Black bone syndrome in broilers fed ethanolic extract of mango seeds

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ABSTRACT This study aimed to evaluate the incidence of black bone syndrome (**BBS**) in broiler chickens fed with ethanolic extract of mango seed (**EEMS**). A total of 504 one-day-old male broilers were used in a completely randomised design assigned with 7 experimental diets and 6 replicates of 12 broilers per experimental plot. The experimental diets consisted of: diet without addition of synthetic antioxidant; diet with addition of synthetic antioxidant (200 ppm); and 5 levels of EEMS: 200 ppm, 400 ppm, 600 ppm, 800 ppm, and 1,000 ppm. Two methods of cooking (roasted and boiled) were used to prepare thigh samples. According to the results, the diets did not significantly influence the performance of the broilers. BBS incidence was higher in broilers fed a diet without antioxidants and was reduced with EEMS dietary inclusion, with the lowest incidence occurring with the inclusion of 1,000 ppm. The synthetic antioxidant butylated hydroxytoluene in the diet promoted a significantly higher BBS incidence than that obtained with 800 and 1,000 ppm EEMS and did not differ from the other diets. Of the cooking methods, a higher BBS incidence was observed for the boiled method. For the meat coloration and bone parameters, there were no significant interactions between the factors, diets and cooking methods. There was a linear reduction in the darkening score and linear increase in the luminosity (L*) of the meat with increasing EEMS in the diet. With regard to the cooking method, the boiled thighs had lower luminosity (L*), higher parameter a*, and lower parameter b* values because of more pronounced meat darkening. The roasted bones were less heavy, dense, and flexible. A negative correlation was observed between the degree of darkening of the meat that characterizes the BBS with the luminosity (L*) and intensity of yellow. We concluded that the addition of EEMS contributes to a reduced darkening of meat that characterises the BBS and recommend the dietary inclusion of 1,000-ppm EEMS.

Key words: antioxidant, black bone, bone quality, darkening meat, ethanolic extract

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INTRODUCTION

Advances in the rapid growth of broiler chickens have resulted in a higher incidence of physiological disorders, which can cause disorders of the locomotory system (Nääs et al., 2012), as well as negatively affect muscle tissue and, thus, the quality of the meat from these animals. The incidence of deformation and bone fragility due to low bone mineralization increases the mortality of poultry on the farm and the condemnation of carcasses, with subsequent economic and animal welfare impacts (Rath et al., 2000). In turn, physiological disturbances can also result in deviations in the quality of commercial cuts of broiler chickens, for example, the darkening of the tibia and adjacent muscle tissues, which results in an unappetizing appearance to the meat (Mota et al., 2019). This problem that has been described as black bone syndrome (**BBS**) (Whitehead, 2009).

BBS is characterised by redness of the flesh and darkening of the bone that occurs due to blood extravasation from the bone marrow at the proximal region of the tibia and femur during freezing of the commercial cuts, and it may be aggravated by cooking (Smith and Northcutt, 2004). The occurrence of this anomaly, especially in the drumsticks and thighs, promotes worsening of the visual feature in the flesh *in natura* which presents implications on appearance and texture and results in higher consumer rejection rates (Baldo et al. 2013). Moreover, this problem also results in an increase in

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prooxidant agents in the tissues, reducing the oxidative stability and the shelf life of the *in natura* and processed meat (Vasquez and Soto-Solanova, 2009; Korver, 2010).

Bone structure characteristics have been reported to predispose for the development of BBS, in particular, the increase of bone porosity associated with deficient bone mineralization (Whitehead, 2009; Korver, 2010). Commercial lines with a rapid growth rate show a decrease in tibial mineralization and an increase in porosity, which negatively compromise bone density (Williams et al., 2004). Therefore, in the short term, the addition of nutrients to the diet could improve the bone quality of broiler chickens and reduce the incidence of BBS (Baldo et al. 2013).

In this context, the addition of dietary antioxidants to the rations for broilers has been reported to reduce the effects of oxidative stress (Post et al., 2003; Abioja et al., 2012), with changes in bone quality associated with increased excretion of minerals, such as calcium, iron, and zinc, in heat-stressed broilers. Moreover, the addition of antioxidants in the diet may indirectly contribute to bone quality by influencing cell oxidation, reducing the production of free radicals (Arijmanji et al., 2002), as well as sequestering oxygen molecules and, thus, preserving the colour by maintaining iron in its reduced form (Lu et al., 2014).

