University of Agronomic Sciences
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Engineering and Management

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# SUMMARY

## I. SESSION GENETICS AND BREEDING



## II. SESSION NUTRITION

## III. SESSION REPRODUCTION, PHYSIOLOGY, ANATOMY

## IV. SESSION TECHNOLOGIES OF ANIMAL HUSBANDRY

## V. SESSION TECHNOLOGIES OF THE AGRO FOOD PRODUCTS PROCESSING

## VI. SESSION WILD LIFE MANAGEMENT, FISHERY AND AQUACULTURE

# GENETICS AND BREEDING

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# APPLICATIONS AND PRACTICAL IMPLICATIONS OF ASSISTED REPRODUCTION AND MOLECULAR GENETICS TOOLS IN ACCELERATING GENETIC GAIN IN GOATS

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***Abstract***

*Acceleration of genetic gain for some valuable traits represents for some local animal breeds an upmost necessity, especially when unimproved breeds are in competition with more productive ones. The progress in DNA and assisted reproduction technologies could represent a valuable tool to overcome these limitations. In many cases, valuable DNA mutations can have a low frequency, making the breeding process costly and time consuming. Therefore, artificial insemination can contribute to increase the frequency of these mutations in a population. We tested the viability of this combined approach on a Carpathian goat population. In this respect, the potential Carpathian goat males, candidates for semen collection needed for the artificial insemination experiments, were first selected from the herd book based on their known origin and phenotype. To confirm their parentage, we used a panel of 22 microsatellites markers. On the other hand we genotyped the remained candidate males for the alpha s1 casein gene, which is significantly associated with milk casein content and cheese yield. Only four males, with AA, BB and AB genotypes (positively associated with these traits) were retained for semen collection. A number of 450 Carpathian goat females were prepared for insemination using hormonal induction and synchronization of oestrus with fluorogestone acetate impregnated sponges (Chronogest sponges, with FGA 20 mg). In the 9th day after the sponges insertion, pregnant mare serum gonadotropin PMSG (Folligon) was injected in a dosage of 400 UI. The semen was collected from the selected males using an artificial vagina and was subsequentlyanalysed and processed for insemination. The goats were artificial inseminated in fixed point, 45 ± 2 hours after the sponges removal, with freshly diluted semen that was collected and diluted 2-3 h before insemination. Fecundity calculated after the end of parturition was 84.65%.*

***Key words****: artificial insemination, DNA markers, Carpathian goats.*

## INTRODUCTION

In most European countries, there is an increased demand for high quality goat milk products. France is by far the most important producer of high-quality genetic stock and goat milk products. Saanen and Alpine goats are by far the most productive breeds, which suitable for intensive breeding. However, the main goat population, composed of unimproved local breeds, is located mainly in the Eastern and Southern European countries. Although are less productive, local goats could represent a viable solution to satisfy this increased demand for high quality goat milk products, due to the fact that they valorise a high-quality food from hilly pastures. Moreover, they can survive under harsh environmental conditions with low inputs, as compared with exotic breeds (Chemineau et al., 1997). It was shown that simple improvement of the technological parameters can lead to an increase milk yield and lactation period.

Genomic selection became in some farm species (Ex. cattle) an important tool to predict the breeding value of sires. In small ruminants the process is still very costly and in applied only at small scale and in some breeds with a high commercial value.

However, in some cases, the polymorphism of major genes can have a significant influence on phenotypes or production traits. Therefore, target genotyping of the animals for these specific markers, can have a beneficial influence in acceleration of the genetic gain for these traits. For example, selection of bucks

based on alfa S1 casein (*CNS1S1*) genotypes has already a long history in Alpine and Saanen goat breeds. In goat milk this protein normally represents around 32% from the whole casein fraction. The casein content of goat milk can significantly vary due to an increased polymorphism of *CNS1S1* gene, with at least 20 alleles currently known to date (Marletta et al., 2007; Bâlteanu et al, 2015). They are associated with four different expression levels *i.e.* strong alleles: A, B, C producing 3.5g $\alpha_{S1}$-CN /l; intermediate alleles: E with 1.1g $\alpha_{S1}$-CN /l; weak alleles: F with 0.45g αS1-CN /l and null alleles characterized by the absence of $\alpha_{S1}$-CN in the milk of homozygous animals (Martin et al., 1999). Association studies highlighted a major positive effect of strong expression alleles on milk quality (casein content), rheological properties or cheese yield, as compared with week alleles and it is well documented in various breeds (Delacroix-Buchet, 1996; Caravaca et al., 2009; Yue et al., 2011; Bâlteanu et al., 2012).

When we talk about unimproved local breeds, Romania can be a suggestive example. Currently the goat population accounts for 2 million heads. The majority of these goats (about 90%) are represented by Carpathian breed. Indeed, improving milk production traits by crossing with improved breeds can represent a convenient solution for commercial farms. But, in many cases this is not a solution since the preservation of local genetic stock is essential.

Acceleration of the genetic gain in Carpathian goat, especially for milk production traits, represents an upmost necessity. In Carpathian goat it was shown that the variability in milk casein contents and cheese yield can be significantly affected by a high frequency of defective E and F alleles (Bâlteanu et al., 2012). They have an estimated frequency of around 45% in this breed (Bâlteanu et al., 2015).

Artificial insemination can significantly contribute to increase the frequency of strong expression *CSN1S1* alleles (A or B), with a beneficial effect of milk casein content and cheese yield. We tested the viability of this combined approach in some Carpathian goat populations.

## MATERIALS AND METHODS

*Bucks selection, blood sampling, parentage testing and CSN1S1 genotyping*

The potential Carpathian bucks candidates for semen collection needed for the artificial insemination experiments, were first selected from the Carpathian breed herd book, based on their known origin and phenotype. A number of 36 males between 8 to 40 months of age were first selected from six farms. Blood samples were collected in $K_3$-EDTA coated tubes from jugular vein. Additionally, to verify the parentage, blood samples were collected from their presumed parents.

DNA samples were purified from 100µl whole blood using Quick-gDNA MiniPrep kit and according to the manufacturer instructions (Zymo Research Corporation, USA). The DNA concentration and purity was determined on a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA).

To confirm their parentage we used a panel of 22 microsatellites markers (including the recommended ISAG panel). Amplified multiplex PCR reactions were analysed in Applied Biosystems 3500 device. Parentage verification was done by comparing the size of the generated microsatellites fragments for each marker, using GeneMapper software (Applied Biosystems, USA).

Genotyping of candidate males for the A, B, E or F *CSN1S1* alleles was done by PCR-RFLP and AS-PCR. Two distinct PCR reactions were prepared for each goat.

To discriminate between A, B/E and F, a small polymorphic fragment containing the entire exon 9 and partial regions from introns 8 and 9 was amplified in 25 µl final volume reaction containing 1X Tissue Green PCR Master Mix (Fermentas, Lithuania), 10 pmol of each primer and 50 ng of genomic DNA. The thermal profile was as follows: 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 58 °C for 1 min, 72°C for 1 min and a final extension step of 72°C for 7 min. Amplicons were digested with 10 U of *XmnI* endonuclease (Thermo Scientific, USA) for 15 min at 37°C and analysed on a 3% agarose gel stained in containing 1X GelRed.

To discriminate between E allele from non-E alleles, DNA samples were amplified in the same conditions, but using a specific primer set flanking a region from exon 19th, were a 457 bp LINE element specific to E allele can be inserted or not.

*Oestrus synchronisation, semen collection and artificial insemination*

A number of 450 Carpathian goat females from four distinct farms located Tulcea County were prepared for insemination.

Hormonal induction and synchronization of oestrus was done with fluorogestone acetate impregnated sponges (Chronogest sponges, with FGA 20 mg). In the 11th day after sponges insertion they were removed and pregnant mare serum gonadotropin (Folligon) was injected in a dosage of 400 UI.

Several hours before artificial insemination, the semen of four males, selected based on genetic criteria (parentage and *CSN1S1* genotypes), was collected using artificial vagina. Subsequently the semen was evaluated and prepared for artificial insemination of selected females.

The goats were inseminated in fixed point, 45 ± 2 hours after sponges removal, with freshly diluted semen that was collected and diluted 2-3 h before insemination. The fecundity was calculated after the end of parturition.

## RESULTS AND DISCUSSIONS

Parentage verification of the candidates bucks was performed based on the genotypes obtained for the 22 microsatellites, by comparing them with the genotypes of the presumed parents.

Allelic sizes for some of these markers are highlighted as an example for two presumed families in Table 1.

Table 1. Allelic sizes obtained for five microsatellites markers is two presumed Carpathian goat families; F1= family 1, M=mother; F=father; O=offspring

| Specie | Breed | Tag | Sex | ID | Microsatellite markers (size in base pairs) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | BM1329 allele 1 | BM1329 allele 2 | BM1818 allele 1 | BM1818 allele 2 | CSRD247 allele 1 | CSRD247 allele 2 | HSC allele 1 | HSC allele 2 | MM12 allele 1 | MM12 allele 2 | n..... |
| Capra hir | Carpathia | RO2565 | F | F1-M | 173 | 181 | 254 | 258 | 234 | 242 | 278 | 294 | 98 | 114 | ..... |
| Capra hir | Carpathia | RO2565 | M | F1-F | 171 | 171 | 258 | 268 | 230 | 232 | 280 | 286 | 104 | 104 | ..... |
| Capra hir | Carpathia | RO2551 | M | F1-O | 169 | 181 | 254 | 258 | 230 | 234 | 278 | 292 | 98 | 98 | ..... |
| | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | |
| Capra hir | Carpathia | RO2560 | F | F2-M | 171 | 177 | 254 | 268 | 230 | 234 | 278 | 300 | 94 | 106 | ..... |
| Capra hir | Carpathia | RO2560 | M | F2-F | 169 | 177 | 260 | 260 | 234 | 238 | 272 | 293 | 98 | 98 | ..... |
| Capra hir | Carpathia | RO2560 | M | F2-O | 169 | 171 | 254 | 260 | 230 | 234 | 293 | 300 | 98 | 106 | ..... |

In the first presumed family (F1), the comparative analysis of fragment sizes, obtained in all microsatellites markers of the offspring (F1-O) and its presumed mother (F1-M), shown 100% compatibility. In contrast, the presumed father (F1-F) of the offspring (F1-O) showed incompatibility for several markers. For example, the presumed father is homozygous for the BM1329 marker (171 bp), fragment which is not present in the offspring. The same situation was found for other markers (ex: HSC, MM12 etc; Table 2).

In the second family (F2) we observed 100% compatibility between the offspring and its parents.

Only the goats that passed the parentage test were further keept as potential candidates for semen collection and were further submitted to *CSNS1* genotyping.

The identification of the main *CNS1S1* genotypes of the analysed bucks was done base on the correlation of the electrophoresis profiles obtained for the two distinct DNA tests performed for each goat.

The first PCR-RFLP test allows the discrimination between A, B/E and F allele. In the case of A allele there is a 11 bp deletion located in intron 9, which is absent in B/E and F allele.

Digestion of the fragment amplified from A allele with *Xmn*Iendonuclease generates two fragments of 150bp and 63bp. In the case of B/E alleles, which are similar in this amplified region, one of the fragments 11bp longer (161 bp), as compared with that obtained in the case of A allele.

Furthermore, the deletion of the 23rd nucleotide (a cytosine) from exon 9 in the case of F allele,

abolishes the *XmnI* restriction site, generating an undigested fragment.
The second AS-PCR testallowed discrimination between E and non-E alleles, based on the 457 bp LINE element insertion from the 19th exon that is characteristic to E allele.

Different combinations of these patterns that correspond either with homozygous or heterozygous genotypes and are highlighted in Figure 1.
Allele and genotypes frequencies at the CSN1S1 locus were calculated for in this bucks population (Table 2).

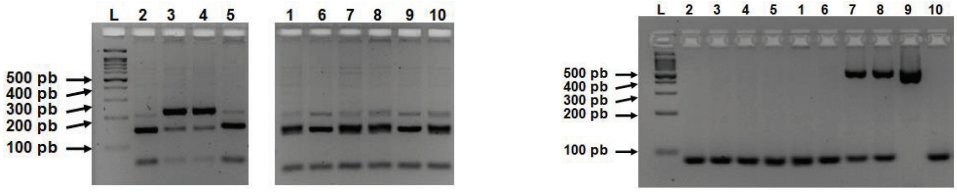


Figure 1. Identification of CSN1S1 genotypes by PCR-RFLP (left pictures) and AS-PCR (right pictures). Samples genotypes 1-AB; 2-AA; 3-AF; 4-AF; 5-BB; 6-BB; 7-AE; 8-AE; 9-EE; 10-AB

Table 2. Allelic frequency at *CSN1S1* in the analyzed Carpathian goat bucks population

| Locus | No. of candidate bucks | Allele frequency | | | | Genotypes frequency | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *CSN1S1* | 36 | A | B | E | F | AA | AB | BB | EE | FF | AE | BE | AF | BF |
| | | 0.35 | 0.40 | 0.07 | 0.18 | 0.17 | 0.25 | 0.14 | 0.03 | 0.03 | 0.03 | 0.06 | 0.08 | 0.22 |

The cu frequency of defective allele (E and F) was much lower (0.25) compared with strong expression allele (0.75). However, we found a significant frequency of genotypes carriers of at least one defective allele (0.39).
The cumulated frequency of strong genotypes (AA, AB and BB), significantly associated with higher milk casein content and cheese yield, was 0.56. Only four males from this category were further retained for semen collection.
The synchronization of oestrus and ovulation was accomplished using a hormonal treatment protocol based on progesterone and prostaglandins.
Hormonal treatment is necessary if artificial insemination is used either in natural or out of season oestrus induction. According to literature the use of one dose of 20mg of FGA can lead to a higher rate of kidding than the use of other doses (Barbosa et al., 2009).
The results obtained in the current study based on the cervical opening degree showed that the females acquired a synchronized oestrus and the average percent of non-returned to oestrus goats after two oestrous cycles was 91.28%.
The rate of females with third degree cervical opening (intrauterine deposition of sperm) was 51%, second degree 38% and first degree 10%.
The reproduction indices in she-goats used for artificial insemination during induced oestrus are shown in Table 3.
In the first farm we registered a 88 % rate of non-returning goats to oestrus after two cycles, a fecundity of 82% and a prolificacy 173.4% in goats inseminated with freshly collected and diluted semen.
In the second farm the rate of non-returning goats was 92%, with a fecundity of 88% and prolificacy 168.2%.
In the third farm the rate of non-returning goats was 88%, with fecundity rate of 79% and prolificacy 170.23% and in the fourth farm these indices were 92,5%, 84,81% and 170.98%, respectively.

Table 3. Reproduction indices of Carpathian she-goats used for artificial insemination

| Group | No. of inseminated goats | % of goats non-returned to oestrus | Fecundity % | Prolificacy % |
|---|---|---|---|---|
| Farm I | 90 | 88.63 | 82.25 | 173.40 |
| Farm II | 90 | 90.00 | 85.55 | 168.88 |
| Farm III | 150 | 94.00 | 86.66 | 170.66 |
| Farm IV | 120 | 92.50 | 84.16 | 191.66 |

Similar results using an artificial insemination protocol with diluted semen were obtained in other studies (Faigl et al., 2012).

According other studies, there is a direct connection between the fecundity and semen deposition, uterine insemination being associated with a higher fecundity rate.

The results obtained in our study point out that the uterine deposition (third degree cervical opening) of the semen is associated with a higher kidding rate, as demonstrated in other studies (Salvador et al., 2005).

## CONCLUSIONS

In this study we tested the possibility to use DNA and assisted reproduction technologies to spread valuable mutations associated with milk traits in goats. We tested the viability of this combined approach in four populations belonging to unimproved Carpathian goat breed.

The potential candidate bucks for semen collection were submitted for parentage testing and for *CNS1S1* genotyping.

Only four males with confirmed genetic origin and valuable *CSN1S1* genotypes (associated with higher milk casein content and cheese yield) were used for the artificial insemination of 450 oestrus synchronised she-goats.

The high fecundity rate of oestrus synchronised goats calculated after the end of parturition was registered (84.65%).

By using freshly collected semen exclusively from *CSN1S1* genotyped goats we proved the viability of this model to spread valuable genes at a higher rate in Carpathian breed.

In this particular case this combined approach can substantially contribute to the improving of casein contents and cheese yield and at the same time to the preservation of the valuable gene pool of this local breed.

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# SELECTION OF KARAKUL LAMBS AFTER THE LENGTH OF PILOUS FIBERS

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***Abstract***

*The purpose of this research was to elucidate the particularities of the correlative links of the length of the pilous fibers of the Moldavian Karakul lambs with their other morpho-productive characters. The length of the pilous fiber at Karakul lambs was defined as the absolute distance between the surface of the skin and the peak of the stretched fiber. This length was determined by the practical method during the evaluation under farm conditions, according to the Instructions of Karakul Sheep Evaluation. The method provides the determining the relative length of the fibers (the average length of a fiber tuft, mainly consist of thick and less of intermediate fibers) to the live lamb. The method of measurement consists in applying perpendicularily to the skin a millimeter ruler, with the sharp end in the seam of the curls, and with the tweezers the fiber tuft is stretched on the ruler graduation, fixing the indices at the peak of the fibers with the precision of 1mm. The scientific researches have demonstrated that the length of the pilous fibers in lambs of Moldavian Karakul is an important morpho-productive character, which has a significant impact on furskins quality. The optimal length of pilous fibers at lambs of superior elite class is within 7-12 mm. At lambs with valuable curling types (jacket, coastal, flat), the length of the fibers is shorter (9,80-11,53 mm), which is beneficial to the quality of the furskin. The costal and flat type of furskins have the shortest fibers (9.80-10.52 mm) compared to the jacket and kaukasian type (11.53-14.46 mm). Positive correlations between fiber length and mass and body length have been identified, and negative correlations with their length and modeling, which disadvantage the process of selecting of corpulent lambs - in the first case, and favor the process of selecting lambs with a valuable curling - in the second case. These correlations have been taken into account in the lambs selection. In the heterogeneous pairing of black and grayish parents, fellow descendants are obtained the grayish descendants with the shorter by 11.7% (P <0.1) of black fiber were obtained, thus with the more valuable furskin qualities, compared to the homogeneous pairing of both grayish partners. Pairing of sheep, with the jacket curling type, with rams, with coastal curling type, contributes to shortening of the length of the fibers at the descendents by 10.4% (P <0.1), thus improving the qualities of the furskin, compared to the mating of parents with jacket curling.*

***Key words****: length, fibers, furskins, lambs, Moldavian Karakul*.

## INTRODUCTION

Pilous fibers of Karakul lambs have attracted the attention of several researchers and specialists in the field, since their arrangement on the surface of the furskin forms the curls of different types and sizes, which as a whole form the curling, determining its ornamental beauty (quality) (Иванов М.Ф., 1964; Дъячков И.Н., 1980; Гигинейшвили Н.С., 1976; Шеффер Х., 1977; Нел Дж.А., 1975; Матер Х.Э., 1975).

Multiple researchers, independently of each other, have demonstrated that fiber length is a very important selection feature, which greatly influences the quality of curling and the value of the furskin as a whole (Бердалиева А.М. и др., 2015; Ержепов С., 2016; Прманшаев М. и др., 2016; Лаханова К.М., 2014). At the Karakul lambs, this character correlates with a series of properties of the furskins, knowledge of which would allow to conduct the selection process in the desired direction.

According to Zahariov M.D. (1987), the length of the fibers depends, firstly, on the duration of the intrauterine period of development of fetus and on the specification of the furskin. Regardless of the color of the pilous cover, at the furskins obtained from the fetuses in the early stages of intrauterine development, the fibers are much shorter and in the late stages - much longer. The shortest fibers are Golyak type (1.89 mm). The Karakulcea and Karakul-Karakulcea furskins have an intermediate length of fibres, between the Golyak and Karakul furskins. Until birth, the daily increase in fiber length is on average about 0.25 ± 0.08 mm. If to consider that the length of the black

fibers in the Karakul furskins is 100%, then their length in Karakul-Karakulcea is 27.3 - 47.9%, in Karakulcea it is 20.3 - 30.4%, in Golyak – 15.8 - 18.4%, and in Yahobab - 170-186%. The length of the fibers is related to the furskin assortment, with the type and shape of the curls, with their color. The shortest fibers had the sorts of furskins "Moscow Jacket", "Thin Coastal I", "Thin Flat I" and "Jacket I". At black furskins of these sorts, the average length of thick fibers is: 9.3; 9.3 to 9.9; 9.8 and 10.9 mm, respectively. At greysh furskins of these sorts, the length of thick black fibers is on average: 7.4; 9.1 to 10.0; 9.2 and 9.5 mm, respectively, at the gray-colored furskins, the length of thick fibers is on average 9.7; 9.8 to 10.1; 10.1 and 11.2 mm, respectively. The longest fibers are mentioned at furskins "Jacket thick", "Coastal thick I", "Thick flate I", "Kaukasian thick I" and lower sorts (II, III, defect). Thus, the average length of thick black fibers in these sorts was: 13.6; 13.2; 13.3 and 17.3 mm respectively. The length of the thick black fibers of grayish furskins of these sorts is 11.7; 13.2 to 15.1; 12.8 and 17.2 mm, respectively. At the grey furskins of these sorts, the length of the thick fibers is: 14,1; 13.7 to 18.2; 14.3 and 18.3 mm, respectively. The shortest fibers have the valuable curls: the tubular, coastal and flattened waves, at the ridges - narrow furrows, at moire curling. The longest fibers were observed at the worthless or defective curls (rings, half-rings, peas, corkscrew, snail). The black and gray furskins have shorter fibers than grayish pink and white furskins. The fluff type fibers, in all sorts and specifications of furskins, are always on 3-4 times shorter than rough and intermediate fibers.

The length of the fibers is hereditary conditioned and influenced by environmental conditions, especially by the ewes nutrition during the gestation period. A number of researchers (Аверьянов И. Я., Ибрагимов И. М., 1968, Дъячков И. Н., 1980, Кошевой М. А., 1975) demonstrated that poor nutrition conditions of pregnant sheep leads to the obtaining of lambs with shorter fibres and as a result with more valuable furskin qualities. At the same time, the Austrian professor Adametz L. (1911), many years ago, as a result of his research concluded that: „*The poor diet of pregnant Karakul sheep not only affects the quality of buckling at lambs, as someone thought, but on the contrary, such nutrition proves to be quite unfavorable, because in these cases the furskins gets bad quality*".

Another part of the researchers (Ескара М.А., 2014; Дюсегалиев М.Ж., 2010; Алибаев Н.Н. и др., 2014; Надвитов Н.К. et al., 2012) demonstrates that the qualities of hair fibers (including their length) in Karakul lambs have some particularities, depending on their belonging to the intra-racial types of sheep, created in different geographical areas, which also have some morpho-biological differences, obtained as a result of the specific selection, in addition to the pedoclimatic conditions of the respective areas. Therefore, in some types of newly created Karakul sheep, the relationship between fibers length and other morpho-productive selection characters can be distinguished, having specific configurations to those generally known.

In this context, the revealing of the particularities of the correlative links between the length of the hair fibers of the Moldavian Karakul lambs with their other morpho-productive features, presents a current problem that would make the selection process more efficient in the direction of increasing the quality of the furskins.

The purpose of this research was to elucidate the particularities of the correlative links of the length of the pilous fibers of the Moldavian Karakul lambs with their other morpho-productive characters (types of curling, length of curls, modeling of curling, color of parents, type of parents' curling).

## MATERIALS AND METHODS

The researches were carried out on a batch of Moldavian Karakul lambs from sheep flock of former sovhoz "Kotovskii", Cainari district and later of the National Institute of Animal Husbandry and Veterinary Medicine from Maximovca village, Anenii Noi district. In our research, the *length* of the hair fiber at Karakul lambs was defined as the absolute distance between the surface of the skin and the peak of the stretched fiber. This length was determined by the *practical method* during the evaluation under farm conditions, according to the

Instructions of Karakul Sheep Evaluation with Amelioration Principles in the Republic of Moldova (Buzu I., Zelinschi N., Evtodienco Silvia, 1996). The method provides the determining the relative length of the fibers (the average length of a fiber tuft, mainly consist of thick and less of intermediate fibers) to the live lamb. The way of measurement consists in applying perpendicularily to the skin a *millimeter ruler*, with the sharp end in the seam of the curls, and with the tweezers the fiber tuft is stretched on the ruler graduation, fixing the indices at the peak of the fibers with the precision of *1mm*.

Usually, the length of the fibers is measured on the croup, being the most important region, where the fibers are the shortest of all body regions. To determine the uniformity of the length of the fibers on the body surface, the measurement is also carried out on the withers. The smaller the difference between these two dimensions, the more uniform the length of the fibers is considered.

According to the evaluation instructions in force, the fibers (by length) can be: short (6-9 mm), medium (9-13 mm), long (14-18mm) and very long (> 18mm). In the practice of evaluation also *very short fibers of < 6 mm* are differentiated.

The Pearson linear correlation coefficient ($r_{xy}$) between the length of the fibers, on the one hand, and the mass and corporal length of the lambs, on the other hand, was calculated using the computerized software "STATISTICA-12".

The data obtained as a result of the researches were statistically processed, and their certainty assessed according to the variational biometric statistics after the methods of Плохинский Н.А (1989).

## RESULTS AND DISCUSSIONS

The results of our research (Buzu I., 2012; Богданович Н.И., Бузу И.А., 1982а, 1982б; Buzu I., 1997) have shown that the length of pilous fibers at Moldavian Karakul lamb has, indeed, specific morphological particularities formed both under the influence of pedo-climatic conditions, in which this new type of sheep was created, and under the pressure of the selection process applied to its creation.

We have found that the length of lamb's fibers of Moldavian Karakul is significantly higher than that of lambs from Central Asia, researched by a number of authors (Table 1).

Table 1. The length of the fibers at black Karakul lambs of various types of curling

| Length of fibers (mm) at lambs with type of curling: | | | | The author of the source |
|---|---|---|---|---|
| jacket | coastal | flat | kaukasian | |
| 11.53 ± 0.16 | 9.80 ± 0.19 | 10.52 ± 0.23 | 14.46 ± 0.20 | Buzu I. A. (1997) |
| 7.50 ± 0.05*** | 6.70 ±0.08*** | 6.70 ± 0.09*** | 10.20 ±0.11*** | Дъячков И.Н. (1963) |
| 10.20 ± 0.07*** | 7.80 ±0.09*** | 8.50 ± 0.08*** | 14.7 ±0.12 | Бадалбаев Н.С. (1966) |
| 9.30 ± 0.04*** | 8.30 ± 0.12*** | 8.40 ±0.06*** | 13.40 ± 0.10*** | Кошевой М.А. (1975) |
| 10.14 ± 0.06*** | 9.11 ± 0.11** | 9.39 ±0.09*** | 14.59 ± 0.13 | Исаянц Б.Л. (1971) |

Remark: ** P<0.01; *** P<0.001, compared to our data.

This difference in fiber length was observed at lambs with all types of curling, except Asian lambs of kaukasian type, investigated by Исаянц Б.Л. (1971), where the difference in fiber length practically is not found.

From the presented data it can be noticed, that the length of the lambs fibers at Moldavian Karakul lambs is obviously in relationship with the type of curling. It has been found that at lambs with valuable curling type, the length of hair fibers is shorter and lambs with worthless curling type (kukasian) have longer fibers. Lambs with coastal and flat curling type possess the shortest fiber. Lambs with the kaukasian type of curling have the longest fibers. Lambs with the jacket type of curling usually have the medium length of the fibers. In continuation, we find that the length of the fibers correlates with other furskin features such as: curl length, modelling, luster, silky, etc. The shorter the fibers, the longer the curls are. With the increase of the fibers length, the curls length become shorter, the modeling, the luster and the silky are diminished. The short and medium length of the fibers usually coincide with excellent modeling, good

pigmentation, high luster and silky. In the end result, the length of the fibers is correlated with the commercial qualities (features) of the furskins. At the furskins of sort I, the length of the fibers correlates positively with the surface of the furskin, its mass, the thickness of the dermis, fiber thickness, curl size and length. At the same time, the fiber length is in negative correlation with fiber density, shape and type of curls, curls length, curling uniformity and curls modeling. Our research has shown that the length of the fibers is corelates linearly positive with the lamb's body mass ($r_{xy}$ = 0.22 ± 0.09 – 0.31 ± 0.09) and body length ($r_{xy}$ = 0.15 ± 0.07 - 0.16 ± 0.07). This means that lamb selection in the direction of decreasing fiber length can lead to lower body development parameters.

We took this unfavorable relationship into account when the individual lamb selection was performed. For breeding, only individuals with well combination of short fibers and high body mass were selected.

We found that between the length of the fibers and the length of the curls at the Moldavian Karakul lambs there is an obvious negative phenotypical correlation (Tab. 2).

Table 2. The length of the pilous fibers depending on the length of the curls at Moldavian Karakul lambs

| Length of curls | N | Length of fibers, mm | | |
|---|---|---|---|---|
| | | M ± m | σ | Cv % |
| Very long (> 50 mm) | 48 | 8.58 ± 0.26*** | 1.80 | 21.0 |
| Long (30 – 50 mm) | 144 | 11.11 ± 0.19** | 2.24 | 20.2 |
| Medium (20 – 30 mm) | 350 | 11.25 ± 0.13** | 2.42 | 21.5 |
| Short (12 – 20 mm) | 76 | 12.21 ± 0.29 | 2.51 | 20.6 |
| Very short (< 12 mm) | 8 | 16.00 ± 0.54*** | 1.52 | 9.5 |

Remarc: **- P<0.01; ***- P<0.001, – compared to the short length of the curls.

The results of the research have shown that the shortest pilous fibers (8.58 ± 0.26 mm) were recorded in lambs with very long curls. The longest fibers (16.00 ± 0.54 mm) were found in lambs with very short curls. With increasing of the curls length at the lambs, from short to very long, the length of the fibers decreased by 3.63 mm or 29.7% (P <0.001).

Thus, compared with the batch of lambs with short curls, the fiber length of the lambs with medium curls was smaller by 0.96 mm or 7.9% (P <0.01), at the lambs with long curls were shorter with 1.10 mm or 9.0% (P <0.001) and at lambs with very long curls - by 7.42 mm, or 46.4% (P <0.001).

Therefore, we can conclude that, with increasing of curls lengths, fibers become shorter, thus increasing the quality of modeling, luster and silk.

This phenotypically negative correlation, in fact, is favorable for amelioration the quality of the furskins in the herd.

Thus, choosing for breeding the lambs with short fibers, we have indirectly accumulated in the herd individuals with long and very long curls, desired to improve the quality of furskins in the sheep population. The length of the fibers correlates quite close to the modeling of the curling (Table 3).

Table 3. The length of the fibers depending on the modeling of the curling at Moldavian Karakul lambs

| Curling modeling | N | Fibers length, mm | | |
|---|---|---|---|---|
| | | M ± m | σ | Cv % |
| Excellent | 195 | 10.46 ± 0.16*** | 2.19 | 20.9 |
| Appropriate | 247 | 11.24 ± 0.18* | 2.80 | 25.1 |
| Weak | 82 | 12.07 ± 0.31 | 2.80 | 23.2 |
| Insufficient | 9 | 12.78 ± 1.46 | 4.12 | 32.2 |

Remarc: *- P<0.05; ***- P<0.001, compared to weak modeling.

We have found that lambs with excellent curling modeling possess the shortest fibers and those with insufficient curling modeling - the longest hair fibers. With increasing of curling modeling, from insufficient to excellent, fiber length decreases from 12.78 ±

1.46 mm to 10.46 ± 0.16 mm or 18.2% (P <0.001). The lambs with excellent curling modeling have shorter lengths of pilous fibers compared to lambs with weak curling modeling with 1.61 mm or 13.3% (P <0.001) and compared to lambs with the appropriate modeling - by 0.78 mm or 7.0% (P <0.001). Therefore, the more valuable the modeling, the shorter the length of the fibers, and vice versa, the weaker the modeling, the longer is the length of the fibers. The length of the lamb's fibers also depends on the parent's mating variants by color. In case of the market requires grayish furskins, the breeders of Karakul sheep apply the heterogeneous mating after color of the black ewes with grayish rams and homogeneously of both grayish partners, with the purpose obtaining of grayish descendens. In addition, by homogeneous mating of grayish sheep, the selectors aim to increase the rate of the furskins of desired colors (bluish, marbled, etc.) with excellent and appropriate uniformity, applying the ITV method of evaluation (early identification of viability).

In these situations, it is important to know the impact of different variants of sheep mating after color - homogeneous and heterogeneous, both on the length of the fibers and on the quality of the furskin curling as a whole (Table 4).

Table 4. The length of fibers at Moldavian Karakul lambs, obtained as a result of the homogeneous and heterogeneous pairing of the parents by color, mm

| The color of the parents | | N | Croup | | Withers | |
|---|---|---|---|---|---|---|
| Ewe | Ram | | M ± m | Cv,% | M ± m | Cv,% |
| Black lambs | | | | | | |
| Black | Black | 48 | 9.79 ± 0.38 | 27.2 | 14.44 ± 0.45 | 21.6 |
| Black | Grayish | 159 | 10.36 ± 0.28 | 24.1 | 14.81 ± 0.25 | 21.1 |
| Grayish lambs, black fibers | | | | | | |
| Black | Grayish | 52 | 10.98 ± 0.37* | 24.6 | 15.23 ± 0.49 | 23.3 |
| Grayish | Grayish | 9 | 12.44 ± 0.80** | 19.3 | 15.66 ± 1.05 | 20.1 |
| Grayish lambs, white fibers | | | | | | |
| Black | Grayish | 52 | 14.77 ± 0.49 | 23.1 | 20.54 ± 0.54 | 10.2 |
| Grayish | Grayish | 9 | 14.12 ± 1.16 | 24.6 | 20.78 ± 1.22 | 17.6 |

Remarc: *- P<0.05; **- P<0.01, compared to homogeneous pairing „Black x Black".

The results of the researches have shown that the shortest fibers both in the croup region (9.79 ± 0.38 mm) and in the withers (14.44 ± 0.45 mm) possess the lambs obtained by homogeneous mating of the black parents.

As a result of the heterogeneous pairing of black parents with grayish, compared to the homogeneous pairing of black partners, there is a tendency of fibers elongation in descendants of both black and grayish lambs. The longest black fibers on both the croup and the withers were recorded at the lambs obtained from the pairing of both grayish parents. Thus, the black fibers from the croup of grayish lambs, born from the heterogeneous mating of the parents, were longer compared to the lambs born from the homogeneous pairing of the black parents with 1.19 mm or 12.2% ($t_d$ = 2,25; P < 0.05). And the black fibers of lambs born from the homogeneous mating of the grayish sheep were longer compared to the same contemporaries by 2.65 mm or 27.1% ($t_d$ = 3.01; P <0.01). Therefore, we can say that the heterogeneous mating of black sheep with grayish, as well as homogeneous mating of grayish sheep, leads to elongation of the fibers in the progeny, with a possible negative impact on the qualities of curling as a whole. Knowledge of these aspects requires the disclosure of breeders with specific heredity genotype in the progeny of the length of the fibers to attenuate this impact by applying appropriate mating by color of the sheep.

The length of lamb's fibers in Moldavian Karakul is related to the type of parental curling (Table 5).

Research has shown that use of one of the partners with the coastal type of curling for mating allows, in a directed manner, to reduce fibers length in descendants. The shortest fibers

were recorded in the lambs obtained by "♀coastal x ♂jacket", "♀jacket x ♂coastal" and ♀kaukasian x ♂coastal mating. The longest fibers were found in the lambs obtained from the matings "♀jacket x ♂jacket" and "♀jacket x ♂flat".

Knowledge of the mode of transmission by heredity of fiber length in different sheep breeding variants in certain populations (flocks) allows the selection to be routed in the desired directions.

Table 5. The length of the fibers in Moldavian Karakul lambs according to the type of parents' curling, mm

| Type of parents' curling | | N | M ± m | |
|---|---|---|---|---|
| Ewe | Ram | | Croup | Withers |
| Coastal | Jacket | 46 | 10.1 ± 0.79* | 14.0 ± 0.81 |
| Kaukasian | Coastal | 39 | 11.0 ± 0.80 | 14.9 ± 1.08 |
| Kaukasian | Jacket | 47 | 10.9 ± 0.62 | 15.1 ± 0.85 |
| Jacket | Jacket | 108 | 11.3 ± 0.24 | 14.5 ± 0.29 |
| Jacket | Flat | 55 | 11.3 ± 0.71 | 15.0 ± 1.53 |
| Jacket | Coastal | 66 | 10.5 ± 0.47* | 14.1 ± 0.51 |

Remarc: *- P=0.1 compared to mating type „Jachet x Jachet".

The pairing of partners with certain fiber lengths, taking into account the specific character of transmission by heredity of this character, allows to increase the probability of obtaining the progeny with the desired length of the fibers.

Based on the results of these researches, concrete proposals for mating the sheep in a homogeneous and heterogeneous system were developed, according to the color of the pilous cover, taking into account the length of the fibers, especially of the rams at birth. Using the guided matings, taking into account the color of the pilous cover and the curling type of sheep, the annual increase of lambs of superior classes (elite and class I) in proportion of 3-5% in the selected flocks were established. The most valuable results were obtained when for the mating the rams with jacket and coastal curling were used, with short, excellent silknes and luster fibers. We have found that for lambs of certain classes and types of curling are characteristic a certain length of fiber. The length of the fibers has an intermediate heredity character, so, in descendants, the fiber length tends to the average of the parents. Generalizing the results of the research, we can conclude that the optimal length fibers at lambs of the superior and elite classes is within the limits of 7-12 mm. At lambs with the coastal curling type, the optimal length of the fibers is <10 mm, at those with the flat curling type it is 10 to 11 mm, and in the ones with the jacket type of curling is between 11-12 mm. In lambs with the not valuble kaukasian curling type, the length of the fibers is the longest (13.0-15.0 mm). Therefore, the length of fibers at lambs with required curling types (jacket, coastal, flat) is shorter, which favors the formation of valuable curls.

On the basis of the results of these researches, the parameters of fiber differentiation according to length were developed, included in the Instructions of Karakul Sheep Evaluation with Amelioration Principles in the Republic of Moldova (1996); were elaborated efficient sheep mating procedures according to the color of the pilous cover and the type of parental curling, which were included in the Recommendations on the technology of the sheep products in the Republic of Moldova.

## CONCLUSIONS

The length of the pilous fibers in lambs of Moldavian Karakul is an important morpho-productive character, which has a significant impact on furskins quality.

The optimal length of pilous fibers at lambs of superior elite class is within 7-12 mm. At lambs with valuable curling types (jacket, coastal, flat), the length of the fibers is shorter (9,80-11,53 mm), which is beneficial to the quality of the furskin.

The coastal and flat type of furskins have the shortest fibers (9.80-10.52 mm) compared to the jacket and kaukasian type (11.53-14.46 mm).

Positive correlations between fiber length and mass and body length have been identified, and negative correlations with their length and modeling, which disadvantage the process of selecting of corpulent lambs - in the first case, and favor the process of selecting lambs with a valuable curling - in the second case. These correlations have been taken into account in the lambs selection.

In the heterogeneous pairing of black and grayish parents, the grayish descendants with the shorter length 11.7% ($P < 0.1$) of black fiber were obtained, thus with the more valuable furskin qualities, compared to the homogeneous pairing of both grayish partners.

Pairing of ewes, with the jacket curling type, with rams, with coastal curling type, contributes to shortening of the length of the fibers at the descendents by 10.4% ($P < 0.1$), thus amelioration the qualities of the furskin, compared to the mating of parents with jacket curling.

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# PARTICULARITIES OF NATURAL *Varroa* - RESISTANCE OF HONEYBEES POPULATION OF CARPATHIAN RACE FROM THE CODRI OF THE REPUBLIC OF MOLDOVA

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***Abstract***

*The aim of the researches was to reveal the particularities of natural varroa-resistance of honey bees of Carpathian race from the Moldavian Codri and their use in the selection and genetic amelioration of the local bee subpopulation. Scientific researches were a part of the SMARTBEE / FP7.Eu-KBBE.2013.1.3-02 Sustainable Management of Resilient Bee Populations project, in collaboration with the Institute for Beekeeping Research and Development in Bucharest, Romania. In order to achieve the purpose, at the experimental apiary of the Institute of Zoology of the Academy of Sciences of Moldova an experiment was carried out, on a batch of 25 bee families, choosed randomly, supplemented with young queens, obtained conducting from the same apiary. The experiment lasted three years (March 2015-March 2018), in which the queens were not replaced, and the bee families during the entire experimental period were not subjected to any anti-varroa or other disease treatment, nor additional feeding in spring or autumn with nutritional stimulators has not been applied. It was found that under natural conditions, without anti-varroa drug interventions and without the change of queens, from 25 colonies at the begining of experiment only 11 survived, which is 44%. In the bee families that survived in the third year of experiment, the index of natural Varroa mite fall during September-October was higher, compared to the first-year bee families by 42.7-43.0% ($t_d$=2.3-1.3; P<0.05 and P>0.1) and 2.7-3.0 times compared to the bee families of the second year ($t_d$=4.9-3.0; P<0.001 and P<0.01). Infestation degree of bees with Varroa mite increased, reaching peak values in the third experimental year, up to 4.81 ± 1.00 mites/10 grams of bees, which led to the inhibition of the main physiological functions of reproduction and developing of bee families. The infestation of capped brood cells with Varroa mite progressed in the third year of experiment, being significantly higher in September by 11.5% (td=2.9; P<0.01), compared with the same month of the previous year, which lead to a decrease in the quantity of capped brood, of the colonies strength and the weakening of the vital activity of the bee families as a whole. Queens prolificity declined significantly in October from 700 eggs/24 hours in the first year, to 234 eggs/24 hours in the third year, being less in the last year with 466 eggs/24 hours, or 66.6% (td=5.1; P<0.001). Out of the 11 bee families, which survived in natural conditions for three years, 5 more valuble families were selected in the breeding batch for reproduction. The bee families of the breed batch significantly exeeded the families from experimental batch after the queens prolificity – with 18.4% (P<0.05), the amount of capped brood – with 18.6% (P<0.05) and honey production – with 6.8% (P<0.01). At the same time, in the bee families of the breeding batch, the degree of infestation with mites was lower, the index of natural mites fall and the colonies strength – higher, as well as the boosted wintering resistance, ranging from 82.3-89.0%.*

***Key words****: bees, natural varroa-resistance, longevity, honey.*

## INTRODUCTION

*Varroa destructor* mite is part of one of the most invasive parasitic species that attacks the most valuable, from a productive-useful point of view, species of insects such as *Apis mellifera* L. The *Varroa* mite's female, parasiting on the body of drones and worker bees and feeding the hemolimph of these hosts, lay their eggs in the combs with uncapped brood cells, preferring the drone brood. The larvae of the mite, feeding intensely with the hemolimph of the larvae and nymphs of bees, reaches within a very short time (4-7 days) the adult form.

Their mating occur in the brood cell. With the bee hatching, the *Varroa* female migrates throughout the hive, and sitting on the body of the drones and bees, the cycle of metamorphosis is continued again. Infested bees have a shorter life and their productivity is diminished. Brood with low infestation of

mites gives rise to small bees with low viability. Heavy *Varroa* mite infestation leads to the appearance of unviable bees with undeveloped wings, deformed head and feet. They fall on the bottom of the hive and are removed outside by healthy bees.

The heavily infested drones lose their mating behavior and the queens remain unfertilized (Ogradă, 1986; Calderon et al., 2010; Guzman-Novoa et al., 2010; Neuman et al., 2010). In addition, by perforating the bee's cuticle, *Varroa* mite inoculates a series of viruses and fungi, triggering the development of a range of quite contagious diseases at bees (Savu, 2012; Dainat et al., 2012; Nazzi et al., 2016; Guzman-Novoa et al., 2012).

The damaging impact of this invasive parasite species on the apiculture branch, as a whole, is of enormous dimensions (Lee et al., 2015; Buchler et al., 2014).

Our previous researches (Cebotari et al., 2013) demonstrated that the intensity of this invasion (calculated by the number of mites per 100 drone brood cells) in *Apis mellifera* bees families averages from 20.7±2.1% in apiaries where attention is paid to prophylaxis and disease control, up to 28.0±1.5% in apiaries where less attention is paid to prophylaxis and disease control measures. The degree of infestation, calculated from the number of mites localized on the bee's body, per 100 worker bees, averaged from 8.9±0.9% to 14.8±0.4% on the studied apiaries. Heavy *Varroa* mite infestation has a negative impact on the vital activity and productivity of the bee family. The coefficient of regression of the amount of honey extracted from the nest, depending on the infestation degree of bee family by *Varroa* parasite is: $R_{x/y}=1.60\pm0.07$ ($t_r=24.2$; $P<0.001$). This means that as the *Varroa* infestation rate increases by 1%, the amount of honey extracted from the nest is reduced by 1.6 kg. For these reasons, beekeepers, specialists and scientists in the field always are looking for effective methods to control and combat this disease in the honey bee (DeGrandi-Hofman et al., 2017; Wantuch et al., 2009).

Some specialists (Oddie et al., 2017; Buchler et al., 2014) drew attention to the ability of bees to respond to the invasive attack of the *Varroa destructor* mite, thereby highlighting their natural resistance against the aggressive parasite.

The natural varroa-resistance of melliferous bees is a phenotypic trait (variability), as in any animal species, resulting from the interaction of genetic variability of the animal population with environmental conditions (Runderer, cited by Siceanu, 2012). At any apiary, under similar conditions of maintenance, exploitation and environment, a variability of bee families is observed after a string of morpho-productive characters, such as: wintering resistance, queen prolificity, amount of capped brood, colony strength, and, finally, the production of honey. The manifestation of these morpho-productive characters in bee families is dependent on their ability to resist against pathogens of different origins: virotic, bacterial, fungal, parasitic, etc. Even under the conditions of systematic drug treatments, some bee families are more vulnerable, others more vigorous against actions of pathogenic factors. These skills are genetically inherited naturally.

According to some researchers in the field (Rosenkranz et al., 2010; Buchler et al., 2014), in European honey bees races, about 5-20% of mites, which infest a bee family, remain infertile after entering in the brood cells. Moreover, there is delayed laying of eggs in some mites, correlated with the development of bee brood, or lay only eggs from which only males come out because they are not fertilized. These situations limit the reproductive success of *Varroa*, which can be measured as the number of female daughters resulting from an adult female (founder) at the time of the brood emerging. Some researchers (Harbo et al., 2015; Panziera et al., 2017) have identified in some bee families a relatively high percentage of non-reproductive mites, which is a heritable trait of the honey bee and which they called "*Suppression of Mite Reproduction - SMR*". It was found that the low percentage of fertile mites was due to the bee's preferential removing of reproductive mites, therefore, this character was renamed "*Varroa sensitive hygiene - VSH*". The investigations of these authors have completed the idea that SMR can also come from other mechanisms, such as the ability of the brood to limit reproduction of the mites. So, some of the bee families may have high levels of mite resistance. They are of

interest in the selection of colonies with natural varroa-resistance.

According to Locke et al. (2012), in some populations selected by *Apis mellifera* in Europe, non-reproductive mites were found in a significant proportion (40-50%), a character appropriated by heredity. Therefore, it is about varroa-resistance of bees.

At the same time, existing researches in this field does not provide sufficient information on the conditions in which bee populations with high varroa-resistance were selected (with or without drug treatments, duration of selection period, longevity of bee colonies in natural conditions without changing queens, evolution of the main morpho-productive characters, etc.) as well as how this high resistance to the productivity of bee families is reflected. In this context, the aim of our researches was to reveal the particularities of natural varroa-resistance of honey bees of Carpathian race from the Moldavian Codri and their use in the selection and genetic amelioration of the local bee subpopulation.

## MATERIALS AND METHODS

The scientific researches were carried out on bee families *Apis mellifera carpatica,* at the experimental apiary of the Institute of Zoology of the Academy of Sciences of Moldova, located in the central part of Moldovan Codri, Forest District Ghidighici, Canton no. 8, Forest Sector no. 21. The main honey plants sources in the area were white acacia (*Robinia pseudoacacia*), large-leaved linden (*Tilia platyphyllos*) and polyphlora of wild plants growing around the forest massifs.

For investigation, in the spring of 2015, an experimental batch of 25 similar bee families was formed, in which young queens were introduced. The bee families of this batch were grown and exploited until spring (March) 2018 without anti-varroa drug treatments and without changing the queens. In the experiment the evolution of natural varroa-resistance of bee colonies without human drug intervention was monitored. During the experimental period (March 2015 - March 2018), a series of indicators and morpho-productive characters for each bee family, wich characterize the natural varroa-resistance and productive performance were evaluated, such as: index of the natural fall of the *Varroa* parasite, the degree of infestation of bees with parasites, the degree of infestation of the brood with *Varroa,* queen prolificity, family strength, the amount of capped brood, wintering resistance of bee colony, the survival of bee families throughout the experiment and the production of honey accumulated in the nest.

The natural fall index of the *Varroa* parasite was assessed on the sticky board with greased paper, located in each hive for a 48 hours, after that the mites were counted.

To determine the degree of infestation of bees with the *Varroa* parasite, the Icing Sugar method was applied. For this, from the honey combs 40-50g of bees were taken of each bee family. The bees were placed in plastic recipients about 750 ml covered with a rare sieve cover through which they did not pass, being weighed with the container. Then, in a 150 ml plastic beaker, 5 tablespoons of powdered sugar were added and poured over the bee pot nets, shaking lightly so that the sugar bears the bees well. For 3 minutes the bee and sugar recipient was shaken from time to time. After this treatment, all mites detach the bees and fall into powder. Subsequently, the recipient was inverted and shaken on a 2.8 mm mesh sieve. After sifting, powdered a czut, and the mites remained on the sieve, being easily counted on a white foil. The bees left in the container were shaken in the family from which they were extracted. The degree of infestation of bees with the *Varroa* parasite was calculated by multiplying the number of mites, found in the jar to 10, and the obtained result was reported to the net bee mass in the recipient.

To determine the degree of infestation of the brood in the nest, a comb with capped brood in the young puppy stage (white or pink eyes) was selected, and about 50 cells were uncapped linearly, in which was then verified the presence or absence of *Varroa* mites. The number of cells infested with *Varroa* in relation to the number of investigated cells is the brood infestation degree, expressed as a percentage.

The queen prolificity was determined according to our methods (Cebotari et al., 2010) described in the Zootechnical norme regarding evaluation of bee families, the

growth and certification of genitor beekeeping material, approved by Government Decision no. 306 of 28.04.2011.

The amount of capped brood was determined with Netz frame (5x5 cm) of the surface occupied by capped brood, expressed in the number of Langstroth frames with the precision of tenths of the frame.

Strength of the bee family was determined by appreciating the number of compact occupied by bees intervals in the nest, expressed in tenths of frames. The wintering resistance of the bee families was assessed by the amount of surviving bees during wintering, using the data from the autumn revisions of the previous year and the spring of curent year. The ratio of the amount of bees after wintering to the amount of bees at the begining of the winter, expressed as a percentage, represents wintering resistance.

Survival of bee families has been evaluated in the spring of each year during the entire experiment period. The ratio of survived bee family in the spring of this year to the number of bee families existing at the start of the experiment, expressed as a percentage, shows the survival index of bee families.

Honey production was evaluated by the method of examining the amount of capped honey accumulated in the nest after each basic harvest and the conversion of the number of honey frames into kilograms.

Selection of the most valuable bee families in the breeding group was performed taking into account longevity, stability of queen prolificity, varroa resistance, winter resistance, colony strength and honey production.

The data obtained as a result of calculating the average value of morpho-productive characters and comparing the differences of two variables were statistically processed using the computerized software "STATISTICA-12" and evaluated their certainty, according to variation biometric statistics, by methods of Плохинский (1989).

## RESULTS AND DISCUSSIONS

The results of the research have shown that bee families, studied for three years of growth, without anti-varroa medication, showed a natural varroa resistance specific to the population of the ecotype formed in this area. From the beginning it should be mentioned that under these conditions, after the first year of experiment, 23 families out of the 25 have survived, which represents 92% (Table 1).

Table 1. Indices of natural *Varroa* resistance and bee family development characters at different times of the year

| Investigated characters | 08 August | 29 August | 19 September | 10 Octomber | 29 Octomber | 20 March of next year |
|---|---|---|---|---|---|---|
| Year 2015 (N=25) | | | | | | (N=23) |
| Natural fall of mites in 48 hours, mites / bee family | 15.6±1.4 | 15.9±1.3 | 15.9±1.2 | 15.1±1.3 | 14.1±1.3 | 5.3±0.3 |
| Degree of infestation of bees, mites / 10 g bees | 3.03±0.28 | 3.28±0.29 | 3.00±0.26 | 2.81±0.24 | 2.94±0.33 | 0.96±0.07 |
| Degree of infestation of brood, % of infested cells | 29.9±2.4 | 34.4±2.8 | 38.2±3.1 | 37.3±4.3 | - | 8.0±0.5 |
| Queen prolificity, eggs / 24 h | 2370±60 | 2500±59 | 2080±55 | 700±78 | - | 1598±24 |
| The amount of capped brood, no. frames with the brood | 4.74±0.12 | 5.00±0.12 | 4.16±0.11 | 1.40±0.16 | - | 3.20±0.05 |
| Colony strength, no. frames with bees | 14.8±0.2 | 11.8±0.2 | 8.1±0.1 | 6.7±0.2 | 6.1±0.2 | 5.0±0.1 |
| Year 2016 (N=23) | | | | | | (N=16) |
| Natural fall of mites in 48 hours, mites / bee family | 5.2±0.6 | 7.7±0.6 | 8.5±0.9 | 7.2±0.9 | 7.1±0.8 | 2.6±0.7 |
| Degree of infestation of bees, mites / 10 g bees | 1.60±0.23 | 1.06±0.19 | 1.09±0.20 | 0.74±0.17 | 0.80±0.14 | 0.97±0.16 |
| Degree of infestation of brood, % of infested cells | 4.1±0.7 | 5.4±0.5 | 5.7±0.9 | 4.5±0.9 | 5.3±1.2 | 3.4±1.2 |
| Queen prolificity, eggs / 24 h | 1719±31 | 2091±44 | 1391±37 | 650±53 | 119±32 | 909±65 |
| The amount of capped brood, no. frames with the brood | 3.44±0.06 | 4.18±0.09 | 2.78±0.07 | 1.30±0.11 | 0.24±0.06 | 1.82±0.13 |
| Colony strength, no. frames with bees | 13.6±0.2 | 9.5±0.2 | 7.8±0.2 | 6.4±0.2 | 5.7±0.3 | 5.1±0.2 |

| Year 2017 (N=16) | | | | | | (N=11) |
|---|---|---|---|---|---|---|
| Natural fall of mites in 48 hours, mites / bee family | 3.1±0.7 | 10.8±1.8 | 22.7±2.7 | 21.6±4.8 | 14.5±5.0 | 5.1±2.1 |
| Degree of infestation of bees, mites / 10 g bees | 1.55±0.14 | 2.36±0.24 | 4.54±0.69 | 4.81±1.00 | 4.03±1.03 | 1.04±0.21 |
| Degree of infestation of brood, % of infested cells | 4.1±1.6 | 11.4±3.3 | 17.2±3.9 | 9.0±2.7 | 4.7±1.5 | 4.9±1.1 |
| Queen prolificity, eggs / 24 h | 1672±67 | 1969±99 | 1953±103 | 234±48 | 89±0 | 1355±76 |
| The amount of capped brood, no. frames with the brood | 3.34±0.13 | 3.94±0.20 | 3.91±0.21 | 0.47±0.10 | 0.18±0.0 | 2.71±0.15 |
| Colony strength, no. frames with bees | 8.8±0.1 | 7.9±0.3 | 7.0±0.3 | 5.9±0.4 | 5.8±0.4 | 5.6±0.2 |

After the second year of experiment, 16 families of bees survived, representing 64% of the 25. After the third year of the experiment, only 11 bee families survived, which is 44%. These colonies were most resistant in the to natural growth conditions, without anti-varroa antropic intervention. We found out that in the first year of the experiment the bee families removed *Varroa* mites quite actively, which could be noticed on the sticky board, especially installed in the hive. Thus, the index of natural fall of mites varied in the first year from 14.1 to 15.9 mites in 24 hours/bee family. The number of naturally fallen mites in the second year of experiment decreased by about two times, being from 5.2 to 8.5 mites in 24 hours/bee family, and then again increased in the third year of experiment, reaching the level of the first year and being in different periods of the year from 3.1 to 22.7 mites in 24 hours/bee family.

It was found that over the three years of experiment, the index of natural *Varroa* mite fall had the highest values each year in September, constituting 15.9±1.2 in 2015, 8.5±0.9 - 2016 and 22.7±2.7 mites in 24 hours/bee family in 2017. We should be mentioned that, the natural fall of mites in the second year of experiment was lower than in the first year with 7.4 mites or 46.5% ($t_d$=4.9; P<0.001). In all research years, since March, the index natural *Varroa* mites fall was increasing until the end of September, after which a decrease was observed. If, in 2015, the natural fall of mites increased insignificantly (only by 1.9%) during August-September, then in the following year (2016) it increased by 63.5% and in the the third year (2017) - by 7.3 times. After September, the index of natural Varroa mites fall decreases by the end of October by 11.3% - in the first year, by 16.5% - in the second year and by 36.1% in the third year of experiment. We found that in bee families that remained alive in the third year of experiment, the index natural mites fall during September-October is higher compared to first-year bee families by 6.8-6.5 mites, or 42.7-43.0% ($t_d$=2.3-1.3; P<0.05 and P>0.1) and, compared to the second year bee families, by 14.1-14.4 mites, or 2.7-3.0 times ($t_d$=4.9-3.0; P<0.001 and P<0.01).

On the basis of these data, we can conclude that at the bee families that remained alive in the third year of experiment, whithout anti-varroa drug treatment, the instinct of natural cleansing (discharging) by *Varroa* mites is quickens, compared with the first bee families and two experiment. Or, after three years of experimentation, we can say that the greatest chances of survival, given the lack of anti-varroa treatments, have those colonies where the cleasing behavior instinct against *Varroa* mites is more developed. We believe that they have a higher natural varroa-resistance.

Analyzing the degree of infestation of bees with *Varroa* mite we found that, every year after the wintering period, at the first spring revision (March), the degree of infestation of bees with mites is small and constitutes 0.96-1.04 mites/10 g bees. However, as the air temperature rises, the degree of infestation of bees with *Varroa* mite increases, reaching values ranging from 2.81-3.28 mites/10 g of bees in 2015 in August, 0.74-1.60 mites/10 g bees in 2016 and 1.55 to 4.81 mites/10 g bees in 2017. In the first two years of experiment, the degree of bee infestation decreased, from 3.03-3.28 mites/10 g bees in August to 2.81-2.94 mites/10 g bees in October of 2015 and from 1.60-1.06 mites/10 g bees in August to 0.74-0.80 mites/10 g bees in October 2016. In the third year of experiment (2017), the degree of infestation of bees with *Varroa* mite increased significantly from 1.55-2.36 mites/10

g of bees in August to 4.81-4.03 mites/10 g bees in October. This demonstrates that, despite the fact that bees possess the natural cleasing behavior against *Varroa* mite, the invasion of this aggressive parasite under natural conditions, without drug interventions, extends predominantly. In the autumn of the third experimental year, at the beginning of October, the infestation rate of bees with *Varroa* mite reached peak values, which averaged over the total remaining bee families (N=16) 4.81±1.00 mites/10 g bees. This leads to inhibition of the main physiological functions of reproduction and development of bee families.

Analyzing the degree of infestation with the *Varroa* mite of capped brood cells, we found that during the year the evolution of this indicator is increasing from March (spring revision) to August-September, with a further decrease in the end of October. The experiment data showed that the highest infestation rate of the brood was established in the first year. Of the total brood cells investigated in the first year, they were infected with the parasite *Varroa* 34.4% at the end of August, 38.2% in September and 37.3% at the beginning of October. In the second year of experimentation, the degree of infestation with the *Varroa* mite of brood cells, in the remaining in the experiment bee families (N=23), decreased to 4.1-5.7%. In the third year of the experiment, in the remained alive bee families (N=16), this indicator rose again to 4.7-17.2%. During 3 years of research it was established that the infestation of brood cells with *Varroa* mite reached the highest values in September. Thus, the *Varroa* infestation degree of brood cells in September 2015 was higher by 8.3% compared to the beginning of August ($t_d$=2.1; P<0.05). In 2016, infestation with the Varroa mite of cell brood in September had a tendency to be higher than that at the beginning of August. In 2017, the infestation degree with *Varroa* mite of brood cell in September was significantly higher than at the beginning of August, with 13.1% ($t_d$=3.1; P<0.01). Based on the obtained experimental data, we can say that despite the fact that the honey bees possess the instinct of the hygiene preferentially remove of reproducrive mites, also called *Varroa sensitive hygiene* - VSH, however, without the anti-varroa treatments, infestation of the brood progresses threateningly in the third year of experiment. Thus, the degree of infestation with *Varroa* of brood cells in August 2017 was higher with 6.0% ($t_d$=1.8; P<0.1) compared with August 2016, and that of September 2017 was significantly higher than in the same month of 2016, by 11.5% ($t_d$=2.9; P<0.01).

Increasing the *Varroa* mite infestation of bees and brood lead, finnaly, to the weakening of the vital activity of bee families, expressed by diminishing of the prolificity of the queens, the amount of capped brood and the straigth of the colonies.

Thus, the queens prolificity during three years of experiment decreased significantly in August from 2370 eggs/24 hours in the first year, to 1672 eggs/24 hours in the third year of experiment, being lower by 698 eggs/24 hours, or 29.5% ($t_d$=7.7; P<0.001). In September, the prolificity of queens in the third year of experiment continued to decline. In October, the queen prolificity decreased considerably from 700 eggs/24 hours in the first year of experiment, to 234 eggs/24 hours in the third year of that period, being less in the last year with 466 eggs/24 hours, or 66.6% ($t_d$=5.1; P<0.001).

Over the three years of experiment, the amount of capped brood, grown in the nest of the colonies in August, decreased from 4.74 frames with brood in the first year up to 3.34 frames in the last year of experiment. The decrease of this morphological character was in this period on average with 1.4 frames with brood, or 29.5% (td = 8.2; P <0,001). In September, the amount of capped brood in the third year of experiment continued to decline. In October, the quantity of capped brood in the family nest decreased from 1.40 frames in the first year of experiment to 0.47 frames in the third year of this period, being lower in the last year by 0.93 frames, or 66.5% ($t_d$=4.9; P<0.001).

As a result of the decrease of the queens prolificity and the quantity of capped brood, the number of bees (family strength) hatched in the nest has also suffered. Thus, bee family strength over the entire experiment period declined significantly in August, from 14.8 bee frames in the first year of the experiment to 8.8 frames in the third year of experiment, the decrease being 8.6 frames, or 40.5% ($t_d$=39.3;

P<0.001). In September, the strength of bee colonies has fallen from 8.1 bee frames in the first year of experiment to 7.0 bee frames in the third year, declining by 1.1 bee frames, or 13.6% ($t_d$=3.4; P<0.01). In October, the decrease in bee family strength continued throughout the experiment period, constituting 0.8 bee frames, or 12.0% ($t_d$=1.8; P<0.1).

Same time, despite the stress conditions, in which bee families have been subjected during three years of experimentation, without anti-varroa drug treatments and without changing of queens, however, some of the colonies (11 families) survived until the spring 2018. We consider that these bee families have a natural resistance that allowed them to survive in extreme conditions. From this livestock, 5 breeding bee families were selected in the breed batch, with the numbers: 1, 7, 8, 33 and 49, intended for the collecting of genetic material and directed growth of the queens. We consider that these bee families have the most valuable indices of morpho-productive characters and natural varroa resistance, compared to the other families in the experimental batch (Table 2).

Table 2. The value of the morpho-productive characters and *Varroa* resistance of bee families selected in the breed batch, compared to the experimental group

| Investigated characters | August 5015 (N=25) | | September 2017 (N=16) | | March 2018 (N=11) | |
|---|---|---|---|---|---|---|
| | Experimental batch N=20 | Breed batch N=5 | Experimental batch N=11 | Breed batch N=5 | Experimental batch N=6 | Breed batch N=5 |
| Natural fall of mites in 48 hours, mites / bee family | 15.6±1.4 | 13.8±3.4 | 25.5±3.2 | 16.4±4.2* | 5.0±3.0 | 5.2±3.2 |
| Degree of infestation of bees, mites / 10 g bees | 3.03±0.28 | 2.38±0.69 | 5.30±0.80 | 2.88±1.06* | 1.11±0.27 | 0.94±0.35 |
| Degree of infestation of brood, % of infested cells | 29.9±2.4 | 25.6±4.2 | 20.5±5.1 | 10.0±4.7 | 4.7±1.8 | 5.2±1.4 |
| Queen prolificity, eggs / 24 h | 2370±60 | 2600±169 | 1863±123 | 2150±169 | 1292±119 | 1530±43* |
| The amount of capped brood, no. frameswith the brood | 4.74±0.12 | 5.20±0.34 | 3.73±0.24 | 4.30±0.34 | 2.58±0.24 | 3.06±0.09* |
| Colony strength, no. frames with bees | 14.8±0.2 | 15.4±0.5 | 6.7±0.4 | 7.4±0.3 | 5.5±0.4 | 5.8±0.2 |
| Wintering resistance, % | 80.0±1.6 | 78.8±3.6 | 79.8±0.9 | 82.3±1.2* | 89.0±1.1 | 89.0±1.6 |
| Survival of bee families, % | 100 | 100 | 64.0 | 100 | 44.0 | 100 |
| Honey production, kg | 44.7±0.9 | 46.3±2.2 | 35.3±0.7 | 37.7±0.6** | - | - |

Remarc: * - P<0,05 compared to the experimental batch.

At the basis of the principles of selection of bee families for breeding batch, the criteria of the development of their main morpho-productive characters have been set, such as: survival under natural maintenance conditions without antivarroa drug treatments and without changing the queen, stability of the queens prolificity over several years, the amount of capped brood in the nest, the colonies strength (the amount of bee present in the nest), the wintering resistance and the production of honey accumulated in the nest at the base harvest.

From the presented data it can be seen, at the end of the experiment, which coincided with the spring revision (March 2018), the bee families of the breed batch (N=5) significantly exeeded the families from experimental batch (N=6) after the queens prolificity - 238 eggs/24 hours, or 18.4% ($t_d$=1.9; P<0.05) and the amount of capped brood - by 0.48 frames with brood, or 18.6% ($t_d$=1.9; P<0.05). Same time, in the bee families of the breeding batch, the degree of infestation with mites was lower, but the index of natural mites fall and the colonies strength - higher. These bee families had a significantly higher honey production in 2017, compared to the families in the experimental group (N=11), with 2.4 frames, or 6.8% ($t_d$=2.6; P<0.01), as well as increased wintering resistance, ranging from 82.3-89.0%.

Analyzing the evolution of the morpho-productive characters of bee families in the breed group, compared to the families in the experimental group, during the three years of experiment, we found that the first had tendencies to overcome the level of character manifestation throughout the period. In our opinion, this is due to the fact that the bee families selected in the breed batch have a

strong natural resistance to *Varroa*. This allows us to predict that from the genetic material of these colonies will get a descendents with boosted potential of natural varroa-resistance and high productivity skills.

## CONCLUSIONS

The natural varroa-resistance of honeybees of the Moldovan Codri population allows survival of 44% of the bee families, maintained for three years in natural conditions, without anthropic anti-varroa drug treatments and without changing the queens.

During the experiment (3 years), it was established that the index of natural fall of *Varroa* mites in bee families is increasing from spring until the end of September, after which the decrease is occur until the end of October.

The index of natural mitesfall in bee families was higher in the first year of the experiment (14.1-15.9 mites per 24 hours/bee family), decreasing in the second year of experiment (up to 5.2 -8.5 mites in 24 hours/bee family), then increased significantly in the third year of experiment, reaching a maximum of 22.7 mites in 24 hours/bee family. In the bee families that survived in the third year of experiment, the index of natural *Varroa* mite fall during September-October was higher, compared to the first-year bee families by 42.7-43.0% ($t_d$=2.3-1.3; P<0.05 and P>0.1) and 2.7-3.0 times compared to the bee families of the second year ($t_d$=4.9-3.0; P<0.001 and P<0.01). After three years of experiment, in which no anti-varroa drug treatments and no change of queens in bee families were applied, can survive only colonies with developed cleasing behavior instinct against *Varroa* mites.

Despite the fact that bees possess cleasing instinct against *Varroa* mite, however, under natural conditions without drug intervention, the degree of infestation of bees with mites increases, reaching peak values in the third experimental year up to 4.81±1.00 mites/10 g of bees, which leads to the inhibition of the main physiological functions of reproduction and development of bee families.

In the absence of anti-varroa medication, infestation of capped brood cells with *Varroa* mite progresses menacing in the third year of experiment, being significantly higher in September by 11.5% ($t_d$=2.9; P<0.01), compared with the same month of the previous year, which leads to a decrease in the quantity of capped brood, of the colonies strength and the weakening of the vital activity of the bee families as a whole.

Maintenance of bee families over three years under natural conditions without anti-varroa treatments and without change of queens leads to a significant decrease in prolificity of queens in October from 700 eggs/24 hours in the first year to 234 eggs/24 hours in the third year, being less in the last year with 466 eggs/24 hours, or 66.6% ($t_d$=5.1; P<0.001).

Out of the 11 bee families, which survived in natural conditions for three years, without anti-varroa drug treatments and without changing the queens, 5 families were selected in the breeding batch for reproduction.

The bee families of the breed batch significantly exeeded the families from experimental batch after the queens prolificity – with 18.4% (P<0.05), the amount of capped brood – with 18.6% (P<0.05) and honey production – with 6,8% (P<0,01). At the same time, in the bee families of the breeding batch, the degree of infestation with mites was lower, the index of natural mites fall and the colonies strength – higher, as well as the boosted wintering resistance, ranging from 82.3-89.0%.

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# EVALUATION OF HETEROSIS EFFECT ON MILK PRODUCTION AT HALF-BREED FEMALES RESULTED BY CROSS-BREEDING OF CARPATINA BREED WITH OTHER BREEDS

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***Abstract***

*The effectuated research aimed to analyse the improvement possibility of milk production at goats on the basis of the application of a cross-breeding programme between Carpatina breed domestic females with male reproducers belonging to some more productive breeds and which proved that have a higher capacity for improving the milk production at half-breed populations. Biological material was represented by goat populations which belong to different breeds, respectively autochthonous Carpatina breed (LM) as well as other half-breed groups of first generation resulted by cross-breeding domestic breed females with male reproducers belonging to Anglo-Nubian breed ($L_1$) and French Alpine ($L_2$). To compare the results and ameliorative effect due to cross-breeding, especially on milk production capacity, were utilise3d data obtained after application of performances productive control. The applied working methods for estimation of milk production were based on technical norms which regulate effectuation of official control for milk production at farm animals accepted in experimental technique and recommended by International Committee for Animal Recording. Based on applied productive control was observed that between $L_1$and LM exist a real difference for total milk production of 17.74 kg milk, difference which have a high degree of statistical significance for P>1%. In case of cross-breeding of batch $L_2$ in comparison with autochthonous goats the real difference between level of mean milk production is 23.30 kg milk being distinct significant for P>1%.*

***Key words****: Carpatina goat, milk goat, improvement, heterosis*

## INTRODUCTION

Role and importance of goat rearing represent a basic activity for many farmers. At world level is estimated that nowadays will be around 703.39 millions of goats, from which almost 95% of them are in developing countries from Asia and Africa (Zaman et al., 2002).

Based on an ample study, Pulina et al. (2018), show that at world level breeders of small ruminants for milk production represent almost 21% and produce around 3.5% from world milk production, from which 2.7% are processed in industrial units. The highest rate of goat breeders is founded in temperate and subtropical areas from Asia, Europe and Africa. In Europe goats are reared for milk and are concentrated around Mediterranean Sea and in regions from Black Sea, where dairy products are typical ingredients for humans and are part of daily diet.

Regarding productions obtained from goats at world level but also in European countries could be observed an increasing trend for cheeses obtained from milk processing (Pascal et al., 2017). To face the demands imposed by market all the countries in which goats are reared must find technical solutions for sustain the increasing of milk production based mainly on application of some breeding programmes and less on increasing of flocks.

In this context, the effectuated research aimed to enlighten the productive capacity as effect of heterosis manifested in expression of lactogen capacity at two populations of half-breed females.

## MATERIALS AND METHODS

Biological material was represented by goat populations which belonged to different breeds, respectively autochthonous breed Carpatina (LM) as well as some groups of first generation resulted by cross-breeding of autochthonous breed females with male reproducers belonging to Anglo-Nubian ($L_1$) and French Alpine ($L_2$) breeds.

Option face to those breeds was sustained by the fact that in many scientific works is shown that those ones have a high degree of genetic combination and proved that have a pronounced influence in breeding of characters specific for milk production (Serradilla, 2001).

At the moment of first lactation debut to eliminate the influence of some external factors on productive performances were established homogenous batches belonging to all studied populations, each of those being formed by 25 females.

Also, maintenance, nourishment conditions as well as the evaluation period for the aimed characters were realised in the same time interval.

To compare the results and the breeding effect due to cross-breeding, mainly on milk production capacity, were utilised data obtained after application of productive performances control.

The working methods applied for estimation of milk production were based on technical norms which regulate effectuation of official control for milk production at farm animals accepted in experimental technique and by International Committee for Animal Recording.

$$\text{Milk yield [kg]} = L_1 . int_1 + \sum_{i=2an} \left( \frac{L_i + L_{i-1}}{2} . int_i \right) + L_n . 14$$

where:

$L_1$ = milk yield in 1st monthly test;

$L_i$ = milk yield in $i^{th}$ monthly test (i = 1,..., n);

$L_n$ = milk yield of the last test;

$int_1$ = number of days from kidding to 1st monthly test;

$int_i$ = number of days between monthly tests (i-1) and i (i = 1,...,n);

n = total number of monthly test for a specific animal.

Data were statistically evaluated with the algorithm REML (REstricted Maximum Likelihood), which provides the achievements of the statistical parametric estimators within the normal range.

Estimation of heterosis effect was realised using bi-variant mixed models inside breed and between populations included in research and was based on analysis and interpretation of obtained data at official control applied for evaluation of lactogen potential.

The effect due to application of a cross-breeding between populations with a different genetic structure to obtain half-breed populations was enlightened by heterosis coefficient, calculated in according with the formula:

$$H_{F1}\ \% = \frac{\overline{X}_{F1} - \overline{X}_P}{\overline{X}_P} \text{ x } 100;$$

in which: $\overline{X}_{F1\ \%}$ = character mean at half-breed females; $\overline{X}_P$ = character means at parents.

## RESULTS AND DISCUSSIONS

Effect due to manifestation of heterosis could represent a maintain way of the characters targeted for change at an upper level and on a greater period of time when are manifested as an unexpected deviation face to mean of those two parental populations (Dikerson 1975; Wakchaure et al., 2015).

In many areas of the world, to shorten the interval in which are obtained new goat generations, more performing, are utilised cross-breeding between local goats with other goat breeds, the most utilised ones being: Sannen, French Alpine, Anglo-Nubian, Toggenbourg and others. Interest face to those breeds, which were formed and evaluated in other areas, is due to the fact that have a high adaptability degree to the new pedo-climatic conditions and have remarkable qualities regarding amelioration of milk production (Pascal et al., 2017). In the context of goal the utilisation of those breeds for cross-breeding with the local ones is for capitalization of effect due to heterosis which is manifested at a high level in the first generations or for being included in formatting schemes of new breeds, with productive valences superior to the local breeds.

So in animals' rearing by cross-breeding could be achieved a safe way for increasing of performances for some characters because maximized pheno-typical effects resulted by application of pairing between individuals, populations, lines, which are gene-type

different. In these conditions the main consequence of unrelated pairings is represented by increasing of frequency of heterozygous level in descendant generation, at the same time with decreasing of homozygous level and as a consequence exactly reverses to related pairing system.

In the same way if homozygous produced by inbreeding causes pheno-typical effects for inbreeding depression, heterozygous determine apparition of a reverse pheno-typical effect named heterosis effect (Negruţiu et al., 1975; McAlister, 2002). The cause of heterosis is due to exercised actions of some non-additive genes (dominant, supra-dominant and epitasis) and isn't observed heterosis for features governed by action of additive gene.

Evaluation of heterosis effect for milk production, as effect of realised cross-breeding, represented the main target of research. Were subjected to appreciations half-breed females from first generation ($F_1$) because it is well known the fact that this phenomenon appear and has a more intensively manifestation at first half-breed generation. This aspect is due to the fact that after cross-breeding in gene-type of new populations are established some genetic interactions which have the role to allow an increased influence on the externalization way of certain productive and reproductive characters (Pascal, 1997). Value and expression of a quantitative character is determined by the effect of several gene types which when are combined in different forms and gene-types could give different effects (Pipernea, 1979; Popescu Vifor, 1978).

To estimate the effect due to heterosis were utilised data collected after application of productive control and by statistical processing was observed a manifestation tendency for positive heterosis type for milk quantity at both half-breed populations. This affirmation is sustained by the fact that both batches of half-breed females provided milk productions higher with 10.44% for the ones resulted by cross-breeding with Anglo-Nubian breed bucks and with 11.56% at batch obtained with French Alpine (figure 1).

In case of analysis effectuated to determine the breeding effect for milk production obtained from first half-breed females' generation resulted by cross-breeding of Anglo-Nubian breed bucks with autochthonous females could be observed a real difference for total milk production, per controlled lactation, of 17.74 kg milk, difference which have a higher degree of statistical significance for P>1%. In case of cross-breeding of French Alpine breed bucks with autochthonous females the real difference between level of milk mean production is 23,30 kg milk being distinct significant for P>1% (Table 1).

All those differences were recorded in conditions in which all microclimate, technological, maintenance and nourishment parameters were identical. Also, to eliminate the influence of season on performances obtained by females from first generation (F1) research was carried out simultaneously in the same time interval.

Into a similar study in which local goats Carpatina was cross-breed with Saanen breed bucks at half-breed females $F_1$ mean milk production was superior with almost 30% face to total mean quantity obtained from a batch formed from females belonging to the local breed (Taftă, 1996).

Regarding the effect due to heterosis could be observed that this one have a higher manifestation on productive performances at females from $F_1$ resulted by cross-breeding of Carpatina breed females with bucks belonging to French Alpine breed. In according with this observation could be affirmed that French Alpine breed have a better degree for genetic combination with Carpatina domestic breed.

The effect of manifested heterosis in this case could be explained by the fact that in expression of lactogen capacity at this two half-breed females' generations took place an increasing of the number of involved loci, consequence of genetic differences between those breeds regarding the frequency of relevant alleles at the level of those loci.

Table 1. Heterosis effect for milk quantity

| Gene-type | n | Character | MU | Milk mean production at Carpatina | Milk mean production at $F_1$ half-breeds | Difference due to heterosis | | Signific. difference |
|---|---|---|---|---|---|---|---|---|
| | | | | | | absolute kg | $\frac{\overline{X}_{F_1} - \overline{X}_P}{\overline{X}_P}$ x 100 | |
| Anglo-Nubian x Carpatina | 25 | Milk quantity | kg | 153.14 | 169.88 | 17.74 | 11.66 | ** |
| French Alpine x Carpatina | 25 | Milk quantity | kg | 178.25 | 201.55 | 23.30 | 13.07 | ** |

Note: * significant (P<5%); ** distinct significant (P>1%).

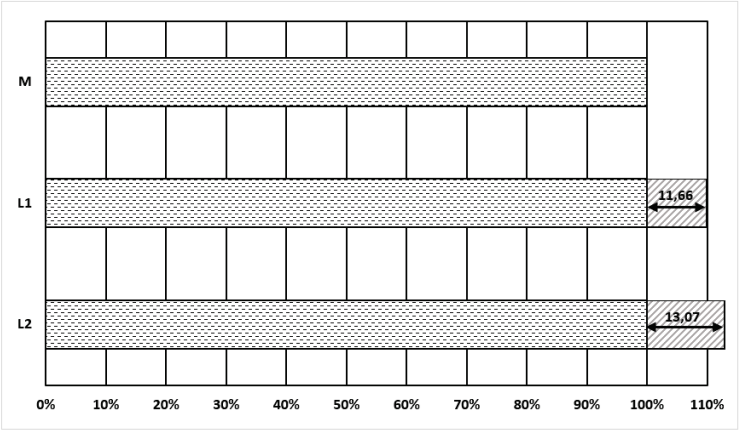
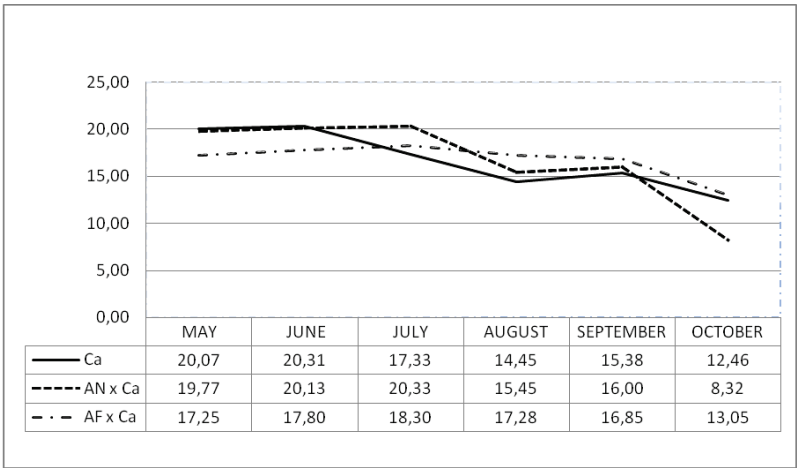



Figure 1. Graphical design of effect due to heterosis on milk production




| | MAY | JUNE | JULY | AUGUST | SEPTEMBER | OCTOBER |
|---|---|---|---|---|---|---|
| Ca | 20,07 | 20,31 | 17,33 | 14,45 | 15,38 | 12,46 |
| AN x Ca | 19,77 | 20,13 | 20,33 | 15,45 | 16,00 | 8,32 |
| AF x Ca | 17,25 | 17,80 | 18,30 | 17,28 | 16,85 | 13,05 |

Note: Ca = Carpatina; AN x Ca = F1 Anglo Nubian with Carpatina; AF x Ca = F1 French Alpine with Carpatina

Figure 2. Rate of monthly production from total quantity obtained in controlled lactation (%)

The existence of a higher level for heterosis manifestation of first generation females resulted by cross-breeding of Carpatina breed goats with French Alpine bucks is due to the fact that between them exist a greater genetic difference, aspect which induce also a higher genetic frequency and implicit a more intense expression of effect due to heterosis.

Also on the base of those data we could affirm that application of cross-breeding represent a more rapid and efficient solution to obtain some new type populations at which to be observed an improvement of characters which require a longer time for improving from pure breed selection and by application of some

breeding programmes based on selection (Popa et al., 2012).

The goals aimed in the applied research are into the international tendency by which it is desired to improve the genetic quality of local breeds based on application of breeding programmes or by application of amelioration cross-breeding at which to participate genitors of breeds which proved that have the improvement capacity for specific characters of basic productions of goats, especially the milk one. Connected with this, in 2001, Serradilla show that performing breeds are introduced in other areas for replacing the local ones which are less productive or to be utilised in amelioration cross-breeding for improving their production performances. Comparing the obtained productions from exotic breeds, local and transversal is always difficult due to strong management differences of all information regarding environment and rearing technologies conditions and breeding methods.

Also, application of cross-breeding to obtain the effect due to heterosis is more pronounced when the aim is represented by improving of features with a low heritability, such as milk production, resistance and fertility.

On the based of effectuated observations we noticed that heterosis effect is manifested also by a better homogeneity, maturity and uniformity for the majority of characters and provide a higher growing intensity at kids being in the first neonatal periods. Higher precocity, an increased ecological plasticity and superior quality are also, important characteristics for hybrid vigour.

In figure 2 is presented the level of monthly production based on determination of rate from obtained milk production from each batch in the lactation subjected to control. From analysis could be observed that at those two batches formed by half-breed females, in each month the obtained milk quantity is superior in comparison with the obtained data for the batch formed only by females from Carpatina local breed.

In case of those two batches formed by half-breed females of first generation ($F_1$) it could be observed that amplitude of lactation curve have a reduced decreasing, confirming that those ones have a superior milk production face to the local breed.

## CONCLUSIONS

By the fact that at both half-breed batches is observed a manifestation of heterosis positive type for obtained milk quantity at first lactation we could affirm that the aim of research was fulfilled and the participant breeds were correctly chosen.

However, because we are speaking about different gene-type also the effect due to heterosis manifested for milk production was different. To justify this affirmation from the obtained data could be observed that at batch represented by half-breed females $F_1$ resulted from cross-breeding of Capatina local females with bucks belonging to French Alpine breed the value of determined heterosis was higher with 1.41 and had a high degree of statistical significance.

Batch formed by half-breed females provided milk production higher 10.44% in case of the ones resulted by utilisation of Anglo-Nubian breed bucks and with 11.56% at batch obtained with French Alpine.

The fact that at half-breed females population obtained by utilisation at cross-breeding of French Alpine breed bucks was realised a production higher with 200 l into a normal lactation recommend the utilisation of those one for improving of this character at domestic goats.

Analysis of monthly mean productions by determination of rate from total milk quantity show that at those two batches formed by half-breed females from first generation ($F_1$) lactation curve had levels situated over the values determined at the batch formed only by local females, confirming the fact that those ones have a superior milk production face to the local breed.

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# RECENT DISCOVERIES IN *Varroa destructor* TREATMENT, PREVENTION AND PARASITE-HOST INTERACTION

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***Abstract***

*The European honey bee, Apis mellifera, as most insects of the world, is currently facing major difficulties and, particularly for honey bees, this results in significant colony losses. One of the most stressful factors for A. mellifera is the ectoparasitic mite, Varroa destructor. V. destructor invasions are largely treatable and preventable, however they bring forth great challenges to A. mellifera populations and breeders, making apiculture increasingly time and resource consuming. The global research in apiculture pathology is mostly focused on varroa. This review will be focusing on the recent literature in varroa treatment, prevention and parasite-host interaction.*

***Key words****: Apis mellifera, Varroa destructor, pathology, host-parasite interaction.*

## INTRODUCTION

*Varroa destructor's* original host was the Asian honeybee, *Apis cerana* which through coevolution was able to develop tolerance toward the mite. This trait was not carried over to the western honeybee, as the host-parasite relationship between *Apis mellifera* and *V. destructor* is relatively recent (Le Conte et al., 2007). A varroa infestation can, therefore, eradicate a colony of *A. mellifera* within 1-3 years, if left untreated. The lack of a balanced host-parasite relationship between the European honeybee and the mite has facilitated a world-wide spread of varroa, within a relatively short period of time. A codependent relationship with humans means they always receive adequate treatment against infestations, in order to keep the colonies healthy and productive. At first glance, this relationship may seem advantageous for the bees, because they don't have to suffer major losses or be weakened by varroa infestations, however at a second glance, such grooming hides a darker side. Namely, it breaks the cycle of natural selection which is required to become tolerant to pathogens. Thus, under constant treatment, any individual who can reproduce, regardless of its genetic sensitivity, is able to pass on its genes, which hinders the possibility of host adaptability. Examples of *V. destructor* resistant *A. mellifera*populations can be found all over the world.

Indeed, cases of *A. mellifera* resistance to *V. destructor* were found in most parts of the world (DeJong et al., 1997; Fries et al., 2006; Le Conte, 2007). These honey bee populations prove that through long-term exposure to the varroa mite, resistance can be developed.

This review will focus on recent discoveries in the host-parasite relationship of *Varroa destructor* and *Apis mellifera*, new treatment methods and the underlying mechanisms of resistance towards the mite.

## MATERIALS AND METHODS

### *VARROA DESTRUCTOR* AND VIRAL INFECTIONS

In addition to the numerous negative effects varroa directly inflicts upon *A. mellifera*, mite infestations are usually also associated with viral infections (reviewed by Tantillo et al., 2015). Recent research has helped shed light on varroa's role as a viral vector and how infections can become a contributing factor in colony losses.

Deformed wing virus (DWV) copy number in honeybee pupae is directly associated with the copy number in infesting *V. destructor* (Wu et

al., 2017). The presence of large DWV copies induces immunosuppession in the honeybee in order to more easily replicate (Di Prisco et al., 2016), which acts as an additional stressor and adds to the likelihood of a colony to perish.

Studies suggest that a longer phoretic stage does not necessarily mean a more successful reproductive cycle but that the longer the phoretic stage lasts, there is a higher chance of deformities to appear on the young honeybee. Additionally, DWV load increases with the time spent in the phoretic stage, thus leading to more frequent and severe infections (Piou et al, 2016).

DWV severity, transmitted by *V. destructor* can be dependent on the climate. Overt infections are much more common in temperate climates than they are in tropical climates, without any differences in infestation rates (Anguiano-Baez et al, 2016). This could happen in part because varroa is a better vector for viruses in temperate climates. This theory is supported by Giacobino et al. (2016), who showed that colonies in temperate climates had a much higher viral load compared to colonies in subtropical climate.

This study, however, also reports that varroa infestation levels were higher in temperate climate compared to tropical climate, as was the case for viral load. Currently there is no knowledge of DWV in honeybees in Australia (Roberts et al., 2017). This could be due in part because *Varroa destructor* has only recently been able to spread to this continent and because Australia's climate is partly tropical and mostly arid, which, as established above, are poor conditions for the DWV. The fact that *V. destructor* infestations are milder in Australia and usually doesn't lead to colony losses supports the idea that honeybee mortality is a result of multiple stress factors working together against the bees.

## RESULTS AND DISCUSSIONS

### FRESH INSIGHTS IN METHODS OF VARROA CONTROL

As far as varroa control goes, the most efficient and widely used methods consist of either synthetic 'hard' chemicals or plant based 'soft' chemicals (Rosenkranz et al., 2010). These treatments function as miticides against varroa and, although effective, they also bring numerous negative side effects for the honeybees, including mortality (Gregorc et al., 2018). Severity of these effects is dependent on the age of the bees and on the level of social interaction (Van Buren et al., 1992). An additional disadvantage to chemical treatments is that varroa can become resistant, which is why efficient management practices are equally as important in varroa control (Thoms et al., 2018). Environmental conditions seem to be the predominant factor in mite infestation levels, followed closely by beekeeper management (Giacobino et al., 2017).

No new active compounds against varroa were discovered in the past 25 years (Mutinelli, 2016), although some recent studies present promising results. Lithium salts were shown to completely eliminate varroa mites in caged environments, without affecting worker bee mortality as compared to untreated controls (Ziegelmann et al., 2018).

Plant extracts offer a great alternative to conventional chemical treatments. These "soft" chemicals offer a similar antiacaricidal effect and are potentially less toxic.

Fumigation with oregano essential oil can rid a colony of varroa within the first two weeks of treatment, while not showing toxic effects towards the honey bees. The constant output of essential oil through fumigation results in a more efficient treatment (Sabahi et al., 2017). Plant based extracts such as *Thymus algeriensis* essential oil also offer a great solution against varroa. This extract contains large quantities of thymol, which is a known antivarroa agent (Noureddine et al., 2016) and has been shown to reduce mite populations by 32.6%, without harming the honeybees (Kouache et al., 2017). Mild acaricide effect was shown in sage - *Salvia officinalis* L. (Lamiaceae) - essential oil (Bendifallah et al., 2018) and costic acid extracted from *Dittrichia viscosa* proved itself to be 80% as effective as commercial acaricides (Sofou, 2017).

In addition to good management practices and chemical treatments, the use of biotechnological methods, like the removal of drone brood (Wantuch and Tarpy, 2009) offers an efficient and cost effective solution against varroa. Irradiation of honeybee colonies did not

seem to influence varroa infestation levels and overall effectiveness in pest control could be described as mild, at best (de Guzman et al., 2019).

The use of *Stratiolaelaps scimitus*, a mite that feeds on small insects, showed promising effects against varroa infestations. This method isn't 100% safe though, since the mite also consumed honeybee eggs in lab conditions, but not in the hive (Rondeau et al., 2018) and treatment applied in late or early fall was not efficient in controlling varroa (Rondeau et al., 2019).

*Bacillus thuringiensis*is is virulent and pathogenic in small insects and acarids, including varroa (reviewed by Chandler et al., 2001), however, it does not affect the honeybee and can be used alongside conventional treatments for varroa control (Alquisira-Ramírez et al., 2017). Overall, bacteria, especially from the *Bacillus* and *Lactobacillus* genus, act as probiotics and bring important benefits for the honeybees by increasing the immune response, stimulating queen egg laying and significantly increasing honey yield (reviewed by Audisio, 2017)

Entomopathogenic fungi could also reduce varroadamage to honeybee brood by both infecting the parasite and preventing varroa-associated suppression of honeybee immunity. Three immune genes of the honeybee, hymenoptaecin, pUf68 and BlCh, are usually suppressed by varroa. When inoculated with *Metarhizium anisopliae* and *Beauveria bassiana*, varroa cannot suppress the expression of these genes (Hamiduzzaman et al., 2012).

The sensory limitations of the varroa mite can be used against it. Given the lack of sight, the varroa mite is dependent on chemoreceptors to find its next host (Dillier et al., 2006). By inhibiting the chemoreceptors, varroa will have difficulties in choosing the right host. One way in which olfactory detection can be inhibited is through the use of racemic compounds (Govardhana et al., 2016)

In addition to grooming and hygienic behaviors, honeybees were also found to change normal behavior in order to alleviate pathogenic pressures. *A. mellifera* colonies have been found to change foraging patterns as a response to pressure from varoosis. Colonies infested with *V. destructor* increased the number resin foragers, thus increasing the quantity of collected resin as a means of self-medication (Pusceddu et al., 2019).

## TREATMENT RESISTANT MITES

Chemical treatments offer the most effective solution for treating varoosis but they also bring forth multiple downsides amongst which toxicity for the honeybees, pollution of bee products and development of treatment resistance in varroa (Rosenkranz et al., 2010). While product pollution and toxicity are negligible in terms of severity and economic impact, the spreading of treatment resistant varroa mites could be disastrous for honeybee populations. The following part of the review will be focusing on recent scientific discoveries in resistance to treatment.

Evidence for resistant varroa populations has started to emerge at the end of the 20th century (Lodesani et al., 1995; Hillesheim et al., 1996; Milani, 1999) and continue to emerge to this day. Recent studies have helped shed light on resistance mechanisms. A link was found between two novel mutations at Leucine 925 of the Voltage-Gated Sodium Channel gene (L925M, L925I) and resistance to pyrethroids, tau-fluvalinate and flumethrin, in USA (Gonzales-Cabrera et al., 2016). Mutations at this residue were also found in Pyrethroid resistant mites from Southern England (Gonzales-Cabrera et al., 2013) and in the Czech Republic (Stara et al., 2018; Hubert et al., 2014). This mutation was found in 98% of mites that went through miticide treatment and in only 45% of non-treated individuals which means that when selective pressure is applied, mite populations can develop resistance to the treatment. A connection between point mutations at position 925 in the sodium channel gene and treatment resistance has been confirmed in a biological assay (Stara et al., 2019).

*Varroa destructor* is a highly inbred species, due to its reproductive mechanism. Genetic diversification only occurs once the varroa population grows, in the middle of the honeybee productive period, when brood cells are populated by more than one founders. Applying antivarroa treatments before this stage, when the varroa population is low and goes through a "bottleneck" could help with

fixating variants responsible for miticide resistance (Beaurepaire et al., 2017).

These findings are alarming considering the slow development of new control methods and the fast spreading of the mite. Hierarchical genetic variation can be found at a colony level, which indicates that varroa transmission doesn't only happen vertically from one generation to the next but also horizontally, between hives and apiaries (Dynes et al., 2016). Horizontal transmission is facilitated by varroa's capacity to quickly climb on its host (Peck et al., 2016). *V. destructor* has also been found in drone congregation areas, which increases the mite's transmission capabilities even further (Mortensen et al., 2018).

Luckily, though, bee populations have a few aces up their sleeves.

## WESTERN HONEYBEE RESISTANCE AGAINST VARROA

The oldest Western honeybee population resistant to varroa was recorded in 1997 by De Jong and coworkers. Twenty Italian honeybee colonies infested with varroa were brought in 1984 to the Island of Fernando de Noronha, off the coast of Brazil. They were genetically isolated, as to prevent genetic contamination and were left to face varroa without any treatment. This population survived the infestation and is still alive to this day (De Mattos et al., 2016).

The first experimental insight on *A. mellifera* resistance to *V. destructor* was brought forth in 2006 by Fries et al. After three years a *V. destructor* infested, untreated *A. mellifera* population of 150 colonies had an 80% mortality rate during winter. This rate decreased to 13% and 19% in the 5$^{th}$ and 6$^{th}$year respectively, while infestation levels in the fall also significantly decreased. This is a great example of adaptability by *A. mellifera* and *V. destructor*, and proves that coevolution is possible when selective pressure is applied.

Varroa surviving colonies also show a similar mortality rate when compared to treated colonies, at the expense of lower honey productivity (Le Conte et al., 2007).

When compared to control populations, *V. destructor* resistant colonies have a similar hygienic and grooming behavior but the reproductive success of varroa is significantly reduced (Locke and Fries, 2011). When compared to *A. mellifera,* mites infesting *Apiscerana* had similar reproductive initiation success, because infested individuals would be removed. Consequently, affected brood in *A. cerana*, was not able to reach maturity, supporting the idea that resistance is based on behavioral traits (Lin et al., 2018).

A *V. destructor* surviving *A. mellifera* population from Norway was analyzed in order to find traits which helped reduce the reproductive success of varroa. A 10% shorter than normal post capping period was found to differentiate resistant colonies from susceptible ones (Oddie et al., 2018). Spermatozoa capacitation in inseminated mites takes 5 days. This could explain, as the phoretic phase is not vital (Ruijter, 1987), why a shortened post capping period would be problematic for varroa (Häußermann et al., 2016).

## CONCLUSIONS

Although behavior traits seem to offer a complete explanation of defense mechanisms for varroa resistant honey bees (i.e. *Apis mellifera scutellata)*, most comparative studies link resistance to physiological traits. While it is currently unknown what the molecular basis for resistance against *V. destructor* is, studies suggest that interferences in the moulting hormone biosynthesis are a likely cause. Further research is needed to fully understand these mechanisms.

Additionally, in order for the two species, *A. mellifera* and *V. destructor,* to coevolve and create a balanced host-parasite relationship, selective pressure needs to be applied. The success of breeders in obtaining resistant *A. mellifera* populations should inspire global programs of resistance-based selection.

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# NUTRITION

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# THE WISDOM OF USING INSECTS AS ANIMAL FEED ON DECREASING COMPETITION WITH HUMAN FOOD

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***Abstract***

*This article presents a brief study on a wisdom of using insects in animal livestock especially as animal feed to reduce competition with human need. The aim of the study is to find out the recent situation of development conditions using insects as animal feed in relation to human food supply. The method used is a comparative study using primary and secondary data from various sources. The growth in the human population in many countries is relatively very fast, which means an increasing in human food needs. This increase in needs is absolutely necessary, followed by efforts to increase food production. On the other hand, the expected increasing in human population from 6.9 billion in mid-2011 to 9.3 billion in 2050 requires various breakthroughs in preparing sufficient food. The use of insects as animal feed is not functioning only as an alternative feed but is an option that could contribute to decreasing competition of food need in relation with human population numbers in the world that are raised in various parts of the world today. Livestock business like this is not only oriented to business but on efforts to build and have insight into the environment. The conclusion is that the development of good insect cultivation needs to be developed even with simple methods to be applied in farms today by considering environmental management aspects.*

***Key words****: insect, animal feed, human, competition.*

## INTRODUCTION

The use of insects for livestock is progressing as appeared in various countries today. In line with these changes, this use for livestock activities is mainly related to benefits as animal feed, besides that it is also convenient as immunogen as well as bio-degradation agents for livestock waste.

The beneficial insect species have begun to be used as alternative feeds (Toar et al., 2018) and were proven to improve livestock production performance, for example by using maggot from various species ofDiptera Order such as *Hermetia illucens* and *Musca domestica* (Sogbesan et al*.,* 2006; Veldkamp et al*.,* 2012) and the Order Coleoptera for example *Rhynchophorus phoenicis* f. from the family Curculionidae (Omotoso and Adedire, 2007).

The content of nutrients, especially protein, makes this alternative feed material attract an attention in the world of animal husbandry. Another reason of this resource is not commonly used as a human food source, even its utilization in livestock will reduce the use of feed ingredients which are also human food ingredients such as corn, soybeans, fish and so on. The policy of applying insects as food in Europe has progressed. Fernandez (2016) states that: "recent changes to European regulations may be a sign that insect protein will soon be entering the animal feed market". This policy could be considered as entry point for insect rearing to fulfil the need of protein insect as animal feed in Europe and as its consequence is to minimize the use the feed raw material as human food.

## MATERIALS AND METHODS

A comparative study approach by using primary and secondary data from various literature sources was used to review the current conditions for application of insect cultivation in livestock development, which affected an impact on reducing the use of

animal feed ingredients that competed with human food needs.

## RESULTS AND DISCUSSIONS

Global challenges in improving the quality and quantity of livestock production to meet world food needs, especially in Asia and Africa (Van Huis A, 2013) moreover the consumption of livestock products will increase up to 70%. The use of insects for livestock can be one of the keys to the development of food from animal products in terms of nutrition and fodder (Stammer, 2105), health livestock (Rumokoy et al., 2015).

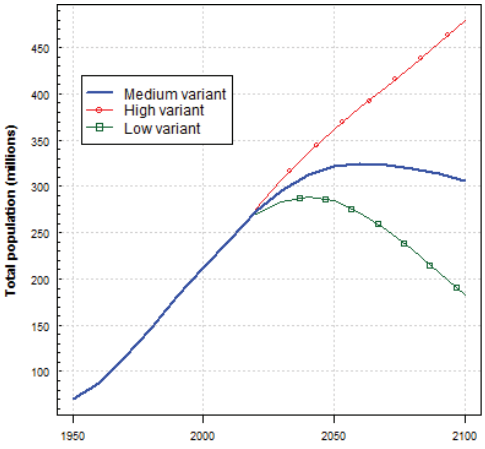


Figure 1. The population in Indonesia.
Sources: World Population Prospect in Indonesia (United Nation, 2017)

Population growth in many countries continues to grow strongly, for example in Indonesia. As shown in Chart 1 in 2019, it reached 260 million, an increase of about 40 million over 2000, but less than 220 million. Even though Indonesia is an agrarian country, to meet national food needs, it is necessary to import foods such as rice from abroad (Rahayu, 2018), even if national food security experiences positive changes (Tarigan, 2018). In a situation where a country still has a poverty rate whose number cannot be ignored, various policies must be applied to anticipate food problems.

The important effort in reducing hunger and malnutrition could be realized by promoting food production. The graph in Figure 2 shows that the population in China has been estimated to reach around 1.4 billion habitants in 2018 according to the United State (2017) followed by various strategies and policies to meet their food needs following the development of the population which tends to increase, among others develop livestock activities and production as reported by McMillan (2018).

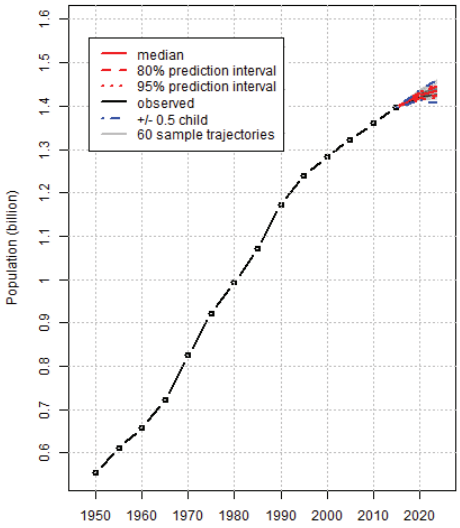


Figure 2. The Total Population in Chine.
Sources: United Nation (2017)

Other sources stated that medium variant of the 2010 Revision of World Population Prospects, the world population was expected to increase from 6.9 billion in mid-2011 to 9.3 billion in 2050 and to reach 10.1 billion by 2100 (United Nation, 2011).

The use of insects as an alternative food contributes to replacing conventional ingredients, thus minimizing the use of food products as livestock feed, such as types of cereals for example maize, rice, wheat and sorghum.

In addition, fishmeal, which is used as the main source of protein for animal feed, can be replaced by insect meal proteins.

The Altech Global Feed Survey, released in January 2017reported that world feed production for the first time exceeded one billion tonnes.

These numbers are related to the number of animals raised. The larger the human population, the greater the number of animal livestock to meet the food consumption needs of these farms.

Table 1. Livestock Slaughtered Number in Indonesia

| Livestock | 2013 | 2014 | 2015 | 2016 | 2017 |
|---|---|---|---|---|---|
| Beef cattle | 1.326.395 | 1.088.140 | 1.207.170 | 1.163.459 | 1.114.748 |
| Buffalos | 41 974 | 36 145 | 34 960 | 37 797 | 32 909 |
| Horses | 3 368 | 3 358 | 3 292 | 3 162 | 3 094 |
| Goats | 274 943 | 211 590 | 212 589 | 186 628 | 193 649 |
| Sheep | 142 736 | 93 578 | 99 987 | 93 342 | 107 704 |
| Pigs | 538 101 | 458 153 | 474 277 | 546 650 | 518 602 |

Source: BPS (2018)

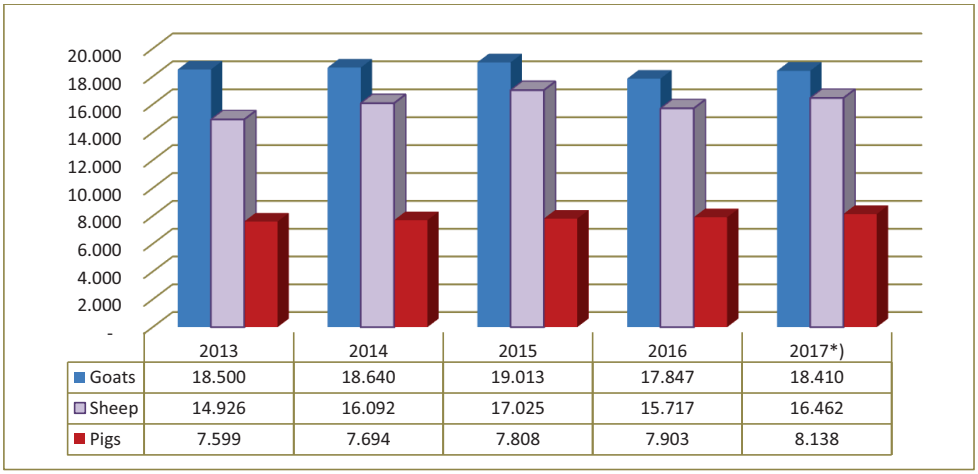


Figure 3. Population of Small Livestock (x 1,000 heads) in Indonesia.
Source: Direktorat Jenderal Peternakan dan Kesehatan Hewan (2017)

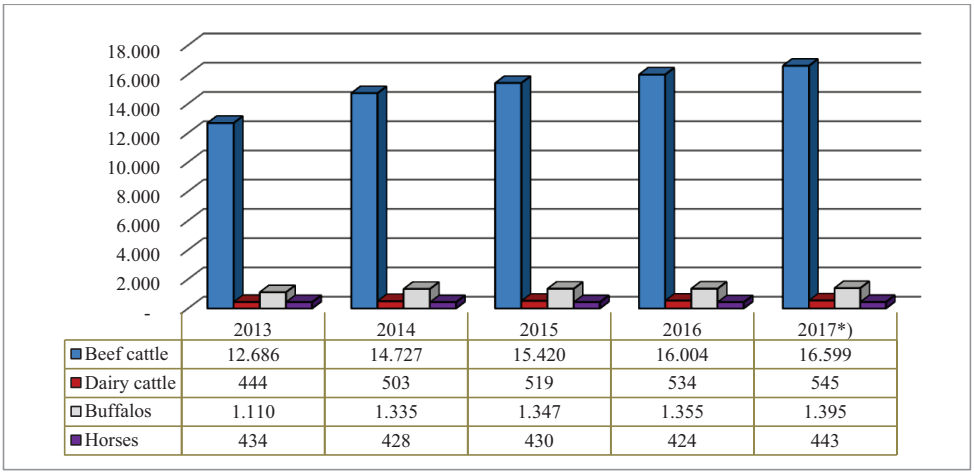


Figure 4. Population of Large Livestock (x 1,000 heads) in Indonesia.
Source: DirektoratJenderalPeternakandanKesehatanHewan (2017)

Insect rearing technology can produce high-quality alternative feeds to eliminate some feed ingredients that compete with human food needs (Rumokoy et al., 2018), for example by utilizing various types of insects: black soldier fly *(Hermetia illucens),* common house cricket *(Acheta domesticus)*, field cricket *(Gryllus bimaculatus),* bamboo caterpillar *(Omphisafus*

*cidenttalis)* pupae silkworm *(Bombyx mori),* Palm weevil larvae (*Rhynchophorus ferrugineus),* South American palm weevil *(Rhynchophorus palmarum),* Oriental migratory locust *(Locusta migratoria)*, house fly *(Musca domestica)* and termites *(Isoptera).* Furthermore, van Huis (2013) reported various insect orders that can be used as animal feed as shown in figure 5 below.

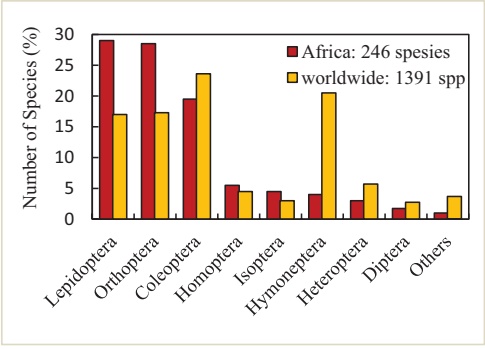


Figure 5. Several insects orde which are potential to be used as alternative feed. Source: van Huis (2003)

A promising prospect in the use of insects because it is easy to breed to be cultivated with relatively low prices can be carried out in larger quantities to be used as animal feed. In various African countries are known as edible insects for animal feed (Ayieko et al., 2010; Hanboonsong et al., 2013).

Various results of research have proven that maggot meal has a significant influence on weight gain and broiler feed conversion value. Insect larvae cycles as animal feed should be carried out at the moment when accumulated insect biomass and metabolic regulation are in the highest range to obtain maximum nutritional benefits, for example those sourced from BSF (Liu et al*.,* 2017). The use of insect larvae for organic livestock can be given fresh to poultry and in the form of flour. The role of the entomology field in alternative feed aspects becomes more complete if accompanied by the development and application of rearing technology in producing larvae or maggots ranging from small scale to large scale.

The results of insect cultivation development studies involve the rearing process to produce quality and continuously available maggots for livestock (Hussein et al*.,* 2017) have raised a business of black soldier fly *(Hermetia illucens)* to large-scale insects in various countries. This BSF cultivation does not require special facilities that are expensive, moreover the organic waste that exists in the livestock environment or from household kitchen waste can be converted into a BSF mass.

The role of insects is not just to be used as animal feed but has other advantages: as decomposers in livestock in relation to livestock waste management. The ability of BSF *(H. illucens)* to degrade livestock waste organic matter can overcome the problem of pollution generated from these wastes (Nguyen et al*.,* 2015).Other insects that can be used as decomposers for livestock waste, for example, are various species from the families of Scarabaeidae, Geotrupidae, Hydrophilidae, Histeridae (Pimsler, 2007) and Calliphoridae namely *Chrysomya megacephala* (Mendonça et al*.,* 2009). Wang et al. (2018) reported that *C. megacephala* fly larvae can significantly reduce the population of pathogen bacteria in the cow manure while being able to degrade and to reduce methane ($CH_4$) emissions and dinitrogen monoxide ($N_2O$) from manure.

A big challenge in animal husbandry is facing potential parasitic infections (Rumokoy et al., 2018b), as well as pathogenic microbes originating from the environment when insects can enhance the immune system in livestock (Rumokoy and Toar, 2015). The use of manufacturing antibiotic substances growth promoters in livestock is not allowed anymore because various considerations, especially the potential of microbial resistance even though antibiotics at GP level give a positive response to growth.

The livestock which born in hypo-gammaglobulinemia conditions are at high risk of passive immunoglobulin transfer or FTP (passive failure of transfer) failure, and become complex when they have to be exposed to pathogenic micro-organisms in the environment that can result in death (Rumokoy and Toar, 2014 ). In a situation like this another alternative is needed in an effort to minimize the risk. The function of immunogens substances derived from insects in enhancing animal immunity becomes a great force to minimize the economic loss risk in the development of organic livestock production.

## CONCLUSIONS

The development of the human population in the world has increased so sharply that it requires various efforts to fulfil the food supply in accordance with its population. The policy of using insects as animal feed can contribute to this effort.

Other benefits of insects as well as slag feed, can be used to improve the livestock immunity system and environmental management of livestock.

It is wise to promote the use of insects as natural resources for animal feed in supporting sufficient food supplies to face a very rapid increase in the human population.

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# THE GREEN MASS YIELD AND THE SILAGE QUALITY OF PERENNIAL SORGHUM, *Sorghum almum,* GROWING UNDER THE CONDITIONS OF THE REPUBLIC OF MOLDOVA

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***Abstract***

*Agricultural production plays an integral role in the regional economy, under the conditions of climate change with uneven distribution of rainfall and extreme heat, expansion of areas with degraded soils, affecting agricultural productivity. The identification of alternative crops that need less water and produce increased yield of organic matter per unit of water is important for agricultural sustainability. Adequate animal nutrition is one of the vital prerequisites to enhance the productivity and the performance of ruminants in terms of milk and meat production. Silage is one of the key components of the feed for herbivorous domestic animals. Sorghum species have recently gained popularity due to their numerous advantages, such as heat and drought tolerance and resistance to specific diseases and pests. Besides, they are well adapted to a wide range of soil types and recover easily after grazing or multiple harvests. The aim of this study was to determine the green mass yield, the silage quality, the nutrient content and the fodder value of the non-native, perennial, rhizomatous grass – Sorghum almum, grown in an experimental field of the National Botanical Garden (Institute), Chişinău. In the third growing season, the perennial sorghum was characterized by high growth rate and regenerative capacity after being cut. The annual productivity, from three harvests, was 6.1 kg/m$^2$ green mass or 1.4 kg/m$^2$ dry matter, surpassing the productivity of maize by 35 %. It was determined that the quality of the silage varied depending on the harvest time: pH 3.8-4.5, lactic acid 24.7-45.5 g/kg, acetic acid 6.3-9.1 g/kg, butyric acid 0.2g/kg, organic matter 892.4-922.8 g/kg, crude protein 50.7-95.2 g/kg, crude fats 20.6-36.8 g/kg, crude cellulose 402-428.9 g/kg, nitrogen free extract 330.5-407.2 g/kg, carotene 15.67-47.17 mg/kg, calcium 3.7-5.5 g/kg and phosphorus 1.8-2.7 g/kg. The fodder value of the prepared silage was 0.14-0.18 nutritive units/kg and 1.47-1.82 MJ/kg metabolizable energy. The potential methane yield of Sorghum almum silage substrates varied from 233 to 242 l/kg. The silage obtained from Sorghum almum, according to organoleptic characteristics (smell, colour and consistency) and biochemical indices (pH, content of organic acids and their correlation, chemical composition), largely meets the standards and can be used as alternative feedstuff.*

***Key words****: fodder value, green mass yield, methane yield, silage quality, Sorghum almum.*

## INTRODUCTION

Agricultural production plays an integral role in the regional economy, under the conditions of climate change with uneven distribution of rainfall and extreme heat, expansion of areas with degraded soils, affecting agricultural productivity. The identification of alternative crops that need less water and produce increased yield of organic matter per unit of water is important for agricultural sustainability. Forages and fodders have attained a special status as animal feed resource for being nutritious, economical and they have other associated advantages like the ease of growing and feeding. Adequate animal nutrition is one of the vital prerequisites to enhance the productivity and the performance of ruminants in terms of milk and meat production. Silage is one of the key components of the feed for herbivorous domestic animals and also feedstock for the production of a second-generation fuel – bio methane. Traditionally, in many parts of Europe, Northern and Southern America, maize silage is the major source of energy in

animal feed and substrates in biogas plant, but frequent droughts, rising prices of seeds, agricultural equipment, fuel and fertilizers have a negative impact on its productivity and cost.

Sorghum species have recently gained popularity due to their numerous advantages, such as heat and drought tolerance, resistance to specific diseases and pests. Besides, they are well adapted to a wide range of soil types. Sorghum grains can be used to produce gluten-free foods, can be given to sheep, pigs and even poultry, but are usually ground for cattle. The whole plant can be used for the production of syrup, building material and fencing, animal feeds and feedstock for renewable energy (Cattani et al., 2017; Herrmann et al., 2016; Marin et al., 2016; Trulea et al., 2013).

*Sorghum almum* Parodi, family *Poaceae,* native to Argentina, is a $C_4$ type photosynthesis plant, robust, erect, tussocky perennial grass with numerous tillers and thick short rhizomes, which curve upwards to produce new shoots near the parental stool, sometimes reaching a height of 300 cm. It is more tolerant to drought than maize, Sudan grass and Johnson grass and survived in areas receiving 200 mm of annual rainfall. This species has been cultivated in the United States of America since 1943, in Romania it has been researched since 1962, in several scientific centres: Fudulea, Caracal, Lovrin (Popescu and Albu, 1970). Depending on the variety, age and the manner of exploitation, the productivity of *Sorghum almum*, under the conditions of the Ukraine, was 20 t/ha dry matter (Rakhmetov and Rakhmetova, 2008), but in Uzbekistan, under irrigation conditions, the green mass productivity reached 211 t/ha (Avutkhonov et al., 2016).

The aim of this study was to determine the green mass yield, the silage quality, the nutrient content and the fodder value of perennial sorghum, *Sorghum almum*, as well as its potential as substrate for the production of biogas.

## MATERIALS AND METHODS

The cv. 'Argentina' of perennial sorghum, *Sorghum almum*, created in the National Botanical Garden (Institute) Chişinău, which was cultivated in the experimental plot of the Plant Resources Laboratory of the National Botanical Garden (Institute), N 46°58′25.7″ latitude and E 28°52′57.8″ longitude, served as subject of the research.

The green mass of three-year-old plants of *Sorghum almum* was cut manually for the first time in the middle of June, the second cut – at the end of July and the third cut – at the end of the September. The harvested green mass was weighed.

The preparation of silage and the evaluation of its quality were carried out in the Laboratory of Nutrition and Forage Technology of the Scientific and Practical Institute of Biotechnologies in Animal Husbandry and Veterinary Medicine, in accordance with the methodological indications and the requirements of the Moldavian standard SM 108.

The harvested green mass was chopped to 1.5-2.0 cm with a forage chopper and compressed in well sealed glass containers, stored at ambient temperature (18–20$^{o}$C) for 45 days, to allow complete fermentation to occur. Following the 45 days fermentation period, each glass container was opened and the content was visually examined, the colour and the aroma were recorded.

The pH of the samples of silage was measured immediately after removal from the containers. At the same time, samples were taken to determine the content of organic acids (lactic, acetic and butyric) in free and fixed state.

The dry matter content was detected by drying samples up to constant weight at 105 C; crude protein – by Kjeldahl method; crude fat – by Soxhlet method; crude cellulose – by Van Soest method; ash – in muffle furnace at 550$^{o}$C; nitrogen-free extract (NFE) was mathematically appreciated, as the difference between organic matter values and analytically assessed organic compounds; organic dry matter was calculated through differentiation, the crude ash being subtracted from dry matter. The calcium concentration of the samples was determined by using atomic absorption spectrometry method, phosphorus concentration – by spectrophotometric method.

The biogas and the biomethane, litre per kg of organic dry matter, were calculated using the gas forming potential of nutrients according to Baserga (1998), corrected by the nutrient digestibility.

## RESULTS AND DISCUSSIONS

The results of our previous study concerned the agro biological features, the green mass yield and its structure, depending on the harvesting period of the *Sorghum almum* plants (Ţîţei et al., 2015). In the third year of growth, when the plants were cut for the first time, in mid-June 2018 y., they were 196 cm tall, with a moderate proportion of leaves – of 30 %, and the productivity reached 2.85 kg/m$^2$ of green mass or 0.67 kg/m$^2$ dry matter. In spite of the favourable weather conditions in June-July 2018, with considerable amount of rainfall and moderate temperatures 22-25$^o$C, the plants recovered well after the harvest, thus, several new shoots developed and, at the end of July, the height of the plants was 160-165 cm and 1.72 kg/m$^2$ natural fodder was harvested, with reduced dry matter content (18.6%), but higher proportion of leaves (49%). After the second cut, the growth and the development of plants were slower in the August, but then they intensified and, until the end of September, the shoots reached a height of about 153 cm and 30% of the plants were in the stage of panicle development. The yield at the third cut was 1.53 kg/m$^2$ green mass or 0.41 kg/m$^2$ dry matter. The annual productivity from three harvests was 6.1 kg/m$^2$ green mass or 1.4 kg/m$^2$ dry matter, surpassing the productivity of maize by 35%.

The production of well-preserved, high-quality silages depends mainly on the composition of the forage used for ensiling and the application of appropriate silage-making practices. When opening the glass vessels with silage made from green mass of *Sorghum almum* obtained after the first and third harvests, there was no gas or juice leakage from the preserved mass, but from the vessels with silage made from green mass obtained after the second harvest, carbon dioxide – a by-product of fermentation – was moderately eliminated. The forage materials obtained after all the harvests resulted in silages with agreeable colour and aroma, the consistency was retained, in comparison with the initial green mass, without mould and mucus. During the organoleptic assessment, it was found that the colour of the silage obtained after the first cut was homogeneous green-yellow with pleasant smell, specific to pickled vegetables, but the silage made from green mass obtained after the second cut – green-olive leaves and yellow-green stems with pleasant smell like fresh grass. In the silage obtained after the third cut, the stems were yellow and the leaves – yellow-green with specific smell of pickled vegetables. The materials consolidated well and the fermentation was complete, with pH values 3.77-4.50, the silage obtained after the first cut had the lowest pH, the silages harvested later had higher pH 4.0-4.50. It has been determined that the amounts of organic acids, in the prepared silages, differed essentially depending on the period of harvesting (Table 1). The content of fixed lactic acid decreased from 33.8 g/kg to 17.6 g/kg DM and free lactic acid from 11.7 to 7.1 g/kg DM; total acetic acid increased from 6.3 to 9.1 g/kg DM and free acetic acid from 3.4 to 3.5 g/kg DM, respectively, in the silage obtained after the second cut. The butyric acid content was below the detected level in fixed form (0.2 g/kg DM) in the silage obtained after the first cut. The concentrations of lactic acid varied from 73.1 to 87.5 % of organic acids.

Table 1. The fermentation quality of the investigated *Sorghum almum* silages

| Indices | First cut | Second cut |
|---|---|---|
| pH index | 3.77 | 4.50 |
| content of organic acids, g/kg DM | 52.0 | 33.8 |
| free acetic acid, g/kg DM | 3.4 | 3.5 |
| free butyric acid, g/kg DM | 0 | 0 |
| free lactic acid, g/kg DM | 11.7 | 7.1 |
| fixed acetic acid, g/kg DM | 2.9 | 6.6 |
| fixed butyric acid, g/kg DM | 0.2 | 0 |
| fixed lactic acid, g/kg DM | 33.8 | 17.6 |
| total acetic acid, g/kg DM | 6.3 | 9.1 |
| total butyric acid, g/kg DM | 0.2 | 0 |
| total lactic acid, g/kg DM | 45.5 | 24.7 |
| acetic acid, % of organic acids | 12.12 | 26.93 |
| butyric acid, % of organic acids | 0.38 | 0 |
| lactic acid, % of organic acids | 87.50 | 73.07 |

The dry matter content in the investigated *Sorghum almum* silages and its biochemical composition significantly varied depending on the harvest time of green mass (Table 2). The dry matter content in the silage obtained from green mass after the first cut was 21.07%, the lowest, 18.07%, was in the silage obtained after the second cut and the highest, 25.85%, was in the silage obtained after the third cut. It was determined that the biochemical composition of the silage varied depending on

the harvest time: crude protein 50.7-95.2 g/kg, crude fats 20.6-36.8 g/kg, crude cellulose 402-428.9 g/kg, nitrogen free extract 330.5-407.2 g/kg and ash 77.2-107.6 g/kg. The amount of protein and fats was high in the silage obtained after the second cut, and low – in the silage obtained after the third cut. The crude cellulose concentration in all the silages was significantly higher. The amount of nitrogen free extract was very low in the silage obtained after the second cut.

Table 2. Dry matter content, biochemical composition, nutritive and energy value of the investigated silages

| Indices | First cut | Second cut | Third cut |
|---|---|---|---|
| Dry matter, g/kg | 210.70 | 180.70 | 258.50 |
| Crude protein, % | 7.58 | 9.52 | 5.07 |
| Crude fats, % | 3.20 | 3.68 | 2.06 |
| Crude cellulose, % | 41.00 | 42.99 | 42.89 |
| Nitrogen free extract, % | 40.50 | 33.05 | 40.72 |
| Ash,% | 7.72 | 10.76 | 8.67 |
| Nutritive units/ kg silage | 0.17 | 0.14 | 0.22 |
| Metabolic energy, MJ/kg silage | 1.80 | 1.47 | 2.18 |
| Calcium, % | 0.48 | 0.55 | 0.37 |
| Phosphorus, % | 0.19 | 0.27 | 0.18 |
| Carotene mg/ kg silage | 35.70 | 47.17 | 15.67 |

Minerals have a disproportionate effect on animal production relative to their low concentration in total diets. The macro-minerals calcium and phosphorus are especially important in milk production. They are also vital for the skeleton and the function of nerve impulses. Phosphorus is the mineral included in the body's energy metabolism, ATP system, affects acid-base balance and plays a role in the detoxification process. The concentrations of minerals, calcium (3.7-5.5 g/kg) and phosphorus (1.8-2.7 g/kg), in the Sorghum silages were acceptable. The concentrations of calcium were higher in the silage made from green mass obtained after the first and second cuts, but phosphorus – in the silage prepared after the second cut.

Vitamins are essential for total body function. In ruminants, as in other animals, carotenes are precursors of Vitamin A –retinol. A deficiency in retinol may reduce reproductive efficiency in dairy cows, especially through impaired ovarian function and increased incidence of abortion. Together with Vitamin E and polyphenols, carotenoids are natural antioxidants in the diet of ruminants. They play a role in cell communication and immune function by protecting cells against free radicals. Plant carotenoids affect the colour of milk and dairy products, particularly of butter, some cheeses, and also body fat. It was determined that the carotene content in silage varied significantly in dependence of the time of harvesting green mass: 35.70 mg/kg in silage from the first cut, 47.17 mg/kg in the silage from the second cut and 15.67 mg/kg – from the third cut.

Some authors mentioned various findings about the quality of *Sorghum* silage. Aminah et al. (2001) determined that *Sorghum bicolor* and *Sorghum almum* produced silage with pH 4.0-4.4 and lactic acid amounts of 3.75%, and also 8.7% protein, 2.6% fats, 33.4% cellulose, 51.0% nitrogen free extract and 4.2% ash, 0.47% Ca and 0.17% P. According to Kallah et al. (1997) the forage, at ensiling, declined in moisture content (85.0 to 56.0%) and leafiness (46.0 to 26.0%) with advancing stage of maturity of *Sorghum almum*, and the chemical composition of the silage changed: 6.4-14.7% protein, 5.3-7.7% fats, 5.3-8.5% ash, 72.6-78.8% NDF, 38.8-49.5% ADF, 0.23-0.58% Ca, 0.12-0.21%. Alpizar et al. (2014) mentioned, that *Sorghum almum* plants produced silage with pH 3.8, 7.92% protein, 60.70% NDF, 36.49% ADF, 24.21% hemicellulose, 9.01% ash. Muhammad et al. (2008) determined that silage from pure *Sorghum almum* plants had pH level 5.5, 11.0% protein, 17.5% fats, 40% cellulose, 6.1% ash, 25.1% nitrogen free extract. Under the climatic conditions of Poland, Ksiezak et al. (2012), reported that the silage from *Sorghum saccharatum* cv. *Sucrosorgo* 506 was characterized by 6.32-6.82% crude protein, 2.80-3.06% crude fats, 31.60-31.82% fibre, 5.48-5.93% ash, 0.34-0.39% calcium, 0.14-0.21% phosphorus, 55.50-56.90% digestibility of dry matter, but maize silage contained 6.57-6.63% crude protein, 2.91-3.16% crude fats, 17.26-17.62% fibre, 5.89-6.70% ash, 0.23-0.26% calcium, 0.13-0.22% phosphorus, 66.35-68.18% digestibility of dry mass. The results obtained in Romania, by Voicu et al. (2013), for the silage prepared from sorghum cultivars *F 436* and *F 465*, ensiled in the milk-dough stage, were 6.39-6.74% crude protein, 1.23-1.38% crude fats, 36.8-39.3% crude fibre, 45.1-48.4% nitrogen free extract, 7.03-7.58% ash, 0.34-0.39% calcium, 0.14-0.21% phosphorus,

55.50-56.90% digestibility of dry mass, for maize silage – 6.57-6.63% crude protein, 2.91-3.16% crude fats, 17.26-17.62% fibre, 5.89-6.70% ash, 0.23-0.26% calcium, 0.13-0.22% phosphorus, 66.35-68.18% digestibility of dry matter.

The content of nutrients and their digestibility influence the fodder and energy value of the *Sorghum almum* silages. So, 100 kg of silage obtained at the first cut contained 17.3 nutritive units and 180 MJ metabolizable energy, at the second cut- 14.2 nutritive units and 147 MJ metabolizable energy; the silage at the third cut was characterized by the highest dry matter content which had a beneficial impact – 22.2 nutritive units and 218 MJ metabolizable energy for cattle.

The rapid increase in population and the substantial burning of fossil fuels have contributed to an increase in global warming because of greenhouse gas (GHG) emissions. Hence, renewable sources of energy can be a key option as a potential substitute for fossil fuels. Energy production from biomass at a large scale without affecting environment and human activity has been encouraged. Anaerobic digestion process is a promising method of volatile solid conversion to gaseous fuel and manure as degraded by-product, thereby solving ecological and agrochemical issues.

The stability and the productivity of anaerobic digestion are mostly influenced by the content of organic matter, its biochemical composition, biodegradability and ratio of carbon and nitrogen (Herrmann et al., 2016).

The content of organic digestible substances in the studied *Sorghum almum* silages ranged from 563.2 to 575.2 g/kg, the gas forming potential varied from 399 to 461 l/kg, the methane yield 232-242 l/kg, respectively (Table 3).

Table 3. The gas forming potential of the fermentable organic matter from *Sorghum almum* silage substrates

| Indices | First cut | Second cut | Third cut |
|---|---|---|---|
| Organic digestible matter, g/kg | 575.2 | 563.2 | 569.1 |
| Digestible proteins, g/kg | 33.4 | 41.9 | 22.3 |
| Digestible fats, g/kg | 20.5 | 23.6 | 13.2 |
| Digestible carbohydrates, g/kg | 521.3 | 497.7 | 533.6 |
| Carbon and nitrogen ratio | 42 | 33 | 63 |
| Biogas, l/kg ODM | 461 | 399 | 454 |
| Biomethane, l/kg ODM | 242 | 237 | 232 |
| Methane, % | 52.6 | 59.4 | 51.1 |

In Cadriano Italy, Barbanti et al. (2014) found that, in the substrate from perennial inter-specific hybrid Sorghum Silk, the carbon and nitrogen ratio (C/H) was 108 and the methane yield was 271 l/kg, in the substrate from annual Sorghum varieties, they were 49-54 C/H and 251-268 l/kg, but in maize substrate – 40 C/H and 316 l/kg, respectively.

In Germany, Herrmann et al. (2016) determined that the silage substrate from a Sudan grass hybrid was characterized by 7.5% crude protein, 1.8% crude fats, 53.5% nitrogen free extract, 58.7% NDF, 36.6% ADF, 4.7% ADL, carbon and nitrogen ratio 39 and methane production was 288.9 l/kg, for comparison, the methane production potential of winter barley silage was 320.1 l/kg.

Under the climatic conditions of Poland, Ksiezak et al., (2012), determined that the methane production potential of sorghum substrate was 232-268 l/kg, but of maize substrate 280-285 l/kg dry matter.

## CONCLUSIONS

In the third growing season, the cv. 'Argentina' of *Sorghum almum*, was characterized by high growth rate and regenerative capacity, the productivity from three cuts was 6.1 kg/m$^2$ green mass or 1.4 kg/m$^2$ dry matter, surpassing the productivity of maize by 35%.

The results of this study indicate that satisfactory silages were obtained: pH 3.8-4.5, lactic acid 24.7-45.5 g/kg, acetic acid 6.3-9.1 g/kg, organic matter 892.4-922.8 g/kg, crude protein 50.7-95.2 g/kg, crude fats 20.6-36.8 g/kg, crude cellulose 402-428.9 g/kg, nitrogen free extract 330.5-407.2 g/kg, carotene 15.67-47.17 mg/kg, calcium 3.7-5.5 g/kg and phosphorus 1.8-2.7 g/kg.

The fodder value of the prepared silage was 0.14-0.22 nutritive units/kg and 1.47-2.18 MJ/kg metabolizable energy.

The gas forming potential of the digestible organic dry matter from *Sorghum almum* silage substrates varied from to 399 to 461 l/kg with 51.3-59.9% methane content.

The silage obtained from *Sorghum almum* largely meets the standards and can be used as alternative feedstuff for cattle and as feedstock for the production of biogas.

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# CHEMICAL AND NUTRITIONAL PROPERTIES OF POTENTIAL PHYTOADDITIVES USED IN ANIMAL NUTRITION

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***Abstract***

*Phytoadditives are used in animal nutrition for prevention and treatment of potential disease, as antioxidants and digestion stimulants. Three medicinal plants: mint (Mentha longifolia), fennel (Foeniculum vulgare) and white willow bark (Salix alba)) were analysed for their proximate composition, minerals, fatty acids and amino acids concentrations. The results revealed an important concentration of crude protein in menthe and fennel (20.72% and 18.89%), while fennel seeds have significant quantities of crude fiber (27.31%). White willow bark have a low content in crude protein (0.18%) and in crude fat (0.01%). Mint is good source of fatty acids with significant quantities of ω3 and a ω3/ω6 ratio below 1. The medicinal plants investigated are good sources of minerals, higher concentrations was found in menthe regarding concentration in Fe (1408.98 ppm), Mn (106.91 ppm) and Ca (1.50%). The results indicated important values of essential amino acids in mint and fennel (9.036 and 7.655 g/100g DM) and semi-essential amino acids (2.891 and 2.986 g/100 g DM). The importance of chemical constituents was evaluated with respect to the role of these plants in animal nutrition.*

***Keywords:*** *amino acids, fatty acids, minerals, plants, proximate composition.*

## INTRODUCTION

In recent years, phytogenic feed additives have attracted increasing interest as an alternative strategy to replace growth promoters like antibiotics in farm animals (Cheng et al., 2014). In the European Union antibiotics have been banned completely from use as additives in livestock feed since 2006 because of a suspected risk of generating microbiota with increased resistance to the antibiotics used for therapy in humans and animals (Windisch et al., 2008).

Herbs have been used for medicinal purposes for centuries with a significant role in maintaining human health (Ertaset al. 2005). Plants and plant extracts are used in animal nutrition as appetite, digestion, physiological functions stimulants, as colorants and antioxidants or for prevention and treatment of certain pathological conditions. Keeping farmanimals healthy is necessary to obtain healthy animal products (Frankič et al., 2009). Phytogenic feed additives are plant-derived products used in animal feeding in order to improve performance of agricultural livestock and they have recently gained increasing interest, especially for use in monogastric animals (Windisch et al. 2008).

Fennel (*Foeniculum vulgare* Mil., *Apiaceae*) is a Mediterranean aromatic plant, which is used in traditional medicine and as a spice. Many studies have shown the diuretic, analgesic and antipyretic, anti-inflammatory, antimicrobial and antiviral, antimutagenic, antispasmodic, and antioxidant effect in the fennel fruit (Badgujar et al., 2014; Oktay et al., 2003). Kazemi Fard et al. (2013), reported thatthe addition of 50 mg/kg *Foeniculum vulgare* supplementation increased egg production, improved performance, hatchability and immune response in post molted broiler breeder hen.

The wild mint (*Mentha longifolia* L. family *Lamiaceae*) grows extensively in Mediterranean regions. *M. longifolia* is used in

the pharmaceutical and food industries. Different parts of the plant including its leaves, flower, stem, bark, and seeds have been also used for a long time in traditional folk medicine as antimicrobial, carminative, stimulant, antispasmodic and for the treatment of various diseases such as headaches and digestive disorders (Stamenkovic et al., 2005; Naghibi, et al., 2010). Durraniet *al*. (2007) reported that administration of *M. longifolia* to broiler chickens resulted in enhancement of weight gain, feed intake, water intake, feed conversion productivity, dressing percentage and weight of different body organs, and also significantly decreased mortality.

*Salix alba* L. it is known as the willow tree. The willow bark extract has a long history as a herbal remedy against fever, pain and inflammation, a given effect of salicylic acid (a precursor of aspirin) and the content in polyphenols and flavonoids (Anilkumar, 2010; März and Kemper, 2002; Nahrstedt et al., 2007).Saracila M. et al., 2018) found out that the dietary inclusion of willow bark extract (1%) in broilers diets did not show significant differences of the growth performance, but have shown a positive effect ($P<0.05$) compared with control, in reducing the proliferation of pathogenic bacteria (*Enterobacteriaceae, E.coli, staphylococci*) in the broiler caecum, under heat stress conditions.

The purpose of this study was to characterize some medicinal plants, from physico-chemical point of view and their potential to be used as a supplement in monogastric animal nutrition.

## MATERIALS AND METHODS

*Plant material*

The studied plants used for the study were obtained from local pharmacies, dried, grounded and packed.

The choice of the plants investigated was based on the properties known and their use in the traditional medicine: mint *(Mentha longifolia)* leaves, fennel *(Foeniculum vulgare)* seeds and powder and white willow *(Salix alba)* bark extract.

*Chemical analysis*

Chemical methods were used to determine the concentration of the main nutrients from feeds according to Regulation (EC) no. 152/2009: gravimetric method for dry matter determination; Kjeldahl method for crude protein determination; the crude fat was determined by extraction in organic solvents; the crude fibre was determined by successive hydrolysis in alkali and acid environment and gravimetric method for crude ash determination.

The samples were analysed for Ca, Cu, Fe, Mn, Zn concentrations applying flame atomic absorption spectrometry (FAAS) after the microwave digestion. Each sample was processed as described previously (Untea et al., 2012) and a blank digest was carried out in the same way. Each sample was quantitatively transferred with 7 mL mixture of 65% $HNO_3$ : 30% $H_2O_2$ (5:2, v/v) into a 60 mL Teflon DAP – 60K vessels used fordigestion. Digestion conditions: 8 min at $130^0$C, 80% energy; 5 min at $155^0$C, 80% energy; 12 min at $170^0$C, 80% energy. Maximal microwave oven power was 1000 W. After full cooling at room temperature the solutions were filtered through filter paper in a 50 mL volumetric flasks using boiling deionized water. The phosphorus content was determined by UV-Vis spectrophotometry according to Regulation (CE) nr. 152/2009.

In order to determine the amino acids profile of samples, an HPLC Surveyor Plus Thermo Electron (Massachusetts, United States), and HyperSil BDS C18 column (Thermo Electron, Massachusetts, United States), dimensions 250mm × 4.6 mm × 5 μm were used. The samples were prepared as described by (Varzaru et al., 2013).

Gas chromatograph Perkin-Elmer Clarus 500 (Perkin-Elmer, USA), fitted with Flame Ionization Detector (FID) and capillary separation column was used in order to determine the fatty acids composition of plant samples.

Each sample was prepared as described previously by (Panaite et al., 2016). The working principle is the saponification of the sample followed by extraction is petrol ether, concentration and addition of chloroform. The sample is split in the GC, it is separated in the chromatographic column, and the results are

compared with the standard chromatograms by measuring the peak area.

## RESULTS AND DISCUSSIONS

Table 1 shows the concentration of the main nutrients (proximate composition) of the studied plants. The table shows that the highest level of crude protein is encountered in mint, followed closely by the fennel seeds.

The highest value of fiber it is found in fennel seeds (27.31%).

White willow bark had the highest level of dry matter (99.26%), and the lowest concentration in crude protein (0.18%).

The highest crude fat content was found in fennel powder (18.11%), while the lowest value was found in white willow bark extract (0.01%).

The highest value of ash content was 10.54% in mint and the lowest (0.46%) in white willow bark extract, fennel seeds and powder have similar levels (7.86% and 8.36%).

These results are in agreement with the literature on the protein content and crude fat in fennel (15.8 % and 14.87%), crude fat and fibre in mint (0.94% and 8.0%) reported by USDA, (2018).

Table 2 shows macro elements and trace elements concentrations in analysed samples. It can be seen that the highest concentration in Ca (1.50%), Fe (1408.98 ppm) and Mn (106.91 ppm) is encountered in mint.

Fennel seeds have important concentrations in Cu (14.97 ppm) and Zn (58.93 ppm), while fennel powder shows important concentrations in Fe (221.19 ppm) and Mn (52.88 ppm).

White willow bark extract does not have a rich content of macro and microelements. There were little variations of Ca, P, Cu, Zn content between fennel seeds, fennel powder and mint.

The Fe content shows great variations between the different plant species, from 3.41 ppm (willow) to 1408.98 ppm (mint).

Comparing the values obtained with the data presented in the literature, it can be observed no major differences on manganese and zinc (107.0 and 35.6 mg/kg) content in mint reported by Gogoasa et al., (2013).

Özcan and Akbulut, (2008) found the fennel content in Cu, Zn and Mn being 8.28 ppm, 20.8 ppm and 33.4 ppm respectively, the results being in the same range with our values.

Table 1. Proximate composition in analysed samples

| Specification | Fennel seeds | Fennel powder | Mint leaves | White willow bark extract |
|---|---|---|---|---|
| Dry matter % | 90.64 | 90.06 | 91.83 | 99.26 |
| Crude protein % | 18.89 | 17.18 | 20.72 | 0.18 |
| Crude fat % | 4.88 | 18.11 | 1.57 | 0.01 |
| Crude fibre % | 27.31 | 14.86 | 10.57 | 1.89 |
| Crude ash % | 7.86 | 8.36 | 10.54 | 0.46 |

Table 2. Macroelements and trace elements concentration in analysed samples

| Specification | Fennel seeds | Fennel powder | Mint | White willow bark extract |
|---|---|---|---|---|
| Ca % | 0.84 | 0.95 | 1.50 | 0.02 |
| P % | 0.53 | 0.49 | 0.29 | 0.39 |
| Cu ppm | 14.97 | 14.55 | 19.36 | - |
| Fe ppm | 37.85 | 221.19 | 1408.98 | 3.41 |
| Mn ppm | 27.11 | 52.88 | 106.91 | 0.41 |
| Zn ppm | 58.93 | 42.50 | 36.24 | 0.02 |

Table 3. Amino acids concentration in the analysed samples (%)

| Amino acids | Fennel seeds | Fennel powder | Mint |
|---|---|---|---|
| Aspartic acid | 2.614 | 2.543 | 2.702 |
| Glutamic acid | 4.170 | 4.189 | 3.136 |
| Serine | 1.296 | 1.186 | 1.271 |
| Glicycine | 1.387 | 1.401 | 1.346 |
| Threonine | 0.996 | 0.857 | 1.097 |
| Arginine | 1.235 | 1.126 | 1.226 |
| Alanine | 1.052 | 0.952 | 1.389 |
| Tyrozine | 0.157 | 0.115 | 0.215 |
| Valine | 1.239 | 1.099 | 1.652 |
| Phenylalanine | 1.104 | 0.920 | 1.494 |
| Isoleucine | 0.916 | 0.753 | 1.001 |
| Leucine | 1.483 | 1.231 | 2.081 |
| Lysine | 1.158 | 1.021 | 1.017 |
| Cystine | 0.298 | 0.254 | 0.179 |
| Methionine | 0.759 | 0.641 | 0.694 |
| The total quantity of amino acids | 19.865 | 18.289 | 20.498 |
| Essential amino acids | 7.655 | 6.522 | 9.036 |
| Semi-essential amino acids | 2.986 | 2.681 | 2.891 |

In Table 3 it can be seen that the highest values of essential amino acids like threonine, valine, phenylalanine, isoleucine and leucine are encountered in the mint. The content of lysine is higher in the fennel seeds (1.158%) than in the mint (1.017%) and fennel powder (1.021%). In the case of semi-essential amino acids, we noticed that, the glutamic acid is found in larger quantities in fennel powder and in fennel seeds than in mint. Cystine and methionine have higher concentrations in fennel seeds and mint have important alanine values.

As regards of total concentrations of essential amino acids, mint has the most important values semi-essential amino acids (9.036%), fennel seeds recording higher values than fennel powder. We can also see that fennel seeds have the highest value in semi-essential amino acids (2.986%) followed closely by mint (2.891%) and fennel powder (2.681%), values encountered in Table 4.

Table 4. Fatty acids concentration in the analysed samples (g/100 g fat)

| Fatty acids | | Fennel seeds | Fennel powder | Mint | White willow bark extract |
|---|---|---|---|---|---|
| Butiric | C 4:0 | 0.12 | 0.00 | 0.24 | 0.67 |
| Caproic | C 6:0 | 0.47 | 0.10 | 1.91 | 6.72 |
| Caprilic | C 8:0 | 1.20 | 0.13 | 8.32 | 0.24 |
| Nonanoic | C 9:0 | 0.00 | 0.00 | 0.00 | 0.00 |
| Capric | C 10:0 | 0.32 | 0.17 | 4.87 | 5.59 |
| Undecanoic | C 11:0 | 0.03 | 0.39 | 0.22 | 0.03 |
| Lauric | C 12:0 | 0.02 | 0.00 | 0.47 | 0.21 |
| Tridecanoic | C 13:0 | 0.04 | 0.00 | 0.16 | 0.00 |
| Myristic | C 14:0 | 0.71 | 0.82 | 8.51 | 10.36 |
| Miristoleic | C 14:1 | 0.00 | 0.00 | 0.53 | 0.64 |
| Pentadecanoic | C 15:0 | 0.21 | 0.08 | 0.82 | 1.38 |
| Pentadecenoic | C 15:1 | 0.07 | 0.00 | 1.14 | 0.27 |
| Palmitic | C 16:0 | 8.29 | 6.28 | 26.09 | 30.33 |
| Palmitoleic | C 16:1 | 0.72 | 0.60 | 1.22 | 1.57 |
| Heptadecanoic | C 17:0 | 0.11 | 0.00 | 0.41 | 0.69 |
| Heptadecenoic | C 17:1 | 0.15 | 0.00 | 0.37 | 0.26 |
| Stearic | C 18:0 | 1.96 | 1.42 | 7.11 | 9.37 |

| | | | | | |
|---|---|---|---|---|---|
| Oleic cis | C 18:1 | 64.25 | 74.97 | 17.54 | 25.12 |
| Linoleic cis | C 18:2n6 | 15.60 | 13.12 | 4.29 | 4.00 |
| Arachic | C 20:0 | 0.02 | 0.00 | 0.03 | 0.27 |
| Eicosenoic | C20 (1n9) | 0.05 | 0.03 | 0.10 | 0.00 |
| Linolenic α | C 18:3n3 | 1.19 | 0.44 | 11.12 | 0.75 |
| Octadecatetraenoic | C18:4n3 | 0.58 | 0.27 | 0.55 | 0.29 |
| Eicosadienoic | C20(2n6) | 0.11 | 0.04 | 0.08 | 0.00 |
| Behenic | C 22:0 | 0.33 | 0.18 | 0.19 | 0.00 |
| Eicosatrienoic | C20(3n6) | 0.09 | 0.08 | 0.06 | 0.00 |
| Erucic | C22 (1n9) | 0.65 | 0.37 | 0.00 | 0.00 |
| Eicosatrienoic | C20(3n3) | 0.67 | 0.06 | 0.10 | 0.00 |
| Arachidonic | C20(4n6) | 0.07 | 0.00 | 0.08 | 0.12 |
| Docosadienoic | C22(2n6) | 0.30 | 0.12 | 0.33 | 0.00 |
| Tricosanoic | C 23:0 | 0.00 | 0.00 | 0.52 | 0.00 |
| Eicosapentaenoic | C20(5n3) | 0.13 | 0.00 | 0.07 | 0.00 |
| Lignoceric | C24:0 | 0.13 | 0.00 | 0.07 | 0.00 |
| Nervonic | C24 (1n9) | 0.00 | 0.00 | 0.04 | 0.00 |
| Docosatetraenoic | C22(4n6) | 0.34 | 0.00 | 0.44 | 0.00 |
| Other fatty acids | | 1.08 | 0.31 | 1.62 | 1.09 |
| *Total fatty acids* | | **100** | **100** | **100** | **100** |
| SFA | | 13.96 | 9.58 | 59.96 | 65.88 |
| MUFA | | 65.88 | 75.98 | 21.30 | 27.86 |
| PUFA | | 19.08 | 14.13 | 17.12 | 5.17 |
| Ω3 | | 2.57 | 0.77 | 11.84 | 1.04 |
| Ω6 | | 16.52 | 13.36 | 5.28 | 4.12 |
| Ω6/Ω3 | | 6.44 | 17.39 | 0.45 | 3.95 |

Similar results as regards isoleucine have been obtained by (Badgujar et al., 2014) in fennel (0.73%).

The fatty acids profile of analysed plants (Table 4) shows that the concentration of linoleic acid (C18:2n6) an omega 6 acid, was highest in the fennel seeds and fennel powder (15.60 g/100 g fat and 13.12 g/100 g fat) close values have been recorded for mint and white willow bark extract (4.29 g/100 g fat and 4.00 g/100 g fat). The concentration of linolenic acid (C18:3n3), omega 3 acid, was highest in mint (11.12 g/100 g fat).

The data of concentration of saturated fatty acids and polyunsaturated fatty acids (PUFA) has the greatest value in fennel seeds. Mint is good source of fatty acids with significant quantities of ω3 (11.84g/100g fat)and a ω3/ω6 ratio below 1 (0.45).

## CONCLUSIONS

The mechanism for phytoadditives effects is not fully understood, but, based on chemical composition of plants, some of them can be considered to be used in animal nutrition. From the studied plants, it can be noticed that mentha is a very important source of trace minerals, essential amino acids and Ω3 fatty acids.


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# CARCASS CHARACTERISTICS OF NATIVE CHICKENS CONSUMED *Abelmoschus manihot* LEAVES JUICE IN DRINKING WATER

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***Abstract***

*The study aimed to determine the carcass characteristics of native chickens consumed gedi (Abelmoschus manihot (L) Medik) leaves in drinking water. A total of 100 unsexed DOC super native chickens were used. The treatments were: water without gedi leaves; 10 mL of gedi leaves juice (GLJ)/L; 20 mL of GLJ/L and 30 mL of GLJ/L. Treatments began to drink to chickens 6 weeks of age, for 5 weeks. Diet was 73% commercial diet plus 10% yellow corn and 17% rice bran. The study used a CRD consisting of 4 treatments and 5 replications. The data have been processed into the following indicators: carcass yield (carcass weight, percentage of carcass, and percentage of carcass commercial pieces), and carcass characteristics (percentage of heart, liver, gizzard, pancreas, and abdominal fat). Results showed that the administration of GLJ up to 30 mL had a non-significant different effect on carcass yield and carcass characteristics except in liver was significantly different. GLJ did not have a negative effect on livestock, there was even a tendency to reduce the abdominal fat, and economically still profitable. As a conclusion, GLJ can be used as an alternative additive in drinking water of native chicken up to 30 mL/L.*

***Key words****: drinking water, gedi leaves, native chicken, water additive.*

## INTRODUCTION

Demand for poultry meat was increasing in line with increasing in income and public awareness of the fulfillment of animal protein needs. The role of broiler chickens was very dominant in providing animal protein, but the role of broiler chickens is very vulnerable, because of the dependency on imported of feedstuffs and birds, so the risk of production failure was very high. While local chicken was very low in productivity, but the level of dependence on foreign countries was small, because the birds are native to Indonesia and have adapted to the environment, so that they are able to utilize local feed ingredients and agricultural by-products as well as agricultural industries which are abundant in the vicinity (Suprijatna, 2010).

The effort that can be done to increase the productivity of poultry was by providing additional herbal plant as feed additive. The scientists have been giving their attention on medicinal plants to achieve the targeted nutritional and health status of poultry. The herbal products and spicy have been used in animal feed as digestion stimulants and growth promoters (Frankic et al., 2009). The use of feed additive can increase immunity, growth, appetite, and meat production. Additional herbal feed was considered safer when compared to the use of antibiotics. The use of antibiotics can produce residues in poultry meat so that it can cause resistance to antibiotics if the poultry meat was consumed. In January 2006, the European Union banned the use of antibiotic growth promoters in animal feeds, which shifted the producers' attention to plant-based supplements (Lipiński et al., 2017).

Gedi plants (*Abelmoschus manihot* (L.) Medik) and their benefits to broilers have been studied by Mandey et al. (2013); Mandey et al. (2014); and Mandey et al. (2015). The results of the study found the chicken meat which was low-fat, safe and healthy, and it was because of gedi leaf juice contained bioactive compounds that have anti-oxidant potential, anti-microbial, hepatoprotective, as a growth promoter. However, from a commercial point of view, it was not optimal to produce chicken meat according to market demand, because gedi leaves contain high mucilage. Subsequent research in the form of gedi leaf juice given

through drinking water in broilers results in higher body weight than when given through diets (Mandey and Pontoh, 2016).

Depending on market demand, poultry can be sold as a whole, ready-to-cook bird, split into two halves, separated into different parts such as wings, whole breast, deboned fillets, drumstick, thigh, whole leg, etc. It is determined that some factors such as line, sex, age, health, nutrition, body weight, fattening period before slaughtering influenced these carcass parts (Nikolova and Pavlovski, 2009). Summers (2004) stated that meat at the most carcasses was deposited on the breast, upper thighs (thighs) and lower thighs (drumsticks). About 70% of the thorax and upper thighs were flesh and less in the lower thighs.

Super-native chickens were the crossing of male native chickens that have a large posture with female laying hens (Salim, 2013). Super-native chickens have faster growth than native chickens. Researchers have suggested that although the growth performance of local chicken is less efficient than that of commercial broiler, the quality of their meat is more appropriate for premium chicken meat (Choo et al., 2014). Research on the use of gedi leaves in super-native chickens has never been done, therefore this study aimed to determine the production of super-native carcasses yields and characteristics given gedi leaves juice through drinking water.

## MATERIALS AND METHODS

The study was conducted using 100 of one-day-old super-native chickens. The treatment used was gedi leaves which were made juice and given through drinking water, with the following treatments arrangement: T0 = drinking water without gedi leaves; T1 = 10 ml of gedi leaf juice (GLJ) / L drinking water, T2 = 20 ml of GLJ / L drinking water and T3 = 30 ml of GLJ / L drinking water. The treatment began to be given to chickens at 6 weeks of age, and data collection was carried out for 5 weeks. The dietwas commercial feed 73% plus 10% corn and 17% rice bran, and the nutrients composition: crude protein 19.49%, crude fiber4.66%, fat 3.63%, Ca 1.02%, P 0.66 %, and metabolizable energy 2920 Kcal/kg, which was given *ad libitum*. The preparation of gedi leaf juice based on Alom (2013).After washing, the fresh leaves were cut into small pieces by scissors and water was added at 1:10 ratio. Then juice was prepared by blending the leaves with pestle and motor and stored in a refrigerator at 4°C.

This study used a completely randomized one-way design (CRD) (Steel and Torrie, 1982) consisting of 4 treatments and 5 replications. The variables measured were carcass yield (slaughter weight, carcass weight, percentage of carcass and commercial cutting: breast, back, thigh, drumstick, and wing), carcass characteristics (percentage of abdominal fat, heart, liver, gizzard, pancreas), and IOFCC (income over feed and chick cost).The carcass was cut into commercial pieces (Irham, 2012), namely: the chest was separated at the tip of the scapula and dorsal ribs, chest weight was measured by weighing on the chest after being separated from the carcass; thighs (separated in the acetabulum, pelvix muscles were included while the pelvix bone does not participate in the thorsal and dorsal end of the tarsus metatarsus bone); the back was separated from the pelvix bone, the tip of the scapula dorsal from the ribs and the posterior part of the neck; the wings can be separated through pieces of the shoulder joints.

The value of carcass percentage was obtained by comparing the weight of the carcass (g) with the slaughter weight (g) multiplied by 100%. Individual part yields were obtained as: (part weight / carcass weight) ×100 (Sariözkan et al., 2016). The percentage of abdominal fat was obtained by comparing the weight of abdominal fat (g) with the slaughter weight (g) multiplied by 100%. The liver, heart, pancreas, and empty gizzard weight were recorded. Income over feed and chick calculation was obtained using the formula: (average body weight x Price per Kg weight of life) - ((average of feed intake x price per Kilogram ration) + price of D.O.C.) (Prawirokusumo, 1990). The data was then analyzed using IBM SPSS 24 software.

## RESULTS AND DISCUSSIONS

The use of GLJ in drinking water and its effect on the carcass yield and characteristics, and IOFCC of super-native chickens showed in

Table 1 and Table 2. The results showed that treatment until 30 ml GLJ/L drinking water had no significantly different (P> 0.05) on carcass yield and carcass characteristics (heart, gizzard and pancreas) but had a significant effect (P <0.05) on the percentage of liver. The IOFCC value was also not significantly different.

This result was consistent with the result from An et al. (2015) who found no significant difference in the body weight, carcass cutting (breast, thigh, leg), and abdominal fat, except in liver weight of white mini broilers fed diet with 0.3% or 0.5% onion extract. White mini broiler is a local mixed breed produced by crossbreeding between meat-type male breeder and egg-type hens. Moreover, the weight of body, carcass cutting and abdominal fat in this study were higher than An et al. (2015) reported.

Factors that influence the percentage of carcass were breed, age, sex, ration, and slaughter weight (Abubakar, 2003).

Table 1. Effect of gedi leaves as water additive on carcass yield and IOFCC of super-native chicken

| Variables | Treatments | | | | SEM | p Value |
|---|---|---|---|---|---|---|
| | 0 ml GLJ | 10 ml GLJ | 20 ml GLJ | 30 ml GLJ | | |
| Slaughter Weight (g) | 1124.8 | 1098.8 | 1095.2 | 1085.6 | 10.29 | 0.61 |
| Carcass Weight (g) | 836.2 | 818.0 | 816.0 | 804.8 | 7.79 | 0.59 |
| Carcass Percentage (%) | 74.34 | 74.12 | 74.68 | 74.12 | 0.25 | 0.73 |
| Breast (%) | 23.93 | 24.25 | 22.84 | 22.16 | 0.43 | 0.30 |
| Back (%) | 29.51 | 30.64 | 30.42 | 30.95 | 0.26 | 0.25 |
| Thigh (%) | 16.10 | 16.20 | 16.46 | 16.66 | 0.14 | 0.53 |
| Drumstick (%) | 15.94 | 16.20 | 16.61 | 16.73 | 0.15 | 0.23 |
| Whole Chicken Leg (%) | 32.04 | 32.40 | 30.55 | 32.93 | 0.47 | 0.34 |
| Wing (%) | 13.22 | 13.29 | 13,.4 | 13.66 | 0.19 | 0.86 |
| IOFCC (Rp) | 8.401 | 8.670 | 8.60 | 8.964 | 142,00 | 0.67 |

SEM = standard error of mean; GLJ = gedi leaves juice

The carcass component that consists of muscles, fat, skin, and bones have different growth speeds. And, the good carcass was characterized by the maximum amount of meat, the minimum amount of bone and the optimum amount of fat.

Carcass weight was closely related to the percentage of carcass. In this study giving 30 ml of GLJ/L drinking water gave no significant different effect on the percentage of carcass. The average percentage of carcasses in this study ranged from 74.12 to 74.68%. This data was higher than the results of Usman et al. (2016) in broilers that were given the prebiotic Immuno Forte in drinking water. This may also be due to the good quality of the dietused in this study. According to Gultom et al. (2012) that protein was known as one of the constituent of body cells and tissues which showed that protein plays an important role in achieving the desired carcass weight.

According to Soeparno (2005), that there was a close relationship between carcass weight and carcass parts with slaughter weight, so if the slaughter weight and carcass weights were not significant effect then the results were not much different on the parts of carcass. Whole carcasses were usually cut according to the customer's order, the usual carcass was cut into nine parts consisting of 2 lower thighs, 2 upper thighs, 2 wings, 2 breast chests and 1 middle chest. According to Merkley et al. (1980), carcasses were divided into five major parts of commercial pieces, namely the chest, wings, back, thighs and drumstick. Chest was part of the body with the most of meat.

The size of the chest was used as a measure of the quality of trade because most of the muscles that were the largest carcass component were around the chest (Jull, 1979). The average percentage of chest weight in this study ranged from 22.16 to 24.25%, lower than that reported by Usman et al. (2016) in broilers that were given the prebiotic Immuno Forte in drinking water, which was 32.7 to 35.7%.

The upper thighs and lower thighs were usually associated with oxidative metabolism of the muscles, because the use of fat as an energy substrate supported their development (Temim et al., 1999; 2000).

The percentage of whole chicken legs in this study (30.55 to 32.93%) compared to Usman et al. (2016) (26.8 to 28.2%) may be caused by the size of the bone. Muryanto et al. (2002) stated that the small amount of meat deposited in carcass parts was strongly influenced by the percentage of bone. The higher the percentage

of carcass, the higher the percentage of thigh pieces produced.

The relatively similar results in the percentage of back showed that the JDG treatment in drinking water had no significant effect (P> 0.05). The percentage of back weight in this study ranged from 29.51 to 30.95%, higher than Usman et al. (2016). Basoeki (1983) suggested that broiler backs contain a lot of bone tissue, so the mineral content in the ration has more influence on back weight compared to protein.

Research showed that the treatment of GLJ in drinking water has no significant effect on the percentage of wings (P> 0.05). The mean value of wing percentage ranged from 13.22 to 13.66%.

Table 2. Effect of gedi leaves as water additive on carcass characteristics of super-native chicken

| Carcass Traits | Treatments | | | | SEM | p Value |
|---|---|---|---|---|---|---|
| | 0 ml GLJ | 10 ml GLJ | 20 ml GLJ | 30 ml GLJ | | |
| Heart (%) | 0.67 | 0.83 | 0.65 | 0.67 | 0.04 | 0.26 |
| Liver (%) | 2.37[a] | 2.91[b] | 2.53[a] | 2.50[a] | 0.07 | 0.04 |
| Gizzard (%) | 3.35 | 3.02 | 2.97 | 2.97 | 0.11 | 0.64 |
| Pancreas (%) | 0.31 | 0.32 | 0.29 | 0.25 | 0.01 | 0.30 |
| Abdominal fat (%) | 1.67 | 1.48 | 1.44 | 1.43 | 0.10 | 0.53 |

SEM = standard error of mean; GLJ = gedi leaves juice

The administration of GLJ in drinking water did not affect the weight of the heart, gizzard and pancreas, but had a significant effect on liver weight. This result was different from the results of the Tahalele (2018) that the provision of herbal ingredients up to 5 ml added to drinking water did not cause changing in the percentage of carcass and liver, but at 5 mL administration there was a decreasing in the percentage of abdominal fat in super-native chickens. According to the study of Sulistyoningsih (2015) giving herbal variations significantly affected body weight, broiler liver weight but did not affect the weight of the heart, gizzard, intestines, and spleen.

Liver is an important organ involved in various metabolic path ways regulating growth and productivity in poultry. It has a wide range of functions, and it is vulnerable to various diseases. Phytobiotics are plant derivatives such as herbs, plant extracts or spices. They have a wide range of activities *viz.* stimulation of feed intake, growth and endogenous secretions in the gut. Phytobiotics possess hepatoprotective and hepatogenic properties, which tone up liver resultingin increased nutrient utilization and better performance (Bhattacharyya et al., 2015).

One of the few parts of the body used to store fat in broilers was the part around the abdomen called abdominal fat. The average percentage of abdominal fat in this study ranged from 1.43 to 1.67%. The results showed that the treatment had no significant effect (P> 0.05) on the abdominal fat percentage, but the data showed a downward trend. Furthermore, the average percentage of abdominal fat in this study was lower than that reported by Bilgili et al. (1992), that the percentage of broiler abdominal fat was 2.6 - 3.6%. This was partly due to differences in strains and nutritional content of rations. The nutrient content in all treatments in this study together caused the same feed intake which will affect carcass weight.

Abdulkarimi et al. (2011) reported that adding 0.6% thyme extract to drinking water significantly reduced the accumulation of fat in the abdominal areas of broiler chickens. The reduction in the abdominal fat traits caused by thyme supplementation may have been attributable to the saponins in thyme (Abdulkarimi et al., 2011), which have inhibitory effects on lipogenesis (Qureshi et al., 1983).

Many scientists have explored ways to decrease the abdominal and/or carcass fat in poultry. It has become clear that fat accretion is closely related to the rate of gain (Lin, 1981), and nutritional and management practices.

The value of IOFCC in this study was not affected by the administration of JDG in drinking water, but there was a tendency to increase with increasing levels of GLJ in drinking water.

## CONCLUSIONS

The weight of the carcass pieces, namely the thighs, chest, back, and wings of the super-native chickens that were given GLJ in drinking water increase the weight range of the standard appearance of super free-range chickens, likewise, with the weight of the heart, gizzard and pancreas, except liver weight. Abdominal fat spread around the abdomen was

not significantly different from the increased level of administration of GLJ in drinking water. The value of IOFCC was also not affected by the increase in the provision of GLJ. Based on the results of this study, GLJ did not have a negative effect on livestock, there was even a tendency to reduce the abdominal fat, and economically still profitable. As a conclusion, GLJ can be used as an alternative additive in drinking water of native chicken up to 30 mL/L.

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# THE EFFECTS OF RICE BRAN FERMENTED WITH *Aspergillus niger* ON QUALITY OF PIG PRODUCT TO ENSURE FOOD SECURITY

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***Abstract***

*In Indonesia rice bran as agricultural waste available abundantly and as major component in pig ration. The limited factor of this material has a high content of phytic acid, inhibitor of phosphorus metabolism. The objectives of this research was to study the effects of rice bran fermented with Aspergillus niger fungus on back fat thickness, meat cholesterol content, bone hardness degree and feed efficiency. Thirty-two castrated pig crossbred Spoted Poland China and Chester White, at12-week-old with live weight of between 32-44 Kg, put in individual cages. The treatments were R1 = 40% rice bran nonfermented, R2 = 40% rice bran fermented 2 days, R3 = 40% rice bran fermented 4 days, R4 = 40% rice bran fermented 6 days. Treatment was given to 4 groups of pigs with different body weight each B1 = 32 kg; B2 = 36 kg; B3 = 40 kg; B4 = 44 kg. The experimental design used was Randomized Block Design. The results shows that utilization of fermented rice bran along 6 days (R4) has back fat thick and meat cholesterol content significant (P<0,05) lower than the other treatments. On the other hand treatment R4 has bone hardness and feed efficiency significant better (P<0.05) compared to the other treatments. The conclusion was utilization of 40% of rice bran fermented along 6 days with Aspergillus niger were able to improved feed efficiency and quality product of pig meat for human health.*

***Key words*** *: rice bran, A.niger, pig, quality.*

## INTRODUCTION

Indonesian rice production were surplus in 2016/2017 around 82,2 million ton and from that amount approximately 60% was rend amen of endosperm, rice bran content around 11% or equivalent to 6.3 million ton of rice bran production. It is common used rice bran in pig fattening ration around 60-80% (Awuy, 2011) which is produced meat with pale in color.

It was reported the pig is able to utilized only around 15% of rice bran phosphorus content (Cromwel et al, 1993; Wahyuni., 2003) due to the presence of phytate (Woyengo et al., 2013). Phytic acid is the primary phosphate storage compound in seeds, typically contributing 50-80% of total phosphate in plant seeds.

The salt form of phytic acid is called phytate, and almost all phytic acid is present as a mixed salt (phytin). Phytate P is poorly available to animals and can reduce the digestibility of other nutrients and the performance of animals owing to its anti nutritional effect (Woyengo et al., 2013).

Phytate also non-selectively binds (Despande et al., 1984) to proteins and has been shown to inhibit enzymes including trypsin and α-amylase, thus reducing protein digestibility in animals Microbial phytase is the most commonly used exogenous enzyme in the feed for monogastric animals.

Phytase can reduce the anti nutritional effect of phytate and improve the digestibility of phosphorous (P), calcium, amino acids and energy, as well as reduce the negative impact of inorganic P excretion to the environment. Phytase (myo-inositol hexa bisphosphate phosphohydrolase) catalyzes the stepwise removal of phosphate from phytic acid or its salt phytate (Wyss et al., 1999; Yu et al., 2012). Phytase activity is measured as a phytase unit. In the official standardized phytase activity measurement, 1 unit is the amount of phytase that liberates 1 mmol of inorganic phosphate per minute from 0.0051 mol $L^{-1}$ sodium phytate at pH 5.5 and at a temperature of 37°C (AOAC, 2000). Phosphorus content in rice bran is about 1.44%, but 89% is unavailable P in

form phytate-bound P as inositol Phosphat-6. In broilers and laying hens, it was observed that the rate of hydrolysis of IP6 and total P retention differed significantly between feed ingredients. Weaning piglets fed with a diet corn–SBM registered a decreasing Ca:P ratio from 1.8 to 1.2, which improved body weight gain and feed efficiency (Leske et al., 1999; Adeola et al. 2006).

Increasing dietary Ca: available-P ratio in the absence of phytase reduced bone ash, but in the presence of phytase bone ash was increased (Amerah et al, 2014; Selle et al., 2009). The objective of this research was to study the effects of phytase enzyme of *Aspergillus niger* fungus could be improved the bioavailability of phosphorus in rice bran.

## MATERIALS AND METHODS

Thirty-two castrated pig crossbred Spoted Poland China and Chester White, 12 weeks old, initial BW were: block I - 32 ± 0.81 kg BW; block II - 36 ± 0.79 kg BW; block III - 40 ± 0.83 kg BW; block IV – 44 ± 0.78 kg BW were treatments randomly arranged in a 4 x 4 block design with 2 replication.

The treatments were differentiated in terms of fermentation duration of *Aspergillus niger* as follows R0 = not fermentation as control, R1 = 2 days fermented, R2 = 4 days fermented and R3 = 6 days fermented. Pigs were given one week to adapt to experimental diets.

Animals were fed twice daily at 8.00 pm and at 16.00 am with free access to fresh water throughout the trial.

Pigs were housed in individual pens (100 cm x 250 cm). Feed intake was measured daily and pigs were weighed weekly.

Animal were cared according to a recommended code of practice of animal welfare. They were fed the one ration common practiced by farmer (Table 1).

Table 1. Ingredient and chemical composition of diets

| | (%) | | | |
|---|---|---|---|---|
| | T0 | T1 | T2 | T4 |
| Items | | | | |
| Yellow corn | 38.0 | 38.0 | 38.0 | 38.0 |
| Rice bran*) | 40.0 | 40.0 | 40.0 | 40.0 |
| Fish meal | 12.0 | 12.0 | 12.0 | 12.0 |
| Coconut meal | 7.5 | 7.5 | 7.5 | 7.5 |
| Premix | 2.5 | 2.5 | 2.5 | 2.5 |
| *). Rice bran content in ration is same but difference according to the treatment (duration of fermentation) | | | | |
| Chemical composition of rations (%) | | | | |
| Crude protein | 15.58 | 16.58 | 18.48 | 19.78 |
| Crude fiber | 11.51 | 11.20 | 10.13 | 8 .95 |
| Ether Extract | 11.77 | 10.38 | 9.32 | 8.40 |
| NFE | 53.34 | 52.47 | 52.12 | 52.81 |
| Ash | 7.80 | 9.37 | 9.95 | 11.06 |
| Ca | 0.95 | 0.96 | 1.14 | 1.17 |
| P | 0.59 | 0.65 | 0.76 | 0.80 |
| GE (kg/g) | 4113 | 4208 | 4289 | 4302 |

## RESULTS AND DISCUSSIONS

The effects of treatments on all variables have been measured (Table 2). In our experiment all variable measured affected significantly higher by duration time of fermentation at treatment T3 or fermented up to six days compared to TO and T1. Except some variables from T2 treatment were not difference significantly compared to T3 treatment in term of energy intake, CP intake, Ca and P digestibility, and muscle cholesterol.

Data from this experiment showed the effective time for incubation was at day 4th to 6th which is agree to the earlier finding reported by (Kurniawan et al., 2016; Sands et al., 2009).

Phytate is a polyanionic molecule with the potential to chelate positively charged nutrients, which is almost certainly funda-mental to the anti nutritive properties of phytate.

But since almost of variables measured in our experiment increased significantly up to six days of fermentation time it is a proved that

phytate compromises the utilization of energy, protein and mineral (Selle and Ravidam, 2007). Moreover, it was recently reported that dietary phosphate deficiency has an immediately depressing effect on appetite, growth rate and feed efficiency of swine (Sefer et al., 2012).

Table 2. The effect of treatments on all variables measured

| Variable | Treatments | | | |
|---|---|---|---|---|
| | TO | T1 | T2 | T3 |
| Energy Intake (kcal/kg) | 9563.00[b] | 9783.00[b] | 9971.00[a] | 10654.00[a] |
| Crude Protein Intake (g/h/d) | 336.00[b] | 335.00[b] | 401.00[a] | 406.00[a] |
| Average Daily Gain (g/h/d) | 663.00[c] | 702.00[c] | 723.00[b] | 760.00[a] |
| Feed Efficiency | 2.75[c] | 2.63[c] | 2.16[b] | 1.89[a] |
| Ca Digestibility (%) | 65.54[c] | 70.86[b] | 73.03[a] | 72.56[a] |
| P Digestibility (%) | 53.32[b] | 55.19[b] | 63.66[a] | 64.26[a] |
| Bone Hardness (kN) | | | | |
| • Metacarpal | 0.87[c] | 1.33[b] | 1.50[b] | 1.72[a] |
| • Metatarsal | 1.05[d] | 1.54[c] | 1.65[b] | 1.90[a] |
| • Femur | 1.57[d] | 2.00[c] | 2.27[b] | 2.50[a] |
| Back-fat Thickness (cm) | 2.75[c] | 2.63[c] | 2.16[b] | 1.89[a] |
| Blood Cholesterol (mg/dl) | 152.50[c] | 148.70[c] | 138.20[b] | 121.90[a] |
| Muscle Cholesterol (mg/100 g) | 160.00[c] | 149.00[b] | 120.00[a] | 116.00[a] |

Different superscript in same row significant ($P<0.05$)

Average daily body weight gain is considered a reliable indicator of feed quality, especially in the investigation of phosphorus bioavailability in feedstuffs. Feed efficiency as an interaction between body weight gain and amount of feed consumed was significant higher at day 6th of incubation time or treatment T3 compared to other treatments.

