Effects of dietary supplementation of a probiotic (*Bacillus subtilis*) on bone mass and meat quality of broiler chickens

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ABSTRACT The aim of this study was to investigate the effect of a dietary probiotic supplement on bone mass and meat quality of broiler chickens. Two hundred ten 1-day-old male Ross 708 broiler chicks were divided among 21 floor pens (10 chicks per pen). The pens were randomly distributed to 1 of 3 dietary treatments containing a probiotic, *Bacillus subtilis,* at 0 (control), 0.25 (**0.25X**), and 0.5 (**0.5X**) g/kg (n = 7). Gait score, footpad dermatitis (**FPD**), leg straightness, and hock burn (**HB**) were examined at day 33, and a latency-to-lie test was performed at day 34. At the end of the experiment (day 35), plasma, right leg, and litter samples were collected for mineral contents, meat quality, bone morphometric parameters, and litter quality assessments. The results indicated that probiotic-fed birds stood much longer during the latency-to-lie test with a greater tibial length, weight, and strength as well as higher plasma levels of calcium and phosphorus compared with the controls. In addition, probiotic-fed birds' leg muscle had higher color lightness at both 30 min and 5 h postmortem and greater water-holding capacity with a trend for less cooking loss ($P = 0.056$) and lower pH values ($P < 0.05$) at 5 h postmortem. Probiotic-fed birds' leg meat was tastier ($P < 0.05$) at 24 h after slaughter. These probiotic effects were greater in the 0.5X group than in the 0.25X group. There were no treatment effects on other measured parameters including gait score, HB, FPD, tibial lateral and medial wall thickness, diaphysis and medullary canal diameters, robusticity and tibiotarsal indexes, plasma magnesium concentrations, and litter moisture and pH values ($P > 0.05$). These findings indicate that the probiotic supplement could be a useful management tool for improving broiler production and welfare by enhanced bone mass and meat quality.

Key words: broiler, probiotic, bone health, meat quality, welfare

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INTRODUCTION

Skeletomuscular disorders and related welfare and economic impacts have been causing a great concern to the broiler industry globally (Skinner-Noble and Teeter, 2009; Yan et al., 2018). Lameness (or paralysis), for example, is one of the top welfare crises, resulted from leg pain–associated immobility and related starvation and dehydration. Lameness accounts for approximately 60% of the skeletal diseases in broilers (Julian, 2005) and has been recognized as one of the main reasons causing the economic loss of the poultry meat industry (McNamee et al., 1999). Estimably, an annual loss of $0.16 per broiler is faced by the poultry industry owing to leg disorders (Cook, 2000), and it is likely much higher in today's market based on the rising production costs and inflation.

Today's broilers raised for meat production are particularly susceptible to leg disorders owing to selective breeding for fast growth and rapid weight gain with large breast muscle, resulting in an imbalance between the body size and the weight-supporting skeletal system (Meyer et al., 2019). The weakness in the low limbs

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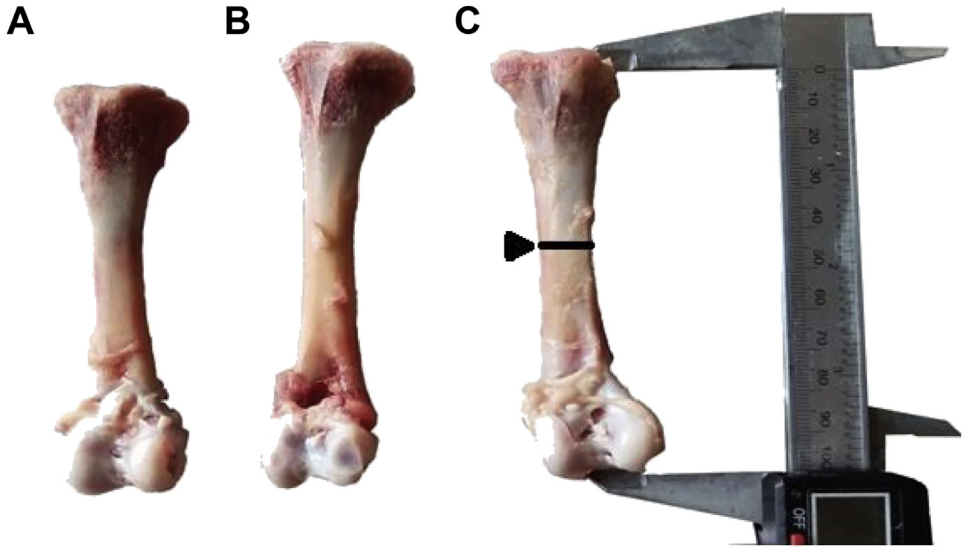

Figure 1. Morphometric analyses of tibia. Tibiotarsal length and tibiotarsal breaking strength at the midshaft (arrowhead) were measured as shown in the photo; and then, the bone was cut horizontally at the midshaft and the diaphysis diameter, medullary canal diameter, and thickness of the medial wall and the lateral wall were measured using a digital caliper, and from them, robusticity index and tibiotarsal index were calculated. Examples, Tibia from (A) a control broiler; (B) a 0.25X broiler; and (C) a 0.5X broiler.

makes broilers stressed out with reduced bone mineral density and related pathologic damage (Shim et al., 2012), leading to downgraded meat quality (Karaoglu and Durdağ, 2005).

Broiler health and welfare are further challenged by ambient environments owing to litter management and climatic changes. Keeping litter dry at a great quality is a critical issue affecting bird performance (Wadud et al., 2012; Dunlop et al., 2016). Wet litter promotes pathogenic proliferation and amplifies emitting of ammonia gas, as a result, increasing the incidence of contact dermatitis and related footpad lesion and breast blisters (Shepherd and Fairchild, 2010). Footpad dermatitis (**FPD**) and hock burn (**HB**), the skin of the foot and hock inflamed with necrotic lesions, have been recognized as the main reasons causing lameness in broilers (Hossain et al., 2018).