Although synthetic antioxidants, such as butylhydroxy-anisole, butylated hydroxytoluene (**BHT**), tert-butylhydroquinone, and propyl gallate, are widely used in the food industry (Harnedy and FitzGerald, 2012), the potentially negative effects of prolonged intake, such as the growth of cancer cells in the stomach, liver, and reproductive system of animals, has led to the search for alternatives, such as the natural antioxidants (Niki et al., 1991; Li et al., 2010). Hence, ethanolic extracts of mango have been studied for use as antioxidants in poultry feed and were found to effect the reduction in the lipid oxidation of the meat and improve the colouring parameters of the breast meat when frozen for 90 D (Freitas et al., 2012, 2015). In addition to their protective qualities, some antioxidants frequently found in the ethanolic mango extracts, including quercetin and kaempferol (Shah et al., 2010; Ma et al., 2011), have activities that promote bone quality, modulate osteoclastogenesis, and regulate several local factors and systemic factors, such as hormones, inflammatory cytokines, and tumour necrosis factor in bone cells (Oliveira et al., 2010). In addition, the extract contains the phenolic compound mangiferin, which can inhibit bone loss by its antireabsorption action, induced by parathyroid hormones (Li et al., 1999).

Therefore, this study aimed to evaluate the performance and incidence of BBS and bone parameters in broilers fed rations containing different levels of ethanolic extract of mango seed (**EEMS**), under 2 cooking methods.

MATERIAL AND METHODS

Purchase of Mango Residue and Extract Preparation

The mango residue, constituted of seeds, was obtained from the extraction of pulp and purchased *in natura* form from a fruit-processing company for extraction of the pulp. Then, the material was subjected to drying and milling, and the extract was obtained by the cold method, using the organic solvents hexane and ethanol (adapted from Freitas et al., 2015). The obtained ethanolic extract was conditioned in a glass container and identified for subsequent use.

Determination of the Antioxidant Potential, Total Phenolics, and Total Antioxidant Activity of Ethanolic Extract from Mango Seed

Aliquots of the extract were evaluated for antioxidant potential, total antioxidant activity, and phenolic compounds (Table 1), with BHT used as standard. The antioxidant potential of the extract was measured by its capacity to sequester the radical 2,2-diphenyl-1-picrylhydrazyl, according to the procedure described by Brand-Willians et al. (1995); the results are expressed in mg/L, referring to half of the maximal inhibitory concentration. The total antioxidant activity was determined with the ABTS free radical + [2,2′-azinobis (3-ethylbenzthiazoline-6-sulphonic acid)] test and the result expressed in μmol of Trolox equivalent antioxidative capacity per g extract (Rufino et al., 2007). The phenolic compounds were analysed using the Folin-Ciocalteau method, expressed as mg of gallic acid equivalent per g extract (Folin and Ciocalteau, 1927; Mueller-Harvey, 2001).

Experimental Design, Treatments, and Experimental Diets

The experimental procedures in this project was approved by the Committee on Ethics in Animal Research under protocol 22/2013 and carried out in an experimental shed for raising broilers, divided internally into 48 boxes of 1.5 m × 1.0 m (1.5 m^2).

To conduct the experiment, 504 one-day-old male chicks of the Ag Ross 308 lineage were purchased and immunised in the hatchery for diseases of Marek and Gumboro. The broilers were housed in a masonry shed of 15 × 10 m, covered by clay tiles, with a cemented floor, a ceiling height of 3.5 m, oriented longitudinally in the east-west. All the boxes contained a pendulum drinker and a tubular feeder, and the floor was covered with wood shavings. The broilers were subjected to the same handling conditions.

The experimental design was completely randomised, with 7 treatments and 6 replicates, each with 12 broilers.

Table 1. Antioxidant potential, total antioxidant activity, and phenolic compounds of the ethanolic extract of mango seed.

Antioxidant	DDPH[1] (mg/L)	ABTS[2] (μM TEAC/g)	Total phenolics[3] (mg GAE/g)
BHT	289.17	350.83	-
EEMS	175.66	518.68	9.50

Abbreviations: BHT, butylated hydroxytoluene; EEMS, ethanolic extract from mango seed; GAE, gallic acid equivalent; TEAC, Trolox equivalent antioxidative capacity.
[1]Radical compound 2.2-diphenyl-1-picrylhydrazyl.
[2]Radical 2.2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid).
[3]Phenolic compound.

