comparing these data with others from two protected areas, we observed that in ROSCI0359 Prigoria-Bengești 228 individuals of *Lucanus cervus* were identified in 59 investigated transects and in ROSCI0354 Platforma Cotmeana 314 individuals (Manu et al., 2027; Bărbuceanu & Truţă, 2021). Considering the small area of Crâng forest, we consider that the population of *Lucanus cervus* is well represented in this location.

On the other hand, another two coleopterans with conservative value were identified, in Crâng forest: *Oryctes nasicornis* and *Dorcus parallelipipedus*. Species *Oryctes nasicornis* was identified in 7 transects, which represent 45% from all investigated ones (Figure 2A, B). The total number of individuals was 44 individuals (27 females and 17 males; 2 live specimens and 42 exuvia), with a higher abundance in T7, T19, T24 and T25. The lowest numerical densities were obtained in T10 and T16 (Table 1). The majority of specimens were identified in 91I0* Euro-Siberian steppic wood with *Quercus* spp., but it was also identified in urban forest ecosystem. If we consider the sex ratio, the highest number of females were recorded in T7 (5 specimens) and T24 and T25 (9 specimens each), on opposite being transects T10, T16 and T21, with any females. The transects where males were dominant were T7 and T19 (4 specimens each).

Species *Dorcus parallelipipedus* was identified in 2 transects, which represent 8% from all investigated ones (Figure 2C, D). The total number of individuals was 2 individuals (one females and one males; 2 live specimens), in T12 and T20 (with one specimen each) (Table 1). These 2 specimens were identified in 91I0* Euro-Siberian Steppic woods with *Quercus* spp.



A. Distribution map for *Oryctes nasicornis*

B. Species *Oryctes nasicornis* - males and females

C. Distribution map for *Dorcus parallelipipedus*

D. Species *Dorcus parallelipipedus* - male

Figure 2: Distribution and habitat of species *Oryctes nasicornis* and *Dorcus parallelipipedus* in Crâng forest, Buzău, 2025 (original)

Table 2: The pressures and threats (with their intensities) for Coleoptera species from Crâng forest, Buzău county (Cc = *Cerambyx cerdo*, Lc = *Lucanus cervus*, On = *Oryctes nasicornis*, Dp = *Dorcus parallelipipedus*)

PRESSURES	Code	Species	Intensity
Removal of dead or dying trees	B02.04	*Cc, L.c, Dp*	Medium
		On	Low
Forest road, tourist trail, footpaths, cycle paths	D01.01	*Cc, Lc, On, Dp*	Medium
National road	D01.02	*Cc, L.c*	Medium
Continuous urbanization	E01.01	*Cc, L.c, On*	Medium
Dispersed housing	E01.03	*Cc, L.c, On*	Medium
Household waste/recreational waste disposal	E03.01	*Cc, On*	Medium
		Lc, Dp	Low
Walking, horse riding and non-motorized vehicles	G01.02	*Cc, L.c, On, Dp*	Low
Motorized vehicles	G01.03	*Cc, L.c*	Low
Other forms of air pollution	H04.03	*Cc, L.c*	Low
Non-native invasive species	I01	*Cc, L.c, Dp*	Medium
		On	Low
Fires, bonfires	J01.01	*Cc*	Medium
		Lc, On, Dp	Low
Groundwater abstraction for public water supply	J02.07.02	*Cc, L.c*	Medium
Drought and reduced rainfall	M01.02	*Cc, L.c, On, Dp*	High
THREATS	**Code**	**Species**	**Intensity**
Removal of dead or dying trees	B02.04	*Cc*	Medium
		Lc	Low
Using chemicals to treat the insects attacks	B04	*Cc, L.c, On, Dp*	High
Forest road, tourist trail, footpaths, cycle paths	D01.01	*Cc, Dp*	Low
		Lc, On	Medium
Specimen collection	F03.02.01	*Cc, L.c, On*	Low
Dispersed housing	E01.03	*Cc, L.c, On*	Low
Household waste/recreational waste disposal	E03.01	*Cc, L.c, On, Dp*	Medium
Walking, horse riding and non-motorized vehicles	G01.02	*Cc, L.c, On, Dp*	Low
Motorized vehicles	G01.03	*Cc, L.c*	Low
Non-native invasive species	I01	*Cc, L.c, On, Dp*	Medium
Fires, bonfires	J01.01	*Cc, L., Onc*	Medium
Groundwater abstraction for public water supply	J02.07.02	*Cc, L.c*	High
Drought and reduced rainfall	M01.02	*Cc, L.c, On, Dp*	High

The code of pressures and threats are according with Order No. 304/2018 from Romania's Ministry of Environment, which approved the Guide for the development of management plans for protected natural areas.

The distribution modeling of species with conservative value represents one of the first step in their ecological knowledge (habitat requirements, conservation measures), especially in urban forests, a less study ecosystems (Onete et al., 2025; Mihai et al., 2025)

In order to protect these species, we must to identify and quantify the intensities of pressures and threats for each protected species. A complete list of them is presented in table 2, being quite similar for all 4 species, but they manifest with different intensities. In total, 13 pressures and 12 threats were identified (Table 2).

We consider that preserving characteristic habitats and applying "minimal intervention" forest management, preserving mature/old trees in their habitat are the main protection measures. We also recommend preserving existing forest trails in optimal/natural conditions, so as not to increase their number (which will lead to habitat fragmentation) or carry out major/aggressive interventions, which involve the destruction of vegetation in adjacent areas and even interventions at tree level. Tourist trails be laid

out in such a way as to protect mature/old trees. If there are old trees with rotten branches that could pose a danger to tourists on tourist routes (alleys/paths) that cannot be reconfigured, they should be selectively cut down. The ongoing urbanization process and scattered settlement must be kept under control so that the structure of the forest ecosystem is maintained at least at its current level, with the preservation of mature trees and reforestation. Proper waste management is another protection measure. The higher number of tourists at the end of the week must be taken into account, ensuring special places for waste storage. The management of invasive non-native species involves constant monitoring and surveillance of areas where they have been identified in order to identify invasive species at an early stage. Once detected, invasive species must be controlled by various methods, such as mechanical removal, biological control (such as mowing, uprooting, and cutting invasive plants), without the use of chemicals. Avoid the use of chemicals as a method of controlling forest pests. If chemical control is absolutely necessary, it should be carried out on small/targeted areas and with selective substances (Purice et al., 2025). In the context of climate change, when drought is becoming increasingly prevalent (with air temperatures reaching up to 38.7°C in Crâng forest), the existence of groundwater catchments/wells can drastically change the hydrological regime of the area, accentuating the drying process of the soil and, implicitly, of the studied forest ecosystem studied. The presence of fire pits and even burned areas in the Crâng forest indicates the presence of another factor disturbing the forest. Fires are strictly prohibited in the forest, and it is recommended that strictly monitored areas be set up for barbecuing or camping. Lighting fires and smoking will only be permitted in specially designated areas; periodic checks will be carried out on those camping within the forest perimeter to verify compliance with fire prevention regulations and to penalize violations. Species as *Cerambyx cerdo* and *Lucanus cervus* are crepuscular invertebrates, which mean that they are mainly active at dusk and dawn. Their activity increases sharply before dusk, reaching peaks at night. Therefore, we consider that public lighting in forest areas poses a threat to this species.

CONCLUSIONS

In Crâng forest from Buzău city, two Natura 2000 species were identified: *Lucanus cervus* and *Cerambyx cerdo*. In the same time, another two indicator species for mature forest were identified: *Oryctes nasicornis* and *Dorcus parallelipipedus*.

Identification of these protected species demonstrated the presence of favorable, characteristically habitats, old/mature deciduous forests and urban forest area with secular oaks, with tree circumferences between 92-411 cm.

On the other hand favorable microhabitats for these species were identified, as: dry logs and dead trees of *Quercus robur* and live trees of the same species.

In the same time the 13 pressures and 12 threats were evaluated, indicating their intensity. The main pressures were: removal of dead or dying trees; forest road, tourist trail, footpaths, cycle paths; urbanization, non-native invasive species, groundwater abstraction for public water supply; drought and reduced rainfall.

The main threats were: using chemicals to treat the insect's attacks; household waste/recreational waste disposal; walking, horse riding and non-motorized vehicles; specimen collection; drought and reduced rainfall. In order to maintain viable populations some specific conservation measures were established, in correlations with identified pressures and threats. Protecting their habitats and microhabitats we will conserve the coleopterans species. A proper forest management, waste management, invasive species management and a controlled urbanization process are few conservation measures. From conservative point of view, we consider that Crâng area represent an important forest ecosystem for Coleoptera species.

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HISTOMORPHOLOGICAL HEPATIC ALTERATIONS IN *Squalius cephalus* (Linnaeus, 1758) EXPOSED TO HEAVY METALS: A CROSS-WATERSHED COMPARATIVE STUDY OF TWO ROMANIAN RIVERS

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Abstract

Heavy metal pollution poses a major threat to freshwater ecosystems and aquatic organisms. This study investigates liver histomorphology in chub (Squalius cephalus), a sentinel species, from two Romanian rivers with contrasting contamination levels: the Arieș River in the Roșia Montană area, a basin heavily affected by long-term mining, and the unpolluted Hășdate River. Water analyses confirmed significant metal contamination in the Arieș River and clean conditions in the Hășdate River. Hepatic tissue was collected, histologically processed by the routine paraffin embedding technique, and stained with Goldner's trichrome to assess structural integrity. Livers from Hășdate fish displayed normal hepatic architecture, whereas hepatic samples collected from the Arieș River exhibited histopathological alterations, including vascular adaptations (hyperaemia), degenerative lesions (vacuolar/lipidic or hydropic degenerations) and extended necrotic lesions. These findings demonstrate that chronic exposure to heavy metal-contaminated waters induce clear structural changes in fish livers, highlighting hepatic histology as a sensitive biomarker for aquatic pollution. The study provides insights into ecosystem health and supports the use of fish liver histopathology for environmental monitoring and risk assessment, offering a model for evaluating pollution impacts in freshwater systems globally.

Key words*: freshwater ecosystems, heavy-metal contamination, histopathology, sentinel species.*

INTRODUCTION

Aquatic ecosystems are increasingly threatened by anthropogenic pollution, particularly by heavy metals originating from industrial and mining activities. In recent years, fish histopathology has become a widely recognized and reliable tool for ecological risk assessment and biomonitoring of aquatic environments (Yancheva et al., 2016; Reddy & Rawat, 2013). Once toxicants cross biological barriers and enter the bloodstream, they are distributed to internal organs, where they may accumulate and induce structural damage (Yancheva et al., 2016). Among fish organs, the liver is considered one of the most informative targets for environmental assessment due to its central role in detoxification, metabolism, and excretion of xenobiotics (Hinton & Laurén, 1990; van der Oost et al., 2003; Salamat & Zarie, 2012; Yancheva et al., 2016). Owing to its high vascularization and metabolic activity, the liver is frequently identified as a primary organ for bioaccumulation and toxicant-induced injury (Yancheva et al., 2016; Georgieva et al., 2016). Histopathological evaluation provides a sensitive and integrative biomarker of exposure, reflecting both short- and long-term toxic effects, and has therefore been incorporated into environmental monitoring

programs worldwide (Yancheva et al., 2016; Reddy & Rawat, 2013).

In healthy teleost fish, the liver exhibits a typical parenchymatous architecture without pathological abnormalities (Vicenti et al., 2005; Figueiredo-Fernandes et al., 2007; Yancheva et al., 2016). The hepatic parenchyma is primarily composed of polygonal hepatocytes containing a centrally located, large spherical nucleus and homogeneous cytoplasm. These cells are arranged in cord-like structures separated by sinusoidal capillaries, whose lumen predominantly contain erythrocytes (Yancheva et al., 2016). The sinusoids are lined by reticuloendothelial cells and are supplied by venous blood entering caudally from the intestine via the hepatic portal vein system (Yancheva et al., 2016). Preservation of this structural organization is generally indicative of good environmental quality and physiological homeostasis.

Exposure to metals and other xenobiotics can induce a wide spectrum of hepatic alterations in fish, many of which are considered reliable biomarkers of toxic damage (Stentiford et al., 2003; Yancheva et al., 2016). Degenerative changes such as vacuolar, hydropic, and fatty degeneration are frequently reported and are associated with biochemical disturbances including impaired protein synthesis, altered enzymatic activity, disruption of ion regulation, and energy depletion (Monteiro et al., 2005; Rajeshkumar & Munuswamy, 2011; Yancheva et al., 2016). Hydropic vacuolation may result from disruption of volume-regulating ATPases and ionic imbalance (Hinton & Laurén, 1990; Yancheva et al., 2016) and has been documented in multiple fish species exposed to contaminants (Stehr et al., 1998; Lang et al., 2006; Yancheva et al., 2016). Lipid accumulation within hepatocytes, characterized by clear cytoplasmic vacuoles, has similarly been associated with toxicant exposure and metabolic disturbance (Marty et al., 2003; Pinto et al., 2009; Yancheva et al., 2016). More severe alterations include hyperemia, necrosis, inflammatory infiltrates, and architectural disorganization of the hepatic parenchyma (Yancheva et al., 2016; Reddy & Rawat, 2013). Necrosis, in particular, is regarded as one of the most serious structural changes and is commonly associated with metal exposure (Yancheva et al., 2016). Previous investigations conducted in Romanian rivers have demonstrated correlations between metal accumulation and histopathological alterations in the liver of European chub *Squalius cephalus* (Linnaeus, 1758) and other indigenous species (Triebskorn et al., 2008).

The Roșia Montană area, situated within the Arieș River Basin in Romania, represents one of Europe's most historically significant mining regions, with exploitation activities dating back to the Dacian Kingdom and intensifying during Roman times (Bănăduc et al., 2021). Mining activities continued throughout the Middle Ages and into the modern era, leaving persistent environmental impacts (Bănăduc et al., 2021). Metal pollution derived from ore extraction and processing is of particular concern due to the persistence, stability, and bioaccumulative properties of heavy metals (Georgieva et al., 2016). Metals released from mining operations can enter surface waters, where they accumulate in sediments and aquatic organisms, posing risks to ecosystem integrity and fish health (Georgieva et al., 2016).

Given the documented sensitivity of fish liver histopathology to metal exposure and the historical mining pressure in the Roșia Montană region, the present study aimed to comparatively evaluate hepatic histological alterations in European chub (*Squalius cephalus*) collected from two rivers differing in heavy metal pollution status. Specifically, we compared the liver architecture of fish inhabiting a river affected by industrial and mining-derived contamination with that of fish from a reference, non-polluted river, in order to assess the extent to which environmental metal exposure is reflected in hepatic structural integrity.

MATERIALS AND METHODS

Sampling site

With the help of thorough bibliographic studies (Glevitzky et al., 2025; Levei et al., 2011; Dumitrel et al., 2015; Bird et al., 2005; Bănăduc et al., 2021), several geographical areas crossed by major watercourses and sharing similar climatic characteristics were identified. Subsequently, field surveys were

carried out in these areas. Two Romanian rivers with contrasting contamination levels were selected: the Arieș River in the Roșia Montană area, a basin heavily affected by long-term mining (Bănăduc et al., 2021), and the unpolluted Hășdate River. In order to have a comparable environment from a climatic point of view, before selecting the rivers, inclusion and exclusion criteria were created. The main characteristics considered were the pollution potential of the area (taking into account whether the watercourse crosses an industrial area upstream—such as mining operations, coal-fired power plants, or waste disposal facilities), ease of access for different fishing methods (parameters such as water depth, discharge, and bank slope were analyzed), as well as the possibility of retaining catches of legal size limit for each specimen. Specifically, the inclusion and exclusion criteria for each river category are presented in Table 1.

Table 1. Specific and common inclusion and exclusion criteria for the selection of two contrasting rivers in terms of industrial pollution

	River category			
	Positive control (industrially polluted with heavy metals)		**Negative control** (not industrially polluted with heavy metals)	
	Inclusion criteria	Exclusion criteria	Inclusion criteria	Exclusion criteria
Specific	Crosses an industrial area upstream	Absence of a clearly identifiable industrial area upstream	Does not cross an industrial area upstream	Presence of a clearly identifiable industrial area upstream
Common	Inclusion criteria		Exclusion criteria	
	Easy access		Lack of access to the watercourse	
	Possibility of retaining catches		Biological conservation area or catch-and-release (C & R) fishing area	
	Climate & hydrology – moderate continental		Different climatic regime	
	Approximate altitude – 400-600 m		High altitude (mountain areas) or low altitude (near 0 m)	
	Aquatic fauna – chub (*Squalius cephalus*) present in fish surveys		Area unsuitable for the presence of chub	

Water evaluation

In situ analyses - determination of temperature and pH: Water temperature was measured using a portable thermometer (Hanna Checktemp 4, model HI151), while pH was determined with a portable pH meter (Hanna pHep+ model HI98108). Both parameters were measured in triplicate at the sites from which water and substrate samples were subsequently collected.

Sample collection for heavy metal pollution assessment: For the determination of heavy metal concentrations, three water samples and three substrate samples were collected from each river site. Water samples were collected in accordance with the guidelines provided by the reference laboratory, using sterile glass containers. Substrate samples were collected using hermetically sealed plastic bags. All collected samples were refrigerated and transported to the laboratory on the day of sampling. The water samples were filtered through 0.45 µm cellulose acetate membrane filters and acidulated with 65% HNO_3 to pH<2. An amount of 3 g sample was digested with aqua regia (HCl 37%: HNO3 65%, 3:1, v:v), filtered and diluted to 100 mL with ultra-pure water (Elga Veolia, High Wycombe, UK) according to ISO 11466. Each sample was prepared in triplicate. A Thermo Fisher Scientific iCAP TQ inductively coupled plasma mass (ICP-MS) spectrometer was used for elemental analysis. The instrument was tuned before the batch run and optimized for high signal and low noise, while making sure the double ionization and oxides level were kept under control. Multi-element ICP-MS standard was purchased for Perkin Elmer for calibration solutions and a 4-point plus blank calibration curve was used for the analysis (with respective concentration of 10-20-50-100 ppb for each element). To ensure no polyatomic or isobaric interference would alter the result, two interference removal techniques were used in the collision reaction cell: kinetic energy discrimination (KED, by using a Helium flow inside the cell) for most elements and mass-shift (by using an Oxygen flow inside the cell)

for elements where KED is known to be ineffective, namely Sc, V, As, Se, Sr.

Biological material

The European chub *Squalius cephalus* (Linnaeus, 1758) has been shown to function effectively as a sentinel species in environmental monitoring studies (Hájková et al., 2007; Havelkova et al., 2008; Krywult et al., 2008). A total of 15 European chub were collected for the present study between July and September 2025 using spinning gear with artificial lures. Only adult specimens exceeding 25 cm in total length (the minimum legal size) were selected. The fish were humanely euthanized immediately after capture by administerring an overdose of *Eugenia cariophilata* extract in water. Shortly thereafter, anatomical dissections were performed and liver tissues were excised. All collected samples were promptly fixed in 10% buffered formalin.

Histological assessment

Histological analyses were performed in the histology laboratory of the Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania. Following collection, samples were fixed for five days, after which liver tissues were processed according to standard paraffin-embedding protocols. Serial sections with a thickness of 5 µm were obtained using a rotary microtome (Leica rotary microtome, Germany). The sections were subsequently stained using Goldner's trichrome method, following established procedures (Suvarna et al., 2018). Microscopic examination was carried out using an Olympus BX41 light microscope equipped with an Olympus SC180 digital camera (Olympus, Japan), and image acquisition and analysis were performed with Olympus CellSens software.

Histopathological evaluation: All microscopic preparations were independently examined by two pathologists, and the observed lesions were identified, recorded, and classified accordingly.

Statistical analysis

The obtained data were processed statistically processed, usingMS Office/Excel and GraphPad Prism 8 software.

Heavy metal concentrations below the analytical *limit of detection* (LOD) were treated as left-censored data and substituted with LOD/2 prior to statistical analysis. This approach was applied when the proportion of values below LOD was low and is commonly used in environmental contamination studies to reduce bias associated with zero substitution (Hornung & Reed, 1990). Heavy metal concentrations (determined in µg/L for water samples and mg/kg for sediment/soil samples) often exhibit high variability and are typically positively skewed, with a small number of samples showing very high concentrations while most remain low. All raw concentration data were log-transformed ($\log_{10}$) prior to analysis. This transformation also helps to reduce the influence of extreme outliers and stabilizes variance across the data range (Reimann & Filzmoser, 2000). Due to the high dimensionality of the dataset (25 elements) and the wide range of concentrations spanning several orders of magnitude, all raw data were subjected to a logarithmic transformation ($y = \log_{10} y$) prior to analysis. This procedure was essential to normalize the data distribution and ensure homoscedasticity across the variables.

To evaluate the environmental dynamics between the polluted (Positive Control River - PCR) and control (Negative Control River - NCR) rivers, three distinct sets of comparisons were conducted: (a) partitioning between the water column and the substrate within each site, (b) comparison of dissolved metals between sites, and (c) comparison of substrate-bound metals between sites.

Statistical significance for each of the 25 elements was determined using Multiple t-tests (one per row). To account for the multiple testing problem and control the family-wise error rate, the Benjamini-Hochberg FDR was applied to adjust *p*-values and identify significant "discoveries." The threshold for statistical significance was set at $p < 0.05$. All results are expressed as Mean Standard Error of the Mean (SEM) (n = 3). Graphical representations were generated as interleaved bar charts on a scale to facilitate the visual assessment of multi-elemental partitioning.

RESULTS AND DISCUSSIONS

Positive control river (industrially polluted with heavy metals)

Following field surveys and the application of the previously presented inclusion/exclusion criteria, the Arieș River in the Roșia Montană area (at the confluence with local tributaries – Abrudel, Roșia, Valea Șesii), was selected as the positive control hydrographic basin (industrially polluted with heavy metals). From a climatic and hydrological perspective, this watercourse exhibits a regime characterized by a moderate continental climate and a pluvio-nival hydrological influence. In this region as well, these environmental characteristics are conducive to the presence of chub (*Squalius cephalus*) in the aquatic fauna (Mireșan et al., 2016).

Negative control river (not industrially polluted with heavy metals)

Following field surveys and the application of the previously presented inclusion/exclusion criteria, the Hășdate River was selected as the negative control hydrographic basin (not industrially polluted with heavy metals). This river spans altitudes ranging from approximately 400 m at the exit of the gorge to over 600-700 m in the mountainous sector. The region experiences a moderate continental climate, with substantial precipitation in spring and summer and winters characterized by a moderate snow cover (approximately 25-65 days per year). The hydrological regime is mixed, pluvio-nival, with potential flood events occurring in spring due to snowmelt and after intense summer rainfall. These hydrological and ecological characteristics are also conducive to the presence of chub (*Squalius cephalus*) (Mireșan et al., 2016).

Water parameters

Water temperature: In the positive control watercourse, the mean recorded water temperature was 20.46 ± 0.12°C (Figure 1). For the negative control watercourse (Hășdate River), the mean water temperature was 20.53 ± 0.04°C. Measurements were carried out at an interval of 10 days, both in June 2025, under sunny conditions and in the absence of precipitation in the preceding days.

Water pH: In the positive control watercourse the mean pH was 6.33 ± 0.36, whereas in the negative control watercourse, the mean pH value was 7.40 ± 0.08 (Figure 1). As for temperature, pH measurements were performed 10 days apart, both in June 2025, during sunny weather and with no rainfall recorded in the days prior to sampling.

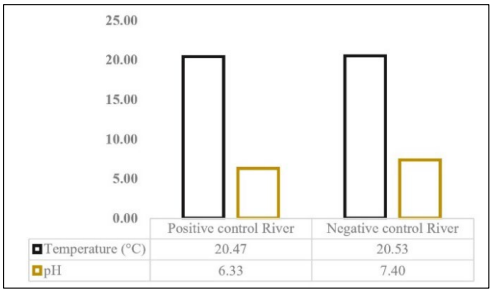


Figure 1. Comparison of mean water temperature and pH between the positive (industrially heavy metal–polluted) and negative (unpolluted) control rivers

Heavy metals contamination: In terms of heavy metal concentrations in water samples from the positive control river, manganese (Mn) and iron (Fe) levels markedly exceeded the threshold values established for Class V (very poor quality) surface waters (MMGA, 2006). Specifically, Mn reached 1429.4 µg/L, compared with the Class V limit of >1000 µg/L, while Fe concentrations were 7937.7 µg/L, substantially surpassing the corresponding limit of >2000 µg/L. In contrast, concentrations of the analyzed elements in the negative control river were considerably lower, resulting in its classification within Class I or, at most, Class II water quality, indicative of an essentially unpolluted system. Furthermore, comparative analysis between the two rivers revealed higher concentrations of Li, Mg, Al, Co, Ni, Cu, Zn, and As in the positive control river relative to the negative control. For both watercourses, concentrations of Be, Co, Ga, Se, Ag, Cd, In, Cs, Tl, Bi, and Hg were below the analytical limit of detection (<1.0 µg/L) (Table 2).

Positive control river results (water vs. sediment)

The comparison between dissolved metals in the water column (W-PCR) and the sedimentary substrate (S-PCR) at the polluted site

revealed a strong preferential accumulation in the solid phase. Statistical analysis using Multiple t-tests identified several significant 'discoveries' where substrate concentrations far exceeded those in the water column ($p < 0.001$). A prominent example was gallium (Ga), which exhibited a highly significant difference ($p = 0.000076$), with substrate concentrations approximately 40 times higher than those recorded in the water column (Difference = -1.597log10 units). Similar trends of sedimentary enrichment were observed for major elements such as aluminium (Al), iron (Fe), and lead (Pb). This distinct partitioning pattern confirms that the substrate in the PCR river acts as the primary reservoir for the heavy metal load, effectively sequestering contaminants from the aqueous phase (Figure 2).

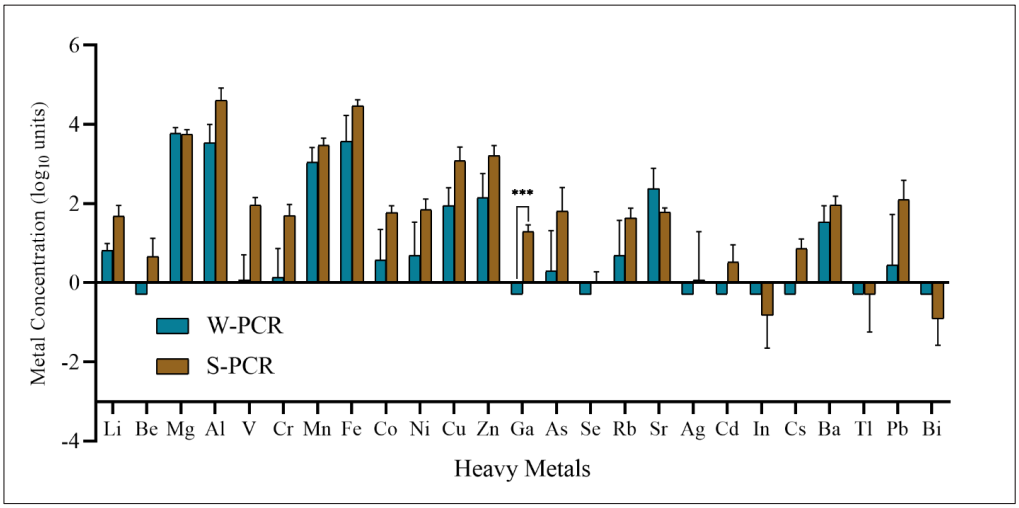


Figure 2. Comparative analysis of heavy metal concentrations in water (W-PCR) and sediment (S-PCR) samples from the Positive Control River. Data are presented on a logarithmic scale (log10 units, µg/L for water samples and mg/kg for sediment samples) to account for the wide range of elemental concentrations. Error bars represent the standard deviation (SD)

Negative control river results (water vs. sediment)

At the NCR site, statistical analysis using Multiple t-tests identified a specific group of trace elements that remained predominantly in the aqueous phase rather than accumulating in the substrate. Selenium (Se), silver (Ag), indium (In), strontium (Sr), cesium (Cs), and thallium (Tl) all exhibited significantly higher concentrations in the water column compared to the sedimentary matrix ($p < 0.01$). The most robust statistical differences were recorded for Se, Ag, and In, which maintained concentrations in the water exactly one order of magnitude higher than in the substrate (Difference = 1.00 log10 units, $p < 0.00000.1$). Strontium (Sr) showed the most substantial divergence, with aqueous levels being approximately 26.5 times higher than those found in the substrate (Difference = 1.424 log10 units, $p < 0.0001$). Furthermore, thallium (Tl) and cesium (Cs) concentrations were 14.2 and 5.5 times higher in the water phase, respectively ($p < 0.002$), indicating a high degree of elemental persistence in the water column of the NCR site (Figure 3).

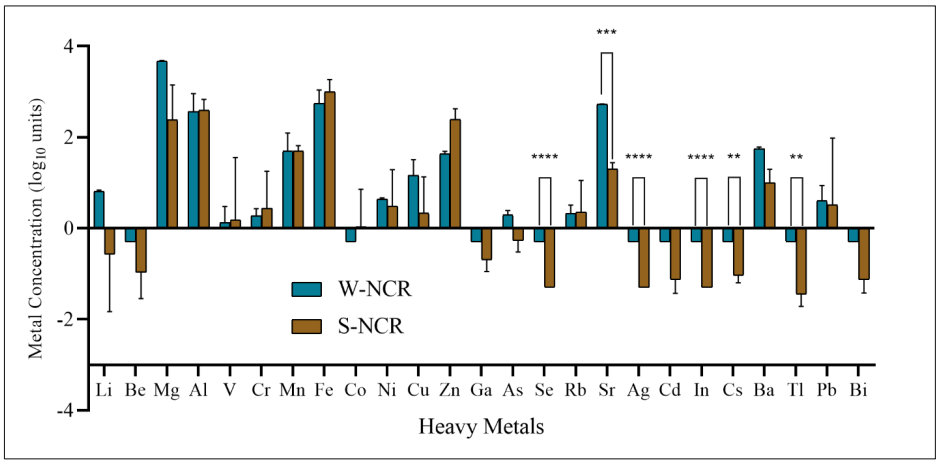


Figure 3. Comparative analysis of heavy metal concentrations in water (W-NCR) and sediment (S-NCR) samples from the Negative Control River. Data are presented on a logarithmic scale (log10 units, µg/L for water samples and mg/kg for sediment samples) to account for the wide range of elemental concentrations. Error bars represent the standard deviation (SD)

Positive control river vs. negative control river water samples comparisons

The comparison of dissolved metal concentrations between the polluted river (PCR) and the negative control river (NCR) showed that, although several elements exhibited higher mean values in the polluted site, no statistically significant 'discoveries' were identified after False Discovery Rate (FDR) correction ($q > 0.05$). Visually, manganese (Mn), aluminium (Al), and iron (Fe) showed the most prominent increases in the W-PCR samples compared to W-NCR. Specifically, manganese reached a *p*-value of 0.0126 and aluminium 0.0512. However, these did not meet the stringency requirements for multiple comparisons across all 25 elements. For highly toxic elements such as cadmium (Cd), lead (Pb), and arsenic (As), concentrations remained relatively low or below the limit of detection (LOD) in both river systems, suggesting that the dissolved phase might not fully reflect the anthropogenic impact at the time of sampling (Figure 4).