This finding is agreed with previous research report (Woyengo et al., 2008). Bone Hardness is an reel indicator the availability and effectiveness work of phytase.

In our research showed that harness of bone of metacarpal, metatarsal and femur were significantly higher at treatment T3 compared the other treatments which is in agree with previous reported (Selle et al., 2009; Amerah et al., 2014).

From lipid parameter point of view in term of back-fat thickness, blood and muscle cholesterol at treatment T3 showed all significantly higher compared the other treatments.

Those phenomenon were probably close related with the yield of lipase enzyme produced by fermentation at day 4th of *Aspergillus niger* as high as 1.8 U/ml or equal to 420 U/g enzyme (Kurniawan et al., 2016).

This high amount of lipase was strongly stimulate by the presence of some $Ca^{2+}$ and $Mn^{2+}$ released from that fermentation (Adam and Ahmed, 2009).

## CONCLUSIONS

Based on these results it could be concluded that utilization of 40% of rice bran fermented incubation along 6 days with *Aspergillus niger* able to improved feed efficiency and quality product of pig meat for human health.

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# EFFECTS OF DIETARY OREGANO ESSENTIAL OIL ON PERFORMANCE, EGG QUALITY AND EGG SHELL BACTERIAL CONTAMINATION IN LAYING HENS HOUSED IN FREE-RANGE

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***Abstract***

*This study was conducted to determine the effects of dietary oregano essential oils (OEO) on performance, egg quality and eggshell bacterial contamination in laying hens housed in free-range system. A totally of 300, 31 weeks of age laying hens (Atak-S) were allocated into two groups: negative control (NC; basal diet) and oregano essential oils (basal diets plus 150 mg/kg oregano essential oils) for 6 months. Totally 30 eggs were collected from each trial group on the last day of each week to determine the egg quality characteristics during the experiment. The highest egg weight and lowest feed conversion ratio were measured in OEO group compared to control (P<0.05). OEO significantly (P<0.05) reduced the number of eggshell contaminated with the contamination of Enterobacteria, Coliform and Escherichia coli. However, no significant differences on egg quality parameters were noticed between OEO and control groups throughout the experiment (P>0.05). In conclusion, the supplementation of OEO to diet may reduce the total bacteria contamination on the egg shell surface obtained laying hens housed in free range systems.*

***Key words****: Free-range, oregano essential oil, egg quality, egg shell bacterial contamination, laying hens.*

## INTRODUCTION

Rearing system has been one of the most frequently addressed issues in poultry farming after European Directive 1999/74/EC requires the ban of conventional cages for housing laying from 2012. Since this request, some alternative rearing systems such as enriched cages, aviary and free-range systems have been proposed. Housing in the cage is the most common system for growing of laying hens (Denli et al., 2018).

Alternative housing systems have been evaluated in terms of production performance (Denli et al., 2018), egg quality (Guesdon et al., 2006) welfare (Abrahamson and Tauson, 1998) and bacterial contamination of eggshell (Mallet et al., 2006).

Ellen et al. (2000) reported that dust concentrations in the air were higher in percheries and aviaries systems than cage systems. Eggs produced in aviary and free-range systems may have higher aerobic bacteria on the shell than eggs from conventional and furnished cages systems (De Reu et al., 2005). De Buck et al (2004) reported that eggshell bacterial load could have an impact on shelf life and food safety and bacteria present on its surface may lead to actual contamination of the egg.

Rearing systems and feed ingredients have important effects on egg quality and hygiene (shell microbial contamination) (Hidalgo et al., 2008; Holt et al., 2010). Tactacan et al (2009) reported that the number of dirty eggs in conventional cages is lower than that in enriched cages. Contamination of *Salmonella enteritis* is the most common microbial contamination in eggs. Kinde et al. (1996) found that 0.3% of the eggs produced in a farms were exposed to *Salmonella enteritidis* contamination. In another study, it was determined that the total number of Gram negative bacteria found in the eggs of the hens raised in the mid-range system was higher than the cages (De Reu et al., 2005). Similarly,

Parisi et al. (2015) reported that eggs produced in free-range system was more polluted in terms of microbial contamination than those produced in cage systems. These microbial contamination causes disease in poultry and serious risks to human health (Havelaar et al., 2010; Arnold et al., 2014). Therefore, reducing this risk without using antibiotics in animal feeds has been one of the most researched subjects in our time.

Phytogenic feed additives are a recent class of alternatives to growth promoters, originating principally from herbs, spices and their products, which have gained wide attention in the feed industry in recent years (Wenk, 2003). Oregano *(Origanum vulgare L.)* essential oils, a phytogenic additive, are an aromatic plant that is indigenous to the Mediterranean region (He et al., 2017). Aromatic plants and essential oils (EO) extracted from these plants have become more important because of their antimicrobial actions, stimulating effects on the digestive systems of animals and antioxidant properties (Wenk, 2003).

Oregano essential oil is one of the more effective substance which has antimicrobial effects. Carvacrol, thymol, p-cymene and γ-terpenene are the major components of oregano essential oil (Kosar et al., 2003). Up to now, many beneficial effects of oregano essential oil on performance and health of animal have been reported (Lee et al., 2003). Dietary oregano essential oil decreased the effects of coccidiosis infection (Giannenas et al., 2003), increased egg production performance and hatchability in hens (Radwan et al., 2008).

In this study we aimed to determine the effects of dietary oregano essential oils (OEO) on performance, egg quality characteristics and bacterial egg shell contamination in laying hens housed in free-range systems.

## MATERIALS AND METHODS

Totally three hundred 31 weeks-old-age of Atak-S laying hens were allocated into two groups in free-range systems (n= 300; 10 house pens; 15 hens per pen; floor space 200 $cm^2$/hen). Control group received the basal diet and oregano essential oils (basal diets plus 150 mg/kg oregano essential oils) for 24 weeks. Laying hens were fed the same diet formulated was based on National Research Council (NRC, 1994) containing 17.5% CP, 2750 ME/kg, 3.7% Ca and 0.45% available P. Throughout the experiment lights were on a 16L:8D schedule.

Feeders were filled manually every day and egg collection was conducted daily during the morning hours. Egg weight, feed intake and feed efficiency were determined weekly during the all experiment period. Egg production per group, per-cage-hen-day production and quality parameters were performed weekly. Totally 30 eggs were collected (in the morning) from each group for 2 consecutive days and stored at 4°C overnight and then broken onto a level surface. Percentage of cumulative mortality of laying hens were recorded during the laying periods. Egg height, width and shell thickness (mm) were measured by using micrometer screw from Mitutoya. The height of the albumen and yolk were measured by using tripod micrometer. The width of the albumen and yolk were measured by using a standard caliper. Yolk color was measured with a Roche yolk color fan scale (Roche scale). Totally 15 eggs were collected from each group (1 egg per hen) and pooled in sterile plastic bags singularly for eggshell bacterial contamination analysis. Total aerobic populations were determined by duplicate spread plating 100 μL of the serial dilutions made from the rinse solution on to plate count agar. Plates were incubated at 35°C for 48 h before enumeration. Coliforms were enumerated by dispensing 1 mL of appropriate dilutions from shell emulsions into violet red bile agar pour plates with overlay. Duplicate plates per sample were incubated at 37°C for 18 to 20 h before typical colonies were counted.

Statistical analysis was performed using the mixed model and t-test procedure of SPSS 18.0. Tukey's test was used to separate group means. A significant difference was at $P<0.05$

## RESULTS AND DISCUSSIONS

Housing system has an important influence on the egg quality parameters of laying hens (Vits et al., 2005). Effects of dietary inclusion OEO on egg production, feed consumption, feed conversion rate and mortality are presented in

Table 1. The supplementation of 150 mg/kg OEO significantly improved feed conversion ratio compared with the control group (P<0.01). Similar to our results, Radwan et al. (2008) observed the use of 1.0% OEO in the diet improved feed conversion rate in laying hens. The beneficial effects OEO on feed conversion rate may be due to the antimicrobial activity of its phenolic compounds (Bozin et al., 2006). These phenolic compounds (carvacrol and thymol) may be improve the feed conversion rate by increasing feed utilization (Lee et al., 2003). However, egg production, feed consumption and mortality were statistically not affected by OEO supplementation throughout the experimental period (P>0.05).

Shell and internal quality of egg is important for the economic success of a producer and also consumer demands (Singh et al., 2009). Egg quality may be influenced by several factors including housing regimen, hen strain and nutritional values. The effect of OEO supplementation on egg weight, egg shape index and eggshell thickness are presented in Table 2. Egg weight was significantly higher in group received the diet containing 150 mg/kg OEO than the control at 1 or 3 weeks and all the period (P<0.05). However, we no found significant difference between control and OEO group regarding the egg shape index, shell weight and shell thickness regarding appearance (P>0.05) for 1 to 6 months. Beneficial effects of the many kinds of herb essential oils alone or mixture on egg weight and egg quality characteristics were observed. In our study, the dietary inclusion of EOE significantly increased egg weight at 1, 3, 5 months and total (P<0.05). These results are similar to the findings that Bolukbasi et al., (2008) increases the egg weight when diets were supplemented with 200 mg/kg EO of thyme, sage or rosemary over a period of 12 weeks. These results may be due to the addition of OEO to the diet of laying hens may enhances intestinal digestive function by increasing the secretion of digestion enzymes.

Other dietary effects of OEO on egg internal quality parameters including albumen height, albumen width and yolk height and yolk width are presented in Table 3. None of these parameters was significantly affected with the dietary OEO treatment (P>0.05). These results are in agreement with previous reports (Florou-Paneri et al., 2005) showing no significant differences in egg internal quality when laying hens fed a diets supplemented 50 and 100 mg/kg oregano essential oils. Contrary to our results, He et al. (2017) observed increases of the percentage of yolk ratio and egg shape index in laying hens fed diet supplemented at 50 mg/kg oregano essential oil. The differences between the studies may be due to the OEO levels supplemented.

Table 1. Effects of dietary inclusion OEO on egg production, feed consumption, feed conversion rate and mortality in laying hens housed in free-range system for 6 months

| Period (months) | Egg Production (%) | | Feed Consumption (FC, g/hen/day) | | Feed Conversion Rate (FCR) | | Mortality (%) | |
|---|---|---|---|---|---|---|---|---|
| | Control | OEO | Control | OEO | Control | OEO | Control | OEO |
| 1 | 84.3±1.22 | 86.6±0.54 | 106.4±1.38 | 108.2±1.44 | 2.26$^{a}$±0.02 | 2.16$^{b}$±0.02 | 0.26±0.10 | 0.20±0.10 |
| 2 | 85.9±0.42 | 86.3±0.42 | 111.0±1.89 | 113.7±2.26 | 2.36$^{a}$±0.04 | 2.27$^{b}$±0.04 | 0.74±0.10 | 0.36±0.10 |
| 3 | 86.5±+0.42 | 86.2±0.70 | 114.7±1.84 | 115.3±1.65 | 2.44$^{a}$±0.04 | 2.30$^{b}$±0.03 | 1.04±0.10 | 1.11±0.10 |
| 4 | 83.5±1.24 | 83.4±0.65 | 117.3±1.47 | 118.2±1.44 | 2.49$^{a}$±0.03 | 2.36$^{b}$±0.03 | 0.93±0.10 | 0.47±0.10 |
| 5 | 81.7±0.76 | 81.5±0.52 | 119.5±1.48 | 118.7±1.38 | 2.54$^{a}$±0.03 | 2.36$^{b}$±0.03 | 0.94±0.10 | 1.40±0.10 |
| 6 | 81.4±0.42 | 85.5±0.79 | 120.7±2.04 | 121.5±1.41 | 2.56$^{a}$±0.04 | 2.42$^{b}$±0.03 | 0.67±0.10 | 0.69±0.10 |
| Periods Average (1 to 6) | 83.7±0.74 | 84.9±0.60 | 114.9±0.56 | 115.9±0.67 | 2.44$^{a}$±0.01 | 2.31$^{b}$±0.01 | 0.76±0.10 | 0.70±0.10 |

$^{a,b}$Means± SE within each period with different superscript letters are significantly different ($P$<0.05), OEO: Oregano Essential Oil

Table 2. Effects of dietary inclusion OEO on weight, shape index and shell thickness of eggs of laying hens housed in free-range system 6 months

| Period (months) | Egg weight (g) | | Egg Shape Index | | Shell Thickness (mm) | |
|---|---|---|---|---|---|---|
| | Control | OEO | Control | OEO | Control | OEO |
| 1 | 55.8[b]±0.15 | 57.9[a]±0.12 | 75.0±0.91 | 75.3±0.99 | 0.40±0.01 | 0.39±0.01 |
| 2 | 58.6±0.13 | 60.5±0.17 | 74.1±0.49 | 75.3±0.80 | 0.36±0.01 | 0.35±0.007 |
| 3 | 60.3[b]±0.19 | 61.6[a]±0.07 | 74.6±0.92 | 74.9±1.11 | 0.33±0.01 | 0.33±0.005 |
| 4 | 60.6±0.14 | 61.7±0.10 | 74.5±0.65 | 74.2±0.95 | 0.34±0.006 | 0.33±0.004 |
| 5 | 61.3±0.09 | 61.5±0.09 | 73.8±0.51 | 74.9±0.61 | 0.34±0.007 | 0.33±0.006 |
| 6 | 61.6±0.08 | 62.6±0.09 | 73.9±0.63 | 73.1±0.57 | 0.35±0.006 | 0.35±0.006 |
| Periods Average (1 to 6) | 59.7[b]±0.16 | 62.8[a]±0.11 | 74.3±0.28 | 74.6±0.31 | 0.35±0.003 | 0.34±0.004 |

[a,b]Means± SE within each period with different superscript letters are significantly different (P<0.05), OEO: Oregano Essential Oil

Table 3. Effects of dietary inclusion OEO on height and width of albumen and yolk of eggs of laying hens housed in free-range system 6 months

| Period (months) | Albumen height (mm) | | Albumen width (mm) | | Yolk height (mm) | | Yolk width (mm) | |
|---|---|---|---|---|---|---|---|---|
| | Control | OEO | Control | OEO | Control | OEO | Control | OEO |
| 1 | 8.3±0.48 | 8.5±0.35 | 40.1±0.43 | 40.1±0.43 | 16.5±1.04 | 18.4±0.44 | 40.1±0.43 | 41.5±0.50 |
| 2 | 7.6±0.29 | 7.8±0.23 | 40.7±0.31 | 40.7±0.31 | 17.6±0.40 | 18.8±0.31 | 40.7±0.31 | 40.9±0.41 |
| 3 | 6.4±0.17 | 6.3±0.20 | 41.1±0.25 | 41.1±0.25 | 17.5±0.30 | 17.0±0.26 | 41.1±0.25 | 41.8±0.35 |
| 4 | 6.8±0.17 | 6.9±0.20 | 40.9±0.61 | 40.9±0.61 | 18.0±0.26 | 18.2±0.34 | 40.9±0.61 | 40.2±0.45 |
| 5 | 6.0±0.24 | 6.1±0.25 | 40.2±0.24 | 40.2±0.24 | 17.8±0.22 | 18.0±0.31 | 40.2±0.24 | 40.6±0.18 |
| 6 | 7.1±0.12 | 6.6±0.17 | 41.6±0.43 | 41.6±0.43 | 17.8±0.71 | 17.1±0.27 | 41.6±0.43 | 41.8±0.40 |
| Periods Average (1 to 6) | 7.2±0.10 | 6.4±0.17 | 40.9±0.18 | 40.9±0.18 | 17.5±0.25 | 17.8±0.15 | 40.9±0.18 | 41.4±0.19 |

[a,b]Means± SE within each period with different superscript letters are significantly different (P<0.05), OEO: Oregano Essential Oil

Table 4. Effects of dietary inclusion OEO on egg shell bacterial contamination in laying hens housed in free-range system 6 months

| Period (months) | Enterobacteria (positive/total, %) | | Coliform (positive/total, %) | | *E. coli* (positive/total, %) | |
|---|---|---|---|---|---|---|
| | Control | OEO | Control | OEO | Control | OEO |
| 1 | 6/10 (60) | 1/10 (10) | 3/10 (30) | ND | 1/10 (10) | ND |
| 2 | ND | ND | ND | ND | ND | ND |
| 3 | 5/10 (50) | 2/10 (20) | 5/10 (50) | 2/10 (20) | 5/10 (50) | 2/10 (20) |
| 4 | 7/10 (70) | 5/10 (50) | 7/10 (70) | 5/10 (50) | 4/10 (40) | ND |
| 5 | 9/10 (90) | 2/10 (20) | 2/10 (20) | 2/10 (20) | 2/10 (20) | 1/10 (10) |
| 6 | 7/10 (70) | 7/10 (70) | 7/10 (70) | 7/10 (70) | 1/10 (10) | 2/10 (20) |
| Periods Average (1 to 6) | 68/60 (56.6) | 17/60 (28.3) | 24/60 (40) | 16/60 (26.6) | 16/60 (21.7) | 4/60 (6.7) |

[a,b]Means± SE within each period with different superscript letters are significantly different (*P*<0.05), OEO: Oregano Essential Oil

Many researchers indicated that oregano had antibacterial activity against *E. coli* and Salmonella (Ouwehand et al., 2010; Mathlouthi et al., 2012). This antimicrobial activity may be due to their major active components such as thymol and carvacrol (He et al., 2017). The results of eggshell bacterial contamination analysis are summarized in Table 4. The positive number of eggshells contaminated by Enterobacteria, Coliform and *Escherichia coli* were significantly decreased for laying hens fed diet supplemented with 150 mg/kg of OEO versus another group.

These results are in agreement with Turcu et al. (2014), who observed the inclusion of OEO in to broiler diets significantly reduced Enterobacteriaceae, *E. coli* and staphylococci in the intestinal microflora compared to the control group ($P \leq 0.05$). In addition, Criste et al. (2017) reported a significant decrease of *Escherichia coli* colony in the intestinal microflora of broilers reared under heat stress ($32^{o}C$) and fed with diets that included 2% oregano powder ($P \leq 0.05$).

## CONCLUSIONS

The supplementation of OEO increased average of egg weight, improved feed conversion rate and reduced the total bacteria contamination on the egg shell surface obtained laying hens housed in free range systems.

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# BIODEGRADATION OF LEACHATE BY CONSORTIUM OF MICROORGANISM INDIGENOUS

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***Abstract***

*Leachate is a liquid that occurs as a result of mixing rainwater runoff with rubbish that has decayed and contains very fine suspended substances and pathogenic microbes. Leachate can cause potential contamination for both surface and groundwater. In this study biodegradation of leachate by microorganisms was carried out to reduce the content of organic and harmful inorganic substances. The purpose of this study was to evaluate the potential biodegradation of bacterial isolates isolated from landfill leachate. The method used in this study is an experimental method with three repetitions. The parameters observed included the levels of Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Total Suspended Solid (TSS) and ammonia, for 14 days the biodegradation process. Data from the research results were analyzed using Variety Analysis (ANOVA) followed by Duncan's multiple distance test. The results showed that a consortium of Bacillus subtilis, Bacillus licheniformis, Bacillus pumilus, Pseudomonas putida, Nitrobacter and Nitrosomonas (K3) capable of reducing BOD levels by 68%, reducing COD levels by 89%, TSS by 71%, and Ammonia by 92 %, reducing 90 % of chrome and 90 % of lead.*

***Key words****: leachate, biodegradation, landfill, consortium.*

## INTRODUCTION

Leachate is a liquid resulting from the decomposition of waste and rinsing by percolation by rainwater through landfills. Leachate defined as a liquid which filters through waste and decomposition results, and extracts dissolved material so that it suspended in the liquid. In another definition, leachate is a liquid waste formed by the entry of external water into landfills, dissolving and rinsing dissolved materials including organic and inorganic compounds resulting from the decomposition process (Meeroff and Lakner, 2014). Landfill utilization will always coincide with the production of leachate because the decomposition of waste and leachate occurs due to rain percolation which is the process of flowing water by gravity from the soil layer to the layer below it. Leachate washing will cause adverse effects such as pollution of groundwater and the environment and produce health risks for living organisms. Landfill leachate components include biodegradable and non-biodegradable products including organic matter, phenols, nitrogen ammonia, phosphate, heavy metals, sulfide, various heavy metal compounds.

In addition, leachate also contains pathogenic and non-pathogenic microorganisms.

In leachate, which also contains high ammonia nitrogen, the harmful impact of high ammonia nitrogen entering the waters is eutrophication which can cause algae blooms and siltation of waters.

The biodegradation process requires a long time to reach a high and active population. Bioremediation of leachate with bioaugmentation by the consortium of trading bacteria considered more economical and practical.

Because in the consortium, bacteria in high populations and various enzymes will produce which can work synergistically. A high population indicates that bacteria can use leachate as a nutrient. According to Doroty (2011), a number of species that can be found in leachate water include *Pseudomonas* sp*., Bacillus* sp*., Cellulomonas* sp*., Staphylococcus*

sp., *Acinetobacter* sp., *Actinobacillus* sp., *Alcaligenes* sp., *Klebsiella* sp., *Flavobacterium* sp., *Enterobacter* sp., *Serratia* sp., *Shigella* sp., *Moraxella* sp., *Pasteurella* sp., *Proteus sp., Yersinia* sp. The ability to degrade organic compounds is different because the enzymes produced are also different by each genus of microorganisms. Therefore, the use of consortium cultures on leachate bioremediation is considered more effective considering the variety of compounds contained in leachate.In this study biodegradation of leachate was carried out by using consortium isolates from leachate. This study aims to analyze the ability of the consortium of species of microorganisms to degrade leachate containing organic and inorganic materials. The parameters measured include Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD) Total Solid Suspension (TSS), and Ammonium, Chromium (Cr) and lead (Plumbum; Pb) levels contained in leachate.

## MATERIALS AND METHODS

Materials: Nutrient Agar (NA), sulfuric acid, starch indicators, Na-thiosulfate, Kl, $MgCl_2$, $FeCl_3$, sulfate buffer, $H_2SO_4$, and $MnSO_4$; $Ag_2SO_4$, distilled water, 0.01N $KMnO_4$, $H_2C2O_4$, hydrogen peroxide ($H_2O_2$).

Methods: The method used in this study is the experimental method with Completely Randomized Design (CRD), the factorial pattern of the AxB with three repetitions. The factor I is a different bacterial consortium. Factor II is the length of biodegradation time. Each treatment repeated three times. The consortium used is: (k0): Leachate; (k1): Leachate inoculated by *Bacillus subtilis*, *B. licheniformis*, and *B. Pumilus*; (k2): Leachate inoculated by *B. subtilis*, *B. licheniformis*, *B. pumilus*, and *Pseudomonas putida;* (k3): Leachate is inoculated by *B. subtilis*, *B. licheniformis, B.pumilus, Pseudomonas putida*, *Nitrosomonas* sp., and *Nitrobacter* sp.

The parameters measured were the number of bacteria, pH, levels of Biochemical oxygen demand (BOD) (SNI 6989.72.2009, BSN, 2004), Chemical oxygen demand (COD) (SNI 6989.2.2009,BSN, 2004) Total solid suspension (TSS) (SNI 06-6989.3-2004,BSN, 2004), Total plate counts andammonia(SNI 06-6989.30-2005, BSN, 2005) Chromium and Pb levels as measured by Atomic Absorption Spectroscopy (AAS). Biodegradation of leachate is carried out for 14 days. The data obtained were tested statistically using variance analysis (ANOVA).

## RESULTS AND DISCUSSIONS

### Growth of bacteria during the biodegradation process

The number of bacteria that grows in the biodegradation process is calculated using the Total Plate Count (TPC) method every 48 hours for 14 days. Bacterial growth during the biodegradation process was analyzed using variance analysis (ANOVA) and Duncan's Multiple Distance Test. ANOVA results and Duncan's Multiple Distance Test in Table 1. show that all consortiums can grow and use nutrients found in leachate. However on the sixth day, the bacteria grows and reaches the maximum population and subsequently decreases the population. The high population of bacteria found in the *B. subtilis* consortium, *B. licheniformis, B. pumilus, P. putida, Nitrosomonas* sp., and *Nitrobacter* sp. which shows that bacterial species in the consortium grow synergistically.

Bacillus generally has relatively fast growth which causes *Bacillus sp*. widely used in the biodegradation process. Landfill leachate characterized by high organic and inorganic pollutant concentrations and is extremely toxic to the environment.

The constituent in landfill leachates include organic materials such as aromatic groups, chlorinated aliphatic, phenols, phthalates, pesticides, and even ammonia, inorganic salts, such as Chromium, lead and copper, are acidic, but also extremely high concentrations of ammonia and organic nitrogen (Purwanta and Susanto, 2017; Mukherjee et al., 2014).

Tabel 1. Bacterial growth (log10 CFU / ml) during leachate biodegradation process

| Consortium | Time of **Biodegradation (days)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 2 | 4 | 6 | 8 | 10 | 12 | 14 |
| Control | 0 a A | 5.33 b A | 7.30 c A | 10.3 g A | 10.0 fg A | 9.6 f A | 8.06 d A | 8.7 e A |
| *B. subtilis, B. licheniformis, B. pumilus* | 0 a A | 9.53 b C | 12.6 d BC | 22.0 g B | 15.8 f B | 11.7 c C | 9,8 b B | 15.1 d D |
| *B. subtilis, B. licheniformis, B. pumilus, P. putida* | 0 a A | 9.10 b B | 12.4 e B | 24.8 h C | 14.3 g C | 11,5 d C | 10.0 c BC | 13.8 f C |
| *B. subtilis, B. licheniformis, B. pumilus, P. putida, Nitrosomonas, Nitrobacter* | 0 a A | 11.46 d D | 19.2 g C | 30.6 h D | 15.8 f C | 10.6 c B | 11.26 e C | 9.6 b B |

Microorganism-induced degradation of organic materials depends on the activity of various hydrolytic enzymes. Bacillus genus such as *Bacillus licheniformis, B. cereus, B. subtilis, B. coagulans, B. pumilus, B. smithii, B. brevis* produces various enzymes such as cellulase, proteases, amylase to decompose various components contained in waste. Pseudomonas putida produces several enzymes, including lipase, chitinase, and xylanase (Schallmey et al., 2004; Inca-Torres et al., 2018). According to Sunar et al., (2014) *P. putida* is an effective biodegradation agent and can multiply in waste. The Bacillus genus has interesting physiological properties because each species has different abilities, including being able to degrade organic compounds such as proteins, starch, cellulose, hydrocarbons, and dyes, produce antibiotics, play a role in nitrification and denitrification, nitrogen binding, selenium oxidizing, oxidizing and reducing manganese (Mn) (Bhatnagar and Kumari, 2013). Nitrosomonas and Nitrobacter are chemoautotrophic organisms found in soil and water, and are responsible for the oxidation of ammonium to nitrite (Nitrosomonas) and nitrite to nitrate (Nitrobacter) or called nitrification.

**Reduction of Biochemical Oxygen Demand (BOD)**

Biological Oxygen Demand (BOD) shows the amount of dissolved oxygen needed by living organisms to break down or oxidize waste materials in water. BOD examination is required to determine the pollution load and to design a biological treatment system. Decomposition of organic waste through the process of oxidation by microorganisms in water is a natural process that readily occurs when the wastewater contains sufficient oxygen.In sewage, organic pollutants are naturally described by existing bacteria. If there is enough oxygen, the bacteria will decompose aerobically, but if the bacteria runs out of oxygen, the decomposition will be carried out by anaerobic bacteria.

Table 2. Decrease in BOD during the biodegradation process (mg/l)

| **Treatment** | **Initialof BOD (mg/l)** | **Duration of Biodegradation** | | **Reduction** |
|---|---|---|---|---|
| | | Day 0 | Day 14 | **(%)** |
| Leachate without the addition of microorganisms | 940,00 | 940,00 a A | 805,00 b A | 14% |
| Consortium of *B.subtilis + B.licheniformis + B.pumilus* | 940,00 | 937,33 a A | 473,33 b AB | 49% |
| *Consortium of B.subtilis + B.licheniformis + B.pumilus + P. putida* | 940,00 | 935,00 a A | 461,33 b B | 50% |
| Consortium *B.subtilis + B.licheniformis + B.pumilus + P.putida + Nitrosomonas + Nitrobacter* | 940,00 | 932,00 a A | 301,00 b C | 67% |

Note:
The same lowercase letter read horizontally shows no significant difference (p> 0.05)
The same capital letter read towards the vertical shows that it is not significantly different (p> 0.05)

From the results of BOD measurements (Table 2), biodegradation of BOD by *Bacillus subtilis* consortium, *Bacillus licheniformis, Bacillus pumilus, Pseudomonas putida*, *Nitrosomonas*, was able to reduce BOD levels more than other consortiums namely from BOD 940 mg/l to 301.00 mg/l or 67% within 14 days. The only biodegradation by the genus Basilus decreases only 49%. Biodegradation with the consortium is more beneficial because it is synergistic because it produces various enzymes that can decompose the organic matter contained in

leachate. *Bacillus licheniformis* produces amylase; *Bacillus subtilis* produces amylase and protease. *Bacillus pumilus* produces cellulase, and *Pseudomonas putida* produces lipases (Thakur, 2012). According to Safitri, et al. (2015) *B. pumilus* is an ammonia oxidizing bacterium. The synergistic ability of the consortium of *Bacillus*, *Pseudomonas*, *Nitrosomonas* and *Nitrobacter* species on leachate bioremediation resulted in effective leachate degradation ability. Reduction of Chemical Oxygen Demand (COD) levels during the biodegradation process.

Chemical Oxygen Demand (COD) COD is the amount of oxygen needed to oxidize organic substances contained in liquid waste by utilizing potassium dichromate oxidizer as a source of oxygen.

Table 3. COD levels in the biodegradation process by three types of consortium (mg/l)

| Treatment | Initialof BOD (mg/l) | Duration of Biodegradation | | Reduction (%) |
|---|---|---|---|---|
| | | Day 0 | Day 14 | |
| Leachate without the addition of microorganisms | 3050.00 | 3050.00 a A | 2220.00 b A | 27% |
| *B.subtilis, B.Licheniformis, B.pumilus* | 3050.00 | 3000.00 a A | 849.0 b B | 71% |
| *B.subtilis, B.licheniformis, B.pumilus Pseudomonas putida* | 3050.00 | 3010.00 a A | 667.0 b B | 77% |
| *B.subtilis + Bacillus licheniformis + Bacillus pumilus + Pseudomonas putida + Nitrosomonas + Nitrobacter* | 3050.00 | 3015.00 a A | 576.0 b B | 80% |

Note:
The same lowercase letter read horizontally shows no significant difference (p> 0.05)
The same capital letter read towards the vertical shows that it is not significantly different (p> 0.05

COD is a measure of water pollution by organic substances that can naturally be oxidized through biological processes and can cause reduced oxygen dissolved in water.

The results of COD analysis during the biodegradation process in Table 3 show that the *Bacillus subtilis* consortium, *Bacillus licheniformis, Bacillus pumilus, Pseudomonas putida*, *Nitrosomonas* and *Nitrobacter* were able to reduce COD levels higher than other consortiums namely from COD levels 3015 mg/l to 576 mg/l or 80%. All consortia can reduce COD levels. This decrease shows that every bacterial species in the consortium produces enzymes that can degrade organic elements in leachate. Microorganisms in the consortium use organic ingredients as nutrients for their growth. According to Ajao et al., (2011) research, *Bacillus subtilis* was able to reduce COD levels of waste by 86% within 15 days. *Bacillus* sp. Isolated from areas contaminated with oil spills can produce proteases and lipases (Lee et al., 2015). *Nitrosomonas* sp. convert ammonia compounds into nitrite compounds, and *Nitrobacter* converts nitrite to nitrate. Therefore biodegradation by species consortium is more effective because it will describe the diversity of pollutant compounds in leachate.

**Total Suspended Solid (TSS)**

Total Suspended Solid (TSS) are solid substances that are suspended in waters and cause turbidity in the waters.

TSS values during the process of biodegradation of leachate by the consortium of *B. subtilis, B. licheniformis, B. pumilus, P. putida*, *Nitrosomonas* and *Nitrobacter* (Table 4) showed that the consortium was able to reduce TSS levels compared to other consortiums, TSS levels of 1005 mg/l to 329 mg/l or decreased by 67%. This result showed that bacterial species in the consortium could use organic matter in their waste as nutrients.

The *Bacillus* genus can decompose crude fibers and lignin which are difficult to decompose and hydrolyze cellulose so that solid organic matter dissolved in waste in the form of lignin, lipids, and cellulose can reduce. *Bacillus pumilus* has the high cellulolytic ability, so dissolved solids containing cellulose can be broken down. The consortium of *B. pumilus, B. subtilis, P. amylolyticus* and *Nitrosomonas* sp. Bacteria resulted in the highest reduction in TSS levels of 85% for 20 days (Zaira, 2014). This result showed that bacterial consortium interaction synergistically so that bacteria can utilize and decompose the substrate of organic matter in leachate.

According to Safitri et al. (2015), reduced suspended solids caused by the degradation of organic compounds by bacterial enzymes of degrading bacteria, during the biodegradation process. The ability of bacteria to decompose organic matter causes the suspended solids to decrease, and the value of TSS is also lower.

Table 4. Levels of total suspended solid (TSS) (mg/l) during the process of biodegradation of leachates by three consortium types

| Treatment | Initial of BOD (mg/l) | Duration of Biodegradation | | Reduction (%) |
|---|---|---|---|---|
| | | Day 0 | Day 14 | |
| Leachate without the addition of microorganisms | 1112,00 | 1112.00 a A | 1059.00 a A | 4% |
| *B.subtilis, B.Licheniformis, B.pumilus* | 1112,00 | 1010.00 a A | 550.0 b B | 45% |
| *B.subtilis, B.licheniformis, B.pumilus Pseudomonas putida* | 1112,00 | 1020.00 a A | 407.0 b B | 60% |
| *B.subtilis + Bacillus licheniformis + Bacillus pumilus + Pseudomonas putida + Nitrosomonas + Nitrobacter* | 1112,00 | 1005.00 a A | 329.0 a B | 71% |

Note:
The same lowercase letter read horizontally shows no significant difference (p> 0.05).
The same capital letter read towards the vertical shows that it is not significantly different (p> 0.05).

**Ammonia**

Ammonia concentration is very high in leachate landfills due to the accumulation of organic waste including protein - ammonia produced from the decomposition of nitrogen-containing organic compounds and hydrolysis of urea in wastewater. Ammonia arises from the ammonification process, namely the process of utilizing organic compounds from dead living things such as proteins and amino acids by decomposing bacteria, also, ammonia can also derive from organic nitrogen originating from urine and livestock feces or excreta poultry. Fertilizers, feed, and various organic materials are sources of ammonia. According to Mpenyana et al., (2008), ammonia concentrations (> 10 mg N $L^{-1}$) produce some problems including eutrophication in waters because N is a vital growth nutrient for plants. Leachate contaminates water underground, when entering the river, and the lake will affect aquatic animals, which is due to reduced oxygen dissolved in the receiving water body, ammonia will be oxidized to nitrite, nitrite to nitrate and nitrate will be converted into nitrogen gas by denitrification bacteria. Ammonia and nitrite are toxic to fish, shrimp, and other aquatic fauna.The results of the ammonia concentration (Table 5) showed that the *Bacillus subtilis* consortium, *Bacillus licheniformis, Bacillus pumilus, Pseudomonas putida,* Nitrosomonas and Nitrobacter were able to reduce Ammonia levels greater than other consortiums namely Ammonia levels 1597 mg / l to 115 mg / l or equal to 92% on day fourteen. The decrease in ammonia is due to the presence of *Nitrosomonas*, and *Nitrobacter* as nitrifying bacteria. Nitrifying bacteria get nutrients from the decomposition of organic compounds into simpler compounds by bacterial species in the consortium, so that they can work more effectively to reduce ammonia in leachate.

Table 5. Ammonia levels during the biodegradation process

| Treatment | Initialof BOD (mg/l) | Duration of Biodegradation | | Reduction (%) |
|---|---|---|---|---|
| | | Day 0 | Day 14 | |
| Leachate without the addition of microorganisms | 1597 | 1597 a A | 1330,00 a A | 16% |
| *B. subtilis, B. licheniformis, B. pumilus* | 1597 | 1597 a A | 1030 b A | 35% |
| *B. subtilis, B. licheniformis, B. Pumilus, Pseudomonas putida* | 1597 | 1597 a A | 516 b B | 67% |
| *B. subtilis + Bacillus licheniformis + Bacillus pumilus + Pseudomonas putida + Nitrosomonas + Nitrobacter* | 1597 | 1597 a A | 115 b B | 92% |

Note:
The same lowercase letter read horizontally shows no significant difference (p> 0.05)
The same capital letter read towards the vertical shows that it is not significantly different (p> 0.05).

**Ability to bind heavy metals**

Heavy metals often found in leachate are lead (Pb) and chromium (Cr). Heavy metal lead (Pb) and chromium (Cr) contained in leachate derived from waste that has disposed of to landfill (TPA). The highest decrease in heavy metal content occurs after day 14 of the consortium can be seen in Figures 1 and 2.

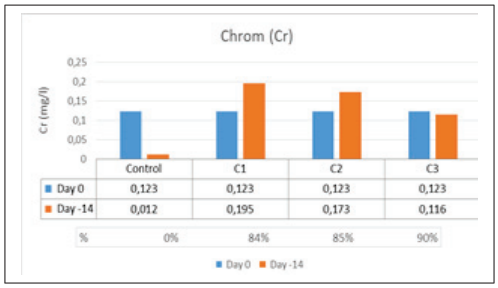


Figure 1. Decrease in Chrom (Cr) during the biodegradation process of leachate for 14 days

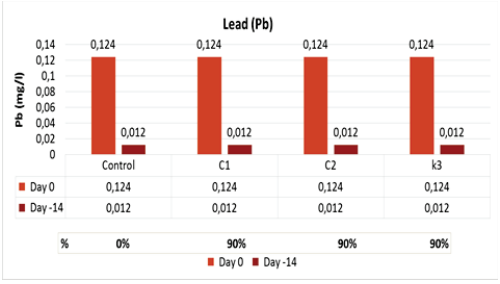


Figure 2. Decrease in Lead (Pb) during the biodegradation process of leachate for 14 days

The reduction of Chrom and Lead, heavy metals (Figure 1 and Figure 2) was carried out by all consortia, but *Bacillus subtilis* consortium, *Bacillus licheniformis, Bacillus pumilus, Pseudomonas putida*, *Nitrosomonas*, and *Nitrobacter* can reduce Cr 90% and 90% Pb respectively. Microorganisms have a strategy to tolerate heavy metals by transforming dangerous elements into safe forms, binding intra-extracellular metals and actively transporting metals out of the cytosol cells (Monachese et al., 2012).

According to Agostinho (2012), *Bacillus* sp. and *P. aeruginosa* can reduce 66% Cr content found in hospital waste. The mixture of *Pseudomonas pseudomallei* and *Pseudomonas aeruginosa* bacteria can reduce the highest levels of lead metal (Pb) in treatment with a decrease of 65% (Khoiroh, 2015). *Bacillus subtilis* was able to interact with a range of toxic metals, including copper, iron, magnesium, gold, and leads - charge of bacteria and cationic charge of many metals.

Gastrointestinal microorganisms also provide the body's first defense by converting toxic Cr (VI) to a less-toxic Cr (III). There are three types of mechanisms for binding heavy metals to bacterial cell walls, namely through (i) ion exchange reactions with peptidoglycan and teichoic acid, (ii) precipitation through nucleation reactions, and (iii) complexation with nitrogen and oxygen ligands. Gram-positive bacteria, particularly Bacillus spp., Have high adsorptive capacity due to high cholesterol and acid content in their cell walls of cells (Monachese et al., 2012).

## CONCLUSIONS

The most effective consortium for degrading leachate is *Bacillus subtilis* consortium, *Bacillus licheniformis, Bacillus pumilus, Pseudomonas putida*, *Nitrosomonas*, *Nitrobacter* (C3) can reduce BOD by 67%. The level of COD reduction was 89%, reducing TSS levels by 71%, reducing ammonia levels by 92%, decreasing Cr and Pb levels by 90%.

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# CHEMICAL COMPOSITION OF THE PERENNIAL PLANT SORGHUM AND FODDER PREPARED AND HAY

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***Abstract***

*It has been investigated the non-traditional perennial fodder plant sorghum (Sorghum almum) for its use in feed for farm animals, fresh and preserved through the preparation of fodder. The content of nutrients in the green mass of the sorghum perennial plant: Moisture-78.25%, crude protein in absolutely dry substance -10.16%, crude fat-3.0, crude cellulose-38.95%, nutritive units-0,18, metabolic energy-8.59 Mj/kg. Organic acids had been formed in the process of green mass fermentation, which has helped to maintain the quality of the green mass and obtaining a high quality fodder. The nutrient content of the perennial herbal plant sorghum is close to the corresponding indices of the initial green mass. The green mass of the perennial plant sorghum is well suited to the preparation of fodder. Fodder is yellowish green, pleasantly smelled of pickled fruit and is well consumed by farm animals.*

***Key words****: animals, nutrients, green mass, organic acids, chewing.*

## INTRODUCTION

In order to diversify the spectrum of fodder crops and to insure farm animals from R. Moldova with rough and juicy qualitative fodder we started research on the non-traditional perennial fodder plant sorghum (*Sorghum almum*). According to the publications of several authors (Cucu et al., 2004; Teleuta, 2010; Teleuta et al., 2015; Petukhov et al., 1989; Marin et al., 2016; Titei et al., 2018), this plant has increased resistance to drought, pests and various diseases, is relatively tolerant to soil quality, has a high regenerative capacity and growth after mowing, is adapted to traditional crop cultivation, harvesting and preparation technologies. The plant is multiannual and can be exploited on the same site for a long time (5-6 years). The green mass of the plant harvested in different phases of vegetation can be used in animal feed both fresh and preserved by the preparation of hay, fooder. During the green season it is possible to be harvested 2-3 times, in high humidity years it grows constantly and can be harvested every 30-35 days.

## MATERIALS AND METHODS

The experimental researches of the perennial plant sorghum (*Sorgum almum*) (Figure 1) werecarried out in the central area of the Republic of Moldova in climatic conditions characteristic to the geographical area. The green mass of the perennial plant sorghum was harvested for the chemical analysis and determination of the nutritional value of theparcels sown in the current year and in the previous years.

Figure 1. The perennial plant sorghum (*Sorgum almum)*

Laboratory analyzes were performed to determine the following indices: Initial and Hydroscopic Humidity, Nitrogen and Crude Protein, Gross Fat, Gross Pulp, Crude Ash, Unsaturated Extractives, Carotene. In laboratory and semi-production conditions, the green mass of this plant was preserved by preparing the hay. Subsequently, the chemical composition of the hay was determined according to the hints shown above and other parameters characterizing the preserved fodder

by fermentation: pH index, organic acid content - lactic, acetic, butyric. The quality of the hay by organoleptic indices was also assessed: smell, color, consistency. Analyzes were performed according to classical methods (Petukhov et al., 1989.) Fodder humidity estimation was performed by drying the samples at 60-65°C, hydroscopic humidity at 100-105°C for 2.5-3 hours. Nitrogen (for crude protein determination) was evaluated using the Kjeldahl method, Gross fat content according to S. V. Ruşkovski's method. The method is based on the excretion of fats with organic diluents. Gross cellulose was evaluated according to the modified Henneberg and Stohmann method. Non-nitrogen extractive substances were calculated by subtracting from 100%: moisture indices, crude protein ash, raw pulp, crude fat, expressed as a percentage. The crude ash was evaluated by burning samples in stoves at 450-500°C. Organic acids content was determined by Lepper-Flig methods.

## RESULTS AND DISCUSSIONS

After harvesting the green mass of the perennial plant sorghum, it was left in the furrows to wipe off to reduce the moisture, then chopped and crushed into 100-150 kg barrels. After a storage period necessary to complete the fermentation processes, the barrels were opened and hay analyzed. After the organoleptic analysis of the hay, it was found that its color deviated from green to yellow, yellow-grey with a pleasant smell of pickled vegetables and fruits and the consistency was identical to the initial green mass prior to preservation.

Table 1. Chemical composition and nutritive value of perennial plant sorghum depending on the vegetation phase

| Indices | | | Green mass, plant growth phase, h = 60-80 cm | The green mass, the pre-emergence phase (the panicle out of the sheath) | Green mass, phase of panicle formation |
|---|---|---|---|---|---|
| Humidity ,% | | first | 85.70 | 76.62 | 76.13 |
| | | hygroscopic | 5.30 | 6.58 | 6.52 |
| | | total | **86.46** | **78.16** | **77.69** |
| Dry Substance,% | | | 13,54 | 21.84 | 22.31 |
| Nitrogen,% | % | in DS | 2.10 | 1.24 | 1.16 |
| | | in the absolutely dry substance | 2.22 | 1.33 | 1.24 |
| | | with natural moisture | 0.30 | 0.29 | 0.28 |
| Crude protein | % | in DS | 13.13 | 7.75 | 7.25 |
| | | in the absolutely dry substance | **13.86** | **8.30** | **7.76** |
| | | with natural moisture | 1.88 | 1.81 | 1,73 |
| | g | | 18.77 | 18.12 | 17.31 |
| Digestible protein, g/kg | | | 12.96 | 12.50 | 11.94 |
| Gross fat | % | in DS | 4.32 | 2.48 | 1.99 |
| | | in the absolutely dry substance | **4.56** | **2.65** | **2.13** |
| | | with natural moisture | 0.62 | 0.58 | 0.48 |
| | g | | 6.18 | 5.80 | **4.75** |
| Cellulose brute | % | in DS | 30.39 | 34.77 | 36.51 |
| | | in the absolutely dry substance | **32.09** | **37.22** | **39.06** |
| | | with natural moisture | 4.34 | 8.13 | 8.71 |
| | g | | *43.46* | 81.29 | 87.15 |
| Gross ash | % | in DS | 11.19 | 6.40 | 6.23 |
| | | in the absolutely dry substance | **11.82** | **6.85** | **6.66** |
| | | with natural moisture | 1.60 | 1.50 | 1.49 |
| NES (non-nitrogen extractive substances),% | % | in DS | 35.68 | 42.02 | 41.50 |
| | | in the absolutely dry substance | **37.67** | **44.98** | **44,40** |
| | | with natural moisture | 5.10 | 9.82 | 9,91 |

| | | | | |
|---|---|---|---|---|
| UN (nutritonal units) | With natural humidity | **0.12** | **0.19** | **0.20** |
| ME (metabolisable energy), Mj/kg | in dry substance | **8.90** | **9.00** | **8.93** |
| | With natural humidity | 1.27 | 2.10 | **2.13** |
| Carotene, mg/kg | | 28.67 | 36,0 | 27.0 |
| Calcium, % | in DS | 0.56 | 0.35 | 0.36 |
| Phosphorus, % | in DS | 0.30 | 0.14 | 0.14 |

The study of dynamics and chemical composition of nutritive value for perennial plant sorghum in dependence on the vegetation phase revealed considerable changes (Table 1). Thus, for young plants, in the beginning of growing period humidity was 86.46% then the ones in the pre-mature phase decreased to 78.16% and the ones in the mature phase to 77.69%.

Respectively dry matter content increases from 13.54 to 22.31%. Instead, the crude protein content of the absolutely dry substance drops from 13.86% in the first case to 8.30 and 7.76%, respectively.

At the same time the digestible protein level in one kg of green mass with natural moisture changes, but less than 12.96 g in the growth period to 12.50 g in the pre-mature phase and 11.94 g in the mature period. With the aging of the plant, increases the level of crude cellulose (from 32.09 to 39.06%) and non-nitrogenous extractive substances (from 37.67 to 44.98%).

All these changes in the chemical composition also lead to a change in the nutritional value of the plants.

Table 2. Fodder and ratio of stalks and leaves mass to plant Sorg depending on the number of harvesters

| Indices | Harvest I | Harvest II | Harvest III | Average |
|---|---|---|---|---|
| Plant height, cm | **196** | 163 | 153 | 171 |
| Fodder mass, green, t/ha total, t/ha | 28.3 x | 17.23 x | 15.31 x | 20.28 60.84 |
| Stalks,g | 23.2 | 16.4 | 8.5 | 16.0 |
| Leaves, g | 11.3 | 11.0 | 7.1 | 9.8 |
| Total, g | 34.5 | 27.4 | 15.6 | 25.8 |
| Ratio, stalks/leaves% | 67.3/ 32.7 | 59.9/ 40.1 | 54.5/ 45.5 | 62.1/ 37.9 |

For example, the energy value increases from 0.12 UN/kg in the plant growth period to 0.20 UN/kg in the matture phase. The data obtained further reveals that the differences between the chemical composition of the plants harvested in the pre-mature and mature phase are insignificant with only one important difference which needs to be taken into consideration when determining the harvesting period, this difference refers to digestible protein content to a nutritional unit. If during the period of plant growth this index is 108 g/UN, before mature phase - 66 g/UN, then drops to 54 g/UN or 18.2% during the mature period.

The technology is harvesting the plants and drying them in furrows up to 55-65% humidity (Figure 2). At this humidity stage the plants are gathered from the furrows, shredded and transported to storage capacities.

Figure 2. Perennial plant sorghum harvested in furrows

To determine the ratio of the stalk mass to the leaves of the perennial plant sorghum, the straw were separated from leaves, weighed and the correlation between them was calculated and presented (Table 2). This procedure has been applied to plants harvested from 3 times consecutive in a season.

As Table 2 shows, as plant is getting higher, the more fodder it gives per hectare. At a height of 196 cm, the fodder is 28.3 t/ha, and at the height of 153.0 cm, the fodder is only 15.31 t/ha. The ratio stalks / leaves averaged 62.1 / 37.9% of the weight of the fresh harvested plants (Table 3).

At the same time this ratio increases from the first to the second harvest from 67.3 / 32.7% to 59.9 / 40.1% and at the third harvest to the 54,5 / 45,5%, not taking into account that the harvest of

green mass decreases from 28.3 t / ha at first harvest to 17.23 at the second and go down to 15.31 at the third harvest.

Table 3. Chemical composition and nutritional value of the green mass and hay of perennial plant sorghum (*Sorgum almum*)

| Indices | Green mass | Hay |
|---|---|---|
| Total humidity, % | 78.25 | 59.74 |
| Dry substances, % | 21.75 | 40.26 |
| | % in dry substances | |
| Nitrogen | 1.63 | 1.3 |
| Crude protein | 10.16 | 8.1 |
| Gross fat | 3.0 | 2.81 |
| Gross pulp | 38.95 | 39.23 |
| Gross ash | 8.34 | 9.24 |
| SEN | 32.51 | 36.43 |
| EM, Mj/kg | 8.53 | 8.59 |
| Ca | 0.42 | 0.49 |
| P | 0.26 | 0.19 |
| Carotene, mg/kg | 53.67 | 32.25 |

Of course, here it has influenced when the plant was harvested, because the plants in the third harvest are finer and has more leaves as seen from the ratio of stalks/leaves of 54.5 / 45.5% in this harvest. So we lose in quantity, but we grow in quality because leaves are much more nutritious than the stalks, which contain predominantly cellulose. As a result, the fodder prepared from the third harvest will be much more qualitative than the one made from first harvest.

During storage, mucosities have not formed and mold has not developed (Figure 3).

Figure 3. The green table *Sorgum almum*

For the determination of the nutrient content and the appreciation of the nutritives values, chemical analysis of the green mass and the hay prepared from the perennial plant sorghum were carried out (Table 3).

The data obtained and presented in Table 3 demonstrate that in the growing stage, at the height of perennial plant sorghum (*Sorgum almum*) 60-120 cm, the total humidity constituted 78.25%, and hay prepared from it 59.74%. Analyzing the content of essential nutrients in the green mass and the hay made from it, we find a very small difference between these two fodders. Thus, the amount of nitrogen in the green mass is 1.63%, and in hay 1.3%, the corresponding crude protein 10.16% and 8.1%, the crude fat 3.0% and 2.81% and so on. The exception is the amount of carotene, which in the green mass is higher at 53.67 mg / Kg, and in hay is only 32.25 mg/kg. It is natural, that in the process of fermentation and storage some of the carotene is lost. The metabolic energy expressed in Mj/kg was practicaly identical, respectively, 8.53 and 8.59.

In order to determine how the fermentation processes were carried for fodder preparation, the chemical analysis of the finished product was performed. Thus the percentages of the basic organic acids (lactic, acetic and butyric) in the free and fixed state, the active acidity (pH index) in the prepared pellet were evaluated (Table 4).

Table 4. The content of free and fixed organic acids in the perennial plant sorghum (*Sorgum almum*

| Indices | Fodder from plant Sorg |
|---|---|
| pH | 4.52 |
| Free: acetic ;% | 0.45 |
| butyric,% | 0 |
| lactic, % | 1.0 |
| Fixed: acetic, % | 0.66 |
| butyric,% | 0 |
| lactic,% | 2.0 |
| Total: acetic, % | 1.1 |
| butyric, % | 0 |
| lactic, % | 3.0 |
| Sum: % Lactic+butyric+acetic | 4.16 |
| Correlation of acids, in % | |
| Acetic | 26.62 |
| butyric | 0 |
| lactic | 73.25 |

The data of Table 4 elucidates the amount of organic acids eliminated in the fermentation process, shows that predominantly lactic acid was produced in total of 3.0%, both in free (1.0%) and fixed (2.0%). Acetic acid wasproduced in moderate quantities, a total of1.1% and the butyric acid that is undesirable

infodder, basically did not produced. This amount of organic acids eliminated as a result of fermentation, but especially the share of 73.35% lactic acid provided a dominant acid-lactic fermentation of the green mass and contributed to the production of the fodder (Figure 4).

Figure 4. The hay of the perennial plant sorghum

As a result, the development of the fermentation process under favorable conditions of the development of the lactobacteria led to the achievement of a superior quality fodder.

## CONCLUSIONS

1. The perennial plant sorghum (*Sorgum almum*) is in the state of research in the Republic of Moldova and is mainly cultivated on experimental parcels.
2. The green mass of the perennial plant sorghum (*Sorgum almum*) can be used freshly in animal feed and is well suited to preservation by preparing the fodder, thus offering the opportunity to expand the spectrum of feed used in farm animal rations.
3. The content of nutrients in the plant sorghum (*Sorgum almum*): total moisture - 73.77%, dry matter - 26.23%, crude protein - 10.57%, gross fat - 2.26%, raw pulp - 38.88%, SEN - 32.51%. Nutrient value in nutrient units averaged 0.20 UN/kg; in metabolisable energy 8.53 Mj/kg of dry matter. Average carotene content was 53.67 mg/kg; mineral substances - calcium 0.42%; phosphor - 0.26%. These indices fall within the average normative parameters of fodder plants used in animal feed.
4. Fodder prepared from perennial plant sorghum according to organoleptic characteristic has the greenish-yellow color, the pleasant smell of pickled vegetables or fruits, well conserved consistency of the original plants, without mold and mucus.
5. The content of fixed and free organic acids with a share of lactic acid up to 80-87% of the total amount of acids ensured a beneficial process of acid-lactic fermentation of the green mass, from the perennial plant sorghum, which led in obtaining a quality fodder.
6. Nutritional value and chemical composition of the fodder prepared from the non-traditional perennial plant sorghum (*Sorgum almum*) was close to the same indices of the initial vegetal mass.

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# CHEMICAL COMPOSITION AND NUTRITIONAL EVALUATION OF SOYBEAN MEAL

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***Abstract***

*Soybean represent the most important protein source for poultry and swine rearing but its producing in Romania and even in Europe is at a quite low level face to demands. To obtain complete mixed fodders are recurred imports from other world countries where are cultivated different soybean sorts under different pedo-climatic conditions, fact which made that products entered on Romanian market to present a great variability regarding chemical and nutritional features. In the current paper we analysed from chemical and nutritional point of view the quality of 10 soybean lots imported from Brazil. Besides basic chemical analysis were effectuated analysis to determine content in amino acids which allow a further protein nutritional evaluation by calculating chemical indices, essential amino acidsindex, protein efficiency rate, biological value and nutritional index. Proteins' nutritional evaluation from analysed soybean meal was realised using as etalon egg protein, amino acids requirements for swine, for broiler chickens and adult persons. Chemically speaking analysed soybean meal had values into limits imposed by literature (90.1% DM, 41.24% CP, 2.47% CF, 4% CA, 4.8% Ash and 37.59% NES). Nutritional evaluation show that analysed soybean meal lots had a good content in protein and even more, a good content in amino acids. This conclusion resulted because EAAI was>0.70, PER was>2.7 and P-BV was>70%.*

***Key words****: soybean meal, nutritional evaluation, amino acids, biological value.*

## INTRODUCTION

Known and cultivated with thousands years ago, soybean was considered, in origin areas from Asia, as a "holy" plant. Idolizing from those ages was transformed during time into an incontestable recognition of its value among plants with an agricultural importance and not only, making that soybean to be considered as "plant of the future" being presented nowadays on all continents. Soybean crop was imposed by its remarkable value being utilised as fodder, oleaginous source, protein source and raw material source for certain industries. Soybean is the leguminous whit the highest content in essential amino acids, which are into a better equilibrium, in comparison with other leguminous and with some animal products (Stan et al., 2005; Halga et al., 2005; Pop et al., 2006).

In according with Stoica (2001), soybean "plant of the future", as it is characterized by experts' in human nourishment have a high protein content (32-35%), fat (16-20%), non-nitrogenous extractive substances (24-30%) and a not such higher content in crude cellulose (8-9%). Protein from soybean is mainly composed by glycine, which due to its features is quite similar with milk casein. Can be soluble in phosphohydric environments and could be re-synthesised in acid environment.

Soybean meal represent the most important protein source for poultry and swine rearing but unfortunately in Romania and even in Europe it is a quite low level face to consumption. To obtain complete mixed fodders are recurred imports from different world areas (USA, Argentina, China, Brazil) where are cultivated different soybean sorts in different pedo-climatic conditions fact which made that products entered on Romanian market to present a great variability regarding chemical and nutritional features.

Literature which has tables with nutritive values for raw fodder material (NRC, 1994; FEDNA, 2010; Rostagno et al., 2011) present

generally nutritive value for soybean meal and amino acids profile but without having in view the influence of sort, provenance area or processing conditions on chemical composition (Serrano et al., 2012).

In establishing of recipes for mixed fodders beside crude protein content is taking in account also amino acids content, parameters which are quite variable function of source and implicitly by sort(Irish et al., 1993; Dudley-Cash, 1997). In this context, was proved that many factors could affect chemical composition of soybean meal such as gene-type (Cromwell et al., 1999; Palacios et al., 2004), soil type, latitude, localization and environmental conditions (van Kempenet al., 2002; Goldflusetal., 2006; Thakuret al., 2007), source and origin country (Waldroupet al., 1985; Parsons etal., 1991; Karr-Lilienthal et al., 2004; de Coca-Sinova et al., 2008). Having in view the above mention things by the current paper we aimed to analyse from chemical and nutritional point of view several soybean meal imported from Brazil, by one of the largest importers from Romania.

## MATERIALS AND METHODS

Analyses were effectuated on soybean meal samples imported from Brazil. The analysed material was constituted by 10 samples which were gathered from 10 imported lots of soybean meal. From each lot was gathered a number of samples and after that was formed an average sample in according with Regulation (EU) nr. 691/2013 (Murariu et al., 2013).

Chemical composition was established as follows: determination of dry matter was realised using AOAC nr. 925.30 method (AOAC, 1990; Benţea et al., 2015;Lazăr et al., 2015), and water content was the difference in according with the formula:Water(%) = 100% – DM(%); protein content was calculated by multiplication of total nitrogen content with 6.25, and for determination of total nitrogen was utilised Kjeldah method in according with AOAC nr. 925.31 method (AOAC, 1990; Szakacs et al., 2016;Lazăr et al., 2014a); content in lipids was determined by Sohxlet method in conformity with AOAC nr. 925.32 method (AOAC, 1990;Benţea et al., 2013; Lazăr et al., 2014b); content in total mineral substances was realised by samples' carbonization and after that their calcinations in according with AOAC 900.02 method (Lazăr et al., 2013); non-nitrogenous extractive substances were calculated by difference in according with the formula:NES(%) = 100% – (Water% + Ash% + CP% + CF%) (Stoica et al., 2001; Dolişet al., 2018).

Determination of amino acids was done by liquid chromatography which presumed the detachment of amino acids from protein molecule by utilisation of acid hydrolysis. Aminoacidsare determined after derivation of samples ortophtalaldehide and detection at 338 µm. Method wasrealised in conformity with SR EN ISO 13903:2005 standard, calculus for concentration being made by rating of drops' area to calibration curve.

The results of analysis were processed being statistically calculated position and variation estimators (arithmetic mean, standard deviation of mean S and variation coefficient V%) (Sandu, 1995; Doliş et al., 2017; Lup et al., 2017).

For determination of soybean meal energetic value was realised the calculus of caloricity using the theoretical formula based on quantity of gross energy liberated at burning of 1 g of proteins, fats and carbohydrates in calorimeter bomb, in concordance with the relation: GE (kcal/100 g) = 5.70 kcal x g proteins + 9.50 kcal x g fat +4.2 kcal x NES (Stoica et al., 2001; Halga et al., 2005; Simeanu, 2017).

Quality of proteins was appreciated with chemical methods which evaluate their value on the basis of content in essential amino acids. At the end of amino acids determination, appreciation was done by calculating the chemical indexes, relating amino acids of studied protein to the ones from etalon protein (Stoica et al., 2001; Marin et al., 2013; Simeanu, 2015; Mierliţă et al., 2018):

$$CI = \frac{\text{content in amino acid A of studied protein}}{\text{content in amino acid A of etalon protein}} \times 100.$$

The nutritional values were referred to the whole egg protein amino acid standard (Standard 1: Lysine - 7, Methionine+Cysteine - 5.7, Threonine - 4.7, Isoleucine - 5.4, Tryptophan - 1.7, Valine - 6.6, Leucine - 8.6, Histidine - 2.2, Phenylalanine+Tyrosine - 9.3;

EAA=51.2 g/16 g N (NRC, 1989)), the standard for mature human (Standard 2: Lysine - 5.5, Methionine+Cysteine - 3.5, Threonine - 4, Isoleucine - 4, Tryptophan - 1, Valine - 5, Leucine - 7, Phenylalanine+Tyrosine - 6; EAA=36 g/16 g N –(FAO/WHO, 1991; FAO, 2007; Murariu et al., 2018)) and to two different standards for animal feeding. The protein usability for animal feeding was estimated on the basis of standard for 20-50 kg growing pigs (Standard 3: Lysine - 7, Methionine+Cysteine- 3.6, Threonine - 4.5, Isoleucine - 4, Tryptophan - 1.2, Valine - 5.2, Leucine - 8, Histidine - 2.5, Phenylalanine + Tyrosine - 8; EAA=44 g/16 g N (Boisen et al., 2000; Marin et al., 2016; Mierliţă et al., 2018)) as well as the standard for 6-8 weeks chicken broilers (Standard 4: Lysine - 4.7, Methionine+ Cysteine - 3.3, Threonine - 3.8, Isoleucine - 3.4, Tryptophan - 0.9, Valine - 3.9, Leucine - 5.2, Histidine - 1.5, Phenylalanine+Tyrosine - 5.8; EAA=32.5 g/16 g N (NRC, 1994; Murariu et al., 2013; Mierliţă et al., 2018)).

After calculation of chemical indexes for essential amino acids we calculated Oser index (Oser, 1959) or EAAI (*Essential AminoAcid Index*) (Sujak et al., 2001; Kotlarz, 2011; Simeanu, 2015, Simeanu et al., 2017):

$$EAAI = \sqrt[n]{CI1 \times CI2 \times CI3 \times \ldots \times CIn}.$$

Protein efficiency ratio (PER) of soybean meal was calculated according to the equations developed by Alsmeyer et al., 1974: PER = 0.06320 [$X_{10}$] - 0.1539, where $X_{10}$ = Threonine + Valine + Methionine + Isoleucine + Leucine + Phenylalanine + Lysine + Histidine + Arginine + Tyrosine.

Biological value (BV) was calculated in conformity with the method described (Oser, 1959; Marin et al., 2017; Mierliţă et al., 2018), in according with the following relation:

$$BV = 1.09\ (EAAI) - 11.7.$$

Nutritional index (NI) for analysed soybean meal was calculated in according with the formula described by Crisan and Sands (Crisan et al., 1978; Mierliţă et al., 2018):

$$NI\ (\%) = \frac{EAAI \times \%\ protein}{100}.$$

## RESULTS AND DISCUSSIONS

Chemical composition and urease activity of analysed soybean mean are presented in Table 1. The obtained results show the fact that analysed soybean meal have a chemical composition close to the one presented in literature (Halga et al., 2005; Valencia et al., 2008; 2009; Stefanello et al., 2016) (Table 1).

Table 1.Chemical quality indicators of soybean meal

| Trait | Means (%) | ±Mean error | V% | Min. | Max. |
|---|---|---|---|---|---|
| Water | 9.9 | 0.05 | 1.69 | 9.60 | 10.20 |
| Dry matter | 90.1 | 0.05 | 0.19 | 89.80 | 90.40 |
| Organic matter | 85.3 | 0.07 | 0.26 | 84.90 | 85.67 |
| Gross energy (kcal/kg) | 4290 | 6.82 | 0.54 | 4250.66 | 4329.34 |
| Proteins | 41.24 | 0.21 | 1.59 | 40.50 | 42.40 |
| Lipids | 2.47 | 0.02 | 2.74 | 2.31 | 2.54 |
| Crude fibre | 4.00 | 0.05 | 3.69 | 3.72 | 4.15 |
| Crude ash | 4.80 | 0.05 | 3.06 | 4.60 | 5.08 |
| Calcium | 0.38 | 0.01 | 7.41 | 0.33 | 0.41 |
| Phosphorous | 0.65 | 0.01 | 5.42 | 0.61 | 0.70 |
| NES | 37.59 | 0.16 | 1.34 | 36.81 | 38.21 |
| Urease activity (mg N/g) | 0.042 | 0.001 | 5.95 | 0.04 | 0.04 |

Speaking about dry matter content could be observed that the obtained value was 90.1±0.05%; being quite good and placed into the limits founded in consulted literature (88-92.1%).

Protein content of analysed soybean meal wasn't a very good one because the value of 41.24±0.21% even if was into the limits imposed by literature (40.4-43.5%), was placed at the lower side of the interval.

Speaking about proteins the analysed soybean meal wasn't one with a very good quality but regarding crude fat content we noticed that the obtained value 2.47±0.02% is with around 60% higher that the one reported by Halga et al. (2005), which made that analysed soybean

meal to have a good energetic value (4290 kcal GE/kg).

Also, at those energetic value also contributes non-nitrogenous extractive substances which had a value of 37.59±0.16%, with around 20% higher than the values founded in consulted literature.

About crude cellulose content, the analysed soybean meal had only 4±0.05% while the values from literature are higher (4.48-6%).

Urease activity index was very low 0.042±0.001 mg N/g, value which indicates a correct applied thermal treatment to soybean grains in order to inhibit the anti-nutritional factors. The value of variation coefficient of only 5.95% indicates a constant process for obtaining a good quality soybean meal in the processing unit.

Chemical analysis for studied soybean meal continued with determination of amino acids content, which is presented in Table 2.

Table 2.Amino acids content of soybean meal

| **Aminoacids** | **Mean (g/100 g)** | **±Meanerror** | **V%** | **Min.** | **Max.** |
|---|---|---|---|---|---|
| Tryptophan | 0.558 | 0.009 | 5.37 | 0.512 | 0.611 |
| Threonine | 1.644 | 0.026 | 5.01 | 1.524 | 1.760 |
| Isoleucine | 1.911 | 0.032 | 5.26 | 1.791 | 2.071 |
| Leucine | 3.153 | 0.029 | 2.89 | 3.024 | 3.316 |
| Lysine | 2.635 | 0.043 | 5.19 | 2.427 | 2.815 |
| Methionine | 0.536 | 0.008 | 4.78 | 0.506 | 0.584 |
| Phenylalanine | 2.180 | 0.029 | 4.28 | 2.031 | 2.319 |
| Valine | 2.040 | 0.019 | 3.01 | 1.968 | 2.137 |
| Histidine | 1.148 | 0.009 | 2.48 | 1.107 | 1.198 |
| Arginine | 2.928 | 0.036 | 3.85 | 2.794 | 3.137 |
| Glycine | 2.048 | 0.021 | 3.25 | 1.913 | 2.135 |
| Serine | 2.378 | 0.021 | 2.80 | 2.273 | 2.451 |
| Tyrosine | 1.489 | 0.021 | 4.55 | 1.412 | 1.627 |
| Cysteine | 0.657 | 0.008 | 3.99 | 0.618 | 0.687 |
| ∑AA essential | 19.389 | - | - | - | - |

Sum of essential amino acids in case of analysed soybean meal samples was 19,389 g/100 g, value which is with 1.2% higher than sum of essential amino acids presented by Steffanelo et al, in 2016 but with 6.6% lower than the value reported by Halga et al., in 2005.

Nutritional values of proteins from analysed soybean meal (EAA, CS, EAAI, BV and NI) were calculated based on nutritional standards for broiler chickens aged 6-8 weeks (NRC, 1994, Mierliţă et al., 2018) and nutritional standard for rearing of swine with a corporal mass between 20 and 50 kg (Boisen et al., 2000; Mierliţă et al., 2018). The analysed soybean meal was compared with standards based on nutrients necessary for adult (FAO/WHO, 1991; Simeanu, 2015; Simeanu et al., 2017; Mierliţă et al., 2018).

Content in essential amino acids related to protein content (in g/16 g N equivalent with g/100 g protein) of studied soybean meal and chemical indexes calculated function of mentioned standards are presented in Table 3.

Table 3. Amino acids content andchemical indexesfor studied soybean meal

| Aminoacids (g/16 g N) | | Chemicalindexes | | | |
|---|---|---|---|---|---|
| | | *Standard 1* | *Standard 2* | *Standard 3* | *Standard 4* |
| Tryptophan | 1.353 | 79.59 | 135.31 | 112.75 | 150.34 |
| Threonine | 3.986 | 84.82 | 99.66 | 88.59 | 104.91 |
| Isoleucine | 4.631 | 85.77 | 115.79 | 115.79 | 136.22 |
| Leucine | 7.645 | 88.90 | 109.22 | 95.57 | 147.03 |
| **Lysine** | 6.389 | **91.28** | **116.17** | **91.28** | **135.95** |
| **Methionine + Cystine** | 2.893 | **50.75** | **82.65** | **80.36** | **87.66** |
| Phenylalanine + Tyrosine | 8.899 | 95.69 | 148.32 | 111.24 | 153.43 |
| Valine | 4.947 | 74.95 | 98.93 | 95.13 | 126.84 |
| Histidine | 2.784 | 126.53 | - | 111.35 | 185.58 |
| EAA | 43.527 | - | - | - | - |

Protein from analysed soybean meal is characterized by a low value in comparison with animal origin protein. This fact is confirmed in the current study by content in exogenous amino acids (EAA) which is 43.527 g/16 g N, value with around 15% lower than content in amino acids in hen egg which was taken as standard (NRC, 1989; Mierliță et al., 2018; Simeanu et al., 2017).

Calculation of chemical indexes by relating to standard protein from egg show the fact that the most reduced chemical index is the one for methionine and cystine (50.75%) and the highest one for histidine (126.53%); otherwise, chemical index for histidinewas the only one which passed the level of 100.

In case of calculation of chemical indexes by relating to standard protein for chicken broilers aged 6-8 weeks, we observed that only in case of methionine and cystinethe value was under the level of 100. This fact enlighten that tio-amino acids from soybean meal became limitative in chicken broiler rearing so it must be utilised synthetic methionine and cystine to balance the amino acids share.

The same aspect was observed also in case of chemical indexes calculated by relating to standard protein for piglets with 20-50 kg corporal mass where chemical index for methionine and cystine was 80.36 – the lowest value from all chemical indexes calculated for swine. At this animals' breed and category was observed that are more amino acids which not fulfil the demands fact which impose that soybean meal to be used in mixture with other fodder raw materials so to be well covered the requirements. For this category of swine is imposed utilisation of synthetic amino acids (L-Lysine, DL-Methionine and L-Threonine) in making of mixed fodders.

Sulphuric amino acids are limitative also in case in which such a soybean product will be used in adult human nourishment. From this reason is mandatory that soybean products to be used in human nutrition only in association with those foods which have a better content in methionine, such as rice.

Nutritional value for protein from analysed soybean meal is presented in Table 4.

After applying the formulas for appreciation of proteins' nutritional value from analysed soybean meal we observed, once more, that this one represent a good protein source for chicken broilers (EAAI=133.64%, P-VB=133.96 and NI=55.11%).

Analysed soybean meal could be a good protein source for pigs with a corporal mass between 20 and 50 kg because calculated values were lower than the ones calculated for chickens with 25.55% for EAAI%, 27.78% for P-VB and with 25.54% for NI%. This fact is due to the lower content in sulphuric amino acids ($CS_{Met+Cys}$=80.36).

Regarding the comparison with standard for adult persons, we observed that this soybean product covered well the necessary for amino acids – EAAI%=11.58 and P-BV=109.92.

Table 4. Proteins' nutritional values of studied soybean meal

| Specification | | *Standard 1* | *Standard 2* | *Standard 3* | *Standard 4* |
|---|---|---|---|---|---|
| P-PER | 2.859 | - | - | - | - |
| EAAI (%) | - | 84.36 | 111.58 | 99.49 | 133.64 |
| P-BV | - | 80.25 | 109.92 | 96.74 | 133.96 |
| Nutritional index (%) | - | 34.79 | 46.02 | 41.03 | 55.11 |

[1]Based on egg standard (NRC, 1989);
[2]Standard based on nutrient requirement for mature human (FAO/WHO 1991);
[3]Standard based on nutrient requirement for growing pigs 20-50 kg (Boisen et al., 2000);
[4]Standard based on nutrient requirement of 6-8 weeks chicken broilers (NRC, 1994);
P-BV - Predicted-Biological Value; P-PER - Predicted-Protein Efficiency Ratio.

Having in view the above presented values we could affirm about analysed soybean meal that have a good content in protein, and more over, a good content in amino acids. This conclusion could be generated because, nutritional a protein source with a good value is when essential amino acids index (EAAI) is>0.70, protein efficiency rate (PER) is>2.7 and predicted biological value (P-BV) is>70% (Mierliță, 2018). Even if analysed soybean meal had a good protein and essential amino acids content, however couldn't be utilised in nourishment of young animals without addition of synthesis amino acids.

## CONCLUSIONS

Research realised on 10 imported soybean meal lots shown that chemically speaking the analysed soybean meal had values into limits imposed by literature (90.1±0.05% DM, 41.24±0.021% CP, 2.47±0.02% CF, 4±0.05% CC, 4.8±0.05% C.Ash and 37.59±0.16% NES), and lots were very homogenous (V%=0.19-7.41). Urease activity index was very low 0.042±0.001 mg N/g, value which indicate a correct thermal treatment applied to soybean grains to inhibit anti-nutritional factors.

Appreciation of proteins' nutritional value from analysed soybean meal shown, once more, that this one represent a good protein source for chicken broilers aged 6-8 weeks (EAAI=133.64%, P-VB=133.96 and NI=55.11%) and a good protein source for pigs with a corporal mass between 20 and 50 kg because the calculated values were lower that the ones calculated for chickens with 25.55% for EAAI%, 27.78% for P-VB and with 25.54% for NI%.

This fact is due to the lower content in sulphuric amino acids ($CS_{Met+Cys}$=80.36). Even if analysed soybean meal had a good content in proteins and essential amino acids, however couldn't be utilised in nourishment of young animals without addition of synthesis amino acids.

Regarding the comparison with standard for adult persons, we observed that this soybean product covered well the necessary for amino acids – EAAI%=11.58 and P-BV=109.92.

Having in view the above presented values we could affirm about analysed soybean meal that have a good content in protein, and more over, a good content in amino acids because essential amino acids index (EAAI) is>0.70, protein efficiency rate (PER) is>2.7 and predicted biological value (P-BV) is>70%.

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# THE USE OF WHEY FOR HONEY BEE FEEDING AND OBTAINING OF PROTEIN-CARBOHYDRATE BEE FEED

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***Abstract***

*The content and quality of the dietary proteins determines its preferences and accessibility for honey bees, and represent the basis for the elaboration of protein additives for supplementary bee feeding, which is increasingly applied in beekeeping practices to compensate the amino acid deficiency when natural pollen is scarce. The use of sugar and whey syrup as a protein supplement for early spring feeding stimulates the growth of bee colonies by 13.1-14.5% and increases productivity in harvesting acacia honey by 24.7-44.8%. The result is explained by the fact that whey contains a higher amount of essential amino acids compared to pollen. The whey also contains all vitamins of group B, enzymes and minerals that increase the working capacity of bees, ensure an intensification of the queen's egg laying, improve the broad rearing. At the same time, sweetened whey may be used to obtain easily assimilable protein-carbohydrate food (honey) with a high content of amino acids for the nutrition of bee colonies in pollen-deficient periods in nature.*

***Key words****: honey bee, protein, amino acids, whey.*

## INTRODUCTION

For normal growth and development, bees need proteins, carbohydrates, lipids, vitamins, minerals and water. The main sources of these nutrients are honey and pollen. If the honey is permanently present in the hive, then pollen (or bee bread) at some periods of the year may be in insufficient amounts, which may cause protein deficiency in the bee's nutrition, which in turn affects their health and resistance to infections and parasites. To compensate these deficiencies in bee's nutrition, the protein supplemental bee feeding are largely used depending on the seasonal specificity of the bee functional state and the bee colony's protein needs: in spring – for feeding and brood growth, and in autumn – for the accumulation of reserves in the fat body (fat bees), which ensures better survival of colonies during the winter (Brodschneider and Crailsheim, 2010; Eremia, 2009).

Some supplements for honeybees are also intended to enhance disease control, but their efficacy is not always scientifically confirmed (Botíasa et al., 2013).

Beekeeping specialists have been carried out investigations in pollen substitute proteins that would supplement the amino acid deficiency of bee colonies, especially in early spring. Various processes and technologies for obtaining protein supplements based on soybean, peas, corn, oats, barley meal, egg powder and egg whites, integrated milk or powdered milk, fish meal, dry yeast, etc. are known to be administered either in the form of "candy", which is a mixture of honey, powdered sugar and protein flour, or in the form of sugar syrup with the addition of proteins or amino acids (Malaiu, 1976; Standifer et al., 1977; Brodschneider and Crailsheim, 2010; Fleming et al., 2015).

However, these substituents did not have the same effect on various vital processes in the bee organism, and as a result contradictory data on the effects of protein supplements feeding on the growth and productivity of bee colonies were obtained. When different protein additives are analyzed, it is recommended to consider the biological value of the proteins and the amino acid content and composition compared to pollen or bee bread. Following these parameters, among the protein sources tested, the most positive results were obtained using dried yeast, soy bean meal and milk (Brodschneider and Crailsheim, 2010, Matilla and Otis, 2006). Also the supplements can be obtained by mechanical mixing of the selected components, without taking into account the functional

peculiarities of food consumption of adult bees. It is known that working bees only consume liquid food; solid protein particles (as flour) are hardly assimilated by bees (Wright et al., 2018). At the same time, the majorities of protein-enriched candies are costly and take a long time to get the final product. On the other hand, in the case of syrup administration in low temperature conditions of early spring and late autumn, the bees lose a lot of energy for its processing (evaporation of the syrup fluid), which in turn causes bee colonies weakening.

Recently, researchers and specialists in nutriation pay close attention to milk whey as a source of biologically and physiologically valuable protein. For these reasons whey proteins are currently used for various therapeutic purposes, as well as a basic component of balanced diet (Krissansen, 2007; Gupta and Prakash, 2017). However, data on the use of whey as a protein additive for bee feeding are fragmented or even lacking.

Thus, the aim of this study work was to reveal the influence of whey sugar syrup on the honey bee colony development and honey production and the use of whey syrup to obtain protein-carbohydrate food (honey) with a high content of amino acids for bee feeding.

## MATERIALS AND METHODS

In order to reveal the influence of whey-based syrup (sweetened whey) on the honey bee colony development and honey production, the experiments were performed on a group of 8 bee colonies: 4 – the control group and 4 – the experimental group.

Syrup was administered in the spring (year 2017). Colonies in the control group were given sugar syrup in the ratio 1:1 water and sugar, and colonies in the experiment group– sweet whey sugar syrup in the same proportion (1:1 sweet whey and sugar). Each colony consumed 3 liters of syrup, given at most 300 ml at an interval of 2-3 days for one month. The recordings were performed until and after stimulation feeding. The size of bee colony was measured by the number frames with bees in hive and by the amount of capped brood cells.

To obtain the protein-carbohydrate food (honey) the bee colonies were feeding with whey sugar syrup in the period: from the end of the picking of acacia honey to the beginning of harvesting of lime honey, daily 3-5 liters per colony in the evening.

Determination of the free amino acid content in obtained honey was performed at the amino acid analyzer AAA-339M (Czech Republic) by the ion exchange chromatography method (Moore et al., 1958).

The analysis is performed in the standard procedure for the determination of free amino acids using lithium buffer solutions, pH 2,90; 2,95; 3,20; 3,80 and 5,00; with a flow rate of 12,0 ml/hr. On the basis of the qualitative calculation of amino acid content in the liquid studied it is stated that the amount of an amino acid in the sample is proportional to the surface of the pick of the chromatogram. The calculation consists in the fact, that sample and standard mixture of amino acids with the same content is analyzed. The amount of amino acids dosed on the ionic column in the test sample is given by the formula below:

$$C_{i(doz.)}=k \cdot n \cdot S_{i(prob.)}/S_{i(st.)} \cdot M_i \cdot 10^{-6} \text{ (mg)}$$

where $C_{i(doz.)}$ – the ionic concentration of amino acids in the volume of the dosed node; n – the amount of the amino acids in the analyzed mixture; $S_{i(prob.)}$ — the tip(pick) surface of the amino acids in analyzed mixture; $S_{i(st.)}$ – the tip(pick) surface of the amino acids in standard mixture; k – correction coefficient considered to be changing the detector sensitivity; $M_i$ – the ionic molecular weight of the amino acid.

The automatical analyzer AAA-339M detects ninhydrin positive components within 1-100 nanomoles concentration. The duration of the analysis of the physiological fluids is 3.5 hours.

## RESULTS AND DISCUSSIONS

For normal growth and development, bees need ten (10) essential amino acids, namely valine, leucine, isoleucine, threonine, methionine, lysine, arginine, phenylalanine, tryptophan, and histidine (De Groot, 1953).

The main source of amino acids and proteins for bees is pollen. However, the pollen from different floral sources has different nutritional values for bees (Corby-Harris et al., 2018).

In the conditions of lack of pollen in nature, there is more than necessary early spring

protein feeding to supplement the protein and amino acids deficiency in food, which are also necessary for the honey bee colony growth and increasing of acacia honey production. Such feeding is a frequent apicultural practice for spring build-up of bee colonies, that stimulate the egg laying by queen in early spring, restore the number of bees in colony and start developing more rapidly (Eremia, 2009; Fetea et al., 2011).

It should be noted that when replacing pollen with other protein-rich feed, it is advisable to consider their nutritional value derived from the amino acids pattern, as well as their amount, especially of essential ones.

On the other hand, Herbert et al. (1977) demonstrated that the optimum protein content in bee feed should be 20-30%. At the same time the 50% level should be avoided.

Choosing of milk whey as the basis for the syrup for honey bee feeding has emerged from the exclusively significance of whey as a dietary super food of new generation (Barth & Behnke, 1997).

Whey proteins make up 20% of milk proteins (the remaining 80% is casein) and have biological and physiological importance (Krissansen, 2007, Gupta & Prakash, 2017). Thus, the whey is valuable not only in terms of the amount of protein but also of its quality. Whey proteins have various therapeutic applications for humans due to its immuno-regulatory, detoxifying, anti-inflammatory, antimicrobial and antioxidant properties (Krissansen, 2007).

Comparative analysis of whey and pollen amino acid patterns revealed that sweet cow's milk whey contains all the spectrum of amino acids present in the pollen. However, their amount in whey is higher than in pollen from different plant species (Table 1).

In whey, as in pollen, a higher content of glutamic and aspartic acids was established. It is considered that glutamic acid is important for the formation of bee's olfactory memory (Locatelli et al., 2005), and glutamine serves as a "fuel" for rapidly proliferating cells, and is considered "conditional essential" in metabolic stress conditions (Krissansen, 2007).

Whey proteins contain a significant amount of branched chain amino acids (BCAAs) – leucine, isoleucine and valine. It was determined that leucine and isoleucine enhance protein synthesis (Kimball & Jefferson, 2006), and for bees, they are key amino acids in the formation of haemolymph proteins. Also these amino acids ensure the functional balance of the internal secretion glands and play an important role in the transition from larva to pupa. Valine is also essential in function of nervous system (Malaiu, 1976; Woltedji et al., 2013).

Table1. The content of some amino acids (mg/g dry weight) in pollen from various plant species (Szezęsna, 2006) and milk whey (Markus et al., 2002; Nilsson et al., 2007)

| **Amino acids** | **Pollen from flower sources** | | | | | **Milk whey** |
|---|---|---|---|---|---|---|
| | *Onagraceae* | *Caryophyllaceae* | *Brassica* | *Sinapis alba* | *Chelidonium maius* | |
| **Essential** | | | | | | |
| Valine | 11.93 | 9.81 | 10.70 | 11.79 | 11.60 | 59.3 |
| Leucine | 16.09 | 18.80 | 21.49 | 22.26 | 23.10 | 79.8 |
| Isoleucine | 10.23 | 8.82 | 8.92 | 9.88 | 9.72 | 57.3 |
| Threonine | 9.62 | 9.53 | 12.50 | 11.65 | 11.44 | 61.1 |
| Methionine | 2.65 | 3.33 | 4.30 | 4.16 | 4.52 | 19.4 |
| Lisine | 14.22 | 14.19 | 20.23 | 21.14 | 16.19 | 76.1 |
| Arginine | 9.45 | 8.80 | 11.00 | 11.26 | 10.72 | 22.0 |
| Phenilalanine | 10.24 | 9.71 | 10.75 | 11.46 | 11.08 | 21.3 |
| Histidine | 4.63 | 6.16 | 5.35 | 5.57 | 5.72 | 18.7 |
| Tryptophan | 2.0 | | | | | 12.3 |
| **Non eseential** | | | | | | |
| Alanine | 11.47 | 10.41 | 12.61 | 12.94 | 12.54 | 42.1 |
| Cysteine | 3.4 | | | | | 22.8 |
| Tyrosine | 5.20 | 4.77 | 5.87 | 5.62 | 5.85 | 20.8 |
| Glycine | 9.76 | 9.58 | 11.47 | 12.76 | 10.79 | 13.8 |
| Proline | 24.97 | 28.79 | 28.02 | 26.98 | 19.18 | 46.7 |
| Serine | 10.43 | 11.43 | 13.27 | 14.20 | 11.94 | 38.8 |
| Aspartic acid | 24.85 | 21.52 | 25.45 | 30.22 | 27.01 | 94.1 |
| Glutamic acid | 26.43 | 22.47 | 26.89 | 29.25 | 27.90 | 141.4 |

In whey (compared to pollen), there is a higher amount of methionine and histidine. It is known that methionine is actively involved in the regulation of protein and lipid metabolism, and in the neutralization of toxic substances, and histidine is particularly necessary for growing brood (Malaiu, 1976; Di Pasquale et al., 2016).

As well, as a result of decarboxylation, histidine is converted to histamine, which is a component of bee venom (De Groot, 1953). In whey, a higher level of tryptophan was found. In pollen only trace amount of this amino acid was detected. Tryptophan is important in maintaining of reproductive functions, producing of nicotinic acid, synthesizing of proteins for larvae feeding and contributing to

pigmentation of the bee's body (Zhao et al., 2015). Some of non-essential amino acids – glycine and proline (that have a higher content in whey in comparison with pollen), exert a stimulating effect on growth in unfavorable conditions and on the flying capacities of honey bees (Haydac, 1970; Malaiu, 1976; Micheu et al., 2000; Di Pasquale et al., 2016). The ratio of lysine and arginine determines the nutritional value of bees' protein (Szezęsna, 2006). In pollen from various floral sources this ratio is 1,87, while in whey – 3,45, which is another argument in using whey as a protein pollen substitutes.

Based on the nutritional potential of whey, the experiments of bee colonies feeding with whey-based syrup in early spring were conducted (Derjanschi et al., 2014). Recordings were made until and after stimulation feeding.

The analysis of the obtained results reveals that the use of sugar and whey syrup stimulates the growth of bee colonies and the brood growth with 13,1-14,5% compared to the control group which receiving water sugar syrup (Table 2).

Table 2. Capped brood and honey production after stimulation feeding of bee colonies with whey sugar syrup

| Variants | Capped brood | | Honey production | |
|---|---|---|---|---|
| | Cells x $10^2$ | % | kg | % |
| Control | 124.1 | 100 | 10.5 | 100 |
| Experimental group | | | | |
| 1 | 142.1 | 114.5 | 15.2 | 144.8 |
| 2 | 140.5 | 113.2 | 13.6 | 129.5 |
| 3 | 141.6 | 114.1 | 14.8 | 140.9 |
| 4 | 140.4 | 113.1 | 13.1 | 124.7 |

Similar data were obtained by other authors, who noted the increase in the number of rearing broods in bee colonies feeding with pollen supplements or pollen protein substitutes in early spring (Doull, 1980; Mattila and Otis, 2006). Previous studies have established that various protein substitutes as well as bee brad contribute to the protein content in haemolymph, development of hypopharyngeal gland, and bee life span (Algarni, 2006; De Jong et al., 2009).

It should be noted that balanced feeding with supplements or with protein substitutes influences not only the brood rearing and some bee morphological and physiological parameters, but also the production of honey.

Thus, it was established that in the experiment with whey-based syrup administration, the productivity of acacia honey increased by 24.7-44.8% compared to the control variant (Table 2). So, we can assume that whey as a source of valuable protein can influences the colony's gathering capacity (Derjanschi et al., 2014).

Some authors consider that increasing honey production in colonies fed with protein supplement may be the result of increasing the bee longevity (Doull, 1980; Mattila and Otis, 2006). Other authors state that honey production is closely correlated with the some physiological abilities of bees, which depends on the feed protein content (Al-Sherif et al., 2012; Malaiu, 1976; Ohashi, 1997). As mentioned above, proteins are absolutely essential for the hypopharyngeal gland development and for the enzyme secretion respectively, which in turn determine the amount of harvested nectar. A direct correlation between the activity of the secretory glands (enzyme activity), the ability to nectar process and the production of honey has been demonstrated (Jerebkin, 1965).

The obtained results are explained by the fact that whey contains the same nutrients that pollen, but in higher quantities (Table 1). The whey also contains B complex vitamins, calcium, potassium and other mineral, which can influence the bee's working capacity, activate the egg laying by queen and brood growth.

Taking into account the nutritional qualities of whey and the high content of essential amino acids for bee growth and development it have been carried out the experiments to obtain whey protein-based food (honey) that corresponds to the natural nutrition of the bees and can be proposed as a protein supplement in the absence of pollen in nature.

The protein-carbohydrate bee feed (whey honey) is obtained as a result of whey sugar syrup administration to bee colonies between the end of the acacia honey harvesting and the beginning of lime honey harvesting and the extraction of "whey honey" after 5-7 days of the last syrup administration. The expected result was to obtain easily assimilable bee food (honey) with an increased amino acid content or protein-carbohydrate bee feed (Vrabie et al., 2019).

In the obtained "whey honey", the content of free amino acids was determined and analyzed in comparison with sunflower honey, most frequently administered to bee colonies during the cold period of the year (January-February) and pollen, which is the main source of protein for bee nutrition (Table 3).

Table 3.The content of free amino acids in protein-carbohydrate bee feed, obtained from whey syrup, in sunflower honey and in pollen (mg/g dry weight)

| Aminoacids | „Whey honey" | Sunflowerhoney | Pollen |
|---|---|---|---|
| Aspartic acid + Asparagine | 0.372 | 0.197 | 13.967 |
| Threonine | 0.156 | 0.059 | 6.950 |
| Serine | 0.176 | 0.091 | 6.598 |
| Glutamic acid + Glutamine | 0.429 | 0.256 | 16.222 |
| Proline | 0.736 | 0.479 | 19.142 |
| Glycine | 0.088 | 0.055 | 6.772 |
| Alanine | 0.141 | 0.084 | 8.092 |
| Valine | 0.135 | 0.075 | 5.894 |
| Cysteine | 0.030 | 0.017 | 1.464 |
| Methionine | 0.003 | 0.001 | 1.432 |
| Isoleucine | 0.148 | 0.056 | 4.485 |
| Leucine | 0.220 | 0.092 | 1.092 |
| Tyrosine | 0.028 | 0.009 | 3.434 |
| Phenylalanine | 0.116 | 0.149 | 5.903 |
| Lysine | 0.180 | 0.087 | 6.722 |
| Histidine | 0.053 | 0.027 | 2.282 |
| Arginine | 0.058 | 0.042 | 3.584 |
| Tryptophan | 0.007 | 0 | 0 |
| Σ of free amino acid | 3.076 | 1.776 | 114.03 |
| Σ of essential amino acids | 1.076 | 0.588 | 38.344 |
| Σ of non essential amino acids | 1.999 | 1.189 | 75.691 |
| Σ of immunoactive amino acids | 1.446 | 0.779 | 59.187 |
| Σ of BCAAs | 0.503 | 0.223 | 11.471 |

The protein-carbohydrate bee feed (whey honey) has a higher free amino acid content compared to sunflower honey.

The ratio of free essential amino acids to the total content of free amino acids in honey obtained from whey is 35.0%, in sunflower honey – 33.1% and in pollen –33.6%; of the immunoactive amino acids respectively is in "whey honey"– 47.0%, in sunflower honey – 44.0% and in pollen – 51.9%.

A significant index for bee's vital activity is the content of branched chain amino acids (valine, leucine and isoleucine), which in "whey honey" is 16.35%, in sunflower honey – 12.55% and in pollen – 10.06%.

Also, isoleucine is the major limiting factor in bee nutrition, which should be not less than 4% of the protein content (Stace et al., 1994). In the protein-carbohydrate bee food obtained from whey the isoleucine content is 4.8%, in the sunflower honey – 3.1%, and in the pollen – 3.9% of the total amino acid content.

It was determined that the lysine-arginine ratio as well as the high lysine content is an important index that determines the protein quality required for bee feeding and the bees' preferences for certain types of pollen (Szezęsna, 2006). In "whey honey" this index is 3.1, in sunflower honey – 2.0 and in pollen – 3.0.

Thus, the protein-carbohydrate bee feed (honey) obtained from the sweetened whey, by exploiting the physiological-metabolic potential of the bee colonies, is an organically valuable product in terms of amino acid content to fill its needs for bees in pollen-deficient periods in nature, which is also accessible and inexpensive.

## CONCLUSIONS

Whey contains all the spectrum of amino acids present in the pollen.

The amount of free amino acids in whey is higher than in pollen from different plant species.

Due to the balanced content of proteins and amino acids in comparison with pollen whey can be used for bee stimulating feeding in early spring.

The administration of whey as a protein substitute encourage the brood rearing and, respectively, the bee colonies size in the spring, which contributes to the increase of acacia honey production.

Protein-carbohydrate bee food (whey honey) obtained from sweetened whey has an increased content of amino acids, is easily assimilable, inexpensive for bee colony stimulating feeding.

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# THE EVOLUTION OF THE BLOOD SERUM INDICATORS DURING THE TRANSITION PERIOD IN DAIRY COWS

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***Abstract***

*The aim of this research trial was to assess the nutritional and health status during the transition period of dairy cows. The transition period is characterized by increased energy requirements and a reduction in the intake of dry matter of the lactating cows. The study was carried out at the Experimental Farm of the Research and Development Institute for Bovine Balotesti, Romania. Blood samples were collected from a number of 50 purebred Romanian Black and Spotted dairy cows during the last 3 pre-partum and first 3 post-partum weeks (3 weeks pre-partum, 1 week pre-partum, at calving, 1 week post-partum and 3 weeks post-partum). A total of 250 blood samples were analysed for serum proteins (total proteins, albumin, urea) and mineral indicators (total calcium, phosphorus and magnesium). During the transition period, the obtained serum values for protein profile were not significantly (P>0.05). The concentration of calcium and phosphorus of blood did not show differences (P>0.05) between sampling period, except the level of magnesium (P<0.05) after partum period comparative with pre-partum period. Current results revealed the importance of the serum indicators as a potential tool for monitoring the nutritional status of the dairy cows at farm levels.*

***Key words****: dairy cows, blood, nutritional status, transition period.*

## INTRODUCTION

Several nutritional strategies have been elaborated and studied in the last years with the aim of optimizing transition cow nutritional status, health and productivity.

One such nutritional strategy involves controlled or restricted energy feeding during the dry period (Cardoso et al., 2013; Roche et al., 2013; Goff, 2006; Goff and Horst, 1997; Bell, 1995; Grant and Albright, 1995; Grummer, 1995). Dairy cows go through physiological changes to prepare themselves for the onset of lactation and the climb to peak milk production (Abuelo et al., 2019).

The transition period for dairy cows has been defined as the period from 3 weeks pre-calving to 3 weeks post-calving (Van Saun, 2016; Quiroz-Rocha et al., 2009 a; Drackley, 1999). The period is characterized by marked changes in the endocrine status of the animal, and a reduction in the intake of dry matter and an increase in energy requirements of the cow for milk production (Huzzey et al., 2005; Smith and Risco, 2005; Cook and Nordlund, 2004; Grummer, 2004; Grummer, 1993).

The transition period is generally associated with poor health and reduced reproductive outcomes (Sheehy et al., 2017), periparturient management is essential for productivity and profitability of dairy farming (Reddy et al., 2016; Ferguson, 2001).

The periparturient period is considered the most critical period in the lactation cycle (Huzzey et al., 2005; Grummer, 1995) because 50 % until 75 % of this may be affected by disease during the first month after calving (Singh et al., 2015; LeBlanc, 2010).

These diseases can be of a metabolic, nutritional, or infectious nature (Andrieu, 2008; Mulligan and Doherty, 2008): ketosis, fatty liver, acidosis, displaced abomasum, milk fever, hypophosphatemia, hypomagmesaemia mastitis, metritis, endometritis and retention of fetal membrane.

It is imperative that nutritional strategies used for transition cows do not result in negatively altered calcium status. Several studies showed that serum calcium levels are important for cow health.

Concentrations lower than 2.0 mmol/L are associated with metritis, displaced abomasum, reduced milk production, reduced pregnancy rates to first service (Chapinal et al., 2012 b; Martinez et al., 2012) and have a detrimental effect on neutrophil function (Martinez et al.,

2014). The aim of this research work was to assess the nutritional and health status during the transition period of dairy cows.

## MATERIALS AND METHODS

The experiment was performed in accordance with the Romanian Law no. 43/2014 and the Council Directive 2010/63/EU on the protection of animals used for scientific purposes.

Fifty purebred Romanian Black Spotted dairy cows were screened for blood serum indicators during the transition period, atthe following intervals: 3 weeks pre-partum, 1 week pre-partum, at calving, 1 week post-partum and 3 weeks post-partum.

The analyses were carried out in the Animal Physiology and Biochemistry Laboratory of the Research and Development Institute for Bovine Balotesti, Romania.

Blood samples were collected aseptically from the jugular vein (9 ml), in vacutainer tubes without anticoagulant for serum separation by centrifugation at 3000 rpm for 15 min and stored in aliquots at –20 °C till further analysis.

The serum proteins (total protein, albumin, urea) and mineral indicators (total calcium, inorganic phosphorus, magnesium) were estimated using a semiautomated biochemical analyzer StarDust MC 15 and DiaSys reagents in dedicated kits. Means ± standard deviations and coefficients of variation (CV) of blood indicators were calculated using Minitab® Statistical Software, version 18.

The Tukey's test was applied to obtain the significance of difference between analyzed intervals. The differences between mean values in the periods were considered significant at $P<0.05$.

## RESULTS AND DISCUSSIONS

The total protein, albumin and urea values of dairy cows during the transition period are presented in Table 1. The means of total protein concentrations were 7.18 g/dL (3 weeks pre-partum), 7.07 g/dL (1 week pre-partum), 6.98 g/dL (calving), 7.21 g/dL (1 week post-partum) and 7.98 g/dL (3 weeks post-partum) without statistically significant differences ($P>0.05$) between periods. The obtained means for serum albumin level (3.54 g/dL-3 weeks pre-partum, 3.37 g/dL-1 week pre-partum, 2.95 g/dL-calving, 3.50 g/dL-1 week post-partum and 3.63 g/dL-3 weeks post-partum did not differ significantly ($P>0.05$) during the transition period. In the assessed period, normal values of the urea were registered, without statistical significance ($P>0.05$).Non-protein nitrogen circulating in blood it has effects on the integrity of the liver and mammary tissue, and alter the reproductive behaviour with decreases in the pregnancy rates and an elevation of the uterine pH after oestrus (Biswajit et al., 2011). These values may reflect the good nutritive diet given to the dairy cows examined during the present work. The obtained serum protein indicators in the present investigation were in agreement with the reports of Coroian et al., 2017. Also, similar values of serum protein indicators were reported by Onita and Colibar (2009), in the peripartum period (for albumin, the obtained values were between 2.4-2.6 g/dL and 22.6-24.3 mg/dL for urea).

Table 1. Serum protein indicators in dairy cows during the transition period (n=50)

| Period/Serum indicators | Total Protein (g/dL) | | Albumin (g/dL) | | Urea (mg/dL) | |
|---|---|---|---|---|---|---|
| | X̄±sd | CV% | X̄±sd | CV% | X̄±sd | CV% |
| 3 weeks pre-partum | 7.18±0.70[a] | 9.75 | 3.54±0.49[a] | 13.84 | 27.36±5.22[a] | 19.08 |
| 1 week pre-partum | 7.07±0.68[a] | 9.62 | 3.37±0.51[a] | 15.13 | 27.06±5.43[a] | 20.07 |
| Calving | 6.98±0.64[a] | 9.17 | 2.95±0.52[a] | 17.63 | 26.94±5.24[a] | 19.45 |
| 1 week post-partum | 7.21±0.60[a] | 8.32 | 3.50±0.52[a] | 14.86 | 27.28±5.94[a] | 21.77 |
| 3 weeks post-partum | 7.98±0.62[a] | 7.77 | 3.63±0.56[a] | 15.43 | 27.52±5.19[a] | 18.86 |
| Reference values | 6.80 – 8.40 | | 2.90- 3.80 | | 20.00 – 40.00 | |

means that do not share a letter are significantly different

The values for the coefficient of variation, for total protein, were below that critical threshold of 10%, indicated a very homogeneous population. The coefficient of variation calculated for albumin was lower than 17.63%, expressing a homogeneous population. However, for the urea, the coefficient of variation was between 18.86 and 21.77%. The means values of

serum calcium, inorganic phosphorus and magnesium in dairy cows during the transition period are presented in Table 2.

Low calcium, inorganic phosphorus and magnesium levels in the blood of peripartum cows can lead to a decrease in food intake, low ruminal activity and intestinal motility (Goff, 2006).

The calcium, phosphorus and magnesium requirements, depending on the body weight of the animal, production, milk composition and the pregnancy stage (Parvu et al., 2003; Dumitru, 1996). The homeostasis of calcium is important for neuromuscular excitability and for hormonal secretion (Wu et al., 2008).

Serum calcium concentration decreases at calving (7.99 mg/dL) and increases (8.24 mg/dL) at the 3 weeks post-partum period (P>0.05).

This evolution is a result of calcium passing in the mammary gland, using it for milk synthesis and consumption in other tissues (Onita and Colibar, 2009).

In the present study, were not found subnormal values of calcemia during the transition periods.

The coefficient of variation calculated for serum calcium was lower than 10%, expressing a very homogeneous population.

Table 2. Mineral indicators in dairy cows during the transition period (n=50)

| Period/Serum indicators | Total Calcium (mg/dL) | | Phosphorus (mg/dL) | | Magnesium (mg/dL) | |
|---|---|---|---|---|---|---|
| | X̄±sd | CV% | X̄±sd | CV% | X̄±sd | CV% |
| 3 weeks pre-partum | 8.09±0.71$^{a}$ | 8.78 | 4.14±1.37$^{a}$ | 33.09 | 1.96±0.68$^{ab}$ | 34.69 |
| 1 week pre-partum | 8.07±0.86$^{a}$ | 10.66 | 3.94±1.24$^{a}$ | 31.47 | 1.97±0.71$^{ab}$ | 36.04 |
| Calving | 7.99±0.73$^{a}$ | 9.14 | 3.84±1.23$^{a}$ | 32.03 | 1.73±0.71$^{b}$ | 41.04 |
| 1 week post-partum | 8.12±0.72$^{a}$ | 8.87 | 3.92±1.26$^{a}$ | 32.14 | 1.91±0.73$^{ab}$ | 38.22 |
| 3 weeks post-partum | 8.24±0.74$^{a}$ | 8.98 | 4.06±1.29$^{a}$ | 31.77 | 2.19±0.62$^{a}$ | 28.31 |
| Reference values | 8.00-11.00 | | 4.60-7.00 | | 2.10-2.80 | |

means that do not share a letter are significantly different

About 80% to 86% of phosphorus present in the body of the animal is found in bones, teeth, and in soft tissue (Álvarez, 2001). In the present work, the inorganic phosphorus (Figure 1) had lower values than normal physiological limits (3.84-4.14 mg/dL) without statistical differences between periods (P>0.05). The risk of phosphorus deficiencies increases at the beginning of lactation when calcium and phosphorus are mobilized through bone resorption to meet the demand for those nutrients (Ekelund et al., 2006).

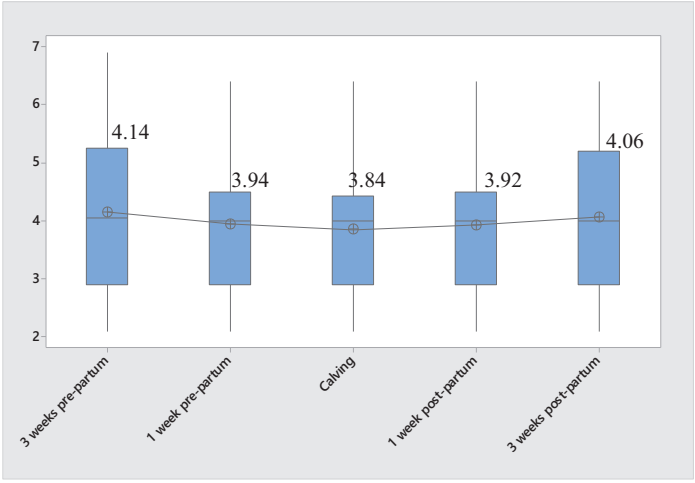


Figure 1. Average values of serum phosphorus in dairy cows during the transition period (mg/dL)

The requirement for magnesium depends on age, production and biological availability of the mineral in the diet, but also on the levels of phosphorus and calcium in the provided feed, if

these levels increase, then the need for magnesium also increases (Álvarez, 2001). Maintaining the plasma concentration of magnesium depends more on the constant flux of this mineral present in the diet offered to the animal than on the mobilization from the skeletal system.

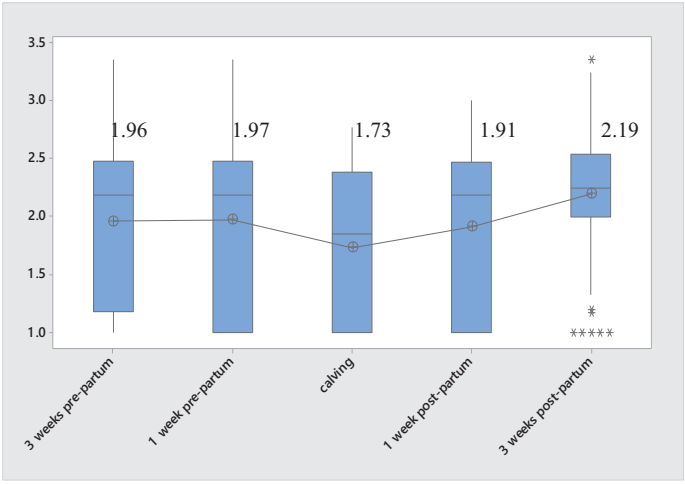


Figure 2. Average values of serum magnesium in dairy cows during the transition period (mg/dL)

The registered values for the serum magnesium level (Figure 2) during the transition period were below normal physiological limits followed by an increase ($P<0.05$) of 3 weeks post-partum (2.19 mg/dL). For inorganic phosphorus and magnesium, the coefficient of variation was situated between 31.47-33.09% (phosphorus) and 28.31-41.04% (magnesium).

## CONCLUSIONS

In the present research trial, the obtained serum values for serum proteins were situated in normal physiological limits, without significant differences between periods. The level of calcium and phosphorus of blood did not show differences between sampling period, except the level of magnesium after partum period comparative with pre-partum period. Current results revealed the importance of the serum indicators as a potential tool for monitoring the nutritional status of the dairy cows at farm levels. For a more complex assessment of nutritional status in dairy cows, research will continue with other investigations (haematological profile, energy profile, enzymatic profile) on a higher number of animals in order to develop a guide of reference values for dairy cows.

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# EFFECTS OF SUPPLEMENTATION OF COW'S MILK AND SOYBEAN MILK FERMENTED WITH PROBIOTIC BACTERIA ON BLOOD LIPID LEVELS AND MEAT QUALITY OF BROILER CHICKEN

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***Abstract***

*The aim of this study was to determine the supplementation of cow's milk-based probiotics and fermented soybean milk on blood lipids profile such as cholesterol levels, triglycerides, and meat cholesterol levels of broiler chicken. The study was conducted from January to February 2018. The method used was experimental with Completely Randomized Design (CRD) and to see the treatment effects. The data were analyzed using Analysis of Variance followed by Duncan's multiple range test. The treatment consisted of five treatments with four replications, namely P0 = control diet/ without giving probiotics, P1 = 100% fermented cow's milk, P2 = 50% fermented cow's milk + 50% soy milk fermentation, P3 = 75% fermented cow's milk + 25% soy milk fermentation. The results showed that the administration of probiotics had a significant effect (P <0.05) on a decrease in blood triglyceride levels and meat cholesterol compared with other treatments. There were no significant effect (P>0.05) on blood cholesterol levels among treatments. In conclusion, the administration of 100% fermented cow's milk and 75% fermented cow's milk + 25% fermented soy milk reduced blood triglyceride levels and meat cholesterol which is improved meat quality.*

***Key words****: fermented milk, blood cholesterol, meat cholesterol, blood triglycerides, broiler chickens, probiotics.*

## INTRODUCTION

Chicken meat is one of the main protein sources for humans. The price of chicken meat is lower compared with the price of beef or mutton. Therefore, the demand for chicken meat continues to increase as a result of the poultry industry in Indonesia is growing. Chicken meat, including poultry products which are foodstuffs, must be safe for consumption. People crave animal foods, especially poultry with low cholesterol and fat content.

High levels of cholesterol and triglycerides in diet can have a negative impact on health such as causing symptoms of pancreatitis and increasing the risk of arteriosclerosis (Wijaya et al., 2013).

Efforts to reduce cholesterol and triglyceride levels in livestock, especially in broiler chickens need attention, one of which is by providing probiotics.

Probiotics are living organisms that are commonly used as additional feed (feed additives), which when consumed can improve the health of livestock by balancing the microflora in the digestive tract.

Fermented products with milk-based probiotic bacteria and soy milk are known to reduce cholesterol and triglyceride levels. The consumption of probiotics is beneficial for the ecosystem balance in the intestinal tract by increasing the population of lactic acid bacteria and decreasing the population of pathogenic bacteria that have an impact on the process of absorption and improving animal health.

Some studies have been carried out to investigate the role of probiotics in lipid metabolism. Adriani et al. (2018) studied the use of fermented cow and soybean milk with *Lactobacillus acidophilus*, and *Bifidobacteria* in broiler's diet had to reduce the blood cholesterol and triglyceride levels.

The purpose of this study was to determine the effect of fermented cow and soybean milk with probiotic bacteria on meat cholesterol levels, cholesterol, and blood triglyceride levels on broiler chickens.

## MATERIALS AND METHODS

This study involved 100 broiler chickens. Probiotics are given at the age of 3 to 45 days. The type of probiotic bacteria consists of

*Streptococcus thermophillus, Lactobacillus bulgaricus, Lactobacillus acidophilus,* and *Bifidobacterium*. Probiotics used in the form of yogurt with the force-feeding method given 2% of the average body weight of chickens (Adriani et al., 2015).

Sampling was carried out at the end of the study, taken from blood and broiler meat. One bird per treatment and replication was taken for sample. Meat samples were taken from the chest and thighs.

Determination of blood and meat cholesterol levels was carried out by the CHOD-PAP (Cholesterol Oxidase Phenylperoxidase Amino Phenozonphenol) method (Richmond, 1983) and triglyceride

analysis using the GPO (Glycerol-3-Phosphate Oxidase) method.

Data obtained from the results of the analysis were tested using the one-way analysis of variance test (ANOVA) and significant results were then tested using the Duncan's multiple range test. The study was conducted by experimental method using a Completely Randomized Design (CRD) with 4 treatments 5 replications, 20 experimental units, bringing a total of 100 tails.

The study was the effect of fermented cow and soybean milks with probiotic bacteria on the meat cholesterol levels, cholesterol and blood triglyceride levels on broiler chickens so that the following experimental treatments were obtained:

P0= Basal ration (without fermented milk)
P1= Basal ration + 100% fermented cow's milk
P2= Basal ration + probiotic 50% fermented cow's milk + 50% fermented soy milk
P3= Basal ration + probiotics 75% fermented cow's milk + 25% fermented soy milk

## RESULTS AND DISCUSSIONS

The results of the analysis of meat cholesterol levels, blood cholesterol, and triglyceride levels in broiler chickens are presented in Table 1.

Table 1. Effects of fermented milk and soybean using probiotic on blood cholesterol, meat cholesterol, and blood triglyceride in broiler

| | P0 | P1 | P2 | P3 |
|---|---|---|---|---|
| Blood Cholesterol (mg/dL) | $150.99 \pm 6.73^{a}$ | $135.49 \pm 7.19^{a}$ | $147.35 \pm 19.92^{a}$ | $141.34 \pm 14.04^{a}$ |
| Meat Cholesterol (µg/mg) | $13.93 \pm 1.87^{b}$ | $10.64 \pm 1.97^{a}$ | $12.19 \pm 1.40^{ab}$ | $10.65 \pm 0.90^{a}$ |
| Blood Triglyceride (mg/dL) | $93.00 \pm 9.67^{b}$ | $53.50 \pm 7.30^{a}$ | $72.67 \pm 21.85^{ab}$ | $65.17 \pm 17.91^{a}$ |

**Blood Cholesterol Level**

The average results show that blood cholesterol level treatments are in the normal range, except those without probiotics (P0) are above the normal range. According to Mitruka (1981) in Manoppo, et al. (2007) that the normal total blood cholesterol level in broiler chickens ranges from 52-148 mg/dL.

The results showed that the supplementation of probiotic-based fermented milk gave the same response to the average blood cholesterol level, but overall the average of each treatment given probiotic-based fermented milk tended to decrease the total blood cholesterol level.

The average blood cholesterol level which was given probiotic-based fermented milk treatment was relatively lower than P0 (without treatment) indicating that the supplementation of probiotics was able to balance the microflora in the digestive tract. According to Surono (2004) and Lengkey and Adriani (2013) states that the cholesterol reduction process is one of them because of the activity of lactic acid bacteria that produce enzymes that hydrolyze bile salt hydrolase or sever the bond of C-24, N-acyl amides formed between bile acids and amino acids in the conjugated bile salts. Lactic Acid Bacteria (LAB) produces the enzyme bile salt hydrolase (BSH), which is a deconjugated bile salt to release the glycine or taurine of steroids to produce bile salt-freeor cause to form colic acid which is poorly absorbed by the small intestine. This result shows that probiotics can increase the synthesis of bile salts which results in more cholesterol needed to synthesize bile salts so that it will reduce

cholesterol levels in the blood, but this study has not had a significant effect. This is supported by the study by Sumardi et al. (2016), which confirms the supplementation of probiotics is not significantly different in reducing blood cholesterol levels.

Decrease in blood cholesterol levels, due to the presence of compounds produced such as short-chain fatty acids from the fermentation process by LAB which competes with Hydroxy Methyl Glutatyil-CoA reductase (HMG CoA reductase) which plays a role in mevalonate formation in the cholesterol synthesis process, so cholesterol synthesis will be hampered (Voet et al., 1999; Sudha et al., 2009). One of the LAB components is propionic acid. Propionate can inhibit the incorporation of acetate into plasma triacylglycerol. This element will result in decreased cholesterol synthesis because acetate is a precursor to cholesterol formation (Marie et al., 2000).

**Meat Cholesterol Level**

The results of statistical analysis of meat cholesterol levels ranging from the highest to the lowest P0 = 12.93 µg/mg, P2 = 12.19 µg / mg, P3 = 10.65 µg / mg, and P1 = 10.64 µg / mg. Statistical analysis showed that the results were significantly different ($P < 0.05$) to decrease meat cholesterol levels.

The results showed that P2 treatment had no significant effect, but treatment P1 and P3 significantly affected the treatment of P0 in reducing cholesterol levels in broiler chicken meat. This finding might indicates that probiotics are capable of producing cholesterol reductase enzymes. The cholesterol reductase enzyme can convert cholesterol to coprostanol, a type of sterol that cannot be absorbed by the intestine. Coprostanol is a natural steroid that can be produced by bacteria in the lower intestine of humans or animals and released through feces (Andi et al., 2015).

The treatment of P1 (100% fermented cow's milk) and P3 (75% of fermented cow's milk and 25% of fermented soy milk) reduced broiler chicken meat cholesterol level. Previous study showed that the higher percentage of cow's milk the higher value of lactic acid levels study (Abu Bakar and Syalawudin, 1999). The difference in basic materials will affect microbial activity in culture, because the basic ingredients affect the growth of lactic acid formation (Tamime and Deeth, 1980).

Flavonoid compounds contained in soy milk are also able to reduce cholesterol levels in meat. Flavonoid is one of the phytochemical groups that have the same structure, namely polyphenols, whose mechanism can reduce cholesterol levels due to HMG-CoA (Hydroxy Methyl Glutatyil-CoA)reductase activity, reduce the activity of the enzyme acyl-CoA cholesterol acyltransferase (ACAT), and reduce cholesterol absorption in the digestive tract (Choi et al., 2008).

Isoflavone compounds in soy milk are also reported to reduce cholesterol levels in addition to flavonoids. Isoflavones are included in the class of flavonoids which are polyphenolic compounds in soy milk. The mechanism for reducing cholesterol by isoflavones is explained by the effect of increasing fat cell catabolism for energy formation which results in a decrease in cholesterol content (Sekiya 2000; Pawiroharsono, 2007).

The conclusion shows that blood cholesterol levels decrease while meat cholesterol levels decrease and are significant. This finding occurs in P1 and P3 treatments, which is 100% fermented cow's milk, and 75% fermented cow's milk + 25% fermented soy milk, respectively (Figure 1).

**Blood Triglyceride**

The average blood triglyceride level of broiler chickens <150 mg/dL (Basmacioglu and Ergul, 2005), Other previous study of Melluzi et al. (1992) reported that normal averages of triglyceride levels were 47.2-162 mg/dL. This shows that blood triglyceride levels in each treatment are in the normal range.

The decrease of triglyceride levels blood in broiler chicken the supplementation of 100% fermented cow's milk (P1) in the diet in the present study maybe due to the ability of probiotic bacteria to produce statins that play a role in the biosynthesis of triglycerides. In line with the statement of Cavallini et al. (2009) that probiotics can produce statins, namely 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors (HMG-CoA reductase) which are cholesterol biosynthesis regulating enzymes, lowering LDL, VLDL, and blood triglyceride levels.

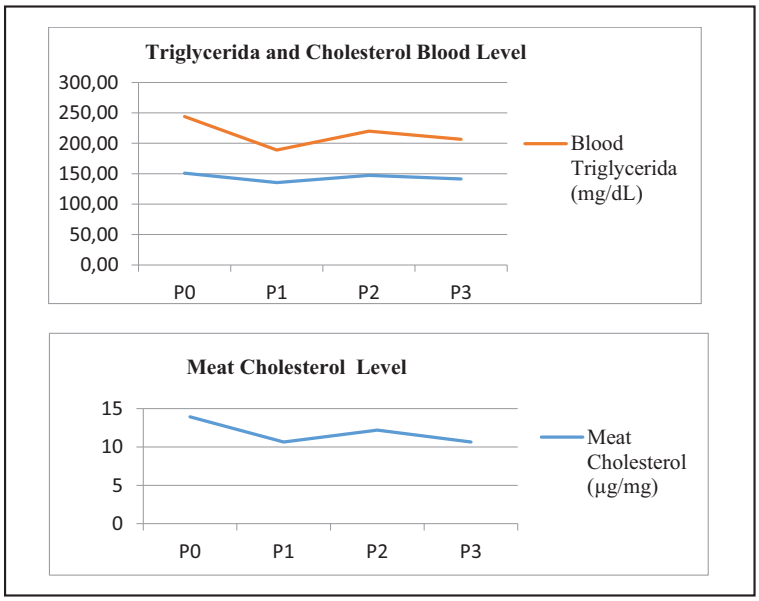


Figure 1. Mean blood cholesterol, blood triglycerides, and meat cholesterol

The mechanism of decreasing triglyceride levels by statins begins when the inhibitor reduces cholesterol concentration in hepatocytes and increases the performance of LDL-receptors that are closely related to VLDL components so that triglycerides will be reduced (Grundy, 1988). The occurrence of inhibition of triglyceride synthesis in the liver and small intestine will result in a decrease in blood triglyceride levels. Scorve et al. (1993) reported that the reduction of fatty acid synthesis in the liver is the main factor causing the decline in triglyceride synthesis in the liver which results in a reduction of the concentration of triglycerides in plasma.

Supplementation of 75% fermented cow's milk + 25% fermented soy milk (P2) decreased triglyceride in plasma due to the closely related decrease in the number of pathogenic bacteria in the intestine. Adriani, et al. (2008) reported that probiotic bacteria such as *Lactobacillus acidophilus* and *Bifidobacterium* can inhibit the growth of pathogenic bacteria. Moreover, Purwati et al. (2005) showed that probiotic administration created a balance of intestinal microflora because the presence of BAL in the intestine which creates an acidic atmosphere that suppresses the growth of pathogenic bacteria in the small intestine. The acidic environment inhibit the secretion of the lipase enzyme, therefore the synthesis of fatty acids in the digestive tract decreases and causes fat to be brought to the liver to decrease which results in a reduction in blood triglyceride levels.

Abu Bakar and Syawaludin (1999) reported that the manufacture of fermented milk with the addition of soy milk. That study recommended that the addition be done at a maximum of 20% of the amo,,unt of cow milk that will be made yogurt, so that the lactic acid level and pH of the fermented milk are optimal. Therefore, supplementation to 50% fermented cow milk: 50% fermented soy milk (P2) does not have a significant effect in reducing blood triglyceride levels. It assumed that carbohydrates contained in soybeans are a group of oligosaccharides that are underutilized as an energy source or as a carbon source for LAB so that the fermentation process is not perfect and causes the LAB population in broiler digestive tract lesser than normal condition, so it cannot inhibit the absorption of lipids correctly.

Flavonoid in soybean is another factor to reduce triglyceride levels in blood. Flavonoid in soybeans can reduce the activity of Glycerol-3-Phosphate Dehydrogenase (GPDH), which is an enzyme that plays a role in the synthesis of

triglycerides. Ta'inindari and Sopandi (2013) reported that flavonoids could inhibit the activity of the GPDH enzyme in adipocytes. Other active substances in soybeans are isoflavones (genistein and daidzein). Isoflavones belong to the flavonoid group. The amount of free isoflavones (aglycones) contained in fermented soy milk is high so that it can activate Peroxisome Proliferator-activated α receptors (PPAR α) (Medjakovic et al., 2010; Kersten, 2001; Kartika and Siti., 2016). PPAR α plays a role in decreasing gene activity which produces triglyceride availability for Very Low-Density Lipoprotein (VLDL) and increases lipoprotein lipase activity. Lipoprotein lipase has a role in lipolysis of triglycerides in chylomicrons and VLDL. The increase in these activities occurs the breakdown of triglycerides into fatty acids and glycerol. This finding is consistent with research by Rayalam and Mary Anne (2007) reporting that injection of genistein and daidzenin can stimulate the occurrence of lipolysis in mice.

## CONCLUSIONS

Supplementation of basal ratio with cow's milk and fermented milk can reduce blood cholesterol levels, blood triglycerides, and cholesterol in broiler chicken meat.
Supplementation of probiotics-based fermented milk with 100% fermented cow's milk and 75% fermented cow's milk + 25% fermented soy milk can increase blood triglyceride levels and broiler chicken fat optimally.

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# FEEDING VALUE OF LOCAL PHYTO-ADDITIVES, POTENTIAL INGREDIENTS IN POULTRY DIETS

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***Abstract***

*Four plants have been characterized physico-chemical as vegetal phytoadditives (blueberry leaves, walnut leaves, marigold and buckthorn meal) in order to determine their nutritional value for inclusion in laying hens diets as possible alternatives to the use of antibiotics in poultry feed. Chemical determinations revealed a variable protein content ranging between 6.76% (blueberry leaves) and 14.14% (buckthorn meal). Marigolds had significantly higher iron content (1262.54 ppm) and blueberry leaves showed high concentrations of manganese (1410.10 ppm) and zinc (40.37 ppm). The walnut leaves were characterized by high concentrations of: calcium (2.01%), lutein + zeaxanthin (264.10 ppm), total polyphenols (53.94 mg EAG/g) and polyunsaturated fatty acids, especially α-linolenic acid (13.45 g/100 g total fatty acids). The studied phytoadditives will be evaluated in a digestibility trial for their nutritional assessment in laying hens diets.*

***Key words****: phytoadditives, plants, nutritional value, hens.*

## INTRODUCTION

The ban on antibiotics as growth promoters in poultry diets made the nutritionists look for alternatives such as probiotics, organic acids, oligosaccharides, botanic materials or plant extracts, which will probably change the microbial profile of the gut content of hens reared under intensive conditions (Yegani and Korver, 2008). Phytoadditives are among the most recent alternatives to antibiotics used as growth promotersfor farm animals, having a beneficial effect on production (Windisch et al, 2008; Kroismayer et al., 2008) and animal health (Athanasiadou et al., 2007; Peric et al., 2010), improving the intestinalmicroflora (Mitsch et al., 2004), enhancing nutrient digestibilityand changing the morphology of the digestive secretion (Kreydiyyeh et al., 2003; Jamroz et al., 2003). There are many plants rich in important nutrients, which seem to have a highly positive effect on animal health and productivity, even on the human health. The walnut (*Juglans regia* L.) contains important amounts of phenol compounds (Labuckas et al., 2008). The highest the content of phenols, the strongest is the antioxidant activity. Their active principles support the bactericide, bacteriostatic, astringent, slightly hypotensive, hypoglicemiant, calming, cicatrizing, emollient, antitoxic, antimitotic, antiperspirant, antiexantematic and antirheumatic activities. The blueberry (leaves and fruits) contains a wide variety of antioxidant substances (Dulebohn et al., 2008; Castrejón et al., 2008; Piljac-Zˇegarac et al., 2009) which help preventing the cardiovascular disorders and protect against cancer (Smith et al., 2004; Seeram, 2008; Neto, 2007) and against cerebral vascular accidents (Wang et al., 2005), also preventing of urinary tract infections (Jepson and Craig, 2007). More recently, strong oxygen radical absorbance capacity (Ehlenfeldt and Prior, 2001), hypotensive effects (Sakaida et al., 2007), hypolipidemic effects (Nagao et al., 2008) and antileukemic activity (Skupień et al., 2006) of the leaves have been reported. However, the detail of the chemical constituents of the leaves has not yet been clarified. Many reports have suggested that blueberry leaves are rich in polyphenol compounds with high antioxidant activity (Wang et al., 2015; Feng et al., 2017). The polyphenols of blueberry leaves are mainly composed of proanthocyanidins, followed by caffeoylquinic acids and flavonolglycosides,

especially the oligomericproanthocyanidins, contribute to the biological activities of the blueberry leaves (Matsuo et al., 2010). According to the literature (Biswas et al., 2010; Kaushaland Sharma, 2011), the leaves, seeds and residues of buckthorn fruits are suitable as animal feeds because they are rich in nutrients (Panaite et al., 2016) and bioactive compounds (Lee et al., 2011) such asvitamins (Luhua et al, 2004; Ranjith et al, 2006), amino acids (Repyakh et al., 1990), lipids (Bekker and Giuschenkova, 1997), sugars (Yang, 2009) and flavonoids (Hakkinen et al., 1999). Some studies have also shown the presence of antioxidants (Püssa et al, 2007;Geetha et al., 2009). They are rich in carotenoids, xanthophyll, phenols andflavonoidsand a high content of essential oils (Yang et al., 2000; Singh et al., 2006). The feeding of buckthorn, in different forms (leaves, seeds or buckthorn fruits residues) produced a significant body weight increase of the animals (Hu and Guo, 2006; Biswas et al., 2010).

The use of 0.2% buckthorn flavonesin broiler diets improved the intestinal absorption of the proteins, lipids, Ca and P. Broiler performance was also improved, as shown by larger eviscerated carcasses, less abdominal fat (Michel et al., 2012).

The marigolds contain flavonoids, carotenoids, vitamin C, etheric oils, bitter substances, triterpene saponins, resins and mucilage.

They are a natural resource of de xanthophyll for broiler diets. In broilers, thezeaxanthin-influences the yellow value in all tissues, particularly in the abdominal fat, lutein and zeaxanthin being stored in the skin and adipose tissues in a proportion of 8-12% and 4-9%, respectively (Hamelin and Altemueller, 2012).

Furthermore, the carotenoids are essential to the immune system, have antioxidant effects and as cannot be synthesized by the birds, they have to be supplied in the diets (Breithaupt, 2007; Jung et al., 2012). The marigold extract (lutein) is a xanthophyll with strong antioxidant capacity, frequently used in layer diets (Koutsos et al., 2006).

Starting from the premises that the above-mentioned plants can be seen as Phytoadditives with positive effects on animal health and productivity, the purpose of the feeding trial was to evaluate the feeding properties of the plants in a digestibility trial for their nutritional assessment in laying hens diets.

## MATERIALS AND METHODS

Our study characterised physically and chemically four plant phytoadditives (marigold flowers, blueberry leaves, walnut leaves and buckthorn meal) as possible alternatives to antibiotics in poultry feeding. The marigold flowers, blueberry leaves and walnut leaves were purchased from a company specialised in processing medicinal plants. The buckthorn meal was purchased from a company producing edible cold pressed oils. The samples were ground in a laboratory mill (Grindomix – GM 200) for 3 minutes, at 6500 rotations/min, homogenized and dried in a drying cabinet for 48 hours (T = $65^0$C) and 24 hours (T = $103^0$C). We used standardized analytical methods, according to Regulation (CE) no. 152/2009 (Sampling and analytical methods for the official inspection of feeds) and ISO standards.

***Determination of the gross chemical composition of the plants***

*The dry matter* (DM) was determined according to ISO standard 6496/2001 using the gravimetric method, by drying at 65-103$^0$C (Sartorius analytical scaleand BMT model ECOCELL Blueline Comfort); *crude protein* (CP) was determined according to ISO standard 5983-2/2009, using the Kjeldahl method (semiautomatic KJELTEC auto 1030 – Tecator); *ether extractives* (EE) was determined according to ISO standard 6492/2001 by extraction in organic solvents (SOXTEC-2055 FOSS – Tecator); *crude fibre* (ISO 6865/2002) was determined by intermediary filtration (FIBERTEC 2010–Tecator) and the *ash* (ISO 2171/2010 was determined using the gravimetric method (Caloris furnace CL 1206). By calculation, we determined the organic matter (formula 1) and the nitrogen-free extractives (formula 2).

$$OM\ (\%) = DMreal\ (\%) - Ash\ (\%) \quad \text{(formula 1)}$$

$$NFE\ (\%) = OM\ (\%) - (CP + EE + CF) \quad \text{(formula 2)}$$

Where: OM = organic matter; DMreal = real dry matter; Ash = ash; NFE = nitrogen-free extractives; CP= crude protein; EE = ether extractives; CF = crude fibre (formula 2)

***Determination of the amino acids***

Amino acids from samples were determined by high performance liquid chromatography (HPLC), using a method optimised and validated by Varzaru et al. (2013), and HPLC system Finnigan Surveyor Plus, HyperSil BDS C18 column, size 250 × 4.6 mm, 5µm (Thermo-Electron Corporation, Waltham, MA).

***Determination of the minerals***

Plant samples of 0.4 g each were processed as described previously (Untea et al., 2012) and analyzed for Ca, Mg, Cu, Fe, Mn, Zn concentrations applying flame atomic absorption spectrometry (atomic absorption spectrometer Solaar M6 Dual Zeeman Comfort (Thermo Electron Ltd., Cambridge, UK) after the microwave digestion (Speedwave MWS-2 Comfort, Berghof, Eningen, Germany). The phosphorus content was determined by UV-Vis spectropho-tometry (UV-Vis spectrophotometer Jasco V530 Tokio, Japan).

***Determination of the fatty acids in the plants***

The fatty acids were determined by gas chromatography, as shown by Panaite et al., (2016), by transformation in methyl esters of the fatty acids from the sample, followed by the separation of the components in the chromatographic column, identification by comparison with standard chromatograms and quantitative determination of the fatty acids according to SR CEN ISO/TS 17764 -2: 2008.

***Determination of the polyphenol concentration***

The total phenol content of plants extracts was measured spectrophotometrically according to the Folin-Ciocalteu's method, as described by (Conrad et al., 2001) with slight modifications. The absorbance was measured at 732 nm and the results were reported as mg gallic acid equivalents per 100 mL of sample (mg GAE/ mL).

***Determination of the total Antioxidant Capacity (TAC) by phosphomolybdenum method***

The total antioxidant capacity of the plant extracts was evaluated by the method of Prieto et al. (2010). The antioxidant activity was expressed for the samples as ascorbic acid equivalents.

**RESULTS AND DISCUSSIONS**

Table 1 shows the basic chemical composition of the phytoadditives. The crude protein ranged between 6.76% CP (blueberry leaves) and 14.14% CP (buckthorn meal). Also the blueberry leaves had the highest concentration of fibre (33.66% CF), whole the buckthorn meal had a high content of fat (15.38% EE) and the lowest proportion of calcium (0.06%), manganese (19.60%) and zinc (27.76 %).

Table 1 data shows that the buckthorn meal is rich in protein and fat. The buckthorn meal protein content was lower than the values reported by (Kaushal and Sharma, 2011) who reported values of 27.7% to33.2%for crude protein, of 15.0% to 21.9% for crude fibre, and of2.7% to 3.6% for the ash. Sharma (2010) reported 90.06%dry matter (DM); 26.00% crude protein (CP); 4.50% ether extractives (EE); 14.00% crude fibre (CF); 2.50% ash; 53.0% NDF; 0.75% calcium (Ca); 1.25% phosphorus (P) and 2906 kcal/kg metabolisable energy (ME) in the buckthorn meal. Similar values for the buckthorn meal chemical composition were also reported by (Fanatico et al.,2005; Fanatico et al., 2006).

Compared to the buckthorn meal (0.06%), the marigold flowers (0.49%) and blueberry leaves (0.50), the concentration of calcium in the walnut leaves (2.01%) was much higher, similar to the findings of other studies (Ercisli et al., 2005). The marigold flowers had the highest concentration of copper (12.16%) and iron (1262.54 mg), while the blueberry leaves had the highest concentration of manganese (1410.10 mg) and zinc (40.37 mg). The iron concentration in the blueberry leaves (62.86 mg) was lowest of all studied plants. Nevertheless, this value is in agreement with the data reported by Criste et. al., (2013) in an inter-laboratory study in Romania, in which participated seven laboratories, the results ranging between 61.43 to 100.86 mg/kg. It is well known that the iron efficiency uptake in wild plants depends on the Fe source, which is different from most greenhouse experiments (Criste et al., 2013).The concentration of lutein-zeaxanthinin the analysed plant material was highest in the walnut leaves and buckthorn meal, followed by the blueberry leaves. Forty-one different carotenoids have been reported in

various cultivars of sea buckthorn berries, the major types being zeaxanthin, cryptoxanthin, and carotene (Pintea et al., 2005). On the other hand, Andersson et al. (2009) showed that the concentration of xanthophyll in the white buckthorn fruits varies with the geographical area. The lowest concentration of lutein and zeaxanthin was determined in the marigold flowers (11.221ppm). As it is known, lutein has a yellow-orange color, and it has been used for many years in poultry diets as a mean to pigment egg yolks. The content of polyphenol (Table 1), both in the walnut leaves(53.94 mg EAG/g) and in the blueberry leaves(52.82 mg EAG/g) shows that both of them are rich in polyphenol.

Walnut leaves constitute a good source of phenolic compounds, suggesting that it could be useful in the prevention of diseases in which free radicals are implicated. The walnut leaves also are a rich source of polyphenol, where flavonols are major compounds, varying between 54.8% and 62.9% of total phenolics (Pereira, et al., 2007). Bilberries are rich sources of various phenolic compounds and carotenoids (Zotatti et al; 2016).

In the other study, Hokkanen et al. (2009) detected several bioactive compounds in bilberry leaves, such as flavan-3-ols, isomers of cinchonain, proanthocyanidins and coumaroyliridoids. On the other hand, bilberry leaf aqueous extracts are useful as antibacterials and against inflammation, especially inflammation of the oral cavity (Wang et al., 2000).

Table 2 shows that the amino acids (essential – lysine, valine and isoleucine, and nonessential – glutamic acid, arginine and tyrosine) content of the buckthorn meal was higher than the concentration of amino acids determined in the walnut leaves, marigold flowers and blueberry leaves. Close values for lysine (0.780%) in the buckthorn meal, were also determined in the walnut leaves (0.688%), which also had the highest concentration of methionine (0.433%) and cystine (0.133%).

The poultry cannot synthesize essential amino acids, which is why these amino acids have to be included in poultry diets for body proteins synthesis, supporting thus the growth and development of the body mass (Mehri et al., 2012; Kheiri and Alibeyghi, 2017).

Table 1. Chemical composition of the plant materials

| Item | Marigold leaves | Blueberry leaves | Walnut leavses | Buckthorn meal |
|---|---|---|---|---|
| **Basic chemical composition, (%)** | | | | |
| SU | 89.81 | 88.37 | 88.99 | 90.20 |
| SO | 79.02 | 86.93 | 79.27 | 88.48 |
| PB | 13.78 | 6.76 | 12.83 | 14.14 |
| EE | 5.55 | 1.38 | 2.21 | 15.38 |
| CF | 15.09 | 33. 66 | 17.41 | 21.19 |
| NFE | 44.6 | 45.13 | 46.82 | 37.77 |
| Ash | 10.79 | 1.44 | 9.72 | 1.72 |
| **Minerals, (% or mg)** | | | | |
| *Macrominerals, (%)* | | | | |
| Ca | 0.49 | 0.50 | 2.01 | 0.06 |
| P | 0.28 | 0.19 | 0.30 | 0.30 |
| *Trace minerals, (mg)* | | | | |
| Cu | 12.16 | 6.95 | 7.11 | 9.02 |
| Fe | 1262.54 | 62.87 | 366.54 | 405.35 |
| Mn | 35.24 | 1410.10 | 159.31 | 19.60 |
| Zn | 29.56 | 40.37 | 30.17 | 27.76 |
| **Xanthophyll, (ppm)** | | | | |
| Lutein+zeaxanthin | 11.221 | 70.591 | 264.096 | 168.757 |
| **Total polyphenols, (mgEAG/g)** | | | | |
| Polyphenols | 13.55 | 52.82 | 53.94 | 31.9 |

Where: DM - dry matter; OM–organic matter; CP– crude protein; EE – ether extractives; CF – crude fibre; NFE – nitrogen-free extractives;; Ca - calcium; P – phosphorus; Cu – copper; Fe – iron; Mn – manganese; Zn – zinc; *Analyses performed by the Laboratory of Chemistry and Nutrition Physiology – IBNA Balotești.

In terms of arginine content, an α-aminoacid used for protein synthesis, the buckthorn meal had the highest concentration, 1.526 % arginine, from the total amount of protein. In poultry nutrition, arginine:lysine ratio is very important,influencing meat quality and the appearance or severity of muscle myopathy in broiler breast (Zampiga et al., 2018).

Table 2. Amino acid profile in the plant materials

| Item | Marigold leaves | Blueberry leaves | Walnut leavses | Buckthorn meal |
|---|---|---|---|---|
| Essential amino acids, (%) | | | | |
| Threonine | 0.523 | 0.498 | 0.713 | 0.557 |
| Valine | 0.811 | 0.385 | 0.724 | 0.760 |
| Phenylalanine | 0.631 | 0.420 | 0.968 | 0.855 |
| Isoleucine | 0.523 | 0.322 | 0.692 | 0.737 |
| Leucine | 0.797 | 0.685 | 1.411 | 1.304 |
| Lysine | 0.397 | 0.451 | 0.688 | 0.780 |
| Methionine | 0.372 | 0.261 | 0.433 | 0.410 |
| Nonessential amino acids, (%) | | | | |
| Ac. aspartic | 2.461 | 0.970 | 1.643 | 1.732 |
| Ac. glutamic | 2.397 | 1.148 | 2.156 | 2.918 |
| Serine | 0.706 | 0.511 | 0.920 | 0.889 |
| Glycine | 0.572 | 0.601 | 0.883 | 0.584 |
| Arginine | 0.555 | 0.359 | 0.831 | 1.526 |
| Alaina | 0.624 | 0.447 | 0.933 | 0.775 |
| Tyrosine | 0.163 | 0.156 | 0.430 | 0.465 |
| Cystine | 0.112 | 0.082 | 0.133 | 0.122 |
| Total | 11.643 | 7.296 | 13.559 | 14.415 |

* Analyses performed by the Laboratory of Chemistry and Nutrition Physiology – IBNA Balotești.

The most important polyunsaturated fatty acid, theα-linolenic acid, was determined in the

highest amount in the walnut leaves (13.45%), followed by the marigold flowers (5.54%) and blueberry leaves (5.47%). However, the blueberry leaves had the best omega6/omega3 ratio, of 1.22, followed by the walnut leaves, with 0.75, and marigold flowers, with 1.6. Walnuts contain about 10% linolenic acid which has been associated with reduced risk in several prospective studies possibly due to antithrombotic and antiarrhymic effects of thelinolenic acid (Dolecek, 1992; Ascherto et al., 1996).

The fatty acids concentration of the plant materials (Table 3), shows that the highest concentration of caproic acid (C6:0) was in the blueberry leaves (3.10%) and in the walnut leaves (3.94%), being absent in the buckthorn meal. Some studies show that the supplements ofcaproic acid (3g/kg feed) for broilers, decreased significantly the number of colony-forming unis in the caecum of broilers, 3 days after the birds were challenged with *Salmonella Enteritidis* (Van Immerseel et al., 2004)

Table 3. Fatty acids profile of the plant materials

| Item | | Marigold leaves | Blueberry leaves | Walnut leavses | Buckthorn meal |
|---|---|---|---|---|---|
| Butyric | C 4:0 | 0.00 | 0.58 | 0.72 | - |
| Caproic | C 6:0 | 1.76 | 3.10 | 3.94 | - |
| Caprilic | C 8:0 | 8.36 | 0.71 | 0.05 | - |
| Nonanoic | C 9:0 | 2.94 | - | - | - |
| Capric | C 10:0 | 4.13 | 3.96 | 0.08 | 0.04 |
| Undecanoic | C 11:0 | 0.48 | 0.00 | 0.08 | - |
| Lauric | C 12:0 | 3.56 | 0.31 | 0.00 | - |
| Tridecanoic | C 13:0 | 0.16 | - | - | - |
| Miristic | C 14:0 | 19.14 | 8.48 | 0.73 | 0.41 |
| Miristoleic | C 14:1 | 0.63 | 1.07 | 0.00 | |
| Pentadecanoic | C 15:0 | 0.41 | 1.10 | 0.10 | 0.00 |
| Pentadecenoic | C 15:1 | 0.40 | 1.17 | 0.07 | 22.01 |
| Palmitic | C 16:0 | 25.38 | 46.51 | 29.84 | - |
| Palmitoleic | C 16:1 | 0.91 | 0.43 | 1.73 | 13.46 |
| Heptadecanoic | C 17:0 | 0.29 | 0.24 | 0.84 | 0.00 |
| Heptadecenoic | C 17:1 | 0.42 | 0.00 | 0.25 | 0.00 |
| Stearic | C 18:0 | 3.95 | 3.84 | 11.29 | 1.91 |
| Oleic cis | C 18:1 | 6.02 | 9.02 | 22.09 | 36.85 |
| Linoleic cis | C 18:2n6 | 7.22 | 4.53 | 10.02 | 21.14 |
| Arachic | C 20:0 | 0.08 | 0.00 | 0.10 | - |
| Eicosenoic | C20 (1n9) | 0.08 | 0.00 | 0.08 | - |
| Linolenic α | C 18:3n3 | 5.54 | 5.47 | 13.45 | 2.34 |
| Heneicosanoic | C 21:0 | - | 0.14 | 0.00 | - |
| Octadecatetraenoi | C18:4n3 | 0.55 | 1.22 | 0.85 | 1.36 |
| Eicosadienoic | C20(2n6) | 0.00 | 0.00 | 0.15 | 0.50 |
| Behenic | C 22:0 | 2.06 | 0.83 | 0.00 | - |
| Eicosatrienoic | C20(3n6) | 0.00 | - | - | - |
| Erucic | C22 (1n9) | 0.00 | - | - | - |
| Eicosatrienoic | C20(3n3) | 0.00 | - | - | - |
| Arachidonic | C20(4n6) | 0.14 | 0.18 | 0.00 | - |
| Docosadienoic | C22(2n6) | 0.45 | 1.62 | 0.75 | - |
| Tricosanoic | C 23:0 | 0.00 | 1.04 | 0.00 | - |
| Eicosapentaenoic | C20(5n3) | 0.37 | 0.00 | 0.17 | - |
| Lignoceric | C24:0 | 0.61 | 0.00 | 0.26 | - |
| Nervonic | C24 (1n9) | 0.00 | 1.79 | 0.97 | - |
| Docosatetraenoic | C22(4n6) | 2.54 | 1.81 | 0.00 | - |
| Altiacizigrasi | | 1.44 | 0.85 | 1.40 | - |
| Total acizigrasi | | 100 | 100 | 100 | 100 |
| *Clasele de acizigrasi din grasime* | | | | | |
| SFA | | 73.31 | 70.84 | 48.01 | 24.37 |
| MUFA | | 8.45 | 13.48 | 25.20 | 50.30 |
| PUFA, din care: | | 16.80 | 14.83 | 25.39 | 25.33 |
| Ω3 | | 6.46 | 6.69 | 14.47 | 3.70 |
| Ω6 | | 10.34 | 8.14 | 10.92 | 21.63 |
| Ω6/Ω3 | | 1.60 | 1.22 | 0.75 | 5.85 |

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## CONCLUSIONS

The data reported in this paper support the fact that the analysed plants can be seen as phytoadditives with positive effects on animal health and productivity, even on human health. Furthermore, it results that they are rich sources of nutrients and that they meet the nutritional requirements for use as ingredients in layer diets.

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# EFFECT OF A BLEND OF COMMERCIAL OILS ON GROWTH PERFORMANCE AND INTESTINAL MICROFLORA POPULATION IN BROILER CHICKENS

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***Abstract***

*The present study was designed to evaluate if the use of a blend of commercial oils (BCO) obtained from different plants could affect growth performance and intestinal microflora of the gut in growing-finishing broiler chickens. A total of 80 Cobb 500, 14-day-old broiler chickens, with initial body weight 360 ±0.38g, were assigned to two groups, basal diet (C) and the basal diet supplemented (E) with 0.50% BCO. Throughout the entire feeding period (14-42 days), there were significant differences (P<0.05) in final body weight, daily weight gain, and for daily feed consumption (35-42days), for group C compared with BCO. At 35 and 42 days, respectively, 6 chicks/group were slaughtered and samples of caecal and intestinal contents were collected for bacteriological examination. Weights of digestive organs including the liver, gizzard, intestine and pH from cecum and intestine were not affected by the dietary treatment. The colony forming units (CFU) of Escherichia Coli and Staphylococcus spp. in the digesta of caecum in the BCO group showed a significantly (P≤0.05) lower number compared with that in the C group. The CFU of Staphylococcus spp. in intestine was significantly (P≤0.05) lower in BCO group compared with C. Salmonella was absent in all cases. The inclusion of 0.5% BCO in the chicken's diet (14-42 days) it has reduced the proliferation of pathogen bacteria and has stimulated the increase of favourable bacteria like Lactobacillus spp. in the intestine and cecum of BCO group.*

***Key words****: broilers; intestinal microflora; essential oil blend; antibiotics; Lactobacillus spp.*

## INTRODUCTION

The increased use of antibiotics has given rise to a fear of the development of resistant pathogenic bacterial strains (Vondruskova et al., 2010) and the contamination with antibiotics of the food chain (Chen et al., 2005). Antibiotics, by being banned from the use as feed additives, has accelerated and led to investigations of alternative feed additives in animal production (Laxminarayan et al., 2013). The concerns about possible antibiotic residues and disease resistance have aroused great caution in the usage of antibiotics in the animal industry (Dibner and Richards, 2005) within the European Union since 2006. The need of new alternatives to replace them has gained increasing interest in animal nutrition. Plants and oil extracts have played a significant role in maintaining human health and improving the quality of human life. Also, they have served humans well for a wide variety of purposes for many thousands of years (Jones, 1996) like flavouring drinks (Lawless, 1995), application for the preservation of stored food (Mishra and Dubey, 1994). Also, as valuable components of seasonings, beverages, cosmetics, dyes, and medicines. Bedford (2000) pointed out that the growth-promoting effects of antibiotics in animal diets are clearly related to the gut microflora because they exert no benefits on the performance of germ-free animals. The manipulation of gut functions and microbial habitat of domestic animals with feed additives has been recognized as an important tool for improving growth performance and feed efficiency (Lee et al., 2001). As one of the alternatives, essential oils are already used as feed supplements to improve growth performance under intensive management systems (William and Losa, 2001). Generally, these essential oils are admitted as safe by the Food and Drug Administration (FDA). They inhibit microbial growth in the gut and enhance nutrient digestibility. Dietary supplementation of some oils has also a beneficial effect on intestinal microflora (Helander et al., 1998). In particular, the antimicrobial activity of plant

oils and extracts has formed the basis of many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies (Lis-Balchin and Deans 1997). While some of the oils used based on their reputed antimicrobial properties have well documented in vitro activity, there are few published data for many others (Kamazeri et al., 2012). Some studies have concentrated exclusively on one oil or one micro-organism, while specific blends of oils (commercial or essential) appear also to control coccidia infection (Saini et al., 2003a) and consequently may help to reduce necrotic enteritis (Saini et al., 2003b). The broiler diets are often supplemented with oils to meet the high energy demands of modern genotypes (Loetscher et al., 2013). Rosehips oils contains carotenoids and phenols, which are important antioxidants (Loetscher et al., 2013; Vlaicu et al., 2017), sesame oil has high phytic acid content is deficient in lysine but high in other essential amino acids (Ahammad et al., 2003). The sea buckthorn oils are rich in vitamins E, K (Vlaicu et al., 2017; Zeb, 2006), carotenoids (lycopene, β-carotene), tocopherols (α-tocopherol is the most abundant), tocotrienols and sterols (β-sitosterol, cholesterol, campesterol, stigmasterol) (Cenkowski et al., 2006; Kumar et al., 2011). Nut oils have extremely variable nutrient levels (protein, lipids and fibre), depending on the extraction process (Panaite et al., 2017). Grape oil contains a wide range of bioactive compounds (polyphenols and flavonoids) which can offer many beneficial properties (Turcu et al., 2018, Olteanu et al., 2017). In the present study, was tested the effects of a blend of commercial oils (BCO) on growth performance and intestinal microflora in growing-finishing broiler chickens.

## MATERIALS AND METHODS

The feeding trial was conducted in the experimental halls of the National Research-Development Institute of Animal Biology and Nutrition (IBNA-Balotesti, Romania) according to a protocol approved by the Commission of Ethics of the institute. The feeding trial was conducted on 80 Cobb 500 broiler chicks (14-42 days), weighed individually, housed in an experimental hall with floor rearing, under 27.10±2.62˚C air temperature, 60.03±11.24% humidity, 32.71±23.38% ventilation and with 23 hours light regimen. The hall was split into 2 experimental compartments (3.5 sq. m/rearing area), each experimental compartment having a capacity of 16 chicks/ sq. m. The broiler chicks were reared on permanent litter of wood shaves (10-12 cm thick). Feed was provided *ad libitum* in some common feeders and water was supplied through automatic nipples with free access. The conventional diet formulation (group C) had corn and soybean meal as basic ingredients (Table 1).

The diet formulation for the experimental group (E) included, unlike the C diet 0.50% BCO in both phases growing and finisher. The BCO was composed of 20% rosehip oil, 20% sesame oil, 20% buckthorns oil, 20% nut oil and 20% grapeseed oil.

Diets formulations were calculated using the results of the chemical analysis of the feed ingredients and according to the nutritional requirements (NRC., 1994) of Cobb 500 hybrid management guide.

Table 1. Compound feeds formulation

| Ingredients | C | E | C | E |
|---|---|---|---|---|
| **%** | **Growing** | | **Finisher** | |
| Corn, | 62.00 | 62 | 60.5 | 60.5 |
| Soybean meal, | 26.58 | 26.58 | 25.46 | 25.46 |
| Gluten, | 4.00 | 4.00 | 6.00 | 6.00 |
| Oil, | 2.50 | 2.00 | 3.75 | 3.25 |
| Blend of commercial oils, (BCO) | 0.00 | 0.50 | 0.00 | 0.50 |
| Calcium carbonate, | 1.40 | 1.40 | 1.33 | 1.33 |
| Monocalcium phosphate, | 1.36 | 1.36 | 1.13 | 1.13 |
| Salt, | 0.37 | 0.37 | 0.33 | 0.33 |
| Methionine, | 0.26 | 0.26 | 0.25 | 0.25 |
| Lysine, | 0.48 | 0.48 | 0.2 | 0.2 |
| Choline, | 0.05 | 0.05 | 0.05 | 0.05 |
| Premix without coccidiosis, | 1.00 | 1.00 | 1.00 | 1.00 |
| Total, | 100 | 100 | 100 | 100 |

| **Calculated:** | | | | |
|---|---|---|---|---|
| Metabolizable energy, kcal | 3140.03 | 3140.03 | 3250.00 | 3250.00 |
| Dry matter, % | 86.48 | 86.48 | 86.49 | 86.49 |
| Crude protein, % | 22.00 | 22.00 | 20.00 | 20.00 |
| Crude fat, % | 4.46 | 4.46 | 5.66 | 5.66 |
| Crude fiber, % | 3.54 | 3.54 | 3.56 | 3.56 |
| Calcium, % | 0.84 | 0.84 | 0.78 | 0.78 |
| Total Phosphorus, % | 0.75 | 0.75 | 0.39 | 0.39 |

1kg premix contains: = 1100000 IU/kg vit. A; 200000 IU/kg vit. D3; 2700 IU/kg vit. E; 300 mg/kg vit. K; 200 mg/kg vit. B1; 400 mg/kg vit. B2; 1485 mg/kg pantothenic acid; 2700 mg/kg nicotinic acid; 300 mg/kg vit. B6; 4 mg/kg vit. B7; 100 mg/kg vit. B9; 1.8 mg/kg vit. B12; 2000 mg/kg vit. C; 8000 mg manganese /kg; 8000 mg iron / kg; 500 mg copper /kg; 6000 mg/ zinc kg; 37 mg/ cobalt kg; 152 mg/ iodine kg; 18 mg selenium /kg

Where: C= basal diet; E= basal diet +0.50% blendof commercial oils (BCO).

The following parameters were monitored throughout the experimental period: body weight (g), average daily feed intake (g feed/chick/day), average daily weight gain (g/chick/day) and feed conversion ratio (g feed/g gain). At 35 and 42 days of age, according to the experimental protocol, six broilers per group were slaughtered by sectioning the jugular vein and carotid artery, after which they were let to bled for 2 minutes, scalded in hot water and manually defeathered. Immediately after were performed measurements to determine the relative weight of some internal organs of broilers (liver, heart, bile, gizzard), by using Kern scales (0.001% precision, Germany) and the pH from cecum and intestine. The pH value of the intestinal and caecal contents was determined with a Mettler Toledo pH-meter. Samples of caecal and intestinal content were collected, in sterile tubes, from the slaughtered chicks, for bacteriological examination (determination of the *Escherichia Coli*, *Salmonella* spp., *Staphylococcus* spp. and *Lactobacillus* spp.).

Samples were collected from each batch of compound feed, for each group, and assayed for the basic chemical composition.

- the dry matter (DM) was determined with the gravimetric method, according to SR ISO 6496:2001;
- crude protein (CP) was determined with the Kjeldahl method, according to SR EN ISO 5983-2:2009;
- the fat was determined by extraction in organic solvents according to ISO 6492/2001;
- the crude fiber (CF) was determined by successive hydrolysis in alkaline and acid environment, according to SR EN ISO 6865:2002;
- the ash (Ash) was determined with the gravimetric method, according to SR EN ISO 2171:2010;
- calcium (Ca) by titrimetric method and phosphorus (P) by spectrophotometry.

*Escherichia Coli* was determined using a classical medium, G.E.A.M. or Levine. The samples were first soaked in enrichment medium (Lauryl-sulphate broth), homogenized and left for 20-30 minutes at room temperature (23-24$^{o}$C). Decimal dilutions were made up to 10-5 in the Lauryl-sulphate medium. The dilutions of $10^{-2}$ – $10^{-5}$ were used to seed 3 Petri dishes each per dilution, on selective medium used.

The Petri dishes were incubated for 48 h at 37$^{o}$C and the colonies were count. Escherichia Coli was interpreted by appearance of dark violet with metallic shine colonies.

The other Staphylococcus spp. formed either dark red opaque colonies (lactic-positive species) or pale pink semi-transparent or colourless colonies (lactic-negative species). *Lactobacillus* spp. were determined on selective medium (MRS broth and MRS agar Merck). The colonies counter was determined by Scan 300, INTERSCIENCE (France).

The effects of treatments were analyzed using one-way variance (ANOVA) with STATVIEW for Windows (SAS, version 6.0). The experimental results were expressed as mean values and the differences being considered statistically significant for $P < 0.001$.

## RESULTS AND DISCUSSIONS

Table 2 data show that the body weight and the daily weight gain recorded for the entire experimental period (14-42 days) were signifycantly ($P \leq 0.05$) higher in C group compared with E group.

Amerah et al., (2011), stated that some essential oils supplementation significantly improved weight gain.

Table 2. Broiler performance (average values/group)

| Item | Days | C | E | SEM | P Value |
|---|---|---|---|---|---|
| Body weight (g) | 14 | 360.21 | 360.55 | 5.634 | 0.9761 |
| | 35 | 2183.09 | 2109.4 | 27.04 | 0.1749 |
| | 42 | 2822.2$^{b}$ | 2669.2$^{a}$ | 38.03 | 0.0435 |
| Daily weight gain (g/day/bird) | 14-35 | 86.96 | 82.87 | 1.249 | 0.1020 |
| | 35-42 | 91.30$^{b}$ | 79.98$^{a}$ | 6.429 | 0.3825 |
| | 14-42 | 88.05$^{b}$ | 82.15$^{a}$ | 1.387 | 0.0324 |
| Daily feed consumption (g CF/bird/day) | 14-35 | 125.22 | 123.21 | 4.451 | 0.8250 |
| | 35-42 | 156.52$^{b}$ | 145.88$^{a}$ | 2.717 | 0.0458 |
| | 14-42 | 133.87 | 129.47 | 3.662 | 0.5539 |

*Where: a-b Mean values within a row having different superscripts are significantly different by least significant difference test (P<0.05); SEM-standard error of the mean; *CF: consumption feed.

Several studies have reported beneficial effects of many combinations of essential oils (mix, blends or just oils) on weight gain (Bento et al., 2013; Péron et al., 2009; Yang et al., 2009), while others reported no effect (Jang et al., 2007; Vlaicu et al., 2017). These differences have been attributed to the type of essential oils used and inclusion level (Cross et al., 2007). Hernandez et al., (2004) reported that an BCO, containing oregano, cinnamon, and pepper and *Labiatae* extract from sage, thyme, and rosemary extracts, fed to broilers gave good performance levels like those of the antibiotic growth promoter, Avilamycin.

The effect of BCO on feed conversion ratio (FCR) is presented in Figure 1. For the entire trial period, FCR was higher in the E group compared with C.

Osman et al., (2005) improved FCR by approximately 12%, by using essential oils in broiler diets. These differences among the researcher's results may be due to different active ingredient from used plants.

The weight of the internal organs (Table 3) didn't show significant differences between the two groups, for any growing period. The measurements performed after slaughter (35 days) show that the relative weight of the liver, heart, bile and the gizzard was lower in the E group compared with C, but not statistically significant.

The same differences were found also after slaughter at 42 days. Amerah et al. (2010), said that essential oils supplementation increased the relative gizzard weight and reduced the caecal weight in birds.

Hernandez et al. (2004), by using a blend of three extract of essential oils didn't find any differences for some internal organs like: proventriculus, gizzard, liver, pancreas, or large or small intestine weight, concluding that the use of BCO had no effects on organ weights.

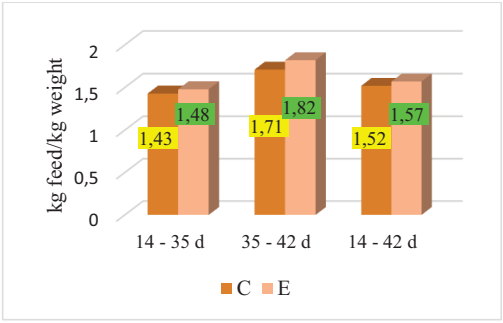


Figure 1. Feed conversion ratio

Table 3. Effect of the BCO on development of the internal organs

| Group / Organs | C | E | SEM | P Value | C | E | SEM | P Value |
|---|---|---|---|---|---|---|---|---|
| | 35 days | | | | 42 days | | | |
| | grams | | | | | | | |
| Liver, | 42.20 | 38.45 | 1.404 | 0.2077 | 52.02 | 47.78 | 0.099 | 0.1864 |
| Heart, | 9.40 | 9.01 | 0.287 | 0.5302 | 12.42 | 11.28 | 0.554 | 0.3548 |
| Bile, | 3.17 | 2.66 | 0.299 | 0.4291 | 2.08 | 2.74 | 0.394 | 0.4956 |
| Gizzard, | 35.53 | 39.76 | 2.643 | 0.4496 | 53.83 | 46.15 | 2.981 | 0.2120 |

Where: a-b: Mean values within a row having different superscripts are significantly different by least significant difference test (P<0.05). SEM: standard error of the mean;

Tables 4 and 5 show the results of the bacteriological determinations on ileal and caecal contents collected after slaughter at 35 and 42 days. The concentration of the analyzed microorganisms *Escherichia Coli*, *Staphylococcus* spp., *Lactobacillus* spp. and*Salmonella* spp. (table 4and 5) are within normal limits (Gournier-Chateau et al., 1994). Regarding the effects of BCO on the intestinal microbial population of broilers (table 4), at 35 respectively 42 days, the number of *Staphylococcu*s spp. CFU was significantly lower (P≤0.05) in E group compared to C group. *Escherichia coli* count was also significantly lower in E group compared with C group. The *Lactobacillus* spp. count was significantly higher (P≤0.05) in the samples collected from E group at 35 and 42 days. Mead & Adams (1975), stated that the intestinal bacterial community of broilers changes with age as indicated by both culture-based and culture-independent studies (Gong et al., 2002a; Wise and Siragusa 2007). Also, the intestinal bacterial composition and activity in broilers has been found to be influenced by the composition and physical structure of the feed (Engberg et al. 2002, 2004).

Table 4. Effects of the BCO on the intestinal microbial population of broilers

| Items | C | E | SEM | P value |
|---|---|---|---|---|
| 35 days | | | | |
| CFU/g intestinal content | | | | |
| *Escherichia Coli* | 6.102[b] | 6.018[a] | 0.013 | <0.0001 |
| *Staphylococcus* spp. | 5.528[b] | 5.374[a] | 0.027 | 0.0003 |
| *Lactobacillus* spp. | 6.189[b] | 6.802[a] | 0.093 | <0.0001 |
| *Salmonella* spp. | absent | absent | NA | NA |
| 42 days | | | | |
| *Escherichia Coli* | 6.360[b] | 6.340[a] | 0.004 | <0.0001 |
| *Staphylococcus* spp. | 6.160[b] | 6.130[a] | 0.006 | <0.0001 |
| *Lactobacillus* spp. | 7.420[b] | 7.450[a] | 0.004 | <0.0001 |
| *Salmonella* spp. | absent | absent | NA | NA |

The results were expressed as logarithm of colony forming units/ml.Where: a-b: Mean values within a row having different superscripts are significantly different by least significant difference test (P<0.05). SEM: standard error of the mean; NA=not applicable

As it can be noticed, the number of *Escherichia coli* CFU in the caecal content at 35 and 42 days of broilers (Table 5) was significantly (P≤0.05) lower in E group compared with group C. Also, at 42 days, the number of Staphylococcus spp. has significantly (P≤0.05) decreased. *Lactobacillus* spp. count was significantly (P≤0.05) higher in the samples collected at 35 and 42 days in cecum from E group. *Salmonella* spp. was absent in all cases.

Table 5. Effects of the BCO on the caecal microbial population of broilers

| Items | C | E | SEM | P value |
|---|---|---|---|---|
| 35 days | | | | |
| CFU/g caecal content | | | | |
| *Escherichia Coli* | 10.04[b] | 9.98[a] | 0.010 | 0.0009 |
| *Staphylococcus* spp. | 8.750 | 8.74 | 0.005 | 0.3871 |
| *Lactobacillus* spp. | 11.40[b] | 11.44[a] | 0.006 | <0.0001 |
| *Salmonella* spp. | absent | absent | NA | NA |
| 42 days | | | | |
| *Escherichia Coli* | 10.30[b] | 10.16[a] | 0.057 | 0.2359 |
| *Staphylococcus* spp. | 8.830[b] | 8.72[a] | 0.017 | <0.0001 |
| *Lactobacillu*s spp. | 11.56[b] | 11.80[a] | 0.037 | <0.0001 |
| *Salmonella* spp. | absent | absent | NA | NA |

The results were expressed as logarithm of colony forming units/ml.Where: a-b: Mean values within a row having different superscripts are significantly different by least significant difference test (P<0.05). SEM: standard error of the mean; NA=not applicable

It is suggested that the establishment of *Lactobacillus* spp. prevents the colonization of pathogenic bacteria by competitive exclusion (van der Wielen et al., 2002). *Lactobacilli* and

bifidobacteria compete against potential pathogens for nutrients and binding sites, thereby reducing the intestinal population of pathogens (Rolfe, 2000). Furthermore, lactobacilli and bifidobacteria produce organic acids and other bactericidal substances (Jin et al., 1998) all of which can suppress the colonization of the intestine by pathogenic bacteria. According to Hammer et al., (1999), when comparing data obtained in different studies, most publications provide generalizations about whether or not a plant oil, extract or blend/mix possesses activity against bacteria. Some publications (Saracila et al., 2018; Turcu et al., 2018; Vlaicu et al., 2017) also show the relative activity of plant oils and extracts by comparing results from different oils tested against the same organism(s), but in different growing conditions. Comparing the data obtained in this study with other published results could be problematic. As stated by Hammer et al., (1999), the composition of plant oils and extracts is known to vary according to local climatic and environmental conditions (Sivropoulou et al., 1995). Furthermore, some oils with the same common name may be derived from different plant species (Reynolds 1996). Unfortunately, reports on the value of commercial oils used as blends in poultry are limited.

## CONCLUSIONS

This study showed that the supplementation of 0.50% BCO (include seabuckthorn, sesame, rosehip, nut and grape oils) in broiler diets significantly improves the health of intestinal microbiota. BCO could be considered as a potential growth promoter for poultry due to digestive stimulating effect, and antimicrobial effect.

## ACKNOWLEDGEMENTS

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# THE QUALITATIVE AND QUANTITATIVE ANALYSIS OF CBD IN HEMP OILS BY UHPLC WITH PDA AND APPLICATIONS

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***Abstract***

*Cannabidiol (CBD) is the major non-psychotropic cannabinoid compound derived from the plant Cannabis sativa L. CBD was first isolated in 1940 and its structure and stereo chemistry determined in 1963. The cannabinoid CBD, a non-psychoactive isomer of the more infamous tetrahydrocannabinol (THC), is available in a growing number of administration modes, but the most commonly known is CBD oil. In just a few years, cannabidiol (CBD) has become immensely popular around the world, CBD is now sold and used to treat a wide range of medical conditions and lifestyle diseases(Hazekamp, 2018). Hemp oil from Cannabis sativa L. is a very rich natural source of important nutrients, not only polyunsaturated fatty acids and proteins, but also terpenes and cannabinoids, which contribute to the overall beneficial effects of the oil. In the European Union (EU), the cultivation of certain cannabis varieties is granted provided they are registered in the EU's Common Catalogue of Varieties of Agricultural Plant Species and the THC content does not exceed 0.2% of the dried flowers of the plant(European Commission website, 2018). There are hundreds of producers and sellers of CBD oils active in the market, and their number is increasing rapidly. Various studies done on CBD oils products around the world have come to similar conclusions about incorrect label information. For that reason, thorough analytical testing of final products by certified third-party labs is an essential tool to guarantee the safety and composition of CBD oils. Hence, it is important to have an analytical method for the determination of these components in commercial samples and their applications. The present work describes a technique for the monitoring the cannabidiol-CBD present in 3 commercial hemp oils, by UHPLC with PDA detection.*

***Key words:*** *Cannabinoids, Cannabis sativa, CBD oil, HPLC, UHPLC.*

## INTRODUCTION

The present work describes a technique for the monitoring of the cannabidiol-CBD present in 3 commercial hemp oils, by UHPLC with PDA detection, for the qualitative and quantitative determination of the cannabidiol (CBD).Using chromatographic methods: HPLC and UHPLC allow the determination of the original composition of cannabinoids in oil by direct analysis.CBD oil is actively marketed for use by children (for Dravet's syndrome, ADHD, autism) (Devinsky et al., 2018), the elderly (Alzheimer's disease, dementia, Parkinson's disease, cardiovascular diseases, inflammatory diseases) (Chagas et al., 2014), patients who suffer from complications (cancer, multiple sclerosis, chronic pain,diabetic complications, arthritis, epilepsy) (Śledziński et al., 2018, Cuñetti et al., 2018, Hunter et al., 2018) and even for pets (anxiety, appetite, sleep, osteoarthritis) (Gamble et al., 2018; Scott et al., 2019); for this reason qualitative and quantitative certification is required by a selective, simple and rapid method. CBD-rich oil has become increasingly popular and is administered via sublingual drops, gel capsules or as a topical ointment (Shimadzu, 2018).

The main source of CBD-rich oil is industrial hemp. CBD oil is derived as a concentrate from $CO_2$ or the butane extraction of hemp, sometimes followed by steam distillation or ethanol distillation for purification (Shimadzu, 2018). Currently, the market is developing further towards more sophisticated and patentable products, including oral capsules, liposomal products, skin creams, and chewing gums containing CBD (Hazekamp, 2018).

CannaLean has developed a novel formulation of Cannabidiol (CBD), with chitosan, a biocompatible, non-toxic, and non-immunogenic compound that enhances the potential of CBD to significantly reduce cholesterol and triglycerides (Apietroaiei et al., 2016, 2018). This method is necessary to determine potency, and ensure the quality and safety of these oils.

## MATERIALS AND METHODS

Chromatographic separation was achieved using a PerkinElmer Brownlee Analytical C18 column (50 mm × 2.1 mm, 1.9 µm) or equivalent, using and gradient elution with 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B. The flow rate was 0.4 mL/min and the injection volume was 5µL. For quantification, the detection wavelength was set at 210 nm (Figure 1).

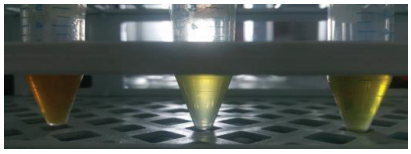

Figure 1. Appearance of samples

All solvents, reagents, and diluents used were HPLC-grade or better.

Solvents, Standards and Samples:

- Cannabidiol Solution, 1.0mg / mL SLBM6755V Analytical Batch Standard Sigma Aldrich
- Absolute methanol, for HPLC, LiChrosolv®
- Acetonitrile, for HPLC, LiChrosolv®
- Formic acid for LC / MS, Fischer Chemical®
- 2-Propanol, for HPLC, LiChrosolv®
- 3 variants of hemp oils from Romania and Holland.

The analytical platform was an PerkinElmer Flexar UHPLC System, including:

- Column Oven
- PDA Plus Detector
- Solvent Manager
- UHPLC Autosampler
- UHPLC Pump

It was used 3 variants of oils marketed on the internet, which, in order to be easier so named:
*Sample 174*-declared product containing 1350 mg/100 ml total cannabinoids concentration (label information),
*Sample 175*-product declared with a content of 2.5% CBD (label information),
*Sample 181*-product declared with 8% CBD content, 4 mg/drop (label information).
The UHPLC method parameters are shown in Table 1.

Table 1. The UHPLC method parameters

<table>
<tr><td>Column</td><td>Column PerkinElmer Brownlee DB , C18(50mm×2,1mm , 1,9µm) (lot# 130114Q)</td></tr>
<tr><td>Mobile Phase</td><td>Solvent A: Water with 0.1% formic acid<br>Solvent B: Acetonitrile with 0.1% formic acid<br>
<table>
<tr><th colspan="4">Program</th><th colspan="3">Solvent Reservoir</th></tr>
<tr><th>Step</th><th>Type</th><th>Time (min)</th><th>Flow (mL/min)</th><th>A</th><th>B</th><th>Curve</th></tr>
<tr><td>0</td><td>Equil</td><td>1.000</td><td>0.4</td><td>50</td><td>50</td><td>0</td></tr>
<tr><td>1</td><td>Run</td><td>0.100</td><td>0.4</td><td>50</td><td>50</td><td>1</td></tr>
<tr><td>2</td><td>Run</td><td>3.000</td><td>0.4</td><td>2</td><td>98</td><td>1</td></tr>
<tr><td>3</td><td>Run</td><td>6.000</td><td>0.4</td><td>2</td><td>98</td><td>1</td></tr>
<tr><td>4</td><td>Run</td><td>1.000</td><td>0.4</td><td>50</td><td>50</td><td>1</td></tr>
<tr><td>5</td><td>Run</td><td>3.000</td><td>0.4</td><td>50</td><td>50</td><td>1</td></tr>
</table></td></tr>
<tr><td>Pressure</td><td>18000 psi/ maximum</td></tr>
<tr><td>Oven Temp</td><td>30ºC</td></tr>
<tr><td>PDA Detection Wavelength</td><td>210 nm</td></tr>
<tr><td>Injection Volume</td><td>5 µL</td></tr>
<tr><td>Sampling (Data) Rate</td><td>5 pts/sec</td></tr>
</table>

The stationary phase in the column must be kept moist before operating the UHPLC. Following an extensive literature study, it was found that the CBD had the maximum absorption in methanol at 210nm wavelength, so this wavelength was chosen for subsequent determinations. To check if the detector works, we took a sample of ultrapure water (about 60 ml). A sample of water was taken, passed through the detector, and then measured.