The treatments consisted of: T1 = negative control–ration without addition of synthetic antioxidant; T2 = positive control–ration with addition of synthetic antioxidant (200 ppm of BHT); T3 = ration with addition 200 ppm of EEMS without synthetic antioxidant; T4 = ration with addition 400 ppm of EEMS without synthetic antioxidant; T5 = ration with addition 600 ppm of EEMS without synthetic antioxidant; T6 = ration with addition 800 ppm of EEMS without synthetic antioxidant; T7 = ration with addition 1,000 ppm of EEMS without synthetic antioxidant.

The feeding programme was divided into 3 phases: initial (1–21 D), growth (22–35 D), and final (36–45 D). The experimental rations (Table 2) were formulated according to the nutritional requirements recommended by the NRC (1994) and were calculated to be isoenergetic and isonutritive, considering the values of the food composition proposed by Rostagno et al. (2011). The inert ingredient of the control ration of each phase was replaced by the antioxidants, according to the treatments.

The environmental conditions inside the poultry shed were monitored by a Data logger (Data Logger U12-012; HOBO, Bourne, MA) installed at the height of the broilers. During the experimental period, the average temperature of the shed was 28.4°C and relative air humidity was 72.2%. The broiler litter was manually rotated twice a week, starting at 8:00 am, and fans were turned on during higher temperature schedules (9 am to 3 pm) from 28 D of age.

Broiler Performance

The performance variables analyzed were feed consumption (g/bird), weight gain (g/bird), and feed conversion (g/bird).

Evaluation of the Incidence of BBS

At 45 D of age, after a 6-h feed fast, all poultries were weighed, and 3 broilers were selected from each experimental unit, with the live weight closest to the average plot weight, totaling 126 broiler chickens. These broilers were euthanised by electronarcosis, followed by bleeding, scalding, and plucking.

After slaughter and evisceration, the carcasses were weighed, and the thighs were separated, identified, weighed, and frozen (−20°C) for later analysis. After 30 D, the thighs were removed from the freezer and thawed in a refrigerator at 4°C for 24 h.

The thighs of each broiler were identified and to be subjected to different cooking methods. The right thighs were roasted in an electric oven preheated to 180°C, and the left thighs were cooked in boiling water, for the same 30-min period.

After cooking, we evaluated the samples for colouring scores to verify the incidence of BBS, adopting the methodology of macroscopic evaluation of the darkening of the meat and attributing the following scores: 0, 1, or 2, according to appearance. A score of 0 was classified as acceptable (region close to the bone without darkening), a score of 1 as intermediary (less than 50% of the compromised region near the bone), and a score of

Table 2. Composition of control diet[1] for broilers in the initial (1–21 D), growth (22–35 D), and final (36–42 D).

	Period		
Ingredients (kg)	Initial	Growth	Final
Corn	56.61	61.51	63.66
Soybean meal (45%)	36.50	31.44	28.47
Soy oil	2.70	3.31	4.34
Bicalcium phosphate	1.87	1.65	1.56
Calcitic limestone	0.92	0.83	0.79
Common salt	0.43	0.41	0.38
DL, methionine	0.30	0.25	0.26
L- lysine	0.27	0.21	0.28
Mineral and vitamin supplement[2]	0.20	0.20	0.10
Inert[3]	0.10	0.10	0.10
Choline chloride	0.05	0.05	0.05
Anticoccidian	0.05	0.05	0.00
Calculated nutritional level			
Metabolizable energy (kcal/kg)	3.000	3.100	3.200
Crude protein (%)	21.40	19.41	18.31
Dry matter (%)	88.67	88.64	88.70
ADF (%)	4.85	4.62	4.45
Neutral detergent fiber (%)	11.78	11.67	11.52
Calcium (%)	0.92	0.82	0.78
Available phosphorus (%)	0.46	0.41	0.39
Sodium (%)	0.19	0.18	0.17
Chlorine (%)	0.30	0.30	0.28
Total lysine (%)	1.36	1.18	1.16
(%) Methionine + total cistine (%)	0.95	0.85	0.83
Total methionine (%)	0.60	0.53	0.53
Total threonine (%)	0.83	0.76	0.71
Total tryptophan (%)	0.26	0.24	0.22