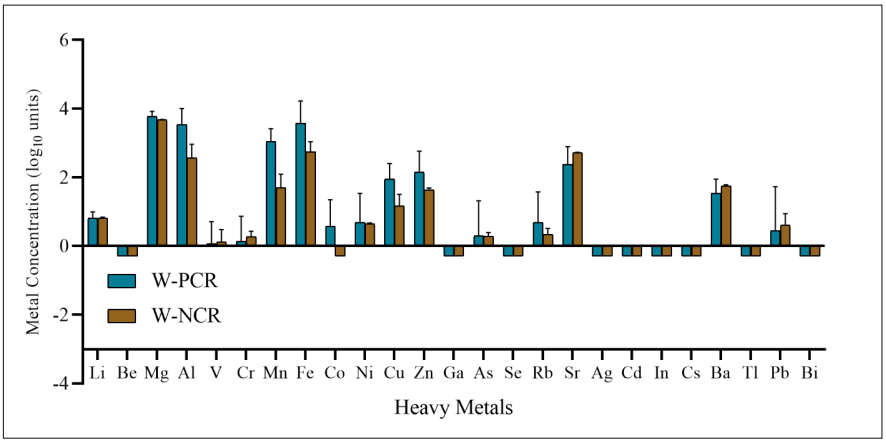


Figure 4. Comparative analysis of heavy metal concentrations in water samples from the Positive Control River (W-PCR) and the Negative Control River (W-NCR). Data are presented on a logarithmic scale (log10 units, µg/L for water samples and mg/kg for sediment samples) to account for the wide range of elemental concentrations. Error bars represent the standard deviation (SD)

Positive control river vs. negative control river sediment samples comparisons

The analysis of the sedimentary substrate provided the most definitive evidence of environmental contamination at the polluted PCR site. Unlike the water column, the substrate samples exhibited a distinct 'pollution fingerprint' for several key elements. Statistical analysis using Multiple t-tests on log_{10}-transformed data identified six statistically significant discoveries (Al, Mn, Fe, Ga, Se, and Cs) with $q < 0.05$ (FDR corrected). The most dramatic enrichment was observed for aluminium (Al) and gallium (Ga), which exhibited concentrations approximately 100 times higher in the S-PCR substrate compared to the NCR reference site (Differences of 2.02 and 1.99 log_{10} units, respectively; $p < 0.001$). Furthermore, manganese (Mn) and cesium (Cs) showed massive accumulation in the polluted sediments, with concentrations nearly 60 to 80 times higher than those in the clean river ($p<0.0005$). Other major elements, such as iron (Fe), also showed significant enrichment in the PCR site (Difference = 1.469; $p = 0.0012$), confirming the role of the PCR substrate as a major long-term sink for anthropogenic pollutants (Figure 5).

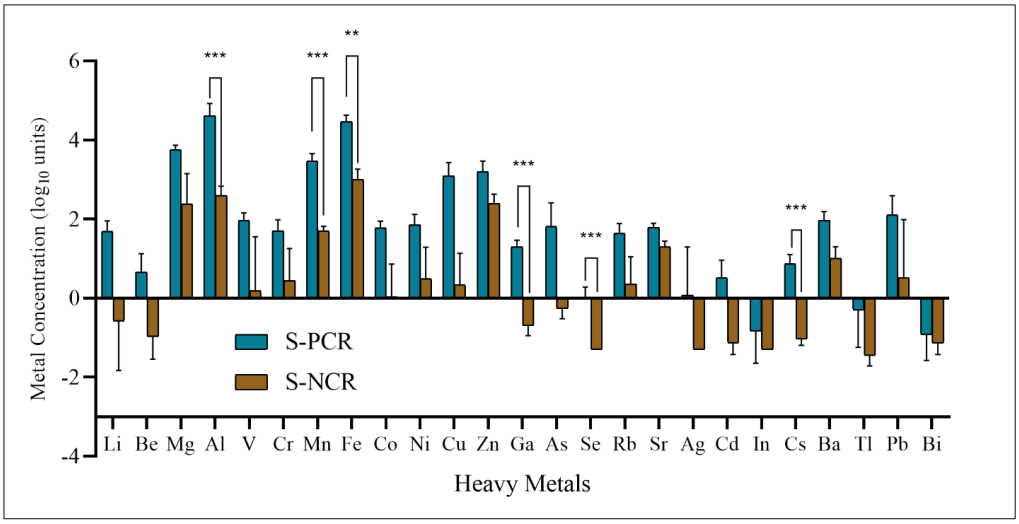


Figure 5. Comparative analysis of heavy metal concentrations in sediment samples from the Positive Control River (S-PCR) and the Negative Control River (S-NCR). Data are presented on a logarithmic scale (log10 units, µg/L for water samples and mg/kg for sediment samples) to account for the wide range of elemental concentrations. Error bars represent the standard deviation (SD)

Regarding heavy metal concentrations in sediment samples, current legislation does not specify threshold values for pollution classification. Nevertheless, the results of the present study indicate that, in comparative terms, metal concentrations in sediments from the positive control river were consistently higher than those measured in the negative control river. Furthermore, in the positive control river, none of the analyzed elements were below the analytical limit of detection (0.10 mg/kg), whereas in sediments from the negative control river, selenium (Se), silver (Ag), and indium (In) were below the limit of detection (Table 2).

Table 2. Heavy metal concentrations in water and sediment samples from the positive and negative control rivers

	Positive control river (PCR)		Negative control river (NCR)	
	Water samples W-PCR (ug/L)	Sediment samples S-PCR (mg/kg)	Water samples W-NCR (ug/L)	Sediment samples S-NCR (mg/kg)
Li	6.79	54.86	6.48	7.34
Be	<LOD	6.31	<LOD	0.50
Mg	6129.67	5785.22	4706.32	675.14
Al	4857.72	47241.84	458.27	435.17
V	6.38	97.69	2.13	13.74
Cr	9.52	57.62	1.95	8.54
Mn	1429.36	3125.62	61.91	50.90
Fe	7937.67	30546.79	638.10	1130.08
Co	10.89	63.06	<LOD	3.49
Ni	15.04	80.94	4.40	9.27
Cu	124.17	1506.10	18.31	6.49
Zn	260.13	1791.64	43.47	272.74
Ga	<LOD	20.81	<LOD	0.23
As	29.81	99.87	1.98	0.59
Se	<LOD	1.18	<LOD	<LOD
Rb	17.27	48.27	2.25	5.35
Sr	392.62	62.47	529.27	20.66
Ag	<LOD	6.94	<LOD	<LOD
Cd	<LOD	4.32	<LOD	0.16
In	<LOD	1.28	<LOD	<LOD
Cs	<LOD	8.15	<LOD	0.10
Ba	43.76	100.63	56.40	11.74
Tl	<LOD	2.24	<LOD	0.02
Pb	83.89	174.19	4.81	54.55
Bi	<LOD	0.70	<LOD	0.16

Note: LOD - limit of detection (1.0 µg/L for water samples and 0.1 mg/kg for sediment samples).

Histological results

Histopathological examination of liver tissue from chub (*Squalius cephalus*) revealed clear differences between specimens collected from the negative and positive control rivers (Figure 6). Fish from the negative control river exhibited normal hepatic architecture, characterrized by well-organized hepatocytes arranged in cord-like structures and separated by narrow sinusoidal capillaries (Figure 6A).

In contrast, liver tissue from fish collected in the positive control river showed multiple and pronounced pathological alterations. Hepatocytes exhibited both lipid (steatotic) and hydropic degeneration, evidenced by marked cytoplasmic vacuolization and cellular swelling (Figure 6B, C, F, I; black arrows). Vascular disturbances were frequently observed, including sinusoidal dilation and congestion consistent with hyperemia (Figure 6D, E, F, G, H; empty arrows).

Numerous hepatocytes displayed morphological features indicative of apoptosis (Figure 6G; arrowheads), while focal areas of hepatocellular necrosis were also present (Figure 6G, H, I; dashed rectangles). These degenerative and vascular changes were accompanied by a loss of normal hepatic architecture, with disruption of the cord-like arrangement of hepatocytes and a markedly disorganized parenchyma (Figure 6H; dashed circle). In more advanced lesions, these alterations frequently co-occurred within the same tissue sections, indicating progressive hepatic damage.

Collectively, these alterations indicate substantial structural impairment of liver tissue in fish from the heavy metal-polluted river. In contrast, liver sections from chub sampled in the negative control river showed preserved histological organization and features consistent with normal hepatic tissue. Hepatocytes were arranged in regular cords separated by narrow, non-dilated sinusoidal capillaries, with no evidence of vascular congestion. The cells displayed a typical polygonal shape, an acidophilic cytoplasm, and centrally located

spherical nuclei, present as either single or occasionally binucleated. No degenerative, apoptotic, or necrotic alterations were detected in these samples (Figure 6).

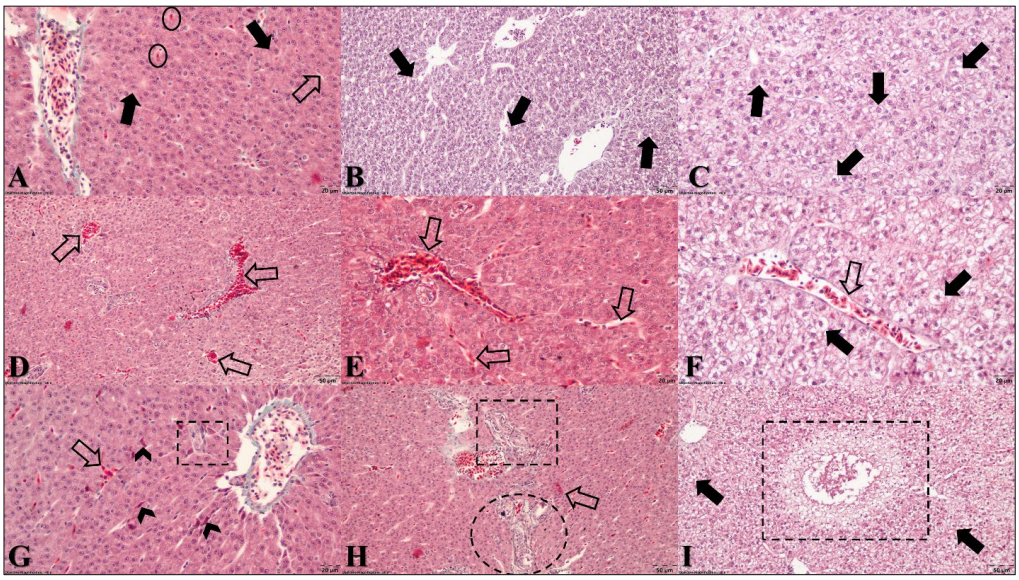


Figure 6. Representative histological sections of liver tissue from chub (*Squalius cephalus*) collected from the negative (A) and positive (B–I) control rivers. (A) Normal hepatic architecture observed in fish from the negative control river; (B–C) Hepatocellular degenerative changes; (D–E) Vascular alterations; (F) Combined hepatocellular and vascular lesions; (G–I) Advanced hepatic lesions, including necrosis and architectural disruption; Black arrows indicate hepatocellular alterations (e.g., vacuolization, degeneration); empty arrows indicate vascular structures and sinusoidal dilation; arrowheads indicate apoptotic cells; dashed rectangles indicate necrotic areas; dashed circles indicate loss of normal hepatic architecture

DISCUSSIONS

The heavy metal analysis clearly differentiated the two rivers in terms of contamination status. In the positive control river, Mn (1429.4 µg/L) and Fe (7937.7 µg/L) exceeded the thresholds established for Class V (heavily polluted) waters, while the negative control river fell within Class I–II quality, indicating minimal impact. Elevated Fe and Mn concentrations are frequently reported in mining-affected catchments due to metal mobilization from historical ore exploitation (Georgieva et al., 2016; Bănăduc et al., 2021), and similar contamination profiles have been associated with increased bioavailability and toxicity in freshwater ecosystems (Yancheva et al., 2016). Additionally, higher levels of Li, Mg, Al, Co, Ni, Cu, Zn, and As were detected in the polluted river, reflecting a multi-metal exposure scenario typical of mining-derived pollution. Such mixed-metal contamination has been linked to hepatic degenerative and necrotic lesions in fish (Mela et al., 2007; Carrola et al., 2009; Yancheva et al., 2016), supporting the histopathological alterations observed in the present study.

Moreover, the present study demonstrates marked differences in hepatic architecture between European chub (*Squalius cephalus*) collected from a heavy metal-impacted river (positive control) and those from a reference, non-polluted river (negative control). Fish from the contaminated site exhibited a wide spectrum of histopathological alterations, including hydropic and lipid degeneration, vascular hyperemia, apoptosis, necrosis, and disruption of normal parenchymal organization. In contrast, specimens from the reference site displayed preserved hepatic architecture, characterized by polygonal hepatocytes with centrally located spherical nuclei arranged in cord-like structures separated by non-dilated sinusoids containing erythrocytes. These

findings are consistent with the established role of fish liver histopathology as a sensitive biomarker of environmental contamination (Yancheva et al., 2016; Reddy & Rawat, 2013). The normal hepatic morphology observed in fish from the negative control river corresponds to descriptions of healthy teleost liver structure reported in previous studies (Vicenti et al., 2005; Figueiredo-Fernandes et al., 2007; Yancheva et al., 2016). The preservation of hepatocyte polarity, cytoplasmic homogeneity, and sinusoidal organization suggests physiological homeostasis and absence of significant toxicant-induced stress. Such structural integrity is generally indicative of good environmental quality and supports the classification of this river as minimally impacted.

In contrast, the liver alterations recorded in fish from the positive control river reflect a pattern typically associated with chronic exposure to heavy metals and other xenobiotics. Hydropic and lipid degeneration, characterized by cytoplasmic vacuolation and ballooning of hepatocytes, were among the most frequent lesions observed. Similar degenerative changes have been widely reported in fish exposed to metals and organic contaminants (Monteiro et al., 2005; Rajeshkumar & Munuswamy, 2011; Yancheva et al., 2016). Hydropic vacuolation has been linked to ionic imbalance and disruption of membrane-bound ATPases involved in cellular volume regulation (Hinton & Laurén, 1990; Yancheva et al., 2016), while lipid accumulation may reflect metabolic disturbances and impaired lipid processing under toxic stress (Marty et al., 2003; Pinto et al., 2009; Yancheva et al., 2016). The presence of these degenerative patterns in our study strongly suggests sublethal but persistent toxicant exposure in fish inhabiting the positive control river.

Marked vascular alterations, particularly sinusoidal dilation and hyperemia, were also observed in the samples from the positive control river. Increased vascular congestion in the hepatic parenchyma has been described as a common response to environmental stressors and may reflect circulatory disturbances or inflammatory processes triggered by toxic exposure (Yancheva et al., 2016; Reddy & Rawat, 2013). The pronounced sinusoidal dilation observed in our specimens indicates altered hepatic hemodynamics, potentially secondary to endothelial damage or impaired outflow, conditions frequently associated with contaminant-induced hepatotoxicity.

More severe lesions, including a high number of apoptotic hepatocytes and focal areas of necrosis, were identified exclusively in fish from the polluted river. Necrosis is considered one of the most critical histopathological endpoints in toxicological studies and has been repeatedly associated with heavy metal exposure (Mela et al., 2007; Carrola et al., 2009; Yancheva et al., 2016). The occurrence of necrotic foci and parenchymal disorganization in the present study indicates advanced cellular injury and loss of tissue integrity. Such alterations likely result from prolonged oxidative stress, mitochondrial dysfunction, and disruption of cellular homeostasis induced by metal accumulation. Previous investigations in European freshwater systems, including Romanian rivers, have similarly reported correlations between tissue metal burden and hepatic necrosis in chub and other cyprinid species (Triebskorn et al., 2008).

The combination of degenerative, circulatory, and necrotic changes observed in the positive control group reflects a continuum of hepatic injury ranging from reversible cellular alterations to irreversible structural damage. This lesion pattern aligns with the known bioaccumulative and persistent nature of heavy metals in mining-impacted aquatic ecosystems (Georgieva et al., 2016; Bănăduc et al., 2021). Given the documented history of mining activities in the Roșia Montană region (Bănăduc et al., 2021), the hepatic pathology identified in this study likely represents the biological consequence of long-term environmental contamination.

Overall, the clear contrast between the two rivers confirms that hepatic histopathology in *Squalius cephalus* serves as an effective biomarker for assessing ecological impact in metal-polluted freshwater systems. The concordance between our findings and previously published data further strengthens the use of fish liver structural alterations as an integrative indicator of environmental quality (Yancheva et al., 2016; Reddy & Rawat, 2013). These results highlight the importance of

continued biomonitoring efforts in historically impacted mining regions and support the inclusion of histopathological endpoints in comprehensive environmental risk assessment frameworks.

The massive enrichment of trace elements in the substrate compared to the water column at the PCR site indicates a high degree of sedimentary retention. In fluvial systems affected by anthropogenic discharge, heavy metals tend to undergo rapid adsorption onto fine-grained minerals, organic matter, and Fe-Mn oxyhydroxides, leading to their accumulation in the riverbed.

The case of gallium (Ga) and other poorly soluble metals highlights the role of the substrate as a 'long-term memory' of pollution. While the water column reflects the immediate and transient state of the river, the substrate provides a cumulative record of contamination. However, this high sedimentary load poses a latent ecological risk; changes in the river's physico-chemical parameters (such as pH or redox potential) could potentially trigger the remobilization of these sequestered metals back into the water column, increasing their bioavailability and toxicity to the aquatic ecosystem.

The significant prevalence of Se, Ag, In, Sr, Cs, and Tl in the water column at the NCR site suggests that these elements possess high geochemical mobility and low affinity for the local sedimentary matrix. In an anthropogenic context, the fact that these elements are not effectively sequestered by the substrate is concerning; it indicates that they remain in a dissolved or colloidal state, which directly increases their bioavailability and potential toxicity to aquatic organisms.

The high mobility of strontium (Sr) and thallium (Tl) in the aqueous phase of the NCR river suggests a lack of active precipitation or adsorption processes that would typically remove these metals from the water column. These results highlight a critical environmental risk: while other heavy metals might be 'trapped' in the sediment, this specific group remains a persistent threat to the water quality of the NCR system. This high solubility must be considered when evaluating the overall ecological impact of pollution in this river segment.

The lack of statistically significant differences in the water column between the polluted (PCR) and clean (NCR) rivers, despite visual trends, can be attributed to several environmental factors. Metals in the aqueous phase are highly dynamic and subject to rapid fluctuations due to flow rate, pH changes, and dilution effects. The fact that elements like Al, Mn, and Fe showed near-significant increases in PCR suggests a state of chronic low-level enrichment rather than acute contamination in the dissolved phase. It is highly probable that the bulk of the heavy metal load in the polluted PCR system has partitioned into the solid phase. Therefore, the sedimentary substrate likely acts as the primary long-term sink for these pollutants, making the comparison of substrate samples (S-PCR vs. S-NCR) critical for a definitive assessment of the environmental degradation in these two river systems.

The stark contrast between the lack of significant findings in the water phase and the heavy contamination found in the S-PCR substrate highlights the critical role of sediments as the 'environmental memory' of fluvial systems. While dissolved metals are subject to rapid dilution and transport, the substrate acts as a permanent reservoir for contaminants through adsorption and precipitation processes.

The extreme enrichment of aluminium and gallium in the PCR sediments suggests specific industrial or geogenic signatures that are effectively sequestered by the mineral matrix. Furthermore, the high concentrations of manganese and iron are indicative of the formation of Fe-Mn oxyhydroxides, which are known to act as scavenging agents for other trace elements like selenium and cesium indicating a significant ecological risk.

CONCLUSIONS

The present study demonstrates a clear association between heavy metal contamination and hepatic histopathological alterations in European chub (*Squalius cephalus*). The river impacted by mining-derived pollution exhibited markedly elevated concentrations of Mn, Fe, and several other metals, corresponding to a heavily polluted (Class V) status, whereas the

reference river showed values consistent with good ecological quality. Concordantly, fish collected from the polluted site displayed a broad spectrum of hepatic lesions - including hydropic and lipid degeneration, hyperemia, apoptosis, necrosis, and architectural disorganization - while specimens from the reference site maintained normal liver morphology.

The analysis reveals that the Positive Control River (PCR) exhibits significantly higher heavy metal loads across both water and sediment matrices compared to the Negative Control River (NCR). Sediments in the PCR act as the primary sink for pollutants, showing a marked accumulation of elements like Fe, Al, and Mn that far exceed the levels found in the control site. The widespread statistical significance observed in the sediment-to-sediment comparison confirms a high degree of environmental contamination in the PCR system. Ultimately, these results highlight the role of riverbed sediments as critical indicators for long-term monitoring of heavy metal pollution.

These findings reinforce the value of fish liver histopathology as a sensitive and integrative biomarker for assessing ecological risk in metal-contaminated freshwater ecosystems. The concordance between chemical data and biological responses highlights the ongoing impact of historical mining activities in the study area and underscores the importance of continued biomonitoring and environmental management strategies in affected watersheds.

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GROWTH PERFORMANCE OF JUVENILE CARP (*Cyprinus carpio*) REARED USING SLUG (*Arion vulgaris*) MEAL AS AN ALTERNATIVE PROTEIN SOURCE IN FISH FEED

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Abstract

Alternative protein sources to fish meal in aquaculture feeds are being researched for decades. This continuous race is driven by the problems that surround fish meal: high cost, availability issues, environmental impact. Throughout the world, gastropods (class Gastropoda) are a common occurrence, and have been researched as candidates to substitute fish meal in aquaculture feeds. Among gastropods, research did not focus on slug species for their potential as alternative protein sources to fish meal, although they are widespread and in some regions are manifesting as invasive species. In this research slug meal obtained from the invasive species Arion vulgaris, was tested as an alternative protein source in an experimental feed on the growth of juvenile carp (Cyprinus carpio) in parallel to a feed containing fish meal. The results displayed no statistically significant differences between the experimental feed and the control feed, showing the potential of slug meal as an alternative protein source.

Key words*: aquaculture nutrition, fish meal, Gastropoda, growth dynamics, invasive.*

INTRODUCTION

Aquatic organisms represent an important food source for the world population. Aquaculture products' availability is increasing along with the continuous rise in production driven by the high demand of fish, shellfish or various other aquatic organisms (FAO, 2022). The economic importance of aquaculture became unquestionable when aquaculture productions surpassed for the first time capture fisheries productions in 2020, with 57 percent of the aquatic animals consumed by humans being aquaculture produced (FAO, 2024). Market growth and importance is also demonstrated by the consumers who are looking for better, safer and higher quality products (Freitas et al., 2020; Lațiu et al., 2024).

The increase in aquaculture production triggers and stimulates aquaculture research. Feed costs are the highest expenditure for the majority of aquaculture systems and research for improving feed and feed production is ongoing worldwide, addressing the issue of continuously rising prices of feed ingredients and the shortages of some raw materials (Rana et al., 2022; Baki & Yücel, 2017). Protein requirements of aquaculture feed are strict, and feed must contain the appropriate amount and type of protein for the aquatic animal which it is destined for. Fish meal is the most utilized protein source in aquaculture feed for decades (FAO, 2004), with excellent nutritional properties, but ever-increasing costs are making it harder to obtain (Nagappan et al., 2021). Substitutes to fish meal are heavily researched for years. Alternative protein sources of vegetal and animal origin are the most studied (Nagappan et al., 2021; Serra et al., 2024). Animal protein sources are more desirable, due to their amino acids composition, which makes them more appropriate for consumption by aquatic animals, and alternative sources studied vary from food industry by-products to insects and other invertebrates (Vrabec et al., 2015; Luthada-Raswiswi et al., 2021; Nagappan et al., 2021; Serra et al., 2024).

The use of gastropods (class Gastropoda) as alternative protein sources in animal feeds has been documented in the speciality literature since the 1950's (Mead & Kemmerer, 1953; Mead, 1961). Aquatic and terrestrial snails and slugs have been used as a feed ingredient in poultry growing (Diarra, 2015), swine growing (Kaensombath, 2005) and their potential was also studied for aquatic animal's feed, with at least 36 studies mentioning the use of gastropods in aquaculture (Muntean et al., 2024a). The most utilized species as alternative protein source are generally terrestrial snails, like the golden apple snail *Pomacea canaliculata* (Lamarck, 1822) (Phonekhampheng et al., 2009; Visca et al., 2018), or the African garden snail *Limicolaria aurora* (Jay, 1839) (Adéyèmi et al., 2020; Sogbesan et al, 2006).

Among the gastropod species studied as alternative protein sources, there is a lack of research regarding slugs, the gastropods that do not have hard shells. Terrestrial slug species are found throughout the world and in many cases, they are considered pests for agricultural crops (Das et al., 2020). Tshernyshev et al. (2022) study highlights the potential of rearing *Limacus flavus* (Linnaeus, 1758) and analyses their nutritional value, indicating they could be utilized for human consumption or animal feed. More in-depth research on the topic of slug potential as feed ingredients in animal rearing are necessary.

Central and Eastern Europe faces an invasion of the Spanish slug *Arion vulgaris* Moquin-Tandon, 1855. This species is often confused with *Arion lusitanicus* Mabille, 1868 and it is hard to differentiate between them morphologically, and it is considered that in most cases across Europe *Arion vulgaris* is present (Zając et al., 2017). Research suggests that the species could be native to Central Europe, based on genetic studies, and that it has manifested as an invasive species in the last decades due to various factors such as phenotypic plasticity, climate change or rapid response to natural selection. Regardless of origin, the species clearly acts as invasive in various regions (Pfenninger et al., 2014; Zając et al., 2017). The presence of *Arion vulgaris* was reported in Romania as well for at least ten years, with a very aggressive spread across the country, and it is considered a pest (Păpureanu et al., 2014). This species was considered a suitable candidate for our research, being easily available to collect and to implement an experiment in which Spanish slug meal was utilized as an alternative protein source in aquaculture feed. Prior to this experiment, the Spanish slug meal was tested for its chemical composition and nutritional value (Muntean et al., 2024b) along with other gastropod sourced meals. Slug meal had a protein content of 47.98%, 4.6% fat and around 8% ash content. Based on these results, the Spanish slug meal was chosen to continue the research, because it displayed an agreeable nutritional spectrum, it is considered a pest with no other use and creates environmental damage. An experiment was developed to analyse the fish growth dynamics when using a feed that contains slug meal as an alternative protein of animal origin, and to compare it in laboratory conditions to a feed that contains standard fish meal as an animal protein source.

The purpose of the experiment was to see if statistically significant differences occur in growth dynamics between the fish reared using the slug meal based feed and the fish reared using the fish meal based feed. The present research aim is to assess slugs' potential as an alternative protein source in aquaculture.

MATERIALS AND METHODS

Obtaining slug meal

Research took place between July 2024 and February 2025. The gathering and processing of slugs (*Arion vulgaris*) and the fish growing experiment were approved by the Bioethics Commission of the University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca through the decision of the Bioethics Commission number 401 from the 21st of September 2023. The research was conducted in compliance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes and Law 43/2014 enforced in Romania.

Slugs (*Arion vulgaris*) were gathered in a 30-day period during July 2024 and August 2024 from the wooded areas of the campus of the University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca. Slugs

were captured using specially designed traps made from covered plastic containers with longitudinal 2 cm wide slots cut at the top of the container. The traps were baited with beer, which attracted the slugs to the trap. A number of 10 to 20 traps were set daily around noon and left until the following morning, when the slugs were collected and the traps reset with fresh bait. Collected slugs were then taken to the Animal Physiology Laboratory of the Faculty of Animal Science and Biotechnologies of the University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca. In the laboratory, the slugs were humanely euthanized using a solution of 50% ethanol and 50% water, then were thoroughly rinsed of any debris and stored in a freezer until the necessary amount of slugs were gathered and further processing could take place.

Slugs were processed and slug meal was obtained in the laboratories of the INCDO-INOE 2000, Research Institute for Analytical Instrumentation of Cluj-Napoca. After defrosting the slugs, they were dried using a drying oven (UFE 400 oven from Memmert, Büchenbach, Germany) at a temperature of 60oC. To determine if the dehydration process was complete, mass monitoring was conducted repeatedly using an analytical balance (WSP4000/C/2, Partner Radwag, Radom, Poland). Dehydrated slugs were then ground to a fine meal using a mill (PM 100, Retsch GmbH, Haan, Germany).

Feed manufacturing process

The experiment required the development of two feeds, an experimental feed that contained slug meal as an animal protein source and a control feed that contained fish meal as an animal protein source. Feeds were developed using complimentary ingredients of vegetal origin and vitamin-mineral premix. Following the modified method of Masithoh et al. (2020), using the Fourier transform near-infrared (FT-NIR) technology with the Tango Brucker spectrometer (Billerica, MA, USA), slug meal, fish meal and various vegetal ingredients were tested. This non-destructive analytical method allows rapid determination of proximate components such as protein, fat, moisture, fibre, and ash based on the absorption of near-infrared light by molecular bonds (mainly C–H, O–H, and N–H). Samples were scanned in reflectance mode, and spectra were analysed using instrument-calibrated models for feed matrices. This technique provided fast and reliable estimates, facilitating the formulation of nutritionally equivalent experimental and control diets. The complimentary ingredients chosen to develop the feeds were: sunflower meal, soybean meal, wheat bran, oat bran, corn germ, tiger nut flour and vitamin-mineral premix Romzeofort (Romvac, Romania). The experimental feed and the control feed recipes were made using the same amount of animal origin meal, slug meal or fish meal (30%) and using the same complimentary ingredients, but with different proportions for each recipe. We used different proportions of the complimentary ingredients because we strived to obtain two similar feeds regarding their protein content. The exact formulation of the experimental feed and the control feed are presented in Table 1. The feeds were produced through a cold pelleting process using an Agrofortel AGF-150M pelleting machine (Romania) equipped with a 2 mm diameter die. All solid raw materials were subjected to a preliminary grinding step using a hammer mill fitted with fine screens (≤1 mm) to reduce particle size. This step enhanced the homogeneity of the mixture, ensuring uniform nutrient distribution and facilitating consistent extrusion during pelleting. Subsequently, the ingredients were accurately weighed according to the formulated composition and thoroughly mixed to obtain a uniform blend. The moisture content of the mixture was adjusted to approximately 15% by adding water, to optimize pellet formation. Pelleting was conducted at a processing capacity of 60-80 kg/h under semi-cold conditions, without applying high temperatures, thereby preserving the nutritional and functional integrity of thermosensitive compounds such as vitamins, polyphenols, and hydrolysed proteins. The resulting cylindrical pellets (2 mm diameter, 3-5 mm average length) were air-dried at ambient temperature for 24 to 48 hours and stored under cool, dry, and dark conditions until use.

Table 1. Experimental feed and control feed formulation

Ingredient (%)	Experimental feed	Control feed
Slug meal (%)	30	0
Fish meal (%)	0	30
Sunflower meal (%)	5	5
Soybean meal (%)	20	10
Wheat bran (%)	5	10
Oat bran (%)	5	15
Corn germ (%)	15	10
Tiger nut flour (%)	19	19

Quality assessment of feed

The pellets from each recipe were tested in triplicate for their proximate content using the same method as for testing the ingredients (Table 2).

Table 2. Experimental and control feeds proximate composition

Nutrient (%)	Experimental feed		Control feed	
	M ± SEM	SD	M ± SEM	SD
Ash (%)	5.8 ± 0.01	0.01	7.91 ± 2.15	3.04
Carbohydrates (%)	7.37 ± 0.28	0.40	18.10 ± 5.50	7.78
Dry matter (%)	95.02 ± 0.04	0.06	95.82 ± 0.07	0.10
Dietary fiber (%)	32.83 ± 0.09	0.12	28.80 ± 0.17	0.24
Fat (%)	6.8 ± 0.10	0.14	7.53 ± 0.07	0.09
Protein (%)	29.76 ± 0.05	0.07	28.53 ± 0.09	0.13
Total sugars (%)	3.47 ± 0.03	0.05	4.9 ± 0	0.00

Legend: M – mean; SD – standard deviation of the mean; SEM – standard error of the mean.

The water dispersibility of the slug-based feed pellets was evaluated gravimetrically by measuring mass loss over time. A quantity of 5 grams of dry pellets (m_0) was weighed and introduced into a 500 mL Berzelius beaker filled with water at room temperature. After 30 minutes of immersion, the pellets were removed, dried in a desiccator until a constant weight was reached, and reweighed (m_1). Water dispersebility was calculated using the following formula:

$$Water\ dispersibility\ (\%\ loss) = \frac{m_0 - m_1}{m_0}\ x\ 100$$

In aquaculture, high-quality pellets should maintain their structural integrity in water for at least one hour without excessive breakdown. Mass losses below 10–15% are generally considered acceptable. This test was conducted to assess the physical stability of the pellets under simulated aquaculture conditions, where feed must remain intact long enough to be consumed by fish, while minimizing organic matter release into the aquatic environment.

The slug meal-based feed showed a mass loss of 7.12%, indicating a good water stability profile within the optimal range for aquaculture feeds (<10-15%).