To save time, it was purged with a 25 ml syringe, and to speed up the process it was directly connected the detectors to the pump. We did the detection with the lamp on average detection = 0.45. So, we determined that the detector is in optimal parameters, so we can introduce the samples.

It was set the tray temperature to 5°C and column temperature to 30°C. We gave a flush to the autosampler for 4 times a with 250µL.

For quantification, the detection wavelength was set to 210nm.

Since we work V/V, 0.5 ml of water was removed from phase A and filled with 0.5 ml of formic acid, and from phase B, we removed 0.25 ml of water and filled with 0.25 ml of formic acid.

The mobile phases were placed on UHPLC. Standard CBD, 1.0 mg/ml in methanol, standard for batch drug analysis SLBMM6755V, Sigma-Aldrich, registered was used as a sample 176 from which we made a dilution in methanol of 100 ppm, 100 µg/mL (complete V/V with 900 µL absolute methanol for HPLC, LiChrosolv®).

It was homogenized everything with a Vortex Genius 3 for 30 seconds.

To check the equipment status, first it was performed an injection of absolute methanol solvent for HPLC, LiChrosolv®.Then it was diluted the samples as follows:

sample 174-0.2 ml oil + 1.8 ml isopropanol (2-propanol) - for HPLC, LiChrosolv®).

sample 175-0.2 ml oil + 1.8 ml isopropanol (2-propanol) - for HPLC, LiChrosolv®)

sample 181-0.2 ml oil + 1.8 ml isopropanol (2-propanol) - for HPLC, LiChrosolv®).

It was developed 7 variants, but we used variant no.6.A PerkinElmer Flexar™ UHPLC system was used, including a quaternary pump, and a PDA (photodiode array) detector.

## RESULTS AND DISCUSSIONS

Sample analysis is significantly reduced (Table 2), while the validation of the method has confirmed that the method generates repeated and accurate results. It was developed an optimized UHPLC-PDA method with low extraction time and more environmentally friendly solvents for adoption in CBD determination laboratories. Sample preparation eliminates the use of chloroform, which has been commonly used in cannabinoid analysis, reducing materials costs, using greener solvents, and improving laboratory safety. Figure 2 shows the cromatograms of a mixture containing CBD, all separeted in a less then 4 minutes.

This method can be used in a variety of settings, from clinical trials, research, quality control, and normative assessment of this growing industry.

Table 2. The accurate quantitation of CBD for the hemp oil samples

| Sample Name | Avg. Amount | Units | Avg. Plates (Foley-Dorsey) | Avg. Tailing Factor | Avg. Resolution | Avg. Area |
|---|---|---|---|---|---|---|
| standCBD 20ppm | 0.0000 | µg/mL | 11,217 | 1.407 | 2.27 | 2,203,748.7 |
| CBD 174 D100 | 35.2245 | µg/mL | 7,385 | 1.083 | 1.25 | 2,803,382.6 |
| CBD175 D100 | 48.3351 | µg/mL | N/A | 0.000 | 1.09 | 3,319,760.2 |
| CBD181 D4500 | 27.3011 | µg/mL | 6,110 | 0.995 | 0.00 | 2,491,310.3 |

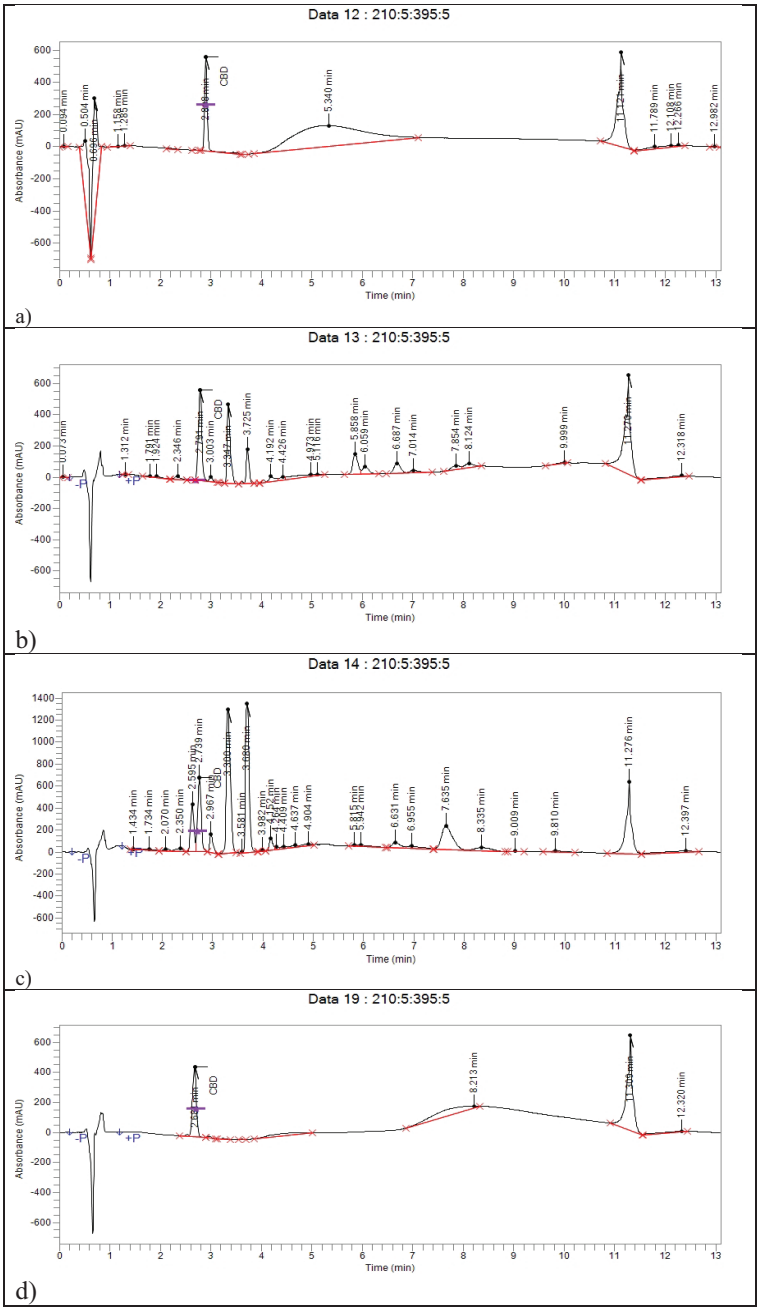


Figure 2. Chromatogram for CBD a) standard 20ppm; b) 174D100; c) 175D100; d) 181 D4500

**Qualitative Analysis of Hemp Oil**

Analysis of the three samples of CBD oil (Table 2) showed differences in the declared concentrations and the results of the study. Off the three analyzed samples: two samples were far below label claim and one sample was well above the label, up to 200%. To solve the problem, some manufacturers simply add to the CBD and CBD-acid content to have a higher CBD-total content on the label. When

purchasing CBD oils, one should consider: label claim, actual concentration, and the selling price.

## CONCLUSIONS

The perspective of the study is to apply this method to different pharmaceutical forms but also to other types of samples (biological, soil, water etc.).It is also an interesting alternative for routine analyzes in forensic sciences, the analytical method easily characterizes and quantifies CBD in hemp oils available from commercial sources to provide a robust tool for potency, safety, and quality determinationswith uses in both human and veterinary medicine.

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# PHYSICAL-CHEMICAL CHARACTERIZATION AND BIOLOGICAL EVALUATION OF CHITOSAN EXTRACTED FROM MARINE WASTE

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***Abstract***

*Chitosan is a well known biopolymer with applications in various areas, and especially in pharmaceutical and medical fields. The main source of chitosan is represented by the marine waste. The present study was focused on chitosan extraction from shrimp waste using a classical chemical procedure. The obtained chitosan was characterized regarding its physical-chemical properties,and also the antioxidant activity and hemolytic activity were evaluated. In this study, the chitosan samples were dissolved in diluted solutions of acetic acid and lactic acid. The results showed that the best antioxidant activity was obtained for the chitosan dissolved in acetic acid. Also, the chitosan samples produced significant effects on the red blood cells activity. All these results suggest that new potential applications could be envisaged.*

***Key words****: antioxidant activity, biological evaluation, chitosan, hemolytic activity.*

## INTRODUCTION

The marine waste is a source of pollution because of its perisability and its high pollution effect if discarded off-shore (Morganti, 2013). Thus, the waste has valuable components such as proteins, salts and chitin that could be recovered for further uses (Dima et al., 2017). The most well-known derivative of chitin ischitosan, which presents biocompatible and biodegradable properties andis obtained by *N*-deacetylation of chitin (Sagheer et al., 2009; Mohanasrinivasan et al., 2014). Chitosan is used in many biomedical applications due to its biocompatibility capacity (Shigemasaand Minami, 1996) and the research carried out over the time exhibited good results of chitosan in treating wound shealing (Alsarra, 2009; Wiegand et al., 2010) or its use as tissue adhesives (Barton et al., 2014). Regarding the chemical structure of chitosan, although the active hydroxylandamino groups in the polymerchains represent the origin of its scavenging capacity, also the deacetylation degree and the molar mass affect the antioxidant activity. Chitosan with very low deacetylation degree has little chelating activity while chitosan with high molecular mass has less scavenging activity because of the strong intramolecular hydrogen bonding that act restrictive to the oxidative agents exposure (Alishahi, 2012).

Chitosan may also behave as hemostatic agent, as previously reported (Nuntanaranont et al., 2018; You, 2016). The hemostatic mechanism of chitosan involves the agglutination of red blood cells, mainly because chitosan is a positively charged polymer that attracts the negatively-charged red blood cells to agglutinate and promote clotting (Zhou et al., 2017).

The main purpose of this study was to extract chitosan using shrimp waste, to evaluate its physical-chemical properties and also the ability to capture long and short term life radicals using ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) assay, respectively chemiluminiscence method. Furthemore, the hemolytic activity of the studied chitosan samples was also tested for

identification of biological effect with different further applications.

## MATERIALS AND METHODS

*Characterization of raw material*

Marine waste was used in this study and the samples consisted in exoskeleton fragments from *Palaemon elegans* (Rathke, 1837) species, which is also known as the rock shrimp (Bacescu, 1967). The samples were collected as waste from the seafood restaurants situated on the Romanian Black Sea littoral zone (Figure 1).

Figure 1. Shrimp waste used as raw material

*Extraction of chitosan*

For the chitosan extraction it wasused a traditional process involving chemical methods (Montilla et al., 2014). Raw material was dried using an oven and was converted into powder using a grinding machine. The obtained powder subjected to demineralization treatment and was performed by immersing the shrimps powder in a solution of 4% HCl (1:13, w/v) for 50 min at room temperature (25°C), while for deproteinization a NaOH solution of 5% (1:20, w/v) for 2 h at 65°C was used. The obtained material was washed once with acetone and rinsed for three times with bidistilled water. Chitindeacetylation was conducted in NaOH solution of 40% (1:15, w/v), for 1 h at room temperature and then 1 h at 95°C. The deacetylation process was repeated using the same experimental conditions in order to obtain a material with less impurities and a higher deacetylation degree. After each chemical treatment the obtained powder was washed with bidistilled water until neutral *p*H was reached, then dried until constant mass.

*Characterization of chitosan*

Physical-chemical characterization of the obtained chitosan was realized in terms of deacetylation degree, viscosity and ash content. The deacetylation degree was determined using the potentiometric titration method (Dima et al., 2017). The viscosity was determined using a mixture of 2% acetic acid solution and KCl 0.1 M as solvent for chitosan (Sagheer et al., 2009), the measurements were performed at 25 ± 1°C using an Ostwald viscometer, and the data was calculated using the Mark-Houwink equation (Hossain and Iqbal, 2014;Moura et al., 2011). The ash content analysis was performed using a furnace from Caloris Group S.A., model 1206M, L Microterm instrument type and was determined according to F2103–01 standard (2006). For chitosan extraction and characterization, hydrochloric acid, puriss. p.a., and acetone, puriss. p.a., from Sigma-Aldrich, lactic acid from Mayam (p.a. 80%, $\rho$ = 1.15 g/cm$^3$), sodium hydroxide pellets, glacial acetic acid and potassium chloride from ChimReactiv S.R.L. were used.

*Biological evaluation of chitosan*

The antioxidant activity of chitosan was tested using two different methods: ABTS and chemiluminiscence. The $ABTS^{\bullet+}$ radical cation solution was prepared according to Rasti et al. (2017) and the sample solutions were prepared using a modified procedure. Volumes of 2 mL $ABTS^{\bullet+}$ and 2 mL of each chitosan sample (0.5-4 g/L) previously dissolved in 1% acetic acid/lactic acid were used. The measurements were realized against a blank sample prepared with 2 mL $ABTS^{\bullet+}$ solution and 2 mL of 1% acetic/lactic acid. Absorbance was measured at 734 nm at time intervals of 4 min using an UV-Vis-NIR Spectrophotometer instrument from Jasco, V-570 type. The analyses were performed in duplicate.

For chemiluminiscence, a Chemiluminometer Turner Design TD 20/20 (USA) instrument was used to scavenge the free oxygen radicals. A system solution containing luminol, Tris-HCl, $H_2O_2$ and 1% acetic acid/lactic acid was used for the preparation of the blank solution. The samples were prepared similarly, using chitosan dissolved in 1% acetic acid/lactic acid. The tests regarding the hemolytic activity are used to evaluate damage to erythrocytes in the

form of membrane damage as a consequence of physical and chemical interactions with the environment (Hasirci and Hasirci, 2018). The hemolysis assay was realized using a modified method from Hasirci and Hasirci (2018). The blood was collected on EDTA to prevent coagulation, from healthy volunteers with their agreement. The blood sample was centrifuged for 5 minutes at 4000 rpm and the plasma was carefully removed using a micropipette. The erythrocytes were washed several times with saline solution (NaCl 0.9%) until the supernatant became clear. The erytrocitar sediment was diluted with 10x phosphate buffer saline (PBS) in a ratio of 1:9 (v/v), and the obtained solution was diluted again with PBS in a ratio of 1:15 (v/v). The obtained erytrocitar solution was added in volumes of 1 mL in each centrifuge tube. The chitosan samples were previously dissolved in 1% acetic acid and in 1% lactic acid and the measurements were realized in triplicate. The prepared samples were kept at 37°C for 12 h, then were centrifuged for 10 min at 4000 rpm and the clearsupernatant solutions were measured using a Jasco V-630 UV-Visspectrophotometer at $\lambda$ = 540 nm (Zhou et al., 2017).

## RESULTS AND DISCUSSIONS

The physical-chemical characteristics of the extracted chitosan sample were performed in duplicate and are presented as follows: deacetylation degree of 94.1 ± 3.5%, viscosity of 209 ± 13 mL/g and ash content of 0.29 ± 0.1%. Consequently, the obtained sample presents a high deacetylation degree and a medium molar mass, and the results are comparable with those obtained by Kucukgulmez et al. (2011) in their study. The high deacetylation degree gives a more reactive character to chitosan in acidic medium, thus contributing to an increase of the antioxidant activity (Rasti et al., 2017). Furthermore, the deacetylation degree values have a significant influence on the biological activity of the extracted chitosan and on its solubility in various acidic solutions (Soares et al., 2009). The viscosity gives informations about the average molar mass and the aggregation capacity of the biopolymer and it strongly depends on the solvent used for solubilization (Younes and Rinaudo, 2015). Generally, the ash content gives information about the total amount of inorganic material, thus, the low ash content obtained for the extracted chitosan could be suitable for biomedical applications (F2103–01, 2006).

Therefore, the obtained properties of chitosan led to the hypothesis for testing its antioxidant and hemolytic activities.

Acetic acid is the most used solvent for chitosan solubilization but also the lactic acid, which is widely used in biological applications (Younes and Rinaudo, 2015). Therefore, both solvents were compared for the antioxidant activity evaluation of chitosan.

Two methods for the antioxidant activity evaluation of chitosan were used. First, the antioxidant activity was determined by ABTS method which consisted in subjecting the chitosan samples to long-life radicals generated by the reaction of ABTS with an oxidizing species represented by the potassium persulfate, then the solution was normalized to 0.7 ± 0.1 absorbance at 734 nm. The absorbance of the chitosan solutions was measured at 734 nm and the results showed that chitosan dissolved in 1% acetic acid presented a higher inhibition percent than chitosan dissolved in 1% lactic acid, for chitosan concentrations ranging between 250 and 2000 µg/mL. Though, both solvents presented similar values for the determination coefficients of $R^2$ = 0.99 (Figure 2).

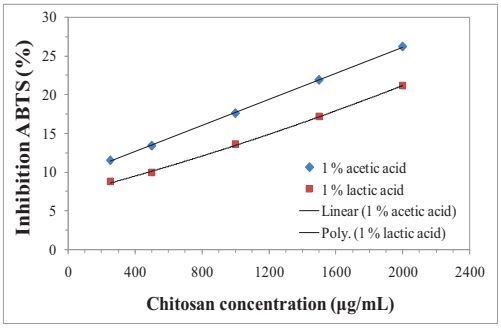


Figure 2. ABTS method evaluated for chitosan dissolved in acetic acid and lactic acid

Chemiluminiscence detection is an extremely sensitive and selective method (Klampfl, 2005), compared to other methods. The

chitosan samples were evaluated in terms of the ability to scavenge the free radicals of short life generated by the reaction of luminol with hydrogenperoxide in the presence of a catalyst to yield 3-aminophthalate in an excited electronic state, which is a light-producing emitter. By hydrogen atom donation the antioxidant samples can quench the activity of the hydrogen peroxide and, thus, lead to the inhibition of the hydrogen peroxide-induced chemiluminiscence (Klampfl, 2005; Zhong and Shahidi, 2015).

The results showed that chitosan dissolved in 1% acetic acid presented a higher inhibition percent than chitosan dissolved in 1% lactic acid, for the same chitosan concentrations used at chitosan concentrations ranging between 50 µg/m Land 400 µg/mL. The equation obtained for acetic acid had a determination coefficient of $R^2$ = 0.98, while for the lactic acid it was obtained $R^2$ = 0.99, which reveals a better correlation of the experimental data (Figure 3). Usually, the increse of chitosan concentration leads to an increase of the antioxidant activiy (Rasti et al., 2017; Abdel-Ghany and Salem, 2019), but also the antioxidant activity is strongly influenced by the solvent used for solubilization (Charernsriwilaiwat et al., 2012).

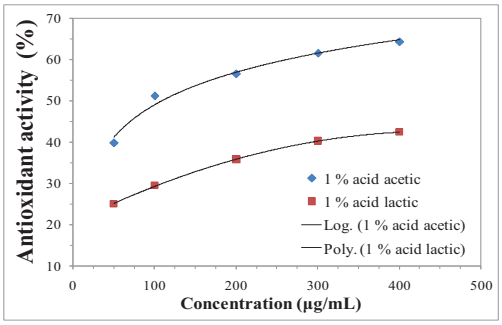


Figure 3. Chemiluminiscence method evaluated for chitosan dissolved in acetic acid andlactic acid

The preliminary results showed that chitosan captures the long and also the short term life radicals and, based on these results, it could be used in aquaculture as feed additive in fish diets due to its capacity of improving health by reducing or preventing the oxidation processes (Abdel-Ghany and Salem, 2019).

Hemolysis represents an important test for biomaterials evaluation and it is calculated as lysis percent of red blood cells (RBCs) produced as a result of the interaction with the biomaterial. The hemolytic activity for chitosan dissolved in 1% acetic acid (AC) solvent showed a more intense activity compared with 1% lactic acid (AL) solvent. For the same chitosan concentration it can be noticed that acetic acid produced a 2-3 times higher hemolytic activity than the lactic acid. The obtained results are presented in Figure 4.

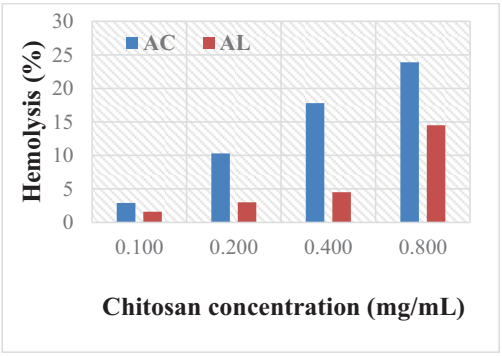


Figure 4. The hemolyticactivity (%) evaluated for the extracted chitosan sample

The activity values below 5% (F756–00, 2000) are classified as slightly hemolytic for chitosan dissolved in lactic acid at concentrations below or equal than 0.4 mg/mL, but also for the chitosan samples dissolved in acetic acid at 0.1 mg/mL concentration. Similar results of hemolytic activity were obtained by Zhou et al. (2017) for chitosan dissolved in solutions of acetic acid. Though, according to F756–00 standard (2000) the values that are higher than 5% classifies chitosan as having hemolytic effects, which occurs for the chitosan samples dissolved in acetic acid at concentrations higher or equal than 0.2 mg/mL.

Therefore, the obtained results highlighted a potential use of chitosan in biomedical applications at certain concentrations that do not affect the integrity of the red blood cells. Also, the solvent used for chitosan sample preparation is an important factor that needs to be chosen deppending on the applications and the expected results.

Though, the hemolytic effect may be lowered if chitosan could be combined with materials such as polyethylene that have no interaction or low interaction with the red blood cells (F756–00, 2000).

## CONCLUSIONS

The marine waste leads to environmental problems because of its high pollution capacity. Therefore, by using the traditional chemical procedure that involves low financial resources the shrimp waste was successfully converted into chitosan.

The antioxidant capacity was evaluated using ABTS and chemiluminiscence methods, and the obtained chitosan presented the highest antioxidant activities for the samples dissolved in acetic acid for both methods used.

Hemolytic activity evaluation showed that chitosan is recommended to be used in situations that involves direct contact with blood, but only in lactic acid and at low concentrations.

The preliminary studies showed that potential applications could be envisaged by using chitosan in aquaculture as food additive, in agriculture as fertilizer or in medical field as bandage for potential infections.

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# CELL DIFFERENTIATION PROCESS OF *Artemia* sp. LARVAE TOOLS FOR NATURAL PRODUCTS TESTING

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***Abstract***

*Morphogenesis and cellular differentiation present a remarkable interest in studies of cellular regeneration, cellular responses to xenobiotics or the evaluation of tumorigeneses and cytotoxicity induced by substances with therapeutic potential. The study of the process of cellular differentiation and its modifications under the influence of natural extracts was made on the larvae (naupliar stages) of Artemia salina. As a result of high sensitivity and accessibility in laboratory manipulation, the larvae of Artemia are used as animal models in the aquaculture, the assessment of acute cytotoxicity and brine shrimp lethality assay (BSLA). The larvae in the growth period pass through a lot of moulting processes which are associated with epithelium cell divisions and the rearrangement of these in tissues structures within a period of several hours. The assessment of cellular differentiation was followed after the exposure of freshly hatched larvae to natural extracts. The effects of the compounds were measured by determining the survival rate of larvae and microscopically observing visible changes. Natural extracts from Taraxacum officinale F.H. Wigg, Chelidonium majus L., Tragopogon dubius Scop, Usnea barbata L., Galanthus elwesii Hook. were analyzed. A cytological study performed on these biotesters indicates a significant correlation between cell differentiation and metabolism. Some potentially cytotoxic compounds induce changes in organogenesis. The results highlight shape changes of the epithelial cells, alteration at intercellular connections, rearranging and reorganizing the primordium appendices cells and altering membrane and blocking cellular differentiation.*

***Key words:*** *Artemia sp., biotester, cell differentiation.*

## INTRODUCTION

The evaluation of cytotoxicity on *Artemia salina* larvae (BSLA) is a test developed and adapted as one of the fastest and most effective due to larval sensitivity to a variety of applications (Michael et al., 1956; Togulga et al., 1998; Telens-Perales et al., 2017). The test is an efficient, cheap and relatively quick way to detect the effect of toxic compounds, requiring only small amounts of samples, i.e., <20 mg.

The species is used in studies aimed at testing the potential cytotoxic activity of various plant extracts (Solýs et al 1993; Meyer, 1982; Rajeh et al., 2012; Sirinthipaporn et al., 2016) and the toxic activity of some mycotoxins (Prior, 1978; Harwig and Scott, 1971). The test also allows rapid and meaningful information to be obtained in cases of teratogenic or potentially mutagenic phenomena (Kerster, 1983; Milhem et al., 2008).

The method has the advantage that the larvae used as a tester have a rapid growth rate. These larvae, in 24 hours, pass to another stage through moulting which activates cell division. For these reasons, larval mortality is associated with phenomena that block the cell cycles during the moulting period.

Furthermore, the simplicity of the larva makes this system comparable to a cellular stem cell complex. In the first 24 hours of hatching the cells are not differentiated, and also, the digestive tract is not open, which strictly involves membrane changes. The larva's transparency allows for the visualization of morphological and cytological details in vivo, which is a significant advantage for the evaluation process.

The study has attempted to test plant extracts known in traditional therapy, and that are described as phytochemical: *Taraxacum officinale* F.H. Wigg, *Chelidonium majus* L., *Tragopogon dubius* Scop*, Usnea barbata* L., *Galanthus elwesii* Hook.

In similar studies, the biotest was used to identify plant extract activities by analysing larval survival, and the results have shown a correlation with cytotoxicity on human cell cultures (Jamieson et al., 2014; Carballo et al., 2002).

Our study specifically focuses on the rapid identification of cytological changes induced by plant extracts in a much simpler and more efficient economical way. The aim is to use these tests as preliminary cytotoxicity studies in the evaluations of substances with toxicological and pharmacological activity, in animals.

The method can be adapted in approaches to assessing the effects of substances such as additives, dietary supplements, medicines or disinfectants used in aquaculture. It also has multiple applications in understanding the effects that certain substances with toxicity risks have when accidentally releasing in the environment.

## MATERIALS AND METHODS

Plant hydroalcoholic extracts are either marketed (*Taraxacum officinale*, *Chelidonium majus*) or obtained through specific laboratory extraction procedures from three vegetables species *Tragopogon dubius, Usnea barbata* (acetone extract), *Galanthus elwesii*. Approximately 100 grams of dried and ground vegetable product was extracted with a solvent (alcohol, acetone) in a Soxhlet installation at about 70°C for 8 hours. After evaporating the solvent through rotavapor, a dry extract was obtained. It was stored in a freezer at a temperature below -20°C until it was used. Stock solutions for each extract were prepared by dilution in saline water (3‰).

*Artemia* cysts hatched in water at 35-36‰, at 25°C (thermostat), with continuous bubbling and artificial illumination (2000 lx). When the larvae appeared, bubbling was interrupted. The larvae were placed in plexiglass for testing (1 ml volume, culture plates). There were 10-20 specimens/samples on each plate. For each concentration, four repetitions were performed. For testing, the extracts were diluted in saline water (Table 1), depending on predicted effects of the extracts.

Table 1. The plant extracts protocol prepared for test

| Plant analysed extracts | Extract type (w:v ratio and solvent used) | Dilutions in saline water |
|---|---|---|
| *Taraxacum officinale* | 40% alcoholic (1:1 ratio) | 1:100 |
| *Chelidonium majus* | 40% alcoholic (1:1 ratio) | 1:100 |
| *Tragopogon dubius* | 40% alcoholic | 1:2, 1:6; 1:10 |
| *Usnea barbata* | DMSO (0,1%) | 1:2, 1:4, 1:10 |
| *Galanthus* | 40% hydroalcoholic (1:3) | 1:2; 1:5, 1:10 |

The larvae that were exposed in the analysed solutions for 24-48 h were subsequently quantified for survival and cytological changes. Results on cytotoxicity assessment were expressed by larval mortality in 24 h. These were compared with control samples, meaning salt water or solvents (alcohol, acetone, DMSO) depending on the extract, in a volumetric solvent: water ratio, comparable to samples containing vegetable extract.

For *in vivo* evaluation and cytological analysis, the nauplii were placed on the slide without fixation and colouring since evaluation though transparency was possible. An Optika 350 microscope with Optikam photo capture System was used.

## RESULTS AND DISCUSSIONS

Survival of larvae measured at 24 h revealed the following:

The cytotoxic activity measured by the BSLA test indicates higher toxicity in the case of *T. officinale, C. majus, G. elwesii* extracts. The correlation between the quantified effects (mortality in 24 h) and the dose is evident in all performed analyses.

As a reference in the cytological evaluation, the visible organogenesis processes in the primordial region of appendages (Figure 1 a, b), as

well as the characteristics of the subcuticular cell lines visible in the posterior part of the larva (Figure 1, a, c) were taken into account.
These epithelial cells are disposed in a monolayer, and the cells are polarized and attached to the cuticle. Microscopic observations allow visualization of the layout in longitudinal or cross-sectional lines (Figures 1, c).

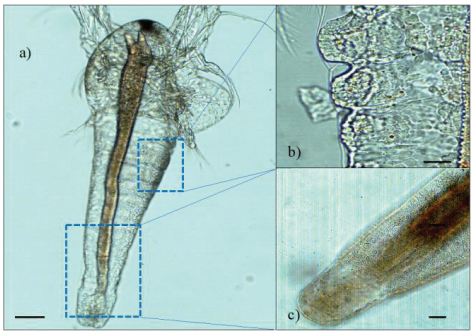


Figure 1. *Artemia* larvae in the control sample: (a) larvae body (the black bar correspond to100 μm), (b) Morphogenesis details of the thorax segment region (the black bar correspond to 20 μm), and (c), subcuticular epidermal cells from the posterior region, arrangements details (the black bar correspond to 40 μm)

Exposure in analysed solutions allowed the tracking and evaluation of cellular changes (arrangement, cell appearance, cell and nuclear volume changes, intercellular attachment loss) induced in these areas and visible *in vivo*. All of these changes were classified as major by affecting cellular differentiations and organogenesis and implicitly stopping larvae from passing through subsequent stages of development (stage II and III).
The visible processes induced by the *C. majus* and *G. elwesii* extracts consisted of stopping the formation of the limb primordium (Figure 2 a, c). The explanations of this phenomenon are related to the content of the analysed extracts.
Thus, phytochemical studies show that *Chelidonium majus* contains little known toxicological alkaloids. Human cell studies that indicate antiproliferative activity for human keratocytes (Vavreckova et al., 1996), and anti-leukaemic activity of protoberberine alkaloids (Smekal et al., 1984) could correlate to our studies.
Other studies indicate that chelidonins are identified as telomerase inhibitors in tumour cells. The alkaloid also inhibits tubulin polymerization by inducing mitosis blockade (Biswas, 2013).
These studies examples correlate with the results of observations on the effect of *Chelidonium* extract analysed on *Artemia* larvae and support the claim that the organogenesis of these larvae is a possible model of cytotoxicity analysis.
The *Galanthus* hydroalcoholic extract exhibits very high acute toxicity in 24-30 hours. The *Galanthus* extract also induces major effects on cell activity in the thoracic segment (Figure 2, c) at the larvae II and III stages.
In addition, there is a loss of contact between the cuticle and the subcuticular cells. Explanations could be related to the combined effect of the complex mixture of alkaloids contained in the *Galanthus* extract.
Phytochemical studies indicate more than 90 alkaloids in the *Galanthus* species.
Of these, some are studied and are known to have a pharmacological activity such as galantamine, which is a competitive inhibitor to acetylcholinesterase (AChE) and an allosteric nicotinic receptor modulator for acetylcholine (ACh). For these properties, the extract finds applications in the treatment of Alzheimer's.
Tyramine is another alkaloid that exhibits structural similarities to adrenaline and leads to cortisol release, inducing high toxicity in animals (Clement et al., 1998).
Lycorine is one of the most common alkaloids in Amaryllidaceae and possesses a wide range of biological properties. It has been reported as a potent inhibitor in ascorbic acid synthesis, in cell growth and division, and organogenesis in higher plants, algae and yeasts, and it inhibits cell cycles during interphase (Bastida et al., 2006).
In addition, lycorine exhibits antiviral, antifungal (*Saccharomyces cerevisiae*, *Candida albicans*), and antiprotozoal (*Trypanosoma brucei*) activity and is more potent than indomethacin acting as an anti-inflammatory agent (Citoglu et al., 1998).
Cellular effects induced by *Taraxacum officinale* and *Usnea barbata* indicate changes in the rearrangement of epidermal invasion (Figure 2, b and d) and blocking morphogenesis mechanisms activity.
Extracts of *Taraxacum* and *Usnea*, tested on human cell cultures, are known to have

inhibitory effects on cancer cells (EMA/HMPC, 2008; Lei Guo, 2008). These inhibitions are related to decreasing phosphorylation at FAK and src levels and reducing the extracellular matrix (*T. officinale)* or blocking mitochondrial activity in the *Usnea* extract (Lei Guo, 2008).

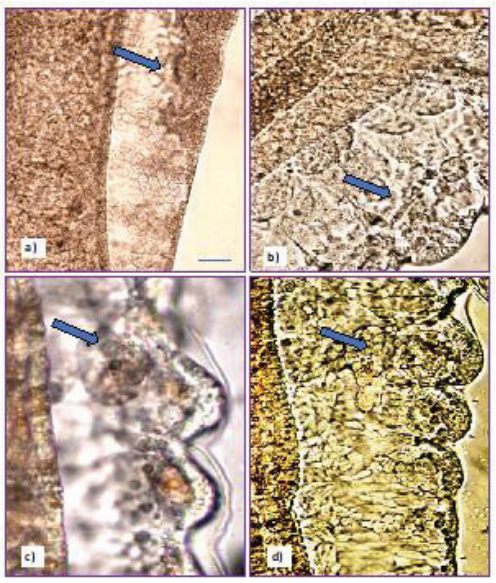


Figure 2. The region of morphogenesis of the thorax segment at brine shrimp larvae, in 24 h exposure to extracts; the arrow indicates the region with amplified abnormal cell arrangement in the lateral germs: *C. majus*; b) *T. officinale* extracts; c) *G. elwesii.* (bulbs extracts; d) *U. barbata* (the blue bar corresponds to 20 μm)

Examination of epidermal lines reveals a loss of arrangement and cell density in solutions with *Chelidonium* extract comparative with control samples (Figure 3, a, b).

Also, phenomena such as: exacerbation of cellular volume and nuclear deformation at exposure to *Tragopogon dubius* extract (Figure 3, c) occur. In traditional Romanian medicine, the *Tragopogon* decoction is used for the skin smoothing (Moromete at al., 2016).

Our observations of the osmotic changes that resulted in cellular growth could be based on the same mechanisms as those revealed in the human skin.

Other cytological phenomena noted at the epithelial level are: cell deformation and loss of intercellular adhesion or linkages between the cuticle and the cellular layer (Figure 3, d) respectively. Observations were made on nauplii introduced into solutions containing the *Usnea barbata* extract.

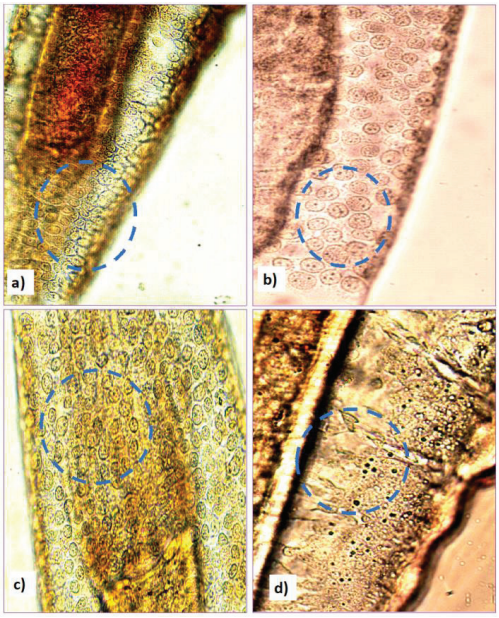


Figure 3. The epidermal cells details with alteration of cell neighbours at 24h of exposure in extracts (mark circle): a) control sample; b) *C. majus;* c) *T. dubius* and loss of connections between cells; d) *Usnea barbata* (the blue bar corresponds to 20 μm)

## CONCLUSIONS

Our results describe a cytotoxic evaluation of plant extracts using the *Artemia salina* biotest. The technique highlights the fact that extracts penetrating into the larvae's body alters the growth and differentiation of cell populations in the area of the thoracic appendix buds and subcuticular epithelial cells.

The rapid highlighting of cellular changes related to shape, density, and the reorganization of elements targeting organogenesis stages by microscopic evaluation, without special preparation techniques, makes the *Artemia* test a fast cytotoxicological test with comparable relevance to animal cell cultures.

The test allows in vivo evaluation of possible substances with cytotoxic and teratogenic potential, providing a good insight into applications in aquaculture, ecotoxicology and pharmacology.

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# REPRODUCTION, PHYSIOLOGY, ANATOMY

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# PRESERVATION OF RAMS' SPERM AT +2-+4°C REGION OF MOLDOVA

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***Abstract***

*Were studied quantitative and qualitative indicators of sperm obtained from rams Karakul Moldovenesc. The average volume of the ejaculate was 0,84 ml, 91% mobility and 2,5mlrd / ml concentration. The action of the BD-1 preparation synthesized at the Institute of Microbiology and Biotechnology of the Academy of Sciences of the Republic of Moldova was experimented as an additional component introduced into the basic environment regarding the quality of the ram seminal material, preserved at 2-4 ° C. It has been found that the introduction in the composition of the basic medium for 6% dilution of seminal material of BD-1 has a positive influence on the preservation of the semen. After 144 hours of incubation at 2-4 °C the sperm mobility was 68% compared to the control group where this index was 45%. artificial insemination of the sheep with refrigerated sperm after 144 hours allowed 71,9% fecundity sheeps. The proposed technology for conserving the semen of ram at 2-4 °C which composition proposes the dilution medium for semen, containing: glucose, sodium citrate, egg yolk, BD-1, allows the results of artificial insamination of sheep to be made more efficient*

***Key words:*** *ram, breed, sperm, dilution medium, preservation, fecundity.*

## INTRODUCTION

Theoretical studies in the biology of animal sperm have opened up great prospects for opportunities not only in the accelerated development and widespread introduction of the method of artificial insemination of farm animals, but also in the preservation of genetically most valuable and endangered species and species of animals.

Methods of storing sperm in a non-organism are based on a decrease in the metabolic processes of spermatozoa, which allows them to increase the time of their survival and preserve their fertilizing ability (Gvozdeckij, 2017). Currently, the most widely used in sheep farming is the short-term storage of diluted sperm at a temperature of 2-4°C (Erohin, 2003). To store sperm at a temperature of 2-4°C, it is diluted with a special medium (GTsZH) prepared according to the following recipes, per 100 ml of bidistilled water: glucose-0.8 g, sodium citrate-2.8 g, yolk-20 ml. The shelf life of sperm at 2-4°C is very small and as a rule they are used during the day. Even if the spermatozoa have a progressive movement after 3 days or more, their fertilizing ability is sharply reduced. Such a short period of storage of sperm causes difficulties in the work of items of artificial insemination of sheep (Aybazov, 2011).

This requires the improvement of this method in order to increase the shelf life of sperm without reducing the fertilizing ability of spermatozoa.

In this regard, further research is needed in the field of improving synthetic media for storing sperm, both in freezing conditions and in the frozen state. It is possible to increase the effectiveness of artificial insemination of sheep by using various biological active compounds in environments (Nauk, 1991).

## MATERIALS AND METHODS

The object of research was Moldavian-type rams of Karakul breed. In the experiments used clinically healthy rams - producers. Sperm was taken into an artificial vagina, the quality of freshly obtained sperm was determined by standard methods for volume, concentration, and motility was determined using the computer program "CEROS". For the experiments, sperm with a mobility of at least 80% and a concentration of at least 2.5 billion / ml was used.

All the original components intended for the preparation of synthetic media for dilution of sperm had a purity of "HCH" or "analytical grade" and were tested for harmlessness to sperm in accordance with approved quality control methods. Environments were prepared according to the standard technique; their quality was checked by the method of biocontrol.

As an additional component introduced into the composition of the basic medium (GTsZH), the drug BD-1 was developed, which was developed at the Institute of Microbiology and Biotechnology of the Academy of Sciences of the Republic of Moldova in order to increase the shelf life of semen of rams. The drug BD-1 was tested with its introduction into the composition of the main medium in different concentrations from 1 to 10%. All comparative experiments in studying the action of the drug BD-1 were studied on separate ejaculates. The quality of the stored sperm at 2-4°C in each prepared medium after dilution of the sperm was tested for motility every 24 hours, using the CEROS program.

## RESULTS AND DISCUSSIONS

Further improvement of the method of storing sperm involves the selection of rams, the sperm of which is suitable for use and does not reduce the loss of spermatozoa during storage, improve the safety of biological usefulness and, accordingly, the effectiveness of artificial insemination (Kasymov, 1990).

Values of the level of semen products of rams, allows you to send in the right direction their use, which is very important in the effectiveness of their use.

During the experiments, special attention was paid to the study of the quantity and quality of sperm obtained from sheep of the Moldavian type of Karakul breed, as well as improving the synthetic environment for diluting the ram sperm and its protective properties.

At first, the assessment of rams producers of the Moldavian type of Karakul breed on the quality of sperm production (Table 1) was reviewed and verified.

Table 1. Average data of indicators of freshly received rams semen

| | *units* | *Statistical Parameters* | | | | |
|---|---|---|---|---|---|---|
| | | n | M±м | V % | V min | V мах |
| *amount* | ml | 15 | 0.84±0.06 | 28.53 | 0.4 | 1.2 |
| mobility | % | 15 | 91±1.01 | 4.38 | 90 | 100 |
| concentration | milliard/ml | 15 | 2.78±0.05 | 24.48 | 2.06 | 2.92 |
| The total number of spermatozoa in the ejaculate | milliard | 15 | 2.59±0.05 | 13.38 | 2.42 | 2.86 |

It has been established that rams of the Moldavian type of Karakul breed is characterized by variability of sperm values. The data presented in the table show that the average volume of ejaculate in ram producers was 0.84 ± 0.06 ml, with fluctuations between rams from 0.4 ml to 1.2 ml. The mobility of freshly obtained sperm was 91.0 ± 1.01%. The concentration of sperm in 1 ml of sperm was 2.78 billion, and the total number of sperm in the ejaculate averaged 2.59 billion / ml.

The experimental data obtained show that the sperm production of ram-makers of the Moldavian type of Karakul breed is lower compared to the standard indicators of the Karakul breed.

Biocontrol quality of diluents allows to determine the effect of the developed media on the mobility and survival of spermatozoa outside the body.

Another series of experiments was carried out to improve the technology of preserving sperm during cooling. After collecting the semen, the ejaculates were subjected to microscopic and macroscopic analysis using the CEROS program. Ejaculates approved for treatment were diluted with GTG medium, which included BioR with membranotropic and antioxidant properties, synthesized in concentrations from 1 to 10%, developed by the Institute of Microbiology and Biotechnology of the Academy of Sciences of Moldova.

Biocontrol quality of diluents allows to determine the effect of the developed media on the mobility and survival of spermatozoa outside the body. The mobility and survival of sperm at 2-4$^{o}$C are presented in Table 2.

Table 2. Mobility and experience of spermatozoa at + 2- +4°C

| Time between researchings, h | indices, % | **control GTJ** | BD-1 concentration | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1% | 2% | 3% | 4% | 5% | 6% | 7% | 8% | 9% | 10% |
| After dilution | motility | 81.3 ±4.6 | 82.0 ±4.7 | 72.0 ±14.5 | 84.0 ±6.3 | 86.8 ±1.5 | 84.8 ±4.8 | 84.3 ±2.2 | 88.3 ±1.5 | 84.5 ±3.7 | 85.3 ±4.8 | 79.3 ±4.9 |
| | progressive | 39.3 ±2.3 | 40.8 ±2.7 | 47.0 ±6.9 | 38.3 ±3.3 | 4.3 ±3.0 | 42.3 ±1.6 | 38.5 ±3.2 | 40.8 ±3.4 | 41.0 ±5.0 | 45.5 ±4.3 | 38.5 ±4.3 |
| 24 | motility | 81.3 ±3.2 | 797 ±6.4 | 80.3 ±5.0 | 82.3 ±3.5 | 88.3 ±3.2 | 83.3 ±3.7 | 81.0 ±3.6 | 82.3 ±3.8 | 80.3 ±2.3 | 83.7 ±0.9 | 82.7 ±4.4 |
| | progressive | 33.0 ±1.0 | 32.2 ±3.7 | 32.0 ±2.1 | 31.0 ±2.1 | 34.0 ±3.1 | 38.3 ±4.4 | 37.7 ±5.9 | 35.3 ±3.7 | 31.7 ±2.2 | 35.0 ±3.5 | 35.3 ±7.5 |
| 48 | motility | 81.7 ±3.7 | 71.0 ±5.1 | 82.0 ±6.8 | 73.0 ±2.9 | 76.0 ±2.3 | 78.0 3±5.2 | 77.0 ±5.1 | 84.0 ±5.5 | 84.3 ±6.1 | 81.7 ±3.2 | 80.0 ±3.2 |
| | progressive | 38.7 ±3.4 | 28.0 ±4.0 | 33.3 ±6.4 | 31.0 ±3.5 | 34.0 ±4.3 | 37.0 ±4.6 | 35.3 ±4.3 | 41.0 ±7.8 | 33.0 ±3.6 | 36.0 ±5.0 | 28.7 ±4.7 |
| 72 | mobility | 71.3 ±1.8 | 65.7 ±5.9 | 77.7 ±3.4 | 70.3 ±0.9 | 76.3 ±4.5 | 73.3 ±6.2 | 74.7 ±2.7 | 78.0 ±4.0 | 77.7 ±1.8 | 73.0 ±3.0 | 75.3 ±5.5 |
| | progressive | 28.3 ±1.2 | 18.7 ±1.7 | 25.0 ±2.5 | 31.3 ±3.5 | 29.7 ±4.1 | 31.3 ±5.8 | 30.7 ±2.3 | 32.7 ±5.6 | 27.0 ±1.5 | 32.0 ±3.1 | 27.3 ±4.8 |
| 96 | mobility | 72.3 ±5.5 | 60.3 ±12.4 | 72.0 ±5.8 | 73.3 ±2.9 | 73.7 ±6.4 | 73.7 ±8.4 | 73.7 ±4.8 | 77.7 ±5.0 | 78.3 ±3.8 | 81.0 ±3.8 | 81.3 ±5.6 |
| | progressive | 28.3 ±6.1 | 24.0 ±5.5 | 23.0 ±4.7 | 24.3 ±3.3 | 28.7 ±4.3 | 29.0 ±3.5 | 26.7 ±0.9 | 29.7 ±4.8 | 28.3 ±1.8 | 28.3 ±2.4 | 29.7 ±9.6 |
| 120 | mobility | 52.0 ±6.0 | 52.5 ±12.9 | 53.5 ±5.2 | 64.3 ±5.0 | 65.3 ±3.7 | 68.5 ±6.2 | 67.3 ±8.8 | 74.0 ±4.9 | 69.3 ±9.4 | 68.8 ±4.2 | 65.5 ±4.8 |
| | progressive | 12.5 ±3.0 | 12.5 ±4.6 | 10.8 ±3.3 | 21.3 ±3.0 | 18.3 ±3.4 | 27.3 ±2.8 | 21.0 ±5.1 | 18.5 ±1.8 | 23.5 ±8.4 | 23.5 ±4.6 | 22.3 ±6.2 |
| 144 | mobility | 45.7 ±8.2 | 45.7 ±8.2 | 31.7 ±16.6 | 36.3 ±7.4 | 37.0 ±4.7 | 60.3 ±7.6 | 69.0 ±2.5 | 68.0 ±5.0 | 56.3 ±3.3 | 61.0 ±3.6 | 61.7 ±0.3 |
| | progressive | 8.7 ±5.2 | 3.0 ±2.5 | 4.3 ±1.9 | 8.7 ±2.0 | 6.3 ±2.4 | 13.0 ±6.6 | 16.0 ±6.0 | 14.7 ±0.3 | 14.0 ±1.5 | 15.0 ±3.5 | 13.3 ±4.5 |
| 168 | mobility | 25.8 ±10.0 | 33.5 ±13.9 | 21.5 ±11.0 | 45.3 ±8.4 | 43.0 ±18.4 | 47.5 ±8.9 | 48.8 ±6.9 | 57.8 ±2.5 | 56.5 ±7.5 | 45.8 ±6.1 | 48.8 ±12.8 |
| | progressive | 6.0 ±5.4 | 3.8 ±1.9 | 2.5 ±2.2 | 6.8 ±4.9 | 13.3 ±7.5 | 12.0 ±5.6 | 5.5 ±1.7 | 11.8 ±5.7 | 7.5 ±2.0 | 4.0 ±1.3 | 6.3 ±2.8 |
| 192 | mobility | 15.3 ±8.3 | 12.7 ±7.2 | 21.0 ±3.8 | 31.0 ±6.9 | 37.0 ±14.0 | 44.3 ±5.8 | 50.3 ±6.1 | 53.0 ±3.5 | 45.3 ±11.8 | 29.0 ±5.7 | 44.3 ±9.8 |
| | progressiveessive | 1.0 ±0.6 | 1.0 ±0.6 | 1.3 ±0.3 | 1.7 ±0.7 | 5.7 ±3.7 | 6.0 ±2.0 | 7.0 ±4.5 | 8.0 ±3.5 | 8.7 ±5.4 | 2.0 ±0.6 | 3.7 ±1.5 |

The experimental data obtained show that the tested drug BD-1 is not toxic to spermatozoa in the tested concentration ranges. After dilution of the sperm with various test media, the motility of the sperm was within 80%, and the number of spermatozoa with straight-line movement was within 40%.

With an increase in the storage time of diluted sperm at 2–4°C, these figures sharply decreased. After 144 hours of sperm storage, sperm motility in the experimental group, where the concentration of the drug BD-1 was in the range of 6-7% was 68-69%, whereas in the control group this indicator decreased and amounted to only 45.7 ± 8.1% .

Similar changes have occurred with the number of sperm with a straight-line movement (Magomedov, 2008). In the experimental group, where the concentration of BD-1 was 6-7% after 144 hours of sperm storage, this indicator ranged from 16 to 15%, whereas in the control group, this indicator was 4.7%.

Investigation of the effect of seed dilution on the safety of spermatozoa by cooling was carried out using spermatozoa from designated rams. Ejaculates were diluted 1: 3 and 1: 4. Samples were kept at four degrees Celsius, sperm motility was determined regularly at established intervals (24 hours) until it reached zero. The breeding medium was prepared in the laboratory of the Scientific and Practical Institute of Biotechnology in Animal Husbandry and Veterinary Medicine.

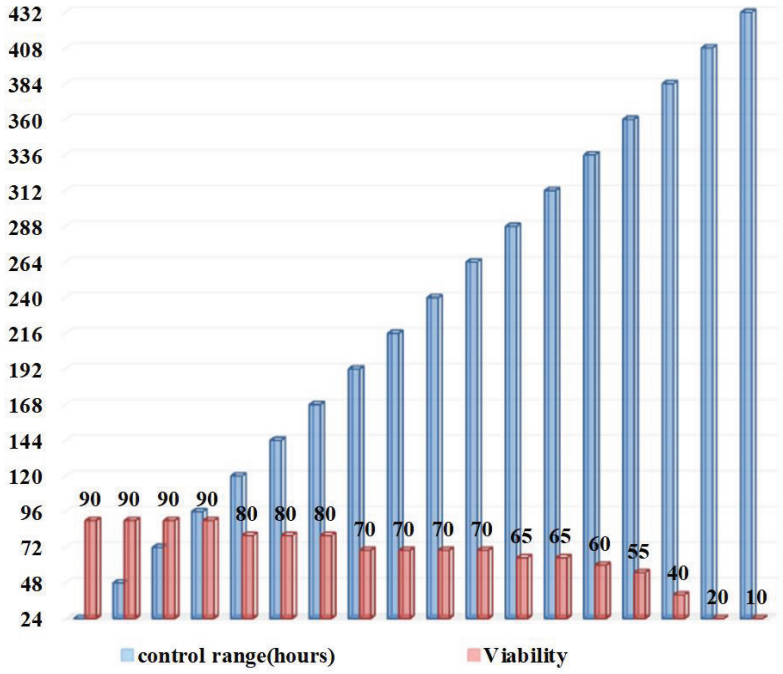


Figure 1. Viability of diluted sperm 1: 3

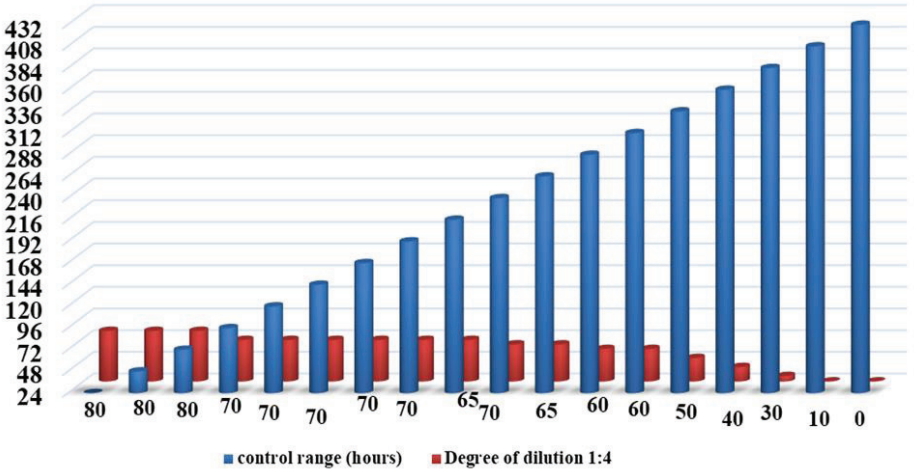


Figure 2. Viability of diluted sperm 1: 4

From the data in Figures 1 and 2 it can be seen that, regardless of the degree of dilution, the ability to maintain sperm motility has longer periods.

The best results are achieved if the dilution is 1: 3. In this case, after 6 days, the mobility of chilled sperm is still 80%, decreasing to 55% on the 15th day and finally 10% (18th day)

As a result of the research, an improved synthetic medium of the following composition was proposed: for 100 ml of bidistilled water, glucose 0.8 g, sodium citrate - 2.8, chicken egg yolk - 20 ml, BD-1 - 6% and antibiotics.

The proposed improved environment was tested under production conditions by artificial insemination of sheep. After production, the

sperm was diluted 1: 2 and 1: 3 and stored in a refrigerator at 2-4C. The heat period at sheeps was detected by the special ram-detector. Insemination is twofold. Data on the results of artificial insemination of sheep are presented in Table 3.

Table 3. Results of artificial insemination of sheep

| Inseminated sheep, heads | camed back in the period of estrus | |
|---|---|---|
| | head | % |
| 74 | 30 | 40.5 |

The data presented show that 30 days after the last insemination 30 heads came to the hunt again, which is 40.5% of the initially inseminated.

## CONCLUSIONS

Based on the results of the research it was found:

1. Our improved environment contributes to better preservation and functional usefulness of sperm.

2. Our improved environment contributes to better survival of sperm after thawing (this is confirmed by the results of sperm motility after thawing).

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# STUDY REGARDING THE MORPHOLOGY OF PUBLIC MOUNT STALLIONS POPULATION FROM TULUCEȘTI STUDFARM

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***Abstract***

*The study was based on 34 Gidran, Lipizzaner, Semigreu Românesc and Pure Arabian stallions from Public Mount Unity of Tulucești studfarm, Galați county, Romania. These horses were reared in troops where they have been used or assessed as public mount stallions after promoting the specific ranking activity. They were appreciated regarding the usual measurements used on horses (height, thorax perimeter and cannon girth) at current criteria evaluation, where they obtained very satisfying marks. The results show that the stallions can be included, based on body measurements, in Elite class, which proves that they should continue to reproduce in Tulucești National studfarm; thus a high biological material will be obtained, which indicates that the main purpose of studfarm will be achieved.*

***Key words****: horses, stallions, studfarm, measurements.*

## INTRODUCTION

Public mount stallions are horses of high genetic value, reared in National studfarms, which have obtained satisfying marks at current criteria evaluation to be framed in Elite class (Velea, 1980).

These stallions are billeted in National Stallion Stores and Public Mount Stallions Unities that exist beside studfarms, like Tulucești unity; their purpose is mating free of charge with households mares regarding equine amelioration. Likewise, these stallions can represent a precious source in stud selection.

The purpose of this study is to add a small contribution in the morphology domain, which can directly influence the performances of every horse.

## MATERIALS AND METHODS

The biological material was represented by 34 stallions from Public Mount Stallion Unity within the Tulucești studfarm, which was registered at the end of 2018. These horses were 5 to 18 years old and 8 of them were Gidran breed, 12 Lipizzaner, 4 Semigreu Românesc and 10 Pure Arabian. We analyzed the withers height, the thorax perimeter and the cannon girth, using the standard equipment like zoometer and metric tape (Doliș et al., 2008; Doliș, 2009; Doliș, 2011; Doliș, 2011; Doliș et al., 2014, Doliș et. al., 2017; Dulugeac, 2005; Georgescu et al., 1990; Mărginean et al., 2012; Moldoveanu et al., 1961). Data obtained was statistically processed (Cucu et al., 2004).

## RESULTS AND DISCUSSIONS

Data obtained through measurements was statistically processed and centralized for every breed (Tables 1-4; Fig. 1-3).

The minimum absolute value for withers height was 151 cm (Pure Arabian stallion) and the maximum absolute value for this parameter was 170 cm (Semigreu Românesc and Lipizzaner) (Fig. 1).

Regarding the thorax perimeter the minimum absolute value was 151 cm (Pure Arabian stallion) and the maximum value for this parameter was 189 cm (Gidran stallion) (Fig. 2)

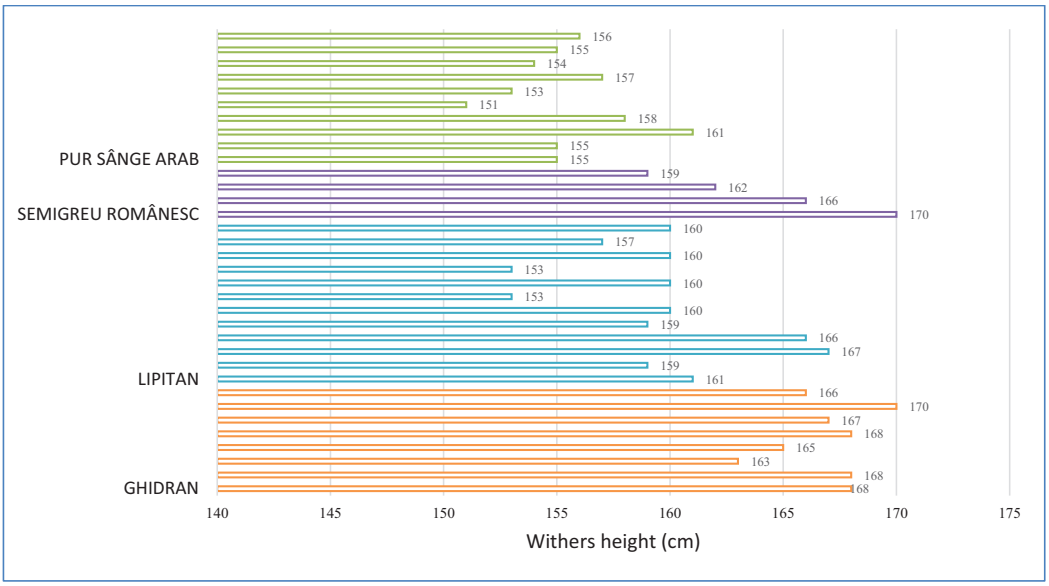


Figure 1. Withers height (cm) of public mount stallions reared in Tulucești studfarm

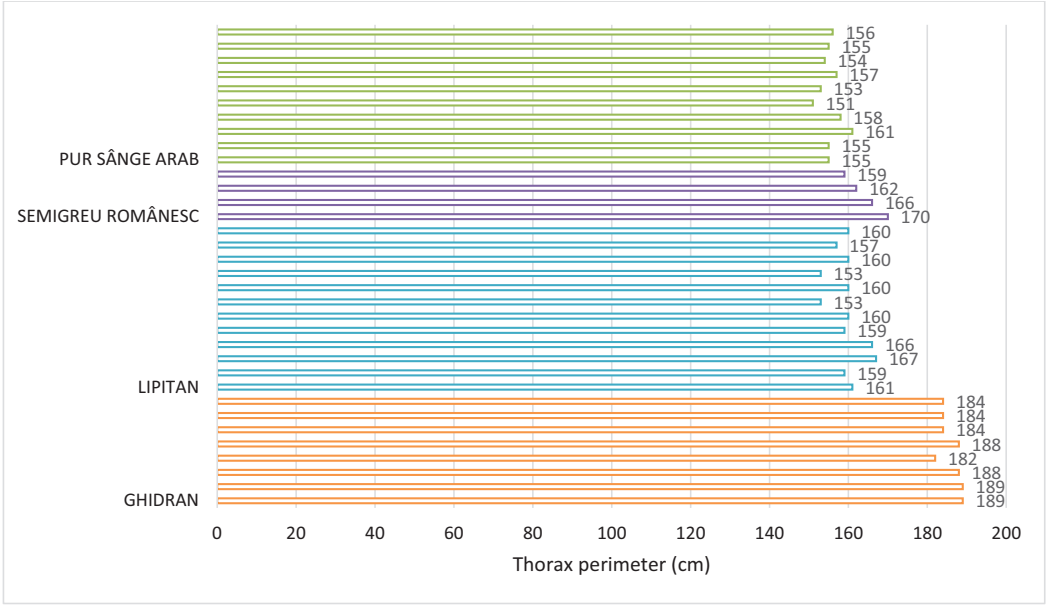


Figure 2. Thorax perimeter (cm) of public mount stallions reared in Tulucești studfarm

The minimum value of cannon girth found at analyzed stallions was 20 cm (Pure Arabian) and the maximum value was 25 cm (Semigreu Românesc) (Fig. 3).

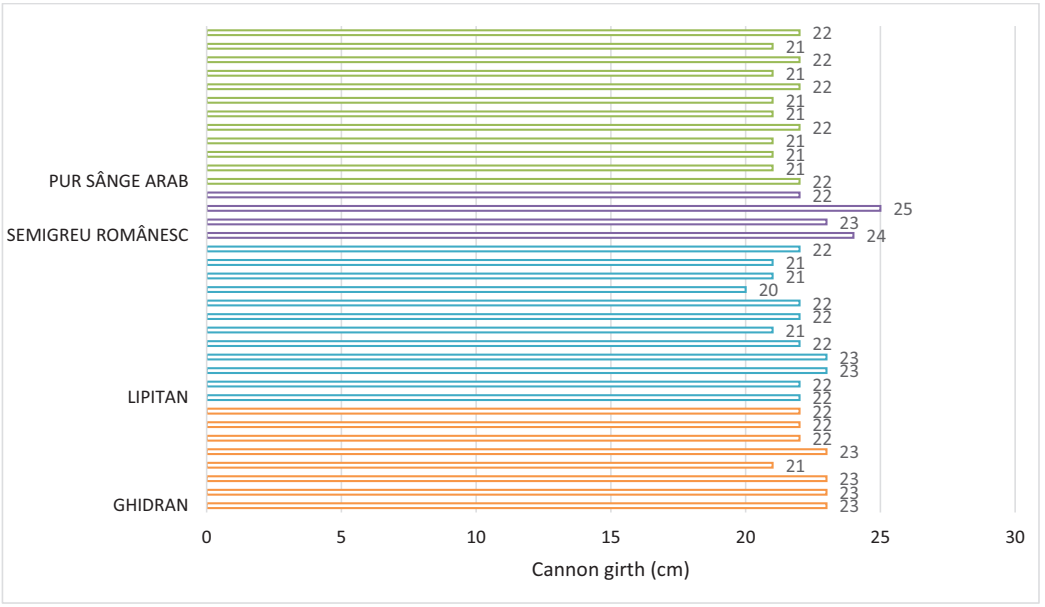


Figure 3. Cannon girth (cm) of public mount stallions reared in Tulucești studfarm

According to the results, all 8 Gidran stallions had an average value for height parameter of 166.88±0.77 cm, a minimum value of 163 cm and a maximum value of 170 cm.

For the thorax perimeter, the absolute value had ranges of 182-189 cm and the average value was 186±0.98 cm; the cannon girth had an average value of 22.38±0.26 cm, with a minimum value of 21 cm and a maximum of 23 cm. The coefficient of variation for all three studied parameters was 1.49% and 3.33%, indicating very homogeneous characters in every assessed case.

Regarding calculated body indexes, the average value of digital-thorax index was 12.03±0.08 cm (minimum of 11.54 cm and maximum of 12.23 cm), while the average value of bone index was 13.41±0.16 cm (minimum of 12.73 cm and maximum of 14.11 cm); the massiveness index had a minimum value of 108.24 cm, a maximum of 115.34 cm and an average of 111.48±0.75 cm, which indicated that the population was homogenous (Table 1).

Table 1. Average values of main body measurements of Gidran public mount stallions from Tulucești studfarm

| Breed | Specification | Withers height (cm) | Thorax perimeter (cm) | Cannon girth (cm) |
|---|---|---|---|---|
| Gidran | n | 8 | | |
| | $\bar{X}$ | 166.88 | 186.00 | 22.38 |
| | $\pm s_{\bar{x}}$ | 0.77 | 0.98 | 0.26 |
| | s | 2.17 | 2.78 | 0.74 |
| | V% | 1.30 | 1.49 | 3.33 |
| | Minimum | 163 | 182 | 21 |
| | Maximum | 170 | 189 | 23 |

For all 12 Lipizzaner stallions, the withers height parameter oscillated between 153-167 cm, the thorax parameter had ranges of 182-192 cm and the cannon girth registered values between 20-23 cm.

The statistical estimators for the Lipizzaner public mount stallions, indicated average values of 159.58±1.21 cm for withers height, 189.67±1.28 cm for thorax perimeter and 21.75±0.25 cm for cannon girth. The studied

group had a coefficient of variation which oscillated between 2.34% and 3.96% revealing homogenous characters for the analyzed parameters (table 2).

Table 2. Average values of main body measurements of Lipizzaner public mount stallions from Tulucești studfarm

| Breed | Specification | Withers height (cm) | Thorax perimeter (cm) | Cannon girth (cm) |
|---|---|---|---|---|
| Lipizzaner | n | 12 | | |
| | $\bar{X}$ | 159.58 | 189.67 | 21.75 |
| | $\pm s_{\bar{X}}$ | 1.21 | 1.28 | 0.25 |
| | s | 4.19 | 4.44 | 0.87 |
| | V% | 2.62 | 2.34 | 3.98 |
| | Minimum | 153 | 182 | 20 |
| | Maximum | 167 | 197 | 23 |

Regarding body indexes, the average value for digital-thorax formula was 11.47±0.11 cm, while the average value for bone index was 13.63±0.11 cm and 118.92±1.21 cm for massiveness index; these results show that the population is highly homogenous (the coefficient of variation had values 2.24%, 3.36% and 3.69% for every character mentioned).

The Semigreu Românesc public mount stallions registered a minimum withers height value of 159 cm, a maximum of 170 cm and an average value of164.25±2.39 cm; regarding the thorax parameter, the average value was 194.25±4.55 cm, with limits of variation comprised between 184-206 cm.

The cannon girth had an average value of 23.50±0.65 cm, while the absolute values oscillated between 22-25 cm.

The coefficient of variation for the studied characters was underneath 5.50% indicating a homogenous group (Table 3).

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Table 3. Average values of main body measurements of Semigreu Românesc public mount stallions from Tulucești studfarm

| Breed | Specification | Withers height (cm) | Thorax perimeter (cm) | Cannon girth (cm) |
|---|---|---|---|---|
| SemigreuRomânesc | n | 4 | | |
| | $\bar{X}$ | 164.25 | 194.25 | 23.5 |
| | $\pm s_{\bar{X}}$ | 2.39 | 4.55 | 0.65 |
| | s | 4.79 | 9.11 | 1.29 |
| | V% | 2.91 | 4.69 | 5.49 |
| | Minimum | 159 | 184 | 22 |
| | Maximum | 170 | 206 | 25 |

The digital-thorax index had an average value of 12.10±0.15 cm, while the average value of bone index was 14.31±0.38 cm and the massiveness index was 118.32±2.09 cm. The coefficient of variation was underneath 5.30%.

Regarding the main body measurements of Pure Arabian public mount stallions, the average values were: 155.50±0.87 cm for withers height, 181.00±1.51 cm for thorax perimeter and 21.40±0.16 cm for cannon girth. The absolute values for withers height oscillated between 151-161 cm, for thorax perimeter were between 172-186 cm and for cannon girth were 21-22 cm.

The studied characters showed that the group was homogenous (the coefficient of variation was 1.77-2.64%) (Table 4).

Table 4. Average values of main body measurements of Pure Arabian public mount stallions from Tulucești studfarm

| Breed | Specification | Withers height (cm) | Thorax perimeter (cm) | Cannon girth (cm) |
|---|---|---|---|---|
| Pure Arabian | n | 10 | | |
| | $\bar{X}$ | 155.50 | 181.00 | 21.40 |
| | $\pm s_{\bar{X}}$ | 0.87 | 1.51 | 0.16 |
| | s | 2.76 | 4.78 | 0.52 |
| | V% | 1.77 | 2.64 | 2.41 |
| | Minimum | 151.00 | 172.00 | 21.00 |
| | Maximum | 161.00 | 186.00 | 22.00 |

As shown in the table 4, the average values of the analyzed indexes were: 11.83±0.11% for digital-thorax index, 13.77±0.16% for the bone index and 116.63±0.96% for the massiveness index; these results showed that the group was homogenous also regarding the calculated indexes.

Generally speaking, the body measurements obtained for the 34 stallions were included in the limits found in the literature or close to it, proving that they had to be promoted in the Public Mount Stallions Unity of the National Tulucești studfarm (Doliș et al., 2008; Doliș, 2011; Doliș et al., 2014; Doliș et al., 2018; Dulugeac, 2005; Furtunescu, 1971; Georgescu et al., 1982; Georgescu et al., 1990; Mărgărint et al., 2012; Suciu et al., 1975; Velea et al., 1980).

There is obvious exceeding of these limits, like a higher withers height then 170 cm for Semigreu Românesc, but this aspect can be positive because it can be used in future amelioration programs considering that this breed it is not fully consolidated.

Data obtained regarding body indexes revealed that, depending on every breed, stallions have a harmonious constitution, representative for the morphological type that they belong to.

## CONCLUSIONS

The analyzed stallions presented values found in breed standards for every studied measurements (withers height, thorax perimeter, cannon girth);

Regarding the data obtained for body indexes (digital-thorax index, bone index, massiveness index), the studied stallions presented an harmonious development, typical to every breed, exactly to the morphological type that they represent.

Data obtained from the statistic program indicate that all breeds have homogenous characters in all analyzed cases.

Regarding the studied characters, the analyzed stallions can be included at least in Elite class.

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# SPECIES FEATURES OF THE CONTENT AND CRYOGENIC CHANGES IN THE PROCESS OF PRESERVATION OF SPERM OF FARM ANIMALS

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***Abstract***

*Experimental studies by using the spectrophotometric method revealed that lipid peroxidation is species specific. At the same time, the number of diene conjugates in rooster sperm is more than twice than in the bull sperm. The content of this product is not subject to cryogenic changes. The amount of hydroperoxides in rooster sperm is an order of magnitude higher than in bull sperm. It should be noted that cryogenic changes of this indicator are observed only in the bull sperm. While the content of malondialdehyde increased in the sperm of both species in the process of cryopreservation. In the process of technological treatment of sperm of various species of animals, an increase in the content of lipid peroxidation products is caused mainly by malonic dialdehyde, and not to diene conjugates and hydroperoxides, and is also species-specific; the effect of the influence of certain antioxidants on the functional state of gametes depends on the degree of stabilization of intermediate and final products of lipid peroxidation. The conclusion is made about the cryolability of hydroperoxides and malonic dialdehyde, the content of diene conjugates is not affected by cryogenic changes.*

***Key words****: cryopreservation, gametes, lipid peroxidation.*

## INTRODUCTION

The reproductive cells of animals basically perform the same functions, the main of which is the transfer of the genome to the future generation. However, they differ significantly in morphology, the number and structure of the constituent components, which determines their cryoresistance.

At the same time, lipids, and especially phospholipids, are the main components of biological membranes, they are involved in such central biological phenomena as biosynthesis, reception, intercellular interactions and signaling, biological transport, regulation of membrane-bound enzymes activity, formation of the immune response, bioenergetic transformations and other (Наук, 1991; Рогинский, 1990). The understandings of the character of lipid peroxidation in biological objects are based on a large experimental material. However, under the influence of various factors lipids may be subjected to peroxidation.

According to the research of (Владимиров et al., 1975), the main reactions at lipid peroxidation are proceeding in a certain sequence (Fig. 1).

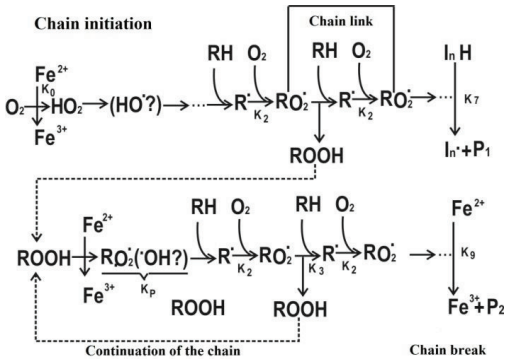


Figure 1. Scheme of reactions of lipid peroxidation (Владимиров, Ю.А. et al., 1975).

Where, RH - unsaturated residue in phospholipids; ROOH - corresponding hydroperoxide; R˙ - lipid radical; ROO˙ - lipid peroxide radical; HO˙$_2$ - superoxide radical; InH - radical reaction inhibitor; In - inhibitor radical; $K_0$, $K_2$ … - the individual reaction rate constants.

As can be seen from this scheme, lipid peroxidation is a chain of successive alternating reactions, the links of which are the stage of

initiation, branching, continuation and termination of the chains.

The only nonpolar cell regions are the middle hydrophobic zones of biomembranes, in which unsaturated fatty acids are located - the most susceptible to oxidative damage. Therefore, it becomes clear the value of lipid peroxidation in membrane destabilization and general cell pathology, since as a result of this process in the hydrophobic chains of fatty acids, hydrophilic areas are formed that significantly change the functioning of membrane structures (Владимиров et al., 1975).

Lipid peroxidation is a universal phenomenon of life, present everywhere, where there is molecular oxygen and where its active forms are formed under normal conditions: $O_2$, $H_2O_2$, OH, i.e. practically in every living cell and, first of all in biological membranes.

Lipid peroxidation is a normal process. Thus, free radical reactions, supported by special regulatory systems at a low stationary level, take part in normal metabolic processes and regulatory functions of the cell. By inhibiting or, conversely, accelerating lipid peroxidation can change the composition of cell membranes, their structural organization and functional activity of the cell. It was also established that the activity of membrane enzymes depends to a large extent on the lipid environment, therefore a change in the composition of biological membranes as a result of modification of lipid peroxidation processes causes inhibition of the activity of some and activation of other membrane-bound and membrane-dependent enzymes. The important physiological role of lipid peroxidation processes is confirmed by a number of studies (Бурлакова et al., 1985).

It is shown that lipid peroxidation is a mechanism of membrane disassembly and renewal. The dependence of the rate of lipid peroxidation reactions is demonstrated not only on the degree of saturation of fatty acids, but also on the structural organization of the lipid phase of biological membranes, which is the molecular mobility of lipids, the strength of lipid-lipid and protein-lipid interactions. In this case, covalently bound associations of membrane proteins can form, localization of integral proteins in the hydrophobic interior of membranes can change (Бурлакова et al., 1985).

Thus, lipid peroxidation is not only a universal modifier of the properties of biological membranes, but also an important physiological regulator of their structure and function, a factor establishing and maintaining the stationary functioning of lipid-dependent enzymes, channel formers, receptors, etc. This provision must be considered when developing techniques for modifying membranes in order to create membrane structures resistant to the action of factors of cryopreservation.

Free radical reactions of peroxidation are most effectively developed in lipid (phospholipid) structures and, first of all, in the lipid bilayer of membranes. At the same time, glutathione peroxidase, which adequately destroys lipid peroxides, does not penetrate into the hydrophobic zones, i.e. inside the lipid bilayer. Only the presence in it of tocopherols and carotenoids in it limits the development of lipid peroxidation. Possibly, the concomitant lipid peroxidation activation of phospholipase hydrolysis contributes to the elution of lipoperoxides from the membranes and their subsequent decomposition by glutathione peroxidase (Scherer et al., 1958).

So, in normal conditions of vital activity, in the functioning of living systems in conditions of physiological optimum, there is a pro- and antioxidant balance, which is the most important mechanism of oxidative homeostasis. This equilibrium is mobile in nature, it is an resultant of oppositely directed processes and is characterized by an oscillatory mode of functioning within the limits compatible with the preservation of homeostasis. Extreme conditions and any kind of structural damage of the living system are inevitably accompanied by activation of lipid peroxidation, a shift of pro- and antioxidant equilibrium (Boronciuc et al., 2003; Boronciuc et al., 2005). Moreover, under stress conditions caused by changes in environmental factors, decay processes are enhanced, the amount of oxidized or partially oxidized, including radical products, active forms of oxygen increases, and lipid peroxidation is activated. At the same time, antioxidant systems limit this activation, preventing the continuation and branching of the free radical oxidation chains, keeping the pro- and antioxidants balance within the limits of the optimum functional activity, within the

normal reaction. Only after exhaustion of the power of protective systems, with prolonged loads, when the consumption of antioxidant exceeds its reserve, the number of harmful peroxidation products increases, which leads to the development of damage associated with oxidative destruction of cellular structures, and, above all, biological membranes.

The main factors leading to the initiation of lipid peroxidation processes in the lipid bilayer of membranes during refrigeration, freezing and thawing are hypoxia, the lipotropic action of hypoconcentrated solutions and breach of the structural integrity of membranes. As a result of the action of these factors, in the membranes is observed an accumulation of the anion radical ($O_2$-), which, under the action of NADH, activates the processes of lipid peroxidation (Tynynyka, 2000).

Based on the above, the purpose of the research, the results of which are presented in this paper was to study the content of lipid peroxidation products and their cryogenic changes in the process of preservation of seed material.

## MATERIALS AND METHODS

The object of the study was the sperm of roosters of the Rhode Island breed and the semen material of bulls of the Black-Motley breed which were kept in conditions corresponding to veterinary requirements. In the experiments for dilution and freezing of roosters semen was used maltose-arginine-petumoside-glycerol-yolk medium and for bulls sperm was used lactose-glycerol-yolk medium.

Semen freezing was carried out on the surface of the fluoroplastic plate with a volume of 0.1-0.2 ml, in vapors of liquid nitrogen, at a temperature of minus 110-120°C, followed by the transfer of granules into liquid nitrogen.

So, as in the process of lipid peroxidation there is a transmission of electrons from the donor to the acceptor, we can talk about donor-acceptor interactions, which were judged by the amount of malonic dialdehyde which was determined by the method of (Владимиров et al., 1972) in the modification of the collaborators of our laboratory, which consists in determining the concentration of gametes of the studied samples instead of protein, and concretizing the calculation formula.

Wherein the concentration of malonic dialdehyde was determined in nanomoles in the calculation of $10^9$ gametes, taking the extinction coefficient equal to $1{,}56 \times 10^{-5}$ $M^{-1}$ $cm^{-1}$., diene conjugates were determined spectrophotometrically at a wavelength of 233 nm by the method described by (Стальная, 1977), the content of lipid hydroperoxides was also determined spectrophotometrically at a wavelength of 480 nm by the method described by (Романова, 1977; Стальная, 1977).

Statistical processing of digital material was performed using the Student's t-test.

About donor-acceptor interactions were judged by the intensity of lipid peroxidation, by studying the cryogenic changes of content in the gametes of the final product of this process - malonic dialdehyde.

## RESULTS AND DISCUSSIONS

Lipid metabolism, the functioning of lipid-dependent enzymes, proliferation rate and membrane permeability are determined by the level of lipid peroxidation. However, the increase in its speed relative to the norm may be the cause or a contributing factor of the damage of the gametes.

In the conducted experiments, about the intensity of lipid peroxidation in the bull and rooster semen was judged by the content of its primary, intermediate and final products. The results are presented in Table 1.

Table 1. The content of products of lipid peroxidation in the bull and rooster semen during the stages of cryopreservation

| Technological stage | Lipid oxidation products | | |
|---|---|---|---|
| | Diene conjugates, conventional units | Hydroperoxide, extinction units | Malonic dialdehyde, nm/billion. |
| | M ± m | M ± m | M ± m |
| Bull semen | | | |
| Dilution | 1.6 ± 0.44 | 0.03 ± 0.004 | 20.4 ± 1.34 |
| Refrigeration | 1.3 ± 0.32 | 0.20 ± 0.02* | 30.8 ± 3.02 |
| Thawing | 1.4 ± 0.11 | 0.87 ± 0.09* | 50.5 ± 2.01* |
| Rooster semen | | | |
| Dilution | 3.8 ± 0.39 | 0.26 ± 0.20 | 38.6 ± 7.82 |
| Refrigeration | 2.1 ± 0.06 | 0.3 ± 0.02 | 40.2 ± 1.08 |
| Thawing | 4.0 ± 0.47 | 0.72 ± 0.02 | 67.0 ± 2.48* |
| Note: * Cryogenic changes are statistically authentic | | | |

From the table it follows that lipid peroxidation is of a species-specific nature. At the same time, the number of diene conjugates in the rooster semen is more than twice its amount in the bull semen. The content of this product is not subject to cryogenic changes. The amount of hydroperoxides in the rooster sperm is much higher than in the bull sperm. It should be noted that the cryogenic changes of this indicator are observed only in the bull semen. While the content of malonic dialdehyde increases in the semen of both species in the process of its cryopreservation.

In the process of technological treatment of semen of various species of animals, the increase in the content of lipid peroxidation products is due mainly to malonic dialdehyde, rather than diene conjugates and hydroperoxides and is also species-specific character; the effect of certain antioxidants on the functional state of gametes depends on the degree of stabilization of intermediate and final products of lipid peroxidation (Boronchuk and Balan, 2005).

Due to the fact that the content of malonic dialdehyde is more susceptible to cryogenic changes, in the following experiment was conducted a comparative study of the intensity of lipid peroxidation in bull and boar sperm (Table 2).

Table 2. The content of malonic dialdehyde in the gametes during cryopreservation of bull and boar sperm, nmol/billion gametes

| Species of animals | Sperm processing stage | | |
|---|---|---|---|
| | Dilution | Refrigeration | Thawing |
| | M ± m | M ± m | M ± m |
| Bull | 13.7 ± 0.75 | 18.3 ± 2.13 | 22.3 ± 2.10* |
| Boar | 22.5 ± 3.10* | 26.4 ± 1.70 | 37.8 ± 3.80* |
| Note: * Cryogenic changes are statistically authentic | | | |

The data of the table show that the greatest amount of malonic dialdehyde is found after thawing of boar sperm, in the gametes of the bull its amount is almost two times less. Consequently, the gametes of the first are more sensitive to peroxidation during the process of sperm cryopreservation.

In the process of refrigerating and freezing-thawing of the semen of the studied animal species, as in the previous experiment, the results of which are presented in Table 1, lipid peroxidation is enhanced in comparison with freshly diluted material by a statistically authentic value in all variants of the experiment.

Extreme environmental factors causing a disturbance of the homeostatic balance between pro- and antioxidant factors in cells contribute to the activation of lipid peroxidation, autocatalytic accumulation of peroxides, epoxides, oxidative radicals, etc. Obtained data convincingly show the need for regulation of lipid peroxidation of animal's semen.

Thus, lipid peroxidation is an important element in the cryo-damage of cells due to changes in the state of the membranes. Intensive development of lipid peroxidation deeply affects the basic functional systems of cells, causing a decrease in viability or complete destruction. However, lipid peroxidation can be regulated both endogenously due to the whole system of protective mechanisms and exogenously by the use of antioxidants and the creation of favorable conditions for the manifestation of the cell's own protective functions realized through donor-acceptor interactions.

## CONCLUSIONS

At the regulation of the process of lipid peroxidation, the most likely are methods, as well as substances, that eliminate the initiation of chain branching and in particular its rupture.

Lipid peroxidation occurs at all technological stages and reaches the highest activity after freezing-thawing of the semen.

The change in the content of lipid peroxidation products in the process of cryopreservation is mainly due to malonic dialdehyde.

The more intense accumulation of lipid peroxidation products in the boar semen is presumably due to the higher content of unsaturated fatty acids in their lipids, whose double bonds are easily broken under the influence of various environmental factors.

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# VITAMINS - AS POSSIBLE COMPONENTS OF CRYOPROTECTIVE MEDIUMS FOR PRESERVATION OF BOAR SPERM

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***Abstract***

*This paper presents some methods and approaches for cryopreservation of boar semen. Despite the long history of experimental research, most of these methods are still not effective enough for routine use in pig farms. The aim of our research was to identify the most effective traditional methods of cryopreservation of reproductive material and to determine the effectiveness of the inclusion of vitamins in the composition of synthetic mediums for dilution and freezing of boar sperm of Landrace breed. Through physiological and biochemical methods it has been established that vitamins of different nature and structure can be included in the composition of cryoprotective mediums, depending on the purpose of the study and their physicochemical properties. Experimental studies have revealed that the tested vitamins have a cryoprotective effect consisting in increasing of the absolute survival rate of reproductive cells when keeping them at room temperature. One of the mechanisms of the cryoprotective effect may be inhibition of the process of lipid peroxidation, which is confirmed by a decrease in the amount of malondialdehyde (MDA). As a result of the study of included vitamins, it was concluded that vitamin E (tocopherol) prevail from the tested vitamins. The antioxidant effect of vitamins, presumably, may be due to their inclusion in the composition of the active center of peroxidation enzymes that decompose reactive oxygen species.*

***Key words****: vitamins, cryoprotective mediums, sperm, antioxidant effect.*

## INTRODUCTION

Studies of recent years (Нарижный et al., 2001; Нарижный et al., 2014; Яковлев et al., 2007) show that among the fundamental problems of livestock biotechnology is an increase in the efficiency of animal reproduction by maximizing the use of breeding producers. In addition to biological indicators of sperm, there are factors that affect the effectiveness of insemination of frozen-thawed sperm. It is known that cryopreservation stimulates lipid peroxidation in boar semen. Thus are damaged the important structures of spermatozoa involved in the regulation of metabolism and fertilization processes of oocytes. This may be one of the reasons for the decline in the fertilizing ability of frozen-thawed boar semen.

According to (Милованов, 1962; Нарижный et al., 2014) among the substances included in the protection system, antioxidant membrane-protective role of vitamin is substantiated. Their properties are manifested by a number of complex effects at all levels of organization - from membrane formations to the organism as a whole. It was found that the use of antioxidant vitamins contributed to increasing the mobility of boar spermatozoa immediately after thawing and increased their survival at 39°C. In addition, their introduction has contributed to improving the preservation of spermatozoa membrane structures during freezing. The cryopreservation method is widely used both in modern reproduction biotechnology and in solving problems of biodiversity conservation.

One of the main technological stages of sperm cryopreservation is its dilution with synthetic mediums, the improvement of which allows to increase the efficiency of long-term preservation of biological objects at ultra-low temperatures. Given the fact that in any direction of research is constantly searching for better methods to improve product quality, in these studies, during freezing and thawing of

sperm, antioxidants vitamins were tested because the question of increasing the fertilizing ability of frozen-thawed sperm is relevant.

## MATERIALS AND METHODS

As an experimental material was used the sperm of Landrace breeding boars, which were kept in the conditions of the State breeding enterprise "Moldsuinhibrid" in accordance with zoo-veterinary requirements. Sperm motility was evaluated by viewing in an "Ampleval" microscope of the manufacturer Carl Zeiss, at 200-fold magnification. The absolute survival rate of spermatozoa was calculated using the generally accepted method (Милованов, 1962). Glucose-chelate-citrate medium (GCC) was used as a base medium (BM) for dilution and storage of sperm. In the experimental variants has been studied the efficacy of different vitamin concentrations obtained by the method of counter series by Milovanov V.K. (Милованов, 1962). Determination of the content of one of the products of lipid peroxidation, malonic dialdehyde, was carried out according to the method of Vladimirov Y.A. and A.I. Archakov (Владимиров, 1972), in the modification of the researchers of our laboratory, which consists in determining the concentration of gametes of the studied samples, instead of protein, and clarifying the calculation formula. At the same time, the concentration of malonic dialdehyde was determined in nanomoles per $10^9$ cells, taking the molar attenuation coefficient equal to $1.56*10^{-5}$ $M^{-1}\cdot cm^{-1}$.

Statistical processing of the digital material was carried out by analyzing the data of the computer program Microsoft Excel 2010, using the Student's t-test.

## RESULTS AND DISCUSSIONS

At technological processing of sperm, diluting it with synthetic mediums and storing it in a cooled or deep-frozen state, occurs significant structural and biological damage of spermatozoa, which leads to disruption of plasma membrane permeability and the exit of a number of enzymes and other components of cellular metabolism from spermatozoa, which significantly reduces sperm fertility (Нарижный et al., 2001; Нарижный et al., 2014). As our working hypothesis, we used the properties of vitamins, which are biologically active substances (Combs et al., 2012; Овчинников, 1987), in connection with which they can influence the process in the composition of mediums for diluting and freezing the sperm of Landrace breeding boars.

It is well known that the reaction of free radical oxidation are of particular importance in the regulation of the functional activity of spermatozoa. This is due to the fact that the marked reactions are a necessary stage of various metabolic processes, the cause or consequence of pathological changes at the cellular level. Excessive accumulation of lipid peroxidation products, triggered by the presence of reactive oxygen species, leads to a change in the intermolecular interactions of the lipiprotein complex of biological membranes, an increase in their permeability to ions and water, a decrease in the activity of membrane-bound enzymes, and the appearance of transmembrane defects that can cause spermatozoa destruction. Reinforcement of lipid peroxidation can occur under the influence of various environmental factors, including temperature changes.

In our studies, to reduce the intensity of lipid peroxidation during the process of diluting and storing of boar semen under hypothermal conditions, was determined the optimum concentration and efficiency of using vitamins as antioxidants in synthetic mediums. The results of experimental studies using vitamin E are presented in Table 1.

Table 1. The effect of vitamin E on the physiological parameters of diluted sperm of breeding boars

| № | Concentration of vitamin, mg/ml | Motility after dilution, points | Absolute survival rate, conventional units | |
|---|---|---|---|---|
| | | | after 12 hours | after 24 hours |
| 1 | BM - control | 7.1 ± 0.23 | 85.2 ± 1.34 | 163.2 ± 6.84 |
| 2 | BM + 0.25 | 7.4 ± 0.13 | 88.8 ± 2.50 | 170.4 ± 2.68 |
| 3 | BM + 0.5 | 7.6 ± 0.11 | 91.2 ± 1.90* | 177.6 ± 2.68 |
| 4 | BM + 1.0 | 7.6 ± 0.11 | 91.2 ± 1.90* | 175.2 ± 3.29 |
| 5 | BM + 2.0 | 7.0 ± 0.08 | 84.0 ± 0.11 | 163.2 ± 3.29 |
| 6 | BM + 4.0 | 7.0 ± 0.08 | 84.0 ± 0.11 | 156.0 ±4.24 |

* The differences are statistically authentic

From the data of Table 1 it follows, that from the tested concentrations in the range of 0.25-4.0 mg/ml the third variant of the experiment turned out to be optimal, when the content of the tested vitamin was 0.5 mg/ml. Sperm motility after dilution was 7.6±0.11 points, the absolute survival rate after 12 hours reached to 91.2±1.90 c.u. and after 24 hours it was 177.6±2.68 c.u., while in the control variant these indicators, respectively, amounted to 7.1±0.23; 85.2±1.34 and 163.2±6.84.

The effectiveness of the studied vitamin can be due to the fact that vitamin E is the main natural antioxidant. Its functions are to protect cells from damaging reactions (peroxidation) resulting from a number of normal metabolic processes and from endo -, exogenous toxic products. The protective effect of vitamin E is mainly directed at biological membranes.

Vitamin E (tocopherol) is an important element of the antioxidant system: it prevents damage to cell walls by neutralizing hydrogen peroxide and other reactive oxygen species; it is necessary for the growth of new cells, for the normal functioning of the immune system.

Vitamin E intake reduces the “severity” of oxidative stress in testicular tissue, increases spermatozoa motility, and positively affects their ability to penetrate the egg (Рузаев, 2015).

Vitamin E is synergistic with retinol and selenium, i.e. by a simultaneous intake these substances exhibit marked efficacy at lower doses than when used separately, due to the mutual prevention of oxidation in the intestine and in tissues.

In the next series of experiments was used vitamin C (ascorbic acid) because it has an important role in human and animal life. Vitamin C exhibits antioxidant properties, participates in the regulation of carbohydrate metabolism and blood clotting, promotes tissue regeneration, increases the body's resistance to infections, reduces the human need for some vitamins (Фролова, 2009).

In addition, vitamin C being an antioxidant, it can inhibit the initiation of the process, stop the chain reaction, destroy hydroperoxides, and also have a neutralizing effect, i.e. it can act on almost all phases of lipid peroxidation, can act as a synergism of vitamin E (Узбеков, 2016).

Since ascorbic acid (vitamin C), together with formed from it the dehydroascorbic acid make up the redox system, which transfers hydrogen. This vitamin can participate in many biochemical processes of the cell. In this regard, vitamin C has been tested by us as an antioxidant in the medium for dilution and storage of boar semen. The results of the experiments are presented in Table 2.

Table 2. The effectiveness of the use of vitamin C in the composition of the medium for dilution of boar sperm

| № | Concentration of vitamin, mg/ml | Motility after dilution, points | Absolute survival rate, conventional units | |
|---|---|---|---|---|
| | | | after 12 hours | after 24 hours |
| 1 | BM - control | 5.7 ± 0.29 | 68.4 ± 3.42 | 112.6 ± 2.16 |
| 2 | BM + 0.063 | 6.3 ± 0.14 | 74.4 ± 2.68 | 150.8 ± 3.21* |
| 3 | BM + 0.125 | 6.0 ± 0.01 | 72.0 ± 0.01 | 140.9 ± 2.68* |
| 4 | BM + 0.250 | 4.5 ± 0.28 | 58.8 ± 3.29 | 121.3 ± 4.24 |
| 5 | BM + 0.500 | 4.0 ± 0.01 | 48.0 ± 0.01 | 90.2 ± 10.16 |
| 6 | BM + 1.0 | 2.8 ± 0.22 | 31.2 ± 3.35 | 63.1 ± 9.18 |

* The differences are statistically authentic

The data of the Table 2 show that the best variant is a medium containing 0.063 mg/ml. Its use allows increasing the absolute survival rate of spermatozoa by a significant difference compared to the control variant. Further increase in the concentration of the test substance causes a decrease in motility and absolute survival rate of spermatozoa. The protective effect of ascorbic acid, both inside and outside the cell, can be explained by the recovery of oxygen free radicals in the presence of glutathione and alpha-tocopherol. When combined with vitamin A, $B_6$, iron ions and selenium, their effects are enhanced (Combs et al., 2012).

Due to the fact that folic acid is necessary for the formation of maintenance in a healthy state of new cells, as well as the normal formation of spermatozoa, it is advisable to study the effectiveness of this vitamin in the development of mediums for dilution and storage of sperm of farm animals. The results of our researches are presented in Table 3.

Analysis of the data presented in Table 3 allows us to note that the concentration of the tested drug is in the range of 0.025-0.4 mg/ml.

Table 3. The effectiveness of the use of vitamin $B_9$ in the composition of the medium for dilution of boar sperm

| № | Concentration of vitamin, mg/ml | Motility after dilution, points | Absolute survival rate, conventional units | |
|---|---|---|---|---|
| | | | after 12 hours | after 24 hours |
| 1 | BM - control | 6.5 ± 0.43 | 75.6 ± 2.85 | 144 ± 13.63 |
| 2 | BM + 0.025 | 7.4 ± 0.27 | 86.4 ± 2.68 | 156 ± 10.39 |
| 3 | BM + 0.05 | 7.4 ± 0.27 | 87.6 ± 2.68* | 160.8 ± 21.47 |
| 4 | BM + 0.1 | 7.3 ± 0.22 | 87.6 ± 2.68* | 160.8 ± 21.47 |
| 5 | BM + 0.2 | 6.8 ± 0.52 | 82.8 ± 4.90 | 144 ± 22.04 |
| 6 | BM + 0.4 | 6.6 ± 0.48 | 70.8 ± 6.48 | 141 ± 17.18 |

* The differences are statistically authentic

Herewith the optimum is the 3rd variant with a vitamin concentration of 0.05 mg/ml. It is in this variant that a higher survival rate of spermatozoa is observed after 12 hours of sperm storage at 18-20°C, which is more on 14% than the experimental version. A similar trend is observed after 24 hours. The effectiveness of folic acid use may be due to the fact that in the presence of vitamin C, folic acid is converted into its main active form - tetrahydrofolic acid, the synthesis of which is carried out with the participation of the enzyme dehydrofolate reductase. In addition, folic acid and its derivatives have acceptor properties in relation to the hydrogen and are involved in the transfer of one-carbon groups, for example, methyl and formyl groups, from one organic compound to another, which determines its participation in redox processes (Владимиров, 1972). Another one feature has vitamin $B_9$. It refers to the compounds with conjugate bonds that predetermine the energy and thermodynamic stabilization of the system. This stability can give a variety of properties to synthetic mediums and cause an increase the resistance of spermatozoa to environmental factors. Together with vitamins $B_{12}$, C, $B_6$, $B_2$ and iron preparations exhibits increased activity.

Of particular interest is vitamin BT, one of the most important functions of which is the transmembrane transport of medium and long chain fatty acids in the mitochondria, where occurs their beta-oxidation to acetylcoenzyme A, which is a substrate for the formation of ATP in the carboxylic acid cycle (Harmeyer, 2002). Besides this L-carnitine performs several other functions such as: improving performance, accelerating growth, increasing strength and muscle mass, regulation of lipid metabolism, reducing cholesterol levels, antioxidant and antihypoxic effect, neuro-, hepato-and cardioprotective action, improve digestion, contribute to the normalization of the main metabolism, stimulates the nervous system, immunity and spermatogenesis. The main physiological function of L-carnitine and its acyl derivatives is the transfer of fatty acid residues from the cytoplasm to the mitochondrial matrix through the internal mitochondrial membrane. This is necessary for the formation of energy that is spent on the life support of the body's cells. By participating in the mitochondrial synthesis of ATP, L-carnitine and acetyl-L-carnitine are able to protect these organelles from oxidative stress by removing toxic acyl groups. The presence of an additional acyl group allows L-carnitine to more easily penetrate into the mitochondria and, as a result, more effectively perform its functions (Иванов et al., 2012).

In this regard, vitamin BT served as the object of studying the effectiveness of its use in the composition of the medium for the dilution of boar semen. The generalized results of such experiments are presented in Table 4.

Table 4. The effect of vitamin BT on the physiological parameters of diluted sperm of boars

| № | Concentration of vitamin, mg/ml | Motility after dilution, points | Absolute survival rate, conventional units | |
|---|---|---|---|---|
| | | | after 12 hours | after 24 hours |
| 1 | BM - control | 6.8 ± 0.42 | 74.7 ± 5.02 | 132.1 ± 18.00 |
| 2 | BM + 0.02 | 7.6 ± 0.27 | 88.8 ± 5.37 | 165.6 ± 10.73 |
| 3 | BM + 0.04 | 7.6 ± 0.27 | 91.2 ± 3.29* | 177.6 ± 6.57* |
| 4 | BM + 0.08 | 7.6 ± 0.27 | 91.2 ± 3.29* | 177.7 ± 6.57* |
| 5 | BM + 0.16 | 7.0 ± 0.35 | 84.0 ± 4.24 | 160.8 ± 9.10 |
| 6 | BM + 0.32 | 6.9 ± 0.27 | 81.6 ± 4.03 | 160.8 ± 9.10 |

* The differences are statistically authentic

From the data of table 4 it follows that 0.08 mg/ml is the optimal concentration of vitamin BT, the use of which allows to increase the absolute survival rate of spermatozoa after 12

hours of storage by 22%, and after 24 hours - by 35% compared with the control variant. The positive effect of carnitine, apparently, is largely due to its ability to form complexes with various organic compounds, which are intermediate products of oxidative processes. These substances accumulating in the cell have a membrane-toxic effect and inhibit the activity of a number of enzymes. Removal of toxins from cells is made in the form of acylcarnitines. The positive effect of the studied vitamins may be due to their participation in redox reactions. However, might work and another mechanism. Therefore, in the next series of experiments was determined the amount of malonic dialdehyde, which is one of the main products of lipid peroxidation (Table 5).

Table 5. The effect of vitamins on the content of malonic dialdehyde in boar semen

| № | Experience variants | Content of vitamin, mg/ml | Content of malonic dialdehyde, nm/billion | | Malonic dialdehyde,% |
|---|---|---|---|---|---|
| | | | after dilution | after 24 hours | |
| 1. | BM + vitamin E (alpha-tocopherol acetate) | 0.5 | 12.8 ± 0.86 | 28.6 ± 1.87* | 123.4 |
| 2. | BM + vitamin C (ascorbic acid) | 0.06 | 12.8 ± 0.86 | 35.3 ± 2.63 | 175.8 |
| 3. | BM + vitamin $B_9$ (folic acid) | 0.05 | 12.8 ± 0.86 | 29.1 ± 2.11* | 127.3 |
| 4. | BM + vitamin BT (carnitine) | 0.08 | 12.8 ± 0.86 | 30.6 ± 3.16* | 154.3 |
| 5. | BM, without vitamins, control | 0 | 12.8 ± 0.86 | 41.3 ± 3.51 | 222.6 |

* The differences are statistically authentic compared with the control variant

From Table 5 it follows that vitamins E, C, $B_9$ and BT have antioxidant properties, as evidenced by the lower rates of accumulation of malonic dialdehyde in boar semen in the case of its storage at 18-20°C.

## CONCLUSIONS

Vitamins E, C, $B_9$ and BT in addition to known properties, also have specific antioxidant features, as evidenced by the different rates of accumulation of malonic dialdehyde in the process of dilution and storage of boar semen.

Whether all vitamins have antioxidant properties is to be studied in perspective using a wider and new arsenal of research methods.

Antioxidants, and in particular vitamins, never completely block the formation of lipid peroxidation products, they only slow down this process.

There is an inversely proportional relationship between the absolute survival rate of gametes and the malonic dialdehyde content.

## ACKNOWLEDGEMENTS

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# MOLECULAR, HISTOPATHOLOGICAL AND ULTRASTRUCTURAL STUDIES OF LANGENDORFF RAT HEART. THE IMPACT OF ISCHEMIC PRECONDITIONING

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***Abstract***

*The potential clinical relevance of ischemic preconditioning (IP) on ischemia-reperfusion injury is a major object of clinical research to find new methods of cardioprotection in humans.The aim of our study was to characterise the Langendorff model of rat heart investigating different durations of ischemia and reperfusion and (IP) protocol on myocardial ischemia reperfusion injury, to test the potential clinical relevance of (IP) phenomenon on infarct size, to assess genomic DNA integrity and histological and ultrastructural changes in heart muscle. Our results revealed that as ischemia and reperfusion time is extended,the level of infarction is also increased.We concluded that 20 minutes stabilisation and 45 minutes ischemia followed by 30 minutes reperfusion seems to povide a compliant platform to study ischemia reperfusion injury. Protection using (IP) was induced by 2 cycles 5` ischemia and 5` reperfusion prior lo lethal ischemia reperfusion protocols. The reduction in infarct size in IP hearts pointed out that inhibition of apoptosis is one of the mechanisms which participate in elicting the increased rezistance to ischemia.*

***Key words****: Langendorff rat hart, ischemia reperfusion, ischemic preconditionining (IP), infarct size, apoptosis, necrosis.*

## INTRODUCTION

It has been established that atherosclerosis a systemic process is the main factor implicated in the onset of cardiovascular and cerebrovascular disease; it afects vascular teritories esspecially at the branch: of coronary tree with risk for myocarial infarction (MI) as well as carotidian tree with risk for stroke (Revnic et al., 2007).

Cardiovascular disease is the leading cause of death in civilised world, over 3.8.milion men and 3.4 million women die annually because of the coronary artery disease (Yellon et al., 2007; Marin et al., 2018b).

The number of known risk factors associated with the development of cardiovascular disease (CVD) are ever increasing and currently include: hypertension (Marin and Goga, 2018a), hypercholesterolemia (Revnic et al., 2007), increased low density lipoprotein (LDL), decreased high density lipoproteins (HDL), obesity, diabetes mellitus, (Revnic et al., 2007), smoking and physical inactivity (Revnic et al., 2007).

Severe abrogation of blood supply (ischaemia) to a region of the heart can cause cell death, which is known as myocardial infarction (MI); this is detrimental and can induce tissue damage and consequentially the initiation of ischaemic heart disease.

Clinicians often observed this condition in patients who develop angina, which can be caused by the narrowing or obstruction of coronary arteries due to debris, such as a thrombus and/or atherosclerotic plaque formation. Ultimately, this ischaemic period initiates the process of tissue damage that is associated myocardial infarction (MI) (Revnic et al., 2007). Reperfusion of the ischaemic area is required to prevent further tissue damage however, paradoxically; the act of reperfusing the ischaemic area can itself induce additional damage and can affect cardiac function thereafter damage is referred to as ischaemia-reperfusion or reperfusion-induced injury work load and minimise ischaemia-reperfusion injury.

Current best practice for the treatment of patients suffering with a MI is to restore blood

flow using thrombolytic therapy or to use surgical interventions, such as balloon catheter angioplasty or coronary artery bypass surgery (Laskey et al., 2005). While these interventions have proven successful, some are invasive and may result in further tissue damage and reoccurrence of MI later in life.

Therefore, alternative strategies to enhance tissue viability post MI are required to help address this unmet clinical need (Dauterman and Topol, 2002; Ferdinandy et al., 2007). Much research is directed at investigating the cellular pathways involved in ischaemia-reperfusion injury and the cell death resulting in the formation of infarcted tissue. The period of ischaemia and the reperfusion of an ischaemic area, together, contribute to cell death and to the severity of myocardial injury (Hausenloy et al., 2005).

Myocardial ischemia-reperfusion (IR) injury may result from pathological processes such as atherosclerotic coronary artery disease and acute myocardial infarction and/or be secondary to surgical processes such as operating on the arrested heart or cardiac transplantation.

In contrast to "ischemic injury" which occurs when oxygen demand exceeds the available blood supply and is associated with cell necrosis, IR injury occurs upon return of blood supply after a period of ischemia and is usually associated with apoptosis (i.e. programmed cell death).

When compared to endothelial cells (ECs), cardiomyocytes (CMs) are more sensitive to ischemic injury and have received the most attention in the quest for preventing myocardial IR injury.

However, several studies suggest that ECs are more sensitive to IR injury than CMs and that they might be a critical mediator of IR injury in the heart (Arun et al., 2010).

Patients succumbing after developing this syndrome generally had infarcts of over 30% of the left ventricular mass at autopsy (Bolli et al., 2004). Therefore, reduction in infarct size in the presence of coronary oclusion is potentialy of great significance.

Recently it has been shown that necrosis following experimental myocardial ischemia can be reduced by pharmacological or hemodinamic factors. Myocardial ischemia-reperfusion injury occurs upon return of blood supply after a period of ischemia and is usually associated with apoptosis. Cell–cell interactions between blood cells and vascular endothelial cells and the release of cytokines and generation of reactive oxygen species from activated neutrophils (PMNs), endothelial cells and myocytes during reperfusion have been proposed as triggers in the induction of apoptosis. These interactions are initiated within the early movements of reperfusion, and may continue for hours and days. Apoptosis is a process of programmed cell death and is under strict genetic control, the apoptotic stimuli determine a change in cell suppressive influences, manifested by biochemical modifications that include activation of proteases and nucleases (Revnic et al., 2007).

The phenomenon of ischemic preconditioning has been recognised as one of the most potent mechanisms to protect against ischemia reperfusion injury. Preconditioning of the myocardium with short episodes of sublethal ischemia will delay the onset of necrosis during a subsequent lethal ischemic insult. It seems to involve a variety of stress signals which include activation of membrane receptors and signalling molecules such as PKC, mitogen activating protein kinases, opening of ATP sensitive K channels and expression of many protective proteins (Revnic et al., 2009).

Efforts to prevent ischemic injury have focused on finding ways to block events associated with irreversible ischemic injury. In 1986, Murry et al. (1986) described a classic phenomenon termed (IP) for the first time, as "multiple brief ischemic episodes that might actually protect the heart from a subsequent sustained ischemic insult". It was originally thought that each ischemic episode caused a cumulative ATP depletion.

While intermittent reperfusion would wash out the ischemic catabolites, surprisingly levels were not depleted by subsequent ischemic challenges and no infarct occurred. This observation led the same scientist group to test the hypothesis that the preservation of highly energy phosphate was due to a slowing consumption during ischemia associated with a rapid and protective adaptation of the myocite. They tested the hypothesis by subjecting the myocardium to a series of 45 minutes coronary branch occlusion each separated by 5 minutes of reperfusion. This rendered the myocardium

more resistant to a subsequent sustained 45 minutes ischemic insult; the infarct size has been reduced to 25% of that seen in control group.
This phenomenon is called preconditioning with ischemia; the classic IP is short lived and fast decayed with antiischemic effects disappearing completely within 2 hours.
The evolution of necrosis is delayed but not prevented; precondition will limit infarct size during a temporary coronary occlusion, but not during a prolonged or permanent occlusion.
The stimulus for preconditioning is a critical reduction in myocardial blood flow and the end point is infarct size; the optimal duration in ischemia is species dependent also the cellular basis of the mechanism underlined preconditioning is not fully understood. Preconditioning results in activation of a number of receptors such as: adenosine (Cohen et al., 2008), alfa adrenergic, delta opioids and bradikinin (Gross et al., 2008). Preconditioning stages can be applied prior to a planned procedure involving a potentially injurious ischemic insult.
Ischemic preconditioning provides a degree of protection against myocardial ischemia-reperfusion by reducing the number of myocites damaged by the above mentioned mechanisms and improved ventricular ejection fraction after reperfusion a clear relationship between preconditioning and apoptosis after myocardial ischemia- reperfusion has not been established yet.
One promising aproach which considerably decreases infarct size following coronary oclusion as demonstrated by enzymatic and hystologic criteria is ischemic preconditioning either mechanicaly or pharmachologicaly.
Ischemic preconditioning stands virtually alone in its ability to limit infarct size in the controlled setting of the experimental laboratories. Understanding the basic mechanisms of ischemia reperfusion injury is critical to developing clinically applicabe strategies to minimize myocardial reperfusion injuries.

**Objectives of paper:**
1.To characterise the Langendorff model of rat heart using different durations of ischemia and reperfusion from the ischemic precondition protocol (IPC) assessing the myocardial ischemia reperfusion injury.
2.To test the potential clinical relevance of ischemic preconditioning (IP) phenomenon on the finall infarct size, (the golden standard).
3.To assess genomic DNA integrity, the histological and ultrastructural changes of ischemic-reperfused myocardium to get insights into molecular mechanisms of IP to limit infarct size.

## MATERIALS AND METHODS

**Biological material**
This study was conducted on 25 white male Wistar rats aged 12 months old , weighting between 250-300g, kept in standard laboratory conditions, divided into five groups of 5 rats each: group 1- control, group 2 - 45 minutes ischemia followed by 30 minutes reperfusion, group 3 - ischemic preconditioning (2 cycles of 5min of ischemia followed by 5 minutes reperfusion) applied prior to 45 minute ischemia and 30 minutes reperfusion, group 4 - 5 minutes ischemia followed by 120 minutes of reperfusion, group 5 - preconditioning (5 minutes of ischemia followed by 5 minutes reperfusion applied prior to the 45 minutes of ischemia and 120 minutes reperfusion.
The animals were anesthetized i.p. with sodium pentobarbital (60 mg/kg) and then received heparin (300 U IP).
After the reflexes were abolished, hearts were excised and placed in perfusion medium on ice and then were quickly mounted in retrograde Langendorff perfusion system (Fig. 1) at a constant pressure (75 mm Hg) according to the published protocol (Revnic et al., 2005).
In order to avoid excessive hydration due to crystalline solution infusion, hearts were immersed in the perfusion liquid.
The perfusion liquid was the Krebs Henseleit bicarbonate buffer with the following composition (nmol/l) NaCl 118.5, NaHCO 25, KCl 4.8, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $CaCl_2$ 1.7, Glucose 1.2, and pH is 7.4 (Fig.2).

**Ischaemia-reperfusion injury: analysis of infarct size**
At the end of the reperfusion period, hearts were taken off the perfusion apparatus and perfused, through the aortic cannula, with 1% pre-warmed triphenyltetrazolium chloride (TTC) which acts as a viability dye.

Hearts were subsequently immersed in TTC and incubated at 37°C for 10 min, after which they were weighed and frozen at -20°C for 24h. While still frozen, hearts were sliced from base to apex at a thickness of ≈1mm; the slices were fixed in 10% formalin for 12h to define the stain borders.

The TTC is a redox indicator and can stain areas of viable myocardium red; this is because viable cells (with intact membranes) retain the dye where it can react with dehydrogenases and NADPH.

In the presence of oxygen these enzymes can reduce TTC, causing a conversion into a red colour (Klein et al., 1981).

Areas of tissue containing non viable/infarcted cells have lost their dehydrogenases, due to wash out in reperfusion, and are unable to retain or convert the dye and therefore emerge unstained and white in appearance.

Heart slices were then photographed on a perspex mounting block using a digital EsKape (Eskape, NY, USA) fixed camera.

NIH Image 1.63 software was used to calculate the infarcted areas and the results were expressed as a % of the whole heart at risk of damage induced by ischaemia-reperfusion injury (I/R%) and presented as means ± standard error of the mean (SEM).

A t-test or a one way ANOVA test were used to assess the differences between groups, as described in the statistics section further in this chapter. The results were considered signifycantly different when $p \leq 0.05$.

**Detection of Genomic DNA Fragmentation**

For detection and qualitative evaluation of DNA fragmentation, we examined whether genomic DNA isolated from ischemic hearts from groups: 1, 2, 3, 4, and 5 produced a typical "ladder" pattern (≈180-bp multiples) when analyzed on an agarose gel DNA fragmentation was investigated usingthe DNA laddering kit Cat.Nr.TA 4630 , R&D Systems England according to the published method (Revnic et al., 2004) in all 5 groups of rats.

**Methods**

TACS apoptotic DNA laddering kit was used for the evaluation of the left ventricular apoptosis by internucleosomal DNA fragmentation and appearance of the DNA laddering.

During apoptosis, the endonucleases produce double chain breaks in DNA, generating DNA fragments, these fragments are isolated from the tissue and optimized.

TACS DNA isolation reagents produce the inactivation of the endogenous nucleases, and the DNA fragments with different molecular weight are rapidly recovered.

The isolated DNA is fractionated according to size by gel electrophoresis and visualized Etd.Br.

**Experimental Procedure**

Cardiac tissue (left ventricle) of rat hearts from: group 1- controls, group 2 - 45 minutes ischemia followed by 30 minutes reperfusion , group 3- ischemic preconditioning (5 minutes of ischemia followed by 5 minutes reperfusion) applied prior to 45 minutes ischemia and 30 minutes reperfusion, group 4 - 45 minutes ischemia followed by 120 minutes of reperfusion, group 5 - ischemic preconditioning (5 minutes of ischemia followed by 5 minutes reperfusion applied prior to the 45 minutes of ischemia and 120 minutes reperfusion was chopped into small pieces and frozen in liquid nitrogen, then 1-2 g frozen tissue was ground to a powder was obtained which was then resuspended in 200 µl buffer.

20µl of 10X buffer was added and incubated at 50 C for 12-18 hours with mild shaking.

At 100µl tissue suspension, 100µl lytic solution from TACS apoptotic DNA Laddering Kit was added.

DNA isolation was done in accordance with the instructions in the user guide. We used 1 µl DNA that was diluted in distilled water without DNAse.

DNA electrophoresis was done according to the protocol described in the user manual of the kit, and then the gel was stained for 15 minutes with 0.5 µg/l EtidiumBromide.

DNA visualization was performed using a transluminal UV photographs that were taken with yellow filter 22A KodakWratten.

For optical microscopy, myocardial transmural sections from groups 1, 2, and 4 were fixed in formaldehyde (100 ml/L) inclusionated in paraffin. After fixation, frontal sections of the heart, including the ventricles and interventricular septum, were embedded in paraffin (Samir, 2015). Sections were cut at 5

μm and stained with hematoxylin and eosin, periodic acid-Schiff, and Gomori's Trichrome. Sections were scored in a blinded fashion by a veterinary pathologist, using the method as follows: grade 0, no lesions; grade 1, focal lesions of the subendocardial portion of the apex and/or the papillary muscle, composed of fibroblastic swelling or proliferation and accumulation of histiocytes; grade 2, focal lesions extending over a wider area of the left ventricle with right ventricular involvement; grade 3, confluent lesions of the apex and papillary muscles, with focal lesions involving other areas of the ventricles and the auricles; and grade 4, confluent lesions throughout the heart, including infarct-like massive necrosis, with occasional acute aneurysm, myocardial function after ischemia-reperfusion and cut into sections of 5 um thick and coloured with hematoxylin-eosine.

For electron microscopy studies were collected samples from the anterior and posterior papilary muscle from the same groups (1, 2, 3 and 4), the tissue was fregmented in 1 mm fragments that were fixed in glutaraldehyde (30 ml/L), in 0.1 ml/L of Na cacodilat buffer, pH 7.4, then fixed in 10 g/L osmium thetraoxide. Dehydration was done using varying degrees of alcohol, after that the inclusion in Spurr resin with low viscosity was done. Sections were made with Sorval Poter Blum ultramicrotome, using a diamond knife. These sections of 80 nm were collected on copper grids, stained with Uranyl acetate Lead citrate according with the published method.and examined in a EM Phylips 200 la 60 keV electron microscope for presence or absence of glycogen, interfibrillar edema, nuclear changes, wide I bands, intramitochondrial amorphous dense bodies and breakes within the sarcolemal membrane (Revnic et al., 2002).

## RESULTS AND DISCUSSIONS

### Study 1: Langendorff control ischemia-reperfusion protocol

Prolonged and unresolved regional myocardial ischemia without reperfusion leads to myocyte death. Although the early restoration of blood flow to the ischemic myocardium is necessary to salvage myocytes from eventual death, abundant evidence indicates that reperfusion after even a brief period of ischemia has additional deleterious effects on the ischemic myocardium that are not expressed during ischemia. IP refers to a process in which a brief, reversible period of ischemia followed by reperfusion enhances myocardial resistance to a subsequent longer period of ischemia. In every animal species studied, IP has produced the most reproducible reduction in experimental necrosis, studies have been undertaken to confirm its protective effect and possible mechanisms involved since Murry et al. first described this endogenous protective mechanism in limiting infarct size (Murry, 1986).

### The evaluation of infarct size

Infarct size was measured as the percentage of infarction to risk area (I/R %), at the end of the reperfusion period, using triphenyltetrazolium chloride (TTC) (white areas represents the infarct and red areas the viable myocardium. Infarct size (I/R% measurement using TTC staining).

### Infarction and duration of ischemia reperfusion

Further optimisation experiments were performed using Langendorff rat heart mode in which the hearts were subjected to various durations of ischemia and reperfusion. 35` ischemia was associated with an augmented infarct size to 39.23$\pm$3.5% and 45` global ischemia was associated with an augmented infarct size to 50.26$\pm$2.61%, $p<0.05$.

The infarct size developed in the risk area subsequent to 20 minutes stabilisation and 45` ischemia followed by either 30 minutes of reperfusion or 120 minutes of reperfusion showed a significant increase (60.62$\pm$3.16) at 120 minutes. These results account for the negative effect of duration of reperfusion on myocyte. IP consisting of 2 cycles of 5` ischemia and 5` reperfusion applied prior to 45` ischemia and 30 minutes reperfusion signifycantly reduced the infarct size to 28.24$\pm$2.3% and to 45.24$\pm$2.42% in hearts with 45` global ischemia and 120 minutes reperfusion.

### Signal transduction pathways involved in the induction of apoptosis

It is generally accepted that the process of apoptosis involves the activation of death

receptor -dependent and -independent signal transduction pathways.
The binding of pro-apoptotic ligands to their receptors initiates a process that results in an imbalance in regulating proteins (i.e. Bcl-2 family) and an activation of cytosolic proteases (i.e. caspase family) (Revnic et al., 2007).
It has been confirmed that the change in status of caspases from the inactive to the active form by both stimulating pathways is the key step to induction of apoptosis.

**Study 2: Evaluation of apoptosis by detection of genomic DNA fragmentation**

Apoptosis is a programmed process that develops simultaneously with necrosis principally during reperfusion, but with a time-course that is slower (days) than the development of necrosis (hours). Several mechanisms trigger apoptosis after ischemia and reperfusion: the generation of cytokines and reactive oxygen substances from endothelium, myocytes or cell–cell interactions between inflammatory and endothelial cells, imbalance in regulation of anti-apoptotic and pro-apoptotic proteins; activation of downstream caspases (Revnic et al., 2007), and the release of cytochrome C from mitochondria. Stimulation of PKC isozymes and the opening of mito-$K_{ATP}$ channels have been shown to be associated with a reduction in apoptosis in addition to necrosis (Revnic et al., 2009).
DNA nucleosomal fragmentation of myocyte in non-ischemic control group 1 is no visible DNA 'ladders' were found. In contrast, genomic DNA isolated from the ischemic zone produced a typical 'ladder' pattern from all 5 animals after ischemia and reperfusion (Fig. 1a).The presence of 'ladder' pattern (line 4`) in the hearts subjected to ischemia for 45 minutes and 120 minutes reperfusion; hearts subjected to two cycles of 5 minutes ischemia followed by 5 minutes of reperfusion before global ischemia of 45 minutes followed by reperfusion for 120 minutes did not show 'ladder' pattern.
We have found that in a rat model of ischemia and reperfusion, two cycles of 5 minutes of IP preceding 45 min global ischemia significantly reduced the intensity of DNA ladders in the area at risk in preconditioned myocardium.
Myocardial ischemic preconditioning techniques demonstrated the decrease of the destruction of myocytes by cell apoptosis following ischemia-reperfusion process, this effect is shown by reducing DNA fragmentation in the group of rats that received ischemic preconditioning compared to those which have not received ischemic preconditioning.

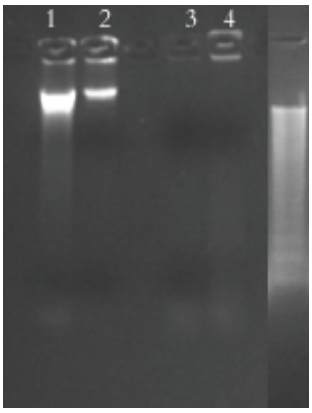


Figure 1a. DNA gel electrophoresis pattern of rat heart from groups:1, 4, 5, subjected to 45 minutes ischemia and 120 minutes reperfusion and IP. Control-group -1, group 4 with 45 minutes ischemia and 120 minutes reperfusion, group 5 with 45 minutes ischemia and 120 minutes reperfusion and ischemic preconditioning

Ischemic preconditioning reduces the myocardial apoptosis after ischemia-reperfusion by several factors including PKC isoforms, reduced inflammation and oxidative stress and $K_{ATP}$.
IP induces a biphasic pattern of myocardial protection. Following the acute phase of protection by early IP, a delayed phase, termed the 'second window of protection' appears between 12 and 24 h after the initial IP stimulus, which lasts up to 72 hours. Ischemia–reperfusion induces myocardial apoptosis and this is mediated by translocation of PKCδ in mitochondria and activation of apoptotic effectors implying Cytochrome C liberation and activation f Caspase 3 (Revnic et al., 2007) and fragmentation of DNA. $K_{ATP}$ channels have been implicated in myocardium recovery following ischemic aggression. It has been suggested that phosphorilation of $K_{ATP}$ following activation of protein kinase C via diacyl glycerol, leads to closing the K channels. Activation of protein kinase C abolishes the bloking effect of K channels by glyblenclamide in such a way that at the beginning of reper-

fusion cardiac frequency increases above control values leading to a slight decrease in time.
Literature data have shown that treatment of hearts with δV1-1 for inhibiting translocation of PKCδ leads to a marked inhibition of DNA laddering (Revnic et al., 2009).
As mentioned above, PCK plays an important role in ischemic preconditioning, experimental studies have used selective PKC inhibitors have shown an increase in myocardial apoptosis after the ischemia reperfusion cardioprotective effect was abolished.
An imbalance between anti- and pro-apoptotic proteins, an increased release of cytochrome C from mitochondria, increased caspase activetion, and activation of protein kinase C isozymes have been proposed as primary signal pathways involved in the induction of apoptosis after initiation of the death signal stimulus. Through altering these pathways, early IP has shown a profound effect on reducing myocardial apoptosis and infarct size.
Although most studies to date have shown that 'classic' or 'early'IP reduces necrotic and apoptotic cell death, more studies are needed to clarify the protective effect of delayed IP on apoptosis and related mechanisms.
From a clinical standpoint, it must be determined whether these interventions delay or permanently reduce apoptosis. However, there are no studies so far showing that early IP permanently reduces apoptosis after a longer period of reperfusion due to the short period of observation in acute experimental studies.
Furthermore, it is not clear whether a reduction in apoptosis contributes to the overall reduction in infarct size after prolonged reperfusion. In addition, it is also unknown whether a short treatment with a caspase inhibitor permanently attenuates apoptosis in the later phase of reperfusion.
Initial studies hold promise of such a translational benefit. If physiological outcomes are improved, then a limitation of apoptosis may offer an opportunity for treatment of ischemic heart disease, heart failure and other cardiac diseases. Although most studies to date have shown that 'classic' or 'early' IP reduces necrotic and apoptotic cell death, more studies are needed to clarify the protective effect of delayed IP on apoptosis and related mechanisms.
Ischemic preconditioning provides a degree of protection against myocardial ischemia-reperfusion by reducing the number of myocites damaged by the above mentioned mechanisms and improved ventricular ejection fraction after reperfusion a clear relationship between preconditioning and apoptosis after myocardial ischemia- reperfusion has not been established yet.
Ischemic preconditioning stands virtually alone in its ability to limit infarct size in the controlled setting of the experimental laboratories.
Understanding the basic mechanisms of ischemia reperfusion injury is critical to developing clinically applicabe strategies to minimize myocardial reperfusion injuries.

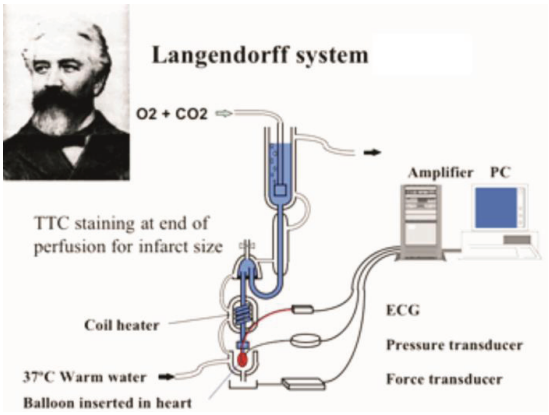


Figure 1b. The Langendorff retrograde perfusion system

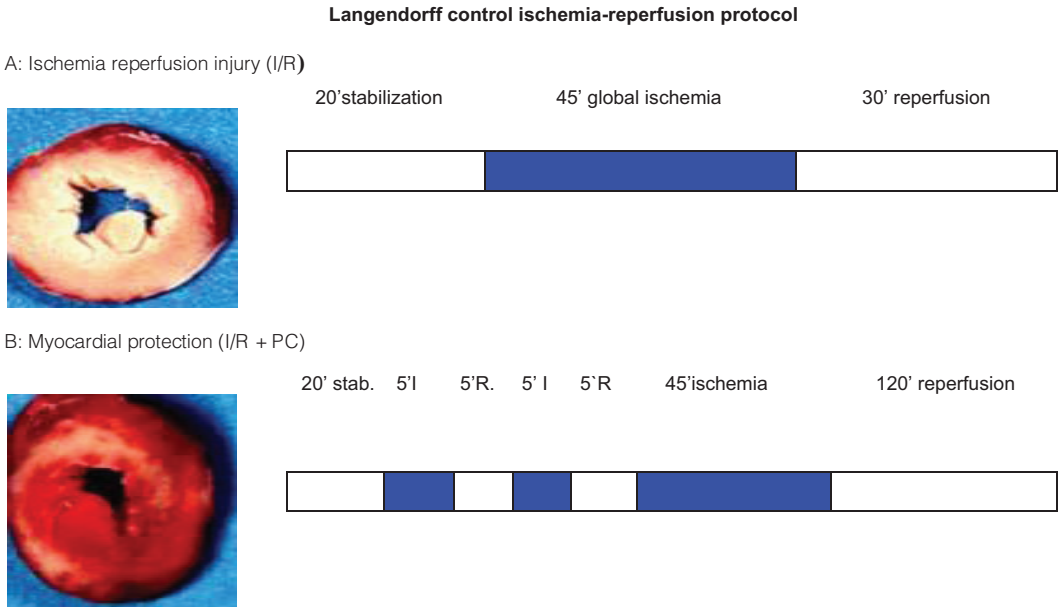


Figure 2. Rat myocardium subjected to: A) Global ischaemia-reperfusion only and B) Global ischaemia-reperfusion with ischaemic preconditioning as a cardioprotective strategy

**Study 3: Optical and electron microscopy results**

The image presents the myofibrils with normal architecture (Fig. 3).
Perinuclear mitochondria rich in glycogen, with intact double membrane, ordered and compact criste, dense and homogeneous matrix (Fig. 4).
Sarcomeres are uniform with Z bands arranged in register in adiacent sarcomeres.
Mitochondra are intact with regularly arranged cristae. Glycogen granules are abundant in perimitochondrial space. Ocasional lipid vaculoes are present (Fig. 5).

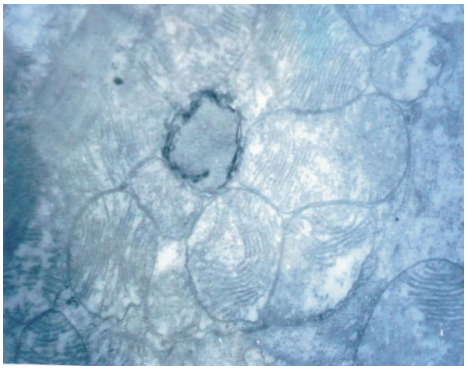

Figure 4. Electron micrograph (22000x) of control non ischemic myocardial tissue

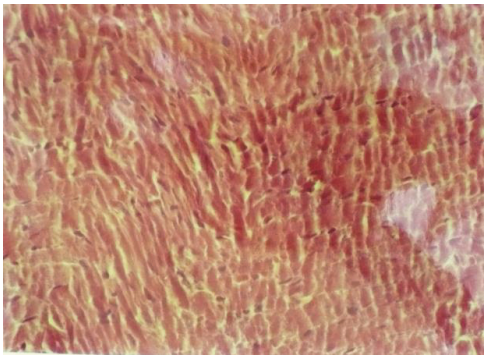

Figure 3. Optical micrograph of rat control myocardium

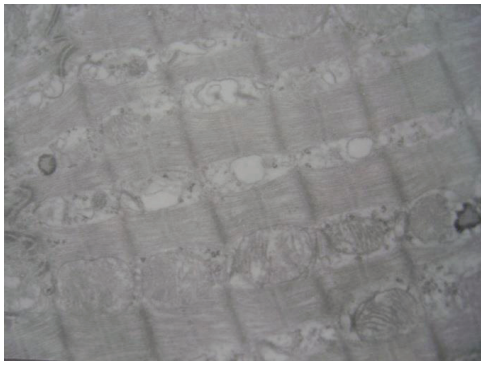

Figure 5. Electron micrograph of control myocardium

Some muscle fibers are vacuolated with pyknotic nuclei or otherwise anucleated, with perimuscular connective tissue destruction. There are accumulations of muscle cells, some muscle cells are poorly colored, which advocates for intracellular edema, scattered as they are form of small islands (Fig. 6). In some areas of the myocardium is less compact with increasing distance between myocytes which advocates for increased extracellular volume.

Severe ischemia is evidenced by the complete absence of glycogen and swelling of mitochondria, there is evidence for irreversible damage. Mitochondria show intact double membranes, some have ordered dense criste and compact matrix homogenous, others have disorganised crest (Fig. 7).

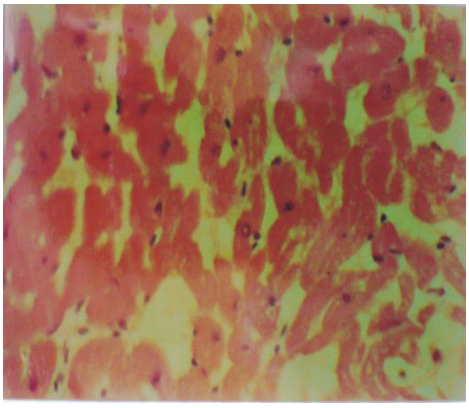

Figure 6. Optical micrograph of myocardium subjected to 45` ischemia and 30 minutes reperfusion.

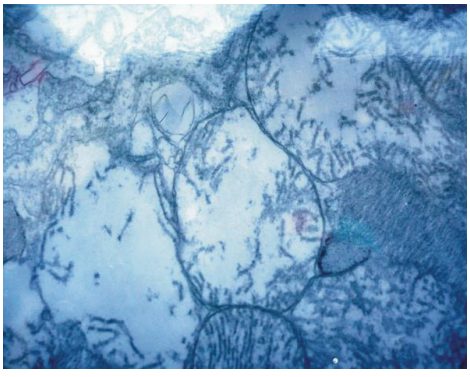

Figure 7. Electron micrograph (22000x) of rat myocardium subjected to 45` ischemia and 30 minutes reperfusion

Alterations classified as minimal. There is a decrease in glycogen granules.

The mytochondria are swollen and reveal the loss of matrix density. Cristae are generaly intact. The cytoplasm is severely edematous and sarcomeres are broken sepeared by an increased tissue space.

The tubules T of the transverse tubular system are dilated. Lysosome are present around the mitochondria. Nuclear cromatin clumping and margination, interfibrilar edema, wide I bands suggesting relaxation.

No amorphous intramitochondrial material or sarcolema breaks were found (Fig. 8).

The visual inspection by optical microscopy on the surface of endocard until the mid-ventricular anterior wall portion, of rat heart subjected to 45` ischemia and 120` reperfusion, the infarct was in some sections transmurally focused (Fig.9).

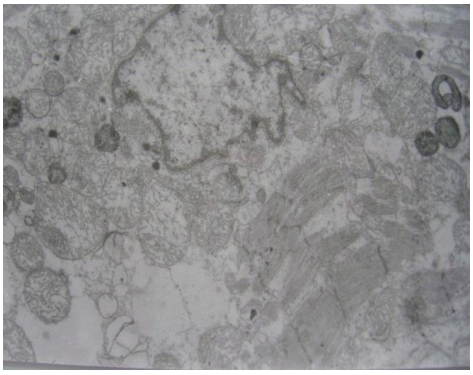

Figure 8. Electron micrograph (11000x) of preconditioned myocardium (group 3)

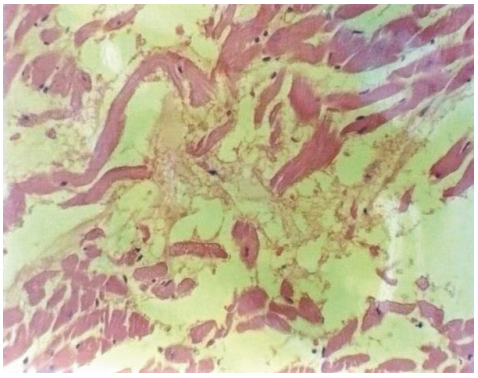

Figure 9. Optical micrograph of cardiac muscle group 4

In the necrotic myocardial tissue, the edema is present together with diffuse haemorrhage and contraction bands (Fig. 10).

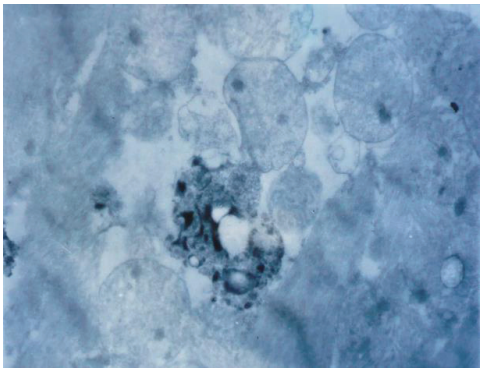

Figure 10. Electron micrograph (11000x)
of infarcted myocardium after 45 minutes ischemia
and 120 minutes of reperfusion

Myocardial tissue is irreversibly damaged, sarcomeres are expanded and hyperextended, mitochondria are swollen and fragmented, they lack matrix density, and cristae are disorganised, some are vacuolated and contain one or more amorphous dense bodies, which constitute signs of irreversible injury. Cell membrane integrity was disrupted. The are discontinuities in the plasma membranes, the cytoplasm is severely edematiated.

## CONCLUSIONS

Ischemia followed by reperfusion in rat heart caused significant changes in the ultrastructure of myocardial cell architecture in particular on cell organelles such as mitochondria that loose their ability to produce ATP, the energy source for cell life. Our results have shown that as ischemia and reperfusion time is extended, the level of infarction is also increased

A significant DNA “ladder” pattern has been observed in hearts exposed to 45 minutes ischemia and 120 minutes reperfusion accounting for an increased apoptosis process which develops simultaneously with necrosis principally during reperfusion.

Myocardial protection achieved by (ICP) induced by 2 cycles of 5` ischemia and 5` reperfusion prior to lethal ischemia reperfusion protocols, accounted for the reduction in infarct size, the golden standard, accompanied by an inhibition of apoptosis, one of the mechanisms that participate in elicting the increased rezistance to ischemia.

Ischemic preconditioning (IP) provides a degree of protection against myocardial ischemia-reperfusion by reducing the number of myocites damaged by the above mentioned mechanisms and improved ventricular ejection fraction after reperfusion a clear relationship between preconditioning and apoptosis after myocardial ischemia- reperfusion has not been established yet.

However, the more basic question remains whether a reduction in apoptosis translates into improvement of clinically relevant outcomes such as infarction, incidence of arrhythmias, global contractile performance, or survival.

The potential clinical relevance of IP on ischemia-reperfusion injury is the object of clinical research to find new thods of cardioprotection in humans

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# TECHNOLOGIES OF ANIMAL HUSBANDRY

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# GHG MITIGATION AND EFFICIENCY IMPROVEMENTS FOR A SUSTAINABLE SHEEP SUPPLY CHAIN: THE SHEEP TOSHIP LIFE STRATEGY

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***Abstract***

*Sardinia (Italy),one of the main European regions for sheep milk production and where a broad variety of dairy sheep farming systems coexist, can represent a special context for testing strategies of climate change mitigation for the small ruminant sector. The Sheep ToShip LIFE - Looking for an eco-sustainable sheep supply chain is a EU project launched in 2016 to develop and implement an intervention model for the sheep-dairy supply chain of Sardinia, able to reduce GHG emissions by 20% over the next 10 years through eco-innovative actions. The core of Sheep ToShip LIFE strategy is the evaluation, with a Life Cycle Assessment (LCA) approach, of the environmental impacts of the main Sardinian dairy sheep farming and manufacturing systems by using a case study methodology. The project's goal seems technically feasible by increasing farm efficiency at both flock and field levels. However, the greening of the dairy sheep sector strongly depends on attractive agro-environmental measures (based on effective eco-innovation criteria) within the next Rural Development Plan.*

***Key words****: eco-innovation, climate change, dairy sheep sector, LCA, GHG mitigation.*

## INTRODUCTION

Within the increasingly public scrutiny of livestock sector as one of the main anthropogenic source of greenhouse gases (GHG) contributing to climate change, Mediterranean sheep supply chain can contribute to boost animal agriculture in the transition towards a bioeconomy-based society. GHG mitigation is highly correlated with increasing production system efficiency and profitability (Jones et al., 2014), therefore improving the environmental performance of sheep farming could not only help combat climate change by reducing GHG and maximising ecosystem services, but also enhance socio-economic sustainability of local supply chains. This is a key point since in a context of structural economic crisis of the EU sheep sector, the risk that an effort to improve environmental performance would be perceived by farmers as a threat to their livelihood should be avoided. Understanding the drivers of GHG emissions within a farming system following a Life Cycle Thinking approach could be useful for defining sustainability strategies in an economically feasible way. In particular, addressing the trade-off between agricultural intensification and benefits of multiple services of livestock systems, a crucial topic of the greening agenda, Mediterranean dairy sheep farming system should represents an interesting case study. As Sardinia is the leading sheep milk producer in Europe (Rural Development Programme of Sardinia - RDP, 2014-2020), a proactive benchmark of climate change mitigation strategies for the dairy sheep sector in Sardinia could contribute to this debate. The Sheep ToShip LIFE (www.sheeptoship.eu), a 4-years (from July 2016 to June 2020) project financed by the EU LIFE Programme Climate

Action 2014-2020 and aimed to improve the environmental sustainability of the dairy supply chain in Sardinia, clearly points in this direction. The overall objective of the project is to reduce by 20% in 10 years GHG emissions from the Sardinian dairy sheep sector. Its actions promote the inclusion of environmental strategies for the sheep sector into rural development programmes, focusing on i) efficiency of production systems and ii) valorisation of the ecosystem services provided by pasture-based farms. The immediate goals of the project are to identify - by a Life Cycle Assessment (LCA) approach - and apply innovative solutions for the reduction of GHG, and to demonstrate the environmental and socio-economic benefits deriving from eco-innovation in the dairy sheep supply chain. The end goal of the project is to transfer the knowledge generated into an Environmental Action Plan for the sheep sector of Sardinia, which harmonizes the project's intervention strategy with regional policies to mitigate climate change. Furthermore, one of the project scopes is to increase the level of knowledge and awareness of stakeholders and general public regarding the environmental quality of products made from sheep's milk and their contribution to the mitigation of climate change. The inclusion of policy makers involved in environmental, climate and rural development sectors at regional, national, and European levels is essential for guaranteeing the project's sustainability and replicability. To achieve their ambitious goals the project cannot ignore the importance of involving policy makers and key stakeholders to ensure that climate change mitigation and adaptation is fully accepted and integrated as part of the regional development strategies for the sheep sector. In line with the project strategy, the Sheep ToShip LIFE partnership involves local authorities responsible for the definition and implementation of policies on environment and livestock production systems (Sardinia Region Department for the Environment), regional agencies for research and assistance services in agriculture (Agris Sardegna and Laore Sardegna), local University (two departments of University of Sassari) and national research center (two institutes of National Research Council of Italy).

## MATERIALS AND METHODS

The evaluation, with an LCA approach, of the environmental implications of the main Sardinian dairy sheep farming and manufacturing systems by using a case study methodology represents the basis of the Sheep ToShip LIFE logical framework (Figure 1). The project adopts this metric procedure to determine the environmental hotspots of the sheep's milk business in Sardinia, including the environmental impacts of Sardinian Protected Designation of Origin (PDO) sheep's cheeses. A cradle-to-farm gate LCA was conducted in 2017/2018, according to international standards (European Commission Recommendation 2013/179/EU).The LCA study analyzes the impacts of 20 sheep farms located in contrasting pedo-climatic zones of Sardinia and representing the main sheep farming systems in Sardinia, as described by Molle et al. (2018). Innovative solutions, based on the preliminary results of this LCA study, are being tested in 10 case study farms with the aim of demonstrating effective way to reduce GHG emissions maintaining quantity and quality standards of milk. In the next year, a Sardinian Environmental Action Plan aimed to reach the general objective of the project (-20% of GHG emissions in 10 years) will be definedon the basis of the assessment of the environmental and socio-economic effects of the Sheep ToShip LIFE implementation actions.The Action Plan will establish priorities and iterative roadmap of sustainable mitigation measures for the Sardinian dairy sheep sector in a way that that it will continuously update/grade the existing regional policy tools such as the Rural Development Programme and the Regional Strategy for Climate Change Adaptation (https://portal.sardegnasira.it/piano-regionale-di-adattamento). As massive adoption of innovations is dependent, among others, on farmers' and other stakeholders attitudes (i.e. beliefs and opinions) towards climate change, a survey was carried out, in 2018, on a sample of 238 stakeholders in order to map the general perceptions and goals related to climate change and their business. These information are propaedeutic to the design and communication of the Environmental Action Plan.


Figure 1. Sheep ToShip LIFE logical framework structured with a Deming cycle approach

## RESULTS AND DISCUSSIONS

The LCA study, identifying the main sources of GHG emissions and technical areas limiting efficiency of milk production, allowed to highlight best practices as well as to define a preliminary mitigation strategy. Moreover, it represents the first step to looking specifically the environmental footprint of the whole Sardinian dairy sheep supply chain.

Diets with greater GHG-generating potential per kilogram, directly related with enteric methane emission (the largest single source of emissions, by far), and off-farm produced protein-based feed represent the key areas of sheep farming to target for mitigation efforts. These results are in agreement with several studies on dairy sector and sheep farming (FAO, 2006; Marino et al., 2016; O'Brien et al., 2016).

Considering that the emissions baseline of Sardinian sheep sector (calculated "from cradle to farm gate") resulted equal to 1,407 kt of $CO_2$-eq (attributable for 80% to milk and 20% to meat) (Atzori et al., 2017), the Sheep ToShip LIFE target reduction is about 280 kt of $CO_2$-eq in 10 years (Table 1).

Table 1. Sheep ToShip LIFE plan for reducing GHG emissions in 10 years of Sardinian sheep sector

| Year | 2017 | 2027 | Variation |
|---|---|---|---|
| Ewe productivity (kg milk/year per present ewe) | 150 | 185 | + 35 |
| Sheep heads (thousand units) | 3,300 | 2,660 | - 640 |
| Total annual milk production (kt) | 315 | 315 | 0 |
| GHG emissions (kt $CO_2$-eq) | 1,407 | 1,127 | -280 |

The outline of the technical approach adopted by the project for reducing environmental and economic costs of sheep farming systems are reported below:

### Flock management

- Monitoring of reproduction performance to increase fertility.
- Monitoring of milk production to improve culling strategy.
- Disease control/prevention.
- Feed quality improvement (use of forage legumes, feedstuff analysis to better balance sheep diet, feed blocks for improve the digestibility of straw and cereal stubbles).

### Land use

- Introduction of native self-regenerating legumes-grasses mixtures and Sulla (a biannual forage).
- Low-input agricultural practices (minimum tillage, direct sowing, reduced use of fertilizers, etc.).
- Soil and water analysis to better drive pasture fertilization.

The survey on stakeholders' attitude found that on climate change related topics, sampled farmers have homogeneous favourable attitude, but on the general topic of innovation they are deemed "conservative" and have heterogeneous attitude on environmental conservation. If on "adaptation" and "effects of farming on climate change" farmers display general agreement, their attitude on causes of climate change and innovation may hinder adoption. Additionally, some different attitudes and perceptions between farmers, researchers and extension

officers were observed. For instance, regarding the importance of experience on farm efficiency improvement (Figure 2), the survey highlighted that farmers and extension officers on one side, and researchers on the other, have diverging opinions. This result indicates that there could be some obstacles in transferring knowledge to improve efficiency from research laboratories to the farm.

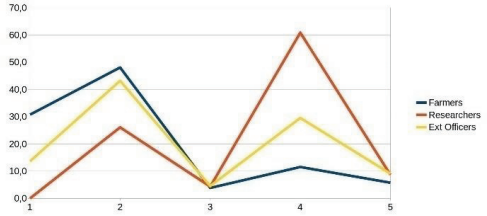


Figure 2. Neyman-Pearson Lemma test on importance of experience on farm efficiency improvement. The score 1-5 indicates not favourable and extremely favourable, respectively

Given the significance and representativeness of the Sardinian sheep sector at European level, Sheep ToShip LIFE proposed Sardinia as a European lab for climate change mitigation and, for this reason, the strategy of the project put special emphasis on networking, communication and dissemination of its results. Therefore, a key aspect is the engagement with European stakeholders as well as governance actions, since the integration between agricultural and environmental policies represents a pillar of the long-term sustainability and replicability of the project. The transferability of the Sheep ToShip LIFE model is essentially based on the following factors: i) the high interest demonstrated by the sector operators, smallholder farmers as well as medium and large dairy companies, towards environmental improvement and valorisation of the traditional livestock products, ii) the analysis of the local and international market trends, where green (and genuine) products are gaining ever-growing importance, iii) the actual guidelines of the European policy on agricultural and food sector, which strongly stresses on innovation and environmental efficiency of the production systems.

Among communication and networking activities, an important place had the first meeting with EU institutions and stakeholders, titled *Environmental actions for the EU sheep sector* and held on January 23, 2019 at the premises of the Autonomous Region of Sardinia in Brussels. About 40 representatives of the following organizations attended the meeting: European Institutions (DG AGRI, DG CLIMA, ENRD, EASME/LIFE Programme, European Shepherds Network, ENVE Commission of the Committee of the Regions); delegates of 5 EU projects (focused on sustainability of livestock production systems); the Italian Ministry for Agriculture, Forestry and Tourism; the Autonomous Region of Sardinia (Agriculture and Relationships with EU departments); the Permanent Delegation of Castilla y Leon (Spain) and Occitanie (France) Regions to the EU

The meeting highlighted that the scientific efforts by European projects for improving overall efficiency of livestock production systems could provide a basis of knowledge and data to boost the greening of the future Rural Development Programmes.

## CONCLUSIONS

The reduction of GHG by 20% in 10 years in Sardinia seems technically feasible by increasing farm efficiency at flock and field levels. However, new policies are needed to support GHG abatement within and out with the next Rural Development Programme. They should be possibly driven by the evaluation of farm environmental performance through a LCA-based metric. Rural development measures should support actions aimed at increasing animal productivity, quality of forages and reduction of input at field level. Moreover, measures should be tailored as much as possible to background systems and co-designed by the stakeholders (farmers *in primis*) using an approach similar to European Innovation Partnership (EIP), and its impact should be evaluated using smart indicators (effective and cheap).

The Sheep ToShip LIFE initiative can thus serve as a model of good practices for other European contexts, and can contribute to improve the environmental performances of production processes and products of the European small ruminant sector.

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# STUDIES CONCERNING THE EFFECT OF THE INBREEDING ON THE PROLIFICITY AND HATCHING PERCENTAGE AT SILKWORMS (*Bombyx mori* L.)

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***Abstract***

*The practicing of mating the individuals with a certain kinship degree leads to ostaining of inbreed lines through which crossing are ostained descendants characterized by performances superior to parental populations average.Using the inbreed lines in the hybridization process has as final result the obtaining of commercial hybrids. This is easily achievedin silkworms, species characterized by a short interval between generations and by high prolificity. Thirty silkworm inbreed lines were studied during six generations and the effect of the inbreeding on some physiological characteristics. The prolificity ranged between 632-494 eggs/layinginside the White Baneasa lines and 748-522 eggs/layingin Baneasa 75 lines. The number of eggs/layingwas lower at $I_6$whit 10.39% in Baneasa 75 lines and 11.41% in White Baneasa lines by comparision with $I_0$. Hatching percentage was affected by inbreeding depression beginning with $I_3$ in both lines groups. This was lower with 6.10-15.66 percentages in White Baneasa lines and 6.80-15.90 percentages in Baneasa 75.*

***Key words****: silkworm, inbreed lines, inbreeding depression.*

## INTRODUCTION

The inbreeding represents the process by which there are mated individuals more close related between them than the population kinship average. The genetic effect of inbreeding consists in changing genotypes freqvency, in the sense of increasing the homozygous genotypes freqvency and implicitly of decreasing the heterozygous genotypes freqvency.

The inbreeding degree is measured by the inbreeding coefficient (Wright). In silkworm case is most practiced the brother x sister mating system (incest).

Following the application of a higher number of generations it appears the inbreeding depression, phenomenon opposite to heterozys and which consists in decreasing the production and reproduction performances for a population. In silkworm, the inbreeding depression is manifested by prolificity, hatching percentage and larvae viability decreasing.

From the researches of Craiciu and Tițescu (1971) results that after six generations of inbreeding, the hatching decreases with up to 2%, reducing larvae viability with up to 18% and increasing the larvar period with 1-2 days. The inbreeding favourable effect has been manifested in some technological characters, as silk percentage and filament length, situations in which the control ($I_0$) has been exceeded with 1.3-4.7% for the first character and with 7-10% in the case of the second character (Table 1).

The experiences also demonstrated that after the sixth generation, the inbreeding favourable effect is no longer noted.

Concerns related to obtaining silkworms inbreed lines used to achieve hybrids for production, were reported Romania since 1969 (Craiciu and Otărășanu, 1969; Craiciu and Tițescu, 1971; Craiciu et al., 1974; Braslă and Matei, 1992).

The mentioned papers specifies the inbreeding effect and respectively of hybridization on some characters important for the sericicultural production, as hatching percentage, silk cocoons weight, silk percentage, etc.

Table 1. The inbeeding effects on some biological and technological characters*)

| Inbreed line | Character/Inbeeding generation | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Hatching (%) | | Larvae viability (%) | | Silk percentage(%) | | Fibre length(m) | |
| | $I_0$ | $I_9$ | $I_0$ | $I_9$ | $I_0$ | $I_9$ | $I_0$ | $I_9$ |
| C1 7/2 | 97.1 | 95.1 | 90.0 | 85.4 | 17.7 | 22.4 | 805 | 973 |
| C10 1/7 | 97.2 | 96.6 | 91.0 | 77.8 | 17.9 | 20.2 | 715 | 965 |
| AB 2/5 | 98.4 | 99.3 | 87.2 | 80.2 | 18.3 | 22.1 | 855 | 978 |
| AJ 1/1 | 98.0 | 99.5 | 83.4 | 78.8 | 18.6 | 19.9 | 750 | 898 |

*) Craiciu et al., 1971

There haven't been approached yet subjects of research with fundamental character, as would be the inbreeding and hybridization consequences on the physiological and biochemical processes which are happening at organism level (modification of protein spectrum under quantitative and qualitative point of view, of nucleic acids content, enzyme activity, intensity of some metabolic processes).

In general, from the papers consulted and presented in refferences, it results that the improvement process by inbreeding is used in creation of new populations, on a limited number of generations, usually no more than three.

## MATERIALS AND METHODS

The material used in the obtaining of inbreed lines was representend by two founding races, White Băneasa and Băneasa 75, the main active races used as parents to obtain industrial hybrids.

The creation of inbreed lines in order to manifest heterozys phenomenon by crossing, has been achieved in several stages, as follows:the extracting of lines and their inbreeding up to a variable number of generations; testing their specific combining capacity by dialele crossing; reproduction of high combining capacity lines; obtaining hybrids by crossing inbreed lines.

The inbreeding lines from the two founding races have been obtained by practicing the related crossings of brother x sister type during six successive generations. To avoid the effects of too accentuated inbreeding depression, it has been practiced the inter-family selection (linear) on the basis of their own performances in each family. The number of lines (families) on which the selection works have been expanded varied in accordance with the technological stage and the specific selection criterion.

The inbreeding coefficient/generation has been calculated with Wright formula:

$$F_x = (1/2)^{n_1+n_2+1}(1 + F_A)$$

in which:

$F_x$ – the inbreeding coefficient of the individual X;

$n_1$, $n_2$ – number of generation exchange between mother or father and common ancestor;

$F_A$ – the inbreeding coefficient of the common ancestor.

The prolificity has been determined by counting the eggs from each laying and the hatching by the ratio between the number of hatched eggs and the total numberof eggs from laying.

## RESULTS AND DISCUSSIONS

### The inbreeding effect on prolificity

The prolificity expressed by the number of eggs layed by a female represents a character taken into conssideration in particular in the works of silkworm reproduction, this influencing the specific number of silk cocoons needed to obtain 1 kg of eggs. The silk moth is characterized by high prolificity, a female producing 400-800 eggs, the number being influenced by the quality of larvae food, moth age, and also by the temperature and humidity conditions during ovogenesis and laying formation (Haniffa etal., 1992).

The prolonged inbreeding also represents a factor that may influence the prolificity, thing observed in the case of 30 inbreed lines studied. The inbreed lines coming from White Băneasa have presented a prolificity between 632-494 eggs/laying during the six inbreeding generations (Table 2).

Table 2.The inbreeding effect on prolificity in White Băneasa inbreedlines (number eggs/laying)

| Line | Inbreeding generation | | | | | | |
|---|---|---|---|---|---|---|---|
| | $I_0$ | $I_1$ | $I_2$ | $I_3$ | $I_4$ | $I_5$ | $I_6$ |
| AB – 1/1 | 588 | 596 | 601 | 598 | 583 | 576 | 545 |
| AB – 3/2 | 636 | 628 | 613 | 611 | 605 | 587 | 572 |
| AB – 4/3 | 612 | 615 | 617 | 618 | 603 | 608 | 596 |
| AB – 5/4 | 592 | 604 | 606 | 603 | 606 | 596 | 545 |
| AB – 7/5 | 638 | 626 | 612 | 616 | 596 | 588 | 576 |
| AB – 8/6 | 616 | 612 | 605 | 588 | 587 | 591 | 590 |
| AB – 9/7 | 602 | 605 | 597 | 566 | 559 | 548 | 552 |
| AB – 10/8 | 594 | 604 | 592 | 598 | 576 | 556 | 536 |
| AB – 12/9 | 642 | 632 | 612 | 594 | 582 | 552 | 514 |
| AB – 14/10 | 632 | 616 | 603 | 610 | 606 | 596 | 530 |
| AB – 15/11 | 597 | 606 | 585 | 591 | 585 | 591 | 527 |
| AB – 18/12 | 624 | 610 | 606 | 603 | 593 | 556 | 536 |
| AB – 20/13 | 612 | 603 | 593 | 595 | 576 | 544 | 514 |
| AB – 22/14 | 598 | 595 | 577 | 588 | 581 | 532 | 494 |
| AB – 25/15 | 622 | 614 | 612 | 603 | 601 | 596 | 532 |
| Average | 614±4.68 | 611±2.85 | 602±2.93 | 599±3.38 | 589±3.55** | 576±5.65** | 544±7.48** |

*P<0.05; **P<0.01; ***P<0.001.

Following the evolution of this parameter it is noticed that with the increasing of inbreeding coefficient its value is reducing, especially in $I_4$-$I_6$generations, which present differences in minus to the control ($I_0$), statistically significant. If in $I_0$average of 15 inbreed lines highlighted a number of 614 eggs/laying, in $I_4$-$I_6$ generations this represents 589, 576, 544 eggs/laying, finding a reduction with cu 4.08-11.41% of the analyzed character's value.

A similar evolution regarding the prolificity have registred the inbreed lines coming from Băneasa 75 race, the number of eggs/laying ranging between (Table 3).

Table 3.The inbreeding effect on prolificity in Băneasa 75 inbreedlines (number eggs/laying)

| Line | Inbreeding generation | | | | | | |
|---|---|---|---|---|---|---|---|
| | $I_0$ | $I_1$ | $I_2$ | $I_3$ | $I_4$ | $I_5$ | $I_6$ |
| B75 – 2/1 | 635 | 632 | 627 | 629 | 624 | 585 | 544 |
| B75 – 4/2 | 689 | 674 | 683 | 675 | 666 | 632 | 596 |
| B75 – 5/3 | 713 | 716 | 707 | 693 | 692 | 612 | 588 |
| B75 – 6/4 | 612 | 610 | 589 | 585 | 587 | 576 | 555 |
| B75 – 8/5 | 756 | 748 | 702 | 702 | 696 | 644 | 636 |
| B75 – 10/6 | 712 | 715 | 693 | 695 | 673 | 632 | 596 |
| B75 – 11/7 | 525 | 565 | 589 | 601 | 572 | 538 | 522 |
| B75 – 13/8 | 621 | 606 | 632 | 594 | 586 | 572 | 552 |
| B75 – 15/9 | 668 | 636 | 672 | 633 | 640 | 636 | 614 |
| B75 – 16/10 | 702 | 698 | 699 | 645 | 636 | 628 | 610 |
| B75 – 18/11 | 531 | 536 | 594 | 588 | 582 | 576 | 540 |
| B75 – 19/12 | 596 | 603 | 575 | 576 | 566 | 563 | 553 |
| B75 – 21/13 | 636 | 621 | 632 | 604 | 598 | 594 | 562 |
| B75 – 23/14 | 632 | 618 | 630 | 628 | 614 | 606 | 593 |
| B75 – 24/15 | 648 | 646 | 631 | 632 | 615 | 610 | 602 |
| Average | 645±16.86 | 642±15.18 | 644±11.82 | 632±10.93 | 623±11.10 | 600±8.09 | 578±8.47** |

*P<0.05; **P<0.01; ***P<0.001.

Analyzed on inbeeding generations, in $I_1$-$I_3$were obtained values close to $I_0$, to whom the differences in minus have represented only 0.47-2.02%. Although in $I_4$and $I_5$the differences in minus to $I_0$are amplifying, being of 3.42-6.98%, they are not statistically significant. Only in the last inbreeding generation ($I_6$) the average of the 15 inbreed lines (578 eggs/laying) presents significant differences (P<0,05) to the control (645 eggs/laying).

Analyzing the prolificity inside each line from the two groups and comparing its evolution from a generation to another, in most cases we find differences in minus which value is smaller in the first inbreeding generations and higher in the last 2-3 generations.

**The inbreeding effect on hatching**

Presenting as well as prolificity, a low heritability, the hatching was affected by the inbreeding depression. During the six generations, the hatching has varied between 98.60-80.14% in lines of White Băneasa group and between 99.15-82.10% in lines Băneasa 75 group (Tables 4 and 5).

Table 4. The inbreeding effect on hatching percentage inWhite Băneasa inbreedlines (%)

| Line | Inbreeding generation | | | | | | |
|---|---|---|---|---|---|---|---|
| | $I_0$ | $I_1$ | $I_2$ | $I_3$ | $I_4$ | $I_5$ | $I_6$ |
| AB – 1/1 | 96.36 | 94.40 | 93.01 | 93.20 | 91.90 | 85.96 | 83.40 |
| AB – 3/2 | 97.42 | 94.66 | 95.20 | 94.90 | 92.86 | 91.60 | 89.20 |
| AB – 4/3 | 95.80 | 93.30 | 91.60 | 92.13 | 90.16 | 84.60 | 84.42 |
| AB – 5/4 | 97.40 | 95.84 | 96.40 | 94.16 | 93.00 | 93.20 | 91.30 |
| AB – 7/5 | 99.27 | 97.70 | 98.60 | 95.20 | 88.46 | 88.32 | 87.16 |
| AB – 8/6 | 97.35 | 96.44 | 93.20 | 94.40 | 92.30 | 89.60 | 88.15 |
| AB – 9/7 | 98.27 | 97.30 | 94.40 | 93.80 | 91.40 | 90.13 | 90.40 |
| AB – 10/8 | 98.50 | 95.18 | 95.20 | 92.90 | 92.00 | 91.12 | 90.26 |
| AB – 12/9 | 98.60 | 94.16 | 93.30 | 92.10 | 87.60 | 85.30 | 88.30 |
| AB – 14/10 | 94.40 | 93.23 | 92.20 | 91.40 | 85.30 | 82.20 | 81.40 |
| AB – 15/11 | 93.20 | 96.66 | 97.30 | 95.20 | 87.20 | 85.31 | 82.60 |
| AB – 18/12 | 94.80 | 93.40 | 92.40 | 93.30 | 88.16 | 84.60 | 83.30 |
| AB – 20/13 | 95.20 | 94.60 | 93.66 | 93.10 | 91.36 | 87.32 | 85.20 |
| AB – 22/14 | 96.14 | 95.80 | 95.40 | 94.14 | 93.14 | 91.12 | 89.16 |
| AB – 25/15 | 95.80 | 96.60 | 96.20 | 95.80 | 87.13 | 83.12 | 80.14 |
| Average | 96.43± 0.426 | 95.28± 0.318 | 95.54± 0.521 | 93.72± 0.331* | 90.13± 0.666** | 87.57± 0.883** | 86.29± 0.925** |

*P<0.05; **P<0.01; ***P<0.001.

Table 5. The inbreeding effect on hatching percentage in Băneasa 75 inbreedlines (%)

| Line | Inbreeding generation | | | | | | |
|---|---|---|---|---|---|---|---|
| | $I_0$ | $I_1$ | $I_2$ | $I_3$ | $I_4$ | $I_5$ | $I_6$ |
| B75 – 2/1 | 98.99 | 98.10 | 98.40 | 97.94 | 93.32 | 92.80 | 89.96 |
| B75 – 4/2 | 98.12 | 97.80 | 97.53 | 97.05 | 97.05 | 92.36 | 91.32 |
| B75 – 5/3 | 98.72 | 98.36 | 93.15 | 89.90 | 83.87 | 83.50 | 84.40 |
| B75 – 6/4 | 97.30 | 94.60 | 97.04 | 93.36 | 94.47 | 90.96 | 89.60 |
| B75 – 8/5 | 99.16 | 98.12 | 95.50 | 95.20 | 93.52 | 92.16 | 86.72 |
| B75 – 10/6 | 98.10 | 96.32 | 97.60 | 89.86 | 82.46 | 83.22 | 82.20 |
| B75 – 11/7 | 97.75 | 95.18 | 96.07 | 88.14 | 83.78 | 84.36 | 85.40 |
| B75 – 13/8 | 98.76 | 97.72 | 98.60 | 93.32 | 91.66 | 89.98 | 86.22 |
| B75 – 15/9 | 97.75 | 95.66 | 94.95 | 93.60 | 92.10 | 90.92 | 88.66 |
| B75 – 16/10 | 98.72 | 97.33 | 94.48 | 91.16 | 90.88 | 88.77 | 88.20 |
| B75 – 18/11 | 97.80 | 96.90 | 97.98 | 94.66 | 93.80 | 91.26 | 87.46 |
| B75 – 19/12 | 99.10 | 97.80 | 99.15 | 95.30 | 92.20 | 91.76 | 89.90 |
| B75 – 21/13 | 97.73 | 95.60 | 94.73 | 89.80 | 85.30 | 86.83 | 87.23 |
| B75 – 23/14 | 98.16 | 97.90 | 96.89 | 88.62 | 82.10 | 83.10 | 84.60 |
| B75 – 24/15 | 97.96 | 97.12 | 94.33 | 87.76 | 84.60 | 82.86 | 83.20 |
| Average | 98.27± 0.151 | 96.97± 0.311 | 96.43± 0.471 | 92.38± 0.850** | 89.41± 1.313** | 88.32± 1.006** | 87.60± 0.691** |

*P<0.05; **P<0.01; ***P<0.001.

In the first two generations of inbreeding there are noted close values of hatching percentage 95.28 and 94.54% in White Băneasa race and 96.97% and 96.43% respectively, in Băneasa 75 race, the differences in minus to the $I_0$ representing 1.15-1.89 percents. Starting with $I_3$, the differences in minus to the control ($I_0$) become significant for both groups of races, the hatching percentage decreasing with the inbreeding depression's increasing, such that in the last generation ($I_6$) were recorded values lower to $I_0$with 10.52% in the group of White Băneasa races and with 10.86 % in the group of Băneasa 75 races. Following the evolution of hatching percentage from a generation to another it is observed that although between them there are differences in minus, these are not statistically significant, excepting $I_3$which presents significant differences in minus to $I_2$for the both groups of races. Analyzing the

hatching percentage for each line during the six inbreeding generations it is noted a descending evolution of it from a generation to another, in most cases.

In the group of White Băneasa lines, the most affected by the inbreeding depression for this character was the line AB-25/15 which in $I_6$has achieved a hatching of 80.14% compared to 95.80% în $I_0$, the difference in minus being of 15.66 percentes. Less influenced was the line AB-5/4 in which the difference in minus $I_0$-$I_6$was 6.10 percentes. Values of hatching percentage higher than 90.00% in $I_6$ were noted in the case of 3 of 15 lines from the White Băneasa group, the maximum value (91.30%) being recorded by line AB-5/4.

The same descending evolution presented the hatching percentage for the group of Băneasa 75 lines, in which the differences in minus $I_0$-$I_6$ranged between 6.80 percents (B75-4/2) and 15.90 percents (B75-10/6). The maximum value of hatching percentage in $I_6$ belongsto B75-4/2 (91.32%), and the minimum to B75-10/6 (82.20%).

## CONCLUSIONS

The prolificity varied with the inbreeding generations between 632-494 eggs/laying in the lines from White Băneasa race and between 748-522 eggs/laying in those from Băneasa 75 race and it was negatively influenced by the inbeeding process. Comparing to $I_0$the prolificityis low in $I_6$ with 10.39% in Băneasa 75 lines and with 11.41 % in White Băneasa lines.

The hatching percentage has been affected by the inbreeding depression starting with $I_3$, in both groups of races. The differences in minus between $I_6$and $I_0$ have varied between 6.10-15.66 percents in the group of White Băneasa lines andbetween 6.80-15.90 percentsin the group of Băneasa 75 lines.

The experimental works which aimed the study of inbeeding and hybridization effect in silkworms allowed the selection of a number of 6 inbreed lines and 8 hybrids characterized by superior biological and technological parameters, recommended for the sericicultural production.

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# RESPONSES OF FARMERS REGARDING THE ROLE OF FLY INSECTS AGAINST SKIN DEFECTS OF LOCAL CATTLE

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***Abstract***

*Recently the average of national production of local beef cattle in Indonesia tends to decline. On the other hand, insect infestations, especially flies in cattle become one of the problems that cause a damage in cow skin and also inhibits local cattle production. This research aimed to evaluate the responses of small-scale local cattle farmers regarding infestation of flies and their consequences which they traditionally manage. The research method used was the descriptive method through a field surveys conducted in 12 small-scale local cattle farmers in Minahasa area and 12 others in Tomohon and North Minahasa. The results showed that the highest number of 42% of respondents who were disagree to say that flies have a potential to transmit disease to cattle, while 25% strongly were agree, the rest give varied opinions. Various responses to flies play a role in skin defects: The highest percentage who agreed with the number are those who say they disagree, that is 33%, who agree to 25%. Through an interview, it was directly shown that limit knowledge about insects resulted low understanding on the potential role of flies that caused a skin defect. However, 25% of respondents were aware that when in such cases appeared than a consultation with livestock health officers was needed.*

***Key words****: insect, skin defect, farmer, local cattle.*

## INTRODUCTION

Local beef cattle production in various regions such as in North Sulawesi Province of Indonesia has great potential in supporting various breakthroughs for national food development for the community, as stated by Tarigan (2018). Moreover, it was observed that these cattle had a type of mix-farming activity. However, until now the farmer of this type of livestock have not a maximum benefit. In this current era of global markets which have begun to take place in the Southeast Asia region, the existence of livestock products faces a challenging because they have to compete with global markets.

The development of beef cattle production quality will be able to provide maximum profits to farmers both from the main results in the form of meat and by-products, especially leather. Local cattle ranches in the Minahasa region of North Sulawesi Province that we explored, showed all of them were small scale and traditionally managed. Kaosa-ard and Rerkasem (1999) constated that: "Traditional farming is based on systems with minimal or no imported inputs and where livestock and crop activities are integrated. Farm products are mainly for domestic consumption and the excess was sold locally". These cows were important in their daily activities because they are used as working cattle and cut. Livestock work because they help in hijacking in the farm or was used a transportation equipment from the farm to the house in their village.

One of the problems found in cows maintained by these farmers was a skin disorders that caused injury. These wounds were often become a place of insect activity, especially flies (Rumokoy et al., 2018a). These insects have been known for their role as pathogenic agent vectors that have an impact on the wound or on environmental health, especially to the animal's body. Maintenance and handling of cow's skin health problems in local cows depended on the perspective, knowledge and insights and experiences of the farmer himself. Newborns have clean skin conditions without a skin defect compared to those over one year, as a consequence of the function of colostrum in

calves as in other mammals such as horses (Rumokoy and Toar, 2014). Injuries to the skin that were not resolved completely resulted an increasing of size of the wound and damaged to the skin permanently which were called a skin defect. Toar et al. (2013) suggested that there were various bio-active ingredients such as citronella oil and papain obtained from several types of local plants in the tropics that have been developed to make fly repellent in livestock.

The problem in this traditional livestock system was a threat of contamination with pathogenic micro-organisms in their environment (Rumokoy and Toar, 2015).

In order to be able to support precisely in overcoming the problems of these traditional farmers in the current conditions, we need a variety of the latest scientific information, especially regarding their responses to the role of fly insects on local cow skin defects that they traditionally raise.

## MATERIALS AND METHODS

A descriptive method was used in this research through a field surveys conducted in 12 small-scale local cattle farmers in centre of Minahasa area and 12 farmers with the same type outside of centre of Minahasa (Tomohon, and North of Minahasa).


Figure 1. Survey Locations Map, (Google Map, 2019)

The survey realized by an interview technical to get the response from farmer aboutthe role of flies on their cattle' skin defect. The first parameter was the opinion about flies as pathogenic agent transmitter and the second was farmer response to overcome this skin defect.

The instrument in this study was questioner by using Likert scale to measure the response. The first parameter was explored by proposing four questions to detect the opinion farmer concerning the flies and cattle skin defect:

P1 (flies were able to transmit pathogenic agent); P2 (many species flies infested in skin defect in cattle); P3 (flies were able to lead a skin defect); P4 (a skin defect could be more serious by a simultaneous infection of virus, bacteria, and parasite).

The second parameter was elaborated to detect their opinion of the way to overcome cattle suffered with a skin defect: rather to consult with skilled person or use their own traditional manner by using a question:

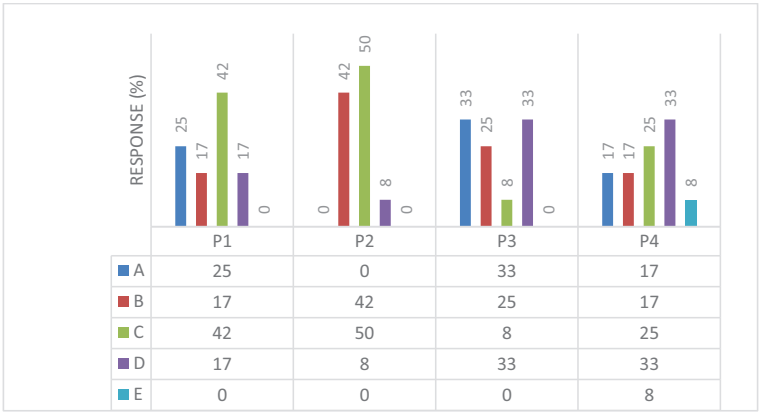
Do you think that consulting with people who are competent in the field of livestock health is important to overcome skin defects rather than using their own traditional methods?