[1]Control-ration without antioxidant.
[2]Guarantee levels per kg of product: vitamin A (min) 5500000 IU vitamin B1 (min) 500 mg, vitamin B12 (min) 7500 mcg, vitamin B2 (min) 2502 mg, vitamin B6 (min) 750 mg, vitamin D3 (min) 1000000 IU, vitamin E (min) 6500 IU, vitamin K3 (min) 1250 mg, biotin (min) 25 mg, niacin (min) 175 g, folic acid (min) 251 mg, pantothenic acid (min) 6,030 mg, cobalt (min) 50 mg, copper (min) 3,000 mg, iron (min) 25 g, iodine (min) 500 mg, manganese (min) 325g, selenium (min) 1,0005 mg, zinc (min) 22.49 g.
[3]Inert-Washed sand.

2 as unacceptable (more than 50% of the compromised region near the bone).

Soon after the allocation of scores, the colouring parameters in the thigh meat were measured. Three different reading points per sample were measured using CR410 Konica Minolta's colorimeter (Osaka, Japan). The results were expressed as L* (brightness), a* (red - green component), b* (yellow - blue component) of CIELAB colour system.

Evaluation of Bone Parameters

For measurement of bone parameters, the femur was properly identified and cleaned using a scalpel, according to methodology described in and adapted from the study by Bruno et al. (2000). Femur length was measured using digital callipers and weighed using a precision scale (0.01 g). Bone density was evaluated through the Seedor index, obtained by dividing the weight (mg) by the length (mm) of the bone (Seedor, 1991). The bone strength and deformity of the femur were determined using a Testo/Ronald Triaxial mechanical press (Ronald Top Ltda., Rio de Janeiro, RJ, Brazil) with a 150-kg capacity.

Statistical Analysis

A statistical analysis of the data was performed using the Statistical Analysis System, version 9.3 (SAS Institute Inc., Cary, NC). For the evaluation of BBS, the body weight data for the selected broilers were submitted to ANOVA and comparison of averages by the Student-Newman-Keuls test.

Statistical analysis of the incidence of BBS scores among treatments was performed using the chi-squared test. Subsequently, for the analysis of the average scores, the real values of the data were submitted to the radical transformation ($\sqrt{x} + 1$). This procedure was adopted to meet the premise of the ANOVA (Sampaio, 1998).

The data of the transformed scores, meat colour and bone quality, were submitted to ANOVA according to a 7×2 factorial model. There were 7 rations and 2 thigh cooking methods (roasted and boiled). The averages of the treatments were compared with the Student-Newman-Keuls test at a 5% probability.

A regression analysis was performed, considering only the data of the treatments with the inclusion of EEMS, to estimate the best EEMS level in the ration. We also performed a Pearson correlation analysis to verify the associations among the intensity of the scores, meat colouring parameters, and quality of the thigh bone after cooking.

RESULTS AND DISCUSSION

Broiler Performance and Evaluation of the Incidence of BBS

The feed intake, weight gain, and feed conversion of broilers selected for slaughter and evaluation of the incidence of BBS (Table 3) did not vary significantly among the different treatments, indicating that the addition of EEMS or BHT had no effect on the performance and final slaughter weight. The results obtained for body weight corroborate with those obtained by Freitas et al. (2012), who tested the addition of 200 ppm and 400 ppm of EEMS and found no significant influence of the addition of EEMS on the performance of broilers at 42 D.

In the evaluation of the incidence of BBS in broiler thigh samples by the chi-square test (Table 4), it was observed that there was a difference between the cooking methods and the rations received by the broilers ($P < 0.05$). There was an increase in the incidence of BBS (score 1, intermediate; score 2, not acceptable) when the thighs were boiled. The roasted thighs had the highest proportion of samples considered acceptable or normal (score 0).

For the effect of the treatments (Table 4), observed that the proportion of samples identified with darkening score of 1 (intermediary), which characterises the incidence of BBS, was higher for the thighs of broiler chickens fed without addition of antioxidant. However, these values obtained differed significantly only in relation to those obtained with the addition of 400 ppm or more EEMS. The addition of BHT resulted in a significantly higher incidence of BBS only in relation to the 800 ppm and 1,000 ppm EEMS doses. Among the levels of EEMS, the lowest proportion of BBS was obtained with 1,000 ppm, although it did not differ significantly to that obtained with 800 ppm. In turn, the addition of 800 ppm EEMS to the ration promoted a lower BBS incidence in relation to 200 ppm. The incidence of BBS for 200 ppm, 400 ppm, and 600 ppm EEMS in the ration did not differ ($P > 0.05$).