Experimental design

The fish growing experiment was conducted on juvenile carp (*Cyprinus carpio* Linnaeus, 1758) obtained from a classic system farm from Cluj County. The experiment took place in a controlled environment at the INCDO-INOE 2000, Research Institute for Analytical Instrumentation of Cluj-Napoca. The carp juveniles were acclimated to laboratory conditions for five weeks prior to the experiment and were fed with pelletized feed used in the farm of their provenance. After the acclimation period, four groups of fish were created with 50 specimens each. The fish were individually weighed and based on their weight were attributed to a group, to obtain groups as uniform as possible. The mean weight of the specimens ranged between 20.43 g to 20.53 g, with a minimum weight of 16.1 g to a maximum weight of 27.9 g across all four groups, thus ensuring a tight similarity between groups. The uniformity of the groups at the initial stage was confirmed by the variance which ranged across groups from 6.52 to 8.78. The groups were labelled as Treatment A, Treatment B, Treatment C and Treatment D. Treatment A, Treatment B and Treatment C, were fed with 2% of their biomass daily using the slug meal experimental feed and were considered as the experimental treatments. Treatment D was fed with 2% of the biomass daily using the fish meal control feed and was considered the control treatment. A feeding plan for the entire experiment period was calculated based on their biomass. A fasting day was set for all groups each week, and the experiment went on for 40 days. The amount of daily feed for each treatment was fed half at the beginning of the light period and half at the end of the light period, which lasted for 8 hours daily. Fish were fed by hand by spreading the pellets in the same area of the tank at each feeding, opposite to where the submersible pump was set in the tank. For each treatment we utilized a 750-litre circular tank, with 600 litres of water and a depth of the water column of 65 cm. Each tank was fitted with an individual pump and external filtration system which facilitated mechanical filtration, biological filtration and UV

sterilization. Supplementary aeration was supplied to each tank trough a 50 mm air stone. Each tank had an underwater digital thermometer, and the temperature ranged during the experiment across treatments between 19.3OC and 21.3OC. pH was sampled in the morning of each feeding day with a portable pH-meter and the values of the pH ranged during the experiment across treatments between 7.18 and 7.37. The dissolved oxygen (O2) content was sampled weekly and tested through laboratory analysis and ranged across treatments between 7.96 mg O2/L and 9.08 mg O2/L.

Evaluation of growth performances

At half of the experiment period, an intermediary measurement of each individual was conducted and also at the end of the experiment period. Each of the measurement phases, at the beginning of the experiment (initial measurement), at half of the experimental period (intermediary measurement) and at the end of the experiment (final measurement), took place in the following manner. Each specimen of juvenile carp body weight (W) (g) was determined individually using a 0.1 g precision balance (Adam Dune DCT 2000, Adam Equipment, UK). Each specimen was photographed on its left side in a small fish tank equipped with a millimetric scale and a black plastic sheet that allows to photograph the fish against the aquarium glass, and a camera was set to be always at the same distance and position against the fish tank. Using the photographs, we determined several somatic measurements using the AmScope ToupView 3.7 software following an established morphometric method (Lațiu, 2025). The somatic measurements determined were: total length (Tl) (cm), standard length (Sl) (cm), commercial length (Cl) (cm), fork length (Fl) (cm), maximum height (H) (cm), minimum height (h) (cm), head length (Hl) (cm), caudal peduncle length (Pl) (cm).

Weight gains and the specific growth rate (SGR) were calculated at the end of the experiment for each treatment. Total gain (TG) (g) was calculated using the following formula:

$$TG = Final\ Biomass - Initial\ Biomass$$

Daily weight gain (DG) (g) was calculated using the following formula:

$$DG = \frac{TG}{number\ of\ experiment\ days}$$

The specific growth rate (SGR) was calculated using the following formula:

$$SGR\ (\%) = \frac{ln_{Final\ Biomass} - ln_{Initial\ Biomass}}{number\ of\ experiment\ days}\ x\ 100$$

Data analysis

Growth dynamics for body weight and each of the somatic measurements was analysed across treatments, to see if statistically significant differences occur between treatments during the intermediary measurement phase or the final measurement phase. Descriptive statistics was conducted for the data and the data were tested for their normality using the Shapiro-Wilk test. For normally distributed data the ANOVA test was applied paired with the post-hoc Tukey test for multiple comparisons and for non-normally distributed data the Kruskal-Wallis test was applied paired with the post-hoc Dunn test for multiple comparisons. The significance threshold was set at $\alpha = 0.05$. Data was centralized using Microsoft Office Excel software and statistical analysis and together with the graphical representation of the results was conducted using GraphPad Prism 8.0 software. During the experiment, no fatalities were recorded, the 200 specimens from all treatments survived the 40 days of experiment.

To better display the results in regard to treatment and phase, body weight and somatic measurements abbreviations were followed by a subscript with the letter of the treatment (A, B, C, D) if it represented determinations from the initial phase; were followed by a subscript beginning with letter "i" followed by treatment letter (iA, iB, iC, iD) if it represented determinations from the intermediary phase; were followed by a subscript beginning with letter "f" followed by treatment letter (fA, fB, fC, fD) if it represented determinations from the final phase.

RESULTS AND DISCUSSIONS

Total weight gain (TG) (g), daily weight gain (DG) (g) and specific growth rate (SGR) (%) for each treatment are presented in Table 3. The biggest weight gain for an experimental treatment was achieved by Treatment B with a

total weight gain of 98.90 g and a daily weight gain of 2.47 g. The control group had a total weight gain of 116.10 g and a daily weight gain of 2.90 g. The mean total weight gain across groups was 86.28 g and the mean daily weight gain was 2.16 g. The biggest SGR for an experimental treatment was achieved by Treatment B, with a SGR of 0.23%, and the biggest SGR across all treatments was achieved by Treatment D with a SGR of 0.27%. The mean SGR across all treatments was 0.20%.

Table 3. Total weight gain (TG), daily weight gain (DG) and specific growth rate (%) for all treatments

	Final Biomass (g)	Initial Biomass (g)	Total Gain (g)	Daily Gain (g)	SGR (%)
Treatment A	1098.10	1026.70	71.40	1.78	0.17
Treatment B	1120.80	1021.90	98.90	2.47	0.23
Treatment C	1083.90	1025.20	58.70	1.47	0.14
Treatment D	1141.80	1025.70	116.10	2.90	0.27
Minimum	1083.90	1021.90	58.70	1.47	0.14
Maximum	1141.80	1026.70	116.10	2.90	0.27
Mean	1111.15	1024.88	86.28	2.16	0.20
SD	25.47	2.08	26.02	0.65	0.06
SEM	12.73	1.04	13.01	0.32	0.03
CV%	2.29%	0.20%	30.16%	30.12%	29.07%

Legend: SD – standard deviation of mean; SEM – standard error of mean; CV% - coefficient of variation; SGR – specific growth rate.

Body weight (W) growth dynamics are represented in Figure 1 and Table 4.

In regard to body weight and based on the statistical analysis, no statistically significant differences were recorded between treatments during any of the phases of the experiment. Mean individual body weight at the end of the experiment ranged between 21.68 g and 22.84 g, with the biggest mean weight recorded for the control group. In experimental Treatment B, it is noticeable that the biggest specimen from the experiment was recorded at 35 g, meaning that one specimen from Treatment B grew at least 7.9 g, taking into consideration that the biggest specimen at the beginning of the experiment from Treatment B weighed 27.10 g.

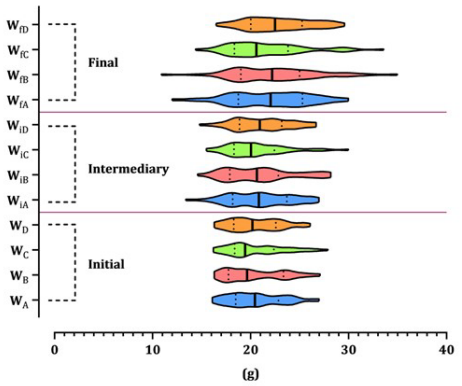


Figure 1. Growth dynamics regarding body weight (W) across treatments and experiment phases

Table 4. Descriptive statistics of growth dynamics regarding body weight (W)

	W_A (g)	W_B (g)	W_C (g)	W_D (g)	W_{iA} (g)	W_{iB} (g)	W_{iC} (g)	W_{iD} (g)	W_{fA} (g)	W_{fB} (g)	W_{fC} (g)	W_{fD} (g)
Minimum	16.10	16.30	16.20	16.30	13.40	14.60	15.50	14.80	12.00	10.90	14.40	16.50
Median	20.45	19.65	19.45	20.20	20.85	20.65	20.05	20.95	22.05	22.20	20.60	22.50
Maximum	27.00	27.10	27.90	26.10	27.00	28.20	30.00	26.70	30.00	35.00	33.60	29.60
Mean	20.53	20.44	20.50	20.51	20.96	21.01	20.55	21.17	21.96	22.42	21.68	22.84
SD	2.76	2.99	2.92	2.58	3.24	3.60	3.04	2.91	4.06	4.60	4.04	3.33
SEM	0.39	0.42	0.41	0.36	0.46	0.51	0.43	0.41	0.57	0.65	0.57	0.47

Legend: SD – standard deviation of mean; SEM – standard error of mean.

Total length (Tl) growth dynamics are represented in Figure 2 and Table 5. In regard to total length and based on the statistical analysis, no statistically significant differences were recorded between treatments during any of the phases of the experiment. Mean individual total length at the end of the experiment ranged between 11.32 cm and 11.52 cm, with the biggest mean total length recorded for the control group. In experimental Treatment C, it is noticeable that the biggest measurement for a specimen from the experiment was recorded at 13.38 cm, meaning that one specimen from Treatment C grew at least 1.95 cm, taking into consideration that the biggest value for total length at the beginning of the experiment from Treatment C was 11.43 cm.

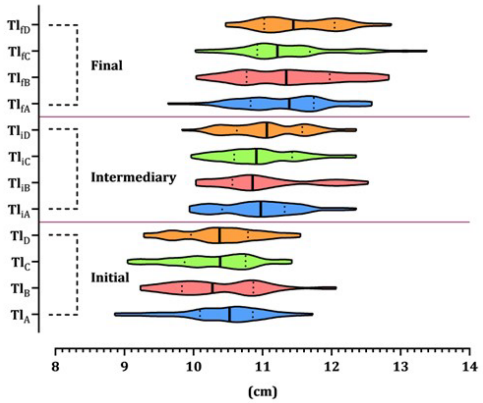


Figure 2. Growth dynamics regarding total length (Tl) across treatments and experiment phases

Table 5. Descriptive statistics of growth dynamics regarding total length (Tl)

	TlA (cm)	TlB (cm)	TlC (cm)	TlD (cm)	TliA (cm)	TliB (cm)	TliC (cm)	TliD (cm)	TlfA (cm)	TlfB (cm)	TlfC (cm)	TlfD (cm)
Minimum	8.86	9.23	9.05	9.28	9.94	10.03	9.97	9.84	9.63	10.04	10.03	10.47
Median	10.52	10.27	10.39	10.38	10.98	10.86	10.91	11.06	11.39	11.34	11.22	11.45
Maximum	11.73	12.07	11.43	11.55	12.36	12.53	12.36	12.36	12.59	12.83	13.38	12.87
Mean	10.41	10.37	10.30	10.39	10.93	11.05	10.98	11.07	11.32	11.41	11.37	11.52
SD	0.63	0.66	0.61	0.58	0.58	0.70	0.57	0.57	0.67	0.74	0.72	0.59
SEM	0.09	0.09	0.09	0.08	0.08	0.10	0.08	0.08	0.09	0.10	0.10	0.08

Legend: SD – standard deviation of mean; SEM – standard error of mean.

Standard length (Sl) growth dynamics are represented in Figure 3 and Table 6. In regard to standard length and based on the statistical analysis, no statistically significant differences were recorded between treatments during any of the phases of the experiment. Mean individual standard length at the end of the experiment ranged between 9.08 cm and 9.31 cm, with the biggest mean standard length recorded for the control group. In control Treatment D, it is noticeable that the biggest measurement for a specimen from the experiment was recorded at 10.75 cm, meaning that one specimen from Treatment D grew at least 1.35 cm in standard length, taking into consideration that the biggest standard length for a specimen at the beginning of the experiment from Treatment D was 9.40 cm.

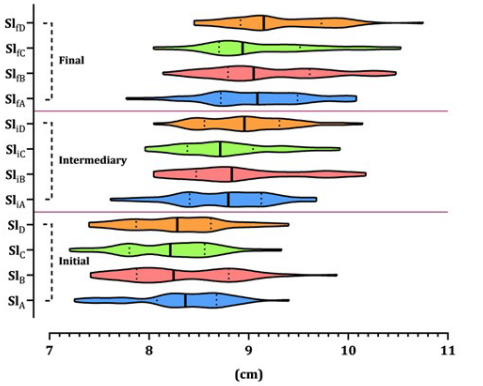


Figure 3. Growth dynamics regarding standard length (Sl) across treatments and experiment phases

Table 6. Descriptive statistics of growth dynamics regarding standard length (Sl)

	SlA (cm)	SlB (cm)	SlC (cm)	SlD (cm)	SliA (cm)	SliB (cm)	SliC (cm)	SliD (cm)	SlfA (cm)	SlfB (cm)	SlfC (cm)	SlfD (cm)
Minimum	7.25	7.41	7.20	7.40	7.61	8.05	7.96	8.05	7.77	8.14	8.05	8.45
Median	8.37	8.25	8.21	8.28	8.80	8.83	8.71	8.96	9.09	9.05	8.94	9.15
Maximum	9.41	9.89	9.33	9.40	9.68	10.18	9.92	10.15	10.08	10.48	10.53	10.75
Mean	8.32	8.35	8.20	8.29	8.75	8.94	8.78	8.97	9.08	9.21	9.13	9.31
SD	0.49	0.57	0.47	0.49	0.48	0.60	0.48	0.45	0.54	0.59	0.60	0.52
SEM	0.07	0.08	0.07	0.07	0.07	0.08	0.07	0.06	0.08	0.08	0.08	0.07

Legend: SD – standard deviation of mean; SEM – standard error of mean.

Commercial length (Cl) growth dynamics are represented in Figure 4 and Table 7. In regard to commercial length and based on the statistical analysis, no statistically significant differences were recorded between treatments during any of the phases of the experiment. Mean individual commercial length at the end of the experiment ranged between 6.18 cm and 6.45 cm, with the biggest mean commercial length recorded for the control group. In experimental Treatment C, it is noticeable that the biggest measurement for a specimen from the experiment was recorded at 7.59 cm, meaning that one specimen from Treatment C grew at least 1.10 cm in commercial length, taking into consideration that the biggest commercial length for a specimen at the beginning of the experiment from Treatment C was 6.49 cm.

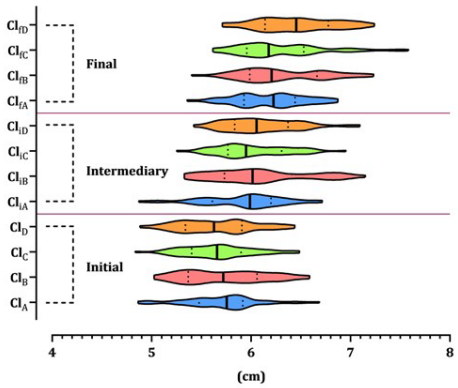


Figure 4. Growth dynamics regarding commercial length (Cl) across treatments and experiment phases

Table 7. Descriptive statistics of growth dynamics regarding commercial length (Cl)

	ClA (cm)	ClB (cm)	ClC (cm)	ClD (cm)	CliA (cm)	CliB (cm)	CliC (cm)	CliD (cm)	ClfA (cm)	ClfB (cm)	ClfC (cm)	ClfD (cm)
Minimum	4.87	5.03	4.84	4.88	4.87	5.33	5.25	5.42	5.36	5.40	5.62	5.71
Median	5.76	5.72	5.66	5.63	5.99	6.02	5.95	6.06	6.23	6.21	6.18	6.45
Maximum	6.69	6.59	6.49	6.44	6.72	7.15	6.96	7.10	6.88	7.24	7.59	7.24
Mean	5.71	5.74	5.66	5.66	5.92	6.09	6.01	6.11	6.18	6.31	6.30	6.45
SD	0.38	0.42	0.35	0.38	0.40	0.48	0.36	0.37	0.35	0.43	0.45	0.39
SEM	0.05	0.06	0.05	0.05	0.06	0.07	0.05	0.05	0.05	0.06	0.06	0.06

Legend: SD – standard deviation of mean; SEM – standard error of mean.

Fork length (Fl) growth dynamics are represented in Figure 5 and Table 8. In regard to commercial length and based on the statistical analysis, no statistically significant differences were recorded between treatments during any of the phases of the experiment. Mean individual commercial length at the end of the experiment ranged between 10.08 cm and 10.34 cm, with the biggest mean fork length recorded for the control group. In experimental Treatment C and the control Treatment D, it is noticeable that the biggest measurements for specimens from the experiment were recorded at 11.72 cm, meaning that one specimen from Treatment C grew at least 1.41 cm in fork length, taking into consideration that the biggest fork length for a specimen at the beginning of the experiment from Treatment C was 10.31 cm, and one specimen from Treatment D grew at least 1.51 cm in fork length, taking into consideration that the biggest fork length for a specimen at the beginning of the experiment from Treatment D was 10.21 cm.

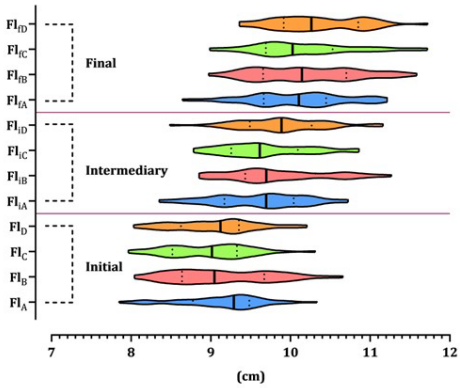


Figure 5. Growth dynamics regarding fork length (Fl) across treatments and experiment phases

Table 8. Descriptive statistics of growth dynamics regarding fork length (Fl)

	FlA (cm)	FlB (cm)	FlC (cm)	FlD (cm)	FliA (cm)	FliB (cm)	FliC (cm)	FliD (cm)	FlfA (cm)	FlfB (cm)	FlfC (cm)	FlfD (cm)
Minimum	7.85	8.04	7.97	8.03	8.35	8.85	8.78	8.49	8.64	8.97	8.99	9.36
Median	9.29	9.05	9.02	9.12	9.69	9.70	9.61	9.89	10.11	10.14	10.03	10.26
Maximum	10.34	10.66	10.31	10.21	10.72	11.27	10.86	11.16	11.21	11.59	11.72	11.72
Mean	9.13	9.14	8.97	9.05	9.60	9.84	9.68	9.89	10.08	10.21	10.15	10.34
SD	0.54	0.64	0.51	0.53	0.58	0.63	0.54	0.55	0.61	0.65	0.64	0.55
SEM	0.08	0.09	0.07	0.07	0.08	0.09	0.08	0.08	0.09	0.09	0.09	0.08

Legend: SD – standard deviation of mean; SEM – standard error of mean.

Maximum height (H) growth dynamics are represented in Figure 6 and Table 9. In regard to the maximum height and based on the statistical analysis, no statistically significant differences were recorded between treatments during any of the phases of the experiment. Mean individual maximum height at the end of the experiment ranged between 3.34 cm and 3.48 cm, with the biggest mean maximum height recorded for the control group. In experimental Treatment C, it is noticeable that the biggest measurement for a specimen from the experiment was recorded at 4.01 cm, meaning that one specimen from Treatment C grew at least 0.39 cm in maximum height, taking into consideration that the biggest maximum height for a specimen at the beginning of the experiment from Treatment C was 3.62 cm.

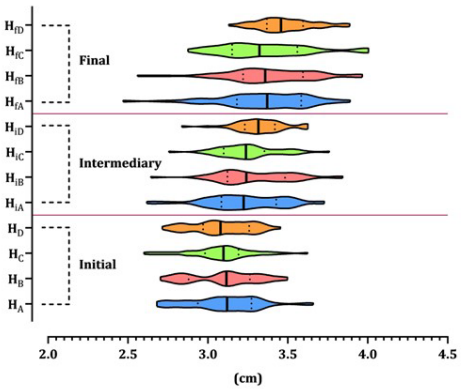


Figure 6. Growth dynamics regarding maximum height (H) across treatments and experiment phases

Table 9. Descriptive statistics of growth dynamics regarding maximum height (H)

	HA (cm)	HB (cm)	HC (cm)	HD (cm)	HiA (cm)	HiB (cm)	HiC (cm)	HiD (cm)	HfA (cm)	HfB (cm)	HfC (cm)	HfD (cm)
Minimum	2.68	2.70	2.60	2.72	2.62	2.64	2.76	2.84	2.47	2.56	2.88	3.13
Median	3.12	3.12	3.10	3.08	3.22	3.24	3.24	3.32	3.37	3.36	3.32	3.46
Maximum	3.66	3.50	3.62	3.46	3.73	3.84	3.76	3.63	3.89	3.97	4.01	3.89
Mean	3.10	3.10	3.08	3.09	3.23	3.29	3.25	3.33	3.34	3.38	3.36	3.48
SD	0.22	0.22	0.20	0.19	0.24	0.23	0.19	0.16	0.28	0.29	0.28	0.17
SEM	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.02	0.04	0.04	0.04	0.02

Legend: SD – standard deviation of mean; SEM – standard error of mean.

Minimum height (h) growth dynamics are represented in Figure 7 and Table 10. In regard to the minimum height and based on the statistical analysis, no statistically significant differences were recorded between treatments during any of the phases of the experiment. Mean individual minimum height at the end of the experiment ranged between 1.29 cm and 1.34 cm, with the biggest mean maximum height recorded for the control group. In experimental Treatment C, it is noticeable that the biggest measurement for a specimen from the experiment was recorded at 1.54 cm, meaning that one specimen from Treatment C grew at least 0.24 cm in minimum height, taking into consideration that the biggest minimum height for a specimen at the beginning of the experiment from Treatment C was 1.30 cm.

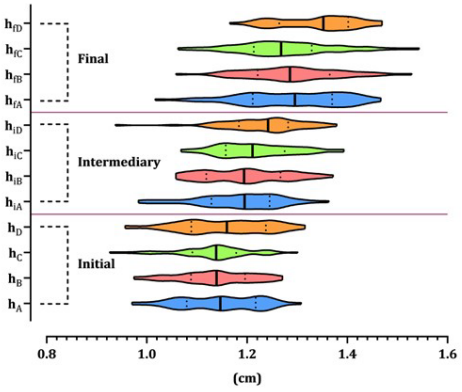


Figure 7. Growth dynamics regarding minimum height (h) across treatments and experiment phases.

Table 10. Descriptive statistics of growth dynamics regarding minimum height (h)

	hA (cm)	hB (cm)	hC (cm)	hD (cm)	hiA (cm)	hiB (cm)	hiC (cm)	hiD (cm)	hfA (cm)	hfB (cm)	hfC (cm)	hfD (cm)
Minimum	0.97	0.97	0.93	0.96	0.98	1.06	1.07	0.94	1.02	1.06	1.06	1.17
Median	1.15	1.14	1.14	1.16	1.20	1.19	1.21	1.24	1.30	1.29	1.27	1.35
Maximum	1.31	1.27	1.30	1.32	1.36	1.37	1.39	1.38	1.47	1.53	1.54	1.47
Mean	1.15	1.14	1.13	1.15	1.18	1.20	1.22	1.23	1.29	1.29	1.28	1.34
SD	0.08	0.07	0.08	0.09	0.08	0.08	0.08	0.08	0.10	0.09	0.10	0.08
SEM	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

Legend: SD – standard deviation of mean; SEM – standard error of mean.

Head length (Hl) growth dynamics are represented in Figure 8 and Table 11. In regard to the head length and based on the statistical analysis, no statistically significant differences were recorded between treatments during any of the phases of the experiment. Mean individual head length at the end of the experiment ranged between 3.03 cm and 3.18 cm, with the biggest mean head length recorded for the Treatment C. In experimental Treatment C, it is noticeable that the biggest measurement for a specimen from the experiment was recorded at 3.87 cm, meaning that one specimen from Treatment C grew at least 0.65 cm in head length, taking into consideration that the biggest head length for a specimen at the beginning of the experiment from Treatment C was 3.22 cm.

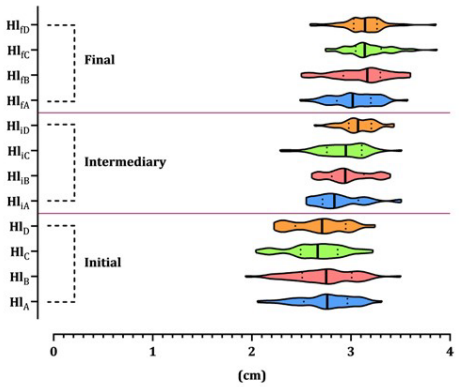


Figure 8. Growth dynamics regarding head length (Hl) across treatments and experiment phases.

Table 11. Descriptive statistics of growth dynamics regarding head length (Hl)

	HlA (cm)	HlB (cm)	HlC (cm)	HlD (cm)	HliA (cm)	HliB (cm)	HliC (cm)	HliD (cm)	HlfA (cm)	HlfB (cm)	HlfC (cm)	HlfD (cm)
Minimum	2.06	1.94	2.04	2.22	2.55	2.61	2.29	2.63	2.49	2.50	2.74	2.59
Median	2.76	2.75	2.67	2.71	2.83	2.94	2.95	3.07	3.02	3.17	3.14	3.14
Maximum	3.31	3.51	3.22	3.24	3.51	3.40	3.51	3.44	3.57	3.60	3.87	3.86
Mean	2.75	2.73	2.65	2.68	2.89	2.97	2.92	3.08	3.03	3.10	3.18	3.13
SD	0.28	0.32	0.28	0.27	0.24	0.22	0.24	0.17	0.23	0.29	0.23	0.21
SEM	0.04	0.04	0.04	0.04	0.03	0.03	0.03	0.02	0.03	0.04	0.03	0.03

Legend: SD – standard deviation of mean; SEM – standard error of mean.

Caudal peduncle length (Pl) growth dynamics are represented in Figure 9 and Table 12. In regard to the caudal peduncle length and based on the statistical analysis, statistically significant differences were identified during the final measurements, between Treatment A and Treatment C (**, $p < 0.0001$) and between Treatment B and Treatment C (*, $p = 0.0309$). For the purpose of analyzing the performances of slug meal as an alternative protein source to fish meal, these statistically significant differrences are not relevant, because the differrences are between two experimental groups which were fed the same diet containing slug meal, and not between an experimental group and the control group. Also, the measurement of the caudal peduncle length is susceptible to human error, due to the fact that you must rely on an imaginary line described from the end point of the base of the anal fin towards the top of the caudal peduncle, which stands as the anterior point of measurement from where you measure towards the middle of the base of the caudal fin. Mean individual caudal peduncle length at the end of the experiment ranged between 1.63 cm and 1.80 cm, with the biggest mean caudal peduncle length recorded for the Treatment A. In experimental Treatment A, it is noticeable that the biggest measurement for a specimen from the experiment was recorded at 2.24 cm, meaning that one specimen from Treatment A grew at least 0.22 cm in caudal peduncle length, taking into consideration that the biggest caudal peduncle length for a specimen at the beginning of the experiment from Treatment A was 2.02 cm.

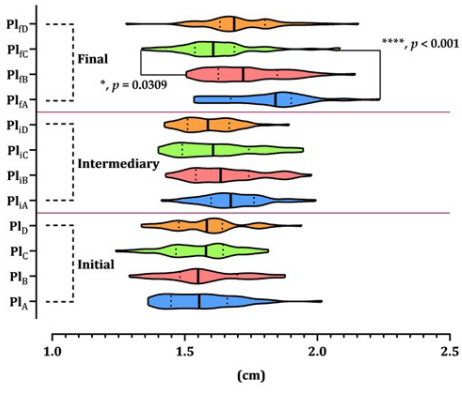


Figure 9. Growth dynamics regarding caudal peduncle length (Pl) across treatments and experiment phases

Table 12. Descriptive statistics of growth dynamics regarding caudal peduncle length (Pl)

	PlA (cm)	PlB (cm)	PlC (cm)	PlD (cm)	PliA (cm)	PliB (cm)	PliC (cm)	PliD (cm)	PlfA (cm)	PlfB (cm)	PlfC (cm)	PlfD (cm)
Minimum	1.36	1.29	1.24	1.34	1.41	1.43	1.40	1.42	1.54	1.51	1.34	1.28
Median	1.55	1.55	1.58	1.58	1.67	1.64	1.61	1.59	1.84	1.72	1.61	1.69
Maximum	2.02	1.88	1.82	1.94	2.00	1.98	1.95	1.89	2.24	2.14	2.09	2.16
Mean	1.57	1.58	1.56	1.58	1.68	1.66	1.63	1.60	1.80	1.74	1.63	1.71
SD	0.14	0.14	0.13	0.13	0.12	0.14	0.15	0.10	0.16	0.15	0.16	0.15
SEM	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.02	0.02	0.02	0.02

Legend: SD – standard deviation of mean; SEM – standard error of mean.

Discussions

Palatability is a crucial characteristic of any animal feed. Aquaculture feeds with palatability issues are easily refused or not consumed in sufficient amounts, directly affecting the growth process (Dworjanyn et al., 2007). Without prior knowledge, if fish accept feed containing slug meals, the palatability of the experimental feed was not demonstrated until we tested it. From how the experiment went on, we can state that feed containing slug meal as an alternative protein source is readily accepted by carp (*Cyprinus carpio*). The fish from the experiment consumed the feed throughout the experiment and were observed to come to the feed every time with the same drive as the fish from the control treatment. Previous studies that utilized other gastropods as alternative protein sources for aquaculture feeds highlight success in having the alternative feed accepted by the reared organisms (Bombeo-Tuburan et al., 1995; Santanumurti et al., 2022).

Evaluating body weight gain and morphometric developments represents valuable insight on how feed influences growth. The results of the growth dynamics analysis for body weight gain and body measurements development when comparing feed containing slug meal as an alternative protein source to feed containing fish meal as a protein source shows no relevant statistically significant differences. This shows that the potential of slug meal is real and can successfully be used as an alternative protein source in aquaculture feeds. Previous studies show the potential of other gastropods to be utilized as alternative protein sources to conventional ingredients in rearing various aquaculture species (Jintasataporn et al., 2004; Suresh, 2007; Ovie & Adejayan, 2010; Muntean et al., 2024a), and this opens the way for slug meal to be tested on the growth of more aquatic animals, and to enrich the knowledge on this protein source.

Optimization of animal feeds is a key aspect in delivering the best results in growth (Sirdeshmukh & Shembekar, 2019). After seizing the potential of a new protein source, in depth research on it needs to be conducted to see how it can be properly processed and incorporated in feed. The feeds utilized in this research were not optimized for the growth of juvenile carp. The research purpose was to have an experimental feed and a control feed that are as similar as possible, except their animal protein ingredient, for the best initial comparison of consecrated fish meal with slug meal as an alternative protein source. Further research on slug meal must be conducted, to completely understand what makes it a good protein source and enhance its results and what can inhibit growth and how to mitigate negative effects.

Terrestrial slug species are found throughout the world, in various climates, and this enhances their potential to be utilized as alternative protein sources. Some species, like in the case of the Spanish slug (*Arion vulgaris*) are considered invasive and pests in many regions (Zemanova et al., 2016), causing environmental and agricultural damage. Practical use for them can help the fight against their spread by collecting them and not utilizing environmentally unfriendly methods (Schüder et al., 2003), which put at risk native lifeforms.

Finding truly successful alternatives to fish meal, cost and economic aspects are paramount. The nature and scale of the present study is not shedding enough light on these aspects. The high availability of invasive slugs is a favourable economic aspect, but economic research and insight must advance further regarding this topic. Technological research is required as well for the improvement of slug collection and processing to slug meal.

CONCLUSIONS

Utilization of slug (*Arion vulgaris*) meal as an alternative protein source in an aquaculture feed tested on juvenile carp (*Cyprinus carpio*) showed no statistically significant differences when compared to a similar feed that contained fish meal as a conventional protein source. The experimental feed was accepted in the same manner as the control feed, thus highlighting the palatability potential of slug meal. All specimens of carp survived the experiment in its entirety. These results and the lack of knowledge for the topic, motivate the need for further research, but the potential of slug meal as an alternative protein source for aquaculture feeds is there to be explored.