## RESULTS AND DISCUSSIONS

Various of farmer's response related to question P1: Were flies able to transmit pathogenic agents? Although not exceeding 50%, it turned out that in the field most farmers gave a neutral response (42%).

When they were asked that the existing of flies infesting in the skin defects or wounds? As many as 42% agreed, while 50% were still hesitant. They were more likely to generalize flies. Based on the statement that "flies were able to lead a skin defect, gave a divers response of the farmer connected which showed that the same proportion between to whom responded: strongly agree compared to the response disagree.

In this point 25% respondents were agreed. Concerning to the question P4 showed the same presentation (33%) for both the person who were strongly agreed and agreed reached to say that a skin defect could be more serious by a simultaneous infection of virus, bacteria, and parasite, while 25% respondents were neutrals, but 33% of respondent were not agreed as shown in Figure 1.




| | P1 | P2 | P3 | P4 |
|---|---|---|---|---|
| A | 25 | 0 | 33 | 17 |
| B | 17 | 42 | 25 | 17 |
| C | 42 | 50 | 8 | 25 |
| D | 17 | 8 | 33 | 33 |
| E | 0 | 0 | 0 | 8 |

Figure 2. Bar chart of farmer's opinion on flies and skin defect in cattle

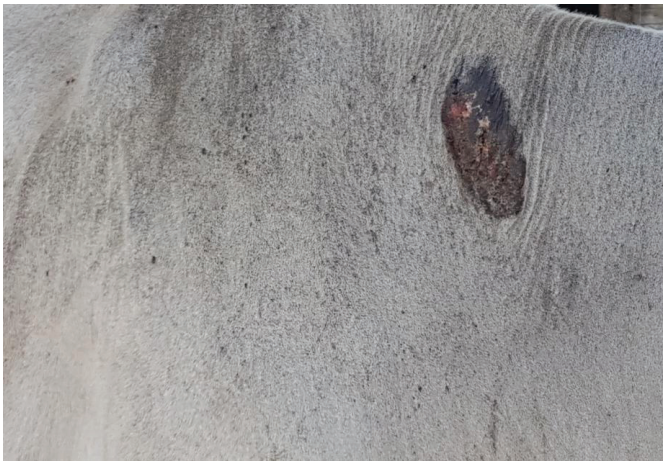


Figure 3. Skin defect of a cattle

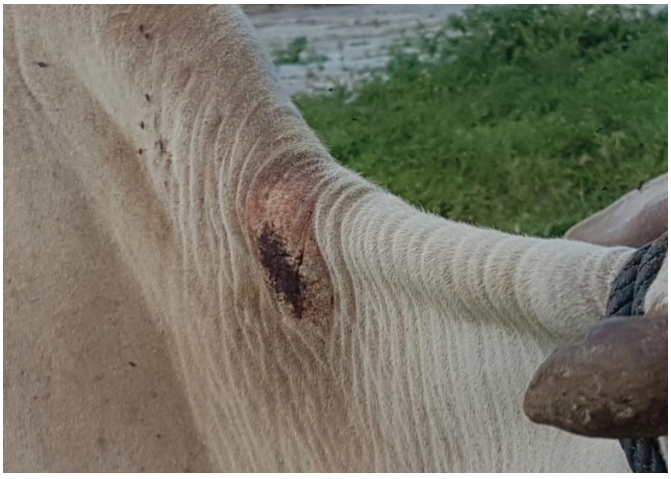


Figure 4. Skin defect of a cattle with flies infestation

The farmer's responses concerning the manner to stun the cases skin defect of their cows were varied (Figures 2, 3, 4, 5). In location A (Centre of Minahasa area) showed that a largest part (34%) agreed that it was better to consult with the trained person rather than used the traditional manner, followed by the response that they preferred to apply a local traditional manner for this case (33%) and 17% of respondent expressed a neutral decision to choose an opinion to overwhelm the problems of skin defect (Figure 6).

In this areal of observation, a same proportion appeared from the respondents who had answered strongly disagreed compared to those who were strongly disagree.

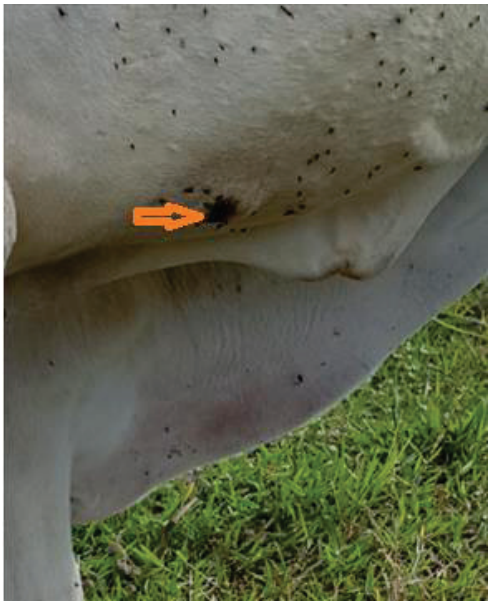


Figure 5. Flies activities in a skin wound of cattle maintained with traditional manners

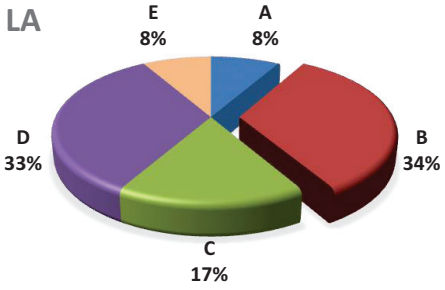



Figure 6. Farmer's opinion in location A (LA)

The respondent of farmers in location LB showed a different opinion: a portion of 25% revealed from the respondent who was strongly agreeing that needed to a trained person to resolve their cow's skins defect (Figure 7). The answer "agree" was also 25% found in the location LB, while 33% could not decide to choose a negative or positive opinion.

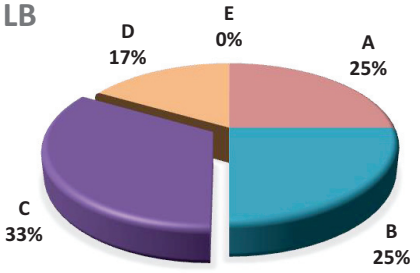



Figure 7. Farmer's opinion in location B (LB)

From the results as mentioned above, illustrates various points in the fields of agriculture and livestock in locations where the surveys were realized:

Technical Support: Farming in Asian countries can be characterised as smallholding agriculture (Ye and Pan, 2016) included the farming surveyed in this research. A technical support seemed to be needed for farmers surveyed to overcome the problem of skin defects of their cows.

Another work of Toar et al. (2018) showed that most beef cattle experienced skin defects associated with infestations of various species of fly even though in this recent survey rose a 42% of respondents gave otherwise respons. The intended support will be needed because some of these breeders were still unaware that flies have the potential to transmit disease agents through injured skin.

Various local natural resources could be used to treat various conditions of infections which spread by insects such as flies, as related to the reports of Rumokoy et al (2018b). Therefore, supports oriented to increase agricultural production including livestock products and the crops as managed by the farmer. Devendra (2012) reveals that a breaking of challenges will be able to increase productivity, improve the quality of life and environment sustainability in the future.

The connection of knowledge of livestock skin health with cattle production gave a consequence to the farming condition which was detected through constant number of cattle in regions do survey. If an extension of technical assistance is programmed periodically, it is highly probable that the farmer can improve their cattle production.

Cultural Context: It was also noted, that families who carried out cattle having a high mobility in their work and run a multi-job of work in agriculture domain, for example as a farmer, a fisherman, a carpenter. In generally this multi-jobs have been done for generations, while their cattle used to be as laborers in their agriculture fields and also as a transport vehicle between their fields and homes.

Livestock ownership in the areas of survey has a social perspective value even though only a few heads of animal. This condition can trigger an increase in the quantity and quality of cow production that intersects with agricultural programs from the government in the region.

Economic Drivers: This type of cattle farming could be as one of the economic growth drivers in the community, especially for those who in the same time have coconut plantations and other perennials as well as vegetable crops. Technical assistance destinate to middle-income farmers can help mind-sets to develop from aspects of production and income. According to Widiatmanti (2015) the household income in the middle level are needed to be support in order to reach in a high net worth individual.

## CONCLUSIONS

Cattle farming in the Minahasa region have important geographical and cultural values in order to improve regional economies and contribute to the national food supply. The effort to increase the local cattle farms production in this area needs to be accompanied by technical assistance, especially in overcoming the problem of skin defects associated with the role of flies.

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# UTILIZATION OF ORGANIC FERTILIZER ON GROWTH, PRODUCTION AND QUALITY OF *Brachiaria humidicola* AND *Pennisetum purpureum* UNDERNEATH COCONUTS BASED FARMING SYSTEM

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***Abstract***

*Utilization of inorganic fertilizer is more simple but costly and has some negative environmental impact. Livestock waste available abundantly and sometime promote soil and air contamination. The aims of this research was to studied the effects of organic fertilizer utilization on growth, production and quality of two common tropical grasses known as Brachiaria humidicola cv. Tully and Pennisetum purpurem cv. Mott grown underneath mature coconuts plantation. Treatments consisted of two kind of organic fertilizer called "bokashi" based on manure of chicken and ruminant fermented with effective microorganism (EM4). Treatments were put as factorial arrangement based on completely randomized design. The variables measured were dry matter production, quality of forages and predicted carrying capacity. Data was using ANOVA followed by HSD. The results shown all variable were significant higher of both kind of grasses with organic fertilizer at level of 20 tons ha$^{-1}$. It could be concluded that both species of grasses response positively utilization of organic fertilizer bokashi, but in term of DM and CP content utilization of bokashi based on chicken manure fermented with effective micro-organisms EM4 was better than those of ruminant manure. Utilization of organic fertilizer in form of bokashi could provide forages to ruminant production integrated with coconuts plantation. By that way could be enhance economic value of this integrated systems.*

***Key words****: fertilizer, quality, B. humidicola, P. purpureum, coconuts.*

## INTRODUCTION

Indonesia is one of the among five country with big human population in the world, still import red meat to meet demand of this meat in country, since the price of this commodity is higher than those import. The problem is supply of forages is insufficient due to limitation of space for forage production. On the other hand there is some under utilize space in coconut plantation since Indonesia is the largest production of coconuts in the world. Furthermore, some tropical grasses have been selected as species tolerance growth under shade environment in coconuts plantation (Rumokoy et Toar, 2014). Dry matter (DM) production of *P. purpureum* including dwarf genotype is enhanced by high input of inorganic fertilizer (Hasyim et al., 2014), but this increases the cost of forage production. Chemical fertilizer is widely used in agriculture. However, in recent years, serious concern has arisen about long-term adverse effects of continuous and indiscriminate use of chemical fertilizer in intensive agriculture on the deterioration of soil structure and function and environmental pollution (Farede et al., 2010). Livestock manure is an organic fertilizer that plays a key role in chemical and biological soil functions of intensively cropping fields under sustainable and environmentally harmonized herbage production. Since manure has a high concentration of organic matter, its application as a fertilizer helps decelerate depletion of organic matter in arable land, especially when there is a high frequency of heavy erosion (Prasad et al., 2002). It also increases the soil levels of the macro elements

of nitrogen (N), phosphorus (P), and potassium (K) (Kaligis et al., 2013; Kaligis et al., 2012). improves soil physical properties, enhances DM yield, and improves the crude protein concentration of herbages (Kaligis et al., 2017). Utilization of in-organic fertilizer for forages production is costly for smallholder farmers and risks environmental pollution by rapid nutrient leaching under heavy rainfall. On the other hand organic manure application has lower risk of nutrient leaching by mineralization when compared with chemical fertilizer input. The objectives of this research were to study the effects organic fertilizer application on growth, yield and estimated carrying capacity of two tropical grasses grown integrated in coconut based farming system.

## MATERIALS AND METHODS

The plant material used in this study was tillers of both species. Tiller was put in individual poly bags (1 plant/poly bag) which were filled with 2 kg growing media. The plants were nursed for 3 months in growing media. After 3 months of the nursery period, these plants has been trimming to get homogeny re-growth, then were transplanted to experimental plot in the field since February 2017 until August 2017. Experimental site receives an average rainfall of 2700 mm, and the distribution fairly even, except for the period of lower rainfall by 100-150 mm monthly, from July to September. The pH of the fertile, sandy loam soil is around 6. Light transmission at 10.00 am on a sunny day as PAR underneath mature tall coconuts averaged 73%. The soil has an average pH of 6 and its color was dark brown clay. Precipitation peaks took place in January, with high rainfall intensity. This caused high relative humidity (80%). Air temperature ranged from $25^0$C to $37^0$C.The *P. purpureum* cv. Mott grass plant space was 100 x 100 cm apart and *B. humidicola* space was 50 x 50 cm apart. This experiment using completely randomized factorial design with 2 factors. Treatments consisted of different level of two kind of organic fertilizer called "bokashi" based on chicken and ruminant manure fermented with effective microorganism (EM4).The first factor was two species of grasses being evaluated were *B. humidicola* cv. Tully and *P. purpurem* cv. Mott. The second factor was two sources of bokashi based on chicken manure (bokashi-1) and ruminant manure (bokashi-2), Three different levels of bokashi applications (B) where B1 = 5 tons, B2 = 10 tons, and B3 = 20 tons $ha^{-1}$. Each treatment was allocated randomly at experimental plots in the field. The variables include fresh weight yield (tons/ha), dry weight yield (tons/ha), crude protein, crude fiber and ash content. Dry matter yield of each plot was calculated through the value of green forage production and dry-weight percentage. Combining the dry matter yield with crude protein, crude fiber, and ash content data allowed us to calculate the mean crude protein, crude fiber, and ash yield. Predicted carrying capacity was determined by the information obtained from the forage harvested; it was collected from productivity estimation of each plot and converted to one ha. Available forage was calculated based on 70% of the total used factor. It is assumed that animal consumes 6.29 kg DM of forage/day/head (Indonesian condition). The plot size was 10 x 10 m. The total number of plots was 60 consisting of both grasses x 2 sources of bokashi x 3 level of bokashi x 5 replications. Data were then statistically analyzed by using analysis of variance (ANOVA) by means of MINITAB (Version 16). Honestly Significance Difference (HSD) was applied to determine the difference among treatments. Differences were considered at $P<0.05$. Harvesting biomass of *B. humidicola* cv.Tully and *P. purpurem* cv. Mott was done simultaneously when the ages of plants has arrived 35 days after replanting time in the field. *P. purpurem* cv. Mott was defoliated at first node from the soil surface (approximately 10 cm above ground). *B. humidicola* was defoliated at 10 cm level above ground. To get sample of *B. humidicola* has been used square 1 x 1 meter. This square was placed two time in the middle of each plot to avoid the border effects. Sample of *P. purpurem* has take five plants in two difference places in each plot so there were 10 plants as sample in each plot. Sample was dried at $60^0$C or about 48 h to determine the dry weight. The samples were analyzed for dry matter, crude protein, crude fiber and ash according to the standard procedure of Association of Official Analytical Chemists.

## RESULTS AND DISCUSSION

The effect of treatments on quality of grasses has been measured (Table 1). Data showed the effects of application both types of bokashi at 20 tons $ha^{-1}$ produced dry matter (DM) content of both grasses significantly higher compared to other level.

Table 1. Dry matter (DM), crude protein (CP), NDF and ash content of both grasses under different kind and levels of bokashi

| Attribute (%) | Treatments (bokashi tons.$ha^{-1}$) | Treatment | | | |
|---|---|---|---|---|---|
| | | Bokashi-1 | | Bokashi-2 | |
| | | Bh | Pp | Bh | Pp |
| DM | 5 | 16.75[b] | 24.01[b] | 15.71[b] | 20.01[b] |
| | 10 | 17.32[b] | 24.35[b] | 16.82[b] | 19.15[b] |
| | 20 | 17.61 [a] | 28.72[a] | 18.51 [a] | 23.22[a] |
| | Significant | * | * | * | * |
| | | 17.23[b] | 25.69[a] | 17.01[b] | 20.79[a] |
| | | 21.46[a] | | 18.90[b] | |
| CP | 5 | 10.73 | 9.03 | 9.75 | 8.05 |
| | 10 | 11.31 | 9.71 | 11.01 | 9.11 |
| | 20 | 11.47 | 10.13 | 12.07 | 11.13 |
| | Significant | NS | NS | NS | NS |
| | | 11.17[a] | 9.62[b] | 10.94 | 9.43 |
| | | 10.39 | | 10.18 | |
| NDF | 5 | 66.31 | 68.53 | 63.31 | 67.63 |
| | 10 | 65.17 | 67.71 | 64.16 | 65.71 |
| | 20 | 65.87 | 67.54 | 66.77 | 66.14 |
| | Significant | NS | NS | NS | NS |
| | | 65.78 | 67.93 | 64.75 | 66.49 |
| | | 66.85 | | 65.62 | |
| Ash | 5 | 11.04 | 11.20 | 10.84 | 10.70 |
| | 10 | 10.15 | 10.76 | 10.35 | 11.16 |
| | 20 | 10.27 | 11.32 | 10.23 | 10.52 |
| | Significant | NS | NS | NS | NS |
| | | 10.49 | 11.09 | 10.47 | 10.79 |
| | | 10.79 | | 10.60 | |

Note: Bh= B. humidicola cv. Tully; Pp = P. purpureum cv. Mott; NDF=Neutral Detergent Fiber; NS = Non Significant Difference. Symbols with different letters were significantly different among treatments by the least significant difference (LSD) method at the 5% level

There were not significantly effects of treatment on all attributes quality of forages in this research, in term of crude protein (CP), neutral detergent fiber (NDF) and Ash.

In general CP content of both species was also high enough for quality feeding above the minimum level required to fulfill the needs of functioning of rumen microbes (Van Soust, 1994). Even though, CP content of *B. humidicola* (11.17%) with bokashi-1 application was significant higher than*P. purpureum* (9.62%). More over DM content of both grasses was significant higher effected by bokashi-1 (21.46%) than bokashi-2 (18.90%). Growth attribute in term of plant height, number of tiller and ground cover has been measured (Table 2).

Data showed growth attributes of plant height did not different significantly on *P. purpureum* (Pp) except application of both type of bokashi at 20 tons $ha^{-1}$ plant height has significant higher compared to other levels, but among them bokashi-1 has significant higher of plant height than bokashi-2.

The effects of treatment on number of tiller of Pp grass in each plant sample or mother plant (MP) was significantly higher at 20 tons of both type of bokashi application than other levels, and again bokashi-1 showed significant higher number of tiller than the other type. Tiller number increase was consistent with seasonal changes in this attribute with the progression of cutting practice (Hasyim et al., 2014). There was no determine these variable to *Brachiaria* (Bh) since it has growth habits as prostrate species, but there was ground cover information of this species which is did not different significantly among treatments.

Table 2. Plant height and number of tiller of *P. purpureum* cv. Mott

| Variable | Treatments (bokashi tons.ha$^{-1}$) | Treatment | | | |
|---|---|---|---|---|---|
| | | Bokashi-1 | | Bokashi-2 | |
| | | Bh | Pp | Bh | Pp |
| Plant height (cm) | 5 | _ | 130$^{b}$ | -- | 120$^{c}$ |
| | 10 | _ | 130$^{b}$ | -- | 133$^{b}$ |
| | 20 | _ | 162$^{a}$ | -- | 150$^{a}$ |
| | Significant | * | | * | |
| | | 140.6$^{a}$ | | 134.3$^{b}$ | |
| Number of tiller (MP$^{-1}$) | 5 | _ | 19$^{c}$ | -- | 15$^{c}$ |
| | 10 | _ | 24$^{b}$ | -- | 19$^{b}$ |
| | 20 | _ | 35$^{a}$ | -- | 28$^{a}$ |
| | Significant | * | | | |
| | | 26.00$^{a}$ | | 20.67$^{b}$ | |
| Ground cover (%. M$^{-2}$) | 5 | 70 | _ | 73 | -- |
| | 10 | 75 | _ | 76 | -- |
| | 20 | 85 | _ | 80 | -- |
| | Significant | NS | | NS | |
| | | 76.66 | -- | 76.33 | |

Note: Bh= *Brachiaria humidicola* cv. Tully; Pp = *Pennisetum purpureum* cv. Mott; NDF=Neutral Detergent Fiber; NS = Non Significant Difference; MP = mother plant; M $^{-2}$ = meter square. Symbols with different letters were significantly different among treatments in each harvesting time by the least significant difference (LSD) method at the 5% level.

Table 3. Dry matter (DM) Crude protein (CP), Neutral Detergent Fiber (NDF) yield and predicted of carrying capacity (CC) of both grasses

| Variable | Treatments (bokashi tons ha$^{-1}$) | Treatment | | | |
|---|---|---|---|---|---|
| | | Bokashi-1 | | Bokashi-1 | |
| | | Bh | Pp | Bh | Pp |
| DM (tons ha$^{-1}$) | 5 | 3.15$^{b}$ | 14.11$^{b}$ | 2.95$^{b}$ | 13.41$^{b}$ |
| | 10 | 4.10$^{a}$ | 15.19$^{b}$ | 4.02$^{a}$ | 14.79$^{b}$ |
| | 20 | 4.25$^{a}$ | 17.75$^{a}$ | 4.15$^{a}$ | 17.05$^{a}$ |
| | Significant | * | * | * | * |
| | | 3.83 | 15.68 | 2.32 | 15.08 |
| | | 9.75 | | 8.70 | |
| CP (tons ha$^{-1}$) | 5 | 0.320 $^{c}$ | 1.017$^{c}$ | 0.310 $^{c}$ | 1.210$^{c}$ |
| | 10 | 0.361$^{b}$ | 1.235$^{b}$ | 0.343$^{b}$ | 1.305$^{b}$ |
| | 20 | 0.407$^{a}$ | 1.419$^{a}$ | 0.411$^{a}$ | 1.381$^{a}$ |
| | Significant | * | * | * | * |
| | | 0.363 | 1.224 | 0.355 | 1.298 |
| | | 0.793 | | 0.826 | |
| NDF (tons ha$^{-1}$) | 5 | 1.895$^{c}$ | 8.275$^{b}$ | 1.791$^{c}$ | 7.775$^{b}$ |
| | 10 | 2.031$^{b}$ | 8.316$^{b}$ | 1.931$^{b}$ | 8.216$^{b}$ |
| | 20 | 2.153$^{a}$ | 9.854$^{a}$ | 2.055$^{a}$ | 9.354$^{a}$ |
| | Significant | * | * | * | * |
| | | 2.026 | 8.815 | 1.925 | 8.448 |
| | | 5.421 | | 5.186 | |
| CC (head year$^{-1}$) | 5 | 5.09$^{b}$ | 18.31$^{c}$ | 4.89$^{b}$ | 17.31$^{c}$ |
| | 10 | 5.35$^{a}$ | 19.98$^{b}$ | 5.05$^{a}$ | 19.08$^{b}$ |
| | 20 | 5.47$^{a}$ | 24.34$^{a}$ | 5.23$^{a}$ | 23.39$^{a}$ |
| | Significant | * | * | * | * |
| | | 5.303 | 20.87 | 5.05 | 19.92 |
| | | 13.08 | | 12.48 | |

Note: Bh = *B. humidicola* cv. Tully; Pp = *P. purpureum* cv. Mott; NDF = Neutral Detergent Fiber; NS = Non Significant Difference; CC = carrying capacity. Symbols with different letters were significantly different among treatments by the least significant difference (LSD) method at the 5% level.

Presumably this species grown with aggressively root development after defoliation (Anis et al., 2011; Anis et al., 2015) and produced abundant tiller (Kaligis et al., 2013) and persist under free grazing (Kaligis et al., 2012). Yield and predicted of carrying capacity has been measured in this experiment (Table 3). Data showed both species of grasses which were received both type of *bokashi* application at 20 tons ha$^{-1}$ produced all variable measured yield of DM, CP, NDF and predicted CC significant higher compared to other level.

High yield was presumably achieved due to increased nutrient absorption capacity release from organic fertilizer (Utamy et al., 2018). Efficiencies used of nitrogen in soil by chemical compound released called *brachialactone* through root exudate of *B. humidicola* (Subarao et al., 2009) and of a high density of roots under regularly defoliated of this species (Anis et al., 2012; Anis et al., 2015) and the ability adaptation of *P.purpureum* (Utamy et al., 2011; Utamy et al., 2018) due to improved soil physical properties (Fukagawa et al., 2000) and continuous nutrient absorption from earlier manure input. (Farede et al., 2010)

## CONCLUSIONS

It could be concluded that both species of grasses response positively utilization of organic fertilizer bokashi, but in term of DM and CP content utilization of bokashi based on chicken manure fermented with effective micro-organisms EM4 was better than those of ruminant manure. Utilization of organic fertilizer in form of bokashi could provide forages to ruminant production integrated with coconuts plantation. By that way could be enhance economic value of this integrated systems.


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# WORLD WIDE USED TRADITIONAL MEDICINAL PLANTS AGAINST *Staphylococcus aureus* STRAINS. A REVIEW

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***Abstract***

*Staphylococcus aureus is an extraordinarily adaptable pathogen with a proven ability to develop resistance. It is notorious for its ability to become resistant to antibiotics. Infections that are caused by antibiotic-resistant strains often occur in epidemic waves that are initiated by one or a few successful clones. Also, it is a virulent pathogen that is currently the most common cause of infections in hospitalized patients. S. aureus infection can involve any organ system. The success of S. aureus as a pathogen and its ability to cause such a wide range of infections are the result of its extensive virulence factors. It is well-known that from ancient times the herbal world was the answer for many bacterial diseases. Throughout the years numerous investigations concerning the inhibition of S. aureus by spices, herbs, their extracts, essential oils and various constituents have been reported. Many of these plant extracts possess significant antimicrobial activity, which in many cases is due primarily to a particular constituent: polyphenols, flavonoids, alkaloids. Interpretation and results comparison of various studies is complicated by variations in the methodology used for the determination of antimicrobial activity.*

***Key words****: plant extracts, S. aureus, antimicrobial activity.*

## INTRODUCTION

Humans are dependent upon plants. Directly or indirectly, plants provide food, clothing, fuel, shelter, and many other necessities of life.

Ever since ancient times, in search for rescue for their disease, the people looked for drugs in nature (Petrovska, 2012). Nature has bestowed on us a very rich botanical wealth and a large number of diverse types of plants grow in different parts of the world.

Since time immemorial people have tried to find medications to alleviate pain and cure different illnesses. In every period, every successive century from the development of humankind and advanced civilizations, the healing properties of certain medicinal plants were identified, noted, and conveyed to the successive generations. The benefits of one society were passed on to another, which upgraded the old properties, discovered new ones, till present days. The continuous and perpetual people's interest in medicinal plants has brought about today's modern and sophisticated fashion of their processing and usage (Petrovska, 2012).

Approved by nature, extracted by science and confirmed by scientists, aromatic and medicinal plants have been used as traditional treatments for numerous human diseases for thousands of years and in many parts of the world. *Staphylococcus* is part of the human indigenous microflora and is carried asymptomatically in a number of body sites. Transmission from these sites causes both endemic and epidemic diseases. *Staphylococcus aureus*, a member of the *Staphylococcaceae* family, appears as Gram-positive cocci in clusters. *S. aureus* infection is a major cause of skin, soft-tissue, respiratory, bone, joint, and endovascular disorders. Many strains of *S. aureus* are developing resistance to available antibacterial

agents, creating a serious problem in medical microbiology. The ß-lactam antibiotics are the drugs of choice for the treatment of *S.aureus* infections. Resistance to ß-lactam compounds has been reported for methicillin, oxacillin, nafcillin, cloxacillin, and dicloxacillin. Methicillin resistant *S. aureus* (MRSA) infections can cause abroad range of symptoms depending on parts of the body that are infected. These may include surgical wounds, burns, catheter sites, eyes, skin and blood. Infections often result in redness, swelling and tender-ness at the site of infection and possibly progress to severe diseases. Methicillin resistance is most commonly mediated by the *mecA* gene, which encodes for a single additional penicillin binding protein, PBP2a, with low affinity for all ß-lactams. *S. aureus* is also resistant to other commonly used antimicrobial agents including aminoglycosides, macrolides, chloramphenicol, tetracycline, and fluoroquinolones.

Medicinal plants have been used as remedies for infectious diseases in many tropical countries, providing a rationale for investigating natural products for the treatment of MRSA infection.

The uncertainty evolving around micro- and macrolevel determinants influencing antimicrobial resistance makes long-term prediction challenging. Although simulation studies may provide guidance about short-term trends, long-term predictions about the future of antimicrobial resistance are fraught with difficulties, as shown by a look back in history. When the antimicrobial drug era began, scientists were impressed by the milestones of antimicrobial agent discovery and issued predictions about the future of antimicrobial resistance that seem overly optimistic today. (Harbarth and Samore, 2005).

This bibliographic study aims to present a brief review of the most important scientific findings about medicinal herbs that possess antimicrobial activity against *S. aureus* (MRSA).

The study is focused on the medicinal plants from four continents, two of them considered developed (Australia and North America) and others developing-countries (Africa, Asia) and it shows the interest for traditional medicine offered by nature itself.

## ANTIBACTERIAL CHARACTERISTICS OF ACTIVE COMPOUNDS FOUND IN PLANT EXTRACTS

The natural products derived from medicinal plants have proved to be an abundant source of biologically active compounds, many of them being the basis for the development of new chemicals for pharmaceuticals. With respect to diseases caused by microorganisms, the increasing resistance in many common pathogens to currently used therapeutic agents, such as antibiotics and antiviral agents, has led to renewed interest in the discovery of novel anti-infective compounds (Palombo and Semple, 2002).

In nature there are a large number of different types of antimicrobial compounds (phytoalexins) that play an important role in the natural defence of all kinds of living organisms. This research is focused on phenolic phytoalexins, such as e.g. flavonoids.

The flavonoids constitute a large group of secondary plant metabolites that are ubiquitous among higherplants.

They are polyphenolic compounds which generally occur as glycosylated derivatives. As dietary compounds, they are widely known antioxidants that inhibit the oxidation of low-density lipoproteins and reduce thrombotic tendencies (Hertog et al., 1993). Attention has also been paid to their antimicrobial activity, but no dramatic evidence of their effectiveness has been reported (Mori et al., 1987; Barnabas and Nagarajan, 1988; Tsuchiya et al., 1996; Rauha et al., 2000). Plant extracts have the ability to hamper the growth of a diverse range of pathogens because of the presence of natural compounds produced by the plant organs. The result of phytochemical screening revealed the presence of flavonoids and tannins in all extracts. Flavonoids and tannins have been reported to possess antimicrobial activity, the antimicrobial activity of flavonoids is due to their ability to complex with extracellular and soluble protein and to complex with bacterial cell wall while that of tannins may be related to their ability to inactivate microbial adhesions, enzymes and cell envelop proteins (Cowan, 1999).

When comparing data obtained in different studies, most publications provide

generalizations about whether or not a plant oil or extract possesses activity against Gram-positive and Gram-negative bacteria and fungi. However, not all of the studies provide details about the extent or spectrum of this activity.

Some publications also show the relative activity of plant oils and extracts by comparing results from different oils tested against the same organism(s).

Due to the large amount of extraction methods and techniques used by different authors in their studies, the comparation of the results is problematic, with previously published results is problematic. First, the composition of plant oils and extracts is known to vary according to local climatic and environmental conditions (Janssen et al. 1987; Sivropoulou et al., 1995). Furthermore, some oils with the same common name may be derived from different plant species (Windholz et al. 1983; Reynolds 1996; Hammer et al., 1999).

Secondly, the method used to assess antimicrobial activity, and the choice of test organism(s), varies between publications (Janssen et al., 1987).

A method frequently used to screen plant extracts for antimicrobial activity is the agar disc diffusion technique (Morris et al., 1979; Smith-Palmer et al., 1998; Yuniati et al., 2018; Chew et al., 2018).

The usefulness of this method is limited to the generation of preliminary, qualitative data only, as the hydrophobic nature of most essential oils and plant extracts prevents the uniform diffusion of these substances through the agar medium (Janssen et al., 1987; Rios et al., 1988). Agar and broth dilution methods are also commonly used.

The results obtained by each of these methods may differ as many factors vary between assays (Janssen et al., 1987; Hili et al., 1997).

These include differences in microbial growth, exposure of micro-organisms to plant oil, the solubility of oil or oil components, and the use and quantity of an emulsifier.

Table 1. Chemical composition of certain plant extracts and their antibacterial activity against *S. aureus* strains

**Africa**

| Herbs – plant origin (country) | Plant source | Extraction method | Active phytochemicals | *S.aureus* strains |
|---|---|---|---|---|
| **Nigeria** | | | | |
| (Okigbo & Mmeka, 2008)<br>*Vernonia amygdalina*<br>*Garcinia kola*<br>*Cymbopogon citratus* | <br>leaves<br>seeds<br>leaves | <br>ethanol<br>cold water<br>hot water | - not determined and specified | *S. aureus* |
| (Akinyemi et al., 2005)<br>*Terminalia avicennioides* Guill & Perr.<br>*Phylantus discoideus* Müll. Arg.<br>*Ocimum gratissimum* Linn.<br>*Acalypha wilkesiana* Müll. Arg. | <br>barks<br>barks<br>leaves<br>leaves | <br>ethanol<br>water | alkaloids, tannins, saponins, anthraquinone flavonoids, reducing and non-reducing carbohydrates | MRSA |
| (Aliyu et al., 2008)<br>*Acacia albida* Del.<br>*Anchomanes difformis* Engl.<br>*Boscia senegalensis* (PERS) Lam.<br>*Moringa oleifera* Lam.<br>*Momordica basalmina* Linn<br>*Pavetta crassipes* K. Schum.<br>*Phyllanthus amarus* Schumach & Thonn.<br>*Vernonia blumeoides* Hook. f. | <br>stem bark<br>roots<br>roots<br>leaves<br>whole plants<br>leaves<br>whole plants<br>aerial parts | <br><br><br><br>methanol<br>ethanol | <br><br><br><br>alkaloids, anthraquinone flavonoids, cardiac glycosides, tannins, saponins, | <br><br><br><br>MRSA |
| **South Africa** | | | | |
| (Eloff, 1998)<br>*Combretum molle* R. Br. ex G. Don (Combretaceae) | <br>leaves | acetone | not determined and not specified | *S. aureus* ATCC 29213 |
| (Aiyegoro et al., 2009)<br>*Helichrysum pedunculatum* | <br>leaves | methanol | not determined and not specified | *S. aureus* ATCC 6538 |

**Asia**

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|---|---|---|---|---|
| **China** | | | | |
| (Zuo et al., 2008)<br>*Anemone rivalry* Buch.-Ham.<br>*Biota orientalis* (L.) Endl.<br>*Conyza blinii* Levl.<br>*Dendrobenthamia capitata* (Wall.) Hutch.<br>*Dichrocephala chrysanthemifolia* (Bl.) DC.<br>*Duchesnea indica* (Arulr.) Forke<br>*Elsholtzia blanda* Benth.<br>*Elsholtzia rugulosa* Hemsl.<br>*Gaultheria yurmaneiisis* (Fr.) Rehd.<br>*Geranium strictipes* K. Kunth<br>*Keineckea carnea* (Andr.) Kunth.<br>*Physalis alkekengi* L.<br>*Polygonum multiflorum* Thunb.<br>*Potentilla fulgens* Wall.<br>*Rosa laevigata* Michx.<br>*Rubia cordifolia* L.<br>*Schizandra spaeraridra* Stapf.<br>*Senecio scandens* Buch.-Ham.<br>*Tetrastigma hypoglaucum* PI. | <br>rhizomes<br>aerial parts<br>aerial parts<br>aerial parts<br>whole plants<br>whole plants<br>aerial parts<br>whole plants<br>whole plants<br>roots<br>whole plants<br>fruits<br>rhizomes<br>fruits<br>rhizomes<br>whole plants<br>aerial parts<br>whole plants<br>roots | ethanol | not determined and not specified | MRSA |
| **Iran** | | | | |
| (Tohidpour et al., 2010)<br>*Thymus vulgaris*<br>*Eucalyptus globulus* | aerial parts | essential oils | thymol, ρ-Cymene, γ-Terpinene, Eucalyptol, (+) Spathulenol, α-Pinene, | MRSA ATCC 33592<br>*S. aureus* ATCC 25922<br>14 MRSA strains |
| (Mansouri, 2008)<br>*Menta viridis* L.<br>*Myrtus communis* L.<br>*Glycyrrhiza glabra* L.<br>*Eucalyptus globolus* Labill.<br>*Satureia hortensis* L.<br>*Teucrium polium* L.<br>*Achillea santolina* L. | <br>leaves<br>leaves<br>rhizomes<br>leaves<br>leaves<br>flowers<br>flowers | ethanol | not determined and not specified | *S. aureus* ATCC 25923<br>*S. aureus* ATCC 9144<br>*S. aureus* ATCC 29737<br>*S. aureus* ATCC 12596<br>*S. aureus* Bristol A 9596<br>489 *S. aureus* strains |
| **Palestine** | | | | |
| (Abu-Shanab et al., 2004)<br>*Syzyium aromaticum* (Myrtaceae)<br>*Cinnamomum cassia* (Lauraceae)<br>*Salvia officinalis* (Lamiaceaea)<br>*Thymus vulgaris* (Lamiaceaea)<br>*Rosmarinus officinalis* (Labiatae) | <br>seeds<br>barks<br>leaves<br>leaves<br>leaves | hot water<br>ethanol<br>methanol | not determined and not specified | MRSA |
| (G. Adwan & Mhanna, 2008)<br>*Psidium guajava*<br>*Rosmarinus officinalis*<br>*Salvia fruticose* | <br>leaves<br>leaves<br>leaves | | | |

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|---|---|---|---|---|
| *Majorana syriaca*<br>*Ocimum basilucum*<br>*Rosa damascene*<br>*Laurus nobilis*<br>*Syzygium aromaticum* | leaves<br>leaves<br>flowers<br>leaves<br>dried flowerbuds | hot water | not determined and not specified | 4 MSSA<br>MRSA |
| (Abu - Shanab et al. , 2006)<br>*Althaea officinalis*<br>*Mentha longifolia*<br>*Melissa officinalis*<br>*Rosa damascene* | aerial parts<br>aerial parts<br>aerial parts<br>flowers | hot water<br>ethanol | corilagin, tellimagrandin | MRSA |
| (Jarrar et al., 2010)<br>*Rosmarinus officinalis* | leaves | ethanol | flavonoids, phenolic acids (caffeic, chorogenic and rosmarinic), essencial oils (camphor and cineole), diterpenes (carnosol) | 5 MRSA strains<br>*S. aureus* ATCC 25923 |
| (Adwan et al., 2008)<br>*Rhus coriaria*<br>*Psidium guajava*<br>*Lawsonia inermis*<br>*Sacropoterium spinosum* | leaves<br>leaves<br>leaves<br>seeds | ethanol<br>water | not determined and not specified | 4 MRSA strains |
| **Thailand** | | | | |
| (Chomnawang et al., 2009)<br>*Barleria lupulina*<br>*Eupatorium odoratum*<br>*Garciniaman gostana*<br>*Hibiscus sabdariffa*<br>*Lawsonia inermis*<br>*Psidium guajava*<br>*Senna alata*<br>*Tagetes erecta* | fruit hulls | ethanol | α- mangostin | *S. aureus* ATCC 25923<br>MRSA strain |
| (Voravuthikunchai & Kitpipit, 2005)<br>*Acacia catechu*<br>*Garcinia mangostana*<br>*Impatiens balsamina*<br>*Peltophorum pterocarpum*<br>*Psidium guajava*<br>*Punica granatum*<br>*Quercus infectoria*<br>*Uncaria gambir*<br>*Walsura robusta* | not specified | water<br>ethanol | Tannins | *S. aureus* ATCC 25923<br>MRSA strain |
| **Turkey** | | | | |
| (Özkan et al., 2004)<br>*Rosa damascene* Mill. | flowers | methanol | phenolic compounds, essential oil | S. *aureus* Cowan 1 |
| (Erdoğrul, 2002)<br>*Artemisia absinthium* (Compositae/Asteraceae)<br>*Rosmarinus officinalis* L. (Labiatae/Lamiaceae) | whole plants<br>leaves | ethyl acetate<br>methanol<br>chloroform<br>acetone | essential oil: α-fenchene, β-myrcene, *endo*-bornyl acetate, and β-pinene<br>essential oil: α-pinene, borneol, 1,8-cineol, camphor, α-terpineol, camphene, and β-pinene | *S. aureus* ATCC 25923 |
| **Bangladesh** | | | | |
| (Alam et al., 2009)<br>*Swertia chirata* | leaves and stems | ethanol | flavonoids, xanthones, terpenoids, iridoid and secoiridoid glycosides | *S. aureus* |
| **India** | | | | |
| (Anas et al., 2008)<br>*Psidium guajava* Linn. | leaves | acetone<br>methanol<br>-water | Tannins | Multi drug resistant *S. aureus* |
| (Parekh & Chanda, 2008) | | | cellogenamide-a cyclic | |

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|---|---|---|---|---|
| *Celosia argentea* L.<br>*Vernonia anthelmintica* (L.) Willd.<br>*Balanites aegyptiaca* (L.) Del.<br>*Spathodea campanulata* Beauv.<br>*Cassia fistula* L.<br>*Beta vulgaris* L.<br>*Rourea santaloides* (Vahl.) Wight &. Arnott<br>*Cressa cretica* L.<br>*Lepidium sativum* L.<br>*Lagenaria vulgaris* Seringe<br>*Momordica charantia* L.<br>*Mukiamadera spatana* (L.) M. Roem.<br>*Cyperus scarious* R. Br.<br>*Cordia dichotoma* Forst.<br>*Ricinus communis* L.<br>*Arachis hypogaea* L.<br>*Vigna radiata* L.<br>*Fumaria indica* (Haussk.) Pugsley.<br>*Mesua ferra* Linn.<br>*Ocimum kilimanjaricum* L.<br>*Cinnamom umtamala* Nees & Ebern.<br>*Wood for diafruticosa* Kurz.<br>*Thespesia populnea* (L.) Sol ex Correa.<br>*Artocarpus hetrophyllus* Lam.<br>*Gardenia resinifera* Roth.<br>*Manilkara hexandra* (Roxb.) Dubard. | whole plants<br><br>whole plants<br>whole plants<br>aerial parts<br>leaves<br>leaves<br>roots<br>roots<br>seeds<br>fruits<br>fruits<br>aerial parts<br>seeds<br>leaves<br>leaves<br>leaves<br>whole plants<br>seeds<br>seeds<br>whole plants<br>leaves<br>flowers<br>leaves<br>whole plants<br>gum exudate<br>leaves | water<br>methanol<br>ethanol | peptide, phenols, flavonoids, resin, essential oil, saponin, argenic acid, mucilage, sugar,fatty acids, glucosides, phenols, tannins, anthraquinone derivatives, gluten, sugar, gum, betin, rourinoside, rouremin, alpha-tocopherol, ascorbic acid, benzyl-isothiocyanate, beta-sitosterol, iodine, niacin, linoleic acid, fixed oils, saponins, vitamins, minerals, 5-hydroxytryptamine, alkaloids, ascorbic acid, beta-carotene, citrulline, cryptoxanthine, diosgenin, lanoscharantin, cryptoxanthin, lutein, lycopene, momordicin, niacin, stigmasterol, zeaxanthin, zeinoxanthin, spinasterol, dihydrospinasterolglucoside, fatty acids, myristic,stearic acid, b-selinne, cyperenone, catharin, gum ash, ricin, ricin oil, palmitin, sterine, palmitic acid, oleic acid, protein, vitamin B1, B2, B6 and containslecithin, proteins, arachidic acid, arginine, ascorbic acid, genstein, shikimic acid, mesuanic acid, mesuaferol, mesuaferrone-A&B, β-sitosterol, xanthones, coumarins, methyl cinamate, camphor, eugenol, terpene, cinnamic aldehyde oil saffral, naturally acquired yeast microflora, gossypol, herbacetin, kaempferol, gyanomacloin, starch, ash fibre, resinous gum called dikamali, | *S. aureus* ATCC 25923 |
| (Aqil et al., 2005)<br>*Allium sativum(Liliaceae)*<br>*Camellia sinensis (Theaceae)*<br>*Citrus sinensis (Rutaceae)*<br>*Delonix regia (Leguminosae)*<br>*Holarrhena antidysenterica (Apocyanaceae)*<br>*Lawsonia inermis (Lythraceae)*<br>*Ocimum sanctum (Labiatae)*<br>*Punica granatum (Punicaceae)*<br>*Terminalia belerica (Combretaceae)*<br>*Terminalia chebula (Combretaceae)* | bulbs<br>leaves<br>rinds<br>flowers<br>barks<br>leaves<br>leaves<br>rinds<br>fruits<br>fruits | ethanol | phenols, glycosides, saponins, alkaloids, phenols, flavonoids | MRSA<br>MSSA |
| (Jahan et al., 2011)<br>*Syzygium cumini* (Jamun)<br>*Lawsonia inermis* (Mehndi)<br>*Zizyphus mauritiana* (Ber)<br>*Ocimum sanctum* (Tulsi)<br>*Ficus religiosa* (Peepal) | leaves | ethanol | flavanoids, tannins, alkaloids, anthocyanin, phenols, xanthoproteins, carboxylic acid, coumarins, sterols, saponins, glycosides | MRSA<br>MSSA |

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|---|---|---|---|---|
| (Duraipandiyan et al., 2006)<br>*Acalypha fruticosa* Forsskal Euphorbiaceae<br>*Albizia procera* Benth. Mimosaceae<br>*Cassia alata* L. Caesalpiniaceae<br>*Cassia auriculata* L. Caesalpiniaceae<br>*Cassia auriculata* L. Caesalpiniaceae<br>*Peltophorum pterocarpum* (DC.) Backorex. K. Heyne. Fabaceae<br>*Punica granatum* L. Punicaceae<br>*Syzygium cumini* Skeels. Myrtaceae<br>*Syzygium lineare* Wall. Myrtaceae<br>*Toddalia asiatica* Pers. Solanaceae | aerial parts<br>stem barks<br>leaves<br>leaves<br>flowers<br>flowers<br>roots<br>seeds<br>leaves<br>leaves | hexane<br>methanol | tannins, essential oils | *S. aureus*<br>ATCC<br>25923 |
| (Mehrotra et al., 2010)<br>*Emblica officinalis*<br>*Azadirachta indica*<br>*Aloe vera*<br>*Camellia sinensisassamica*<br>*Syzygium aromaticum* | fruits<br>leaves<br>leaves<br>leaves<br>buds | ethanol | not determined and not specified | MRSA |
| (Thosar et al., 2013)<br>*Melaleuca alternifolia*<br>*Lavandula officianalis L. angustifolia* or *L. vera*‑*Labiatae/Lamiaceae*<br>*Thymus* spp., *T. citriodorits, T. vulgaris*‑*Labiatae/Lamiaceae*<br>*Mentha piperita*‑*Lamiaceae/Labiatae*<br>*Eugenia caryophyllata* | whole plants<br>flowers<br>leaves +<br>flowers<br>leaves<br>buds, stems,<br>weeds | essential<br>oils | terpinen‑4‑ol, α‑terpineol and 1,8‑, monoterpens, oxides,linalyl, geranyle esters, geraniol, linalool, thymol and carvacrol with borneol, cineol, linalool, menthone, B‑cymene, pinene and triterpenic acid, monoterpenic alcohols-menthol, ketones-menthones, tannin complex, gum, resin, glucosides of sterols, eugenol (4‑allyl‑2‑methoxyphenol), acetyleugenol, gallic acid, sesquiterpenes, furfural, vanillin, methyl‑n‑amyl ketone, flavonoids, carbohydrates, lipids, oleanolicacid, rhamnetin and vitamins | *S. aureus*<br>ATCC<br>25923 |

**Australia**

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|---|---|---|---|---|
| (Hammer et al., 1999)<br>*Anibaros aeodora*<br>*Apium graveolens*<br>*Boswellia carterii*<br>*Cananga odorata*<br>*Cedrus atlantica*<br>*Citrus aurantifolia*<br>*Citrus aurantium*<br>*Citrus aurantium var. bergamia*<br>*Citrus limon*<br>*Citrus x paradisi*<br>*Citrus reticulate var.madurensis*<br>*Commiphora myrrha*<br>*Coriandrum sativum*<br>*Cucurbita pepo*<br>*Cupressus sempervirens*<br>*Cymbopogon citratus*<br>*Cymbopogon martini*<br>*Cymbopogon nardus*<br>*Daucus carota*<br>*Eucalyptus polybractea*<br>*Foeniculum vulgare* | woods<br>seeds<br>resins<br>flowers<br>woods<br>fruits<br>peels, leaves and twigs<br>peels<br>peels<br>peels<br>peels<br>resins<br>seeds<br>seeds<br>leaves and twigs<br>leaves<br>leaves<br>leaves<br>seeds<br>leaves and twigs<br>seeds | | | |

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| *Gaultheria procumbens* | herbs | | | |
| *Juniperus communis* | berries | | | |
| *Lavandula angustifolia* | flowers | essential | not determined | *S. aureus* |
| *Macadamia integrifolia* | nuts | oils | and not specified | NCTC |
| *Melaleuca alternifolia* | leaves and twigs | fixed oils | | 6571 |
| *Melaleuca cajuputi* | leaves and twigs | | | |
| *Melaleuca quinquenervia* | leaves and twigs | | | |
| *Mentha x piperita* | herbs | | | |
| *Mentha spicata* | herbs | | | |
| *Ocimum basilicum* | herbs | | | |
| *Oenotherabiennis* | seeds | | | |
| *Origanum majorana* | herbs | | | |
| *Origanum vulgare* | herbs | | | |
| *Pelargonium graveolens* | herbs | | | |
| *Pimpinella anisum* | seeds | | | |
| *Pimenta racemosa* | leaves | | | |
| *Pinus sylvestris* | needles | | | |
| *Piper nigrum* | berries | | | |
| *Pogostemon patchouli* | leaves | | | |
| *Prunus armeniaca* | seeds | | | |
| *Prunus dulcis* | seeds | | | |
| *Rosmarinus officinalis* | herbs | | | |
| *Salvia officinalis* | herbs | | | |
| *Salvia sclarea* | herbs | | | |
| *Santalum album* | woods | | | |
| *Syzygium aromaticum* | buds | | | |
| *Thymus vulgaris* | herbs | | | |
| *Vetiveria zizanioides* | leaves | | | |
| *Zingiber officinale* | rhizomes | | | |
| (Palombo & Semple, 2002) | | | | M67638 |
| *Amyema quandang* (Loranthaceae) | leaves | | | M67783 |
| *Eremophila alternifolia* (Myoporaceae) | leaves | | | M99320 |
| *Eremophila duttonii* (Myoporaceae) | leaves | ethanol | not determined | M173525 |
| *Lepidosperma viscidum* (Cyperaceae) | stem bases | | and not specified | M180920 |
| | | | | M183909 |
| | | | | *S. aureus* |
| | | | | ATCC |
| | | | | 12600 |

**North America**

| **Canada** | | | | |
|---|---|---|---|---|
| (Omar et al., 2000) | | 1. | 7. | 13. |
| *Acer rubrum* L. | | 2. | 8. | 14. |
| *Acer saccharum* L. | | 3. | 9. | 15. |
| *Betula papyrifera* Marsh. | | 4. | 10. | 16. |
| *Carya cordiformis* K. | | 5. | 11. | 17. |
| *Carya ovata* K. | | 6. | not determined | 18. |
| *Fagus grandifolia* Ehrh. | barks and woods | ethanol | and not specified | MSSA |
| *Juglans cinerea* L. | | | 12. | |
| *Prunus serotina* Ehrh. | | | | |
| *Populus* sp. | | | | |
| *Quercus rubra* L. | | | | |
| *Tilia americana* L. | | | | |
| *Ulmus americana* L. | | | | |
| (McCutcheon et al., 1994) | | | | |
| *Rhus glabra* | barks | | | |
| *Oplopa naxhorridum* | inner barks | | | |
| *Asarum caudatum* | whole plants | | | |
| *Mahonia aquifolium* | roots | | | |
| *Alnus rubra* | barks | | | |
| *Alnur rubra* | catkins | | | |
| *Betula papyrifera* | branches | | | |

| | | | | |
|---|---|---|---|---|
| *Lonicera ciliosa* | branches | | | |
| *Sambucus caerulea* | branches | | | |
| *Sambucus racemosa* ssp. *pubens* | barks | | | |
| *Symphoricarpos albus* var. *laevigatus* | branches | | | |
| *Achillea millefolium* ssp. *lanulosa* var. *lanulosa* | whole plants | | | |
| *Ambrosia chamissonis* | aerial parts | | | |
| *Antennario microphyila* | whole plants | methanol | not determined | MSSAP00017 |
| *Arnico sororia* | aerial parts | | and not specified | MRSAP00017 |
| *Artemisia ludoviciana* var. *latiloba* | aerial parts | | | |
| *Artemisia michauxiana* | aerial parts | | | |
| *Artemisia tridentata* ssp. *tridentata* | branches | | | |
| *BaIsamorhiza sagittata* | aerial parts | | | |
| *Balsamorhiza sagittata* | roots | | | |
| *Chaenactis douglasii* | whole plants | | | |
| *Chrysothamnus nauseous* var. *abicaulis* | branches | | | |
| *Erigeron filifolius* | aerial parts | | | |
| *Gailiardia aristata* | aerial parts | | | |
| *Conocephalum conicum* | thalluses | | | |
| *Cornus canadensis* | aerial parts | | | |
| *Capsella bursa-pastoris* | whole plants | | | |
| *Cardamine angulata* | roots | | | |
| *Juniperus communis* | branches | | | |
| *Empetrum nigrum* | branches | | | |
| *Arctostaphylosuva-ursi* | branches | | | |
| *Arctostaphylosuva-ursi* | roots | | | |
| *Kalmia microphylla* ssp. *occidentalis* | branches | | | |
| *Ledum groenlandicum* | branches | | | |
| *Moneses uniflora* | aerial parts | | | |
| *Monotropauniflora* | whole plants | | | |
| *Ribes sanguineum* | branches | | | |
| *Philadelphus lewisii* | branches | | | |
| *Hypericum perforatum* | aerial parts | | | |
| *Lupinussericeus* var. *sericeus* | aerial parts | | | |
| *Lycopodium clavatum* | branches | | | |
| *Fauria crista-galli* | aerial parts | | | |
| *Nuphar polysepalum* | roots | | | |
| *Nuphar polysepalum* | rhizomes | | | |
| *Epilobium minutum* | whole plants | | | |
| *Larix occidentalis* | branches | | | |
| *Pinuscontorta* var. *contorta* | branches | | | |
| *Pinus ponderosa* | branches | | | |
| *Plantago major* | whole plants | | | |
| *Eriogonum heracleoides* | aerial parts | | | |
| *Eriogonum heracleoides* | roots | | | |
| *Polystichum munitum* | rhizomes | | | |
| *Delphinium nuttallianum* var. *nuttallianum* | whole plants | | | |
| *Ceanothus velutinus* | branches | | | |
| *Amelanchieral nifolia* var. *humptulipensis* | branches | | | |
| *Aruncus sylvester* | branches | | | |
| *Crataegus douglasii* | branches | | | |
| *Fragaria chiloensis* | leaves | | | |
| *Fragaria vesca* | leaves | | | |
| *Geum macrophyllum* | roots | | | |
| *Holodiscus discolor* | branches | | | |
| *Potentilla arguta* | roots | | | |
| *Potentilla pacifica* | branches | | | |
| *Prunus virginiana* var. *demissa* | branches | | | |
| *Prunus virginiana* var. *virginiana* | branches | | | |
| *Rubus parviflorus* | branches | | | |
| *Spiraea pyramidata* | branches | | | |
| *Heuchera cylindrica* | roots | | | |
| *Penstemon fruticosus* | branches | | | |
| *Verbas cumthapsus* | leaves | | | |

| | | | | |
|---|---|---|---|---|
| *Glehnialittoralis* ssp. *leiocarpa*<br>*Heracleum lanatum*<br>*Heracleum lanatum*<br>*Lomattum dissectum* var. *multifidum*<br>*Osmorhiza purpurea* | roots<br>aerial parts<br>roots<br>roots<br>roots | | | |
| **United States of America** | | | | |
| (Frey & Meyers, 2010)<br>*Achillea millefolium*<br>*Hieracium pilosella*<br>*Ipomoea pandurata*<br>*Solida gocanadensis*<br>*Silene virginica* | flowers<br>flowers and leaves<br>flowers and leaves<br>leaves<br>leaves | water | not determined and not specified | *S. aureus* [Presque Isle No.4651] |

## NEW ERA: NEW SYNTHETIC DRUGS, MORE RESISTANT BACTERIAL STRAINS

While the intense selective pressure of antimicrobial drug use has been an important factor in the emergence of resistance, the inconsistent application of infection control guidelines by hospital personnel largely accounts for the dissemination of resistance in the healthcare setting.

Infection control measures to limit the spread of antimicrobial resistance are being increasingly well defined. Despite the increase in the prevalence of resistance of several important pathogens, there has been some success in controlling its clinical impact. Several countries have recently reported a stabilization or decrease in infection rates due to multidrug-resistant *Staphylococcus aureus* (Schrijnemakers et al., 2004).

Novel anti-MRSA modalities of plant antimicrobials such as alteration in efflux pump, inhibition of pyruvate kinase, and disturbance of quorum sensing in MRSA are also summarized which may be promising alternatives to antibacterial drug development in future(Li et al., 2018).

MRSA, a virulent and difficult-to-treat "superbug," can optimize its gene content and expression to create new strains with augmented virulence and colonization capabilities. Being an extraordinarily adaptable pathogen with the proven ability to develop resistance, MRSA is considered an urgent threat to public health (Lakhundi and Zhang, 2018).

## CONCLUSIONS

Herbal plants are an important source of new chemical substances with medicinal potential uses.

The increased interest on plant medicines in today's world is from the belief that green medicine is safe and dependable, compared with costly synthetic chemicals that have different adverse effects (Nair and Chanda, 2006).

The present study suggests that plant extracts certainly possess some chemical constituents with antimicrobial properties and these findings are very important in discovering new drugs for the therapy of infectious diseases.

However, further studies are required to isolate and characterize the active constituents responsible for the antimicrobial property of all the plants studied.

So far plants could be the ideal potential sources to explore novel antibacterial drugs even against antibiotic-resistant bacterial strains (Davidson, 2001; Ceylan and Fung, 2004; Tayel et al., 2018).

Also, the resurgence of interest in natural therapies and increasing consumer demand for effective and safe natural products, meaning that quantitative data on plant oils and extracts are required.

In summary, this study confirms that many essential oils and plant extracts possess *in vitro* antibacterial activity against *S. aureus*. However, if plant oils and extracts will be used for food preservation or medicinal purposes, safety and toxicity studies both *in vitro* and *in vivo*, must be made.

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# STUDY REGARDING THE QUALITY OF MILK FROM COWS REARED ON THE REDIU FARM

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***Abstract***

*Assuring of raw material with superior quality, have particular importance because on this depends the obtaining of superior quality products as well as a realization of a superior capitalization index of raw material. Qualitative reception of milk was daily effectuated, through three control periods, as follows: 1st period: 23.12.2017 – 6.01.2018, 2nd period: 24.02 – 10.03.2018, 3rd period: 7 – 21.04.2018; gathering samples on which were determined: fat, protein titer, acidity, density as well as somatic cells. Statistical analysis of the main physical-chemical features show differences between those three control periods regarding milk fat content, modifications which reflected also on density. So, milk gathered in the first period recorded a mean value of 4.23±0.02%. For milk gathered in the second period, fat content had a lower value (4.03±0.03%) due to a change in cows' nourishment from preserved fodders to green ones. Milk analyzed in the third period, suffered some qualitative and quantitative modifications; animals recorded a higher milk production in comparison with the winter season, but fat content was lower. Generally, we could say that milk obtained at Rediu Farm, was in accord with the norms stipulated into quality standards.*

***Key words****: milk, quality, fat content, minerals.*

## INTRODUCTION

Success in dairy cows farming due to the multiple factors related to animal husbandry technologies, company management and hygienic and veterinary prophylactic measures (Carlsson et. al., 1995).

Certain main objectives are to be followed in cows farming, such as: better exteriorizing of animals' genetic potential into the main product, maximizing the cows' productive longevity, turning their yield into efficient production, improving the nutritional quality of the milk and decreasing the production costs (Heck et. al., 2009).

Milk is a natural product with a complex chemical composition, secreted in the mammary gland on the basis of carbohydrates, proteins, vitamins, and minerals extracted from the circulating blood and converted into specific milk nutrients by the cells in the udder epithelium. (Pereira, 2014).

According to Codex Alimentarius (CODEX STAN, 1999), milk is defined as the product secreted by the mammary gland of one or more healthy, well rested and appropriately fed cows, obtained through a complete and hygienic milking.

Nutritionally, milk is considered one of the most important food matters in rational human nutrition, due to its complex chemical composition and to its biological value (Matte et. al., 2014).

Milk chemical composition could be influenced by both genetic and environmental factors (Miller et. al., 1970; Ujică and Maciuc, 2000; Bernabucci et. al., 2002).

Most of the dry matter in milk is represented by nitrogenous substances, most of them (95%) being proteins and 5% being non-protean nitrogenous compounds (Bille et. al., 2009; Harding, 1995).

The milk protein comprises casein (70 -80%) and serum (whey) proteins, such as lactalbumin and lactoglobulin (3.5% of the whole nitrogen in milk and 12% of that in colostrum) (Harding, 1995). The milk also comprises proteo-peptones (4 - 5%), such as creatine, creatinine,

urea, uric acid, and guanidine. These ones originate in blood and are part of the lipid goblets membranes in milk, as glycoproteins (Imran et. al., 2008; Ozrenk and Inci, 2008).
The fat in milk is synthesized in the mammary gland and is the milk compound presenting the highest variability as the proportion (3 – 5.4%) (Amitot et. al., 2002; Michalski et. al., 2005).
Lactose is the main milk carbohydrate, also synthesized by the mammary epithelium, starting from blood originating glucose (Norberg, 2005).
Most of the minerals in milk are found up to 0.7% and contain chlorides, phosphates and calcium citrates (Kittivachra, 2007).
The Ca/P ratio is quite relevant technologically because it interacts directly with the milk coagulation behavior (Blewu and Aiyegbusi, 2004).
Milk enzymes have an endogenous origin (bloodstream) (Andrews, 1992; Brew, 2003; Calore and Vingola, 2002).
Vitamins content is also important in milk, especially for the newborn and is strongly influenced by the diet of the lactating female (Schrodes, 1982; Florence, 2010).
Providing high-quality milk, as raw matter in the food industry is essential in order to guaranty the manufacturing of products presenting superior quality and high conversion efficiency (Borkova and Snaselova, 2005).

## MATERIALS AND METHODS

Qualitative reception of raw matter milk was organized daily throughout three control timeframes: period I, 23.12.2017 - 6.01.2018; period II, 24.02 - 10.03.2018; period III, 7 - 21.04.2018.
Daily analytical assessments were carried on the sampled milk, in order to measure the raw matter density (g/cm3), acidity (˚T), total lipids (%), total proteins (%) and casein content (%).
By the end of each control period the samples were investigated for their content in crude ash (total minerals) and of certain subsequent macro elements such as Ca (mg/L), Mg (mg/L), Na (mg/L) and K (mg/L).
Milk density was measured using a thermolactodensitometer. This physical trait represents the ratio between the milk mass at +20˚C and the mass of the same water volume at a +4˚C temperature (SR 143:2008; SR 2418:2008).
Milk acidity was assessed via the Thörner method – neutralizing of organic acids with NaOH (0.1N) titration, using phenolphthalein as witness pigment (SR 2418:2008; SR 143:2008).
Total lipids content was quantified by the acid-butyrometric Gerber method (digestion of milk proteins with sulfuric acid followed by separation of lipids via centrifugation, under the influence of isoamyl alcohol and 65˚C temperature) (ISO 2446:2009; ISO 3433:2009 STAS 6352-1:1988).
Total protein content was measured through the Schúrltz titrimetric method: milk treatment with formaldehyde that locks the protein amino groups, followed by NaOH (0.143N) titration of the free carboxyl groups resulting in a direct value of protein percentage (Merliță et. al., 2018; Rațu et al., 2017).
Crude ash content was assessed via incinerating at 550ºC, in a Super Therm C311 oven after prior combustion with a Bunsen funnel, until samples ceased to smoke according to AOAC, No. 945.46 (2005).
The macro element's contents - Ca, Mg, Na and K - were quantified via atomic absorption spectrometry. The Atomic Absorption Spectroscopy (AAS) is an analytic technique widely used in research studies, which aim to determine inorganic ions in solution. The determination is both qualitative and quantitative. This method is based on the quantification of the energy released by an atom when it passes from an excited state to the ground state (Carroll et al., 2006; Summer et al., 2009).
Collected data were subjected to statistical computation, using the ANOVA one-way algorithm included in MsExcel, to calculate the descriptive statistics (mean, standard error) and find out whether there were significant differences and upgraded with PostHoc Daniel's XL Toolbox version 4.01 (http://xltoolbox.sf.net), to identify the differences (Radu-Rusu et. al., 2014).

## RESULTS AND DISCUSSIONS

Certain differences with different degrees of statistical significance were found between the

average values of the physical and chemical milk traits measured in the three control periods. Thus, for the total lipids content, the average value was 4.26±0.02% in the first period ($P_1$), 4.03±0.03% in the second period ($P_2$) and 3.91±0.02% in the third period ($P_3$). Highly significant differences were identified for all three comparisons. The $P_1$ vs $P_2$and $P_2$ vs $P_3$ comparisons were found statistically significant ($P < 0.05$), while the P1 vs. P3 comparison was found as highly significant ($P < 0.001$) (Table 1). According to the quality standards, the milk fat content should not be less than 3.2%. All the samples analyzed by us, surpassing this concentration (Figure 1).

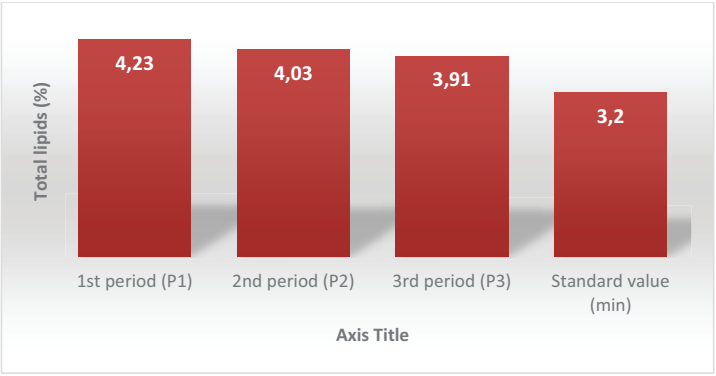


Figure 1. Total lipids content (%)

Milk titratable acidity reached 17.68±0.09˚T in P1 samples, 18.39±0.08˚T in P2 ones and 18.66±0.09˚T in P3 milk, with no statistical differences occurring between the three moments of analysis.

The total proteins assessment did not reveal statistical significance between the samples, while the average values reached 3.37±0.03% in P1, 3.34±0.02% in P2 and 3.31±0.02% in P3 samples (Table 1).

Table 1. Means (± SD) for the chemical composition of raw milk

| Quality parameters | 1st period (P1) | 2nd period (P2) | 3rd period (P3) | ANOVA computation and analysis | | |
|---|---|---|---|---|---|---|
| | | | | Compared period | P value | Significance |
| Total lipids (%) | 4.23±0.02 | 4.03±0.03 | 3.91±0.02 | P1 vs.P2 | 0.0024 | ***(P<0.001) |
| | | | | P1 vs. P3 | 0.0029 | ***(P<0.001) |
| | | | | P2 vs. P3 | 0.0022 | ***(P<0.001) |
| Density ($g/cm^3$) | 1.0299±0.0001 | 1.0287±0.0001 | 1.0282±0.0001 | P1 vs.P2 | 0.0153 | *(P <0.05) |
| | | | | P1 vs. P3 | 0.0003 | ***(P<0.001) |
| | | | | P2 vs. P3 | 0.0192 | *(P <0.05) |
| Acidity (˚T) | 17.68±0.09 | 18.39±0.08 | 18.66±0.09 | P1 vs.P2 | 0.4480 | ns (P >0.05) |
| | | | | P1 vs. P3 | 0.2386 | ns (P >0.05) |
| | | | | P2 vs. P3 | 0.6313 | ns (P >0.05) |
| Total proteins (%) | 3.37±0.03 | 3.34±0.02 | 3.31±0.02 | P1 vs.P2 | 0.4908 | ns (P >0.05) |
| | | | | P1 vs. P3 | 0.0914 | ns (P >0.05) |
| | | | | P2 vs. P3 | 0.2867 | ns (P >0.05) |

ANOVA within rows: ns=not significant ($P > 0.05$); *=significant($0.01 < P < 0.05$), **=distinguished significant ($0.001 < P < 0.01$); ***=highly significant ($P < 0.001$)

The analysis of the crude ash content indicated 0.722±0.005% in P1 samples, 0.726±0.001% in P2 and 0.734±0.007% in P3, while the differences between these average values did not pass the statistical significance threshold.

Compared to blood, milk contains more K, Ca and P, and less Na and Cl, due to the Na-K pump that regulates osmotic pressure between the cytoplasm of blood cells and cytoplasm of epithelial cells that secrete milk. At the same

time, Ca is transported from the basal membrane to cytosol and onward into the Golgi apparatus of the alveolar cells in the mammary glands to be incorporated into casein micelles (Paulina and Bencini, 2004).

Serial dilutions were made from total ash in order to quantify the major mineral elements in milk.

The Ca content reached 1.194±0.001 mg/L in P1, 1.193±0.001 mg/L in P2 and 1.193±0.001 mg/L in P3 (Table 2) samples.

Calcium is named also "the mineral of milk" (Cashman, 2006) and close values to our findings were measured by Soliman (2005), which reported a Ca content of 1.19 mg/L, while Zamberlin et al. (2012) identified 1.07 – 1.33 mg Ca/L cow milk.

In milk, all of these macro-elements are distributed differently into soluble and insoluble fractions (essentially casein micelles). Potassium, sodium, and chloride ions are essentially soluble, while calcium, inorganic phosphate, and magnesium are partly bound to the casein micelles, therefore mostly insoluble (Guancheron, 2012).

The Mg content reached 115.8±0.374 mg/L in P1 samples, 116±0.707mg/L in P2 samples and 116.4±0.509mg/L in P3 samples.

Magnesium is a ubiquitous food mineral. Milk is a good source of Mg with an average content of 117 mg/L (De Marchi et. al., 2014).

The average Na content was of 529.6±0.50 mg/L in P1, 528.8±0.374 mg/L in P2 and 528.2±0.663mg/L in P3 milk samples.

Sodium is a monovalent cation mainly located in extracellular fluids.

If compared to other major minerals, its concentration in bovine milk is relatively low, with an average of 531 mg/L (De Marchi et. al., 2014).

Table 2. The mineral content of milk

| Quality parameters | 1st period (P1) | 2nd period (P2) | 3rd period (P3) | ANOVA computation and analysis | | |
|---|---|---|---|---|---|---|
| | | | | Compared period | P value | Significance |
| Crude ash (%) | 0.722±0.005 | 0.726±0.001 | 0.734±0.007 | P1 vs.P2 | 0.6195 | ns (P >0.05) |
| | | | | P1 vs. P3 | 0.2165 | ns (P >0.05) |
| | | | | P2 vs. P3 | 0.3773 | ns (P >0.05) |
| Calcium (Ca) (mg/L) | 1.194±0.001 | 1.193±0.001 | 1.193±0.001 | P1 vs.P2 | 0.7690 | ns (P >0.05) |
| | | | | P1 vs. P3 | 0.6181 | ns (P >0.05) |
| | | | | P2 vs. P3 | 0.8066 | ns (P >0.05) |
| Magnesium (Mg) (mg/L) | 115.8±0.374 | 116.1±0.707 | 116.4±0.509 | P1 vs.P2 | 0.8088 | ns (P >0.05) |
| | | | | P1 vs. P3 | 0.3705 | ns (P >0.05) |
| | | | | P2 vs. P3 | 0.6585 | ns (P >0.05) |
| Sodium (Na) (mg/L) | 529.6±0.509 | 528.8±0.374 | 528.2±0.663 | P1 vs.P2 | 0.2415 | ns (P >0.05) |
| | | | | P1 vs. P3 | 0.1328 | ns (P >0.05) |
| | | | | P2 vs. P3 | 0.4534 | ns (P >0.05) |
| Potassium (K) (mg/L) | 1.539±0.003 | 1.540±0.003 | 1.539±0.005 | P1 vs.P2 | 0.6938 | ns (P >0.05) |
| | | | | P1 vs. P3 | 0.6665 | ns (P >0.05) |
| | | | | P2 vs. P3 | 0.3465 | ns (P >0.05) |

ANOVA within rows: ns=not significant (P > 0.05); *=significant(0.01 <P <0.05), **=distinguished significant (0.001 < P<0.01); ***=highly significant (P <0.001)

The average K content reached 1.539±0.003 mg/L in the milk collected in P1, 1.540±0.003 mg/L in P2 milk and 1.539±0.005 mg/L in P3 samples (Table 2).

Potassium is one of the most important intracellular cations, but in a lower concentration is present also in the extracellular fluids. Potassium is found in cow milk, mainly in the aqueous phase, with an average concentration of 1.550 mg/L (De Marchi et al., 2014).

All the differences concerning the contents of the macro element in the milk sampled in the three control moments were not statistically (P >0.05) significant (Table 2).

## CONCLUSIONS

The analyses run in our study revealed that the proximate composition of milk modifies upon season. The most significant differences

occurred for total lipids content and for milk density.

So, for example, the milk collected in the 1st period (P1) has the highest lipid content (4.25±0.02%). The milk collected in the 2nd period recorded a fat content was less than 0.2% compared to that of P1. For the milk collected in the 3rd period, the fat content was less than 0.32% compared to that of P1 and 0.12 compared to that of P2.

Acidity and density of analyzed milk were also inside the values indicated by SR 2418.

Despite the fact that the crude ash content and the individual analyzed macro-elements contents were different between the control moments, they did not differ significantly from the statistical point of view, hence the lack of seasonality influence on these quality traits.

So, for calcium content, the mean obtained by us had values between 1.193–1.194 mg/L. Magnesium content was 115.8±0.374 mg/L for milk collected in the 1st period, 116±0.707 mg/L for the for milk collected in the 2nd period and 116.4±0.509 mg/L for milk collected in the 3rd period. Content in sodium varied between 528.2±0.663 mg/L and 529.6±0.509 mg/L.

The potassium content varied between 1.539±0.003 mg/L and 1.540±0.003 mg/L.

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# JAPANESE KNOTWEED (*FALLOPIA JAPONICA*): LANDSCAPE INVASIVE PLANT VERSUS HIGH QUALITY HONEY SOURCE

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***Abstract***

*Scientific studies show that the darker the honey, the higher its bio-active properties are. Fallopia japonica (Japanese knotweed) is an invasive plant, growing shrub reaching heights of 3 m. Flowering occurs in late summer, when small, greenish-white flowers develop in long panicles in the axils of the leaves. Plants are dioeciously with flowers intensely visited by the bees, the honey obtained from nectar of this plant being a mild-flavored version of buckwheat honey; dark in color, appearing dark red when held to light. The present study aim to determine the chemical composition of Japanese knotweed honey and also their bioactive properties derived from the chemical composition. Different physico-chemical, gravimetric, spectrophotometric and chromatographic determinations were used in the study. Analyzed honey samples are very good sources of minerals, especially K and Na (1187-6196 mg/kg K and 58.8-68.8 mg/kg Na). Also high amounts of Ca were determined. High amounts of total polyphenols and flavonoids were determined in Japanese knotweed honey samples, from several western places of Romania. This could reduce the impact that Fallopia japonica invasive plant have on the habitat, and if this plant is kept under observation and far from the inhabited areas, it could be highly valuable for beekeepers and honey production.*

***Key words****: Fallopia japonica, plant, honey, chemical composition, antioxidant effect.*

## INTRODUCTION

Japanese knotweed (*Fallopia japonica* Houtt., *Reynoutria* spp.), is one of the most troublesome exotic species in Europe and elsewhere (Saintenoy-Simon, 2003; Weber, 2003; Muller, 2004). It has been included in the list of the "100 of the World's Worst Invasive Alien Species" (ISSG). Originating from East Asia, it was introduced in Europe at the end of the 19th century and is now found in many regions and countries. *F. japonica* is a shrub-like rhizomatous geophyte and thus belongs to a functional type not represented in the native vegetation. In Europe, the reproduction of *F. japonica* is only vegetative. Fragments of rhizomes and stems easily re-sprout and can be carried by streams or animals (Weber 2003). The main dispersion agent is human activity through the movement of topsoil containing plant fragments (Dassonville et al., 2007, Fennel et al., 2018).

Knotweeds are perennial herbs with long branched rhizomes, multiple high erect stems and large leaves with ovate or broadly elliptic blade. Inflorescences are axillary or terminal with small white-yellowish flowers (Figure 1). Flowering occurs in late summer, when the flowers develop in long panicles in the axils of the leaves. Plants are dioeciously with flowers intensely visited by the bees. Flowers are functionally monosexual, male with long stamens and short pistils, female with short stamens and distinct pistils (Patocka et al., 2017). The management of this species is very difficult and most of the time not successful. *F. japonica* has been extensively studied. Published studies concern its past and present distribution (Pysek et al., 2001; Mandák et al., 2004), possible impact of climate change on its future distribution (Beerling et al., 1995), genetic diversity (Hollingsworth & Bailey, 2000), impacts on native plant and animal communities (Maertz et al., 2005) and management (Child et al., 2001).

The invasive, strong root system may damage concrete foundations, buildings, roads, paving and architectural sites.

In Romania, this plant grows mainly on the riverbanks of western part of the country, but

also in other places in Transilvania (Dumitraşcu et al., 2012, 2014).
Scientific studies show that the darker the honey, the higher its bio-active properties are. Honey obtained from nectar of this plant being a mild-flavoured version of buckwheat honey, is dark in colour (Figure 1), appearing dark red when held to light. The present study aim to determine the chemical composition of Japanese knotweed honey and their bioactive properties derived from the chemical composition.

Figure 1. *Fallopia japonica* invasive plant and honey

## MATERIALS AND METHODS

*Honey samples. Fallopia japonica* declared honeys (three samples) from the western part of Romania were collected from beekeepers. The botanical origin of honey samples was determined by pollen and physico-chemical analysis. Each honey sample was kept in closed containers in the dark at 4$^{o}$C until analysis and testing.
*Honey Analysis.* Honey analysis were carried out in the Laboratory for Quality Control of Bee Products and Bee Diseases in the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. The physicochemical parameters were determined according to Romanian and International Legislation for honey or food ingredients, following standard methods or original methods developed in the lab. Palinological analysis is used for botanical origin determination (Louvreaux et al. 1978). Sugar profile was determined by high performance liquid chromatography (HPLC) with refractive index detection, following the method described in International Honey Commision Methods (Bonta et al., 2008). For the quantification of main sugars, a calibration curve in the range 4–0.5 g/100 g, with regression coefficient of $R^2$=0.9982 for a mixture of 9 standards (glucose, fructose, saccharose, trehalose, maltose, turanose, isomaltose, erlose, melezitose) was used. Results were expressed in g/100g honey. Water and HMF content, electrical conductivity and diastazic index, were determined following the methods from International Honey Commission, methods also validated in APHIS Laboratory, USAMV Cluj-Napoca (Bobiş et al., 2010; Cimpoiu, et al., 2013). The content of total lipids was determined using Soxhlet method (Soxtherm, Gerhardt, Germany), with an adapted method from literature (Almeida Muradian et al., 2005). Protein content was determined by Kjeldahl digestion , distillation and titration, following an adapted method of Lujerdean and Varga (2002). Total Phenolic Content (TPC) in extracts was determined according to the Folin-Ciocâlteu procedure described by Singleton et al. 1999 with some modifications. Briefly, an aliquot of 25 µL honey solution was mixed with 125 µL Folin Ciocâlteu 0.2N and incubated at room temperature for 5 min. Next, 100 µL of sodium carbonate solution (75g/L) was added and allowed to stand for 2 h at room temperature in the dark. The absorbance of the reaction mixture was read at 760 nm using multichannel spectrophotometer (model Sinergy 2 Biotek). A methanolic gallic acid solution (0.001- 0.15 mg/mL) was used for calibration curve. The $AlCl_3$ method was used for estimation of flavone/flavonol content of the samples (Meda et al., 2005). 150 µL of honey solution was mixed with the same amount of 2% $AlCl_3$ methanolic solution. The mixture was shaken and after 10 min of incubation, absorbance was read at 415 nm in the Sinergy 2 Biotek spectrophotometer. Flavonoid content was calculated from the calibration curve of quercetin standard and expressed as mg quercetin equivalents/g sample. Different essential and trace elements such as Ni, Cr, Fe, Mg, Ca, Mn, Pb, Na, Cd and K in honey samples were analysed using atomic absorption spectrophotometer (Aanalyst 800, CromatecPlus, U.S.A) equipped with graphite furnace. The inert argon gas flow and the temperature parameters were followed as recommended by the manufacturer. The absorption wavelength for determination of each element together with its linear working

range and correlation coefficient of calibration graphs are given in Table 1.

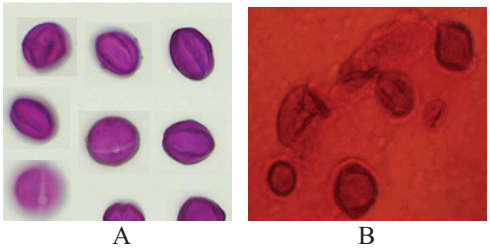
Tab.1. Operating parameters for working elements

| Elements flow | Wavelength (nm) | Slit coefficient (nm) | Width correlation (mA) |
|---|---|---|---|
| Fe | 248.3 | 0.2 | 0.9999 |
| Mg | 285.2 | 0.7 | 0.9988 |
| Ca | 422.7 | 0.7 | 0.9984 |
| Na | 589.0 | 0.2 | 0.9990 |
| K | 766.5 | 0.7 | 0.9967 |
| Pb | 283.3 | 0.7 | 0.9923 |

*Statistical analysis*. All data are expressed as mean from three replicates of every sample. Results were analyzed using Statistical Package for Windows®. Differences between samples are tested by one-way ANOVA. P values of <0.05 were considered significant.

## RESULTS AND DISCUSSIONS

The results of the melissopalynological analyses of honeys declared as Japanese knotweed honey show that the specific pollen was present but not overrepresented (Figure 2).



A B

Fig. 2. Fallopia pollen: A- http://pollen.tstebler.ch; B-knotweed honey pollen

*Fallopia japonica* plant belongs to Polygonaceae family and honey obtained from the nectar of this plant has similar characteristics as buckwheat honey (Panseri et al., 2013). It is known that buckwheat honey is dark in colour; possess a strong taste due to the volatile profile and very good biological characteristics (antioxidant, antibacterial).

Sensory determination of analysed samples show a sweet aromatic honey, dark brown in colour (Figure 1), crystallized with fine uniform crystals. The aroma is different from any other flower honey and different of buckwheat honey (even the plants belong to the same family).

Main chemical composition parameters and bioactive molecules for the analysed samples are presented in Table 2. They are in accordance with the values stated by existing standards or literature studies.

Tab.2. Analyzed chemical parameters of honey samples.

| Parameter | Sample 1 | Sample 2 | Sample 3 |
|---|---|---|---|
| Water content (%) | 18.9 | 20.0 | 15.6 |
| Electrical conductivity(mS/cm) | 0.697 | 0.647 | 0.541 |
| Diastazic index (DN) | 13.34 | 11.52 | 18.74 |
| HMF content (mg/kg) | 9.93 | 0.29 | 2.54 |
| Fructose (%) | 39.83 | 38.91 | 38.90 |
| Glucose (%) | 31.48 | 29.30 | 35.24 |
| Sucrose (%) | 0.29 | 3.18 | 0.67 |
| Turanose (%) | 1.29 | 1.08 | 0.74 |
| Maltose (%) | 1.96 | 1.36 | 2.18 |
| Trehalose (%) | 0.44 | 0.27 | 0.34 |
| Erlose (%) | 0.13 | 0.22 | 0.11 |
| Na (mg/kg) | 64.62 | 58.81 | 68.88 |
| Mg (mg/kg) | 15.74 | 16.27 | 4.86 |
| Ca (mg/kg) | 28.77 | 46.09 | 41.84 |
| Fe (mg/kg) | 3.68 | 2.07 | 3.09 |
| K (mg/kg) | 1187.36 | 1414.56 | 6196.83 |
| Total nitrogen (%) | 1.05 | 0.47 | 0.64 |
| Total lipids (%) | 0.12 | 0.52 | 0.41 |
| Total polyohenols (mgGAE/100g) | 195.0 | 100.0 | 145.0 |
| Total flavonoids (mgQe/100g) | 55.0 | 20.0 | 35.0 |

Water content ranged between 15.6 and 20%, being in the limits of standard. Although Japanese knotweed honey is dark in colour, its electrical conductivity is at the lower limit of the standard for honeydew honey (0.600 mS/cm) (Bogdanov et al., 1997). High diastazic index and low HMF content show an authentic, fresh honey. The main sugars present in the samples were fructose and glucose, with the fructose content higher that glucose (F/G ratio in the analysed samples is higher than 1.2, denoting that honeys remain fluid for a long period of time). The sucrose content is below the upper limit of the standard (5%). In the samples other di and trisaccharides were present, namely turanose (0.74 – 1.29%), maltose (1.36 – 2.18%), trehalose (0.27 – 0.44%), erlose (0.11 – 0.22%).

Lipid content of honey came from the residual pollen present in the sediment. Lipid content of the analysed samples ranged between 0.12 – 0.52%, which is high for honey. The same situation was observed for nitrogen content expressed as total proteins: 0.47 – 1.05%. These parameters make Japanese knotweed honey an important source of protein and amino acids and give this type of honey a high nutritional value.

Mineral content in honey is related to the geographical origin, with the presence of

specific minerals in the soil where the nectar plants are growing. These substances are of nutritional and health importance. Some of the minerals found in honey include calcium, copper, iron, magnesium, manganese, phosphorus, potassium and zinc. Their amounts are different, with geographical and botanical origin of honeys. Five minerals were determined in the honey samples, and the highest mineral present is by far potassium (118.7 – 619.7 mg/100 g). This amount is very high compared to literature (Tuzen et al., 2007; Alvarez Suarez et al., 2012; Ajibola et al., 2012; Solayman et al., 2015). Also high amounts of calcium and sodium were found (2.87 – 4.61 mg/100 g and 5.88 – 6.88 mg/100 g respectively).

High amounts of total phenolics were determined by means of Folin Ciocâlteu method: 100 – 195 mg GAE/100 g honey as well as flavones/flavonols: 20 – 55 mg QE/100 g. These amounts are higher than those found in honeydew honeys and other dark colour honeys (Gheldorf et al., 2002; Yao et al., 2003; Meda et al., 2005; Lachman et al., 2010).

Even for buckwheat honey, Kaškoniene et al. (2009) found lower amounts of polyphenols, which give our honey important qualities that need to be better explored.

## CONCLUSIONS

Honey consumption, as a nutraceutical product, and this type of honey specifically, is associated with various nutritional benefits and therapeutic potential (Cianciosi et al., 2018). The biological activity of honey is determined always by its complex and important components. The composition of honey is strongly influenced by a multitude of factors, which vary with botanical and geographical origins. Minerals are minor constituents of honey, but they play important roles in determining honey quality. The bioactive compounds from the class of polyphenols play also important roles in the honey nutraceutical properties. Further studies are needed on a higher number of samples, but for start, this type of honey, specific to the Western part of Romania need all our attention to elucidate all its properties. This could reduce the impact that *Fallopia japonica* invasive plant have on the habitat, and if this plant is kept under observation and far from the inhabited areas, it could be highly valuable for beekeepers and honey production.

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# PROPOSALS FOR LEGISLATIVE MEASURES TO IMPROVE THE LEGAL FRAMEWORK ON THE PRODUCTION AND PROCESSING OF MILK, ELIMINATION OF FAKE PRODUCTS FROM THE MILK MARKET AND GROWTH OF CONSUMER TRUST. THE MILK LAW PROJECT

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***Abstract***

*The paper aims to carry out an external analysis of Romania's proposal to regulate the regulatory framework in the field of milk production and processing, to establish the way of marketing of dairy products, to increase consumer confidence in domestic dairy products, to eliminate falsified products from the market dairy products, to facilitate the identification of authentic products by consumers and, last but not least, to the increase in milk production due to all the measures mentioned.*

***Key words****: milk, law, chemical hazards, milk products, evolution, food safety, milk production, Romania.*

## INTRODUCTION

Romania became a member of the European Union (EU) on 1 January 2007, following a difficult and painful transition to a market economy.

Beginning in the early 1990s, this process was characterized by slow pace, structural resistance, inconsistent reforms and ad-hoc political decisions.

Economic and financial instability prevailed in the 1990s, with a number of major economic crises. As a result of the reform packages involving the International Monetary Fund and the World Bank, the economy began to recover in the early 2000s, helped by the political factor, forced to focus on joining the EU.

The official opening of negotiations for EU accession in May 2000 was a crucial step towards the objective of reforming the Romanian agricultural policy.

In Romania, agriculture traditionally occupies an important position in the national industry, representing the field that generates food and raw materials for the agro-food industry.

The massive fragmentation of property, the existence of a large number of low-fat dairy cows holdings, reduced productivity and high self-consumption of own products in households generate important structural problems in Romanian agriculture. Due to the low level of labor productivity and given that the Romanian food industry fails to provide enough products to cover the high demand for food products, the Romanian agricultural industry can not compete with some EU Member States that have a developed industry , with high productivity.

In the perspective of joining the European Union, Romania had to pay special attention to the development of a competitive agriculture, based on a private initiative, capable of a long-term uniform evolution capable of ensuring economic and social cohesion, according to the European Union standards.

This was achieved also by adopting an adequate normative framework for the new European dimensions of Romania, ensuring the development of the Romanian agricultural system in order to reduce the differences from the rest of the member states.

Approximately 10 years after accession, the Romanian legislature considered additional measures needed to reduce counterfeits in the dairy market, to increase consumer confidencein domestic dairy products, contributing significantly to increased milk production.

## DE LEGE FERENDA PROPOSAL ANALYSIS AND DISCUSSIONS

The dairy sector is a strategic sector in ensuring the country's food security, as milk and dairy products are important social products and are an indispensable element in the food rations of the population, including children and elders, as well as vulnerable social people.

The way of marketing of milk and dairy products mainly seeks to protect the life, health of the population and the environment, to meet the consumption needs of all categories of consumers.

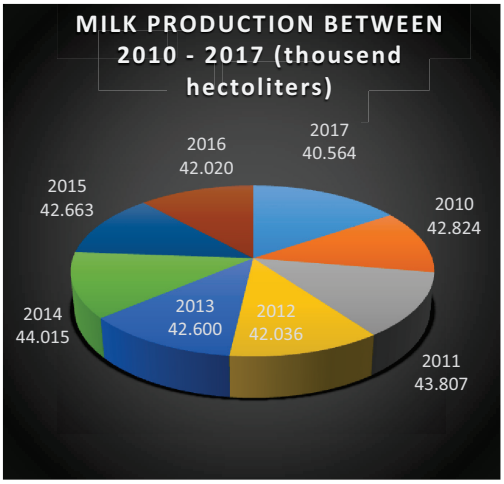


Figure 1. Milk production between 2010-2017

The above figure, based on statistical data provided by the National Institute of Statistics, indicates the tendency for milk production to decrease from 2014, from 44,015 thousand hectoliters in 2014 to 40,564 thousand hectoliters in 2017.

This decrease was one of the reasons why Romania took the decision to develop and promote a bill that would lead, among other things, to increasing consumer confidence in domestic dairy products, thereby contributing to the increase in domestic milk production.

As a result of the control actions carried out by the institutions responsible for verifying compliance with the legal provisions on marketing, compliance, labeling of drinking milk and dairy products, it was found that they contained non-conforming physico-chemical parameters, fat content, protein or moisture content is different from that required by regulatory acts or declared by manufacturers by labeling.

It has also been found that a significant percentage of the verified dairy products do not comply with the quality standards imposed by law or assumed by the producers, the use of dairy dairy product names for commodities, misleading consumers as to the nature of the product.

There are situations where the products obtained from milk and/or casein and vegetable/animal fats are presented under names of cheese marketed under improper names, commercial names that create the idea that they are dairy products, although they are vegetable fats.

The currently used names highlight certain ingredients that by their proportion in composition do not give specificity to the product and create confusion for consumers.

There are some deviations in informing consumers about the marketing of bulk dairy products, such as: lack of mandatory information at the place of marketing, namely the name under which the product is sold, the date of minimum durability or the end-of-life consumption and allergenic ingredients. Exposure of dairy products (with vegetable fat content) to the same dairy area without proper consumer information.

The Romanian agri-food sector continues to face challenges related to compliance with high standards of food safety and quality across the agro-food chain, and through the lege ferenda project, it enables consumers to identify products that have specific qualities, to increase consumer confidence in products domestic dairy products, making an important contribution to increasing milk production.

### The milk law

The proposal of the Law on Milk intends to regulate the way in which the economic agents carrying out activities in the production, processing and marketing of milk and dairy products intended for sale to the final consumer will be registered in the Milk Register, a new way in the Romanian agro-food system. It will be forbidden to activate in this field without being registered in this new register.

The proposal also aims to regulate the milk and dairy sector, milk-based products and milk-based products and creates the legal framework for the presentation, presentation and marketing of milk products.
From the point of view of compatibility with the acquits communitarian, we mention that the law regulates the mandatory information to be printed on the label of consumer milk and dairy products in accordance with Regulation (EU) No. 1.169/2011 of the European Parliament and of the Council of 25 October 2011.
Food security is a major priority for the EU, it affects all citizens and is in close contact with trade policies. EU food safety policy aims to ensure a high level of protection of human life and health, and seeks to protect EU citizens against three types of hazards that may be present in the EU food: physical hazards, biological hazards and chemical hazards.
With regard to chemicals, the EU food safety model is considered to be a global reference and, according to the World Health Organization (WHO), European citizens benefit from one of the highest levels of insurance in the world about the food safety it consumes.
The strength of this model is based on:
(a) its governance structure, in which responsibilities are shared between EU agencies and European Commission: this allows separation of the evaluation risk management risks;
(b) its objective of assessing the safety of chemicals before they are used in the food chain; and
(c) clear allocation of responsibilities between the private sector and public control authorities.
European legislation in the field food aims to guarantee "a high level of protection of human life and health" [1]
The Commission insisted on the importance of this policy, stating the following: "*Guarantee the fact that food products marketed in the EU remain safe is at the center of a Europe that protects* "[2]

All foods contain chemicals. Chemical hazards refer to substances which have the potential to produce adverse health effects and which occur in naturally occurring or which are added during the manufacture or handling of the products food (see Fig. 2).

| | |
|---|---|
| Regulated food Ingredients | Food additives<br>Food enzymes<br>Food flavourings<br>Nutrient sources (food supplements / botanicals) |
| Food chain residues | Feed additives<br>Veterinary medicines<br>Pesticides |
| Contaminants | Environmental pollutants<br>Natural contaminants<br>Process contaminants |
| | Food contact materials |

Figure 2 - Groups of chemical hazards covered by EU legislation

These include some additives, pesticides and pesticide certain metals. residues of certain substances may subsist and may have an impact on the next links of the food chain or different product categories.
For example, pesticide residues which have been used in the cultivation of the intended plants to serve as animal feed can be detected later in the tests performed on food of animal origin.
For this reason, the EU model for food security is based on an integrated approach and includes measures covering the whole food chain: animal feed, animal health, plant protection and food production up to processing, storage, transport, import, export and retailing.
Chemical hazards may be present in all foods, including food (also called "bio" foods)[3], and these practices are misleading consumers.
EU legal corpus governing Chemicals in the context of food security is vast and fragmented. The EU has adopted numerous acts legislation 10, including directives, regulations, decisions and agreements, for each field (food additives, flavorings, feed additives, pesticides, etc.). Overall, this legal body covers about 8,000 chemicals.

[1] Article 5 of Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in food safety.
[2] European Commission, *Food Safety EU budget for the future of the EU*), June 7, 2018 (Https://ec.europa.eu/food/sites/food/files/future_budget_factsheet_en.pdf)
[3] Organic foods are certified as being obtained by complying production methods standards for organic farming. Compliance with these standards does not mean that the presence of any chemical hazards, such as contaminants, is excluded.

Of course, besides the legislation adopted at the level of the European Union, each member state has the freedom to issue legislation regulating the activities of interest, to the limit and without infringing the acquis communautaire.

In this context, Romania has taken the decision to regulate at national level the milk and dairy sector, milk-based products and raw milk products and creates the legal framework for product presentation and marketing mainly regarding the health of the population, meeting the consumption needs of all categories of consumers and respecting hygiene conditions.

In addition, it is intended to regulate the mandatory informations to be entered on the label of consumer milk and dairy products in accordance with Regulation (EU) No. 1.169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) 1.924/2006 and (EC) No. Decision No 1.925/2006 of the European Parliament and of the Council and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directives 1999/10/EC, Directive 2000/13 of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Regulation (EC) No 608/2004, so that consumers are informed directly and specifically of the possible chemical hazards of the food presented for sale.

Within the Ministry of Agriculture and Rural Development, the Milk Registry will be set up within the Ministry's specialized technical directorate, in which economic operators must register for the purpose of carrying out activities in the field of raw milk processing and production of milk and dairy products, intended for marketing.

Registration in the Milk Register is done 60 working days prior to the commencement of marketing of drinking milk or dairy products. By way of exception, economic operators engaged in the processing of raw milk and the production of drinking milk and dairy products intended for marketing before the date of entry into force of this law shall be entered in the Milk Register within 30 working days.

The provisions of the law under discussion do not apply to agricultural producers, persons who obtain agricultural products in their own farm / household and who exceed their own consumption needs and market them on the basis of the producer certificate and the marketing card, according to the Law no. 145/2014 for the establishment of agricultural market regulation measures, as amended and supplemented.

Consumed milk and dairy products in stock prior to the date of entry into force of this law may be marketed with the initially marked identifiers until the stocks are exhausted but no more than 2 months after the entry into force of this to this law.

In addition, the law sets out what changes are allowed for whole milk:

- modifying the natural fat content by removing or adding cream or by adding whole milk, semi-skimmed or skimmed milk in order to ensure the fat content established for drinking milk;
- enrichment of milk with milk proteins, mineral salts or vitamins, in accordance with Regulation (EC) No. 1925/2006 of the European Parliament and of the Council of 20 December 2006 on the addition of vitamins and minerals and of certain other substances to foods;
- reducing the lactose content by changing glucose and galactose. (2)

These changes to the composition of the milk shall be permitted only if they are indicated on the packaging of the product so that they can be easily seen and read and cannot be deleted. However, these indications do not replace the obligation to indicate the nutritional value on the label laid down in Regulation (EU) No. 1169/2011.

If protein is added, the protein content of the enriched milk must be 3.8% or above this value.

As regards drinking milk, the bill establishes the following conditions:

- have a freezing point close to the average freezing point of the raw milk recorded in the area of origin where the drinking milk was collected;
- have a mass greater than or equal to 1,028 grams/liter for milk containing 3.5% (m/m) fat at a temperature of 20°C or an equivalent weight per liter for milk having a different content fat;

- contain a minimum protein content of 2.9% (m/m) for milk containing 3.5% (m/m) fat or an equivalent concentration for milk with a different fat content.
The bill also establishes that the production of dairy products with the addition of hydrogenated fats is processed in production units distinct from milk and dairy processing plants.
Dairy products with the addition of hydrogenated fats will be marketed in a well-defined space, with explicit attention to the buyer's content of these products. The same provisions will apply to the sale of dairy products with the addition of hydrogenated fats sold in bulk.
It will not be allowed to market dairy products with the addition of hydrogenated fats in the same place as milk and dairy products that do not contain hydrogenated fats.
Finished food products that have ingredients as hydrogenated fats will be labelled with the explicit mention of their existence in the product. At their points of sale, consumers will be advised of the existence and designation of the finished foodstuff offered for sale.

**Update - April 2019**

Following the submission of the draft for public debate, it has been modified, so we present the new aspects.
A number of amendments were brought by the officials of the Ministry of Agriculture (MADR), following the analysis of the points of view submitted by the Romanian Employers' Association of the Milk Industry (APRIL), the Federation of Food Industry Employers (Romalimenta) and the Romanian Association of Commerce Networks (AMRCR).
Along with the new bill MADR published also the proposals received and how the normative act was amended on the basis of them.
Among the changes made are the fact that in the Milk Register will be registered not only the local producers, but also the importers:
The economic operators engaged in the processing of raw milk and the production of milk and dairy products, intended for marketing in Romania, including the importers and economic operators engaged in intra-Community trade may market products under the name of milk and dairy products only on the basis of the registration of the product in the Milk Register, prepared by the technical directorate of the Ministry of Agriculture and Rural Development.
MADR rejected the APRIL proposal that the Milk Register should not be set up, the industry argument being that the activity of the Registry overlaps with the activity of National Food Safety and Veterinary Authority (ANSVSA) and Payment and Intervention in Agriculture Agency (APIA).
Registration in the Milk Register shall be made at least 30 working days prior to the commencement of the marketing of drinking milk or dairy products. In a exceptional way, economic operators engaged in raw milk processing and production of drinking milk and dairy products intended for marketing before the date of entry into force of this law will register the products in the Milk Register within 60 working days.
Consumed milk, dairy products and packaging in stock prior to the date of entry into force of this law may be marketed with the initially marked identification until the stocks are exhausted but no more than 12 months after the date of entry into force of this the new law.
For registration in the Milk Register the economic operators submit to the Ministry of Agriculture and Rural Development the following information:
a) name of the economic agent
b) number of the sanitary veterinary authorization issued by ANSVSA
c) name of the product.
Following the enrolment, the economic operator is required to enter the registration number in the Milk Register on the product label. The registration number is placed in the center of a white-red rectangle, preceded by the phrase "Ministry of Agriculture and Rural Development".
The new law maintains provisions to combat dairy counterfeiting. Thus, products containing vegetable fats will be labelled and sold separately from milk-based products without vegetable fats.
The provisions of this law do not apply to agricultural producers, natural persons who obtain agricultural products on their own farm /

household and who exceed their own consumption requirements.
The fines for non-compliance of the new law could reach 50,000 RON (approx. 10,500 EUR), and in cases where the non-observance of the law is repeated, the decision can be taken to ban the carrying out of the activity for which the operator has been sanctioned for 10 years. Furthermore, the prohibition shall also apply to the associate / shareholder / administrator for the economic activities to be carried out from the date of the finding of the contravention in any other form of legal organization. This will prevent the same shareholder, through another firm, from resuming the same activity for which he received the sanction.
In order to be effective, the Law on Milk will be passed to the Parliament where it will be subject to the legislative process.
The initiative will then be submitted to the final vote and, if it is adopted, will have to be promulgated by the President of Romania and published in the Official Gazette to force in to law.

## CONCLUSIONS

With the regards of the topic chosen for the present paper, we consider that, in the absence of the approach of the basic elements in support of milk producers, namely coherent legislation, which maximally speculates the permissibility of the Community legislation, respecting its limits, it is very difficult for the dairy industry to catch up with the highly developed and strong member states on this market, such as Germany, Great Britain, France, Italy, the Netherlands, Poland, which produce about 70% of the EU milk production.
In order to ensure compliance with the provisions of Community law on this matter, the law will be notified to the European Commission in accordance with the provisions of Article 108 (3) of the Treaty on the functioning of the European Union, as amended and supplemented.
From the analysis of the draft law debated, we appreciate that from the point of view of the macroeconomic impact, the application of the measures provided by the draft normative act will allow to improve the way of sell and distribute the milk and milk products and correct and complete information of the population regarding the products which it purchases.
Regarding the identified impact on the business environment, we consider that this proposal complements the regulations in the field of processing and marketing of milk and dairy products, and will be useful to the economic agents that act correctly on the dairy market in Romania.
Regarding the social impact, this law is expected to help ensure compliance with high food safety and quality standards across the agro-food chain and to enable consumers to identify specific quality products, to avoid fake products, to boosts sales of milk products, and off course, to contribute to the raise of the milk production in Romania.

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# RESEARCHES REGARDING THE EVOLUTION OF OVINES MEAT QUALITY DURING REFRIGERATION STORAGE CONDITION

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***Abstract***

*The purpose of this paper is to present the evolution of the pH values during ovine meat refrigeration/maturation. In order to achieve the proposed objectives, investigations were carried on a total number of 104 ovines (52 lambs and 52 adults) taken from the farm of Horleşti, located in the north-east of Romania, in proximity of the Iaşi municiple. The analyzed muscles are characterized by oscillatory amplitude of recorded values, following a descending trend during the first 48 h, then an ascending trend within the time span of 48-120 h, when meat maturation is stimulated. At the end of the rigidity period it is found that in muscle samples taken from the Karakul breed, the highest glycogen values are found in the Longissimus dorsi, as for Ţurcană they are found in Trapezius pars thoracica. pH values during meat maturation fit within qualitative standards for meat, avoiding undesired effects such as PSE or DFD.*

***Key words****: quality, meat acidity, ovines, NE Region, Romania.*

## INTRODUCTION

Quality has a particularly important role in the social and economic challenge, being demanded and imposed by the saturation of agri-foods markets, due to the high efficiency of modern agriculture and zootechnical sector (Crăciun et al., 2011; 2012; Murariu et. al., 2018; Rațu et al., 2018). This is one of the reasons why the quality of meat has occupied a significant place in the research agenda, many years ago, at worldwide level. The structure of the skeletal muscle and its biochemical components influence the transformation of the muscle into meat and sensitively perceptible quality of it including tenderness, color, flavor and succulence (Murariu et al., 2013a;b;c; Lazăr et al., 2011; Frunză et al., 2019a;b). The acidity of the meat is given by the concentration of organic acids from meat (Simeanu et al.2015; Simeanu et al., 2017; 2018), including acidic substances, being measured by the pH value. Knowing the evolution of muscle acidity in post mortem period has a particular importance because it influence the physical properties of the meat, of the water retention capacity and on the product self life (Byrne et al., 2000; Boișteanu, 2002). The most important meat quality index is pH. It reflects the biochemical processes that are taking place in the transformation of muscle into meat (Lup et al., 2018; Murariu et al., 2012). In lambs alive, the pH of the muscle is between 7.1 and 7.3 values. After slaughter, blood circulation stops. Consequently, the intake of oxygen and nutrients is stopped. From this moment on, skeletal muscles are happening a series of irreversible physicochemical and biochemical changes (anaerobes and lactic acid formation) that produce cadaveric rigidity. One of the consequences of this phenomenon is the decrease of the pH values that passes from 7.1 – 7.3 values to values that varies between 5.6 to 6.4 depending on the muscle region. Changes on the pH values during the post-mortem period depend on the glycogen concentration of the muscle after slaughter (Dragomir, 2005). The pH values in *post mortem* stage found in the literature indicate that the ovines species is less sensitive to slaughtering stress than suine and bovine species (Petrescu et al., 2011; Vergara et al., 1999).

Immediately after slaughter, the meat of slaughterhouse animals has an almost neutral reaction. The first enzymes that act are calpaines, the proteolytic enzymes contained in

muscle fiber, that have similar properties to cathepsins, but who act in a neutral and slightly alkaline environment. As the muscular rigidity is installed, the reaction becomes acidic. The main consequence of the accumulation of lactic phosphoric acids is the decrease of the pH to about 5.6.

## MATERIALS AND METHODS

In order to characterize the evolution of meat quality stored in refrigerate condition by its acidity through this paper it aimed to present the evolution of the pH values during ovine meat refrigeration for 120 hours. These assessments are necessary given the pattern of modern consumers model who are increasingly concerned about safe meat production and marketing without adverse effects on their health.

In order to achieve the proposed objectives, investigations were carried on a total number of 104 ovines (52 lambs and 52 adults), respectively 26 lambs of Karakul breed and 26 lambs of Ţurcană breed, 26 adult ovines of Ţurcană breed and 26 ovines of Karakul breed, taken from the farm of Horleşti, located in the north-east of Romania, in proximity of the Iaşi municiple. Harvesting and sampling was made for three muscular tissue, as follows: *Longissimus dorsi, Triceps brachii and Trapezius pars thoracica*.

The determination of the pH values was performed after 6; 12; 24; 48; 72 and 120 hours *postmortem*, according to the analysis principle described by SR ISO 2917:2007. To perform the examinations it was prepared the aqueous meat extract for each sample. For this purpose, the meat sample was cleansed by connective and fatty tissues and finely chopped. From the minced sample it was taken 10 grams with 100 $cm^3$ distilled water in a 250 $cm^3$ Erlenmeyer flask. The mixture was left about 15 – 20 minutes during which it was stirred several times. It was filtered and after the filtrate was subjected to the examination. The Hanna digital pH-meter reads automatically the pH value and the temperature. Measurement was carried out after calibration with 4.01 and 7.01 buffers. After the device balancing, the reading electrode was introduced into the filtered extracts prepared and the values were readed automatically.

## RESUTS AND DISCUSSIONS

Before slaughter stress has significant influences on the ultimate pH value of the muscle, so the tenderness differs between muscles with free shortening and those immobilized from the shortening (Murariu et al., 2014*)*. Stress may be due to the animal transport to the slaughter house, narsh way handling, unfavourable temperature, starvation, and any other factor that could affect the development of glycogen stores before animal slaughter (Muchenje, 2007; Ghimpeteanu et al., 2016).

Muir et al. (1998) found that green – fed animals are more sensitive to stress before slaughter, in association with depletion of glycogen stores compared with animals feed with dietary supplements. In green – fed animals are found higher pH values in relation to animals feed with concentrated feed.

Muscle acidity presents changes during muscle conversion in meat. The evolution of acidity value and its oscillation amplitude have specific influences on the physical properties of meat. Berge et al. (2003) found a direct dependence between the weight of the carcass, with a higher predisposition for cold shortening for muscles from lambs. They recommend to prevent the risk of cold shortening to keeping the carcass at 15 $^oC$ for 6 hours *post mortem*. Thus, is attempted a slower decrease of temperature, without increasing the rate on fall in *rigor mortis* phase (McGeehin et al., 2001). The ultimate pH value, measured at 24 hours after slaughter is influenced by glycolytic potential, namely the amount of glycogen content that could be converted in lactic acid.

In the *prerigor* phase, at 6 hours after slaughter, the muscles collected from Ţurcană breed revealed very significant differences ($p>0.001$) reported on the age category at slaughter, with the mean values ranging from 6.16 (in *Trapezius pars thoracica muscle*) and 6.45 (in *Triceps brachii muscle* and for the muscles samples collected from Karakul ovines there were significant differences ($p>0.05$) at Triceps brachii muscles and distinctly significant differences ($p>0.01$) for *Trapezius pars thoracica muscle* with the mean values ranged between 6.27 (in *Triceps brachii*) and 6.5 for *Trapezius pars thoracica muscle.* This

differences are influenced by the energy reserves (glycogen and phosphocreatine) present in the muscles, the amount of the lactic acid founded in the muscle mass, the health and fatigue states of the ovines, the effect of animal stress and the contraction state of the muscle before slaugter (Strugaru et al., 2010). The moment of muscle rigidity installation at 5 – 6 hours after slaughter is favored by the decreasing of pH values trend and the increasing of actin and myosin atractivness. Therefore, the animals age at slaughter does not show a factor of interest in *rigor mortis* phase, as evidenced by insignificant differences ($p<0.05$), until is reached the meat maturation threshold. This downward trend was recorded up to 48 hours, at which moment the pH values began to increase. These results are in accordance with those fouded in the literature, by Mc Geehin et al. (2001) and Berge at al. (2003) who following a comparative study of muscles composition and meat quality from six different European countries, reported that is no evidence that the age of animals influences the rate of *postmortem* pH decrease in lambs meat. The pH decrease is direct proportional with ATP hydrolysis actvity, being determined by glycogen stores at the moment of animal slaughter. Thus, it take place the formation of actomizinic complex with the actin and myosin accumulate in non contractile mass. Primary statistical estimates calculated do the researches data, which characterize the degree of dispersion of ovines meat acidity values during meat maturation, were low. Thus, the standard error of the average values have oscillated between 0.004 and 0.05, and by coefficient of variation calculating it were express values below 5% limit (0.21 – 2.88%), with one exception of 6.28%, values which evidence a very good homogeneity within the age of ovines and breed (Table 1).

Whereas the weight of carcass is directly dependent on the age of animals, these aspects creating a higher predisposition for cold shortening of the lamb muscles (Berge et al. 2003), in this work reserches it were been taken precautions measures to prevent the risk of cold shortening. Thus, the carcasses were kept at 14$^{o}$C for 6 hours in *post mortem* phase, to perform the wounding, in order to prevent cold shortening of the muscles by a low temperature decrease.

The mean values of ultimate pH ranged from 5.6 to 5.7 beeing generally accepted as normal for ovines meat with a slow entry in rigidity phase.

The ultimate pH values obtained for muscles sampled collected from Karakul breed ranged between 5.65 (*Trapezius pars thoracica*) and 5.71 (*Triceps brachii*) and for muscles collected from Țurcană breed the values ranged from 5.62 (*Triceps brachii*) and 5.67 (*Longissimus dorsi*). These values fall within the normal limits presented in the literature (Harss and Shorthose, 1988; Berge et al., 2003). The meat maturation began to be established in this researches at 48 hours after slaughter, being favored by the pH values increased. This phase is characterized by a low degradation of actomyosin in actin and myosin and of sarcoplasmic proteins, by an increse of hydration capacity and water retention capacity and an improve of sensorial characteristics of ovines meat (tenderness, succulence and flavor). Instead, the muscles collected from Țurcană breed carcasses revealed significant statistical differences ($p>0.05$) for *Longissimus dorsi* muscle at 72 hours after slaughter, distinctly significant differences for *Longissimus dorsi* after 120 hours after slaughter and very significant differences ($p>0.001$) for *Triceps brachii* muscles at 72 and 120 hours after slaughter with the mean values ranging from 5.9 (*Longissimus dorsi*, from adult ovines) and 6.14 (*Triceps brachii*, adult ovines). These results fall within the limits presented in the literature 5.8 – 6.2 (Georgescu et al., 2000). In the maturation phase of the meat, the samples collected from Karakul breed carcasses revealed significant statistical differences ($p>0.05$) for *Longissimus dorsi* muscle and distinctly significant differences for *Triceps brachii* muscles ($p>0.01$) related to age at 120 hours after slaughter, with the mean values ranging from 5.84 (*Longissimus dorsi* muscle from adult ovines) and 6.12 (*Triceps brachii* muscles from adult ovines). At all muscular regions evaluated, collected from the both age groups (*Karakul* and *Țurcană* breeds), the acidity highlight a decrease of the mean values between 0 and 48 hours post mortem, reaching the minimum limit of 5.39 value, being followed by an evolution of the means

values that ranged in the 48 – 120 hours interval, with the final mean values ranged from 5.84 to 6.14 (Figure 1).

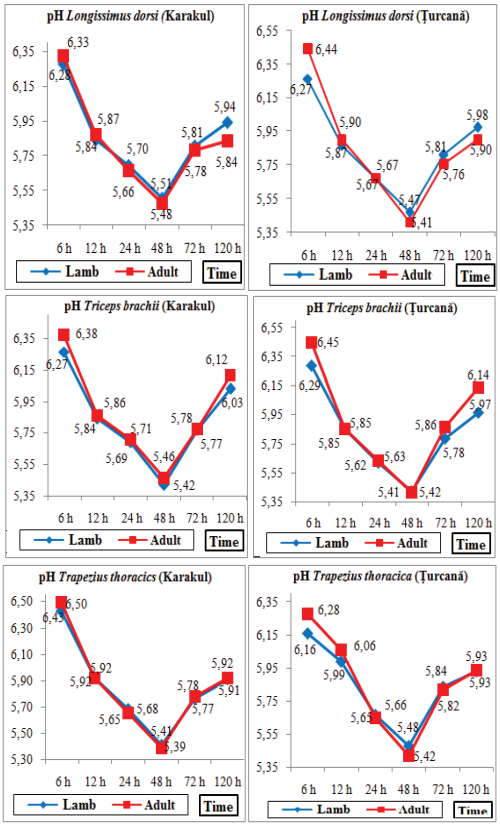


Figure 1. The acidity dynamics of L*ongissimus dorsi, Triceps brachii and Trapezius pars Thoracica* muscles during maturation, depending on age

The pH mean values presented by age groups for the ovines breed analysed highligted variations in the *pre rigidity* phase, uniforming themselve in the same intervals of decrease, thus at 12; 24 and 48 hours the most values have intersected except for those measured at 12 hours for *Trapezius pars thoracica* collected from Ţurcană ovines. In the maturation phase, at 72 hours of slaughter, the upward trend maintains the uniformity of variation except the mean values obtained for the (*Longissimus dorsi* and *Triceps brachii* muscles from Ţurcană breed whose values ranged from 5.76 to 5.81 and 5.78 to 5.86. Therefore, the dynamic of acidity between the two ovines age groups for each breed revealed curvilinear relations within the same areas of variations of the mean values (Figure 1).

The quality of ovines meat according to it's acidity is mainly influenced by intrinsic factors (breed, gender, muscular growth rate, energy reserve) as well as extrinsic ones (intensive exploitation of animals associated with slaughter and marketing methods or slaughtering season).

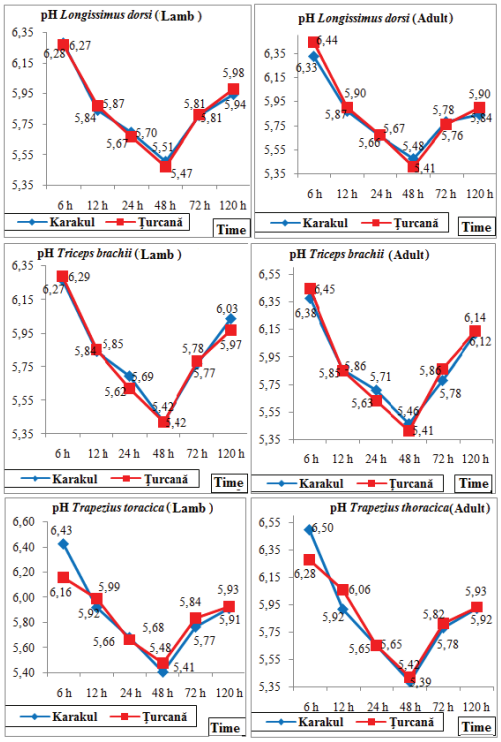


Figure 2. Dynamic of ovines meat mean values for acidity (derived from lambs and adult ovines) during maturation, depending on breeds

The statistical analysis of the pH dynamic for each age group according to breed revealed the recording of significant differences ($p>0,001$) for *Triceps brachii* muscle at the final pH for both age groups and at 72 hours after slaughter when talking about the acidity of the samples collected from *Turcana* ovines (Table 1). The statistical analysis of the *Trapezius pars thoracic* value of muscular acidity showed significant differences according to breed for both age groups in the previous phase and 12 hours after slaughter. Unlike adults, the lambs recorded significant distinct differences ($p>0.01$) at 48 hours after slaughter according

to breed and very significant differences (p>0.001) during maturation (at 72 hours) (Table 1). These differences can be observed in the charts for both age categories of ovines studied comparatively between breeds (Figure 2). Thus, at lambs, the curvilinear relationship between the two breeds fits within very uniform areas of variation for *Longissimus dorsi* and *Triceps brachii* muscles, while mean pH values for the *Trapezius pars thoracic* muscle revealed variations in the *pre-rigor* phase (6.16 - 6.34), which were uniform throughout the rigidity and maturation of the meat (Figure 2). The acidity dynamic of the evaluated meat for adult sheep showed the uniformity of the variation ranges for most of the phases except for *Longissimus dorsi* and *Trapezius pars thoracic* muscles which at the time of *pre-rigidity* showed variations in the range of 6.33 ÷ 6.44 and 6.28 ÷ 6.5 when the rigidity was installed at 12 hours *post mortem* (Figure 2).

Table 1. Statistial estimators and statistical differences between lambs and adult ovines and between Karakul and Țurcană breeds of ovines meat acidity during its maturation

| Muscle region | | Age categ. | Karakul breed | | ANOVA | | Țurcană breed | | ANOVA |
|---|---|---|---|---|---|---|---|---|---|
| | | | $\bar{X} \pm s_{\bar{x}}$ | V% | K vs Ț | T vs A | $\bar{X} \pm s_{\bar{x}}$ | V% | T vs A |
| *Longissimus dorsi* | 6 h | L | 6.28 ± 0.04 | 2.25 | i.s. | i.s. | 6.27 ± 0.01 | 0.81 | *** |
| | | A | 6.33 ± 0.05 | 2.88 | * | | 6.44 ± 0.01 | 0.41 | |
| | 12 h | L | 5.84 ± 0.02 | 1.11 | i.s. | i.s. | 5.87 ± 0.01 | 0.78 | i.s. |
| | | A | 5.87 ± 0.02 | 1.04 | i.s. | | 5.9 ± 0.02 | 0.94 | |
| | 24 h | L | 5.7 ± 0.01 | 0.88 | i.s. | i.s. | 5.67 ± 0.01 | 0.57 | i.s. |
| | | A | 5.66 ± 0.02 | 1.03 | i.s. | | 5.67 ± 0.01 | 0.58 | |
| | 48 h | L | 5.51± 0.02 | 1.3 | i.s. | i.s. | 5.47 ± 0.01 | 0.77 | i.s. |
| | | A | 5.48 ± 0.02 | 1.08 | ** | | 5.41 ± 0.01 | 0.94 | |
| | 72 h | L | 5.81± 0.01 | 0.56 | i.s. | i.s. | 5.81 ± 0.01 | 0.69 | * |
| | | A | 5.78 ± 0.01 | 0.84 | i.s. | | 5.76 ± 0.02 | 1.02 | |
| | 120 h | L | 5.94± 0.01 | 0.56 | i.s. | * | 5.98 ± 0.01 | 0.9 | ** |
| | | A | 5.84 ± 0.01 | 0.8 | * | | 5.9 ± 0.02 | 1.28 | |
| *Triceps brachii* | 6 h | L | 6.27 ± 0.03 | 1.6 | i.s. | * | 6.29 ± 0.02 | 1.24 | *** |
| | | A | 6.38 ± 0.04 | 2.42 | i.s. | | 6.45 ± 0.02 | 0.95 | |
| | 12 h | L | 5.84 ± 0.01 | 0.21 | i.s. | i.s. | 5.85 ± 0.01 | 0.57 | i.s. |
| | | A | 5.86 ± 0.01 | 0.57 | i.s. | | 5.85 ± 0.04 | 2.44 | |
| | 24 h | L | 5.69 ± 0.01 | 0.75 | *** | i.s. | 5.62 ± 0.02 | 1.07 | i.s. |
| | | A | 5.71 ± 0.01 | 0.76 | *** | | 5.63 ± 0.01 | 0.83 | |
| | 48 h | L | 5.42 ± 0.01 | 0.74 | i.s. | * | 5.42 ± 0.01 | 0.94 | i.s. |
| | | A | 5.46 ± 0.01 | 0.9 | * | | 5.41 ± 0.01 | 0.98 | |
| | 72 h | L | 5.77 ± 0.01 | 0.67 | i.s. | i.s. | 5.78 ± 0.02 | 1.1 | *** |
| | | A | 5.78 ± 0.01 | 0.91 | *** | | 5.86 ± 0.01 | 0.51 | |
| | 120 h | L | 6.03 ± 0.02 | 1.3 | ** | ** | 5.96 ± 0.004 | 0.27 | *** |
| | | A | 6.12 ± 0.02 | 0.95 | i.s. | | 6.14 ± 0.02 | 1.06 | |
| *Trapezius thoracica* | 6 h | L | 6.43 ± 0.02 | 0.94 | *** | ** | 6.16 ± 0.01 | 0.69 | *** |
| | | A | 6.5 ± 0.02 | 0.99 | *** | | 6.28 ± 0.02 | 1.27 | |
| | 12 h | L | 5.92 ± 0.01 | 0.81 | *** | i.s. | 5.99 ± 0.01 | 0.8 | *** |
| | | A | 5.92 ± 0.02 | 1.25 | *** | | 6.06 ± 0.01 | 0.85 | |
| | 24 h | L | 5.68 ± 0.01 | 0.82 | i.s. | i.s. | 5.66 ± 0.02 | 1.02 | i.s. |
| | | A | 5.65 ± 0.02 | 1.1 | i.s. | | 5.65 ± 0.01 | 0.85 | |
| | 48 h | L | 5.41 ± 0.02 | 1.26 | ** | i.s. | 5.48 ± 0.02 | 1.03 | i.s. |
| | | A | 5.39 ± 0.01 | 0.94 | i.s. | | 5.42 ± 0+.01 | 0.6 | |
| | 72 h | L | 5.77 ± 0.02 | 1.03 | *** | i.s. | 5.84 ± 0.01 | 0.48 | i.s. |
| | | A | 5.78 ± 0.01 | 0.78 | i.s. | | 5.82 ± 0.01 | 0.9 | |
| | 120 h | L | 5.91 ± 0.1 | 0.53 | i.s. | i.s. | 5.93 ± 0.02 | 1.13 | i.s. |
| | | A | 5.76 ± 0.1 | 6.28 | i.s. | | 5.93 ± 0.01 | 0.76 | |

[1] h – hours from slaughter; [2] A – adult ovines; L – lambs; [3]V% - coefficient of variation

[4] i.s – insignificant statistical differences (p<0.05); * - semnificative differences (0.01>p>0.05); ** - distinct semnificative differences (0.001>p>0.01); *** - very significant differences (p>0.001)

## CONCUSIONS

Varying age or weight at slaughter is one of the most important factors for ovine meat acidity, during the *prerigor mortis* phase. Resulted statistical differences in pH values between the two age categories for muscle samples from the two breeds taken into presented researches (Karakul and Ţurcană) are justified by the level of energetic resources present in muscles due to age, with higher glycogen quantities in adult ovines.

The main consequence of the accumulation of lactic phosphoric acids is the decrease of the pH to about 5.6. When the meat enters in the resolution phase, the reaction tends to neutral again. It is appreciated that there is a strong correlation between the chemical reaction (expressed by pH) and the state of meat tenderness.

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# IMPACT OF CLIMATE CHANGE OF AIR TEMPERATURE ON VITAL ACTIVITY OF THE BEE FAMILIES

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***Abstract***

*The purpose of this paper was to determine the correlation between the monthly average atmospheric air temperature values at different periods of the year and the evolution of the valuie morpho-productive characters of the bee families, thereby elucidating the impact of climate change on the vital activity of bee colonies Apis mellifera. The scientific researches were carried out at the experimental apiary of the Institute of Zoology of the Academy of Sciences of Moldova, located in the central part of Moldavian Codri. Research results have demonstrated that there are positive correlations between the atmospheric air temperature in October of the last year and January of this year and the wintering resistance of the bees colonies ($r_{xy}$ = 0.469 and 0.768). High temperatures of atmospheric air in July, August and September have a negative impact on the wintering resistance of bee families in the next year ($r_{xy}$ = -0.479; -0.699 and -0.494). The prolificity of queens is positively influenced by January temperatures ($r_{xy}$ = 0.464 ± 0.076; $t_r$ = 6.11; P <0.001). Air temperature in February, April and June correlates negatively with the prolificity of queens, estimated in June ($r_{xy}$ = -0.594; -0.795 and -0.461). High temperatures in July and September negatively influence the prolificity of queens in the following year ($r_{xy}$ = -0.531 and -0.711). Colony strength, evaluated in June, is negatively influenced by air temperatures in April and June ($r_{xy}$ = -0.603; -0.691), also correlated with air temperature in September of the last year ($r_{xy}$ = - 0.60; $t_r$ = 2.71; P <0.01), as well as positive one with October of the same year ($r_{xy}$ = 0.517; $t_r$ = 2.00; P <0.05). The viability of the bee brood is positively influenced by the January and February air temperatures ($r_{xy}$ = 0.495 and 0.511), and - negative in the May ($r_{xy}$ = -0.548, $t_r$ = 2.22, P <0.05). Overall, the annual average air temperature positively influence the viability of the brood ($r_{xy}$ = 0.833; $t_r$ = 7.71; P <0.001). Honey production, appreciated at the end of June, tends to be positively influenced by the atmospheric air temperature in January ($r_{xy}$ = 0.488; $t_r$ = 1.81; P <0.1) and, negatively, by the temperature in June ($r_{xy}$ = -0.497; $t_r$ = 1.87; P <0.1). Atmospheric air temperatures in July and September have a negative impact on honey production in the next year ($r_{xy}$ = -0.548 and -0.684; $t_r$ = 2.22 and 3.64; P <0.05 and <0.001); but in October has a positive impact on this production ($r_{xy}$ = 0.513; $t_r$ = 2.00; P <0.05).*

***Key words****: bees, climate change, temperature, air, correlations, characters, morpho-productive.*

## INTRODUCTION

In the last decades of the XX century and the beginning of the XXI century, human society exerts a growing influence on the climate, in particular on Earth's temperature, by burning fossil fuels, deforestation and intensive farming livestock. This adds enormous amounts of greenhouse gases to those naturally occurring in the atmosphere, increasing the greenhouse effect and global warming.

According to a report of the European Commission (Causes of climate change, 2018), the current global average temperature on Earth is 0.85ºC higher than it was in the late 19th century. Each of the past three decades has been warmer than any preceding decade.

Another European source (Consequences of Climate Change, 2018) mentioned that climate change on Earth already have an impact on human health. There has been an increase in the number of heat-related deaths in some regions and we are already seeing changes in the distribution of some water-borne illnesses and disease vectors.

The most affected economic sectors of society are: agriculture, forestry, energy and tourism. Global climate change is happening so fast that the survival of many plant and animal species is threatened.

Many terrestrial, freshwater and marine species have already migrated. Some plant and animal species will be at increased risk of extinction if global average temperatures continue to rise unchecked, according to Paris Agreement - UN Framework Convention on Climate Change (2016) and Council Decision (EU) 2016/1841 (2016).

In Technical Report of Greenpeace Research Laboratories (2013) it was mentioned that: „climate change, such as increasing temperatures, changes in rainfall patterns and more erratic or extreme weather events, will have impacts on pollinator populations. Some of these changes could affect pollinators individually and ultimately their communities, becoming reflected in higher extinction rates of pollinator species".

For example, in Poland it has been documented that honeybees reacts to climate change by making their first cleansing flight (spring cleaning) earlier than normal, which coincides with the phenomenon commonly known as "seasonal change".

The cleansing flight took place one month earlier, compared to the average during the 25-years observation period, which was attributed to the temperature rises (Sparks et al., 2010).

Climate change can lead to the changing of flowering patterns, shifting the flowering period of honey plants that are a major food source for bees, or seasonal change, in which case the flowering period no longer corresponds to the moment of spring awakening of bees (Kremen et al., 2007).

Because of changes in times and patterns of flowering in plants, climate change also affects the interaction between pollinators and their sources of food.

Thus, the researchers (Memmott et al., 2007) demonstrates the reducing of available floral resources to 17-50% of all pollinator species, depending on model of climat change that cause the modification in flowering patterns of plants.

The authors anticipates that the predicted result of these disruptions is the extinction of pollinators, plants and their crucial interactions.

In the abovementioned bibliographic sources, besides the general conclusions made by the authors, concrete information about the influence of climate change on evolution of the morpho-productive characters of bee families is lacking.

At the same time, the above-mentioned bibliographic sources, besides the general conclusions made by the authors, lack information about the concrete influence of climate change on the evolution of morpho-productive characters of bee families.

In this context, the purpose of this paper was to determine the correlation between the parameters of the average monthly atmospheric air temperature values at different periods of the year and the evolution of the morpho-productive characters of the bee families, thereby elucidating the impact of climate change on the vital activity of bee colonies *Apis mellifera*.

## MATERIALS AND METHODS

The scientific researches were carried out on bee families *Apis mellifera Carpatica,* at the experimental apiary of the Institute of Zoology of the Academy of Sciences of Moldova, located in the central part of Moldavian Codri, Forest District Ghidighici, Canton no. 8, Forest Sector no. 21. At the apiary there were a total of 50 bee families. Every bee colony were evaluated for the main morpho-productive reproductive and developmental characters (queen prolificity, family strength), wintering and disease resistance, brood viability, as well as productivity of honey accumulated in nest, according to our methods (Cebotari et al., 2010) described in the Zootechnical norme regarding breeding of bee families, the growth and certification of genitor beekeeping material, approved by Government Decision no. 306 of 28.04.2011 (Official Journal of the Republic of Moldova, 2011). Then the average value of each evaluated morpho-productive characters per apiary was calculated.

To study the impact of climate change on the vital activity of bee families, monthly and annual average of atmosferic air temperature data in the last 8 years (2010-2017), from the nearest hydrometeorological station in Bravicea, Calaras, at a distance of 27 km from the apiary, were used. During this period, for each month, Pirson's linear correlation coefficients were calculated between the monthly average of atmospheric air temperature and the average values per apiary of each of the 6 main morphoproductive characters of bee families, such as: queen prolificity, families strength, wintering colony resistance, disease resistance, brood viability and honey production of bee families. For the first half of the year, correlation coefficients were calculated between atmospheric air

temperature and values of morpho-productive characters, evaluated in the same year at the end of June, with the exception of wintering resistance, which was assessed at the end of March. Given that in the second half of the year the climatic factors don't influence the morpho-productive characters, already evaluated in this year, the atmospheric air temperature variable in July-December was calculated in correlation with the value of the morpho-productive characters of the bee families from next year. The same correlation coefficients were also calculated for the average annual temperature of the atmospheric air and the average values per apiary of the above-mentioned in this 6 morpho-productive characters. For each correlation coefficient ($r_{xy}$) the criterion of certainty of the correlation ($t_r$) and the certainty threshold (P) after Student was calculated in part.

The obtained in experience data were statistically processed using computer software "STATISTICA - 12" and evaluated their certainty, according to variation biometric statistics, by methods of Плохинский Н.А. (1989).

## RESULTS AND DISCUSSIONS

The results of the research have shown that the global warming phenomenon has been manifested also in the area of Bravicea Hydrometeorological Station, located near the site where the experimental apiary is situated. Moreover, in this area the effects of global warming were more evident, compared to data from the European Commission Report (2018). We found that the average annual air temperature rose from 10.4°C in 2010 to 10.9°C in 2017, i.e. by 0.5°C. If we compare the average air temperature during the first three years (2010-2012) of the research period with the average of the last three years (2015-2017), we find significant increase from 9.6°C to 11.1oC, or 1.5°C, which is very much and worrying. Because of this, during the relatively short research period of only 8 years, there were two terrible droughts in this area: one in 2011-2012 and another in 2015. High air temperatures and terrible droughts have had a negative impact on the flora and fauna of the given ecosystem, especially on agriculture in this area. We can suppose that if the warming of the air will continue at this rate, over several decades we will be witness of transformation of this area into an arid and deserted one.

Researches have shown that air temperature is one of the most important climatic factor that influences the vital activity of Apis mellifera bee families. Between this climatic factor and the evolution of the morpho-productive characters of the bee families there are correlative links of different size. The impact on bee families of the climate change in air temperature depends of the year period. It was found that the impact of air temperature on the vital activity of bee families is caused both by monthly average temperatures, in some concrete times of the year, and the annual average of air temperature. Climate change in air temperature in the first half of the year, especially in January, February, April and June, had the greatest impact on the vital activity of bee families (Table 1).

Thus, between the average air temperature in January and the queens prolificity, determined at the end of June, a significant positive correlation was found ($\mathbf{r_{xy}}$=0.464±0.076; $\mathbf{t_r}$=6.11; P<0.001).

This means that with the increase of air temperature in January, there is an increase in the prolificity of queens at the end of spring - early summer. Also, the January air temperature positively influences the wintering resistance of bee families (evaluated at the end of March), brood viability and honey production, appreciated at the end of summer. The correlation coefficients between these characters are of average size and are comprised among 0.469 - 0.495 ($t_r$=1.74 – 1.85; P <0.1).

At the same time, researches have shown that February's air temperature variability has a significant negative correlation on with queens prolificity and positive with brood viability, assessed at the beginning of the summer (June). The correlation coefficient between these variables is of medium size, but quite significant ($\mathbf{r_{xy}}$= -0.594±0.129; $\mathbf{t_r}$=2.59; P<0.05 and $\mathbf{r_{xy}}$=0.511±0.262; $\mathbf{t_r}$=1.96; P<0.05).

This means that the higher is air temperature in February, the lower the queen prolificity in the early summer, and the higher brood viability will be.

About the March air temperature we can not say if it influences in some way the vital activity of the bee families, since the correlation coefficients of this variable with the main morpho-productive characters are not significant, which does not allow us to make any definite conclusions.

Table 1. The correlation coefficient between the average monthly air temperature in the apiary area during the first half of the year and the medium value of the morpho-productive characters of bee families

| Morpho-productive characters | $r_{xy} \pm m_r$ | $t_r$ | P | Morpho-productive characters | $r_{xy} \pm m_r$ | $t_r$ | P |
|---|---|---|---|---|---|---|---|
| **Air temperature in January, $t^0$C** | | | | **Air temperature in February, $t^0$C** | | | |
| Wintering resistance | 0.469±0.275 | 1.74 | <0.1 | Wintering resistance | 0.015±0.353 | 0.04 | >0.1 |
| Queen prolificity | 0.464±0.076 | 6.11 | <0.001 | Queen prolificity | -0.594±0.129 | 2.59 | <0.05 |
| Colony strength | 0.439±0.285 | 1.54 | >0.1 | Colony strength | -0.006±0.353 | 0.01 | >0.1 |
| Disease resistance | 0.269±0.328 | 0.82 | >0.1 | Disease resistance | 0.009±0.353 | 0.03 | >0.1 |
| Brood viability | 0.495±0.267 | 1.85 | <0.1 | Brood viability | 0.511±0.262 | 1.96 | <0.05 |
| Honey production | 0.488±0.269 | 1.81 | <0.1 | Honey production | 0.370±0.303 | 1.21 | >0.1 |
| **Air temperature in March, $t^0$C** | | | | **Air temperature in April, $t^0$C** | | | |
| Wintering resistance | 0.107±0.349 | 0.31 | >0.1 | - | - | - | - |
| Queen prolificity | -0.129±0.347 | 0.37 | >0.1 | Queen prolificity | -0.795±0,130 | 6.12 | <0.001 |
| Colony strength | -0.331±0.314 | 1.05 | >0.1 | Colony strength | -0.603±0,225 | 2.68 | <0.05 |
| Disease resistance | 0.161±0.344 | 0.47 | >0.1 | Disease resistance | 0.070±0,352 | 0.20 | >0.1 |
| Brood viability | 0.449±0.282 | 1.59 | >0.1 | Brood viability | 0.182±0,342 | 0.53 | >0.1 |
| Honey production | 0.401±0.296 | 1.35 | >0.1 | Honey production | -0.222±0,336 | 0.66 | >0.1 |
| **Air temperature in May, $t^0$C** | | | | **Air temperature in June, $t^0$C** | | | |
| Queen prolificity | 0.332±0.314 | 1.06 | >0.1 | Queen prolificity | -0.461±0,278 | 1.66 | <0.1 |
| Colony strength | 0.421±0.291 | 1.45 | >0.1 | Colony strength | -0.691±0,184 | 3.76 | <0.001 |
| Disease resistance | -0.451±0.282 | 1.60 | >0.1 | Disease resistance | -0.350±0,310 | 1.13 | >0.1 |
| Brood viability | -0.548±0.247 | 2.22 | <0.05 | Brood viability | 0.192±0,341 | 0.56 | >0.1 |
| Honey production | 0.038±0.353 | 0.10 | >0.1 | Honey production | -0.497±0,266 | 1.87 | <0.1 |

Research data demonstrates that April air temperature has an obvious impact on vital queen reproduction functions and bee family development. Thus, the air temperature in April is significantly negative correlated with the prolificity of the queen ($r_{xy}$= -0.795±0.130; $t_r$=6.12; P<0.001), and the colony strength ($r_{xy}$= -0.603±0.225; $t_r$=2.68; P<0.05). That is, with increasing of air temperature in April, queens prolificity decreasing will occur in early summer, which will diminish the strength of bee families.

We can say that the air temperature in May influences negatively the viability of the brood. Also, we observed that increased air temperature in May, led to decrease of the viability of brood. The correlation coefficient between these two variables is significant, of medium size and constitutes $r_{xy}$=-0.548±0.247; $t_r$=2.22; P<0.05. As for the impact of air temperature in May on other morpho-productive characters, we can not deduce certain conclusions, as the correlation coefficients between this variable and the main morpho-productive characters are not significant.

It was found that the air temperature in June has a greater impact on vital activity of bee families. Namely, the air temperature in this summer month has an obvious negative impact both on the reproduction and development functions and on the honey production capacity of the bee families. Thus, research data demonstrates that there is a significant negative high-level correlation between the air temperature in June and the colony strength. ($r_{xy}$=-0.691±0.184; $t_r$=3.76; P<0.001). The climatic variability of the air temperature in June has a negative correlation tendency with both the queen prolificity ($r_{xy}$ = -0.461 ± 0.278, $t_r$ = 1.66, P <0.1), as well as honey production ($r_{xy}$= -0.497±0.266; $t_r$=1.87; P<0.1). This means that as the June air temperature increases, there will be a fall in the strength of bee families and the production of honey, accumulated in the nest.

Generalizing the impact of climate changes in air temperature in the first half of the year, we can conclude that the raised temperatures during this period, especially in April-June, negatively influence the vital activity of bee

families. First of all, the negative impact is directly manifested on honey flowers.

Under the action of high temperatures, they bloom suddenly and for a short time, and the removed nectar evaporates rapidly, becoming too consistent and inaccessible to bees. Secondly, excessively high temperatures inhibit the vital activity of nurses and worker bees, which leads to diminishing of the reproduction functions of queens and harvesting functions of worker bees.

This conclusion is in line with some of our previous researches (Cebotari et al., 2013) in which it was demonstrated that the excessively high spring-summer temperatures of a dry year, caused a drastic decrease in morpho-productive indices of bee families.

Starting in the second half of the year (Table 2), the air temperature in July-December has no impact on previously evaluated morpho-productive characters (by the end of June), but can have a direct impact on the vital activity of bees families, which is related to the consolidation of the colonies' strength and their preparation for wintering, as well as, indirectly, to the evolution of morpho-productive characters in the next year.

Table 2. Coefficient of correlation between the average monthly air temperature during the second half of the current year and the value of morpho-productive characters of bee families in the next year

| Morpho-productive characters | $r_{xy} \pm m_r$ | $t_r$ | P | Morpho-productive characters | $r_{xy} \pm m_r$ | $t_r$ | P |
|---|---|---|---|---|---|---|---|
| **Air temperature in July, $t^0$C** | | | | **Air temperature in August, $t^0$C** | | | |
| Wintering resistance | -0.479±0,272 | 1.76 | P<0.1 | Wintering resistance | -0.699±0,181 | 3.86 | P<0.001 |
| Queen prolificity | -0.531±0,254 | 2.09 | P<0.05 | Queen prolificity | -0.146±0,346 | 0.42 | P>0.1 |
| Colony strength | -0.233±0,334 | 0.70 | P>0.1 | Colony strength | 0.040±0,353 | 0.11 | P>0.1 |
| Disease resistance | -0.045±0,353 | 0.13 | P>0.1 | Disease resistance | -0.067±0,352 | 0.19 | P>0.1 |
| Brood viability | 0.157±0,345 | 0.46 | P>0.1 | Brood viability | 0.388±0,300 | 1.29 | P>0.1 |
| Honey production | -0.548±0,247 | 2.22 | P<0.05 | Honey production | -0.206±0,339 | 0.61 | P>0.1 |
| **Air temperature in September, $t^0$C** | | | | **Air temperature in Octomber, $t^0$C** | | | |
| Wintering resistance | -0.494±0,267 | 1.85 | P<0.1 | Wintering resistance | 0.768±0,145 | 5.30 | P<0.001 |
| Queen prolificity | -0.711±0,175 | 4.06 | P<0.001 | Queen prolificity | -0.063±0,352 | 0.18 | P>0.1 |
| Colony strength | -0.606±0,224 | 2.71 | P<0.01 | Colony strength | 0.517±0,259 | 2.00 | P<0.05 |
| Disease resistance | -0.139±0,347 | 0.40 | P>0.1 | Disease resistance | 0.404±0,296 | 1.36 | P>0.1 |
| Brood viability | 0.384±0,301 | 1.27 | P>0.1 | Brood viability | 0.186±0,341 | 0.54 | P>0.1 |
| Honey production | -0.684±0,188 | 3.64 | P<0.001 | Honey production | 0.513±0,261 | 1.97 | P<0.05 |
| **Air temperature in November, $t^0$C** | | | | **Air temperature in December, $t^0$C** | | | |
| Wintering resistance | -0.264±0,329 | 0.80 | P>0.1 | Wintering resistance | -0.223±0,337 | 0.66 | P>0.1 |
| Queen prolificity | 0.108±0,349 | 0.31 | P>0.1 | Queen prolificity | -0.100±0,350 | 0.28 | P>0.1 |
| Colony strength | 0.394±0,299 | 1.32 | P>0.1 | Colony strength | -0.289±0,324 | 0.89 | P>0.1 |
| Disease resistance | 0.222±0,336 | 0.66 | P>0.1 | Disease resistance | -0.061±0,352 | 0.17 | P>0.1 |
| Brood viability | -0.040±0,353 | 0.11 | P>0.1 | Brood viability | 0.402±0,296 | 1.36 | P>0.1 |
| Honey production | 0.260±0,330 | 0.79 | P>0.1 | Honey production | -0.055±0,353 | 0.16 | P>0.1 |
| **Annual average, $t^0$C** | | | | | | | |
| Wintering resistance | -0.528±0.255 | 2.07 | P<0.05 | | | | |
| Queen prolificity | -0.440±0.285 | 1.54 | P>0.1 | | | | |
| Colony strength | -0.295±0.323 | 0.91 | P>0.1 | | | | |
| Disease resistance | 0.178±0.342 | 0.52 | P>0.1 | | | | |
| Brood viability | 0.833±0.108 | 7.71 | P<0.001 | | | | |
| Honey production | -0.121±0.348 | 0.35 | P>0.1 | | | | |

After analyzis of the atmospheric air temperature variability in the second half of the current year, correlated with the evolution of morpho-productive characters of bee families, evaluated in the first half of the next year, we found that this (air temperature) also had an impact quite obviously on the vital activity of bee families.

Thus, the atmospheric air temperature in July has a tendency negative impact on wintering resistance ($\mathbf{r_{xy}}$ = -0.479 ± 0.272; $\mathbf{t_r}$ = 1.76; P <0,1) and a significant negative impact on queens prolificity ($\mathbf{r_{xy}}$ = 0.531 ± 0.254, $\mathbf{t_r}$ = 2.09, P <0.05), as well as on honey production in the following year ($\mathbf{r_{xy}}$= -0.548±0.247; $\mathbf{t_r}$=2.22; P<0.05). This means that with the

increase of atmospheric air temperature in July, a downward trend in wintering resistance of bee colonies, as well as, a significant decrease in the prolificity of queens and honey production, accumulated in the nest, in the next year will occur.

In August, climate change, by increasing atmospheric air temperature, has a significant negative impact on the wintering resistance of bee families the following year. The correlation coefficient between these two variables is high and quite significant, and according to the probability prognosis theory without error by Student, it has the highest threshold of certainty ($\mathbf{r_{xy}}$=-0.699 ±0.181; $\mathbf{t_r}$=3.86; P<0.001). This means that the higher is the atmospheric air temperature in August, the lower will be bee's wintering resistance. About variability of the values of other morpho-productive characters of the bee families, we can not say if they were or not influenced by the atmospheric air temperature in August, because the values of the correlation coefficients between these variables were not significant.

At the same time, the climatic change of atmospheric air temperature in September has an obvious impact on several morpho-productive characters of the vital activity of bee families in the coming year. Thus, the atmospheric air temperature in this autumn month has a negative impact on the wintering resistance of bee colonies ($\mathbf{r_{xy}}$=-0.494±0.267; $\mathbf{t_r}$=1.85; P<0.1) and a significant negative impact on queens prolificity ($\mathbf{r_{xy}}$=-0.711±0.175; $\mathbf{t_r}$=4.06; P<0.001), on the strength of bee families ($\mathbf{r_{xy}}$=-0.606±0.224; $\mathbf{t_r}$=2.71; P<0.01) and amount of honey accumulated in the nest ($\mathbf{r_{xy}}$=-0.684±0.188; $\mathbf{t_r}$=3.64; P<0,001). That is, with the rise of atmospheric air temperature in September, the next year there will be a decrease of wintering resistance, of queens prolificity, of colonies strength and their honey production.

Climate changes in atmospheric air temperature in October havean entirely different (contrariwise) influence on the vital activity of bee families. It has been found that the rise in atmospheric air temperature during this autumn month has a beneficial impact on the main morpho-productive characters of bee families. The high temperatures of this month correlate positively with the wintering resistance of bee colonies ($\mathbf{r_{xy}}$=0.768±0.145, $\mathbf{t_r}$=5.30, P<0.001), their strength ($\mathbf{r_{xy}}$=0.517±0.259; $\mathbf{t_r}$=2.00; <0.05) and honey production in the next year ($\mathbf{r_{xy}}$=0,513±0,261; $\mathbf{t_r}$=1,97; P<0,05). This means that with the rise in atmospheric air temperature in October this year, we will have an amelioration of the values of the main morpho-productive characters of bee families in the next year.

We can not pronounce somehow about the influence of climatic changes of atmospheric air temperature in November and December on the variability of the morpho-productive characteristics of bee families, because the correlation coefficients between these variables are insignificant, which does not allow us to make any clear conclusions.

Regarding the climatic change of the annual average temperature, we found that it has a significant impact on the variability of the values of some morpho-productive characters of bee families in the following year. Thus, the annual average temperature has a significantly negative correlation with the wintering resistance of colony ($\mathbf{r_{xy}}$=-0.528±0.255; $\mathbf{t_r}$=2.07; P<0.05) and significantly positive, very high correlation, with brood viability in the next year ($\mathbf{r_{xy}}$=0.833±0.108; $\mathbf{t_r}$=7.71; P<0.001). That is, once the average annual air temperature is raised, we will see a reduction of wintering resistance of bees (evaluated in March) and the increase of brood viability, assessed in June next year.

Therefore, generalizing the data on the correlation between climate changes of atmospheric air temperature and the values of the main morpho-productive characters of bee families, we can conclude that they (climate change) have a significant influence on the vital activity of bee colonies. The variability of atmospheric air temperature in different months of the year influences differently the development of morpho-productive characters of bee families. Moreover, climatic changes in air temperature in the first half of the year directly affect the variability of morpho-productive characters, evaluated by the end of June, and the air temperature in July-December indirectly influences the variability of their values in the next year. Knowledge of the particularities of impact of climate change of atmospheric air temperature on the variability

of the morpho-productive characteristics of bee families, during concrete months of the year, will allow beekeepers to undertake certain mitigation measures, by applying special care proceedings and additional directed feeding of bee families, depending on the specific periods of the year.

## CONCLUSIONS

The wintering resistance of *Apis mellifera* bees colonies is positively influenced by the atmospheric air temperature in October of last year and January of current year. At the same time, the high air temperatures in July, August and September have a negative influence on the wintering resistance of bee families.

The queens prolificity is positively influenced only by the January temperatures. The high atmospheric temperatures in February, April and June have a negative impact on the queens prolificity, assessed in June. High temperatures during the second half of the year, especially in July and September, have a negative impact on the prolificity of queens in the next year.

The strength of bee families, appreciated in June, is negatively influenced by atmospheric temperatures in April and June. High temperatures in September have a negative impact, and those in October have a positive impact on the colonies strength in the next year.

Atmospheric temperatures in January and February influence positively, and those in May have a negative impact on the viability of bee brood. Overall, the average annual air temperature have a positive impact on the viability of the brood.

Honey production, appreciated at the end of June, is positively influenced by the atmospheric temperature in January and negative - by the atmospheric temperature in June. At the same time, atmospheric temperatures in July and September have a negative impact, and those in October - a positive impact on honey production of bee families in the next year.

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# THE INTERVENTION OF *Hermetia illucense* L. (DIPTERA: STRATIOMYIDEA) IN MANURE EMPOWERMENT ON BLOOD LIPID PROFILE OF NATIVE CHICKEN

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***Abstract***

*The aims of this research were to evaluate the utilization of manure degraded by Hermetia illucens to be used as diets on blood lipid profile of native chicken. The treatments were R1 (control diet with 15% fish meal + 0% MBHI meal), R2 (diet with 10% fish meal + 5% MBHI meal), R3 (diet with 5% fish meal + 10%MBHI meal), and R4 (diet with 0% fish meal + 15% MBHI meal). The data were analyzed using analysis of variance according to Completely Randomized Design (CRD) and means separated by the application of Duncan's multiple range test. Variables measured were triglyceride, LDL, HDL, and cholesterol. The results shown that R3 gave a significant (P<0.05) lower in triglyceride (47,15 -59,66 mg/dl), LDL (45,68 – 81,06 mg/dl),cholesterol (124,98 -155,97 mg/dl) Whereas, R3 gave HDL (75,85 – 86,28 mg/dl) level significantly higher (P < 0.05). High blood HDL level at 10% MBHI meal in the diet not only maintains blood HDL at normal level but also increases native chicken meat quality, as was expected in the present study.*

***Key words****: manure, H. illucense, blood, native chickens.*

## INTRODUCTION

The utilization of insect in animal husbandry become important (Rumokoy et al., 2017) especially as ingredients for feed has gained considerable interest recently, for example *H.illucens* or black soldier fly (BSF) can be used as feeds for animals and human food as well and further stated that BSF is rich in protein and fat (Wang and Shelomi, 2017); while Barragan-Fonseca et al. (2017) reported BSF has a protein content of 37-63%, fat 7-39%, ash 8-25% (DM basis), protein 40.8 ± 3.8%, fat 26.6 ± 8.6% and suited for poultry feed (Nyakeri et al., 2016). BSF lives in organic matter, food waste disposal, and manure, produces enzymes such as: amylase, lipase, and protease to hydolize karbohidrate, fat, and protein into smaller or individual parts as maltose, fatty acids, glycerol, and amino acids respectively (Kim et al., 2011). By doing this, BSF can break down waste disposal to become a high quality feedstuffs and can also reduce pollution (Wang and Shelomi, 2017). This BSF or *H. illucens* has been studied for its capability to convert organic waste to high quality protein, control certain harmful bacteria and insect pests, provide potential chemical precursors to produce biodiesel and for its use as feed for a variety of animals. BSF has been used as feedstuffs and can substitute parts or total proportion of soybean meal or fish meal in layer diets (Maruer et al., 2016; Marono et al., 2017), broiler (Schiavone et al., 2016), and quails (Widjastuti et al., 2014). Moula et al. (2017) reported that native chicken consumed horse manure biodegraded by *H. illucens* resulted in good performances.

Tohala (2010) reported that blood triglyceride, total cholesterol, HDL, LDL,VLDLP level of native chicken consumed high and low energy diets about 53.83 ± 2.24 mg/dL; 139.69±5.82 mg/dL; 57.50±4.39 mg/dL; 75.50±3.71; 10.57±0.46 mg/dL; and 50.17±1.40; 131.83±4.13 mg/dL; 53.33±1.7; 73.50±1.52; 10.03±0.29 mg/dL, respectively. The utilization of chicken manure biodegraded by *H. illucens* is an interesting choice as an inconvensional feed ingredient to replace conventional feed ingredients which are expensive and compete

with human use. Yet little is known about the effect of utilization of chicken manure biodegraded by *H. illucens* in the diets on meat quality of native chicken. Therefore, the present study was arranged to elaborate the utilization of chicken manure biodegraded by *H. illucens* in the diets on blood lipid profiles of native chicken *(Bali chicken)*.

## MATERIALS AND METHODS

The wild adult *H. illucens* or BSF were captured from the chicken farm environment and then thirty pairs flies were placed into each manure box. The manure box was designed with a dimension 100 x 100 x 70cm, each side is made of gauze. Thirty kg of broilers manure, as the BSF larva media, were placed in this manure box. The flies laid down their eggs until day fourth. The BSF larvae were reared in this media to take place the biodegradation of the manure during eight days of their life cycle. The study was conducted for two months. A total of 100 four month-old native chicken *(Nunukan X Kedu)* known as *Bali chicken*, were allocated in twenty 0.7 x 0.5 x 0.5 m battery cages. Birds were randomly divided into 20 experimental units of 5 chicks each and each diet was offered at random. Each pen was equipped with three separate tube feeders and waterers. Fresh water and feed were provided *ad libitum* throughout the experimental period. The dietary treatments were in a completely randomized design with 4 treatments and 5 replications according to Steel and Torrie (1980). Each treatment was formulated in iso-nutrient and iso-caloric arrangement. Treatments were formulated as follow: R1 as control diet with 15% fish meal + 0% MBHI meal; R2 was a diet with 10% fish meal + 5% MBHI meal; R3 was a diet with 5% fish meal + 10% MBHI meal; and R4 was a diet consisted with 0% fish meal + 15% MBHI meal. Composition and nutrient content of treatment diets is presented in Table 1.

**Data collection**

At day 60, 2 chicks from each pen were selected and blood samples collected. The triglyceride (TG), cholesterol (CHOL), high density lipoprotein (HDL) levels were measured using Cholesterol Oxidase-peroxidase amino antipyrine phenol (CHOD-PAP) method (Enzymatic Calorimetric/Method NS), using commercially available kits. (Kit/Spectrophotometry Analysis (Biotech England, 2011).

Table. 1. Composition and nutrient content of experimental diets (%)

| Ingredients (%) | Treatments | | | |
|---|---|---|---|---|
| | R1 | R2 | R3 | R4 |
| Yellow corn | 55 | 55 | 55 | 55 |
| Rice bran | 11.5 | 11.5 | 11.5 | 11.5 |
| Copra meal | 7 | 7 | 7 | 7 |
| Soybean meal | 10 | 10 | 10 | 10 |
| Fish meal | 15 | 10 | 5 | 0 |
| MBHI meal | 0 | 5 | 10 | 15 |
| Bone meal | 1 | 1 | 1 | 1 |
| Vitamin Premix*) | 0.5 | 0.5 | 0.5 | 0.5 |
| *Nutrient assayed* | | | | |
| Crude protein (%) | 20.30 | 20.17 | 19.83 | 19.55 |
| Crude fiber (%) | 4.56 | 5.02 | 5.49 | 5.95 |
| Ether extract (%) | 5.27 | 5.23 | 5.20 | 5.03 |
| Ca (%) | 1.00 | 0.92 | 0.83 | 0.67 |
| P (%) | 0.74 | 0.61 | 0.55 | 0.42 |
| ME (kcal/kg) | 2951.40 | 2934.60 | 2917.25 | 2905.75 |

*)Vitamin premix is a mixture of vitamins and mineral premix supplied the following per kilogram of feed: vitamin A = 6000 IU, vitamin D3 = 1000 IU, vitamin E = 12.50 mg, vitamin K = 1.450 mg, vitamin B1 = 4.50 mg, vitamin B2 = 2.250 mg, vitamin B6 = 0.450 mg, vitamin $B_{12}$ = 2.250 mg, niacin = 5.500 mg, pantothenic acid = 0.150 mg, iron = 18.50 mg, copper = 2.5 mg, manganese = 50.0 mg, zinc = 27.50 mg, iodine = 4.0 mg, and selenium = 0.0500 mg.

**Statistical analysis.** Data were subjected to analysis of variance and significant differences observed in means subjected to Duncan's multiple range test. All data were analyzed for variance analysis using the general linear model (GLM) procedures of the SAS Institute.

## RESULTS AND DISCUSSIONS

The results of the blood lipid indices of the experimental birds are presented in Table 2 and Diagram 1.

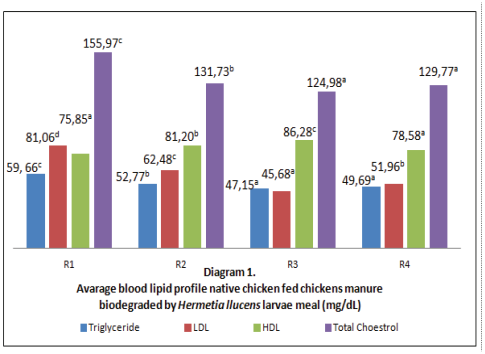
Triglyceride as a lipid component in the blood normally increases drastically soon after meals, especially with simple carbohydrate such as sugars and saturated fat. Since simple carbohydrate cannot be used directly as an

energy sources, instead of being transformed into triglycerides and stored in a form of energy. At the time when body needs energy, triglyceride will be mobilized by hormones.

Table 2. Average blood lipid profile native chicken fed chickens manure mealbiodegraded by *Hermetia illucens* (*Diptera:* L *statiomyidea)* (MBHI) (mg/dL)

| Blood Lipid Profiles | Treatments | | | |
|---|---|---|---|---|
| | R1 | R2 | R3 | R4 |
| Triglycerid | $59.66^{c}$ | $52.77^{b}$ | $47.15^{a}$ | $49.69^{a}$ |
| LDL | $81.06^{d}$ | $62.48^{c}$ | $45.68^{a}$ | $51.96^{b}$ |
| HDL | $75.85^{a}$ | $81.20^{b}$ | $86.28^{c}$ | $78.58^{a}$ |
| Total Cholesterol | $155.97^{c}$ | $131.73^{b}$ | $124.98^{a}$ | $129.77^{a}$ |

$^{a,b,c}$ Means on the same row with different superscripts differ significantly ($P < 0.05$)




Research results showed that blood triglyceride levels in the present study are about 47.15 – 59.66 mg/dL. The highest blood triglyceride level found at R1 (control diet without MBHI in the diet) (59.66 mg/dL; whereas the lowest level of triglyceride found at R 3 (10% MBHI and 5% fish meal in the diet)(47.15 mg/dL. The average level of triglyceride in the present study was in agreement with Tohala (2010) who reported that blood triglyceride level in broiler chicken is about 50.17±1.4 to 52.83±2.44 mg/dL. Blood triglyceride level is determined by feed consumption especially simple carbohydrate such as sugar, saturated fat, high level of circulated free fatty acids, high insulin level, and low glucagon level. Carbohydrate in the liver is broken down to fatty acids and transformed back to triglyceride (Murray, 2012). Chicken manure biodegraded by *H.illucens* (MBHI ) in the present study is assumed to have high enzymes (amilase, lipase, and protease) activity specifically lipase. Initial study (Manangkot, 2014) revealed that at day 7 of manure after being biodegraded by *H. illucens* and day 8 of larvae growth in the manure, gave a highest amylase, protease, and lipase enzymes activity. Lipase showed a value of 44.84±17.39 mg/dL. It is assumed that the higher the level of MBHI in the diets, the higher the lipase activity. Blood LDL level in the present study ranged from 45.68 to 81.06 ml/dL. R1 (control diet without MBHI meal in the diet) gave a highest blood LDL level of about 81.06 ml/dL; whereas R3 (10% MBHI meal in the diet) gave a lowest blood LDL level of about 45.68 ml/dL. Statistical analysis revealed that treatments gave a significant difference ($P < 0.05$) on blood LDL level of native chicken in the present study. It can be said that MBHI has an ability in lowering blood LDL level of native chicken in the present study. Lower blood LDL level in the present study compared with a normal level (46.68 – 81.06 ml/dL vs 95 – 125 ml/dL) is a result of high MBHI meal level in the diets, so that the diets have more active enzyme to mobilize cholesterol and LDL itself. The average blood HDL level in the present study is 75.85 – 86.28 mg/dL. R1 (control diet without MBHI meal in the diet) has a lower ($P < 0.05$) blood HDL level compared with R2, R3, and R4. Blood HDL level of R2 (5% MBHI meal in the diet) was significantly lower ($P < 0.05$) than R3 (10% MBHI meal in the diet). Blood HDL level of R4 (15% MBHI meal in the diet) was significantly lower ($P < 0.05$) than R2 and R3; while blood HDL level of R3 (10% MBHI meal in the diet) was significantly higher ($P < 0.05$) than the rest of treatments. Hariyanto (2017) also reported that blood triglyceride level in broiler chicken ranged from 54.20±20.9 to 84.78±23.6 mg/dL. R1 gave a significantly higher ($P < 0.05$) compared with R2, R3, and R4 treatments. R2 gave a significantly ($P < 0.05$) higher blood triglyceride level than R3; while there was no significant different ($P > 0.05$) was observed on blood triglyceride level between R3 and R4. The results indicated that the higher the MBHI meal level in the diets, the higher the blood HDL level of native chicken in the present study. It can be said that MBHI was able to increase blood HDL level of native chicken in the present study. High blood HDL level can prevent the incidence of atherosclerosis. HDL

is a lipoprotein that function as a carrier for cholesterol from body tissues to liver. The excess of cholesterol is transported by HDL to the liver (Murray et al*.,* 2003) degraded and excreted as bile acids.

Blood cholesterol level in the present study is about 124.98-155.97 ml/dL. R1 gave the highest blood cholesterol level and R3 gave the lowest blood cholesterol level. The average blood cholesterol level found in the present study was in agreement with a finding by Iriyanti et al., (2014) who reported that blood cholesterol level in native chicken ranged from 123.04±7.07 to 170.27±9.68 ml/dL. Statistical analysis revealed that treatments gave a significant difference (P< 0.05) on blood cholesterol level. R1 (control diet without MBHI meal in the diet) had a higher (P<0.05) blood cholesterol level compared with R2, R3, and R4 treatments. R2 (5% MBHI meal in the diet) also gave a higher (P < 0.05) blood cholesterol level compared with R3 and R4 treatments. As mentioned above that MBHI used in the present study is high in enzymes activity. So that the higher the MBHI level in the diets, the higher the enzymes activity (more specifically lipase), in turn the higher the production of fatty acids and glycerol.

Blood triglyceride level is determined by feed consumption especially simple carbohydrate such as sugar, saturated fat, high level of circulated free fatty acids, high insulin level, and low glucagon level. Carbohydrate in the liver is broken down to fatty acids and transformed back to triglyceride (Murray, 2012). Chicken manure biodegraded by *Hermetia illucens* L (*Diptera: statiomyidea)* (MBHI) in the present study is assumed to have high enzymes (amilase, lipase, and protease) activity specifically lipase. Initial study (Manangkot, 2014) revealed that at day 7 of manure after being biodegraded by *Hermetia illucens* L (*Diptera: statiomyidea)*and day 8 of larvae growth in the manure, gave a highest amylase, protease, and lipase enzymes activity. Lipase showed a value of 44.84±17.39 mg/dL. It is assumed that the higher the level of MBHIL in the diets, the higher the lipase activity. Lipase is a group of enzyme that normally hydrolyzes triglyceride to free fatty acids and glycerol. This what was expected to be a key factor in decreasing blood triglyceride level in chicken (Mingrui Yu et al., 2007 in Supriyatna et al., 2015).

**Low Density Lipoprotein (LDL).**Blood LDL level in the present study ranged from 45.68 to 81.06 ml/dL. R1 (control diet without MBHIL meal in the diet) gave a highest blood LDL level of about 81.06 ml/dL; whereas R3 (10% MBHIL meal in the diet) gave a lowest blood LDL level of about 45.68 ml/dL. The normal level of LDL for broiler chicken is about 95 – 125 ml/dL. Statistical analysis revealed that treatments gave a significant difference (P < 0.05) on blood LDL level of native chicken in the present study. The result indicated that the higher the MBHI meal level in the diets, the lower the blood LDL level. It can be said that MBHIL has an ability in lowering blood LDL level of native chicken in the present study. Lower blood LDL level in the present study compared with a normal level (46.68-81.06 vs 95 – 125 ml/dL) is a result of high MBHI meal level in the diets, so that the diets have more active enzyme to mobilize cholesterol and LDL itself. LDL is a lipoprotein that acts as a main carrier for cholesterol to be transported to the tissues. The blood level of LDL is highly influenced by cholesterol itself. Montgomery et al., (1993) expalined that blood LDL level is simultaneously produced with triglyceride and cholesterol.

**High Density Lipoprotein (HDL).** The average blood HDL level in the present study is 75,85 – 86,28 mg/dL. The highest blood HDL level is showed by R3 (10% MBHI meal in the diet), whereas the lowest blood HDL level is showed by R1 (control diet without MBHIL meal in the diet). The average blood HDL level in the present study is in agreement with a research results by Hariyanto et al., (2016) who reported that average blood HDL level in chicken is about 87.26 ±5.16 mg/dL. Statistical analysis revealed that blood HDL level among treatment showed a significant defferences (P< 0.05). R1 (control diet without MBHI meal in the diet) has a lower (P < 0.05) blood HDL level compared with R2, R3, and R4. Blood HDL level of R2 (5% MBHI meal in the diet) was significantly lower (P < 0.05) than R3 (10% MBHI meal in the diet). Blood HDL level of R4 (15% MBHI meal in the diet) was significantly lower (P < 0.05) than R2 and R3; while blood HDL level of R3(10% MBHI meal

in the diet) was significantly high($P< 0.05$) than the rest of treatments. The results indicated that the higher the MBHI meal level in the diets, the higher the blood HDL.level of native chicken in the present study. It can be said that MBHI was able to increase blood HDL level of native chicken in the present study. High blood HDL level can prevent the incidence of atherosklerosis. HDL is a lipoprotein that function as a carrier for cholesterol from body tissues to liver. The excess of cholesterol is transported by HDL to the liver ((Murray et al., 2012) degraded and excreted as bile acids. Approximately 75-80% of cholesterol in HDL particles will be converted to HDL particles (Mayes et al. 1997).

**Cholesterol.** Cholesterol is a main lipid component that functions as a precursor of all steroid hormones and bile acids. Cholesterol is synthesized in the liver. Blood cholesterol level in the present study is about 124.98-155.97 ml/dL. R1 gave the highest blood cholesterol level and R3 gave the lowest blood cholesterol level. The average blood cholesterol level found in the present study was in agreement with a findings by Iriyanti et al. (2014), who reported that blood cholesterol level in native chicken ranged from 123.04±7.07 to 170.27±9.68 ml/dL. Statistical analysis revealed that treatments gave a significant difference ($P < 0.05$) on blood cholesterol level. R1 (control diet without MBHI meal in the diet) had a higher ($P < 0.05$) blood cholesterol level compared with R2, R3, and R4 treatments. R2 (5% MBHI meal in the diet) also gave a higher ($P < 0.05$) blood cholesterol level compared with R3 and R4 treatments. It can be said that the higher the MBHIL meal level in the diets, the lower the blood cholesterol level in native chicken in the present study. It is indicated that MBHIL meal in the diets could reduce blood cholesterol level of native chicken in the present study. The explanation is that changes in blood cholesterol level is a direct response related to the rate of free fatty acids change in the diets as a result of increased MBHIL meal level in the diets. In this case, free fatty acids is converted to acetyl co-A as a main precursor for cholesterol formation (Lovita, 2005). In the body, cholesterol is a precursor for bile acid in producing bile salts to emulsify fats. The production of these bile salts requires cholesterol. So, when level of cholesterol in the liver is low, high density lipoprotein (HDL) will mobilize cholesterol from body tissues to accomplish its requirement for cholesterol (Mayes, 1997). As mentioned above that MBHIL used in the present study is high in enzymes activity. So that the higher the MBHIL level in the diets, the higher the enzymez activity (more specifically lipase), in turn the higher the production of fatty acids and glycerol. As a consequence it trigger the production of bile salt to emulsify fatty acids, then it will increase the use of cholesterol to balance the high secretion of bile salt and the end results is lower blood cholesterol level.

## CONCLUSIONS

Chicken manure biodegraded by *Hermetia illucense larvae* (MBHI )can be used up to 10% in the diets to maintain blood triglyceride, cholesterol, LDL, and HDL in a normal level. High blood HDL level at 10% MBHI meal in the diet not only maintains blood HDL at normal level but also increases native chicken meat quality, as was expected in the present study.

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# BODY CONDITION SCORE AND IT CORRELATION WITH RANK AND AGE OF LACTATION IN HOLSTEIN LIVESTOCK FROM ROMANIA

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***Abstract***

*Body Condition Score (B.C.S.) is a good indicator for estimating the reserves, especially of energy, that the dairy cows have to support milk production. In general, the values that this indicator can take vary between 1 and 5 with the evolution of lactation over time, but also from one cow to another. Good dairy cows have difficulties maintaining B.C.S. at an optimal level, especially during the first and second lactation period, since the ingestion capacity of nutrients they have is exceeded by the body's need for milk production. For this reason, the negative energy balance is installed. In order to cope with this period, animals dispose their body fat, thus setting up the negative energy balance, a phenomenon that significantly affects certain zootechnical efficiency parameters such as productive longevity, lactation duration, calving interval, etc. Nutritionists can mitigate this impact by optimizing ratios also taking into consideration this parameter. The present paper aims to determine the correlation between the rank and age of lactation with the value of B.C.S.*

***Key words****: Body condition score, body fat, good indicator, optimizing ratios, negative energy balance.*

## INTRODUCTION

BCS is a very good indicator for estimating how the dairy cow organism will cope with the production effort and, at the same time, it is also a good parameter for assessing the efficiency of farm feeding technology management (Tedeschi et al., 2006). The ideal situation is where the cows have a body condition score of approx. 3 throughout the productive life (if the reference scale takes values between 1 and 5) and without too much variation correlated with the physiological state.

As we have previously mentioned, the most common method of assessing BCS is to label animals on a scale of 1 to 5 (M'Hamdi et al., 2012), where 1 is associated with an emaciated cow and 5 with an overweight animal that has health and reproduction problems (fig. 1 and 2). The BCS dynamics of a cow can also provide information about its welfare due to the correlations that exist between it and the factors of influence such as food quality and quality, ambient temperature, water quality, vital space, adaptability to the exploitation system, etc.

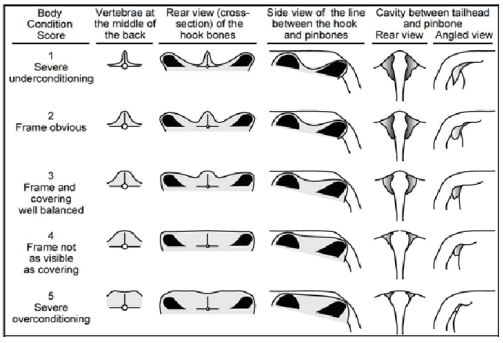


Figure 1. Evaluation of BCS on a scale of 1 to 5 (spiritedrose.wordpress.com)

Classically, animal marking is done with the help of specialists in assessing animal body condition.