The result obtained for the identification of samples considered normal (score 0) was inverse to that described for the effect of the ration on the incidence of BBS, as the incidence of samples scoring 1 decreased because of the higher incidence of score 0. Therefore, with the addition

Table 3. Average weight and standard deviation of broiler chickens selected for evaluation of black bone syndrome.

Diets	Average weight ± standard deviation (kg/bird)
Without SA	3.20 ± 0.22
With SA - 200 ppm	3.18 ± 0.17
200 ppm EEMS	3.20 ± 0.36
400 ppm EEMS	3.09 ± 0.34
600 ppm EEMS	3.27 ± 0.16
800 ppm EEMS	3.19 ± 0.24
1,000 ppm EEMS	3.17 ± 0.20
Average	3.19
SEM	0.0225
ANOVA[1]	*p-valor*
Diet	0.5584
Regression	*p-valor*
Linear	0.7167
Quadratic	0.7302

Abbreviations: EEMS, ethanolic extract from mango seed; SA, synthetic antioxidant.

[1]ANOVA ($P > 0.05$), statistical effect not significant.

Table 4. Number and incidence of samples per coloring scores of the meat of the thighs of the broilers fed with the different diets.

	Cooking methods								
	Roasted			Boiled			Total		
	Score			Score			Score		
Diets	0	1	2	0	1	2	0	1	2
Number of samples per score									
Without SA	0	18	0	0	18	0	0	36	0
With SA - 200 ppm	2	16	0	1	16	1	3	32	1
200 ppm EEMS	1	17	0	1	17	0	2	34	0
400 ppm EEMS	4	14	0	2	16	0	6	30	0
600 ppm EEMS	5	13	0	2	16	0	7	29	0
800 ppm EEMS	6	12	0	3	15	0	9	27	0
1,000 ppm EEMS	8	10	0	6	12	0	12	22	0
Total	26	100	0	15	110	1	41	210	1
Percentage of sample per score									
Without SA	0	100.00	0	0	100.00	0	0.00^{d}	100.00^{a}	0
With SA - 200 ppm	11.11	88.89	0	5.56	88.89	5.56	$8.33^{b,d}$	$88.89^{a,b}$	2.78
200 ppm EEMS	5.56	94.44	0	5.56	94.44	0	$5.56^{d,c}$	$94.44^{a,b}$	0
400 ppm EEMS	22.22	77.78	0	11.11	88.89	0	$16.67^{b,c}$	$83.33^{b,c}$	0
600 ppm EEMS	27.78	72.22	0	11.11	88.89	0	$19.44^{a,b}$	$80.56^{b,c}$	0
800 ppm EEMS	33.33	66.67	0	16.67	83.33	0	$25.00^{a,b}$	$75.00^{c,d}$	0
1,000 ppm EEMS	44.44	55.56	0	33.33	66.67	0	33.33^{a}	61.11^{d}	0
Total	20.63^{A}	79.37^{B}	0^{B}	11.90^{B}	87.30^{A}	0.79^{A}	16.27	83.33	0.40

[a-d, A,B]In the row, values for the same score, followed by a different capital letter, indicate significant difference between the cooking methods, by the chi-squared test ($P < 0.05$). In the column, values for the same score, followed by a different lowercase letter, indicate a significant difference between the rations, by the chi-squared test ($P < 0.05$).

Abbreviations: EEMS, ethanolic extract from mango seed; SA, synthetic antioxidant.

of 1,000-ppm EEMS, the number of normal samples increased to 33% and the incidence of BBS decreased to 61% when the total of samples was considered.

When evaluated the average colouring scores of the meat adjacent to the bone of broiler thighs (Table 5), there was no significant interaction between treatments and cooking methods. However, the results among the treatments and between the cooking methods were different ($P < 0.05$).

As for the cooking methods, we observed a higher average meat colouring score and a higher proportion of samples scoring 1 and 2 when the thighs were boiled than the roasted samples.

