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Dietary phytogenic mixture for broilers reared under thermoneutral and heat stress conditions

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ABSTRACT

During two feeding trials, the effect of dietary phytogenic mixture on the performance and oxidative stress biomarkers in the liver of broilers reared under thermoneutral conditions (TN) and heat stress (HS) was studied. A number of 60 Cobb 500 chicks/ trial were sheltered in environmentally-controlled digestibility cages. On the 14 days of age, the chicks were weighted and assigned to four groups (2 groups/ trial with 30 chicks/ group). In the first trial, two groups (C-TN and PM-TN) were kept in thermoneutral conditions. In the second trial, other two groups were kept (C-HS and PM-HS) in heat stress (32 ±1 °C). The structure of diets was the same in both experiments. Compared with the control diet (C), the experimental diet (PM) contained the addition of 1% phytogenic mixture (bilberry leaves, peppermint leaves, fennel leaves and sea buckthorn meal). Irrespective of temperature conditions, dietary PM did not affect broiler's performance. The dietary supplementation of PM delayed protein and lipid oxidation in the liver tissue of broilers in both trials by increasing the hepatic catalase, glutathione and superoxide dismutase activity.

Keywords: antioxidant, broiler, phytogenic mixture, heat stress, oxidative stress.

INTRODUCTION

Heat stress (HS) represents an acute threat to homeostasis and has negative effects on overall health of poultry (Attia et al., 2017a; Attia et al., 2018a; Attia and Hassan, 2017; Shi et al., 2019; Al-Sagan et al., 2020). In addition, HS is the main environmental cause of oxidative stress (Lin et al., 2006). Oxidative stress implies a disproportion between reactive oxygen species (ROS) and antioxidant defense system that lead to disorder of the structure of proteins, lipids and cell membranes (Tan et al. 2010, Kumbhar et al. 2018). Among other functions, the liver has an important role in keeping body homeostasis (Jastrebski et al., 2017), so it is one of the organs most affected by the action of HS. Under normal temperature conditions, ROS are eliminated by the body's enzyme antioxidant systems such as catalase (CAT), superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GPx) (Zhao and Shen, 2005; Habashy, et al., 2019). They act as ROS scavengers and convert them to less reactive species. The HS conditions increase the cellular ROS level of broilers and antioxidant enzyme systems become inefficient, as a result, the enzymatic antioxidant activities decrease (Altan et al., 2003; Habashy et al., 2019). Thus, there is a crucial need for exogenous supplementation of antioxidants such as phytochemicals, vitamins, etc, to counteract the excessive ROS production (He et al., 2019).

Several studies have reported that dietary supplementation with phytochemicals improved performance (Attia et al., 2017b; 2018b, Attia et al., 2019; Al-Sagan et al., 2020), antioxidant status (Arain et al., 2018), immunity and endocrine function (Mirzaie et al., 2018), gut health (Criste et al., 2017) of broilers reared under HS.

Bilberry (*Vaccinium myrtillus L.*), also known as European blueberry, is a shrub containing high quantities of phenolics such as hydroxycinnamic acids, flavonols, anthocyanins, procyanidins and chlorogenic acid (Martz et al., 2010; Ferlemi and Lamari, 2016). Studies were reported that bilberry leaves possess antioxidant, anti-inflammatory (Ferlemi and Lamari, 2016), antidiabetic properties (Bljajić et al., 2017), antistaphylococcal activity (Sadowska et al., 2014).

Peppermint (*Mentha piperita*) is a medicinal plant rich in essential oils such as menthol, menthone, 1,8-cineole (Schmidt et al., 2009) with undeniable strong antioxidant and antibacterial properties (Singh et al., 2015).

Fennel (*Foeniculum vulgare*) is an aromatic and widely cultivated herb. Their leaves contained high levels of ω-3 fatty acids (Barros et al., 2010) and volatile compounds such as trans-anethole (59.8–90.4%), limonene (0.1–21.5%), neophytadiene (0–10.6%), responsive to their antioxidant and antimicrobial activities, mainly on Gram-positive bacteria (Senatore et al., 2013).