These people assess the degree of development of fat deposits at certain points of the cow's body by means of semiological methods such as inspection and palpation.

This method has some degree of subjectivity. For this reason, with the development of precision technologies, a series of automatic BCS assessment methods have emerged.

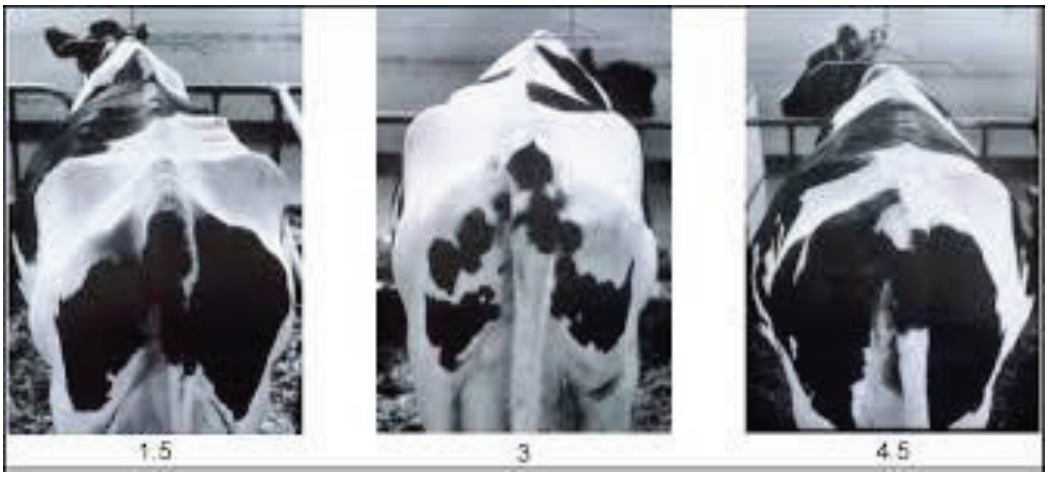


Figure 2. Appearance of some cows and the score obtained as a result of BCS evaluation(bizplan-uz.com)

## MATERIALS AND METHODS

In order to determine the correlation values between BCS and the age of lactation or its rank, 2,564 Holstein lactating cows exploited in 8 farms in Romania (Table 1) were evaluated. Of these, only 1,415 generated accurate information to make calculations, and the rest of the records (27.34%) were removed either due to measurement errors or because of lack of information. The study was conducted on dairy cows being between the first and the eighth lactation, and with a lactation age between the day 1 and day 1,357.

Table 1. Cow distribution on farms

| FARM | Livestock | % |
|---|---|---|
| Farm 1 | 199 | 10.68% |
| Farm 2 | 158 | 8.48% |
| Farm 3 | 16 | 0.86% |
| Farm 4 | 251 | 13.47% |
| Farm 5 | 120 | 6.44% |
| Farm 6 | 198 | 10.63% |
| Farm 7 | 330 | 17.71% |
| Farm 8 | 39 | 2.09% |
| Farm 9 | 199 | 10.68% |
| Farm 10 | 353 | 18.95% |
| TOTAL | 1863 | 100.00% |

BCS measurement was done with a BodyMat V equipment (figure 3) produced by Ingera Company that can accurately estimate the body condition of the cows, as well as weight or other physiological parameters using a picture (fig. 4) analysis algorithm (ingera.ch).

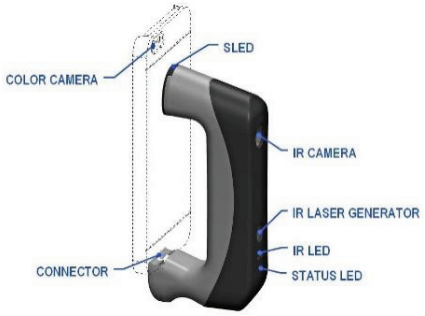


Figure 3. BodyMat V (ingera.ch)

Figure 4. BodyMat V in action (ingera.ch)

The device can connect to a smartphone and features an integrated laser generator, capable of collecting 3D images and interpreting and compiling data about the animal. The package contains the BodyMat Vet, a smartphone, two chargers (one for the actual d, vice and one for the smartphone) and earphones (fig. 5).


Figure 5. BodyMat Vet packge (docs.wixstatic.com)

After downloading the data, they are sent to a computer, and then can be exported to Excel.

## RESULTS AND DISCUSSIONS

The gross data collected from the field were synthesized and systematized in Table 2. Out of the 1,415 cows for which BCS was evaluated, 43.33% were lactating first, 30.88% at second lactation, 12.27% at third lactation, 6.93% at fourth lactation, and 6.15% of herd at fifth lactation or more.

Table 2. Synthesation and systematisation of data

| SPECIFICATION | n | X | s | Sx | V |
|---|---|---|---|---|---|
| **Lactation rank** | **613** | **1.00** | **0.00** | **0.04** | **0%** |
| Cow age (years) | | 3.04 | 0.93 | 0.12 | 31% |
| BCS value | | 2.87 | 0.56 | 0.12 | 19% |
| Lactation age (days) | | 254.60 | 169.56 | 10.28 | 67% |
| **Lactation rank** | **437** | **2.00** | **0.00** | **0.10** | **0%** |
| Cow age (years) | | 3.81 | 0.65 | 0.18 | 17% |
| BCS value | | 2.58 | 0.57 | 0.12 | 22% |
| Lactation age (days) | | 179.78 | 149.25 | 8.60 | 83% |
| **Lactation rank** | **180** | **3.00** | **0.00** | **0.22** | **0%** |
| Cow age (years) | | 5.19 | 1.02 | 0.39 | 20% |
| BCS value | | 2.61 | 0.64 | 0.19 | 24% |
| Lactation age (days) | | 192.22 | 140.42 | 14.33 | 73% |
| **Lactation rank** | **98** | **4.00** | **0.00** | **0.40** | **0%** |
| Cow age (years) | | 6.53 | 0.94 | 0.66 | 14% |
| BCS value | | 2.64 | 0.56 | 0.27 | 21% |
| Lactation age (days) | | 205.52 | 139.04 | 20.76 | 68% |
| **Lactation rank** | **87** | **+ 5** | **0.94** | **0.62** | **16%** |
| Cow age (years) | | 8.50 | 1.49 | 0.91 | 18% |
| BCS value | | 2.60 | 0.60 | 0.28 | 23% |
| Lactation age (days) | | 167.98 | 134.53 | 18.01 | 80% |
| **Lactation rank** | **1415** | **2.06** | **1.33** | **0.05** | **64%** |
| Cow age (years) | | 4.13 | 1.76 | 0.11 | 43% |
| BCS value | | 2.72 | 0.59 | 0.07 | 22% |
| Lactation age (days) | | 214.83 | 159.89 | 5.71 | 74% |

For cows at first lactation, a BCS with an average value of 2.87 ± 0.56 was recorded. The average age of the cows was 3.04 ± 0.93 years and the mean lactation age was 254.90 ± 169.56 days. For BCS a coefficient of variability of approx. 19% was measured, this value meaning that, for this group of cows, BCS has a relatively medium variability.

For second lactation cows, BCS recorded a value of 2.58 ± 0.57, with a 22% variability coefficient (average variability). This can be justified by the fact that at the second lactation the cows are much stronger. Their average age was 3.81 ± 0.65 years, and lactation averaged 179.78 ± 149.25 days.

In the case of the third lactation, there was an average age of 5.19 ± 1.02 years and an average lactation age of 192.22 ± 140.42 days. BCS was 2.61 ± 0.64 with a 24% variability.

Fourth lactation cows recorded an average BCS of 2.64 ± 0.56 with a coefficient of variation of 21%. They have an average age of 6.53 ± 0.94 years and an average lactation age of 205.52 ± 139.04 days.

For cows that were in the fifth or more lactation, similar data were recorded, BCS having a value of 2.60 ± 0.60 and a coefficient of variation of 23%. The average age of cows was 8.50 ± 1.49 years, and the average lactation age was 167.98 ± 143.53 days.

Overall, the study population recorded an average BCS of 2.72 ± 0.59 with a 22% variability coefficient. The average age was 4.13 ± 1.76 years, and the mean lactation was at the 214.83 ± 159.89 day.

Figure 6 shows the visual relationship between the BCS value and the age evolution of the animals. Considering its aspect, it can be stated that the studied cows have, throughout their productive life, optimum conditions of exploitation although trend line has a slightly downward trend.

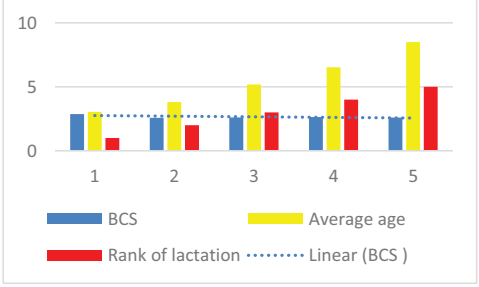


Figure 6. Evolution of BCS over age

Considering its aspect, it can be stated that the studied cows have, throughout their productive life, optimum conditions of exploitation though trendline has a slightly downward trend.
Table 3 summarizes the BCS situation in the 8 farms.

Table 3. BCS situation in the studied farms

| Specification | X | s | Sx | V |
|---|---|---|---|---|
| **Farm 1** | | | | |
| Cowas age | 4.84 | 2.04 | 0.34 | 42% |
| BCS | 2.58 | 0.61 | 0.18 | 24% |
| Average lactation age | 241.49 | 173.90 | 17.12 | 72% |
| Lactation rank | 2.52 | 1.62 | 0.18 | 64% |
| **Farm 2** | | | | |
| **Cowas age** | **4,23** | **1,68** | **0,34** | **40%** |
| **BCS** | **2,37** | **0,52** | **0,19** | **22%** |
| **Average lactation age** | **131,73** | **129,54** | **10,31** | **98%** |
| **Lactation rank** | **2,63** | **1,28** | **0,21** | **49%** |
| **Farm 3** | | | | |
| Cowas age | 6.04 | 3.27 | 1.46 | 54% |
| BCS | 2.65 | 0.58 | 0.64 | 22% |
| Average lactation age | 194.29 | 120.53 | 47.12 | 62% |
| Lactation rank | 3.18 | 2.55 | 0.77 | 80% |
| **Farm 4** | | | | |
| **Cowas age** | **4.32** | **1.97** | **0.27** | **46%** |
| **BCS** | **2.60** | **0.52** | **0.16** | **20%** |
| **Average lactation age** | **222.47** | **155.26** | **14.04** | **70%** |
| **Lactation rank** | **2.33** | **1.46** | **0.15** | **63%** |
| **Farm 5** | | | | |
| Cowas age | 3.08 | 0.68 | 0.22 | 22% |
| BCS | 3.16 | 0.52 | 0.22 | 16% |
| Average lactation age | 217.92 | 111.76 | 15.45 | 51% |
| Lactation rank | 1.22 | 0.58 | 0.09 | 48% |
| **Ferma 6** | | | | |
| **Cowas age** | **5.15** | **1.91** | **0.82** | **37%** |
| **BCS** | **2.58** | **0.34** | **0.41** | **13%** |
| **Average lactation age** | **181.85** | **105.75** | **29.12** | **58%** |
| **Lactation rank** | **1.00** | **0.00** | **0.16** | **0%** |
| **Farm 7** | | | | |
| **Cowas age** | 4.36 | 1.84 | 0.31 | 42% |
| **BCS** | 2.86 | 0.60 | 0.20 | 21% |
| **Average lactation age** | 229.60 | 165.09 | 16.28 | 72% |
| **Lactation rank** | 2.10 | 1.35 | 0.15 | 64% |

Table 3. BCS situation in the studied farms (continuation)

| Specification | X | s | Sx | V |
|---|---|---|---|---|
| **Farm 8** | | | | |
| Cowas age | **3.80** | **1.26** | **0.20** | **33%** |
| BCS | **2.72** | **0.55** | **0.14** | **20%** |
| Average lactation age | **227.12** | **179.06** | **12.09** | **79%** |
| Lactation rank | **1.89** | **0.93** | **0.10** | **49%** |

Concerning the average age of the cows per farm, the minimum was recorded on the farm 5 (3.08 ± 0.68 years) and the maximum in farm 3 (6.04 ± 3.27 years).
The minimum BCS was recorded on farm 2 and had the value of 2.37 ± 0.52 and a coefficient of variability of 40% (high variability). Maximum BCS was recorded on farm 5 and had a value of 3.16 ± 0.52 and a 16% variability coefficient (mean variability).
The mean age of lactation was recorded on farm 2 (131.73 + - 129.54 days), and the maximum on farm 1 (241.49 ± 173.90). Interestingly, there were minimal values in farm 2 for both the median age of lactation and BCS. This is explained by the negative energy balance phenomenon that occurs during the first two stages of lactation and which negatively affects the body condition of the cows. From this point of view, we can divide the lactation curve into 3 phases: the ascending phase (the first 40 to 50 days after calving), the plateau phase (the next 50 days) and the descending phase (from 90 –100$^{th}$ day after calving to the end of lactation) (Grosu and Rotar, 2015).In very good dairy cows, the first 2 phases after calving can cause major nutritional imbalances that can affect the rest of their productive life (fig. 7). During this period, the nutritional needs exceed the intake capacity of the cows and thus the negative energy balance is installed. This means that the cow will consume its own fat reserves to support the evolution of milk production, and in time will lose weight and vigor.
For lactation to have a lesser negative impact on the cow's body, it is recommended to maintain BCS around 2.75-3.25. In the case of the studied livestock, the results are centralized in Table 4.

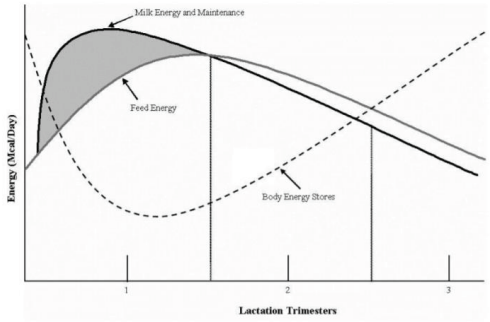

Figure 7. Energy utilisation during lactation (Hoffman et al., 2000)

Table 4. BCS in the 3 phases of lactation

| Specification | n | X | s | Sx | V |
|---|---|---|---|---|---|
| Phase I | 249 | 2.43 | 0.57 | 0.15 | 23% |
| Phase II | 162 | 2.36 | 0.49 | 0.19 | 21% |
| Phase III | 1004 | 2.84 | 0.56 | 0.09 | 20% |

It is noted that between the first two stages of lactation, BCS decreases by about 2.88%, and between the second and third phases it increases by 20.34%. The BCS value during the third lactation phase is 16.87% higher than the value recorded during the first phase.
From the perspective of the correlation between BCS and the age and the rank of lactation, the data from table 5 was obtained.

Table 5. BCS correlations

| with the | Value |
|---|---|
| age of lactation | +0.42 |
| rank of lactation | -0.15 |

As expected, BCS has a positive-medium correlation with age of lactation and negative-low with its rank.

## CONCLUSIONS

BCS is an important indicator for the assessment of lactation evolution.
The livestock studied here has an optimal BCSvalue (2.72 ± 0.59 with a CV of 43%).
BCS has a positive-medium correlation with age of lactation and negative-low with its rank

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# THE CLIMATE FACTORS IMPACT ON THE NECTARIFEROUS QUALITIES OF *Phacelia tanacetifolia* Benth

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***Abstract***

*An analysis of the influence of climatic factors on the honey qualities of Phacelia tanacetifolia Benth was carried out by conducting the field experiments. The main objective is to study the influence of air temperature and humidity on the formation and extraction of nectar from plants flowers. Determine the impact of climatic factors on the flowering duration, the number of plant blooms, the amount of nectar, and the composition of sugars in the nectar.*

***Key words:*** *special honey plants, honey production, Phacelia tanacetifolia, climatic factors, nectar sugar content.*

## INTRODUCTION

Additions to the climate system are signify-cantly faster than those in the past millennium. They cause variations in the amount and distribution of precipitation in many regions, leading to a strong occurrence of very high temperatures, drought or floods. Due to the nonlinear nature of climatic influences and ecosystem sensitivity, even minor temperature fluctuations can have serious consequences.

The higher temperatures can, induce shifts in plant phenology and change the interaction networks between plants and pollinators (Takkis et al., 2015; Schweiger et al., 2010; Petanidou et al., 2014). Most of plants species have an optimum temperatures range for proper development- around 35$^{o}$C. The temperature also directly affects a nectar production and the amount of sugar produced per flower. A number of studies from have found decreased nectar volumes and nectar production rates at higher temperatures under both experimental and natural conditions (Jakobsen and Kristjánsson, 1994; Petanidou and Smets, 1996; Keasar et al., 2008). At the other hand, nectar sugar concentration is usually less dependent on external factors and more constant throughout the flowering season. In relation to this, evaluation of the climate parameters is a very important in the context of global warming. Agro-meteorological and phenological observations are a valuable source of information about the relationship between climate and development of plants during the growing season.

"Special honey plants" are not a large group that is used in some area in case of lack of bee grazing. In beekeeping, they are known as special honey plants, they release a large amount of nectar and pollen. To prevent periods without any harvesting, sowing of special honey plants is very importance.

The *Phacelia* takes the first place in the production of nectar and pollen among honey plants (Bijev, 2003). It is used for green fertilization as an intermediate crop, such as a decorative plant, for fodder and honeybee pasture. The phacelia is an annual dicotyledonous plant of the family *Hydrophilaceae*. Originally from North America, individual species occur in Asia, Africa and Hawaii.

In Bulgaria, the phacelial occurs only as a cultural species. And it is one of the best honey plants with a long flowering period and a large amount of nectar (Talevnina, 2016).

The stem is erect and highly branched, is 50-80 cm high. The leaves are consecutive, pinnate cuties, with short handles, bristly glared. Flowers are normal, two-poles, forming multi-colour thick inflorescences, which are twisted in a spiral (Petkov, 1989).

They have a characteristic aroma that strongly attracts bees (Naumkin, 2001). The *Phacelia* is characterized by a rapid initial development, a

very high degree of coverage and shading of the soil surface, thereby greatly suppressing the growth of weeds. She has a very good tolerance to drought, a strong root with a moderate share of fine roots mainly in the upper soil layer (up to 20 cm). The phacelia is very suitable as a roofing culture. It can be sown by direct sowing (Naumkin, 2001; Lembacher, 2009).

The *phacelia* flowers give a significant amount of nectar of varying intensity during the flowering of each individual flower (Carreck, 1997; Williams, 1991). It can be said that this is an excellent honey plant, emitting large amounts of nectar and pollen. According to Petkov (1989), for the Vidin region of 1 da it was produced from 33 to 36 kg of honey, and for the Sofia region - from 21 to 37 kg.

The global studies give evidence of more than 50-60 kg of honey obtained by 1 da.

These positive characteristic of the phacelia plant have led us to an in-depth study and observe the influence of climatic factors on honey qualities.

According to Tsankov (2004), the extricate of nectar from plants depends on many factors.

One of the main and important factors which directly affect the nectar productiveness is air temperature.

Plants nectar extraction begins above 10-12˚C and increases with increasing of temperature. The most favourable temperature for the release of nectar in plants is 16-25˚C. When the temperature decreases at night, nectar secretion decreases sharply. The optimal temperature is different for a different plants species and is closely related to a number of other factors.

Another important factor is air humidity. The optimal air humidity for most plants is around 60-80%. It has been found that higher humidity increases the amount of nectar but reduces the sugar content in it. Prolonged rainfall reduces photosynthesis and less nectar is formed. The rains stimulate the growth of the green parts of the plants and thus retain the blossom development, on the other hand prolonged rainfall wash the nectar from flowers.

Dry winds, combined with high temperature and low relative humidity, drastically reduce the amount of nectar compartment and even the deformation of nectarines (Petkov, 1989).

In Bulgaria, there are not sufficiently studies related to the impact of climate factor on honey plants and its productivity.

## MATERIALS AND METHODS

The studies were conducted in the experimental field of Vrajdebna - University of Forestry, Sofia, in 2018 on an alluvial meadow soil with an area of 150 $m^2$. The experiment is based on the standard method and scheme in 6 variants as fallow:

I-Control
II-Control + Biochar
III - Manure
IV- Manure + Biochar
V- Compost
VI - Compost + Biochar

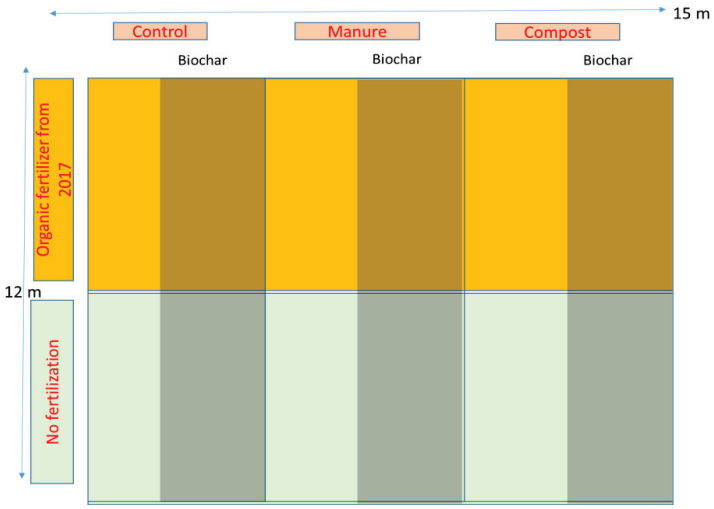


Figure 1. Experimental scheme

The imported BC is 15 kg/da in all variants. The norm is selected based on our previous BC studies conducted on the same field.

The single variants, which are as control, have optimal amounts of used ameliorants. This amounts were determined by literature data. In combine variants, the main point is on the ability of BC to improve soil fertility when is combined with different organic fertilizer types and compositions.

The biochar for the experiment is provided by a licensed manufacturer and is fragmented into a finer fraction before its being introduced into the soil. In order to determine the influence of BC on the soil properties, field experiments with *Phacelia tanacetifolia* Benth were carried out. The sowing data is 20.04.2018.

The followed parameter is: start and duration of flowering; Flowering duration of a separate blossom; Number of blossoms per plant; Amount of nectar of 1 blossom in mg; Percentage of sugar content in nectar (Brix%);

## RESULTS AND DISCUSSIONS

For the purpose of the experiment, information on climatic conditions and changes has been collected. An analysis of the meteorological conditions for the last 31 years was carried out in order to track the climate change for the Sofia field (Fig. 2). Information about the annual rainfall in the Sofia region was also collected.

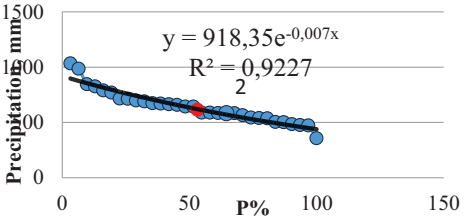


Figure 2. The precipitation provision curve for the period 1987-2018

Information on annual rainfall in the Sofia region is also collected. On the basis of this information curve of probability of precipitation per year has been prepared and according to the results obtained in 2018 it is characterized by secure close to 53%, which determines it as a mean dry year.

The weather conditions during the experimental period (April-September), is presented on Figure 3.

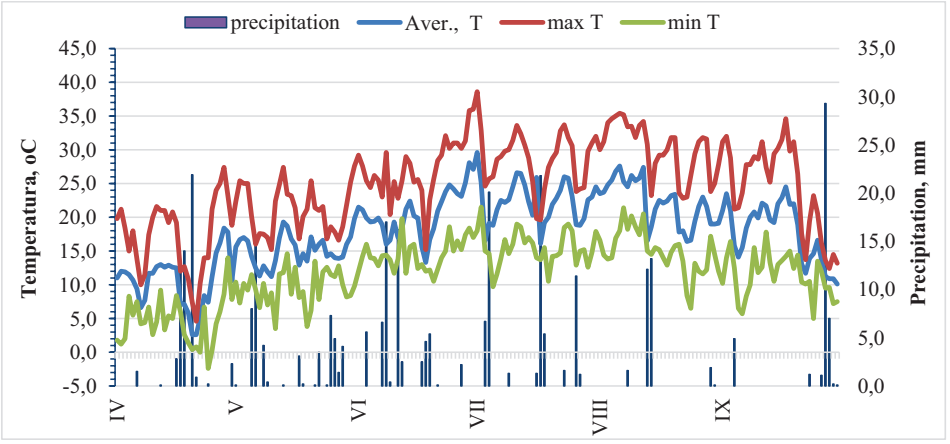


Figure 3. Temperatures and rainfall during the vegetation of phacelia 2018

Higher temperature values are observed, and at the beginning of July, the maximum reading is 38.6°C. The reported temperatures are on average by +1.2°C, higher than the data published by the National Institute of Hydrology and Meteorology, compared to the norms for the period 1961-1990.

Precipitation are relatively uniform, but their amount in most case exceeds 10 mm, this indicating that the plants is under water stress conditions. High temperatures in July and August and water stress alter many physiological processes during the plant life cycle and affect plants at the molecular, cellular, and

organismal level. Water stress leads to stomatal closure, reducing water potential and $CO_2$ uptake, thus leading to inhibition of photosynthesis (Descamps et al., 2018).
Rainfall in April and May is below the average for a long time in the region of Sofia - 54 mm and 72 mm. The average monthly air temperature does not exceed 30°C. At the end of September, there is a sharp drop in temperature (from 16°C to 9°C) and the minimum values are -2.5°C.
The data shown in Figure 4 clearly demonstrates the presence of water deficiency during the growth of the *Phacelia*. The line indicating the average monthly precipitation values is permanently reduced below the average temperature line. This clearly indicates the need for irrigation during the period.
The environmental factors also have a significant impact on the quantity and quality of the nectar produced by the flower. Temperature, solar radiation, vapour pressure, and soil moisture all may affect nectar production (Corbet, 1990; Michaud, 1990).

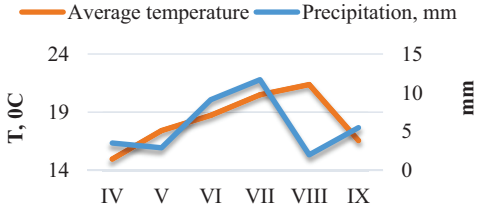


Figure 4. Average temperature and precipitation in failed experiment-2018

The figure 5 represents the average values of relative humidity. The solar radiation and the relative humidity have a direct impact on honey productivity of plants.

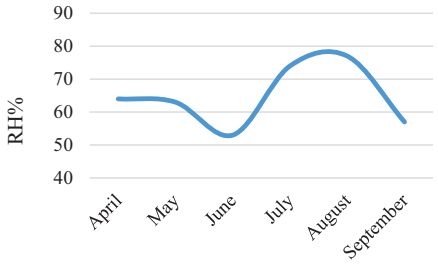


Figure 5. Monthly relative humidity during the failed experiment-2018

The high temperatures during the study period combined with low atmospheric humidity and water deficiency dramatically reduce the separation of nectar. Under these conditions, deformation of the nectarines of the plants is possible; it is not accounted for in this experience.
The amount of nectar increases and the bees find it difficult him collect.
There is a close relationship between solar radiation and relative humidity. The lowest values are in June and the highest first ten days August.
Phase start flowering was recorded one month after sowing the seeds end of May.

Table 1. Number of flower in one inflorescence

| Variant | Number of flowers in initial flowering | Number of flowers in oneplant |
|---|---|---|
| I-Control | 1–2 | 159 |
| II-Control + Biochar | 2 | 162 |
| III - Manure | 3–4 | 200 |
| IV- Manure + Biochar | 6 | 300 |
| V- Compost | 4–5 | 254 |
| VI - Compost + Biochar | 5 | 265 |

Table 1 shows the results obtained for the number of flowers in the beginning and mass flowering phases. Initially, flowering began with variants III and IV with fertilizer and biochar. In these variants, there was a vigorous growth and increased development of the generative organs of the plants. In the initial flowering phase, we observed the blossoming of 1 to 6 of flowers in inflorescence, gradually increasing their number and reaching from 250 to 300 of flowers in one plant. We observed similar results with other variants. In version I and II (Control and Control + Biochar), flowering started later with a smaller number of flowers (1-2). The blossoming of the flowers in the inflorescence occurs from the bottom up.
The long flowering period of this species is because the individual flowers blossom in succession from the base to the top of the inflorescence.
Figure 6 shows the results for the sugar content in the nectar.

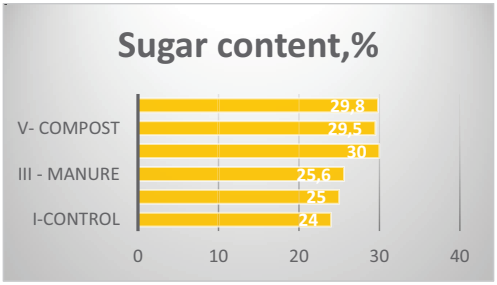


Figure 6. Sugar content, %

Sugar content is higher in variants, III – Manure, IV- Manure + Biochar,V- Compost, VI - Compost + Biochar, from 25.6% to 30.0% and lower in control variants 24-25%.
The presence of manure and biocharis a good solution for improving the soil properties and for honey productivity plants despite the unfavorable climatic conditions.

## CONCLUSIONS

The weather conditions during flowering have a strong influence on the production of nectar from the flowers of the plants. The most favorable temperature is from 16 to 25°C, and the minimum that most plants begin to produce nectar is about 10-12°C. During the development of Phacelia tanacetifolia weather conditions were not suitable for optimal amounts of nectar, reported a water deficit and dry periods. The high temperatures during the study period combined with low atmospheric humidity and water deficiency dramatically reduce the separation of nectar. The concentration of nectar increases and bees find it difficult him collect.

## ACKNOWLEDGEMENTS

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# MONTHLY CHANGES OF BEHAVIORAL CHARACTERISTICS IN HOLSTEIN-FRIESIAN, BROWN SWISS AND SIMMENTAL BULLS

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***Abstract***

*Behaviors of Holstein-Friesian (HF), Brown Swiss (BS) and Simmental (SIM) bulls were determined under the Mediterranean conditions for a period of six months. A total of35 bulls were fed in two groups (10 HF and 8 BS in group I and 10 SIM and 7 HF bulls in group II). At the time of high feed consumption, the tendency to drink water from all breeds was also high and HF bulls had higher drinking and elimination behavior rates than those of BS and SIM bulls especially in hot summer months. All breeds preferred to perform locomotor activities late in the evening during the hot summer months. The bulls decreased feeding, standing and locomotion activities during hot hours at a lower rate or postponed these behaviors to the cooler hours of the day, but they increased lying and rumination activities in those hours. While HF bulls were more affected by higher temperatures than SIM and BS bulls, taking precautions against high temperature on farms level would lead to increase the fattening performance and also the welfare of the bulls.*

***Key words*****:** *behavioral changes ,cattle breeds, fattening, heat stress.*

## INTRODUCTION

In beef production, more importance has been given to the genetic improvement and nutrition, however, environmental factors and animal welfare aspects were generally pushed into the background. It is admitted that the studies on the behaviors of fattening cattle have been neglected for a long time but, there has been an increasing interest on the behaviors of fattening cattle (Brown-Brandl et al., 2006). In earlier studies, under hot environmental conditions, the behavioral changes in fattening bulls (Dikmen, 2013; Rosselle et al., 2013; Zgur et al., 2014), in heifers (Mitlöhner et al., 2001; Mitlöhner et al., 2002), in steers (Tapki, 2012) and in dairy cows (TapkiandSahin, 2006) were determined. In these studies, the changes of losing appetite and decreasing feed intake, activity (Brown-Brandl et al., 2006), increasing water intake (Mitlöhner et al., 2002, Dikmen, 2013) and spending more time for standing (Cook et al., 2007) were mentioned. However, no studies were conducted to determine the behaviors of the three most common cattle breeds, Holstein-Friesian (HF), Brown Swiss (BS) and Simmental (SIM) bulls. Therefore, in this study the effects of environmental factors on the behaviors of fattening bulls and behavioral differences among HF, BS and SIM bulls under the Mediterranean climatic conditions by using scan sampling technique were aimed to be determined.

## MATERIALS AND METHODS

This study was performed with the ethical permission (IX. Session held on October 8, 2013) of ADU-HADYEK. The study was carried out at a farm located at 37°46’55.2’’N and 28°4’9.12’’E in Turkey. Temperature Humidity Index (THI) was calculated by using the temperature and relative humidity records (HOBO U10) in the barn (Kibler1964).

In group I, 10 HF and 8 BS, in group II, 10 SIM and 7 HF bulls aged 8-12 months old were fed in two paddocks. Each paddock area was 120 $m^2$. Rumination, standing, lying, walking, feeding, drinking, mounting, agonistic, defecation and urination behaviors of the bulls were monitored every Monday for one hour at 06:00, 09:00, 12:00, 14:00, 17:00, 20:00 and 23:00 for 10 min period from February to August by using scanning sampling technique (Mitlöhner et al., 2001; Mitlöhner et al., 2002; Dikmen, 2013). The animals were fed with wheat straw, tomato meal (24% DM), barley flakes and concentrates. The feed intake,

nutrient components of ration, fattening performance, carcass and beef quality of the bulls were reported in another study by Çatıkkaş and Koç (2017).

Prior to the statistical analysis of the data, an arcsine-square root transformation was performed on the behavioral data (Mitlöhner et al., 2001).Statistical analysis of data was performed with using PROC GLM procedure of Statistical Analysis System (SAS, 1999). The differences between LSMEANS of the fixed factor levels were taken into account to be statistically significant at P<0.05 (2-tailed) based on Tukey's adjustment type I error rate. Statistical model used for the analysis of data is given in Equation I as follow:

$$y_{ijkl}=\mu+a_i+b_j+c_k+(ab)_{ij}+(ac)_{ik}+(bc)_{jk}+e_{ijkl} \quad (1)$$

where $\mu$ is the overall mean, $y_{ijkl}$ is the observation of the behavior, $a_i$ is the breed effects (i=HF, BS and SIM), $b_j$ is the month effects (j=February, March, …… and August), $c_k$ is the observation hour effects (k=06:00, 09:00, 12:00, 14:00, 17:00, 20:00 and 23:00), $(ab)_{ij}$ is breed (x) month and $(ac)_{ik}$ is breed (x) observation hour, $(bc)_{jk}$ is month (x)observation hour interaction effects and $e_{ijkl}$ is the residual random errors.

## RESULTS AND DISCUSSIONS

***Climatic conditions:*** From June till the end of fattening period, the THI values (Figure 1) were over the threshold level (THI=72) of heat stress in cattle (Ravagnolo and Misztal, 2000; Gantner et al., 2011) and the behaviors of the bulls were affected more or less from the heat stress.

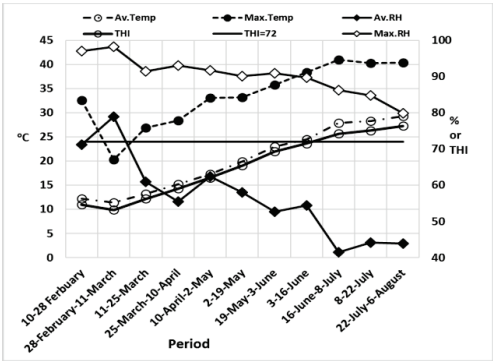
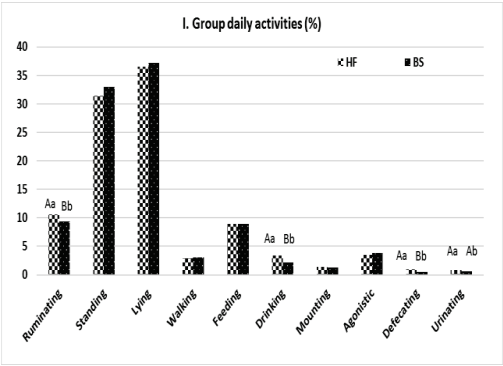



Figure 1. Average and maximum temperature (°C), relative humidity (RH, %) and temperature humidity index (THI) during fattening

It can be said that the bulls in the last three months of the fattening were under thermal comfortless conditions and the physiological, biochemical and behaviors of them could be significantly affected (Rosselle et al., 2013; Umpapol et al., 2014). To decrease the effect of heat stress on the farm level some precautions like providing cool water, changing the ration formulation, establishing evaporative cooling system and etc. need to be takenon this farm and in all the farms of the region.

***Behavioral characteristics*** in group I and II are given in Table 1 and 2, respectively. The daily activities in both groups weremainly similar. The highest daily activities in both groups were lying with about 35% and standing for more than 30% (Figure 2).



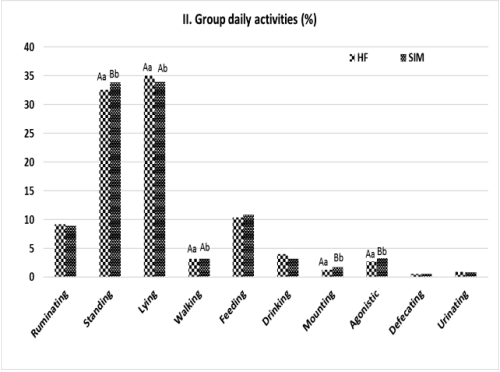




Figure 2. Daily activities (%) of HF, BS and SIM bulls Different letters show differences between the breeds, A, B for P<0.01; a, b for P<0.05

These behaviors were followed by nutritional activities. In both groups, feeding, ruminating and drinking activities occupied more than 20% of the bulls' time.

Table 1. Behavioral characteristics in group I

| Factor | Feeding | Ruminating | Drinking | Walking | Lying | Standing | Mounting | Agonistic | Defecating | Urinating |
|---|---|---|---|---|---|---|---|---|---|---|
| Breed | NS | ** | ** | NS | NS | NS | NS | NS | ** | * |
| HF | 0.089±0.003 | 0.106±0.003$^{Aa}$ | 0.034±0.002$^{Aa}$ | 0.030±0.002 | 0.365±0.007 | 0.314±0.006 | 0.014±0.001 | 0.035±0.002 | 0.009±0.001$^{Aa}$ | 0.008±0.001$^{Aa}$ |
| BS | 0.089±0.003 | 0.094±0.003$^{Bb}$ | 0.022±0.002$^{Bb}$ | 0.031±0.002 | 0.372±0.007 | 0.330±0.006 | 0.013±0.001 | 0.038±0.002 | 0.005±0.001$^{Bb}$ | 0.006±0.001$^{Ab}$ |
| Hour | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** |
| 06:00 | 0.045±0.006$^{ACa}$ | 0.178±0.006$^{Aa}$ | 0.010±0.003 $^{a}$ | 0.010±0.003$^{Aa}$ | 0.580±0.014$^{Aa}$ | 0.153±0.011$^{Aa}$ | 0.004±0.002$^{ABac}$ | 0.004±0.004$^{Aa}$ | 0.005±0.002$^{Aac}$ | 0.004±0.002$^{Aa}$ |
| 09:00 | 0.199±0.006$^{Bb}$ | 0.043±0.006$^{Bb}$ | 0.044±0.003$^{Bb}$ | 0.039±0.003$^{BDbd}$ | 0.133±0.014$^{Bb}$ | 0.471±0.011$^{Bb}$ | 0.015±0.002$^{ADbe}$ | 0.038±0.004$^{BCbd}$ | 0.010±0.002$^{Ab}$ | 0.007±0.002$^{ABab}$ |
| 12:00 | 0.065±0.006$^{AEce}$ | 0.134±0.006$^{ACc}$ | 0.031±0.003$^{Bbc}$ | 0.034±0.003$^{BCDb}$ | 0.222±0.014$^{Cc}$ | 0.434±0.011$^{BDbd}$ | 0.013±0.002$^{ABEab}$ | 0.046±0.004$^{Bb}$ | 0.010±0.002$^{Aab}$ | 0.008±0.002$^{ABab}$ |
| 14:00 | 0.024±0.006$^{Cd}$ | 0.126±0.006$^{Cce}$ | 0.017±0.003$^{ACad}$ | 0.018±0.003$^{ACac}$ | 0.635±0.014$^{Aa}$ | 0.143±0.011$^{Aa}$ | 0.003±0.002$^{Bc}$ | 0.023±0.004$^{ACc}$ | 0.005±0.002$^{Aabd}$ | 0.003±0.002$^{Aa}$ |
| 17:00 | 0.150±0.006$^{De}$ | 0.069±0.006$^{Dd}$ | 0.030±0.003$^{BCbc}$ | 0.030±0.003$^{BCb}$ | 0.281±0.014$^{Cc}$ | 0.391±0.011$^{Cc}$ | 0.006±0.002$^{ABac}$ | 0.029±0.004$^{BCcd}$ | 0.007±0.002$^{Aabd}$ | 0.003±0.002$^{Aa}$ |
| 20:00 | 0.080±0.006$^{Ec}$ | 0.042±0.006$^{Bb}$ | 0.032±0.003$^{Bbc}$ | 0.046±0.003$^{Dd}$ | 0.272±0.014$^{Cc}$ | 0.409±0.011$^{CDed}$ | 0.029±0.002$^{Cd}$ | 0.077±0.004$^{De}$ | 0.004±0.002$^{Aabd}$ | 0.007±0.002$^{ABab}$ |
| 23:00 | 0.057±0.006$^{AEae}$ | 0.100±0.006$^{CDe}$ | 0.027±0.003$^{BCcd}$ | 0.038±0.003$^{BDbcd}$ | 0.453±0.014$^{Dd}$ | 0.247±0.011$^{Ee}$ | 0.023±0.002$^{CDEde}$ | 0.036±0.004$^{Bbd}$ | 0.004±0.002$^{Acd}$ | 0.012±0.002$^{Bb}$ |
| Month | NS | ** | ** | ** | NS | ** | ** | ** | NS | NS |
| February | 0.102±0.006$^{Aa}$ | 0.125±0.006$^{Aa}$ | 0.024±0.003$^{ABab}$ | 0.015±0.003$^{Aa}$ | 0.372±0.015 | 0.321±0.013$^{ACa}$ | 0.010±0.002$^{Aa}$ | 0.018±0.004$^{ADad}$ | 0.008±0.002 | 0.007±0.002 |
| March | 0.094±0.004$^{Aa}$ | 0.095±0.004$^{Aa}$ | 0.023±0.002$^{Aa}$ | 0.031±0.002$^{BCb}$ | 0.347±0.010 | 0.344±0.008$^{ABa}$ | 0.012±0.002$^{Aa}$ | 0.038±0.003$^{BCEbc}$ | 0.008±0.001 | 0.009±0.001 |
| April | 0.082±0.004$^{Aa}$ | 0.102±0.005$^{Aa}$ | 0.016±0.002$^{Aa}$ | 0.042±0.002$^{Bbc}$ | 0.376±0.011 | 0.306±0.009$^{ACa}$ | 0.017±0.002$^{ABab}$ | 0.047±0.003$^{Bbc}$ | 0.005±0.001 | 0.005±0.001 |
| May | 0.085±0.004$^{Aa}$ | 0.076±0.004$^{Bb}$ | 0.033±0.002$^{BCb}$ | 0.042±0.002$^{Bc}$ | 0.383±0.010 | 0.309±0.008$^{ACa}$ | 0.022±0.002$^{Bb}$ | 0.043±0.003$^{Bb}$ | 0.004±0.001 | 0.004±0.001 |
| June | 0.086±0.004$^{Bb}$ | 0.058±0.005$^{Bc}$ | 0.042±0.002$^{Cc}$ | 0.040±0.002$^{Bc}$ | 0.226±0.011 | 0.367±0.008$^{Bc}$ | 0.011±0.002$^{Aa}$ | 0.057±0.003$^{ACac}$ | 0.008±0.001 | 0.006±0.001 |
| July | 0.085±0.009$^{Aa}$ | 0.141±0.009$^{Aa}$ | 0.028±0.004$^{ABCab}$ | 0.016±0.005$^{ACa}$ | 0.407±0.022 | 0.283±0.018$^{Ca}$ | 0.010±0.003$^{Aa}$ | 0.017±0.006$^{DEd}$ | 0.008±0.002 | 0.009±0.002 |
| BreedxHour | NS | NS | NS | ** | ** | NS | NS | ** | NS | NS |
| BreedxMonth | NS | NS | ** | NS | NS | NS | NS | NS | * | NS |
| HourxMonth | ** | ** | ** | ** | ** | ** | ** | ** | * | * |

HF: Holstein-Friesian, BS: Brown-Swiss, NS: not significant, *: p<0.05, **: p<0.01; A,B,C,D,E: Same letter in the column show insignificance for P<0.01, a,b,c,d,e: Same letter in the column show insignificance for P<0.05.



Table 2. Behavioral characteristics in group II

| Factor | Feeding | Ruminating | Drinking | Walking | Lying | Standing | Mounting | Agonistic | Defecating | Urinating |
|---|---|---|---|---|---|---|---|---|---|---|
| Breed | NS | NS | NS | * | * | ** | ** | ** | NS | NS |
| HF | 0.104±0.003 | 0.092±0.003 | 0.040±0.002 | 0.031±0.002$^{Aa}$ | 0.350±0.007$^{Aa}$ | 0.326±0.005$^{Aa}$ | 0.013±0.001$^{Aa}$ | 0.027±0.002$^{Aa}$ | 0.006±0.001 | 0.009±0.002 |
| SIM | 0.108±0.003 | 0.089±0.003 | 0.032±0.002 | 0.032±0.002$^{Ab}$ | 0.339±0.007$^{Ab}$ | 0.338±0.005$^{Bb}$ | 0.017±0.001$^{Bb}$ | 0.033±0.002$^{Bb}$ | 0.006±0.001 | 0.008±0.002 |
| Hour | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** |
| 06:00 | 0.067±0.006$^{Aa}$ | 0.124±0.005$^{Aad}$ | 0.027±0.003$^{Aa}$ | 0.023±0.003$^{Aa}$ | 0.481±0.013$^{Aa}$ | 0.239±0.010$^{AEa}$ | 0.009±0.002$^{Aa}$ | 0.013±0.003$^{Aa}$ | 0.007±0.001$^{ABac}$ | 0.010±0.001$^{ABad}$ |
| 09:00 | 0.232±0.006$^{Bb}$ | 0.033±0.005$^{Bb}$ | 0.047±0.003$^{Bb}$ | 0.035±0.003$^{Bb}$ | 0.110±0.013$^{Bb}$ | 0.482±0.010$^{Bb}$ | 0.016±0.002$^{Bb}$ | 0.035±0.003$^{Bbe}$ | 0.006±0.001$^{ABac}$ | 0.006±0.001$^{ACac}$ |
| 12:00 | 0.041±0.006$^{Cc}$ | 0.129±0.005$^{Aa}$ | 0.035±0.003$^{ACac}$ | 0.038±0.003$^{Bb}$ | 0.352±0.013$^{Cc}$ | 0.339±0.010$^{Cc}$ | 0.012±0.002$^{ABa}$ | 0.028±0.003$^{BCbc}$ | 0.011±0.001$^{Aa}$ | 0.016±0.001$^{Bb}$ |
| 14:00 | 0.017±0.006$^{Dd}$ | 0.117±0.005$^{Aad}$ | 0.039±0.003$^{ACc}$ | 0.021±0.003$^{Aa}$ | 0.544±0.013$^{Dd}$ | 0.227±0.010$^{Ad}$ | 0.008±0.002$^{Aa}$ | 0.021±0.003$^{Aa}$ | 0.002±0.001$^{Bb}$ | 0.004±0.001$^{Cc}$ |
| 17:00 | 0.202±0.006$^{Ee}$ | 0.059±0.005$^{Cc}$ | 0.038±0.003$^{BCbc}$ | 0.024±0.003$^{Aa}$ | 0.287±0.013$^{Ee}$ | 0.353±0.010$^{Cc}$ | 0.010±0.002$^{Aa}$ | 0.019±0.003$^{ACac}$ | 0.004±0.001$^{BCbc}$ | 0.004±0.001$^{Cc}$ |
| 20:00 | 0.121±0.006$^{Ff}$ | 0.062±0.005$^{Cc}$ | 0.032±0.003$^{ACac}$ | 0.050±0.003$^{Cc}$ | 0.213±0.013$^{Ff}$ | 0.426±0.010$^{De}$ | 0.026±0.002$^{Cc}$ | 0.062±0.003$^{Dd}$ | 0.005±0.001$^{BCbc}$ | 0.007±0.001$^{ACacd}$ |
| 23:00 | 0.063±0.006$^{ACac}$ | 0.110±0.005$^{Ad}$ | 0.034±0.003$^{ACc}$ | 0.033±0.003$^{Bb}$ | 0.423±0.013$^{Ag}$ | 0.260±0.010$^{Ea}$ | 0.026±0.002$^{Cc}$ | 0.034±0.003$^{Be}$ | 0.007±0.001$^{ACac}$ | 0.011±0.001$^{ABd}$ |
| Month | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** |
| February | 0.111±0.006$^{ABab}$ | 0.116±0.006$^{ADade}$ | 0.021±0.003$^{Aa}$ | 0.023±0.004$^{Aa}$ | 0.360±0.014$^{ABab}$ | 0.326±0.011$^{Aa}$ | 0.016±0.003$^{ABacd}$ | 0.010±0.004$^{Aa}$ | 0.009±0.002$^{ABa}$ | 0.009±0.001$^{ABac}$ |
| Marc | 0.099±0.004$^{ABa}$ | 0.110±0.004$^{Aa}$ | 0.022±0.002$^{Aa}$ | 0.033±0.002$^{Bb}$ | 0.383±0.009$^{Aac}$ | 0.297±0.007$^{Aa}$ | 0.011±0.002$^{Ab}$ | 0.030±0.002$^{Bb}$ | 0.009±0.001$^{Aac}$ | 0.006±0.001$^{ACac}$ |
| April | 0.098±0.005$^{ACac}$ | 0.100±0.004$^{ADad}$ | 0.019±0.002$^{Aa}$ | 0.039±0.003$^{Bb}$ | 0.363±0.010$^{ACac}$ | 0.306±0.008$^{Aa}$ | 0.019±0.002$^{BCac}$ | 0.049±0.003$^{Cc}$ | 0.004±0.001$^{BCb}$ | 0.004±0.001$^{Aa}$ |
| May | 0.094±0.004$^{ABa}$ | 0.074±0.004$^{Bb}$ | 0.040±0.002$^{Bb}$ | 0.039±0.002$^{Bb}$ | 0.381±0.009$^{Aa}$ | 0.297±0.007$^{Aa}$ | 0.023±0.002$^{Ba}$ | 0.046±0.003$^{Cc}$ | 0.003±0.001$^{Cb}$ | 0.006±0.001$^{ACac}$ |
| June | 0.075±0.005$^{Bb}$ | 0.059±0.004$^{Cc}$ | 0.035±0.002$^{Bb}$ | 0.053±0.003$^{Cc}$ | 0.327±0.010$^{Bb}$ | 0.371±0.008$^{Bb}$ | 0.015±0.002$^{ACce}$ | 0.055±0.003$^{Cc}$ | 0.005±0.001$^{BCcd}$ | 0.008±0.001$^{ACac}$ |
| July | 0.128±0.005$^{CDcd}$ | 0.096±0.004$^{ADde}$ | 0.049±0.002$^{Cc}$ | 0.021±0.003$^{Aa}$ | 0.307±0.010$^{BCb}$ | 0.353±0.008$^{Bb}$ | 0.012±0.002$^{ACbde}$ | 0.013±0.003$^{Aa}$ | 0.008±0.001$^{ADa}$ | 0.013±0.001$^{Bb}$ |
| August | 0.136±0.009$^{Dd}$ | 0.082±0.008$^{Bdbe}$ | 0.066±0.005$^{Dd}$ | 0.018±0.005$^{Aa}$ | 0.289±0.021$^{ABbc}$ | 0.376±0.015$^{Bb}$ | 0.010±0.004$^{ACbc}$ | 0.009±0.005$^{Aa}$ | 0.003±0.002$^{BCDbd}$ | 0.011±0.002$^{BCbc}$ |
| Breed x Hour | NS | ** | ** | * | NS | NS | ** | * | ** | ** |
| Breed x Month | * | ** | ** | ** | NS | * | NS | ** | NS | NS |
| Hour x Month | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** |

HF: Holstein-Friesian, SIM: Simmental, NS: not significant, *: p<0.05, **: p<0.01; A,B,C,D,E: Same letter in the column show insignificance for P<0.01, a,b,c,d,e,f: Same letter in the column show insignificance for P<0.05.

*Nutritional, standing and lying behaviors* of HF, BS and SIM bulls were mainly similar. The feed intake behavior increased at 09:00 and 17:00 and the bulls preferred drinking at the time when they had higher feeding rates. Ruminating behavior rate was lower at 09:00 and 17:00 and 20:00 due to higher feeding activities at this time (Figure 3).Except for August in group II, HF bulls had lower standing rates than those of BS and SIM bulls. HF bulls had higher lying rate in July and in August than those of the BS and SIM bulls and unlike the group I, the lying rates in group II were decreased from May to August for HF and SIM breeds.

As the ruminating and feeding behaviors decreased gradually from February to July, except for BS bulls in July, the drinking activity was increased in hot summer months for all breeds. Similar to Dikmen (2013) a higher drinking rate for HF bulls than those of BS bulls was detected.

The lower ruminating behavior rate at 09:00 and 17:00 and 20:00 due to feeding activities at this time (Figure 3)agree with Zgur et al. (2014) with the study about Sloven Cika and SIM bulls and Dikmen (2013) with the study about HF and BS bulls. The higher ruminating rates found for HF and BS in group I than those of HF and SIM bulls in group II could be due to longer fattening time of group II in hot weathers. During this time, the animals tend to decrease feed intake especially forages to decrease the heat load. The higher feeding rate found in the morning and in the evening in this study was also similar to Mitlöhner et al. (2001).

The lower standing rate found early in the morning and late in the evening and higher standing rates at 09:00 and 17:00-20:00 agree with Dikmen (2013).Similar to Platz et al. (2007)the lying behavior is the highest daily behavior and similar to the Dikmen (2013)the different lying behavior rates between HF and BS breeds were detected.

*Locomotor behaviors* in all breeds decreased significantly in July and August (Figure 4), due to higher temperature seen in the region. In these months because of THI >72, the bulls might have heat stress and in order to decrease heat load on their bodies, they reduced their locomotor activities. Similar to Mitlöhner et al. (2001) and Dikmen (2013), the locomotor activities of the bulls were intense during the evening hours.

*Eliminating behaviors:* For almost all hours the defecation and urinating rates for HF bulls in group I were higher than those of BS bulls ($P<0.05$). In group II, only in the evening the elimination rates were obviously higher in HF bulls than that of SIM bulls. In hot summer months, the elimination rates were higher in HF bulls than those of BS and SIM bulls. The higher eliminating behavior found for HF bulls than BS bulls disagrees with the results of Dikmen (2013).

## CONCLUSIONS

The increase in THI in hot summer months showed that the animals were exposed to heat stress and as a response to heat stress the bulls performed some of their behaviors like decreasing feeding, standing and locomotion and increasing drinking and eliminating behaviors or postponing these behaviors to the cooler hours of the day. In terms of drinking, defecation and urination, BS bulls could be more resistant to higher environmental temperatures and relative humidity than those of HF bulls, however the behavioral differences were not obvious between HF and SIM bulls.

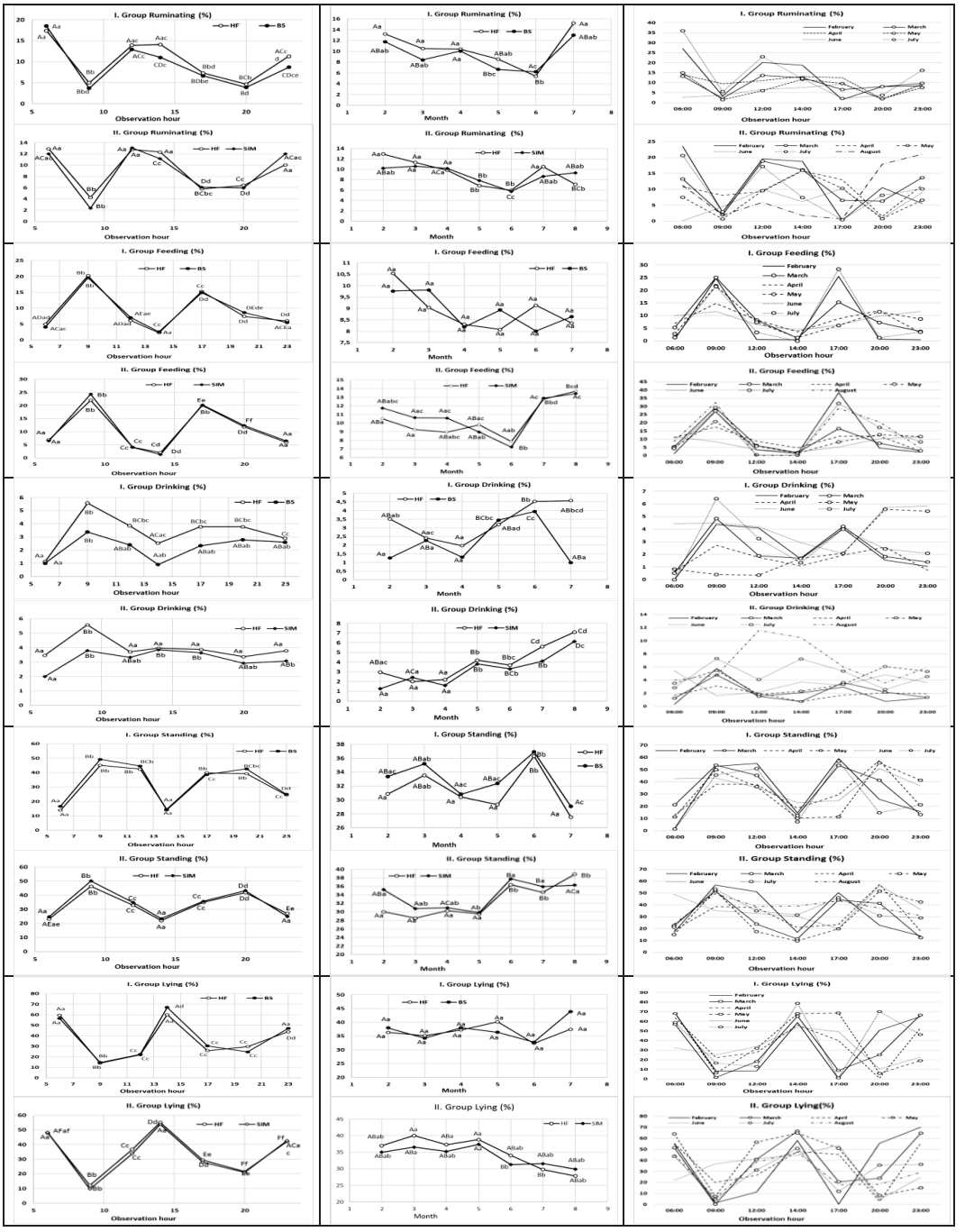



Figure 3. Changes of nutritional, standing and lying behaviors of HF, BS and SIM depending on observation hour and month

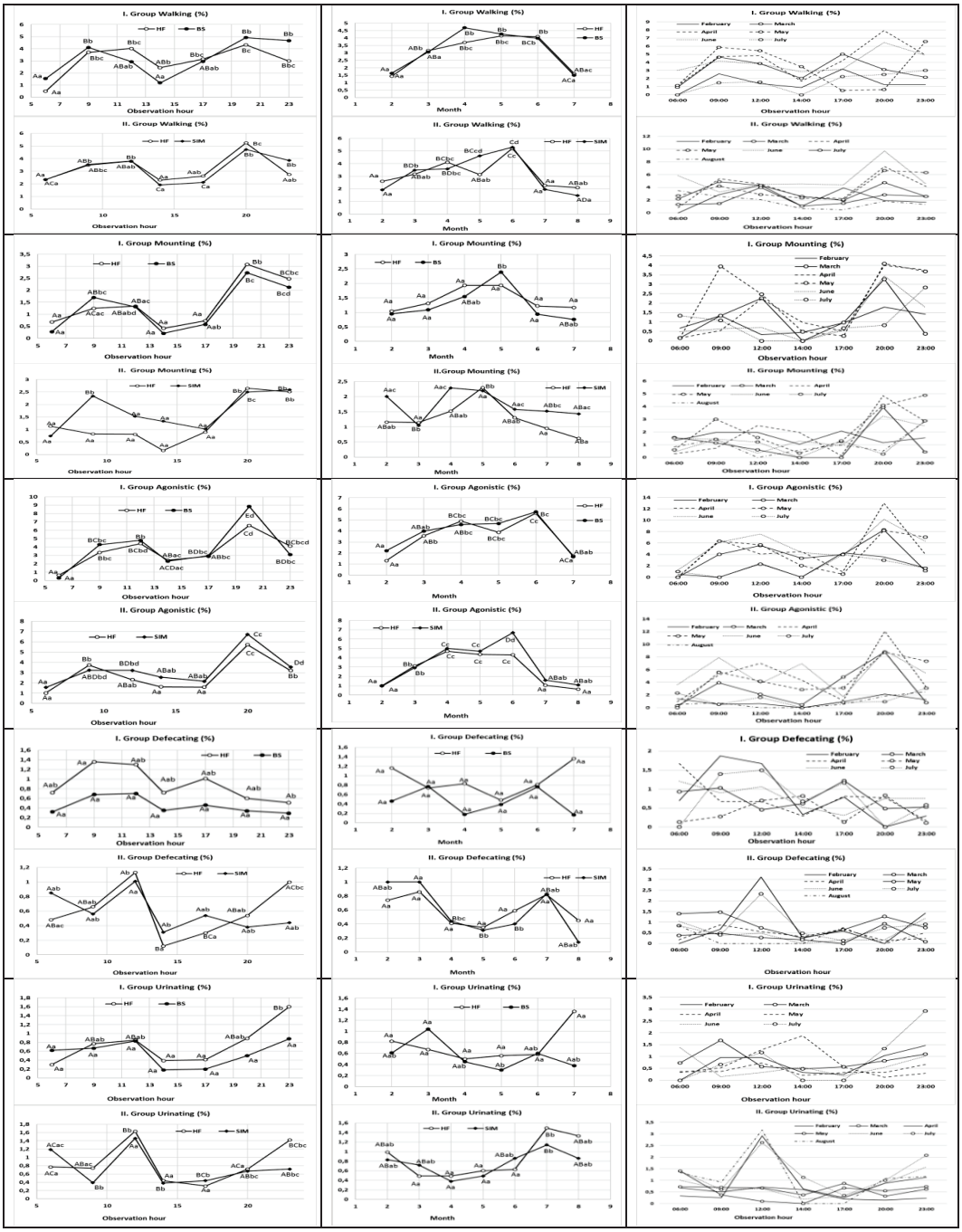



Figure 4. Changes of locomotion and eliminating behaviors of HF, BS and SIM depending on observation hour and month

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# ASSESSMENT OF BUFFALO MILK NUTRITIONAL COMPOSITION USING FT-IR SPECTROSCOPY

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***Abstract***

*In the last decade, FT-IR spectroscopy has been introduced in methodological portfolio specific for analyzing food products, because it is a very efficient, and non-destructive analytical tool. Vibrational spectral techniques, as FT-IR, offer several advantages in the context of current research and using this techniques we can identify molecular components in the studied samples. In this study FT-IR spectroscopy technique is applied to detect fat, protein, and lactose content of buffalo milk, and compositional differences between samples corresponding to different lactations. The results emphasizes specific evolutions corresponding to the increase of protein, lactose, and fat buffalo milk contents, from the first lactation up to the fifths/fourths lactations.*

***Key words****: buffalo milk, stage of lactation, FT-IR.*

## INTRODUCTION

Buffalo milk is characterized by specific chemical composition. Fat, lactose and protein content, which vary function of different factors such as area (Tăpăloagă et al., 2012) breeding, or stage of lactation, confer the special traits of the milk. In order to be used in different aims (consumption, raw material for cheese production, etc.) the buffalo milk must be collected properly and it also has to be free of colostrum.

Fourier - transform infrared spectroscopy (FT-IR) is one the most widely used method for detection of the compositional differences between samples, and it relies on the basic vibration of various chemical groups at specific wave lengths within the interval 400-4000 $cm^{-1}$.

This technique may be considered, by the food industry, as potential tool for testing the quality of food products, based on the fingerprint region (1800-200 $cm^{-1}$) because it can provide large information about functional groups characterizing the chemical composition of samples (Babushkin et al., 2016; Ketty et al., 2017; Kučević et al. 2017).

FT-IR technique, which is a non-invasive method, and involves minimum effort for sample preparation, may be considered a simple and fast alternative to other laborious and expensive analyze techniques.

For this reason it is used in many fields such as food science, chemical industry, pharmaceuticals study, Chinese medicine, food control, medicine (Liu at al., 2006; Xu et al., 2006; Andronie et al., 2011; Geghardt et al., 2011).

FT-IR technique was applied with great success for classification of raw of milk, collected from cooperatives located in three areas of Morocco (Elbnassbasi et al., 2010).

Lei Yu et al. (2010) performed a study where IR spectroscopy was used in combination with two dimensional (2D) correlation infrared spectroscopy for analysis of crystalized lactose, protein and fat in milk powder.

Grewal et al. (2017) used the advantages of this method in order to detect physico-chemical changes that lead to sedimentation or gelation under accelerated storage temperature in UHT (ultra-high temperature) milk and was used by for their study.

The aim of this study was to investigate the buffalo milk quality, in terms of fat, protein, and lactose content, depending on the stages of lactation, using FT-IR spectroscopy.

## MATERIALS AND METHODS

The trial was carried out on a private farm located in Zalău, Sălaj County, Romania (47°11'28''N, 23°3'26''E). 50 buffalo females (*Bubalus bubalis* L.), Romanian buffalo breed, in different stage of lactation, were used. They were milked twice daily, in the morning and in the afternoon. Function of lactation, the buffalo females were divided in five groups.

The milk was centrifuged at 13,000 x g for 5 minutes, previously to further analysis. Lactose, protein, and fat, from the fresh buffalo milk samples were analyzed function of lactation, using FT-IR spectroscopy.

FT-IR spectroscopy was performed with Nicole FT-IR spectrophotometer equipped with Horizontal Attenuated Total Reflectance (HATR) with ZnSe accessory. IR frequencies are expressed by a light number that is directed to the sample. When radiant energy is equal to the vibrational frequency of the molecule, it realizes the suction and vibrating. Absorption intensity for each frequency of vibration is monitored by a detector. Specific footprint is a specific combination between molecular vibration and rotational vibration and has a great significance to identify specific molecules. Measurements were carried out on infrared scale of 650-4000 $cm^{-1}$, 100 scans per sample at 2 $cm^{-1}$ resolution (Fig. 1).

These spectra were analyzed by comparing the obtained vibrational bands with those of similar functional groups from the literature (Ley el.al., 2010; Murphy et a., 2014; Jaiswal et. Al. 2015; Mendelsohn et.al., 2010).

The IBM SPSS v.19.0 for windows, was used for statistical analysis. Basic statistics, was implemented in order to emphasize the mean (X), standard deviation (SD) and coefficient of variation (CV%), of protein, fat, and lactose, by lactations. The mean concentration of milk components were compared across the various lactations using one-way analysis of variance (one-way ANOVA). Differences of the means were considered to be significant when p-value < 0.05 (Kittivachra et al., 2007). The Box-Plot diagrams were used for showing the distributions of the lactose, protein, and fat, by lactations. Minimum, maximum concentration values, and quartiles (first, median, third, and forth) are emphasized.

## RESULTS AND DISCUSSIONS

The FT-IR spectra of milk in different stage of lactation present the same band positions and relative intensities with some differences.

The obtained FT-IR spectra emphasize the main types of structures present in the analyzed milk samples.

In order to determine the fat content of milk is very important to check the intensity of the peak 1743 $cm^{-1}$ and 1161 $cm^{-1}$. In the region between 3000-2800 $cm^{-1}$ are reported substantial changes in intensity, ad IR spectra show two peaks at 2920, 2852 $cm^{-1}$ rending to fat content in the buffalo milk. The content of fat decreases in concentration depending on lactation. Both, peak characteristic for C=O bond in fat, corresponding to 1743 $cm^{-1}$, and peak representing C-O vibration in fat, corresponding to 1161 $cm^{-1}$ are noticeably decreasing in intensity from lactation 1 compared to lactation 5 (Fig. 1).

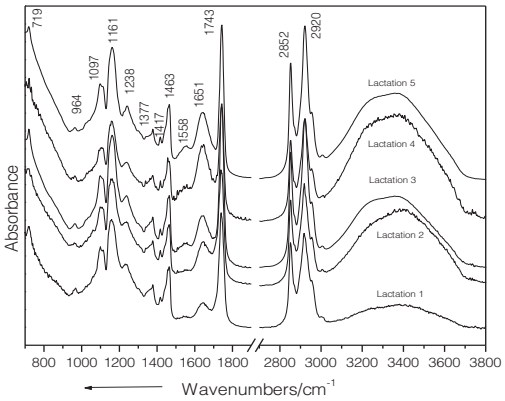


Figure 1. FT-IR spectra of milk in different stages of lactation

The region 1700-1500 $cm^{-1}$ has two main parts, namely amide I (1700-1600 $cm^{-1}$) corresponding to C=O stretching mode of the peptide bonds, and amide II (1600-1500 $cm^{-1}$) attributed to C-N stretching vibration (Fig. 1). Another peak that increases in intensity at lactation 5 was found at 1651 $cm^{-1}$ for the vibration of amide I. The peak reported at 1558 $cm^{-1}$ was more emphasized in the spectrum that corresponds to lactation 5 (Fig. 1).

Region 1500-1200 $cm^{-1}$, amide III spectral region, respectively, also corresponds to secondary structure of proteins. In this region

was reported a peak at 1463 $cm^{-1}$ were the increase in intensity is not very emphasized (Fig. 1).

For lactose, specific spectrum ranges from 1150 to 1030 $cm^{-1}$ and the peak corresponding to C-O groups of the lactose is reported at 1097 $cm^{-1}$. The peak reported at 964 $cm^{-1}$, characterized by very low intensity was assigned to C-O vibration for carbohydrates (Iñón et. al. 2004). The mean content of protein in buffalo milk gradually increases from the lactation 1, when 4.98% content is reported, to the lactation 5, when 6.44% content is emphasized, by 1.48%, respectively.

The differences between mean protein buffalo milk content, function of lactations were not statistically assured at significance threshold 1%, between lactations 1, 2, and 3, on one hand, and 4, and 5, respectively, on other hand, but they were assured at significance threshold of 1% between lactation 1 (4.98%), and lactation 4 (6.07%), and at significance threshold of 0.1%, between lactation 1 and lactation 5 (6.44%), respectively. The standard deviation emphasizes a normal distribution of the individual protein concentration, and the values of coefficients of variations within the interval CV=1.51% (Lactation 3), and CV=4.57% (Lactation 1), confirm the representativeness of the means (Table 1).

Concerning the mean lactose content in buffalo milk, our study also emphasizes a gradual increase from lactation 1 to lactation 5, by 0.62%. The differences between mean lactose buffalo milk content, function of lactations were statistically assured at significance threshold 1%, only between lactation 1 (4,69%), and lactation 5 (5.62%).

The standard deviation, in this case, too, emphasizes a normal distribution of the individual lactose concentrations.

The coefficients of variations with values within the interval CV = 2.55% (Lactation 1), and CV = 3.47% (Lactation 6), emphasizes the representativeness of the means (Table 1).

The buffalo milk analyzed our study had a fat content, which increase from the lactation 1, when a mean of 8.33% is reported, to lactation 4, when it is reported a mean of 9.02%, by 0.69%, respectively. In lactation 5, the mean lactose content slowly decrease to 8.96%, by 0.62% higher, compared to lactation 1.

Table 1. The basic statistics and significance of differences, for protein, lactose, and fat content quantified in buffalo milk, function of lactation (%)

| Issue | | Protein (%) | Lactose (%) | Fat (%) |
|---|---|---|---|---|
| Lactation 1 | n | 10 | 10 | 10 |
| | X | **4.98$^{abc}$** | **4.69$^{ab}$** | **8.33$^{ab}$** |
| | SD | 0.22 | 0.12 | 0.7 |
| | CV% | 4.57 | 2.55 | 8.34 |
| Lactation 2 | n | 10 | 10 | 10 |
| | X | **5.36$^{a}$** | **4.98$^{a}$** | **8.64$^{a}$** |
| | SD | 0.14 | 0.16 | 0.21 |
| | CV% | 2.69 | 3.12 | 2.50 |
| Lactation 3 | n | 10 | 10 | 10 |
| | X | **5.64$^{a}$** | **5.14$^{a}$** | **8.90$^{a}$** |
| | SD | 0.09 | 0.15 | 0.44 |
| | CV% | 1.51 | 2.90 | 4.90 |
| Lactation 4 | n | 10 | 10 | 10 |
| | X | **6.07$^{ba}$** | **5.31$^{ab}$** | **9.02$^{ab}$** |
| | SD | 0.17 | 0.16 | 0.32 |
| | CV% | 2.87 | 2.92 | 3.57 |
| Lactation 5 | n | 10 | 10 | 10 |
| | X | **6.44$^{ca}$** | **5.62$^{ab}$** | **8.96$^{ab}$** |
| | SD | 0.15 | 0.20 | 0.42 |
| | CV% | 2.3 | 3.47 | 4.63 |

X – mean; SD – standard deviation; CV – coefficient of variation;
a – $p > 0.05\%$, b – $p < 0.05\%$; c – $p < 0.01\%$.

The differences between mean fat buffalo milk content, function of lactations were statistically assured at significance threshold of 1% between lactation 1 (8.33 %), and lactation 4 (9.02%), and 5 (8.96%), respectively.

The standard deviation emphasizes a normal distribution of the individual protein concentration, and the values of coefficients of variations within the interval CV = 2.50% (Lactation 2), and CV = 8.34% (Lactation 1), confirm the representativeness of the means (Table 1).

According to Box-Plot diagram (Fig. 2), different individual values are recorded for mean protein content identified in buffalo milk, function of lactations, but equilibrate distributions are emphasized only for lactations 2, 3, and 5, emphasizing similar individual values, meaning high samples homogeneity.

In lactations 1 and 4, the protein contents show asymmetric distributions, which suggest high variability of individual values.

In lactation 1, where the quartiles 1, and 2 are considerably bigger, predominate smaller individual values, compared to the mean, while

in lactation 4, where third quartile is bigger, slight predominance of bigger values compared to the mean, is suggested.

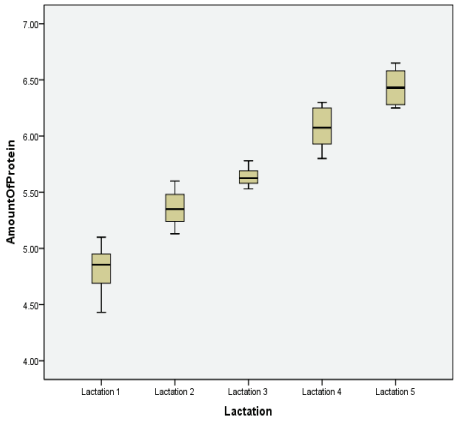


Figure 2. Comparison of protein content in different lactation stages

The distributions of the individual values of the lactose content in buffalo milk, by lactations, are predominant asymmetric. One exception is recorded, and it corresponds to lactation 2, where Box-Plot diagram emphasize equilibrate quartiles.

In lactations 1, and 3, quartiles 1 and 4 are bigger, and this signifies that smaller individual values are predominant, compared to the mean.

Corresponding to lactations 3, and 5, quartile 4, and 3 and 4, respectively, are more extended, meaning that, in this case, bigger individual values are predominant, compared to the mean (Fig. 3).

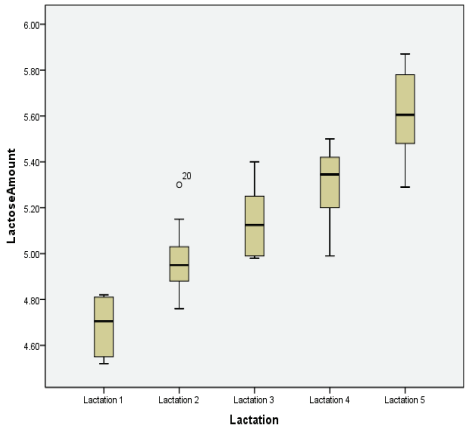


Figure 3. Comparison of lactose content in different lactation stages

The mean fat content shows different individual distributions, by lactation, as emphasized by Box-Plot diagrams (Fig. 4).

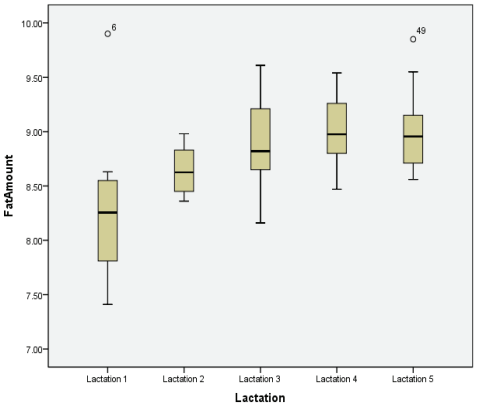


Figure 4. Comparison of fat content in different lactation stages

In lactation 2 a symmetric distribution is recorded.

Lactation 1 is characterized by asymmetric distribution, where quartiles 1, and 2 are bigger, meaning predominant lower values compared to the mean.

Lactations 3, and 4, also with asymmetric distributions, have the biggest extent correspondent to quartile 3. Lactation 5, also characterized by asymmetry, is characterized by the predominance of mean fat content values corresponding to quartiles 1, and 4.

## CONCLUSIONS

The protein is the nutritional trait of buffalo milk, which is most affected by the lactation stage, exhibiting an increase of 1.48%, from lactation 1 to lactation 5. Less affected by lactation is fat content, where an increase of 0.69% is recorded between lactation 1, and lactation 4, and lactose content, where an increase of 0.62% is reported from lactation 1 to lactation 5. Continuous increase of protein and lactose buffalo milk content is reported function of lactations, while concerning lactose, discontinuous evolution is observed, with an increase from lactation 1 to lactation 4, followed by a slight decrease in lactation 5. Based on the results of our study, it can be concluded that the FT-IR spectroscopy is a reliable instrumental technique for the

determination of lactose, protein and fat in milk. This method offer highly specific, precise, and accurate and linear data across the analytical range. The study has show that the FT-IR method is indeed a acceptable instrumental technique for analyzing the chemical parameters characterizing the buffalo milk in different stages of lactation.

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# CATTLE FARMS GROSS MARGIN - THE CASE OF ALBANIA AND KOSOVO

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***Abstract***

*The aim of this paper was to analyze the economic efficiency of dairy cattle farms in Albania and Kosovo. It's a descriptive and quantitative survey and the target populations were the dairy farmers in six regions of Albania and seven regions of Kosovo. The random sampling technique was used to select the respondents in both countries (in Albania 79 farms and in Kosovo138 farms), in Albania from the list of the Regional Agricultural Directories and in Kosovo from the list of Paying Agency. Two methods of data analysis were used, namely: descriptive statistics, and gross margin analysis. The objective of this study was the comparison of the performance of the 5-10 cows farms (considered small farms) and 11cows farms (considered medium/large sized dairy farms). Data on: milk production, farm expenses and returns, use of milking machine, artificial insemination, fodder production, and feed bought in the market for each farm were recorded during the period 2015-2016. In Kosovo, the Gross Margin per Farm from milk and meat and Gross Margin per Farmfrom milk for the farms with 5-10 milking cows have negative values -318 Euro/farm and -673 Euro/farm, respectively, while the 11+ cows farms have positive values (3743 Euro/farm and 922 Euro/farm, respectively). While in Albania, as average the GMpF milk+meat and GMpF milk in both type of farms have positive results, however 15.8 percent of the 5-10 cows are showing negative results for the GMpF milk (loosing money), while for the 11+ cows farms this indicator is 31.7 percent, as the cost of production is very high. Taking into account the results obtain is a must that extension service to train the farmers for better: management of their farm, feeding system, fodder production, animal health etc.*

***Key words:*** *dairy farm, gross margin per farm, income per farm, milk cost.*

## INTRODUCTION

Both countries continue to be predominantly rural economies with about 20 percent of the Gross Domestic Product (GDP) generated by agriculture in Albania, and 13% in Kosovo. Agriculture is also the largest employing sector, accounting for it employs about 42 percent of the active force in Albania and approximately 35 percent in Kosovo. (MAFRD, 2017; INSTAT, 2018).

The government of Albania considers priority the dairy sector (MBZHRAU, 2014)[1].The cattle sector is one of the most important sub-sectors in agriculture since it provided during 2017- 85 percent of milk, and 44.7 percent of meat production (INSTAT, 2017a).

Small-scale farming system is dominant for milk and beef production. Most of the cattle small sized farms continue to produce in the traditional way and market their animal origin products through informal channels. The number of cows is approximately 349,200, and one farm family as average is managing 2.29 cattle or 1.65 cows (INSTAT, 2017a). Cow milk production is 983 000 ton, but only ½ of the production reach the markets while the other half is used for consumption (by animals or processed on the farm). From the part of milk that reach the market only 11.2% (110,000 tons) is processed by the dairy industry, while the remaining 38.8% (381,000 tones) reaches consumers directly. The cow milk yield in 2016 reached 2815 kg/cow/year (INSTAT, 2017 b).

In Kosovo, livestock production is the most profitable activity and is of economic importance, and cattle milk dominates raw milk production. According MAFRD (2017), the

[1]Ministry of Agriculture full name was Ministry of Agriculture, Food and Consumer Protection(MAFCP) until 2013, and as of 2013, following institutional changed, it is named Ministry of Agriculture, Rural Development and Water Administration (MARDWA), since 2017 and on the name is Ministry of Agriculture and Rural Development (MARD).

cattle fund is approximately 265,000 heads of which 136,780 are cows and milk production is 285,000 tones. The cow milk yield in 2016 reached 2085 kg/cow/year. Small and middle-sized farms are the dominant farms and most small dairy farms produce for self-consumption. On average, the farm size is 3.2 ha agricultural land (including common land/pasture), about 3.9 cattle (about 2 dairy cows), and it is estimated that today there are about 91,200 livestock farms in Kosovo (ASK, 2015). According to MAFRD (2014) small farms (1-4 cows) account for 94.2 percent of farms that breed dairy cows. During 2014, there were 5,472 commercial farms (5.8 percent of dairy farms) that have more than 4 dairy cows and are the main suppliers in the dairy industry with a total of about 62 million liters of milk per year; or about 18 percent of domestically produced milk (AZHB, 2015; MBPZHR, 2015a).

Many small sized farms in Albania and Kosovo have experience in growingthe local breed or their crosses which are smaller in size (body weight), produce less milk and has modest feeding requirements compare with pure milk breeds. This is one of the main causes behind the low production performance and high production cost.

However the success of dairy farms largely depends on the effective management of operations. Returns in dairy farming are deeply determined by variable cost and production cost and the correlations existing between farm size, milk yield, variable cost, total cost and milk price are important to be studied and keep under control by farmers (Dhuyvetter, 2010).

According to Popescu (2012) the advantage of gross margin is the fact that it allows the comparison, in terms of profitability, between various activities running in a farm. Serban (2010) cited by Popescu (2012) is mentioning that the higher production performance and the lower variable costs, the higher gross margin.

In addition, Popescu (2009) is emphasizing that dairy farm structure is close related to the economic efficiency, being well known the fact that the higher the farm size and milk yield, the higher the economic efficiency. While Alvarez et al. (2014) points out that the gross margin reveals a direct link between production efficiency and characteristics for each dairy farm such as farm size, milking system, used feeding system, and so on. According to Keskin and Dellal (2011), the gross margin is recognized as an important benchmark for success in determining competitive production capability, and is used in comparing enterprise across the EU within the Farm Accountancy Data Network (FADN).

Moran (2009) is mentioning that milk price generally has the biggest influence on farm profits.

Ford and Shonkwiler (1994) emphasized that financial structure, labor efficiency, and milk per cow was very important characteristics of managerial ability. Increasing dairy managerial ability would have a larger impact on profitability for many farms than increasing herd size. Nastić et al. (2011) is mentioning some of the factors affecting milk production such as natural conditions; prices of crop products used for feeding of milking cows; prices of other inputs used for the fodder and all the production chain, subsidies; breeds that are managed, etc.

This paper aimed to make a comparison between the profitability of the dairy cattle farms in Albania and Kosovo. In addition, the paper presents an analysis of milk production in various farms of different herd size and also the main aspects of economic efficiency in order to put in evidence the importance of farm size for increasing profitability and competitiveness in dairy farming. For this purpose, the data were collected from farms of seven regions of Kosovo and six regions of Albania. They were processed according to the specific methodology for calculating the gross margin and profit.

## MATERIALS AND METHODS

This study, in both countries, was conducted to collect farm data pertaining to revenue and expenses on farms managing 5-10 cows and those with 11+[2] cows to make an economic analysis based on gross margin. For this purpose, the gross margin was used as comparison criteria. The gross margin is calculated as the difference between total

[2]In Albania were interviewed farms with 11+ cows (11-170) while in Kosovo 11-50 cows

income and the variable cost. Variable cost includes the cost of:

- feed (from farm fodder production and feed bought in the market),
- labor (from family member and hired labor),
- veterinary service (including and insemination),
- water,
- electricity,
- transportation, and miscellaneous.

For Kosovo was used the list of dairy farms, breeding more than four cows, which is available from the Ministry of Agriculture, Forestry and Rural Development (MAFRD) based on the direct payments. The random sampling techniques were used to select the respondents. One hundred and thirty-eight dairy farm were monitored and interviewed in the seven regions of Kosovo (or 2.6 percent of all farms with over 4 cows)[3].

While in Albania were monitored 79 dairy cattle farms (or 2.1% of all Albania's farms, with over 4 cows) randomly selected from the list of farms that breed more than four cows, and are available from the Regional Agricultural Directorates (DRBs)[4].

In both countries the interviewed took place during the period December 2015 - April 2016.

***Data collection*:** In both countries, a structured questionnaire was used for collection of all information related to dairy farming. The questionnaire was discussed with a panel of four specialists, to verify its content and validity, as well was tested with seven farmers, to avoid confounding questions and for clarity. Face-to-face interviews were conducted. According to the questionnaire the following data were recorded:

- Income and expenses: (i) Milk yield per cow; (ii) Milk production per farm; (iii) Quantity of milk sold; (iv) Price of milk sold; (v) Quantity and price of meat sold (live bodyweight); (vi) Expenses for the fodder production; (vii) Expenses for the animal feed bought in the market; (viii) Expenses for veterinary service and cow's insemination; (ix) Expenses for fuel, electricity, water, and trips; (x) Estimated cost of labor needed to take care of the herd per year;(xi) Gross Margin per Farm from sales of milk and meat (GMpF milk+meat); (xii) Gross Margin per Farm from sales of milk (GMpF milk).
- Technical data, such as: Insemination (artificial or natural mating); milking (by machine or by hand); and type of animal feed used (including microelements or premix).

***Data analysis*:** For data analysis was developed a model in Microsoft Excel program, while the statistical data processing was done with Statgraphics Centurion XVI.

## RESULTS AND DISCUSSIONS

In Table 1 (data for Albania) and Table 2 (data for Kosovo) below are summarized the data of farms with 5-10 dairy cows and those with 11+ milking cows:

- Number of cattle and cows per farm
- Milk yield/cow/year
- Income per Farm (IpF milk+meat, and IpF milk),
- Variable cost per farm
- Gross Margin per Farm (GMpF milk+meat and GMpF milk)
- Milk cost per liter
- Ratio milk sold in the market vs total milk production
- Prices of milk and meat sold
- Milking and insemination method
- Feed expenses vs variable cost.

As far as the number of heads is concerned, on farms 5-10 heads, it is clear from the label itself that this is a variation of heads for farms, while at 11+ cows farms this variation is 11-170 cows.

On frms with 5-10 heads of cows rmilk yield ranging from 3400 to 7500 kg/cow/year while milk yield of farms with 11+ cows varies from 3000 to 7700 kg/cow/year or 15.7% higher than the first.

The income of dairy farms, studied, comes from milk and meat. But most of the income for all farms comes from milk 80.14 percent (81% on 5-10 cows farms and 80% on 11+ cows farms) and the rest from calves.

IpF milk+meat for 11+ cows farms are 5.29 times higher than 5-10 cows farms (69,639 Euro vs 13,147 Euro), while the IpF milk of 11+ cows farms is 5.23 times more than the 5-10 cows farms (55,711 Euro vs 10,646 Euro).

[3]Gjilan, Gjakove, Ferizaj, Mitrovice, Peje, Prishtine, and Prizren.

[4]Elbasan, Lushnje, Fier, Shkodra, Korça and Tirana.

## 1. Albania

Table 1. Technical data of dairy farms in Albania

| Number of heads | **No of farms** | **No of cattle per farm** | **No of Cows per farm** | **Milk yield (liter)** | **IpF (milk + meat) Euro** | **IpF (milk) Euro** | **Variable cost per farm** | **GMpF (milk + meat) Euro** | **GMpF (milk) Euro** |
|---|---|---|---|---|---|---|---|---|---|
| 5-10 cows | 38 | 12.02 | 8.1 | 4307 | 13147 | 10646 | 8996 | 4154 | 1650 |
| 11+ cows | 41 | 56.92 | 37.95 | 4984 | 69639 | 55711 | 53199 | 16440 | 2512 |
| Total/ average | 79 | 35.33 | 23.6 | 4658 | 42465 | 33634 | 31342 | 11123 | 2292 |

**Table 1 continue**

| Number of heads | **Milk cost (Euro/kg)** | **Milk sold vs milk produced (%)** | **Price of milk sol (Euro)** | **Price of meat sold (Euro)** | **Milking** By hand | by machine | **Insemination** Naturale | Artif. | **Feed expense vs variable cost (%)** |
|---|---|---|---|---|---|---|---|---|---|
| 5-10 cows | 0.246 | 88.0 | 0.348 | 3.68 | 25.8 | 74.2 | 0 | 100 | 62.9 |
| 11+ cows | 0.238 | 99.7 | 0.321 | 3,40 | 9.6 | 90.4 | 0 | 100 | 71.2 |
| Total/average | 0.242 | 94.1 | 0.334 | 3,54 | 17.4 | 82.6 | 0 | 100 | 67.2 |

These significant differences are coming as the result of the number of cows, milk yield, the price of milk and meat sold, that in most of the cases are higher at 11+ cows farms compare with 5-10 cows farms.

The IpF milk+meat, IpF milk, GMpF milk+meat and GMpF milk are higher at 11+ farms compared with 5-10 cows farms. However 15.8 percent of the 5-10 cows farmare showing negative results (loosing money) for the GMpF milk, while for the 11+ cows farms this indicator is 31.7 percent, as the cost of production is very high. Other authors (Szalka, 2002; Demircan et al., 2006; Günden et al., 2010; Terin et al., 2017), report on losses of farms especially from dairy production. Authors emphasized that the lack of accurate knowledge about input usage among some of the farmers may be one of the main obstacles to efficient input use. Knowledge about production techniques is also needed along with efficient inputs.As Frank (1996) says, therefore, farmers should not continue to increase their business without understanding how to control costs, and once they have it in control to continue business growth.

Whereas, according to Dijkhuizen and Huirne, (1997) gross margin analysis is used to calculate farm milk profits because it is the simplest and most viable method of assessing enterprise profitability and is widely used in the farm management economy. When the size of the dairy cattle farm is statistically significant ($p = 0.000$) and its coefficient is positive, meaning that the increase with each cow unit results in an increase in the milk production profitability. This is in line with what Cain et al. (2007) emphasized that the benefit of a dairy enterprise is highly related to the size of the herd.

The milk cost of 5-10 cows farms is 3.4 percent higher than 11+ cows farms, while the milk price of 5-10 cows farms is 8.4 percent higher than the 11+ cows farms. The highest selling price from 5-10 cows farms comes as a result of selling milk (65-100 percent of sales) to the people houses or in the market where they have the highest price. 42.1 percent of farms with 5-10 heads of cows sell milk to the house or market versus 21.9 percent of farms 11+ cows.

In variable cost, feed took the highest share by 67.2 percent, ranging from 62.9 percent for the 5-10 cows farms to 71.2 percent of the 11+ cows farms, because the last ones are spending more on concentrate feed as the milk yield is higher compared with 5-10 cows farms.

Gloy et al. (2002) says that in terms of milk, some studies have found a negative relationship between spending on purchasing feed for cows and financial benefit measures.

While several studies carried out in different countries (Moran, 2005; Aktürk et al., 2010; Keskin and Dellal 2011; Semerci et al., 2014; Beldman et al., 2017; Zeqiri 2018; Krasniqi et al., 2018),the authors report that feed costs account for 57.0-87.5 percent of variable costs. Both type of farms use only artificial insemination for their cows.

Knowing that in our country the spread of artificial insemination covers 62 percent of the

cows population, it is understood that on the farms taken in the study this indicator is very positive in favor of artificial insemination since the regions where surveillance is conducted are the main regions of milk production, and should be considered that all farms are lying in the field area.

Regarding the method of milking by hand or by using milking machines, at 11+ cows farms, this indicator is higher in favor of milking machines, compared with 5-10 cows, indeed, we have 90.4 percent versus 74.2 percent, respectively. In 5-10 cows farms, all work is carried out by family members, while in the case of farms with 11+ cows in 22 percent of them, apart from family members, there are also employed workers because of the large number of heads.

Statgraphics Centurion XVI was used for statistical data processing, to compare 5-10 cows farms and 11+ cows farms for: GMpF milk+meat vs. Number of cows/year;

GMpF milk vs. Number of cows/year; and results are shown below:

**GMpF milk+meat vs number of cows**

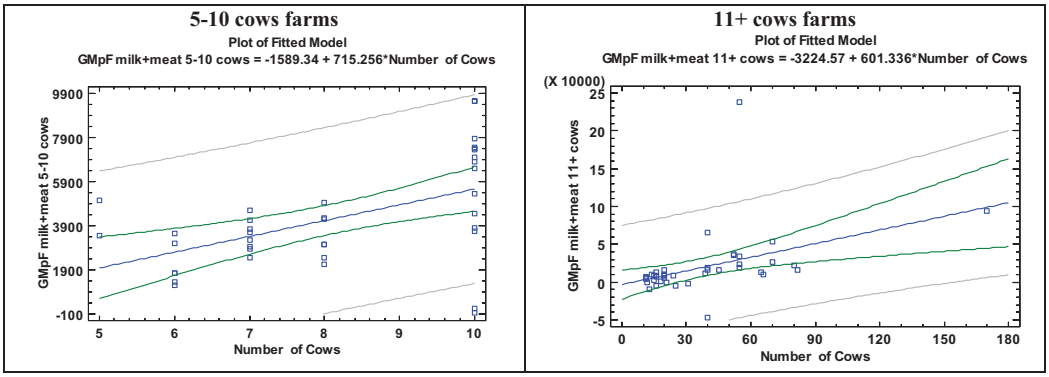


Figure 1. 5-10 and 11+ cows farms GMpF milk+meat vs Number of Cows

**5-10 cows farms:**GMpF milk+meat 5-10 cows = -1589.34 + 715.256*Number of Cows. The correlation coefficient equals 0.519478, indicating a moderately strong relationship between the variables. Since $p<0.05$(0.0008) there is a statistically relationship between GMpF milk+meat 5-10 cows and Number of Cows at the 95.0% confidence level.

**11+ cows farms**: GMpF milk+meat 11+ cows = -3224.57 + 601.336*Number of Cows. The correlation coefficient equals 0.438626, indicating a relatively weak relationship between the variables. Since $p<0.05$ (0.0041), there is a statistically relationship between GMpF milk+meat 11+ cows and Number of Cows at the 95.0% confidence level.

**GMpF milk vs number of cows**

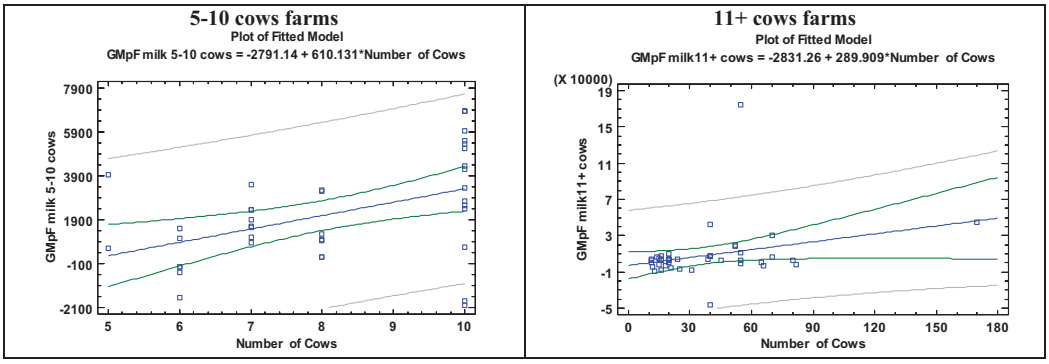


Figure 2. 5-10 and 11+ cows farms GMpF milk vs Number of cows

**5-10 cows farms:** GMpF milk 5-10 cows = -2791.14 + 610.131*Number of Cows. The correlation coefficient equals 0.457578, indicating a relatively weak relationship between the variables. Since $p<0.05$ (0.0039), there is a statistically relationship between GMpF milk 5-10 cows and Number of Cows at the 95.0% confidence level.

**11+ cows farms**: GMpF milk11+ cows = -2831.26 + 289.909*Number of Cows. The correlation coefficient equals 0.289602, indicating a relatively weak relationship between the variables. Since $p<0.05$ (0.0663), there is not a statistically relationship between GMpF milk11+ cows and Number of Cows at the 95.0% or higher confidence level.

## 2. Kosovo

Table 2. Technical data of dairy farms in Kosovo

| Number of heads | No of Farms | No of cattle per farm | No of Cows per farm (average) | Milk yield (liter) | IpF (milk + meat) Euro | IpF (milk) Euro | Variable cost per farm | GMpF (milk + meat) Euro | GMpF (milk) Euro |
|---|---|---|---|---|---|---|---|---|---|
| 5-10 cows | 68 | 12.54 | 7.25 | 3166 | 8168 | 7813 | 8486 | *-318* | *-673* |
| 11+ cows | 70 | 37.85 | 20.93 | 3932 | 28736 | 25915 | 24993 | 3743 | 922 |
| Total/average | 138 | 25.38 | 14.19 | 3555 | 18601 | 16995 | 16859 | 1742 | 136 |

**Table 2:** Continue

| Number of heads | Milk cost (Euro/kg) | Milk sold vs milk produced (%) | Price of milk sold (Euro) | Price of meat sold (Euro) | Milking | | Insemination | | Feed expense vs variable cost (%) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | by hand | by machine | Naturale | Artif. | |
| 5-10 cows | 0.369 | 62.96 | 0.347 | 2.69 | 44.1 | 55.9 | 44.1 | 55.9 | 66.4 |
| 11+ cows | 0.298 | 71.60 | 0.333 | 3.02 | 11.4 | 88.6 | 37.1 | 62.9 | 71.8 |
| Total/average | 0.333 | 68.2 | 0.336 | 2.85 | 27.5 | 72.5 | 40.6 | 59.4 | 69.2 |

The milk yield of 11+ cows farms (ranging from 2000 to 6238 kg/cow) is 24.2 percent higher than the 5-10 cows farms (ranging from 1633 to 5742 kg/cow). The milk cost of 5-10 cows farms is 23.8% higher than 11+ cows farms, while the milk price of 5-10 cows farms (ranging from 0.25 to 0.60 euro cents/kg) is 4.2% higher than the 11+ cows farms (ranging from 0.22 to 0.50 euro cents/kg). About 12% of the farms in both type of farms are selling the milk directly tothe market and getting the highest price of 0.50-0.60 euro cents/kg.

In variable cost, feed took the highest share by 69.2%. The same result is reported by Popescu (2009) where feeding keeps the highest share within Variable Costs: 75.44 - 77.36%. While for Kosovo is reported more than 60% (Zeqiri, 2018; Krasniqi et al., 2018).

The farms with 11+ cows had better access to artificial facilities (62.9% vs 55.9%) and milking machine (88.6 percent vs 55.9 percent) compare to the 5-10 cows farms. In addition, 25% of 5-10 cows farms and 57.1 percent of 11+ farms raise heifers in their herds. The returns of the dairy farms came from the sale of milk and meat. The highest share of total returns for all categories of farms came from the sale of milk (93.7-95.5%) and sale of calves (4.5-6.3%).

The GMpFmilk+meat and GMpF milk have negative values for the 5-10 cows farms (-318 Euro/farm and -673 Euro/farm, respectively), while the 11+ cows farms have positive values (3743 Euro/farm and 922 Euro/farm, respectively). However, for the all farms monitored, the values are positive 1742 Euro/farm for the GMpFmilk+meat and 922 Euro/farm for the GMpF milk. According to MBPZHR (2015b) analysis of competitiveness of agriculture of Kosovo shows that currently only a small share of farms can compete in the regional market, EU and international level.

In addition, several studies have found a negative relationship between expenditures for purchase feed per cow and measures of financial profitability (Gloy et al., 2002). Some

other studies have reported that the highest share in the milk production cost is represented by feeding, which is more sensitive to variation than average milk cost; therefore, milk economics deeply depend on feed cost (Sandor, 2003; Popescu, 2008; Popescu, 2009; Bytyqi et al., 2014). According to Popescu (2014) higher milk yield requires a higher production cost, an aspect that farmers should take into consideration and handle in the most efficient way.

The farm inefficiency decreased as farm size increased, it means that there is ample scope to raise farm profitability by improving economic efficiency and minimizing profit loss. According to Gloy "et al" (2002) many studies have reported a positive relationship between milk production per cow and various measures of financial success.

Statgraphics Centurion XVI was used for statistical data processing, to compare 5-10 cows farms and 11+ cows farms for:

- GMpF milk+meat vs. Number of cows;
- GMpF milk vs. Number of cows/year;

and results are shown below:

**GMpF milk+meat vs number of cows**

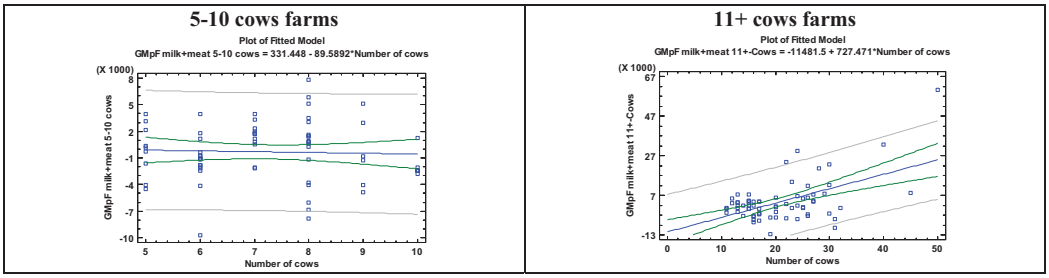


Figure 3. 5-10 and 11+ cows farms GMpF milk+meat vs Number of cows

5-10 cows farms: GMpF milk+meat 5-10 cows = 331.448 - 89.5892*Number of cows. The correlation coefficient equals -0.041, indicating a relatively weak relationship between the variables. Since $p>0.05$(0.7383), there is not a statistically relationship between GMpF milk+meat 5-10 cows and Number of cows at the 95.0% or higher confidence level.

11+ cows farms: GMpF milk+meat 11+-Cows = -11481.5 + 727.471*Number of cows. The correlation coefficient equals 0.54, indicating a moderately strong relationship between the variables. Since $p<0.05$(0.0000), there is a statistically relationship between GMpF milk+meat 11+-Cows and Number of cows at the 95.0% confidence level.

**GMpF milk vs number of cows**

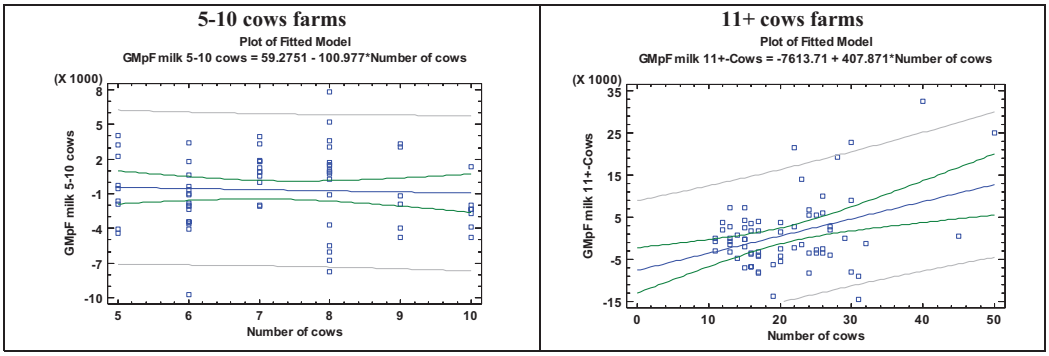


Figure 4. 5-10 and 11+ cows farms GMpF milk vs Number of cows

5-10 cows farms: GMpF milk 5-10 cows = 59.2751 - 100.977*Number of cows. The correlation coefficient equals -0.047, indicating a relatively weak relationship between the variables. Since $p>0.05$ (0.7043), there is not a statistically relationship between GMpF milk

5-10 cows and Number of cows at the 95.0% or higher confidence level.

11+ cows farms: GMpF milk 11+-Cows = -7613.71 + 407.871*Number of cows. The correlation coefficient equals 0.379263, indicating a relatively weak relationship between the variables. Since $p<0.05$ (0.0012), there is a statistically relationship between GMpF milk 11+-Cows and Number of cows at the 95.0% confidence level.

These data of our study show that 11+ cows farms had better results than the 5-10 cows farms for milk yield, production cost, gross margin per cow, use of artificial insemination and milking machine.

Cocchi et al. (1998) found that small dairy farms were 12 to 20 percent less efficient than larger dairy farms. While Delgado (2008) says that smaller farms typically receive lower prices for their output and still manage to realize higher unit profits in some cases is only partly explained by not costing family labor, which lowers the unit costs of smallholder producers more than those of large producers. However, the differential impact across sizes of farm was different in different countries and for different commodities.

According to Nastić et al. (2011) competitiveness of milk production is largely dependent on access to price competitive and high quality feed inputs and quality cattle. In the variable cost structure, the most significant are the costs of animal feed, whose share is 46.93%, than the costs of operating machines (29.4%) and other costs (18.99%), while the costs of veterinary services and artificial insemination are less than 3%.

## CONCLUSIONS

Cow milk is the most important livestock production in Albania and Kosovo.

Most farmers intuitively think about farm costs and returns. However, greater use should be made of ways to make them become aware of the relative importance of all their financial inputs, in terms of their contribution to the cost of production per kilogram of milk produced on the farm.

The economic results of our study (in both countries) are much better for 11+ cows farms than for 5-10 cows farms such as milk yield; milk cost; better access to artificial facilities and use of milking machine.

In Kosovo, the GMpFmilk+meat and GMpF milk for the farms with 5-10 dairycows have negative values (-318 Euro/farm and -673 Euro/farm, respectively), while the 11+ cows farms have positive values (3743 Euro/farm and 922 Euro/farm, respectively). The efficiency of the farm has increased with the increase farm size, it means that there is ample scope to raise farm profitability by improving economic efficiency and minimizing the profit loss.

While in Albania, as average the GMpF milk+meat and GMpF milk in both type of farms have positive results, however 15.8 percent of the 5-10 cows are showing negative results for the GMpF milk (loosing money), while for the 11+ cows farms this indicator is 31.7%, as the cost of production is very high.

As the 5-10 cows farms, in Kosovo, and several in Albania have negative incomes for the milk production is a must for extension service to train farmers to keep the financial record separate for milk, and other crops. In addition the extension service needs to train the farmers for better: management of their farm, feeding system, fodder production, animal health etc.

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# BREEDING TECHNOLOGY INFLUENCE ON SPERM CONCENTRATION IN BROILER BREEDERS

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***Abstract***

*Researches performed in this study aimed to study semen quality and breeding efficiency and other characters that in conjunction are shaping the fecundity of breeding cocks in ROSS 308 breeding cocks under the influence of some microclimatic factors (light intensity, birds density). Observations during the three trials (A – with analyze parameters sub-standard and litter made of chopped straws B – with analyze parameters above standard and litter made of rice hulls and C – with analyze parameters at the level recommended by the manufacturer of biological material and litter made of wood shavings) were performed during 3 control weeks (25, 35 and 45) during production period (19-64 weeks). The values obtained for sperm concentration were between 0.730 ±0.02 billion/ml, in week 45 – series C of trials and 1.044 ± 0.07 billion/ml in week 35 – series A of trials. Therefore stress due to microclimatic parameters stepping up manifests itself through slight decrease in the concentration but secures a consistency of its value on a longer period which would positively influence the effectiveness of breeding.*

***Key words****: litter, breeding cocks, density, light intensity, concentration of sperm.*

## INTRODUCTION

Concentration in sperm is the number of sperm per volume unit of sperm and varies depending on some factors influenced by species, race, maintenance conditions and not lastly by animal (Bunaciu, 2009; Vacaru Opriș, 2002).
There are large differences between species about spermatogenesis and so about concentration in sperm of the ejaculate. Thus average values are between 9.46 and 3.68 x $l0^9$ sperm/ml (Fujihara, 1991).
Data are showing that there is a positive correlation between sperm volume and sperm concentration (Sonseeda et al, 2013; Churchil, 2014).
Sperm concentration is also dependent on the interval between collections so on intensity of use of the breeding; when pause between collections increases sperm concentration also increases (Bunaciu, 1989).
Sperm is diluted during ejaculation due to sperm liquid consisting of fluids secreted by accessory reproductive organs (Fujihara, 1991).
The concentration of sperm in semen correlates negatively with volume which demonstrates that sperm concentration decreases as semen volume increases, (Bunaciu, 1992; Hermiz et al., 2016).
This phenomenon occurs due to increase secretions attachments not as a consequence of intensifying the spermatogenesis process.
It is therefore necessary that to measure the volume and to determine sperm concentration when males are chosen for breeding purposes.
Male body development has no influence on sperm concentration (Bunaciu, 2009).
Sperm quality and sperm capacity of fertilization are influenced by dietary deficiencies as lack of vitamin E or essential fatty acids (Jensen, 1968), presence of aflatoxin in feed, (Yaroshen et al., 2003) or low calcium level (Bunaciu, 2009).

## MATERIALS AND METHODS

Semen quality is often defined by four characteristics: volume, concentration, viability

of sperm (% of live sperm) and sperm motility (Parker et al., 2000; McGovern, 2002).
The number of sperm deposited in the female genital apparatus (concentration of sperm in semen) is of particular significance. Although a single sperm and ovule copulates in the intimate fecundation process a far greater number of seminal cells must attend to this process.
This is because sperm once around follicular development are releasing the ovule from follicular cells which make up the radiated Crown by releasing hyaluronidase. These cells are united between them with a substance containing hyaluronic acid.
The presence of a greater number of sperm around ovule causes the release of a much larger amount of hyaluronidase and so fecundation process is favored.
As shown in numerous studies cited in this paper the ability of fertilization of cocks directly depends on the quality of semen (volume, concentration, mobility, etc.). Technological factors (temperature, humidity, density, intensity and duration of light, litter quality, etc.) may affect the ability of fertilization of cocks.
As in females a significant decline in semen parameters is found from cocks in some stress conditions determined by microclimate factors.
Considering the mentioned facts our own research undertaken in this thesis aimed to study the quality of semen and breeding efficiency in ROSS 308 hybrids cocks in terms of the influence of microclimate factors (light intensity, birds density) and other characters that collectively are determining the fertilization ability of cocks.
Works have been undertaken within the framework of three units one for each series of experiments: Avicola Călăraşi, S.C. Agrafood S.A. and Avicola Focşani and observations and records were carried out in 3 weeks for control (25, 35 and 45), during production period (19-64 weeks) during two years an on an effective of 25 males and 250 females for each experimental series.
Microclimate parameters considered for the series A of trials are:

- litter: chopped straws;
- luminous intensity under the standard: 30 lux;
- birds density under the standard: 3 males/$m^2$;
- the ratio of sexes under the standard: 25 weeks - 8 heads, 35 weeks – 7.5 heads, 45 weeks – 6.5 heads.

Microclimate parameters considered for the series B of trials are:

- litter: rice husks;
- luminous intensity above the standard: 70 lux;
- birds density above the standard: 5 males/$m^2$;
- the ratio of sexes above the standard: 25 weeks - 9 heads, 35 weeks – 8.5 heads, 45 weeks – 7.5 heads.

Microclimate parameters considered for the series C of trials are:

- litter: wood sawdust;
- luminous intensity standard: 40 lux;
- standard birds density: 4 males/$m^2$;
- the ratio of sexes standard: 25 weeks - 8,5 heads, 35 weeks - 8 heads, 45 weeks -7 heads.

Raising the birds was carried out in uniform conditions in the three units corresponding to those three series of experiments on permanent bedding (wide captivity) and in upgraded sheds and feed and water have been provided under the technical card of the hybrid. The individuals analyzed in the three series of experiments have benefited from the same feeding conditions in order to ensure comparability of results.
The pointer being chased during production was the quality of semen (sperm concentration) and this indicator has been determined using a spectrophotometer.
Classical statistical methods were used to characterize the phenotypic testing of batches (Sandu, 1995) and Student's test has been used to study the variation of parameters showing a normal distribution for comparing the two samples homogeneity environments (Sandu, 1995).

## RESULTS AND DISCUSSIONS

Sperm concentration (or sperm density) is an important indicator of the quality of semen and the setting is the defining reproductive capacity of males and representing the starting point

within the technology of conservation in order to establish the degree of dilution of sperm. Subjectivity is removed entirely in appreciation of this character as evaluation of ejaculates in this direction is done by spectrophotometry.

At this level, however, there is a problem which could justify any variances in relation to semen fecundation ability concerned that the analysis of sperm concentration with spectrophotometer it doesn't take into account mobility and anomalies of morphology.

It is known that a high concentration of semen can be also determined by a large proportion of sperm immobile or with abnormal movements without the opportunity to participate in intimate of fecundation.

In table 1 and the graph in Figure 1 are presented the values obtained for sperm concentration at individuals in the A series of trials durint the 3 control weeks.

Table 1. Average values of concentration of sperm for first experience series

| Week | n | $\bar{X} \pm s_{\bar{X}}$ (bl/ml) | s | c.v.% |
|---|---|---|---|---|
| 25 | 25 | 1.050 ± 0.07 | 0.3378 | 32.17 |
| 35 | 25 | 1.044 ± 0.07 | 0.3555 | 34.05 |
| 45 | 25 | 0.770 ± 0.06 | 0.2764 | 35.89 |

Data presented in table 1 and the graph in Figure 1 are revealing that the concentration of sperm in the semen has values that can be assigned in the normal range of the species with a large variability in all three control weeks.

It is found a dense sperm confirming the practical correlation between pH values and semen quality (in the series A of experiments has been recorded the lowest pH value).

It has been noticed the significant fall in sperm concentration in week 45 which is also highlighted very well by the pH value in this control week.

The differences observed between the concentrations of sperm in the three control weeks have been tested as statistical significance using the Student test and values are shown in table 2.

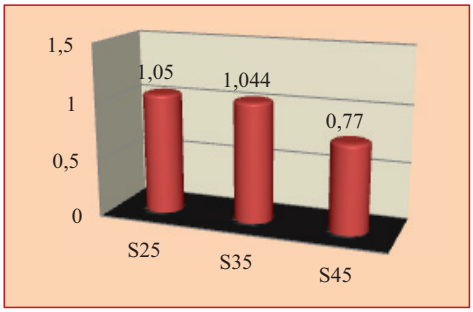


Figure 1. Average values of concentration of sperm for first experience series

Table 2. Testing the significance of differences observed between the three weeks in terms of concentration of sperm, first series

| Specification | S25 | S35 | S45 |
|---|---|---|---|
| S25 | - | $0.87^{NS}$ | $3.25^{***}$ |
| S35 | | - | $3.08^{***}$ |

Data in table 2 are showing that there are differences between average values of sperm concentrations during the three control weeks inside series A without statistical significance between weeks 25 and 35 and very significant as for the rest. Differences in terms of statistical significance of the differences between the pH and the concentration in the sperm can be explained by the different degrees of variability of the two characters in the analyzed samples.

The values obtained for the concentration of sperm in the coming of the individuals within the B series of experiments from the period of adult has been presented in table 3 and graph in Figure 2.

Table 3. Average values of concentration of sperm for second experience series

| Week | n | $\bar{X} \pm s_{\bar{X}}$ (mld/ml) | s | c.v.% |
|---|---|---|---|---|
| 25 | 25 | 1.037 ± 0.05 | 0.2516 | 24.26 |
| 35 | 25 | 1.032 ± 0.06 | 0.3201 | 31.02 |
| 45 | 25 | 1.014 ± 0.06 | 0.2924 | 28.84 |

The data reveals the existence of sperm concentration in values in the normal range of the species with a great variability of observations during the three control weeks like in trial series A.

Concentrations in sperm obtained in series B are correlated with high pH recorded from these individuals and there are smaller than

those obtained inside series A with the exception of week 45.

Observed differences between character averages inside the three weeks have been tested for statistical significance and calculated values of Student test are shown in table 4.

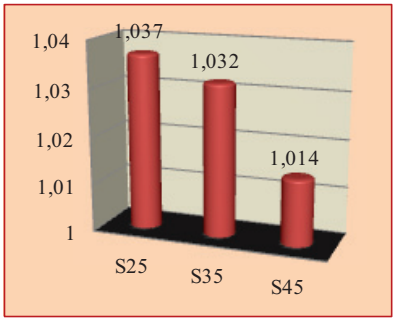


Figure 2. Average values of concentration of sperm for second experience series

Table 4. Testing the significance of differences observed between the three weeks in terms of concentration of sperm, second series

| Specification | Week 25 | Week 35 | Week 45 |
|---|---|---|---|
| Week 25 | - | $0.11^{NS}$ | $1.93^{*}$ |
| Week 35 | | - | $1.72^{*}$ |

Calculated values of the Student test (table 4) are revealing the existence of significant differences in relation to the amount of sperm concentration between week 45 and the other two weeks otherwise they will be caused of random or individual variation without meaning in terms of statistically with no statistical significance.

The smaller values of sperm concentration inside trial series B are correlated with values of pH to individuals in this series. The lower values of concentration might also be due to a higher volume of the ejaculate obtained from cocks in series B. Considering that during the trial the same feeding conditions have been maintained results of sperm concentrations is not in measure to highlight a certain influence of the values of technological parameters and the type of litter (husks of rice) on sperm quality and so further investigations are necessary.

The values obtained for the concentration of sperm in the coming of the individuals in the series C of experiments, from the period of adult, has presented in table 5 and graph in Figure 3.

Table 5. Average values of concentration of sperm for third experience series

| Week | n | $\bar{X} \pm s_{\bar{X}}$ (mld/ml) | s | c.v.% |
|---|---|---|---|---|
| 25 | 25 | 1.042 ± 0.02 | 0.11 | 10.54 |
| 35 | 25 | 1.036 ± 0.02 | 0.12 | 11.27 |
| 45 | 25 | 0.730 ± 0.02 | 0.11 | 15.32 |

Presented data are revealing the existence of sperm concentration values inside the normal range with the slightest degree of variability registered amongst all trial series most probably due to environmental conditions at standard values and usage of a classical sawdust litter.

Observed differences between average concentrations of semen inside the three control weeks of the period of adult have been tested for statistical significance and calculated values of Student test are shown in table 6.

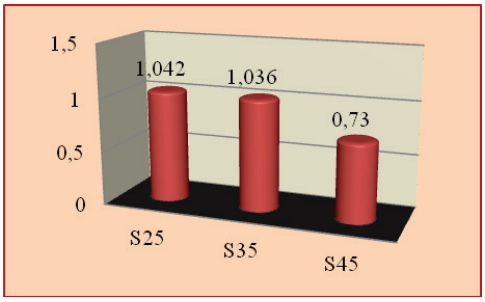


Figure 3. Average values of concentration of sperm for third experience series

Table 6. Testing the significance of differences observed between the three weeks in terms of concentrate of sperm, third series

| Specification | Week 25 | Week 35 | Week 45 |
|---|---|---|---|
| Week 25 | - | $0.17^{NS}$ | $1.85^{*}$ |
| Week 35 | | - | $1.69^{*}$ |

Calculated values of Student test (table 6) are revealing the existence of differences statisticaly significant between the three control weeks inside series C with the exception of weeks 25 an 35. Drastic decrease in sperm concentration in week 45 of control is most likely correlated with a higher volume of ejaculate recorded in the same period.

In the graph in Figure 4 we are showing the observed differences between registered averages in the three trial series concerning the concentration of sperm in semen.

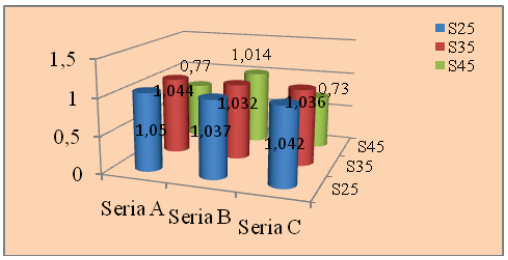


Figure 4. Comparative between the three expreimental series on concentration of sperm

Analyze of values are revealing some aspects of sperm concentration of semen. Thus firstly it is observed that values obtained inside trial series B were not the highest values of the analyzed character but they were the most constant during the three control weeks. This is somewhat in contrast to the pH values obtained under the same series but it seems that the higher values of these influenced just slightly the order of magnitude of concentration in sperm and not at all their frequency. Such results seem to advocate in favor of the usage of microclimate parameters at values above the technological standard and of a litter of rice husks. Secondly during trial series A and C has been noticed a decrease of sperm concentration values in week 45 of life. Results obtained during trial series B are suggesting that stress caused by intensification of microclimate parameters is indeed manifested through a slight decrease of concentration but assures a consistency of its value on a longer period which would beneficially influence biological and economical efficiency of breeding activity.

There were tested for statistical significance the observed differences between averages of sperm concentration from the three trial series during the whole control period with the aim to verify the influence of microclimatic parameters and of the ratio of sexes and of litter type on the quality of semen. We are showing in tables 7-9 calculated values of Student test and their significance.

Calculated values of Student test are showint the existence of some differences with different degrees of statistical significance between averages of sperm concentration from the three trial series. It is noticed the fact that there are differences significant from statistical point of view only during week 45 when sperm concentration is decreasing during series A and C. This fact allows us to state that exposing individuals to values above standard of technological parameters and using rice husks as litter are contributing to stepping up physiological processes which are controlling breeding activity with favourable influences on the effectiveness of the unit.

Table 7. Testing of significance for differences between experimental series, 25th week, for concentration of sperm

| Specification | t test value |
|---|---|
| A-B | $0.31^{NS}$ |
| A-C | $0.54^{NS}$ |
| B-C | $0.09^{NS}$ |
| $t_{49;0,05} = 1.68$; $t_{49;0,01} = 2,40$; $t_{49;0,001} = 3,50$ | |

Table 8. Testing of significance for differences between experimental series, 35th week, for concentration of sperm

| Specification | t test value |
|---|---|
| A-B | $0.07^{NS}$ |
| A-C | $0.06^{NS}$ |
| B-C | 0 |
| $t_{49;0,05} = 1.68$; $t_{49;0,01} = 2,40$; $t_{49;0,001} = 3,50$ | |

Table 9. Testing of significance for differences between experimental series, 45th week, for concentration of sperm

| Specification | t test value |
|---|---|
| A-B | $7.18^{***}$ |
| A-C | $0.91^{NS}$ |
| B-C | $6.98^{***}$ |
| $t_{49;0,05} = 1.68$; $t_{49;0,01} = 2,40$; $t_{49;0,001} = 3,50$ | |

But we are saying again that a high sperm concentration of semen does not necessarily correspond to with a high reproductive capacity because the spectrophotometry does not necessarily notice the difference between viable spermatozoids and those morphologically abnormal or with aberrant movements and so supplementary investigations are necessary.

## CONCLUSIONS

Certain values associated with the effectiveness of the biological characters and economical effectiveness of breeding activity has been determined during the production period. Following conclusions might be drawn concerning the sperm concentration:

- in the series A it is found that there is a dense sperme which is practicly confirming the correlation between pH values and semen quality;
- It is found that there are some differences with no statistical significance between average values of sperm concentration during the three control weeks inside series A between weeks 25 and 35 and very significant as for the rest;
- concentrations in sperm obtained in series B are correlated with the high pH recorded at these individuals and are smaller than those obtained inside series A with excepting week 45;
- Calculated values of Student test are revealing the existence of significant differences concerning sperm concentration inside series B between week 45 and the other two weeks otherwise they will be caused by random or individual variation with no statistical significance.
- The lowest degree of variability between all trial series has been obtained in the series C most probably because of the environmental conditions at standard values and usage of a classical sawdust litter.
- the results obtained in experimental series B suggests that stress caused by intensification of microclimate parameters are indeed manifested by lightly decreasing sperm concentration but this provides more than its value to a consistency which would positively affect biological and economic efficiency of breeding activity;
- Calculated values of Student test are revealing the existence of differences with varying degrees of statistical significance between average sperm concentrations of the three experimental series.


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# STUDY ON IMPROVING BEEF PRODUCTION THROUGH INDUSTRIAL CROSSING

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***Abstract***

*This paper aimed to assess the improvement of beef production using industrial crossbreeding. The research was conducted on 101 individuals allotted in four groups, L1-Romanian Spotted cattle (RS) – control 1; L2 Limousine (L) x Romanian Spotted cattle; L3-Romania Black and White cattle (RBW) – control 2 and L4-Limousine x Romanian Black and White cattle. Body development of half-bloods from experimental groups was higher than the maternal breeds, showing pronounced aptitudes for beef production, especially in L x RS cross, which performed a body weight by 18 months old of 689.37 kg (+14.36% compared to maternal breed). An obvious improvement of beef yield was registered also in L x RBW group (603.13 kg live weight at 18 months old, +15.38% vs. the maternal breed). BS x L half-bloods had an average daily gain above 975 g, fitting into the beef morphological type. In the study, we found a good ability of body development for half-bloods and a pronounced heterosis effect compared to maternal races.*

***Key words****: cattle, production, beef, improvement, half bloods.*

## INTRODUCTION

Beef production could be regularly improved by increasing weight at slaughter and adjusting husbandry technological factors or through genetic-breeding methods, such as crossing with beef specialised breeds (Maciuc et al., 2018; Ujică et al., 2011; Phocas et al., 2005).

The use of first generation crossbreeds between bulls from beef breeds with cows from mixed and dairy breeds for increasing beef production has expanded in all advanced countries in the world, due to the achieved economic efficiency. Cows with low milk production, with udder diseases, reformed cows after 1st calving that do not interest in selection and dairy cows that constitute a surplus for the farmer are used as maternal receptacles in such crossings (Barwick et al., 2005; Crump et al., 1997; Pribylova et al., 2004).

The economic advantages of first generation crossbreeds are: better ability of gaining weight; the use of the heterosis effect to achieve high weight gains; higher beef production, in terms of yield and quality indexes (Maciuc et al., 2018; Vidu et al., 2013; Ujică et al., 2011).

Worldwide, the trends are to increase beef production, both quantitatively (especially in those countries with food deficit) and qualitatively (goal to be achieved by the countries more developed economically).

Most research, such as those in Denmark on crossbreeds between dairy and beef races, demonstrates the possibility of improving meat production by obtaining commercial hybrids with beef attributes (Bignon, 2008; Eriksson et al., 2004, Onaciu et al., 2016).

Out of the numerous data presented in the scientific literature it rise the importance and utility of crossings of local breeds with beef specialized breeds, due to the occurrence of the heterosis effect which is translated into: better feed conversion, especially of forages and pasture species; increasing of the average daily gains by 6 to 10%; increasing of the live weight at slaughter and, subsequently, of the dressed percentage value; higher participation of great quality meat in carcass and better protein levels in the proximate beef composition; better caloric value of carcasses; inheritance of carcass quality traits via paternal genetic lines; exceptional potential in genetic combinability of Simmental breed in crossings with beef

breeds; overall improvement of meat yields, vitality and resistance against various diseases.
Also, there are in the national herd many cows with hypermetric body development. It is also known that the beef oriented breeds are better in energy conversion therefore in overall energetic balance of the production ecosystem, hence they do not require high amounts of feed to convert into the main product, due to 8-12% lower feed conversion ration value, in comparison with the non-beef oriented breeds (Agus et al., 2018; Aiello et al., 2018; Disking et al., 2018; Kelsey et al., 2018; Koenig et al., 2018; Li et al., 2018; Vendramini et al., 2018; Zhao, 2019).
Usage of industrial crossings, as way to amplify the heterosis effect remains one of the main challenges in cattle farming, worldwide. The perpetual importance of this technique is given by the permanent need in increasing the beef yield (Chilimar, 2006; Nelson et al., 2018; Onaciuc et al., 2016; Walmsley et al., 2018).
The research is part of the current national guidelines in order to know the effect of industrial crosses with specialized breeds and improve beef production in cattle using the biological potential that we have (Vidu et al., 2013; Fogh, 2007).

## MATERIALS AND METHODS

In our country, it has been undertaken relatively little research to test the combining capacity of indigenous breeds (Romanian Spotted, Black and White Romanian, Brown of Maramureş, Pinzgau of Transylvania, Grey Steppe) with beef specialized, especially with modern breeds.
That's why we wanted to know the combinative capacity of the national breeds with Limousine breed and to use for Tow-breed Terminal Cross cows from Romanian Spotted and Black and White Romanian races.
Adult cows with unaltered reproductive function and without incidents in precedent parturitions were chosen to become crossbreeds' mothers. They were inseminated with semen from valuable bulls belonging to beef breeds.
Milk yields of mothers cows were good in Romanian Spotted breed and very good in Black and White Romanian Breed, being able to provide enough milk to the suckling calves, that induced them a better body development.
One hundred and one individuals were used as biological material, assigned to four groups: $L_1$ Romanian Spotted – control group; $L_2$ Limousine x Romanian Spotted; $L_3$ Black and White Romanian – control group and $L_4$ Limousine x Black and White Romanian. Subsequently, the issued crossbreeds were raised separately in accordance with their age group (calving, 6 months, 12 months, 18 months) within the same farming conditions, the half-intensive production system.
The diets were balanced accordingly, to comply the average daily gains requirements in each specific rearing stage and age maintenance needs of the breeding stock.
The feed rations were formulated in accordance with the existing feed raw matters, in order to cover the energy, protein, minerals and vitamins needs and were provided to both crossbreeds' groups and to pure breeds-control groups.
Within our research, the assessment of beef production was measured through fattening (growth) indexes, as related to body weight, as well as through the measurements of the main body dimensions, applied in all groups and age categories.
There were carried on weightings and body measurements such as: height at withers, height at hips, body length, depth of chest (heart girth), head length, head width, chest (thorax) circumference, cannon circumference.
Primary acquired data were systematised in a database then statistically processed. The statistical descriptors (estimators) that characterise a normal distribution are represented by both median and mean, as well as by the dispersion indexes, such as the variance, standard deviation of each analysed traits. They were noted with Latin and Greek letters, as following: arithmetic mean ($\overline{X}$), variance ($s^2$), standard deviation (s), theoretical mean ($\mu$), variance ($\acute{o}^2$) and standard deviation ($\acute{o}$).
The S.A.V.C. software package (Statistics, Analysis of Variance and Covariance, 2003) was used to compute the arithmetic mean ($\overline{X}$), standard mean error ($\pm s_{\bar{x}}$), standard deviation (s), coefficient of variation (V %) as well as the

significance of the differences between means (ANOVA and *p* values).
Data analysis was correlated with the field observation at farm levels and in accordance with the European Union requirements and normatives.
Due to the real interest manifested by the cattle farmers for the results acquired in the crossbreedings between local Romanian breeds and the two tested beef specialised breeds, the follow-up of the research was to investigate the usage of other beef specialised breeds in crossings, especially in those individual farms from hillous and mountainous areas.

## RESULTS AND DISCUSSIONS

The average values and variability of body weight at birth are shown in Table 1.
Analysing body weight at birth, in relation with gender and cross-breeding, revealed that Lx RS half breeds had intermediate average values of 40.83 kg for males and 37.80 kg for females. The average value for both groups was 40.57 ± 0.412 kg.
In the cross-breeding variant of RBWx Limousine, the calves had lower average birth weights than those resulting from the first crossbreeding variant RS x L. RBW breed registered an average weight of 36.76 kg for males and of 35.08 kg for females.
Average value for both (RBW males and females) was 35.34±0.280 kg. Compared to half-breeds F1 L x RS, L x RBW half breed had lower body weight.

Table 1 The mean values and variability of the body weight at birth

| Group | Gender | n | $\overline{X} \pm s_{\overline{x}}$ | s | V% | Min | Max |
|---|---|---|---|---|---|---|---|
| $L_1$ RS | M | 14 | 38.42 | - | - | - | - |
| | F | 10 | 35.73 | - | - | - | - |
| | Total | 24 | 36.52±0.421 | 2.06 | 5.58 | 33 | 40 |
| $L_2$ L x RS | M | 15 | 40.83 | - | - | - | - |
| | F | 10 | 37.80 | - | - | - | - |
| | Total | 25 | 40.57±0.412 | 2.06 | 5.28 | 35 | 42 |
| $L_3$ RBW | M | 15 | 36.76 | - | - | - | - |
| | F | 12 | 35.08 | - | - | - | - |
| | Total | 27 | 35.34±0.280 | 1.45 | 4.07 | 33 | 38 |
| $L_4$ L x RBW | M | 13 | 38.97 | - | - | - | - |
| | F | 12 | 36.56 | - | - | - | - |
| | Total | 25 | 38.09±0.304 | 1.51 | 4.08 | 35 | 40 |
| **Total** | | **101** | **37,93±0,215** | **2.64** | **6.98** | **33** | **46** |

Depending on the crossbreed variant, the ANOVA test indicated very significant differences between the L x RBW group and the RBW group, between the L x RS group. The L x RS half-breeds had an average body weight at birth with 2.08 kg higher then RS breed ($p<0.001$).
Analysing body weight variability at birth, depending on crossbreeding variant we notice that maximum value for standard deviation for total group was 2.06 kg and for coefficient of variance was 5.58%.
Analysing dispersion indices we can conclude that for body weight at birth the groups were homogeneous.
Following body development of experimental groups at 6 months old, 12 months old and 18 months old, there were found the following results: at 6 months old, all males from groups had a body weight above 200 kg, with the exception of the RBW group.
Higher development was noticed in L x RS with an average body weight of 237.53±1.636 kg, followed by L x RBW with an average body weight of 219 kg (tab. 2).
For overall groups, average body weight at 6 months old was 223.74 kg, suggesting thus a good body development.

Table 2. The mean values and variability of body weight at 6 months (males, n=15 per group)

| Group | $\overline{X} \pm s_{\overline{x}}$ | s | V% | Min | Max |
|---|---|---|---|---|---|
| $L_1$RS | 209.57±1.113 | 4.16 | 1.98 | 203 | 215 |
| $L_2$ L x RS | 237.53±1.636 | 6.33 | 2.82 | 211 | 262 |
| $L_3$RBW | 188.20±0.932 | 3.61 | 1.91 | 182 | 195 |
| $L_4$ L x RBW | 219.31±0.536 | 1.93 | 0.91 | 209 | 230 |
| Total | 223.74±1.994 | 18.38 | 8.48 | 182 | 262 |

The most significant weight differences were recorded between the RBW group and the L x RS group (49.33 kg) and L x RBW (31.00 kg) half-breeds.
At 6 months old, the groups were homogeneous enough, the standard deviation values being between s = 1.93 kg for L x RBW group and s = 6.33 kg for L x RS group.
The variability amplitude had a minimum limit of 188.20 kg (RBW) and a maximum of 237.53 kg (L x RS). At this age, the average weight on all groups was 223.74 ± 1.994 kg.
For the 12-month age, body weight indices are shown in Table 3.
It was found that the groups L x RS and L x RBW exceeded the weight of 400 kg, while the RS and RBW groups achieved average weights close to the 400 kg threshold.

The weakest body development was found in RBW group, of just 335.14 ± 1.082 kg, much lower than the other experimental groups.
The best body development was achieved for Lx RS, which was 237.53 ± 1.636 kg. Also, the average body weight for all groups studied was 427.68 ± 1.223 kg.
The dispersion indices depict a good homogeneity of all groups, standard deviation values being between 5.17 kg (RBW) and 9.52 kg (L x RS), while the coefficient of variation oscillated between 0.57% (L x RS) and 2.29% (RS).
At 12 months old, L x RS group is ranked first with an average body weight of 463.37 kg, followed by the L x RBW group with 417.38 kg, keeping the same sequence from the previous ages (birth, 6 months).

Table 3 The mean values and variability of body weight at 12 months (males, n=15 per group)

| Group | $\overline{X} \pm s_{\overline{x}}$ | s | V% | Min | Max |
|---|---|---|---|---|---|
| $L_1$RS | 397.36±1.419 | 9.05 | 2.29 | 380 | 410 |
| $L_2$ L x RS | 463.37±0.652 | 9.52 | 0.57 | 437 | 446 |
| $L_3$ RBW | 335.14±1.082 | 5.17 | 1.55 | 323 | 340 |
| $L_4$ L x RBW | 417.38±1.049 | 6.66 | 1.72 | 375 | 400 |
| **Total** | **427.68±1.223** | **47.87** | **11.68** | **323** | **498** |

The amplitude of variability had the lower limit of 335 kg (RBW) and the upper limit of 463.37 kg (L x RS).
The average values and the significance of differences for the live weight at 18 months old are presented in Table 4.

Table 4 The mean values and variability of body weight at 18 months (males, n=15 per group)

| Group | $\overline{X} \pm s_{\overline{x}}$ | s | V% | Min | Max |
|---|---|---|---|---|---|
| $L_1$ RS | 589.44±1.209 | 11.72 | 2.01 | 568 | 625 |
| $L_2$ L x RS | 689.37±1.774 | 8.32 | 1.26 | 648 | 700 |
| $L_3$ RBW | 510.33±1.167 | 6.500 | 1.29 | 488 | 520 |
| $L_4$ L x RBW | 603.13±1.287 | 6.468 | 1.12 | 565 | 615 |
| **Total** | **619.21±1.133** | **8.532** | **1.41** | **488** | **712** |

From the comparative analysis of the body weight at 18 months old it was found that the L x RS group achieved the highest weight (689.37 kg) while the L x RBW half-breeds achieve the lowest one (603.13 kg).
Compared with males from maternal breeds, Lx RS and L x RBW half-breeds performed higher weights, with very significant differences as result of ANOVA ($p < 0.001$).
Not significant differences were recorded only between the RS and RBW groups and the Lx RBW half-breeds exceeded the weight of RS males with 13.69 kg.
The largest differences were recorded between the L x RS group and RBW group (179.04 kg), but also between the L x RBW group and RBW group (92.80 kg).
The individual variability in the groups was reduced, the groups being sufficiently homogeneous, as indicated by dispersion indices (s and V%), the maximum standard deviation being recorded for the RS group (s=11.72 kg and V% 2.01).
The maximum variability amplitude was recorded for the L x RS group (689.37 kg) and the minimum for the RBW group (510.33 kg).
Following the presentation of the results on the evolution of body weight from birth to 18 months old, it resulted that half-breeds issuing from RS x Limousine had higher growth energy than the RBW x Limousine half-breeds.
The dynamics of body weight by age and genotype is shown in Figure 1.

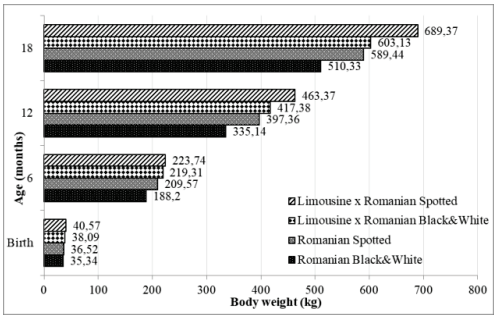


Figure 1. Body weight dynamics, according to age and genotype

In both cases, however, the half-breeds had higher body weights than maternal breeds (RS and RBW), which fully justifies the use of these cross-breeds to improve beef production. The chart representation clearly highlights the superiority of beef half-breeds as compared to maternal breeds throughout the fattening period.

## CONCLUSIONS

Absolute and relative values show that F1 half-breeds calves had better body development at birth compared to maternal breeds (RS and RBW).

The body development of the half-breeds in the experimental groups is higher to the maternal races, highlighting the pronounced skills for beef production, especially in Lx RS half breeds.

Thus, the Lx RS half-breeds achieved the body weight of 689.37 kg, exceeding the maternal breed with 99 kg (14.36%). The RS x L half-breeds achieved an average daily gain of more than 975 g/day, fitting into the morphological type of meat.

An obvious improvement in beef yield was also achieved in the case of L x RBW half-breed, which achieved at 18 months old a body weight of 603.13 kg, higher than the native breed with 92.80 kg (15.38%).

The economic advantages of industrial crosses result from the better ability of the half-breeds to fattening and use the heterosis effect to achieve high weight gains, as well as by higher indexes in the quantity and quality of beef.

The main conclusion is that all tested breeds could be successfully used in crossing. Those cows having a higher body development could be inseminated with sperm produced by Charolaise and Limousine bulls, while those with lower development are to receive Hereford sperm, knowing that interracial hybrids with Charolaise and Limousine tend to develop more muscle mass during fetal stage, inducing, therefore, possible calving issues.

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# RESEARCH ON THE FATTY ACID COMPONENT OF GOAT MILK, OBTAINED IN DIFFERENT BREEDING SYSTEMS

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***Abstract***

*The aim of the research was to evaluate the productive performance of Saanen goats by applying an optimizing modern food rations formula. The experiments were realized between 1.05-2015-2.11.2016 in 2 farms of Constanta County, Romania. Experiments were conducted on two groups of 30 females in full lactation with an average weight of 55 kg, breeding in anintensive system(Group I) and semi-intensive system (Group II). The experiment lasted 130 days; during this period the milk production was recorded and samples were taken monthly for analysis, in particular the concentration of fatty acids and food consumption. Milk samples were processed by gas chromatography.Between the two groups at the same time changes in fatty acids were recorded for significantly increasing concentrations of monounsaturated fatty acids (MUFA-28.94 g FAME / 100 g fat) and polyunsaturated fatty acids (PUFA-4.86 g FAME / 100 g), as well as an improvement of the Ω3: Ω6 ratio to the second group (20.66). In conclusion the breeding system, energetic and protein level of food ratio was influenced the milk production and the fatty acids composition.*

***Key words****: goat, nutrition, milk production, fatty acids.*

## INTRODUCTION

In recent years, the goat sector in Romania has continuously grown, so from 750,000 goats in 1997, the total number of goats has increased to over 2,000,000 goats in 2017. At the same time, the breed structure has changed due to the massive import of goats specialized in milk production, among which the most numerous are Saanen and Alpine breeds. These breeds are intensively used to improve milk and meat production in Carpatina and Alba de Banat local goat breeds. The main motivation for numerical growth of goats is: 1) the existence of favorable breeding conditions in Romania; 2) the increase in consumption of milk and processed products from goat milk due to its special qualities for human health; 3) more accessible reproductive and growth conditions for goat farmers compared to dairy cows. The nutritional composition of goat's milk is influenced by different factors: season, stage of lactation, breed, genetics, nutrition, environmental factors. Goat milk contains about 87% water, 4% carbohydrates, 4% lipids, 3-4% protein, about 0.5% minerals (including 120 mg of calcium) and vitamins (Morand-Fehr et al., 2007; Park et al., 2007). The whole goat milk contains about 35 g/l of fat composed of 99.5% lipids and 0.5% liposoluble substances (cholesterol, vitamins A, D). Lipids essentially have an energetic role (9 kcal/g). Goat milk contains a wide variety of fatty acids (FA), classified according to the length of their carbon chain and the number of double bonds. Goat FA contains about 65-70% of saturated FA and 30-35% unsaturated FA (USDA 2004). The large amount of medium chain fatty acids has benefits to human health.

The quality of the goat milk, especially on fatty acidcomposition is influenced by leguminous plants (Shingfield et al., 2008). Goat milk contains a higher amount of short chain fatty acids, being richer in butyric acid (C4:0), caproic (C6:0), capric (C8:0), capric (C10:C18:1), myristic (C14:0), palmitic (C16:0), linoleic (C18:2), which have numerous benefits for human health (Park, 2007; Haenlein, 2004; Tudisco et al., 2010; Kučević et al., 2016). The objectives of this study was to evaluate the milk productions and biochemical composition of milk fatty acid on Saanen imported goats, breeding inintensive and traditional systems.

## MATERIALS AND METHODS

The experiments was made in 2015 in two farm of Dobrogea County on imported Saanen goats, at their 3th lactations. At Elcomex Agroindustrial SA farm the Saanen goats were reared in intensive system of breeding.The goats received one feed ration which contained 2.86 UNL, calculated for >3 kg milk production/day. The feed ratio was formulated by a computer program and it contains a daily intake of 2.86 UNL, 208 g PDIN, 205 g PDIE, 18.7 g Ca and 8.25 g P. At Ciocarlia farm, lot 2 of the Saanen goats were reared in traditional system of breeding.

The goats received one feed ration which contained 2.27 UNL calculated for 2 kg/day milk production. The feed ratio had a daily intake of 2.27 UNL, 147 g PDIN and 146 g PDIE, 15.19 g Ca and 4.41g P. The goats grazed daily 6 hours, the cereals were administered during milking and the hay and the straw after the evening milking.

Experiment lasted 130 days; during this period the milk production wasrecorded and average milk samples were collected at the beginning, mid and end of lactation, determining physicochemical parameters using the Funke Gerber automatic milk analyzer. All fatty acids (FA) were determined by gas chromatographic method (GC Perkin Elmer-Clarus 500). The results were statistically analyzed with the ANOVA test.

## RESULTS AND DISCUTIONS

The lambing season started at the begining of February till at March 15. The milk collection was started on March. The experimental data are presented in Tables 1-3 and figures 1-2.

Table 1. Dynamics of goat milk fatty acids in terms of milk and protein production; the energy value of reports at the beginning of lactation *(Total FAME g/100 g fat)*

| System | I | II |
|---|---|---|
| Milk (l) | 3.59 | 1.58 |
| UNL | 2.86 | 2.27 |
| SFA* | 72.35 | 69.69 |
| MUFA* | 21.78 | 25.53 |
| PUFA* | 5.49 | 4.55 |
| UFA* | 27.27 | 30.08 |
| SFA/UFA | 2.653 | 2.317 |
| PUFA/MUFA | 0.252 | 0.178 |
| Total FA | 99.62 | 99.77 |
| Non-detected FA | 0.38 | 0.23 |

Goat milk is an important source of protein and fat in human nutrition. The mean values of the main fatty acid groups, representing values of FAME total (g FAME/100 g of fat), show variations depending on the lactation stage (at the beginning, middle or end of lactation), age, nutrition, season (Park, 2007; Haenlein, 2004). The analysis of the fatty acid profile of goat's milk was carried out by evaluating the main fatty acid (FA) groups such as saturated fatty acids (SFA), monosaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), unsaturated fatty acids (UFA), ratios between SFA/UFA, PUFA/MUFA, Ω3, Ω6 and the ratio Ω6/Ω3.

Protein and energy levels of the Saanen goat ratios grown in industrial and traditional systems were 2.86-2.27 UNL and were calculated for medium production of 3.5 liters and 2 liters of milk respectively.

Under these conditions, the SFA profile showed high values at the beginning of lactation (72.35/69.69 g FAME/100 g fat), which decreased in the middle of lactation in both groups of animals. The decrease was very high in Group I vs. Group II production of SFA increased to the end of lactation in both groups, showing values of 69.30 and 69.69 g of FAME/100 g of fat.

Table 2. Dynamics of goat milk fatty acids in terms of milk and protein production; the energy value of reports at the at the middle of lactation *(Total FAME g/100 g fat)*

| System | I | II |
|---|---|---|
| Milk (l) | 3.59 | 1.58 |
| UNL | 2.86 | 2.27 |
| SFA* | 46.73 | 52.58 |
| MUFA* | 36.38 | 40.65 |
| PUFA* | 15.11 | 6.10 |
| UFA* | 51.49 | 46.75 |
| SFA/UFA | 0.908 | 1.125 |
| PUFA/MUFA | 0.415 | 0.150 |
| Total FA | 98.22 | 99.33 |
| Non-detected FA | 1.78 | 0.67 |

Table 3. Dynamics of goat milk fatty acids in terms of milk and protein production; the energy value of reports at the end of lactation *(Total FAME g/100 g fat)*

| System | I | II |
|---|---|---|
| Milk (l) | 3.59 | 1.58 |
| UNL | 2.86 | 2.27 |
| SFA* | 69.30 | 69.69 |
| MUFA* | 23.02 | 22.84 |
| PUFA* | 5.92 | 5.92 |
| UFA* | 28.94 | 28.76 |
| SFA/UFA | 2.394 | 2.423 |
| PUFA/MUFA | 0.257 | 0.259 |
| Total FA | 98.25 | 98.4 |
| Nondetected FA | 1.75 | 1.55 |

In the SFA group there are short chain fatty acidswhich have an even number of carbon atoms, among which there are four fatty acids characteristic of goat's milk: capric acid (C10:0), myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0). Cis oleic acid (C18:1cis-9) is added from the MUFA acid group, which together holds 75% of the total fatty acids in the milk.

In smaller quantities are caproic, caprylicand lauric acids, which are also characteristic of goat milk, giving it a particular taste (Park, 2004; Goudjil et al., 2004). SFA values in Group II were lower, as goats grazed between May and October. Studies have shown that SFAs have lower values because grazing causes a decrease in saturated fatty acids in goat milk (Chiliard, 2004). The MUFA profile is opposite to SFA, meaning that its highest value was placed in the middle of lactation, when the values of the two groups were close, respectively 36.38 and 40.65 g FAME/100 g fat. From this group are highlighted monosaturated fatty acids myristate, pentadecanoic, palmitoleic, heptadecenoic and trans oleic acids. The PUFA profile has a linear pattern in Group II with values between 4.55 and 6.10 g FAME/100 g of fat, while in Group I the highest value was presented in the middle of lactation in the summer time, reaching 5.49; 15.11 and 5.92 g of FAME/100 g of fat.

The SFA / UFA and PUFA / MUFA reports are very close among the two milking groups at the beginning, middle and end of lactation. The PUFA fatty acid group varies between 3-5%. Of all fatty acids, alpha and gamma linoleic acids, eicosatrienoic and CLA (conjugated linoleic acid) are the most important goat's milk acids, because they have benefic effects on human health. The majority of FA, from acetic acid (C2:0) to arachidic acid (C20:0),contains an even number of carbon atoms.Five fatty acids (C10:0, C14:0, C16:0, C18:0 and C18:1) represent> 75% of total FA in goat's and sheep's milk. Higher levels of valuable fatty acids with short and medium chain, such as caproic, caprylic, capric and lauric acids, have a much higher rate in goat milk than in cow's milk (Alonso et al., 1999; Goudjil et al., 2004). These FAs are associated with the characteristic flavors of goat cheese and are used to detect milk mixes from different species (Chilliard, 2004). Regardingthe values for Ω3 and Ω6 and the Ω6/Ω3 ratio, the results obtained are presented in Figures 1 and 2.

The values for Ω3 and Ω6 were close between the two groups, 0.6 and 4.5, which determined that the ratio of Ω6 / Ω3 to be 8.35 for goats grown in industrial system and 6.99 for those grown in traditional system.

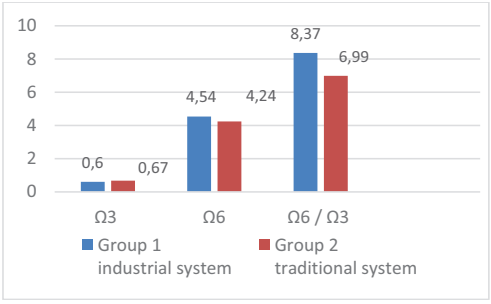


Figure 1. Histogram of fatty acids in relation to milk production

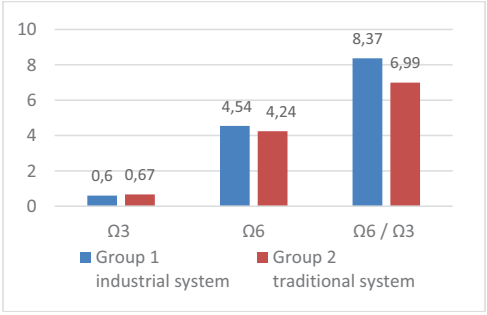


Figure 2. Histogram of Ω3 and Ω6 fatty acids in relation to milk production

Lipids and protein are the most important components of milk in terms of costs, nutrition, as well as the physical and sensory characteristics it confers on goat dairy products. After Park (2006) and Haenlein (2007), the basic composition of goat milk (Mean values per 100 g) are the following amount of constituents: fat (g) 3.8; protein (g) 3.5; lactose (g) 4.1; ash (g) 0.8; total solids (g) 12.2. Goat's milk is less caloric compared to sheep and goat milk and contains only 70 calories per 100 ml; in terms of its composition, it is very close to female milk containing more lactose and fewer proteins (Zamfirescu, 2017).

The biochemical profile of the main biochemical components (fat, protein and dry

substance) from goat's milk obtained from goats in the two growth systems is presented in Tables 4-7.

The goat's milk fat in industrial lot 1 is of maximum value in March (4.08 ± 0.026) and remains high in April and May, then decreases significantly in July and August.

Fat in September and October rose to 3.85% - 3.94% (Table 4). The milk fat profile of the goats kept in the traditional system showed the same fat profile, which had a maximum value in March and April (4.18%-3.91%), after which it declined and remained flat in May (3.6%-3.7%) and reached the initial value in the last lactation month (3.87 ± 0,014%) (Table 6).

The lipid fraction of goat milk is relatively high in saturated fatty acids, which is typical of milk fat from all ruminants.

The average size of goat milk fat globules is about 3.5 micrometers and is characterized by its high homogeneity that provides lipases with greater surface area of fat for enhanced digestion.

The smaller fat globules found in goat milk allows for better fat dispersion and poor creaming ability of the milk, which provides a natural homogenization that is beneficial to human health (Haenlein et al., 1984; Park, 2006).

Regarding the total protein profile in the milk collected from the two groups of goats, it had characteristic values for the species, namely 3.31-3.52% for the goats in the industrial system and 3.31-3.42% for the goats kept in the traditional system (Tables 4 and 6).

Table 4. The monthly distribution of fat and protein in milk (1, industrial system)

| Month | Fat% x±sx | Protein% x±sx |
|---|---|---|
| 03 | 4.08±0.026 | 3.33±0.024 |
| 04 | 3.96±0.025 | 3.41±0.018 |
| 05 | 3.98±0.026 | 3.39±0.013 |
| 06 | 3.88±0.022 | 3.31±0.008 |
| 07 | 3.78±0.011 | 3.33±0.009 |
| 08 | 3.74±0.014 | 3.40±0.012 |
| 09 | 3.85±0.0372 | 3.52±0.011 |
| 10 | 3.94±.0.024 | 3.49±0.050 |

The five major proteins in goat milk are β-lactoglobulin, α-lactalbumin, κ-casein, β-casein, and α2-casein (Carles, 1986; Haenlein and Caccese, 1984; Mikkelsen et al., 1987).

Table 5. The monthly distribution of SU% and fat/protein ratio in milk (1, industrial system)

| Month | SU% Min/max/x±sx | Fat/protein ratio Min/max/x±sx |
|---|---|---|
| 03 | 12.38±0.039 | 1.22 |
| 04 | 12.61±0.061 | 1.16 |
| 05 | 12.51±0.045 | 1.17 |
| 06 | 12.41±0.041 | 1.17 |
| 07 | 12.25±0.024 | 1.13 |
| 08 | 12.21±0.028 | 1.10 |
| 09 | 12.53±0.062 | 1.09 |
| 10 | 12.72±0.055 | 1.13 |

Table 6. The monthly distribution of fat and protein in milk (2, traditional.system)

| Month | Fat% Min/max/x±sx | Protein% Min/max/x±sx |
|---|---|---|
| 03 | 4.18±0.016 | 3.38±0.019 |
| 04 | 3.91±0.015 | 3.42±0.021 |
| 05 | 3.76±0.033 | 3.39±0.011 |
| 06 | 3.61±0.029 | 3.31±0.018 |
| 07 | 3.65±0.018 | 3.32±0.010 |
| 08 | 3.73±0.022 | 3.31±0.020 |
| 09 | 3.76±0.031 | 3.34±0.017 |
| 10 | 3.87±.0.014 | 3.39±0.031 |

Table 7. The monthly distribution of SU% and fat /protein ratio in milk (2, traditional system)

| Month | SU% x±sx | Fat/protein ratio x±sx |
|---|---|---|
| 03 | 12.39±0.036 | 1.23 |
| 04 | 12.55±0.044 | 1.14 |
| 05 | 12.50±0.042 | 1.11 |
| 06 | 12.41±0.021 | 1.11 |
| 07 | 12.36±0.028 | 1.10 |
| 08 | 12.38±0.029 | 1.13 |
| 09 | 12.42±0.041 | 1.13 |
| 10 | 12.59±0.048 | 1.14 |

The results obtained are similar to those reported by Bruhn (2000), Chiliard (2003) Park (2006) and Haenlein (2004).

The total dry substance ranged between 12.21%-12.72% for the goats in group 1 and 12.39%-12.59% for the goats in group 2.

The fat / protein ratio in group 1 (Table 5) had a similar value to reference data for goat milk. This indicator was below 1.2 because of the lower fat content during the summer period. The fat/protein ratio is an important indicator for the transformation of milk into cheese (Ricordeau, 1967; Meyer 1998).

Kučević (2016) reports that growth systems influence the quality of milk. In our experiments, the fat, protein, and dry substance profile had close values; they had lower values

during the summer and increased to the end of lactation.

## CONCLUSIONS

Growth systems did not have any major influence on the biochemical composition of Saanen's goat milk grown in industrial and traditional systems.
The determination of the fatty acid profile of goat's milk is influenced by the lactation stage.
The SFA general profile was lower in traditional grown, demonstrating that grass can influence the decrease in saturated fat content.
The ratio of fatty acids Ω6 and Ω3 was very high in the Saanen goat milk grown in the two systems, far exceeding the admissible values.
The profile of the main biochemical components in milk, namely fat, protein and dry matter, analyzed over 10 months of lactation, was similar except for fat that had lower values during the summer. This decrease led to a lower fat / protein ratio in the milk collected from the goats raised in the traditional system.

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# GEMOTHERAPY, AN ALTERNATIVE TO TREATMENT OF ANIMAL DISORDERS. ACTUAL TRENDS ON THE PHARMACEUTICAL MARKET

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***Abstract***

*The pharmaceutical market faces a new trend that has both social and economic effects. Thus, in addition to the quantitative and value-development of the normal market held by medicines and drug solutions, this is multiplied by the development of the niche of food supplements but also of gemotherapeutic products. In the category of consumers, besides traditional ones representing human patients, it can be found with a growing share and users like animals. If, in the patients' situation, their use has become quite accurate, medical practice has allowed us to extend this use to animals as an effective and safe alternative to administering different gemotherapies in accordance with their constitutions. Gem derivatives can also be used effectively in animals as they are no toxicity and are easy to administer, prescribed individually or in combination. Thus, gemotherapy can be a therapeutic method for animals not only for humans, because they use diluted decimation solutions of hydroglycero alcoholic macerates from fresh vegetable extracts represented by meristematic tissues: jams, young branches, buds, young roots, avenues, internal roots of roots , shell of young branches, seeds or other embryonic plant tissues found in the growing phase. The use of gemoderivatives in the treatment of effects in animals allows the limitation of the use of chemically synthesized drugs which, despite their effectiveness, lead to the occurrence of side effects. Thus, the alternative to treatment with gemotherapies and animals is one with various implications both in terms of diversity and uniqueness of treatment, as well as financial implications for those who use them, but also for the economic entities that produce them, as well as for the pharmacies that issue and collect the price them.*

***Key words:*** *animal therapy, economic efficiency, gemmotherapies.*

## INTRODUCTION

As with human patients, the body of animals no matter where they live, is in permanent contact with "foreign" organisms such as bacteria, viruses, parasites. They can benefit from their body by participating, for example, in the digestion of ingested products, in producing vitamins, or causing parasitic diseases as a result of infestation with various parasites (Laudato and Capasso, 2013).

The most common parasitic diseases encountered in animals are: toxoplasmosis, coccidiosis, babesiosis, tenosis, ancilostomosis, uncinariosis, trichurosis, strongiloidosis, spirocercosis, ascaridosis, trichocephalosis, dirofilariosis, trichinelosis (Rajeev Singh et al., 2008).

These parasitic diseases develop in the intestinal epithelial cells and bile duct cells, urinary tubes or other tissues, red blood cell elements or muscle tissue (Nițu, 2009).

Treatment of these diseases with broad spectrum anthelmintic drugs with active substance albendazole and levamisol or avermectin, are indicated in pet animals, dogs and cats in internal and external deworming.

Classic treatment is a precondition for both internal and external pests, both in animals and humans.

Internal parasites have various localizations (muscle, digestive, respiratory, excretory, etc.), causing the animals various parasitic diseases: protozoa (protozoa); nematodes (caused by parasitic cylindrical bodies, spread throughout the globe and parasitizing many animal species, including humans); trematodoses (caused by trematodes, parasites with elongated oval body) and cestodoze (parasitic diseases whose body is flat in the form of ribbon) (Rajeev Singh et al., 2008).

Canidae, including the dog, are the definitive hosts for the caterpillars and can cause illness to various animal species, including humans.

External parasites (fleas, lice, ticks, worms, etc.) cause some clinical symptoms in animals: anxiety, pruritus, anemia, slimming, sting abscesses, urticaria and alopecia (Blăjeni, 1994).

## METHODS AND MATERIALS

To characterize the evolution of gemoterapeutic demand that can be used in animals, we used the information provided by a pharmacy.
We have used quantitative value charts of gemotherapeutic products released in the pharmacy.
The study used gemetherapy sales as a study period, which can be used in animals between May 2018 and May 2019.
The method of study used was the quantitative value analysis, but also the commercial advantage achieved during the analyzed period.

## RESULTS AND DISCUSSIONS

Deepening the use of gemo-therapies in treating various diseases in humans leads us to extinguish the study on their use in animals as well.
The study was addressed both in terms of content in mineral elements, both from the point of view of the therapeutic action and also from the economic point of view, of the sales made by the economic entities.
1. Thus, complex gemo-therapies of the type Polygemma, administered as such, but also in combination with other gemtherapies with strict therapeutic action on a particular condition can be used (Towle, 2002).
Polygemma is an original gemotherapeutic complex composed of combinations of extracts from different parts of plants. These complexes can often be used in detoxification cures for certain levels of the human and animal body. Thus, Polygemma 8 - psycho-physical asthenia and memory, is a combination of gemotherapies from Birch seeds, Oak shoots, Rosmarin shoots and extract from root of Siberian Ginseng. It can combat the fatigue states commonly encountered in parasitosis or post parasitism and are given 2 ml twice a day, 2-4 weeks (López-Malo et al., 2005).
Polygemma 15 Intestine can be used for detoxification (Oak Mugs, Cranberry and walnut shoots) - replenishes the intestinal flora, anti-inflammatory, tonic.
Polygemma 17 - immunity is a combination of gemoterapies from Cats, Porumbar, Maces and Echinacea purpurea juice, supports the restoration of the body and a shorter convalescence.
Administer 2 ml 3 times a day in 1-2 month treatments after untreated or inadequately treated parasites (López-Malo et al., 2005).
From the first signs of infestation with intestinal parasites, Giardinophyt will be administered. You can associate cures and other natural preparations, depending on the unpleasant manifestations that may occur. The extract from the Merişor branches is administered at noon and evening if the sensitivity is mostly intestinal, or there are transits disorders (López-Malo et al., 2005).
Walnut bud extract is administered at noon and evening in case of intestinal infections. Fig buds extract is administration noon and night, if present heartburn, and if signs of parasites are strong (large deletions), we add wild garlic tincture, 20-30 drops 3 times a day. The black currant bud extract is administered in the morning and at noon in case of allergic, respiratory or cutaneous reactions (López-Malo et al., 2005).
Tamarix seed extract and Porcupine bud extract are administered twice daily for weak or anemic patients.
Extract from Porumbar or Polygemma 8 shoots - in case of asthenia, in case of prolonged asthenia or after a parasite cure (López-Malo et al., 2005).
Polygemma 17 - Immunity is given for an immune cure. Only one gemoterapic or 2-3-combination combination may be given for 4-6 weeks - either beginning with the administration of Giardinophyt or after the Giardinophyt cure **(**European Pharmacopoeia, 2007**).**
If we alter the use of gemoterapics in the treatment of diseases that are potent and in animals, economically, the situation of gemotherapeutic sales for parasitosis treatment is presented as in the table below.

Table no. 1 Gemotherapy issued for treatment of intestinal paragues

| No. crt | Name extract | Price entry | Price exit | Amount exit/pc | Value entry | Sales value | Comm. Addition |
|---|---|---|---|---|---|---|---|
| **1.** | Mlada Cranberry | 16.99 | 22.50 | 16 | 271.84 | 360.00 | **88.16** |
| **2.** | Walnut Buds | 16.99 | 22.50 | 6 | 101.94 | 135.00 | **33.06** |
| **3.** | Fruit buds | 13.86 | 18.50 | 2 | 27.72 | 37.00 | **9.28** |
| **4.** | Polygema 8 | 13.98 | 18.50 | 4 | 55.92 | 74.00 | **18.08** |
| **5.** | Polygema 17 | 14.00 | 18.00 | 8 | 112.00 | 144.00 | **32.00** |
| **6.** | Blackcurrant Buds | 15.78 | 20.50 | 4 | 63.12 | 82.00 | **18.88** |
| **7.** | Fountains by Tamarix | 15.78 | 20.50 | 13 | 205.14 | 266.50 | **61.36** |
| **8.** | **Giardinophyt** | 12.30 | 16.70 | 59 | 725.70 | 985.30 | **259.60** |
| **9.** | Poplar buds | 15.02 | 20.00 | 12 | 180.24 | 240.00 | **59.76** |
| | **TOTAL** | - | - | - | **1631.62** | **2105.80** | **474.18** |

Source: Gemotherapeutic products during the analyzed period

As can be seen from the table above, the Giardinophyt gemoterapeutic product was approved and requested to be released in a quantity of 59 bottles with a release value of 985.30 ls, respectively a commercial addition of 259.60 lei . The value of gemotherapies with released anti-parasitic action was 2105.80 lei, with a commercial loom in absolute value of 474.18 lei.

From this study it appears that the release of gemoterapeutics with antiparasitic activity can be appreciated as an effective alternative to their human patient prescription and possible administration to animals.

Gemoterapics can also be recommended for animals to treat inflammatory conditions

Inflammation of a joint may have multiple causes, from exposure to adverse environmental factors to microtraumatisms and exaggerated stresses of the same joints and other complicated infectious diseases with joint attachment like streptococcal infection (WHO guidelines, 2004).

Thus, local treatment in animals aims to restart the affected joint by applying ointments or creams with anti-inflammatory and anti-algic effects, applications of local warm or cold compresses, depending on existing ways or treating the existing infection in the body.

The current trend is to orient the treatment of various acne to the natural variants including animals, and lately there is an orientation towards treatments with gemoterapies in the form of tinctures or various extracts.

To relieve pain caused by inflammation of the joints, extracts and tinctures obtained from buds and juveniles of various plants are used as adjunct treatment **(**Farmacopeea europeană, 2007).

Thus, for the treatment of osteoarthritis, black currant extract is used which neutralizes the inflammation of affected joints. The effect is similar to that of anti-inflammatory drugs.

This plant extract does not have any inconvenient iatrogenic side effects that we can encounter in anti-inflammatory drug therapies.

The anti-inflammatory action of black currant extract acts on tendons and ligaments and improves their flexibility even in animals (WHO guidelines, 2004).

It also has a distinct antiallergic activity. It is indicated in any inflammatory condition in which there is an accelerated rate of inflammation and allergies of any system.

Another gemoterapist used is the extract from Janeapan buds that acts on articular cartilage to maintain bone durability. It can also be given to animals to prevent fractures (De Smet, 2005).

It can also be used to treat chronically non-inflammatory rheumatic diseases in the spine, knee or hip. This treatment can be combined with Rinoplasty Tincture to stimulate lymphatic drainage around the joints and to help regenerate the cartilage (Sirois, 2009).

The live vine extract slows joint deformation along the joint and can be used for any painful arthritis, even in animals. The extracts presented may be given singly in the morning -

at noon - or during the night, or together using 2-4 ml each (Sirois, 2009).
There were no contraindications for this treatment, and treatment is usually continued two months before and one month after the symptoms disappears.
This gemoterapeutic treatment can be given alone or with other homeopathic and biotherapeutic remedies.
This gem therapy recommended for arthritis can also be associated with the fluffy blast extract known for its in-depth action on the reticulo-endothelial system and arterial circulation (Rajeev Singh et al., 2008).
This is a universal drencher and at one time works on several systems.
Knee arthritis can be treated with the same extracts to which can also be used and Janeapan sprout extract, also associated with the extract from the Macecs mildew for an anti-inflammatory effect on the knee joint.
Also for articular affections, the Mur extract of extract, which helps to restore cartilage and to treat osteoarthritis, can also be used.
In the treatment of aggressive inflammatory conditions such as rheumatoid arthritis, ankylosing spondylitis or psoriatic arthritis, black currant extracts, wild vines with tendon and ligament action, as well as bone deformity and sclerosis can be administered (Pitera, 2004).
As a result of the results of the study, the treatment of arthritis and inflammatory diseases by administering gemo-therapeutic preparations to both humans and animals should be addressed also from the financial point of view and from the point of view of the commercial advantages of the pharmaceutical entity.
In the following table we presented gemoterapeutic preparations with anti-inflammatory action released in the studied pharmacy.
Considering prescribing and administering anti-inflammatory drugs to humans and animals over a long period of time leads to side effects, the alternative to gemoherapies is one of quicker, less pungent treatment (Fig. 1).
The most liberated gemoderivate extracts with the most effective anti-inflammatory action were those in the Honey Mug extract with a total of 55 bottles, followed by the 28-cell Cranberry extract extract and the 23-vial Aarin white bud extract (López-Malo et al., 2005).

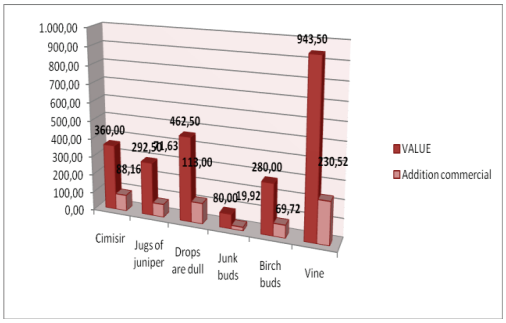


Figure 1. The commercial additions made to gemotherapeutic sales for the treatment of arthritis
*Source: Gemotherapeutic products during the analyzed period*

From the financial point of view it must be stated that the patient fully supports the value of the therapies with germo-therapy products, which can be appreciated as a considerable financial effort, knowing that the treatments are for longer periods of time.
From the point of view of the financial aspects of the pharmaceutical entity, it can be appreciated that the release of gemo-therapeutic products brings an additional of the immediate financial resources, totally collected at the time of the issue.
This is a financial aspect that is taken into consideration by the individual pharmaceutical entities when they need to be supplied in quantity, quality, and assortment structure that responds to patients' treatment needs.
Thus, from the release of anti-inflammatory anti-inflammatory gematherapeutics, the pharmaceutical entity achieved a commercial surcharge of 883.68 lei, which can be appreciated as a favorable one for this therapy group (Fig. 2).

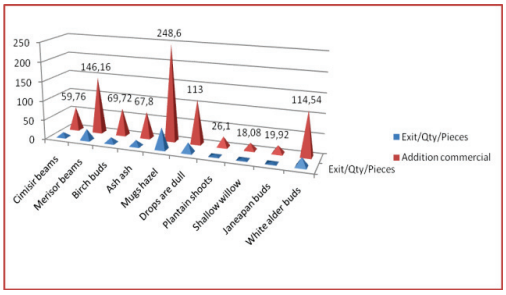


Figure 2 Commercial Addition
to anti-inflammatory gemotherapeutic sales
*Source: Gemotherapeutic products during the analyzed period*

This financial aspect determines the pharmaceutical unit to source natural products, both from domestic manufacturers and from external producers.
The promotion of alternative therapies to allopathic therapies is a way to stimulate the development of research and production of such products at the national pharmaceutical market, but also to stimulate the engagement of the specialized labor force in this field.
In addition to the economic advantages, there are some therapeutic advantages of gemoterapics as follows:
- cellular detoxification by chemical reaction to purify and clean cells;
- supports organ drainage by stimulating the removal of toxins by body fluids such as blood and lymph;
- regenerates dead cells, oxygenates cells, stimulates the functions of various organs and glands, feeds / provides a full spectrum of antioxidants, hormones, enzymes, vitamins, minerals, trace elements, amino acids, rejuvenate organs, skin tissue and cells by stimulating blood and lymphatic activities and by providing antioxidants in the system.
Thus, studies conducted in the field have made it possible to use gemo-therapeutic products as presented for both humans and animals.

## CONCLUSIONS

The study of the investigational pharmacy on the release of gemoderivates for the treatment of arthritis, inflammation and antiparasitic activity in both humans and animals influences the research activity on this niche of future pharmacists.
Thus, in addition to the release of medications with and without prescription, pharmacies are also interested in the sale of dietary supplements or other parapharmaceuticals.
If the release of prescription drugs involves delayed settlement of their value by the County Health Insurance House within 60-90 days, the release of OTC and parapharmaceuticals as they are and gemoterapics, provides the pharmacy with additional immediate financial resources.
Thus, cash receipts facilitate payment of obligations to suppliers of goods and services, payment of salaries and their obligations towards the state budget and social security budget.
Supplementing pharmacy incomes from drug delivery with parapharmaceutical product sales, including gemotherapeutic products, ensures the achievement of the objective of each economic entity, namely the realization of profit.
The achievement of the activity of the pharmacy, i.e. the realization of the profit is the only one that ensures the development of an economic entity, the expansion of the patrimony, the increase of the salary of the specialists at the level of the European pharmaceutical market, as well as the provision of quality services to the patients.
In conclusion, the expansion of the gemotherapeutic market in both humans and animals can be an additional source of financial resources for pharmacies, but also a means of developing the pharmaceutical industry at national level, promoting national professional values and developing pharmaceutical research in this field.
As animals are more and more present in people's lives, finding less toxic alternatives to treating their illness has become more and more present in studies exploring the use of plate products.
Studies should also be approached from an economic point of view, correlated with the commercial advantages of the pharmaceutical entities that produce them, as well as with the entities that release these products.