Was also observed a linear reduction (Y = 1.02–0.0004x; R2 = 0.87) in the meat colouring scores with an increase in EEMS. Thighs of broilers not fed antioxidant in diets had greater colouring scores, differing from those fed 800-ppm and 1,000-ppm EEMS. The use of BHT only resulted in a significantly higher darkening score than the addition of 1,000 ppm EEMS. Among the EEMS levels, the lowest average darkening score was obtained with 1,000 ppm, but did not differ from that obtained with 800 ppm. In turn, there was no difference in the meat-colouring scores for broilers fed with 200-ppm, 400-ppm, 600-ppm, or 800-ppm EEMS ($P > 0.05$).

For all colouring parameters (L*, a*, b*) of the thigh meat (Table 6), there was no significant interaction between the treatments and thigh cooking methods. However, there was a significant difference between the results obtained with the treatments for the luminosity (L*) ($P < 0.05$). Between the cooking methods, there was a difference for the luminosity (L*) and the parameters a* (red–green component) and b* (yellow-blue component) ($P < 0.05$).

Although there was no effect of antioxidant addition on the colour components a* and b*, there was a linear increase (Y = 54.12 + 0.0017x; $R^2 = 0.76$) in the luminosity value (L*) of the meat with increasing EEMS levels in the ration. Also observed lower values for luminosity (L*) in the thighs of broilers not fed antioxidants differed significantly from those broilers fed rations

Table 5. Average results for the coloring scores of broiler's thighs fed with ethanolic extract of the mango seed in the diets.

	Scores values	
Diets	Real	Transformed
Without SA	1.00^{a}	1.40^{a}
With SA - 200 ppm	$0.91^{a,b}$	$1.37^{a,b}$
200 ppm EEMS	$0.94^{a,b}$	1.38^{a}
400 ppm EEMS	$0.83^{a,b}$	$1.33^{a,b}$
600 ppm EEMS	$0.86^{a,b}$	$1.34^{a,b}$
800 ppm EEMS	$0.75^{b,c}$	$1.30^{a,b}$
1000 ppm EEMS	0.6^{c}	1.24^{b}
Cooking methods		
Roasted	0.79^{b}	1.32^{b}
Boiled	0.90^{a}	1.36^{a}
Average	0.86	1.34
SEM	0.0362	0.0145
ANOVA	*p*-valor	
Diet	<0.001	
Cooking methods	0.0208	
Diets × cooking methods	0.6190	
Regression	*P* value	
Linear	0.0003	
Quadratic	0.4493	

[a-c]In the column, values followed by different lowercase letter indicate significant statistical difference ($P < 0.05$).

Abbreviations: EEMS, ethanolic extract from mango seed; SA, synthetic antioxidant.

containing 800-ppm and 1,000-ppm EEMS. The addition of BHT to the broiler rations resulted in a lower luminosity (L*) of the meat than those fed 800-ppm and 1,000-ppm EEMS, which did not differ from the other treatments.

With regard to the cooking methods, the roasted thighs showed higher luminosity (L*), lower component a*, and greater component b* values, indicating that roasting resulted in lighter meat with a lower red and higher yellow content. Therefore, the thighs subjected to this cooking method showed lower average darkening scores, a characteristic of BBS.

Pearson's correlation analysis (Table 7) of the darkening and colouring scores of the meat revealed a negative correlation among the darkening that characterizes BBS, luminosity (L*), and intensity of yellow. This indicates that the increase in scores from 0, 1, and 2 is determined by a lower luminosity (L*), less colour yellow, and more colour blue, determined by the reduction of the value of component b*.

These results confirmed the association between the instrument-determined meat colouring scores and the visual perception of darkening during the evaluation of the scores that characterise BBS, suggesting that the visual allocation of scores that characterise this syndrome indicates true colour changes in the meat.

Evaluation of Bone Parameters

For the bone parameters of the thighs (Table 8), we found that, for all variables, there was no significant interaction between treatments and cooking method, as well as no effect significant for the different rations ($P > 0.05$). However, between the cooking methods, there were differences for weight, Seedor index, and bone deformity ($P < 0.05$).

Table 6. Average results for the color parameters of thighs of birds fed with EEMS in the diets.