Sea buckthorn (*Hippophae rhamnoides*) is a shrub with a long tradition, but which has only recently been planted as a new berry crop to obtain bioactive compounds such as flavonoids, phenolic acids, carotenoids, etc (Wani et al., 2016). Many *in vitro* and *in vivo* studies (conducted on animals) have showed that these compounds contained by sea buckthorn berries and leaves exhibit antioxidant (Goran et al., 2008; Papuc et al., 2008; Criste et al., 2020), anti-inflammatory (Ganju et al., 2005), and hypocholesteromic effects (Tereshchuk et al., 2020). Some reports have shown that constituents such as phenolics, flavonoids, and carotenoids can act synergistically (Shi et al., 2004; Hajimehdipoor et al., 2014; Attia et al., 2017b, 2018b, 2019; Phan et al., 2018). Given the content of bioactive compounds and the antioxidant properties of plants, the addition of bilberry, peppermint, fennel leaves and sea buckthorn meal in the mixture was pursued, in the premise that together can potentiate the effects.

The aim of this study was to highlight the effects of dietary antioxidant plant mixture (bilberry, peppermint and fennel leaves and sea buckthorn meal) on performance and oxidative stress parameters in the liver of broilers reared under thermoneutral and heat stress conditions.

MATERIALS AND METHODS

Ethical approval

The experimental study was consistent with the Directive 2010/63/EU guidelines and the protocol was approved by the Ethics Commission of the National Research Development Institute for Biology and Animal Nutrition.

Birds and management

Two feeding trials lasted for 28-days were carried out on 60 Cobb 500 broilers/ trial, sheltered in environmentally- controlled digestibility cages. Until 14 days of age, the chickens were fed a commercial diet (based on corn, gluten and soybean meal) with 22% CP and 3102 kcal/kg ME. On the 14 days of age, the chicks were allotted to four homogeneous groups (2 groups/ trial with 30 chicks/ group). In the first trial, two groups (C-TN and PM-TN) were reared in thermoneutral (TN) conditions, according to the Management guide of the Cobb 500 hybrid. In the second trial, other two groups (C-HS and PM-HS) were subjected to heat stress (HS) conditions (32 ±1 °C). The light regimen was 23h light/ 1h darkness. Feed (mash form) and water were administered *ad libitum*. Compared with the control diets (C-TN; C-HS), the experimental diets (PM- TN; PM-HS) included the addition of 1% phytogenic mixture (40% bilberry leaves, 20% peppermint leaves, 20% fennel leaves and 20% sea buckthorn meal) (Table 2). The percent of inclusion was based on the antioxidant capacity of plants reported by literature: bilberry leaves (Panaite

et al., 2019; Popescu et al., 2020; Untea et al., 2020; Varzaru et al., 2020); fennel (Nagy et al., 2014); peppermint (Brown et al., 2019); sea buckthorn meal (Panaite et al., 2016). Also, from our analyses, bilberry leaves showed the highest antioxidant capacity and therefore we included in a higher rate in the phytogenic mixture.
The bilberry, peppermint leaves and fennel powder used for the study were purchased from local pharmacies, dried, grounded and packed. Sea buckthorn meal was obtained from a local producer (E-Prod SRL, Teleorman, Romania), dried, grounded and packed.

Table 1. Proportion of plant inclusion in the phytogenic mixture

Ingredient	Proportion of plant inclusion in the mixture (%)
Bilberry leaves*	40
Fennel powder	20
Peppermint leaves*	20
Sea buckthorn meal	20
Phytogenic mixture	**100**

Antioxidant activity of plant mixture

The methanolic extract of plants and phytogenic mixture (n=6) were used to analyse the total polyphenols (TP) and total antioxidant capacity, following the methods described by Untea et al., (2018). The antioxidant capacity was assessed by two different assays (ABTS and DPPH). Total polyphenols were estimated following the Folin-Ciocalteu's assay. The values were reported as mg/g gallic acid equivalents (GAE). The antioxidant activity of samples was estimated by plotting inhibition of ABTS and DPPH radical (%) against Trolox. The values were expressed as mmol Trolox equivalents (TE)/kg sample.

The effect of phytogenic mixture on performance was investigated recording the bodyweight (BW, g), average daily feed intake (ADFI, g feed/broiler/day) and calculating the average daily weight gain (ADWG, g/broiler/day), and feed conversion ratio (FCR, g feed/g gain).

At 42 days of age, 6 chicks/group were slaughtered by cervical dislocation. After bleeding, the internal organs and gut were excised. Liver samples were collected (n=6) and preserved in the freezer (−80 °C) until further analysis.