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IMPACT OF HABITAT ORIGIN ON THE MICRO AND MACROELEMENT PROFILE OF EUROPEAN CATFISH (*Silurus glanis*)

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Abstract

This study examines how habitat origin influences the macro- and microelement composition of wild and farmed catfish, with emphasis on the distribution of toxic metals across tissues. Mineral levels in liver, bones, head, skin, muscle, and fins were quantified by AAS and ICP-MS. Ca was the dominant microelement, with concentrations up to 4.5% higher in aquaculture fish, followed by potassium (7200 ppm), Na (6066.5 ppm), and Mg (4108 ppm). Fe was the predominant microelement, reaching 363.05 ppm in the liver of wild catfish compared with 342.55 ppm in farmed specimens. Zn and Cu showed the largest habitat-related differences, with levels approximately 19.8% and 16.3% higher, respectively, in aquaculture fish, while manganese remained similar across samples. Microelements accumulated mainly in the liver, followed by skin and fins, and were lowest in muscle and head. Hg occurred only in the head and skin, with levels 2.3 times higher in skin and 2.1 times higher in the head of wild fish. Habitat origin was associated with differences in shaped elemental profiles, underscoring the importance of selecting raw material for safe catfish products.

Key words*: aquaculture catfish, habitat, heavy metal, mineral profile, wild catfish.*

INTRODUCTION

Silurus glanis, known as the European catfish (wels, sheatfish), is one of the largest freshwater fish species in the world, reaching lengths of up to 5 m and body masses exceeding 300 kg (Copp et al., 2009). *Silurus glanis* L., is the principal European representative of the order Siluriformes, noted for its ecological and economic relevance (Saez-Gomez, 2022). They exhibit strong environmental adaptability and occur predominantly in the freshwater systems, although they are occasionally recorded in the salty waters of the Black Sea and Baltic Sea (Severov, 2020; Bergström et al., 2022). The species is native to Eastern Europe and Western Asia, being abundant in the Danube and Volga river basins, but it has been widely introduced into numerous European aquatic systems due to its commercial value and popularity in recreational fishing (Britton, 2007; Carol et al., 2007, 2009). European catfish farming has expanded steadily in recent years, driven by three main factors such as: i) the species have highly palatable flesh, which is particularly valued by consumers in Eastern Europe and Asia (Panicz, 2017; Lyach, 2021; Bergström et al., 2022); ii) its suitability for polyculture systems, where it contributes to maintaining a healthy biocenosis (Vejrik et al., 2019; Linhart et al., 2020); iii) its strong appeal in recreational fisheries in countries such as Spain, the Netherlands, France, and Italy (Rees et al., 2017; Lyach & Remr, 2019; Říha et al., 2022), owing to its impressive size and the notable challenge it presents during capture. From an economic

perspective, *S. glanis* is a species of major importance for both commercial fisheries and aquaculture (Copp et al., 2009). In recent years, the intake of catfish has grown rapidly, driven by its accessibility, reliable quality, and nutritional advantages (Hoke et al., 2000). In general, fish is a nutrient-dense dietary resource, supplying essential vitamins and minerals and offering proteins with a complete and well-balanced profile of indispensable amino acids and catfish also belongs to this category (FAO, 2001). Fish represent an excellent dietary source of macro minerals such as calcium (Ca), magnesium (Mg), sodium (Na), potassium (K), and phosphorus (P) as well as microminerals including iron (Fe), zinc (Zn), copper (Cu), selenium (Se), iodine (I), and manganese (Mn) (Porto et al., 2016). Fish acquire minerals both through dietary intake and directly from the surrounding water, with absorption processes largely influenced by environmental factors, particularly mineral concentrations (Terech-Majewska et al., 2016). Depending on the harvesting environment or the composition of aquaculture feed, fish may also expose consumers to toxic trace elements (Türkmen et al., 2009). In this context, fish are also commonly used as bio-indicators of aquatic ecosystem quality because they are abundant and can readily accumulate heavy metals (Luczynska et al., 2022, Şirin et al., 2024). The distribution of minerals within fish tissues is strongly influenced by habitat and feeding regime. In aquatic environments, both essential and toxic trace elements originate from natural and anthropogenic sources, and the extent of their accumulation in fish tissues varies with pollutant type, species, sampling location, trophic level, and feeding behaviour (Allinson et al., 2009). On the other hand, trace element pollution in aquatic environments is a major concern because it can cause long-lasting damage to ecosystems and endanger human health when these substances enter the food chain (Ali et al., 2016; Chowdhury et al., 2016). Rising levels of trace elements can have toxic effects on invertebrates, fish, and humans due to bioaccumulation and biomagnification. Some of the most harmful trace elements for aquatic ecosystem health and stability include Ni, Zn, Pb, Cr, Cu, Hg, As, and Cd (Martín et al., 2015; Sharma et al., 2023). Impact of 70 years urban growth associated with heavy metal pollution. Environmental pollution, 196, 156-163. The present study aims to highlight the differences in the micro and macroelement composition of farmed and wild catfish harvested in Romania, emphasizing the influence of habitat and feed on the mineral profile of this species.

MATERIALS AND METHODS

Biological material

This study focused on European catfish (*Silurus glanis*) reared/originating from two different environments: 1) an indoor recirculating aquaculture system (RAS), and 2) the wild, natural environment. In the first experimental group, tissue samples were collected in September from 5 European catfish specimens, aged 2 years and 3 months, which had been reared exclusively indoors in a recirculating aquaculture system (RAS). Throughout their entire life cycle, the fish were maintained under indoor farm conditions at the Pădurea Verde Fish Farm, belonging to the University of Life Sciences "King Mihai I" from Timișoara. The fish were fed exclusively with pelleted compound feeds and, during the last year of life, received a Supreme 10 diet with a pellet size of 6 mm (Alltech Coppens, Germany). The body weight of the fish used in the study was 2.362 ± 0.109 kg, and their total length was 68.4 ± 2.074 cm. In the second experimental group, tissue samples were collected from 5 European catfish specimens originating from the Danube River in the Orșova area of Mehedinți County (44°43′31″ N, 22°23′46″ E). These fish were caught in September, at the same time as the farmed fish were sampled. The size of the fish caught and retained for the study was similar to that of the fish from the first experimental group. Thus, their mean body weight was 2.237 ± 0.0912 kg, and their total length was 65.2 ± 2.031 cm. The fish were purchased fresh, cut into anatomical sections, and the following tissues were collected: liver, muscle, skin, bone, fins, and head. All samples were analyzed for moisture and ash content (the results are expressed as wet-weight basis), as well as for the concentrations of microelements (Cu, Zn, Fe, Mn), macroelements (K, Ca, Mg, Na), and trace toxic elements (Hg).

Moisture and ash contents

The moisture and ash content of catfish samples were determined according to ISO standards: moisture was measured following SR 91/2007, section 10, while ash content was assessed using the SR ISO method (SR 91/2007 pct.10 humidity; SR ISO 2171/2010, ash).

Determination of mineral content

Macro and microelement concentrations were determined after sample calcination at 650°C in a muffle furnace (Nabertherm GmbH, Lilienthal/Bremen, Germany) until a constant white ash was obtained. Subsequently, extraction was performed using 20% hydrochloric acid (HCl) (Sigma-Aldrich Chemie GmbH, Munich, Germany). The elemental concentrations: cooper (Cu), zinc (Zn), iron (Fe), manganese (Mn), potassium (K), calcium (Ca), magnesium (Mg), sodium (Na) were quantified by atomic absorption spectrometry (AAS) using a Varian Spectra 240 FS (USA) spectrophotometer. The experimental parameters are detailed in Table 1.

Table 1. The experimental parameters for Varian Spectra 240 FS spectrophotometer

Metal	λ (nm)	Lamp current (mA)	Slit width (mm)	Quantification (LOQ) limit
Cu	324.8	4	0.5	0.0012µg/L
Zn	213	5	1	0.008µg/L
Fe	248.3	5	0.2	0.06mg/L
Mn	279.5	5	0.2	0.025mg/L
Ca	422.7	10	0.5	0.10mg/mL
Mg	282.5	4	0,5	0.011mg/mL
K	414	4	0.5	0.252mg/mL
Na	589	8	0.5	0.028mg/L

Hg was quantified by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) using a PlasmaQuant Elite instrument equipped with an AIM3300 autosampler (Analytik Jena, Germany) according to Djedjibegovic et al., (2012). All analyses were performed in triplicate, and results were expressed in ppb (µg/kg) (González et al., 2025).

RESULTS AND DISCUSSIONS

Tables 2 and 3 present the moisture and ash content of fish tissues originating from wild and aquaculture catfish samples. A substantial variation was also observed in ash content, with the highest value recorded in the bones (56.989%), followed by the head and fins, while the lowest value was found in the muscle (1.279%). The ash content of the skin and liver showed closely similar values.

Table 2. Moisture and ash content of fish tissues harvested from aquaculture catfish samples

Fish tissue	Moisture (% wwb)	Ash (%wwb)
Fins	56.25±1.420	14.096±0.420
Bones	26.989±0.563	56.989±0.563
Skin	54.266±1.770	5.121±0.024
Muscle	78.731±2.435	1.279±0.075
Liver	77.178±2.731	3.038±0.333
Head	61.213±1.699	20.411±0.692

Consistent with the aquaculture specimens, the muscle exhibited the highest moisture level (77.060%), whereas the bones contained the highest proportion of ash (56.091%). Conversely, the bones showed the lowest moisture content (27.019%), while the muscle presented the lowest ash concentration (1.385%).

Table 3. Moisture and ash content of fish tissues harvested from wild catfish samples

Fish tissue	Moisture (%wwb)	Ash (%wwb)
Fins	52.252±1.029	17.2496±0.512
Bones	27.019±0.563	56.091±0.502
Skin	51.343±1.963	5.612±0.030
Muscle	77.060±2.317	1.385±0.086
Liver	71.451±1.922	3.798±0.513
Head	64.385±1.699	18.852±0.486

A comparative assessment of the values determined for tissues belonging to wild and farmed catfishes shows that moisture levels of the bones and head in wild-caught catfish was lower than of the farmed ones, whereas all other analyzed tissues parts showed a slightly higher water content in those belonging to the farmed fish. The ash content of the analyzed catfish samples shows an inverse relationship with their moisture levels, such that tissues with lower moisture exhibited higher ash concentrations (bones and head), whereas tissues with higher moisture displayed correspondingly lower ash levels (all the other analyzed tissues).

The obtained results for the moisture and ash content of the muscle are consistent with those

reported by Mecatti et al. (2017), Pyz-Łukasik & Paszkiewicz (2018), and Barbacariu et al. (2023). No published data were found regarding the moisture content of catfish bones as an individual anatomical tissue.

Mineral content of wild and aquaculture catfish

Figure 1 presents the distribution of macroelements in wild and aquaculture catfish and shows the difference between the values determined for Ca, K, Mg, and Na in the catfish samples originating from the two habitats.

Calcium (Ca) was the most abundant macroelement identified in all samples, occurring predominantly in the bones of catfish from both habitats. The highest Ca concentration was observed in the bones of aquaculture-raised catfish, where levels were approximately 4.5% higher than those measured in wild individuals. Elevated Ca levels were also detected in the skin, followed by the fins and head. Potassium (K) was the next most abundant macroelement, with a mean concentration of 7200 ppm across all tissues in aquaculture catfish and 7091 ppm in wild specimens, showing the highest accumulation in the liver and muscle and the lowest in the skin. Sodium (Na) followed, with average concentrations of 6066.5 ppm in farmed catfish and 5869 ppm in wild catfish, with the greatest levels recorded in the bones, skin, and head. Magnesium (Mg) exhibited the lowest concentrations among the macroelements analyzed, averaging 4108 ppm across all tissues in aquaculture catfish and 3869 ppm in wild catfish, with the highest values found in the bones and skin and the lowest in the head and muscle.

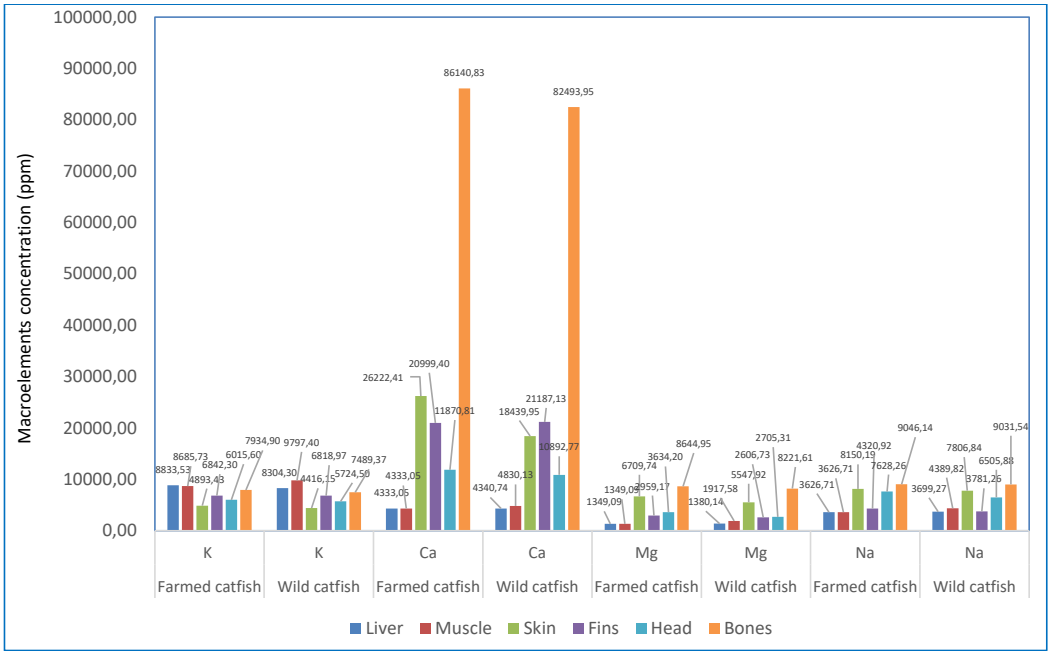


Figure 1 Distribution of macroelements in wild and aquaculture catfish

The obtained results indicate that the concentrations of the analyzed macroelements were higher in all tissues of aquaculture catfish, with an average increases of approximately 10% for Mg, 4.5% for Ca, 3% for Na, and 2% for K. Values reported in other studies reach up to 304.82 mg/kg for Ca, 142.42 mg/kg for Na, and 112.8 mg/kg for Mg in catfish muscle, expressed on a dry-weight basis (Pyz-Lukasik & Chalabis-Mazurek, 2019; Abdel-Mobdy et al., 2021). Furthermore, Matloob (2023) reported concentrations in catfish muscle ranging from 9014 to 10,879 mg/kg for K, 999 to 2039 mg/kg for Na, 797 to 3081 mg/kg for Ca, and 1206 to 1819 mg/kg for Mg, values that are more closely aligned with those obtained in the present study.

The reviewed literature does not report macro-element concentrations in the same tissues that we examined. Figure 2 presents the distribution of microelements in wild and aquaculture catfish, which shows the difference between the values determined for Zn, Fe, Cu and Mn in the catfish samples originating from the two habitats.

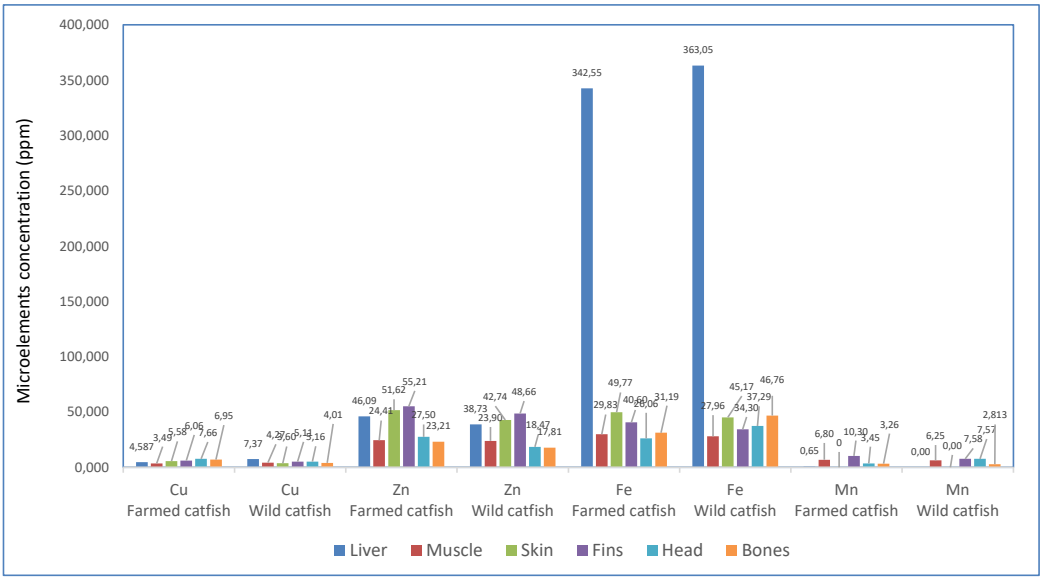


Figure 2. Distribution of microelements in wild and aquaculture catfish

Iron (Fe) was identified as the predominant microelement, with the highest concentration detected in the liver of wild catfish (363.05 ppm), approximately 6% higher than in farmed catfish (342.55 ppm). The lowest Fe concentration was detected in the head of farmed specimens (26.06 ppm). Fe was followed by Zn, with the highest concentrations recorded in fins, skin, and liver. The accumulation pattern was generally similar in the two catfish groups, although most microelements were slightly higher in farmed specimens, except for Fe, which was higher in wild specimens. The greatest habitat-related differences were observed for Zn and Cu, whereas Mn showed only minor variation between groups. Analysis of microelement distribution in catfish tissues showed that the highest concentrations accumulated in the liver, followed by skin and fins, while the lowest concentrations were found in muscle and head.

The values reported in previous studies ranged from 6.73-11.99 mg/kg Fe, 7.09-9.132 mg/kg Zn, 1.15-1.657 mg/kg Cu and 0.34-0.418 mg/kg Mn (Pyz-Lukasik & Chalabis-Mazurek, 2019; Abdel-Mobdy et al., 2021). Jovičić et al. (2015) reported comparable values determined by ICP-MS, namely 19.46 µg/g Fe, 0.670 µg/g Mn, 0.950 µg/g Cu, and 19.62 µg/g Zn in muscle, and 745.7 µg/g Fe, 4.570 µg/g Mn, 5.095 µg/g Cu, and 93.14 µg/g Zn in liver. In another study, Subotić et al. (2013) found (using ICP-MS method) 396.16 µg/g Fe, 1.65 µg/g Mn, 8.37 µg/g Cu and 41.52 µg/g Zn in liver, respectively 27.06 µg/g Fe, 0.44 µg/g Mn, 1.42 µg/g Cu and 20.81 µg/g Zn in muscle. No reported values were found for some of the other catfish tissues examined in the present study.

Hg content of wild and farmed catfish tissues is presented in Table 4.

The low concentrations of mercury detected in the analyzed catfish tissues suggest a low toxicological concern for consumers, particularly given that measurable levels were limited to the head and skin and these tissues are generally less commonly consumed than muscle tissue. In all other tissues Hg was undetectable.

Table 4. Hg in fish tissues collected from aquaculture and wild catfish samples

Fish tissue	Aquaculture catfish (ppb)	Wild catfish (ppb)
Fins	nd	nd
Bones	nd	nd
Skin	0.1±0.026	0.23±0.062
Muscle	nd	nd
Liver	nd	nd
Head	0.557±0.111	1.183±0.047

nd= undetectable

The consulted literature reported Hg concentration of 0.34 mg/kg in muscle and 0.25 mg/kg in liver determined by Direct Mercury Analyser (Squadrone et al., 2013). Also, Alomar et al (2016), detected 0.84 mg/kg (by AAS) in catfish liver, and Subotić et al (2013) reported 1.90 μg/g Hg in liver, respectively 1.63 μg/g Hg in muscle (by ICP-MS).

CONCLUSIONS

This study reveals clear habitat-related differences in the distribution of macro and microelements in wild and aquaculture catfish. Calcium was the dominant macroelement, especially in bones, while iron was the predominant microelement, with the highest levels found in the liver of wild individuals. Microelements generally accumulated in the liver, followed by the skin and fins, and were least abundant in the muscle and head, reflecting consistent tissue-specific patterns.

Habitat influenced several elemental concentrations, with notable differences observed for Fe, Zn, and Cu, whereas Mn remained relatively stable across samples. Mercury was detected only in the head and skin of both catfish groups, and exclusively at low levels that remained well below European and international regulatory limits (FAO, 2017). Hg was undetectable in all other tissues, indicating minimal risk for consumers, particularly since the tissues containing measurable Hg are not typically consumed.

Collectively, these findings provide new insights into mineral distribution in catfish tissues and underscore the role of habitat conditions in shaping elemental profiles, while also confirming the overall safety of the analyzed specimens with respect to mercury contamination.

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EVALUATING HEMPSEED MEAL BY-PRODUCT AS A FUNCTIONAL ADDITIVE IN *Cyprinus carpio* var. *specularis* DIETS

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Abstract

Hempseed meal, a nutrient-rich by-product resulted from oil extraction from Cannabis sativa L., represents a promising candidate for high value functional feed additive due to its favorable amino acid profile, increased levels of essential fatty acids, and availability as an agro-industrial residue. This study investigates the nutritional value and functional potential of hempseed meal as a partial supplementary alternative to traditional protein ingredients in the diet of common carp (Cyprinus carpio), one of the most economically important freshwater species in global aquaculture. The analysis focuses on the biochemical composition, its digestibility, and its influence on growth performance, feed utilization, and metabolic efficiency. Preliminary evidence suggests that the inclusion of moderate levels of hempseed meal in carp diets can enhance feed efficiency without compromising growth or health, while supporting a more sustainable production system through the valorization of agricultural by-products. The findings highlight the relevance of hempseed meal as a functional ingredient that aligns with circular economy principles and contributes to reducing the environmental impact of aquaculture.

Key words*: common carp, functional ingredients, hempseed meal.*

INTRODUCTION

The rapid growth of aquaculture necessitates a transition toward ecologically responsible, cost-efficient feed alternatives to mitigate the high costs and environmental burdens associated with traditional fishmeal and soybean meal (Borrelli et al., 2013; Henriksson et al., 2018).

A critical challenge in contemporary fish nutrition is the replacement of conventional protein sources, such as fishmeal, with sustainable and cost-effective alternatives (Sainsbury et al., 2000; Van Huis et al., 2013). Current research increasingly focuses on the synergistic effects of diet composition and environmental variables on the life traits and heritability of growth-related parameters (Mansilla et al., 2017; Mohamed & House, 2024). Understanding the digestive physiology of this species, particularly its reliance on alkaline enzymatic hydrolysis, is fundamental for formulating feeds that maximize nutrient retention and minimize nitrogenous waste discharge into aquatic ecosystems. In this context, industrial hemp (*Cannabis sativa* L.) derivatives, specifically hemp seed oil and meal, have emerged as promising candidates.

Integration of advanced processing technologies, such as cold-pressing and pelletizing equipment tailored for small-to-medium scale operations, is essential for localizing production and reducing the carbon footprint of the feed supply chain (Cassano et al., 2013; Catchpole et al., 2011; Nenciu et al., 2022).

Unlike traditional plant-based ingredients, hemp products offer a unique profile of bioactive compounds, essential fatty acids, and highly digestible proteins that may enhance the immune response and metabolic health of teleost fish.

For the Mirror Carp variety specifically, dietary formulation is highly important due to its rapid growth potential and unique physiology. Unlike other carp varieties, the *specularis* variety often exhibits faster mass accumulation and a higher tendency for lipid deposition. Consequently, their diets must be carefully balanced with lipid levels between 8% and 12% to ensure high-quality muscle development rather than excessive abdominal fat. For Mirror Carp, the digestive process is highly temperature-dependent; their metabolic rate and enzyme activity, such as amylase and cellulase, peak in warmer waters (20-25°C), making them exceptionally efficient at processing the plant-based carbohydrates found in hemp and other oilseed meals. To support their robust frame and rapid weight gain, Mirror Carp require elevated levels of bioavailable phosphorus (0.6-0.7%). Without this mineral, the fish are prone to structural issues that can decrease their commercial value and welfare (Andre et al., 2009; House et al., 2010).

By-products from the agricultural and food sectors offer a viable pathway toward a circular bioeconomy by repurposing residues into high-value aquafeeds (Kroes et al., 2021; Majewski & Jurgoński, 2021). Among these, hemp (*Cannabis sativa* L.) has emerged as a particularly versatile candidate (Campbell et al., 2001; Nenciu et al., 2023; Palmegiano et al., 2006). The defatted meal resulting from cold-press oil extraction, containing 30-35% protein alongside essential fatty acids and bioactive compounds, remains an underexploited nutritional resource (Naylor et al., 2021; Olusegun et al., 2017). Given its favorable amino acid profile and potential palatability enhancers, hempseed meal is well-suited for omnivorous species like carp (Gibb et al., 2015; Oprescu et al., 2023; Oyugi et al., 2011). Empirical evidence suggests that partially substituting conventional proteins with hempseed derivatives does not adversely affect growth or feed conversion in salmonids and cyprinids (Callaway, 2004; Gengmao et al., 2015; Karimi et al., 2020). Furthermore, the inclusion of omega-3 and omega-6 fatty acids from hemp may bolster lipid metabolism and immune function (Elzinga et al., 2015; Ross et al., 2000). Beyond nutrition, the integration of this industrial waste stream aligns with sustainable development goals by reducing reliance on marine stocks and leveraging the agronomic benefits of hemp, such as carbon sequestration and minimal pesticide requirements (Kocour et al., 2010; Makkar et al., 2014). Despite this potential, there is a scarcity of field-scale data regarding the application of cold-pressed hempseed meal in pond-based carp culture (Ahmed et al., 2019; Russo & Reggiani, 2015). This study addresses this gap through a proof-of-concept investigation involving *Cyprinus carpio* var. *specularis*. By evaluating young specimens (100 g each) over a 90-day period, this research bridges the gap between laboratory potential and applied feasibility. The objective is to validate hempseed meal as a functional, circular ingredient that enhances feed sustainability without compromising fish performance or environmental integrity.

MATERIALS AND METHODS

The research was performed at the National Institute of Research-Development for Machines and Installations Designed for Agriculture and Food Industry (INMA Bucharest), utilizing controlled pond systems during a trial period from May to July 2025. A completely randomized block design was applied with four diets containing increasing levels of hempseed meal, each trial being replicated in three separate ponds to improve statistical robustness and reduce environmental effects. Over a 90-day nutritional study, environmental parameters and water quality metrics were strictly regulated to ensure they remained within the ideal thresholds for carp aquaculture.

Hemp seeds were processed by mechanical cold pressing at temperatures below 50°C to protect heat-sensitive constituents, including omega-3 and omega-6 fatty acids, tocopherols, and other natural antioxidants. The method produces an unrefined oil with preserved sensory qualities, nutritional value, and good oxidative stability, suitable for food and cosmetic uses. In contrast to thermal extraction methods, cold pressing preserves the chemical stability of the meal by avoiding heat-induced breakdown and impurities, offering an eco-friendlier production route.

The hemp seeds used belonged to the Jubileu Secuieni cultivar, (SCDA Secuieni, Romania), with a typical production of 1200-1600 kg/ha. Seeds were pre-dried before processing to improve extraction efficiency calculations. Cold pressing produced an average oil yield of about 29%, corresponding to roughly 84% of the total seed oil, while the remaining fraction formed hempseed cake containing approximately 10-12% residual oil.

The hemp meal has a promising fish fodder value due to its balanced composition: 30-35% crude protein, 8-12% lipids, 20-25% fiber, 5-7% ash, and 25-30% nitrogen-free extract. Its protein provides a favorable amino acid spectrum, particularly arginine, methionine, and glutamic acid, supporting fish growth and metabolism. The residual lipid fraction is rich in polyunsaturated fatty acids - mainly linoleic (50-60%) and α-linolenic acid (15-20%) - which contribute to membrane function, immunity, and overall physiological performance. Natural antioxidants, including tocopherols and phenolics, further enhance oxidative stability and may promote fish health when included in feed formulations.

To produce the oil for this research, a specialized mechanical pressing unit - designed and developed by INMA Bucharest - was employed to ensure controlled extraction parameters.



Figure 1. Industrial cold-pressing installation for extraction of hemp oil from seeds (original source)

The mechanical extraction of lipids via cold pressing represents a highly efficient, non-thermal processing technique that simultaneously yields two distinct fractions: cold-pressed hemp oil and residual hemp meal. Both derivatives exhibit exceptional nutritional profiles characterized by high biological value for both human and animal consumption. The lipid fraction is particularly distinguished by its favorable ratio of polyunsaturated fatty acids, while the resulting meal serves as a concentrated source of high-quality vegetable protein and dietary fiber. By maintaining low processing temperatures, this method ensures the preservation of thermosensitive bioactive compounds, including tocopherols and phenolic antioxidants, thereby enhancing the functional properties and oxidative stability of the final products within a circular bioeconomy framework.



Figure 2. Primary products obtained through cold-pressing process: hemp oil and hemp meal (original source)

The experiment lasted three months and evaluated the influence of hempseed meal inclusion in diets on somatic growth of mirror carp (*Cyprinus carpio* var. *specularis*). Four feeds containing graded hempseed meal levels (0-20%) were formulated to examine effects on nutrient composition and performance. Each treatment was tested in triplicate, with fish of similar initial size distributed evenly among tanks (100 individuals per tank) to maintain uniformity. Fish were fed twice daily, with rations adjusted to biomass, while water temperature, dissolved oxygen, and pH were kept stable throughout the trial.

Body weight was recorded monthly from the start (month 0) to the end (month 3). For each diet and replicate, batch averages were calculated, generating longitudinal data at four time points across four treatments (R1-R4) with three replicates each. Individual ponds served as experimental units (n = 3 per treatment), and growth parameters were expressed as pond-level means derived from all fish within a unit.

Juvenile fish with an initial body mass of 100 g each specimen, were stocked in ponds with a volume of 3 m³. Prior to the experiment, fish underwent a two-week acclimation period

during which they received a control diet without hempseed meal. Throughout the experimental phase, essential aquatic parameters - including pH, temperature, dissolved oxygen, and ammonia levels - were strictly regulated and held within the ideal thresholds for carp aquaculture.

The tanks operated within a recirculating aquaculture system equipped with a filtration unit (Hailea G12000) manufactured by Guangdong Hailea Group Co., Ltd. in China, an automated feeder (Eheim Twin Duo) produced by EHEIM GmbH & Co. KG. in Germany, and a monitoring system (Atlas Scientific Wi-Fi Hydroponics Kit) developed by Atlas Scientific Environmental Robotics in United States. These components enabled stable environmental control throughout the experimental period.



Figure 3. Experimental installation for juvenile fish development (original source)

Composition and Preparation of Experimental Rations

Four experimental diets (R1-R4) have been formulated to investigate the effects of hempseed meal on the development of juvenile cyprinids, with inclusion levels of 0% (control), 5%, 10%, and 20%. Hempseed meal partially replaced conventional plant protein sources while maintaining isonitrogenous and isoenergetic compositions. Crude protein content was maintained at 30%, a level considered optimal for juvenile carp with elevated metabolic requirements, by gradually decreasing the inclusion of pea flour and soybean meal as the proportion of hempseed meal increased. This approach preserved amino acid balance and metabolizable energy consistency. The hemp by-product, derived from defatted seeds, contributed 25-50% protein along with balanced amino acids and polyunsaturated fatty acids.

Constant proportions of wheat bran (15%), corn flour (10%), and alfalfa meal (15%) were retained to ensure similar pellet structure, density, and acceptability across diets. Minor differences in lipid (2.23-3.52%), fiber (9-10.3%), and ash were considered beneficial for energy supply, digestion, and gut function. Dry ingredients were thoroughly blended using a high-shear mixer, cold-extruded through a 3-mm mesh, air-dried to 8-10% moisture, sieved for uniformity, and stored under refrigeration until use. Fish were fed at 09:00 and 17:00 h at 3% of their average body weight, with rations adjusted weekly based on biomass estimates. Feeding activity and consumption were monitored to detect any influence of hemp-derived components on palatability.