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# VARIABILITY IN NUMBER OF ANTRAL FOLLICLES IN HOLSTEIN FRIESIAN HEIFERS AND ITS ASSOCIATION WITH REPRODUCTIVE AND PRODUCTIVE PERFORMANCES AFTER THE END OF FIRST LACTATION

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***Abstract***

*In cattles the small antral growing follicles develop from a reserve of primordial follicles constituted early in life. The antral follicle count (AFC, follicles ≥ 3 mm in diameter) is determined by ovarian ultrasound measurement. In this study 13 to16 months old Holstein Friesian heifers were subjected to a single ultrasound measurement of the number of follicles ≥3 mm in diameter. Heifers were classified into a low (≤ 5 follicles) mid (16-24 follicles) and high (≥25 follicles) follicle number group (FNG). We then compared the reproductive and productive performances at the end of first lactation. Results showed that heifers in the high FNG had a higher pregnancy rate, a shorter calving to conception interval, and a higher culling rate, compared with heifers in the low FNG. Because this study was made in a single herd with limited animal numbers (n=100) it is premature to make an industry-wide recommendation to select the dairy heifers in a herd based on single AFC measurements.*

***Key words:*** *antral follicles, ultrasound measurement, reproductive performances, culling rate.*

## INTRODUCTION

Recently, there have been an increasing interest in studies concerning antral follicle count (AFC) and its association with reproductive performances in dairy cattle (Ireland et al., 2011; Pontes et al., 2011; Rico et al., 2012; Silva Santos et al., 2014; Morotti et al., 2015). The AFC represents the member of follicles visualized by ultrasound evaluation in the ovaries.

In cattle, calculations based on the number of granulose cells in follicles of various classes and from the time required to double the number of cells within a follicle, indicate that a follicle takes 27 days to grow from 0.13 to 0.67 mm, 6.8 days from 0.68 to 3.67 mm and 7.8 days from 3.68 to 8.56 mm, indicating that growth rates varied with the size of the follicle (Lussier et at., 1987). A period equivalent to oestrus cycles would therefore be required for a follicle to grow through the antral phase, i.e. from 0.13 mm to preovulatory size .

Antral follicles grow in a wave-like pattern (Ginther et al., 1989; Fortune et al., 1991). In cows there are two or three waves of follicular development during an oestrus cycle (Fortune et al., 1991; Ginther et al., 1996).

The numbers of follicles ≥ 3 mm in diameter recruited in each wave have been counted on different days of the oestrus cycle, in dairy heifers and postpartum dairy cows (Singh et al., 2004; Ireland et al., 2011). The AFC is highly variable among animals but very highly repeatable (0.85 to 0.95) within individuals (Jimenez-Krassel et al., 2009; Mossa et al., 2010).

This persistence in AFC in the same individual becomes a good information for the classification of females as low-, intermediate- or high AFC by a simple ultrasound evaluation during follicular waves. (Burns et al., 2005; Ireland et al., 2007, 2011; Mossa et al., 2012.)

Studies have shown that AFC can be affected by maternal environmental conditions, such as health and nutritional status during pregnancy or by lactation status and milk quality. The relationship between AFC and genetic characteristics in dairy cattle is an issue that needs to be better understood.

In this paper the hypothesis that AFC is positively associated with fertility in Holstein Friesian cattle was tested. The objective was to determinate if AFC, utilizing a single ultrasound measurement of number of follicles ≥ 3mm in diameter at puberty age, was associated with

several measures of reproductive and productive efficiency after the end of first lactation.

## MATERIAL AND METHODS

### Animals

Dairy cattle used in this experiment were located at Agriculture Research and Development Station (ARDS) Simnic Craiova Romania.

The experiment was performed in compliance with the European Union Directive 86/609/EC, on Holstein – Friesian cattle- that belong to a long large genetic improvement program.

Holstein Friesian (HF) heifers with adequate body condition score (BCS) and normal health status were selected for the experiments .This project was initiated in January 10, 2015.

### Measurement of Follicle Numbers

Cycling NF heifers (n=107) 13-15 month old were subjected to 2 intramuscular injections of PGF 2∝ administrated 15 mounts old were subjected to 2 intramuscular injections of PGF2α administrated 11 days apart to synchronize estrous cycle.

At 96 hours after the last PGF2∝ injection, a single ultrasound measurement was used to count number of follicles ≥ 3mm in diameters (Burns et al, 2005). Ovaries in each heifer were scanned with Veterinary Ultrasound scanner Ecoson 80+.

Each ovary was scanned from end to end to identify the positions of the antral follicles and the corpus luteum. Video images for each ovarian section were captured on a computer monitor. The lactations of each antral follicle ≥ 3 mm in diameter in each section were drown an ovarian map (Burns et al., 2005). Two separate of diameters were averaged for each follicle and recorded on each ovarian map. Total number of antral follicle ≥ 3mm in diameter per pair of ovaries for each animal was determined by counting the number of follicle ≥ 3 mm in diameter on each map for each animal. Heifers were classified into follicular number group (FNG) based on number of follicles as follows: low (13-15 follicles) mid (16-24follicles) and high (≥ 25 follicles).

### Reproductive management

After induced estrous cycle, heifers were observed for signs of oestrus 3 times per day, and then were subjected to artificial insemination (AI) after standing oestrus. Diagnosis of pregnancy was done by palpation of uterine contents between50 to 60 day after AI. The heifers diagnosed as not pregnant were subjected again to AI.

After first calving, lactating primiparous cows, were subjected to AI, after a voluntary waiting period (VWP) of 60 days postpartum. The cows diagnosed with metritis (n=3) were treated with prostaglandin $F_2\alpha$ ($PGF_2\alpha$) at 23 ± 3 days postpartum.

Cows returning to estrus before pregnancy diagnosis were inseminated at standing oestrus. Pregnancy status was assessed by rectal palpation of uterine contents at 60 days after AI. The primiparous cows diagnosed non pregnant were subjected to Ai at standing estrus, after either a single $PGF_2\alpha$ injection if a corpus luteum (CL) was present or rechecked 7 days later if a CL was not present. This breeding procedure in cows was repeated for same animals up to 5 times.

The reproductive performance, level of milk production, health, and reasons why animals were removed or culled from the herd, of each individual cow were recorded daily.

The involuntary terms used to describe why cows were removed or culled from the herd were: reproductive problems (≥ 5 inseminations without pregnancy, cystic follicles, abortions), mastitis, calving injury, retained placenta or metritis, displaced abomasum, death, lameness, metabolic syndrome, infections.

The voluntary terms used were: low milk production, poor conformation and sold to other dairy farmers.

The proportion of cows removed from the herd for each involuntary or voluntary reason was associated with each FNG.

To study the association between AFC and fertility, the following reproductive performance were calculated and analyzed: pregnancy rate at first service, pregnancy rate after all services (up to 5) and number of services per conception as heifers, and pregnancy rat at first service, pregnancy rate after all services (up to 5), mean length of the

service period, and number of services per conception as primiparous cows.

**Statistical analysis**

All statistical analysis were done using Microsoft Office Excel procedures. Analysis of variance was used to determine if significant ($p \leq 0.05$) overall differences existed among different FNG for all reproductive parameters. The correlation between number of ancestral follicles and level of milk was determined by Pearson correlation analysis.

## RESULTS AND DISCUSSIONS

Based on the AFC, utilizing a single ultrasound measurement of number of follicles ≥ 3mm in diameter, distribution of heifers is shown in figure 1.

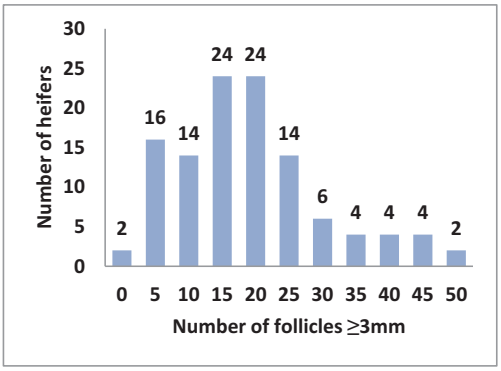


Figure 1. Freqvency distribution for number of heifers with different number of follicles ≥ 3mm in diameter determined by a single ultrasound examination

Table 1. Characteristics at single ultrasound measurement of number of follicles ≥ 3 mm in diameter and reproductive performances in each follicular number group of Holstein Friesian young animals

| Item | Follicle number group00 | | | |
|---|---|---|---|---|
| | Low | Mid | High | Level of significance |
| Characteristics at scanning:<br>Number of heifers (%)<br>Mean antral follicle count (mm)<br>Range (mm) | 42<br>(42%)<br>10.47[a]<br>3-15 | 36<br>(36%)<br>19.38[b]<br>16-24 | 22<br>(22%)<br>34.72[c]<br>25-50 | -<br>< 0.001<br>- |
| Reproductive performance as heifers:<br>Pregnancy rate at first service (%)<br>Pregnancy rate after all services (%)<br>Number of services per conception | 62.2[a]<br>84.4[a]<br>2.2 | 75.4[b]<br>88.6[ab]<br>1.8 | 68.1[ab]<br>96.1[b]<br>1.8 | < 0.005<br><0.05<br>NS |
| Reproductive performance as primiparous cows:<br>Pregnancy rate at frst service (%)<br>Pregnancy rate after all service (%)<br>Mean length of service period (days)<br>Number of services per conception (nr) | 52.2<br>72.8<br>118[a]<br>2.4 | 65.1[ab]<br>86.8[ab]<br>104[ab]<br>2.2 | 68.0[b]<br>90.0[b]<br>100[b]<br>2.0 | <0.05<br><0.05<br><0.05<br>NS |

[a b] different superscripts within a row indicate. That the high-differs from mid- or low - FNG groups: NS = non significant

Table 2. Productive performances and culling rates and reason of culling during first lactation in each follicle number group of Holstein Friesian young animals

| Item | Follicle number group | | | | | | |
|---|---|---|---|---|---|---|---|
| Milk production with 3,5 % fat corrected milk (kg/d) | Low | | Mid | | High | | Level of signi ficance |
| | n | % | n | % | n | % | |
| | 42 | 29.4 | 36 | 31.2 | 22 | 30.8 | NS |
| Culling reason:<br>Involuntary Reproduction | 2<br>1 | 4.7[a]<br>2.3 | 2<br>1 | 5.6[a]<br>2.8 | 3<br>1 | 13.6[b]<br>4.5 | < 0.05<br>NS |
| Death | - | - | 1 | 2.8 | - | - | - |
| Health | 1 | 2.3[a] | - | - | 2 | 9.1[b] | < 0.05 |
| Voluntary | 2 | 4.7 | 2 | 5.5 | 1 | 4.5 | NS |
| All reason | 4 | 9.4[a] | 4 | 11.1[a] | 4 | 18.1[b] | < 0.05 |

[a b] different superscripts within a row indicate that the high differ from mid ar low FNG groups. NS = non significant

Based on the AFC heifers were assigned to the low group (≤ 15 follicles) 42 (42%) to the mid group (16 to 24 follicles) 36 (36%), and 22 (22%) to the high (≥ 25 mm) group (Table 1). The association between AFC and fertility of heifers is summarized in Table 1. The actual pregnancy rates of heifers at first service were 62.2%, in the low group, 75.4% in the mid group and 68.1% in the high group. The actual pregnancy rates of heifers at first service were 62.2%, in the low group, 75.4% in the mid group and 68.1% in the high group. The actual pregnancy rates overall were 84.4% in the low group, 88.6% in the mid group and 96.1% in the high group of heifers, and number of services per conception was 2.2, 1.8 and 1.8 in the low, mid and high groups respectively. The association between AFC and fertility in the

first lactation is summarized in Table 1. As primiparens cows the actual pregnancy rates at first service was 52.2%, 65.1% and 68% in the low, mid and high groups respectively. After all services the actual pregnancy rates was 8.4%, 88.6% and 96.1% in the low, mid and high groups respectively. The mean, length of service period was 118 days, 104 days and 100 days in the low, mid and high groups respecttively (table 1). Primiparous cows with e low AFC received more services per concepttion compared with primiparous cows with mid and high AFC (2.6 vs. 2.2 and 2.0 respectively).

The association between AFC and productive performances and culling rates after first lactation of dairy cows is summarized in table 2.

No difference was evident in milk production with 3.5% fat corrected milk per day among group (Table 2).

The FNG classification scheme used in this study relied on a single ultrasound measurement of follicle numbers at unknown stage of follicular wave. In a previous study, Burns et al., (2005), used daily ovarian ultrasound evaluation throughout most days of consecutive follicular waves to classify cattle based on peak number of antral follicles ≥ 3mm in diameter into low (≤ 15 follicles, mid (16-24) or high (≥ 25 follicles) AFC groups. A single ultrasound measurement could have been made at the nadir or peak (or in between) for AFC during a wave. If the single ultrasound determination is made at the nadir for AFC during follicular wave, which can be 50% lower than the peak, an individual with an AFC of 25 or more must have a consistently high peak AFC. A cow with an AFC of 8 after a single determination could be in the low (≤15 follicles ≥3 mm in diameter) or mid (16-24 follicles) AFC group. A cow with an AFC of 24 after a single measurement could have been in the high or mid AFC group. The low and mid FNG contain a mixture of animals with a low, intermediate or high AFC during follicular waves. In our study we compared high FNG with the mid or low FNG.

In this study animals with high AFC were superior in several characteristics related to fertility, such as high pregnancy rate, or short length of service period as heifers, and as primiparous cows. This result is consistent with some previous reports (Mossa et at., 2012, Koyama et al., 2018).

In the present study culling rates the end of first lactation were greater for cows in the high, compared with low FNG (18.1% vs. 9.4%). Because the proportion of cows with an AFC ≥ 25 follicles was 22% of the cows enrolled in this study, we could not assess the adverse effects of the higher AFC on dairy cows. The high variation in follicle numbers may not only be reflective of reproductive disorders, but could be the evidence to indicate that it may be associated with alterations in the function of other non-reproductive systems that may have effects on animals health.

## CONCLUSIONS:

We confirmed that the reproductive performance of cows with an AFC of ≥ 25 follicles was better than of cows with mower AFCs.

Because this study was made in a single herd with limited animal numbers (n=100) it is premature to make an industry-wide recommendation to select the dairy heifers in a based on single AFC measurements.

Future studies should, therefore, verify if the high variation in follicle numbers in dairy cows could be associated with alterations in the function of non-reproductive systems.

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# CLASIFICATION OF SELECTED ROMANIAN BREEDS AND HYBREDS OF SILKWORM USING MULTIPLE TRAIT EVALUATION INDEX METHOD

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***Abstract***

*Mulberry silkworm (Bombyx mori L.) is a species with high economical and scientific value, acknowledged at international level, as well as at national level. Extensive research was conducted on the local silkworm breeds and hybrids, on both biological and technological parameters, yet no classification was done. The main purpose of the current research is to attempt to implement a classification of 17 breeds and hybrids of the silkworm using a statistical analysis method called Evaluation Index Method that takes into consideration multiple economical and technological traits. The biological and technological traits that were taken into consideration are: prolificacy (number of eggs/laying, hatching percent), evaluation of the larvae ( weight), duration of the larval period, evaluation of the raw silk cocoon and the dried silk cocoon (dimension, weight of the cocoon, weight of chrysalis, weight of the silk shell). The results of the present study will result in a clear classification, based on their economical value and the breeds and hybrids that have a cumulative value of the index higher than 50, will be considered economical valuable.*

***Key words****: silkworms, parameters, evaluation, classification*

## INTRODUCTION

Silk is a sought after textile due to its elegance, softness and ecological nature it detains. The silk is widely used, from textile industry to the medical and technological industries, making it a valuable animal product worth rearing.

The origin of the silk fabric is the silk cocoon, which, are produced mostly by the silkworm *Bombyx mori* L, mainly known as the mulberry silkworm (Popescu, 2013).

Not only it is a source for important textile fabrics, but this insect is also considered an important biological model used in many fields of research, including genetics (Furdui et al, 2010, Furdui et al., 2014).

In order to assure the best results by obtaining good and healthy larvae and valuable cocoons, it is important that the proper rearing technique is implemented, the needed microclimate conditions are assured and feed requirements must be met (Furdui et al, 2010). In the case of the Romanian Sericulture industry, it can be stated that is as known a long development with its success and failures, its development being aided by a favorable environmental conditions, suitable for silkworm rearing. After 1990, the industry has continued to decline and, in the present time, the main activities are focused preservation of the biological silkworm breeds and mulberry breeds) that, despite being rather productive, do need improvements focused on increased silk production capacity. The silkworm breeds are currently managed by the Global Center of Excellence for Advanced Research in Sericulture and Promotion of Silk Production, (GCEARS–PSP) established at the University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca

(Mărghitaș et al, 2005, Dezmirean el al., 2013; Dezmirean et al., 2018).
The sericultural gene pool plays a pivotal role in conservation or/and diversification of species. The GCEARS–PSP creates programs for local adapted biological material preservation (Matei et al, 2008).
The classification of the silkworm breeds and hybrids plays an important economical role as it offers a perspective of the valuable technological characteristics of the analyzed breeds and hybrids (focused on silk and on important byproducts).
The present study has the role to present the results obtained from the rearing of breeds and hybrids of silkworms. The obtained results will provide a classification trough Multiple Trait Evaluation Index, a classification method that takes into consideration a cumulus of economical and technological traits (Sudhkara et al, 2001; Buhroo et al, 2017).
The importance will be focused on the breeds with maximal values as they will be considered economical important.

## MATERIALS AND METHODS

The experiments took place at the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, within the Global Center of Excellence for Advanced Research in Sericulture and Promotion of Silk Production, (GCEARS–PSP). Rearing of the silkworms was done in a specially arranged room (magnanery) at the Faculty of Animal Husbandry and Biotechnologies/ Sericulture Department, under proper implemented rearing techniques (Mărghitaș et al., 2003), respecting the microclimate requirements as stated in Table 1:

Table 1. Optimal rearing temperature and humidity requirements (Furdui et al., 2010)

| Instar | Temperature | Humidity |
|---|---|---|
| I-II | 26-28°C | 80-85% |
| III | 25-26°C | 75-80% |
| IV-V | 23-24°C | 65-70% |

The breeds and hybrids, part of the genetic sericultural resources of the University of Agricultural and Veterinary Medicine Cluj-Napoca, selected for the current research are as follows (Mărghitaș et al, 2009, Dezmirean et al., 2010; Dezmirean et al., 2018, ,):

- White cocoon breeds: AB, AO 33, B1, C122, J90, JH3, N5, P4/T, S8;
- Coloured cocoon Breeds: Galben de Băneasa, Auriu Chinezesc, RG 90, E27;
- White cocoon commercially established hybrids: AC/t, AC29/T, AJ 5F, CTK.

The measurements were performed for the evaluation of the following biological and technological characteristics:

- Eggs: number of eggs/laying, hatching percent;
- Larvae: larval duration, larvae weight (5$^{th}$ instar, day 7);
- Chrysalis: weight; raw and dried silk;
- Cocoons: cocoon weight, chrysalis weight, silk shell weight, silk ratio (%).

The results were analysed statistically and then analysed under the Evaluation Index (a novelty classification method in Romania, but an established statistical analysis method in India) for the classification of the analysed breeds and hybrids.
The formula of the Evaluation index is as follows (Sudhkara et al, 2001; Buhroo et al, 2017):

$$EI = \frac{A - B}{C} X10 + 50$$

Where:
A = the mean value of a trait within a species,
B = the mean value of a trait for all breeds and hybrids analyzed,
C = the standard deviation of a feature for all breeds and hybrids,
10 = standard unit,
50 = fixed value.

In the case of negative traits, such as larval period, the formula changes as follows (Sudhkara et all, 2001; Buhroo et al, 2017):

$$EI = \frac{B - A}{C} X10 + 50$$

According to this Indicator, breeds and hybrids that have a value of the EI index higher than 50, are considered of great economical value (Sudhkara et al, 2001; Buhroo et al, 2017).

## RESULTS AND DISCUSSIONS

The obtained results concerning fecundity and hatching percent are presented in Table 2.

Table 2. Fecundity and Hatching percent of studied silkworm breeds and hybrids (2017)

| | | Fecundity | Hatching (%) |
|---|---|---|---|
| Breeds (White cocoon) | AB | 641 | 94.93 |
| | AO33 | 568 | 97.23 |
| | B1 | 645 | 93.61 |
| | C122 | 648 | 93.65 |
| | J90 | 620 | 90.48 |
| | JH3 | 684 | 89.77 |
| | N5 | 650 | 94.61 |
| | P4/T | 647 | 96.56 |
| | S8 | 611 | 91.49 |
| Breeds (coloured cocoon) | Auriu Chinezesc | 522 | 95.21 |
| | E27 | 479 | 84.98 |
| | Galben de Băneasa | 575 | 90.14 |
| | RG 90 | 442 | 93.16 |
| Hybrids | AC/T | 695 | 92.36 |
| | AC29/T | 654 | 95.16 |
| | AJ5/F | 612 | 91.78 |
| | 4. CTK | 634 | 95.73 |
| $\bar{X}$ | | 607.47 | 92.99 |
| $S_{\bar{x}}$ | | 69.79 | 3.04 |

From the analysis of these parameters it can be stated that, from the white cocoon breeds, JH3 have the higher prolificacy with 684 eggs/laying, followed by N5 with 650 eggs/laying and P4/T 647 egg/laying. The breeds C122 with 648 eggs/laying and a hatching percent of 93.65% and AB with 641 eggs/laying and a hatching percent of 94.93% appear to be most valuable.

The lowest values are registered by AO 33 with 568 eggs/laying with a high hatching percent of 97%.

In the case of coloured cocoon breeds, taking into account both fecundity and hatching, it can be stated that the valuable breeds are Auriu Chinezesc with 522 eggs/laying and a hatching percent of 95.21% and Galben de Băneasa with 575 eggs/laying and a hatching percent of 90.14%

In the case of the analysed hybrids, in can be observed that the number of eggs/laying and hatching percent are rather valuable as all hybrids registered high values in both parameters.

Other researchers have analysed white cocoon breeds and white cocoon hybrids.

On the fecundity parameters most the breeds and hybrids recorded values slightly higher than in other studies, but in terms of hatching percent, the recorded values are close, with variations of only 1 to 6%.

The average values in terms of the profilacity and hatchability parameters, diferences in time placement of the study affects these parameteres, as it is stated by Dezmirean et al, 2015.

Acording to studies done on the entire silkworm *Bombyx mori* gene pool, the fecundity values lye inbetween 318 and 584 eggs/laying, average values lowered by the tropical orgin silkworm breeds. Localy adapted and/or obtained breeds and highbreeds record values above the mentiod average. As these breeds have been localy adapted, their values have grown closer to the native group values (490-710 eggs/laying). For the hatching (%) parameter in can be stated that all the studied breeds and hybrids have values close to the whole gene pool hatching average value of 83.9-99.1%, with the exception of the AB breed and RG 90 breed (Matei, 2007; Matei et al, 2009; Mărghitaș et al., 2009 ; Dezmirean et al., 2010; Furdui, et all, 2010; Dezmirean et al, 2015; Dezmirean et al, 2018).

Table 3. Duration of the larval stage of studied silkworm breeds and hybrids

| | | Larval period (Days) |
|---|---|---|
| Breeds (White cocoon) | AB | 33.060 |
| | AO33 | 31.956 |
| | B1 | 32.959 |
| | C122 | 32.941 |
| | J90 | 32.957 |
| | JH3 | 32.956 |
| | N5 | 32.958 |
| | P4/T | 32.951 |
| | S8 | 32.956 |
| Breeds (colored cocoon) | Auriu Chinezesc | 32.959 |
| | E27 | 28.942 |
| | Galben De Băneasa | 31.958 |
| | RG 90 | 32.038 |
| Hybrids | AC/T | 32.953 |
| | AC29/T | 32.958 |
| | AJ5/F | 32.956 |
| | CTK | 32.956 |
| $\bar{X}$ | | 32.554 |
| $S_{\bar{x}}$ | | 1.007 |

From the table 3, it can be stated that all the studied breeds and hybrids have similar larval period, with the exception of the coloured breed E27, where the shortest larval stage is registered with the value of 28.942 days.

The recorded larval periods are longer by an average of 2 to 4 days in comparison to other studies, close to the values registered by the native breeds and Japanese breeds from the silkworm native gene pool (Matei et al, 2009; Mărghitaş et al., 2009; Dezmirean et al, 2010).
The average weight of the silkworm larvae can be economical valuable, especially in the food and animal fodder industry, therefore from this perspective, the average weight it's not only a trait for comparison, but a trait to consider from an economical perspective.

Table 4. Average larval weight studied silkworm breeds and hybrids

| | | Larvae weight |
|---|---|---|
| Breeds (White cocoon) | AB | 2.9607 |
| | AO33 | 2.8074 |
| | B1 | 3.1972 |
| | C122 | 2.7078 |
| | J90 | 3.0987 |
| | JH3 | 2.9270 |
| | N5 | 3.2894 |
| | P4/T | 3.2728 |
| | S8 | 3.0178 |
| Breeds (collored cocoon) | Auriu Chinezesc | 2.4508 |
| | E27 | 2.5645 |
| | Galben de Băneasa | 2.7473 |
| | RG 90 | 3.5567 |
| Hybrids | AC/T | 3.2738 |
| | AC29/T | 3.8585 |
| | AJ5/F | 2.8167 |
| | CTK | 2.6852 |
| $\bar{X}$ | 3.0137 | 3.0137 |
| $S_{\bar{x}}$ | 0.5407 | 0.5407 |

From the table above, it can be stated that from the white cocoon breeds the most valuable are N5 with an average weight of 3.2894 g, followed by P4/T (3.2728 g) and J90 (3.0987 g). The lowest average weight is registered by C122 with the average weight of 2.7078 g. From the colored cocoon breeds, the most valuable breeds are RG 90 (3.5567) and Galben de Băneasa (2.7473g), and the lowest value is registered by Auriu Chinezesc breed with the average value of 2.4508 g.
From the studied hybrids the most valuable one is AC29/T with an average weight of 3.8585 g and least valuable hybrid is AJ5/F with the average value of 2.8167g.
Taking into consideration other studies done on selected breeds and hybrids, where similar values were obtained, with the exceptions of a few breeds (B1 and S8) and hybrids (AC/T), the registered values were lower, on average by 1 gram in comparison with the recorded values of other research projects (Matei, 2007; Furdui et al., 2010).
Differences in weight can also be influenced by the day of sample collection. In the present study, samples were collected and measurements made in the 7$^{th}$ day of the 5$^{th}$ instar of the larval period.

Table 5. Average values of raw silk cocoon parameters for the studied silkworm breeds and hybrids

| | | Cocoon Weight (g) | Chrysalis weight (g) | Silk Shell Weight (g) | Silk Ratio (%) |
|---|---|---|---|---|---|
| Breeds (White cocoon) | AB | 1.5083 | 1.1810 | 0.3274 | 23.46 |
| | AO33 | 1.8190 | 1.4530 | 0.3579 | 19.95 |
| | B1 | 2.0052 | 1.5996 | 0.3900 | 19.56 |
| | C122 | 1.7357 | 1.3930 | 0.3322 | 19.25 |
| | J90 | 1.6988 | 1.3458 | 0.3475 | 20.72 |
| | JH3 | 1.7822 | 1.4437 | 0.3366 | 18.92 |
| | N5 | 1.8003 | 1.4477 | 0.3412 | 19.13 |
| | P4/T | 1.8210 | 1.4539 | 0.3554 | 19.97 |
| | S8 | 1.7937 | 1.4814 | 0.3067 | 17.18 |
| Breeds (collored cocoon) | Auriu Chinezesc | 1.7958 | 1.4876 | 0.2987 | 16.78 |
| | E27 | 1.1904 | 1.0212 | 0.1710 | 14.48 |
| | Galben de Băneasa | 1.7234 | 1.4041 | 0.2557 | 15.17 |
| | RG 90 | 1.9263 | 1.5819 | 0.3343 | 17.42 |
| Hybrids | AC/T | 1.7171 | 1.3698 | 0.3394 | 19.93 |
| | AC29/T | 2.2207 | 1.7935 | 0.4167 | 18.87 |
| | AJ5/F | 1.8843 | 1.5112 | 0.3672 | 19.59 |
| | CTK | 2.0498 | 1.6577 | 0.3810 | 18.59 |
| $\bar{X}$ | | 1.7902 | 1.4473 | 0.3321 | 18.66 |
| $S_{\bar{x}}$ | | 0.3383 | 0.2830 | 0.0706 | 3.00 |

After the results from the table 5 were analyzed, it can be observed that from the white silk cocoon breeds, the breeds with the heavier silk cocoons are B1 with an average weight of 2.0052 g, followed by P4/T (1.8210 g) and AO33 (1.8190 g). AB and S8 have cocoon weight values well above 0.300 g, a very valuable aspect.
The most valuable breed with coloured cocoons is RG 90 with an average cocoon weight of 1.9263 and the least valuable breed is E27 with the cocoon average weight of 1.1904 g. From the white cocoon hybrids, the most valuable is CTK with a cocoon weight of 2.0498 and the least valuable hybrid is AC/T with a cocoon average weight of 1.7171 g.
The chrysalis may be important economical by-products as it is a source of natural fat and protein content. Economically, the most important white cocoon breeds are B1 (1.5996 g), followed by S8 (1.4876 g) and P4/T (1.4816g). In the case of the coloured cocoon

breeds, the average chrysalis weight is between 1.0212 g (E27) and 1.5819 (RG90) . In the case of the hybrids, the average chrysalis weight is registered between 1.3698 (AC/T) and 1.7935 (AC29/T).

In terms one of the most important economical traits, the weight of silk shell, it can be stated that for the white cocoon breeds, the minimal value is recorded by S8 breed with a minimal value of 0.3067 g and the maximal value is recorded by the breed B1 with the average weight of 0.3900 g.

In the case of the coloured cocoon breeds the values are registered between 0.1710 for E27 and 0.3343 g for RG 90.

For the analysed hybrids the differences between them are quite visible as well as the average shell weight is situated between 0.3394 for AC/T and 0.4169 g for AC29/T.

In the case of the silk content percent, for the white silk cocoon breeds the values are between 17.18% for S8 and 23.46% for AB, for coloured silk cocoons breeds the values are situated between 14.8% for E27 and 17.42%. for RG90 and for the hybrids are between 17.42 for AC/T and 19.93% for AC 29/T.

Table 6. Average values of dried silk cocoon parameters for the studied silkworm breeds and hybrids

| | | Dried Cocoon Weight (g) | Dried Chrysalis weight (g) | Dried Silk Shell Weight (g) | Silk Ratio (%) |
|---|---|---|---|---|---|
| Breeds (White cocoon) | AB | 0.6077 | 0.3032 | 0.3012 | 51.18 |
| | AO33 | 0.6662 | 0.3284 | 0.3328 | 51.95 |
| | B1 | 0.7983 | 0.4198 | 0.3643 | 45.79 |
| | C122 | 0.5814 | 0.2923 | 0.2833 | 48.47 |
| | J90 | 0.6536 | 0.3216 | 0.3230 | 49.13 |
| | JH3 | 0.5640 | 0.2794 | 0.2738 | 48.77 |
| | N5 | 0.6893 | 0.3483 | 0.3332 | 48.48 |
| | P4/T | 0.6893 | 0.3483 | 0.3332 | 48.48 |
| | S8 | 0.6364 | 0.3227 | 0.2986 | 48.13 |
| Breeds (coloured cocoon) | Auriu Chinezesc | 0.6430 | 0.3571 | 0.2802 | 43.99 |
| | E27 | 0.3974 | 0.2265 | 0.1636 | 41.31 |
| | Galben de Băneasa | 0.5559 | 0.3182 | 0.2298 | 40.97 |
| | RG 90 | 0.5980 | 0.3233 | 0.2657 | 44.50 |
| Hybrids | AC/T | 0.6011 | 0.2977 | 0.2943 | 49.06 |
| | AC29/T | 0.7780 | 0.3795 | 0.3900 | 50.15 |
| | AJ5/F | 0.7403 | 0.3687 | 0.3536 | 47.82 |
| | CTK | 0.6577 | 0.3301 | 0.3164 | 48.25 |
| $\bar{X}$ | | 0.6370 | 0.3273 | 0.3005 | 47.12 |
| $S_{\bar{x}}$ | | 0.1365 | 0.0740 | 0.0728 | 4.73 |

Concerning the weight of silk cocoon, for the white cocoon breeds the values are situated between 0.5814 g for C122 and 0.7983 g for B1, for the coloured cocoon breeds the values are situated between 0.3974 g for E27 and 0.6430 g for Auriu Chinezesc and in the case of the hybrids the values registered are between 0.6011 g for AC/T and 0.7780 for AC29/T.

In the case of the dried chrysalis weight, for the white cocoon breeds the values are situated between 0.2794 g for JH3 and 0.4198 g for B1, for the coloured cocoon breeds the values are situated between 0.2265 g for E27 and 0.3571 g for Auriu Chinezesc and in the case of the hybrids the values registered are between 0.2977 g for AC/T and 0.3795 for AC29/T.

In the case of the dried silk shell weight, for the white cocoon breeds the values are situated between 0.2833 g for C122 and 0.3643 g for B1, for the coloured cocoon breeds the values are situated between 0.1636 g for E27 and 0.2802 g for Auriu Chinezesc and in the case of the hybrids the values registered are between 0.2943 g for AC/T and 0.3900 for AC29/T.

In the case of the silk ratio percent, for the white cocoon breeds the values are situated between 45.79% for B1 and 51.95% for AO 33, for the coloured cocoon breeds the values are situated between 40.97% for Galben de Băneasa and 44.50% for RG90 and in the case of the hybrids the values registered are between 47.82% for AJ 5F and 50.15% for AC29/T.

In the case of other studies made on the current breeds, it can be stated, that, in the case of the white cocoon breeds and hybrids, the overall results registered are similar with many other performed studies on selected breeds and hybrids, mainly the following parameters: raw cocoon weight, raw silk shell weight and dried silk shell weight.

In the case of the dried cocoon parameters, the overall cocoon weight differs, as one influencing factor is the duration of the drying period and the implemented protocol. (Bențea et all, 2006; Matei, 2007; Furdui et al. 2010; Pașca et al., 2010; Mărghitaș et al, 2011; Dezmirean et al, 2018;).

According to the latest studies, the results, focused on all analysed traits excluding the weight of the chrysalis, obtained in the current study and the cited research are overall similar (Dezmirean et al, 2018).

The similarity in registered values can also be observed when a comparison is done with averages values of the studied parameters for

the silkworm breeds and hybrids of the native gene pool.

The raw cocoon average weight registers values between 1.538 g and 2.144 g. The studied breeds and hybrids recorded similar values.

The raw cocoon shell weight registers values between 0.306 g and 0.469 g. The studied breeds and hybrids recorded similar values with the exception the following breeds: E27 and Galben de Baneasa (Coloured cocoon breeds).

The raw cocoon silk ratio registers values between 17,60% and 23.59%. The studied breeds and hybrids recorded similar values with the exception the following breeds: E27 and Galben de Baneasa (Coloured cocoon breeds).

The dried cocoon average weight registers values between 0.778 g and 1.105 g. The studied breeds and hybrids recorded similar minimal values with the exception of the coloured cocoon breeds.

The dried cocoon silk ratio registers values between 37.1% and 43%. The studied breeds and hybrids recorded similar values or higher (Matei et al, 2008; Matei et al, 2010).

When observing the results from the current study and the results from previous studies, although the microclimate conditions were rather similar, differences can still be observed.

Annual and seasonal differences in climate can affect the technological parameters and biological properties of the silkworm breeds and hybrids. Variations in humidity and heat, parameters of the rearing room microclimate can also affect the silkworm and their biological and technological parameters. Another factor that influences the development of the silkworm development is the quality of mulberry leafs (Rahmathulla, 2012).

It is easy to look at a number of breeds and hybrids of the silkworm *Bombyx mori L*. and identify per trait promising lines, but from an economical perspective, when by-products are considered important to it is necessary to have an inclusive classification of the analysed breeds and hybrids, in order to identify the most successful one. In this context, an inclusive index has been applied, Multiple Trait Evaluation Index, what has been widely used for classification and selection of promising breeds and hybrids of silkworms. After the calculation of the individual index (per trait, per breed/hybrid), the average value of the considered traits is calculated and analysed breeds and hybrids are calculated. If the average value is greater than 50, the breed is considered valuable and suitable for commercial exploitation (Buhroo et al., 2017). This method of statistical classification was applied to the current study as well.

Table 7. Evaluation Index Method for fecundity and hatchability for the studied silkworm breeds and hybrids

| | | Fecundity | Hatching (%) |
|---|---|---|---|
| Breeds (White cocoon) | AB | 54.80 | 56.37 |
| | AO33 | 44.34 | 63.93 |
| | B1 | 55.38 | 52.03 |
| | C122 | 55.81 | 52.16 |
| | J90 | 51.80 | 41.75 |
| | JH3 | 60.97 | 39.41 |
| | N5 | 56.09 | 55.32 |
| | P4/T | 55.66 | 61.73 |
| | S8 | 50.51 | 45.07 |
| Breeds (coloured cocoon) | Auriu Chinezesc | 37.75 | 57.29 |
| | E27 | 31.59 | 23.67 |
| | Galben de Băneasa | 45.35 | 40.63 |
| | RG 90 | 26.29 | 50.55 |
| Hybrids | AC/T | 62.54 | 47.93 |
| | AC29/T | 56.67 | 57.13 |
| | AJ5/F | 50.65 | 46.02 |
| | CTK | 53.80 | 59.00 |

Fecundity and hatchability can be used to asses and forecast the cocoon production, therefore at this stage all breeds and hybrids are valuable, the coloured breed has low values as it competes with white cocoon breeds, and falls behind Galben de Băneasa.

Table 8. Evaluation Index Method for larval period and silkworm's larvae weight for the studied silkworm breeds and hybrids

| | | Duration of the larvae period | Larvae weight |
|---|---|---|---|
| Breeds (White cocoon) | AB | 54.80 | 56.37 |
| | AO33 | 44.34 | 63.93 |
| | B1 | 55.38 | 52.03 |
| | C122 | 55.81 | 52.16 |
| | J90 | 51.80 | 41.75 |
| | JH3 | 60.97 | 39.41 |
| | N5 | 56.09 | 55.32 |
| | P4/T | 55.66 | 61.73 |
| | S8 | 50.51 | 45.07 |
| Breeds (coloured cocoon) | Auriu Chinezesc | 37.75 | 57.29 |
| | E27 | 31.59 | 23.67 |
| | Galben de Băneasa | 45.35 | 40.63 |
| | RG 90 | 26.29 | 50.55 |
| Hybrids | AC/T | 62.54 | 47.93 |
| | AC29/T | 56.67 | 57.13 |
| | AJ5/F | 50.65 | 46.02 |
| | CTK | 53.80 | 59.00 |

The larval period doesn't vary a lot, the only exception being the E27 breed. The Index for the larval weight reflects the results obtained in Table 4.

Table 9. Evaluation Index for raw cocoon parameters for the studied silkworm breeds and hybrids

| | | Cocoon Weight | Chrysalis weight | Silk Shell Weight | Silk Ratio (%) |
|---|---|---|---|---|---|
| Breeds (White cocoon) | AB | 41.67 | 40.59 | 49.34 | 66.01 |
| | AO33 | 50.85 | 50.20 | 53.66 | 53.39 |
| | B1 | 56.35 | 55.38 | 58.21 | 52.64 |
| | C122 | 48.39 | 48.08 | 50.01 | 51.60 |
| | J90 | 47.30 | 46.41 | 52.18 | 55.98 |
| | JH3 | 49.76 | 49.87 | 50.64 | 50.75 |
| | N5 | 50.30 | 50.01 | 51.29 | 50.97 |
| | P4/T | 50.91 | 50.23 | 53.31 | 52.86 |
| | S8 | 50.10 | 51.20 | 46.41 | 44.80 |
| Breeds (collored cocoon) | Auriu Chinezesc | 50.16 | 51.42 | 45.27 | 43.24 |
| | E27 | 32.27 | 34.94 | 27.19 | 35.67 |
| | Galben de Băneasa | 48.02 | 48.47 | 39.19 | 37.26 |
| | RG 90 | 54.02 | 54.76 | 50.31 | 45.64 |
| Hybrids | AC/T | 47.84 | 47.26 | 51.03 | 53.68 |
| | AC29/T | 62.72 | 62.23 | 61.98 | 50.35 |
| | AJ5/F | 52.78 | 52.26 | 54.97 | 52.76 |
| | CTK | 57.67 | 57.43 | 56.93 | 49.76 |

Table 10. Evaluation Index Method for dried cocoon parameters for the studied silkworm breeds and hybrids

| | | Cocoon Weight | Chrysalis weight | Silk Shell Weight | Silk Ratio (%) |
|---|---|---|---|---|---|
| Breeds (White cocoon) | AB | 46.75 | 50.04 | 58.21 | 50.43 |
| | AO33 | 50.15 | 54.40 | 55.68 | 52.42 |
| | B1 | 62.50 | 58.73 | 46.79 | 55.07 |
| | C122 | 45.28 | 47.58 | 53.14 | 48.81 |
| | J90 | 49.23 | 53.06 | 54.59 | 50.15 |
| | JH3 | 43.53 | 46.28 | 52.80 | 48.53 |
| | N5 | 52.85 | 54.46 | 52.37 | 52.58 |
| | P4/T | 52.85 | 54.46 | 52.37 | 53.45 |
| | S8 | 49.38 | 49.69 | 49.44 | 48.56 |
| Breeds (collored cocoon) | Auriu Chinezesc | 54.03 | 47.16 | 42.56 | 46.66 |
| | E27 | 36.39 | 31.11 | 37.62 | 37.21 |
| | Galben de Băneasa | 48.77 | 40.22 | 37.97 | 44.05 |
| | RG 90 | 49.47 | 45.16 | 44.32 | 48.97 |
| Hybrids | AC/T | 46.01 | 49.10 | 53.64 | 50.79 |
| | AC29/T | 57.06 | 62.28 | 56.04 | 58.82 |
| | AJ5/F | 55.60 | 57.26 | 51.18 | 51.81 |
| | CTK | 50.38 | 52.14 | 51.88 | 52.30 |

Analyzing these values, it can easily be seen the most valuable breeds and hybrids, in terms of silk content (as it is one of the most important economical traits): in the case of the raw cocoon, for the white cocoon breeds is B1, followed by AO33 and AB breeds, for the coloured cocoons is RG90, and for the hybrids is AC 29/T and in the case of the dried cocoon, for the white cocoon breeds is B1, N5, P4/T, followed by AB and AO33, for the coloured cocoons are Auriu Chinez (due to the possibility of lower water loss during drying process) and RG90, and for the hybrids is AC 29/T

As the values of the trait index are calculated for each breed and hybrid is it logical to asses that it reflects the situation discussed above, when the analysis of the values of the traits was made.

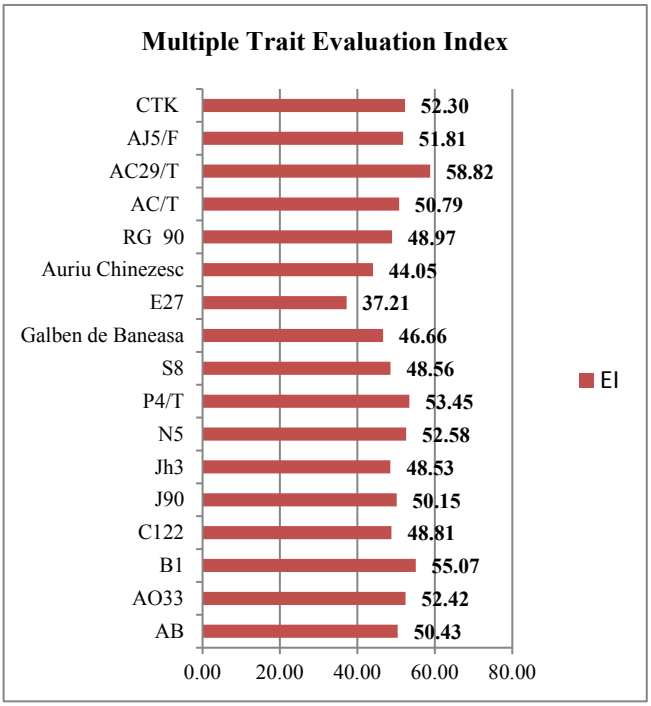


Fig. 1. Multiple Traits Evaluation Index. Classification of the analysed breeds and hybrids

The productivity of chosen breeds used to be attributed mainly to the ability to produce silk itself (silk shell and silk content ratio), but in recent studies it has been noticed that the overall productivity of a breed or hybrid is affected by totality of 21 component traits, therefore it is important to use an Index that takes into account more than one trait at a time. In this context, to respond to the need to proper identify successful breeds and hybrids, the Multiple Trait Evaluation Index was developed and implemented.

A classification of all analysed breeds and hybrids has been made under the argument that all analysed breeds and hybrids have been well established, and despite the differences given by the specific characteristics, the most

important aspect for a farmer it is the overall productivity (Bhat et al,.2017).
Overall almost all breeds and hybrids had values of their afferent index over 50, providing evidence of the value of the local sericultural *Bombyx mori* gene pool.
Based on the values obtained, the following can be stated: the most valuable white cocoon breed is B1 (55.27), the most valuable coloured silk cocoon breed is Auriu Chinezesc (47.27), followed by RG90 (46.81), and the most valuable hybrid is Ac29/T (58.29 – highest recorded value of the Index.

## CONCLUSIONS

The Multiple Trait Evaluation Index Method offers the possibility to easily oversee the classification not only on their overall economical value, but for individual or subgroups of biological and technological traits as well.
Therefore in can be stated that most valuable breeds per trait for the eggs parameters:

- No eggs/laying: white cocoon breeds – all but AO33 , coloured cocoon breeds – Galben de Baneasa and Auriu chinez, white cocoon hybrids – AC/T and AC29/T;
- Fecundity: white cocoon breeds – all but J90, JH3 and S8, coloured cocoon breeds – Galben de Baneasa and Auriu chinez, white cocoon hybrids – AC/T and AC29/T.

In the case of the larval duration, the differences are minimal, being shorter for the coloured cocoon groups.
Concerning the larval weight, if by-products are an important aspect of it, from the white cocoon breeds, N5 and AO33 are rather valuable. From the hybrids AC29/T is the most valuable. From the coloured cocoon group, RG has recorded values over 50.
In terms of the raw cocoon parameters, considering all afferent traits, the most valuable white cocoon breeds are considered all valuable, as only S8 has visible lower values; the most valuable coloured cocoon breeds are the native breeds Galben de Baneasa and RG 90; the most valuable white cocoon hybrids are considered all valuable.
In terms of the dried cocoon parameters, considering all afferent traits, the most valuable white cocoon breeds are the most valuable white cocoon breeds are considered all valuable, as only C122 ad J90 breeds have visible lower values; the most valuable coloured cocoon breeds are the native breeds Galben de Baneasa and RG 90; the most valuable white cocoon hybrids are considered all valuable.
Differences between the raw cocoon parameters and dried cocoon parameters classification can be given by the rate and amount of lost water during the cooking process.
More studies need to be performed on the coloured cocoon breeds and the breeds that have scored an EI under 50, but the silk shell Index is over 50, such as AO 33, C122 and AJ 5F.
Taking into consideration the overall economical value, looking at the values obtained, the hybrids have maximal values, the top being represented by AC29/T.
From the white cocoon breeds, the commercial established breeds like AB. AO33 or B1, maintain their economical value over time, as their overall score of the Index is situated over 50, their value being in their high production stability in different rearing periods.
Newly or less studied breeds like N5 or P4/T also have relative high scores.
When compared to other performed studies, we may conclude, even though some exceptions occurred, that the development of the breeds and hybrids was rather normal.
The differences between the groups are due to different genotypes of the simple breeds, as well as differences in genotypes between the parental breeds of the commercial hybrids and those of the coloured cocoon breeds.
As a classification method, the Multiple Trait Evaluation Index is suitable and flexible for the Romanian *Bombyx mori* gene pool, but more studies done replications in different environmental conditions are needed to establish a long lasting classification of the silkworm breeds and hybrids.

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# TECHNOLOGIES OF THE AGRO FOOD PRODUCTS PROCESSING

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# TECHONOLGIES CONCERNING THE PROCESSING AND CONSERVATION OF NATURAL CASING INTENDED FOR FOOD INDUSTRY

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***Abstract***

*Coming from animals with economic interest, small and large intestines are what we call the "natural casings" and they are characterized by being strong enough to withstand the pressure produced by filling them, they are permeable to water vapour and gas, absorb fumes and impregnate with a pleasant flavour, expand or contract in accordance with the amount of added content and can be closed at the ends by binding or clipping. They are subjected to a calibration process after which they must comply with the stipulations of technical specifications or manufacturing standards. Natural casings are preserved by drying and salting and must meet a number of legal requirements. The purpose of this research is to identify and monitor the obtaining and preservation of natural casings for the meat industry. Natural casings that were processed in specialized units and then traded on the domestic market were analyzed. Differences in technology are found depending on the species from which the natural casings originate, but also according to the intestinal segment subjected to processing phases. Processing defects such as holes or tearing in the natural casings can be ascertained, but also defects in preservation that may lead to downgrading or even the marketing withdrawal of the incriminated products. Salt casings can be packed in plastic or barrels with different capacities. Separately packed are wrinkled pork guts, small pork guts, small sheep guts etc. To ease the insertion in the spur pipe, the casings can be sprouted onto the plastic sticks, resulting in tubular casings. Although technology development makes it possible to produce artificial casings such as collagen, cellulose and fibrous membranes, meat products in casings from natural intestines are preferred by consumers.*

***Key words****: casings, meat products, food safety.*

## INTRODUCTION

The casing is an assembly of proteins and lipids, having specific properties of permeability, whereby subdivision of the living matter is accomplished (Costea et al., 2004; Dubei, 2009; Marcu et al., 2009).

Natural casings are represented by small and large intestines from animals of economic interest (cattle, sheep, goats, pigs) that differ in length and caliber (Priseceanu et al., 2015; Tăpăloagă et al., 2016). They are used in the meat-making industry as guts (Petcu, 2015).

The natural casings are preserved by drying and salting and must meet the following requirements: be tied up in ties (salted ones) or packets (dried ones), be degreased, not to smell of rancid, sour or rotten, not to show holes (Georgescu, 2000).

They are characterized by being strong enough to with stand the pressure produced by filling them, they are permeable to water vapour and gas, absorb fumes and impregnate with a pleasant flavour, expand or contract in accordance with the amount of added content and can be closed at the ends by binding or clipping.

Although technology development makes it possible to produce artificial casings such as collagen, cellulose and fibrous membranes, natural casings are especially preferred for products consumed with edible wrapping, such as sausages (Bakker et al., 1999; Zengin, 2010).

Historically, intestinal segments of lamb, beef, or pig, such as the stomach, the small and the large intestines or bladder, used to be filled with the sausage mix. Being one of the most commonly consumed meat products, sausages and salamis are typically obtained from red meat or poultry meat, by chopping and mixing with appropriate spices (Zengin, 2010; Ghimpeţeanu et al., 2016).

## MATERIALS AND METHODS

The purpose of this research is to identify and monitor the obtaining and preservation of natural casings for the meat industry.
In the period 2017-2018, natural casings processed in specialized units and then traded on the domestic market, were analyzed.
Depending on the species from which the natural casings originate, as well as the intestinal segment subjected to processing, differences in technology can be found.
After the processing, the natural casings of the animals intestines (guts), are salted or dried,after which they are packaged and stored until filled with a meat composition (Petcu, 2015).
Before processing, the intestines are subjected to veterinary control and only those that have been foundappropriate are processed for use in the food industry (Pavel et al., 2007; Laslo et al., 2009).
Through an organoleptic examination, appreciation is made after the following criteria: the outer appearance of the casings, the colour, the consistency and the smell.
Verification of physico-chemical characteristics refers to the strength of the casings walls at an air pressure required for calibration, as well as the Nessler reaction and the determination of hydrogen sulphide ($H_2S$), which must be negative.
Processing defects such as holes or tearing in the natural casings can be ascertained, but also defects in preservation that may lead to downgrading or even the marketing withdrawal of the incriminated products (Arduser et al., 2005).

## RESULTS AND DISCUSSIONS

Natural casings are calibrated and must meet the requirements of technical specifications or manufacturing standards.
Salt casings can be packed in plastic or barrels with different capacities.
Separately packed are wrinkled pork guts, small pork guts, small sheep guts etc.
Lately, to ease the insertion in the spur pipe, the casings can be sprouted onto the plastic sticks, resulting in tubular casings (Figure 1).

Figure 1. Tubular natural casings (www.marchard.ro)

**Processing defects**
Holes result from the evisceration process, pulling guts from the mesenter, the manual or mechanical degrease performed faulty.
External dirt and debris of intestinal contents are defects produced bynon-compliance with hygiene and processing technology.
Fat remnants have the appearance of fat islands or a continuous fat line (on the intestine and mesenter joining line).
The defect can be avoided by pulling the guts from the mesenter correctly, cooling the guts for further degreasing, and using special equipment or a wide knife for the best grinding of fat (Mincu, 2001; Arduser et al., 2005).

**Preservation defects**
Casings redness is caused by certain salt-resistant microbial species that grow to over 15°C (*Micrococcus rozeus, Bacillus serratium*). Reddish guts give off a characteristic smell. Defects can be avoided by observing technology and applying rigorous hygiene measures (Pop et al., 2005).
Rust is a common defect in pigs and sheep casings.It appears on the surface of the casings, having the appearance of a lick, similar to fish scales, yellowish-brown to greyish. Rust is produced by bacteria that metabolize the iron and the calcium from salt.
Colour change is a defect of chemical nature. It occurs when casings are not well covered with salt, which causes a grey or even blackish appearance.
Casings putrefactions caused by the multiplication of putrefaction bacteria due to faulty processing and storage.The process of alteration is similar to post-mortem

putrefaction, resulting in a smell of ammonia and hydrogen sulphide, and on the surface of the casings appear sulphmethaemoglobin spots, black or bluish, of different shades (Ciobotaru, 2013).

Salt stains appear of different shapes and sizes, located on the surface of the casings preserved with large granule salt, which stick to the walls of the casings, due to the fact that the granules remain undissolved. For prevention, only salt with the right granule must be used (Parker, 2001).

Due to weakness against swelling by air or water, the weakening of casings walls strength usually accompanies the process of putrefaction. Sometimes, the resistance is also weakened by the incomplete scraping operation.

Souring is a slow acid fermentation that changes the colour of the casings from pink to grey, accompanied by the appearance of a sour odour. The phenomenon occurs in insufficiently cleaned and salted casings. The gases released dislodge the tunics that make up the walls of the casings and cause them to lose their strength, rendering them unusable.

The lack of gloss (in dry casings) is caused by the surface deposition of protein substances resulting from insufficient softening in drainage basins or as a result of drying at temperatures too low. In order to prevent the defect, it is recommended to work correctly, soaking the casings in basins where water changes frequently, drying at temperatures above 10$^{o}$C.

Wrinkling is a defect present in dry casings that have not been tightly connected to the ends nor have small unobservable holes, favouring gradual loss of air during the drying process (Savu and Petcu, 2002).

Excessive drying occurs in casings subjected to the drying process at too high temperature and too low humidity or in the casings that have been exposed to direct sunlight. As a result, casings become friable and brown. Drying of casings should be done only in ventilated areas and away from direct sunlight.

**Storage defects**

Mildew process is a defect that occurs in dry casings, processed in improper conditions, and stored in damp and non-aerated rooms. The moulds penetrate into the walls of the casings and form a velvety white-greenish on the surface layer that emits a specific odour.

Destruction caused by insects (moths and bugs) is found in dry, dirty, poorly defatted and stored in improper conditions casings. For the purpose of prevention, perfect hygiene, good ventilation and compliance with pest control programmes, mandatory for all units of the food industry, will be maintained (Neagu, 2010).

**Results obtained from the examination of small and wrinkled pork guts**

Small pork guts (derived from the processing of small intestines) are used for different types of cooked sausage, fresh sausage, liverwurst etc. Pig intestines have a diameter from 28/30 mm to 42/44 mm (Neagu, 2010). Wrinkled pork guts (derived from the processing of large intestines) are used for the liverwurst, dried sausages etc. For commercialization, small pork guts are divided into large, medium or small diameters.

After being obtained and cleaned, these have been subjected to a salting treatment (Figure 2).

Figure 2. Salt treatment of pork casings

The quality verification rules imply: organoleptic verification (Table 1), product temperature at reception (+2 ... +10$^{o}$C), shelf

life verification on the label and visual inspection of packaging.
Usually, packing of small pork guts is made in plastics, each box having 10m of intestines. Packing of wrinkled pork guts is made in plastics, each box having 0.70m of wrinkled guts. The storage temperature is between 2 and 10°C, to avoid any changes of the products. The shelf life of salted wrinkled pork guts is two years.

Table 1. Organoleptic and physico-chemical properties of natural casings from pig intestines

| Organoleptic properties | |
|---|---|
| Appearance | natural |
| Colour | transparent white |
| Consistency | firm and elastic |
| Smell | pleasant, characteristic, no foreign smell |
| Physico-chemical properties | |
| Having walls resistant to air pressure necessary to determine the calibre and sorting by product type (Figure 3). | |

Figure 3. Checking the calibre of natural casings in pig intestines

During salt treatment and packaging, the products will be handled quickly to avoid interruption of the cold chain.At the end of the shelf life, freshness parameters of the products were tested and corresponding results were obtained at all tested samples.

**Results obtained from the examination of cattle guts**

The natural cattle casings are used for different types of sausages and for traditional products. The small intestines are 36/38 mm in diameter, medium intestines are 38/40 mm in diameter, and the large ones have a diameter of 40/43mm.
After being obtained and cleaned, these have been subjected to a salting treatment. Organoleptic properties of natural casings from cattle intestines are presented in table 2.

Table 2. Organoleptic and physico-chemical properties of natural casings from cattle intestines

| Organoleptic properties | |
|---|---|
| Appearance | natural |
| Colour | yellow |
| Consistency | firm and elastic |
| Smell | characteristic, no foreign smell |
| Physico-chemical properties | |
| Having walls resistant to air pressurenecessary to determine the calibre. | |

Most commonly, the cattle casings are packed in bundles, each one having 30m.The storage temperature is 2-10°C.During the 2 years of the study, no deviations from the set temperature values have been identified in the processing unit. Salted cattle casings have a shelf life of 2 years. The products will be handled quickly during salt treatment and during packaging to avoid interruption of the cold chain.

**Results obtained from the examination of sheep guts**

Natural sheep's casings are used for various types of sausages. Sheep intestines have a diameter from 16/18 mm to 28 mm (Banu, 1996). Table 3 presents the organoleptic and physico-chemical properties of natural casings from sheep intestines.

Table 3. Organoleptic and physico-chemical properties of natural casings from sheep intestines

| Organoleptic properties | |
|---|---|
| Appearance | natural |
| Colour | varies from white to grey |
| Consistency | firm and elastic |
| Smell | characteristic, no foreign smell |
| Physico-chemical properties | |
| Having walls resistant to air pressurenecessary to determine the calibre (Figure 4). | |

Figure 4. Checking the calibre of natural casings in sheep intestines

The results and discussions on the microbiological analysis of the intestine samples for the meat industry are presented in Table 4.

Table 4. Microbiological analyses of intestinal samples for the meat industry

| Product | Test / Analysis Method | | |
|---|---|---|---|
| | Enterobacteriaceae by ISO 21528-2/2017 | Total number of germs by SR EN ISO 4833-1/2014 | Salmonella spp. by SR EN ISO 6579-1/2017 |
| Pork casings 10m casserole | <10 ufc/g | <10 ufc/g | Undetected / 25 g |
| Wrinkled pork casings 0.70m | <10 ufc/g | <10 ufc/g | Undetected / 25 g |
| Hindgut pork casserole | <10 ufc/g | <10 ufc/g | Undetected / 25 g |
| Sheep casings 10m casserole | <10 ufc/g | <10 ufc/g | Undetected / 25 g |
| Pork natural casing for hog's pudding | <10 ufc/g | <10 ufc/g | Undetected / 25 g |
| Pork casings 10m, casserole | <10 ufc/g | <10 ufc/g | Undetected / 25 g |
| Wrinkled pork casings 0,70m | <10 ufc/g | <10 ufc/g | Undetected / 25 g |
| Pork casings 10m, casserole | <10 ufc/g | <10 ufc/g | Undetected / 25 g |
| Wrinkled pork casings 0.70m | <10 ufc/g | <10 ufc/g | Undetected / 25 g |

Samples of the packaged products are harvested and subjected to laboratory analysis. The results obtained fall within the accepted limits for the products under study.

**Results and discussions on meat traceability for the meat industry**

The way the product is batched and identified must prove good traceability (Figure 5). Therefore, when a large batch of raw materials is received in the casings processing unit to obtain meat products, the workforce must strictly follow the recommendations and directions received from the food safety manager. Smaller batches of casings, which have undergone similar conditions of temperature, humidity and hygiene, in a single working day, have been formed most of the time. There were no instances of non-compliance with traceability.

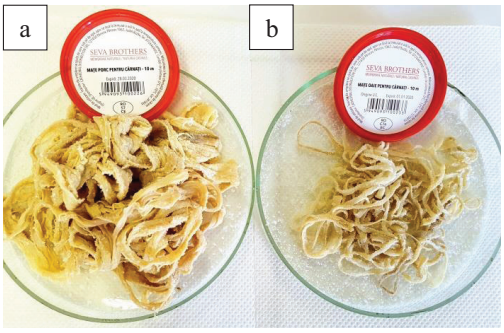


Figure 5. Salt pork casings (a) and salt sheep casings (b)

## CONCLUSIONS

Although technology development makes it possible to produce artificial casings such as collagen, cellulose and fibrous membranes, meat products in casings from natural intestines are preferred by consumers, which is why the monitoring of their quality must be carried out rigorously in all stages of their processing.

The monitoring of the storage temperature of the casings during the research has demonstrated compliance of the values established by the technical specifications, for all assortments of casings.

The microbiological analyses performed for samples of pork and sheep casings have demonstrated compliance with the values required by the technical specifications and also with the freshness parameters that have

been maintained throughout the shelf life for all the batches analyzed.

Traceability has been respected among all batches of processed casings, meaning that the staffis properly trained and aware of the importance of traceability in the food industry.

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# UTILIZATION EFFECT OF DOG ROSE (*Rosa canina*) EXTRACT ON YOGURT QUALITY

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***Abstract***

*The current paper aimed to study the utilization impact of dog rose (Rosa canina) extracts on yogurt quality. Our research was carried out on three types of yogurt: classic yogurt (control – LC), yogurt with 0.5% hydro-alcoholic extract of dog rose (L1) and yogurt with 2% liposoluble extract of dog rose (L2). It was realized a sensorial and physical-chemical analysis for all those three yogurt assortments and the tests were done during 15 days of storage (day 1, 7, 9, 12 and 15). The raw material was represented by cow milk. Sensorial analysis of yogurt presumed the evaluation of some sensorial characteristics such as: external aspect, consistency, taste, smell and colour. The best results were obtained by yogurt with 0.5% hydro-alcoholic extract of dog rose followed by yogurt with 2% liposoluble extract of dog rose and classic yogurt. Regarding physical-chemical characteristics were analyzed: dry matter content, acidity, pH value, syneresis index, viscosity and microbiological activity. The highest rate of dry matter was founded in yogurt with 2% liposoluble extract of dog rose (9.1%) because yogurt contains a higher quantity of oil due to vegetal extracts. The acidity of yogurt increased in time being almost constant in the first days and in the last day (day 15) reaching the values of 111°T for yogurt with 0.5% hydro-alcoholic extract of dog rose and 109°T for yogurt with 2% liposoluble extract of dog rose. By adding of vegetal extracts the pH value decreased in time reaching in the fifteenth day of experiments the values of 4.38 for yogurt with 0.5% hydro-alcoholic extract of dog rose and 4.4 for yogurt with 2% liposoluble extract of dog rose. The highest values for syneresis index were obtained for yogurt with dog rose extract in comparison with the classic one. The viscosity index is directly proportional with syneresis index, so while syneresis index increased the viscosity decreased. In comparison with the classic yogurt (981.3 mPa*s) the lowest value for viscosity was obtained at yogurt with 0.5% hydro-alcoholic extract of dog rose (768 mPa*s) followed by yogurt with 2% liposoluble extract of dog rose with 896 mPa*s. Microbiological activity had a linear increasing, for classic yogurt, from day 1 to day 12 and after that remained constant. Regarding the yogurt with dog rose extracts could be observed an inhibition of microbiological activity in comparison with control sample.*

***Key words****: analyses, dog rose, extract, quality, yogurt.*

## INTRODUCTION

In the last decades demands of consumers' regarding food production know a considerably change (Avarvarei, 2013). Nowadays consumers are more and more aware that foods have a great and directly contribution to their health (Mollet and Rowland, 2002; Simeanu, 2005). Today foods have not only the role to satisfy hunger or to provide necessary nutrients for humans, but also have the role in prevention of nutrition-related diseases and in improving consumers' physical and mental well-being state (Roberfroid, 2000; Menrad, 2003).

The European Commission Concerted Action of Functional Food Science in Europe (FUFOSE) statute that a food could be considered "*functional*" if it can be proper proved that, besides the nutritional effects, it is favourably influencing one or more "*target*" functions in the organism (Mocanu et al., 2010). Therefore, it improves the general health state of humans and/or can decreases the risks of illnesses (Costin et al., 2007).

Acid diet products are very popular in the world due to their pleasant sensorial characteristics, as well as due to potential for maintaining or improving consumers' health (Avarvarei et al., 2014). Consumption of dairy products in generally and acid dairy products in particular reached a new level in the last years, due to their benefit effects on humans' health.

Obtaining of new products with a superior quality, diversification of the assortment range for acid dairy products supposes utilization of new technologies and ingredients. Yogurt, considered to be one of the oldest acid dairy products was accidentally discovered (due to acidification of milk stored in goat skin or in clay pots), almost 10,000 years ago, on the nowadays territory of Turkey and Iran (Usturoi, 2007; 2012a). Other authors consider that an ancestral version of yogurt probably appeared 9,000 or 8,000 years BC in Mesopotamia and Egypt and subsequently spread in the North-East of Africa, in the Middle East, in Central Asia, and later in Balkan countries, offering a large variety of "fermented milks" (Corrieu and Béal, 2016) During time were observed, studied and further exploited the benefit properties which yogurt have on consumers' longevity and health state, as well as its role in natural detoxification of human organism. Acid dairy products are obtained by milk fermentation with lactic cultures and in some cases with lactic bacteria associated with some yeast species (mixed fermentation). Yogurt is an acid dairy product which is obtained by fermentation of milk inoculated with a mixed culture composed by two species of thermophilic bacteria *Streptococcus salivarius* and respectively *Lactobacillus bulgaricus* (Usturoi, 2012b).

The utilization of medicinal plants aiming to heal different types of human affections, dates back from ancient times when humans who were living in the middle of nature, trying to ensure the existence, had observed that some plants are good to be eaten or used to heal certain diseases and others have a toxic effect. A study made by WHO (World Health Organization) concluded that 75-80% of worlds' population treat themselves by using natural means.

The role of antioxidants on health of humans begins to be researched starting with the second half of $20^{th}$ century when was discovered the so-called free radicals (Yilmaz and Ercisli, 2011). Free radicals are atoms or molecules which are formed in human body as a result of oxidation processes of essential nutritional ingredients. "Oxidative stress" represents the negative effects of free radicals (Zhelyazkova and Taneva, 2016).

Regarding the vegetal extracts from dog rose (*Rosa canina*) its properties are well-known; because chemical composition of it is rich in vitamin C and this one assure a good function at cellular level, having a fundamental role in deployment of metabolic processes, in increasing of resistance of sanguine vessels, being also known for its anti-diarrheic and diuretic properties (Yilmaz and Ercisli, 2011).

*Rosa canina*, is a spontaneous species, presented in Europe, West Asia and North Africa. In Romania is known as dog rose, brier, eglantine, wild rose, etc. Dog rose (*Rosa canina*) was considered, for a long period of time, to belong to the group of harmful plant species for farming and forests. Due to the progresses made by pharmaceutical, agricultural and forestry sciences, dog rose were reconsidered, being a stabilization factor and fighting against soil erosion, it is also a source of fruit vitamins, of flowers for jam and perfume.

Botanists have identified more than 200 varieties, forms and even hybrids of the species. *Rosa canina* is a xeromesophytic pentaploid species which can reach 3-5 m height; its stems and branches are covered with small sharp prickles; could be founded up to 1200 m altitude (Pârvu, 2000; Tiţă, 2003; Petrova et al., 2007; Kiliçgun and Dehen, 2009; Ghiorghiţă et al., 2012).

The nutritive and therapeutic value of dog rose hips is residing in their content in: sugars, vitamin C, $B_1$, $B_2$, K, PP, D and E, organic acids, pectins, carotenoids (β-carotene, lycopene and isomers of rubixanthin), flavonoids, tannins, macro-elements, micro-elements etc. (Pârvu, 2000; Demir and Ozcan, 2001; Tiţă, 2003; Stănescu et al., 2004; Arsenescu-Popa, 2008; Orhan et al., 2009; Ghiorghiţă et al., 2012).

The dog rose seeds contain oil and minerals; fatty acids within the dog rose oil are mainly represented by linoleic, oleic, linolenic, palmitic, stearic and arachidonic acid, (Ozcan, 2002; Selahvarzian, 2018).

Combining the intake of fruit and yogurt could provide probiotics, prebiotics, high-quality protein, important fatty acids, and a mixture of vitamins and minerals which have the potential to exert synergistic effects on health. Yogurt consumption has been associated with reduced weight gain and a lower incidence of diabetes, whereas fruits have

effects on reducing cardiovascular risk of (Fernandez and Marette, 2017).
For above mentioned reasons in the current paper we aimed to study the utilization impact of dog rose (*Rosa canina*) extracts on yogurt quality.

## MATERIALS AND METHODS

Our research was carried out on three types of yogurt: classic yogurt (control - LC), yogurt with 0.5% hydro-alcoholic extract of dog rose (L1) and yogurt with 2% liposoluble extract of dog rose (L2). It was realized a sensorial and physical-chemical analysis for all those three yogurt assortments and the tests were done during 15 days of storage (day 1, 7, 9, 12 and 15). The raw material was represented by cow milk. Sensorial analysis of yogurt presumed the evaluation of some characteristics such as: external aspect, consistency, taste, smell and colour. Regarding physical-chemical characteristics were analyzed: dry matter content, acidity, pH value, syneresis index, viscosity and microbiological activity. Samples were stored into a fridge during our research.
All the analysis, both sensorial and as well the physical-chemical ones were realised in according with the AOAC norms (AOAC, 2016).
Manufacturing of yogurt as well as sensorial and physical chemical analysis were effectuated at Laboratory for Milk and dairy products quality control which also has a micro-production line for milk and dairy products, UASVM Iaşi and analysis regarding dog rose (*Rosa canina*) extracts were mare at Center for Horticultural Research, Faculty of Horticulture, UASVM Iaşi
Technological flow for yogurt with dog rose (*Rosa canina*) extracts has the following stages: standardized milk - homogenization (55-65$^{o}$C, 15-20 MPa) - pasteurization (95$^{o}$C for 3 minutes) - cooling to incubation temperature (45-48$^{o}$C) - addition of starter culture (2-3%) - incubation - cooling (4-8$^{o}$C) - adding of dog rose (*Rosa canina*) extracts (0.5% hydro-alcoholic extract or 2% liposoluble extract) - stirring - packing - cold storage.
*Appreciation of sensorial quality* was realised on the basis of a scoring scale (0 to 5 points) in according with ISO 6658:2005, by a team of 6 judges. This appreciation has the following steps:
1. Preparation and presentation of samples.
2. Examination of sensorial properties in the following order: external aspect, colour, consistency, smell and taste.
3. Completing of appreciation files for products' sensorial quality.
4. Calculus of weighted means score $P_{mp}$ using the formula: $P_{mp} = P_{mnp} \times f_p$, where:
$P_{mnp}$ is the obtained un-weighted mean score (arithmetic mean of results);
$f_p$ is weighted factor (describe the participation of a sensorial feature to product quality).
5. Calculus of weighted total score $P_{tp}$ with the relation: $P_{tp} = \Sigma P_{mp}$.
*Examinations on horticultural extracts* supposed determination of antiradical activity by using DPPH free radicals, determination of phenolic compounds, and determination of tannins content.
Vegetal extracts from dog rose fruits were obtained by extraction. As solvent was used a 50% ethanol solution at 15 hydro-module (means that added solvent content for extraction is equal with content of grated dog rose fruits multiply with 15). Dog rose fruits, in dried state, were grated, for increasing the contact area with ethanol solution, so was facilitated a more rapid extraction. Time for this process is 1 hour and after that extract is filtered.
Determination of antiradical activity by using DPPH$^{*}$ free radicals was realised by using Brand-Williams method (Brand-Williams et al., 1995). Spectrophotometer method with DPPH$^{*}$ free radical (2,2-Diphenyl-1-picrylhydrazyl) is characterized by diminishing of radical absorbance in the presence of antioxidants. DPPH$^{*}$ is a stable radical, resulted by deloca-lisation of an unpaired electron from the whole molecule. Delocalisation of unpaired electron is characterized by apparition of a violet colour, and this one form an absorption stripe, which have a maximum situated at almost 520 nm. Wave length which is mostly used in research is $\lambda$ = 515 nm. To read the indications of spectrophotometer was made a solution, resulted by mix of 3.9 ml DPPH$^{*}$ with a 60 μM concentration, previously diluted in methanol and after that 0.1 ml from filtrated sample is added. Reaction lasts 30 minutes, in dark

conditions. After 30 minute the results are read.
Methods for assessment of *total content of phenolic compounds* and determination of their antioxidant capacity are mostly based on oxidizing–reducing properties, possibility of phenolic compounds functioning as reduction agents and offering hydrogen radical or electron (Huang et al., 2005). A Folin–Ciocalteu (FCM) method (Singleton et al., 1999; Stratil et al., 2007) is commonly used only for assessment of the sum of phenolic compounds in plant extracts.
The method is based on the mix of phosphomolybdic acid ($H_3PMo_{12}O_{40}$) and phosphotungistic acid ($H_3PW_{12}O_{40}$) into an alkaline environment and in the presence of phenolic compounds from extracts is reduced to tungsten ($W_8O_{23}$) and molybdenum ($Mo_{12}O_{23}$), this ones being blue oxides. Absorption length is 750 nm and this one is given by blue coloration. This light coloration/absorption is proportional with the content in total phenolic compounds of vegetal extracts.
*Determination of tannins content* is based on the fact that Folin-Ciocalteu reagent oxidise the totality of phenolic compounds and so appears a blue coloration, its intensity is direct proportional with the content in phenolic compounds (Brand-Williams et al., 1995; Singleton et al., 1999; Huang et al., 2005; Stratil et al., 2007). For this determination was utilised a Hach Lange DR-5000 spectrophotometer. In a 50 ml bowl are added 0.5 ml extract and after that are introduced 25 ml of distillate water. Content is homogenized and after that is added 2.5 ml Folin-Ciocalteu reagent and 10 ml sodium carbonate solution with 20% concentration. Water is added till sign and it is homogenized and left 30 minutes for resting. After that the absorbance is measured at spectrophotometer at a wave length of 750 nm. Calculus formula is:

$$m_t = C_t M_t 10^{-3} 50 V_{ext} 10^3 \text{ where,}$$

$C_t$ is concentration in tannins (mol/l);
$M_t$ is molar mass of tannins (g/mol);
$V_{ext}$ is the utilised volume of extract (ml).
*Determinations realised on yogurt* (with or without addition of dog rose extracts) aimed the following parameters: acidity, pH value, syneresis index, viscosity, microbiological analysis (AOAC, 2016).

## RESULTS AND DISCUSSIONS

Regarding the analysis of antiradical activity using DPPH* free radicals, content of biological-active substances from dog rose fruits extracts are presented in Table 1. To find the biological-active compounds from hydro-alcoholic and liposoluble extracts was measured their optical density at spectrophotometer at a wave length of λ = 200-1200 nm.

Table 1. Content of biological-active substances in dog rose fruits extracts

| Content of phenolic compounds $mg_{gallic\ acid}/g_{extract}$ | Content of tannins $mg/g_{extract}$ | Antiradical activity DPPH* (%) | Index of total polyphenols |
|---|---|---|---|
| 26.98±0.38 | 106.41±1.34 | 85.11±0.02 | 69.08±0.25 |

Could be observed that extracts from *Rosa canina* fruits have a high antiradical activity (85.11±0.02), because those extracts have a high content in phenolic compounds and tannins. After measuring the optical density of extracts at spectrophotometer, respectively at λ = 200-1200 nm (exception being β-carotene and lycopene content which were measured at λ = 448-452 nm) were obtained the following values for physical-chemical indicators of dog rose extracts at 30°C (Table 2).

Table 2. Physical-chemical indicators for dog rose extracts at 30°C

| Nr. | Index | Values of dog rose extract |
|---|---|---|
| 1 | Acidity index, mg KOH/g oil | 0.25±0.04 |
| 2 | Peroxide index, mmol/g oil | 1.94±0.2 |
| 3 | Content of diene, μmol/g oil | 14.93±0.07 |
| 4 | Content of conjugated triene , μmol/g oil | 15.05±0.09 |
| 5 | p-anisidine index, u.c | 4.18±0.03 |
| 6 | β-carotene, mg/100 g dog rose | 13.85 |
| 7 | Lycopene content, mg/100 g dog rose | 15.15 |

From the presented data could be observed that studied dog rose extracts had a mean acidity index of 0.25±0.04 mg KOH/g oil. Regarding peroxide index, this one offers information about oxidation degree of vegetal oils and had a mean value of 1.94±0.2 mmol/g oil. Dog rose extracts were analysed at several temperatures, respectively at 30°C, 45°C and 65°C. The highest value for oxidation degree being recorded at temperature of 65°C, which demonstrate that oxidation process, is more rapidly, with increasing of temperature. Content in diene and triene influence the

oxidation process of vegetal oils, this one varying between 14.93 and 17.82 μmol/g oil for diene and 15.05±0.09 μmol/g oil for tiene. P-anisidine index is represented by aldehydes and ketones and had values between 1.96 and 5.60 u.c. Aldehydes and ketones influence the taste and smell of oild being responsible of their rancidity. Content in carotenes, such as β-carotene and lycopene varied between 13.85 and 15.15 mg/100 g dog rose.

The results of the sensorial evaluation are presented in Table 3, for all those three yogurt assortments: classic yogurt (control - LC), yogurt with 0.5% hydro-alcoholic extract of dog rose (L1) and yogurt with 2% liposoluble extract of dog rose (L2). Tests were done during 15 days of storage (day 1, 7, 9, 12 and 15). Sensorial quality was realised on the basis of a scoring scale (0 to 5 points) in according with ISO 6658:2005, by a team of 6 judges. Samples were stored into a fridge during our research.

Table 3. Sensorial analysis of yogurt

| Nr. | Day 1 (score) | Day 7 (score) | Day 9 (score) | Day 12 (score) | Day 15 (score |
|---|---|---|---|---|---|
| LC | 16.42 | 16.22 | 16.02 | 15.67 | 14.67 |
| L1 | 16.75 | 16.45 | 16.25 | 15.95 | 14.95 |
| L2 | 16.43 | 16.23 | 16.03 | 15.68 | 14.68 |

In according with the data presented in table 3, the analysed product obtained in the first day a score between 16.42 points (LC) and 16.75 points (L1) and in the last day (day 15) the score was between 14.67 points (LC) and 14.95 (L1). The score for the firsts days is good, which means that product have specific positive sensorial feature, well outlined, with small and insignificant defects. In day 15 the score is a satisfactory one being characteristic to a product to a product with weak outlined sensorial characteristics.

Must be mentioned that the highest score and which was given by the majority of judges was obtained by yogurt with 0.5% hydro-alcoholic extract of dog rose (L1), fact which demonstrate the fact that yogurt with natural colorants summed a series of remarkable sensorial properties.

Products which were obtained were subjected to several analyses which aimed to determine the following parameters: acidity, pH value, syneresis index, viscosity, microbiological analysis. Those analyses were effectuated during 15 days, respectively day 1, day 7, day 9, day 12 and day 15. Also for those analysis samples were kept into a fridge.

In table 4 are presented the results obtained for acidity.

Table 4. Yogurt acidity during research period ($^oT$)

| Nr. | Day 1 | Day 7 | Day 9 | Day 12 | Day 15 |
|---|---|---|---|---|---|
| LC | 84 | 88 | 89 | 100 | 111 |
| L1 | 85 | 90 | 90 | 100 | 111 |
| L2 | 84 | 88 | 88 | 98 | 109 |

Studies demonstrate that phenomenon of acidity increasing is due to transformation process of reducing carbohydrates, stage which starts, just after adding of starter cultures and continue, during fermentation process, inclusively in storage stage. Also, acidity evolution, could be correlated with intensity of lactic fermentation, products with dog rose vegetal extracts both hydro-alcoholic as well as liposoluble have a great importance, and also influence the development and viability of probiotics. From the data presented in table 4 could be noticed that together with intensity of fermentation process, acidity of yogurt, increase. In the first days is constant and after that reaching higher values, difference being visible.

Literature (Usturoi, 2007; 2012a; 2012b) specify that yogurt acidity, for all assortments, must not be higher than $140^oT$, and the highest values obtained by us during research period were $111^oT$ in day 15. The highest acidity belongs to yogurt with hydro-alcoholic dog rose extract.

Regarding value of pH (Table 5) in according with literature (Usturoi, 2007; 2012a; 2012b), as in case of acidity, its evolution is correlated with intensity of lactic fermentation. By adding of vegetal extracts the pH value of samples decreased.

Table 5. pH value of yogurt during research period

| Nr. | Day 1 | Day 7 | Day 9 | Day 12 | Day 15 |
|---|---|---|---|---|---|
| LC | 4.54 | 4.52 | 4.46 | 4.46 | 4.41 |
| L1 | 4.49 | 4.46 | 4.44 | 4.41 | 4.38 |
| L2 | 4.51 | 4.48 | 4.46 | 4.45 | 4.40 |

From the data presented in table 5 could be observed the constant decreasing of pH value. The highest value for pH was reached in day 1 for classic yogurt (pH = 4.54).

Syneresis is defined as a process of spontaneous separation of whey on surface of coagulated product, being considered a defect. Regarding the studied products syneresis process wasn't a spontaneous one, but was a forced syneresis process, because samples were subjected to centrifugation for 5 minutes at 1000 rpm. Whey quantity eliminated by forced syneresis varying between 5.45 (day 1 - L2) and 67.18 (day 15 - L1) (Table 6).

Table 6. Syneresis index yogurt during research period

| Nr. | Day 1 | Day 7 | Day 9 | Day 12 | Day 15 |
|---|---|---|---|---|---|
| LC | 8.47 | 36.31 | 62.67 | 64.31 | 65.69 |
| L1 | 7.78 | 60.89 | 64.60 | 66.47 | 67.18 |
| L2 | 5.45 | 59.50 | 64.95 | 64.95 | 66.83 |

Process of spontaneous syneresis could be eliminated or reduced by increasing of dry matter content in milk, till reaching a value of almost 15%. Syneresis could also be influenced by products' composition, content in mineral substances, acidity and last but not least by the added starter culture. In our study the highest values for syneresis index were obtained for yogurt with 0.5% hydro-alcoholic extract of dog rose (L1).

Viscosity of the studied products was determined with a DV-III ULTRA Brookfield viscometer and the results are presented in Table 7.

Table 7. Viscosity of yogurt during research period (mPa*s)

| Nr. | Day 1 | Day 7 | Day 9 | Day 12 | Day 15 |
|---|---|---|---|---|---|
| LC | 2133 | 1195 | 1024 | 981.3 | 981.3 |
| L1 | 1728 | 768 | 750 | 725.3 | 597.3 |
| L2 | 1813 | 1024 | 938.7 | 896 | 768 |

From table 7 could be observed that viscosity is direct proportional with syneresis index. While syneresis index increases, products' viscosity decreases. This fact is due to incapacity of extracts to retain liquids at their surface.

From Table 8 could be observed that took place a rapid increasing of micro-organisms' number.

Table 8. Influence of extracts on microbiological activity

| Nr. | Day 1 | Day 7 | Day 9 | Day 12 | Day 15 |
|---|---|---|---|---|---|
| LC | $6x10^2$ | $70x10^2$ | $150x10^2$ | $275x10^2$ | $275x10^2$ |
| L1 | $4.5x10^2$ | $30x10^2$ | $70x10^2$ | $150x10^2$ | $160x10^2$ |
| L2 | $4x10^2$ | $50x10^2$ | $90x10^2$ | $200x10^2$ | $210x10^2$ |

Control sample have a linear increasing starting from day 1 till day 12 and after that number of micro-organisms remain constant till day 15. Regarding yogurt with dog rose extracts, in according with the data presented in table 8, could be observed an inhibition of increasing of micro-organisms in comparison with control sample (LC).

Analysis of antioxidant capacity shown that dog rose extracts have an antioxidant capacity which reached the value of 85.11±0.02, while for classic yogurt (LC) antiradical capacity is a level of 54.26±6.41.

## CONCLUSIONS

The studied dog rose extracts had a mean acidity index of 0.25±0.04 mg KOH/g oil. Peroxide index had a mean value of 1.94±0.2 mmol/g oil.

Content in diene and triene varied between 14.93 and 17.82 μmol/g oil for diene and 15.05±0.09 μmol/g oil for tiene.

P-anisidine index had values between 1.96 and 5.60 u.c.

Content in carotenes, such as β-carotene and lycopene varied between 13.85 and 15.15 mg/100 g dog rose.

The analysed product obtained in first day a score between 16.42 points (LC) and 16.75 points (L1), in the last day (day 15) the score was between 14.67 points (LC) and 14.95 (L1).

The highest values obtained by us during research period for acidity were $111^oT$ in day 15. The highest acidity belongs to yogurt with hydro-alcoholic dog rose extract.

The highest value for pH was reached in day 1 for classic yogurt (pH = 4.54).

Whey quantity eliminated by forced syneresis varied between 5.45 (day 1 - L2) and 67.18 (day 15 - L1).

The highest values for syneresis index were obtained for yogurt with 0.5% hydro-alcoholic extract of dog rose (L1).

Viscosity is direct proportional with syneresis index, while syneresis index increases products' viscosity decreases.

For yogurt with dog rose extracts, could be observed an inhibition of increasing of micro-organisms in comparison with control sample (LC).

Control sample have a linear increasing of microbiological activity starting from day 1 till day 12 and after that number of micro-orga-

nisms remain constant till day 15. Regarding yogurt with dog rose extracts, it could be observed an inhibition of increasing of micro-organisms in comparison with control sample (LC).

Analysis of antioxidant capacity shown that dog rose extracts have an antioxidant capacity which reached the value of 85.11±0.02, while for classic yogurt (LC) antiradical capacity is at a level of 54.26±6.41.

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# ANTIMICROBIAL EFFICACY OF APICULTURAL PRODUCTS AGAINST SOME PATHOGENIC BACTERIA AND *Candida albicans*

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***Abstract***

*Results are presented showing the antimicrobial effects of bee venom, pollen, propolis and honeys (of thyme, oil-seed rape, acacia and lime) against a selection pathogenic bacteria and Candida albicans. Bee venom was found to show the strongest antimicrobial activity against all species. In the case of Staphylococcus aureus the inhibition zone for colony growth was even greater than the control antibiotic used for comparison (cefuroxin). The strains of bacteria studied showed sensitivity to propolis extract, the greatest growth inhibition being observed for Staphylococcus aureus. Pollen was found to exert a large antimicrobial effect, as evidenced by a large growth inhibition zone, on Pseudomonas aerugnosia. Lime flower honey showed moderate inhibitory effects on the growth of Staphylococcus pyogenes and Staphylococcus aureus and was the most potent of the honeys studied.*

***Key words****: antimicrobial effect, apicultural products, pathogenic bacteria.*

## INTRODUCTION

Apicultural products have been used since ancient times for their therapeutic properties. Depending upon the variety of honey a variety of therapeutic effects have been claimed including antimicrobial, expectorant, anti-inflammatory, diuretic and laxative ones. Antimicrobial activity of honey against bacterial and fungal pathogens has been shown by many researchers (Alzahrani et al., 2012; Ghabanchi et al., 2010; Molan, 1992; Molan, 2007; Zaghloul et al., 2001). The antimicrobial effects of honey are due to the presence of its various chemical components and are affected by the botanical origin of any given honey (Bogdanov, 1997). Yatsunami, 1984, consider that the lower pH of honey is responsible for it antimicrobial activity while (Mundo, 2004) have drawn attention to the high sugar content as a possible antibacterial factor. According to Molan (2007), the antimicrobial activity is due to a combination of the osmotic effect, lowered pH and the presence of inhibitors, phenolic acids and flavonoids.

Pollen may contain as many as 185 nutritive components, including 22 amino acids, 27 mineral salts and a wide range of vitamins, hormones, carbohydrates, lipids, enzymes and coenzymes as well as bactericidal substances. The complex composition of pollen gives it properties which are antifungal, antimicrobial, anti-inflammatory, anti-viral and immunostimulatory (Almaraz-Abarca, 2004; KomosinskaVassev, 2015; Kroyer, 2001). Studies of (Kaćaniová, 2012) have shown antimicrobial effects of pollen against *Clostridium butyricum, Clostridium hystoliticum, Clostridium intestinale, Clostridium perfringens* and *Clostridium ramosum.*

Propolis is appreciated as one of the most valuable natural products due to the following therapeutic effects: antimicrobial, antibiotic, antifungal, anti-inflammatory, analgesic, antioxidant and anti-tumoral. It is a mixture of the different plant resins collected by foraging worker bees and contains essential oils, wax, amino acids, minerals, vitamins and flavonoids. There is a close correlation between the chemical composition of propolis and the area from which it has been collected, the season, and weather conditions (Szweda, 2017). The antimicrobial activity of propolis is attributed to the presence of phenolic and flavonoid compounds. The phenomenon of increased resistance of bacteria to antibiotics provides a

reason for raised current interest in these antimicrobial properties.

Bee venom is an apicultural product secreted from the venom glands of worker bees which has the important property of triggering human defence responses, stimulating antimicrobial functions. It contains a wide range of biologically active peptides, enzymes and amines (Dotimas, 1987) as well as toxins with specific actions (Zolfagharian, 2016) and is an important source of pharmaceutical compounds.

The purpose of our study was to evaluate the antimicrobial activity of honeys, pollen, propolis and honey bee venom against a number of gram positive and gram negative bacteria, as well as against the pathogenic microfungus *Candida albicans.*

## MATERIALS AND METHODS

Research was carried out using materials from the hives of the Animal Science and Biotechnology Faculty of Banat's University of Agricultural Sciences and Veterinary Medicine "King Michael I of Romania" from Timisoara, Romania between the dates of 20.04.2018 and 20.05.2018. Apicultural products studied were: bee venom, propolis (in alcoholic extract 1:5), pollen, and four samples of honey (thyme, oilseed rape, acacia and lime).

Venom collection was carried out using BeeWhisper v5.1 (2016 model); pollen was collected with the aid of pollen collectors mounted at the narrow part of hive entrances and samples of honey came from centrifugal extractors in the Apiculture laboratory.

Freshly inoculated nutrient broth cultures were grown for each of the six bacterial species in the study (*Escherichia coli, Salmonella spp., Bacillus cereus, Pseudomonas aeruginosa, Streptococcus pyogenes, Staphylococcus aureus*), with Sabouraud liquid medium being used for *Candida albicans*. Evaluation of the antimicrobial activity of the sample material was carried out by the disc diffusion method according to the scheme in Table 1 using, as controls, antimicrobial susceptibility discs (CT0127, 30 µg using for the bacteria cefuroxin, oxycilin or gentamycin depending on the species, and CT0073B 100 units Nystatin for cultures of *C. albicans*).

Table 1. Scheme of organisation of the experiment

| Experimental treatment | *Escherichia coli* | *Salmonella* spp. | *Staphylococcus aureus* | *Streptococcus pyogenes* | *Bacillus cereus* | *Pseudomonas aeruginosa* | *Candida albicans* |
|---|---|---|---|---|---|---|---|
| Control | Cefuroxin 30 µg | Cefuroxin 30 µg | Cefuroxin 30 µg | Cefuroxin 30 µg | Oxycilin 30 µg | Gentamycin 30 µg | Nistatin 100 units |
| 1 | Pollen | Pollen | Pollen | Pollen | Pollen | Pollen | Pollen |
| 2 | Bee venom | Bee venom | Bee venom | Bee venom | Bee venom | Bee venom | Bee venom |
| 3 | Tyme honey | Tyme honey | Tyme honey | Tyme honey | Tyme honey | Tyme honey | Tyme honey |
| 4 | Rape honey | Rape honey | Rape honey | Rape honey | Rape honey | Rape honey | Rape honey |
| 5 | Acacia honey | Acacia honey | Acacia honey | Acacia honey | Acacia honey | Acacia honey | Acacia honey |
| 6 | Propolis extract | Propolis extract | Propolis extract | Propolis extract | Propolis extract | Propolis extract | Propolis extract |
| 7 | Lime honey | Lime honey | Lime honey | Lime honey | Lime honey | Lime honey | Lime honey |

All inoculated cultures were maintained in a thermostat at 37˚ C and on the second day Mueller-Hinton agar Petri culture plates of uniform depth (4mm) were poured under aseptic conditions, with similar thickness sterile plates of Sabouraud agar medium being prepared for culture of *C. albicans*. Once set plates, with lids displaced, were allowed to surface dry for 10-15 minutes in a sterile cabinet. Seeding was effected by surface flooding with pipetted liquid culture inoculum using standard aseptic technique, with surplus fluid being drawn off by sterile pipette if necessary after a few minutes. Seeding density was done in such a way as to produce closely-spaced but distinct colonies. Once the seeded gel surfaces had been allowed to dry control discs of antibiotic or, as appropriate, antifungal substance were positioned, followed by equally spaced sterile discs which had been impregnated with the apicultural products used in the study. The resulting diffusion zones have a concentration of the relevant substance in inverse proportion to the position along the

radius of the diffusion zone, that is the distance from the edge of the disc. Results were read by measuring the diameter of the zones of inhibition using a calibrated grid.

## RESULTS AND DISCUSSIONS

*E. coli* showed a higher level of sensitivity to venom and propolis as evidenced by the larger zones of growth inhibition measured. *E. coli* showed less sensitivity to pollen and honey with inhibition zones measuring between 2.4 mm for lime honey and 4.0 mm for pollen (as shown in Figure 1).

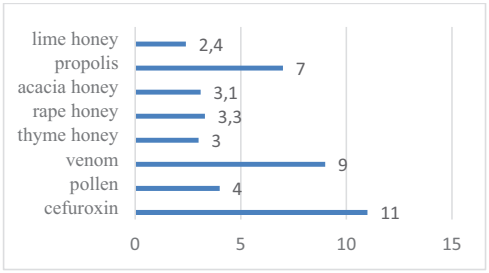


Figure 1. Microbial sensitivity to apicultural products against *Escherichia coli*

For *Salmonella sp.* the largest zone of inhibition was observed for venom (7.0 mm) followed by propolis (5.8 mm) and then acacia honey (5.0 mm)(as shown in Figure 2). For pollen and the three other kinds of honey inhibition zones between 2.0 mm (for pollen and lime honey) and 4.0 mm (for thyme honey) were recorded.

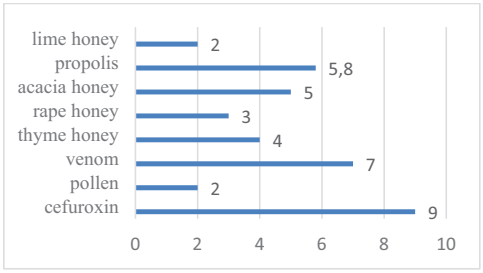


Figure 2. Microbial sensitivity to apicultural products against *Salmonella sp.*

*Staphylococcus aureus* showed marked sensitivity to venom (inhibition zone diameter 25.0 mm) – greater even than to the dose of antibiotic used for comparison (cefuroxin) (as shown in Figure 3). The pollen and the four samples of honey inhibition zone diameters ranged from 2.0 mm (for thyme honey) to 4.0 mm (for lime honey).

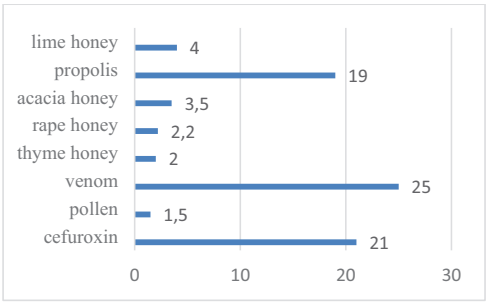


Figure 3. Microbial sensitivity to apicultural products against *Staphylococcus aureus*

For *Streptococcus pyogenes* the greatest inhibition was found for bee venom (16.0 mm) followed by propolis (14.0 mm) (as shown in Figure 4). Sensitivity of this bacterium was observed in the case of honeys of lime (inhibition zone 7.0 mm) thyme (6.0 mm) and rape (5.0 mm). Low antimicrobial activity was found for pollen (1.5 mm inhibition).

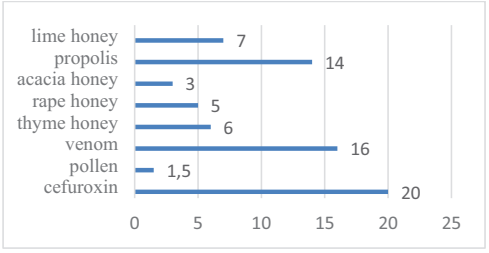


Figure 4. Microbial sensitivity to apicultural products against *Streptococcus pyogenes*

*Bacilus cereus* showed a raised susceptibility to venom and propolis extract but low susceptibility to pollen and the four types of honey (as shown in Figure 5).

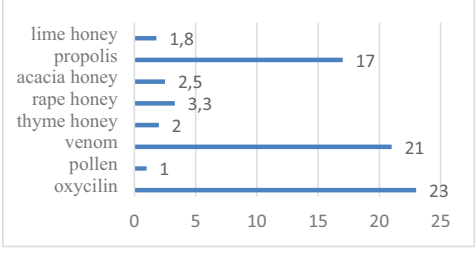


Figure 5. Microbial sensitivity to apicultural products against *Bacillus cereus*

The most pronounced antibacterial effect for *Pseudomonas aeruginosa* was found for venom (18.2 mm) followed by pollen (13.0 mm) and propolis extract (11.5 mm). Tests using honey gave low degrees of inhibition (between 1.0 mm for thyme and 4.0 mm for rape) (as shown in Figure 6).

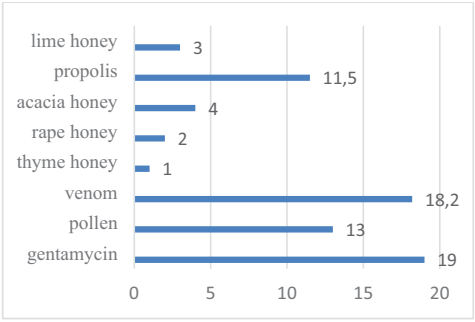


Figure 6. Microbial sensitivity to apicultural products against *Pseudomonas aeruginosa*

*Candida albicans* showed raised sensitivity to venom (inhibition zone 13.5 mm) and propolis (11.0 mm) but was little affected by honey (inhibition zones between 2.0 and 3.5 mm) (as shown in Figure 7).

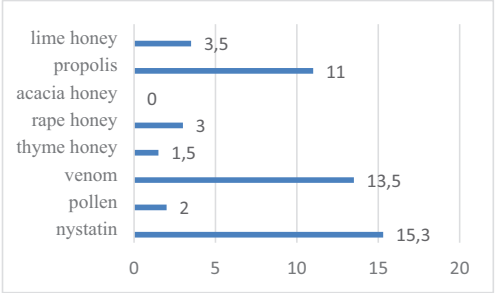


Figure 7. Microbial sensitivity to apicultural products against *Candida albicans*

Similar results reflecting antimicrobial effects of bee venom on *E. coli* have been reported by others (Zolfagharian et al., 2016; Hegazi et al., 2015).Similar results for bee venom have been reported by (Hegazi et al., 2014). Raised sensitivity to propolis extract was also observed (inhibition zone 19.0 mm) confirming the observation of (Kaćaniová et al., 2014), who found large inhibition zone in cultures of *Staphylococcus aureus* when using 70% ethanolic extracts of propolis. Results of experiments carried out by (Fiordalisi et al., 2016; Santana et al., 2012) have shown the efficacy of propolis extract for the treatment of mastitis caused by *Staphylococcus aureus*.

Ani et al. (2018) observed a synergy between alcoholic extract of propolis and the antibiotics vanomycin and oxycilin against *Streptococcus pyogenes*.

Studies of Al-Waili et al. (2012), confirm the effect of propolis extract in inhibiting the growth of *C. albicans* in both pure and mixed cultures.

## CONCLUSIONS

Bee venom showed the greatest inhibitory effect on the growth of the bacteria studied (*Escherichia coli, Salmonella* spp., *Bacillus cereus, Pseudomonas aeruginosa, Streptococcus pyogenes, Staphylococcus aureus)* and of the fungal species *Candida albicans*.

The strength of the antibacterial effect of venom is highlighted by the finding that it had an even stronger inhibitory effect on one species (*Staphylococcus aureus*) than the cefuroxin used as a control comparison.

The strains of bacteria studied all showed sensitivity to propolis extract, as evidenced by the presence of growth inhibition zones. The greatest sensitivity was found for *Staphylococcus aureus*.

With the exception of *Pseudomonas aeruginosa*, where a growth inhibition zone of 13.0 mm was observed, pollen extract was observed to have only a small inhibitory influence on the growth of most bacterial strains studied.

In general low sensitivity of bacteria was found to the different honeys in the study, with the exception of lime flower honey which showed a moderate inhibitory effect on the growth of *Staphylococcus pyogenes* and *Staphylococcus aureus* and was overall more inhibitory than the other honeys tested.

## ACKNOLEDGEMENTS

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# QUALITY ASSESSMENT OF THE COW MILK TRADED ON THE IASI MARKET

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***Abstract***

*This paper presents the data of a cross-sectional study on the commercial quality of fresh cow`s milk marketed in Iasi. The biological material came from three bovine farms, distributed in the chilled state through milk dispensers. From each source, two litres of milk, in sterile containers, were purchased for five consecutive days, from which the laboratory samples were homogenized and maintained until the analysis, as indicated by the manufacturers. Ten samples were dosed from each farm and subjected to physiochemical analysis by means of isometric, gravimetric, titrimetric, potentiometric and ultrasonometric methods. Regarding the freshness indicator, acidity, the highest value was obtained for the milk of the F3 group where the average was 18.1 ± 0.21°T, while for the milk of the group F1 and F2 the mean values were 16.28 ± 0.20°T and 17.24 ± 0.31°T respectively. Regarding the chemical composition, determinations were made for the determination of SUNG, GB, PB and lactose. In terms of fat content, the mean values were 4.05 ± 0.01% for F1, 3.27 ± 0.01% for F2 and 4.03 ± 0.01% for milk from F3. The milk was not suspected of being falsified because no added water was detected in the analyzed samples and the density was within the normality range of at least 1.029 g/cm$^3$. The results of the researches carried out indicated that the marketed milk is in compliance with the quality standard in force, even if there were significant differences between the qualitative parameters analyzed.*

***Key words****: milk, quality, ultrasonometric.*

## INTRODUCTION

In the current paper, we aimed to realise a qualitative analysis of milk raw material came from three bovine farms, distributed in the use of milk and milk products as human food has got a very long history. Human rational nutrition couldn't be conceived without milk and dairy products due to its exceptional nutritive value and accessibility (Bartowska et. al., 2006). As first class complete food milk could be fully considered a strategic food, contributing to the improvement of life quality and at assuring of food safety by covering the numerous nutritive demands of humans (Rațu et al., 2017). Globally, consumers pay great attention to food and its composition due to a pivotal relationship between diet and human health (Rafiq et. al., 2016).

The milk, as it is meant to be the first and sole food for offspring of mammals, is an almost complete food.

The quality of milk products is reliant on milk composition that varies with stage of lactation, milking methods, environment, season, diet, feeding system, breed and species (Kittivachra et al., 2007). However, the composition of milk fluctuates markedly among different species (Pavic et al., 2002; Ahmad et al., 2008).

Also, these proteins are ranked as quality proteins with the highest biological value, good digestibility (97% to 98%), rapid absorption and utilization in the body (Schaafsma, 2000). One of the most important protein is caseins (Bos et al., 2000).

Quantity and quality of proteins in milk influence the yield, technological and health-beneficial properties of milk. The value of milk proteins is more than twice that of milk fat. The amount of whey proteins produced by cows depends strongly on many factors, including cows' diet, health, stage of lactation, breed and time of year (Kuczynska et al., 2011).

The amino acids profile of caseins and whey proteins occupy a unique position in human nutrition. It contains in a balanced form all the necessary and digestible elements for building and maintaining the human and animal body. In

addition, it contains immunoglobulins which protect the newly born against a number of diseases (Kittivachra et al., 2007).
Milk is the best diet for human health because it contains a good source of essential minerals such as calcium and phosphorous (Rațu et al., 2018).
Due to the nutritional importance milk is consumed at large scale in recent time. Milk is also considered a raw material formed by animals.
A good understanding of the properties of milk minerals is important for fundamental research but also for the development of dairy products in which this fraction appears to be complex, dynamic, and in strong interaction with the chilled state through milk dispensers.

## MATERIALS AND METHODS

### Collection of milk samples

The biological material came from three bovine farms, distributed in the chilled state through milk dispensers. From each source, two litres of milk, in sterile containers, were purchased for five consecutive days, from which the laboratory samples were homogenized and maintained until the analysis, as indicated by the manufacturers. These samples were labelled, ice packed and transported to the laboratory. All milk samples were then placed in the refrigerator at 4°C for further analysis.

### Physicochemical analysis

Determination of fat content was realised using acid-butyrometric method (dissolution of protein substance from milk in the presence of sulphuric acid and far separation by centrifugation, using heat and isoamyl alcohol) (ISO 488/2009).
**Total dry matter** (T**DM**) was determined by oven drying method (Simeanu et al., 2018; Nacu et al., 2018).
**Water content** was established by difference using the formula:
**Water (%) = 100 – DM(%)** (ISO 488/2009)**.**
**Non-fat dry matter** (NFDM) was determined by using the relation:
**NFDM (%) = TDM – G**where TDM = total dry matter and G = fat content of milk (Mierliță et al., 2018).
**Lactose (%)** contents were determined according to standard protocol of SR ISO 5548:2008.
**Acidity** was determined by using Thörner method - – neutralizing of organic acids with NaOH (0.1N) titration, using phenolphthalein as witness pigment (SR ISO 11869; 2000; SR ISO 6091),.
**Milk density** was determined with a thermo-lacto-densimeter, this physical parameter representing the rate between milk mass at +20°C and mass of the same water volume at a temperature of +4°C (STAS 2418:2008).
The **ash** content was estimated by incineration of samples in muffle furnace at 550°C for 6 hours, as given in AOAC, No. 945.46 (2005).

### Nitrogenous fractions

The crude protein (CP), true protein (TP), casein, noncasein-nitrogen (NCN), whey proteins and non-protein nitrogen (NPN) contents were determined by using Kjeldahl method according to standard protocol of IDF (1993).
Protein (nitrogen) fractions were calculated as:
TP = CP–NPN,
Casein (N %) = Total protein (N%)–NCN (N %)
Whey protein = NCN–NPN.

### Statistical analysis

Collected data were subjected to statistical computation, using the ANOVA one-way algorithm included in MsExcel, to calculate the descriptive statistics (mean, standard error) and find out whether there were significant differences and upgraded with PostHoc Daniel's XL Toolbox version 4.01 (http://xltoolbox.sf.net), to identify the differences (Radu-Rusu et al., 2014).

## RESULTS AND DISCUSSIONS

The first quality parameters analyzed for the milk from the three dispensers in Iași consisted of determining the fat content, density and acidity.
For the fat content, the average of the milk collected from the F1 dose was 4.05 ± 0.01%, 3.27 ± 0.01% for the F2 picker and 4.03 ± 0.01% for the milk at the F3 metering unit.
Calculation of differences between batches revealed that there was a very significant difference between F1 vs. F2 (P-value = 1.1556), the same difference being noted between F1 vs. F2. F3 (P-value = 2.5888). Comparison of F1 vs. F3 revealed insignificant differences (P-value = 0.2252).

For density, we obtained a mean value of 1.0300±0.0003 g/cm$^3$ for milk collected on F1, 1.0290±0.0002 g/cm$^3$ for milk collected on F2 and 1.0296±0.0002 g/cm$^3$ for milk collected on F3. Statistically there were no differences in statistical significance between the three groups analyzed (P> 0.05) (Table 1).

To highlight the milk freshness state, acidity was determined by the tittering method. The mean obtained value was 16.28±0.31°T for milk collected on the F1 milk dispensers, 17.24±0.29°T for milk collected on the F2 milk dispensers and 18.10±0.05°T for milk collected on the F3 milk dispensers.

In terms of the statistical analysis of the data, there were no significant differences between F1 vs. F2 (P value = 0.0550), very significant between F1 vs. F3 (P value = 0.0004) and significant between F2 vs. F3 (P value = 0.0202) (Table 1).

Table 1. Physical-chemical parameters for milk, distributed in the chilled state through milk dispensers

| Qualiy parameters | F1 | F2 | F3 | ANOVA computation and analysis | | |
|---|---|---|---|---|---|---|
| | | | | Compared period | P value | Significance |
| Fat content (%) | 4.05±0.01 | 3.27±0.01 | 4.03±0.01 | F1 vs.F2 | 1.1566 | ***(P<0.001) |
| | | | | F1 vs. F3 | 0.2252 | ns (P>0.05) |
| | | | | F2 vs. F3 | 2.5888 | ***(P<0.001) |
| Density (g/cm$^3$) | 1.0300±0.0003 | 1.0290±0.0003 | 1.0296±0.0002 | F1 vs.F2 | 0.0557 | ns (P >0.05) |
| | | | | F1 vs. F3 | 0.3465 | ns (P >0.05) |
| | | | | F2 vs. F3 | 0.1720 | ns (P >0.05) |
| Acidity (˚T) | 16.28±0.31 | 17.24±0.29 | 18.10±0.05 | F1 vs.F2 | 0.0550 | ns (P >0.05) |
| | | | | F1 vs. F3 | 0.0004 | ***(P<0.001) |
| | | | | F2 vs. F3 | 0.0202 | * ( P <0.05) |
| NFDM (%) | 8.76±0.10 | 9.06±0.11 | 8.75±0.11 | F1 vs.F2 | 0.0842 | ns (P >0.05) |
| | | | | F1 vs. F3 | 0.9642 | ns (P >0.05) |
| | | | | F2 vs. F3 | 0.0894 | ns (P >0.05) |
| DM (%) | 12.82±0.11 | 12.34±0.12 | 12.78±0.12 | F1 vs.F2 | 0.0178 | * ( P <0.05) |
| | | | | F1 vs. F3 | 0.8305 | ns (P >0.05) |
| | | | | F2 vs. F3 | 0.0287 | * ( P <0.05) |
| Water (%) | 87.18±0.12 | 87.66±0.12 | 87.22±0.12 | F1 vs.F2 | 0.0178 | * ( P <0.05) |
| | | | | F1 vs. F3 | 0.8305 | ns (P >0.05) |
| | | | | F2 vs. F3 | 0.0287 | * ( P <0.05) |
| Lactose (%) | 4.80±0.01 | 4.25±0,02 | 4.75±0,02 | F1 vs.F2 | 1.7816 | ***(P<0.001) |
| | | | | F1 vs. F3 | 0.1171 | ns (P>0.05) |
| | | | | F2 vs. F3 | 1.6729 | ***(P<0.001) |

ANOVA within rows, between groups for different superscripts, one by one comparison: ns: not significant; significant = * (P <0.05); distinguished significant = ** (P <0.01); highly significant = *** (P <0.001).

Also, in order to determine the milk quality parameters, NFDM was determined, a parameter for which the mean values calculated by us were 8.76 ± 0.10% for milk collected from F1, 9.06 ± 0.11 for the collection from F2 and 8.75 ± 0.11% for that collected from F3. Statistically, no differences in statistical significance were reported for this indicator (Table 1).

Milk of dairy cows is a biological solution containing approximately 12.8% of dry matter. Milk dry matter consists of proteins, carbohydrates, fats, minerals and vitamins (Coballero et al., 2003; Roginski et al., 2003).

As for the DM content, the highest value was found in milk from F1, the average being 12.82 ± 0.11%, followed by the milk collected from F3 (12.78 ± 0.12%) and then the F2 collected, where the value mean was 12.34 ± 0.12%.

On the comparison of data, for this parameter it were found significant differences between F1 vs. F2 and F2 vs. F3; between the F1 vs. F3 reported differences were insignificant. The same differences were also highlighted in the case of the milk content of the milk analyzed by us (Table 1).

For lactose content, the mean calculated by us was 4.80±0.01% for milk collected on the F1 milk dispensers, 4.25±0.02% for milk collected on the F2 milk dispensers and 4.75±0.02% for milk collected on the F3 milk dispensers.

Regarding the differences between the three analyzed lots, these were very significant between F1 vs. F2 and F2 vs. F3 (P <0.001) and insignificant between F1. vs. F3 (P> 0.05).
Protein is an important constituent of milk which contains about 95% of the total nitrogen present. In the current exploration, protein fractions like CP, TP, caseins and whey proteins, NCN and NPN contents showed significant differences (p<0.05) between the milk collected. The CP (3.398%±0.02%), TP (3.084%±0.03%), caseins (2.656%±0.02%) and NPN (0.314%±0.002%) contents were relatively higher in milk collected from the F1.
Concerning the comparative analysis of CP data, the differences between the F1 vs. F2 and F2 vs. F3 (P <0.05) and insignificant among the F1 vs. F3.

Regarding the TP content (representing the difference between CP and NPN) the mean values obtained were 2.990 ± 0.02% for F2 and 3.076 ± 0.03% for F3. The ANOVA test revealed significant differences (P <0.05) between F1 vs. F2 and insignificant (P> 0.05) between F1 vs. F3 and F2 vs. F3.
For the casein content, the lowest level was found in the milk collected from the F2 doser, ie 2.574 ± 0.02%, followed by the milk collected from the F1 doser (2.656 ± 0.02%) and then the milk collected from F3 (2.664 ± 0.03%). Following the ANOVA test, significant differences (P <0.05) between F1 vs. F2 and insignificant (P> 0.05) between F1 vs. F3 and F2 vs. F3 (Table 2).

Table 2. Milk protein fractions of different milk, distributed in the chilled state through milk dispensers

| Qualiy parameters | F1 | F2 | F3 | ANOVA computation and analysis | | |
|---|---|---|---|---|---|---|
| | | | | Compared period | P value | Significance |
| Crude protein-CP (%) | 3.398±0.02 | 3.286±0.02 | 3.382±0.03 | F1 vs.F2 | 0.0195 | * (P <0.05) |
| | | | | F1 vs. F3 | 0.7208 | ns (P >0.05) |
| | | | | F2 vs. F3 | 0.0431 | * ( P <0.05) |
| True protein-TP (%) | 3.084±0.03 | 2.990±0.02 | 3.076±0.03 | F1 vs.F2 | 0.0422 | * ( P <0.05) |
| | | | | F1 vs. F3 | 0.8615 | ns (P >0.05) |
| | | | | F2 vs. F3 | 0.0635 | ns (P >0.05) |
| Casein (%) | 2.656±0.02 | 2.574±0.02 | 2.664±0.03 | F1 vs.F2 | 0.0498 | * ( P <0.05) |
| | | | | F1 vs. F3 | 0.8519 | ns (P >0.05) |
| | | | | F2 vs. F3 | 0.0517 | ns (P >0.05) |
| Whey protein-WP (%) | 0.428±0.004 | 0.416±0.006 | 0.412±0.006 | F1 vs.F2 | 0.1894 | ns (P >0.05) |
| | | | | F1 vs. F3 | 0.0497 | * ( P <0.05) |
| | | | | F2 vs. F3 | 0.6453 | ns (P >0.05) |
| Non-casein nitrogen-NCN (%) | 0.742±0.003 | 0.712±0.003 | 0.718±0.005 | F1 vs.F2 | 0.0004 | ***(P<0.001) |
| | | | | F1 vs. F3 | 0.0019 | ** (P <0.01) |
| | | | | F2 vs. F3 | 0.2896 | ns (P >0.05) |
| Non-protein nitrogen-NPN (%) | 0.314±0.002 | 0.296±0.004 | 0.306±0.002 | F1 vs.F2 | 0.0049 | ** (P <0.01) |
| | | | | F1 vs. F3 | 0.0497 | * ( P <0.05) |
| | | | | F2 vs. F3 | 0.0655 | ns (P >0.05) |

ANOVA within rows, between groups for different superscripts, one by one comparison: ns: not significant; significant = * (P <0.05); distinguished significant = ** (P <0.01); highly significant = *** (P <0.001).

Several findings concerning the protein content of cow milk proteins (Ozrenk et al., 2008; Shamsia, 2009) have shown harmony with present research.
Similarly, the TP contents of cow milk, are in line with the investigations of Pirsi et al. (2000). The findings of previous studies are comparable with the results of current exploration concerning the casein contents of cow milk (Imran et al., 2008.).

It is also known that proteins are an important factor affecting the quality of dairy products as the reduction in proteins and casein (α- and β-casein) contents results in poor cheese making properties (Bernabucci et al., 2002). The findings of Borkova and Snasolva (2005) have shown that cow milk contains 0.47%±0.01% whey proteins.
Regarding the WP values obtained by us, mean values were 0.428 ± 0.004% for milk collected

from F1, 0.416 ± 0.006% for that collected from F2 and 0.412 ± 0.006% for F3 collected.
The results of the ANOVA test on the WP milk content analyzed by us showed insignificant differences (P> 0.05) between F1 vs. F2 and F2 vs. F3 and significant differences (P <0.05) between F1 vs. F3.
Concerning the NCN content (%), the average values obtained by us oscillated between 0.712 ± 0.003% as obtained for the milk collected from F2 and 0.742 ± 0.003% as obtained from the milk collected from F1. The ANOVA test revealed very significant differences (P <0.001) between F1 vs. F2, distinctly significant (P <0.01) between F1 vs. F3 and insignificant between F2 vs. F3.
A final parameter analyzed was NPN (%) where the averages obtained were 0.314 ± 0.002% for milk collected from F1, 0.296 ± 0.004% for F2 and 0.306 ± 0.002% for that collected from the F3 doser 2).
The NPN obtained within the study is higher than one in American researches (Raden and Powell, 2009) – 0.19%. While NPN content found in research conducted in the Netherlands was lower (Heck et al., 2009) than in this study (0.182%).
Researches performed prior in other countries affirm changes in non-protein nitrogen content depending on holding, breed, lactation, day in lactation and season (Ng-Kwai-Hang et al., 1985); therefore, the author of this paper has evaluated results of this research considering all the factors above.

## CONCLUSIONS

The breed from which it originates and the type of diet administered to the animals may influence the quality of the milk. Consequently, following the determinations made by us, significant strength differences were noted in the case of fat content. Significant differences were also noted for the lactose content where the mean values were 4.80% for milk collected from F1, 4.25% for the F2 collected and 4.75% for the collection from F3.
For protein content, the differences noted were significant between F1 vs. F2 and F2 vs. F3.
The data indicated in this studio indicate that milk distributed in the city of Iasi through tonometers is of superior quality.
Also, the present investigation would be useful for the dairy processing industries to formulate nutritionally enhanced milk based functional products for the vulnerable segment of the population.

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# *Nigella sativa* SEED OIL ANTIMICROBIAL ACTIVITY AGAINST *Staphylococcus* spp. IN A FOOD MATRIX

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***Abstract***

*The antimicrobial effects of Nigella sativa have been studied in vitro and in vivo, against various microorganisms such as Enterobacteriaceae, Staphylococcus, Streptococcus, Salmonella etc. Recent studies proved antimicrobial effects of Nigella sativa seed oil (NSSO) against various contaminant bacteria in cheese. Due to promising results concerning NSSO effect against bacteria in brined cheese, this study tested NSSO effect against naturally occurring Staphylococcus spp. which contaminates kneaded, sheep´s milk Romanian cheese. Three batches of traditionally manufactured raw milk kneaded cheese were considered: control cheese without NSSO and cheese samples enriched with 0.1 and 0.2 w/w NSSO. Staphylococcus spp. enumeration revealed a descending trend in CFU/g throughout the ripening period, for all batches of cheese. The counts were lower for the 0.1% w/w NSSO cheeses than for the control batch, but no statistical significance could be attributed to this difference (p-value - 0.57). However, for the 0.2% w/w NSSO batch of cheeses, Staphylococcus count registered noticeable decrease, and the results were statistically significant (p-value - 0.048), and no colonies were obtained by the end of the ripening period.*

***Key words:*** *kneaded cheese, Nigella sativa seed oil, ripening, Staphylococcus spp.*

## INTRODUCTION

Raw milk traditional cheeses have been often associated with food borne infections or intoxications (Öner, 2006; Little, 2008; Choi et al., 2016; Prates, 2017; Bintsis, 2002; Gao, 2017). If most hard or semi-hard raw milk cheeses are usually ripened long enough to be safe for consumption, traditional soft cheeses continue to be a real hazard for public safety, as they provide appropriate environment for pathogens survival and development (Eck et al., 2000; Fox et al., 2000; Tăpăloagă, 2017; Ilie (a,b), 2017). A continuous search of antimicrobial solutions, adequate as natural additives in foods in general and in cheeses, in particular, has increasingly been reported by recent studies (Fadavi, 2015; Amatiste, 2014; El-Dahma, 2017; Wahba, 2010; Gouvea Fabiola dos Santos, 2017; Darwish, 2017).

Black cumin (*Nigella sativa*) seeds have been extensively used as spices in a wide range of foods and beverages especially in Middle and Far East countries, being appreciated for a wide range of pharmacological actions, such as anti-diabetic, anti-cancerous, immunity modulator, analgesic, antimicrobial, anti-inflammatory, spasmolytic, bronchodilator, antioxidant etc. (Ahmad, 2013; Gholamnezhad, 2016). Most of *Nigella sativa* therapeutic properties have been attributed to thymoquinone, considered the most significant bioactive component of the essential oil (Ijaz, 2017). *Nigella sativa* is commonly added to food, as seeds or essential oil (Hassanien, 2015; Ramadan, 2016; Abedi, 2017), for various benefficial effects.

Recent literature provides studies of *Nigella sativa* seeds or cold pressed oil effects on the overall quality of cheeses (Hassanien, 2014; Hassanien, 2015; Mahgoub, 2013; Cakir, 2016).

The antimicrobial effects of *Nigella sativa* have extensively been studied *in vitro* (Muhammet, 2005; Utami, 2016; Forouzanfar, 2014; Bakal, 2017) and *in vivo* (Rafati, 2014) against various microorganisms such as *Enterobacteriaceae, Staphylococcus, Streptococcus, Salmonella, Helicobacter, Listeria, Pseudomonas, Klebsiella, Proteus* etc.

In this context, the paper attempted an assessment of *Nigella sativa* seed oil (NSSO) antimicrobial activity, against *Staphylococcus spp.* contaminating traditionally manufactured raw milk kneaded cheese.

## MATERIALS AND METHODS

The experiment included three batches of cheese: control cheese without NSSO, 0.1% w/w NSSO enriched cheeses and 0.2% w/w NSSO enriched cheeses. Commercially available NSSO was purchased from Aghoras Invent SRL company, of Bucharest.

The NSSO was added to the mildly heated milk in the respective concentrations, before renneting. The content of the Ideal® rennet dose (8 g) was diluted in 250 mL warm distilled water and 25 mL solution were added to 10 l warm milk (30-35°C), under continuous manual mixing for 10 minutes.

Coagulation time was 30-45 minutes. The soft curd was left for further solidification needed for processing, for 15-30 minutes. Further stages of the technological process were followed according to the usual, traditional Burduf cheese manufacturing and included pressing, wriggling, bursting, resting, ripening in fir woodandfilling (fig.1) (Tăpăloagă, 2013; Tăpăloagă, 2018).

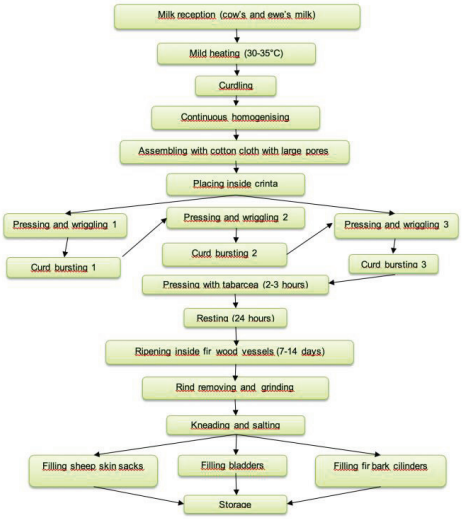


Figure 1. Soft kneaded raw milk cheese manufacture diagram

For this experiment, fir bark cylinders were filled with cheese and kept at dark, cold environment (8-10°C), according to traditional custom, throughout the experiment (for 42 days).

Experimental kneaded cheese samples were analyzed in duplicates for *Staphylococcus* spp. count, at 0, 14, 21, 28, 35 and 42 days of ripening. *Staphylococcus* spp. enumeration was performed using 3M™ Petrifilm™ Staph Express Count System (St. Paul, Minnesota, USA) and Petrifilm Staph Express disk (AOACOfficial Method of Analysis 2003.08, for dairy foods) (www.eoma.aoac.org).

Data analysis was performed by One way analysis of variance (ANOVA) using SAS(ANOVA version 9.1. SAS institute Inc., Cary, USA, 2003) (53). The threshold of significance level was $p<0.05$. The repetitions (duplicates) of determinations were not considered in statistical significance calculations.

## RESULTS AND DISCUSSIONS

*Staphylococcus* spp. enumeration revealed lower counts for the 0.1% w/w NSSO cheeses than for the control batch, but no statistical significance could be attributed to this difference (p-value - 0.57) (Table 1). However, for the 0.2% w/w NSSO batch of cheeses, *Staphylococcus* count registered noticeable decrease, with strong statistical significance (p-value - 0.04), no colonies being obtained by the end of the ripening period (Table 2).

The antimicrobial effect of *Nigella sativa* oil and extracts on *Staphylococcus* spp. is extensively cited throughout literature, both in vitro (Uzair, 2017; Emeka, 2015; Forouzanfar, 2014) and in vivo experiments (Hannan, 2008; Rafati, 2014; Bakathir, 2011). *Staphylococcus* spp. is a commonly found contaminant of raw milk cheeses, especially those processed through traditional methods, as revealed in recent studies (Taban, 2017; Kav, 2011) As staphylococci can grow at high sodium chloride concentrations, brined cheeses are commonly associated with *Staphylococcus* contamination from milking and processing environment and personnel, staphylococcal toxins being a frequent cause of food borne intoxications (Bianchi, 2014). Therefore it was expected to find natural staphylococcal contamination of raw milk kneaded cheese (Ilie, 2018), but the revealed counts were not high enough for enterotoxin production (Fig. 2). For all cheese samples, *Staphylococcus* counts dropped starting with the 7th day of ripening, which may also be correlated with the dropping trend

of pH values in all cheeses, as staphylococcal growth is limited at pH values below 5.8-6 (Delbes, 2006).

NSSO was significantly associated with lower staphylococcal counts than the ones noticed for control samples, throughout ripening: the *f*-ratio value was 0.32 for 0.1% w/w NSSO batch and 4.84 for 0.2% w/w NSSO cheese batch, compared with control, while the *p*-value was 0.57 for 0.1% w/w NSSO batch and 0.04 for 0.2% w/w NSSO cheese batch, compared with control (Fig. 2).

These findings are in agreement with other studies which report significant reducing effect against the proliferation of *S. aureus* by addition of NSSO (Hassanien, 2014). Other similar studies reveal significant antibacterial activity against *S. aureus* only at doses of 0.2% NSSO and not at lower levels.

Table 1. Data analysis for 0.1% w/w NSSO cheeses compared to control

| k | Treatments | | |
|---|---|---|---|
| | Control | 0.1% w/w NSSO | |
| N | 7 | 7 | |
| ∑X | 15.29 | 13.74 | |
| Mean | 2.18 | 1.96 | |
| ∑X2 | 35.68 | 31.01 | |
| St.dev. | 0.61 | 0.82 | |
| | Result details | | |
| | SS | Df | MS |
| Between treatments | 0.176 | 1 | 0.1716 |
| Within treatments | 6.3385 | 12 | 0.5282 |
| Total | 6.5101 | 13 | |
| f-ratio value | 0.32489 | | |
| p-value | 0,579202 | | |

Another opinion phrased by similar research states that both 0.1% and 0.2% NSSO supplementation induce significantly reduced counts in *S. aureus* and *E. coli,* but 0.2% concentration showed the most intense effect (Mahgoub, 2013).

Most authors consider a decrease of 1.3-1.5 log $CFUg^{-1}$, by the 21$^{st}$ day of ripening, as being significant (Hassanien, 2014).

This study analyzed the degree of significance in terms of difference in count dynamics between treatment groups and control, throughout ripening, as this comparison was considered useful for assessing the impact of NSSO on natural contaminating microflora in regular ripening conditions and not the antimicrobial capacity of *Nigella sativa* seed oil on its own.

Table 2. Data analysis for 0.2% w/w NSSO cheeses compared to control

| Data analysis item | Treatments | | |
|---|---|---|---|
| | Control | 0.1% w/w NSSO | |
| N | 7 | 7 | |
| ∑X | 15.29 | 8.48 | |
| Mean | 2.18 | 1.21 | |
| ∑X2 | 35.68 | 16.18 | |
| St.dev. | 0.61 | 0.993 | |
| | Result details | | |
| | SS | Df | MS |
| Between treatments | 3.3126 | 1 | 3.312 |
| Within treatments | 8.2079 | 12 | 0.684 |
| Total | 11.5204 | 13 | |
| f-ratio value | 4.84304 | | |
| p-value | 0.048077 | | |

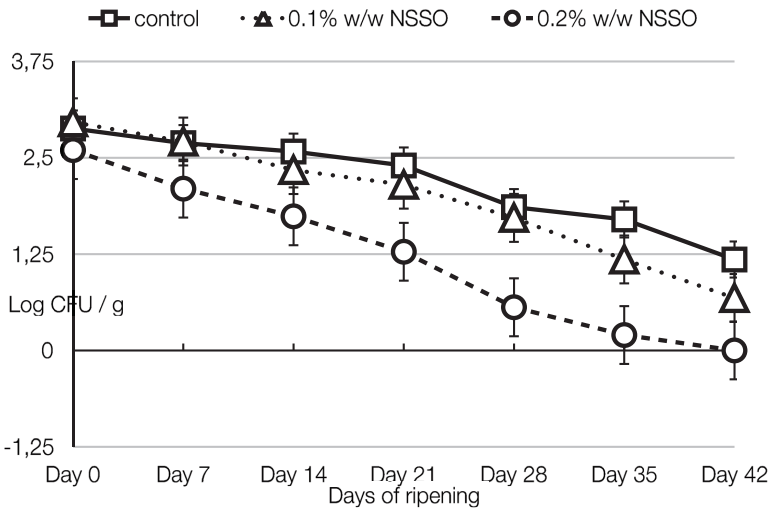


Figure 2. *Staphylococcus* spp. population fluctuation in NSSO enriched cheeses, compared to control batch, over ripening

## CONCLUSIONS

*Staphylococcus* spp. enumeration revealed a descending trend in CFU/g throughout the ripening period, for all batches of cheese. The counts were lower for the 0.1% w/w NSSO cheeses than for the control batch, but no statistical significance could be attributed to this difference (p-value - 0.57).

However, for the 0.2% w/w NSSO batch of cheeses, *Staphylococcus* count registered noticeable decrease, and the results were statistically significant (p-value - 0.048), and no colonies were obtained by the end of the ripening period. Thus, NSSO could be a good option as additional measure for the hygiene control of traditionally manufactured raw milk cheeses.

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# THE RELATIONSHIP BETWEEN FOOD SAFETY, FOOD QUALITY AND CUSTOMER SATISFACTION

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***Abstract***

*The nutrition of all people is oriented, generally, on a basic food, which provides the daily needed nutrients. By their main components, meat products contribute to the growth of human body cells, to recover the damaged tissues, to maintain health and work capacity. For the satisfaction of the growing and diversified requirements needed for a modern diet, today there is a wide variety of food assortments. The purpose of this market study was to identify consumer preferences in terms of meat products consumption. The study includes consumer research, based on a questionnaire, which was completed by the direct interview method. The target group of respondents included people from urban and rural areas, young people, adults and retirees, respecting a certain equality of gender. Starting from these objectives, a number of working hypotheses have been established to see whether they are verified or not. The results show the existence of some discrepancies between the perception and interest for quality and safety food. The consumers are concerned about food safety, but this is not a priority in the process of purchasing food, the sensorial characteristics and the cost price underlying the acquisition process.*

***Key words:*** *customer satisfaction, food quality, food safety, meat products*

## INTRODUCTION

Over the centuries, humans have selected and bred livestock species, creating a range of breeds with special traits, adapted to specific environments, for the conversion of particular types of vegetation and feed into locally-distinct foods, or for the production of specific products (Tăpăloagă, 2016; Tăpăloagă et al., 2016). Valuable animals, intensively bred to supply uniform products under controlled management conditions – exist alongside multipurpose breeds kept by small-scale farmers and herders, mainly in low external input production systems (Tăpăloagă, 2014; Tăpăloagă et al., 2008). The body homeostasis depends on the character of nutrition, influencing the human system functions, through enzymatic and hormonal factors (Ilie, 2007).

Through this research, the analysis of the preferences and consumption habits of interviewed people was pursued, in order to determine the meat products consumption choice and finding out the desires and possible discontents about certain types of meat products, existing on the market. Food safety concept used today includes the whole food chain intended for consumption by animals or humans (Ilie, 2013).

The aim of the study was to achieve the following objectives: determining the profile of meat products consumer; identifying the type of meat product frequently consumed; identifying the place of purchase; determining the motivations underlying the consumption of meat products; identifying the importance of the presentation of meat products; identifying the consumer's level of information, taking into account the content of the label and the main quality characteristics; determining the level of meat products consumption; identifying the main quality features which drive consumers to purchase a certain product.

## MATERIALS AND METHODS

The questionnaire was realized in accordance with the research objectives and with the information which needs to be collected. In its achievement, the purpose of the research was taken into account and also of the main objectives pursued. The questions provide the possibility to choose the answer that best suits the consumer's claims, but at some questions the respondent was left to express his / her

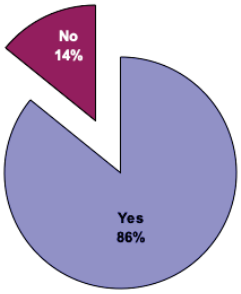
opinion, when his variant was not among those presented.
The questionnaire contained questions about the choice of meat products preferred by the consumers, the way of products presentation, questions about the frequency of meat products consumption and the preferred place of purchase. There are also questions about the consumer's interest on the information content of the label, important issues in the purchase decision.
The market survey was conducted on a sample of 114 people. Their answers to the questionnaire questions were recorded, together with the spot observations and possible reflections that have emerged later.
The target group of respondents was made up of people residing in both urban and rural areas, friends and gentiles, but also among unknown people, young people, adults and retirees, respecting a certain equality of gender, considering the fact the meat products are consumed by a large population category.

## RESULTS AND DISCUSSIONS

To assess the qualities consumers most value in meat products, survey respondents were presented with a list of questions.

1. Are you consuming meat products?

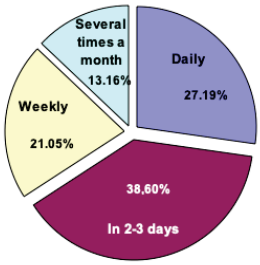
| | | |
|---|---|---|
| Yes | 98 | 85.96% |
| No | 16 | 14.04% |




Of the 114 respondents, it is found that almost 86% are consumers of meat products, a finding that supports the assumption that they are products consumed by a large number of people, so the effects of possible food nonconformities may be ample among the consumers.

2. How often do you buy meat products?

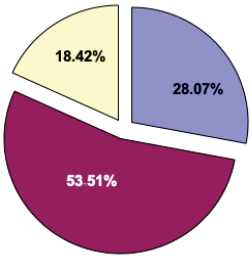
| | | |
|---|---|---|
| daily | 31 | 27.19% |
| once in 2-3 days | 44 | 38.60% |
| weekly | 24 | 21.05% |
| several times a month | 15 | 13.16% |




In relation to the frequency of consumption, it can be said that the majority (38.60%) of population consumes meat products at a range of 2-3 days and only 13.16% of respondents only a few times in a month.

3. How much meat products do you buy?

| | | |
|---|---|---|
| under 0.5 kg | 32 | 28.07% |
| 1-2 kg | 61 | 53.51% |
| over 2 kg | 21 | 18.42% |




In terms of quantities, it turned out that most consumers (53.51%) use an average of 1-2 kg of meat products per month.

4. Where do you buy most of the meat products? (more possible answers)

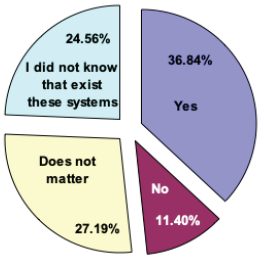
| | | |
|---|---|---|
| directly from the manufacturers | 37 | 20.22% |
| food stores | 62 | 33.88% |
| hypermarkets | 56 | 30.60% |
| traditional fairs | 28 | 15.30% |

Regarding the places to buy meat products, consumers are frequently attracted by the food stores, follow very closely by the hypermarkets.


Very few consumers choose the traditional fairs to purchase these products, as the first cause is the convenience of consumers and second, the inconsistency in organizing these fairs.

5. Do you prefer to buy meat products from the manufacturers that have implemented and certified a quality management system?

| | | |
|---|---|---|
| Yes | 42 | 36.84% |
| No | 13 | 11.40% |
| Does not matter | 31 | 27.19% |
| I did not know that these systems exist | 28 | 24.56% |

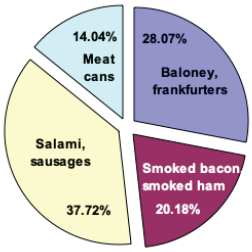



Related to the existence of systems and levels that protect the safety and health of consumers, insufficient information about the prevention of food risks, ignorance towards this essential information, and lack of significance given to these systems by the consumers, were the main findings. Thus, 63.16% of consumers do not know about the existence of these systems or do not give them the proper importance. Similar approach of consumers towards food safety and food defense systems are mentioned by other authors (Georgescu, 2013a; Georgescu, 2013b).

6. What types of meat products do you prefer to consume?

In the category of „meat products types" there is an increased demand for the assortments "ready to eat" which can easily represent the basis of a sandwich.

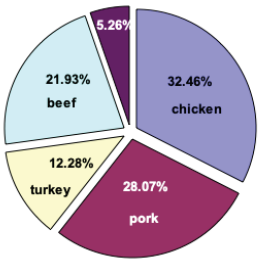
| | | |
|---|---|---|
| Baloney, frankfurters | 32 | 28.07% |
| Smoked bacon, smoked ham | 23 | 20.18% |
| Salami, sausages | 43 | 37.72% |
| Meat cans | 16 | 14.04% |




In 2003, in British Food Journal, Tihomir Vraneševic, discussed about „The effect of the brand on perceived quality of food products" He considered that the chosen marketing strategy (including the branding as its integral parts) is highly important in the process of assessing meat quality (Vraneševic and Stančec, 2003). The brand becomes one of the basic motives for the consumers' choice of a particular food product. The importance of the product brand shall be seen primarily in its impact on consumers' choice and their loyalty through identifying and differentiating quality and origin, as well as creating additional values. The author analyzes the sales of tin cans as well as explores the effect of the product brand on sales. The main conclusions of the paper are that consumers do not value products based exclusively on their physical characteristics and that in the process of making a purchasing decision when choosing an alternative, consumers will first perceive the brand as "a sign of quality" and then other evaluation criteria (physical appearance and packaging, price, the reputation of the retail network).

7. Which type of meat does your choice meat product contain?

| | | |
|---|---|---|
| chicken | 37 | 32.46% |
| pork | 32 | 28.07% |
| turkey | 14 | 12.28% |
| beef | 25 | 21.93% |
| others | 6 | 5.26% |




From the category of favorite meats, the chicken meat is desired by 32.46% of consumers due to the relatively low price compared to the turkey meat, and pork is in front of beef as meat preferences.

Other authors also communicated results of surveys about consumer preferences in terms of meat products (Pirvutoiu, 2013; Raita, 2014, 2018). Thus, in the paper "Research on Consumer Behavior in Bucharest Poultry Meat Market", the authors Pirvutoiu and Popescu, (2013) stated the consumer preference for various meat sorts. Poultry meat was mentioned by 82%, respondents, pork by 71% respondents, fish by 68%, beef by 39%, turkey by 19% and lamb by 14%. This showed that the interviewed persons used to consume various sorts of meat along the year, but the most preferred were chicken, pork and fish. Meanwhile, regarding the consumer preference for the amount of purchased chicken meat, about 62% respondents preferred to buy 1-3 kg chicken meat both with bones and without bones in order to assure a varied menu for their family, 20% respondents used to buy 0,5-1 kg meat, 11% over 3 kg and just 7% less than 0.5 kg. The smallest amount of meat was justified by the reduced number of members in the family, consumption frequency and budget allotted for food. Most of consumers preferred to buy fresh meat and mainly every 2-3 days in order to cover the weekly need of their family.

8. What is the main criterion for choosing a meat product?

| | | |
|---|---|---|
| the price | 41 | 35,96% |
| the quality | 24 | 21,05% |
| the packaging and the product appearance | 16 | 14,04% |
| the higher availability | 33 | 28,95% |


The main criterion for choosing a meat product is correlated for 35.96% of respondents with their salary level, so with the cost price and only on the third place is the quality of the purchased food.

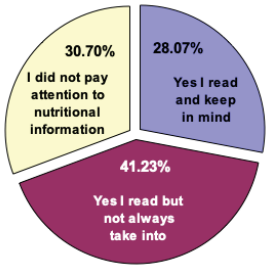
"Consumer Preferences for Meat Attributes", a review published by Kynda (2006), also approached the consumers preferences regarding meat quality. In that paper, while survey respondents rated freshness and taste/flavor as the most important factors on their meat purchasing decisions, 55% of the respondents rated natural production as having an extreme or very important influence on their meat purchasing decisions and 36% of respondents rated local production as having an extremely or very important influence on their purchasing decisions (Kynda et al., 2006).

The highest premium consumers in their study were willing to pay pertained to high-grade beef products, but all meat products bearing both the grass-fed and locally grown labels received willingness to pay premiums over the standard meat products. This indicates that the use of these two labels together will bring a added value compared to individual labels. Furthermore, at least 65% of the respondents were willing to pay extra for the labeled products discussed.

9. Do you read and keep in mind the nutritional information of a product when you buy it?

| | | |
|---|---|---|
| yes I always read and keep in mind | 32 | 28.07% |
| yes I read but not always take into account | 47 | 41.23% |
| I did not pay attention to nutritional information | 35 | 30.70% |

It should be noted that all 114 study participants had knowledge about nutritional information, but only a little over a quarter of them took these into account.




10. In your opinion, what manufacturers should do in the future to meet your exigencies?
Regarding the suggestions of those interviewed to the producers of meat products, the highest percentage of them recommends the increasing of products quality, even if the price would be higher and also, new products (Curtis et al., 2006). So, it is worth noting that for consumers the quality of food is a desideratum, but few of them do actually persuade it.

| | | |
|---|---|---|
| To focus more on quality, even if the price would be higher | 38 | 33.33% |
| To communicate better with customers | 22 | 19.30% |
| To introduce new products | 31 | 27.19% |
| To focus more on packaging | 14 | 12.28% |
| Others | 9 | 7.89% |


In a survey made in U.S.A., by Dr. Shang-Ho Yang and his collaborators, a web-based survey was completed by 3802 consumers distributed across Kentucky, Tennessee, Ohio, Illinois, and Indiana in the autumn of 2015 stated that meat shoppers have very different perspectives in their perceptions of where to source quality and what sorts of services they prefer. While the vast majority of consumers look to the traditional grocer for their meat, other meat marketing formats are also popular. Consumers were also asked to provide their perception of the highest quality source of raw meat. The authors conclude that different meat merchandising strategies are going to be effective targeting different age groups and geographic populations. Many retailers have figured this out already. Their data suggests decent opportunities for targeted branding and service that could more effectively reach certain segments.

## CONCLUSIONS

The results show that there are gaps in the perception and the care of food quality and safety. Even though the manufacturers are making big efforts to increase the food safety of the products they offer, adopting a number of good practices in this respect, their effort to inform the consumers is relatively low.
From the point of view of the perception towards quality and food safety systems for meat products, the consumers have a great emphasis on freshness of products. Despite the fact that the consumers are concerned about food safety, this is not a priority in the acquisition process, the sensorial characteristics and the cost price underlying the acquisition process.
The Romanian consumers have an adequate level of information on the quality characteristics of the consumed products, the label and the nutritional information on it. Being responsible and interested in what they consume, will also lead to an increase in the producers' interest to offering products on high quality standards.

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# THE NUTRITIONAL VALUE OF MEAT AS SEEN THROUGH THE VARIOUS POULTRY FOOD SPECIES – A COMPARATIVE ANALYSIS WITH A FOCUS ON PROTEINS, FATTY ACIDS AND MINERAL CONTENT

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***Abstract***

*Poultry meat represents a very important part of the human diet, being preferred to „read" meat due to the low content in cholesterol and the high digestibility. Further on, it provides a valuable source of proteins, their quality being reflected in a high content of essential amino acids. The paper represents a review of the main sources cited recently considering the nutritional value of poultry meat. The various poultry species has been mentioned, in comparison to the well-known and preferred chicken and turkey: duck, goose and ostrich. Their nutritional value is presented by highlighting mostly the amino acids, fatty acids and mineral content.*

***Key words****: poultry meat, nutritional value, amino acids, fatty acids.*

## INTRODUCTION

Meat represents "the edible parts removed from the carcass of animals used for food […]". It is considered an important part of the human diet, providing energy, vitamins, minerals and fatty acids.

The main so-called "white" meat comes from poultry, among the species currently chosen more and more would be chicken and turkey. However, duck, goose and ostrich meat have different nutritional aspects that could be taken into consideration in order to complete the human diet with a sufficient source of fatty acids and proteins (amino acids).

In this paper, the aim was to highlight the importance of poultry meat through the essential amino acids, fatty acids and mineral content, for the main species, chicken and turkey, and to mention, in comparison, what other species might have to offer as nutritional source: duck, goose and ostrich.

## MATERIALS AND METHODS

In order to obtain the main data presented in this paper, a number of articles and books have been consulted online and on paper. Further on, information has been analysed and withdrawn as to make sure the comparison is as objective and accurate as possible.

The main method through which this bibliographic study has been obtained is by starting with the reviews presented recently on the subject and further on analysing several other complementary sources, mainly articles.

## RESULTS AND DISCUSSIONS

### A. Poultry meat as nutritive source in comparison to meat obtained from other food species

According to Wood (2017), on average, for each 100 g meat, the human body will receive 20 g of high biological-value protein and 8 g of fat. Compared to beef and lamb, chicken and turkey have the lowest content of fat, thus being highly recommended for a healthier diet (Lopez-Bote, 2017) (Table 1).

The fat content, which is a subject of great concern for meat consumption, might vary with the species, feeding system and the analysed cut. Therefore, leaner cuts obtained from pork or beef could be included in the human diet for their richness in vitamins and minerals, as they will not differ significantly from the skinless

turkey or chicken cuts (Pereira and Vicente, 2013). Indeed, it seems that the presence of the skin is causing the fat content (g/100 g edible portion) to reach 8.9 in raw chicken breast, while the same skinless cut will only amount to about 1.2 g/100 g. Also, the same authors mention in this paper that the turkey leg portions tend to have a higher fat content than the chicken legs.

Table 1. Content of protein and fat of the most common meat varieties (100 g edible portion) (Wood, 2017)

| | Beef | Pork | Lamb | Rabbit | Chicken | Turkey | Duck |
|---|---|---|---|---|---|---|---|
| Protein (g) | 18.7 | 21.4 | 17.5 | 20 | 20.3 | 21.8 | 18.3 |
| Total (g)fat | 17.1 | 5.7 | 18.7 | 5.5 | 2.7 | 2.9 | 5.9 |
| SFA (g) | 6.9 | 1.9 | 8.1 | 1.7 | 0.7 | 0.9 | 2.3 |
| MUFA (g) | 7.4 | 2.6 | 7.6 | 1.5 | 0.8 | 0.6 | 1.5 |
| PUFA (g) | 0.6 | 0.6 | 1.5 | 1.1 | 0.7 | 0.8 | 0.7 |

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

The leanest cut in chicken and turkey is known to be the breast meat (when skin is not considered). In comparison, beef presents a lower fat content in the round section than the sirloin and flank, while pork meat is lower in fat content on the ribs and leg meat, compared to loin (Wood, 2017) (Table 2).

The mineral and trace elements' content of meat is evident through its richness in iron, zinc and selenium (table 4).

Probst (2009) compared the nutrient values of chicken, beef, lamb and pork cuts.

The author also included tuna as reference for fish (Table 5). Chicken seems to be closer to tuna as cholesterol content, as well as total fat content, compared to the other species presented.

Table 2. Total fat content and fatty acids content in several cuts of beef pork, chicken and turkey (edible portion of 100 g) (Wood, 2017)

| | Beef cuts | | | Chicken cuts | |
|---|---|---|---|---|---|
| | Sirloin | Flank | Round | Breast | Leg |
| Total (g)fat | 5.6 | 7.2 | 4.3 | 1.2 | 3.8 |
| SFA (g) | 1.7 | 3 | 1.5 | 0.3 | 1 |
| MUFA (g) | 2.5 | 2.9 | 1.8 | 0.3 | 1.2 |
| PUFA (g) | 0.2 | 0.3 | 0.2 | 0,3 | 0,9 |
| 6PUFAω (mg) | 20 | 169 | 145 | 170 | 730 |
| 3PUFAω (mg) | 187 | 81 | 10 | 40 | 100 |
| | Pork cuts | | | Turkey cuts | |
| | Loin | Ribs | Leg | Breast | Leg |
| Total (g)fat | 8.2 | 5.6 | 15.7 | 0,7 | 2,4 |
| SFA (g) | 2.8 | 2 | 5.4 | 0,2 | 0,8 |
| MUFA (g) | 3.7 | 2.5 | 7 | 0,1 | 0,5 |
| PUFA (g) | 0.9 | 0.6 | 1.7 | 0,2 | 0,7 |
| 6PUFAω (mg) | 720 | 565 | 1400 | 110 | 570 |
| 3PUFAω (mg) | 30 | 24 | 120 | 2050 | |

Table 3. Vitamin content of different types of meat, according to the species of origin (100 g edible portion) (Wood, 2017 from USDA, 2011)

| Specification | Beef | Pork | Lamb | Rabbit | Chicken | Turkey | Duck |
|---|---|---|---|---|---|---|---|
| Vitamin A (IU) | 0 | 7 | 0 | 0 | 45 | 0 | 79 |
| Vitamin C (mg) | 0 | 0.6 | 0 | 0 | 0 | 0 | 5.8 |
| Vitamin D (IU) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Vitamin E (mg) | 0 | 0.2 | 0.2 | 0 | 0.2 | 0.4 | 0.7 |
| Thiamine (mg) | 0.1 | 1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.4 |
| Riboflavin (mg) | 0.2 | 0.3 | 0.2 | 0.2 | 0.1 | 0.2 | 0.5 |
| Niacin (mg) | 3.2 | 4.9 | 6 | 7.3 | 7.9 | 4.5 | 5.3 |
| Vitamin B6 (mg) | 0.4 | 0.5 | 0.2 | 0.5 | 0.4 | 0.5 | 0.3 |
| Folate (mcg) | 6 | 5 | 23 | 8 | 7 | 9 | 25 |
| Vitamin B12 (mcg) | 3 | 0.6 | 0.7 | 7.2 | 0.4 | 0.4 | 0.4 |

Table 4. The content of minerals for several meat varieties, displayed for a quantity of 100 g (edible portion) (Wood, 2017)

| Specification | Beef | Pork | Lamb | Rabbit | Chicken | Turkey | Duck |
|---|---|---|---|---|---|---|---|
| Calcium (mg) | 7 | 17 | 12 | 13 | 10 | 14 | 11 |
| Iron (mg) | 1.9 | 0.8 | 1.6 | 1.6 | 1 | 1.5 | 2.4 |
| Magnesium (mg) | 19 | 23 | 22 | 19 | 23 | 25 | 19 |
| Phosphorous (mg) | 177 | 211 | 166 | 213 | 198 | 195 | 203 |
| Potassium (mg) | 306 | 389 | 239 | 330 | 238 | 296 | 271 |
| Sodium (mg) | 58 | 52 | 59 | 41 | 75 | 70 | 74 |
| Zinc (mg) | 3.7 | 1.8 | 3.5 | 1.6 | 1.2 | 2.4 | 1.9 |
| Selenium (µg) | 22.4 | 36.1 | 19.7 | 23.7 | 16.9 | 26.5 | 13.9 |

Table 5. Comparison of nutrient content for different types of meat, obtained from food species (100 g edible portion) (Probst, 2009)

| Specification | Chicken (breast, raw) | Lamb (tenderloin, raw) | Beef (eye fillet, raw) | Pork (fillet, raw) | Tuna (raw) |
|---|---|---|---|---|---|
| Total fat (g) | 1.6 | 4 | 3 | 2.3 | 1 |
| Total protein (g) | 22.25 | 19.8 | 22.3 | 22 | 23.4 |
| Cholesterol (mg) | 59 | 70 | 58 | 95 | 45 |
| Sodium (mg) | 41 | 69 | 57 | 54 | 37 |
| Potassium (mg) | 300 | 330 | 380 | 405 | 444 |
| Magnesium (mg) | 28 | 24 | 27 | 26 | 50 |
| Calcium (mg) | 12 | 8 | 6 | 4 | 16 |
| Phosphorous (mg) | 231 | 240 | 230 | 237 | 191 |
| Iron (mg) | 0.4 | 2.1 | 2.2 | 1.1 | 0.7 |
| Zinc (mg) | 0.7 | 2.9 | 3.8 | 1.7 | 0.5 |
| Selenium (µg) | 21.4 | 10 | 12 | 15 | 37 |
| Copper (mg) | 0.03 | 0.13 | 0.15 | 0.09 | 0.06 |
| Manganese (mg) | 1.64 | 0.02 | - | - | 0.02 |
| Vitamin A (IU) | 15.5 | 9 | 2 | - | - |
| Vitamin C (mg) | 0.8 | - | 1 | - | 1 |
| Vitamin B-12 (µg) | 0.38 | 1.3 | 1.9 | 0.3 | 0.5 |

**B. Nutritional composition of chicken meat**

Chicken meat composition might be different when taking into account factors such as the rearing system. Bogosavljevic-Boskovic et al. (2010) showed that the percentage of protein and fat in the breast muscle is different between the meat from chicken reared in extensive indoor system and the one from chicken reared in free range conditions. More precisely, this study has found that both breast muscle samples from free range males and females contained a higher percentage of protein (23.72 % for males and 23.44 %, in comparison to 22.96 % and 22.57 % respectively).

Moreover, the fat percentage was lower in the breast muscle of free-range chicken.

This might be an indicator that the free-range system could be a more favourable one for the nutritional quality of chicken meat. Chen et al. (2016) studied the composition of chicken meat (breast and thigh cuts) obtained from three different types of genetic lines (817 crossbred chicken, Arbor Acres (AA) broiler and Hyline Brown (HB)).

In their study, they concluded that the crossbred chicken lines were more valuable from the nutritional point of view, as well as for maintaining a well-balanced diet.

In chicken meat, the predominant amino acids were identified to be lysine and leucine, among essential and non-essential types (Table 6).

Table 6. Composition in essential amino acids for breast meat and thigh meat* (g/100g dry weight) – three different lines of chicken (Chen et al., 2016)

| Specification | Breast meat | | | Thigh meat | | |
|---|---|---|---|---|---|---|
| | 817 | AA | HB | 817 | AA | HB |
| Arg | 4.692±0.133 | 4.547±0.124 | 4.668±0.082 | 4.377±0.208 | 4.507±0.122 | 4.207±0.089 |
| His | 2.728±0.062 | 2.474±0.062 | 3.390±0.051 | 1.967±0.072 | 1.999±0.040 | 2.048±0.045 |
| Ile | 3.463±0.063 | 3.308±0.072 | 3.396±0.068 | 3.017±0.070 | 3.167±0.087 | 3.004±0.103 |
| Leu | 6.128±0.143 | 5.905±0.132 | 6.107±0.109 | 5.442±0.203 | 5.709±0.115 | 5.428±0.129 |
| Lys | 6.470±0.140 | 6.268±0.103 | 6.405±0.117 | 5.903±0.147 | 6.123±0.139 | 5.825±0.140 |
| Met | 1.925±0.063 | 1.880±0.040 | 1.931±0.068 | 1.560±0.031 | 1.756±0.050 | 1.534±0.056 |
| Phe | 3.048±0.074 | 2.917±0.063 | 2.976±0.072 | 2.613±0.051 | 2.844±0.091 | 2.691±0.081 |
| Thr | 4.172±0.088 | 3.559±0.083 | 3.318±0.076 | 3.466±0.074 | 3.562±0.100 | 3.176±0.044 |
| Val | 3.637±0.079 | 3.462±0.094 | 3.568±0.053 | 3.122±0.107 | 3.256±0.085 | 3.014±0.102 |

*determined with Amino Acid Analyzer L-8900 Hitachi (freeze-dried meat samples)

The composition in amino acids might be affected by preservation techniques, such as freezing. In a study conducted for the evaluation of the effect of frozen storage on the amino acid composition of dark and light chicken meat, Wladyka and Dawson (1968) found that during frozen storage, the structural proteins will suffer modifications, most probably as a result of proteolysis, therefore exuding in the drip.

**C. Turkey meat's nutritional composition and value for human diet**

According to the USDA National Nutrient Database (2018), raw turkey meat (skinless) (100 g edible portion) will contain approximately 22.64 g protein and 1.93 g fat (total content).

The fatty acids composition of turkey meat cuts shows the presence of C18:2ω-5, C18:1ω-9, C16:0, C18:0 and C20:4ω-6 (table 7). Due to its low cholesterol content and high polyunsaturated fatty acids content, the turkey meat represents a good choice for diets aiming to control blood cholesterol levels.

Oblakova et al. (2016) observed a statistically significant content of protein in raw turkey breast muscles (23%) and thigh (20.73%). Also, they have confirmed that in the case of turkey meat, as it shows for other poultry meat species, the breast meat has a relatively higher protein and lower fat content than the thigh meat.

Table 7. Fatty acids composition (mg/100 g) in turkey meat portions (Baggio et al., 2002)

| Fatty acid | Turkey wing | Turkey leg | Turkey breast |
|---|---|---|---|
| C10:0 | 1.5±0.2 | - | 0.5±0.1 |
| C12:0 | 2.6±0.5 | 2.6±0.1 | - |
| C14:0 | 6.5±0.6 | 7.1±0.8 | 2.6±0.3 |
| C15:0 | 40.7±10.2 | 44.7±9.4 | 28.8±5.9 |
| C16:0 | 176.7±12.1 | 201.7±47.2 | 91.4±16.8 |
| C17:0 | 4.9±0.8 | 10.4±2.6 | 7.0±1.1 |
| C18:0 | 107.4±25.7 | 133.2±25.1 | 62.1±9.6 |
| C21:0 | - | 0.5±0.1 | 0.9±0.2 |
| C22:0 | 1.3±0.3 | 1.5±0.2 | 1.1±0.1 |
| C14:1 ω-9 | - | 1.0±0.1 | 0.5±0.2 |
| C16:1 ω-7 | 20.0±1.6 | 26.3±6.8 | 7.0±0.8 |
| C18:1 ω-9 | 189.2±18.5 | 228.1±48.8 | 89.6±8.9 |
| C18:2 ω-6 | 223.0±90.4 | 279.1±46.6 | 127.4±24.3 |
| C18:3 ω-3 | 5.8±1.8 | 8.8±2.9 | 2.6±0.6 |
| C20:2 ω-6 | 3.1±1.2 | 3.1±0.5 | 1.9±0.5 |
| C20:4 ω-6 | 55.5±3.1 | 73.9±8.6 | 40.2±8.3 |
| C22:5 ω-3 | 3.8±1.4 | 4.4±0.9 | 3.0±0.8 |
| C22:5 ω-6 | 3.5±0.4 | 4.8±0.9 | 3.1±0.7 |
| C22:6 ω-3 | 3.1±0.8 | 3.2±0.3 | 2.5±0.7 |
| % SFA | 40 | 39 | 41 |
| % | 25 | 25 | 21 |
| MUFA | | | |
| % PUFA | 35 | 36 | 38 |
| ω-6/ ω-3 | 0.04 | 0.04 | 0.04 |

In 1968, Essary and Ritchey analysed the amino acid composition of raw turkey meat (light meat, front part; dark meat, back part).

The differences between the two types of meat are not significant, eleven of the amino acids targeted for analysis showed slightly higher quantities in the dark meat (Table 8).

Table 8. Amino acid composition of raw dark and light turkey meat (g amino acid/100 g protein) (Essary and Ritchey, 1968)

| Amino acid | g amino acid / 100 g protein | |
|---|---|---|
| | Dark meat | Light meat |
| Aspartic acid | 5.58 | 5.87 |
| Threonine | 1.64 | 1.80 |
| Serine | 1.53 | 1.56 |
| Glutamic acid | 9.33 | 9.07 |
| Proline | 2.87 | 2.71 |
| Glycine | 3.76 | 3.19 |
| Alanine | 3.57 | 3.46 |
| Valine | 3.05 | 3.06 |
| Cystine | 0.96 | 0.90 |
| Methionine | 1.88 | 1.76 |
| Leucine | 4.70 | 4.27 |
| Tyrosine | 2.17 | 2.09 |
| Phenylalanine | 2.75 | 2.61 |
| Lysine | 5.01 | 4.64 |
| Histidine | 2.10 | 2.16 |
| Arginine | 4.80 | 4.48 |

**D. The potential nutritional role of duck meat**

After chicken meat, duck meat is the second most consumed meat in Southeast Asia (Aronal *et al.*, 2012). Duck meat has a higher content oftotal fat, reaching 5.95 g/100 g raw (skinless), while the protein content reaches 18.28 g/100 g for the same type of sample (USDA National Nutrient Database, 2018).

According to Kim and Nam (1977), duck meat presents a general content of protein between 13.61 and 21.19% and 17.32-34.92% fat.

The crude protein percentage determine by the authors was set to 79 %. Except for tryptophan, almost all essential amino acids were analysed. The chosen analysis method was gas chromategraphy and table 9 includes the results found at the time.

Table 9. Percentage of amino acids from duck meat (results obtained by Kim and Nam in 1977)

| Amino acid | Gram (%) |
|---|---|
| Alanine | 6.1 |
| Valine | 2.75 |
| Glycine | 7.13 |
| Isoleucine | 2.2 |
| Leucine | 4.54 |
| Proline | 4.9 |
| Threonine | 5.8 |
| Methionine | 1.15 |
| Hydroxyproline | 3.2 |
| Phenylalanine | 3.01 |
| Aspartic acid | 6.7 |
| Glutamic acid | 12.71 |
| Lysine | 4.95 |
| Arginine | 1.11 |
| Histidine | 5.6 |
| Cystine + cysteine | 4.4 |

As stated by the study of Aronal et al. (2012), duck meat quality depends on the amino acid and fatty acid profiles.

Among the amino acids, they have detected a high concentration of glutamic acid in both lines selected from analysis: Peking and Muscovy.

The highest concentrations among the essential amino acids were found for lysine and methionine (Table 10).

Table 10. Amino acid composition* (g/100 g protein) of Peking and Muscovy duck meat (Aronal et al., 2012)

| Specification | Peking duck meat | | Muscovy duck meat | |
|---|---|---|---|---|
| | Breast | Thigh | Breast | Thigh |
| Cystine | 2.65±0.18 | 2.07±0.45 | 0.07±0.05 | 0.08±0.03 |
| Histidine | 3.23±0.35 | 2.79±0.27 | 2.96±0.22 | 2.74±0.29 |
| Isoleucine | 7.61±0.28 | 7.85±0.18 | 3.44±0.08 | 3.26±0.16 |
| Leucine | 2.79±0.08 | 2.82±0.04 | 7.63±0.20 | 7.24±0.16 |
| Lysine | 9.21±0.38 | 9.12±0.26 | 9.41±0.00 | 8.23±0.56 |
| Methionine | 7.09±1.76 | 10.12±1.63 | 6.15±0.74 | 12.06±2.65 |
| Phenylalanine | 3.22±0.09 | 3.27±0.01 | 3.90±0.05 | 3.72±0.30 |
| Threonine | 4.65±0.15 | 4.70±0.06 | 4.96±0.14 | 4.30±0.84 |
| Tyrosine | 1.84±0.12 | 1.85±0.11 | 3.70±0.09 | 3.85±0.03 |
| Valine | 4.58±0.13 | 4.57±0.06 | 3.49±0.12 | 3.21±0.12 |
| Arginine | 7.07±0.21 | 6.40±0.30 | 7.28±0.13 | 8.40±0.55 |
| Alanine | 6.21±0.49 | 6.02±0.08 | 6.62±0.07 | 5.85±0.15 |
| Aspartic acid | 9.57±0.38 | 9.55±0.38 | 10.01±0.30 | 8.69±0.68 |
| Glutamic acid | 15.21±0.18 | 14.96±0.18 | 15.62±0.45 | 13.71±0.00 |
| Glycine | 6.26±0.62 | 5.53±0.33 | 5.57±0.02 | 5.68±0.57 |
| Proline | 4.23±0.08 | 3.94±0.05 | 4.31±0.05 | 4.29±0.03 |
| Serine | 4.56±0.16 | 4.44±0.30 | 4.87±0.16 | 4.67±0.69 |

Fatty acids composition of meat plays a crucial role in the human diet, as the biological effects of ω-3 long-chain polyunsaturated fatty acids had received a great interest in human nutrition, due to their role in the prevention and management of several pathologies: coronary heart disease, hypertension, type 2 diabetes, renal disease, ulcerative colitis and Crohn's disease.

Duck meat presents a very well-balanced fatty acids' composition, therefore in the future, through the development of specific techniques, the composition in the fatty acids might be improved through the use of dietary oils, such as soybean and fish oils (Schiavone et al., 2010). Qiao et al. (2017) have conducted astudy in order to assess the quality of duck meat destined for processing, in order to obtain meat products.

They have selected Cherry Valley (CV), Spent Layer (SL) and Crossbred (CB) duck lines and have determined the fatty acid composition of breast and thigh muscles, through gas chromatography. In a similar attempt, Aronal et al. (2012) have done a profiling on fatty acid composition on the same cuts, for Peking (PK) and Muscovy (MC) duck meat. Results are included in Tables 11 and 12.

Table 11. Fatty acid composition of breast muscle (% of total fatty acid) for several duck meat lines (after Qiao et al., 2017 and Aronal et al., 2012)

| Specification | Breast | | | | |
|---|---|---|---|---|---|
| | CV | SL | CB | PK | MC |
| C14:0 | 0.26±0.03 | 0.46±0.04 | 0.24±0.01 | 2.74±0.55 | 2.24±0.08 |
| C16:0 | 20.81±0.14 | 22.40±2.09 | 23.06±0.46 | 24.11±3.93 | 22.61±0.06 |
| C16:1 | 0.33±0.02 | 0.51±0.06 | 0.25±0.01 | 0.75±0.15 | 2.25±0.18 |
| C18:0 | 14.21±0.62 | 8.97±0.87 | 14.78±0.30 | 0.00±0.00 | 10.63±0.25 |
| C18:1 ω-9 | 26.20±1.16 | 35.90±1.88 | 22.27±0.55 | 26.89±3.19 | 36.45±1.32 |
| C18:2 ω-6 | 17.31±0.16 | 21.86±1.53 | 13.93±0.55 | 13.28±0.81 | 14.69±0.53 |
| C18:3 ω-6 | 0.04±0.00 | 0.02±0.00 | 0.03±0.00 | 0.03±0.05 | 0.08±0.13 |
| C20:1 | 0.38±0.02 | 0.34±0.02 | 0.31±0.01 | 0.03±0.27 | 0.19±0.17 |
| C20:3 ω-6 | 1.39±0.13 | 0.23±0.01 | 1.19±0.06 | 0.00±0.00 | 0.12±0.12 |
| C20:4 ω-6 | 0.05±0.00 | 0.02±0.01 | 0.05±0.00 | 9.23±1.89 | 4.74±0.58 |
| C22:6 ω-3 | 0.27±0.03 | 0.67±0.06 | 0.88±0.21 | 1.60±0.40 | 0.44±0.38 |
| SFA | 46.82±0.95 | 40.83±3.23 | 53.18±0.36 | 26.85±3.38 | 35.47±0.24 |
| MUFA | 33.40±0.87 | 40.67±1.35 | 30.05±0.41 | 30.22±2.65 | 41.59±1.56 |
| PUFA | 20.00±0.28 | 23.40±1.65 | 17.03±0.46 | 42.47±5.97 | 22.94±1.33 |
| ω-6/ω-3 | 0.15±0.01 | 1.52±0.08 | 0.47±0.04 | 1.22±0.03 | 7.48±0.30 |

Table 12. Fatty acid composition of thigh muscle (% of total fatty acid) for several duck meat lines (after Qiao et al., 2017 and Aronal et al., 2012)

| Specification | Thigh | | | | |
|---|---|---|---|---|---|
| | CV | SL | CB | PK | MC |
| C14:0 | 0.37±0.12 | 0.45±0.02 | 0.40±0.02 | 5.26±1.28 | 2.64±0.07 |
| C16:0 | 19.64±0.16 | 17.78±0.24 | 22.89±0.24 | 20.32±0.83 | 21.38±0.37 |
| C16:1 | 0.38±0.00 | 0.39±0.01 | 0.39±0.01 | 1.80±0.38 | 2.37±0.05 |
| C18:0 | 11.00±0.54 | 6.55±0.22 | 9.93±0.19 | 0.00±0.00 | 3.91±6.77 |
| C18:1 ω-9 | 36.00±0.55 | 41.78±0.79 | 38.14±0.82 | 30.36±4.34 | 40.24±1.07 |
| C18:2 ω-6 | 18.95±0.12 | 24.30±0.88 | 15.32±0.41 | 17.04±0.31 | 12.69±0.03 |
| C18:3 ω-6 | 0.04±0.00 | 0.03±0.00 | 0.04±0.00 | 0.54±0.47 | 0.00±0.00 |
| C20:1 | 0.30±0.00 | 0.49±0.02 | 0.33±0.01 | 0.15±0.13 | 0.21±0.19 |
| C20:3 ω-6 | 0.70±0.04 | 0.15±0.00 | 0.37±0.02 | 0.17±0.19 | 0.00±0.00 |
| C20:4 ω-6 | 0.03±0.00 | 0.02±0.00 | 0.02±0.00 | 6.16±3.65 | 4.78±0.36 |
| C22:6 ω-3 | 0.22±0.01 | 0.37±0.03 | 0.38±0.03 | 1.03±0.47 | 0.95±0.28 |
| SFA | 38.80±0.82 | 30.09±0.36 | 41.82±1.07 | 25.58±2.03 | 27.93±6.69 |
| MUFA | 39.93±0.47 | 44.72±0.72 | 41.78±0.82 | 34.67±5.16 | 42.21±1.21 |
| PUFA | 21.40±0.72 | 25.29±0.86 | 16.49±0.42 | 39.75±7.13 | 26.86±5.68 |
| ω-6/ω-3 | 0.22±0.04 | 1.25±0.08 | 0.75±0.06 | 1.73±0.17 | 2.00±1.26 |

According to these results, duck meat seems to be rich in palmitic acid (16:0), the most abundant SFA, followed by stearic acid (18:0). Among monounsaturated fatty acids, the predominant one is oleic acid (C18:1 ω-9), while for the polyunsaturated category, the highest concentration was that of linoleic acid (C18:2 ω-6). In order to assess further on the quality of duck meat, the ratio of ω-6 and ω-3 was calculated for both breast and thigh samples. The ω-3 and ω-6 fatty acids have a very important role in human nutrition, as they are precursors of eicosanoids, protaglandines, leukotrienes and thromboxanes, which regulate crucial physiological (Qiao et al.., 2017). In the scientific literature, authors have agreed that the value of this ratio should be lower than 5.

**E. Nutritional composition of goose meat and its potential for nutrients source in human diet**

The USDA National Nutrient Database (2018) mentions that 100 g of goose raw meat (skinless) has a high content of protein (22.75 g) and a total fat content of 7.13 g. Also, goose meat seems to be rich in potassium (420 mg/100 g) and phosphorous (312 mg/100 g).

Isguzar and Pingel (2003) analyzed the protein and fat content of breast and leg muscle of different goose genotypes, their findings suggesting that the protein content varies between 18 and 22% (for three different local genotypes). The percentage of fat reached 1,4 % in one of the genotypes, but all three are considered suitable for meat production, as well as usage in commercial crossbred programs.

In an attempt to quantify the nutritional value of the Egyptian goose (*Alopochen aegyptiacus*), Geldenhuys et al. (2013) have evaluated its nutritional value by comparison with other food species, the meat samples being cooked in the oven until the core temperature reached 75$^{o}$C. The samples were then analyzed, parallels being drawn between the breast portions of Egyptian goose, guineafowl, Pekin duck and broiler chicken. As a reference, ostrich meat was used, of which two different cuts were selected: fan fillet (*M. iliofibularis*) and moon steak (*M. femorotibialis*).

The data showed that the lowest protein content was identified in broiler chicken breast, while the highest was of the ostrich fan fillet. On the other hand, Egyptian goose and Pekin duck showed a higher percentage of intramuscular fat, compared to broiler chicken, ostrich cuts and guineafowl (Table 13).