Liver oxidative stress evaluation

The liver homogenate (6 samples/ treatment; 3 samples each) was obtained as Erdogan et al., (2005) described. The supernatant obtained was use to analyse the lipid peroxidation (thiobarbituric acid reactive substances, TBARS), protein carbonyl (PC), glutathione (GSH), total antioxidant capacity (T-AOC) and superoxide dismutase (SOD).

Table 2. Diet formulation (%)

Ingredient	Grower stage (14-35 days)		Finisher stage (36-42 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
Plant 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

Note: *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.
C- control diet; PM- control diet+ 1% pytogenic mixture

Lipid peroxidation assay was performed according to Papuc et al., (2018). The absorbance of samples was read at 532 nm wavelength. The results were reported as nmol malondialdehyde (MDA)/g tissue.

Protein carbonyl (PC) was determined as described by Patsoukis et al., (2004), using a colorimetric method based on the derivatization of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH) and subsequently the production of a stable dinitrophenyl (DNP) hydrazone product. The values were reported as nmol/g tissue.

Glutathione (GSH) was analysed following the protocol detailed by Papuc et al., (2012). The values were reported as µmol/g tissue.

The total antioxidant capacity (T-AOC) of liver extract was evaluated following the colorimetric method detailed by Predescu et al., (2012). The T-AOC of liver extract was expressed as % DPPH scavenging capacity. The MDA/T-AOC ratio was used as antioxidant balance indicator and was calculated as Attia et al., (2020) described.

Superoxide dismutase activity (SOD) was determined using a colorimetric superoxide dismutase assay kit purchased from Fluka. The SOD activity (U/g tissue) was calculated according to the manual provided with the assay kit.

Statistical analysis

The experimental design was 2 × 2 factorial including two environmental temperatures (TN vs. HS), and two experimental diets (C, PM). The effect of PM on performance and oxidative stress parameters of broiler liver was analysed by performing the analysis of variance (two-way ANOVA) using XLSTAT software 2020 version (Addinsoft, Paris, France). The Tuckey test was used to predict differences among the criteria; the effects were considered significant if $p < 0.05$.

RESULTS AND DISCUSSION

The results depicted in Table 3 demonstrate that the bilberry leaves possess the highest antioxidant activity in terms of ABTS and DPPH estimation. The antioxidant capacity of plants expressed by ABTS method was as follows: bilberry leaves> peppermint leaves> sea buckthorn meal> fennel powder. Fennel powder expressed the lowest antioxidant capacity resulted from both methods used.

Table 3. Antioxidant capacity of plants and phytogenic mixture

Variable	**Antioxidant capacity**		
	ABTS mmol TE/kg	**DPPH mmol TE/kg**	**TP mg/g GAE**
Bilberry leaves*	321	256	13.3
Fennel powder	39.5	228	6.42
Peppermint leaves*	286	228	71.9
Sea buckthorn meal	143	168	14.5
Phytogenic mixture	341	247	38.3

* data published by Untea et al., (2018)

ABTS- {2,2'- azinobis- (3-ethyl-benzothiazoline-6-sulphonic acid)}; DPPH- 2,2-Diphenyl-1-Picrylhydrazyl; TP- Total Polyphenols; TE- Trolox Equivalents; GAE- Gallic Acid Equivalents.

Although the highest antioxidant capacity was recorded by bilberry leaves, the highest TP content was obtained for peppermint leaves. Also, the sea buckthorn meal contained higher amounts of TP than bilberry leaves. The explanation might be that bilberry leaves contain bioactive compounds, others than polyphenols, that contribute to their valuable antioxidant capacity. Panaite et al., (2019) showed bilberry leaves and sea buckthorn meal represent a rich source of polyphenols (52.82 mg GAE/g for bilberry leaves and 31.9 mg GAE/g for sea buckthorn meal). All in all, we can conclude that the bilberry leaves had a greater concern to the higher antioxidant capacity of the plant mixture, while the peppermint leaves had to the high level of TP.

Performance

The effects of temperature and diet on broiler performance are shown in Table 4. Irrespective of the temperature conditions, the final BW, ADFI, ADWG and FCR were not influenced ($p>0.05$) by PM supplementation. However, the temperature was exerted a significantly effect on final BW, ADWG and ADFI ($p=0.0001$). A higher final BW, ADFI, ADWG was observed in the experiment conducted in TN than in HS.