Table 1. Ingredient composition (%) and estimated proximate nutritional composition of the experimental diets containing different levels of hempseed meal for mirror carp

Ingredient (%)	Formulation 1 Hempseed Meal 0%	Formulation 2 Hempseed Meal 5%	Formulation 3 Hempseed Meal 10%	Formulation 4 Hempseed Meal20%
Ingredient composition - Composition for 1 kg of feed (g/kg)				
Cannabis sativa seed-meal	0	50	100	200
Pea flour	100	75	50	0
Defatted Soybean meal	500	475	450	400
Wheat bran	150	150	150	150
Maize meal	100	100	100	100
Dehydrated Lucerne meal	150	150	150	150
Estimated proximate composition - for 1 kg of feed (g/kg)				
Proteins	300	300	300	300
Crude lipid	22.3	25.6	28.8	35.2
Fiber content	90	93.3	96.5	103
Mineral content	53	53.6	54.2	55.5
Soluble fraction (NFE)	534.7	527.5	520.5	506.3

Although diets were formulated to be isonitrogenous and isoenergetic, minor differences in lipid (± 1.3%), fiber (± 0.8%), and ash (± 0.6%) occurred due to partial replacement of soybean and pea meals with hempseed meal, which naturally contains higher fiber and residual oil. These small compositional shifts may have affected

digestibility and energy utilization; however, overall growth performance indicated efficient use of the mixed plant protein sources without negative effects on feed conversion. The proportional reduction of soybean and pea ingredients alongside hemp inclusion was intended to reflect practical feed production scenarios, where multiple plant proteins are typically combined to achieve nutritional balance. Total feed for each pond daily was 0.4 kg in the first month, 0.6 kg in the second month and 0.9 kg in the third month.

Growth Performance Calculations

For each experimental unit (replicate tank and batch), growth parameters were calculated and subsequently averaged by treatment based on diet type. When individual-level data were required, body weights recorded at each sampling interval were used.

Absolute weight gain (WG), representing the overall increase in body weight throughout the experimental period, was determined using the following formula:

$$WG = Wf - Wi \quad (1)$$

where Wf is the final body weight (g) and Wi is the initial body weight (g).

Average daily gain (ADG), defined as the mean daily increase in body weight, was determined using:

$$ADG = (Wf - Wi) / t \quad (2)$$

where Wf is the terminal mass of the fish specimens (g), and Wi is the initial body weight (g), and t is the duration of the period (days).

Specific growth rate (SGR), which expresses growth as a percentage of body weight per day while accounting for exponential growth patterns, was calculated as:

$$SGR = [(ln\, Wf - ln\, Wi) / t] \times 100 \quad (3)$$

where Wf and Wi represent final and initial body weights (g), respectively, t is time (days), and ln denotes the natural logarithm.

Feed conversion ratio (FCR), indicating feed efficiency as the quantity of feed required to produce a unit of weight gain, was calculated as the ratio between total feed consumed (TFC and the weight gain (Wg):

$$FCR = T_{fc}/W_g \quad (4)$$

Ethical statement

All experimental procedures were conducted in accordance with EU Directive 2010/63/EU on the protection of animals used for scientific purposes and were approved by the for Ethical Research of National Institute of Research-Development for Machines and Installations Designed for Agriculture and Food Industry-INMA Bucharest, (Protocol Code 02/ May 2021). The stocking density (100 individuals per 3 m^3) was selected based on aquaculture practices and did not induce stress conditions, as confirmed by stable water quality parameters and high survival rates (≥95%) across all treatments.

Statistical Analysis

All data were expressed as expressed as the mean values of the recorded parameters, for each experimental group. Differences between the experimental months were analyzed using one-way Analysis of Variance (ANOVA). When significant differences were detected, Tukey's Honestly Significant Difference (HSD) post-hoc test was applied for multiple comparisons. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using Microsoft Excel 2024 Software.

RESULTS AND DISCUSSIONS

The Mirror Carp's natural behavior as a benthic (bottom) feeder necessitates the use of high-stability, sinking pellets. Their diet often functions as a supplement to the natural protein-rich biota found in pond sediments. By integrating hempseed meal into these sinking formulations, farmers can provide a balanced amino acid profile - specifically supplementing Lysine and Methionine - to mirror the nutrition found in their natural environment.

Figure 4 illustrates the evolution of the average body weight of mirror carp specimens during the three-month experimental period for the four dietary treatments. All groups showed a progressive increase in body weight, indicating normal growth conditions and good feed acceptance. Statistical analysis revealed that fish fed diets R2 and R3 exhibited significantly higher final weights compared to the control group (R1) ($P < 0.05$), while no significant differences were observed between R2 and R3.

The R4 group showed lower values compared to R2 and R3, although differences were not always statistically significant. These results suggest that moderate inclusion levels (5-10%) optimize growth performance.

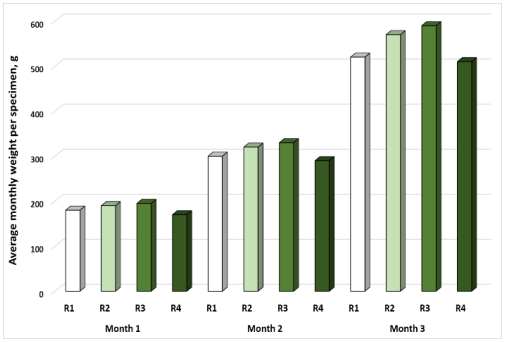


Figure 4. Average monthly weight per specimen for the four feeding recipes for mirror carp

Figure 5 presents the average daily growth rate of mirror carp under the four feeding regimes. Daily growth rate followed a similar trend, with significantly higher values recorded in R2 and R3 compared to R1 ($P < 0.05$). The highest values were observed in R3 across all months. The R4 diet resulted in lower DGR values, supporting the hypothesis that excessive inclusion levels may limit nutrient utilization efficiency. Diets containing moderate levels of hempseed meal tended to produce higher daily growth rates, which may be associated with the balanced amino acid profile and the presence of beneficial polyunsaturated fatty acids in the hemp by-product.

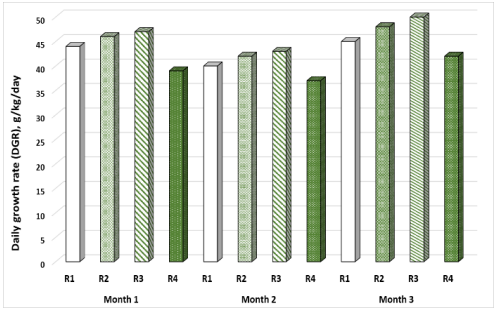


Figure 5. Daily average growth rate depending on the diet for mirror carp

Figure 6 shows the average final body length of mirror carp at the end of the experimental period. Differences among treatments were relatively small, indicating that all diets supported normal somatic development. Nevertheless, fish fed diets supplemented with hempseed meal displayed slightly higher mean lengths, suggesting a positive influence of this ingredient on overall growth performance.

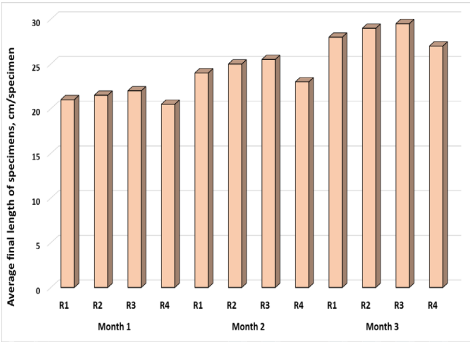


Figure 6. Average final length for mirror carp specimens

Figure 7 presents the survival rate of mirror carp in each experimental treatment. Survival remained high across all dietary groups, indicating that the inclusion of hempseed meal in the feed did not negatively affect fish health or environmental adaptability. The results confirm that hempseed meal can be safely incorporated into carp diets without compromising fish viability.

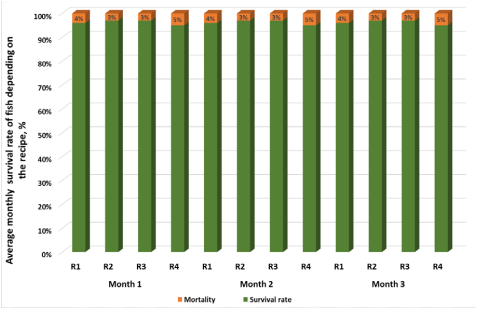


Figure 7. Average survival rate depending on diet in each pool

Figure 8 illustrates the feed conversion ratio (FCR) recorded for each dietary treatment. Feed conversion ratio (FCR) values were significantly improved in R2 and R3 compared to the control group ($P < 0.05$), indicating more efficient feed utilization. In contrast, R4 showed higher FCR values, suggesting reduced efficiency. This trend may be associated with

increased dietary fiber content, which can negatively affect digestibility.

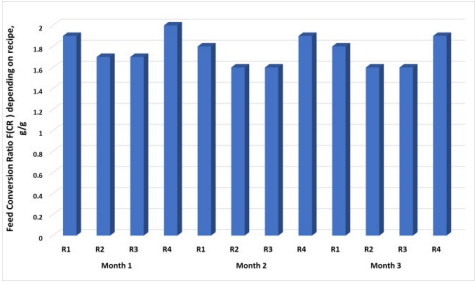


Figure 8. Feed conversion ratio depending on died for mirror carp

The growth performance recorded in the experimental groups indicates that moderate inclusion levels did not negatively affect fish development and, in some cases, even supported slightly improved growth indicators. This effect may be associated with the nutritional characteristics of hempseed meal, which contains a balanced amino acid profile, digestible proteins and beneficial fatty acids. In addition, the presence of bioactive compounds and residual lipids may contribute to improved feed palatability and nutrient utilization. Similar findings have been reported in previous studies investigating plant-based protein sources as substitutes for conventional feed ingredients in carp diets. However, further studies evaluating long-term growth performance, digestibility and carcass composition would be beneficial in order to determine the optimal inclusion level for carp nutrition.

The analysis of growth performance revealed a highly significant progression across all monitored parameters, as evidenced by the robust one-way ANOVA results. The substantial increase in body mass ($F(2, 18) = 624.5$, $p < 0.001$) suggests a steady anabolic trajectory, with Tukey's HSD post-hoc test confirming that each monthly interval represented a statistically distinct developmental stage. This consistent weight gain was mirrored by a significant optimization of the Feed Conversion Ratio ($p = 0.032$), where the transition from the first to the second month marked a shift toward higher nutrient utilization efficiency. While the Daily Growth Rate exhibited minor mid-term fluctuations, the significant recovery observed in the final phase ($p < 0.01$ vs. Month 2) indicates that the specimens reached a state of metabolic equilibrium rather than growth stagnation. Collectively, the convergence of stabilized FCR values and peak DGR in the third month provides a strong statistical basis for the efficacy of the experimental protocol, demonstrating that the culture conditions successfully supported maximum biomass accumulation without compromising metabolic efficiency.

The experimental diets containing different inclusion levels of hempseed meal supported normal development of the fish throughout the experimental period, indicating that this ingredient can represent a viable alternative protein source in aquaculture feeds. Growth indicators such as final body weight, length and daily growth rate showed comparable values among the experimental groups, suggesting that moderate inclusion levels of hempseed meal are well tolerated by mirror carp. In addition, the feed conversion ratio values indicate that the experimental diets were efficiently utilized by the fish, highlighting the nutritional potential of this plant-based ingredient.

Future studies will aim to optimize these formulations through detailed physiological and metabolic analyses. This will allow for the creation of precision diets tailored to the life stages of the Mirror Carp, ensuring that the shift toward sustainability also results in maximum production efficiency and fish health.

The significant increase in body mass, corroborated by the optimized FCR values in the latter stages of the experiment, suggests an efficient metabolic adaptation of the specimens to the diet. The stabilization of the FCR between Month 2 and Month 3 (as shown by the Tukey post-hoc test) indicates that the peak of feed conversion efficiency was reached (for their specific age and environmental conditions), maintaining a steady plateau despite the increase in total biomass.

The reduced performance observed at the highest inclusion level (R4, 20% hempseed meal) may be explained by the increased crude fiber content (10.3%). High dietary fiber is known to reduce nutrient digestibility by limiting enzyme access and accelerating intestinal transit time. Excessive fiber intake may impair protein and energy utilization

efficiency. This likely explains the plateau or slight decrease observed in growth and feed efficiency parameters at higher inclusion levels. While the dietary fiber content appears to be a primary driver of the observed performance trends, several contextual factors inherent to the experimental design must be considered. First, the study utilized juvenile specimens with an initial mean weight of 100 g; it is well-established that metabolic rates and nutrient requirements shift as fish progress through different life stages. Consequently, the observed sensitivity to fiber-rich hempseed meal may vary in larger, more mature individuals with more developed digestive tract capacities.

Furthermore, the three-month experimental duration, while sufficient to capture significant growth trends, represents only a snapshot of the species full production cycle. Long-term effects of high-fiber inclusion on intestinal morphology or chronic nutrient absorption remain to be fully elucidated over an extended period. Lastly, the pond dimensions and stocking density employed in this study provided a specific controlled environment that may influence energy expenditure and social behavior. The physical constraints of the culture system, combined with the juvenile stage of the specimens, create a unique physiological context that should be factored into the generalization of these growth and feed efficiency parameters to larger-scale or different-aged cohorts.

CONCLUSIONS

These findings support the use of hempseed meal as a viable alternative feed ingredient in carp aquaculture, offering promising prospects for improving feed sustainability and resource efficiency in fish production systems. By repurposing this agro-industrial by-product, the study provides a "proof-of-concept" for circular bioeconomy practices that reduce waste and reliance on traditional fishmeal. Moderate inclusion levels (5-10%) proved optimal, improving growth performance and feed conversion efficiency without negatively affecting fish health or survival. In contrast, higher inclusion (20%) resulted in reduced performance, likely due to increased dietary fiber limiting nutrient digestibility. However, the outcomes are influenced by factors such as fish developmental stage, experimental duration, and controlled rearing conditions. Therefore, further long-term studies are recommended to evaluate digestibility, carcass quality, and physiological responses across different life stages and production systems.

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SUBSTITUTION, MISLABELING, AND FRAUD IN THE SEAFOOD INDUSTRY

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Abstract

Illicit practices within global seafood supply chains pose major challenges to economic integrity, environmental sustainability, and public health. This paper reviews and integrates recent scientific investigations that examine the scale, drivers, and consequences of deceptive practices in seafood commerce. By combining evidence from species identification, market surveillance, and regulatory enforcement reports, the analysis demonstrates how opaque trade networks facilitate the commercialization of undeclared or illegally sourced aquatic organisms. These activities result in financial losses for consumers and legitimate operators, while simultaneously undermining fisheries governance. From a conservation standpoint, inaccurate product identification conceals the exploitation of threatened stocks and compromises biodiversity protection by distorting catch statistics and management assessments. Additionally, the circulation of incorrectly identified seafood products raises food safety concerns, including exposure to allergens, unexpected toxins and heavy metals, and inconsistent nutritional values. The paper underscores the growing importance of authentication tools, improved traceability technologies, and coordinated international regulatory frameworks. Addressing these interconnected issues requires an integrated approach that links economic oversight, biodiversity conservation, and food safety policies to promote transparency, sustainability, and consumer confidence in the seafood sector.

Key words*: aquatic biodiversity, economic adulteration, ethics, food safety, seafood fraud, supply chain, traceability.*

INTRODUCTION

Food fraud represents a deliberate act of deception carried out for economic gain through the misrepresentation of food products, ingredients, or labeling (Kennedy, 2021). Institutional reports have suggested that food fraud may generate substantial illicit profits globally, although precise estimates remain uncertain due to the covert nature of fraudulent activities (Kennedy, 2021; FDA, 2025).

In the seafood sector, which includes edible species from both marine and freshwater environments, fraud manifests primarily through species substitution, mislabeling, undeclared processing practices, and falsification of origin or production method (Luque & Donlan, 2019; Silva et al., 2021). Due to the high biodiversity of aquatic species, extensive product processing, and the globalization of supply chains, seafood has been consistently identified as one of the food categories most vulnerable to fraudulent practices (Visciano & Schirone, 2021).

Seafood plays a critical role in global food security, providing approximately 15% of animal protein intake and essential micronutrients for billions of people worldwide (FAO, 2024). Global fisheries and aquaculture production has reached unprecedented levels, totaling 223.2 million tons, including 185.4 million tons of aquatic animals and 37.8 million tons of algae. In 2022, global aquaculture production alone accounted for 130.9 million tons, with an estimated value of USD 312.8 billion, representing approximately 59% of total global fisheries and aquaculture production (FAO, 2024).

The European Union seafood market represents a major global hub for fisheries and aquaculture products, with total trade reaching €38.2 billion and 8.1 million tons in 2023, despite a slight decline in traded volumes. The market is characterized by high import dependency, with an estimated self-sufficiency rate of approximately 38%. In 2024, imports amounted to €29.9 billion, dominated by high-value species such as salmon, cod, and tuna (FAO,

2024). This structural dependence increases supply-chain complexity and exposure to fraud, particularly when traceability information becomes fragmented across multiple actors and jurisdictions (Blanco-Fernández et al., 2021).

Seafood fraud generates a wide range of negative consequences that extend beyond direct economic losses. From an economic perspective, fraudulent practices distort market competition, undermine consumer trust, and disadvantage compliant producers and retailers (Donlan & Luque, 2019). From a public health standpoint, mislabeling and species substitution may expose consumers to unexpected allergens, parasites, toxins, or unauthorized additives, particularly in raw or minimally processed products (Cohen et al., 2009; Asatryan et al., 2023). From a conservation and fisheries management perspective, fraudulent labeling obscures the true exploitation levels of fish stocks, masks illegal, unreported, and unregulated (IUU) fishing, and compromises biodiversity protection and stock assessment processes (Kroetz et al., 2020; Blanco-Fernández et al., 2021).

Despite increasing regulatory efforts and the development of advanced analytical tools such as DNA barcoding, metabarcoding, and complementary chemical approaches, seafood fraud remains widespread across global markets (Galimberti et al., 2013; Ahles et al., 2025). Existing studies often address isolated components of the phenomenon, including mislabeling prevalence, analytical detection methods, or regulatory frameworks, but less frequently integrate economic, food safety, and biodiversity dimensions within a single analytical framework. The objective of this review is to synthesize current scientific evidence on the main forms, drivers, and effects of seafood fraud, with particular emphasis on mislabeling and species substitution, detection methodologies, and traceability challenges within global and European seafood supply chains.

MATERIALS AND METHODS

Literature sources and selection criteria

This study is based on a narrative literature review combined with the analysis of official regulatory and monitoring frameworks related to seafood fraud. Scientific publications were identified through targeted searches in major bibliographic databases, including Web of Science, Google Scholar, and Google, focusing on peer-reviewed articles, review papers, and authoritative institutional reports relevant to seafood mislabeling, species substitution, food fraud, traceability, and authentication methods.

The literature search covered publications released primarily between 2010 and 2025, with earlier references included where necessary to provide historical or conceptual context. Search terms included combinations of keywords such as “seafood fraud”, “fish mislabeling”, “species substitution”, “food authenticity”, “DNA barcoding”, “seafood traceability”, and/or “fisheries fraud”. Studies were selected based on their relevance to the objectives of the review, methodological robustness, and contribution to understanding the economic, food safety, and biodiversity-related dimensions of seafood fraud.

Regulatory and institutional data sources

In addition to scientific literature, official documents and datasets from international and European institutions were consulted to contextualize seafood fraud within existing regulatory and control frameworks. These sources included reports and legislative documents from the Food and Agriculture Organization of the United Nations (FAO), the European Commission, and the U.S. Food and Drug Administration (FDA), as well as information from the EU Agri-Food Fraud Network and the Alert and Cooperation Network (ACN). These materials were used to describe regulatory approaches, monitoring systems, and reported fraud patterns, rather than as sources of quantitative experimental data.

Analytical framework

The collected literature and institutional information were analyzed qualitatively and organized thematically. Identified cases and findings were classified according to major categories of seafood fraud (economic fraud, food safety-related fraud, and fraud affecting biodiversity and fisheries management), types of fraudulent practices (e.g., mislabeling, species substitution, undeclared processing), and detection or authentication methods. This integrative approach allowed for a structured synthesis of current knowledge and facilitated

the identification of recurring patterns, knowledge gaps, and challenges associated with seafood fraud across global and European supply chains.

RESULTS AND DISCUSSIONS

Within the European Union regulatory framework, food fraud is defined as any intentional act or omission involving false, incorrect, or incomplete information, or the non-disclosure of required information, that affects the financial interests of the European Union (Directive (EU) 2017/1371). To address the scale and complexity of such practices in the agri-food sector, Regulation (EU) 2017/625 strengthened official control systems and consumer protection against fraud. In this context, food fraud notifications have been formally integrated into the Rapid Alert System for Food and Feed through the iRASFF platform (Regulation (EU) 2019/1715), highlighting the recognized prevalence and regulatory relevance of seafood fraud within European food control systems.

Forms and drivers of seafood fraud

The review of scientific literature and institutional reports indicates that seafood fraud is a multifaceted phenomenon driven by a combination of economic incentives, supply-chain complexity, and information asymmetry between producers, intermediaries, and consumers. High price differentials between species, market demand for premium products, and limited consumer ability to identify fish species, particularly in processed forms, represent key structural drivers of fraudulent practices (Luque & Donlan, 2019; Visciano & Schirone, 2021).

Seafood products are especially vulnerable to fraud due to the biological diversity of aquatic species, the frequency of processing steps such as filleting, freezing, or cooking, and the globalized nature of fisheries and aquaculture trade. As products move through increasingly complex supply chains involving multiple actors and jurisdictions, traceability information may become fragmented or lost, creating opportunities for intentional mislabeling or substitution (Blanco-Fernández et al., 2021). Fraud tends to concentrate downstream in the supply chain, at the level of wholesalers, retailers, and food service establishments, b where information asymmetry is greatest and direct oversight is limited.

Economic fraud and mislabeling

Economic fraud represents the most frequently reported form of seafood fraud and is primarily manifested through species substitution and mislabeling. Substitution typically involves replacing high-value species with lower-value alternatives that share similar sensory characteristics, such as color or texture, allowing fraudulent products to remain undetected by consumers (Donlan & Luque, 2019). Numerous studies have documented higher mislabeling rates in processed products, including fillets and sushi, compared with whole fish, suggesting that these practices are predominantly deliberate rather than accidental (Ahles et al., 2025).

Price differentials play a central role in motivating economic fraud. Species with high market demand but limited supply are more likely to be mislabeled, while abundant and lower-value species are frequently used as substitutes (Luque & Donlan, 2019). An important criterion for assessing the likelihood of fraud is therefore the price gap between the declared species and potential substitute species. Market prices for commercially important groups, such as white fish, vary substantially across Member States and seasonally, creating persistent economic incentives for species substitution, particularly in price-sensitive market segments. This pattern has been highlighted in official communications from the European Commission's EU Agri-Food Fraud Network, which coordinates actions against fish substitution across Member States (European Commission, https://food.ec.europa.eu/food-safety/eu-agri-food-fraud-network/eu-coordinated-actions/fish-substitution_en.).

Empirical evidence indicates that species substitution occurs at multiple stages of the seafood supply chain, with increasing prevalence downstream. Reported substitution rates rise from approximately 40% at the supplier level to 58% at fishmongers and up to 62% in restaurants, where information asymmetry between sellers and consumers is greatest. Bluefin tuna (*Thunnus thynnus*) is among the most frequently substituted species, accounting for up to 73% of documented cases. Seasonal patterns further reveal instances of

reverse substitution during fishing seasons, potentially indicating the laundering of illegally caught products into legal markets (Gordoa & Carreras, 2017).

In food service establishments, economic fraud is further facilitated using generic or ambiguous commercial names that mask underlying species diversity. Umbrella terms are often employed to obscure differences related to fishing area, stock status, price, or production method, limiting consumers' ability to make informed choices. Restaurant settings are particularly vulnerable, as seafood products are typically served in processed forms and consumers rely almost exclusively on menu descriptions. Consistent with this, mislabeling rates reported for restaurants are substantially higher than those observed in retail outlets, reflecting elevated information asymmetry and reduced opportunities for independent verification (Gordoa & Carreras, 2017; Ahles et al., 2025).

Product form strongly influences mislabeling rates. The highest overall mislabeling has been reported for sushi and sashimi products (67.5%), followed by other raw or minimally processed preparations such as ceviche, poke, roe, and seared products (54.7%). Intermediate rates are observed in cooked products (45.9%) and smoked products (29.4%), while substantially lower mislabeling rates characterize dried or jerky products (21.6%) and canned or retort products (16.5%). These patterns indicate that reduced visual identifiability and higher levels of processing significantly facilitate fraudulent practices, whereas standardized industrial processing and labeling requirements act as partial deterrents (Ahles et al., 2025).

Overall, economic fraud through mislabeling and species substitution results in direct financial losses for consumers and distorts market competition by disadvantaging compliant operators who adhere to labeling and traceability regulations. The concentration of fraudulent practices in downstream market segments, particularly within food service establishments, highlights the limits of upstream controls alone and underscores the need for integrated monitoring strategies that combine economic risk indicators, product-specific vulnerability profiling, and targeted enforcement across the entire seafood supply chain.

Food safety-related fraud

Beyond economic implications, seafood fraud raises significant food safety concerns. Species substitution may expose consumers to unexpected allergens, parasites, toxins, or chemical residues, particularly when substituted species have different biological or ecological characteristics from those declared on product labels (Cohen et al., 2009; Asatryan et al., 2023). These risks are amplified in raw or minimally processed products, such as sushi or sashimi, where pathogens or parasites are not eliminated through thermal processing.

Cases of mislabeling involving toxic or allergenic species, including pufferfish containing tetrodotoxin, illustrate the potential severity of food safety fraud when regulatory controls fail (Cohen et al., 2009). Additionally, undeclared additives or treatments used to enhance appearance or extend shelf life may pose further health risks and erode consumer trust in seafood markets. While not all instances of seafood fraud result in direct health hazards, the unpredictability of exposure underscores the importance of accurate labeling and effective authentication systems.

Fraud affecting biodiversity and fisheries management

Seafood fraud also has substantial implications for biodiversity conservation and fisheries management. Mislabeling and species substitution can obscure the true exploitation levels of fish stocks, thereby compromising stock assessments, management decisions, and conservation measures (Kroetz et al., 2020). By enabling products derived from illegal, unreported, and unregulated (IUU) fishing to be sold under false identities, fraudulent practices effectively facilitate the "laundering" of illegally harvested fish into legitimate markets.

A particularly concerning dimension of seafood fraud involves the substitution of semi-wild, threatened, or protected species with non-threatened ones. Several studies have documented cases in which endangered or heavily regulated species are marketed under alternative names to bypass conservation measures and trade restrictions (Muñoz-Colmenero et al., 2017). Iconic examples include Mediterranean bluefin tuna (*Thunnus thynnus*) and European sturgeon (*Acipenser*

sturio), as well as certain shark species sold as swordfish. In the case of bluefin tuna, long-standing illegal fishing pressure has resulted in actual catches exceeding authorized quotas, in some instances by substantial margins, directly undermining international recovery plans for the species.

In commercial seafood markets, molecular authentication methods, particularly DNA barcoding, have proven effective in detecting the fraudulent substitution of high-value species with lower-value alternatives, such as the replacement of cod or grouper with *Pangasius* (Miller & Mariani, 2010) or *Tilapia* (Pardo et al., 2016). These practices not only distort market dynamics but also conceal the exploitation of species subject to stricter management or conservation measures. DNA-based identification has additionally revealed cases in which bluefin tuna has been misidentified in favor of substitute species associated with food safety concerns, including escolar (*Lepidocybium flavobrunneum*), further illustrating the multifaceted consequences of species substitution (Lowenstein et al., 2009).

Evidence further indicates that species substitution often involves products originating from fisheries characterized by weak management frameworks or limited monitoring capacity. Substitutes such as tuna juveniles sold as anchovy, unregulated hake species (*Merluccius polli*), or other high-value taxa have been linked to IUU fishing activities in African waters, pointing to intentional and organized fraud rather than accidental mislabeling (Blanco-Fernández et al., 2021).

Such practices distort catch statistics, mask overexploitation, and shift fishing pressure onto unreported stocks, thereby impeding sustainable seafood production and consumption.

Incomplete or ambiguous labeling represents an additional challenge for fisheries management. Missing or imprecise information regarding species identity, origin, or production method creates uncertainty that hampers the differentiation between sympatric species and prevents accurate stock-specific management (Kroetz et al., 2020). This lack of transparency reduces the effectiveness of conservation measures and complicates enforcement efforts, particularly for species with overlapping distributions or similar commercial designations (Blanco-Fernández et al., 2021).

From a governance perspective, the complexity of seafood supply chains further exacerbates biodiversity-related fraud risks. As products move from producers to traders, processors, wholesalers, and retailers, control over traceability information diminishes, increasing opportunities for substitution and misrepresentation. While fisheries management and resilience research has traditionally focused on harvesting activities and fishers' livelihoods, comparatively less attention has been given to post-harvest sectors (Moonmoon et al., 2025), including processing, logistics, and retail, where fraudulent practices frequently materialize and where biodiversity impacts ultimately become visible (Rice et al., 2024).

Overall, seafood fraud undermines biodiversity conservation by disconnecting market signals from ecological reality. When species are sold under false identities, exploitation levels are underestimated, management responses are delayed or misdirected, and conservation priorities are distorted. These dynamics highlight the need to prioritize control efforts on highly valued and vulnerable species, strengthen species-specific traceability requirements, and integrate fraud detection more explicitly into fisheries management and biodiversity protection frameworks.

Molecular approaches for seafood authentication

The validation of food authenticity relies primarily on the analysis of protein profiles and/or DNA sequences. Protein-based approaches, including immunological assays and electrophoretic or chromatographic techniques such as high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC), have been successfully applied to fresh or minimally processed products. However, their effectiveness decreases substantially in heavily processed foods, where protein denaturation or degradation limits analytical resolution.

In contrast, DNA-based methods provide higher robustness across a wide range of food matrices, as DNA molecules are generally more stable and can be recovered even from small or degraded biological traces. As a result, molecular

approaches have become central to seafood authentication, particularly in contexts where morphological or biochemical identification is no longer possible (Galimberti et al., 2013).

Among DNA-based techniques, DNA barcoding has emerged as a standard tool for species identification in seafood products (Yang et al., 2024; Burke et al., 2025). This approach typically targets short, conserved mitochondrial markers, most commonly the cytochrome *c* oxidase subunit I (COI) gene in fish, allowing species-level identification through comparison with curated reference databases. DNA barcoding has proven effective for detecting species substitution across a wide range of product forms, including fillets, frozen products, cooked seafood, and sushi, and is widely applied in both research and regulatory monitoring (Galimberti et al., 2013).

Recent reviews indicate that DNA barcoding has expanded beyond single-locus identification to include multi-marker approaches and advanced technologies such as environmental DNA (eDNA) and next-generation sequencing, enhancing species discrimination in complex samples and broadening its applications in molecular authentication and biodiversity monitoring (Odah, 2023; Yang et al., 2024; Rani et al., 2026).

DNA metabarcoding extends the barcoding concept by enabling the simultaneous detection of multiple species within complex or mixed samples. Using high-throughput sequencing and universal primers, this approach allows the identification of entire species assemblages without the need for physical separation of individual components (Zhang et al., 2018; Xiong et al., 2022; Liang et al., 2026). DNA metabarcoding has been increasingly applied to processed seafood products, mixed preparations, and environmental samples, providing valuable insights into biodiversity composition and supporting fraud detection in multi-ingredient products (von Cräutlein et al., 2011; Yang et al., 2024).

From an ecological perspective, DNA barcoding addresses challenges associated with cryptic species complexes, providing evidence that morphologically similar populations may in fact represent distinct taxonomic entities requiring differentiated conservation and management strategies, as demonstrated for scalloped hammerhead sharks *Sphyrna lewini* (Quattro et al., 2013). Moreover, molecular identification remains one of the few effective tools for combating illegal wildlife trade, enabling the detection of protected CITES-listed species in highly processed products such as shark fins and sturgeon caviar (Fields et al., 2018). By exposing species substitution and laundering practices that obscure true exploitation levels, DNA barcoding directly supports biodiversity conservation and strengthens the enforcement of international fisheries management and wildlife protection frameworks.

While DNA-based methods are particularly powerful for species authentication, they do not provide direct information on geographic origin or production methods (Woolfe & Primrose, 2004; Gopi et al., 2018). Consequently, complementary analytical approaches have been explored. Stable isotope analysis has been used to support forensic assessments of catch origin in species such as Atlantic cod, although its accuracy is highly dependent on spatial resolution and baseline characterization (Wilson et al., 2024). Similarly, targeted molecular assays combining mitochondrial and nuclear markers have been applied to distinguish closely related species or detect hybrids, such as in the case of European and American eels, offering effective tools for identifying fraudulent labeling in high-risk markets (Ely et al., 2023; Stein et al., 2021).