Diets	Color parameters		
	L*	a*	b*
Without SA	53.07[b]	7.21[a]	15.13[a]
With SA - 200 ppm	53.42[b]	7.42[a]	14.97[a]
200 ppm EEMS	54.72[a,b]	7.41[a]	15.14[a]
400 ppm EEMS	54.80[a,b]	7.72[a]	14.96[a]
600 ppm EEMS	54.81[a,b]	7.69[a]	14.65[a]
800 ppm EEMS	55.26[a]	7.52[a]	15.11[a]
1000 ppm EEMS	56.23[a]	7.34[a]	15.42[a]
Cooking Methods			
Roasted	54.99[a]	7.29[b]	15.28[a]
Boiled	54.24[b]	7.66[a]	14.83[b]
SEM	0.1867	0.0607	0.0846
ANOVA	*p*-valor		
Diet	<0.0001	0.2159	0.3691
Cooking methods	0.0339	0.0019	0.0088
Diets × cooking methods	0.4240	0.0870	0.9661
Regression	*P* value		
Linear	0.0158	0.6091	0.3670
Quadratic	0.3079	0.1272	0.1224

[a,b]In the column, values followed by different lowercase letter indicate significant statistical difference ($P < 0.05$).

Abbreviations: EEMS, ethanolic extract from mango seed; SA, synthetic antioxidant.

Table 7. Pearson's correlation between the darkening score and the meat coloring parameters.

Coloring parameter	Correlation coefficient	*P* value
L* - luminosity	−0.37	<0.0001
a* - red to green	0.11	0.0791
b* - yellow to blue	−0.18	0.0041

The femur of thighs that were submitted to boiling presented a lower weight, Seedor index, and bone deformity than roasted thighs. However, bone length and bone resistance did not differ between cooking methods. According to the results, the addition of EEMS reduced the percentage of samples affected by BBS and increased the percentage of normal samples, and it also contributed to the darkening of the meat measured by the luminosity parameter (L*).

The reduction in the incidence of BBS and, thus, darkening of the broiler thighs, with the increase in EEMS in the ration, may be associated with its antioxidant action, which has been reported by some researchers (Freitas et al., 2012, 2015). However, the contribution the antioxidant action of EEMS had to these results requires more clarification.

Besides factors related to age, sex, and line of broilers, the diet may also contribute to the higher incidence of this disorder (Korver, 2010). In this sense, the protective effects of antioxidants on the cell membrane and their addition to the broiler ration may provide better marrow cell membrane stability, decreased phospholipases activity (Wood and Enser, 1997), and reduction of lipid oxidation, once may react with free radical of the oxidative process.

Antioxidant addition can also help by sequestering oxygen molecules, thus preserving the colour of meat by keeping the iron in its reduced form (Lu et al., 2014).

This context, the darkening of meat that characterises BBS, has been associated with blood extravasation from the bone marrow during the preparation of the meat for consumption (Whitehead, 2009; Korver, 2010), and the improvement in bone quality is often related to a reduction in the incidence of this syndrome (Whitehead, 2010; Baldo et al. 2013). In turn, the antioxidants of the ration can contribute indirectly to the quality of the bones, by influencing cell oxidation and reducing the production of free radicals (Arijmanji et al., 2002).

Dietary antioxidants also allow a greater availability of vitamin C, which is essential for the conversion of 25-hydroxyalkylol into 1,25-dihydroxicalciferol and, in turn, is critical for the metabolism of calcium and phosphorus, the main minerals in bone formation (Abioja et al., 2012).

Some natural antioxidants, such as quercetin and kaempferol, inhibit bone loss by affecting osteoclastogenesis and regulate various local and systemic factors, including hormones, inflammatory cytokines, and the tumour necrosis factor in bone cells (Oliveira et al., 2010). Whereas, mangiferine has bone antireabsorption actions induced by parathyroid hormones (Li et al., 1999). These compounds are present in the ethanolic extracts

Table 8. Bone parameters of the femur of broilers fed ethanolic extract of mango seed.

