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IMPLICATION OF DIETARY PHYTOGENIC MIXTURE IN MODULATING THE INTESTINAL MICROFLORA OF BROILERS RAISED IN THERMONEUTRAL AND HEAT STRESS CONDITIONS

Mihaela SARACILA[1, 2], Tatiana Dumitra PANAITE[1], Dumitru DRAGOTOIU[2]

[1]National Research-Development Institute for Animal Biology and Nutrition (IBNA),
1 Calea Bucharest, Balotesti, 077015, Ilfov, Romania
[2]University of Agronomic Sciences and Veterinary Medicine of Bucharest,
59 Marasti Blvd, District 1, Bucharest, Romania

Corresponding author email: mihaela.saracila@yahoo.com

Abstract

The paper presents the effect of a dietary phytogenic mixture on intestinal microflora of broilers raised under thermoneutral conditions (TN) and heat stress (HS). The feeding trials were conducted on 120 Cobb 500 broilers (60 chicks/trial) raised in environmentally- controlled digestibility cages. Up to 14 days the chicks were fed a conventional diet. On the 14th day, they were divided in four homogeneous groups (2 groups/ trial, 30 chicks/ group). In the first trial, two groups (C-TN and PM-TN) were kept in TN. In the second trial, two other groups (C-HS and PM-HS) were kept in HS (32°C). Both trials used the same structure of diets. Compared with the control diet (C), the experimental diet (PM) included the addition of 1% phytogenic mixture (bilberry leaves, peppermint leaves, fennel leaves and sea buckthorn meal). Both in TN and HS, dietary PM lowered the number of staphylococci and increased the lactobacilli populations in the intestinal and caecal content. E. coli populations have decreased only in the intestinal content of broilers fed PM diet. In conclusion, dietary PM could be an efficient alternative to modulate the intestinal microflora of broilers even in heat stress conditions.

Key words*: broilers, heat stress, microflora, mixture, phytogenic.*

INTRODUCTION

The balance of the intestinal microflora of broilers is crucially affected by exposure to heat stress. This consequence is important because the intestine health is related to the general health of the broiler. In the recent years, many efforts have been redirected for the improvement of intestine microflora. A common strategy for ensuring gut health has been nutritional manipulation. In this regard, many researchers have focused on the use of several dietary supplements, including phytobiotics considering effective stress-alleviating agents (AL-Sagan et al., 2020; Gheorghe et al., 2019; Saracila et al., 2020). They can be included in the diet for broilers as essential oils, powder from different parts of the plant or a combination of plants or oils. In the poultry industry, herbs and herbal products are used to substitute synthetic products to stimulate or promote the development of the chicken gut, ensuring a proper balance between bacterial communities that colonize the intestine, etc.

Bilberry leaves have attracted attention for inclusion in the broiler diet due to the important content of polyphenols, Zn, vitamin E, lutein and zeaxanthin and important antioxidant capacity (Mäkinen et al., 2020; Untea et al., 2020; Varzaru et al., 2020). Popescu et al. (2020) reported that the dietary bilberry leaves positively influence the microbiota of laying hens. Peppermint is a plant with a long history, with strong antibacterial and antioxidant effects in poultry (Abdel-Wareth et al., 2019). The beneficial effects of using peppermint in the broiler diet have included growth promoting efficacy (Toghyani et al., 2010), reduced abdominal fat deposition, and improved antioxidant status (Khempaka et al., 2013). Fennel contains health-promoting volatile essential oil compounds (e.g., fenchone, anethole, myrcene, etc.), amino acids, phenolic compounds, and flavonoids and have been reported to decrease intestinal *E. coli* populations (Ghiasvand et al., 2021), and have had a beneficial impact on growth performance and carcass quality of broilers under heat stress conditions (AL-Sagan

et al., 2020). Sea buckthorn has been studied extensively due to its antioxidant, anti-inflammatory and immunostimulatory properties. Saracila et al. (2020) did not report effects on the coefficients of absorption and performance of broilers fed diet supplemented with sea buckthorn meal and raised under HS.

The aim of this study was to investigate the efficacy of a dietary phytogenic mixture (PM) on the intestinal microflora of broilers raised under thermoneutral conditions (TN) and heat stress (HS).