Similar with the present results, several studies reported a reduction in performance parameters when broilers were subjected to HS (Attia et al., 2017a; 2018a and Attia and Hassan, 2017). Al Sagan et al., (2020) reported that HS (32 ± 2 °C for 7 h/day, during 19-41 days) significantly decreased ADFI and impaired FCR of Ross 308 broiler chickens compared to the thermoneutral group. Exposing broilers at 34 to 38 °C, during 28 days, Shi et al., (2019) showed impairment in the growth performance. The authors recorded a lower BW (-19.03%) and ADFI (-12.21%) compared with the thermoneutral group. Also, in our study, broilers reared under HS had a lower feed intake than those reared in TN (Table 4). It was reported that the lower feed intake could be the result of HS and reduces the motility in the gastrointestinal tract and extends gastric emptying (Datta, 2001).

To our knowledge there have been no studies on the use of the same plants in the mixture, but on their separate use in broiler diet (Attia et al., 2017b; 2018b and 2019). For example, Ma et al., (2015) showed that under normal conditions of temperature broilers fed diet containing 0.05 to 0.10% flavones of sea buckthorn fruits improved ADFI, ADWG, and final BW. Al-Sagan et al., (2020) showed that dietary 3.2% of fennel seed powder enhanced tolerance to heat stress (32 ± 2 °C for 7 h/day) from 19 to 41 days of age. Arab Ameri et al., (2016) showed that dietary 1 and 2% peppermint powder negatively affected the FCR (21 days) and BW (42 days) of broilers reared at 34 °C for 8 hour/day.

Liver oxidative stress evaluation

Table 5 shows the effect of dietary inclusion of PM on oxidative stress biomarkers in the liver tissue of chicken reared under thermoneutral and HS conditions.

Table 4. Effect of dietary PM on broiler performance

Variable	Thermoneutral		Heat stress		p-values			
	C-TN	PM-TN	C-HS	PM-HS	Overall	Diet	Temp.	Diet x temp.
BW (14d)	360	361	400	400	0.542	0.580	0.328	0.350
BW (42d)	2822[a]	2843[a]	2138[b]	2002[b]	<0.0001	0.343	0.0001	0.085
ADWG	87.9[a]	88.6[a]	62.0[b]	57.2[b]	<0.0001	0.304	0.0001	0.064
ADFI	134[a]	136[a]	103[b]	97.8[b]	<0.0001	0.800	0.0001	0.816
FCR	1.52	1.54	1.67	1.71	0.489	0.808	0.127	0.914

BW- body weight (g/broiler); ADWG- average daily weight gain (g/broiler); ADFI- average daily feed intake (g feed/broiler/day); FCR- feed conversion ratio (g feed/g gain).

Table 5. Effect of dietary PM on liver oxidative status

Variable	Thermoneutral		Heat stress		p-values			
	C-TN	PM-TN	C-HS	PM-HS	Overall	Diet	Temp.	Diet x temp.
MDA	4.81[ab]	3.54[c]	5.85[a]	3.84[bc]	0.001	0.0001	0.013	0.321
T-AOC	0.880[b]	1.14[a]	1.02[a]	0.980[a]	0.123	0.043	0.719	0.201
MDA/T-AOC	5.50[ab]	3.12[c]	5.94[a]	4.16[bc]	0.0001	0.0001	0.070	0.438
PC	0.770[a]	0.520[b]	0.830[a]	0.490[b]	0.003	0.0001	0.653	0.728
CAT	1500[ab]	1825[a]	1104[c]	1198[bc]	0.001	0.011	0.0001	0.667
GSH	25.9[b]	33.6[a]	27.2[b]	32.6[a]	0.039	0.005	0.859	0.844
SOD	200.90[b]	263.07[a]	116.62[c]	155.32[c]	<0.0001	0.001	0.0001	0.063

Note: [a, b, c] Means in the same column with different superscripts differ significantly ($p<0.05$). SEM = standard error of the means; MDA – malondialdehyde (nmol/ g tissue); T-AOC-Total antioxidant capacity [%DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging]; PC- Protein carbonyl (nmol/ g tissue); CAT- catalase (U/mL); GSH- glutathione (μmol/ g tissue); SOD- superoxide dismutase (U/ g tissue)