Table 13. Results of analyses (g/100 g cooked meat cuts) for nutritional quality of Egyptian goose breast compared to other poultry meat cuts (Geldenguys et al., 2013)

| Specification | Egyptian goose breast | Guineafowl breast | Ostrich fan fillet | Ostrich moon steak | Pekin duck breast | Broiler chicken breast |
|---|---|---|---|---|---|---|
| Protein[1] | 30.9±2.6 | 31.9±1.9 | 32.7±2.2 | 32.5±1.6 | 31.4±0.6 | 29.8±1.4 |
| Fat[1] | 5.9±1.9 | 3.2±0.9 | 3.8±0.7 | 3.8±0.8 | 5.8±0.6 | 3.7±0.8 |
| SFA[2] | 37.91±2.22 | 43.63±2.8 | 43.85±1.4 | 45.9±3.92 | 40.87±1.63 | 33.27±4.58 |
| MUFA[2] | 22.24±5.93 | 26.70±3.58 | 27.54±2.39 | 25.71±2.19 | 34.00±2.31 | 22.71±3.06 |
| PUFA[2] | 39.70±3.93 | 29.47±2.99 | 28.33±2.59 | 27.79±3.91 | 24.89±2.40 | 43.86±7.06 |
| ω-6/ω-3[2] | 9.94±1.79 | 8.56±2.37 | 9.60±2.60 | 7.06±1.03 | 17.78±8.09 | 21.83±10.17 |
| P[3] | 192.5±15.6 | 182.4±18.4 | 179.3±9.8 | 181.7±6.5 | 186.5±6.4 | 208.7±16.0 |
| K[3] | 180.1±19.1 | 162.5±15.0 | 171.5±9.6 | 180.1±8.3 | 169.3±13.8 | 189.5±20.8 |
| Ca[3] | 12.3±1.74 | 11.9±1.8 | 11.6±1.8 | 11.6±2.0 | 17.3±1.4 | 10.7±1.5 |
| Mg[3] | 32.5±2.3 | 30.2±5.0 | 32.6±1.3 | 30.7±1.0 | 31.4±2.0 | 36.7±2.7 |
| Na[3] | 22.0±6.0 | 15.8±2.2 | 20.6±0.6 | 24.5±1.9 | 29.0±1.9 | 18.9±2.2 |
| Fe[3] | 7.5±0.59 | 1.8±0.6 | 4.2±0.4 | 3.6±0.4 | 4.6±0.8 | 1.4±0.2 |
| Cu[3] | 0.5±0.14 | 0.2±0.1 | 0.3±0.03 | 0.3±0.03 | 0.4±0.2 | 0.1±0.02 |
| Zn[3] | 2.1±0.40 | 1.2±0.3 | 2.3±0.2 | 5.5±0.4 | 1.9±0.2 | 1.2±0.2 |
| Mn[3] | 0.1±0.01 | 0.04±0.01 | 0.04±0.01 | 0.03±0.002 | 0.04±0.01 | 0.03±0.004 |
| B[3] | 0.03±0.004 | 0.03±0.01 | 0.03±0.01 | 0.03±0.003 | 0.03±0.004 | 0.03±0.003 |
| Al[3] | 2.8±2.2 | 3.1±1.9 | 4.3±0.9 | 4.4±1.0 | 2.7±1.8 | 3.2±1.6 |

[1] g/100 g edible portion (sample); [2] % out of total fatty acids composition; [3] mg/100 g dry basis.

The fatty acid composition showed that broiler chicken breast and Egyptian goose breast were Similar in their concentration of monounsaturated fatty acids and polyunsaturated ones, the latter being the highest among all types of samples subject to analysis.

Considering the mineral composition of the Egyptian goose meat, its iron content is significantly higher than the other's. According to the authors, this might be related to the higher degree of physical activity, compared to the other species. It might be known that the fat content and the fatty acids composition can be influenced by the muscle type fiber, therefore leading to differences between cuts, Because the red muscles have a higher concentration of phospholipids, they will have a higher percentage of polyunsaturated fatty acids. After analyzing this parameter, Oz and Celik (2015) found that indeed the total polyunsaturated fatty acids content (19,97%) was higher in raw leg meat than in the breast cut (14.79%).

Goose breast meat content of saturated fatty acids is 31.38%, 53.81% in monounsaturated fatty acids and 14.79% in polyunsaturated fatty acids (total). Linoleic acid represented a high proportion of the total PUFA content (73%) (Oz and Celik, 2015) The same team mentioned that the leg meat had a content of SFA reaching 38.79%, MUFA 41.24% and PUFA 19.97%, for the latter the biggest portion being taken by docosapentaenoic acid.

Geldenhuys et al. (2015) later on evaluated the differences between the Egyptian goose cuts, breast, drumstick and thigh, considering the fatty acid composition.

They observed that the breast portion contained a higher percentage of polyunsaturated fatty acids, while the thigh portion had the lowest. The drumstick showed a high content of short-chain saturated fatty acids, such as the myristic acid (C14:0).

The authors suggest that these differences are caused firstly by the muscle fiber composition, with consequences on phospholipids.

Further on, the composition of the main lipid fractions (tryacylglycerols) might have an effect on these differences, the physical structure of the thigh, for example, allowing an increased fat deposition, therefore a higher content of tryacylglycerol adipocytes.

**F. Ostrich meat nutritional quality, as reflected through its value for human diet**

Compared to the meat species mentioned so far in this study, ostrich meat seems to have an even higher fat content for 100 g raw portion (skinless) – 8.7 g. The protein content remains high, 20.22 g/100 g (USDA National Nutrient Database, 2018).

Jukna et al. (2012) have evaluated the chemical composition of ostrich by comparison to turkey and broiler meat. By using classical methods of analysis, they have obtained the protein and fat percentage, their results showing just a slight difference between the species considering the protein percentage. The intramuscular fat percentage was higher in broiler chicken (2.20%), compared to ostrich (1.82%), while turkey seems to be the leaner of the three types of meat included in the study (1.21%).

In 1996, Sales and Oliver-Lyons wrote a report on the knowledge of nutritional composition of different muscles which could be included in the human diet, obtained from ostrich. They have included the protein and intramuscular fat percentages for several muscles, as included in Table 14. It seems that there are no significant differences between the types of targeted ostrich muscles.

Table 14. Composition of protein and intramuscular fat (%) as seen in different samples of ostrich muscles (%) (Sales and Oliver-Lyons, 1996)

| Muscle | Protein (%) | Intramuscular fat (%) |
|---|---|---|
| *M. gastrocnemius pars interna* | 20.6 | 0.26 |
| *M. femorotibialis medius* | 20.6 | 0.31 |
| *M. ambiens* | 21.5 | 0.44 |
| *M. iliotibialis lateralis* | 21.2 | 0.40 |
| *M. iliofibularis* | 20.9 | 0.42 |
| *M. iliofemoralis* | 21.9 | 0.69 |
| *M. fibularis longus* | 21.0 | 0.24 |
| *M. iliotibialis cranialis* | 20.0 | 0.52 |
| *M. flexor cruris lateralis* | 21.0 | 0.82 |

Concerning the amino acid composition, the ostrich meat seems to have a high nutritive value. Also, except for a few amino acids, there has been consistency of the pattern between the analyzed muscles (Sales and Oliver-Lyons, 1996) (Table 15).

In a paper aimed to evaluate the fatty acid composition of two different types of ostrich muscles (*M. gastrocnemius* and *M. iliofibularis*), Horbanczuk and Sales (1998)

found a total percentage of saturated and monounsaturated fatty acids to be similar between muscles. They did find differences in palmitic (16:0) and palmoleic (16:1) acids between the two types of muscles.

Also, they have observed a high proportion of 18:1, with no differences between the samples. The same panel of fatty acids has been targeted by Girolami et al. (2003), the results of both studies being included in Table 16.

Table 15. Amino acid composition of ostrich raw muscles (g/100 g edible portion) (Sales and Oliver-Lyons, 1996)

| Component | Muscle | | |
|---|---|---|---|
| | *M. iliofibularis* | *M. femorotibialis medius* | *M. gastrocnemius pars interna* |
| Lysine | 1.65 | 1.67 | 1.61 |
| Threonine | 0.78 | 0.75 | 0.74 |
| Valine | 1.05 | 0.91 | 0.96 |
| Methionine | 0.57 | 0.54 | 0.53 |
| Isoleucine | 0.97 | 0.88 | 0.89 |
| Leucine | 1.79 | 1.69 | 1.64 |
| Phenylalanine | 0.99 | 0.91 | 0.92 |
| Histidine | 0.38 | 0.40 | 0.40 |
| Arginine | 1.48 | 1.30 | 1.30 |
| Aspartic acid | 1.96 | 1.85 | 1.88 |
| Serine | 0.59 | 0.59 | 0.58 |
| Glutamic acid | 3.17 | 3.15 | 3.31 |

Table 16. Total lipid and fatty acid composition of two different ostrich muscles (Horbanczuk and Sales, 1998; Girolami et al., 2003)

| Study | Horbanczuk and Sales (1998) | | Girolami *et al*. (2003) | |
|---|---|---|---|---|
| Muscle | *Musculus gastrocnemius* | *Musculus iliofibularis* | *Musculus gastrocnemius* | *Musculus iliofibularis* |
| 12:0 | 0.11 ± 0.02 | 0.13 ± 0.02 | 0.04 ± 0.001 | 0.05 ± 0.001 |
| 14:0 | 0.91 ± 0.08 | 0.91 ± 0.05 | 0.48 ± 0.02 | 0.70 ± 0.02 |
| 16:0 | 23.15 ± 4.84 | 21.43 ± 1.44 | 17.48 ± 0.71 | 22.89 ± 0.71 |
| 18:0 | 13.52 ± 0.89 | 12.81 ± 1.12 | 11.02 ± 0.37 | 8.87 ± 0.37 |
| 18:1 | 33.13 ± 1.10 | 31.07 ± 1.01 | 29.36 ± 0.56 | 31.58 ± 0.56 |
| 18:2 ω-6 | 14.72 ± 1.80 | 15.76 ± 2.09 | 16.63 ± 0.70 | 16.24 ± 0.70 |
| 18:3 ω-3 | 0.65 ± 0.07 | 5.81 ± 0.29 | 1.50 ± 0.09 | 2.14 ± 0.09 |
| 20:4 ω-6 | 5.38 ± 0.52 | 5.63 ± 0.46 | 11.34 ± 0.54 | 6.50 ± 0.54 |
| 20:5 ω-3 | 0.49 ± 0.06 | 0.39 ± 0.05 | 0.54 ± 0.04 | 0.28 ± 0.04 |
| 22:5 ω-3 | 0.99 ± 0.08 | 0.79 ± 0.10 | 1.40 ± 0.06 | 0.74 ± 0.06 |
| 22:6 ω-3 | 0.80 ± 0.08 | 0.72 ± 0.09 | 0.39 ± 0.03 | 0.21 ± 0.03 |
| SFA | 37.8 ±1.58 | 35.36 ± 1.59 | 29.88 ± 0.47 | 33.31 ± 0.47 |
| MUFA | 36.68 ± 1.35 | 35.51 ± 1.23 | 35.52 ± 0.85 | 39.05 ± 0.85 |
| PUFA | 23.5 ± 1.55 | 29.11 ± 2.96 | 34.60 ± 1.18 | 27.64 ± 1.18 |

## CONCLUSIONS

The sources cited so far lead us to the following conclusions:

1. Compared to beef and lamb, chicken and turkey meat are leaner, therefore much more suitable for diets aiming to lower the cholesterol blood level.
2. Poultry meat cuts which include the skin might have a close level of total fat compared to skinless cuts of pork or beef, thus poultry meat skinless cuts being the only ones suitable when aiming to lower the fat content of human diet.
3. The leanest cut in chicken and turkey is the breast meat (skinless). Compared to that, beef meat presents the lowest fat content in the round cut, while pork has a low-fat content in the ribs and leg meat.
4. Chicken meat is a valuable source of niacin, while duck meat is known to be rich in vitamin A and folate.
5. Chicken meat has a high content of lysine and leucine.
6. Turkey meat presents a high content of PUFA and differences in protein and fat content between the cuts (with higher

amount of protein and lower content of fat in the breast cut).

7. Duck meat is rich in glutamic acid, lysine and methionine. It also represents a well-balanced meat type when considering the fatty acids composition. It is predominantly rich in linoleic acid, oleic acid and palmitic acid.
8. Goose breast meat is rich in linoleic acid as well, this representing 73 % of the total content of PUFA in this type of meat. On the contrary, goose leg meat seems to have a high content of docosapentaenoic acid.
9. Ostrich meat amino acids' composition, analysed on different muscle groups is very consistent, therefore no statistically signify-cant differences have been observed. Its fatty acids' composition is also consistent, overall the amount of oleic acid being higher when compared to poultry species.

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# THE IMPACT OF SOFT CHEESE *Nigella sativa* SEED OIL ENRICHMENT ON MOISTURE PATTERN DURING RIPENING

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***Abstract***

*Recent literature provides studies of the benefits of Nigella sativa cold pressed seed oil (NSSO) cheese enrichment, but insufficient data is available on the effect on the physico-chemical cheese properties. Three batches of traditionally manufactured raw milk brined cheese were considered: control cheese without NSSO and cheese samples enriched with 0.1 and 0.2 w/w NSSO. Experimental cheese samples were analyzed in duplicates for moisture contents, at 0, 14, 28, and 42 days of ripening, according to the AOAC Official Method 926.08. Data indicates moisture loss during ripening, which is consistent with most literature findings, and strong statistical significance could be associated to the trends of moisture decrease (f-ratio value - 59.07682; p-value - 0.00001), results being consistent with other sources which communicated p values <0.05 for moisture decrease findings. However, the slightly higher moisture values for the NSSO added cheeses were positively associated (p-value - 0.05). The data indicates slightly better moisture retention for 0.2% w/w NSSO cheese batch, which might be linked to a possible prevention of loss of body and texture loss during ripening of soft brined cheese.*

***Key words****: moisture, Nigella sativa seed oil, raw milk brined cheese, ripening.*

## INTRODUCTION

Impersonation of refinement and real culinary delicacies in some countries, traditional Romanian cheeses are not only delicious, tasteful and flavorful food, but also powerful historical, social, economical and cultural symbols of the individuality of Romanian people (Tăpăloagă, 2018).

The trend in international scientific world is to understand the composition and dynamics peculiarities concerning the physicochemical, biochemical and microbiological changes that take place in raw milk cheeses during manufacturing, ripening and storing (Georgescu, 2014).

However, raw milk, traditional cheeses are not always safe, being associated with food borne infections or intoxications (Öner et al., 2006; Choi, 2016; Little, 2008; Prates Denise da Fontoura, 2017; Bintsis, 2002; Gao, 2017; Tăpăloagă, 2017; Ilie, 2018).

Various natural antimicrobial solutions have been proposed by extensive literature, for improving the microbiological quality of such cheeses, among which, *Nigella sativa* (Georgescu, 2018a; Georgescu, 2018b; Georgescu, 2018). *Nigella sativa* is known for a large variety of accepted or potential applications in the medical field, as numerous reviews of the literature communicate (Georgescu et al., 2018c).

Nevertheless, the antimicrobial activity of *Nigella sativa* against cheese contaminating microorganisms is not yet completely understood, especially because of the complexity of the microbiological and physicochemical processes which take place during cheese ripening (Georgescu et al., 2015).

Complex ripening processes affecting cheeses are extremely different among cheese types and the pattern of these modifications is difficult to predict, especially when cheeses are enriched with natural antimicrobials such as *Nigella sativa* seed oil (NSSO).

The unexplored implications of the particular microbiological and physicochemical dynamics occurring during ripening of traditional cheese, should also be considered because of the specificity for each cheese type.

The available scientific literature offers data on raw milk quality (Ilie, 2017a; Ilie, 2017b), but does not provide enough reports on this

important topic related to traditional raw milk cheeses.
In this context, the paper presents an assessment of the moisture fluctuation pattern for NSSO enriched cheese, in order to evaluate the impact of such enrichment over the primary composition of the product, during ripening.

## MATERIALS AND METHODS

The experiment included three batches of cheese: control cheese without NSSO, 0.1% w/w NSSO enriched cheeses and 0.2% w/w NSSO enriched cheeses. *Nigella sativa* cold pressed seed oil. *Nigella sativa* (black cumin) cold pressed seed oil (Negriol) was purchased from a company in Romania, Aghoras Invent SRL, Bucharest. The NSSO was added to the mildly heated milk in the respective concentrations, before renneting. The content of the Ideal® rennet dose (8g) was diluted in 250 mL warm distilled water and 25 mL solution were added to 10 l warm milk (30-35°C), under continuous manual mixing for 10 minutes.
Coagulation time was 30-45 minutes. The soft curd was left for further solidification needed for processing, for 15-30 minutes.
Further stages of the technological process were followed according to the usual, traditional Telemea cheese manufacturing (Tăpăloagă, 2013).
The soft curd was processed through repeated pressing and then it was cut into pieces (Figure 1). It was left resting for 20 minutes before cutting to final size cubes (12/12 cm).
The brine concentration used for experimental Telemea was 6-8%. Brining was performed at maximum 16°C, for 24 hours and was followed by drying for 12 hours at 2-8°C on wood shelves. Experimental Telemea cheese was packed in vacuumed plastic bags and kept refrigerated (4-8°C) for 42 days.
Experimental Telemea cheese samples were analyzed in duplicates for moisture contents, at 0, 14, 28, and 42 days of ripening, according to the AOAC Official Methods of Analysis 926.08.
Data analysis was performed by One way analysis of variance (ANOVA) using SAS (ANOVA version 9.1. SAS institute Inc., Cary, USA, 2003). The threshold of significance level was $p<0.05$.

## RESULTS AND DISCUSSIONS

No significant differences in moisture content were noticed between treatment groups of cheeses (the f-ratio value is 0.76808; the p-value is 0.476501) (Table 1), even though results indicated higher average values for NSSO added cheeses (56.43±0.6065 for 0.1% w/w NSSO and 56.49±0.3312 for 0.2% w/w NSSO, compared with 53.19±0.0907 for control, at 42 days of ripening) (Figure 2).

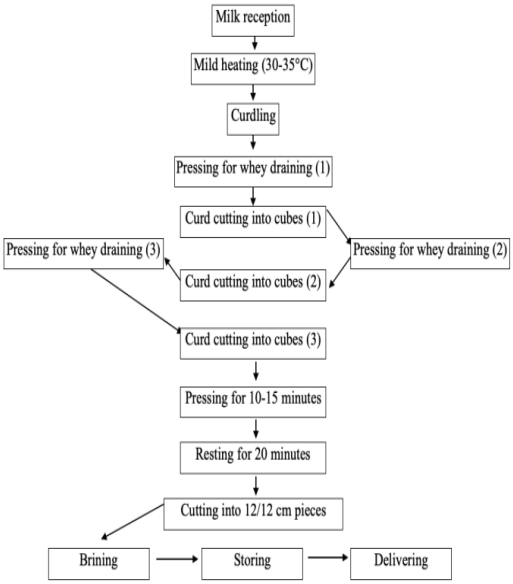


Figure 1. Soft brined raw milk cheese manufacture diagram

Our data indicates moisture loss during ripening, which is consistent with most literature findings (Levkov, 2014), and strong statistical significance could be associated to the trends of moisture decrease (the f-ratio value is 59.07682; the p-value is <0.00001) (table 2).
These results are consistent with other sources which also communicated p values <0.05 for moisture decrease findings (Mahgoub, 2013).
Moreover, most authors report lower moisture values for similar type cheeses throughout processing and ripening (Hassanien, 2014; Hasanzadeh et al., 2017).
This might be explained by not using calcium chloride, not starter culture in cheese manufacturing and by using lower brine concentrations than those reported by the literature (Mestani, 2017; Pappas, 2007).

Table 1. Moisture content of experimental Telemea cheeses over 42 days of ripening (%)

| Assessment time | control | 0.1% w/w NSSO | 0.2% w/w NSSO |
|---|---|---|---|
| **fresh** | 62.64±0.17 | 62.74±0.11 | 63.33±0.07 |
| **Day 14** | 59.21±0.04 | 61.57±0.02 | 62.18±0.12 |
| **Day 28** | 56.47±0.09 | 58.88±0.06 | 59.38±0.03 |
| **Day 42** | 53.19±0.09 | 56.43±0.6 | 56.49±0.33 |

When comparing the values of moisture registered for 0.2% w/w NSSO cheese batch, with those obtained for the control batch, it is obvious that there are slightly higher water percent numbers for the NSSO enriched cheeses. Data analysis indicates that this moisture improvement for the NSSO enriched cheese is strongly associated ($p< 0.05$) (Table 3). This is suggesting that NSSO enrichment might have a positive impact on water loss in such soft cheeses during ripening, which is a positive aspect, strongly related to consumers perception over the sensory qualities of cheese. These results are consistent with similar other research findings provided by the scientific literature (Hassanien et al., 2015).

Table 2. Data analysis for the trends of moisture decrease

| Data analysis item | Treatments | | | | Total |
|---|---|---|---|---|---|
| | Fresh | Day 14 | Day 28 | Day 42 | |
| **N** | 9 | 9 | 9 | 9 | 36 |
| **∑X** | 548.93 | 548.93 | 524.21 | 498.36 | 2139.6 |
| **Mean** | 63.1222222 | 60.9922222 | 58.2455556 | 55.3733333 | 59.433 |
| **St.dev.** | 0.65840295 | 1.35892216 | 1.35096365 | 1.6695134 | 3.2095 |
| | **Result details** | | | | |
| | **SS (sum of squares)** | **Df** | **MS (mean square)** | | |
| **Between treatments** | 305.3921 | 3 | 101.7974 | | |
| **Within treatments** | 55.1403 | 32 | 1.7231 | | |
| **Total** | 360.5324 | 35 | | | |
| **f-ratio value** | 59.07682 | | | | |
| **p-value** | < .00001 | | | | |
| The result is significant at $p < 0.05$. | | | | | |

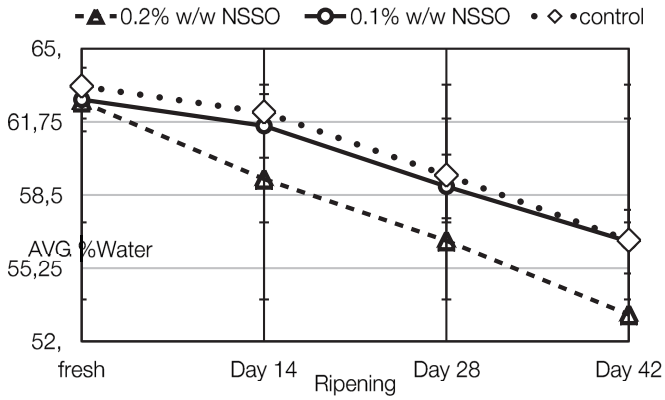


Figure 2. Moisture content of NSSO enriched soft brine cheese over ripening, compared to control cheese batch

Table 3. Data analysis for the moisture retention comparison – control batch versus *0.2% w/w NSSO cheese batch*

| **Data analysis item** | **Treatments** | | | | **Total** |
|---|---|---|---|---|---|
| | **Control** | **0.2% w/w NSSO cheese batch** | | | |
| **N** | 9 | 9 | | | 18 |
| **∑X** | 506.65 | 534.19 | | | 1040.84 |
| **Mean** | 56.2944 | 59.3544 | | | 57.824 |
| **∑X2** | 28576.1187 | 31755.3701 | | | 60331.4888 |
| **Std.Dev.** | 2.611 | 2.4703 | | | 2.9255 |
| | **Result details** | | | | |
| | **SS** | **Df** | **MS** | | |
| **Between treatments** | 42.1362 | 1 | 42.1362 | | |
| **Within treatments** | 103.3578 | 16 | 6.4599 | | |
| **Total** | 145.494 | 17 | | | |
| **f-ratio value** | 6.52277 | | | | |
| **p-value** | 0.021232 | | | | |
| The result is significant at $p < 0.05$. | | | | | |

## CONCLUSIONS

Data revealed slightly higher moisture values for ripened NSSO treated cheeses, which links positively to higher consumers' appreciation of treated cheeses by the end of ripening. However, the slightly higher moisture values for the NSSO added cheeses were positively associated (p-value - 0.05).

The data indicates slightly better moisture retention for 0.2% w/w NSSO cheese batch, which might be linked to a possible prevention of loss of body and texture loss during ripening of soft brined cheese.

This study shows that NSSO could be used as a natural enhancer of Telemea cheese flavor and taste and could be a possible solution for the improvement of the sanitary quality of this artisanal raw milk soft, brined cheese.

Without compromising the original characteristics and nutritional value, NSSO enrichment of cheese could ease the successful promotion of more traditional cheeses, safe for consumption, on the national and international market.

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# INFLUENCE OF SLAUGHTERING TECHNOLOGICAL PARAMETERS ON SENSORIAL QUALITY OF REFRIGERATED POULTRY MEAT

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***Abstract***

*A short review of literature on sensorial analysis of avian products demonstrate a great diversity of sensorial procedures, such kind of different approaches impending realisation of comparisons between studies. To optimize technological parameters involved on slaughtering technological flow and to achieve the proposed aims, was elaborated an experimental protocol, designed for optimization of slaughtering technology into a processing unit from perspective of technical parameters applied at stages with impact on resulted carcasses, the effects being examined by sensorial qualitative parameters. For realization of study were formed three experimental batches, slaughtering technological line being the logistic base which allowed modification of technological parameters in stunning, bleeding, scalding and chilling stages. After thermal treatment, aroma intensity for muscular samples at chest level was described by mean values into interval 5.22±0.449 points (L3) and 7.77±0.375 points (L2), metallic aroma being the most pronounced one, given by means between 2.13±0.443 points (L1) and 3.37±0.593 points (L2), followed in a descendant way by the descriptive score for fried aroma. At the opposite pole, descriptive points for rancid aroma were sub-unitary, fact which shows the incipient stage of oxidation reactions for the adherent fat to analyzed muscular tissue.*

***Key words****: slaughtering, technological, sensorial, poultry.*

## INTRODUCTION

Sensorial analysis is the science which uses human perceptions to realize a characterization of a product in an objective and reproducible way. Measuring instruments are represented by humans, an essential role in analysis being given to description into an exhaustive and detailed way of the utilized methodology for reducing the intrinsic error, which is commonly presented in such kind of measurement (Perlo et al., 2006).

In poultry meat case, to obtain real sensorial descriptors is important to realize the tasting of samples gathered from birds differentially from musculature point of view, parameters which are different applied in processing stages and storage method because lipids' oxidation was associated with unfavourable modifications for aspect, aroma and texture (Jensen et al., 1998).

A short review of literature on sensorial analysis of avian products demonstrate a great diversity of sensorial procedures, such kind of different approaches impending realisation of comparisons between studies (Vermein et al., 2006; Chartrin et al., 2006; Kennedy et al., 2005; Rababah et al., 2005; Liu et al., 2004).

In refrigerate state, poultry meat present an own taste, thermal processing conferring to meat samples a taste specific to recipe (Northcutt et al., 2001). Literature describes refrigeration influence on poultry meat taste (Mielnik et al., 1999), loosening of aromatic compounds taking place, mainly, during chilling by immersion (Barham, 2001; Lawless and Heymann, 2010). The research aimed to evaluate the qualitative parameters for broiler chicken meat from sensorial perspective, determining being the implied factors in slaughtering process from stunning, bleeding, scalding and chilling carcasses stages.

## MATERIALS AND METHODS

Research was focused on a single anatomic region, respectively chest, being realised three experimental batches, and in which were modified the parameters of slaughtering process, as follows: For experimental batch L1, technical parameters for each slaughtering process stage

were: Stunning – was realised at 70 V power voltage, 180 mA intensity and 360 Hz frequency. Conveyer's speed was de 0.16 m/s, and time covered in stunning bath was 22 s/bird.
Bleeding – necessary time for bleeding was 185 s, at a conveyers' speed of 0.16 m/s.
Scalding–water temperature in scalding device was 52°C, for a period of 180 s. Conveyer's speed at scalding were 0.16 m/s.
Chilling of carcasses– air temperature in refrigeration tunnel was 2–4°C, with a 3.5 m/s flow speed of air currents. Also, chilling water temperature had values between 3 and 5°C. Initial temperature of carcasses, at entrance in chilling tunnel, was 35–39°C, and after 105 minutes to reach values of 3–4°C.
In case of experimental batch L2, current voltage in stunning basins was 85 V, at an intensity of 160 mA and a frequency of 380 Hz. Conveyer's speed at stunning was 0.24 m/s, and transition time through stunning bath was 15 s/bird.
The bleeding period of carcasses was 130 seconds at a conveyer' speed of 0.24m/s.
Water temperature in basins for realisation of scalding stage was 54°C for 120 s, at a conveyer's speed 0.24 m/s.
Chilling of carcasses was realised both in cold air flow, at a temperature of 0–3°C and a 3.5 m/s speed for air currents, as well as by pulverization with water at a temperature of 2–4°C. Time duration for chilling of carcasses from 35–39°C to 2–4°C was of 105 minutes.
Regarding experimental batch L3, stunning was realised by immersion in basins with water and the current voltage was 100 V, at an intensity of 150 mA and a frequency of 400 Hz. Conveyer's speed at stunning was 0.28 m/s, and transition time through stunning bath was 13 s/bird.
Bleeding stage was realised on duration of 112 seconds, and at conveyer's speed of 0.28 m/s.
Carcasses'scalding were realised in water at a temperature of 58°C, by immersion of carcasses in basins for 100 seconds, conveyer's speed being 0.28m/s. Air temperature in refrigeration tunnel was 0–2°C, air currents speed was 2.5m/s, carcasses being pulverized with water at the temperature of 1–3°C. Initial carcasses temperature was 35–39°C, and after 90 minutes reached values between 1 and 2°C.
Each experimental batch was constituted by 25 ROSS 308 chicken broiler individuals, aged 42 days. The utilised methodology in sampling was based on own experience corroborated with methodologies mentioned in other studies, sensorial evaluation taking place on chest samples, gathered at 24 h after slaughtering (Sebranek et al., 1979; Civille and Lyon, 1996).
Portioning of meat samples and further technical processing of them into a preheated oven at 120°C for 20 minutes aimed to reach a frying temperature of 75°C in centre of each sample, monitoring being realised with K type thermocouple, at taking out from oven, samples were identified, coded and warm served to tasters in ceramic bowls utilised also during thermal treatment.
To balance the samples' presentation order was utilised the model described by McFie et al., 1989. Establishing the score for analysed characteristics, tasters used a cube from analysed sample for descriptive parameters of aroma and a cube for the texture ones, analysis of sensorial perception being realised in controlled light conditions.
Working methodology imposed rinsing of oral cavity with sparkling water before beginning of sensorial analysis and between samples' tasting. After a preliminary selection and validation phase (method adapted after Meilgaard et al., 1991) were chosen 17 parameters for aspect, aroma and texture characteristics.
Sensorial evaluation took place into a sensorial tasting chamber, equipped with devices for a constant maintaining of air pressure, individual boxes and lights designed to mask the obvious colour differences (ISO, 1988), excluding visual evaluation. The analyses were effectuated in Sensorial Analysis Laboratory UNI-ISN 8589 belonging to Department of Agricultural Sciences and Environment from University of Udine – Italy, for argumentation of sensorial profile being involved 8 qualified tasters. Each of them tasted 3 samples in 5 sessions (repetitions), and their answers being related to a linear scale with 10 points (Ruiz et al., 2001).
Significance of differences between the established means for the samples gathered from those three batches (L1, L2, L3) was calculated with statistic programme IBM SPSS 20.0 using T test with two variables (**T-Test** (2-tailed)).

## RESULTS AND DISCUSSIONS

On a 10 points scale, aspect of meat samples gathered at the level of pectoral musculature from slaughtered chickens' carcasses was described by means situated in interval 1.29±0.301 points (L3) and 2.72±0.428 points (L2). Mean uniformity of colour for analysed samples was characterized by an interval between 7.26±0.457 (L3) – 9.46±0.299 points (L1).

In case of colour aspect, the results of hedonic analysis show a certain uniformity between batches, while colour uniformity enlightened a visual declassification of chest from batch L3 by a difference greater than 2 points between batches L3 and L1, respectively L3 and L2 (Table 1).

Table 1. Sensory descriptive parameters for poultry meat colour (chest muscles) subjected to chilling, depending on technological slaughter regime

| Specification | | Exp. batch | n | $\bar{X} \pm s_{\bar{x}}$ | V% | Min. – Max. | Interpretation of differences T-Test (2-tailed) | |
|---|---|---|---|---|---|---|---|---|
| COLOUR | Aspect of colour | L1 | 25 | **1.56**±0.227 | 56.240 | 0.40 – 3.70 | L1-L2 | t = -2.211; p = 0.044$^{*}$ |
| | | L2 | 25 | **2.72**±0.428 | 61.009 | 0.40 – 5.80 | L1-L3 | t =0.683; p = 0.506$^{ns.}$ |
| | | L3 | 25 | **1.29**±0.301 | 90.724 | 0.20 – 4.70 | L2-L3 | t = 3.287; p = 0.005$^{**}$ |
| | Uniformity of colour | L1 | 25 | **9.46**±0.299 | 12.258 | 5.40 – 10.00 | L1-L2 | t = 0.397; p = 0.697$^{ns.}$ |
| | | L2 | 25 | **9.31**±0.223 | 9.280 | 6.70 – 10.00 | L1-L3 | t = 3.709; p = 0.002$^{**}$ |
| | | L3 | 25 | **7.26**±0.457 | 24.379 | 3.40 – 9.60 | L2-L3 | t = 3.602; p = 0.003$^{**}$ |

**T- test** (2-tailed)– for each analysed character, comparative on experimental batches:
$^{ns.}$insignificant differences (p>0.05); $^{*}$significant differences (p<0.05);$^{**}$distinct significant differences (p<0.01).

At the end of thermal treatment, intensity of aroma for chest muscular samples was described by means into interval 5.22±0.449 points (L3) and 7.77±0.375 points (L2), metallic aroma being the most pronounced one, having means between 2.13±0.443 points (L1) and 3.37±0.593 points (L2), followed in a descendant way by the descriptive score for fried aroma. Opposite, the descriptive points for rancid aroma were much sub-unitary, fact which shows the incipient stage of oxidation reactions for the adherent fat to analyzed muscular tissue.

The obtained results for sensorial analysis of aromatic profile of pectoral musculature gathered from the carcasses of chickens which were subjected to slaughtering enlightened the aromatic superiority of samples from batch L2 due to maximal means for aroma intensity, fried aroma and peanuts aroma, corroborated with sub-unitary values for rancid aroma, followed in descendant way by musculature samples from batch L1 and L3 (Table 2).

Table 2. Sensory descriptive parameters for poultry meat flavour (chest muscles) subjected to chilling, depending on technological slaughter regime

| Specification | | Exp. batch | n | $\bar{X} \pm s_{\bar{x}}$ | V% | Min. – Max. | Interpretation of differences T-Test (2-tailed) | |
|---|---|---|---|---|---|---|---|---|
| AROMA | Intensity of aroma | L1 | 25 | **6.15**±0.426 | 26.801 | 3.20 – 8.70 | L1-L2 | t = -3.428; p = 0.004$^{**}$ |
| | | L2 | 25 | **7.77**±0.375 | 18.688 | 5.10 – 9.60 | L1-L3 | t = 1.472; p = 0.163$^{ns.}$ |
| | | L3 | 25 | **5.22**±0.449 | 33.317 | 2.30 – 7.50 | L2-L3 | t = 4.335; p = 0.001$^{**}$ |
| | Fried aroma | L1 | 25 | **1.49**±0.238 | 61.710 | 0.30 – 3.24 | L1-L2 | t = -2.083; p = 0.056$^{ns.}$ |
| | | L2 | 25 | **2.63**±0.405 | 59.538 | 0.30 – 6.20 | L1-L3 | t = -1.468; p = 0.164$^{ns.}$ |
| | | L3 | 25 | **2.16**±0.334 | 59.939 | 0.20 – 4.30 | L2-L3 | t = 1.424; p = 0.176$^{ns.}$ |
| | Peanuts aroma | L1 | 25 | **0.85**±0.278 | 126.379 | 0.00 – 4.30 | L1-L2 | t = -1.858; p = 0.084$^{ns.}$ |
| | | L2 | 25 | **1.32**±0.346 | 101.630 | 0.00 – 4.80 | L1-L3 | t = 1.152; p = 0.269$^{ns.}$ |
| | | L3 | 25 | **0.56**±0.242 | 167.352 | 0.00 – 3.50 | L2-L3 | t = 3.302; p = 0.005$^{**}$ |
| | Rancid aroma | L1 | 25 | **0.11**±0.031 | 108.311 | 0.00 – 0.40 | L1-L2 | t = 1.821; p = 0.090$^{ns.}$ |
| | | L2 | 25 | **0.05**±0.027 | 198.769 | 0.00 – 0.30 | L1-L3 | t = 3.233; p = 0.006$^{**}$ |
| | | L3 | 25 | **0.03**±0.021 | 238.298 | 0.00 – 0.30 | L2-L3 | t = 1.193; p = 0.253$^{ns.}$ |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Metallic / blood aroma** | **L1** | 25 | **2.13**±0.443 | 80.669 | 0.40 – 5.80 | **L1-L2** | t = -2.028; p = 0.062$^{ns.}$ |
| | **L2** | 25 | **3.37**±0.593 | 68.240 | 0.60 – 7.50 | **L1-L3** | t = -0.983; p = 0.342$^{ns.}$ |
| | **L3** | 25 | **2.95**±0.569 | 74.747 | 0.00 – 6.80 | **L2-L3** | t = 0.671; p = 0.513$^{ns.}$ |

**T- test** (2-tailed)– for each analysed character, comparative on experimental batches:
$^{ns.}$insignificant differences (p>0.05); $^{*}$ significant differences (p<0.05);$^{**}$ distinct significant differences (p<0.01).

After thermal treatment, pectoral musculature from chickens' carcasses of experimental batches was described by a relatively intense sweetish taste, evaluated by means which are into interval 4.93±0.404 (L1) – 5.63±0.485 points (L3) on a 10 points scale. Umami sensation, described by means in interval 1.75±0.283 (L3) – 2.96±0.578 points (L2) is a clue for proteins' presence, this basic taste being especially conferred by monosodium glutamate and ribonucleotides. The results of evaluation show a ranking for batches, sweet taste corroborated with umami one, non-bitter and salty environment placing in favourable zone the muscular samples of chickens' carcase from batch L2, on the second place being chicken meat samples from batch L1, followed by the ones belonging to batch L3 (Table 3).

Table 3.Sensory descriptive parameters for poultry meat taste (chest muscles) subjected to chilling, depending on technological slaughter regime

| **Specification** | | **Exp. batch** | **n** | $\bar{X} \pm s_{\bar{x}}$ | **V%** | **Min. – Max.** | **Interpretation of differences T-Test** (2-tailed) | |
|---|---|---|---|---|---|---|---|---|
| **TASTE** | **Sweet taste** | **L1** | 25 | **4.93**±0.404 | 31.733 | 2.50 – 7.20 | **L1-L2** | t = -0.726; p = 0.480$^{ns.}$ |
| | | **L2** | 25 | **5.37**±0.520 | 37.482 | 3.10 – 8.90 | **L1-L3** | t = -1.064; p = 0.305$^{ns.}$ |
| | | **L3** | 25 | **5.63**±0.485 | 33.370 | 2.90 – 8.50 | **L2-L3** | t = -0.324; p = 0.751$^{ns.}$ |
| | **Umami taste** | **L1** | 25 | **2.01**±0.434 | 83.405 | 0.00 – 7.10 | **L1-L2** | t = -1.686; p = 0.114$^{ns.}$ |
| | | **L2** | 25 | **2.96**±0.578 | 75.649 | 0.00 – 9.20 | **L1-L3** | t = 0.632; p = 0.538$^{ns.}$ |
| | | **L3** | 25 | **1.75**±0.283 | 62.649 | 0.00 – 4.20 | **L2-L3** | t = 2.218; p = 0.044$^{*}$ |
| | **Salty taste** | **L1** | 25 | **0.52**±0.109 | 81.352 | 0.00 – 1.20 | **L1-L2** | t = 0.980; p = 0.344$^{ns.}$ |
| | | **L2** | 25 | **0.41**±0.053 | 50.190 | 0.12 – 0.73 | **L1-L3** | t = 0.414; p = 0.685$^{ns.}$ |
| | | **L3** | 25 | **0.43**±0.163 | 148.103 | 0.00 – 2.10 | **L2-L3** | t = -0.097; p = 0.924$^{ns.}$ |
| | **Acid taste** | **L1** | 25 | **0.68**±0.239 | 136.399 | 0.10 – 3.60 | **L1-L2** | t = -0.159; p = 0.876$^{ns.}$ |
| | | **L2** | 25 | **0.72**±0.172 | 92.754 | 0.00 – 2.00 | **L1-L3** | t = -1.117; p = 0.283$^{ns.}$ |
| | | **L3** | 25 | **1.05**±0.130 | 47.765 | 0.11 – 2.09 | **L2-L3** | t = -1.653; p = 0.121$^{ns.}$ |
| | **Bitter taste** | **L1** | 25 | **0.03**±0.015 | 170.610 | 0.00 – 0.20 | **L1-L2** | t = 1.505; p = 0.154$^{ns.}$ |
| | | **L2** | 25 | **0.01**±0.009 | 263.899 | 0.00 – 0.10 | **L1-L3** | t = -2.199; p = 0.045$^{*}$ |
| | | **L3** | 25 | **0.08**±0.014 | 70.567 | 0.00 – 0.21 | **L2-L3** | t = -4.149; p = 0.001$^{**}$ |

**T- test** (2-tailed)– for each analysed character, comparative on experimental batches:
$^{ns.}$insignificant differences (p>0.05); $^{*}$significant differences (p<0.05);$^{**}$distinct significant differences(p<0.01).

Muscular samples gathered from chicken carcasses belonging to batch L2 obtained the most favourable mean scores, meat being described by an intermediary granulosity, fibrosity, succulence and unctuosity and a minimal adhesiveness. Second place was occupied by texture of muscular samples from carcasses of batch L1, followed by the ones obtained for batch L3, which even if presented means in the inferior zone for granulosity and fibrosity, minimal succulence and maximal adhesiveness countered and cancelled their positivity (Table 4).

Table 4. Sensory descriptive parameters for chilled poultry meat texture, depending on technological slaughter regime (L1, L2, L3 experimental batches)

| | Specification | Exp. batch | n | $\bar{X} \pm s_{\bar{x}}$ | V% | Min. – Max. | Interpretation of differences T-Test (2-tailed) | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| TEXTURE | Granulosity | **L1** | 25 | **5.89**±0.487 | 32.007 | 2.30 – 8.60 | **L1-L2** | t = 1.904; p = 0.078$^{ns.}$ |
| | | **L2** | 25 | **4.65**±0.542 | 45.193 | 1.40 – 8.20 | **L1-L3** | t = 3.156; p = 0.007$^{**}$ |
| | | **L3** | 25 | **3.47**±0.417 | 46.573 | 0.80 – 6.30 | **L2-L3** | t = 1.942; p = 0.073$^{ns.}$ |
| | Adhesiveness | **L1** | 25 | **1.46**±0.302 | 80.077 | 0.20 – 4.20 | **L1-L2** | t = 1.926; p = 0.075$^{ns.}$ |
| | | **L2** | 25 | **0.85**±0.298 | 136.348 | 0.00 – 3.10 | **L1-L3** | t = -0.719; p = 0.484$^{ns.}$ |
| | | **L3** | 25 | **1.84**±0.349 | 73.470 | 0.40 – 4.80 | **L2-L3** | t = -2.245; p = 0.041$^{*}$ |
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| TEXTURE | Succulence | **L1** | 25 | **2.44**±0.364 | 57.737 | 0.20 – 5.10 | **L1-L2** | t = 1.882; p = 0.081$^{ns.}$ |
| | | **L2** | 25 | **1.79**±0.332 | 71.953 | 0.40 – 4.00 | **L1-L3** | t = 1.743; p = 0.103$^{ns.}$ |
| | | **L3** | 25 | **1.56**±0.363 | 90.254 | 0.10 – 5.20 | **L2-L3** | t =0.452; p = 0.658$^{ns.}$ |
| | Fibrosity | **L1** | 25 | **6.15**±0.475 | 29.941 | 3.40 – 8.90 | **L1-L2** | t = 1.249; p = 0.232$^{ns.}$ |
| | | **L2** | 25 | **5.48**±0.558 | 39.466 | 2.20 – 8.70 | **L1-L3** | t = 2.244; p = 0.041$^{*}$ |
| | | **L3** | 25 | **4.73**±0.526 | 43.083 | 2.20 – 8.20 | **L2-L3** | t = 1.054; p = 0.310$^{ns.}$ |
| | Unctuosity | **L1** | 25 | **0.09**±0.041 | 175.933 | 0.00 – 0.60 | **L1-L2** | t = -0.482; p = 0.638$^{ns.}$ |
| | | **L2** | 25 | **0.12**±0.064 | 203.466 | 0.00 – 1.00 | **L1-L3** | t = -1.278; p = 0.222$^{ns.}$ |
| | | **L3** | 25 | **0.19**±0.058 | 121.986 | 0.00 – 0.90 | **L2-L3** | t = -0.768; p =0.455$^{ns.}$ |

**T- test** (2-tailed)– for each analysed character, comparative on experimental batches:
$^{ns.}$insignificant differences (p>0.05); $^{*}$significant differences (p<0.05);$^{**}$distinct significant differences(p<0.01).

## CONCLUSIONS

The research results represent comparison data regarding quality parameters for poultry meat which is commercialized in East area of Romania with the ones presented in literature and are exact data which could be utilised by units' management to adopt some changes regarding productive and qualitative efficiency.
Aiming the general view of analysed sensorial characters we could appreciate the qualitative superiority of pectoral musculature from chickens' carcasses belonging to experimental L2 due to favourable scores for colour and uniformity, corroborated with aroma intensity, the fried and peanuts one, completed by sweet and savoury taste, umami, almost imperceptibly salty, acid and bitter and by intermediary textural parameters. Second place was occupied by meat samples from chicken carcasses belonging to L1, followed by the ones from batch L3.

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# THE AMINOACID CONTENT IN PROTEIN MINERAL CONCENTRATES OBTAINED AT DIFFERENT ELECTROPHYSICAL PROCESSING REGIMES OF WHEY

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***Abstract***

*It was investigated the amino acid content in protein mineral concentrates obtained during electrophysical processing of different types of whey at membrane electrolyzer EDP-4 at current density j=10 mA/cm2 and J=20mA/cm2. It was established that the degree of amino acids isolation in the protein mineral concentrates during electrophysical processing of whey depends on the: current density, duration (time) of processing, and on the type of whey. Varying these parameters, the content of essential and functional amino acids in the protein mineral concentrates during electrophysical processing can be modeled. The maximum degree of isolation of free amino acids, especially of essential amino acids, is recorded during electro-physical processing of whey after the manufacture of the granulated cottage cheese „Grauncior" (company JLC) at current density j=20 mA/cm2 in the 10th min of processing. The most important functional amino acids such as immunoactive, sulfur containing and branched-chain amino acids have the same character of content variations in the protein mineral concentrates as essential amino acids during electrophysical processing of whey.*

***Key words****: whey, amino acids, electrophysical processing.*

## INTRODUCTION

The aminoacid composition is the most important factor in defining food protein quality, followed by the digestibility of the protein and the bioavailability of its amino acids. Due to their aminoacid composition the main bovine milk proteins – caseins and whey proteins, can be regarded as a complete source of aminoacids (Sindayikengera and Xia, 2006; Arghiriade et al., 2013). It is known that whey proteins make up about 20% of the milk proteins. The remaining 80% is casein. But milk whey proteins have been considered superior to casein from the nutritional point of view (Farrell et al., 2011). These proteins present the amino acid profile superior to casein, being similar to human milk (Hambraeus, 1982; Pennings et al., 2011). Whey proteins are also leaders in the content of essential amino acids (EAAs) among other important sources of EAAs and are also rich in the branched chain amino acids (BCAAs) that are physiologically extremely important and confer whey proteins an important biological value and thought to play a role as metabolic regulations in protein and glucose homeostasis, and in lipid metabolism (Morato et al., 2006; Smithers, 2008).

The main whey protein fractions also differ by aminoacids profile. In particularly, β-lactoglobulin (β- Lg), which represents one of the main whey protein fractions, is rich in cysteine residues, an aminoacid bearing a key role in stimulating the synthesis of glutathione (Tavares and Malcata, 2013). The main amino acids in quantitative terms of β-Lg are Ala, Asp, Glu, Ile, Leu, Lys, Val. Another whey protein fraction – α-lactalbumin (α-La), is commercially used in food supplements for babies because of its similarity in structure and composition to human milk proteins. The higher content of all EAAs and BCAAs in α-La makes it also an ingredient of choice in supplements for sportsmen (Tavares and Malcata, 2013). The most significant amino acids contained in α-La are Trp and Cys, Leu, Ile and Val (Etzel, 2004). Together with α-La, β-Lg is a major source of EAAs and BCAAs (Etzel, 2004). In the other whey protein fraction – glycomacropeptide (GMP) a higher amount of Glu, Ile, Pro, Ser, Thr, and Val in

comparison with α-La and β-Lg was revealed (Etzel, 2004).

The protein and respectively amino acid content in whey protein concentrates or isolates depend on the methods of their obtaining (manufacturing).

The whey proteins are isolated by application by different physical, chemical and microbiological methods (principles). The most known of whey protein products are: whey protein concentrates, whey protein isolates and whey protein hydrolysates.

Amino acid profile of whey protein concentrate (WPC) 80 and its hydrolysates consist in a high content of sulfur-containing amino acids – Met and Cys; a high concentration of Leu, Ile, Lys, Thr; and a relatively low content of the aromatic amino acids Phe and Tyr. Generally, high digestibility of milk protein is due to Tyr, Phe, Ile, Leu, and Lys content. By comparing essential amino acid acids pattern of human milk with that of WPC, sodium caseinate and their hydrolysates, it is apparent that WPC 80 and its hydrolysates are more similar to human milk than sodium caseinate and its hydrolysates (Sindayikengera and Xia, 2006).

Whey processing operations could potentially affect the content of amino acids. Amino acid profiles showed that excessive heating of whey (121°C, 5000s) destroys a significant part of Cys at, Lys, and Arg. Excessive heating also decreases the availabilities of Lys, Pro, Asp, Glu, Thr, Ala, Gly and Ser. Severe heating decreased the availabilities of Cys, Tyr and Arg, probably as a result of structural modifications of the protein upon heating (Desrosiers and Savoie, 1991).

Thus the manufacture of protein concentrates requires certain rules in order to maintain a high degree of purity in the protein native form, namely, to exclude thermal denaturation, which, in the case of whey proteins is 55-65$^{o}$C (Etzel, 2004), and chemical denaturation of proteins and chemical modification of amino acids (Desrosiers and Savoie, 1991; Cheftel, 1977).

It was established that electrophysical processing of whey is a wasteless method that allows the valorification of all whey components. Besides, this type of processing allows controlling the content of whey proteins in the obtained concentrates, depending on the processing regime. Extraction of whey proteins and obtaining of protein-mineral concentrates (PMCs) of a high value under the action of an electric current and avoiding the direct usage of chemicals is an advantageous process based on modern principles, which exclude the thermal and chemical denaturation of proteins (Bologa et al., 2009, 2010; Vrabie et al., 2018).

The aim of this study was the investigation of the content of amino acids in protein-mineral concentrates during the electrophysical whey processing at different processing regimes.

## MATERIALS AND METHODS

In the framework of the experiments of electrophysical processing two types of whey provided by the **"**JLC" Joint Stock Company, Chisinau, Republic of Moldova, after the manufacture of the*:* granulated cottage cheese "Grauncior"; "Cottage cheese", 2% fat content were used. The electrophysical processing of whey was performed at the experimental membrane electrolyzer, at j=10-20 mA/cm$^2$, in the stationary regime, specially designed for collecting the samples so as to study amino acids (Maximuc et al., 2008). All PMCs were collected every 5 minutes and in the cathode cell (CC) (Bologa et al., 2009).

The determination of the content of amino acids in the studied samples was done by the ion-exchange chromatography (Moore et al., 1958) at aminoacid analyzer AAA-339M.

## RESULTS AND DISCUSSIONS

In our experiments, 18 proteinogenic aminoacids out of 20 were detected: Asp includes both Asp and Asn and Glu includes both Glu and Gln (in the process of detection Asn is combined with Asp and Gln with Glu and so they have the identical picks that reflect the quantity of extraction).

To determine what is the best (optimal) regime to remove a higher amount of aminoacids, especially essential ones, from two types of whey - after the manufacture of the granulated cottage cheese "Grauncior" and of the „Cottage cheese", 2% fat content – the dynamic of variation of the aminoacid content at different current density: j=10 mA/cm$^2$ and j=20 mA/cm$^2$ was compared (Figure 1).

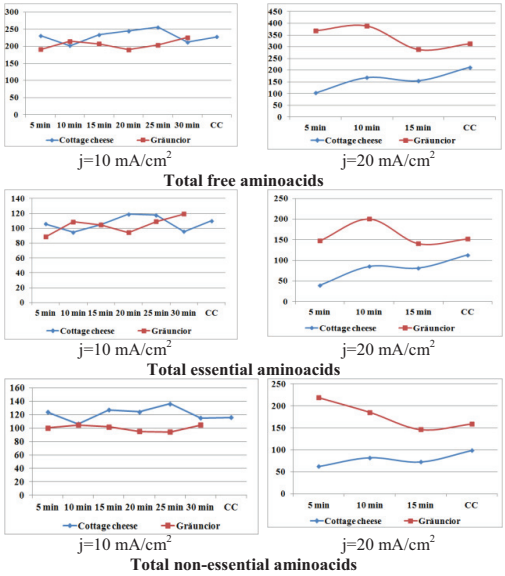


Figure 1. Variations of total free amino acids of whey after manufacture of granulated cottage cheese "Grauncior" and "Cottage cheese", 2% fat content during electrophysical processing, in stationary regime, at current density j=10 mA/cm$^2$ and j=20 mA/cm$^2$

A higher degree of total free aminoacids isolated in the PMCs was established during electrophysical processing of whey:

- after the manufacture of the "Cottage cheese", 2% fat content, at current density j=10 mA/cm$^2$; and
- after the manufacture of the granulated cottage cheese "Grauncior", at current density j=20 mA/cm$^2$.

The maximum content of essential and non-essential amino acids in PMCs was also recorded during electrophysical processing of whey:

- after the manufacture of the "Cottage cheese", 2% fat content, at current density j=10 mA/cm$^2$; and
- after the manufacture of the granulated cottage cheese "Grauncior", at current density j=20 mA/cm$^2$.

Thus, the greatest amount of free, essential and non-essential aminoacids is established at the whey after the manufacture of the granulated cottage cheese "Grauncior", at current density j=20 mA/cm$^2$ in the 5$^{th}$ and 10$^{th}$ minutes of electrophysical processing.

The variation of the content of essential aminoacids in the PMCs during electrophysical processing with membrane electrolyzer of the granulated cottage cheese "Grauncior" in the stationary regime of treatment, at a current density of 10 mA/cm$^2$ is shown in Figure 2.

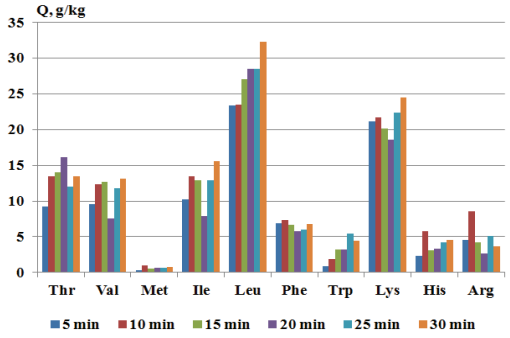


Figure 2. Variations of content of essential aminoacids in PMCs during electrophysical processing of whey after manufacture of granulated cottage cheese „Grauncior" in stationary regime, at current density j=10 mA/cm$^2$

From the presented data in Figure 2 it is seen that during electrophysical processing of whey after the manufacture of the granulated cottage cheese "Grauncior" at a current density of j=10 mA/cm$^2$, all essential aminoacids are extracted in the PMCs, but the degree of isolation of each amino acid in the PMCs varies depending upon the time of electrophysical whey processing: four essential amino acids (Met, Phe, His and Arg) have the maximum degree of isolation at 10 minutes of electrophysical processing, two (Thr and Leu) – at 20 min of processing, two (Trp and Leu) – at 25 min (amount of isolated Leu is identical at 20 and 25 min of processing) and three aminoacids – Val, Ile and Lys – have the maximum degree of isolation at 30 min of electrophysical processing.

The quantitative spectrum of essential aminoacids of whey after the manufacture of the "Cottage cheese", 2% fat content, during electrophysical processing with membrane electrolyzer, at a current density of j=10 mA/cm$^2$ is shown in Figure 3.

It is established that two out of ten essential aminoacids have the highest degree of isolation at 5 min (His and Arg), five – at 20 min of processing (Thr, Val, Met, Ile, Leu), two – at 25 min (Phe and Trp) and one – at 30 min (Trp). Met has the same maximum quantity at 20 and 25 min of processing and Trp – at 25 and 30 min. The maximum content of Lys is detected in the cathode cell, although a higher

degree of this aminoacid is recorded at 20 and 25 min of electrophysical processing.

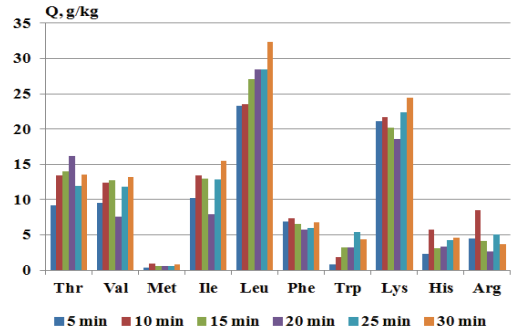


Figure 3. Variations of content of essential aminoacids in PMCs during electrophysical processing of whey after manufacture of "Cottage cheese", 2% fat content, in stationary regime, current density j=10 mA/cm$^2$

Data analyses of variation of essential amino acids content in the whey after the manufacture of the granulated cottage cheese "Grauncior" at a current density of 20 mA/cm$^2$ is shown in Figure 4.

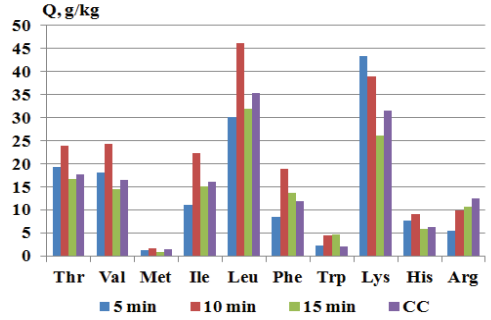


Figure 4. Variations of content of essential aminoacids in PMCs during electrophysical processing of whey after manufacture of granulated cottage cheese "Grauncior", in stationary regime, at current density j=20 mA/cm$^2$

Results revealed that in this type of whey, at current density j=20 mA/cm$^2$, the highest degree of isolation at 10 min of processing have, such essential aminoacids as Thr, Val, Met, Ile, Leu, Phe, Trp; at 5 min – Lys; at 15 min – Trp and Arg (Trp was extracted in the same extent at 10 and15 min of processing, but a higher amount of Arg is found in the cathode cell).

At electrophysical processing of whey after manufacturing of the „Cottage cheese", 2% fat content, at current density j=20 mA/cm$^2$, the majority of essential aminoacids (9 out of 10 – Val, Met, Ile, Leu, Lys , His and Arg) have a higher content in the cathode cell, with the exception of Trp that has the highest degree of isolation at 15 min of processing.

If we compare the process only during 5 min, 10 min and 15 min, then there appears the second peak of quantity that reflects the degree of isolation of Val, Met, Ile, Leu, Lys, His and Arg observed at 10 min of processing (Figure 5).

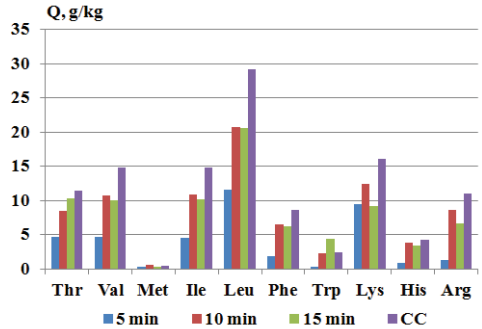


Figure 5. Variations of content of essential aminoacids in PMCs during electrophysical processing of whey after manufacture of "Cottage cheese", 2% fat content, in stationary regime, at current density j=20 mA/cm$^2$

From the obtained data it is clear that the current intensity differently influences the degree of extraction of essential amino acids in the PMCs.

In addition to the essential amino acids, non-essential amino acids were also analyzed, which also have a significant role in nutrition and health. Currently the notion of functional aminoacids is utilized. Within this category of amino acids there are both essential and the non-essential amino acids such as Glu, Pro, Cys, Tyr, etc.

The degree of isolation of non-essential aminoacids of whey after the manufacture of the granulated cottage cheese "Grauncior" at current density j=10 mA/cm is heterogeneous during electrophysical processing: the higher content of Pro was noted at 5 min of processing; Ser, Gly and Glu – at 10 min; Tyr and Cys – at 15 min; Ala and Asp after 30 min of electrophysical processing (Figure 6).

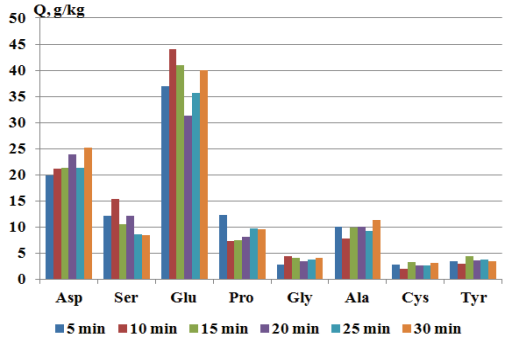


Figure 6. Variations of content of non-essential aminoacids in PMCs during electrophysical processing of whey after manufacture of granulated cottage cheese "Grauncior", in stationary regime, at current density j=10 mA/cm$^2$

The character of non-essential aminoacid isolation during electrophysical processing of whey after the manufacture of the "Cottage cheese", 2% fat content, at current density j=10 mA/cm$^2$, is also heterogeneous as the whey after the manufacture of the granulated cottage cheese "Grauncior" (Figure 7).

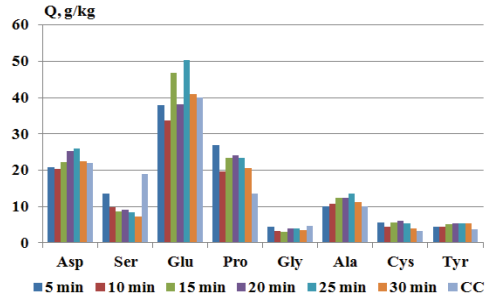


Figure 7. Variations of content of non-essential aminoacids in PMCs during electrophysical processing of whey after manufacture of "Cottage cheese", 2% fat content, in stationary regime, at current density j=10 mA/cm$^2$

After 5 min of electrophysical processing a higher degree of isolation of Ser, Pro and Gly; after 20 min – of Asp, Cys and Tyr; after 25 min – of Glu and Ala was noted (Asp has approximately the same degree of isolation at 20 and 25 min of processing). Also such aminoacids as Ser and Gly have the highest content in the cathode cell *(*Figure 7*)*.

The variation of non-essential aminoacid content of whey after the manufacture of the granulated cottage cheese "Grauncior" at current density j=20 mA/cm$^2$ is shown in figure below.

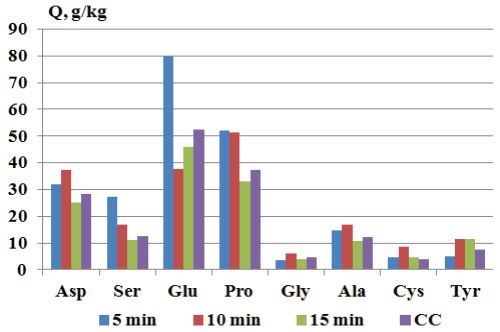


Figure 8. Variations of content of non-essential aminoacids in PMCs during electrophysical processing of whey after manufacture of granulated cottage cheese "Grauncior", in stationary regime, at current density j=20 mA/cm$^2$

A higher degree of non-essential aminoacids isolation is revealed at 5 and 10 min of processing: at 5 min a larger content of Ser, Glu and Pro; at 10 min – of Asp, Gly, Ala, Cys and Tyr was noted.

During electrophysical processing of whey after the manufacture of the "Cottage cheese", 2% fat content, at current density j=20 mA/cm$^2$, the main non-essential amino acids (Asp, Glu, Pro, Gly, Ala, Cys, Tyr) have the highest content in the cathode cell, with the exception of Ser, which has the maximum degree of isolation at 5 min of processing (Figure 9).

If we compare only the time of processing, then we can establish that a higher content of Asp, Glu, Pro, Gly, Ala, Cys, Tyr is at 10 min of electrophysical processing.

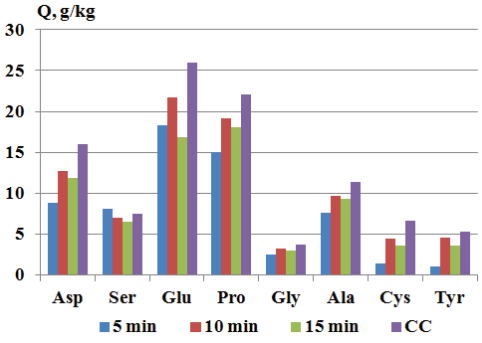


Figure 9. Variations of content of non-essential aminoacids in PMCs during electrophysical processing of whey after manufacture of "Cottage cheese", 2% fat content, in stationary regime, at current density j=20 mA/cm$^2$

From the presented data it can be seen that Glu has the highest content compared with other proteinogenic aminoacids. Glu is a non-essential amino acid but it was established to play a significant role in the main physiological processes of living organism and its nutritional contribution to health (Newsholme et al., 2003; Hertz, 2013). Thus Glu it is considered as a functional one. Thus, the fact that during electrophysical processing of both whey after the manufacture of the granulated cottage cheese "Grauncior" and whey after manufacture of the "Cottage cheese", 2% fat content, with membrane electrolyzer, at current density j=10 mA/cm$^2$ and j=20 mA/cm$^2$ ,a higher degree of Clu is established, denoting the biofunctional value of PMCs. Data referring to Glu include both data about Glu and Gln, because in the process of detection Glu is combined with Gln and so they have the identical picks that reflect the quantity of extraction. The Gln and Glu are closely related in a chemical sense: Gln can produce Glu through the glutamate ammonium ligase. Gln becomes conditionally essential (requiring intake from food or supplements) in states of illness or injury and it is also important for functioning of body in stress conditions (Lacey and Wilmore, 1990).

Thus, the degree of aminoacids isolation in the PMCs during electrophysical processing of whey depends on the: current density, duration (time) of processing, and on the type of whey. Varying these parameters, the content of essential and functional aminoacids in the PMCs during electrophysical processing can be modeled.

The maximum degree of isolation of free aminoacids, especially of essential amino acids, is recorded during electrophysical processing of whey after the manufacture of the granulated cottage cheese „Grauncior" at current density j=20 mA/cm$^2$ in the 10$^{th}$ min of processing.

This allows us to consider that electrophysical processing with membrane electrolyzer at current density j=20 mA/cm$^2$ of whey after the manufacture of the granulated cottage cheese „Grauncior" is the optimal regime (condition) for the maximum content extraction of essential and functional aminoacids in the PMCs.

## CONCLUSIONS

The level of migration of each essential aminoacid and non-essential amino acid in the PMCs is varying in dependence on time of electrophysical processing and current density that can be promising investigations in the direction of the PMCs obtaining with desired amino acids content and spectrum by applying various parameters (regimes) of whey electrophysical processing.

The maximum degree of isolation of free amino acids, especially of essential aminoacids is recorded during electrophysical processing of whey after the manufacture of the granulated cottage cheese „Grauncior" at current density j=20 mA/cm$^2$ t, at 10 min of processing.

## ACKNOWLEDGEMENTS

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# ISOLATION AND IDENTIFICATION OF SOME PATHOGENIC STRAINS FROM RAW AND PROCESSED MEAT SAMPLES

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***Abstract***

*Food poisonings can be lethal situations for digestive tractus, caused by ingestions of food contaminated with microbial germs or with theirs metabolic products. In this paper there were determinate bacteria from Salmonella genus, on the following types of samples, harvested from slaughterhouses and outlets: poultry and bird organs (thighs, gizzard and hearts and bird carcass), raw pig meat (pork neck, pork ribs and pork ham), processed pig meat (mincemeat and sausages). From analysed samples there were isolated: S. enteritidis, S. infantis, S. enterica, S. tennessee, S. saintpaul, S. bredeney, S. typhirium and S. muenchen. 50% of these bacteria were found in processed pork meat samples, followed by poultry and pork meat (raw meat), with 25% each. The isolation and identification technique was done with horizontal method, following these steps: pre-enrichment in unselective liquid mediums, enriching in selective liquid mediums, isolation and identification, identity confirmations.*

***Key words****: isolation, identification, meat sample, pathogenic bacteria.*

## INTRODUCTION

The presence of pathogenic germs, in raw meat and meat products, favors the development of diseases at consumers (Apostu, 2006). Salmonella sp. frequently contaminate the animal carcase, and because of that, they produce food poisonings, which are on top list in most of the countries (Bărzoi, 2002). Salmonella sp. belong to the group of severely pathogenic germs (Apostu, 2004).

Getting ill may be caused by a single serotype or multiple serotypes associated with each other (Tudor, 2002).

As a consequence of some factors as: import of food products, transportation of food products in improper conditions, resistance of bacteria in the external environment, it has come to the situation in which other products, that normally are not favorable environment for salmonella, develops contamination with Salmonella sp. (Ulea, 2017).

In order to keep under control these risk situations, it is necessary to standardize the identification methods, by laboratory analysis, according to ISO regulations (Dan, 2001).

## MATERIALS AND METHODS

In order to establish the frequency of food products contamination with different types of Salmonella, there were collected and analyzed samples in 2017 and 2018, as follows: 367 samples of poultry and bird organs, 244 samples of raw pig meat, 648 samples of processed pig meat, during 2017 and 412 samples of poultry and bird organs, 294 samples of raw pig meat, 673 samples of processed pig meat, during 2018.

The work technique for Salmonella sp. isolation and identification, by horizontal method, assume the following steps:

- Stage I - Pre-enrichment in unselective liquid mediums.

The Salmonella bacteria may be in low number and often accompanied by a large number of Enterobacteriaceae or other bacteria species. Pre-enrichment is necessary to discover the low number of Salmonella sp. or modified Salmonella sp.

Buffered peptone water is inoculated along with sample, then incubated at 37°C ± 10°C for 18 ± 2 h.

- Stage II - Enriching in selective liquid mediums.
The Rappaport-Vassiliadis soybean medium (RVS broth) and tetrathionate/novobiocin Muller-Kauffmann broth (MKTTn broth) are inoculated with bacteria culture medium obtained in buffered peptone water. The RVS broth is incubated at 41.5 ± 1°C for 24 ± 3 hours and the MKTTn broth at 37 ± 1°C for 24 ± 3 hours.
- Stage III - Isolation and identification.
From the culture mediums obtained, two solid selective mediums are inoculated: xylose-lysine-dezoxycholate agar (XLD agar (Figure 1) and Rambach agar (Figure 2).
They are incubated at 37 ± 1°C and examined after 24 ± 3 h.

Figure 1. *Salmonella* colonies on X.L.D Agar

Figure 2. Typical *Salmonella* (red) colonies on Rambach Agar

- Stage IV - Identity confirmation.
Isolated colonies, presumed as Salmonella sp., are confirmed by biochemical and serological tests.

## RESULTS AND DISCUSSIONS

There were collected and analyzed food samples, in order to establish the frequency of food products contamination with different types of Salmonella. In Table 1 and Figure 3, there are presented the tested products type and the number of samples, for 2017 and 2018.

Table 1. Sample category and number of samples collected in 2017 and 2018

| Tested product type | Number of samples | |
|---|---|---|
| | 2017 | 2018 |
| Poultry and bird organs | 367 | 412 |
| Raw pig meat | 244 | 294 |
| Processed pig meat | 648 | 673 |
| Total number of samples | 1259 | 1379 |

In 2017, there were collected 367 samples of poultry and bird organs, 244 samples of raw pig meat, 648 samples of processed pig meat
In 2018, there were collected 412 samples of poultry and bird organs, 294 samples of raw pig meat and 673 samples of processed pig meat.
In 2017, from a total of 1259 collected samples there were found 7 types of *Salmonella*: *S. enterica* – 2 samples; *S. enteritidis* – 3 samples; *S. bredeney* – 2 samples; *S. saintpaul* - 3 samples; *S. ifantis* – 1 sample; *S. tennessee* – 3 samples, *S. typhimurium* – 5 samples.
In 2018, from a total of 1379 collected samples there were found 8 types of Salmonella: *S. enterica* – 2 samples; *S. enteritidis* – 2 samples; *S. bredeney* – 1 sample; *S. saintpaul* - 3 samples; *S. ifantis* – 1 sample; *S. tennessee* – 2 samples, *S. typhimurium* – 3 samples, *S. muenchen* – 1 sample.

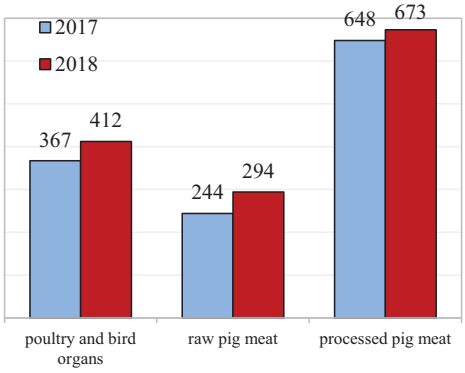


Figure 3. Sample category and total number of samples

In Table 2 and Figure 4 there are presented the number of samples for each the category of poultry and bird organs: thighs - 128 samples, gizzard and hearts – 110 samples and bird carcass – 129 samples, for 2017 and thighs -

153 samples, gizzard and hearts – 127 samples and bird carcass – 132 samples in 2018.

Table 2. Sample category and number of samples collected in 2017 and 2018, for poultry and bird organs

| Sample category | Number of samples | |
|---|---|---|
| | 2017 | 2018 |
| ***Chicken thighs*** | 128 | 153 |
| ***Poultry gizzard and hearts*** | 110 | 127 |
| ***Bird carcass*** | 129 | 132 |
| ***Total number of samples*** | 367 | 412 |

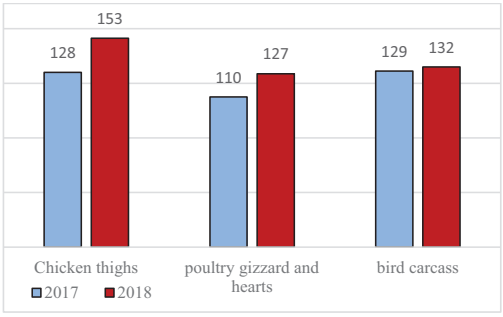


Figure 4. Number of samples for poultry and bird organs

In Table 3 and Figure 5 and 6 there are presented the number and percent of samples found contaminated with different types of Salmonella sp.in poultry and bird organs.

Table 3. Occurrence percent of different types of Salmonella sp. in poultry and bird organs

| Sample category | Salmonella sp. | | | | |
|---|---|---|---|---|---|
| | Stereotype | 2017 | | 2018 | |
| | | No. | % | No. | % |
| ***Chicken thighs*** | *S. enteritidis* | 1 | 0.78 | 1 | 0.65 |
| | *S. infantis* | 1 | 0.78 | 1 | 0.65 |
| | *S. enterica* | 1 | 0.78 | 1 | 0.65 |
| ***Poultry gizzard and hearts*** | *S. tennessee* | 1 | 0.90 | - | - |
| ***Bird carcass*** | *S. enteritidis* | 1 | 0.77 | 1 | 0.75 |
| | | **5** | | **4** | |

In 2017, from a total of 367 tested samples, there were isolated 5 Salmonella types: *S. enterica*; *S. enteritidis*; *S. ifantis*; *S. tennessee*, founded in 5 samples (1.36%).
In 2018, from a total of 412 tested samples, there were isolated 3 Salmonella types: *S. enterica; S. enteritidis; S. ifantis*, founded in 4 samples (0.97%).

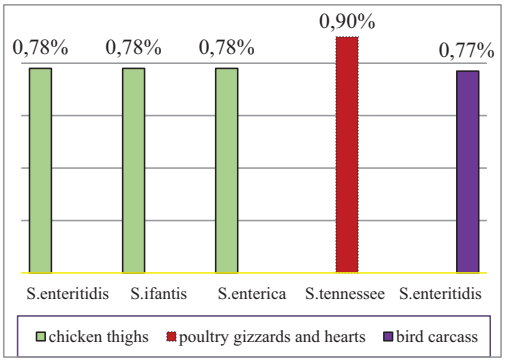


Figure 5. Occurrence percent of different types of Salmonella sp. in poultry and bird organs, in 2017

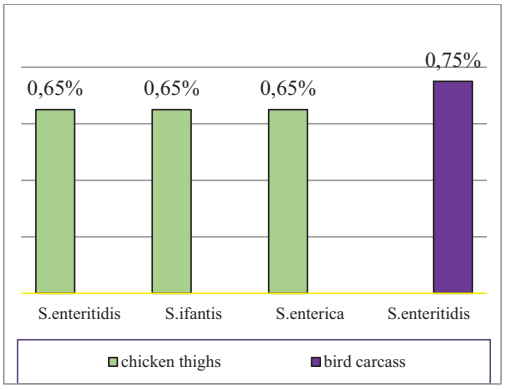


Figure 6. Occurrence percent of different types of *Salmonella* sp. in poultry and bird organs, in 2018

In Table 4 and Figure 7 there are presented the number of samples for each category of raw pork meat: pork neck – 83 samples, pork ribs – 90 samples and pork ham – 71 samples, in 2017 pork neck – 110 samples, pork ribs – 68 samples and pork ham – 116 samples, in 2018

Table 4. Sample category and number of samples collected in 2017 and 2018, for raw pork meat

| Sample category | Number of samples | |
|---|---|---|
| | 2017 | 2018 |
| ***Pork neck,*** | 83 | 110 |
| ***Pork ribs*** | 90 | 68 |
| ***Pork leg*** | 71 | 116 |
| ***Total number of samples*** | 244 | 294 |

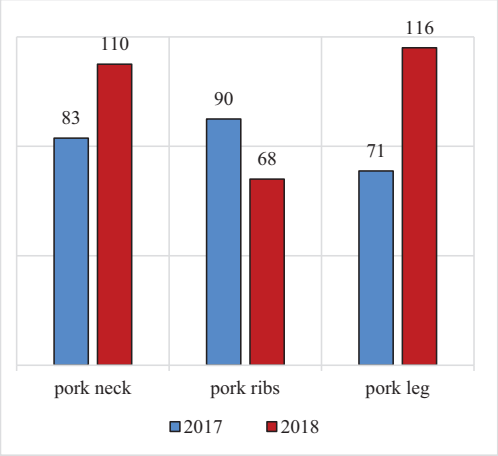


Figure 7. Number of samples, for raw pig meat

In Table 5 and Figure 8 and 9 there are presented the number and percent of samples found contaminated with different types of Salmonella sp. in raw pork meat

Table 5. Occurrence percent of different types of Salmonella sp. in in raw pork meat

| Sample category | Salmonella sp. | | | | |
|---|---|---|---|---|---|
| | Stereotype | 2017 | | 2018 | |
| | | No. | % | No. | % |
| Pork neck | S. tennessee | 1 | 1.20 | 1 | 0.90 |
| Pork ribs | S. bredeney | 1 | 1.11 | - | - |
| | S. typhimurium | 1 | 1.11 | 1 | 1.47 |
| Pork leg | S. saintpaul | 1 | 1.41 | 1 | 0.86 |
| | S. muenchen | - | - | 1 | 0.86 |
| | | 4 | | 4 | |

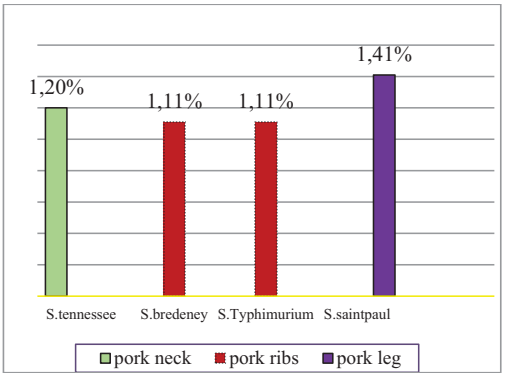


Figure 8. Occurrence percent of different types of Salmonella sp. in in raw pork meat, in 2017

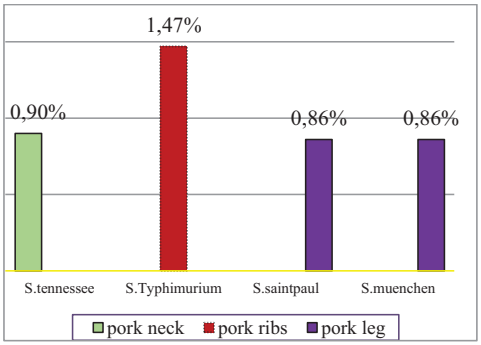


Figure 9. Occurrence percent of different types of Salmonella sp. in in raw pork meat, in 2018

In 2017, from a total of 244 tested samples, there were isolated 4 Salmonella types: *S. bredeney; S. saintpaul, S.typhimurium, S. tennessee*), founded in 4 samples (1.64 %).
In 2018, from a total of 294 tested samples, there were isolated 4 Salmonella types: *S. tennessee, S. saintpaul, S. typhimurium, S. muenchen*, founded in 4 samples (1.36 %).
In Table 6 and Figure 10 there are presented and the number of samples for each category of processed pig meat: mincemeat – 331 samples and sausages – 317 samples, in 2017 and mincemeat – 350 samples and sausages – 323 samples, in 2018.

Table 6. Sample category and number of samples collected in 2017 and 2018, for processed pig meat

| Sample category | Number of samples | |
|---|---|---|
| | 2017 | 2018 |
| Pork - Mincemeat | 331 | 350 |
| Pork sausages | 317 | 323 |
| Total | 648 | 673 |

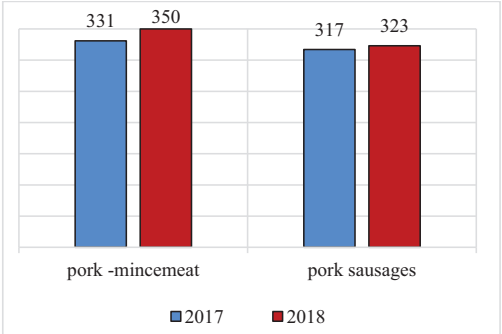


Figure 10. Number of samples, for processed pig meat

In Table 7 and Figures 11 and 12 there are presented the number and percent of samples found contaminated with different types of Salmonella sp. in processed pork meat

Table 7. Occurrence percent of different types of Salmonella sp. in processed pig meat

| Sample category | Salmonella sp. | | | | |
|---|---|---|---|---|---|
| | Stereotype | 2017 | | 2018 | |
| | | No. | % | No. | % |
| Pork - Mincemeat | S. tennessee | 1 | 0.30 | 1 | 0.29 |
| | S. enterica | 1 | 0.30 | 1 | 0.29 |
| | S. typhimurium | 2 | 0.60 | 1 | 0.29 |
| | S. saintpaul | 1 | 0.30 | 1 | 0.29 |
| Pork sausages | S. bredeney | 1 | 0.32 | 1 | 0.31 |
| | S. typhimurium | 2 | 0.63 | 1 | 0.31 |
| | S. saintpaul | 1 | 0.32 | 1 | 0.31 |
| | S. muenchen | - | - | 1 | 0.31 |
| | | 9 | | 8 | |

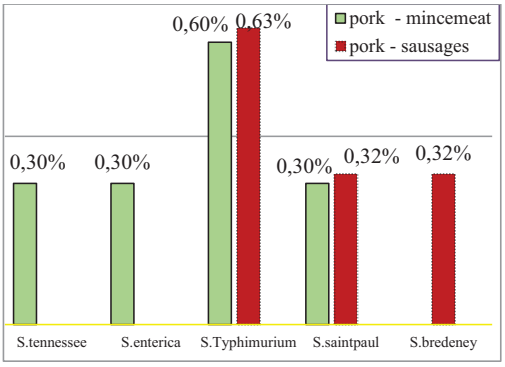


Figure 11. Occurrence percent of different types of Salmonella sp. in processed pig meat, in 2017

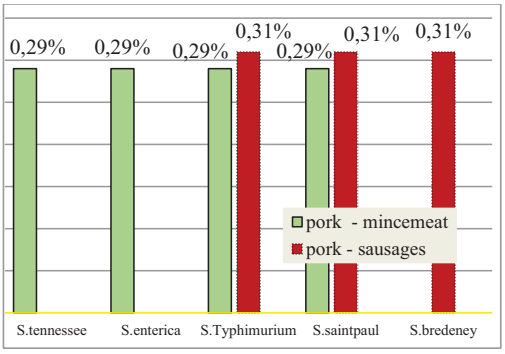


Figure 12. Occurrence percent of different types of Salmonella sp. in processed pig meat, in 2018

In 2017, from a total of 648 tested samples, there were isolated 5 Salmonella types: *S. enterica, S. bredeney, S. saintpaul, S. typhimurium, S. tennessee*, founded in 9 samples (1.39 %).

In 2018, from a total of 673 tested samples, there were isolated 6 Salmonella types: *S. tennessee, S. enterica, S. saintpaul, S. typhimurium, S. bredeney, S. muenchen*, founded in 8 samples (1.19 %).

It can be observed that the dominant types of Salmonella in poultry and bird organs were *S. enteritidis* and *S. enterica*, while in raw pork meat and processed pork meat the dominant types of Salmonella were *S. saintpaul*, *S. typhimurium* and *S. tennessee*.

In 2017, the most of the samples with Salmonella sp. were found in processed pork meat (50%), followed by poultry and bird organs (28%) and raw pork meat (22%). In 2018, 50% of Salmonella bacteria were also found in processed pork meat samples, followed by poultry and pork meat (raw meat), with 25% each (Figures 13 and 14).

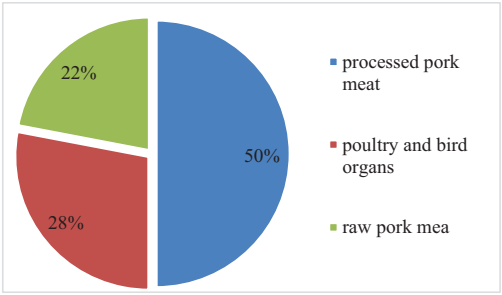


Figure 13. The percent of contamination depending on product type samples, in 2017

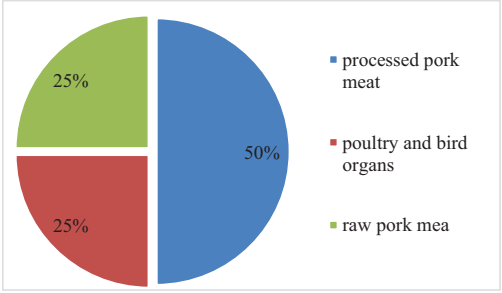


Figure 14. The percent of contamination depending on product type samples, in 2018

## CONCLUSIONS

In 2017 and 2018, from collected samples there were isolated different types of Salmonella: *S. enterica; S. enteritidis; S. bredeney; S. saintpaul; S. ifantis; S. tennessee, S typhimurium*. In 2018 there was isolateted one more: *S. muenchen*.

Both in 2017 and 2018, it can be observed that the most of these contaminated samples, were isolated in processed pig meat (50%), followed by poultry meat (28% and 25% respectively) and raw pig meat (22% and 25% respectively).

The dominant types of Salmonella in poultry and bird organs were *S. enteritidis* and *S. enterica*, while in raw pork meat and processed pork meat the dominant types of Salmonella were *S. saintpaul, S. typhimurium* and *S. tennessee*.

The presence of Salmonella sp. in meat is favored by failure to comply the transport conditions, animals stressing at slaughterhouse, failure to comply the slaughtering technology and programs of hygiene and desinfection.

Food products can be contaminated at all stages of processing, including in the consumer phase. Potential contamination sources are: ingredients, manufacturing procedures, thermal processing (heating or cooling techniques), advanced processing techniques, packaging methods.

In processing industry and especially in slaughterhouses it is imperative to implement and strictly follow the HACCP and hygiene good practice guidelines.

In order to reduce the market penetration of products contaminated with Salmonella, another important objective is to check the carcasses and raw materials at the reception stage.

## ACKNOLEDGEMENTS

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# STUDY REGARDING CONSUMERS BEHAVIOUR TOWARDS INNOVATIVE CONFECTIONERY PRODUCTS

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***Abstract***

*Confectionery is one of the food industry sectors with the greatest dynamics and innovation in Romania. Cooperation among confectioners, ingredient producers and food technologists is useful to the technologies and recipes development and optimisation, and intelligent use of ingredients in order to meet the consumers' expectations and demanding. The present work focused on the investigation of consumer's behaviour regarding purchasing of the confectionary products and to identify the factors affecting the impact on the purchasing decision. Data were obtained based on a questionnaire and the respondents were selected based on conventional sampling procedures. Data analysis and results interpreting were done using statistical techniques.*

***Key words****: behaviour, confectionary products, consumers, innovation, market research.*

## INTRODUCTION

In Romania, the market and consumption per capita of frozen foods are among the lowest in Europe and the highest seasonality. However, the growth potential is high, with the segment growing in a context that is due to the ease and speed of preparing the table for such products. The retail market for frozen confectionery products grows steadily every year, but it is a small segment, considering that there are complementary categories where similar products can be purchased: bulk store bakery, baked goods or even well-known street pastry (Retail Zoom research).

Lack of free time and increased purchasing power make Romanians look for frozen pastry because of eased preparation, but also for the multitude of applications / versatility.

There are several types of desserts that can be frozen, including bakery desserts (cakes, pies, etc. – unbaked, pre-baked or ready-to-eat), starch-based or gelatin-based custards and puddings, raw and prepared fruits, sweetened whipped toppings, and many more.

These products are all characterised by thawing before consumption. In these products, the freezing steps must be optimised to maintain the quality parameters that were inherent in the original product before freezing, to deliver to the consumer a final product that has not been affected by the freezing process.

In addition to infrastructure, frozen products "carry" additional costs of controlled temperature and electricity.

Besides the challenges of the described situation, there is also the need to keep the price of products at a level according to the category they are part of, all under the conditions mentioned above - respectively for fish and seafood, restrictions on fishing areas and annual quotas etc. (Sun Da Wen, 2006).

## MATERIALS AND METHODS

Data regarding the consumer's perception on confectionery products, frozen goods, or innovative sweet products has been collected by filling a questionnaire with 24 questions by 466 respondens.

The questionnaires were completed and uploaded on Google forms document by 30 students of the Faculty of Animal Productions Engineering and Management, participants of the project-contest "Innovation and research in frozen confectionery" developed by the Faculty of Animal Productions Engineering and Management of University of Agronomical and Veterinary Medicine University of Bucharest (Bahaciu et al., 2018).

## RESULTS AND DISCUSSIONS

Data collected via questionnaires was graphic processed and presented based on each characteristic investigated and it will be detailed for each item.

### Consumer's behaviour

Regarding the place to buy confectionery products, 44.47% of the respondents said that they are buying confectionery products from their favourite confectionery store and only 3.9% from online shopping / home delivery (figure 1).

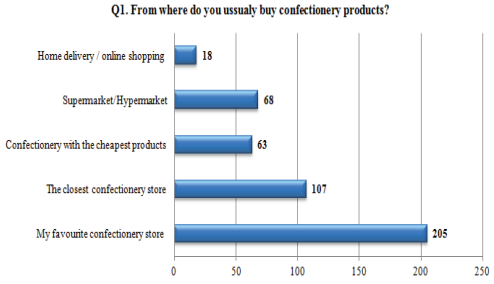



Fig. 1. Preferences on places to buy confectionery products

The most important criteria for choosing the place to buy confectionery products it is also shown in figure 2.

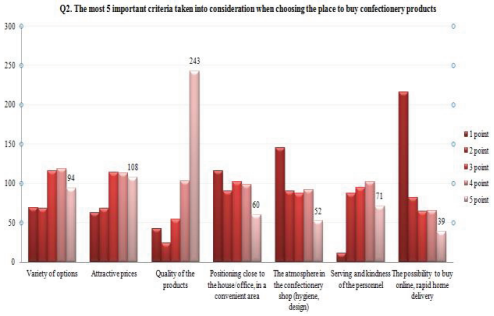



Fig. 2. The most important criteria for choosing the place to buy confectionery products

As it can be observed, the most important was considered the quality of the products (243 respondents gave 5 points of importance); attractive price was the second criteria with 108 answers and serving and kindness of the personnel was the third choice (with 71 answers).

The usual reason for purchasing confectionery products was also investigated in the questionnaire and data is available in figure 3.

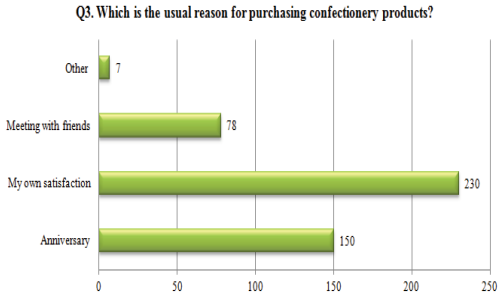



Fig. 3. The usual reason for purchasing confectionery products

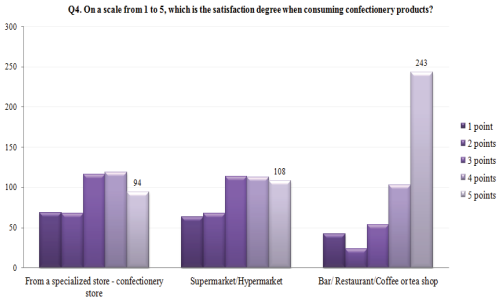



Fig. 4. The satisfaction degree when consuming confectionery products

People buy confectionery products for their own satisfaction (49.46%), followed by anniversary reasons (32.26%). Meeting with friends is the third reason for purchasing choice of confectionery products (16.78%).

The satisfaction degree when consuming confectionery products is also an important item to be tested on consumers, from the producer's point of view and it is shown in figure 4. The highest satisfaction of confectionery products consumption is reached when consumed in bars, restaurants or coffee/tea shops (243 respondents gave 5 points).

When buying confectionery products, consumers take into consideration different criteria and the importance of these criteria are shown in figure 5. The most important are: products freshness (211 of 5 points answers), quality (204 of 5 points answers), ingredients used (95 answers). It is important to observe that buying products with special characteristics (raw-vegan, natural ingredients, with functional

effects ingredients) is considered the last 5 points choice for the consumers (only 56 gave the maximum score).

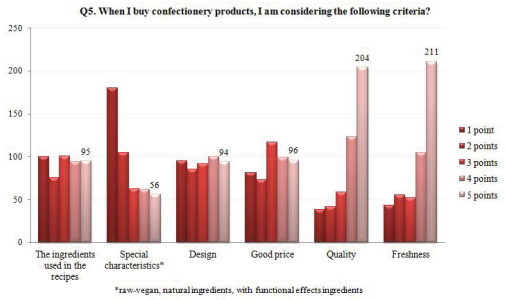



Fig. 5. Criteria taken into consideration when buying confectionery products

**Consumption habits: frequency of buying, preferences on confectionery products**

56.56% of respondents considered that confectionery products are an occasional desert

Thus, 3.66% think that there are a rapid replacement of a meal (figure 6).

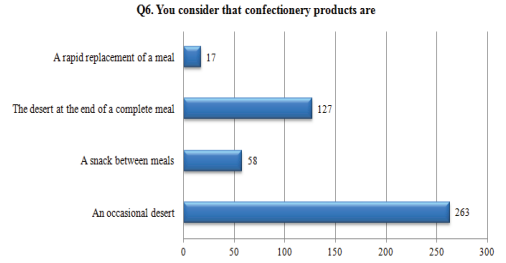



Fig. 6. Considerations on confectionery products

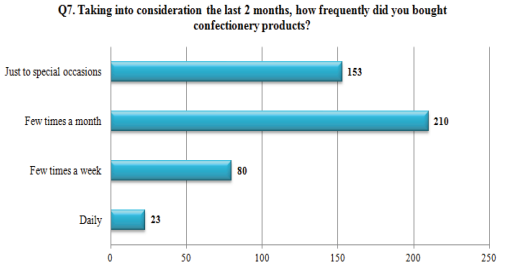



Fig. 7. The frequency of confectionery products buying

The frequency of confectionery products buying is shown in figure 7. It can be observed that 45.06% of the respondents buy confectionery products few times a month, 32.83% just to special occasions, few times a week. There are also 17.17% of respondents that are buying confectionery products daily.

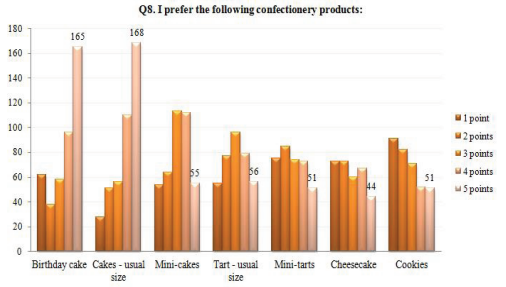



Fig. 8. The most preferred confectionery products

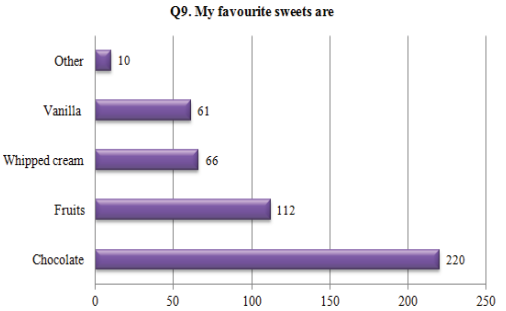



Fig. 9. The most preferred sweets

**Personal Preferences**

The personal preferences of the consumers (types of products, favourite cakes, tastes, decorations) are important parameters to be investigated by producers and marketing departments, so we have introduced these into the questionnaire.

As it can observed from figure 8, the most preferred confectionery products are cakes-usual size (168 responses with high grade), birthday cakes (165). At a long distance, tart-usual size (56), mini-cakes (55), mini tarts and cookies (51) and finally cheesecakes (51) are following. Referring to the most appreciated sweets, 220 consumers have pointed chocolate, which means 46.91% of total respondents. The following preferences were, in order, fruits (23.88%), whipped cream (14.07%), vanilla (13.01%).

Regarding the favourite taste for the confectionery products, in figure 10 it can be shown that the most appreciated taste is sweet and flavoured (26.94% of respondents), followed by sweet (23.28%), sweet and sour

(21.55%). The intense sweet products are appreciated by only 12.28% of the respondents.

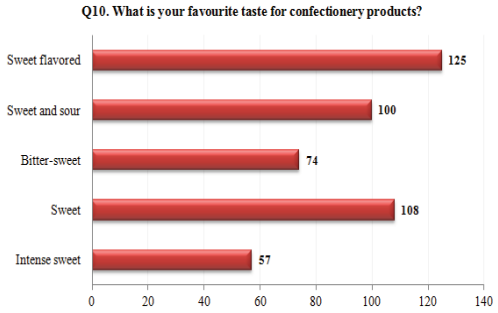



Fig. 10. The favourite taste for the confectionery products

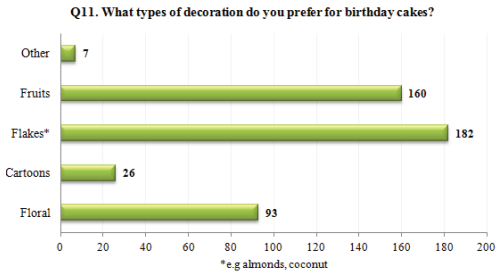



Fig. 11. Decorations preferences of the consumers

Consumers prefered as decoration for birthday cakes (figure 11) the almonds or coconut flakes (38.89%). The next decoration in the preferences of the investigated consumers was fruits (34.19%), followed by floral decorations (19.87%) and cartoons (5.56%). When producers take into consideration their portfolio of products extending, it is very important to have an opinion on consumers opening to new products, new tastes or aroma. This is why in our questionnaire we included some questions to find the eventual consumer's reluctance to new developments. These trends were investigated via questionnaire and the results are shown in figure 12.

Consumers were opened to buy flakes (32.02%), products with unexpected flavours (29.19%), natural flavoured products (14.68%).

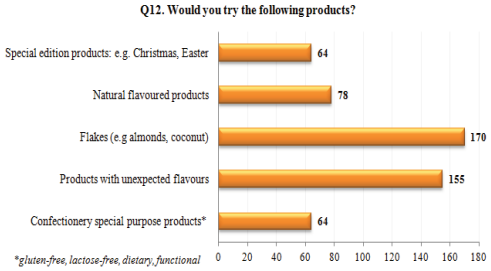



Fig. 12. Opening to buy different types of confectionery products

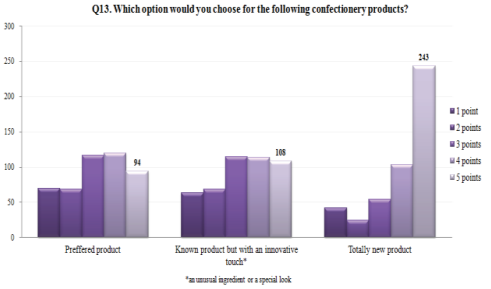
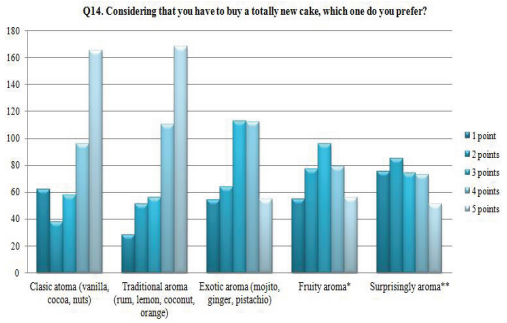



Fig. 13. Choosing option between preferred products or totally new ones

As it can be seen in figure 13, the respondents expressed their opening to totally new products (243 of 445 gave 5 points of importance at this question). This is a very important finding by showing the non conservative attitude of the respondents and their opening to try new things.

**Perception on frozen products**

Classic and traditional aromas were preferred by 165 and respectively 168 consumers (of total 495) in their intention of buying confectionery products (figure 14). On the other hand, exotic and fruity aromas were grated with 5 point on the scale by 55 and 56 consumers (respondents). Meanwhile, the surprisingly aroma like chilli chocolate, salted caramel, lime and coconut, as well as rosemary vanilla were preferred by 51 of the respondents, which means 10.3% of the total respondents.




*blueberries, raspberries, strawberries, bitter cherries*
***chilli chocolate, salted caramel, lime with coconut, rosemary vanilla*

Fig. 14. Opening to new aromas

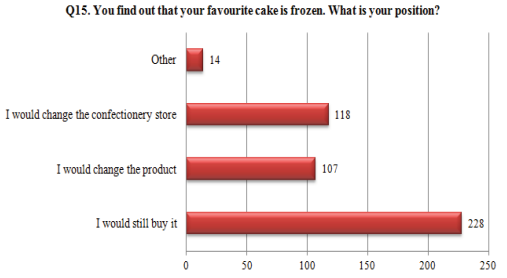



Fig. 15. Perception on frozen products

Almost half of the respondents to our questionnaire (48.82%) have also shown an opening to frozen products. 228 of them were saying that they will still try a cake even if they have found that the cake was frozen. Meanwhile, 48.18% will refuse the products, 118 or respondents will change the store and 107 will change the product (figure 15).

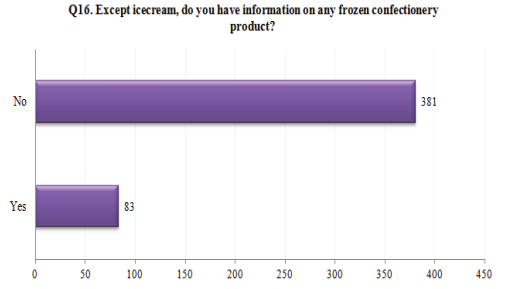



Fig. 16. Information on frozen products

The market of frozen confectionary products is not yet very well known by the Romanian consumers, because, except ice-cream, only 17.89% knew other frozen products (as frozen yogurt, or frozen confectionery products) (figure 16).

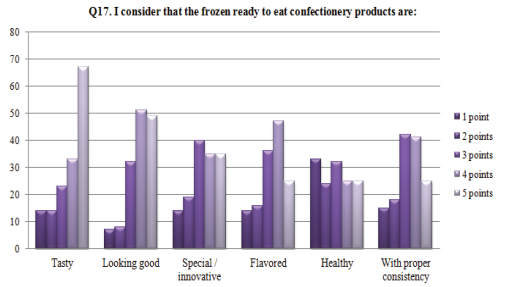



Fig. 17. Perception on frozen confectionery products

## CONCLUSIONS

Changing patterns of consumption, active lifestyles, demand for new recipes, ingredients, but last but not least, convenience contribute to market growth. New product categories, such as prepared, ready-meals, will grow due to the demand for active lifestyle consumers.

1. The reason for purchasing confectionery product is for consumers own satisfaction (49.46%), anniversary reasons (32.26%), or meeting with friends (16.75%)
2. 56.56% of respondents considered that confectionery products are an occasional desert; thus, 3.66% which think that there are a rapid replacement of a meal. This must be taken into attention due to the high rate of obsesity. In 2016, Romania had a prevalence of obesity among adults aged between 20 and 29.9% (Romanian Governement, 2018).
3. Classic and traditional aromas were preferred by 67.28% in total respondents, but also 10.3% of the respondents shown a high interest in surprisingly aroma like chilli chocolate, salted caramel, lime and coconut, as well as rosemary vanilla. This show a great opening to new falvours and aroma
4. Almost half of the respondents (48.82%) have also shown an opening to frozen products.

As with most other frozen products, freezing time and storage temperature are the most important parameters to be optimised. Rapid freezing promotes small ice crystals, which minimise structure disruption and water dislocation. Low and constant storage temperatures maintain this population of ice crystals by minimising ice recrystallisation (Evans, 2008).

Desserts often provide a challenge due to formulation as a result of high sugar content. This creates a low freezing point due to solute effects and freeze-concentration, which results in a high content of unfrozen water at typical storage temperatures, in comparison to products like vegetables or meats. Thus, it is especially imperative that desserts be maintained at the lowest storage and distribution temperatures possible. Functional ingredients, such as polysaccharide stabilisers or modified starches, may be used to help control water redistribution and ice recrystallisation (Goff and Hartel, 2006).

## ACKNOWLEDGEMENTS

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# PRELIMINARY STUDY REGARDING SODIUM BENZOATE AND OTHER FOOD DYES SINERGIC ACTION USING BSLA CITOTOXICITY TEST

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***Abstract***

*Legislation enforce allows the use of food additives that have adverse effects on the human body, such as: asthma, contact hives, allergies, digestive disorders, ADHD, cancer. The toxic effect is particularly enhanced when more than one additive is associated in a food. In this context, we propose to study the effect of benzoate ± sorbate on some food colorants (E129 red allura and E133 bluish blue) from 8 fruit juice samples. Sodium benzoate was dosed by the spectrophotometric method using a standard solution Cs = 0.1 mg/mL. Evaluation of the cytotoxicity of these juice additives was followed using the BLSA (Brine shrimp lethality assay) test. For all analyzed samples, the maximum admissible value for benzoate (200mg/L) is observed to be followed. The fastest cytotoxic effects were recorded in the first 24 hours, but at low concentrations of 50 µL/mL and 100 µL/mL. Processes such as cytoplasmic accumulation of inclusion vesicles, disruption of membrane activity, as well as phenomena associated with cell division and differentiation are identified. In conclusion, although all products contain benzoate levels within acceptable limits, there is a risk of accumulation of larger quantities in the body, by consuming products with this preservative. The combination of these preservatives with dangerous dyes increases the toxic potential, which was also confirmed by the BSLA test. Proper product labeling would inform and guide the consumer / patient to balanced consumption with fewer synthetic additives, with the option of choosing an alternative that does not harm the health.*

***Key words***: *cytotoxicity brine shrimp lethality assay (BSLA), sodium benzoate, food colorants*.

## INTRODUCTION

Currently in our country, the same additives (natural and synthetic), which are admitted by the Ministry of Public Health and the National Medicines Administration, are used both in the pharmaceutical industry and in food industry in accordance with the EU Regulation 1331/2008 and the recommendations of the Committee joint FAO / WHO expert (EU Regulation, 2008). The safety of food additives in Romania is respected and the limit of benzoic acid in fruit juices of 200 mg/L is established. Great attention is paid to the purity of these additives, knowing they have heavy metals traces (EU Regulation, 2012). With regard to the harmful effect of synthetic food additives, specialists warn that exaggerated consumption of these highly-processed and processed foods can cause a number of harmful systemic reactions: bronchial asthma, contact hives, allergies, digestive disorders, hyperactivity in children, headaches, cancer. If to additive foods are added the additives from drugs, adverse reactions may occur by cumulative effect (Banu et al., 2014).

Benzoic acid is one of the chemical presservatives commonly used as antimicrobial agent in the food and beverage industry refreshing, often used in combination with sorbic acid. There are studies reporting that it is carcinogenic and has side effects such as hives, non-immunological contact hives and asthma (Syed, 2011). Benzene which is carcinogenic can be used at a very low level (ppb level) in products containing both benzoate and ascorbic acid. Exposure to heat and light further stimulates this reaction (Kusi and Acquaah, 2014).

Studies also highlight the toxic effect of many food colorants, such as those commonly found in foods associated with benzoic acid (tartrazine - E 102, Ponceau red 4 - E 124, orange yellow - E 110, carmoisine - E 122,

yellow quinoline - E 104 and red Allura - E 129) (Grumezescu, 2018; Brian, 2014). Benzoic acid in combination with at least one of the 6 colorants has synergistic action for ADHD syndrome, especially among children (Brian, 2014; James, 2014). Many studies have reported this synergy of these additives, which reduces food security for the consumer (Lewis, 2018). In order to improve health safety, the European Union requires that the foods in question be labeled as follows: "the name or E number of the dye (s) may negatively affect the child's care and attention" (Annex V to EU Regulation 1333/2008).

The additives labeling is based on the principle of adequate consumer information, that health is protected and that the product can be used without risk (whether there are allergens, durability, storage conditions or user instructions) (Giurea, 2012). Considering the widespread use in the preparation of soft drinks of these additives that are toxic and potentiates ADHD syndrome, we propose that in this paper we determine the benzoate content in some samples of juices available in the Romanian market and stores and evaluate the cytotoxicity of these additives using the BLSA (Brine shrimp lethality assay) test. The *Artemia salina* bioassay was chosen because it is fast and low cost.

The use of additives in veterinary food and their risk to human population and environment is among the international trends in research (Andreu et al., 2013).

Furthermore, there is a good correlation between in vivo and in vitro tests, and this method is a useful tool for predicting oral acute toxicity. It is also a test that can be used to identify cellular effects of very low concentrations of xenobiotic (Martinov, 2018).

## MATERIALS AND METHODS

### 1. Analyzed samples

Eight samples of fruit juices (Romanian and imported juices) from Romanian market were tested. Juices contain preservatives - benzoate / sorbate and colorants of the hazardous category - red ponceau, brilliant blue and caramel (Table 1). Four samples containing different concentrations (c1-20 μg/mL; c2 = 40 μg/mL; c3 = 100 μg/mL; c4 = 200 μg/mL) of benzoic acid were also analysed test control (control benzoic control test = ABCT) considering that 6 of the analyzed samples include sodium benzoate as well (S3-S8).

Tabel 1. The content of juice sample

| Nr. Sample | The content of sample |
|---|---|
| S1 | without preservative, without dye |
| S2 | without preservative, without dye |
| S3 | *B + S; Red allura + Black brilliant |
| S4 | *B +Sr; Red allura+Black brilliant |
| S5 | *B + Blue brilliant |
| S6 | *B |
| S7 | *B + Caramel |
| S8 | *B + Sr |
| S9 | Standard Benzoat |

*B=Benzoat; Sr= Sorbat

### 2. For the spectrophotometric determination of benzoic acid

Following reagents were used: pure benzoic acid Gatt Koller (Germany), Ethyl Ether Sigma Aldrich (Germany), $NaHCO_3 \geq 99.5\%$ (Merck, Darmstadt, Germany); tartaric acid ≥ 99.5%, Sigma Aldrich (Germany), NaOH ≥97.0%, pellets Sigma Aldrich (Germany) and $H_2SO_4$ 98% Sigma Aldrich (Germany).

We used the spectrophotometric method (CAN Standards, 2008; Goma et al., 2013).

Thus, extraction was performed with ethyl ether respecting all the separation steps after eventual neutralization with $NaHCO_3$ and NaOH and dissolution of the filtrate with tartaric acid. The levels of absorbance of ethyl ether were read at 268, 272, 277nm and $A_0$ calculated according to the formula $A_0 = A_2$ (272nm) - [$A_1$ (268nm) + $A_2$ (277nm)] / 2

The sodium benzoate content is calculated using the standard curve, expressed in mg / mL of sodium benzoate.

The calibration curve has good linearity (R2 = 0.9852) and was plotted using the absorbancen values corresponding to the 5 standard solutions prepared, 0.5 concentration; 0.75; 1.00; 1.25; /1.5 mg/mL (Figure 1).

Absorbance readings were performed on the UV 6800PC spectrophotometer; the machine performs triple readings.

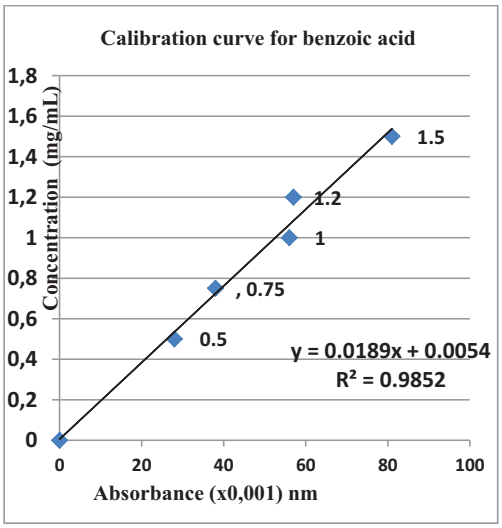


Figure 1. Calibration curve for benzoic acid

**Assessment of cytotoxicity**

*Artemia salina*, in naupliar stage I, II was used. These cysts were subjected hatching in artificial seawater of 35-36‰, at 25ºC (thermostat) with continuous aeration and artificial lighting. *Artemia* larvae, hatched about 5 h, were placed in water of 2-3 ppt.

The experimental containers are made of Plexiglas (cell culture plates) with volumes at 1 mL. In each box was placed an equal volume of saline solution with larvae (10-20 specimens/box). Five repetitions were performed for each concentration. Control samples were made by placing the larvae in sea water with salinity like (Andreu et al., 2013).

The quantification of the effects consisted of measuring the survival of the larvae after 24, 48, hour from the start of the experiment. The cytological study was made at Optika B-350 microscope, with Optika Vision Pro photo capture.

## RESULTS AND DISCUSSIONS

Following the quantitative analysis of benzoate in the samples taken, all samples were found to be within the maximum admissible values - for juices less than 200mg/L allowed (EU Regulation, 2008). The results obtained are presented in Table 2. For each sample, 3 batches were tested at 1-month interval; from each batch, 3 determinations were made.

The results are expressed as the mean ± standard deviation (SD) of the triplicate determinations.

The ANOVA method and the Student'st test were used to test any statistically significant difference. Correlation values were evaluated using the Pearson correlation. Differences that have $p < 0.05$ are considered significant.

As a result of these determinations, we note that for all samples, the admissible values between 0.062 - 0.200 mg/mL are followed; significant differences are found in samples S3, S5, S6 and S7.

The highest values are in the S5 and S6 samples which have only benzoate, but in S4 and S2 the absence of the preservative is confirmed, as stated on the label.

Although the samples do not contain benzoate in concentrations above the maximum permitted levels, they are not free of risks to the health of consumers (they contain benzoate and dangerous dyes - blue brilliant, red allura, caramel).

Table 2. Values obtained for sodium benzoate from the analyzed samples

| Sample | Minimum | Maximum | Mean | | Std. Deviation |
|---|---|---|---|---|---|
| | Statistic | Statistic | Statistic | Std. Error | Statistic |
| S 3 | 0.0570 | 0.0620 | 0.059422 | 0.0005257 | 0.0015770 |
| S 4 | 0.1070 | 0.2050 | 0.136444 | 0.0119816 | 0.0359448 |
| S 5 | 0.1580 | 0.1720 | 0.167111 | 0.0016368 | 0.0049103 |
| S 6 | 0.1380 | 0.1440 | 0.140222 | 0.0007027 | 0.0021082 |
| S 7 | 0.0580 | 0.0640 | 0.060556 | 0.0006261 | 0.0018782 |
| S 8 | 0.0130 | 0.1100 | 0.090222 | 0.0097721 | .0293163 |

In this respect, there are many studies that report the increasing incidence of ADHD among children in the consumption of products with additives (Lewis, 2018; Rian, 2014; Brian, 2014]. The toxic effect of additives due to synergism from the analyzed samples is also supported by BSLA test results, presented below.

**Assessment of cytotoxicity**

The effects of the compounds were measured by determining the survival rate of larvae and observing microscopically visible changes. A moderate cytotoxic effect was found in terms of benzoic acid at 24 h and 48 h respectively.

It decreases larval survival at high concentrations of 0.100 mg/ mL and 0.200 mg/mL, respectively.

These observations indicate a nontoxic effect in the first 24h and a very low cytotoxic effect after an extended 48h exposure. For the analyzed samples (P1-P9) the mortality values (%) recorded in the first 24 h suggest significant differences (Figure 2). Mortality over 50% was recorded in P1, P4, P5, P6, P8. These data indicate the cumulative effects induced by the mixture of dye and benzoic acid in P4, P5. In P6, the recorded mortality correlates with the phenomenon recorded in the ABCT test for benzoic acid solutions whose concentrations are above 0.1 mg/mL.

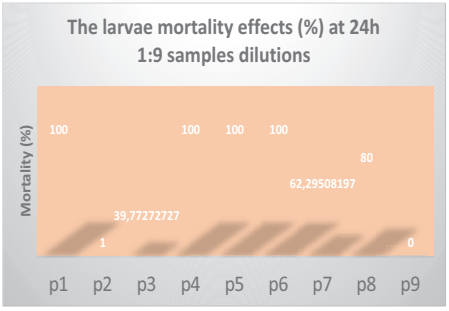


Figure 2. The larvae mortality effects (%) at 24h exposure in solutions samples with 1:9 dilutions

**The microscopically analysis**

Morphological and cytological changes are evident in all samples. The effects of a larger scale are in the samples containing combinations of benzoic acid solutions and dyes. Effects identified based on microscopic observations were classified into two categories:

1. Visible effects at the cellular population (limb germs, subcuticular epithelial layer) (Figures 3, 4). Visible changes are noted for sample S3 and S4. These results are explicable, since S3 contains benzoate and sorbate; even if the benzoate is in a lower concentration than the other samples. Sample S4 has high benzoate values but also two red dyes (red allura and Blue brilliant)

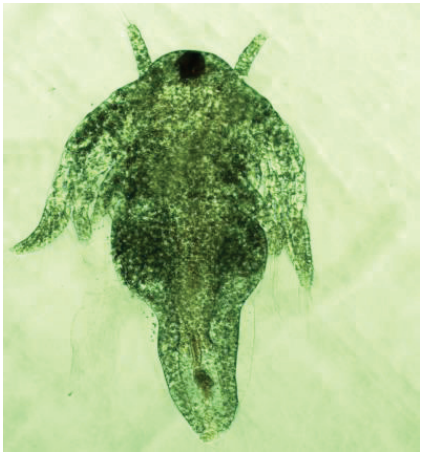

Figure 3. Abdominal and digestive tract morphological changes for P4 sample

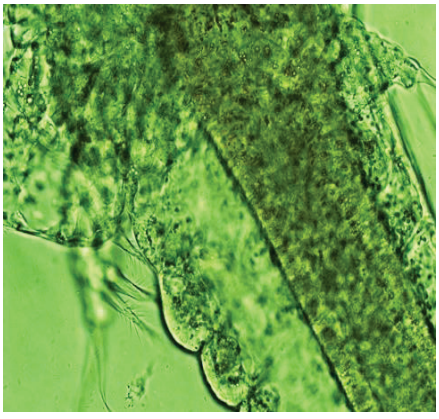

Figure 4. The limb germs inhibitions for larvae from S3 sample

2. Intracytoplasmatic, highlighting numerous cytoplasmic vacuoles, identified for S4 - Figure 5. Some larvae are deformed and the explanation is probably related to osmotic perturbations (Figure 3), but the larvae continued to survive for several hours. The most important cytological phenomena noted were those related to inhibition of organogenesis (Figure 4). These observations also explain the increased mortality of larvae exposed to the tested solutions. Theoretically, in the first 24-48 h, the larvae pass through 2 or 3 successive moults, during which time divisions and respective cell differentiation processes occur that ensure success during growth.

A similar phenomenon has also been described for invertebrates, in literature. Thus, Martinov's

study, 2018, indicates inhibition of growth and reduction in the number of moults in *Tenebrio molitor* larvae.

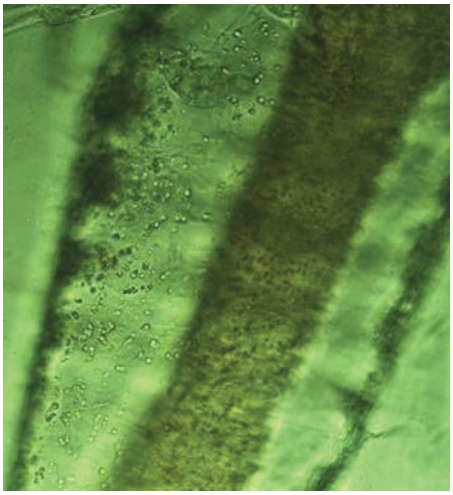

Figure 5. The cytoplasmatic vacuolization's for P4 sample

Phenomena such as growth inhibition and cytokine synthesis, glycolysis alterations are noted in other testing systems such as human lymphocytes (Freeman, 2005) or *Saccharomyces cerevisi*ae (Bruno, 2006) at exposure to benzoic acid solutions.

Cytological studios highlight phenomena that can be used later to understand the mechanisms of action of widely used additives in the food or human food sector.

Extensive morphological changes such as inhibition of cell growth and disruption of membrane integrity are those that explain the decrease in Artemia larva survival after 24 hours of exposure to dyes or admixtures.

As a result, our study suggests that the way conservatives and dyes are associated, would require more attention when establishing the need for additives in veterinary or human food.

## CONCLUSIONS

Following BSLA cytotoxicity tests for fruit juices containing benzoate / sorbate and dangerous dyes responsible for a range of phenomena in humans and animals, the results show that:

• intensively processed products still jeopardize food safety and, implicitly, the health of the body;

• better nutrition education with the involvement of competent institutions and parents is needed if we refer to children;

• cytological changes suggest that additional studies are needed on how benzoate / sorbate combinations and dye combinations work at morphological and cytological level;

• identifying the phenotypes of blocking growth or cytoplasmic functioning or osmotic perturbations could also explain a series of phenomena observed in humans;

• products containing a chemical substance used as additives continue to jeopardize the safety of veterinary and human food;

• these chemical combinations may represent a major risk to human health and the environment.

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# MORPHOLOGICAL CHARACTERISTICS OF THE CARCASES AND RESULTS OBTAINED AT SACRIFICATION PREPARATIONS FROM THE MEAT LINE

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***Abstract***

*Somatometry helps characterize morphological carcasses and their commercial classification. Research has revealed differences in age and sex, both in morphological aspects of the carcass and at slaughter. The measurements on the carcass at the 45-day-old youth showed differences in the size of the thigh, forefoot and forearm in favor of the cocks. The other dimensions, namely the length of the clavicular bone, the width of the casing and its angle as well as the perimeter of the casing had values in favor of the pucks. At the age of 60 days, the measurements showed small differences in the main measurements in the two sexes. It is noted the length of the carcass length that recorded different values in the two age periods, respectively in males, the values oscillated between 107.00 ± 0.71mm at 45 days and at 111.87 ± 1.71, at 60 days, and in females at 107.43 ± 2.12mm, at 123.88 ± 2.61mm, at the same time. Also, the perimeter of the carcass had different values at the two analyzed ages and by sex, so at 45 days the values were in males of 163.50 ± 1.01mm and 60 days respectively of 170.55 ± 1.11mm. In females the values were 170.00 ± 1.11 mm at 45 days and 174.75 ± 0.55mm at 60 days. At sacrifice the differences were determined both the age, sex, and quail weight. Sexual dimorphism is favorable to females, causing the results obtained at slaughter to favor both the weight of the carcasses and its anatomical portions. At the age of 60 days, was determined a higher amount of abdominal fat both females and males, respectively 5.33 ± 0.21 g in females and 3.66 ± 0.11 g in males. In adult quail, carcass dimensions recorded comparable values to those recorded in youth at the age of 60, while gender differences remained. At adult sacrifice, the live weight was over 300g in both genders, so in chickens it was 358.70 ± 7.19g and in males 316.40 ± 5.01 g. We have determined higher weights at the chest with bone and socket harvested from adults, compared to the youth, bouth males and females. We note in this category a beautifully conformed carcass, with a fat content of both the subcutaneous and the abdomen in a larger amount in females than in males, the cause being hormonal.*

***Key words****: carcass, quails, morphological characteristics.*

## INTRODUCTION

In the past 10-15 years, poultry has experienced the greatest development among all branches of animal breeding.

There have been increases in meat and egg production, but the offer for consumers has also varied.

The meat production market offers for consumption turkey, goose, duck, guinea fowl, pheasant, quail, pigeons and ostrich.

Quail meat is produced in Europe and the US, but most consumers are in France, Italy and Spain.

Consumption and production of poultry meat also increased as a result of the health image of meat and its products (Petrac, 2009).

The bird has also the advantage of providing fresh meat because its body weight is low and the time it takes to slaughter is relatively small.

The sensory and dietetic properties of quail are decisive for the consumption of this product. These properties are influenced by many factors including the genotype (Vacaro, 2007), feeding (Ştefănescu, 1960), the age of slaughtering (Minvielle, 2004).

From breeder the point of view, the relationship between the quantity and the quality of the meat is a major problem, because there is not always a positive correlation between these elements (Baston, 2010). Concerning the amount of meat, it results from birds with large muscle mass. Carcasses are sacrificed at the age of 35 days when the average weight is 150-180 g.

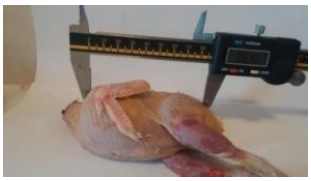
Romanian consumers prefer carcasses weighing over 200 g, which means that the age at slaughtering is between 40-45 days.

The quality of quail meat is also influenced by the appearance of the meat, the carcass, and the

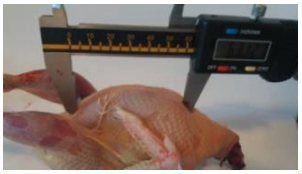
freshness of the meat. This depends on the thickness of the muscle fibers and the ratio of the metabolic types of these fibers (Rouvier, 1965).
According to investigations carried out on meat, the pectoral muscles are composed of 97-98% of muscle tissue, connective tissue and fat 1.1-2.1% 0.2-0.6% (Genchev et al., 2008).
A few years ago, the amount of fat in the carcass constituted a restrictive and even rebuttable factor for a particular consumer group (Sarbulescu et al., 1983).
In the case of quail, the presence of high unsaturated fatty acids contributes to their labeling as dietary. The presence of Omega 3 and 6 fatty acids reduces blood pressure, has beneficial effects in cardiovascular diseases, asthma and oncological diseases, etc. (Banu et al., 2010).
The interest of consumers regarding food quality and in our case of meat is a permanent concern for breeders.
The results obtained from our research can be a landmark for quail farmers to introduce these species as a consumer offer. This assortment of meat is recommended for human nutrition due to its special sensory qualities, but also for its nutritional value.

**Biological material**
The research was carried out on young quails of 45 and 60 days slaughtered for meat and adults who completed their 18-week exploitation cycle.
In order to achieve the proposed goal, two age categories and two sexes were worked out. Measurements have been made on the carcass after sacrifice at which it has been determined both the hot slaughter yield, the edible and inedible parts of the carcass, and the proportion of the valuable anatomical parts of the carcass.

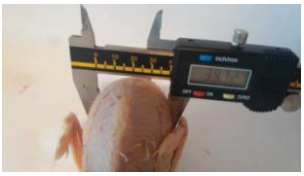
**Working method**
For the morphological characterization of the carcass and to remove the subjectivity of the free estimation method measurements were made and their landmarks will be presented further. Measurements of length, width, perimeters and angles on dewormed carcasses of young and adult quail were made. The measurements made are:
- the length of the case: measured between the scapular-humeral joint and the posterior prominence of the skull;



- chest length: anterior hip and xenophoid appendix;



- chest width: the distance between two scapular-humeral joints;



- the perimeter of the casing: measured with the underlying ribbon under the wings at the base;
- carcass length: coxo-femoral joint and graft
- the length of the calf: the distal and proximal extremity of the tibia;
- the angle of the chest: with the compass between the clavicular bones;
- the length of the jaw (clavicle): the length of the clavicular bones/
For the quantitative evaluation of the meat weighed both the live animal and the carcass, weighed the edible and inedible organs, the abdominal fat, we determined the slaughter yield and the percentage of the weight of the chest and thigh in relation to its weight.

## RESULTS AND DISCUSSIONS

**Morphological characterization of the carcass at the youth of the Pharaoh quail line**
The casing conformation is objectively characterized by body measurements performed thereon. This aspect is important because it allows the identification of different correlations between the principal measurements.
In birds, part of the conformation measurements indicate either the development of the skeleton (whistle length, clavicular bone, etc.)

or the development of the muscles (chest angle, width and lesser girth perimeter).

Practically, somatometry is of real use in defining the morpho-product type. In the case of measurements on the carcass, they help to characterize it morphologically, aspect related to the commercial classification of carcasses (Boișteanu, 2015).

The results of the measurements on housing youth were conducted at two different ages, respectively 45 days when, according to literature states that achieve sexual maturity for both sexes and 60 days old reflecting somatic maturity. Period of installation coincides with the age of sexual maturity which the first egg, which has limitations 40-60 days according to the literature.

This was the reason why we studied the limits of the two ages. The measurements of the measurements made on carcasses on quails aged 45 days are presented in Table 1.

By analysing the table presented we can draw the following conclusions:

- at the age of 45 days, the carcass length has approximately the same value for the two sexes, from 107.00 ± 0.71 mm in males and 107.43 ± 2.12 mm in females;
- there are differences between the sexes in length and chest lengths respectively, the values obtained being 60,97 ± 0,81 mm in males and 59,35 ± 1,44 mm in females;
- Lengths of the thigh and calf length show that in males the thigh is smaller than the female, but the cocks are longer than the chicks;
- Width measurements are bigger in males compared to those recorded in females;
- The length of the clavicular bone and the peg angle at this age are superior to female sex, comparing with the male.

The results obtained from the slaughtering of the young are shown in Table 2.

Table 1. The main dimensions of the carcass of the females and males of the Faraon line 45 days

| No | Specification | UM | Male Youth (N = 35) | | Youth female (N = 35) | |
|---|---|---|---|---|---|---|
| | | | $\bar{x} \pm s\bar{x}$ | V% | $\bar{x} \pm s\bar{x}$ | V% |
| 1 | Length of the casing | mm | 107.00±0.71 | 8.73 | 107.43±2.12 | 4.11 |
| 2 | Chest length | mm | 60.97±0.81 | 7.23 | 59.35±1.44 | 7.17 |
| 3 | Thigh length | mm | 48.5±0.63 | 6.82 | 52.01±1.11 | 6.41 |
| 4 | Length of calf | mm | 54.16±1.09 | 9.51 | 49.01±1.1 | 6.46 |
| 5 | The length of the clavicular bone | mm | 31.70±1.81 | 7.52 | 34.60±1.61 | 5.8 |
| 6 | The forearm length | mm | 42.7±1.13 | 9.03 | 44.05±0.99 | 8.72 |
| 7 | Chest angle | degrees | 29.01±0.79 | 9.72 | 30.01±0.51 | 5.81 |
| 8 | The width of the chest | mm | 33.34 ± 0.76 | 11.63 | 32.44 ±1.75 | 12.71 |
| 9 | Height of the casing | mm | 53.96±.61 | 5.78 | 47.5±0.89 | 4.92 |
| 10 | Housing perimeter | mm | 153.5±1.01 | 13.55 | 170.0±1.01 | 17.59 |

Table 2. Participation of different anatomical parts in the structure of the quail carcass aged 45 days

| No crt | Specification | UM | Female Youth (N = 35) | | Youth male (N = 35) | | Sex differences |
|---|---|---|---|---|---|---|---|
| | | | $\bar{x} \pm s\bar{x}$ | V% | $\bar{x} \pm s\bar{x}$ | V% | |
| 1 | Live weight | g | 265.50±23.94 | 10.23 | 244.30±7.43 | 9.76 | + 21.20 |
| 2 | Carcass weight | g | 208.15±10.3 | 15.01 | 181.03±11.78 | 10.18 | + 21.12 |
| 3 | Chest weight with bone | g | 89.79±3.41 | 15.69 | 73.01±4.32 | 15.49 | + 16.79 |
| 4 | Abdominal fat | g | 3.51±0.12 | 5.73 | 1.87±0.11 | 4.66 | + 1.64 |
| 5 | Meat in the house | g | 106.11±19.91 | 14.69 | 85.56±6.6 | 15.52 | + 20.55 |
| 6 | G. edible parts | g | 15.56±0.29 | 7.51 | 15.94±0.59 | 9.84 | - 0.38 |
| 7 | G. non-edible parts | g | 14.01±0.91 | 13.0 | 12.42±0.78 | 16.61 | + 1.59 |
| 8 | Slaughter yield | % | 78.39±13.1 | 9.13 | 76.56±9.11 | 11.03 | + 1.83 |
| 9 | Thigh + thigh | g | 87.05±3.61 | 16.3 | 61.86±2.26 | 9.65 | + 25.19 |

From analyzing the data in table 2 we note the following:

- The live weight of 45 days of quail is different for the two sexes. being in the advantage of the chickens. so the females reach a weight of 265.50 ± 23.94 g and the males 244.30 ± 7.43 g;
- the weight of the carcass depends on the live weight. so that the females have registered a carcase with a higher muscle mass than the males;

- regarding the carcass weight. the majority its represented by the chest. which in the females was 89.79 ± 3.41 g and 73.01 ± 4.32 g. respectively;
- the weight of the thigh and calf were different for the two sexes with the mention that the females had a higher value, the average of 87.05 g for the females. and for the males of 61.86 g;
- the edible parts of the carcass are close in value for the two sexes. in averaging 15.56 g for females and 15.94 g for males;
- the yield on slaughter differs. being in favor of the chickens. but the differences are small below 1%.

The percentage of participation of the different anatomical parts of the carcase was followed, the data being presented in Table 3.

Table 3. Participation of the various anatomical parts in the structure of the quail carcass aged 45 days

| No crt | Specification | UM | Male (N= 35) | Female (N 35) |
|---|---|---|---|---|
| 1 | Head + legs | g | 4.48 | 7.65 |
| 2 | Blood | % | 2.71 | 2.08 |
| 3 | Edible items | % | 8.52 | 5.51 |
| 4 | Chest with bone | % | 39.34 | 31.81 |
| 5 | Hams | % | 37.07 | 30.86 |
| 6 | Chest box | % | 24.79 | 17.5 |
| 7 | Abdominal fat | g | 0.89 | 1.24 |
| 8 | Yield | % | 76.56 | 77.39 |

- We highlighted some aspects that have not been analyzed so far. namely the percentage of blood that represents 2.71% in females and 2.08% in males of live weight.
- The chest box has a 24.79% female participation percentage. and male 17.5%. This is explained by the fact that organs found in the chest box have a higher weight in females than in males.
- The largest percentage in the carcass is the chest and pulp, which represents 84.95% of the carcass weight in females and 72.1% in males.

The same characters were then analyzed on the carcasses from the young slaughtered at the age of 60 days.

The data on the measurements made on the carcasses of this age group are presented in Table 4.

Table 4. The main dimensions of the carcass at 60 days old quails

| No crt | Specification | U. M | Young quail (60 days) | | | |
|---|---|---|---|---|---|---|
| | | | Female (N 35) | | Male (N 35) | |
| | | | $\bar{x} \pm s\bar{x}$ | V% | $\bar{x} \pm s\bar{x}$ | V% |
| 1 | Chassis length | mm | 123.88±2.61 | 9.09 | 111.87±1.71 | 5.73 |
| 2 | The length of the chest | mm | 60.4±3.72 | 12.73 | 61.98±0.24 | 7.08 |
| 3 | Thigh length | mm | 53.97±1.97 | 7.98 | 50.53±1.63 | 5.51 |
| 4 | Length of throat | mm | 49.75±3.05 | 11.51 | 54.73±3.15 | 7.18 |
| 5 | The length of the clavicular bone | mm | 37.62±1.96 | 10.41 | 39.03±1.99 | 8.86 |
| 6 | The width of the casing | mm | 38.6±1.91 | 4.94 | 39.26±1.69 | 7.46 |
| 7 | Height of the casing | mm | 59.97±3.49 | 13.96 | 54.06±1.99 | 6.52 |
| 8 | Housing perimeter | mm | 174.75±0.55 | 6.73 | 170.55±0.11 | 11.76 |
| 9 | Forearm | mm | 48.73±1.03 | 8.92 | 46.93±1.11 | 8.81 |
| 11 | The weight. housing | g | 221.15±10.3 | 15.01 | 199.51±3.21 | 10.18 |
| 12 | Chest angle | degrees | 31.17±0.59 | 9.17 | 30.71±0.62 | 8.73 |

Analyzing the data. we make the following remarks:
- The length of the casing and the thigh have recorded higher values in the chickens than in the cock;
- Differences obtained for the carcass length were 3.01 mm in favor of the females and 3.44 for the thigh width also for them;
- The length of the chest, calf and clavicular bone recorded close values in males compared to females;
- The dimensions of width, of the shell and the basin have shown values capped at the two genders;
- Thigh and calf dimensions are the same characteristics at the age of 60 days at the age

of 45 days. The drumstick of chicks has a longer thighs and shorter thighs. but these are contrary to the male.

- The height of the carcass is higher in the male, with an average value of 54.6 mm far superior to that of the females that recorded 49.97 mm.

Table 5. Results on slaughter of quails aged 60 days

| No crt | Specification | Female | | Male | |
|---|---|---|---|---|---|
| | | $\bar{X} \pm S\bar{X}$ | V % | $\bar{X} \pm S\bar{X}$ | V% |
| 1 | Live body weight (g) | 282.25±9.35 | 10.23 | 257.66±10.73 | 12.45 |
| 2 | Housing weight (g) | 232.33±10.3 | 15.01 | 204.99±3.21 | 10.18 |
| 3 | Chest with bone (g) | 89.79±3.41 | 15.69 | 83.01±4.32 | 15.49 |
| 4 | Thighs (g) | 87.05±3.61 | 16.3 | 81.86±2.26 | 9.65 |
| 5 | Abdominal fat (g) | 5.33±0. 21 | 7.09 | 3.66±0.11 | 8.33 |
| 6 | Weight of offal (g) | 21.75±0.56 | 5.19 | 20.0±0.48 | 4.28 |
| 7 | Weight of non-edible parts (g) | 14.0±0.91 | 13.01 | 14.33±0.87 | 8.13 |
| 8 | Slaughter yield (%) | 82.39±13.1 | 9.13 | 79.56±9.1 | 11.03 |

- the young quails aged 60 days. the average weight of the female housing was 10.3 g ± 232.33 and 204.99 ± 99 the male slaughter with a yield of 82.39% hot. female and 79.56% in males.
- the amount of abdominal fat was higher in females than in males. the difference between the two sexes being 1.99g.
- as anatomical parts. the chest and pulp represented 84% of the carcass weight in females and 82.63% in males.
- these values show that the parameters analyzed were influenced by gender. for all the characters being tracked.

**Morphological characterization of carcases obtained from adult quails.**

Adults were sacrificed at the end of the operating period. at the age of 1.6 years (78 weeks).

The results obtained from measurements on the housing are presented in Table 6.

Table 6. The main dimensions of the Faraon adult female carcasses

| No crt | Specification | UM | Female (N=35) | | Male (N=35) | |
|---|---|---|---|---|---|---|
| | | | $\bar{x} \pm s\bar{x}$ | V% | $\bar{x} \pm s\bar{x}$ | V% |
| 1 | Length of the casing | mm | 97.35±0.81 | 4.39 | 92.76±1.24 | 4.84 |
| 2 | Chest length | mm | 61.16±0.68 | 6.09 | 62.76±1.73 | 9.94 |
| 3 | Thigh length | mm | 54.94±0.86 | 6.47 | 50.92±10.69 | 7.83 |
| 4 | Length of calf | mm | 50.05±0.01 | 8.47 | 56.61±1.05 | 6.69 |
| 5 | Housing perimeter | mm | 188.3±0.18 | 4.14 | 174.33±1.49 | 3.22 |
| 6 | The length of the clavicular bone | mm | 31.81±0.2 | 7.21 | 34.6±0.59 | 6.77 |
| 7 | The width of the chest | mm | 42.99±0.96 | 12.7 | 39.69±1.56 | 14.18 |
| 8 | Chest angle | grade | 33.35±0.92 | 6.06 | 31.8±0.79 | 5.73 |

It was found that:

- the length of the female housing registered a value of 97.35 mm and 92.76 mm from the male;
- thigh and calf length dimensions show that in females, the thigh is longer than the hammer, the average values being 54.94 mm for the thigh and 50.05 mm for the thigh;
- chest width in females was 42.99 mm. and the basin 40.57 mm. and in males the values were close. the carcass width was 39.69 mm. and the basin 38.66 mm;
- the clavicular bone is longer in male than in female. and in chest angle. it is lower in males;
- the highest variability was recorded in chest and basal width of 14.18%. respectively 13.86% in males and 12.77% in chest width in females. and in other sizes the variability was less than 10%;
- the carcasses obtained from adult females were beautifully conformated with average lengths and widths with a short clavicular bone and a chest angle of 33.3 degrees;
- the male casing was characterized by a more elongated appearance, with the widths close in value. the sock had a longer length. which is more obvious in the case;

- as for breast muscles, females are more developed than males;
- the females chest muscles are more obvious, and that of the copan has small differences between the length and width dimensions;
- the male casing has an elongated shape with less obvious pectoral muscle, and the drumstick musculature more developed in the muscles of the gamba.

The results obtained from the slaughter of adult quail are shown in Table 7.

Table 7. Results on the slaughter of adult quail

| No | Specification | UM | Female (N=35) | | Male (N=35) | |
|---|---|---|---|---|---|---|
| | | | $\bar{x} \pm s\bar{x}$ | V% | $\bar{x} \pm s\bar{x}$ | V% |
| 1 | Live weight | g | 358.7±7.19 | 11.32 | 316.4 ±5.01 | 12.35 |
| 2 | Carcass weight | g | 274.6±5.11 | 8.82 | 234.25±7.95 | 9.09 |
| 3 | Chest weight with bone | g | 113.5±0.62 | 14.3 | 92.44±0.17 | 11.55 |
| 4 | Thigh + thigh weight | g | 87.05±3.61 | 16.3 | 85.85±3.04 | 11.49 |
| 6 | Weight of edible organs | g | 17.54±0.24 | 6.05 | 15.36±0.26 | 4.49 |
| 7 | Incomprehensible weight | g | 27.4±3.24 | 3.57 | 28.35±0.26 | 3.97 |
| 8 | Abdominal fat | g | 6.16±0.11 | 7.7 | 2.3±0.27 | 8.12 |
| 9 | Slaughter yield | % | 76.54±7.11 | 9.19 | 74.42±11.3 | 12.04 |

Following the analysis of the table presented. we conclude that:
- the average adult female carcass weight was 274.6 g. Of this weight 113.5 g was the chest with bone and the thigh and shank recorded 87.05 g;
- the male yield at slaughter in females was 56.54%, superior to that presented by the literature (Genchev et al. 2010; Jiang et al., 2011; Tilski et al., 2011);
- the average carcass weight in males was 234.25 g, of which the chest was 92.44 g and the 85.85 g;
- the yield at slaughter was 74.12%. the highest value recorded for the biological material on which our researches were performed, compared to the ones presented by the literature;
- edible organs (liver, heart, lung, rhinoceros) showed small differences (2.18 g) between the two sexes. Bigger differences were registered in inedible parts, here entering ovary weight and the female reproductive system that weighs more than the testes in males;
- abdominal fat is higher in females. as it has been found that they normally have less metabolic activity than the male, as well as the presence of estrogenic hormones. cause a higher amount of adipose cells (the authors).

The participation of the various anatomical parts in the carcass structure shows differences in the two sexes as shown in the Table 8.

Table 8. The participation of various anatomical parts in the body structure of adult quail

| No crt | Specification | UM | Female | Male |
|---|---|---|---|---|
| 1 | Live weight | % | g | g |
| 2 | Carcass weight | % | 76.54 | 74.12 |
| 3 | Head + legs | % | 11.35 | 8.59 |
| 4 | Blood | % | 2.56 | 2.77 |
| 5 | Edible items | % | 4.89 | 4.85 |
| 6 | Incomprehensible parts | % | 6.52 | 5.48 |
| 7 | Chest with bone | % | 41.33 | 39.50 |
| 8 | Thigh + thigh | % | 31.70 | 36.68 |
| 9 | Chest box | % | 18.75 | 17.91 |
| 10 | Abdominal fat | % | 2.24 | 0.98 |

By analysing the Table 8, we can highlight some important aspects as follows:
- of live weight. head and legs represent 11.35% for females and 8.99% for males;
- the amount of blood in the two sexes does not show any essential differences. with values ranging between 2.56% in females and 2.77% in males;
- small differences between genders were recorded in both offal and non-edible parts;
- over 30% participation in both sexes was recorded at the weight of the thigh and calf. and the bone chest had the highest;

- percentage of participation. with values ranging from 39.5% in males to 41.33% in females.

The following table summarizes the evolution of the carcass dimensions in the three age categories (adults. youth 45 days and 60 days). the two sexes (Table 9).

Table 9. Carcass measurements of studied biological material

| Specifi-cation | Adult quail | | | | Young quails (45 days) | | | | Young quails (60 days) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Female | | Male | | Female | | Male | | Female | | Male | |
| | $\bar{x} \pm s\bar{x}$ | V% | $\bar{x} \pm s\bar{x}$ | V% | $\bar{x} \pm s\bar{x}$ | V% | $\bar{x} \pm s\bar{x}$ | V% | $\bar{x} \pm s\bar{x}$ | V% | $\bar{x} \pm s\bar{x}$ | V% |
| Carcass length | 97.35±5.81 | 4.39 | 92.76±1.24 | 4.84 | 87.43±2.21 | 4.18 | 87.07±2.71 | 8.73 | 93.88±2.61 | 9.09 | 90.87±1.71 | 5.73 |
| Chest length | 61.16±0.68 | 6.09 | 62.76±1.73 | 9.94 | 59.35±1.44 | 7.17 | 60.97±0.81 | 7.23 | 60.4±3.72 | 12.73 | 61.98±0.24 | 7.08 |
| Thigh length | 54.94±0.86 | 6.47 | 50.92±10.69 | 7.83 | 52.01±1.11 | 6.41 | 48.5±0.63 | 6.82 | 53.97±1.97 | 7.98 | 50.53±1.63 | 5.51 |
| Length of throat | 50.05±0.01 | 8.47 | 56.61±1.05 | 6.69 | 49.01±1.10 | 6.46 | 54.16±1.09 | 9.51 | 49.75±3.05 | 11.51 | 54.73±3.15 | 7.18 |
| Long clavicular bone | 41.81±0.2 | 7.12 | 39.6±0.59 | 6.77 | 35.4±1.61 | 5.81 | 38.7±1.8 | 7.52 | 37.62±1.96 | 10.41 | 39.03±1.99 | 8.86 |
| Large. housing | 42.99±0.96 | 12.77 | 39.69±1.56 | 14.18 | 34.44±1.94 | 14.06 | 36.62±0.77 | 10.92 | 38.6±1.91 | 4.94 | 39.26±1.69 | 7.46 |
| Height of the casing | 57.53±0.74 | 6.81 | 57.01±1.13 | 7.15 | 47.5±0.89 | 4.92 | 53.96±0.61 | 5.78 | 49.97±3.49 | 13.96 | 54.06±1.99 | 6.52 |
| Housing perimeter | 188.3±0.18 | 4.14 | 174.33±1.49 | 3.22 | 170.0±1.01 | 17.59 | 163.5±1.01 | 13.55 | 174.75±0.55 | 6.73 | 170.55±0.11 | 11.76 |
| Chest angle | 33.35±0.92 | 6.06 | 31.18±0.79 | 5.73 | 30.01±0.51 | 8.17 | 29.01±0.79 | 9.72 | - | - | - | - |
| Live weight | 358.7±7.19 | 11.32 | 316.43±5.01 | 12.35 | 265.5±23.94 | 18.0 | 244.7±7.43 | 12.95 | 282.25±9.35 | 10.23 | 257.66±10.73 | 12.45 |
| Carcase weight | 274.6±5.11 | 8.82 | 234.25±7.95 | 9.09 | 208.15±10.3 | 15.01 | 189.51±15.78 | 10.18 | 221.15±10.3 | 15.01 | 199.51±3.21 | 10.18 |

From the analysis of the table. we highlight some interesting aspects:

- from the young we can conclude an increase in all dimensions. with age. the changes are different depending on sex;
- the length dimensions of the casing and the chest achieve at the age of 60 days values close to those of the adult. both sexes;
- the length of the thigh and calf have reached 99% in the adult at the age of 60 days. both the male and the female;
- males at the age of 60. it is found that the length of the clavicular bone and the width of the carcass have the same values (with small differences) compared to adult males;

differences between the two sexes occur from the age of 45 days and continue until the adult age. the obvious gender differences are in the chest and limbs.

## CONCLUSIONS

Main characteristics. something that must be used to characterize them frequently.

The dimensions of the housing determine the size of its own weight and of the various portions of commercial

The male and female youth of quails from the pharaoh meat line were analyzed at two different age groups. namely 45 and 60 days. coinciding with the installation of sexual maturity.

At the age of 45 days it was found that measurements of the length of the casing in the two sexes are favorable chicks. with the exception of the length of the carcass. and the length of the forearm which favors the male.

At the age of 45 days the dimen- sions show great differences between genders. the values oscillate between 34.44 mm in the females and 36.62 in the males. and the basin was 32.44 in the chicks and 33.44 in the cocks.

Elevation. angles and perimeter measurements were favorable for females.

Sacrifice at the age of 45 showed that carcases are obtained whose weight was 208,5±15g for females and 181,03 for males.

The slaughter yield was 78.39% in females and 77.56 in males.

The anatomical parts with the highest participation in the carcass structure were the chest and the drumstick with different values for the two sexes. being of value to the puppies. They represent 62.67% in males and 71.35% in females.

Measurements on the sacrificial quail housing at the age of 60 showed that they tended to grow in both females and males.

The length measurements had a larger increase compared to the width. which is closer to the adult value.

In females there is a higher weight gain of non-edible organs and abdominal fat. and in the rest of the components the changes are smaller.

The slaughtering results analyzed on anatomical portions show that the growth tendency is maintained in all carcass components. with the indication that in males

the differences between muscle masses from one period to another are more pronounced than in chickens.

In the population of adult quail, it was found that the chickens achieved higher chest and chest lengths than males. and the width of the basin and chest is superior to the cocks.

The clavicular bone was shorter in adult quail and defines a broader angle. and the male bone is longer. the angle of the chest is sharper, causing the pectoral muscles to change.

Adult slaughtering results show that carcass weight was higher in females than in males, and all the other components analyzed were in favor of females.

Research has shown that there are gender and age differences in all aspects investigated. starting with carcass morphology and ending with slaughtering results.

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# EFFECT OF TEMPERATURE AND EXTRACTION TIME ON THE CHARACTERISTICS OF PIGSKIN GELATIN

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***Abstract***

*The quality of gelatin depends on its physichochemical properties and manufacturing method. The process of gelatin required the extraction step to improve the quality of gelatin. The aimed of this study was to research the effect of temperature extraction and extraction time on the characteristics of gelatin produced from pigskin. This study used Completely Randomized Design (CRD) with two factors 3x3 and three replicates of treatments. The first factor was temperature extraction with 3 levels ($50^oC$, $60^oC$ and $70^oC$). The second factor was extraction time consisting of 3 levels (3 hours, 6 hours and 9 hours). The result showed that the interaction of the extraction temperature and extraction time had significant effect ($P<0.05$) to content of protein, gel strength, yield and viscocity from gelatin of pigskin. The highest amino acid content was glycine. It was concluded that the use of extraction temperature $60^0C$ and time of extraction 6 hours was the best characteristics of pigskin gelatin.*

***Key words****: extraction, gelatin, pigskin, temperature.*

## INTRODUCTION

Gelatin is a denaturalized protein that is derived from collagen and is an important functional biopolymer that has a very broad application in many industrial fields (Cho et al., 2004). Gelatin is a protein of animal origin, that can be obtained from collagen by acidic or alkaline hydrolysis (Said et al., 2011; Ward, 1977). The typical characteristics collagen protein include containing at least 33% amino acid glycine and 22% proline (Karim and Bath, 2008). The functional properties of gelatin are divided into two types. The first properties relate to gel formation processes (gel strength, gel formation time, melting temperature, viscosity, thickness, texture and water content) and the second nature is related to the properties of the gelatin surface shape and stabilization of emulsions, colloid protection, foam stability, film shape andadhesion cohesion. Gel strength is a very important physical property of gelatin, depending on the hydrogen bonds between water molecules and the free hydroxyl group of amino acid groups, the size of the protein chain, concentration and molecular weight distribution of gelatin (Said et al., 2011). Its functional properties depend on processing conditions as well as the raw material (Sobral and Habitante, 2001). Animal age also affect of the protein content of skin collagen, increasing the animal age, protein collagen and fibrous collagen growing stronger (Krochta et al., 1997; Ockerman and Hansen, 2000; Swatland, 1984). Gelatin production required a extraction step to improve quality of gelatine (Sompie et al., 2015). Good quality of gelatin is formed with a low extraction temperature, because at this temperature less hydrolysis of polypeptide chains occurs (Phillips and Williams, 2000). Furthermore, it was stated that at a temperature of $50^oC$ the extraction process can separate the structure of collagen, changing helical shape and hydrolyzed some covalent bonds. High quality gelatin is obtained from low extraction temperatures, but high extraction temperatures will increase yield (Ockerman and Hansen 2000). Yield produced from a gelatin is strongly influenced by the extraction process on collagen protein (Kasankala et al., 2007, Zhou and Joe, 2005).

The effect of extraction time to produce gelatin from pigskin was limited information. Thus, this research was conducted to study the effect of combination between temperature and extraction time on characteristics of pigskin gelatin.

## MATERIALS AND METHODS

**Materials**: Three thousand grams of pigskin were used as a raw material, acetic acid solution and stilled water.

**Procedures**: Gelatine was prepared by the acid extraction method (Ockerman and Hansen, 2000). Acetic acid ($CH_3COOH$ 0.5M) then diluted again with water in of 2%, 4% and 6% (v/v) were used as a treatments. The pigskin was soaked on acetic acid solution 24 hours. After soaked, samples were neutralized to pH 6, weighed and extracted on water bath for 3 hours, 6 hours and 9 hours at $50^0$C, $60^0$C and $70^0$C. Solubilised gelatin was separated from residual skin fragments by filtration through a nylon filter. The extracted gelatin was concentrated at $70^0$C for 6 hours and it was stored in the refrigerator $5^0$C for 30 minutes, then dried at $60^0$C for 24-36 hours until the solution dries and forms a gelatin sheet. Gelatin sheets were milled and packaged in vacuum plastic and stored in a desiccator for subsequent process.

The experiment were determined by analysis of Completely Randomized Design (Steel and Torrie, 1991) with two factors and three replicates of treatments. The first factor was temperaturextraction (50, 60 and 70 degrees Celsius). The second factor was time of extraction consisting of 3 levels (3, 6 and 9 hours). The significant differences of the average were determined using Duncan's new multiple range test.

**Parameters**: Gel strength was determined with a Universal Testing Machine (Zwick/Z.0,5). Gelatin solution of 6.67% w/v (6.67 grams to aquadest 100 ml) was heated at ± 60°C until the gelatin particles dissolved completely. Solution in the container Ø5 cm and height 6 cm was stored at $5^0$C for 16-18 hours. Gelatin was placed at the bottom of the plunger (Ø=13mm). Measurement was conducted at the temperature of $10^0$C and the speed 10 mm/min as deep as 4 mm was used as plunger. The value of gel strength (g Bloom) use the formula = 20 + 2,86 x $10^{-3}$D, where D = F/G x 980; F = height chart before fracture; G = constants (0.07) (Liu et al., 2008., Muyonga et al., 2004., Said et al., 2011[a]).

Viscosity was measured by gelatin powder dissolved in distilled water at a temperature of 40°C with a solution concentration of 6.67%. The values was measured by Stromer Viscosimeter Behlin CSR-10, It was obtained by expressed in centipoise according to the method Gomez (Sompie et al., 2012).

FOSS Kjeltec 2200 was used to determine protein content. A total of 0,5 g of sample + ¼ bussino tablet + 12 ml $H_2SO_4$ was concentrated in the destruction of the tube FOSS at ± $410^0$C for 1 hour. The results of destruction was distilled with thio-NaOH 40% + $H_3BO_4$ 4% + BCGMR indicators. A total of 150 ml was distillated in Erlenmeyer disk and titrated with 0,099 N HCl until the color changed from blue to pink. Five point fifty five was used as the conversion factor of gelatin protein.

The protein content (%) was calculated using the formula (ml HCL – ml Blanko) x N HCL x 14,0008 x 100 x 5,55)/g sample x 1000.

Amino acid analysis (sample preparation ):

1. Weigh the sample 60 mg
2. Add 4 ml of HCl 6 N
3. Reflux for 24 hours with a temperature of $120^o$C
4. Cool to room temperature
5. Neutralize with NaOH 6 N (PH.7) volume of 10 ml
6. Filter with Wattman 0.2
7. Take 50 filtered samples
8. Add an OPA solution of 300 ul
9. Take 20 ulsamples with the syringe into the HPLC

The tool used is LC 10 SHIMADZU HPLC Column: LiChrospher 100 RP-C18 (5 um) Eluent A: 50 mM Natrium Acetate: THF: Methanol (96:2:2) Eluent B: 65% Methanol.

## RESULTS AND DISCUSSIONS

### Protein Content

Gelatin is a source of protein derived from large amounts of collagen and is a group of structural proteins originating from the extracellular matrix (Hidaka and Liu, 2002; Karim and Bhat, 2008).

Statistical analysis on Table 1 indicated that the interaction between temperature and time of extraction had highly significant effect ($P<0.01$) on the protein content of pigskin gelatin. Duncan test results showed that protein

content of gelatin from pigskin had a tended to increase with increasing temperature of extraction.The increase in levels of gelatin protein is related to changes in the amount of amino acid bonding structures that make up collagen proteins. According to Swatland (1984), age slaughter affects the content of collagen in the skin, increasing age increased collagen protein. Protein content ranged 86.14 to 90.24%. That it was not different with protein content from chicken leg skin ranged 83-90 % and commercial gelatin, 91, 63% (Said et al., 2011).

Table 1. The characteristics of pigskin gelatin

| Parameters | Extraction time (hours) | extraction temperature (ºC) + Sd | | |
|---|---|---|---|---|
| | | 50 | 60 | 70 |
| Protein | 3 | 89.04±0.57 | 90.24±0.16 | 90.04±0.07 |
| content | 6 | 88.50±0.27 | 88.30±0.77 | 88.03±0.47 |
| (%) | 9 | 88.16±0.03 | 87.14±0.63 | 86.14±0.23 |
| Gel | 3 | 78.10±0.62 | 78.30±0.22 | 79.91±0.12 |
| strength | 6 | 77.08±0.20 | 78.38±0.21 | 78.84±0.20 |
| (g bloom) | 9 | 77.20±0.02 | 78.20±0.32 | 78.24±0.02 |
| Yield | 3 | 12.87±0.21 | 13.67±0.12 | 13.85±0.37 |
| (%) | 6 | 12.06±0.13 | 13.27±0.27 | 14.53±0.17 |
| | 9 | 12.17±0.05 | 13 .07±0.32 | 14.87±0.17 |
| Viscocity | 3 | 8.21±0.01 | 9.24±0.01 | 9.27±0.01 |
| (cP) | 6 | 8.16±0.16 | 8.17±0.04 | 9.18±0.06 |
| | 9 | 8.93±0.07 | 8.06±0.07 | 8.86±0.07 |

**Gel Strength**

One of the functional properties of gelatin is gel strength (Bergo and Sobral, 2007; Schrieber and Gareis, 2007). The average of gel strength from pigskin gelatin is displayed in Table 1. Statistical analysis indicated the interaction between temperature and time of extraction had significant effect (P<0.05) on pigskin gelatin. The value of gel strength tended to increase with increasing level of extraction temperature. Gel formation occurs due to the development of gelatin molecules during the extraction process. The presence of hydroxyproline caused the stability of the hydrogen bonds between water molecules and free hydroxyl groups of amino acids in gelatin, it is very important for gel strength (Kolodziejska et al., 2003; Wang et al., 2008).The amino acid monomer chains from one another to the next combine to form a continuous three-dimensional structure and bind water to form a compact gel structure (Said et al.,2011). Gel strength values from pigskin gelatin were ranged 77.08 – 79.91 g Bloom, that in line with the criteria of ISO 75-300 g Bloom (Sompie et al., 2014). Bloom is the value used to determine the quality of gelatin.

**Yield**

Yield is the amount of dry gelatin produced from a number of skin raw materials in a clean state through an extraction process (Giménez et al., 2005).Statistical analysis showed that the interaction between temperature and time of extraction gave highly significant effect (P<0.01) on the yield of pigskin gelatin. Duncan test results showed that the yield of gelatin from pigskin have increase with increasing temperature and time of extraction. The yield of gelatin tended to increase. The higher yield of pigskin gelatin was 14.53%.The higher of yield value indicates that the production process becomes more efficient.

**Viscosity**

The average viscosity of pigskin gelatin is displayed in Table 1. Statistical analysis indicated that the interaction between temperature and time of extraction had significant effect (P<0.05) on pigskin gelatin. The value of viscosity tended to decrease at the time of extraction acid increased. In order words, the higher extraction time, the viscosity was tended to decrease. This is because the viscosity of gelatin is directly proportional to the gel strength that was not significantly different between treatments (Astawan et al 2002). Furthermore, Sompie et al. (2015) explained that viscosity is affected by molecular weight and the length of amino acid chain.Increased concentrations of acetic acid in the gelatin production process can reduce the viscosity The curing material has been breaking the peptide bonds of amino acids into short-chain molecule so that its viscosity decrease. Viscosity values from pigskin gelatin were ranged 8.06 to 9.24 cP. It values is included in the ISO range 2.0 to 9.5 cP.

**Amino acid profile**

Amino acids are organic compounds that have a carboxyl functional group (-COOH) and amine (-NH2) in amphoteric solutions (Giménezetal, 2005). The amino acid profile of

pigskin gelatin is shown on Table 2 and Table 3.

Table 2. Amino acid profile from pigskin gelatin (ppm)

| No | Amino Acid | Pigskin Gelatin (ppm) |
|---|---|---|
| 1 | Aspartic | 53.11 |
| 2 | Glutamic | 124.19 |
| 3 | Serine | 37.67 |
| 4 | Hidroxyproline | 42.91 |
| 5 | Proline | 41.10 |
| 6 | Histidine | - |
| 7 | Glycine | 123.81 |
| 8 | Threonine | - |
| 9 | Arginine | 72.24 |
| 10 | Alanine | 63.91 |
| 11 | Tyrosine | - |
| 12 | Methionine | 7.65 |
| 13 | Valine | 13.22 |
| 14 | Phenylalanine | 9.60 |
| 15 | Isoleucine | - |
| 16 | Leucine | 17.06 |
| 17 | Lysine | 21.77 |

Table 3. Amino acid profile from pigskin gelatin (%)

| No | Aminoacid | Pigskin Gelatin (%) |
|---|---|---|
| 1 | Aspartic | 5.75 |
| 2 | Glutamic | 10.95 |
| 3 | Serine | 5.21 |
| 4 | Hidroxyproline | 1.44 |
| 5 | Proline | 1.36 |
| 6 | Histidine | - |
| 7 | Glycine | 37.65 |
| 8 | Threonine | - |
| 9 | Arginine | 7.01 |
| 10 | Alanine | 13.05 |
| 11 | Tyrosine | - |
| 12 | Methionine | 0.56 |
| 13 | Valine | 3.56 |
| 14 | Phenylalanine | 1.90 |
| 15 | Isoleucine | - |
| 16 | Leucine | 3.66 |
| 17 | Lysine | 1.42 |

Based on Tabel 2 and Table 3 indicated that the dominant amino acid profile in gelatin from pigskin was glycine (123.81 ppm). The amino acid glycine can be synthesized in several ways, namely through transaminases from glyoxylates, glutamate and alanine and from choline and serine through the reaction of serine hydroxy metal transferase (Pearson and Dutson, 1992). The amino acid glycine on each position of the three amino acid chains of collagen protein is an absolute requirement for the formation of collagen fibrils during its formation process (Gautieri et al., 2008; Gelse et al., 2003). Gelatin is a type of protein extracted from collagen so that it generally has an amino acid composition resembling collagen (Giménez et al., 2005; Nemati et al., 2003).

Curing has changed due to denaturation of skin collagen proteins and some certain amino acids change chemically (Pearson and Dutson, 1992; Said et al., 2011). Typical properties of collagen protein are high levels of amino acids glycine, proline and hydroxyproline, while amino acids from aromatic and sulfur groups are present in small amounts (Wolf, 2003). Acidic solution is able to convert collagen fibers from triple helix to monohelix whereas alkaline solution is only able to convert into bihelix (Kołodziejska et al., 2003; Said et al., 2011).

## CONCLUSIONS

It was concluded that the use of extraction temperature $60^0$C and time of extraction 6 hours was the best characteristics of pigskin gelatin.

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# BODY THERMAL RESPONSE TO ENVIRONMENT TEMPERATURE IN RAINBOW TROUT (*Oncorhynchus mykiss*) DURING THE SUMMER SEASON

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***Abstract***

*The body temperatures of rainbow trout, Oncorhynchus mykiss were monitored from June through August 2018 in three trout farms from Bistrița-Năsăud County (Romania). The body temperature was measured from 3 different regions of the body: head, trunk (epaxial and hypaxial muscle region) and caudal peduncle. Body temperatures were compared to the basins water temperature. The lowest water temperature in the basins was recorded in May and June, in Șoimul de Jos farm, and the highest temperature in Fiad farm. In July, Strâmba farm, recorded the highest water temperature, and in August, Fiad farm. In all farms studied, the body temperature of the trout was higher than that of the basins water temperature regardless of the studied region. The highest differences between the basins water and body temperature in farms were found in the caudal peduncle and epaxial muscle regions, due to their role in locomotor mechanism. This is due to the high growth density, the feeding and the intensification of metabolism, with energy release, as well as a much higher level of stress than that encountered in the natural environment.*

***Key words***: *body temperature, infrared thermometer, metabolism, rainbow trout, Salmonidae.*

## INTRODUCTION

The Rainbow trout (*Oncorhynchus mykiss)* is one of the most studied fish species from the Salmonidae family, due to their high economic value (Ihuț et al., 2018b;Topuz et al., 2017), growth rate (Hokanson et al., 1977), interesting biologically life cycle and the history of translocation across many continents, alongside other species of high economic value (Nicolae et al., 2018).

Changing environmental conditions (Pörtner and Farrell, 2008) are affecting reproduction (Pankhurst and Munday, 2011; Donelson et al., 2010; Uiuiu et al., 2017), feeding (Fu et al., 2009), swimming (Blake, 2004; Day and Butler, 2005; Green and Fisher, 2004; Imre et al., 2002), physiological traits of fishes by affecting energy sources through the changes in temperature (Brune and Tomasso, 2005) and concentration of dissolved oxygen (Lee et al., 2003, Ihuț et al., 2018a). Digestion, food consumption (Kausar and Salim, 2006), behavior (Wagner et al., 1997), immunity (Cocan et al., 2018), are also influenced by the temperature. Inadequate temperatures can lead to a state of stress, being the precursor of pathological conditions. The immune system of most fish species has optimal performance at about 15°C of water temperature (Schmidt-Nielsen, 1991).

Temperature has great importance and plays a very important role in the life of aquatic poikilotherms organisms (Barton, 1996), which means that their body temperature is the same as the water in which they live or has 0.5 to 1.5°C tolerance below or above the temperature of water (Bidgood, 1980).

In natural habitats, fish can easily tolerate seasonal temperature changes such as 0°C during winter and up to 20-30°C (depending on species) during summer in the temperate continental climate. However, these changes should not be sudden.

Thermal shocks in trout occur when placed in a new environment, where the difference of temperature is higher or lower by 8°C, compared to the initial temperature of the water (Svobodová et al., 1993).

Rainbow trout tolerates diurnal temperature differences above 5°C but generally prefers a constant temperature. Each species has a temperature range in which the metabolic processes run with maximum intensity. Also, the species has lower and upper temperature threshold (lethal temperature). Rainbow trout, along with other species of the Salmonidae family, is a stenotherm species, but however, it exhibits higher plasticity, tolerating higher temperature variations. Optimal temperatures are between9 and 18°C, but feeding and growing at water temperatures of 4 to 20°C takes place in good conditions. At water temperatures below 4°C and above 20°C the intensity of feeding and growing is reduced. Rainbow trout have a specific type of metabolism, their metabolic rate continues at low temperatures, but at high temperatures, usually above 20°C they consume less food and become less active. Temperature above 20°C is not comfortable for trout, the lethal temperature is from 24.9 to 26.3°C (Matschak et al., 1998).

## MATERIALS AND METHODS

The biological material used in this study was sampled from Strâmba, Şoimul de Jos and Fiad trout farms, Bistriţa-Năsăud County (Figure 1). The study took place in the summer of 2018, between May-August. The number of fish specimens from which temperature measurements were taken was 25. Clinically healthy specimens were used in order not to negatively affect the obtained results

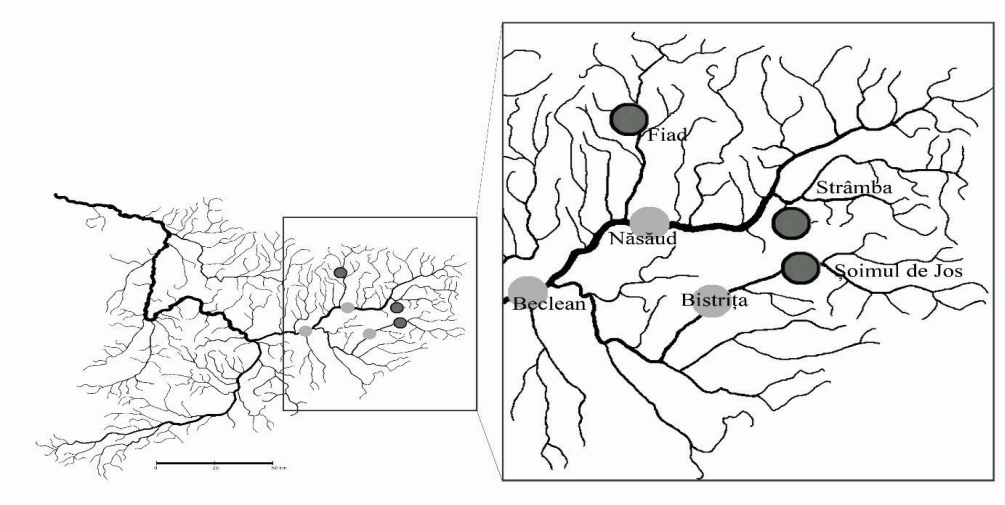


Figure 1. Someş River catchment – Fiad trout farm (Sălăuţa River), Strâmba trout farm (Strâmba River) and Şoimul de Jos trout farm (Şoimu River)

For the determination of body temperature, we used Hannah HI-99551-00 Infrared Thermometer with IR Sensor. The accuracy of the thermometer is± 2%, the range -10 to 300°C and the IR sensor optic coefficient 3:1 (ratio of distance to target diameter). The IR thermometer was compared to a mercury thermometer and it was within his stated accuracy. The body temperature was measured from 4 different regions of the body: head, trunk (epaxial and hypaxial muscle region) and caudal peduncle. For a better manipulation, the trout specimens were anesthetized with clove oil (*Eugenia caryophyllata*), 0.047 ml/L concentration in water, a solution used as a local antiseptic and anaesthetic until the fish lost equilibrium and did not respond to physical stimuli. Clove oil is a natural anaesthetic that under immersion conditions, acts on the somatic nervous system, leaving the functions of the vegetative nervous system active (opercular movement). After anesthetization, the measurements were made in the shortest time possible to reduce the level of stress that they are subjected. Immediately after the measurements were made, the fishes were transferred in tanks with fresh water for easier recovery from anaesthesia.

For the determination of water temperature, we used the Hanna HI 9828/4-01 Multi-Parameter, with a range of -5.00 to 55.00°C, resolution 0.01°C and an accuracy ±0.15°C. The measurements of water temperature were made at the same time as those for the body temperature.

The obtained data was interpreted and processed statistically with the GraphPad Prism v6 software and Pearson Correlation and Scatter Plot Matrix was made with IBM Statistics SPSS 20 software. Drafting, images, graphics, and spreadsheets were edited in Microsoft Word v. 2016. All the methods used are up-to-date and the existing data has been processed in the laboratories of UASMV Cluj-Napoca, Faculty of Animal Science, Physiology of Aquatic Organisms Discipline.

## RESULTS AND DISCUSSIONS

We used the Bivariate Pearson Correlation to produce a sample correlation coefficient that measures the force and direction of linear relationships between pairs of variables. We also analysed the correlations between water temperatures over four consecutive months (May, June, July and August)in the three trout farms (Figure 2). All relations are positive, but not all are statistically significant.

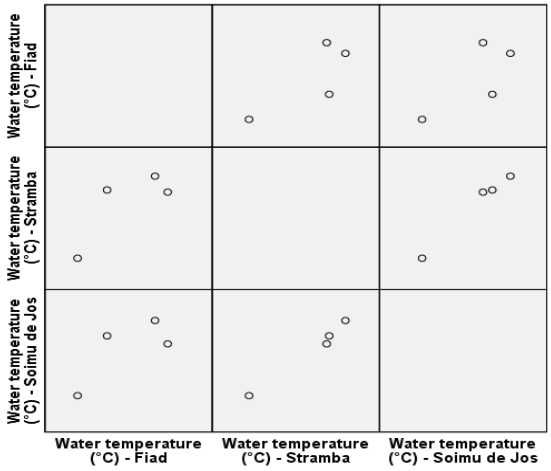


Figure 2. Water temperatures correlations from Fiad (Sălăuța River), Strâmba (Strâmba River) and Șoimul de Jos (Șoimu River) trout farms from Bistrița-Năsăud County, May-August 2018

The strongest correlation and statistically significant water temperature is encountered in the case of Strâmba and Şoimul de Jos trout farms (0.993), resulting in a significance threshold of 1%. This can also be seen from the collected data, the temperature values being very close to each other. Strong correlations are the result of the close proximity of the two farms due to their geomorphological conditions in volcanic mountains. Geographical location and landscape are the main elements that directly influence the climatic and meteorological properties of the area. By its geographical position, the Bârgăului and Călimani Mountains belong to the area with continental moderate climate, often subject to the advection of maritime polar air, with frequent frontal activity. Also, the length of the river (from spring to farm unit emplacement) plays a very important role in water temperature. In Strâmba and Şoimul de Jos trout farms, the distance between the springs and the water emplacement of the farm is relatively short, 5 km (Strâmba) and 4 km (Şoimul de Jos). Fiad farm is supplied by the Sălăuța River, which has a length of 20 km from the spring to the emplacement of the farm.