MATERIALS AND METHODS

The experimental trials were conducted in agreement with the guidelines established by the Ethics Commission of the National Research Development Institute for Biology and Animal Nutrition (IBNA-Balotesti, Romania). Briefly, a total of 120 Cobb 500 broilers were assigned to two feeding trials (for 28 days) with 60 broilers/trial and housed in environmentally- controlled digestibility cages. Until the age of 14 days, a commercial diet (based on corn, gluten and soybean meal) with 22% CP and 3102 kcal/kg ME was administered to broilers. At the age of 14 days, the chicks were weighted and on this criterion were assigned to four homogeneous groups (2 groups/ trial with 30 chicks/group). In the first trial, two groups (C-TN and PM-TN) were raised in thermoneutral (TN) conditions. In the second trial, two other groups (C-HS and PM-HS) were raised in heat stress (HS) conditions (32 ± 1°C). During the experimental period, the light regimen was set to 23h light/ 1h darkness. Feed (mash form) and water were administered ad libitum. Compared to the control diets (C-TN; C-HS) that contained a commercial diet, the experimental diets (PM- TN; PM-HS) included the addition of 1% phytogenic mixture of 40% bilberry leaves, 20% peppermint leaves, 20% fennel leaves and 20% sea buckthorn meal) (Table 1). The mixture is characterized by a high content of polyphenols and a superior antioxidant capacity, as reported by Saracila et al. (2020). The dried and grounded plants used for the mixture were obtained from pharmacies, while sea buckthorn meal was bought from E-Prod SRL, Teleorman, Romania.

Table 1. Diet composition*

Ingredient	Grower stage (14-35 days)		Finisher stage (35-42 days)	
	C	PM	C	PM
Corn	62.00	61.00	60.50	60.00
Soybean meal	26.58	26.58	25.46	25.00
Gluten	4.00	4..00	6.00	6.00
Oil	2.50	2.50	3.75	3.71
Phytogenic mixture (PM)	0	1.00	0	1.00
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	0.20	0.20
Choline	0.05	0.05	0.05	0.05
Vitamin-mineral premix*	1.00	1.00	1.00	1.00
Total	100	100	100	100

*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/kg manganese; 8000 mg/kg iron; 500 mg/kg copper; 6000 mg/kg zinc; 37 mg/kg cobalt; 152 mg/kg iodine; 18 mg/kg selenium.

*Diet structure published previously by Saracila et al. (2020)

At 42 days of age, 6 birds per treatment were slaughtered by cervical dislocation. Consequently, the entire intestine was removed from the oesophagus to the cloaca. Samples of intestinal and caecal contents were collected in aseptic medium, in sterilized plastic tubes and stored at -20ºC until bacteriological tests (*Escherichia coli,* staphylococci, lactobacilli, *Salmonella* spp.).

Bacteriological analyses were performed according to the test described by Saracila et al. (2020). Briefly, for the *E. coli* assay, the decimal dilutions (up to 10^{-5}) in lauryl-sulphate medium, were inoculated in 2 Petri dishes on

Levine medium and incubated, and after this the colonies were counted.

The staphylococci assay was performed by immersing the sample in hyper-chlorinated liquid medium, then inoculating in successive dilutions on hyper-chlorinated solid medium and incubated and finally counted. For the lactobacilli method, MRS broth and MRS agar were used as the selective medium and then the forming colonies were counted. *Salmonella* spp. was determined according to SR EN ISO 6579/2003/A1:2007. Scan 300, Interscience (France) was used to count bacterial colonies. The results were reported as a log base 10 colony-forming units (CFU) per gram of intestinal and caecal contents.

Statistical analysis

Data were analysed by 2-way ANOVA using Graph-Pad Prism v. 9.02 (San Diego, CA), with diet (C, E) and temperature (TN, HS) as factors using the following model:

$$Yij = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon ijk$$

Where: y = the dependent variables; μ = the general mean; α_i and β_j = diet and temperature effects; $(\alpha\beta)_{ij}$ = the interaction between diet and temperature; ε_{ij} = the random error. When significant main effects were detected, means were compared using Tukey's multiple range test. Significance was set at a $p<0.05$.