Due to the fact that it is rich in lipids and involved in vital functions including in maintaining homeostasis, liver is more susceptible to oxidation. It is noticeable that both diet and temperature exerted a significantly effect on MDA concentration. Both under TN and HS, dietary supplementation with PM led to a significantly lower concentration of MDA (as lipid peroxidation product) in the broiler liver than C diet. It is observed that C-HS group registered a higher concentration of MDA than C-TN. Numerous studies reported that phytochemicals lowered the oxidative process of lipids and protein in broiler's tissues (Attia et al., 2017b; 2018b and 2019; Lee et al., 2019; Panaite et al., 2020; Turcu et al., 2020) due to their antioxidant compounds, which scavenge the reactive species that initiate the oxidative reactions in cells.

Adding PM powder in broiler's feed significantly increased T-AOC in the liver from groups reared under TN conditions, probably due to the antioxidant activity. There was no difference between groups reared under HS and TN conditions (Table 5), the temperature did not exert a significantly influence. Varzaru et al., (2020) showed that the extract of bilberry leaves expressed the highest *in vitro* antioxidant capacity on scavenging the hydrogen peroxide (41.09–61.52%) radical and as result, delayed the peroxidation of meat lipids. Papuc et al., (2009) reported in an *in vitro* study that sea buckthorn (*H. rhamnoides*) polyphenolic extract protected the refrigerated meat (beef and pork) against lipid peroxidation. Kalia et al., (2018) reported that *H. rhamnoides* extract is rich in flavonoids and its inclusion in the diet (200 mg/kg) improved the antioxidant defense level, increasing T-AOC and decreasing the MDA in the plasma of Rhode Island Red Cross-bred chicks reared at high altitude cold dessert. The same authors explained that those results could be attributed to the potentially synergistic effect of phenolic compounds and carotenoids contained. The MDA/T-AOC ratio significantly decreased in the group supplemented with PM powder than in C group, meaning that the PM had a positive effect on the antioxidant balance. A significantly influence had the temperature, which increased the MDA/T-AOC ratio (Table 5).

Regarding the concentration of PC (as protein degradation product), it was significantly lower in the groups whose diet was supplemented with PM powder (PM-TN, PM-HS) compared to those fed diet C (C-TN, C-HS). The temperature factor did not exert significantly influence on PC concentration.

The CAT and SOD activities were influenced by diet and temperature. Thus, the CAT and SOD activities increased in PM supplemented group than in C group. However, chicken exposed to HS had a lower CAT and SOD activities than those reared under TN conditions. The activity of GSH was influenced only by the diet, being higher in PM- supplemented group than C group (Table 5).

Several studies have reported improvements in the endogenous antioxidant system when supplementing the diets of broiler chickens with natural sources of antioxidants (Attia et al., 2017b, 2018b; 2019). Panaite et al (2020) showed similar results when broilers were reared under TN and fed 0.25 and 0.5% powder of *Salix alba* bark. Also, under thermoneutral conditions, Dong et al., (2011) reported that the dietary polysavone supplementation of a natural extract from alfalfa (1.5 g/kg) enhanced SOD activity in broiler serum and liver. Shen et al., (2019) showed that supplementing the chicken diet with 1.0, 2.0, 3.0, 4.0, and 5.0g bamboo leaf extract enhanced the antioxidant level both in serum and liver as a result of amplifying the expression of SOD, GSH-Px and CAT mRNA and inhibiting lipid oxidation. Ghabru et al., (2018) showed that including sea buckthorn leaves (10000 ppm) in the broiler diet increased SOD, CAT and GSH activities in liver and blood. However, Table 5 highlights that the HS decreased the activity of CAT and SOD in liver compared with TN. Accordingly, several studies have acknowledged the detrimental influence of HS on the activity of antioxidant enzymes in chickens (Akbarian et al., 2016; Attia et al., 2017a, 2018a; Attia and Hassan, 2017; Hu et al., 2019; Surai et al., 2019), suggesting that PM can be used as tool to improve antioxidant balance under stress and natural condition.

CONCLUSION

Dietary PM did not affect performance of broilers neither under TN nor under HS condition. However, PM could protect against the protein and lipid oxidation in the liver tissue of broilers reared under HS or TN by increasing the activity of antioxidant enzymes (CAT, GSH, SOD). Hence, PM had only a positive effect on the antioxidant status of broilers.

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