More recently, non-molecular techniques such as portable X-ray fluorescence (pXRF) have been proposed as complementary screening tools for fisheries monitoring. By generating rapid, non-destructive elemental profiles, pXRF may support preliminary species discrimination and field-based decision-making, although its application for seafood authentication remains at an exploratory stage and requires further validation (Burke et al., 2025).

Overall, DNA barcoding and related molecular approaches represent core components of modern seafood authentication strategies. Their effectiveness is maximized when integrated with complementary analytical methods, standardized reference databases, traceability systems, and regulatory controls, forming a multi-layered framework for detecting and preventing seafood fraud.

Traceability challenges along the seafood supply chain

Despite advances in analytical authentication, traceability remains a critical challenge in combating seafood fraud. The increasing length and complexity of seafood supply chains, coupled with changing consumer preferences for processed and ready-to-eat products, significantly reduce transparency and complicate enforcement efforts (Luque & Donlan, 2019; FAO, 2024). As seafood products pass through multiple actors and jurisdictions, traceability information may become fragmented or lost, creating structural vulnerabilities that facilitate mislabeling and species substitution (Blanco-Fernández et al., 2021).

Inadequate or incomplete labeling, inconsistent use of commercial and scientific nomenclature, and limited harmonization of traceability requirements across jurisdictions further exacerbate these challenges. Such deficiencies hinder effective monitoring and enforcement, particularly for imported products originating from regions with heterogeneous regulatory frameworks and control capacities (European Commission, 2017; Visciano & Schirone, 2021).

Effective traceability systems therefore require not only technological solutions, such as digital tracking or molecular authentication tools, but also coordinated regulatory frameworks, stakeholder compliance, and enforcement capacity throughout the entire supply chain. To counter information fragmentation along the supply chain, the implementation of Blockchain technologies, as Nicolae et al. (2025) point out, offers a solution through the use of immutable ledgers. They ensure that data entered at the point of origin remains unchanged and verifiable by all actors involved, eliminating the possibility of altering species identification data at critical transfer points and facilitating transparent digital traceability from "ship to plate". Without accurate and complete traceability information, even sophisticated analytical methods may fail to prevent fraudulent products from reaching consumers or to support timely corrective actions (Galimberti et al., 2013; Kroetz et al., 2020). Consequently, strengthening traceability remains central to mitigating the economic, public health, and environmental impacts associated with seafood fraud.

Research limitations

This review is subject to several limitations inherent to its scope and methodological approach. First, the analysis is based primarily on published literature and official institutional reports, which may underrepresent the true extent of seafood fraud due to its covert nature and the likelihood of underreporting. Fraudulent practices that escape regulatory detection or are not documented in peer-reviewed studies are therefore not fully captured.

Second, the reviewed studies show substantial heterogeneity in terms of geographic coverage, sampling design, analytical methods, and product categories. Differences in study objectives, species targeted, market segments investigated, and detection techniques limit the direct comparability of reported mislabeling rates and fraud patterns across regions and time periods.

Third, most available data focus on downstream segments of the seafood supply chain, such as retail and food service establishments, while upstream stages, including harvesting, primary processing, and international trade, remain comparatively underrepresented. As a result, the precise points at which fraudulent practices are introduced into supply chains cannot always be conclusively identified.

Finally, although molecular authentication methods are extensively discussed, this review does not quantitatively compare the performance, cost-efficiency, or scalability of different analytical techniques. The effectiveness of detection tools is therefore evaluated qualitatively rather than through systematic benchmarking.

Challenges in fraud prevention and control

Several persistent challenges hinder effective prevention and control of seafood fraud. A major challenge arises from the complexity and length of seafood supply chains, which often involve multiple intermediaries, cross-border transactions, and heterogeneous regulatory frameworks. As products move from capture or harvest to final consumption, traceability information may become fragmented or lost, creating structural opportunities for mislabeling and species substitution.

Another critical challenge is the lack of harmonization in labeling requirements and

commercial nomenclature across jurisdictions. Inconsistent use of commercial names, incomplete labeling, and insufficient integration of scientific nomenclature reduce transparency and complicate enforcement, particularly for imported products originating from regions with variable control capacities.

From a methodological perspective, while DNA barcoding and metabarcoding provide powerful tools for species identification, they cannot independently resolve questions related to geographic origin, production method, or legality of capture. This limitation necessitates the integration of molecular tools with complementary analytical approaches and regulatory controls, which are not yet consistently implemented.

Enforcement capacity also remains uneven across regions. Limited inspection resources, coupled with the high volume of seafood products traded globally, mean that only a small fraction of products can be subjected to analytical verification. This imbalance between trade volume and control capacity allows fraudulent practices to persist despite advances in detection technologies.

CONCLUSIONS

The fish and seafood sectors represent a particularly vulnerable target for food fraud due to their large trade volume, high economic value, and continuously growing global demand. These characteristics create strong economic incentives for fraudulent practices, while the scale of production and trade limits the proportion of products that can be effectively inspected or analytically verified by control authorities.

This review highlights three major and interrelated dimensions of fish and seafood fraud: economic fraud, public health–related risks, and environmental and biodiversity impacts. While economic fraud primarily affects market transparency and consumer trust, health-related fraud may expose consumers to unexpected hazards, and environmental fraud undermines fisheries management, conservation efforts, and the sustainable use of aquatic resources.

Despite significant advances in analytical detection methods, including DNA barcoding, DNA metabarcoding, and emerging complementary tools such as portable X-ray fluorescence, fraud remains difficult to fully control. The length and complexity of seafood supply chains, often characterized by limited transparency and fragmented traceability from capture or harvest to the final consumer, allow fraudulent practices to persist and evade detection.

Within this context, accurate and standardized labeling remains a cornerstone of effective fraud prevention. The consistent use of country-specific authorized commercial names, complemented by unambiguous scientific nomenclature, would significantly improve product transparency and consumer awareness. Such an approach would facilitate species recognition, support regulatory enforcement, and enhance the effectiveness of analytical authentication methods, thereby contributing to more robust control systems across seafood supply chains.

Mitigating seafood fraud requires an integrated strategy that combines improved labeling practices, strengthened traceability frameworks, targeted analytical controls, and coordinated regulatory enforcement. Through such a multi-layered approach can the economic, health, and environmental impacts of seafood fraud be effectively reduced.

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GROWTH, CONDITION AND POPULATION INSIGHTS OF *Mesogobius batrachocephalus* IN THE ROMANIAN BLACK SEA

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Abstract

Understanding the ecology and population dynamics of Mesogobius batrachocephalus along the Romanian Black Sea coast is essential due to limited available data. This study examined population structure, growth, and condition based on 248 individuals (183 females, 65 males), revealing a female-biased sex ratio of 2.82:1. Morphometric measurements (total length, TL; body weight, W) were collected, and Fulton's condition factor (K) was calculated. TL ranged from 11.5 to 32.4 cm (mean ± SD: 24.06 ± 3.44 cm) and W from 26.22 to 287 g (131.68 ± 50.64 g). K varied between 0.61 and 1.86 (mean ± SD: 0.92 ± 0.17). The length–weight relationship was $W = 0.0457 \times TL^{2.4882}$ *(*$r^2 = 0.8521$*). Von Bertalanffy parameters were* $L\infty = 34.11$ *cm,* $K = 0.370$*, and* $t_0 = -0.355$*. Mortality estimates were* $Z = 1.34$*,* $M = 0.63$*, and* $F = 0.71$*. These findings provide essential baseline information for monitoring and managing M. batrachocephalus populations in the western Black Sea.*

Key words*: condition factor, Knout goby, length-weight analysis, population dynamics, Von Bertalanffy growth.*

INTRODUCTION

The knout goby *Mesogobius batrachocephalus* (Pallas, 1814) is a demersal gobiid widely distributed in the Black Sea, Sea of Azov, and eastern Mediterranean, inhabiting shallow coastal waters with sandy, muddy, or mixed substrates. Along the Romanian coast, Gobiidae represent one of the most diverse benthic fish groups, with over 15 species reported, several of local ecological or commercial interest (Bănaru et al., 2017). Recent taxonomic revisions revealed considerable species-level diversity within morphologically similar gobies (Vasil'eva, 2025), while morphometric studies across the Azov–Black Sea basin have documented pronounced spatial variability within gobiid populations, emphasizing the influence of regional environmental conditions (Belogurova, 2024). These findings highlight the need for species-specific studies of growth and population dynamics. *M. batrachocephalus* is among the largest gobiids, exceeding 30 cm total length, historically captured in artisanal fisheries and as by-catch (Roșca & Mânzu, 2011), and also recorded in experimental coastal gear, including round goby traps (Țiganov et al., 2023). Bulgarian Black Sea catches show that gobies are the second most important fish group after sprat, demonstrating ecological and commercial relevance (Zarev et al., 2013).

Previous studies along the Romanian coast focused on feeding ecology and seasonal diet variation. *M. batrachocephalus* shows an opportunistic benthic diet dominated by crustaceans, polychaetes, and small fishes, with seasonal shifts linked to prey availability (Roșca & Mânzu, 2011). Other benthic fish studies confirm a generalist feeding strategy based on macrobenthic fauna (Roșca & Surugiu, 2010). Morphological analyses, including otolith shape, revealed distinctive sagittal otolith morphotypes in *M. batrachocephalus*, useful for trophic studies and interspecific differentiation (Bănaru et al., 2017). Recent morphometric and

genetic work confirms clear separation from sympatric gobiids (Karadurmuş et al., 2024).

In other Black Sea regions, studies examined growth, condition, reproduction, and length–weight relationships. Bengil & Aydın (2020) reported sex and habitat-related variations in length–weight parameters, condition factor, and feeding patterns, while Aydın (2021) provided detailed biometric assessments of growth and population parameters. Although not a major commercial species, *M. batrachocephalus* is periodically caught in coastal traps and gillnets, with variable biomass contribution depending on year and location (Bengil & Aydın, 2020; Aydın, 2021; Țiganov et al., 2023). Long-term, species-specific datasets along the Romanian coast remain scarce, with fluctuations in catches influenced by environmental and ecological factors.

Given limited updated data for Romania on growth, length–weight relationships, monthly biological variation, and sex-specific condition, this study provides an integrated assessment of *M. batrachocephalus* using recent data. Biometric characteristics, condition indices (including Fulton's K), sex ratio, length-frequency structure, and population parameters are analysed to improve understanding of the species status and provide a baseline for future ecological and fisheries research.

MATERIALS AND METHODS

Specimens of *Mesogobius batrachocephalus* were collected along the Romanian Black Sea coast (Baia Mamaia, across Port Tomis, across Port Constanta and the Tuzla-Costinesti area, at depths between 3 and 25 m) as part of the National Fisheries Data Collection Program conducted by NIMRD "Grigore Antipa". Sampling locations were mapped in ArcGIS (Figure 1).

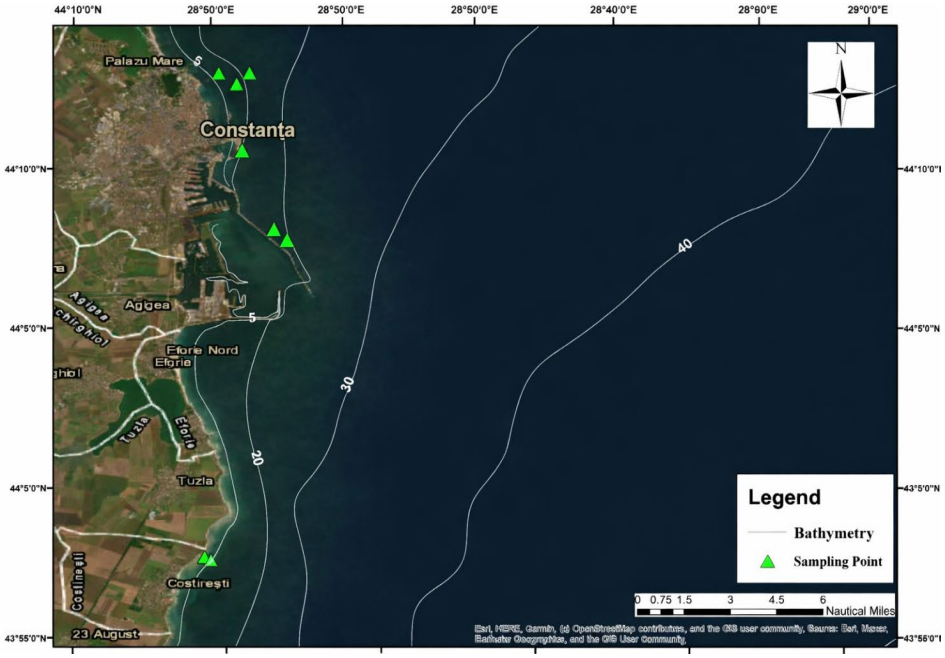


Figure 1. Sampling map (Alexandru Cândescu, NIMRD "Grigore Antipa")

Specimens were captured using 50 round goby traps deployed each month (March, April, September, October, November 2025). Chicken meat was used as attractant. Captured fish were removed from traps and transported to the laboratory in insulated containers, where biometric measurements were performed. In the laboratory, total length (TL, ± 1 mm), total weight (W, ± 0.1 g), and additional morphometric traits were recorded, with photographic documentation (Figure 2).



Figure 2. Total length (TL) measurements of *Mesogobius batrachocephalus* individuals in the laboratory (original by NIMRD)

The length-weight relationship was estimated using the allometric equation (Froese, 2006):

$$W = a \cdot TL^b \qquad (1)$$

with log-transformed linear regression:

$$\log W = \log a + b \cdot \log TL \qquad (2)$$

Fulton's condition factor was calculated as:

$$K = \frac{W}{TL^3} \cdot 100 \qquad (3),$$

following Fulton (1904) and Froese (2006).
Population structure was analyzed using FiSAT II – FAO ICLARM Stock Assessment Tool (Gayanilo et al., 2005) to determine length-frequency distributions, growth parameters ($L\infty$, K), mortality rates (total, natural, fishing), and sex ratio. Additional analyses, including descriptive statistics, t-tests for sex- and month-related differences, and TL-W correlations (Pearson), were performed in Microsoft Excel 365 and XLSTAT (2025.1.3). Graphs generated in Excel included lnTL vs lnW scatterplots, length-frequency distributions, and boxplots of TL, W, and Fulton's K by sex and month.

ETHICAL STATEMENT

Fish were collected through scientific sampling as part of the National Fisheries Data Collection Program conducted by the National Institute for Marine Research and Development "Grigore Antipa". No experimental procedures were performed on live animals. The study was based on specimens obtained from scientific fisheries sampling in accordance with national regulations.

RESULTS AND DISCUSSIONS

A total of 248 *Mesogobius batrachocephalus* individuals were collected from March to November 2025 using round goby traps. Total lengths ranged from 11.5 to 32.4 cm and weights from 26.22 to 287 g, with mean TL 24.06 cm and mean W 131.68 g, indicating a population composed of juveniles and adults (Table 1).
When separated by sex, females were slightly larger and heavier than males, consistent with typical gobiid sexual dimorphism. Maximum values were also higher in females, reflecting their reproductive role.
Sex composition was strongly female-biased (183 females, 65 males; F:M ≈ 2.82:1, 73.8 % females) (Figure 3), comparable to Bănaru et al. (2017, ~2:1) in the northwestern Black Sea. In contrast, southern Black Sea populations show near parity (Aydın, 2021; Bengil & Aydın, 2020). Female predominance may reflect differences in reproductive lifespan, spawning mortality, spatial distribution, or pot selectivity, indicating a population in a stable reproductive phase.

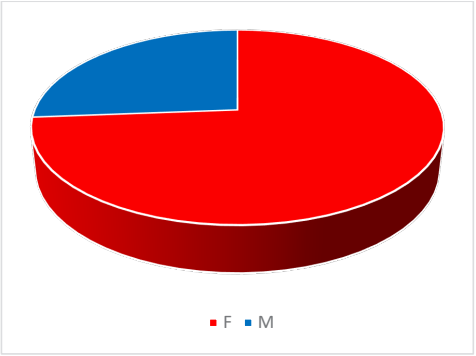


Figure 3. Sex ratio of *Mesogobius batrachocephalus* (original)

Table 1. Descriptive statistics of total length (TL) and weight (W) by sex and overall

Group	N	TL mean	TL SD	TL min	TL max	W mean	W SD	W min	W max
Total	248	24.06	3.44	11.5	32.4	131.68	50.64	26.22	287
F	183	24.03	3.42	11.5	32.4	131.26	50.19	26.22	287
M	65	23.96	3.46	12	31	131.68	50.99	26.3	246.91

Length-frequency analysis shows most individuals in the 22-28 cm range, with minor peaks at 11-20 cm and 31-32 cm (Figure 4), reflecting multiple cohorts. The distribution is slightly left-skewed, suggesting fewer large specimens. The main peak (25-28 cm) represents the dominant size class, while smaller peaks correspond to juveniles. This structure indicates ongoing recruitment and reproductive potential.

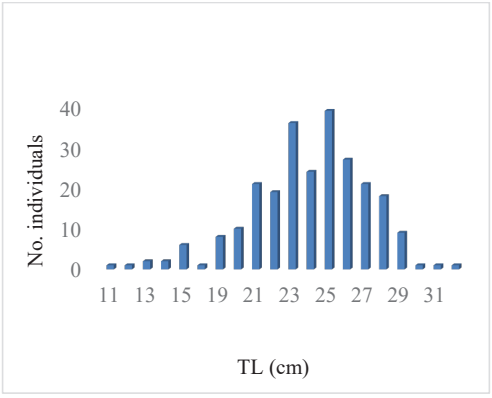


Figure 4. Length - frequency (both sexes)

Overall, the population exhibits a wide size range, female-biased sex ratio, and multiple cohorts, confirming active reproduction and a healthy demographic structure, providing the basis for subsequent growth and condition analyses.

Length-Weight Relationship (LWR)

The length-weight relationship of *Mesogobius batrachocephalus* was analysed to assess growth patterns and population condition, and to compare with previous studies. The overall LWR showed negative allometric growth ($b = 2.4882$; $R^2 = 0.8521$), indicating that weight increases more slowly than length (Figure 5). This aligns with findings from Demirhan & Can (2007), Demirhan et al. (2005), Ak et al. (2009), and Çalık & Erdoğan Sağlam (2017), who also reported $b < 3.0$ in various Black Sea populations (Table 2). Negative allometry suggests energy allocation toward reproduction or maintenance rather than somatic growth (Ricker, 1973; 1975; Weatherley & Gill, 1987; Froese, 2006).

Some studies report isometric growth ($b \approx 3$) (Păun, 2024) or positive allometry ($b > 3$) (Dağtekin et al., 2022; Bengil & Aydın, 2020; Aydın, 2021), highlighting regional variability due to environmental conditions, productivity, or sampling season. The negative allometric

growth observed in the present study (b = 2.4882) differs from several studies in the southern Black Sea where positive allometry was reported (b > 3). This difference may be explained by environmental and seasonal factors, including lower food availability, temperature differences, reproductive investment, and sampling period. The specimens in this study were collected mainly during spring and autumn, periods associated with gonadal development and energy allocation toward reproduction rather than somatic growth. Additionally, habitat conditions along the Romanian coast, including substrate type, productivity, and fishing pressure, may influence growth patterns and result in lower b values compared to other Black Sea regions. Such regional variability in length–weight parameters is common in benthic species and reflects environmental plasticity rather than population decline. Females exhibited negative allometry (b = 2.5128; R^2 = 0.8441) and males similarly (b = 2.3911; R^2 = 0.862) (Figures 5-7). The lnW–lnL regression confirmed linearity of the relationship, showing a clear positive correlation between body weight (lnW) and total length (lnL) (Figure 8). Low scatter around the regression line indicates consistent growth patterns within the population, while outliers likely reflect individual variability related to age, nutritional status, or reproductive stage.

The predominance of negative allometric growth in both sexes suggests a growth strategy characterized by body elongation rather than proportional mass increase, a pattern commonly observed in benthic and demersal gobiid species. Such growth dynamics may represent adaptive responses to habitat structure, variable food availability, or increased energetic investment in gonadal development, particularly during reproductive periods. Similar growth strategies have been reported for gobiid fishes inhabiting shallow coastal environments, where environmental variability influences somatic growth trajectories. The consistency of negative allometry across sexes and size classes indicates a relatively homogeneous growth response within the studied population, despite seasonal environmental fluctuations. This stability enhances the applicability of the derived length-weight parameters for biomass estimation and population monitoring. Therefore, the LWR parameters obtained in the present study provide a robust baseline for interpreting Fulton's condition factor (K) and for use in fisheries-independent assessments and comparative ecological studies.

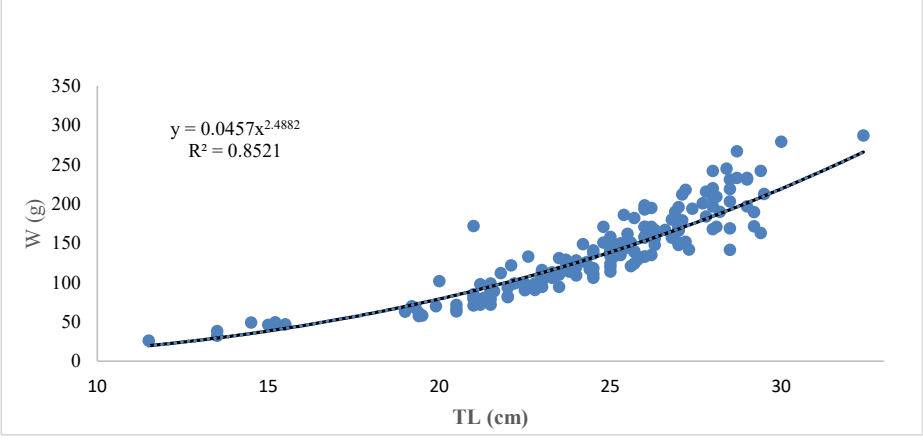


Figure 5. Length-weight relationship for all individuals

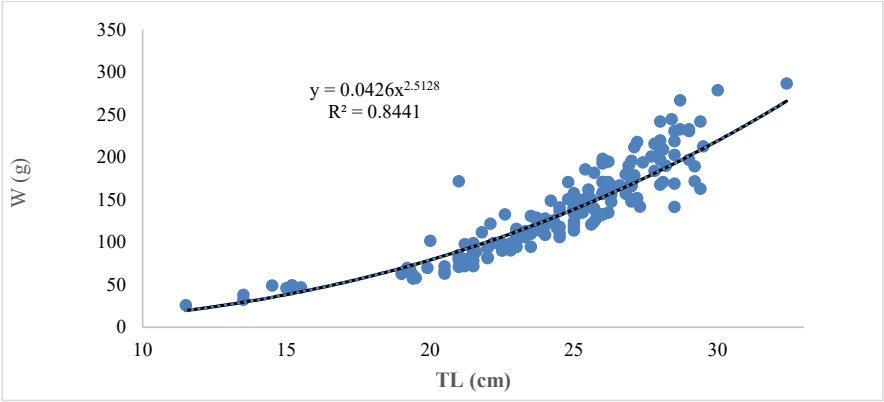


Figure 6. Length-weight relationship for females

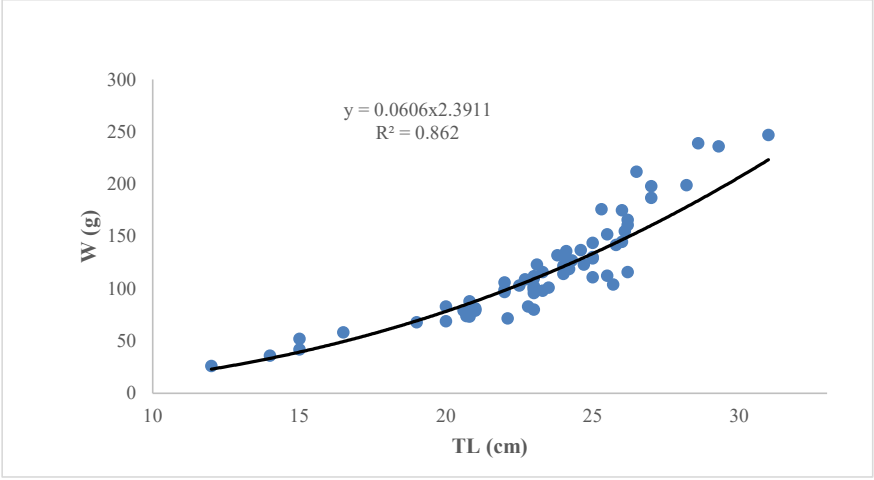


Figure 7. Length-weight relationship for males

Table 2. Previous literature indicating the result of length-weight relationship for round goby in different area

Species	L (min.-max.)	W (min.-max.)	n	a	b	r2	Growth type	Area	References
M. *batrachocephalus*	7.2-13.3	-	37(C)	0.0203	2.75	0.93	A-	South-eastern Black Sea	Demirhan & Can (2007)
	7.2-13.3	4-25.7	40(C)	0.0289	2.6	0.88	A-	South-eastern Black Sea	Demirhan et al. (2005)
	12.6-31.8	12.62-377.54	470(C)	0.0062	3.13	0.9606	A+	Southern Black Sea	Bengil & Aydın, (2020)
	7.4-26.7	4.22-195.78	141 ©	0.0055	3.1566	0.9743	A+	South-eastern Black Sea	Dağtekin et al. (2022)
	5.3-34	1.34-377.54	641(C)	0.0058	3.148	0.9621	A+	Southern Black Sea	Aydın (2021)
	5.5-18.0	1.71-77.0	184(C)	0.024	2.736	0.913	A-	Eastern Black Sea	Ak et al. (2009)
	12.2-23.5	14.0-120.0	35(C)	0.0149	2.7768	0.92	A-	South-Central Black Sea	Çalık & Erdoğan Sağlam (2017)
	19.0-29.3	68.0-242.0	46(C)	0.0093	2.9981	0.8856	I	Black Sea	Păun et al. (2024)
	11.5-32.4	26.22-287	248(C)	0.0457	2.488	0.8521	A-	Black Sea	Present study

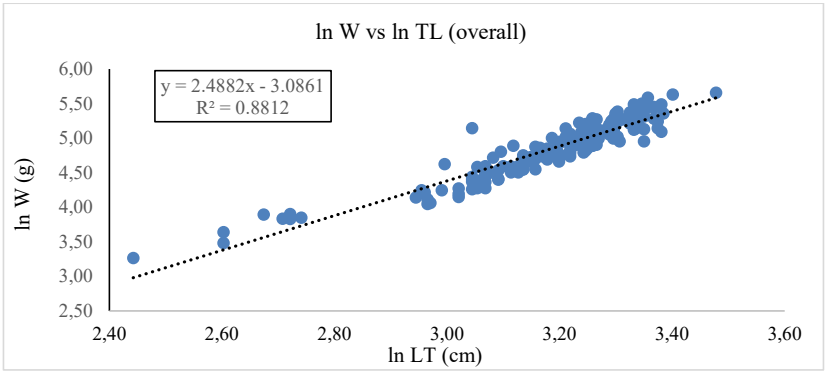


Figure 8. lnW–lnTL relationship (all data)

Condition Factor (Fulton's K)

Fulton's condition factor (K) was calculated following Fulton (1904) and Froese (2006) to assess body condition and energy allocation. The mean K for the population was 0.92 (range 0.61-1.86), indicating generally healthy individuals with moderate robustness (Table 3).

Table 3. Fulton's condition factor (K) by sex and overall

Group	N	Mean K	SD K	Min K	Max K
Total	248	0.92	0.17	0.61	1.86
F	183	0.92	0.17	0.61	1.86
M	65	0.93	0.17	0.61	1.54

Males and females showed similar mean K values (0.92 and 0.92, respectively), though females reached slightly higher maxima, likely reflecting gonadal development. Comparable K values (0.85-1.05) have been reported in other Black Sea populations (Çalık & Erdoğan Sağlam, 2017; Bengil & Aydın, 2020), supporting the interpretation of a healthy population.

Monthly comparisons of TL, W, and K showed no significant sex differences for most months ($p > 0.05$), except in October, where weight and K differed ($p < 0.001$), possibly reflecting autumnal variation in energy allocation prior to winter (Table 4). Similar patterns have been observed in other gobiids (Kornis et al., 2012). These differences likely relate to physiological processes rather than absolute size differences, as sex- and season-specific data for *M. batrachocephalus* are limited.

Table 4. Monthly comparison of TL, W and K between females and males of *M. batrachocephalus*

Month	Variable	Mean F ± SD	N F	Mean M ± SD	N M	p-value	Significance
March	TL	24.17± 3.14	30	21.53±1.35	5	0.14	ns
March	W	121.18±47.05	30	79.46±16.25	5	0.09	ns
March	K	0.82±0.10	30	0.79±0.06	5	0.43	ns
April	TL	20.76±4.33	36	21.24±4.46	14	0.77	ns
April	W	88.71±38.56	36	95.6±46.53	14	0.29	ns
April	K	0.99±0.28	36	0.99±0.27	14	0.48	ns
September	TL	23.99±2.16	25	23.93±2.07	7	0.13	ns
September	W	122.88±31.57	25	121.73±31.46	7	0.09	ns
September	K	0.88±0.1	25	0.87±0.09	7	0.87	ns
October	TL	24.12±2.47	46	24.06±2.51	18	0.0003	*
October	W	128.72±38.03	46	128.21±38.88	18	0.0001	*
October	K	0.90±0.14	46	0.91±0.14	18	0.72	ns
November	TL	24.91±2.42	46	24.96±2.44	21	0.08	ns
November	W	144.81±45.21	46	145.71±45.59	21	0.02	ns
November	K	0.91±0.12	46	0.91±0.12	21	0.01	ns

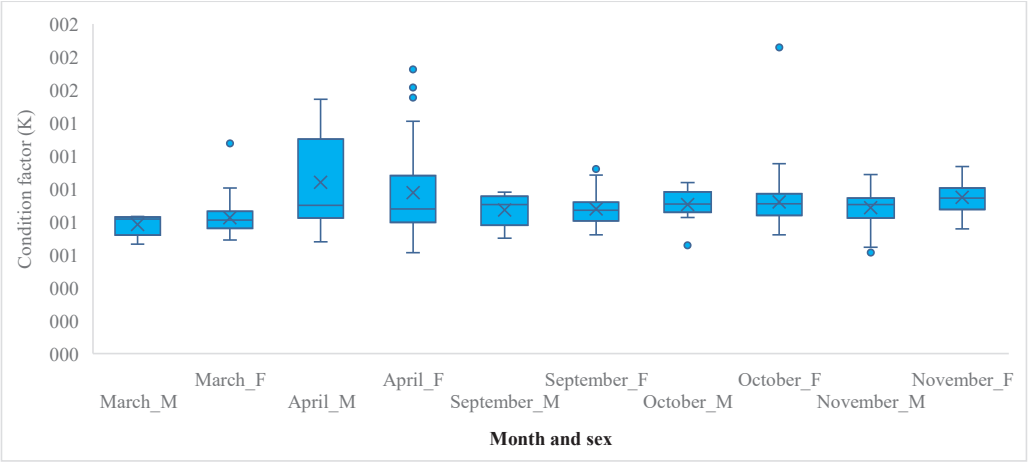


Figure 9. Boxplots of Condition factor (K) in *M. batrachocephalus* by Month and Sex

Boxplots of monthly K values (Figure 9) show largely overlapping interquartile ranges between sexes, indicating comparable physiological status. Slightly higher median K in females in March and October supports the interpretation of similar overall condition. These K patterns provide context for interpreting growth and mortality estimates in subsequent analyses.

These patterns in Fulton's K provide essential context for interpreting growth dynamics and mortality estimates examined in the following sections.

Size Structure and Monthly Variation

Boxplots of total length (TL) and weight (W) by month and sex (Figures 10-11) show that females generally have slightly higher TL and W values and wider ranges than males, although most differences were not statistically significant ($p > 0.05$). Males displayed narrower distributions with fewer extreme values. Weight patterns mirrored TL, with substantial overlap between sexes, indicating similar environmental and nutritional conditions. Female variability likely reflects reproductive cycle and seasonal gonadal development.

Fulton's condition factor (K) was stable across months, with comparable values between sexes and minor fluctuations (slightly higher in females in March and October), suggesting consistent physiological condition and energy allocation. Minimal outliers confirm population stability.