RESULTS AND DISCUSSIONS

Table 2 shows that in HS conditions, dietary PM significantly reduced the number of *E. coli* and staphylococci in the intestinal content. In particular, while staphylococci were significantly lower in the PM-supplemented group than in group C, in TN, *E. coli* populations did not differ between the two groups.

Both diet and temperature significantly influenced the number of *E. coli* and staphylococci populations.

Table 2. Effect of diet supplementation and temperature on intestinal bacterial populations (log10 CFU*/g wet intestinal digesta)

Variable	TN		HS		p-values summary		
	C-TN	PM-TN	C-HS	PM-HS	Diet	Temp.	Diet x temp.
E. coli	6.36[a]	6.35[a]	5.31[b]	5.24[c]	*	***	ns
Staphylococci	6.16[a]	6.15[b]	5.94[c]	5.83[d]	****	****	****
Lactobacilli	7.42[a]	7.43[a]	6.36[b]	6.89[c]	****	****	****
Salmonella spp.	absent	absent	absent	absent	NA	NA	NA

[a, b, c, d]Means in the same column with different superscripts differ significantly ($p<0.05$). Data are presented as mean SEM (n = 6 broilers/group). Asterisks denote statistical significance ($p > 0.1234$ ns, *$p \leq 0.0332$, **$p \leq 0.0021$, ***$p \leq 0.0002$, ****$p<0.0001$).

Interestingly, the lowest populations of *E. coli* and staphylococci were recorded in the intestinal contents of broilers raised under HS. Similarly, in HS the number of lactobacilli was significantly higher in the intestinal content of broilers fed PM diet than in those fed the C diet. If we compare the stress conditions with the normal ones, we see that the lactobacilli have been affected by the applied heat stress, their populations number being smaller.

Ragab et al. (2013) reported a decrease in intestinal pH and the total microflora count of broilers fed a diet supplemented with fennel seeds. The authors explained that these results could be related to the antimicrobial effect of feed seeds. Safaei-Cherehh et al. (2020) showed that the inclusion of 200 ppm fennel extract in broiler (42 days) diet decreased the ileal *E. coli* count due to the antibacterial activity of fennel extract. In a study conducted on Ross broiler chicks, Vase-Khavari et al. (2019) showed that the dietary addition of 0.5% peppermint reduced the total number of *E. coli* and enhanced the caecal populations of lactobacilli.

Vlaicu et al. (2019) reported that a blend of commercial oils (20% rosehip oil, 20% sesame oil, 20% buckthorns oil, 20% nut oil and 20% grapeseed oil) included in the broiler diet (14-42 days) reduced the proliferation of pathogenic bacteria and increased the lactobacilli in the intestine and cecum.

Popescu et al. (2020) explain that dietary bilberry powder influences positively the microbiota by modulating several digestive enzymes that promote the development of lactobacilli and decrease pathogenic bacteria such as *Enterobacteriaceae*.

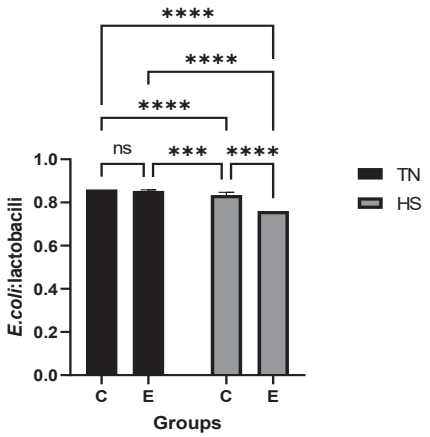

Figure 1. Effect of diet and temperature on *E. coli*: lactobacilli ratio in the intestinal content of broilers

Main effects of diet, temperature, and the interaction are presented in each graph (Prism Graph 9.02). Data are presented as mean SEM (n = 6 broilers/group). Asterisks denote statistical significance (p > 0.1234 ns, *p ≤ 0.0332, **p ≤ 0.0021, ***p≤ 0.0002, ****p<0.0001)

The Figure 1 depicted the influence of dietary PM supplementation and temperature on *E. coli*: lactobacilli ratio. Both temperature and dietary PM supplementation exerted a significant influence on *E. coli*: lactobacilli ratio in the intestinal content.

Under thermoneutral conditions, the ratio was not significant between two groups, but in HS, PM supplementation decreased the ratio. As expected, under HS, the *E. coli*: lactobacilli ratio was lower than that recorded under TN.