In Table 1 are presented the Rainbow trout body and water temperatures obtained from Fiad trout farm located on Sălăuța River, Bistrița-Năsăudand the differences between them, during the summer months. To determine the thermal response tothe environment, the

temperatures recorded from the fish body were compared to those of the water. Body temperature was measured from 4 different regions of the fish's body: head region, trunk region (epaxial and hypaxial muscle region) and caudal peduncle region.

Table 1. Rainbow trout body and river temperatures simultaneously recorded, fromFiad trout farm, Sălăuța River, Bistrița-Năsăud, May- August, 2018

| Month | Water temperature (°C) | | Body temperature (°C) (N=25) | | | |
|---|---|---|---|---|---|---|
| | | | Trunk region (muscle) | | Caudal peduncle | Head region |
| | | | Epaxial | Hypaxial | | |
| May | 13.32 | Mean ± SD | 14.69±0.40 | 14.57±0.40 | 14.80±0.34 | 14.60±0.43 |
| | | SEM | 0.08 | 0.08 | 0.07 | 0.09 |
| | | Min-Max | 14.00-15.50 | 13.80-15.60 | 14.20-15.40 | 13.90-15.90 |
| | | V% | 2.76% | 2.81% | 2.27% | 2.92% |
| | | Dif (Bt-W) | 1.37 | 1.25 | 1.48 | 1.28 |
| June | 17.17 | Mean ± SD | 19.90±0.50 | 19.95±0.40 | 20.32±0.50 | 19.93±0.51 |
| | | SEM | 0.10 | 0.08 | 0.10 | 0.10 |
| | | Min-Max | 19.10-20.80 | 19.40-20.90 | 19.60-21.30 | 19.30-21.30 |
| | | V% | 2.52% | 1.99% | 2.48% | 2.56% |
| | | Dif (Bt-W) | 2.73 | 2.78 | 3.15 | 2.76 |
| July | 14.58 | Mean ± SD | 16.48±0.40 | 16.35±0.55 | 16.93±0.46 | 16.56±0.47 |
| | | SEM | 0.08 | 0.11 | 0.09 | 0.09 |
| | | Min-Max | 15.70-17.30 | 15.40-17.40 | 15.90-17.70 | 15.60-17.30 |
| | | V% | 2.40% | 3.36% | 2.72% | 2.82% |
| | | Dif (Bt-W) | 1.90 | 1.77 | 2.35 | 1.98 |
| August | 16.63 | Mean ± SD | 17.82±0.42 | 17.99±0.36 | 18.45±0.29 | 18.09±0.29 |
| | | SEM | 0.08 | 0.07 | 0.06 | 0.06 |
| | | Min-Max | 17.10-18.60 | 17.30-18.50 | 17.80-18.90 | 17.40-18.50 |
| | | V% | 2.35% | 1.98% | 1.58% | 1.60% |
| | | Dif (Bt-W) | 1.19 | 1.35 | 1.82 | 1.46 |

*Note: N – Number of specimens; SD – Standard deviation; SEM – Standard error of mean; Min/Max – Minimum/Maximum; V% -Coefficient of variation; Dif (Bt-W) – The difference between body and water temperature

The body temperature of fishes was higher in all cases than the water temperatures. As we can notice in all months, the difference between the caudal peduncle and the water temperature is higher than in the hypaxial and epaxial muscle and in the head region. This is due to the fact that the caudal peduncle has a very important role in the mechanism of locomotion, resulting in an increase of the temperature in accordance to Joule's law. Rainbow trout is a fast swimming and powerful fish, due to its natural habitat conditions (high water speed). When we analysed the differences between water temperature and hypaxial/epaxial muscle regions, we noticed when environmental water temperature is lower (May, July) the activity of epaxial muscle region is more intense than that of hypaxial muscle region resulting in an increase in temperature of epaxial muscle region (due to the higher movement and swimming of fishes). When the water temperature is higher (June, August) the activity of epaxial muscle region decreases. Hypaxial muscle region temperature is higher than epaxial muscle region due to the reduction of movement and swimming. This is a consequence of the increasing temperature of water, which has as result a slow metabolic rate.

In Table 2 are presented the Rainbow trout body and water temperatures obtained from Strâmba trout farm located on Strâmba River, Bistrița-Năsăud and the differences between them, during the summer months.

Table 2. Rainbow trout body and river temperatures simultaneously recorded, from Strâmba trout farm, Strâmba River, Bistrița-Năsăud, May-August, 2018

| Month | Water temperature (°C) | Body temperature (°C) (N=25) | | | | |
|---|---|---|---|---|---|---|
| | | | Trunk region (muscle) | | Caudal peduncle | Head region |
| | | | Epaxial | Hypaxial | | |
| May | 11.6 | Mean ± SD | 14.12±0.33 | 14.28±0.47 | 14.37±0.34 | 14.18±0.49 |
| | | SEM | 0.07 | 0.09 | 0.07 | 0.10 |
| | | Min-Max | 13.30-14.70 | 13.50-15.60 | 13.70-15.10 | 13.10-15.20 |
| | | V% | 2.37% | 3.31% | 2.37% | 3.44% |
| | | Dif (Bt-W) | 2.52 | 2.68 | 2.77 | 2.58 |
| June | 15.58 | Mean ± SD | 17.15±0.44 | 17.18±0.47 | 17.56±0.54 | 17.48±0.78 |
| | | SEM | 0.09 | 0.09 | 0.11 | 0.16 |
| | | Min-Max | 16.40-17.90 | 16.20-17.90 | 16.90-18.70 | 16.50-19.00 |
| | | V% | 2.59% | 2.75% | 3.05% | 4.46% |
| | | Dif (Bt-W) | 1.57 | 1.60 | 1.98 | 1.90 |
| July | 15.71 | Mean ± SD | 17.24±0.45 | 17.47±0.41 | 17.72±0.37 | 17.49±0.54 |
| | | SEM | 0.09 | 0.08 | 0.07 | 0.11 |
| | | Min-Max | 16.40-17.90 | 16.80-18.40 | 16.90-18.60 | 16.60-18.60 |
| | | V% | 2.60% | 2.33% | 2.10% | 3.08% |
| | | Dif (Bt-W) | 1.53 | 1.76 | 2.01 | 1.79 |
| August | 16.54 | Mean ± SD | 17.69±0.30 | 17.56±0.32 | 18.04±0.41 | 17.87±0.37 |
| | | SEM | 0.06 | 0.06 | 0.08 | 0.07 |
| | | Min-Max | 17.20-18.30 | 16.90-18.10 | 17.40-19.20 | 17.40-18.80 |
| | | V% | 1.72% | 1.80% | 2.26% | 2.07% |
| | | Dif (Bt-W) | 1.15 | 1.02 | 1.50 | 1.33 |

*Note: N – Number of specimens; SD – Standard deviation; SEM – Standard error of mean; Min/Max – Minimum/Maximum; V% -Coefficient of variation; Dif (Bt-W) – The difference between body and water temperature

Water temperature varies daily and seasonally due to the temperate climate of our country. Both in natural habitats and trout farms, salmonids are exposed to fluctuating water temperatures. The same situation as in Fiad trout farm also occurred in Strâmba farm, where the body temperature of fishes was higher in all cases than the water temperatures.

In May, water temperature was 11.6°C in Strâmba trout farm (Strâmba River) and the mean trunk epaxial muscle region temperature was 14.12°C with 2.52°C higher than water temperature. As for hypaxial muscle region, the temperature was higher (2.68°C). Regarding the caudal peduncle region temperature, the difference was 2.77°C. The same situation was encountered in the head region, where the difference was 2.58°C.

Baird and Krueger (2003) found in a study that took place in the Adirondack River, New York, in the summer of 1997, from June to September, that in the case of Brook trout, *Salvelinus fontinalis*, body temperature was lower with 2.3°C and in Rainbow trout (*Onchorhyncus mykiss*) with 1.5°C than the main river water temperature. Both brook trout and rainbow trout used localized cooler water areas to lower their body temperatures below that of the main river. Stream temperatures may differ at various locations and may include localized cooler water areas that could serve as thermal refuges, allowing the survival of fish that are sensitive to high temperatures. Water which is cooler than mainstream flows can also occur where tributaries or groundwater sources discharge cooler water. In trout farms, these thermal refuges do not exist, so the body temperature is usually higher than that of water. In captivity and under intensive feeding conditions, temperature differences are reversed compared to those from the mentioned study, due to the lack of shade, benthic structures, depth and cooler water areas that could serve as thermal refuges. Also farming

stock density, feeding intensity, stress level compared to natural habitat may be a source of increasing the body temperature. In Table 3 are presented the Rainbow trout body and water temperatures obtained from Șoimul de Jos trout farm located on Șoimu River, Bistrița-Năsăud and the differences between them, during the summer months.

Table 3. Rainbow trout body and river temperatures simultaneously recorded, from Șoimul de Jos trout farm, Șoimu River, Bistrița-Năsăud, May-August, 2018

| Month | Water temperature (°C) | | Body temperature (°C) (N=25) | | | |
|---|---|---|---|---|---|---|
| | | | Trunk region (muscle) | | Caudal peduncle | Head region |
| | | | Epaxial | Hypaxial | | |
| May | 10.57 | Mean ± SD | 13.09±0.33 | 12.92±0.32 | 13.56±0.27 | 13.34±0.39 |
| | | SEM | 0.07 | 0.06 | 0.05 | 0.08 |
| | | Min-Max | 12.60-13.90 | 12.30-13.40 | 12.90-14.10 | 12.70-14.00 |
| | | V% | 2.50% | 2.51% | 1.99% | 2.90% |
| | | Dif (Bt-W) | 2.34 | 2.17 | 2.81 | 2.59 |
| June | 13.35 | Mean ± SD | 15.80±0.39 | 15.87±0.38 | 16.20±0.29 | 15.91±0.36 |
| | | SEM | 0.08 | 0.08 | 0.06 | 0.07 |
| | | Min-Max | 15.00-16.80 | 15.10-16.50 | 15.70-16.80 | 15.10-16.40 |
| | | V% | 2.46% | 2.40% | 1.78% | 2.28% |
| | | Dif (Bt-W) | 2.45 | 2.52 | 2.85 | 2.56 |
| July | 13.75 | Mean ± SD | 15.88±0.34 | 15.88±0.32 | 16.22±0.32 | 15.98±0.29 |
| | | SEM | 0.07 | 0.06 | 0.06 | 0.06 |
| | | Min-Max | 15.10-16.40 | 15.20-16.40 | 15.70-16.70 | 15.40-16.50 |
| | | V% | 2.11% | 2.02% | 1.94% | 1.80% |
| | | Dif (Bt-W) | 2.13 | 2.13 | 2.47 | 2.23 |
| August | 14.52 | Mean ± SD | 16.57±0.32 | 16.70±0.41 | 17.03±0.48 | 16.78±0.46 |
| | | SEM | 0.06 | 0.08 | 0.10 | 0.09 |
| | | Min-Max | 16.10-17.20 | 16.00-17.50 | 16.20-17.90 | 16.00-17.50 |
| | | V% | 1.94% | 2.49% | 2.82% | 2.77% |
| | | Dif (Bt-W) | 2.05 | 2.19 | 2.51 | 2.26 |

*Note: N – Number of specimens; SD – Standard deviation; SEM – Standard error of mean; Min/Max – Minimum/Maximum; V% -Coefficient of variation; Dif (Bt-W) – The difference between body and water temperature

In Șoimul de Jos trout farm, we noticed that in most regions the differences between body and water temperature are higher in caudal peduncle and in the head region. At the level of the head region, we recorded the temperature from operculum, which is a series of bones found in bony fish that serves as a facial support structure and a protective covering for the gills; it is also used for respiration and feeding. The higher is the feeding intensity and the opercular rate, the higher is the temperature in the region.

In the conditions of a salmonid trout farm, over several generations, there is an artificial and phylogenetic adaptation of the fish, where they tolerate larger variables of minimum and maximum temperatures. However, in the natural environment, growth, survival and successful reproduction are more sensitive to thermal tolerances. Fish are able to physiologically adapt to farming thermal conditions due to artificial selection, but in natural habitats, when ecological factors such as food availability, vulnerability to predators, hydrological factors (rainfall, flood and drought) represent the action of natural selection. It is important to remember that fish from the Salmonidae family are physiologically adapted to live in cold water environments and that their capacity to adapt to higher water temperatures is limited due to the degree of

amelioration and genetic evolution of the species.

## CONCLUSIONS

Fish, like other aquatic organisms, are poikilotherms. There are specific adaptations and responses to temperature changes. The results indicate a higher temperature in the caudal peduncle compared to the head region and higher temperatures in the epaxial muscles region compared to the hypaxial muscles region. Improving thermal tolerance can be done through selective reproductive programs. Although the ability to develop a tolerance for some of the variables is still debatable (pH), the selective growth to produce a tolerant high-temperature line appears as feasible within a few generations. However, further research should be undertaken on the tolerances of young stages (embryos, juveniles) to confirm the development of tolerances.

## ACKNOWLEDGMENTS

We are grateful to the technical staff of Strâmba, Șoimul de Jos and Fiad trout farms, Bistrița-Năsăud County for the opportunity to collect data from the trout farms.

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# NEW DATA FOR HELMINTH COMMUNITIES OF *Alburnus alburnus* (Linnaeus, 1758) FROM MARITSA RIVER, BULGARIA

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***Abstract***

*During summer 2018, 29 specimens of bleak (Alburnus alburnus (Linnaeus, 1758)) from Maritsa River were collected and examined with standard techniques for parasites. Helminth parasites were recorded in 24 bleak specimens (82.75%) from Maritsa River. Five species of parasites were identified: one trematode species (Allocreadium isoporum (Looss, 1984)), two cestode species (Caryophyllaeus brachycollis Janiszewska, 1951, Ligula intestinalis (Linnaeus, 1758))*; *one acantocephalan species (Pomphorhynchus laevis (Müller, 1776)) and one nematode species (Rhabdochona denudata (Dujardin, 1845; Raillet, 1916). The analysis of the dominant structure of the found parasite species is presented to the component and infracommunities level. In the component community of Alburnus alburnus from Maritsa River C. brachycollis, P. laevis and, R. denudata are corespecies. L. intestinalisis component parasite species and A. isoporum is accidental parasite species for the helminth communities of bleak. The infracommunities data was used to be fixed principal biotic indices. Bioindicator significance of established parasite species was discussed for ecological evaluation of the state of the studied freshwater ecosystem.New data for the helminths and helminth communities of bleak from Maritsa River are presented.*

***Key words****: Alburnus alburnus; bioindication; helminth communities; Maritsa River.*

## INTRODUCTION

The Maritsa River (Maritsa/Meriç/Evros) is the longest river (539 km, of which 321.6 km on Bulgarian territory) that runs solely in the interior of the Balkan Peninsula. The Maritsa River springs from the Rila Mountains (2378 m altitude) in Western Bulgaria, flowing southeast between the Balkan and Rhodope Mountains and passes through the districts of Pazardzhik, Plovdiv, Stara Zagora and Haskovo. After leaving Bulgaria, the river passes through the north-eastern part of Greece and the European part of Turkey and enters the Aegean Sea. The Maritsa is the fourth longest river in Bulgaria and is included in the National monitoring program (Regulation 1/2011).

Fish parasite communities and biodiversity from the Maritsa River and the state of the freshwater ecosystem, including metal content in the fish host and parasites were studied (Margaritov, 1965; Kirin, 2000a; 2000b; 2001a; 2006; 2013; 2014), but only Margaritov (1965) examined specifically bleak. The aim of this study is to present the diversity and communities of endoparasites of bleak (*Alburnus alburnus* (Linnaeus, 1758)) from Maritsa River, Bulgaria. As a result of this survey new data for helminths and helminth communities of *A. alburnus* is presented.

## MATERIALS AND METHODS

During the summer of 2018, fish and fish parasites were collected and examined from the Maritsa River (in the vicinityof the city of Plovdiv). The city of Plovdiv (42°9′N 24°45′E) is situated on the two banks of the Maritsa River. The region of the city and the riverside are distinguished with a significant diversity of highly protected species and territories declared as protected with national and international nature protective status (Assyov, 2012).

A total of 29 bleak specimens (*Alburnus alburnus* (Linnaeus, 1758)) were collected and examined from the Maritsa River during the summer of 2018. The fish were caught by angling. The scientific and common names of the fish host are used according to the FishBase database (Fröse and Pauly, 2018). The fish

were immediately after their capture examined for gastrointestinal and tissue helminths (an incomplete parasitological study). Helminths were cleaned in a saline solution and fixed in 70% ethanol. Trematodes were fixed as permanent slides after their colouring with acetic carmine, differentiation in 70% acid ethanol, dehydrating in increasing ethanol series, clarifying in eugenol and mounting in Canada balsam (Bykhovskaya-Pavlovskaya, 1985; Georgiev et al., 1986). The samples were counted and identified using keys of Bauer et al. (1981) and Bykhovskaya-Pavlovskaya (1985). Cestodes were stained with acetic carmine and mounted as permanent slides in a Canada balsam according to Georgiev et al. (1986) and Scholz and Hanzelová (1998). Acanthocephalans were examined as temporary slides in ethanol-glycerin and identified (Petrochenko, 1956; Ergens and Lom, 1970; Bykhovskaya-Pavlovskaya, 1985). Nematodes were examined as temporary microscopic preparations in glycerin (Moravec, 1994; 2013).

The dominant structure of the component helminth communities was determined according to the criteria proposed by Kennedy (1993) on the basis of the prevalence (P%): accidental (P%<10), component (10<P%<20) and core (P%>20) species. The ecological terms prevalence, mean intensity are used, based on the terminology of Bush et al. (1997). Analyses of helminth community structure were carried out in both levels: infracommunity and component community. The component data were used to determine the total number of species, Shannon diversity index (H'), Pielou evenness index (E), Berger-Parker dominance index (d) according to Magurran (2004). The infracommunity data was used to calculate the mean number of species, the mean number of helminth specimens, Brillouin diversity index (HB) (Kennedy, 1993; 1997; Magurran, 2004).

## RESULTS AND DISCUSSIONS

A total of 29 specimens of bleak (*Alburnus alburnus* (Linnaeus, 1758)) were collected and examined from the Maritsa River. *Alburnus alburnus* is estimated as least concern species (LC=Least Concern; IUCN Red List Status). Bleak is freshwater, brackish, benthopelagic, potamodromous fish species. This fish species inhabit open waters of lakes and medium to large rivers. Larvae of *A. alburnus* live in the littoral zone of rivers and lakes, while juveniles leave shores and occupy a pelagic habitat, feeding on plankton, drifting insects or invertebrates fallen on the water surface. The diet of this fish includes mainly plankton, as well as crustaceans and insects (Fröse and Pauly, 2018).

Helminth parasites were recorded in 24 bleak specimens (85.75%) from the Maritsa River. Five species of parasites were identified: one trematode species (*Allocreadium isoporum* (Looss, 1984)), two cestode species (*Caryophyllaeus brachycollis* Janiszewska, 1951), *Ligula intestinalis* (Linnaeus, 1758)); one acantocephalan (*Pomphorhynchus laevis* (Müller, 1776)) and one nematode species (*Rhabdochona denudata* (Dujardin, 1845) Raillet, 1916) (Table 1).

All helminth species occurred as adults with the exception of *L. intestinalis*. *A. isoporum, C. brachycollis, P. laevis* and *R. denudate* are autogenic species, matured in fish. *L. intestinalis* is allogenic species. The larval stages of *L. intestinalis* develop in the body cavity of carp fishes – *Abramis brama, A. sapa, S. erythrophtalmus, A. alburnus, A. bipunctatus, Gobio gobio, Rutilus rutilus, Barbus barbus, B. m. petenyi, Leuciscus cephalus, L. idus* and *Phoxinus phoxinus* (Kakacheva-Avramova, 1983). In an adult state *L. intestinalis* parasitized in fish-eating birds, mainly gulls (*Larus*), less commonly in fish-eating ducks (*Bucephala* and *Mergus*) and in *Podiceps* (Kakacheva-Avramova, 1983).

In the component community of *Alburnus alburnus* from the Maritsa River *C. brachycollis* (P%=24.13), *P. laevis* (P%=31.03) and *R. denudata* (P%=37.93) are core species. *L. intestinalis* (P%=10.34) is component parasite species and *A. isoporum* (P%=6.89) is accidental parasite species for the helminth communities of bleak (Table 1).

Table 1. Helminth parasites of *Alburnus alburnus* from Maritsa River (N – number of examined hosts, n – number of infected hosts, p – number of parasites, P – prevalence, MI – mean intensity, MA – mean abundance)

| **Helminth species** | N=29 | | | | | |
|---|---|---|---|---|---|---|
| | N | P | P% | MI±SD | MA±SD | Range |
| *Allocreadium isoporum* (Looss, 1984) | 2 | 3 | 6.89 | 1.5±0.5 | 0.10±0.40 | 1-2 |
| *Caryophyllaeus brachycollis* (Janiszewska, 1951) | 7 | 11 | 24.13 | 1.57±0.49 | 0.37±0.71 | 1-2 |
| *Ligula intestinalis* (Linnaeus, 1758) | 3 | 3 | 10.34 | 1.0±0 | 0.10±0.30 | 1 |
| *Pomphorhynchus laevis* (Müller, 1776) | 9 | 16 | 31.03 | 1.77±0.78 | 0.55±0.93 | 1-3 |
| *Rhabdochona denudata* (Dujardin, 1845) Raillet, 1916 | 11 | 18 | 37.93 | 1.63±0.48 | 0.62±0.84 | 1-2 |

In the component community of *Alburnus alburnus* from the Maritsa River nematodes are presented with the highest number of specimens, with 1 species and 18 specimens. Acanthocephalans are presented with one species and 16 specimens. Cestodes are represented by two species and 14 specimens. Trematodes are represented by one species and 3 specimens.

*Allocreadium isoporum* was found in *A. alburnus* from Danube River (Kakacheva-Avramova, 1977). For Maritsa River, *A. isoporum* was reported as parasite of *Squalius cephalus* (Linnaeus, 1758) (*Leuciscus cephalus*) and *Barbus cyclolepis* (Heckel, 1837) (*Barbus tauricus cyclolepis*) (Margaritov, 1965). For Maritsa River and Stryama River was also reported the subspecies *Allocreadium isoporum macrorchis* with host *Sq. cephalus* (*L. cephalus*) (Kirin, 2000a; 2000b; 2001a; Kirin et al., 2002). *A. isoporum* is an intestinal parasite of many species of family Cyprinidae (Kakacheva-Avramova, 1983). *A. isoporum* develops with the participation of two intermediate hosts: the first are bivalves of the genus *Sphaerium*, and the second is an insect larva of the genus *Ephemera*, *Anabolia* and *Chaetopteryx* (Kakacheva-Avramova, 1983).

Representatives of the genus *Sphaerium* and genus *Anabolia* are bioindicators for α-mesosaprobity and β-mesosaprobity (Johnson et al., 1993). Representatives of the genus *Ephemera* are bioindicators for β-mesosaprobity and oligosaprobity (Johnson et al., 1993). Representatives of the genus *Chaetopteryx* are bioindicators for β-mesosaprobity (Johnson et al., 1993).

*Caryophyllaeus brachycollis* was found in *A. alburnus* from Maritsa River, Danube River and Arda River (Margaritov, 1965; Kakacheva-Avramova, 1977; Kirin et al., 2002). For Maritsa River *C. brachycollis* was reported as parasite of *Sq. cephalus* (*L. cephalus*), *B. cyclolepis* (*B. tauricus cyclolepis*), *Vimba melanops* and *Rutilus rutilus* (Margaritov, 1965; Kirin, 2000a; 2001a; 2014). Intermediate host of *C. brachycollis* is *Limnodrilus hoffmeisteri* and definitive hosts are freshwater fish species from family Cyprinidae (Kulakovskaya, 1961; Kakacheva-Avramova, 1983).

*Limnodrilus hoffmeisteri* is bioindicator for polisaprobity (Johnson et al., 1993).

*Ligula intestinalis* was reported as parasite of *Alburnus alburnus* from Palakariya River, Arda River and Danube River (Kakacheva-Avramova and Nedeva-Menkova, 1978; Kirin et al., 2002; Chunchukova et al., 2018).

*Ligula intestinalis* (Diphyllobothriidae) is widely distributed cestode species with a complex life cycle, which involves a copepod as the first intermediate host, fish as a second intermediate host and an avian definitive host (Dubinina, 1980).

*P. laevis* was reported as a parasite of *A. alburnus* from Struma River and Danube River (Kakacheva-Avramova, 1962; 1977; Margaritov, 1966; Atanasov, 2012; Chunchukova et al., 2018). For Maritsa River *P. laevis* was reported as parasite of *Sq. cephalus* (*L. cephalus*) and *Esox lucius* (Kirin, 2000a; 2000b; 2001a; 2006).

Intermediate host of *P. laevis* is *Gamma ruspulex*, definitive hosts are fish most often from family Cyprinidae and paratenic hosts are small fish of the same family (Kakacheva-Avramova, 1983). *G. pulex* is a bioindicator for β-mesosaprobity (Johnson et al., 1993).

*R. denudate* was reported as a parasite of *A. alburnus* for the rivers Struma, Tundja, Maritsa, Arda, Veleka, Resovka and Danube (Kakacheva-Avramova, 1962; 1970; 1977; Margaritov, 1965; Kirin, 2001b; 2003; Kirin et al., 2002). For Maritsa River, *R. denudate* was reported as a parasite of *Sq. cephalus* (*L. cephalus*), *Vimba melanops* (Heckel, 1837) (*Vimba vimba melanops*), *Alburnus alburnus* and *Barbus cyclolepis* (*B. tauricus cyclolepis*) (Margaritov, 1965; Kirin, 2000; 2001b).

*Rhabdochona denudata* is an intestinal parasite of many species of family Cyprinidae (Moravec, 2013). Intermediate hosts of *R. denudata* are insect larvae: *Heptagenia* sp., *Ephemerella* sp. and *Hydropsyche* sp. (Bauer, 1987; Kakacheva-Avramova, 1983). Moravec (2013) suggested that in addition to mayflies also some other aquatic arthropods may serve as intermediate hosts of *R. denudata.*

Representatives of the genera *Heptagenia* and *Ephemerella* are bioindicators for β-mesosaprobity. *Hydropsyche* sp. is bioindicator for 0-α-mesosaprobity (Johnson et al., 1993). Species richness in infracommunityof bleak ranges from 0 to 3 species. With 1 helminth species were infected 17 specimens of fish (58.62%): 6 bleaks (20.69%) with *R. denudata*; 6 bleaks (20.69%) with *P. laevis*; 4 bleaks (13.79%) with *C. brachycollis* and only one bleak (3.44%) with *L. intestinalis*. With 2 helminth species were infected 6 specimens of *A. alburnus* (20.69%). Only one specimen of bleak (3.44%) was infected with 3 helminth species (*A. isoporum*, *C. brachycollis* and *R. denudata*). Five of the studied bleak specimens were free of parasites (17.24 %).

The largest number of helminth specimens established in a single host specimen is 5. The average species richness (mean number of species for a fish specimen) in infracommunity of bleak is 1.10±0.71 species (Table 2).

Average abundance (mean number of helminths in fish) in these infracommunities is 1.76±1.36. The parasite communities of *A. alburnus* from the Maritsa River showed Brillouin diversity index, HB=1.26 (Table 2).

Table 2. Infracommunities of *Alburnus alburnus* from Maritsa River

| *Alburnus alburnus* | Number of endohelminth species | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | Mean±SD | Range |
| | 5 | 17 | 6 | 1 | 1.10±0.71 | 0-3 |
| *Alburnus alburnus* | Number of endohelminth specimens | | | | | |
| | Total number | | Mean±SD | | Range | Brillouin's index HB |
| | 51 | | 1.76±1.36 | | 0-5 | 1.26 |

In general, the parasite communities of *A. alburnus* are represented by 5 species of parasites belonging to four classes, five orders, and five families. The total number of isolated and studied helminth specimens was 51. The obtained results were related to a Brillouin diversity index HB=1.26, Shannon diversity index H'=1.395, Berger-Parker dominance index d=0.353 and Pielou evenness index E=0.867.

The circulation of parasitic flow in the studied freshwater ecosystem can be represented as follows: class Cestoda: aquatic worms - fish (*Caryophyllaeus brachycollis*) and crustaceans - fish - birds (*Ligula intestinalis*); for class Acanthocephala: crustaceans - fish - fish

(*Pomphorhynchus laevis*); Class Nematoda: insect larvae - fish (*Rhabdochona denudata*).
During this study of helminth communities of *A. alburnus* five parasite species were found. In the only previous research of helminth fauna of bleak from Maritsa River, Margaritov (1965) reported three species (Table 3). In this study, the highest prevalence was detected for *Rhabdochona denudate* (P%=37.93). Margaritov (1965) also reported *R. denudata* as the species with the highest prevalence in helminth communities of bleak, but with higher values (P%=55). This study of endohelminth community of *A. alburnus* from the Maritsa River corresponds only partially with previous survey by Margaritov (1965) (Table 3).

Table 3.Overview of helminth species of *Alburnus alburnus* registered in the Maritsa River

| Authority / Helminth species | Margaritov (1965) | This study |
|---|---|---|
| **Class Trematoda** | | |
| *Allocreadium isoporum* | | ● |
| **Class Cestoda** | | |
| *Caryophyllaeus brachycollis* | ● | ● |
| *Ligula intestinalis* | | ● |
| **Class Acanthocephala** | | |
| *Acanthocephalus anguillae* | ● | |
| *Pomphorhynchus laevis* | | ● |
| **Class Nematoda** | | |
| *Rhabdochona denudata* | ● | ● |

## CONCLUSIONS

This study presents new data forthe bleak's (*Alburnus alburnus* (Linnaeus, 1758)) endohelminth species and structure of helminth communities from Maritsa River, Bulgaria. Helminth parasites were recorded in 24of the examined bleak specimens (85.75%) from the Maritsa River.Five species of parasites were identified: one trematode species (*Allocreadium isoporum* (Looss, 1984)), two cestode species (*Caryophyllaeus brachycollis* Janiszewska, 1951, *Ligula intestinalis* (Linnaeus, 1758)); one acantocephalan (*Pomphorhynchus laevis* (Müller, 1776)) and one nematode species (*Rhabdochona denudata* (Dujardin, 1845) Raillet, 1916).
The established in this study *A. isoporum, L. intestinalis* and *P. laevis* are reported for the first time for *A.alburnus* from Maritsa River.
The obtained results for the parasite communities are related to Brillouin diversity index HB=1.26 and Pielou evenness index E=0.867.
The obtained results for the parasite communities and the bioindicative role of their intermediate hosts are demonstrating a favourable development of the studied freshwater ecosystem.

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# BIODIVERSITY OF THE HELMINTH COMMUNITIES OF *Scardinius erythrophthalmus* (Linnaeus, 1758) FROM MARITSA RIVER, BULGARIA

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***Abstract***

*This study is the first that presents the rudd's (Scardinius erythrophthalmus (Linnaeus, 1758)) endohelminth species and structure of helminth communities from Maritsa River, Bulgaria. During 2018, 13 specimens of Scardinius erythrophthalmus were collected and examined with standard techniques for parasites from Maritsa River. Helminth parasites were recorded in all 13rudd specimens (100%) from Maritsa River.Three species of parasites were identified: one cestode species (Ligula intestinalis (Linnaeus, 1758)), one acantocephalan species (Pomphorhynchus laevis (Müller, 1776)) and one nematode species (Rhabdochona denudata (Dujardin, 1845, Raillet, 1916).The analysis of the dominant structure of the found parasite species is presented to the component level. All established parasite species are core for the component community of Scardinius erythrophthalmus from Maritsa River.The infracommunities data was used to be fixed principal biotic indices. Bioindicator significance of established parasite species was discussed for ecological evaluation of the state of the studied freshwater ecosystem.*

***Key words****: bioindication; helminth communities; Maritsa River; Scardinius erythrophthalmus.*

## INTRODUCTION

The length of the Maritsa River on Bulgarian territory is 321.6 km. Thus, Maritsa is fourth in length among the Bulgarian rivers - after the Danube, Iskar and Tundja. The river is related to Aegean Basin and is included in the National monitoring program (Water Body Type BG3MA350R039 – Major Rivers) (Regulation 1/2011).

Parasites are well established sensitive bioindicators for the aquatic ecosystem state (Marcogliese, 2004). Parasite communities are useful indicators of food web structure and biodiversity (Marcogliese, 2005).

Fish parasite communities and biodiversity, heavy metal content of fish host and parasites and the state of the freshwater ecosystem of the Maritsa River were studied from few authors (Margaritov, 1965; Kirin, 2000a; 2000b; 2001; 2006; 2013; 2014).

This study is the first that presents the results of examinations of the rudd's (*Scardinius erythrophthalmus* (Linnaeus, 1758)) endohelminth species biodiversity and structure of helminth communities from Maritsa River, Bulgaria.

## MATERIALS AND METHODS

During the summer of 2018, fish and fish parasites were collected and examined from the Maritsa River (before the city of Plovdiv). The city of Plovdiv (42°9′N 24°45′E) is situated on the two banks of the Maritsa River. The region of the town and the riverside are distinguished with a significant diversity of highly protected species and territories declared as protected with national and international nature protective status (Assyov, 2012). The Maritsa River springs from the Rila Mountains (2378 m altitude) in Western Bulgaria, flowing southeast between the Balkan and Rhodope Mountains, past Plovdiv to Edrine, Turkey and to Aegean Basin (41 m above sea level) (Dakova et al., 2004). After leaving Bulgaria, the river passes through the north-eastern part of Greece and the European part of Turkey and enters the Aegean Sea. The Maritsa River (Maritsa/Meriç/Evros) is the longest river that runs solely in the interior of the Balkan Peninsula.

A total of 13 rudd specimens (*Scardinius erythrophthalmus* (Linnaeus, 1758)) were collected and examined from the Maritsa

Riverduring the summer of 2018. The scientific and common names of the fish host are used according to the FishBase database (Fröse and Pauly, 2018).

The fish were immediately after their capture examined for gastrointestinal and tissue helminths (an incomplete parasitological study).

Helminthological examinations were carried out following recommendations and procedures described by Bauer et al. (1981), Bykhovskaya-Pavlovskaya (1985), Gusev et al. (1985), Georgiev et al. (1986), Moravec (2001, 2013).

The dominant structure of the component helminth communities was determined according to the criteria proposed by Kennedy (1993) on the basis of the prevalence (P%): accidental (P%<10), component (10<P%<20) and core (P%>20) species.

The ecological terms prevalence, mean intensity are used, based on the terminology of Bush et al. (1997). Analyses of helminth community structure were carried out in both levels: infracommunity and component community.

The component data were used to determine the total number of species, Shannon diversity index (H'), Pielou evenness index (E), Berger-Parker dominance index (d) according to Magurran (2004).

The infracommunity data was used to calculate the mean number of species, the mean number of helminth specimens, Brillouin diversity index (HB) (Kennedy, 1993; 1997; Magurran, 2004).

## RESULTS AND DISCUSSIONS

A total of 13 specimens of rudd (*Scardinius erythrophthalmus* (Linnaeus, 1758)) were collected and examined from the Maritsa River. *Scardinius erythrophthalmus* is estimated as least concern species (LC=Least Concern; IUCN Red List Status). Rudd is freshwater, brackish, benthopelagic, potamodromous fish species. *S. erythrophthalmus* inhabit mainly rich of nutrients, well-vegetated lowland rivers, backwaters, oxbows, ponds and lakes. This fish species feeds on plankton, terrestrial insects and plant material. Rudd can adapt to an unfavourable environmental condition (Fröse and Pauly, 2018).

Helminth parasites were recorded in 13 rudd specimens (100%) from the Maritsa River. Three species of parasites were identified: one cestode species (*Ligula intestinalis* (Linnaeus, 1758)), one acantocephalan (*Pomphorhynchus laevis* (Müller, 1776)) and one nematode species (*Rhabdochona denudata* (Dujardin, 1845) Raillet, 1916) (Table 1). All helminth species occurred as adults with the exception of *L. intestinalis*. *P. laevis* and *R. denudata* are autogenic species, matured in fish. *L. intestinalis* is allogenic species. The larval stages of *L. intestinalis* develop in the body cavity of carp fishes – *Abramis brama, A. sapa, S. erythrophtalmus, A. alburnus, A. bipunctatus, Gobio gobio, Rutilus rutilus, Barbus barbus, B. m. petenyi, Leuciscus cephalus, L. idus* and *Phoxinus phoxinus* (Kakacheva-Avramova, 1983).

In an adult state *L. intestinalis* parasitized in fish-eating birds, mainly gulls (*Larus*), less commonly in fish-eating ducks (*Bucephala* and *Mergus*) and in *Podiceps* (Kakacheva-Avramova, 1983).

In the component community of *Scardinius erythrophthalmus* from the Maritsa River *L. intestinalis* (P%=100), *P. laevis* (P%=61.54) and *R. denudata* (P%=30.77) are core species (Table 1).

In the component community of *Scardinius erythrophthalmus* from the Maritsa River cestodes are presented with the highest number of specimens, with 1 species and 38 specimens.

Table 1. Helminth parasites of *Scardinius erythrophthalmus* from Maritsa River (N – number of examined hosts, n– number of infected hosts, p – number of parasites, P – prevalence, MI – mean intensity, MA – mean abundance)

| **Helminth species** | N=13 | | | | | |
|---|---|---|---|---|---|---|
| | n | P | P% | MI±SD | MA±SD | Range |
| *Ligula intestinalis* (Linnaeus, 1758) | 13 | 38 | 100 | 2.92±1.38 | 2.92±1.38 | 1-5 |
| *Pomphorhynchus laevis* (Müller, 1776) | 8 | 14 | 61.54 | 1.75±0.83 | 1.08±1.07 | 1-3 |
| *Rhabdochona denudata* (Dujardin, 1845) Raillet, 1916 | 4 | 5 | 30.77 | 1.25±0.43 | 0.38±0.62 | 1-2 |

Acanthocephalans are presented with one species and 14 specimens. Nematodes are represented by one species and 5 specimens.

*L. intestinalis* was found in *Scardinius erythrophtalmus, Gobio gobio, Rutilus rutilus* and *Alburnus alburnus* from River Danube (Kakacheva-Avramova et al., 1978; Chunchukova et al., 2018). *L. intestinalis* was reported also as a parasite of *Alburnus alburnus* from Arda River (Maritsa Basin) (Kirin et al., 2002).

*Ligula intestinalis* (Diphyllobothriidae) is widely distributed cestode species with a complex life cycle, which involves a copepod as the first intermediate host, fish as a second intermediate host and an avian definitive host (Dubinina, 1980).

*P. laevis* was found in *S. erythrophtalmus* from Strumeshnitsa River and Danube River (Kakacheva-Avramova, 1962, 1977; Atanasov, 2012). For Maritsa River, *P. Laevis* was reported as a parasite of *Squalius cephalus* (*Leuciscus cephalus*) and *Esox lucius* (Kirin, 2000a; 2000b; 2001; 2006). *P. Laevis* was reported also as parasite of *Sq. cephalus* (*L. cephalus*) from Stryama River (Maritsa Basin) (Kirin et al., 2005).

Intermediate host of *P. laevis* is *Gamma ruspulex* and definitive hosts are fish most often from family Cyprinidae, and less often from families Salminidae, Percidae, Siluridae etc. (Kakacheva-Avramova, 1983). *G. pulex* is a bioindicator for β-mesosaprobity (Johnson et al., 1993).

Paratenic hosts of *P. laevis* are small fish of the family Cyprinidae (Kakacheva-Avramova, 1983). For Bulgaria *P. laevis* was also reported as parasite of Eurasian otter (*Lutra lutra*) originating from the vicinities of the village of Yunatsite (Pazardzhik Region) (Dimitrova et al., 2008). A village of Yunatsite is situated on the southern bank (right) of the Topolnitsa River, which is left tributary of the Maritsa River. Dimitrova et al. (2008) suggested that both postcyclic and paratenic transmission routes seem possible for the establishment of *P. laevis* as parasite of *L. lutra.*

*Rhabdochona denudata* is an intestinal parasite of many species of family Cyprinidae (Moravec, 2013). *R. denudata* was reported as a parasite of *S. erythrophthalmus* for river Strumeshnica, Lake Srebarna and Danube River (Kakacheva-Avramova, 1962; Shukerova and Kirin, 2008; Atanasov, 2012). For Maritsa River, *R. denudata* was reported as a parasite of *Squalius cephalus* (*Leuciscus cephalus*), *Vimba melanops* (Heckel, 1837), (*Vimba vimba melanops*), *Alburnus alburnus* and *Barbus cyclolepis* (Heckel, 1837) (*Barbus tauricus cyclolepis*) (Margaritov, 1965). *R. denudata* was reported also as parasite of *Sq. cephalus* from Chepelarska River, Arda River and Stryama River (all belong to Maritsa Basin) (Kirin, 2002; Kirin et al., 2002; Kirin et al., 2005). *R. denudata* was established as parasite of *A. alburnus* from Arda River (Maritsa Basin) (Kirin et al., 2002; Kirin, 2003).

Intermediate hosts of *R. denudata* are insect larvae: *Heptagenia* sp., *Ephemerella* sp. and *Hydropsyche* sp. (Bauer, 1987; Kakacheva-Avramova, 1983). Representatives of the genera *Heptagenia* and *Ephemerella* are bioindicators for β-mesosaprobity. *Hydropsyche* sp. Is bioindicator for 0-α-mesosaprobity (Johnson et al., 1993).

Moravec (2007) studied experimentally the life cycle of *R. denudata.* The author obtained encapsulated infective larvae of *R. Denudate* in mayfly nymphs *Habroleptoides modesta* and *Habrophlebia lauta.* Moravec (2013) suggested that in addition to mayflies also some other aquatic arthropods may serve as intermediate hosts of *R. denudata.*

Species richness in infracommunity of rudd ranges from 1 to 2species. With 1 helminth species was infected only one fish (7.69%; one rudd with 4 specimens of *L. intestinalis*), with 2 helminth species - 12 fishes (92.31%; 8 rudds (61.54%) - with *L. intestinalis* and *P. laevis*; 4 rudds (30.77%) - with *L. intestinalis* and *R. denudata*).

The largest number of helminth specimens established in a single host specimen is 8. The average species richness (mean number of species for a fish specimen) in infracommunityof rudd is 1.92 species (Table 2). Average abundance (mean number of helminths in fish) in these infracommunities is 4.38±1.68. The parasite communities of *S. erythrophthalmus* from the Maritsa River showed Brillouin diversity index, HB=0.762 (Table 2).

Table 2. Infracommunities of *S. erythrophthalmus* from Maritsa River

| *Scardinius erythrophthalmus* | Number of endohelminth species | | | |
|---|---|---|---|---|
| | 1 | 2 | Mean±SD | Range |
| | 1 | 12 | 1.92±0.27 | 1-2 |
| *Scardinius erythrophthalmus* | Number of endohelminth specimens | | | |
| | Total number | Mean±SD | Range | Brillouin's index HB |
| | 57 | 4.38±1.68 | 2-8 | 0.762 |

In general, the parasite communities of *S. erythrophthalmus* are represented by 3 species of parasites belonging to three classes, three orders, and three families. The total number of isolated and studied helminth specimens was 57. The obtained results were related to a Brillouin diversity index HB=0.762, Shannon diversity index H'=0.829, Berger-Parker dominance index d=0.667 and Pielou evenness index E=0.754 (Figure 1).

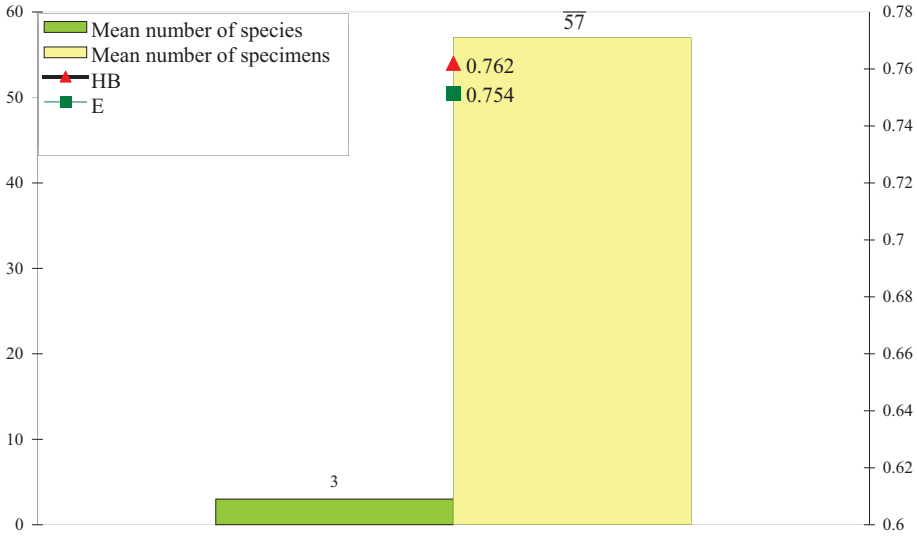


Figure 1. Biodiversity and ecological characteristics of the parasite communities of *Scardinius erythrophthalmus* (Linnaeus, 1758) from the freshwater ecosystem of the Maritsa River

The circulation of parasitic flow in the studied freshwater ecosystem can be represented as follows: class Cestoda: crustaceans - fish – birds (*Ligula intestinalis*); for class Acanthocephala: crustaceans - fish - fish (*Pomphorhynchus laevis*); Class Nematoda: insect larvae - fish (*Rhabdochona denudata*).

## CONCLUSIONS

This study is the first that presents the rudd's (*Scardinius erythrophthalmus* (Linnaeus, 1758)) endohelminth species and structure of helminth communities from Maritsa River, Bulgaria. All of the established parasites are reported for the first time for *S. erythrophthalmus* from Maritsa River.

Helminth parasites were recorded in all 13 examined rudd specimens (100%) from the Maritsa River.

Three species of parasites were identified: one cestode species *Ligula intestinalis* (P%=100), one acantocephalan *Pomphorhynchus laevis* (P%=61.54) and one nematode species *Rhabdochona denudata* (P%=30.77).

All of the established parasites are core for the helminth communities of *S. erythrophthalmus* from Maritsa River.

The obtained results for the parasite communities are related to Brillouin diversity index HB=0.762 and Pielou evenness index E=0.754. The obtained results for the parasite communities and the bioindicative role of their intermediate hosts are demonstrating a favourable development of the studied freshwater ecosystem.

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# GROWTH AND MORTALITY ESTAMATION PARAMETERS FOR THE PIKE-PERCH (*Sander lucioperca*, Linnaeus, 1758*)* POPULATION IN ROMANIAN SECTION OF THE DANUBE RIVER

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***Abstract***

*The objective of this study was to estimate growth and mortality parameters of the Pike perch population (Sander lucioperca, Linnaeus, 1758) in the Danube River (kmD 197 – kmD 170) and to assess the effects of fishing on this stocks. This study was performed on 175 specimens of pike perch caught in 2015.The purpose of this study was to determine the relationships: length – weight (L - W), to estimate the growth parameters (von Bertalanffy) $L_{\infty}$, k, and the mortality rates (Z, M, F) for the pike perch population. The relationship between length – weight (L – W) found is* $W=0.0057*TL^{3.122}$. *The asymptotic length ($L_{\infty}$), growth coefficient (k) were estimated at 89.25 cm, 0.430 per year.The estimated values of the mortality rates for the studied population are high thus: total mortality (Z) is 2.18 per year; the natural mortality (M) reaches 0.512 per year and fishing mortality (F) 1.668 per year. Exploitation rate, E = 0.77 calculated for the pike perch population exceeds the optimum value (0.5) suggesting that the population in the studied area is over fished.*

***Key words***: *growth, exploitation mortality, parameters*.

## INTRODUCTION

Pikeperch *Sander lucioperca* (L.) is a predatory fish, native to Eastern Europe and Western Asia (M'Hetli et al., 2011).

The species is widespread (natural or introduced) in northern to southern European countries (Welcomme, 1988; Lehtonen et al., 1996) in Central Asia (Petr and Mitrofanov, 1998), Western China (Walker & Yang, 1999) and in North Africa (Zaouali, 1981; Meddour et al., 2005).

In Romania it meets in the Danube and in the lower and middle courses of large tributaries, as well as in some hills and lakes (in many cases introduced).In the Danube Delta is found on the Danube River and its arms, Razim - Sinoe Lake and less frequently in the brackish water of the Black Sea.

It is the most valuable percid of our waters, being an important species for both commercial and recreational fishing.

In the commercial catches of this sector, the pike perch held small percentages over time, so between 1972 and 1986 the percentage in multiannual industrial catches was 0.4% and in the period 2006 - 2009 the average percentage was ~ 1.7% (Gheorghe, 2011).

Although, at national and global level, it is not appeared on conservation lists, our country is threatened by pollution and overfishing.

The overfishing and the water degradation lead to a reduction of these populations in the waters of our country.

Sustainability is one of the central concerns in fisheries (Pauly et al., 2002; Gaichas, 2008). There is increasing recognition that it is necessary to manage fisheries in a broader ecological context (Constable, 2001; Garcia et al., 2003; Sainsbury and Sumaila, 2003; Pikitch et al., 2004; Fulton et al., 2005; Fogarty, 2014).

The objective of this study is to estimate the growth and mortality parameters of the population of pike perch to assess the effects of fishing on stocks and to develop coherent management measures.

## MATERIALS AND METHODS

The area of study is represented by the Danube River section between km D 170 – km D 197 (Figure 1).

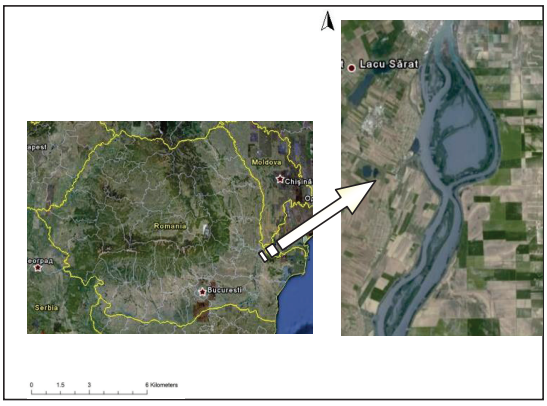


Figure 1. Area of study

Samples of *Sander lucioperca* were collected between April and July 2015usedgillnets (32, 40, 50 mm mesh size). A total of 175 individuals with a total biomass of ~ 143 kg were captured and sampled. The captured exemplars were biometrical and gravimetrical measured. It has been measured the total length (TL; to the nearest lower 0.1 cm) and the total weight (TW; to the nearest 0.01 g) of each specimen.

The relationship between length and weight was described by: $W = a \times L^b$ (Ricker, 1973), where W is the total weight (in grams), "a" is the intercept, "b" is the slope (fish growth rate), and "L" is the total length (in centimetres).

Growth parameters (L∞, k) were estimated by using length frequency analysis with the ELEFAN I which is an analysis of modal progression.

The total mortality rate (Z), natural mortality rate (M), fishing mortality rate (F) and rate of exploitation (E) were estimated by Pauly (1980, 1982) at the mean habitat temperature which was 12 °C.

Also, using length frequency data were estimated: length at first maturity (TL 50), probability of length classes in catches and the exploitation rate that maximizes yield per recruit. The data were analysed using FiSAT II (FAO - ICLARM Stock Assessment Tools).

## RESULTS AND DISCUSSIONS

*Length - weight relationship*

The length of specimens sampled varied between 20.8 ÷ 76 cm with an average value of 44.6 ± 1.64 cm and a weight of 78 ÷ 3700 g with an average of 987.93 ± 104.12 g.

Regression curve shows a positive relationship between the total length (TL) and mass (TW) (r = 0.972).

The relationship between TL and TW for the pike perch population from the studied sectionis: $W=0.0057*TL^{3.122}$ (Figure 2).

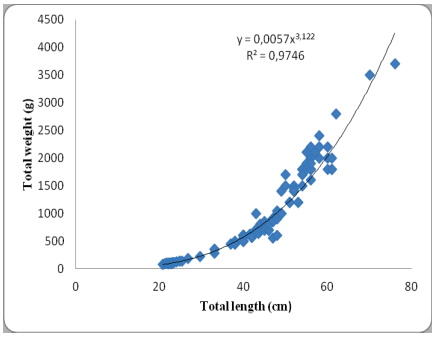


Figure 2. Length – weight relationship of *Sander lucioperca*

The length – weight relationship has a great importance in the ecology and management of the fisheries stocks (Savaş et al., 2011).

This relationship can also be helpful to assess the feeding rate, gonad maturity and metamorphosis of fish (Le Cren, 1951).

The coefficient b from the length – weight relationship according to Tesch (1968), is a measure of the environmental conditions that can be useful to compare different environments conditions, the fatness or the well – being of fish. The growth character revealed by the "b" coefficient value (3.122) shows an alometric growth of the pike perch population meaning that the increase in weight is made faster than the growth in length.

*Estimation of growth parameters*

The asymptotic length (L∞) and growth coefficient (k) were estimated at 89.25 cm, 0.43 cm per year.

The value of asymptotic length (L∞=89.25 cm) which was determined is close to the values found in specialized literature for our country (fishbase.org).

The k parameter value (0.43) is relatively high which indicates that this specie is rapidly approaching the maximum age, so it has a reduced longevity.

*Mortality and exploitation*

The total mortality (Z) of *Sander lucioperca* estimated by the length converted catch curve was 2.18 per year while the natural mortality (M) was found to be 0.512 per year and the estimated fishing mortality (F) was 1.668 per year (Figure 3). The exploitation ratio E was found to be 0.77.

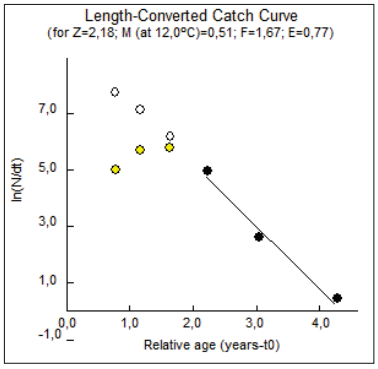


Figure 3. Length converted catch curves of *Sander lucioperca*

Table 1. The values of growth and mortality parameters of *Sander lucioperca*

| Parameters | Value |
|---|---|
| L∞ | 89.25 |
| k | 0.430 |
| Z | 2.18 |
| M | 0.512 |
| F | 1.668 |
| E | 0.77 |

In Table 1 are presented the values of the growth and mortality rates of the pike perch population.

The values obtained show that the pike perch population in this sector has high mortality rates, especially those resulting from fishing. Also, the value of the exploitation rate E (0.765) shows that this species is overfishing in this sector.

*The length of the first catch* (TL50)

TL50 is the length of the first catch or the length at which 50% and of the individuals are held in the net.

TL50 has been suggested as an indicator for both marine and freshwater species (Chen and Paloheimo, 1994; Gangl and Pereira, 2003). The mean length in the catch (Lc) in relation to TL50 has been suggested as a potential indicator of fishing pressure for data-limited stocks. If Lc exceeds TL50, the biomass of a mature stockis probably above that which can produce a maximum sustainable yield (ICES, 2012a), implying that the fishery is probably sustainable.

The estimated value for TL50 is 40.63 cm and the mean length (Lc) is 44.6 cm. It can be noted that Lc is slightly higher than TL50 (only 4 cm), we could say that the mature stock biomass could support sustainable fisheries.

The probability of capture by groups of lengths is shown in Figure 4.

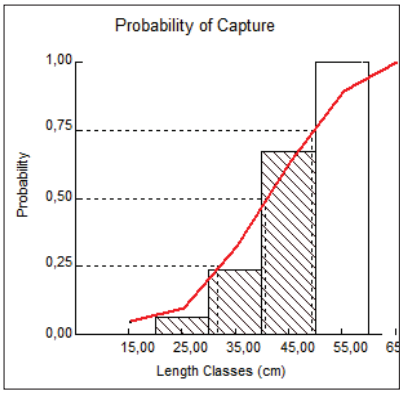


Figure 4. Probability of capture of the *Sander lucioperca*

## CONCLUSIONS

In this study, a total of 175 specimens of *Sander lucioperca* from Danube River were examined.

- The size of the pike perch population recorded in this study ranged from 20.8 ÷ 76

cm for total length and 78 ÷ 3700 g for total weight.

- The length-weight relationship in fish may change with age, season, nutrition, sexual maturity and species (Ricker, 1975; Bagenal, 1978). It was determined that the length - weight relationship was $W=0.0057*TL^{3.122}$.
- The length - weight relationship revealed a highly correlation (r=0,972), and the coefficient b value of 3.122 indicating an alometric increase meaning that the growth in weight increase faster than in length.
- Growth parameters (L∞, k,) have values similar to those from specialized literature.
- k - is considered the growing constant which indicates us the speed with which a fish approaches the asymptotic length (maximum theoretical) and it has been also demonstrated that it is bound to the fish longevity (Lai et.al., 1996). Well, the bigger the value of this constant is, the smaller the longevity is.
- The total mortality (Z) recorded in this study was 2.18. The fishing mortality (1.668 per year) was also higher than natural mortality of 0.512 per year which shows that the fishing activities in this section of riverput pressure on the stock of this population, which is also shown by the value of the exploitation rate E = 0.77.
- • In this study, the estimated length at first capture (L50) was estimated at 40.63 cm which shows that 50% of the catches are individuals of small age.This situation is also described by Frose (2004) as growth overfishing; when fishes are caught before they can realize their full potential.

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# HELMINTS AND HELMINTH COMMUNITIES OF *Squalius cephalus* (Linnaeus, 1758) FROM OSYM RIVER, BULGARIA

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***Abstract***

*During 2018, the first ecologoparasitologycal study of Squalius cephalus from Osym River, a tributary of Danube River, Bulgaria was made. In 20 specimens of examined common chub, 3 species of endohelminths are established (Ichtyocotylurus pileatus (Rudolphi, 1802) Dubois, 1937 Metacercaria; Caryophyllaeus brachycollis Janiszewska, 1951; Rhabdochona denudata (Dujardin, 1845) Raillet, 1916). C. brachycollis and Rh. denudata are autogenic species, whereas I. pileatus isallogenic species. I. pileatus is reported for the first time for the freshwater fish fauna of Osym River. Sq. cephalusis a new host record for I. pileatus in Bulgaria. The basic ecological characteristics and biotic indices of the parasite populations and communities are determined. The dominant structure of the endohelminth communities is presented on the level of the component community.*

***Key words****: helminths, helminth communities, Osym River, Squalius cephalus.*

## INTRODUCTION

River Osam refers to Type R7: Large tributaries of the Danube River in Ecoregion 12 (Pontian province). The freshwater ecosystem is a subject of impact monitoring as a part of the National Environmental Monitoring System (Belkinova et al., 2013; Peev and Gerassimov, 1999). The river ecosystem and its adjacent territories are characterized by great biodiversity. This is the reason for the announcement of the protected areas BG 0000615 Devetashko Plato and BG 0000616 Mikre, associated with the river ecosystem (Directive 92/43; Natura 2000), etc. The fish fauna of the OsamRiverhas been studied by a number of authors (Vassilev and Pehlivanov, 2005; Zarev et al., 2013). At the same time, no studies on parasites and parasite communities of *Squalius cephalus* (Linnaeus, 1758) of the OsamRiverhave been conducted. Parasites are interesting as bioindicators of different biological aspects of fish host's and for environmental quality status (Marcogliese and Cone, 1997; Galli et al., 2001; Tieri et al., 2006). The complex biological cycles of endoparasites reflect the relationships with a number of invertebrate and vertebrate hosts. The species diversity of parasites and characteristics of the endoparasite communities indicated a seasonal variation of water characteristics and state of biodiversity in the environment (Tieri et al., 2006; Lamková et al., 2007). The aim of the study is to present the results from the examinations of the endoparasite species, as well as to study the ecological characteristics of the helminth communities of *Sq. cephalus* of the Osam River (Danube Basin).

## MATERIALS AND METHODS

The River Osam takes it source from Levskipeak (1,821 m above sea level), Balkan mountain and flows into the Danube River, not far away from the town of Nikopol (5 km; 40 m above sea level).The river is 314 km long with 2,838 $km^2$ size of the basin. The studied specimens of chub are collected by angling during 2018 in the vicinity of the town of Lovech ($43^0$08'05"N $24^0$43'02"E; north-central Bulgaria).Town of Lovech is divided into two parts of the Osam River (Statistical Yearbook, 2017). The river is broad, mainly with a slow stream. The river be disband with organic sediments, clay, etc., where it is distinguished by pebble and rocky areas (Belkinova et al., 2013). The water in the upper part of the river is used for electricity and in the middle and lower part for irrigation and for industry

(Statistical Yearbook, 2017).A total of 20 specimens *Squalius cephalus* (Linnaeus, 1758) are examined for endohelminths. The scientific and common names of the fish are presented according to the FishBase database (Fröse and Pauly, 2018). Helminthological examinations are carriedout following recommendations described by Zashev and Margaritov (1966); Byhovskaya-Pavlovskaya (1985); Bauer (1987); Protasova et al. (1990); Moravec (2013). Specimens are fixed and preserved in 70% ethyl alcohol.The specimens of Trematoda and Cestoda are studied by methods of Georgiev et al. (1986); Scholz and Hanzelová (1998). The nematodes are studied on temporary mounts with 5% glycerolin 70% ethanol (Zashev and Margaritov, 1966; Moravec, 2013). Analyses of helminth community structureare carried out in both levels: infracommunity (total number of species; total and mean number of specimens; Brillouin's index of diversity (HB); Pielou index of evenness (E)) and component community (prevalence (P%) and mean intensity (MI) for each species) (Bush et al., 1997; Magurran, 1988).The species are divided into core species (P%>20), component species (P%>10) and accidental species (P%<10) (Kennedy,1997; 1993). The diversity measures are calculated by software products Statistica 10 (StatSoft Inc., 2011) and MS Excel (Microsoft 2010).

## RESULTS AND DISCUSSIONS

### Fish communities

During 2018, a total of 20 specimens of chub (*Squalius cephalus* (Linnaeus, 1758); Cyprinidae) are examined for endohelminths. *Sq. cephalus*is included in the list of IUCNas least concern species (LC=Least Concern; IUCN Red List Status, 2018). The chub is not included in the Red Data Book of the Republic of Bulgaria (Golemanski (Ed.), 2011). The chub inhabits slow to-medium-flowing waters in the lower and middle streams of rivers, irrigation canals, reservoirs. The species is distinguished by specific migrations, pursuing small passages of fish, for example, which in autumn, in periods of low water, leave the shallows and go to greater depths for greater security. The species prefer habitats to steep, steeply descending coasts, as well as pebbles or sandy bottom. *Sq. cephalus* is an omnivorous species. It feeds on small fish, small frogs, crabs and even mice. The chub is predominantly with daily activity, but during the hottest periods of the year shifts its demand for food early in the morning and at night. The species is characterized by year-round activity (Karapetkova and Zhivkov, 2006; Fröse and Pauly, 2018).

### Helminth community structure

During 2018, 20 specimens of chub are infected with 3 species of endoparasites, belonging to the classes Trematoda, Cestoda and Nematoda. They are *Ichtyocotylurus pileatus* (Rudolphi, 1802) Dubois, 1937 (metacercaria); *Caryophyllaeus brachycollis (*Janiszewska, 1951) and *Rhabdochona denudata* (Dujardin, 1845; Raillet, 1916). A total, 28 specimens of parasites are studied. Matures of *I. pileatus* have a site of infection at intestines and a factory bag of seagulls. They are specific definitive hosts of these parasite species. The cycle of development of *I. pileatus* is carried out with the participation of two intermediate hosts: freshwater snail of genus *Valvata* and fish species of the families: Cyprinidae, Percidae, Cobitidae, Gobiidae, Esocidae, Siluridae etc. The fish are intermediate hosts for parasite metacercaria, which grows in the body cavity under the serious cover of digestive organs and in the walls of the vesicle. The high abundance of the parasites causes massive extinction of fish hosts (Bauer, 1987; Kakacheva-Avramova, 1983; Sudarikov, 1984). *C. brachycollis* is intestinal parasite species of fish. The life cycle of *C. brachycollis* Janiszewska, 1951 is accomplished with the intermediate hosts *Limnodrilus hoffmeisteri* and *Tubifex tubifex*. Definitive hosts are fish species belonging to family Cyprinidae. Typical definitive hosts are fish species: *Barbus barbus*, *B. petenyi*, *Sq. cephalus*, *Leuciscus idus* (Kakacheva-Avramova, 1983; Bauer, 1987; Protasova, 1990; Scholz and Hanzelová, 1998; Barčák et al., 2017). Definitive hosts of *R. denudata* are a lot of fish species from family Cyprinidae. *R. denudata* is intestinal parasite species of fish. Intermediate hosts are invertebrates of genera Heptagenia, Ephemerella and of Hydropsyche

(Kakacheva-Avramova, 1983; Bauer, 1987; Moravec, 2013). *C. brachycollis* and *R. denudata* are authogenic parasites. They are species which life cycle is completing within the same freshwater ecosystem. The third species, *I. pileatus* is allogenic species, which use chub and another freshwater fish as intermediate hosts and mature in fish-eating birds – seagulls. *I. pileatus*, *C. brachycollis* and *R. denudata* are generalist, parasitizing in more than one different fish hosts.

**Component communities**

Helminths are fixed in 9 of 20 examined chub (45%). For each endoparasite species, prevalence (P%) and mean intensity (MI) are determined (Table 1). *R. denudata* is a core species (P%=30) of endoparasite communities of *Sq. cephalus* of the Osam River. *I. pileatus* is a component species (P%=10) and *C. brachycollis* is an accidental species (P%=5). With highest mean intensity was represented *I. pileatus* (MI=5), followed by *R. denudata* (MI=2.8). Only one specimen of *C. brachycollis* was established in one infected specimen of fish hosts (Table 1).

Table 1. Species diversity, prevalence (P%) and mean intensity (MI) of the endoparasites of *Squalius cephalus* from the Osam River

| Species of endoparasites | Ecological indices (N[1]= 20) | | | |
|---|---|---|---|---|
| | n[2] | p[3] | P%[4] | MI[5] |
| Class Trematoda<br>Order Strigeidida; Family Strigeidae | | | | |
| *Ichtyocotylurus pileatus* (Rudolphi, 1802) Dubois, 1937 (metacercaria) | 2 | 10<br>4-6 | 10 | 5 |
| Class Cestoda<br>Order Caryophyllaeidea; Family Caryophyllaeidae | | | | |
| *Caryophyllaeus brachycollis* Janiszewska, 1951 | 1 | 1 | 5 | 1 |
| Class Nematoda<br>Order Spirurida; Family Rhabdochonidae | | | | |
| *Rhabdochona denudata* (Dujardin, 1845) Raillet, 1916 | 6 | 17<br>2-6 | 30 | 2.8 |

[1]N = total number of examined fish specimens.
[2]n = total number of infected fish specimens.
[3]p = total number of endoparasite specimens.
[4]P% = prevalence.
[5]MI = mean intensity.

**Infracommunities**

A total of 11 examined specimens of chub are free of parasites (55%). No mixed infection has been established. Maximal numbers of endoparasites of examined specimens of chub are fixed for *I. pileatus* and *R. denudata* (on 6 specimens). The average number of endoparasite specimens, found in the total number of studied fish specimens is low (1.4±1.98). The value of Brillouin's diversity index isHB=0.689 (Table 2).

Table 2. Infracomminities data

| No. of helminth species | | |
|---|---|---|
| Number of fish | 11 | 9 |
| Number of helminth species | 0 | 1 |
| Number of helminth specimens | | |
| Total number | 28 | |
| Mean±SD | 1.4±1.98 | |
| Range | 2-6 | |
| Mean HB±SD | 0.611±0.145 | |

To this time, the endoparasites of *Sq. cephalus* in Bulgaria are presented with 28 species, belonging to the classes Trematoda, Cestoda, Acanthocephala and Nematoda (Table 3).

Table 3. Species of endoparasites of *Sq. cephalus* in Bulgaria

| **Species of endoparasites** | **Authors** |
|---|---|
| **Trematoda** | |
| *Allocreadium isoporum macrorchis* | [4,5] |
| *Allocreadium isoporumdubium* | [10] |
| *Pseudochetosoma salmonicola* | [4,11] |
| *Posthodiplostomum cuticola* | [10,11] |
| *Tylodelphys clavata* | [5] |
| *Nicola skrjabini* | [8] |
| *Apophallus mühlingi* | [7] |
| *Metagonimus yokogawai* | [7,11] |
| *Sphaerostomum bramae* | [10] |
| **Cestoda** | |
| *Caryophyllaeus laticeps* | [10] |
| *Caryophyllaeides fennica* | [1,3,6] |
| *Ligula intestinalis* | [2,6,10] |
| *Proteocephalus torulosus* | [4,6,7,10] |
| *Caryophyllaeus brachycollis* Janiszewska, 1951 | [4,6,10] |
| *Caryophyllaeides fennica* | [4,5] |
| *Cestoidea* gen sp | [6] |
| *Shulmanella petruschewskii* | [10] |
| **Acanthocephala** | |
| *Acanthocephalus lucii* | [1,7,10] |
| *Acanthocephalus anguillae* | [4,6,10] |
| *Paracanthocephalus tenuirostris* | [6] |
| *Pomphorhynchus laevis* | [1,7,9,10,11] |
| *Neoechinorhynchus rutile* | [10] |
| **Nematoda** | |
| *Rhabdochona denudata* (Dujardin, 1845) Raillet, 1916 | [4,5,6,10,11] |
| *Philometra abdominalis* | [3,11] |
| *Philometra ovate* | [10] |
| *Phylometra sp.* | [4] |
| *Rhaphydascaris acus* | [10] |
| *Cuculanus dogieli* | [10] |

[1]Margaritov, 1959.
[2]Bajlyozov et al., 1964.
[3]Margaritov 1964.
[4]Kakcheva-Avramova, 1969.
[5]Margaritov, 1977.
[6]Kakcheva-Avramova&Menkova, 1978.
[7]Kakcheva-Avramova et al., 1978.
[8]Nedeva, 1991.
[9]Nedeva et al., 2003.
[10]Cakis et al., 2004.
[11]Atanasov, 2012.

They are determined as a result of scientific survey of 11 authors in Bulgaria (Table 3).

These species are established mainly from the chub of the Danube River and its tributaries as well as of some lentic ecosystems of the Danube Basin in Northern Bulgaria.

The species *R. denudata* and *P. laevis*, followed by *P. torulosus,* and by *C. brachycollis, C. fennica* and *L. intestinalis* etc. are most frequently reported.

The three endoparasite species, found in the present study of the Osam River, represent only 10.71% of the established species for the country. *I. pileatus* is a new endoparasite species of the chub of the Osam River. *Sq. cephalus* is a new host record for *I. pileatus* in Bulgaria.

The species *C. brachycollis* and *R. denudata* are established of chub and of another fish species (Table 4), but they are reported for the first time of the freshwater ecosystem of the Osam River.

The knowledge of the life cycle of the established endoparasite species is testifying for the following paths for the circulating of the parasitic flow: A. Trematoda: 1. molluscs (*Valvata*) – fish – birds (*I. pileatus,* metacercaria); B. Cestoda: 1. Oligochaeta – fish (*C. brachycollis*); C. Nematoda: 1. larvae'sof Ephemeroptera and Diptera – fish (*R. denudata*).

Probably, the specified groups of intermediate hosts are represented with higher intensities of populations and are dominant species in the food ration of the chub of Osam River.

Tieri et al. (2006) examined metazoan parasites of *Sq. cephalus* of two rivers in Italy.

They reported 7 species of endohelminths, including *C. brachyollis* and *R. denudata*.

The prevalence and mean intensity of *C. brashycollis* showed higher values from both Italian rivers than these from the Osam River. In opposite, the prevalence and mean intensity of *R. denudata* from the Orta River were fixed with higher values than these from the Osam River, but they are more than two times lower of the Pescara River (more polluted), etc.

In general, the species diversity and characteristics of parasite communities of chub of the Osam River are low.

Table 4. Other species of fish in Bulgaria – hosts of the endoparasites found in *Sq. cephalus* from the Osam River

| **Species of endoparasites Fish species** | ***I. pileatus*** | ***C. brachycollis*** | ***Rh. denudata*** |
|---|---|---|---|
| *Alburnus alburnus* (Linnaeus, 1758) | [13,17] | [4,21] | [4,5,8,13,17,21] |
| *Leuciscus aspius* (Linnaeus, 1758) | | | [3,27] |
| *Barbus barbus* (Linnaeus, 1758) | | [5,10] | [1,5,29] |
| *Barbus cyclolepis* Heckel, 1837 | | [3,4,18,22] | [3,4,22] |
| *Barbus petenyi* Heckel, 1852 | | [5, 9,10,14] | [1,5] |
| *Blicca bjoerkna* (Linnaeus, 1758) | [8] | | |
| *Cobitis taenia* Linnaeus, 1758 | | | [10] |
| *Cyprinus carpio* Linnaeus, 1758 | [6,7,11] | | [15] |
| *Gobio gobio* (Linnaeus, 1758) | | | [5] |
| *Leuciscus idus* (Linnaeus, 1758) | [8] | | |
| *Rutilus rutilus* (Linnaeus, 1758) | | [4] | |
| *Sander lucioperca* (Linnaeus, 1758) | [8,12] | | |
| *Scardinius erythropthalmus* (Linnaeus, 1758) | [8] | | [2,26,27,29] |
| *Squalius orpheus* Kottelat & Economidis, 2006 | [14,17,19,21,23, 30] | [3,4,5, 9,10, 14,17,19, 21,23,25,30] | [3,4,10, 14,16,17,19, 20,21,23,25,30] |
| *Perca fluviatilis* Linnaeus, 1758 | [12,27,28] | [12,24] | |
| *Vimba melanops* (Heckel, 1837) | | [4] | [4] |
| *Zingel streber* (Siebold, 1863) | | | [8] |
| *Zingel zingel* (Linnaeus, 1766) | | | [8] |

[1]Margaritov, 1959.
[2]Kakacheva-Avramova, 1962.
[3]Kakacheva-Avramova, 1965.
[4]Margaritov, 1965.
[5]Kakacheva-Avramova, 1969.
[6]Margaritov, 1975.
[7]Margaritov, 1976.
[8]Kakacheva-Avramovaetal., 1978.
[9]Kakacheva-Avramova&Menkova, 1978.
[10]Kakacheva-Avramova&Menkova, 1981.
[11]Margaritov, 1992.
[12]Nedeva&Grupcheva, 1996.
[13]Kirin, 2001.
[14]Kirin, 2001a.
[15]Kirin, 2001b.
[16]Kirin, 2001c.
[17]Kirin, 2001d.
[18]Kirin2002.
[19]Kirin2002a.
[20]Kirin, 2002b.
[21]Kirinetal., 2002.
[22]Kirin, 2003.
[23]Kirin, etal., 2003.
[24]Cakisetal., 2004.
[25]Kirinetal., 2005.
[26]Shukerova&Kirin, 2008.
[27]Shukerova, 2010.
[28]Sukerovaetal., 2010.
[29]Atanasov, 2012.
[30]Kirinetal., 2013; etc.

## CONCLUSIONS

River Osam is a new locality of *I. pileatus*, *C. brachycolli* and *R. denudata* in Bulgaria. *Sq. cephalus* is a new host record for *I. pileatus* in Bulgaria and Bulgarian part of the Danube Basin. *R. denudata* is a core species of the helminth communities.

The determined three endoparasite species represent only 10.71% of the established intestinal species of the chub in the country.

The low characteristic of infection indicated low biodiversity of the studied habitats and showed negative impacts on the areas of the examined freshwater ecosystem.

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# HELMINTHS AND HELMINTH COMMUNITIES OF PERCH (*Perca fluviatilis* Linnaeus, 1758) AS BIOINDICATORS FOR ECOSYSTEM CONDITION OF THE MARITSA RIVER

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***Abstract***

*Maritsa River is related to the Aegean water collecting region. During 2015, a total amount of 23 specimens of perch (Perca fluviatilis Linnaeus, 1758) are investigated. Identified two species of helminths (Proteocephalus percae (Müller, 1780) and Acantocephalus lucii (Müller, 1776)) are reported for the first time as intestinal parasites of perch from the freshwater ecosystem of the Maritsa River. They are core and authogenic species for the helminth communities of P. fluviatilis. Analyses of the helminth communities were carried out at both levels: infracommunity and component community. Basic ecological characteristics and biotic indices were determined to evaluate ecosystem condition of the river. The results of these studies attest to the importance of perch's helminths and helminth communities as sensitive bioindicators.*

***Key words****: bioindication, helminths, Maritsa River, Perca fluviatilis.*

## INTRODUCTION

The Maritsa River is related to the Aegean Basin. The major negative anthropogenic impact on the Maritsa River ecosystem associated with the changes in the studied freshwater communities are farm activities, industry, power production, irrigation, constructions etc. Maritsa River is included in the National monitoring program (Water Body Type BG3MA350R039– Major rivers; river type R12: Large flat rivers in the Ecoregion 7: Eastern Balkans) (Peev & Gerassimov, 1999; Belkinova et al., 2013).

The Maritsa River is a part of the National Ecological network (Natura 2000). In close connection with the protection of the freshwater communities and natural habitats of the Maritsa River and its riversides were declared protected areas BG 0000578 "River Maritsa" according to Directive 92/43 and protected area BG 0002081 "Maritsa – Parvomaj" according Directive 79/409. There are not a few examinations on species diversity, conservation status and characteristics of the freshwater fish communities of the river and its tributaries (Vassilev and Pehlivanov, 2005; Georgiev, 2006; Kolev, 2013; Kolev, 2016).

Parasites and parasite communities of freshwater fish respond differently to changes in habitat conditions and on the other hand they are defined as complex systems affected by many ecological and environmental factors both in time and space (Lafferty and Kuris, 1999; Valtonen, 2001; Kuhn, 2015; Kadlec et al., 2003; Marcoglise, 2016; Lagrue et al., 2018).

The complex life cycles of the development of a large number of intestinal parasites occurring with the participation of a number of species and groups of intermediate hosts are essential for the endoparasites and characteristics of parasite communities of freshwater fish species to be used as bioindicators for the ecological status of the studied habitats (Sures, 2001; Schludermann et al., 2003; Nachev, 2010; Chapman et al., 2015; De Aquino Moreira et al., 2015; Hofmann et al., 2016).

The paper presents the results of the examinations on the intestinal parasites and its endoparasite communities of European perch (*Perca fluviatilis* Linnaeus, 1758) of the Maritsa River and to evaluate their bioindicator role in the studied freshwater ecosystem (region of the town of Parvomaj; Aegean Basin).

## MATERIALS AND METHODS

River Maritsa has a length of 521 km and is the longest river on the Balkan Peninsula. The river springs from Rila Mountains (2°09'40"N, 23°36'00"E, 2378 m altitude, from Maritsa lakes, below Peak Mancho) in Western Bulgaria. It running southeast between the Balkan and Rhodope Mountains, past Plovdiv southeast part of Greece and European Turkey and flows into Aegean Basin (41 m above sea level) (Dakova et al., 2004).

The studied biotope (around the town of Parvomaj, 42.099444N, 25.224167E) is situated on the riverside,about 50 km far away south-eastern from the town of Plovdiv (42.15N, 24.75E). It is characterized by a depth and speedy runningwater, with a sandy bottom. The waterside vegetation is represented mainly by *Salix alba* L., *Populus alba* L., *Populus nigra* L., *Alnus glutinosa* (L.) Gaertn., *Rhobinia pseudoacacia* L. etc.

During 2015, a total of 23 specimens of perch are collected and examined for helminths from the Maritsa River (near the town of Parvomaj; Aegean Basin). Fish names (scientific and common names) are presented according to the FishBase database(Fröse and Pauly, 2018).

Helminthological examinations were carriedout following recommendations described by Zashev and Margaritov (1966), Bykhovskaya-Pavlovskaya (1985), Bauer (1987), Scholz and Hanzelová (1998). Specimens are fixed and preserved in 70% ethyl alcohol. The specimens of Cestoda are studied by methods of Georgiev et al. (1986); Scholz and Hanzelová (1998). The acanthocephalans are studied on temporary mounts with 5% glycerolin 70% ethanol (Petrochenko, 1956; Zashev and Margaritov, 1966). Analyses of helminth community structureare carried out in both levels: infracommunity (total and mean number of species; total and mean number of specimens; Brillouin's index of diversity (HB) and component community (prevalence (P%), mean abundance (MA) and mean intensity (MI) for each species) (Bush et al., 1997; Kennedy, 1993, 1997; Magurran, 1988). The species are classified as core species (P%>20), component species (P%>10) and accidental species (P%<10) (Kennedy, 1993). The diversity measures are calculated by software products Statistica 10 (StatSoft Inc., 2011) and MS Excel (Microsoft 2010).

## RESULTS AND DISCUSSIONS

### Fish communities

A total of 23 specimens of European perch (*Perca fluviatilis* Linnaeus, 1758; Percidae) are collected and studied for intestinal helminths. *P. fluviatilis* is determined as least concern species (LC=Least Concern; IUCN Red List Status, 2019) and isnot included in Red Data Book of the Republic of Bulgaria (Golemanski, 2011).

The perch is wide spread in Europe, including in Bulgaria, but negative environmental impacts have been reported after its introduction. *P. fluviatilis* prefer rivers, lakes, reservoirs, overgrown with aquatic vegetation. The perch is also found in semi-saline waters and habitats of high acidity, with a high population density. The perch is freshwater, brackish, demersal and anadromous fish species (Kottelat and Freyhof, 2007; Fröse and Pauly, 2018).

The fish species is distinguished by age differences in nutrition: small fish feed on zooplankton; adult fish also add bottom invertebrates to their ration. Only the largest specimens of perch (with size around 12 cm) have been found feeding entirely with small fish (mainly sticklebacks, perches and minnows). *P. fluviatilis* is a predatory fish species. The European perch is an important fish species as food and for game fishing (Karapetkova and Zhivkov, 2006; Fröse and Pauly, 2018).

According to this examination, there are no specimens of perch free of intestinal parasites.

### Helminth community structure

23 specimens of perch are infected with two specimens of intestinal helminths: *Proteocephalus percae* (Müller, 1780) and *Acanthocephalus lucii* (Müller, 1776) belonging to two classes, Cestoda and Acanthocephala, respectively.

*P. percae* grows with the participation of intermediate host copepods (*Cyclops strenuous* Fischer, 1851, *C. vicinus* Ulyanin, 1875, *Eucyclops serrulatus* (Fischer, 1851) etc.). The species is ubiquitously represented in the

habitats of the perch (Bauer, 1987). *P. fluviatilis* is a typical definitive host of *P. percae* (Bauer, 1987; Scholz and Hanzelova, 1998).

Reporting of *P. percae* from other hosts outside of Percidae is considered as erroneous species identification. Detection of parasites in another predatory fish (*Esox lucius*, *Lota lota* etc.) is associated with postcyclic parasitism and obtaining the parasite when these fish species eating infected with *P. percae* specimens of perch (Scholz & Hanzelova, 1998).

In Bulgaria, the species *P. percae* was reported of *Gymnocephalus schraester* (Linnaeus, 1758) (=*Acerina schraester* Linnaeus, 1758), *Sander volgensis* (Gmelin, 1789) (=*Stizostedion volgense* Gmelin, 1789), *Gymnocephalus cernua* (Linnaeus, 1758) (=*Acerina cernua* Linnaeus, 1758) from Danube river (in the region of towns Vidin, Silistra and Tutrakan) (Kakacheva-Avramova et al., 1978) as *Proteocephalus cernuae* (Gmelin, 1790) La Rue, 1911; of *P. fluviatilis* from Lake Srebarna (Shukerova, 2010; Shukerova et al., 2010); etc.

The life cycle of *A. lucii* is carried out with the participation of intermediate host species *Asellus aquaticus* (Linnaeus, 1758). Definitive hosts are many freshwater fish species from Cyprinidae (Linnaeus, 1758) (Kakacheva-Avramova, 1983; Bauer, 1987).

In Bulgaria, the species *A. lucii* was reported of *Silurus glanis* Linnaeus, 1758 and *Squalius cephalus* (Linnaeus, 1758) (=*Leuciscus cephalus* Linnaeus, 1758) (Margaritov, 1959) of *P. fluviatilis* (Margaritov, 1966), of *Ballerus sapa* (Pallas, 1814) (=*Abramis sapa* Pallas, 1814), *Sq. cephalus*, *Rutilus rutilus* (Linnaeus, 1758), *S. glanis*, *P. fluviatilis*, *Lota lota* (Linnaeus, 1758), *G. schraester*, *Benthophilus stellatus* (Sauvage, 1874), *Proterorhinus marmoratus* (Pallas, 1814) (Kakacheva-Avramova et al., 1978); of *Sq. cephalus* (Cakic et al., 2004); of *L. lota*, *Zingel zingel* (Linnaeus, 1766) (Atanasov, 2012); of *Abramis brama* (Linnaeus, 1758) and *Alburnus alburnus* (Linnaeus, 1758) (Chunchukova, 2017); of *A. alburnus* (Chunchukova et al., 2018) of the Danube River; of *A. brama* (Linnaeus, 1758) (Chunchukova et al., 2016); of *P. fluviatilis* (Shukerova, 2010; Shukerova et al., 2010) from Lake Srebarna etc.

**Component communities**

The two determined species, *P. percae* and *A. lucii* are generalists for the helminth communities of perch of the Maritsa River. With higher prevalence, mean abundance and mean intensity is distinguished *A. lucii* (Table 1). *P. percae* and *A. lucii* are core species of the helminth communities of *P. fluviatilis* from the freshwater ecosystem of the Maritsa River according to on the criterion of Bush et al. (1997). The two determined species of endohelminths are autogenic species of the helminth communities of the perch from the river.

Table 1. Species diversity, prevalence (P%), mean intensity (MI) of the established endohelminth species of *Perca fluviatilis* of the Maritsa River

| Species of parasites | Intermediate hosts | Definitive host *P. fluviatilis* ($N^1$=23) | | |
|---|---|---|---|---|
| | | $n^2/p^3$ | $^4$P% | MA$^5$ MI$^6$ Rang |
| Cestoda | | | | |
| *Proteocephalus percae* (Müller, 1780) | Copepoda | 11/25 | 47.83 | 1.09 2.27 1-4 |
| Acanthocephala | | | | |
| *Acanthocephalus lucii* (Müller, 1776) | Amphipoda | 23/84 | 100 | 3.65 3.65 1-7 |

[1]N = total number of examined fish specimens.
[2]n = total number of infected fish specimens.
[3]p = total number of endoparasite specimens.
[4]P% = prevalence.
[5]MA = mean abundance.
[6]MI = mean intensity

**Infracommunities**

In 11 of 23, a total examined specimens of perch (47.83%), a mixed invasion was established with the presence of both species of intestinal parasites. All examined fish specimens (23 specimens) were infected with acanthocephalans. The average number of endoparasite specimens found in the total number of studied fish specimens is 4.695±1.717. The minimal number of endoparasite specimens per a fish specimen is 2 and maximal is 9 parasites. The value of Brillouin's diversity index is HB=0.516 (Table 2).

Table 2. Infracomminities data

| No. of helminth species | | |
|---|---|---|
| Total number of species | 2 | |
| Number of fish | 11 | 23 |
| Number of helminth species | 2 | 1 |
| Number of helminth specimens | | |
| Total number of specimens | 109 | |
| Mean±SD | 4.695±1.717 | |
| Range | 2-9 | |
| Mean HB±SD | 0.395±0.053 | |

In Bulgaria, 16 endoparasite species were reported as parasites of parasite communities of *P. fluviatilis*. In this study, the fixed two species of helminths (*P. percae* and *A. lucii*) presents for only 12.5% of those established of the perch for the country. The examinations of parasite and parasite communities of perch are mainly from Bulgarian part of the Danube River and its tributaries, Lake Srebarna and some freshwater ecosystems of the Aegean Basin. *P. percae* and *A. lucii* are reported as parasites of perch in Bulgaria, but they are reported for the first time for Maritsa river ecosystem.

Table 3. Species of endoparasites of *P. fluviatilis* in Bulgaria

| Trematoda | Authors |
|---|---|
| *Nicollas krjabini* | Kakacheva-Avramova et al.,1978 |
| | Kirin, 2005 |
| *Ichthyocotylurus pileatus* | Nedeva&Grupcheva, 1996 |
| | Shuketrova, 2010 |
| | Shukerova et al., 2010 |
| *Bunodera luciopercae* | Kirin, 2005 |
| **Cestoda** | |
| *Caryophyllaeus brachycollis* | Nedeva&Grupcheva, 1996 |
| *Caryophyllaeides fennica* | Shuketrova, 2010 |
| *Proteocephalus percae* | Shuketrova, 2010 |
| | Shukerova et al., 2010 |
| **Acanthocephala** | |
| *Acanthocephalus lucii* | Margaritov, 1966 |
| | Kakacheva-Avramova et al., 1978 |
| | Shukerova et al., 2010 |
| | Shukerova, 2010 |
| *Acanthocephalus anguillae* | Shukerova, 2010 |
| | Kirin, 2005 |
| *Pomphorhynchus laevis* | Kakacheva-Avramova et al., 1978 |
| | Nedeva et al., 2003 |
| | Kirin, 2005 |
| | Shukerova et al., 2010 |
| *Neoechinorhynchus rutili* | Kirin, 2005 |
| **Nematoda** | |
| *Eustrongylides excisus* | Atanasov, 2012 |
| | Nedeva&Grupcheva, 1996 |
| | Kirin et al, 2013a |
| | Kirin et al, 2013b |
| | Shukerova, 2010 |
| | Shukerova et al., 2010 |
| *Eustrongylides tubifex* | Shukerova, 2010 |
| | Shukerova et al., 2010 |
| *Crowcroecoecumskrjabini* | Margaritov, 1966 |
| *Rhabdochona* sp., larvae | Margaritov, 1966 |
| | Kakacheva-Avramova et al., 1978 |
| *Contracoecum*sp., larvae | Kakacheva-Avramova et al., 1978 |
| | Shukerova, 2010 |
| | Shukerova et al., 2010 |
| *Rhaphidascaris acus* | Nedeva&Grupcheva, 1996 |
| | Shukerova, 2010 |
| | Shukerova et al., 2010 |

The most commonly was reported *E. excisus* (in 6 scientific studies), followed by *A. lucii* and *P. laevis* (4 studies) (Table 3). *P. percae*; *Bothriocephalus claviceps* (Goeze, 1782) Rudolphi, 1810; *Glanitaenia osculata* (Goeze, 1782); *Triaenophorus nodulosus* (Pallas, 1781) Rudolphi, 1793 (Cestodaspecies), *A. lucii* (Acanthocephala) and *Camallanus lacustris* (Zoega, 1776) (Nematoda) were reported as a commonly parasites of juvenile *P. fluviatilis* (Kuchta et al., 2009). Wierzbicki (1970) established 13 intestinal species, including *P. percae* and *A. lucii*, also determined as common species of perch. Sobeska and Słomínska (2007) determined 8 endoparasite species of perch of the Odra River in Poland, including *P. percae*. Opposite to higher species diversity, *P. percae* from Odra river was distinguished with lower prevalence and mean intensity than those of *P. percae* from Maritsa river ($P\%_{P.\ percae\_\text{river Odra}}=9.09$; $MI_{P.\ percae\_\text{river Odra}}=1.8$; $P\%_{P.\ percae\_\text{river Maritsa}}=47.83$; $MI_{P.\ percae\_\text{riverMaritsa}}=2.27$, Table 1). The examined perch specimens are from brackish water habitats of the Odra River and probably the lower parameters of infection are associated with higher salinity and worse conditions for the development and abundance of intermediate parasite hosts. The prevalence of *P. percae* is even lower of *P. fluviatilis* from Lake Srebarna than this of the Odra River ($P\%_{P.\ percae\_\text{Lake Srebarna}}=3$; Shukerova, 2010; Shukerova et al., 2010) due to the strong anthropogenic impacts, the high euthrophication, the low oxygen content, etc., with negatively affects the interactions and the development of the organisms. Carney & Dick (1999) compared the endohelmints of *P. fluviatilis* from Europe and *Perca flavescens* Mitchill from North America. For *P. fluviatilis*, they have determined four predictable parasitic species, specific for *P. fluviatilis*: *Bunodera luciopercae* (Müller, 1776), *P. percae*, *A. lucii* and *C. lacustris*. The authors point out, that specificity is not a requirement for predictability. They make a conclusion that the predictability of these parasitic groups is closely related to biology, and especially to the nutrition of the perch. Therefore, they established that respectively, parasites of perch are closely related to certain biological elements in the habitats as intermediate hosts and food. Intermediate hosts of *P. percae* (copepods *C. strenuus*, *C. vicinus*, *E. serrulatus* etc.) and of *A. lucii* (amphipods *A. aquaticus*) probably are represented with the highest density in the

studied habitats of the Maritsa River and are predominant in the diet of the examined specimens of perch. *A. aquaticus* is a tolerant species of a range of pollutants (Maltby, 1991).

## CONCLUSIONS

As a result of the study of 23 specimens of perch, a total of two species of endoparasites (*P. percae* and *A. lucii*) are determined. They are a core species of the helminth communities of *P. fluviatilis* of the Maritsa River. *P. percae* and *A. lucii* are reported for the first time for the freshwater ecosystem of the Maritsa River in Bulgaria. The poor species diversity of intestinal parasites and the high values of the characteristics of infection indicated poor species diversity of the free-living stages of the river section and negative impacts on the ecosystem.

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# ENDOHELMITHS AND ENDOHELMINTH COMMUNITIES OF *Rutilus rutilus* (Linnaeus, 1753) FROM ANTHROPOGENIC LOADED ECOSYSTEM OF THE LUDA YANA RIVER, BULGARIA

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***Abstract***

*During 2018, biomonitoring of the Luda Yana River ecosystem was carried out by examining the biological elements of quality: the freshwater fish species common roach (Rutilus rutilus (Linnaeus, 1753)) and its endohelmiths and endohelminth communities as bioindicators. 45 specimens of common roach are examined for parasites and three species of endohelmiths (Caryophyllaeides fennica (Schneider, 1902) Nybelin, 1922; Acanthocephalus lucii (Mueller, 1776); Rhabdochona denudata (Dujardin, 1845) Raillet, 1916) are fixed. New host and locality records are reported. The analysis of the dominant structure of the established intestinal parasite complex was presented to the level of the component community. For an ecological estimation of the freshwater ecosystem, principal biotic indexes are fixed. The bioindicator significance of the identified parasite populations and communities are discussed.*

***Key words:*** *bioindication, Luda Yana River, parasite communities, Rutilus rutilus.*

## INTRODUCTION

The Luda Yana River is a part of the protected area of the National Network Natura 2000 (BG 0000426 River Luda Yana) according to Directive 92/43/EEC. The protected area is distinguished by great biodiversity. At the same time, the river is influenced by the serious anthropogenic impacts as a result of irrigation, ore mining activities, industrial and municipal waste, etc. (Georgieva et al., 2014). According to other studies, the mining activities, the weathering and the oxidation processes have strong effects on the physicochemical processes in the whole water ecosystem (Rabadjieva et al., 2009). Freshwater fish parasites and its parasite communities are increasing interestbeing used as accumulative bioindicators and for ecological assessment of the ecosystems (Nachev, 2010; Sures et al., 2017; etc.). Parasite communities of *R. rutilus* are indicative of pollutants, eutrophication and fragmentation in the environment (Valtonen et al., 1997; Valtonen et al., 2003; etc.). This paper presents the results from an examination of common roach parasites, dominant structure of fish parasite communities and their bioindicator role for biodiversity condition of the freshwater ecosystem of the Luda Yana River (town of Popinci; Aegean Basin).

## MATERIALS AND METHODS

During April-August, 2018 fish and fish parasites are collected and examined from the Luda Yana River (after village of Popinci). The Luda Yana River is of 73.05 km long and is one of the biggest left tributaries of the Maritsa River (Southern Bulgaria). The River springs from 1423 m, from the west of the Bich peak at Sredna Gora Mountain and flows into the Maritsa River at 195 m altitude. It has a catchment's area of 685 km$^2$, which occupies 1.3% of the Maritsa River catchment (Michev et al., 1980).

The Luda Yana River refers to Type R12: Large Plain Rivers in Ecoregion 7 (Eastern Balkans) (Belkinova et al., 2013). It features a sandy-gravel bottom and a rainy-snowy feeding. It is mainly used for irrigation and industrial water supply, influencing its ecological status (Georgieva et al., 2014).

The studied biotope (42$^0$41'66'67N, 24$^0$28'33'33E, 343 m altitude) is divided into two parts from the river (Michev et al., 1980).A total of 45 freshwater fish specimens belonging

to the species *Rutilus rutilus* (Linnaeus, 1758) are collected and examined for endohelmiths. The fish are caught by angling. The scientific and common names of the fish hostare used according to the FishBase database (Fröse and Pauly, 2018). Helminthological examinations are carried out following recommendations and proceduresdescribed by Petrochenko (1956); Kakacheva-Avramova (1983); Bykhovskaya-Pavlovskaya (1985); Bauer (Ed.) (1987); Protasova et al. (1990); Moravec (2013); etc. Specimens are fixed and preserved in 70% ethyl alcohol. The Cestoda are studied with methods of Georgiev et al. (1986); Scholz and Hanzelová (1998). The acanthocephalans and nematodes are studied on temporary mounts with 5% glycerolin 70% ethanol (Zashev and Margaritov, 1966; Moravec, 2013). The ecological terms prevalence (P%), mean intensity (MI) are presented for each species. Analyses of helminth community structure are carried out in both levels: infracommunity and component community. The infracommunity data are used to calculate the total number of species, the mean number of helminths, etc. (Kennedy, 1993; 1997; Magurran, 1988). The infracommunity data are used to calculate the total number of species, mean number of helminth worms, the Brillouin's diversity index (HB) (Maguran, 1988). The analysis of the dominant structure of the parasite communities is presented to the level of the component communities using the criterion of Bush at al. (1997). The diversity measures are calculated by MS Excel (Microsoft 2010) and Statistica 10 (StatSoft Inc., 2011).

## RESULTS AND DISCUSSIONS

### Fish communities

A total of 45 fish specimens belonging to the species *Rutilus rutilus* (Linnaeus, 1758) are collected and examined from the Luda Yana River. *R. Rutilus*is estimated as least concern species (LC=LeastConcern; IUCN Red List Status, 2019) and isnot included in Red Data Book of the Republic of Bulgaria (Golemanski (Ed.), 2011). The common roach occurs in fresh and brackish waters but is very adaptable and can be found in different freshwater ecosystems (small ponds, lakes, big rivers). *R. rutilus* feeds mainly on crustaceans, insect larvae, oligochaetes, algae and higher aquatic vegetation (Karapetkova and Zhivkov, 2006; Fröse and Pauly, 2018). *R. rutilus* is a part of biological elements for bioindication of the fresh water ecosystems (Belkinova et al., 2013). *R. rutilus* is one of the dominant fish species of the freshwater ecosystem of the Luda Yana River. Two of the examined fish specimens are free of parasites.

### Helminth community structure

From studied 45 specimens of common roach (*Rutilus rutilus* (Linnaeus, 1758)), 3 parasite species are fixed (*Caryophyllaeides fennica* (Schneider, 1902) Nybelin, 1922; *Acanthocephalus lucii* (Mueller, 1776); *Rhabdochona denudata* (Dujardin, 1845) Raillet, 1916). They are belonging to classes Cestoda (1), Acanthocephala (1) and Nematoda (1).

Definitive hosts of *C. fennica* are a number of fish species from Cyprinidae: *Squalius cephalus* (Linnaeus, 1758)*, Leuciscus idus* (Linnaeus, 1758)*, Barbus barbus* (Linnaeus, 1758)*, B. petenyi* Heckel, 1852*, R. rutilus, Abramis brama* (Linnaeus, 1758)*, Scardinius erythrophthalmus* (Linnaeus, 1758)*, Aspius aspius* (Linnaeus, 1758)*, Gobio gobio* (Linnaeus, 1758), etc. (Kakacheva-Avramova, 1983; Moravec, 2001; Bauer, 1987; Protasova et al., 1990;etc.). Development of *C. fennica* is with the participation of an intermediate host – oligochaets of the species *Stylaria lacustris* (Linnaeus, 1767) (Bauer, 1987; Kakacheva-Avramova, 1983; Protasova et al., 1990). *R. rutilus* is a typical host for *C. fennica* (Protasova et al., 1990).

In Bulgaria, the species *C. fennica* was presented of *B. barbus* from Iskarand Tundzharivers; of *B.petrnyi* from rivers Iskarand Palakariya; of *Sq.cephalus* (*Leuciscus cephalus* (Linnaeus, 1758)) from Iskar River (Margaritov, 1959); *Sq. cephalus*, *Barbus cyclolepis* Heckel, 1837 and *Vimba melanops* (Heckel, 1837) from Maritsa and Topolnitsa rivers (Margaritov, 1964); of *B. cyclolepis*, *Sq. cephalus*, *V. melanops* from Asenitsa, Topolnitsa, Syuyutlijka, Sushitsa and Bedechka rivers (Kakacheva-Avramova, 1965); of *B. barbus* and *Sander lucioperca* (Linnaeus, 1758) (*Lucioperca lucioperca* (Linnaeus, 1758)) from the Danube River (Margaritov, 1966); of *B.*

*petenyi* from Nishava, Ogosta, Vodomerka, Buchinska, Vrabnishka, Barziya, Chuprenska, Iskrecka, Botunya, Bebresh rivers; of *B. barbus* from the Bogovina River; of *Sq. cephalus* from Bogovina, Nishava, Ogosta, Vodomerka, Barziya, Botunya, Bebresh rivers; of *G. gobio* from Botunya and Bebresh rivers; of *Chondrostoma nasus* (Linnaeus, 1758) from the Ogosta River; of *Alburnus alburnus* (Linnaeus, 1758) from the Leva River (Kakacheva-Avramova, 1969); of *Sq. Cephalus* and *R. rutilus* from the Shiposhnitsa River and Reservoir Iskar (Margaritov, 1977); of *Vimba vimba* (Linnaeus, 1758), *A.brama*, *Ballerus sapa* (Pallas, 1814) (*Abramis sapa* (Pallas, 1814)), *Ballerus ballerus* (Linnaeus, 1758) (*Abramis ballerus* (Linnaeus, 1758)), *Blicca bjoerkna* (Linnaeus, 1758), *A. alburnus*, *B. barbus*, *S. lucioperca*, *S.erythrophthalmus*, *Pelecus cultratus* (Linnaeus, 1758) (Kakacheva-Avramova and Menkova, 1978); *Sq. cephalus* and *R. rutilus* from the Palakariya River (Kakacheva-Avramova and Menkova, 1978); of *B. barbus* from the Struma River (Kakacheva-Avramova and Menkova, 1981); of *B. petrnyi* from the Mesta River (Kirin, 2001a); of *Sq. orpheus* from the Arda River (Kirin, 2002a; 2002b; Kirin et al., 2003); of *Sq. orpheus* and *A. alburnus* from the Arda River (Kirin et al., 2002); of *A. alburnus* and *B. cyclolepis* from the Arda River (Kirin, 2003); of *Sq. cephalus* from the Stryama River (Kirin et al., 2005); of *Sq. cephalus* from the Danube River (Cakic et al., 2004); of *B. barbus* from the Danube River (Atanasov, 2012); of *Sq. orpheus* from the Tunja River (Kirin et al., 2013), etc.

Definitive hosts of *A. lucii* are freshwater fish species from Cyprinidae, Percidae, Siluridae, Salmonidae, Esocidae, Gadidae, Cobitidae, Anguillidae. Intermediate host are crustaceans *Asellus aquaticus* (Linnaeus, 1758). (Petrochenko, 1956; Kakcheva-Avramova, 1983; Bauer, 1987).

In Bulgaria, the species *A. lucii* was presented of *Silurus glanis* Linnaeus, 1758 from the Danube River and of *Sq. cephalus* from Iskar and Tundzha rivers (Margaritov, 1959); of *Perca fluviatilis* Linnaeus, 1758 (Margaritov, 1966); of *B. sapa*, *Sq. cephalus*, *R. rutilus*, *S. glanis*, *P. fluviatilis*, *Lota lota* (Linnaeus, 1758), *Acerina schraetser* (Linnaeus, 1758), *Benthophilus stellatus* (Sauvage, 1874), *Proterorhinus marmoratus* (Pallas, 1814) (Kakacheva-Avramova et al., 1978); of *Sq. cephalus* (Cakic et al., 2004); of *L. lota* and *Zingel zingel* (Linnaeus, 1766) (Atanasov, 2012); of *A. brama* (Chunchukova et al., 2017); of *A. alburnus* (Chunchukova et al., 2018), from the Danube River; of *P. fluviatilis* (Shukerova et al., 2010) and *A. brama* (Chunchukova et al., 2016), from the Lake Srebarna etc.

Definitive hosts of *R. denudate* are fish species from Cyprinidae. Intermediate hosts are larvae of representatives of the generas *Heptagenia*, *Ephemerella* and *Hydropsyche* (Kakcheva-Avramova, 1983; Bauer, 1987).

In Bulgaria, the species *R. denudata* was presented of *B. barbus*, *B. petenyi* and *Sq. cephalus* from the Iskar River (Margaritov, 1959); of *Sc. erythrophthalmus* from the Strumeshnitsa River (Kakacheva-Avramova, 1962); of *Sq. cephalus*, *A. alburnus*, *Leuciscus aspius* (Linnaeus, 1758) (*Aspius aspius* (Linnaeus, 1758)), *B. cyclolepis* from Trakian's freshwater ecosystems (Kakacheva-Avramova, 1965); of *Sq. cephalus* from Maritsa, Vacha, Chepinska rivers; of *V. melanops* from the Maritsa River; of *A. alburnus* from Maritsa and Chepinska rivers; of *B. cyclolepis* from Maritsa, Chepinska, Vacha and Topolnitsa rivers (Margaritov, 1964); *Sq. cephalus* from Ogosta, Vrabnishka, Barziya, Nishava, Botunya, Leva, Archar, Berkovska, Chuprenska rivers; of *B. peteny* from Chuprenska, Barziya and Leva rivers; of *B. barbus* from the Leva River; of *G. gobio* from the Barziya River; of *A. alburnus* from Ogosta, Lom and Leva rivers (Kakacheva-Avramova, 1969); of *Sq. cephalus* from the Shiposhnitsa River and Reservoir Iskar (Margaritov, 1977); of *A. alburnus*, *Zingel streber* (Siebold, 1863), *Z. zingel* (Kakacheva-Avramova et al., 1978); of *Sq. cephalus* from the Palakariya River (Kakacheva-Avramova and Menkova, 1978); of *Cobitis taenia* Linnaeus, 1758 from State Fish Farming Blagoevgrad; of *Sq. cephalus* from Zheleznitsa, Blagoevgradska, Bistritsa, Gradevska and Strumarivers (Kakacheva-Avramova and Menkova, 1981); of *Sq. cephalus* and *B. cyclolepis* from the Struma River (Nedeva, 1991); of *C. carpio* (Kirin, 2001a) from the Mesta River; of *Sq. cephalus*

and *A. alburnus* from Kardzhali Reservoir (Kirin, 2001b); of *Sq. orpheus* (Kirin, 2002a; 2002b); of *B. cyclolepis* and *A. alburnus* (Kirin, 2003); of *Sq. orpheus* and *A. alburnus* (Kirin et al., 2002) from the Arda River; of *Sq. orpheus* from the Chepelarska River (Kirin, 2002a; 2002b); of *Sq. orpheus* from the Arda River (Kirin et al., 2003); of *Sq. cephalus* from the Danube River (Cakic et al., 2004); of *S. erythrophthalmus* from Srebarna Biosphere Reserve (Shukerova and Kirin, 2008); of *Sq. cephalus*, *S. erythrophthalmus*, *B. barbus* from the Danube River (Atanasov, 2012); of *Sq. orpheus* from the Tunja River (Kirin et al., 2013), etc. *C. fennica*, *A. lucii* and *R. denudata* are intestinal parasites in the body of fishes. For all fixed endoparasite species, the common roach is a definitive host.

**Component communities**

The three determined species, *C.fennica, A. lucii, Rh. denudata*, parasitizing in *R. rutilus* are generalists for the helminth communities of the examined freshwater fish species of the Luda Yana River ecosystem.

With the highest prevalence is distinguished *R. denudata* (P%=62.23), followed by those of *C. brachycollis* (P%=26.67) and *A. lucii* (P%=6.67). This tendency is also preserved in terms of mean intensity (MI) (Table 1).

*R. denudata* and *C. brachycollis* are core species of the parasite communities of common roach of the Luda Yana River. *A. lucii* is an accidental species of these communities (according to the criteria of Bush et al., 1987). The three determined species of endohelminths are autogenic species of the helminth communities of the common roach from the river.

**Infracommunities**

Established parasite species are presented with a total of 130specimens. *R. denudata* is distinguished with the highest number of specimens (88 specimens), and *A. lucii*, with the lowest (7 specimens).There are no mixed invasions. The low values for the mean number of species, mean number of specimens and Brillouin's diversity index (HB) are due to low species diversity, a small number of specimens of fish and low mean intensity of specimen of *R. rutilus* (Table 1).

For Bulgaria there are a few studies on parasites of *R. rutilus* and a total of 15 species of helminthes from the digestive tract of the roach were reported (Atanasov, 2012; Kakacheva-Avramova, 1983; Shukerova, 2010, etc.). Of these, two species are found in the present study. The third species, *R. denudata*, is a new endoparasite species for *R. rutilus* in the country. Consequently, the roach of the Luda Yana River is represented by 18.75% of the total found for *R. rutilus* intestinal parasite species.

Table1. Species diversity, prevalence (P%), mean intensity (MI) of the established endohelminth species of *Rutilus rutilus* from the Luda Yana River

| Species of endoparasites | Ecological indices (N[1]=45) | | | |
|---|---|---|---|---|
| | n[2] | p[3] | P%[4] | MI[5] Range |
| Cestoda | | | | |
| Caryophyllaeidae | | | | |
| *Caryophyllaeides fennica* (Schneider, 1902) Nybelin, 1922 | 12 | 35 | 26.67 | 2.92 1-7 |
| Acanthocephala | | | | |
| Echinoderhynchidae | | | | |
| *Acanthocephalus lucii* (Mueller, 1776) | 3 | 7 | 6.67 | 2.34 1-3 |
| Nematoda | | | | |
| Rhabdochonidae | | | | |
| *Rhabdochona denudata* (Dujardin, 1845) Raillet, 1916 | 28 | 88 | 62.23 | 3.14 1-10 |
| Total number of species (Mean number of species±SD) | 3 (0.95±0.2) | | | |
| Total number of specimens (Mean number of specimens±SD) | 130 (2.88±2.29) | | | |
| Brillouin's diversity index (HB) | 0.74±0.79 | | | |

[1]N = total number of examined fish specimens.
[2]n = total number of infected fish specimens.
[3]p = total number of endoparasite specimens.
[4]P% = prevalence.
[5]MI = mean intensity.

According to a number of authors, the species developed with intermediate hosts were reported with low indices of infection in ecosystems with negative impacts and parasitological studies of specific parasite species of freshwater fish can be used as bioindicators for environmental conditions (Rakauskas and Blaevièius, 2010; Valtonen et al., 2003, etc.).

The results obtained from the study and the knowledge of the biology of the established parasite species reveals the following main pathways of the parasitic flow: A. Cestoda: 1. Oligohaetes – Fishes (*Caryophyllaeides*

*fennica*); B. Acanthocephala: 1. Crustaceans – Fishes (*Acanthocephalus lucii*); C. Nematoda: 1. larvaes of Ephemeroptera and Diptera – Fishes (*Rhabdochona denudata*). Determined parasite species and ecological characteristics of parasite communities show that the larvae's of Ephemeroptera and Diptera are dominant in the nutrition of the roach. Probably their populations are well represented in the studied ecosystem of the river ecosystem.

## CONCLUSIONS

From studied 45 specimens of common roach, 3 parasite species are fixed: *C. brachycollis*, *A. lucii* and *R. denudata*. The Luda Yana River is a new locality for all of them. *R. rutilus* is a new host record for the endohelminth species *R. denudata*. Poor species diversity and low indices of invasion indicate for negative impacts on biodiversity of the Luda Yana River ecosystem.

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# HELMINTHS AND HELMINTH COMMUNITIES OFORPHEUS DACE (*Squalius orpheus* Kottelat & Economidis, 2006) FROM STRYAMA RIVER, BULGARIA

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***Abstract***

*During 2018, studies on the biodiversity and biomonitoring by the biological elements for environmental quality: Squalius orpheus (endemic of Balkan Peninsula) and its helminths and helminth communities were carried out. In 59 specimens of Sq.orpheus, four specimens of intestinal helminths are fixed (Allocreadium isoporum (Kowal et Kulakowskaja, 1957); Caryophyllaeus brachycollis (Janiszewska, 1951); Pomphorhynchus laevis (Müller, 1776); Rhabdochona denudata (Dujardin, 1845)). P. laevis is distinguished with the highest prevalence and mean intensity (66.10% and 1.85, respectively). A. isoporum, P. laevis and Rh. denudata are core species for the helminth communities of Orpheus dace, while C. brachycollis is a component species. The eutrophication effects on the pathways of the parasitic flow and the structure of the helminth communities were traced. The bioindicator significance of the parasitic complexes was discussed.*

***Key words***: *eutrophication effects, helminth communities, Stryama River, Squalius orpheus.*

## INTRODUCTION

Stryama River (110.1 km long) is one of the largest left tributaries of the Maritsa River in Bulgaria. The freshwater ecosystem and the adjacent areas are characterized by great biodiversity, protected areas (BG0000429 Stryama, BG0000289 Trilistnik, etc.), species and habitats (Natura 2000). The Stryama River valley is an important bio-corridor connecting the Upper Thracian valley with the Balkan Mountains. The aim of the study is to explore the state of endoparasites and parasite communities of *Sq. orpheus* of the Stryama River, as well as to discuss their bioindicator role in the eutrophication processes based on the endoparasitic flow.

## MATERIALS AND METHODS

A total of 59 specimens *Squalius orpheus (*Kottelat & Econodimis, 2006) are examined for endohelminths. The scientific and common names of the fish are provided according to the FishBase database (Fröse and Pauly, 2018). Helminthological examinations are implemented following recommendations described by Petrochenko (1956); Zashev and Margaritov, 1966; Bauer, 1987; Moravec, 2013). Specimens are fixed and preserved in 70% ethyl alcohol. The specimens of Trematoda and Cestoda are studied by methods of Zashev and Margaritov (1966); Georgiev et al. (1986); Scholz and Hanzelová (1998) and of Acanthocephala and Nematoda – of Moravec (2013). Analyses of helminth community structure are carried out in both levels: infracommunity (total and mean number of species; total and mean number of specimens; Brillouin's index of diversity (HB)) and component community (prevalence (P%) and mean intensity (MI) for each species) (Bush et al., 1997; Magurran, 1988).The species are divided into core species (P%>20), component species (P%>10) and accidental species (P%<10) (Kennedy, 1997). The diversity measures are calculated by software products Statistica 10 (StatSoft Inc., 2011) and MS Excel (Microsoft 2010).

## RESULTS AND DISCUSSIONS

### Fish communities

Orpheus dace (*Squalius orpheus* Kottelat & Economidis, 2006) inhabits almost all the rivers and reservoirs in Bulgaria. It is a pelagic

species. The Orpheus dace prefers fast-flowing rivers with sandstone bottom. Young fish feeds on algae and crustaceans, and adults - insects and their larvae, fish, frogs and small rodents (Karapetkova and Zhivkov, 2006; Fröse and Pauly, 2018). *Sq. orpheus* is estimated as least concern species (LC=Least Concern; IUCN Red List Status, 2018) and is not included in Red Data Book of the Republic of Bulgaria (Golemanski (Ed.), 2011). *Sq. orpheus* is an endemic fish species of the Aegean Basin (Kolev, 2013).

**Helminth community structure**

From studied 59 specimens of Orpheus dace (*Squalius orpheus* Kottelat & Economidis, 2006), a total of 4 species of helminths are determined: *Allocreadium isoporum (*Kowal et Kulakowskaja, 1957); *Caryophyllaeus brachycollis (*Janiszewska, 1951); *Pomphorhynchus laevis* (Müller, 1776); *Rhabdochona denudata* (Dujardin, 1845).

The first intermediate hosts of *A. isoporum* are snails of genus *Sphaerium* and the second – larvae's of insects of genera *Ephemera*, *Anabolia* and *Chaetopterix*. Definitive hosts are many fish species of Cyprinidae, Percidae, Esocidae, Salmonidae, etc. (Kakacheva-Avramova, 1983; Bauer, 1987). *A. isoporum* was reported of *Squalius cephalus* (Linnaeus, 1758) (*Leuciscus cephalus* Linnaeus, 1758), *Alburnoides bipunctatus* (Bloch, 1782), *Barbus barbus* (Linnaeus, 1758) and *Phoxinus phoxinus* (Linnaeus, 1758) from rivers Dokusak and Resovska (Kakacheva-Avramova, 1960); of *Alburnus alburnus* (Linnaeus, 1758) of the Danube River (Kakacheva-Avramova, 1977); of *Gobio gobio* (Linnaeus, 1758) from rivers of Eastern Staraplanina mountain (Kakacheva-Avramova, 1973), from rivers of Strandzha mountain (Kakacheva-Avramova, 1960), from water basins of Trakia (Kakacheva-Avramova, 1965), from rivers Vrabnishka and Nishava (Kakacheva-Avramova, 1969); of *Barbus petenyi* Heckel, 1852 from rivers Mesta and Struma (Kakacheva-Avramova, 1962), from rivers of Western Staraplanina mountain (Kakacheva-Avramova, 1969), from rivers of Central and Eastern Staraplanina mountain (Kakacheva-Avramova, 1973); of *Barbus cyclolepis* Heckel, 1837 of the Vacha River (Margaritov, 1965), from water basins of Trakia (Kakacheva-Avramova, 1965), of the Tundzha River (Kakacheva-Avramova, 1972); etc. Intermediate hosts of *C. brachycollis (*Janiszewska, 1951) are *Limnodrilus hoffmeisteri (*Claparède, 1862) and *Tubifex tubifex* (Müller, 1774). Definitive hosts are fish species of Cyprinidae. Typical definitive hosts are fish species: *B. barbus*, *B. petenyi*, *Sq. cephalus*, *Leucis cusidus* (Kakacheva–Avramova, 1983; Bauer, 1987; Protasova, 1990; Scholz and Hanzelová, 1998; Barčák et al., 2017). *C. brachycollis* was reported of *B. cyclolepis* and *Sq. orpheus* from rivers Asenitsa, Sushitsa, Syuyutlijka, Chepinska, Bedechka and Topolnitsa (Kakacheva-Avramova, 1965); of *Sq. orpheus* from rivers Maritsa, Vacha and Chepinska; of *Vimba melanops* (Heckel, 1837) of the Maritsa River, of *A. alburnus* from rivers Maritsa and Chepinska, of *B. cyclolepis* from rivers Maritsa, Vacha and Topolnitsa, of *Rutilus rutilus* (Linnaeus, 1758) of the Bistritsa River (Margaritov, 1965); of *Sq. cephalus* from rivers Vrabnishka and Nishava, of *B. petenyi* from rivers Mirkovska, Botunya, Ogosta, Iskar, of *B. barbus* of the Bebresh River (Kakacheva-Avramova, 1969); of *Sq. cephalus* of the Palakariya River, of *B. petenyi* from rivers Devinska and Sarneshka, of *Sq. Orpheus* of the Vacha River (Kakacheva-Avramova and Menkova, 1978); of *B. petenyi* of the Blagoevgradska Bistritsa River, of *B. barbus* of the Struma River, of *Sq. cephalus* from rivers Zheleznitsa, Gradevska, Struma (Kakacheva-Avramova and Menkova, 1981); of *Perca fluviatilis* Linnaeus, 1758 of Reservoir Zhrebchevo (Nedeva and Grupcheva, 1996); of *Sq. orpheus* of the Maritsa River (Kirin, 2000, 2001b); of *Sq. cephalus* (*L. cephalus*) and *B. petenyi* of the Mesta River (Kirin, 2001c); of *Sq. orpheus* of Reservoir Kardzhali (Kirin, 2001b); of *B. cyclolepis* of the Luda Yana River (Kirin, 2002c); of *Sq. orpheus* (Kirin, 2002a), of *Sq. orpheus* and *A. alburnus* (Kirin et al., 2002), of *B. cyclolepis* and *A. alburnus* (Kirin, 2003), of *Sq. orpheus* (Kirin et al., 2003) of the Arda River; of *Sq. cephalus* of the Danube River (Cakic et al., 2004); of *Sq. orpheus* of the Stryama River (Kirin et al., 2005), of the Tunja River (Kirin et al., 2013); of *V. melanops* of the Maritsa River (Kirin,

2014), etc. Intermediate host of *P. laevis* is *Gamma ruspulex* (Linnaeus, 1758). Definitive hosts are mainly freshwater fish species of Cyprinidae and less often - of Salmonidae, Percidae, Siluridae, etc. (Petrochenko, 1965; Kakacheva-Avramova, 1983; Bauer, 1987). *P. laevis* was reported of *Sq. cephalus* of the Iskar River, of *B. barbus* of the Danube River (Margaritov, 1959); of *Acipenser ruthenus* Linnaeus, 1758, *G. gobio*, *B. barbus*, *A. alburnus*, *Blicca bjoerkna* (Linnaeus, 1758), *Pelecus cultratus* (Linnaeus, 1758), *Carassius gibelio* (Bloch, 1782), *Cyprinus carpio* Linnaeus, 1758, *Sabanejewia bulgarica* (Drensky, 1928) (*Cobitis bulgarica*), *Silurus glanis* Linnaeus, 1758, *Sander lucioperca* (Linnaeus, 1758) (*Lucioperca lucioperca*), *Zingel zingel* (Linnaeus, 1766) (*Asprozingel* Linnaeus, 1766), *Zingel streber* (Siebold, 1863) (*A. streber* Siebold, 1863), *Gymnocephalus cernua* (Linnaeus, 1758) (*Acerina cernua* (Linnaeus, 1758)), *Gymnocephalus schraetser* (Linnaeus, 1758)), (*A. schraetser* (Linnaeus, 1758)), *Ponticola constructor* (Nordmann, 1840) (*Gobio cephalarges constructor* Nordmann, 1840), *G. gobio* (*G. fluviatilis* Linnaeus, 1758), *Benthophilus stellatus* (Sauvage, 1874) of the Danube River (Matgaritov, 1966); of *Chondrostomanasus* (Linnaeus, 1758) and *Ph. phoxinus*from rivers Ogosta and Nishava (Kakacheva-Avramova, 1969); of *A. ruthenus*, *A. güldenstädtii* Brandt &Ratzeburg, 1833, *Salmo labrax* Pallas, 1814, *Alosaimmaculata* Bennet, 1835 (*Alosapontica* Bennet, 1835), *Anguilla anguilla* Linnaeus, 1758, *C. carpio*, *C. gibelio*, *V. vimba*, *Abramisbrama*(Linnaeus, 1758), *Ballerussapa* (Pallas, 1814) (*Abramissapa* (Pallas, 1814)), *Ballerus ballerus* (Linnaeus, 1758) (*Abramis ballerus* (Linnaeus, 1758)), *P. cultratus*, *A. alburnus*, *B. bjoerkna*, *G. gobio*, *Romanogobio albipinnatus* (Lukasch, 1933) (*G. albipinatus* (Lukasch, 1933)), *B. barbus*, *Ch. nasus*, *L. idus*, *Scardinius erythrophthalmus* (Linnaeus, 1758), *Sq. cephalus*, *Leuciscus aspius* (Linnaeus, 1758) (*Aspius aspius* (Linnaeus, 1758), *Ctenopharyngodon idella* (Valenciennes, 1844), *Proterorhynus marmoratus* (Pallas, 1814), *S. glanis*, *Lota lota* (Linnaeus, 1758), *Esox lucius* Linnaeus, 1758, *S. lucioperca*, *S. volgense*, *P. fluviatilis*, *G. cernua*, *G. schraester*, *Z. zingel*, *Z. streber*, *Ponticola kessleri* (Günther, 1861) (*Gobius kessleri* (Günther, 1861)), *Lepomis gibbosus* (Linnaeus, 1758), *G. gobio*, *B. stellatus* of the Danube River (Kakacheva-Avramova et al., 1978); of *B. barbus* from rivers Struma, Zheleznitsa, Gradevska, of *A. bipunctatus* from rivers Zheleznitsa and Gradevska, of *Sq. cephalus* of the Struma River (Kakacheva-Avramova and Menkova, 1981); of *C. carpio* and *S. lucioperca* (Nedeva and Grupcheva, 1996), of *C. gibelio* of Reservoir Zhrebchevo (Grupcheva and Nedeva, 1999); of *Sq. Orpheus* of the Maritsa River (Kirin, 2000; 2001); of *Sq. cephalus* of the Danube River (Cakis et al., 2004); of *P. fluviatilis* of the Arda River (Kirin, 2005); of *Sq. orpheus* of the Stryama River (Kirinetal., 2005); of *A. brama*, *B. sapa*, *A. ruthenus*, *A. alburnus*, *A. immaculata*, *B. barbus*, *C. gibelio*, *E. lucius*, *G. schraester*, *Sq. cephalus*, *P. cultratus*, *Pomatoschistus minutus* (Pallas, 1770), *S. lucioperca*, *Sc. erythrophthalmus*, *S. glanis*, *Z. zingel* of the Danube River (Atanasov, 2012); of *Sq. cephalus* of the Tunja River (Kirin et al., 2013), etc. Definitive hosts of *R. denudata* are fish species from Cyprinidae. Intermediate hosts are larvaes of the genera *Heptagenia*, *Ephemerella* and *Hydropsyche* (Kakcheva-Avramova, 1983; Bauer, 1987). *R. denudata* was presented of *B. barbus*, *B. petenyi* and *Sq. cephalus* of the Iskar River (Margaritov, 1959); of *Sc. erythrophthalmus* of the Strumeshnitsa River (Kakacheva-Avramova, 1962); of *Sq. cephalus*, *A. alburnus*, *L. aspius*, *B. cyclolepis* from Trakian's freshwater ecosystems (Kakacheva-Avramova, 1965); of *Sq. orpheus* from rivers Maritsa, Vacha, Chepinska, of *V. melanops* of the Maritsa River, of *A. alburnus* from rivers Maritsa and Chepinska, of *B. cyclolepis* from rivers Maritsa, Chepinska, Vacha and Topolnitsa (Margaritov, 1965); *Sq. cephalus* from rivers Ogosta, Vrabnishka, Barziya, Nishava, Botunya, Leva, Archar, Berkovska, Chuprenska, of *B. peteny* from rivers Chuprenska, Barziya and Leva, of *B. barbus* of the Leva River; of *G. gobio* of the Barziya River, of *A. alburnus* from rivers Ogosta, Lomand Leva (Kakacheva-Avramova, 1969); of *Sq. cephalus* of the Shiposhnitsa River and Reservoir Iskar (Margaritov, 1977); of *A. alburnus*, *Z. streber*, *Z. zingel* (Kakacheva-Avramova et al., 1978); of *Sq. cephalus* of the

Palakariya River (Kakacheva-Avramova and Menkova, 1978); of *Cobitis taenia* Linnaeus, 1758 from State Fish Farming Blagoevgrad; of *Sq. cephalus* from rivers Zheleznitsa, Blagoevgradska, Bistritsa, Gradevska and Struma (Kakacheva-Avramova and Menkova, 1981); of *Sq. cephalus* and *B. cyclolepis* of the Struma River (Nedeva, 1991); of *C. carpio* (Kirin, 2001a) of the Mesta River, of *Sq. cephalus* and *A. alburnus* of Reservoir Kardzhali (Kirin, 2001b); of *Sq. orpheus* (Kirin, 2002a), *B. cyclolepis* and *A. alburnus* (Kirin, 2003), *Sq. orpheus* and *A. alburnus* (Kirin et al., 2002) from the Arda River; of *Sq. orpheus* of the Chepelarska River (Kirin, 2002b); of *Sq. orpheus* of the Arda River (Kirin et al., 2003); of *Sq. cephalus* of the Danube River (Cakis et al., 2004); of *Sq. orpheus* of the Stryama River (Kirin et al., 2005); of *S. erythrophthalmus* and *L. aspius* (*A. aspius*) from Srebarna Biosphere Reserve (Shukerova and Kirin, 2008; Shukerova, 2010); of *Sq. cephalus*, *S. erythrophthalmus*, *B. barbus* of the Danube River (Atanasov, 2012); of *Sq. orpheus* of the Tunja River (Kirin et al., 2013), etc. *C. fennica*, *A. lucii* and *R. denudata* are intestinal parasites in the body of fishes. For all reported endoparasite species, *Sq. orpheus* is a definitive host.

**Component communities**

The found intestinal parasites are generalists for the helminth communities of Orpheus dace of the Stryama River. They are authogenic species for the studied freshwater ecosystem. With the highest prevalence and mean intensity is distinguished *P. laevis* (P%=66.10; MI=1.85), followed by *A. isoporum* (P%=38.98; MI=1.69) (Table 1). *P. laevis*, *A. isoporum* and *Rh. denudata* are core species of the helminth communities of Orpheus dace. Thefourthspecies, *C. brachycollis* is a component species of these communities according to the criterion of Bush et al. (1997).

**Infracommunities**

The established 4 species of endoparasites are presented a total with 142 specimens. Two fish specimens are free of parasites. With the highest number of parasite species are distinguished 5 specimens of Orpheus dace – 3 species, followed by 20 specimens of fish infected with two species of endohelminths. 30 specimens of examined fish are infected with one species of the reported parasites. Mean number of species and specimens of intestinal parasites per specimen of examined fish are fixed (Table 1). Minimal number of endoparasite specimens per a fish specimen is one and maximal is six (2.37±1.03). Brillouin's diversity index is high (HB=1.13) (Table 1).

Table 1. Biodiversity and ecological indices of the helminth communities of *Sq. Orpheus* from Stryama river

| Ecological indices (N = 59) Biodiversity | n p | P% MI Range |
|---|---|---|
| **Trematoda** | | |
| *Allocreadium isoporum* | 23 39 | 38.98 1.69 1-6 |
| **Cestoda** | | |
| *Caryophyllaeus brachycollis* | 11 13 | 18.64 1.18 1-2 |
| **Acanthocephala** | | |
| *Pomphorhynchus laevis* | 39 72 | 66.10 1.85 1-4 |
| **Nematoda** | | |
| *Rhabdochona denudata* | 16 18 | 27.11 1.12 1-2 |
| **Infracommunity data** | | |
| Total number of species | 4 | |
| Mean±SD | 1.75±1.41 | |
| Number of fish | 2 30 | 20 5 |
| Number of helminth species | 0 1 | 2 3 |
| Total number of specimens | 142 | |
| Mean±SD | 2.37±1.03 | |
| Range | 1-6 | |
| HB (Brillouin's diversity index) (Mean±SD) | 1.13 (0.409±0.1) | |

The parasite communities of Orpheus dace from freshwater ecosystems of Bulgaria, to this time, are represented by 23 species of intestinal parasites. The four species of endoparasites, found in this study, are only 17.39% of the established for the country. The species *C. brachycolis*, *P. laevis* and *Rh. denudata* were reported of *Sq. orpheus* of the Stryama River (Kirin et al., 2005). In 2005, the parasite communities of Orpheus dace were presented a total of 8 species of endohelminths. *A. isoporum* is reported for the first time of *Sq. orpheus* of the Stryama River. The prevalences of *C. brachycolis* and *P. laevis* are higher (2.77 and 1.36 times more, respectively) than those of 2005, but in the opposite, the prevalence of *Rh. denudata* is lower (1.70 times less). Intermediate hosts of *A. isoporum* of genus

*Sphaerium* are bioindicators of β-α saprobity and the larvae's of insects of genera *Ephemera*, *Anabolia* and *Chaetopterix* are bioindicators of 0-β, 0-α and 0-saprobity, respectively. *L. hoffmeistri* and *T. tubifex*, intermediate hosts of *C brachycolis* are bioindicators of polysaprobity (p). *G. pulex*, intermediate host of *P. laevis* is a bioindicator of χ-β-mesosaprobity and intermediate hosts of *Rh. denudata* (*Ephemerella* sp. and *Hydropsyche* sp.) are bioindicators of 0-α-mesosaprobity (Rosenberg et al., 1997).Most of the intermediate hosts have extensive ecological tolerance. The highest increase is established for the prevalence of *C. brachycolis*, but the highest prevalence is recorded for *P. laevis*. For the three species of parasites, the lower mean intensity was reported than those from the previous study. Probably *G. pulex* is dominant species in the diet of Orpheus dace from the studied habitats. Similar research and dependencies were traced by Brewster (2016), Goga (2016), etc.

## CONCLUSIONS

*Sq. orpheus* and its parasites along the path of nutritional interactions can have an important role in monitoring the effects of eutrophication in taking ecosystem conservation measures.

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# USE OF CLINOPTILOLITE NATURAL ZEOLITE IN AQUACULTURE - A REVIEW

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***Abstract***

*In 2016, the global aquaculture was 46.82%of total fish production, which means 80 million tonnes of 170.9 million tonnes. Latest researches highlighted that using the natural zeolites in aquaculture in order to maximize the use of resources (water, food, species) and to ensure the lowest negative impact on the environmentwas the most viable solution. The studies on zeolites use, clinoptilolitein particular, were focused on their use as feed additives (up to 2.5% concentration) and also as water quality improvers; this is mainly due to their ability to remove ammonia, its compounds and heavy metals, to reduce water hardness and to consequently prevent diseases and decrease the losses on fish population. A practical and efficient use of natural zeolites in aquaculture will determine increased economic efficiency.*

***Key words****: ammonia, feed, fish, water quality.*

## INTRODUCTION

Aquaculture has the fastest growing rate among the food-producing sectors, especially those producing animal protein (Simeanu et al., 2015). At present, over 50% of the total fish production used as human food comes from this sector and it is expected to increase to 62% by 2030 due to the drastic decrease of wild fishery catches (FAO 2014). In order to ensure a higher productivity, aquaculture technologies need to be more effective and also to reduce the negative impact on the environment with regard of toxic contaminants decreasing both in effluents from recirculating systems and pond wastewater.

Since 1756, over 60 types of natural zeolites have been discovered and more than 150 different types have been synthesized (Ghasemi et al., 2016). In 1857, the reversible dehydration zeolites property was discovered, and in 1858, it was determined that zeolites also have the propriety of cationic exchange in aqueous solution. Due to their porous structure, the specific gravity of zeolites is low. Natural zeolites have considerable variations of chemical composition: water content, cations and the Si / Al ratio (Mishra and Jain, 2011). Due to their accesibility, lower cost and ecological compatibility, zeolites are very important in environment preserving (Bedelean and Avram, 1991). One of the best zeolites used for ammonia removal is clinoptilolite.

Since 2007, zeolite-based additives have been declared safe for end-users of meat, milk or eggs from animals that have received zeolite in feed or mannure. Clinoptilolite zeolite is registered by the European Community as food additive DIN 53 770.

The global use of zeolites (clioptilolitein particular) in bioeconomy has shown a major development. In Romania, since 2000, researchers started to study the use of zeolites in animal husbandry andhave shown favorable effects of their use as nutrients and of feed conversion coefficients (Pogurschi et al., 2017). The use of the Romanian clinoptiloliterich volcanic rock as a feedadditive showed the capacity to improve the milk quality and production, animal health and welfare, ensuring

optimal technological conditions for the environment (Marin et al., 2018).
Researchers studied clinoptilolite as an ion-exchange and adsorbent in waste water purification in recirculation systems due to its affinity for certain cations - especially ammonia, but also for its ability to capture toxic heavy metals. Applications of zeolites, especially clinoptilolite, in the transport of live fish as well as their use as potential feed additives have to be also considered.

## MATERIALS AND METHODS

This paper presents a review of the most relevant literature regarding the influence of natural zeolites, clinoptiloliteespecially, on the assurance of the best medium conditions for fish activity. It was also investigated the effect of clinoptiloliteadding into the diet on the rate of fish growth. The experiments were conducted mainly on fresh water fish species, but marine species have also been studied.

## RESULTS AND DISCUSSIONS

One of the main parameters determining the water quality of water in a fish farm is the concentration of ammonia.
The amount of ammonia in fish ponds is influenced by the feed intensity, the amount of feed protein and wastedfeed that decomposes into the system (Cristea et al., 2002).
Compared to ponds, the carrying capacity of a recirculating system is higher, but the filtering capacity is limited.
Fishes can assimilate the ammonia and the remnant concentration may exceed 0.02 ppm, resulting in a lethargic state and ultimately the death of the fish population.
Even when the concentration of ammonia in the pools does not reach lethal values, a series of sub-lethal effects occur: lower feed conversion, lower growth rate and low immunity to various diseases (Ghasemi et al., 2016).
Total ammoniacal nitrogen (TAN) has two forms: non-ionized ammonia, $NH_3$, and ionized ammonia, $NH_4^+$.
The non-ionized form is extremely toxic for most fish species and its share of TAN depends on pH and water temperature. TAN is initially transformed by nitrifying bacteria into nitrites $NO_2^-$, then nitrates $NO_3^-$, a nitrogen compound with less toxicity for fish.
By combining nitrite with hemoglobin in fish blood, methaemoglobin is formed, thus preventing the transport of oxygen from gills to tissues (Marin et al., 2015).
In order to decrease the level of ammonia from contaminated waters, several processes can be used in farms: replacement of a volume of waste water with fresh water, nitrification or use of ion exchange processes.
The zeolites used in the aquaculture water purification process show high selectivity to ammonia (Beler-Baykal et al., 1996; Booker et al., 1996; Pansini, 1996).
Researches have shown that the ion exchange reaction between zeolite and water components is influenced by the zeolite type, particle and pores size, Si / Al ratio and chemical composition of contaminated water (Koon and Kaufman, 1975; Jorgensen et al., 1976; Klieve and Semmens, 1980; Curkovic et al., 1997).
The kinetic of the ion exchange reaction is influenced by the particle size of the zeolite; the cations from the solution will not reach the inner positions of larger zeolite particles if the contact time between the solution and the adsorbent is not long enough. 0.7-1.0 mm clinoptilolite and mordenite particles showed a greater absorption capacity of ammonia in a recirculating system compared to larger particles, 1.0-1.4 mm, at a certain contact time (Mwale, 2000).

*Improving water quality by using clinoptilolite*
Clinoptilolite is among the zeolites with higher affinity to ammonia, along with mordenite and chabazite.
It is a hydrated sodium and potassium / calcium aluminosilicate. It has a unique crystalline structure, "clino" type, and special properties no metter the size of the particle (Emadi et al., 2001).
This structure resists at extreme pressure, it requires high temperatures to break, similar to those that melt the glass and is not chemically attacked, except for extreme acid or alkaline conditions.
Degradation over time is impossible if one of the above conditions is not met.
The ideal simplified formula of clinoptilolite is (Na, K) $6Si_{30}Al_6O_{72}\_nH_2O$ (Figure 1).

*Removal of ammonia from fish ponds*

Clinoptilolite is a highly effective adsorbent in removing ammonia from water due to the relatively high cation exchange capacity (Figure 2) and alsoto a high selectivity for ammonium ions in the presence of other competing cations (Peters and Bose, 1975).

The differences among natural zeolites of the same type are due to the different environmental conditions, in which they formed, as well as the quantities and types of impurities they include (Hawkins, 1983).

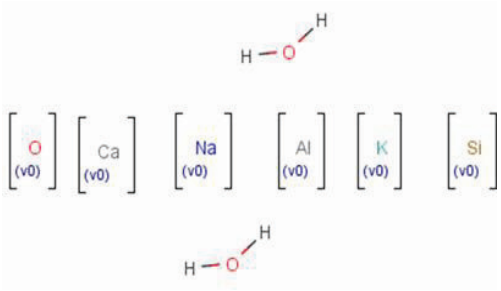


Figure 1. Chemical formula of clinoptilolite (Mutlu et al., 2016)

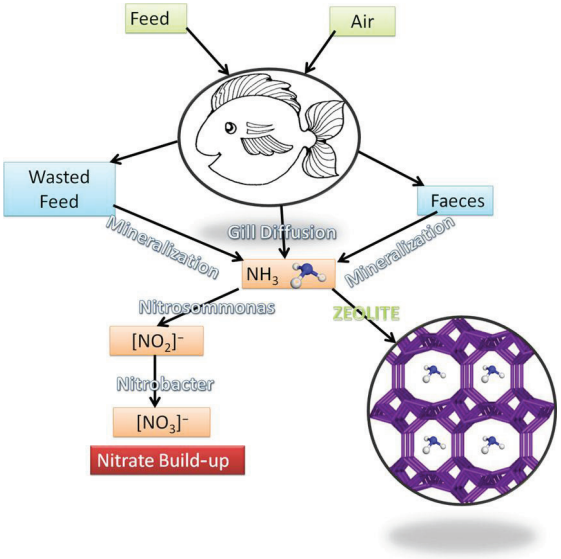


Figure 2. Nitrogen cycle in fish ponds (Durborow et al., 1997)

By using 12 g $L^{-1}$ and 15 g $L^{-1}$,respectively, the survival rate to lethal ammonia concentrations in the case of beluga *(Huso huso)* (Asgharimoghadam et al., 2012; Farhangi et al., 2013) and Persian sturgeon (*Acipenser persicus*) has increased (Farhangi and Rostami-Charati, 2012). The aquarium growth of scalars (*Pterophyllum scalare*) with the addition of 10 g $L^{-1}$ of clinoptilolite was considered optimal for ammonia and water hardness decreasing (Ghiasi and Jasour, 2012). Research on rainbow trout (*Oncorhynchus mykiss*) showed that 15 g $L^{-1}$ of natural clinoptilolite decreased the mortality rate when reaching a lethal concentration (Farhangi and Hajimoradloo, 2011). At thesame time, the effectiveness of a 2.5% natural zeolite diet was demonstrated by lowering the concentration of ammonia by 24% compared to the control group (Ergun et al., 2008).

Clinoptilolite was also used in aquatic growth systems of tilapia (*Oreochromis sp.*) and salad (*Lactuca sativa* var. *Longifolia*) as a bed for salad seedlings, added 10 g each in small cotton bags.

The results have shown that zeolite can improve water quality by lowering the total ammonia concentration (Rafiee and Saad, 2006).

Researchers tried to replace the nitrifying biological filters with natural clinoptilolite and the results are promising.

In a carp recirculating system, the level of nitrate compounds was determined after the

filterring of the effluent through a filter of clinoptilolite and nitrifying bacteria. The ammonia values were significantly lower and the amount of nitrates significantly higher in effluent treated with zeolite and bacteria (Motesharezadeh et al., 2015). Studies on recirculation systems (Bergero et al., 1994) have shown a greater effectivenessin ammonia removal of clinoptilolite and phillipsite compared to chabazite-rich volcanic tuff.

In the experiments conducted at USAMV Bucharest, in an aquarium with crucian carp (*Carassius carassius*), the biological filter was replaced with clinoptilolite, an amount of 6.7 g $L^{-1}$ and particle size of 1-3 mm (Sava et al., 2017).

The results were encouraging and it was found that the admissible level of ammonia and nitrite were exceeded only after 32 hours of experimentation (Table 1).

Table 1. Chemical analysis of the samples (Sava et al., 2017)

| Time, hours | pH | $NH_4$ [mg/l] | $NO_2$ [mg/l] | $NO_3$ [mg/l] | $P_{total}$ [mg/l] | Ca [mg/l] |
|---|---|---|---|---|---|---|
| 0 | 7.67 | 0.035 | 0.026 | 6.643 | 0.096 | 33.5 |
| 4 | 7.72 | 0 | 0.030 | 6.643 | 0.799 | 37.5 |
| 8 | 7.71 | 0 | 0.076 | 5.757 | 1.332 | 42.3 |
| 12 | 7.72 | 0 | 0.056 | 6.200 | 1.788 | 43.1 |
| 16 | 7.76 | 0 | 0.033 | 7.971 | 2.239 | 44.7 |
| 20 | 7.77 | 0 | 0.033 | 8.414 | 2.230 | 45.5 |
| 32 | 7.66 | 0.146 | 0.079 | 11.514 | 4.967 | 48.7 |

By using acolumn of regenerated clinoptilolite, a decrease from 0.28 mg $L^{-1}$ to 0.08 mg $L^{-1}$of the ammonium content was found in the first 12 hours (Nicolae et al., 2017).

In order to increase the anion retention capacity of the clinoptilolite in water from a recirculating system, researches have been conducted on modifying thezeolite with a surfactant. It has been observed that by increasing the temperature from 10°C to 15°C the retention capacity of the modified zeolite increased from 10 to 20 mg $g^{-1}$ in the case of nitrates and from about 0.0 to 1.2 mg $g^{-1}$ in the case of nitrites (Shokouh et al., 2010).

Researches were also been carried out on the possibilities of using synthetic zeolites as such or with natural ones in fish farms (Ariful Islam et al., 2014). Regarding theammonia removal from freshwater culture systems, the results have shown that the overall efficiency of synthetic zeolites is lower and their cost is higher compared to natural clinoptilolite.

*Use of clinoptilolite as a feed additive*

Clinoptilolite is approved by FDA and EU for birds and animals feeding additive, classified as safe from the health and safety point of view. Up to now, no toxic effect of accidental ingestion of zeolite is known. Including of 2-5% clinoptilolite in fish feed showned an increasing of biomass, an encouraging result to be more investigated.

By adding a 2% zeolite in rainbow trout (*Oncorhynchus mykiss*) feed, the biomass productivity increasedof up to 10%. The study was conducted on 100 trouts that were given normal feed with 48% of proteins for 48 days (Ghasemi et al., 2016). By adding a 5% clinoptilolite into the diet of common carp (*Cyprinus carpio*), the apparent digestibility coefficients and biomass growth were positive (Khodanazary et al., 2013). Feed enhanced with 1-2% clinoptilolite was used for feeding redbelly tilapia (*Coptodon zillii*), and this determined an improved feed conversion and growth performance (Yıldırım et al., 2009). There were also researches that shown no influence on fish development after the use of 5% and 10% clinoptilolite in the diet of silver salmon (*Oncorhynchus kisutch*) (Edsall and Smith, 1989), but these can be explained by the use of zeolites from different geological sources, particle size, or zeolite conditioning.

Improvement of growth indices has been associated with the zeolite detoxification effect (Ortatatli&Oguz, 2001; Rizzi et al., 2003), but also by the slower passage of fodder through the intestine, which has led to an improvement in the use of the nutritive substances in feed (Dias et al., 1998, Eya et al., 2008). Therewith,

clinoptilolite captured ammonia, toxic to celular level, which led to a better use of nutrients (Papaioannou et al., 2005).

*Other uses of natural zeolites*

Researches were also directed towards the investigation of the ion exchange capacity of zeolites in order to remove the various cations of toxic heavy metals from the wasted waters in fish farms. By adding 10 g $L^{-1}$ of clinoptilolite to water, the accumulation of cadmium in the common carp (*Cyprinus carpio*) body was reduced (Ghiasi et al., 2011). Prussian carp (*Carassius gibelio*) tissues analysis revealed the reduction of cadmium bioaccumulation by using clinoptilolite which was added in doses of 0.5, 2.0 and 4.0 g $L^{-1}$, the rate of reduction of heavy metal being correlated with the dose used (Nicula et al., 2010).

Clinoptilolite has been successfully used to transportation of live fish. In order to choose the most effective solution for controling the ammonia level in a fish transporting tank, it has to consider a series of parameters, including fish species, fish density, tank volume and transportation time. Studies have shown that the addition of 7 g $L^{-1}$clinoptilolitezeolite is sufficient to reduce ammonia in the transport of rainbow trout (*Oncorhynchus mykiss*) (Oz et al., 2010). By adding the same amount of clinoptilolite, high water quality and reduced ammonia concentration in frycarps transport (*Gibelion catla*, *Labeo rohita* and *Cirrhinus mrigala*) were maintained, which resulted in a higher survival rate (Singh et al., 2004). In the case of Victoria lake ciclide (*Haplochromis obliquidens*), a mixture of clove oil and clinoptilolite introduced into the transportation bags reduced the ammonia level by 82% compared to the bags in which the mixture was not used (Kaiser et al. 2006). Similar results were also reported with the use of clinoptilolite at doses of 10, 20 and 40 g $L^{-1}$ for carriage in polyethylene bags of goldfish (*Carassius auratus*) when the ammonia concentration was reduced by 73%, 87% and 93% (Bower and Turner, 1982). The study was carried out during the transportation of ornamental fish, *Ancistrustriradiatus*, at high temperatures, and showed that the use of 22.7 g $L^{-1}$of clinoptilolite reduced ammonia levels from 51.6mg $L^{-1}$ to 15.5 mg $L^{-1}$ (Ramirez-Duarte et al., 2011). Clinoptilolite has also been successfully used for carp larve transportation in the Kemerevoregion (Polyakov et al., 2009).

Clinoptilolite has a low ammonia absorption capacity in salty waters;this disappears after about 8 hours (Emadi et al., 2001). However, researcheshave shown that clinoptilolitezeolite can be used to transport marine fish over a 24-hour period. The reduction of TAN levels by 18.33% at 8 hours, 34.08% at 16 hours, and 20.96% at 24 hours, was observed after using 40 g $L^{-1}$ (Kanyilmaz et al., 2014). In order to be effective in saline water, clinoptilolite should be added in bigger quantities and the maximum absorption capacity is reached 16 hours after introduction into water.

It can be concluded that for transportation of live fish and the elimination of TAN from fish farms, clinoptilolitezeolite is more effective in freshwater than salty water.

## CONCLUSIONS

The purpose of the present work was to present different applications of clinoptilolitezeolite in the aquaculture industry. Despite its low efficiency when used in salty water, researches and studies in freshwater and fish feed have proven the utility of using this zeolite in fish farms due to its unique structure and physico-chemical properties, in particular the cation exchange. Starting from its high affinity for ammonia, but also for other cations, it is possible to determine the main directions of using of this zeolite in aquaculture. Due to the selective ion exchange process, this is an alternative to the biological filters in the recirculation systems. Clinoptilolite has shown an important role played in improving the water quality in the growth basins and implicitly in the health of fish. In the transportation of live fish, its use contributes to the maintenance of total ammoniacal nitrogen (TAN) in normal limits and along with other zeolites, clinoptilolite offers the possibility of removing heavy metals from the water used in aquaculture but also from the fish body. The use of zeolite in fish diet has beneficial effects and determines the increasing of biomass and maintaining the health of fish populations. Other benefits that recommend using clinoptilolite and other zeolites in fish are low

cost, good accesibility (deposits found in many parts of the world) and that theyt are environmental friendly materials. Researchers will continue to study these "stones of the future" to use them in environmental protection technologies and to obtain high aquaculture production at low cost.

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# INFLUENCE OF SUBSTRATE TYPE ON THE PHYSIOLOGICAL PROFILE OF THE HETEROTROPHIC BACTERIAL COMMUNITY IN RECIRCULATING AQUAPONIC SYSTEMS

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***Abstract***

*Aquaponics incorporate fish culture and hydroponically grown plants into one intensive production system. In this productive system, the microbial community develops everywhere being responsible of carrying out the nutrient between different compartments. Currently, the state of knowledge regarding the dynamics of microbial communities in recirculating aquaponic systems is stilllimited, due to poor understanding of interactions between bacterial population established on different substrates; therefore, in order to clear a number of uncertainties, especially at the level of heterotrophic communities, further studies are required. The present paper assessed the dynamics of heterotrophic microbial communities in recirculating aquaponicsystems. The studyis based on the assessment of activity and, especially, of the metabolic diversity of bacterial community attached to the root area of hydroponically cultivated species. Examination of the data gave the first indications to functional groups of organisms in the different compartments of an experimental aquaponic system.*

***Key words:*** *aquaponic systems, heterotrophic bacteria, substrate type, wastewater treatment.*

## INTRODUCTION

Aquaponic systems are highly engineered aquaculture-agriculture systems that use fish effluent (which comprises both particulate waste solids and dissolved nutrients) from intensive closed aquaculture system as nutrient medium to grow edibleplants in attached hydroponic subsystems (Bartelme et al., 2018). The treatment of waste water from intensive aquaculture, using phytoremediation strategy, is of increasing interest for the international scientific community, as it has been materialized in a series of studies published in prestigious journals in recent years (Graber and Junge, 2009; Sikawa and Yakupitiyage, 2010; Endut et al., 2010; van Kessel et al., 2010; Hu et al., 2015; Suhl et al., 2016; Filep et al., 2016; Forchino et al., 2017).

In addition to the clear advantage of utilizing residual nutrients and purifying technological water (Turciosa and Papenbrock, 2014; Simionov et al., 2017), aquaponic integrated systems provides a significant support surface, represented by plant's root system, to form biofilm. Moreover, this renewable surface ensures a permanent oxygenation of the culture medium while generating a series of valuable organic compounds such as enzymes and vitamins (Bertin et al., 2003). Microbial community (MC) from aquaponic system is the invisible link betwwen fish excrements, highly concentrated in ammonium, and plant fertilizer, which should be a combination of low ammonium and high nitrate (Somerville et al., 2014).

Despite the importance of MC in aquaponic systems onlyfew investigations have been undertaken to determine the microorganisms whitin the systems or microorganisms that colonize the plant rhizosphere and to evaluate their activity (Kuhad et al., 2004; Munguia-Fragozo et al., 2015; Schmautz et al., 2017). In recent years, with the development of modern molecular techniques, it has been possible to elucidate some aspects of biofilm structure attached to different filter media (inert) and characterize bacterial population dynamics in biological filters with different configurations integrated in classical recirculating systems (Brazil, 2006; Malone and Pfeiffer, 2006; Eding et al., 2006). However, these studies involved mainly nitrifying bacteria (ammonium-oxidizing bacteria and nitrite-oxidizing bacteria) or organisms that perform

denitrification, ANAMMOX (Rurangwa and Verdegem, 2015).

Aquaponic systems incorporate different compartments operating under different technological conditions and, therefore, they develop different microbial communities worth studying. Understanding rhizosphere microorganism associations and MC activity can be an important step in optimizing processes within aquaponic systems.

Community-level physiological profiling (CLPP), assessed using BiologEcoPlates, is a technique detecting multiple microbial metabolic activitiesgiving valuable information about functional adaptations over space and time of these communities that can be compared and classified based on sole carbon source utilization patterns. The population of microorganisms gives a characteristic response pattern called a metabolic fingerprint (Gryta et al., 2014).

The present paper assessed the dynamics of heterotrophic microbial communities in different compartments of recirculating aquaponic systems. The main goal was to assess the metabolic activity and diversity of bacterial community attached to the root area of hydroponically cultivated plants.

## MATERIALS AND METHODS

### *Experimental system*

The experiment was carried out in an experimental aquaponic system (AS) with a total volume of 1.8 $m^3$. The aquaponic recirculating system incorporates four fish rearing tanks (268 l) connected to three hydroponic units, each of which consisted of three deep water culture basins. For total solids (TS) control, the recirculating system has been provided with a backwash sand filter where wastewater from the sump tank was continuously pumped with a constant flow of 48 l/min. Water, free of solids, was pumped to the top of the biofilter via a 'spray bar', then trickled across the biological filter medium (0.4 $m^3$, Bactoballs, 200 $m^2/m^3$). Biologically treated water was pumped to hydroponic modules that consist of three units (60x90x30 cm), with independent inlet provided with valves which permitted flow control and adjustments. The flow rate through hydroponic units was 8 l/m. Before returning to the fish tanks, the water was passed through a degassing column for $CO_2$ striping. The sterilization and disinfection process was realized with an UV filter. For the oxygen supply, the recirculation system was provided also with an oxygenation unit represented by one compressor Resun Air-Pump, Model: ACO-018 A with a flow of 260 l/min.

The experiment was designed to characterize microbial communities developed at different compartments of AS (biological filter, mechanical filter, basins, rhizosphere of two types of plants: *Lactuca sativa* and *Ocimum basilicum*). Before starting the experiment, biofilter was activated in order to develop a suitable population of nitrifying bacteria. After three weeks, the system was populated with carp, which was maintained in the system for 2 weeks, sufficient for nutrient accumulation to provide the nutritional needs of the plants used (*Lactuca sativa*, Clarion and Lollo Rosa varieties and *Ocimum basilicum*).

The experiment started by stocking 146 fish of approximately 96 g/ex in the four rearing units (Table 1). The fish were fed with commercial pellets of 41% protein content, in a ratio of 2% of body weight/day. The total amount of food calculated for one day was administered in three meals that were manually distributed over a period of 4-5 hours, starting at 9.00 in the morning.

Table 1. Biometry of carp yearlings used in the experiment

| Tank | Parameter | Med.±Stde. |
|---|---|---|
| **B1** | Weight (g/ex.) | 95.22±48.45 |
| | Lenght (cm) | 18.00±3.13 |
| **B2** | Weight (g/ex.) | 92.10±48.38 |
| | Lenght (cm) | 17.75±3.33 |
| **B3** | Weight (g/ex.) | 98.45±55.16 |
| | Lenght (cm) | 18.08±3.687 |
| **B4** | Weight (g/ex.) | 96.78±55.84 |
| | Lenght (cm) | 18.14±3.31 |

Salad and basil seedlings (4-6 leaves) were planted at a density of 44 plants/$m^2$ (24 plants per unit), each specie/variety being distributed in one of the three hydroponic units connected to the recirculating system. The lighting regime for plant growth was 10 h/day. The water level in each hydroponic unit was maintained at 15

cm. Before being introduced into the system, the plants were weighed and the roots were washed with an antibiotic solution. Dehydrated and sterilized coconut fiber was used as the physical support. All plants were then placed on the floating rafts, suspended at the surface of the water so as to allow 3-4 cm immersion of the container into solution.Nutrient supplements (iron DTPA, microelements) were added to meet the nutrient requierments for plants.

***Sampling***

Samples were taken every week during five weeks.The sample representing the biofilm in the biofilter (BACTO) was scraped off directly from the 10 bioballs (black polypropylene) with a pincer. The sample of plants rootswas assembled by cutting one root hair from 3 random plants in each of the three basins (SR- *Lactuca sativa* variety Lollo Rosa, SV- *Lactuca sativa* variety Clarion, B – *Ocimum basilicum*). All samples were placed immediately into a 50 ml Falcon tube and transferred to the laboratory. For the analysis of the microbial communities from the water, the samples were taken from the outlet of biological filter (FB), from the outlet of mechanical filter (FM) and directly from the fish basins (APA).

The dissolved oxygen (DO), conductivity and pH levels were determined daily with hand-held devices (WTW Oxi 315 i, Conductimeter WTW and WTW pH meter 340 respectively) Determination of $NH4^-$, $NO_2^-$, $NO_3^-$ and $PO_4^{3-}$ was carried out weekly using the spectrophotometric method (Specol UV-VIS). All water parameters were kept within optimal range for plants and carp rearing.

***Microbial community profiling***

To evaluate the metabolic profile and diversity of microbial communities (MC) in different compartments of the recirculating aquaponicsystem (including rhizosphere), Biologecoplates were used. These plates were designed for microbial communities and for microbial ecology studies. The Biolog plates consist of 96 wells, 31 carbon substrates and a three-loop control. The rate of utilization of the carbon sources was pointed by the reduction of tetrazolium violet redox dye, which changed from colorless to purple if added microorganisms utilize the substrate (Pohland and Owen, 2009).

To be able to capture environmental-induced variability 3 replicas/plate were inoculated with the same sample. Water samples were inoculated directly into the wells (100 µl). To assess the microbial communities developed at the rhizosphere, the samples (10 $cm^3$ of root) were transferred to 90 ml of sterilized distilled water enriched with 0.2% nutrient agar. Subsequently, they were shaken at 125 rpm for 15 minutes and held for one hour at room temperature prior to inoculation.The slurry was then poured into sterilize, and then passed through four layers of sterilized gauze and then inoculated into microplates. All instruments, equipment and glassware used were sterilized before use.A Tecan Sunrise reader was used to read the microplates at 24, 48 and 72 hours during incubation ($25^0$C) at an optical density (OD) of 590 nm.

Optical density values obtained at 48 h of incubation represented the optima range of optical density readings, so 48 h of incubation results was used for the assessment of microbial functional diversity and statistical analyses. In Biolog pates, substrates are subdivided into five group substrates represented by carbohydrates, carboxylic and ketonic acids, amines and amides, amino acids, and polymers, according to Weber and Legge (2009). Microbial activity in each microplate was expressed as average well color development (AWCD):

$$AWCDjt = \frac{1}{31}\sum_{i=1}^{31} ODijt$$

where: ODijt is optical density value from each i well and j replicate at time t corrected subtracting the blank well (inoculated, but without a carbon source) values from each plate well.

OD values were standardized according to Grove et al. (2004) with the following formula:

$$\overleftarrow{ODijt} = \frac{ODijt}{AWCDjt}$$

The final values used to indicate activity in each well were obtained after extracting the

OD value of the control.In order to quantify microbial biodiversity Shannon's diversity index (H) was calculated with formula H=−Σpi(lnpi), where pi = proportional color development of the well over total color development of all wells of a plate.

***Statistical analisys***

The statistical analysis was performed using the following programs: SPSS 15.0 for Windows, and Biodiversity Pro 2. The distribution normality was verified using the Kolmogorov-Smirnov Z test. The statistical differences between the variables were tested using ANOVA test (α=0.05). The homogeneity of the variance was tested using the Levene test. The physiological profile of microbial communities has been assessed through the analysis of principal components (PCAs) that allowed microbial samples to be compared based on differences in patterns of use of different carbon sources.

## RESULTS AND DISCUSSIONS

Biological treatment is one of the most important parts of wastewater treatment in a recirculanting aquaponic system. Therefore, knowing the activity of microorganisms developed in different compartiments of an AS may be important for the operational management and for keeping the balance of nutrients within the system. The Biolog plate was used for studying metabolicresponse of microbial communities from an experimental AS.

The analysis of the data recorded during different stages/weeks revealed that, in the case of the studied plants, the mean values of the optical densities differ significantly ($p<0.05$; Anova repeated measures) from one stage to the other, the icreasing rate of the bacterial density being also different among plant speciesor system compartiments (Figure 1). Unlike microbial community developed on plant roots, biofilm developed on the inert support media was characterized by a relatively constant bacterial density over the experimental stages, with mean AWCD values ranging from 1.03 to 1.07. In the last two experimental stages the biological community developed on the roots of the plants (SV and B) is characterized by a higher bacterial density compared to the biological film developed on bactoballs (inert support). The mean AWCD for the last experimental stagerecordedsignificant (post-hoc Anova, $p<0.05$) higher value for B (1.35 ± 0.23) comparing with SV (1.09 ± 0.34), BACTO (1.03 ± 0.37) and SR (0.93 ± 0.42).

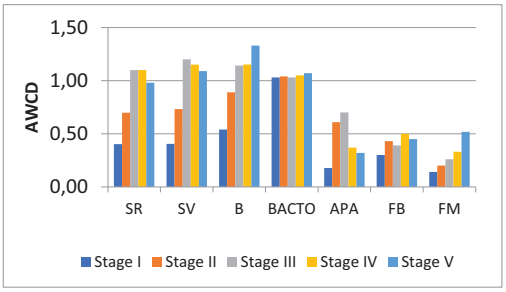


Figure 1. The dynamics of mean AWCD values for all sampling points and stages during experiment

Unlike the water from biological filter, where practically no significant variation in microbial density was registered, in the last week there was a tendency of microbial abundance in the mechanical filter most likely due to increase of nutritive substrate in this compartment.

Concerning the diversity of bacterial communities, this was analyzed using the Shannon (H) diversity index. As can be seen from Table 2, lowest H values were found for the samples taken from the mechanical filter (FM) in the first three experimental stages, for the water samples (APA) and, surprisingly, for the biological filter biofilm (BACTO) samples taken in the third stage.In the fourth stage at the level of the biological filter, both abundance and bacterial diversity are restored. A possible explanation for this phenomenon is that periodically, some of the bacteria attached to the biological film detach themselves and form the so-called planktonic bacterial communities (Michaud et al., 2006). This could be also the explanation for higher bacterial diversity from water samples from biological filter collected in the third stage of the experiment. The greatest microbial diversity is observed, regardless the stage, at the biofilm bound to the plantrizoshere. This implies that the microbial communities from the roots of plants had aheterogenous bacterial comunity.

Over all stages, analysis of the diversity of microbial communities using the Shannon Diversity Index highlights the fact that fixed

microbial community (roots and inert media) are significantly more diverse ($p<0.05$) than MC from water samples (Table 2).
Also, it can be noticed that, over time, microbial diversity is amplified, a sign that they are also composed of fast growing species and slow growing species.

The statistical comparison of the Shannon -H biodiversity index for all sampling points sugest significant differences ($p<0.05$) with BACTO, SV, SR and B framing in the same subset of values, while FM, APA and FB were classified into distinct subsets.

Table 2. Mean values of Shannon index (H) based on 48-h incubation (means±standard errors)

| Shannon H index | SR | SV | B | APA | FB | FM | BACTO |
|---|---|---|---|---|---|---|---|
| **Stage I** | 0.9±0.01 | 0.95±0.02 | 0.98±0.03 | 0.78±0.01 | 0.85±0.01 | 0.57±0.01 | 1.04±0.11 |
| **Stage II** | 1.13±0.04 | 1.31±0.01 | 1.24±0.06 | 0.98±0.04 | 0.96±0.06 | 0.58±0.03 | 1.32±0.08 |
| **Stage III** | 1.28±0.02 | 1.34±0.04 | 1.45±0.07 | 0.78±0.03 | 1.06±0.05 | 0.69±0.04 | 0.73±0.09 |
| **Stage IV** | 1.45±0.03 | 1.46±0.07 | 1.45±0.09 | 0.99±0.05 | 0.95±0.01 | 0.90±0.05 | 1.12±0.10 |
| **Stage V** | 1.43±0.02 | 1.47±0.02 | 1.47±0.02 | 1.09±0.04 | 1.15±0.03 | 1.24±0.01 | 1.15±0.12 |

Regarding the metabolic profile of the microbial communities in the recirculating system, in general, and from the biological film, in particular, it was characterized after the heterotrophic bacteria preference analysis for a particular carbon compound or for one or more groups of compounds, which we will further define as chemical guilds.At the biolog plate, the 31 carbon sources can be grouped into six categories: amines, amino acids, carboxylic acids, carbohydrates, phenols, and polymers.
The patterns of using different carbon sources by the bacterial communities developed at the different compartments of the AS and on the rhizosphere of horticultural plants, cultivated in the hydroponic modules having as nutritive support the waste water from the intensive growth of *Cyprinus carpio*, was analyzed by PCA. This method allows highlighting the pattern of associations (correlations) between variables, but also to determine possible latent variables that would explain a part of the measured variance.
PCA analysis for the entire experimental period shows a clear separation of the seven sampling points along the first two components. In this case, PC1 explains 50.09% of the variance, while PC2 explains 22.74%.
Figure 2 shows that the metabolic profile of bacterial communities in recirculated water (APA), biological filter (FB) and bactoballs (BACTO) is relativelly similar, differing from the metabolic profile of the plant rhizosphere. However, if we consider also nitrifying bacteria, the composition of the microbial community attached to the biofilter differed from the composition of the suspended bacteria (Blancheton et al., 2013).
In the case of a complete analysis, which also takes into account the average AWCD values for different chemical guilds, for each experimental stage, it was observed that there is a similarity of the metabolic profile of all components at the beginning of the experiment, the diversity increasing with the maturation of the biological films.

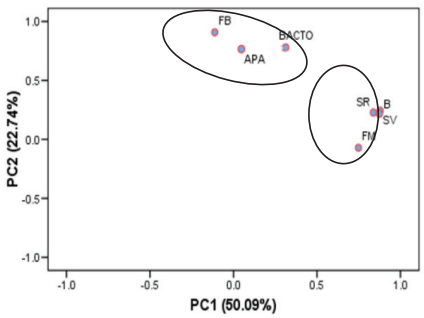


Figure 2. Principal component analysis for AWCD for all samples during experiment

Analysis of AWCD mean values reveals significant differences (Anova, $p<0.05$) when comparing different trophic types preferred by microbial communities in different RAS compartments.
It is also noted that carbohydrates represent, with the exception of FM, the preferred carbon source of MCfromwater or attached as biofilms in the aquaponic recirculating system.

The statistical analysis revealed insignificant (t test, $p>0.05$) differences between the chemical guilds at the root level of the salad, both varieties having relatively the same organizational structure regarding the metabolic profile of the present microorganisms.

Unlike salad, in the rhizosphere of *Ocimum basilicum* species, AWCD average values for phenols and polymers were significantly lower than those for the rest of the substrates. At the level of the biological filter, communities developed as biofilm preferred as nutritive substrat polymers and carbohydrates, followed by amino acids and amines. Also, it was observed that MCs having the preferred substrate phenols have diminished in the last two experimental stages.

The same tendency of decreasingMC that prefers the substrate of phenols is also observed in red salad plants. During experiment, at the level of the rhizosphere, there was a decrease in the response of the wells with source of carbon polymers.

Table 3. Pattern of substrate utilization for anlysed samples

| *Chemical guilds* | **Carbon Sources** | | | | *SR* | *SV* | *B* | *APA* | *FB* | *FM* | *BACTO* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Amines (A)*** | Putrescine | | | | | | | | | | |
| | Phenylethylamine | | | | | | | | | | |
| ***Aminoacids (AA)*** | L-Arginine | | | | | | | | | | |
| | L-Asparagine | | | | | | | | | | |
| | L-Phenylalanine | | | | | | | | | | |
| | L-Serine | | | | | | | | | | |
| | Glycyl-L-glutamic acid | | | | | | | | | | |
| | L-Threonine | | | | | | | | | | |
| ***Carbohydrates (CH)*** | α-D-Lactose | | | | | | | | | | |
| | β-Metil-D-Glicoside | | | | | | | | | | |
| | D- Cellobiose | | | | | | | | | | |
| | D-Mannitol | | | | | | | | | | |
| | i- Erythritol | | | | | | | | | | |
| | Glucose-1-phosphate | | | | | | | | | | |
| | D- Galactonic Acid γ-Lactone | | | | | | | | | | |
| | N-Acetyl-D-Glucosamine | | | | | | | | | | |
| | D,L-α-Glycerol phosphate | | | | | | | | | | |
| | D-Xylose | | | | | | | | | | |
| ***Carboxylic acids (CA)*** | Acid α-Ketobutiric | | | | | | | | | | |
| | D-Glucosaminic acid | | | | | | | | | | |
| | D-Malic acid | | | | | | | | | | |
| | γ-Hydroxybutyric acid | | | | | | | | | | |
| | Pyruvic acid methyl ester | | | | | | | | | | |
| | D-Galacturonic acid | | | | | | | | | | |
| | Itaconic acid | | | | | | | | | | |
| ***Polymers (P)*** | α-Ciclodextrin | | | | | | | | | | |
| | Tween 40 | | | | | | | | | | |
| | Tween 80 | | | | | | | | | | |
| | Glycogen | | | | | | | | | | |
| ***Fenols (F)*** | 2- Hydroxybenzoic acid | | | | | | | | | | |
| | 4- Hydroxybenzoic acid | | | | | | | | | | |

Legend


In this paper, both the structure and the dynamics of the various bacterial communities, both attached and suspended, developed at the different compartments of an aquaponic recirculating system were assesed. The variation in the pattern of metabolic response,

indicated by CLPP, suggested a complex interaction between bacterial communities and the type of support medium, treatment process, biofilm age, and perhaps other environmental variables. Metabolic imprinting using redox technology embedded in Biolog plates has already been demonstrated as an effective method in differentiating heterotrophic microbial communities from different wastewater treatment systems (Hench et al., 2004).

Five weeks after the start of the experiment, significant differences ($p<0.05$) among metabolic profiles of biofilms formed at different compartments of the aquaponic recirculating system were detected. The highest intensity response came from the salad and basil rizosfere microbial community. Analysis of the different ages of biofilms reveals pronounced differences in both, the metabolic profile and the diversity of microbial communities.

These results suggest that the support medium plays an important role in determining the structure of the microbial community in aquaponic systems, and that the incorporation of plants (roots) in the recirculation system configuration could provide unique connectivity sites for certain microbial populations.

The fact that the structure of the bacterial communities differs depending on the media environment, the specific conditions and the operating period of a reactor has already been demonstrated. Also, some studies conducted with the purpose to include different plant species in wetlands and assess their waste water treatment potential have highlighted the fact that macrophytes can stimulate the development of specific microbial communities (Vacca et al., 2005). Vymazal (2010) also showed differences in microbial activity at the root of the different plant species within the same wetlands.

In this study, differences in the metabolic profile of biofilms extracted from different support media were detected although there were no significant differences regarding water parameters from different compartiments. Thus, the main carbon sources preferred by the bacterial communities at the level of the rhizosphere were:

- SR: i-Eritriol (CH), Piruvic Acid Methyl Ester (CA), Tween 40 (P).
- SV: L-Argininеand L-Asparagine (AA), Glucose-1-Phosphate (CH).
- B: L-Asparagine and L-Phenylalanine (AA), D- Cellobiose and Glucose-1-Phosphate (CH).

The metabolic profile of communities in the inert environment is suggested by the following substrates: L-Phenylalanine (AA), Glucose-1-Phosphate (CH), Phenylethylamine (A), α-Cyclodextrine(P) and i-Eritritol (CH).

As for microbial diversity, there were statistical differences ($p<0.05$) between the bacterial films attached to the inert environment comparing with those from the plant rhizosphere. However, among plant species/varieties there were no significant differences ($p>0.05$) regarding diversity of microbial community prolipherating in the rizosfere although the metabolic profile was sligly diferent.

## CONCLUSIONS

The results show the importance of the substratetype in determining the structure of heterotrophic microbial communities in recirculating aquaponic systems. The plant roots (as a support medium) contribute to the microbial community development and thus, indirectly, influences waste water treatment processesin aquaponic systems since microbioms are governing nutrient cycling. The present study suggests microbial niche differentiation within the aquaponics components.

In conclusion, the obtained results highlighted the importance of understanding the metabolic pathways and structure of microbial communities within aquaponic systems. At rizosphere microbiom level may also proliferate plant growth promoting microorganisms which, especially in aquaponic systems, may reduce nutrient supplementation if those communities could be properly manipulated and integrated as processes into AS design. However, the contribution of plant roots (as a support medium) to the efficiency of the process of treatment of aquaculture effluent remains to be elucidated. The way in which heterotrophic bacteria interfere in improving growth conditions is unclear and requires further investigation. Applying modern methods of study to microbial communities will make possible the correlation of microbial systema-

tics and dynamics with management of aquaculture activities.

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