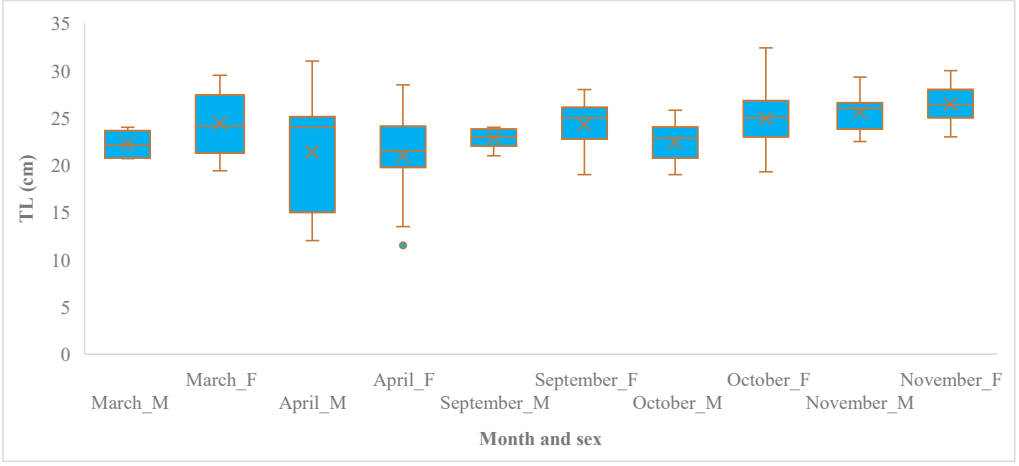


Figure 10. Boxplots of Total Length (TL) in *M. batrachocephalus* by Month and Sex

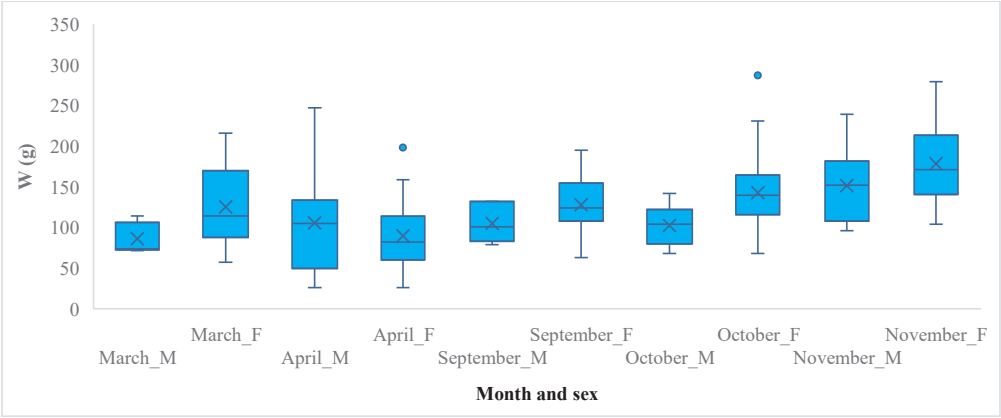


Figure 11. Boxplots of Weight (W) in *M. batrachocephalus* by Month and Sex

These monthly trends complement descriptive statistics and sex ratio patterns, providing a clear overview of population structure and a foundation for interpreting growth parameters, recruitment timing, and mortality in subsequent analyses.

Mortality and Growth Analysis

The length-converted catch curve highlights size at full recruitment, showing that smaller individuals are underrepresented due to gear selectivity (Figure 12). The descending slope indicates higher cumulative mortality in larger fish, reflecting moderate to high fishing or natural mortality, typical for benthic species.

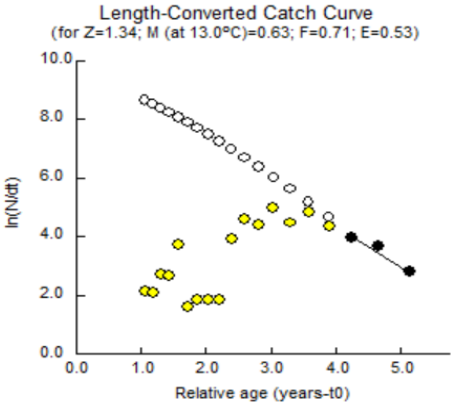


Figure 12. Length - Converted Catch Curve for *M. batrachocephalus*

The probability-of-capture (selectivity) curve shows low catchability for the smallest length classes, with Lc50 marking the size at 50% vulnerability (Figure 13). The steep curve indicates a narrow selectivity window, validating mortality and growth analyses based on larger size groups.

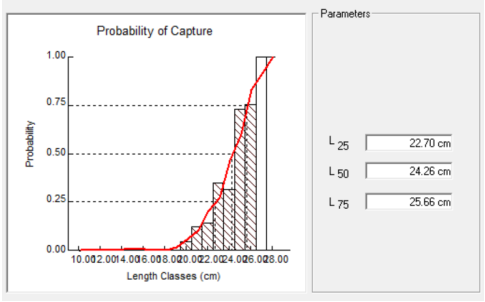


Figure 13. Probability of Capture in *M. batrachocephalus* (Lc50)

K-scan analysis identified a clear optimal von Bertalanffy growth coefficient (K), supporting a single dominant growth pattern (Figure 14). The von Bertalanffy model yielded an asymptotic length $L\infty \approx 34.11$ cm and $K \approx 0.370$ year^{-1}, placing the species in a moderate-growth category typical of demersal gobies. The derived Φ' index confirms biologically reasonable growth and allows comparison with other populations.

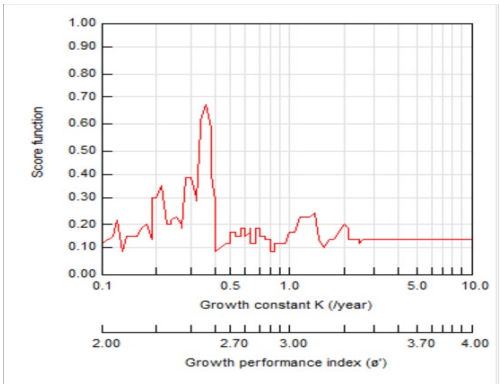


Figure 14. Growth Coefficient Distribution (K-Scan)

Comparison with Aydın (2021) from the southern Black Sea shows $L\infty = 38.2$ cm and $K = 0.245$ year^{-1}, with positive allometric growth ($b \approx 3.15$), contrasting with the negative allometry ($b \approx 2.4882$) observed here. Romanian coast populations grow faster initially but reach smaller maximum sizes, while southern populations grow slower to larger lengths. Fulton's condition factor is similar (~0.92 vs. ~0.913), indicating comparable energy reserves. Mortality estimates here indicate moderate to high pressure on larger individuals, a parameter not reported by Aydın (2021).

These results suggest that spatial variation, environmental factors, seasonality, and local exploitation influence growth patterns and maximum size. The estimated parameters provide a reliable basis for understanding *M. batrachocephalus* ecology along the Romanian coast and inform future monitoring and management.

CONCLUSIONS

The population of *Mesogobius batrachocephalus* along the Romanian coast exhibits a good physiological condition, active recruitment, and a relatively stable population structure.

Growth is characterized by negative allometry and a moderate asymptotic length, with rapid growth during early life stages.

Females predominate in the population, while monthly variations are minor and no significant differences were detected between sexes.

The results suggest the existence of spatial variability in growth patterns within the Black Sea, whereas condition factors appear to remain relatively stable.

These findings provide baseline information for population monitoring, contribute to fisheries management considerations, and emphasize the importance of accounting for spatial and seasonal variability when assessing the ecological dynamics of this species.

Future research should focus on broader temporal and spatial analyses to further refine management and conservation strategies for *M. batrachocephalus* in the Black Sea.

ACKNOWLEDGEMENTS

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ECTOPARASITE FAUNA OF DOMESTIC WATERFOWL IN THE NORTHERN REGION OF THE REPUBLIC OF MOLDOVA

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Abstract

A study on the ectoparasite fauna of domestic waterfowl (ducks and geese) from the Anatidae family in the northern region of the Republic of Moldova showed that domestic ducks (Anas platyrhynchos domesticus, Linnaeus, 1758) were infested with six species of chewing lice: Trinoton querquedulae, Anatoecus icterodes, Anatoecus dentatus, Anaticola crassicornis, Anaticola anserinum, and Menopon obscurum, as well as the gamasid mite Dermanyssus gallinae.
Domestic geese (Anser anser domesticus, Linnaeus, 1758) were found to host ten species of ectoparasites, including eight species of chewing lice: Trinoton querquedulae, Trinoton anserinum, Anatoecus icterodes, Anaticola crassicornis, Anaticola anserinum, Anatoecus adustus, Menopon obscurum, and Lipeurus caponis. In addition, geese were infested with the gamasid mite Dermanyssus gallinae and the argasid tick Argas persicus.These findings indicate a high diversity of ectoparasites infesting domestic ducks and geese in the northern region of the Republic of Moldova, with differences between host species. The presence of lice, mites, and ticks highlights the need for monitoring and controlling ectoparasite populations in order to maintain the health of domestic waterfowl in this region.

Key words*: ectoparasite fauna, habitat, mixtinvasions, monoinvasions, waterfowl*

INTRODUCTION

Domestic waterfowl, particularly ducks and geese belonging to the family *Anatidae*, represent an important component of poultry production systems worldwide. Due to their high adaptability to diverse environmental conditions, their ability to utilize natural aquatic resources, and their economic value as sources of meat, eggs, and feathers, these birds are widely reared in both traditional household systems and intensive commercial farms. In many regions, including Eastern Europe and the Republic of Moldova, domestic waterfowl are commonly raised in close association with natural or artificial water bodies, such as ponds, lakes, irrigation channels, and wetlands. While these environments offer favorable conditions for growth and productivity, they also expose birds to a wide range of parasitic organisms, including ectoparasites, which pose significant health, welfare, and economic challenges.

Ectoparasites are organisms that live on the surface of the host's body or temporarily feed on it, including lice, mites, ticks, fleas, and blood-sucking dipterans. In domestic waterfowl, ectoparasitic infestations are often underestimated compared to endoparasitoses, despite their substantial negative impact on bird health and production. Ectoparasites cause direct damage through blood feeding, skin irritation, and tissue injury, and indirect damage by acting as vectors for bacterial, viral, and protozoan pathogens. Heavy infestations may lead to anemia, stress, reduced body condition, impaired thermoregulation, feather damage, and increased susceptibility to secondary infections. In severe cases, ectoparasitoses can result in decreased productivity, poor growth performance, reproductive disorders, and even mortality, particularly in young or immunocompromised birds (Didă, 1996; Niculescu & Didă, 1998; Marquardt et al., 2000; Erhan, 2024).

The epidemiology of ectoparasitic infestations in waterfowl is closely linked to environmental, climatic, and management-related factors. Warm temperatures, high humidity, and the presence of organic matter favor the survival and reproduction of many ectoparasite species. Aquatic and semi-aquatic habitats provide optimal microclimatic conditions for ectoparasites, while nesting sites, bedding material, and shelters act as reservoirs for different developmental stages. Furthermore, traditional rearing systems, characterized by free-range access, mixed-species flocks, limited biosecurity measures, and close contact with wild birds, significantly increase the risk of infestation. Wild waterfowl often serve as natural reservoirs for ectoparasites and play a key role in maintaining and disseminating parasite populations across different ecosystems (Olteanu et al., 1991; Șuteu & Cozma, 2007; Rusu, 2021a; Erhan, 2024).

From a veterinary and public health perspective, ectoparasites of domestic waterfowl deserve increased attention due to their role in the transmission of zoonotic agents. Several ectoparasite species are known vectors of pathogens such as *Borrelia*, *Rickettsia*, *Salmonella*, *Pasteurella*, and various arboviruses. Through bites, mechanical transmission, or contamination of skin lesions, ectoparasites can facilitate the spread of infectious diseases among birds, livestock, and humans. In rural areas, where close contact between humans and domestic animals is common, the presence of ectoparasites on poultry represents a potential public health risk, especially for farm workers and households involved in traditional waterfowl farming (Hudson, 1996; Erhan, 2020).

In addition to their epidemiological significance, ectoparasites have a pronounced economic impact on poultry production. Infested birds exhibit reduced feed intake, lower feed conversion efficiency, decreased egg production, and inferior carcass quality. Feather damage caused by lice and mites diminishes the commercial value of feathers and down, while stress-induced immunosuppression increases veterinary costs associated with disease prevention and treatment. Moreover, ectoparasitic infestations may lead to increased use of chemical insecticides and acaricides, which raises concerns regarding environmental contamination, development of resistance, and the presence of chemical residues in animal products. These issues highlight the need for sustainable and integrated approaches to ectoparasite control in domestic waterfowl.

The diversity of ectoparasite fauna associated with domestic waterfowl is considerable and varies depending on geographic region, climate, host species, and rearing conditions. Common ectoparasites of ducks and geese include chewing lice (order *Phthiraptera*), such as *Anaticola crassicornis* and *Trinoton anserinum*; mites (order *Acari*), including *Dermanyssus gallinae* and *Knemidocoptes* spp.; ticks (*Ixodidae*), which may parasitize birds during certain stages of their life cycle; and hematophagous insects, such as mosquitoes and biting flies. Understanding the composition and structure of ectoparasite communities is essential for assessing infestation dynamics and identifying species of major veterinary importance (Gherman et al., 2003; Dărăbuș et al., 2006).

In the Republic of Moldova, the study of ectoparasites in domestic waterfowl remains limited, despite the widespread distribution of ducks and geese and the favorable ecological conditions for parasite development. The temperate-continental climate, characterized by warm summers and relatively mild winters, combined with the presence of numerous aquatic ecosystems, creates optimal conditions for ectoparasite survival and transmission. Anthropized habitats, such as agricultural wetlands, irrigation systems, and small rural ponds, further contribute to maintaining ectoparasite populations and facilitating contact between domestic and wild birds. These factors underscore the importance of conducting comprehensive investigations into the ectoparasite fauna of domestic waterfowl at the national and regional levels (Erhan, 2017; Rusu, 2020).

Monitoring ectoparasite fauna provides valuable information for the development of effective control and prevention strategies. Accurate identification of ectoparasite species, assessment of infestation prevalence and intensity, and analysis of seasonal dynamics are essential components of integrated parasite management programs. Such data allow

veterinary specialists to tailor control measures to local conditions, optimize treatment timing, and reduce reliance on chemical control methods. Furthermore, ectoparasitological studies contribute to a better understanding of host–parasite interactions and the ecological factors that influence parasite distribution (Bejsovec, 1972).

In recent years, increasing attention has been directed toward sustainable and environmentally friendly approaches to parasite control. Integrated ectoparasite management strategies combine chemical, biological, and ecological methods, emphasizing prevention, improved hygiene, habitat management, and host resistance. The use of biological products, plant-based insecticides, and natural repellents has gained interest as alternatives to conventional synthetic compounds. These approaches aim to minimize negative environmental impacts, reduce the risk of resistance development, and ensure food safety by avoiding chemical residues in meat and eggs. For domestic waterfowl, which are often raised in close association with natural ecosystems, such strategies are particularly relevant (Olteanu et al., 1993; Erhan et al., 2001; Rusu et al., 2023).

Given the veterinary, economic, and public health significance of ectoparasitic infestations, comprehensive studies on ectoparasite fauna are essential for improving the health and productivity of domestic waterfowl. Detailed knowledge of ectoparasite diversity and epidemiology supports the development of evidence-based control programs and contributes to the sustainability of waterfowl production systems. Moreover, such research aligns with modern requirements for animal welfare, environmental protection, and food safety (Erhan, 2024; Rusu et al., 2020).

The present study aims to investigate the ectoparasite fauna of domestic waterfowl, focusing on ducks and geese reared under different management conditions. By identifying the main ectoparasite species, assessing their prevalence and distribution, and analyzing the factors influencing infestation, this research seeks to provide a scientific basis for improved ectoparasite control and prevention. The results are expected to contribute to a better understanding of ectoparasitism in domestic waterfowl and to support the development of effective, sustainable strategies for enhancing bird health, productivity, and biosecurity (Rusu, 2021a).

The study of ectoparasite fauna in domestic waterfowl is of major scientific, veterinary, and socio-economic importance, as it addresses a complex problem situated at the intersection of animal health, public health, and environmental sustainability. Domestic ducks and geese are frequently reared in semi-intensive and extensive systems, often in close contact with natural aquatic ecosystems, which creates favorable conditions for the persistence and transmission of ectoparasites. Despite this, ectoparasitoses in waterfowl remain insufficiently studied compared to those affecting terrestrial poultry, leading to gaps in knowledge regarding parasite diversity, epidemiology, and control strategies (Olteanu et al., 2001; Şuteu & Cozma, 2003).

From a veterinary health perspective, ectoparasites significantly affect the welfare and productivity of domestic waterfowl. By causing chronic stress, blood loss, skin lesions, and feather damage, ectoparasitic infestations reduce growth performance, feed efficiency, reproductive capacity, and overall resistance to disease. The identification and characterization of ectoparasite species provide essential information for understanding infestation dynamics and for developing targeted, species-specific control measures. Consequently, this study contributes to improving diagnostic accuracy and enhancing the effectiveness of prophylactic and therapeutic interventions (Olteanu et al., 1999; Erhan, 2024; Rusu et al., 2011).

The epidemiological relevance of the study is closely linked to the role of ectoparasites as vectors of infectious agents. Many ectoparasites are capable of transmitting bacterial, viral, and protozoan pathogens, some of which have zoonotic potential. Domestic waterfowl may serve as a bridge between wild avifauna and human-managed environments, facilitating the circulation of pathogens across ecological boundaries. By analyzing ectoparasite fauna and infestation patterns, the study supports the early identification of potential epidemiological threats and strengthens surveillance systems aimed at reducing zoonotic and epizootic risks.

From an ecological standpoint, the research provides valuable insights into host-parasite-environment interactions within anthropized aquatic ecosystems. Ectoparasite populations are strongly influenced by climatic factors, habitat characteristics, and host behavior. Understanding these relationships is essential for predicting changes in parasite distribution under conditions of environmental alteration, climate variability, and intensified agricultural activity. The results of this study may therefore contribute to ecological risk assessments and to the development of adaptive management strategies in waterfowl production systems (Goldova, 2006).

The economic impact of ectoparasitic infestations further underscores the importance of this research. Reduced productivity, increased veterinary costs, and losses associated with poor carcass and feather quality represent significant financial burdens for poultry producers. In traditional and small-scale farming systems, these losses can threaten the viability of waterfowl production. By providing data on ectoparasite prevalence and risk factors, the study supports the optimization of control measures, helping to reduce economic losses and improve farm profitability (Olteanu et al., 1999; Rusu, 2021a).

In the context of food safety and consumer protection, the study has additional significance. The increasing demand for residue-free animal products has highlighted the limitations of intensive chemical control of ectoparasites. Excessive use of insecticides and acaricides may lead to environmental contamination and the accumulation of chemical residues in meat and eggs. The findings of this research can inform the development of integrated and sustainable ectoparasite management strategies, reducing dependence on chemical treatments and supporting the production of safe, high-quality food products (Olteanu, 1991; Erhan, 2020).

Finally, the study holds practical importance for veterinary practice and policy development. By generating region-specific data on ectoparasite fauna in domestic waterfowl, it provides a scientific basis for the formulation of guidelines, recommendations, and biosecurity measures adapted to local conditions. Such evidence-based approaches are essential for improving animal health management, enhancing biosecurity, and ensuring the long-term sustainability of waterfowl farming (Didă, 1996; Olteanu et al., 2001; Gherman et al., 2003).

In summary, the impact of this study extends beyond the descriptive characterization of ectoparasite fauna. It contributes to advancing scientific knowledge, improving veterinary and public health, supporting sustainable agricultural practices, and strengthening food safety. Through its multidisciplinary relevance, the research addresses current challenges in waterfowl production and provides a foundation for future studies and innovative control strategies (Rusu, 2021b; Erhan, 2024).

MATERIALS AND METHODS

Research aimed at establishing the ectoparasite fauna of domestic waterfowl from various anthropized biotopes in the northern region of the Republic of Moldova was conducted during 2024–2025 at the Laboratory of Parasitology and Helminthology of the Institute of Zoology, Moldova State University. Biological material was collected from domestic waterfowl raised in private households in different localities of the northern region, within anthropogenic ecosystems. The study focused on determining the extent and intensity of ectoparasitic infestations in ducks and geese maintained under traditional rearing conditions.

A total of 460 birds were examined, including 240 domestic ducks (*Anas platyrhynchos domesticus*, Linnaeus, 1758) and 220 domestic geese (*Anser anser domesticus*, Linnaeus, 1758). The birds were kept in extensive or semi-intensive systems, with access to outdoor environments and natural water sources. Sampling followed a cross-sectional design, with birds randomly selected from different households. Biological samples were collected at various times of day (morning, midday, and evening) to ensure comprehensive detection of ectoparasites.

Several methods for collecting ectoparasites from birds are described in the scientific literature. The classical manual collection method from dead or hunted animals (Dubinina, 1971) involves mechanically removing ectoparasites using forceps and a scalpel. However, this method is labor-intensive, time-consuming, and does not ensure complete

collection, as many parasites leave the host shortly after death. It is also unsuitable for rare or protected species whose capture or hunting is prohibited.

Other approaches include the prophylaxis and treatment of ectoparasites using plant-based preparations, such as Ectogalimol (short-term invention patent MD 408 Z, 31.03.2012), applied in poultry (Rusu et al., 2011).

The method described by Luncașu and Zamornea (2007) uses ether as an immobilizing agent for live birds. While effective, it has significant disadvantages, including high toxicity, rapid volatilization, and risk to both the operator and the bird, as well as potential loss of collected material in field conditions.

In the present study, a modern biological method, developed and optimized at the Laboratory of Parasitology and Helminthology (Rusu et al., 2021b), was employed. This method allows for efficient collection of ectoparasites from live birds without harming either the parasites or the host. It involves spraying the bird with a 5.0% aqueous solution of Ectogalimol, administered at a dose of 50 ml per bird. Ectogalimol is a biologically active plant extract obtained from the dried aerial parts of *Pyrethrum cinerariifolium*, with proven insecticidal and acaricidal properties.

After spraying, the bird is placed in a nylon bag of appropriate size (20-25 × 30-35 cm or 30-35 × 40-55 cm, depending on body size), with the opening secured around the neck while leaving the eyes and beak exposed. The bird is held horizontally on a flat surface for 5-10 minutes until ectoparasites are immobilized. The bird is then removed, and the ectoparasites are shaken into a white plastic container to avoid material loss.

Collected ectoparasites were transferred to individually labeled test tubes containing 70% rectified ethyl alcohol for preservation. Labels included bird species, date of collection, locality, and the name of the collector.

Ectoparasites were identified in the laboratory using stereomicroscopy and standard taxonomic keys, based on morphological characteristics, at the species level where possible.

Ectoparasites targeted by Ectogalimol include chewing lice (*Cuclotogaster heterographus, Eomenacanthus stramineus, Goniocotes gallinae, Goniocotes maculatus, Goniodes dissimilis, Lipeurus caponis, Menopon gallinae, Menacanthus cornutus, Menacanthus pallidulus*), fleas (*Ceratophyllus gallinae, C. hirundinis*), and gamasid mites (*Dermanyssus gallinae, D. hirundinis*). Experimental studies conducted between 2016 and 2019 demonstrated up to 100% immobilization of ectoparasites within 5-10 minutes, without adverse effects on birds or personnel. These results established the 5.0% concentration of Ectogalimol for use in this study.

Parasitological indices were calculated to evaluate infestation levels, including prevalence (percentage of infested birds), mean intensity (average number of parasites per infested host), and relative abundance. Data were statistically analyzed to describe the structure and distribution of ectoparasite communities.

The method proved to be safe, efficient, and well-adapted for field conditions, ensuring complete collection of ectoparasites while preserving their integrity and posing no risk to birds or operators.

RESULTS AND DISCUSSIONS

As a result of parasitological studies conducted over several years on the ectoparasite fauna, it was established that both domestic and wild birds, as well as mammals, are infested with a wide range of parasitic agents.

Thus, approximately 300 species of mallophagans were identified, belonging to the orders Amblycera and Ischnocera (families Menoponidae and Philopteridae), as well as two species of fleas and two species of gamasid mites. In domestic birds from the Republic of Moldova, 18 species of mallophagans, 2 species of fleas, and 2 species of parasitiform mites had previously been recorded (Luncașu et al., 2007), which confirms the high diversity of the avian ectoparasite fauna.

Parasitological research carried out in 2024 2025 on domestic waterfowl – ducks (*Anas platyrhynchos domesticus*) and geese (*Anser anser domesticus*) – from the northern region of the Republic of Moldova revealed a high level of ectoparasite infestation. In total, 11 species of ectoparasites were identified, belonging to the classes Insecta and Arachnida, classified into 6 families (Trinotonidae, Philopteridae, Menoponidae, Lipeuridae, Dermanyssidae,

Argasidae) and 7 genera (Anatoecus, Anaticola, Trinoton, Menopon, Lipeurus, Dermanyssus, Argas) (Table 1).

Table 1. Taxonomic structure of ectoparasite species identified in ducks and geese

No.	Parasite species	Family	Genus
1.	*Anatoecus icterodes* (Nitzsch, 1818)	Philopteridae	Anatoecus
2.	*Anatoecus dentatus* (Scopoli,1773)	Philopteridae	Anatoecus
3.	*Anaticola crassicornis* (Scopoli, 1763)	Philopteridae	Anaticola
4.	*Anaticola anserinum* (Linnaeus, 1758)	Philopteridae	Anaticola
5.	*Trinoton querquedulae* (Linnaeus, 1758)	Trinotonidae	Trinoton
6.	*Trinoton anserinum* (Fabricius, 1805)	Trinotonidae	Trinoton
7.	*Menopon obscurum* (Piaget, 1880)	Menoponidae	Menopon
8.	*Lipeurus caponis* (Linneus, 1758)	Lipeuridae	Lipeurus
9	*Anatoecus adustus* (Nitzsch in Giebel,1874)	Philopteridae	Anatoecus
10.	*Dermanyssus gallinae* (De Geer, 1778)	Dermanyssidae	Dermanyssus
11	*Argas persicus* (Oken,1818)	Argasidae	Argas

In domestic ducks (*Anas platyrhynchos domesticus*, Linnaeus, 1758), six species of mallophagans and one species of gamasid mites were identified. According to the synthesized data and correlated with the study summary, the most frequent mallophagan species were: *Anaticola crassicornis* – EI 26.0%, *Anatoecus dentatus* – EI 22.0%, *Anaticola anserinum* – EI 16.0%, *Menopon obscurum* – EI 13.0%, *Anatoecus icterodes* – EI 11.0%, *Trinoton querquedulae* – EI 1.0%.

In addition, the presence of the gamasid mite *Dermanyssus gallinae* was recorded, with an infestation extent (EI) of 21.0%. The highest infestation levels were recorded for *Anaticola crassicornis* and *Anatoecus dentatus*, while *Trinoton querquedulae* and *Anatoecus icterodes* showed the lowest values (Figure 1).

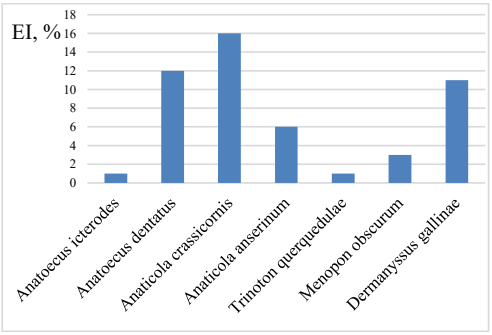


Figure 1. Extent of ectoparasite infestation in ducks (*Anas platyrhynchos domesticus*)

Mixed invasions consisting of two species (*Anaticola crassicornis* + *Trinoton querquedulae*; *Anatoecus dentatus* + *Menopon obscurum*; *Anatoecus icterodes* + *Anaticola anserinum*; *Dermanyssus gallinae* + *Anatoecus dentatus*; *Trinoton querquedulae* + *Anatoecus dentatus*) were recorded in 47.0% of cases.

Three-species parasitic agent combinations (*Anaticola crassicornis* + *Dermanyssus gallinae* + *Anatoecus icterodes*; *Menopon obscurum* + *Anatoecus dentatus* + *A. crassicornis*; *Anatoecus dentatus* + *Anaticola anserinum* + *Trinoton querquedulae*; *Dermanyssus gallinae* + *Anatoecus dentatus* + *Menopon obscurum*) were recorded in 26.0% of cases; four-species combinations (*Trinoton querquedulae* + *Dermanyssus gallinae* + *Menopon obscurum* + *Anatoecus dentatus*; *Trinoton querquedulae* + *Dermanyssus gallinae* + *Anaticola crassicornis* + *Anatoecus dentatus*) were recorded in 5 cases.

In ducks (*Anas platyrhynchos* L.), it was found that 26.4% were infested as mono-invasions, while 73.6% were infested as poly-invasions. Polyparasitic associations consisting of two parasite species were estimated in 44.5% of cases; three-species associations in 24.3% of cases; and four-species associations in 4.8% of cases (Figure 2).

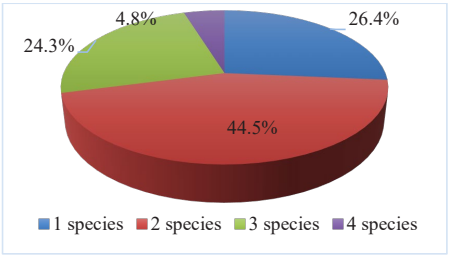


Figure 2. Ectoparasite associations in ducks (*Anas platyrhynchos* L.) from the Republic of Moldova

In domestic geese (*Anser anser domesticus*, Linnaeus, 1758), a higher ectoparasite diversity was observed compared to ducks. Ten ectoparasite species were identified, including eight species of mallophagans, one species of gamasid mites, and one species of argasid ticks. The most frequent mallophagan species in geese were: *Anaticola anserinum* – EI 36.0%, *Anatoecus icterodes* – EI 27.0%, *Anaticola crassicornis* – EI 25.0%, *Trinoton querquedulae* – EI 23.0%, *Lipeurus caponis* – EI 20.0%, *Menopon obscurum* – EI 18.0%, *Trinoton anserinum* – EI 17.0%, *Anatoecus adustus* – EI 14.0%. Additionally, infestation with *Dermanyssus gallinae* (24.0%) and the argasid tick *Argas persicus* (22.0%) was recorded (Figure 3).

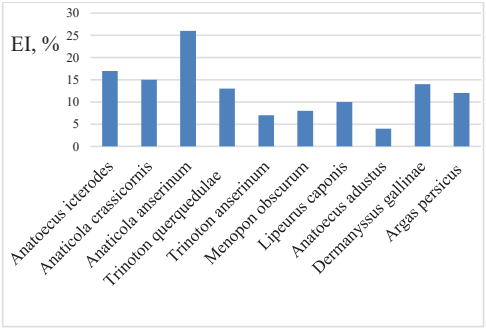


Figure 3. Extent of ectoparasite infestation in geese (*Anser anser domesticus*)

Mixed invasions consisting of two species were recorded in 64.0% of cases: mallophagans (*Trinoton querquedulae* + *Anatoecus icterodes*; *Anaticola anserinum* + *Menopon obscurum*; *Trinoton anserinum* + *Dermanyssus gallinae*; *Lipeurus caponis* + *Argas persicus*; *Anaticola crassicornis* + *Anatoecus adustus*).

Ectoparasite associations consisting of three species (*Menopon obscurum* + *Anaticola anserinum* + *Dermanyssus gallinae*; *Trinoton querquedulae* + *Anaticola crassicornis* + *Lipeurus caponis*; *Argas persicus* + *Trinoton querquedulae* + *Anatoecus icterodes*; *Trinoton anserinum* + *Anatoecus adustus* + *Dermanyssus gallinae*) were recorded in 53.0% of cases, while polyinvasions consisting of four ectoparasite species (*Anatoecus adustus* + *Dermanyssus gallinae* + *Menopon obscurum* + *Anaticola anserinum*; *Anaticola crassicornis* + *Lipeurus caponis* + *Argas persicus* + *Menopon obscurum*; *Dermanyssus gallinae* + *Anaticola crassicornis* + *Anatoecus adustus* + *Trinoton anserinum*) were recorded in 7 cases.