The dietary PM supplementation significantly reduced staphylococci populations in caecal content of broilers raised in either TN or HS conditions (Table 3). But dietary PM supplementation had no effect on the intestinal and caecal number of *E. coli*. Regardless of temperature conditions, the number of lactobacilli was significantly higher in groups fed a diet supplemented with PM (PM-TN; PM-HS) than in those fed a diet C (C-TN; C-HS).

Table 3. Effect of diet supplementation and temperature on caecal bacterial populations (log10 CFU*/g wet caecal digesta)

Variable	TN		HS		p-values summary		
	C-TN	PM-TN	C-HS	PM-HS	Diet	Temp.	Diet x temp.
E. coli	10.30	10.16	10.37	10.33	ns	ns	ns
Staphylococci	8.83[a]	8.75[b]	8.65[c]	8.34[d]	****	****	****
Lactobacilli	11.56[a]	11.79[b]	10.66[c]	10.79[d]	****	****	****
Salmonella spp.	absent	absent	absent	absent	NA	NA	NA

[a, b, c, d]Means in the same column with different superscripts differ significantly (p<0.05). Data are presented as mean SEM (n=6 broilers/group). Asterisks denote statistical significance (p > 0.1234 ns, *p ≤ 0.0332, **p ≤ 0.0021, ***p≤ 0.0002, ****p<0.0001).

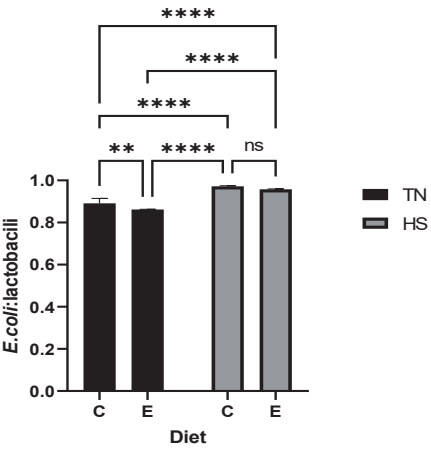

Figure 2. Effect of diet and temperature on *E. coli*: lactobacilli ratio in the caecal content of broilers

Main effects of diet, temperature, and the interaction are presented in each graph (Prism Graph 9.02). Data are presented as mean SEM (n = 6 broilers/group). Asterisks denote statistical significance (p > 0.1234 ns, *p ≤ 0.0332, **p ≤ 0.0021, ***p≤ 0.0002, ****p<0.0001)

The Figure 2 shows that both diet and temperature had a significant influence on the *E. coli:* lactobacilli ratio in the caecal content of broilers. In HS, dietary PM supplementation had no effect on *E. coli:* lactobacilli ratio, while in TN, it showed a significantly lower value. Compared to TN, the values of the *E. coli*: lactobacilli ratio were lower than in HS. This observation confirms the negative effect of HS on the balance of the broiler microflora.

CONCLUSIONS

Dietary PM had a positive effect in reducing *E. coli* populations only in the intestinal contents of broilers subjected to TN and HS. In both HS and TN, the PM diet decreased staphylococci populations and increased lactobacilli in the caecal and intestinal contents of broilers.

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REFERENCES

Abdel-Wareth, A.A., Kehraus, S., & Südekum, K.H. (2019). Peppermint and its respective active component in diets of broiler chickens: growth performance, viability, economics, meat physicochemical properties, and carcass characteristics. *Poultry science*, *98*(9), 3850-3859.

AL-Sagan, A.A., Khalil, S., Hussein, E.O.S., & Attia, Y.A. (2020). Effects of fennel seed powder supplementation on growth performance, carcass characteristics, meat quality, and economic efficiency of broilers under thermoneutral and chronic heat stress conditions. *Animals*, *10*, 206.

Attia, G., El-Eraky, W., Hassanein, E., El-Gamal, M., Farahat, M., & Hernandez-Santan, A. (2017). Effect of dietary inclusion of a plant extract blend on broiler growth performance, nutrient digestibility, caecal microflora and intestinal histomorphology. *International Journal of Poultry Science*, *16* (9), 344-353.