Diets	Weight (g)	Length (mm)	Seedor index (mg/mm)	Resistance (kgf/cm^2)	Deformity (mm)
Without SA	11.81	79.63	148.08	21.14	5.32
With SA - 200 ppm	11.65	79.63	146.21	20.64	5.18
200 ppm EEMS	11.77	79.43	149.57	22.36	5.59
400 ppm EEMS	12.02	79.57	150.85	22.85	5.23
600 ppm EEMS	12.42	80.40	154.45	22.46	5.55
800 ppm EEMS	12.00	80.35	149.32	20.85	5.36
1,000 ppm EEMS	12.05	80.31	150.34	21.81	5.53
Cooking methods					
Roasted	12.27^a	79.78^a	153.86^a	21.78^a	5.58^a
Boiled	11.66^b	80.02^a	145.82^b	21.68^a	5.21^b
SEM	0.0791	0.1555	0.8728	0.2597	0.06862
ANOVA	*P* value				
Diet	0.1555	0.3942	0.2353	0.1638	0.5313
Cooking methods	<0.0001	0.5350	<0.0001	0.8394	0.0077
Diets × cooking methods	0.6646	0.9733	0.6643	0.9650	0.3681
Regression	*P* value				
Linear	0.3566	0.0538	0.9840	0.1548	0.9702
Quadratic	0.1084	0.4194	0.2580	0.8888	0.3875

[a,b]In the column, values followed by different lowercase letter indicate significant statistical difference ($P < 0.05$).
Abbrwviations: EEMS, ethanolic extract from mango seed; SA, synthetic antioxidant.

obtained from the mango (Shah et al., 2010; Ma et al. 2011). Therefore, broilers fed rations supplemented with antioxidants were expected to show an improvement in bone quality.

However, the bone quality parameters after cooking the samples did not differ with the rations offered to the broilers ($P > 0.05$). Whitehead (2009) reported an improvement in bone quality associated with a reduction of the incidence of BBS in bones not subjected to heating. But, in the present study, bone evaluation was performed after the thighs were submitted to the different cooking methods, and heating may have contributed to changes in bone structure, affecting the results.

The effect of the cooking method might be greater if we consider that the thigh samples used were from the same broilers; however, there was a higher incidence of BBS in the boiled samples, evident by the darker meat with a higher content of blue, and with bones that were less heavy, dense, and flexible than the roasted samples. Therefore, we can infer that cooking the thighs in boiling water altered the properties of the bones, changing their flexibility and resulting in greater weight loss, which may be associated with increased blood extravasation from the bone.

Our results corroborate those of Baldo et al. (2013), who reported that the cooking method of the thighs and drumsticks of the broilers had an influence on the appearance of the dark meat that characterises BBS because the method and thawing time, as well as temperature and cooking time, can influence bone structure and blood extravasation from bone to adjacent meat. In this sense, lower cooking temperatures result in a slower temperature increase and tend to increase darkening, as they allow a greater amount of blood extravasation from the bone marrow.

The myoglobin and hemoglobin present in the blood can react with oxygen and change the colour of the meat, as both proteins associate with iron (Venturini et al., 2007). Therefore, the phenolic compounds found in the mango extract may help prevent the oxidation of hemoglobin and myoglobin, as they can react with iron to form a complex with Fe+2, accelerating its oxidation and the formation of the more stable Fe+3 polyphenol complex, beyond participating in other propagation lipid peroxidation reactions (Rodriguez et al., 2006).

In this context, after evaluating the antioxidant potential of extracts from different parts of the mango, Ribeiro et al. (2008) reported that mango seed extract showed a high iron reduction activity compared with BHT and gallic acid, with an increase in antioxidant activity as a function of the amount of extract, correlating with the amount of phenolic compounds present in the extract. From this, it can be inferred that the antioxidant action of EEMS in the iron complex reduces the oxidation of muscle myoglobin and hemoglobin of extravasated blood from the bone, decreasing the darkening of broiler thighs and, thus, the incidence of samples detected as BBS.

The benefits of the antioxidant action of EEMS in reducing broiler meat oxidation have been reported for meat cooled (Freitas et al., 2012) and frozen for up to 90 D, with an additional improvement in the colouring parameters (Freitas et al., 2015). In terms of the possibility of a total reduction in the incidence of BBS in broiler thighs, it should be considered that the research into the improvement in bone quality did not completely eradicate BBS nor did the antioxidant action of EEMS. However, the changes in broiler thighs induced by freezing which increased the incidence of this syndrome can be at least minimized with nutritional strategies to improve bone quality and to prevent oxidative damage during storage.

The addition of EEMS contributes to the reduction in meat darkening that characterises BBS, and the

addition of 1,000-ppm EEMS to the broiler chickens diet has the most effect.

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