In geese (*Anser anser* L.), it was found that 13.9% were infested as mono-invasions, while 86.1% were infested as poly-invasions. Polyparasitic associations consisting of two parasite species were estimated in 44.5% of cases; three-species associations in 36.8% of cases; and four-species associations in 4.8% of cases (Figure 4).

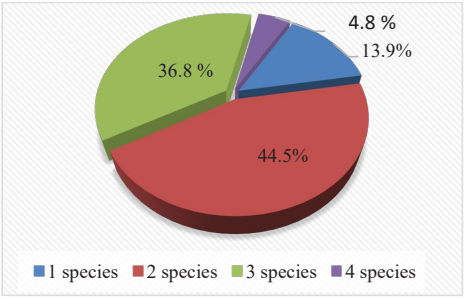


Figure 4. Ectoparasite associations in geese (*Anser anser domesticus*) from the Republic of Moldova

Among the identified species, two mallophagan species stand out: *Lipeurus caponis*, which is common to both domestic and wild gallinaceous birds, and *Trinoton anserinum*, which parasitizes many species of wild waterfowl, including swans and geese. *T. anserinum* also serves as an intermediate host in the life cycle of *Sarconema eurycerca*, a nematode from the family Filarioidea, which causes cardiac infection and subsequently leads to their death.

The gamasid tick *Dermanyssus gallinae* serves as a host for numerous species of wild birds and mammals, including humans. The argasid tick *Argas persicus* identified in geese is hazardous and transmits multiple pathogens (e.g., *Borrelia anserina*) to animals, including humans;

therefore, these parasites pose a significant health risk.

Thus, a high level of infestation of domestic waterfowl with various parasitic agents was established in both natural and anthropogenic ecosystems across different regions of the Republic of Moldova. This further demonstrates that they participate in maintaining the epizootic chain of these diseases and play an important role in the infestation of migratory wild birds. Since controlling parasitic agents is difficult and very costly, preventive measures are the only effective and economical approach. At the same time, biocontrol measures can help minimize the spread of parasitic agents.

CONCLUSIONS

The parasitological investigations conducted on domestic waterfowl (*Anas platyrhynchos domesticus* and *Anser anser domesticus*) from the northern region of the Republic of Moldova revealed a high diversity of ectoparasites, confirming the significant parasitological pressure in both natural and anthropized ecosystems.

A total of 11 ectoparasite species belonging to the classes *Insecta* and *Arachnida* were identified, including representatives of the families *Philopteridae, Trinotonidae, Menoponidae, Lipeuridae, Dermanyssidae,* and *Argasidae*, demonstrating the complex taxonomic structure of ectoparasite fauna in domestic waterfowl.

Domestic geese showed a higher diversity and prevalence of ectoparasitic infestations compared to domestic ducks, which may be associated with species-specific biological traits, rearing practices, and increased contact with aquatic environments.

Among ducks, the highest infestation rates were recorded for the mallophagan species *Anaticola crassicornis* and *Anatoecus dentatus*, while *Trinoton querquedulae* and *Anatoecus icterodes* exhibited the lowest prevalence.

In geese, the dominant ectoparasite species were *Anaticola anserinum, Anatoecus icterodes,* and *Anaticola crassicornis*, alongside a considerable presence of the gamasid mite *Dermanyssus gallinae* and the argasid tick *Argas persicus*, both of which have high epidemiological relevance.

Polyparasitic infestations were predominant in both host species, particularly in geese, indicating frequent associations of two or more ectoparasite species and highlighting the complexity of host–parasite interactions in domestic waterfowl.

The presence of ectoparasite species with zoonotic and vector potential, such as *Dermanyssus gallinae* and *Argas persicus*, emphasizes the veterinary and public health significance of monitoring ectoparasite fauna in domestic waterfowl.

The high infestation levels observed underline the necessity of implementing effective prophylactic, monitoring, and biocontrol strategies aimed at reducing ectoparasite prevalence, improving animal welfare, and limiting the circulation of parasitic agents within anthropized ecosystems.

ACKNOWLEDGEMENTS

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MORPHOLOGICAL AND MERISTIC ANALYSIS OF *Macrobrachium nipponense* POPULATIONS: IMPLICATIONS FOR ECOLOGICAL STABILITY IN LENTIC HABITATS OF THE DANUBE DELTA

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Abstract

This paper analyzes the morphometric and morphological characteristics of the alien species Macrobrachium nipponense (De Haan, 1849) in the Danube Delta ecosystem. The study was conducted on a representative sample of specimens collected from Matița-Merhei lake complex of the Danube Delta Biosphere Reserve, aiming to assess phenotypic variability in the context of acclimatization to local hydro-climatic conditions. The methodology involved measuring 15 morphometric parameters, including total length, carapace length, and claw dimensions, as well as examining the qualitative characteristics of the rostrum The meristic analysis focused on the variability of the rostral formula and pereiopod segmentation, confirming a high adaptive plasticity of the species in lentic habitats. The analysis of morphometric correlations indicates a plastic adaptation of populations to the thermal and hydrological regime of the Danube Delta, confirming the high competitive potential of the species compared to native crustaceans. The data obtained provide essential information for monitoring the population dynamics of this oriental shrimp, contributing to the understanding of the ecological impact of allochthonous species on benthic biodiversity in the region.

Key words*: freshwater, interspecific competition, Matița-Merhei, meristic traits, shrimp.*

INTRODUCTION

Macrobrachium nipponense (De Haan, 1849), commonly known as the oriental river prawn, is a freshwater decapod native to the subtropical and temperate regions of East Asia, including China, Japan, Korea, and Taiwan, where it inhabits a variety of environments ranging from rivers and lakes to low-salinity estuaries (Chen et al., 2015a; Jin et al., 2023). In its native range, the species is characterized by its significant role in benthic food webs and its high commercial value for aquaculture, being well-adapted to fluctuating seasonal temperatures and varying dissolved oxygen levels (Wang et al., 2022).

However, the expansion of non-native species in aquatic ecosystems represents one of the most significant challenges to global biodiversity, carrying the potential to alter the structure of benthic communities and local trophic interactions. In this context, decapod crustaceans, particularly those within the Palaemonidae family, have gained attention for their remarkable invasive capabilities, facilitated by high ecological tolerance and significant dispersal capacity (Ashelby et al., 2013; De Carvalho-Souza et al., 2025).

As a globally successful invader, *M. nipponense* has expanded its range into numerous aquatic systems across Iran (De Grave & Ghane, 2006) and, more recently, Europe, where it continues to spread upstream into countries like Germany and Hungary (Bláha et al., 2025). Recent studies indicate that in newly colonized habitats, such as the Danube Delta, individuals can even exceed the sizes recorded in their native range,

suggesting a high adaptive plasticity (Lepekha et al., 2025). Its success is attributed to a strategic combination of structural stability in its body plan and pronounced phenotypic plasticity—traits that enable it to cope with environmental variability (Ashelby et al., 2013; Chen et al., 2015b).

In the Danube basin, the presence of *M. nipponense* has shifted from isolated reports to a widespread phenomenon, becoming a common element of the local benthic fauna (Zhmud et al., 2021). The species has demonstrated high invasive potential by establishing viable populations in freshwater systems across the lower Danube and Dniester (Nekrasova et al., 2024; Bushuiev et al., 2023). This expansion, which includes the first official record in Romania (Surugiu, 2022) and recent confirmations in the Bulgarian sector (Kutsarov et al., 2025), transforms the Danube River into a critical invasion corridor where the species may directly compete with native fauna.

Morphometric analysis and allometric growth models are essential tools for understanding the adaptation of this species to new ecosystems (Chen et al., 2015b). Differentiating between size-related (structurally integrated) and shape-related (functionally flexible) traits provides valuable insights into energy allocation strategies and sexual maturation (Jin et al., 2023). Furthermore, pronounced sexual dimorphism influences population dynamics and the capacity for persistence in non-native habitats (Jin et al., 2023; Wang et al., 2022).

The present study aims to characterize the morphological structure of an *M. nipponense* population collected from the Matița-Merhei complex (Danube Delta), evaluating the stability of biometric correlations and the variability of functional traits as indicators of adaptive success in this specific deltaic environment.

MATERIALS AND METHODS

Sampling and data collection. A total of 45 individuals of *Macrobrachium nipponense* were collected from lentic habitats within the Danube Delta- Matița Merhei Lake Complex, Miazăzi lake (Figure 1). Surface area estimates were derived using geospatial analysis based on publicly available cartographic data.

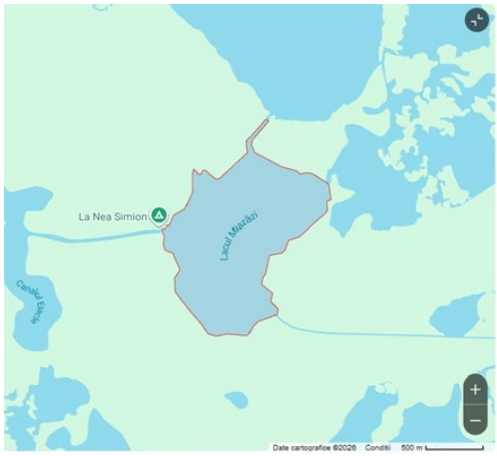


Figure 1. Map with a description of the research area (https://maps.app.goo.gl/5MEdZbMeHJwAD84y5)

Sampling was conducted during the pre-reproductive period (March-April), when individuals undergo gonadal development but prior to active spawning. In temperate regions, the reproductive period of the species typically extends from May to September (Jin et al., 2022). During the pre-reproductive stage, body weight may increase due to energy allocation toward gonadal development, while lacking the additional variability associated with ovigerous females (Hartnoll, 1982; Sastry, 1983).

Sampling was performed using baited crayfish traps (length: 0.40-3.00 m; diameter: 30-50 cm; mesh size: 10 mm; wire thickness: 1.5 mm), ensuring minimal selectivity bias across size classes. All specimens were preserved and transported to the laboratory for subsequent analyses. Sampling and handling procedures followed standard practices for invertebrate research.

Morphological measurements. Each specimen was photographed using a stereomicroscope (OZP 558, KERN & SOHN GmbH, Balingen, Germany) at 40× magnification, equipped with a digital imaging system (ODC 832, KERN & SOHN GmbH, Balingen, Germany). Images were processed and measured using MicroscopeVIS 2.0 PRO software (x64 version), compatible with the imaging system (Figure 2).

The following variables were recorded: Total length (TL, mm), Carapace length (CL, mm), Rostrum length (RL, mm), Carapace width (CW, mm), Body weight (W, g) (Kawamata &

Taino, 2018). Additionally, proportional indices were calculated to describe body shape: CL/TL, RL/CL, CW/CL.



Figure 2. Morphometric measurements of *Macrobrachium nipponense* based on digital image analysis (Source: original)

All measurements were performed using a digital caliper (precision ±0.01 mm) and an electronic balance (precision ±0.01 g). Measurements were performed on calibrated images using integrated measurement software. Values displayed in the figure are expressed in pixels (px) and were subsequently converted to millimeters (mm) based on image calibration (1 mm ≈ 44 px).

Sample size was constrained by field conditions but considered sufficient for multivariate statistical analysis.

Ethical approval. All sampling and handling procedures were conducted in accordance with standard ethical practices for invertebrate research and in line with the general principles of Directive 2010/63/EU. The measurement activities were supervised by the Bioethics Commission of the Research and Development Institute for Aquatic Ecology, Fishing and Aquaculture, Galați (Decision no. 52/16.03.2023).

Descriptive statistics. Descriptive statistics were calculated for all variables, including mean, standard deviation (SD), standard error of the mean (SEM), minimum and maximum values, and coefficient of variation (CV). These parameters were used to characterize the variability and structural organization of the population.

Data distribution and quality assessment. The presence of outliers was assessed using the Grubbs test, while the normality of data distribution was evaluated using the Shapiro-Wilk test ($\alpha = 0.05$). These analyses ensured the reliability of subsequent statistical procedures and informed the selection of appropriate analytical methods.

Frequency distribution analysis. Frequency distributions for total length (TL), carapace length (CL), and body weight (W) were constructed using grouped class intervals to evaluate the size structure and population homogeneity.

Sex-related analysis. Sex was determined for each individual, and the sex ratio (F:M) was calculated. A chi-square (χ^2) test was applied to assess deviations from the expected 1:1 ratio. Differences between sexes in morphological variables were tested using the Mann-Whitney U test. The analysis focused on both size-related variables (TL, CL, W) and proportional indices (CL/TL, RL/CL, CW/CL), allowing the identification of potential sexual dimorphism.

Length-weight and allometric relationships. The relationship between total length and body weight was described using a power function: $W = a \cdot TL^b$, where a is the intercept and b is the allometric coefficient (Ricker, 1975; Froese, 2006; Ko, 2025).

Allometric relationships between morphological traits (e.g., TL–CL, CL–CW, CL–RL) were analyzed using log-transformed linear regressions (Wang et al., 2022). The value of b was used to determine growth patterns (isometric, positive or negative allometry).

Correlation analysis. Relationships between morphological variables were assessed using Pearson correlation coefficients. This approach was selected based on the approximate normal distribution of the data and the expected linear relationships among variables.

Principal component analysis (PCA). Principal component analysis (PCA) was performed on standardized variables using a correlation matrix (Maćkiewicz & Ratajczak, 1993). The aim was to reduce data dimensionality and identify the main axes of morphological variation.

Only the first two principal components (PC1 and PC2) were retained for interpretation, based on their high cumulative explained variance. Factor loadings were used to interpret the contribution of each variable, while individual scores were used to visualize population structure through a PCA biplot.

All statistical analyses were performed using XLSTAT Essentials (Addinsoft, 2026.1.0 version), integrated within Microsoft Excel.

RESULTS AND DISCUSSIONS

A total of 45 individuals of *Macrobrachium nipponense* were collected during the study period, with a cumulative biomass of 167.27 g. Descriptive statistics of the analyzed morphological variables are presented in Table 1.

Table 1. Descriptive statistics of morphological variables of Macrobrachium nipponense populations

Variable	N	Mean ± SEM	Min.–Max.	CV (%)
TL (mm)	45	77.80±1.61	59.56-99.14	13.88
CL (mm)	45	32.01±0.61	22.67-40.09	12.76
RL (mm)	45	21.97±0.68	11.22-30.11	20.77
CW (mm)	45	3.67±0.10	1.79-4.95	17.99
W (g)	45	3.72±0.24	1.19-7.86	43.37
CL/TL	45	0.41±0.004	0.38-0.48	6.01
RL/CL	45	0.69±0.02	0.31-0.96	18.41
CW/CL	45	0.11±0.002	0.08-0.14	13.99

Values are presented as mean ± standard error of the mean (SEM), with minimum and maximum values and coefficient of variation (CV).

The absence of significant outliers (Grubbs test, $p > 0.05$) indicates a homogeneous dataset, supporting the reliability of subsequent analyses. Similarly, the Shapiro–Wilk test confirmed that all variables did not significantly deviate from normality ($p > 0.05$), justifying the use of parametric approaches for further analyses, although the value observed for TL ($p = 0.058$) suggests slight heterogeneity in size distribution.

The population exhibits a heterogeneous pattern of variability across traits, reflecting differences in biological control and functional significance. Body weight (W) showed the highest variability (CV = 43.37%), a result that is biologically expected, as this parameter is strongly influenced by short-term physiological processes such as feeding intensity, energy reserves, and reproductive status. In crustaceans, fluctuations in gonadal development can significantly affect body mass independently of structural dimensions, contributing to the observed dispersion. In *M. nipponense*, rapid gonadal development and early sexual maturation can significantly influence growth dynamics and energy allocation, contributing to variability in body weight and condition (Jin et al., 2022).

Rostrum length (RL) also displayed relatively high variability (CV = 20.77%), suggesting a degree of phenotypic plasticity in this trait. Given its functional role in feeding, defense, and environmental interaction, variability in RL may reflect adaptive flexibility, potentially allowing individuals to exploit a wider range of ecological niches. In invasive species, increased variability in functionally relevant structures is often associated with enhanced ecological performance in novel environments.

In contrast, the CL/TL ratio exhibited very low variability (CV ≈ 5%), indicating a high level of morphological stability in body proportions. This suggests strong developmental or genetic control over the relative growth of the cephalothorax and total body length. Such stability in core structural proportions is essential for maintaining biomechanical efficiency and overall organismal functionality.

The coexistence of highly variable traits (e.g., weight, rostrum length) and highly stable proportions (e.g., CL/TL) highlights a dual pattern of morphological organization: structural stability combined with functional flexibility. This pattern is particularly relevant in the context of invasive species, where the maintenance of a stable body plan, coupled with variability in ecologically relevant traits, may contribute to successful establishment and persistence in new environments.

The successful establishment of *M. nipponense* in non-native habitats has been attributed to its combination of structural stability and phenotypic plasticity, traits that enhance its ability to cope with environmental variability (Ashelby et al., 2013). The recent expansion of *M. nipponense* in the Lower Danube, including its first record in Romania, provides a relevant ecological context for interpreting the morphological patterns observed in this study, as such traits may facilitate its successful establishment and persistence in newly colonized environments (Surugiu, 2022). This pattern is consistent with the global spread of

Macrobrachium nipponense, which has been increasingly reported as a non-native species in various aquatic systems, facilitated by its high ecological tolerance and dispersal capacity (De Carvalho-Souza et al., 2025).

The size structure of the population further supports this interpretation. Total length (TL) and carapace length (CL) exhibited approximately symmetrical distributions, as indicated by low skewness values (TL: 0.18; CL: −0.19) and slightly negative kurtosis. This suggests a relatively homogeneous population structure, without the dominance of a single cohort. In contrast, body weight (W) showed moderate positive skewness (0.63), reflecting a higher frequency of individuals with lower mass and fewer high-weight specimens. This divergence between length-based and weight-based distributions indicates a partial decoupling between structural growth and energetic condition.

The frequency distributions of total length (TL), carapace length (CL), and body weight (W) confirm the observed structural patterns (Figure 3). Both TL and CL exhibit unimodal distributions, with most individuals concentrated in intermediate size classes (approximately 73-80 mm for TL and 31-34 mm for CL), indicating a relatively homogeneous and structurally coherent population.

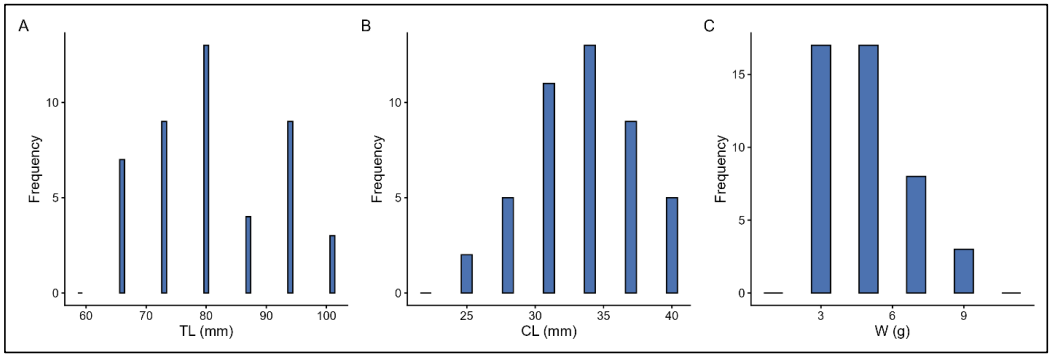


Figure 3. Frequency distributions based on grouped class intervals

In contrast, body weight (W) shows a more uneven distribution, with a concentration in lower and intermediate classes (3-6 g) and a gradual decline toward higher values, suggesting variability in physiological condition. This pattern is consistent with flexible energy allocation among individuals.

The divergence between the relatively compact length distributions and the broader, skewed weight distribution indicates a decoupling between structural growth and energetic status. While body dimensions (TL and CL) appear tightly regulated, body mass varies more substantially, likely reflecting differences in feeding efficiency, metabolic reserves, or reproductive stage. Such a pattern is characteristic of populations with flexible energy allocation strategies and may be particularly advantageous in invasive species, allowing individuals to maintain structural integrity while adjusting physiological condition in response to environmental variability.

Morphometric variability observed in this study is consistent with previous findings showing that *Macrobrachium nipponense* exhibits significant morphological differentiation among populations, likely driven by environmental conditions and local adaptation (Chen et al., 2015b).

Sex-related analyses revealed a balanced sex ratio (F:M = 1.25:1), which did not differ significantly from the expected 1:1 distribution (χ^2 test, $p > 0.05$). However, significant differences were observed between sexes in size-related traits. Males exhibited significantly higher values of total length (84.22 ± 10.27 mm vs. 72.67 ± 8.29 mm; $p = 0.001$) and body weight (4.59 ± 1.72 g vs. 3.02 ± 1.14 g; $p = 0.001$), indicating a pronounced sexual dimorphism. The absence of differences in sex ratio, coupled with significant size differences, suggests that this dimorphism reflects intrinsic biological patterns rather than sampling bias.

These results indicate a clear sexual dimorphism in overall body size, with males exhibiting significantly larger size and higher weight compared to females. The absence of deviation in sex ratio suggests that this pattern is not related to sampling bias but reflects intrinsic biological differences between sexes. This finding is consistent with previous studies reporting faster growth rates and larger body size in male *Macrobrachium nipponense*, driven by differences in reproductive physiology and hormonal regulation (Jin et al., 2022; Chen et al., 2015b).

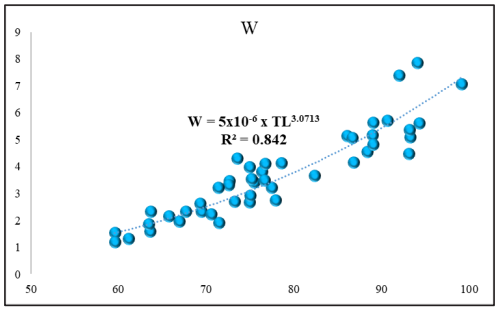


Figure 4. Length–weight relationship for *M. nipponense*

The length–weight relationship was best described by a power function ($W = 5 \times 10^{-6} \cdot TL^{3.0713}$; $R^2 = 0.842$), indicating a strong positive association between size and mass. The allometric coefficient (b = 3.07) suggests slightly positive allometric growth, where weight increases at a marginally higher rate than length (Figure 4).

This pattern reflects efficient energy accumulation and may be associated with favorable environmental conditions or adaptive life-history strategies.

Further analysis of allometric relationships revealed that the TL–CL relationship (Figure 5) follows a negative allometric pattern (b = 0.856; $R^2 = 0.817$), indicating that total length increases faster than carapace length, resulting in body elongation with growth. The CL–CW relationship showed moderate association ($R^2 = 0.416$) and negative allometry (b = 0.447), supporting a consistent elongation trend within a relatively stable structural framework. In contrast, the CL–RL relationship was weak ($R^2 = 0.174$) and characterized by a low allometric exponent (b = 0.226), indicating high variability and reduced structural constraint.

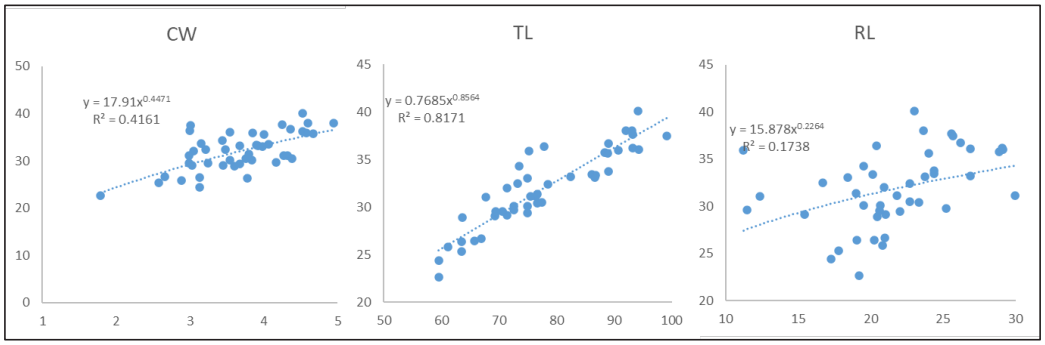


Figure 5. Patterns of Morphological Allometry and Structural Variability in *Macrobrachium nipponense*

Such differences in growth patterns and body proportions are in agreement with studies demonstrating that allometric relationships and morphometric traits in *M. nipponense* vary across populations and environmental contexts (Chen et al., 2015b).

The Pearson correlation matrix supports these findings (Figure 6), revealing strong positive correlations among total length (TL), carapace length (CL), and body weight (W), with TL–CL (r = 0.903), TL–W (r = 0.919), and CL–W (r = 0.850), indicating that these variables define the core structural framework of the organism. Moderate correlations were observed between TL and carapace width (CW) (r = 0.596), as well as between CW and CL (r = 0.644), suggesting that width increases with size but with greater variability compared to longitudinal growth. In contrast, rostrum length (RL) exhibited weaker correlations with structural variables, particularly with CL (r = 0.461) and CW (r = 0.493), while its association with TL was

moderate (r = 0.657), confirming a lower degree of structural integration and higher variability of this trait.

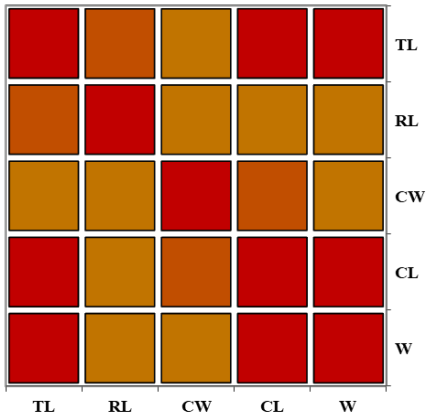


Figure 6. Correlation Matrix of Morphological Variables in *Macrobrachium nipponense*

Overall, these results highlight a clear distinction between tightly integrated morphological traits (TL, CL, W), which define the core body structure, and more variable traits such as RL, which may reflect higher phenotypic plasticity. This combination of strong structural coherence and localized variability may contribute to the adaptive capacity of *Macrobrachium nipponense* in heterogeneous or novel environments.

Principal component analysis (PCA) revealed that the first two components explained 86.04% of the total variance (Figure 7). PC1 (74.16%) was strongly associated with total length (TL), carapace length (CL), and body weight (W), representing an overall body size gradient. PC2 (11.88%) was primarily driven by rostrum length (RL), reflecting variation in body proportions rather than size.

These results indicate that morphological variation in the analyzed population is structured along two main axes: a size-related component characterized by strong integration of core body traits, and a shape-related component associated with increased variability in specific functional structures.

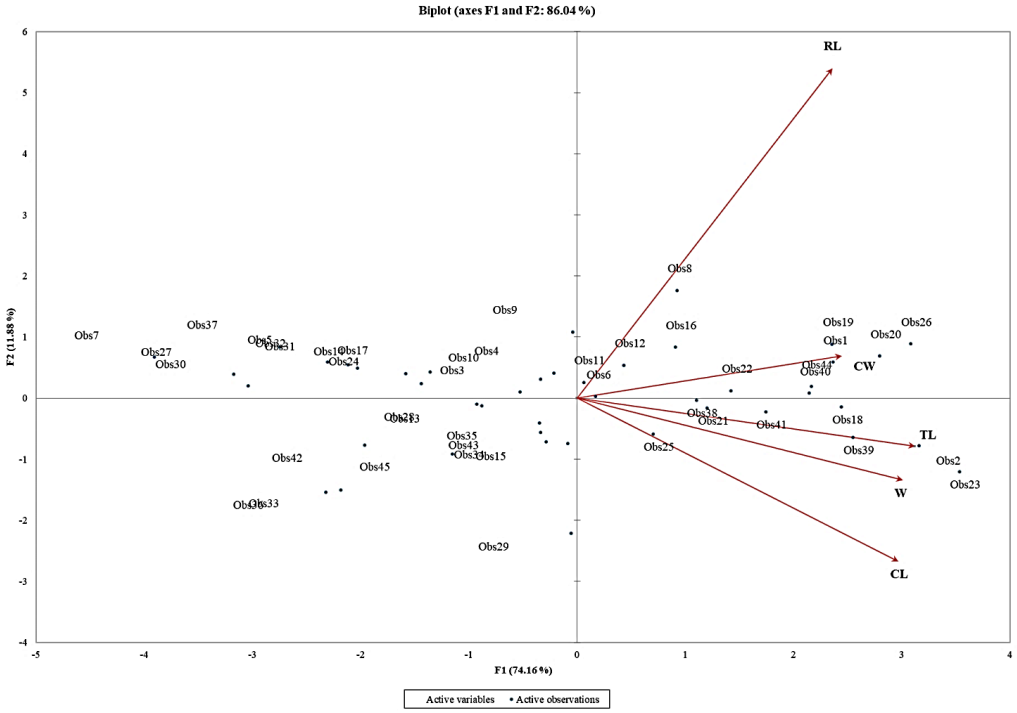


Figure 7. Principal component analysis (PCA) biplot based on standardized morphological variables. Arrows indicate the direction and magnitude of variable contributions, while points represent individual observations (i.e., measured specimens), positioned according to their scores on the first two principal components (PC1 and PC2)

This combination of structural stability and phenotypic plasticity is consistent with patterns reported for invasive populations of *Macrobrachium nipponense*, which exhibit high ecological tolerance and capacity to establish in non-native freshwater systems (Nekrasova et al., 2024).

The differentiation between size-related and shape-related traits identified in this study is consistent with multivariate analyses showing that morphological variation in *M. nipponense* reflects both structural integration and environmental adaptation (Chen et al., 2015b).

From an ecological perspective, this pattern is highly relevant, as it indicates that the species maintains a stable body structure, essential for movement, growth, and survival, while allowing flexibility in specific traits that interact directly with the environment. For instance, variability in rostrum length may reflect differences in feeding strategies or habitat use. This combination of structural stability (size-related traits) and phenotypic flexibility (shape-related traits) is characteristic of species capable of adapting to a wide range of environmental conditions and may partly explain the ability of *Macrobrachium nipponense* to establish and persist in non-native habitats.

Such adaptive capacity has been associated with traits including rapid reproductive development and flexible energy allocation, which enhance population growth and persistence under favorable conditions (Chen et al., 2015a; Jin et al., 2022). In addition, morphometric variation in this species has been linked to environmental conditions and habitat type, suggesting a strong relationship between morphology and ecological adaptation (Chen et al., 2015b).

At a broader scale, the increasing number of records of *M. nipponense* outside its native range indicates an ongoing expansion process, driven by its high ecological tolerance and dispersal capacity (De Carvalho-Souza et al., 2025). In the Danube basin, this trend is already evident (Kutsarov et al., 2025), as the species has shown a high invasive potential, being able to establish viable populations in freshwater systems (Surugiu, 2022). Eastern Europe has been identified as a suitable region for further expansion, particularly under future climate scenarios (Nekrasova et al., 2024).

The establishment of this species may have ecological consequences, including potential impacts on benthic communities and trophic interactions, thereby posing a risk to local biodiversity in the Danube Delta ecosystem. In this context, the Danube River represents an important invasion corridor, and the increasing presence of *M. nipponense* highlights the need for continuous monitoring and assessment of its ecological impact.

In this context, further research is needed to integrate morphological data with environmental variables to better understand the drivers of population variability and expansion. In particular, the relationships between morphological traits and key abiotic factors, such as temperature, dissolved oxygen, and trophic conditions, should be investigated across different habitats in the Danube Delta.

Given the invasive nature of *Macrobrachium nipponense*, priority should be given to assessing its ecological impact, including trophic interactions and potential competition with native species. Complementary approaches, such as stable isotope analysis and eDNA monitoring, may provide valuable insights into its ecological niche and spatial distribution.

From a management perspective, the increasing occurrence of this species highlights the need for early detection and continuous monitoring programs, particularly in ecologically sensitive areas of the Danube Delta. Management efforts should focus on limiting further spread, identifying potential ecological impacts on native communities, and integrating the species into existing biodiversity monitoring frameworks.

CONCLUSIONS

The analyzed population of *Macrobrachium nipponense* exhibits a dual morphological structure, characterized by strong integration of size-related traits and increased variability in functionally relevant features. Significant sexual dimorphism was identified, with males displaying larger body size and higher weight. Allometric relationships indicate differential growth among body components, while PCA confirms the separation between size and shape variation. These characteristics support the species' adaptive capacity and its potential to

establish in non-native environments. Given its increasing occurrence in the Danube Delta, continued monitoring and management strategies are required to assess and mitigate its ecological impact.

These findings support the need for targeted monitoring and management strategies to limit the spread and ecological impact of this invasive species in the Danube Delta.

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