Gheorghe, A., Habeanu, M., Tabuc, C., & Marin, M. (2019). Effects of dietary pea seeds (*Pisum sativum* L. cv. Tudor) on performance, carcass traits, plasma biochemistry and intestinal microflora in broiler chicks. *AgroLife Scientific Journal*, 8(1), 99-106.

Ghiasvand, A.R., Khatibjoo, A., Mohammadi, Y., Akbari-Gharaei, M., & Shirzadi, H. (2021). Effect of fennel essential oil on performance, serum biochemistry, immunity, ileum morphology and microbial population, and meat quality of broiler chickens fed corn or wheat-based diet. *British Poultry Science*.

Khempaka, S., Pudpila, U., & Molee W. (2013). Effect of dried peppermint (Mentha cordifolia) on growth performance, nutrient digestibility, carcass traits, antioxidant properties, and ammonia production in broilers. *J. Appl. Poult. Res.*, *22*, 904-912.

Mäkinen, S., Hellström, J., Mäki, M., Korpinen, R., & Mattila, P.H. (2020). Bilberry and sea buckthorn leaves and their subcritical water extracts prevent lipid oxidation in meat products. *Foods* (Basel, Switzerland), *9*(3), 265.

Popescu, R.G., Voicu, S.N., Gradisteanu Pircalabioru, G., Ciceu, A., Gharbia, S., Hermenean, A., Georgescu, S.E., Panaite, T.D., & Dinischiotu, A. (2020). Effects of dietary inclusion of bilberry and walnut leaves powder on the digestive performances and health of Tetra SL laying hens. *Animals*, *10*, 823.

Ragab, M.S., Namra, M.M.M., Aly, M.M.M., & Fathi, M.A. (2013). Impact of inclusion fennel seeds and thyme dried leaves in broiler diets on some productive and physiological performance during summer season. *Egyptian Poultry Science Journal*, *33*(1), 197-219.

Safaei-Cherehh, A., Rasouli, B., & Alaba, P.A. (2020). Effect of dietary Foeniculum vulgare Mill. extract on growth performance, blood metabolites, immunity and ileal microflora in male broilers. *Agroforest Syst*, 94, 1269-1278.

Saracila, M., Olteanu, M., & Panaite, T.D. (2020). Implications of using some phytoadditives in broiler nutrition-a review. *Scientific Papers. Series D. Animal Science, LXIII*(2), 165-172.

Saracila, M., Panaite, T.D., Tabuc, C., Șoica, C., Untea, A., Varzaru, I., Wojdyło, A., & Criste, R. (2020). Maintaining intestinal microflora balance in heat-stressed broilers using dietary creeping wood sorrel (*Oxalis corniculata*) powder and chromium (chromium picolinate). *Spanish Journal of Agricultural Research*, 18.

Saracila, M., Panaite, T.D., Papuc, CP, Predescu, C.N., & Untea, A. (2020). Dietary phytogenic mixture for broilers reared under thermoneutral and heat stress conditions. *Archiva Zootechnica, 23*(2), 101-116.

Toghyani, M., Toghyani, M., Gheisari, B., Ghalamkari, G., & Mohammadrezaei, M. (2010). Growth performance, serum biochemistry and blood hematology of broiler chicks fed different levels of black seed (*Nigella sativa*) and peppermint (*Mentha piperita*). *Livest. Sci., 129*, 173-178.

Untea, A.E, Varzaru, I., Panaite, T.D., Gavris, T., Lupu, A., & Ropota, M. (2020). The effects of dietary inclusion of bilberry and walnut leaves in laying hens' diets on the antioxidant properties of eggs. *Animals*, *10*(2), 191.

Vase-Khavari, K., Mortezavi, S.H., Rasouli, B., Khusro, A., Salem, A.Z., & Seidavi, A. (2019). The effect of three tropical medicinal plants and superzist probiotic on growth performance, carcass characteristics, blood constitutes, immune response, and gut microflora of broiler. *Tropical animal health and production*, *51*(1), 33-42.

Vlaicu, P.A., Panaite, T.D., Tabuc, C., Soica, C., & Stanel, I. (2019). Effect of a blend of commercial oils on growth performance and intestinal microflora population in broiler chickens. *Scientific Papers. Series D. Animal Science*, *LXII*(1), 130-137.