Modern fast-growing broilers are susceptible to stress, such as social stress caused by reared in high-density environments and thermal stress arisen under hot or cold conditions. Stress in broilers, similar to in humans and rodents, activates the hypothalamic–pituitary–adrenal axis to increase the synthesis of corticosterone in the adrenal glands (Lyasere et al., 2017). Excess corticosterone affects the bone mass by enhancing the osteoclast proliferation and inhibiting the osteoblast osteoblastogenesis (Henneicke et al., 2014) and reducing bone mineral density (Kang et al., 2016). Osteoblasts and osteoclasts function in bone formation, growth, repair, and breakdown (Chen et al., 2018; Kikuta and Ishii, 2018).

Probiotics are live microorganisms which confer health benefits in hosts when administered in appropriate amounts (FAO/WHO, 2001). Several studies that investigated the effect of probiotics on human health have reported that probiotics can be used as a bacteriotherapy for a variety of diseases including psychiatric disorders such as major mental illness (Butler et al., 2019; Nguyen et al., 2019). Similarly, several probiotics, such as *Lactobacillus*, *Bifidobacterium*, and yeasts, have been investigated as diet additives or alternatives to antibiotics for improving production and health in broilers (Pelicia et al., 2004; Alavi et al., 2012; Mohammed et al., 2018). However, the conflicted findings, improvement or no change, have been reported (McCabe et al., 2015; Saiyed et al., 2015; Salehimanes et al., 2016; Sarangi et al., 2016). The differences in the bacterial strains or concentrations of the probiotics used in those studies may be associated with the conflicting results. Furthermore, the effect of the probiotic, *Bacillus subtilis*, on musculoskeletal health and related meat quality in broilers has not been investigated previously, although *B. subtilis* as a probiotic has been widely used in humans and other animals including broilers. In broilers, most of the *B. subtilis* investigations have been focused on production, immune, and endocrine responses under regular production conditions (Goodazi Boroojeni et al., 2018) and a variety of stressful stimulations, such as heat stress (Wang et al., 2018) and *Salmonella* challenge (Abudabos et al., 2019). Therefore, the objective of this study was to investigate the effects of dietary supplementation of *B. subtilis* on bone mass and meat quality of broilers. We hypothesized that the dietary probiotic supplement will improve leg health and related activities as well as meat quality.

MATERIALS AND METHODS

Animals and Housing

Two hundred ten 1-day-old male broiler chicks (Ross 708 strain; Mangabad, Assiut, Egypt) were weighed and allocated to 21 floor pens (10 birds per 100 cm × 100 cm floor pen) with similar average BW in an environment-controlled room (The Animal and Poultry Behavior and

Management Research Unit, the Faculty of Veterinary Medicine, Assiut University, Egypt). Fresh and dry wood shaving as bedding was used at a depth of 10 cm. The bird management was as per the guidelines of Aviagen (2018). Room temperature was gradually decreased from 35°C on day 1 by 0.5°C/d until it reached 26°C, then kept constant until day 35. The RH was approximately 60%. The lighting program was fixed at 30 lx for 23L: 1D until day 3 and then 10 lx for 20L: 4D until day 35 (Mohammed et al., 2018).

All procedures and animal handling were approved by the Animal Care and Use Committee of the Faculty of Veterinary Medicine, Assiut University, Egypt.

Dietary Treatments

The 21 floor pens were randomly allocated to 1 of 3 dietary treatments in 7 replicates of 10 broilers per replicate: a regular diet mixed with the probiotic, *B. subtilis* PB6 (CLOSTAT; Kemin, Europe, NV; Herentals, Belgium) at 0 (control), 0.25 (**0.25X**), and 0.5 (**0.5X**) g/kg feed. The concentrations of CLOSTAT dietary treatments were based on the company's recommendation. The dietary treatment was from day 1 to day 35 when they reached the market weight. The dietary nutrition was provided previously (Mohammed et al., 2018) (Table 1).

Leg Health Indicators

Gait Score, FPD, HB, and Leg Straightness Five broilers per pen (35 birds per treatment) were randomly marked with livestock green spray marker on their backs (Livestock green sharpmark spray paint marker; Cotran Corporation, Portsmouth, Rhode Island). At 33 d of age, the 5 focal broilers' leg strength was examined using a 3-point gait score system (0 = normal gait, 1 = gait with obvious sickness, and 2 = gait with severe sickness) as described previously (Webster et al., 2008; Yan et al., 2018).

A 3-point score system was used to examine FPD injury (0, no footpad lesion; 1, obvious small area injured; and 2, severe large area injured) and HB damage (0, no hock burn; 1, obvious small light lesion; and 2, severe dark-colored lesion). A 2-point score system was used for determining leg straightness (0, legs straight and 1, obvious outward or inward twist at the intratarsal joint) (Rault et al., 2017). For statistical analysis, the proportion of the focal broilers per pen within a score of each test was calculated and expressed as a percentage.

Latency-to-Lie Test At 34 d of age, 2 unmarked broilers per pen were randomly used to examine their leg strength (14 birds/treatment). Briefly, each broiler was put in a bucket filled with 3 cm deep of warm water (28°C). The test was terminated after the bird sat down and touched the water; and the time was recorded. The test was stopped if a broiler still stood after 600 s, and the observation of 600 s was recorded (Berg and Sanotra, 2003).

Sample Collection On day 35, 2 unused broilers were randomly taken from each pen and sedated with sodium pentobarbital (30 mg/mL) for the sample collection (14 birds/treatment). Five milliliter of blood per bird was drawn via cardiac puncture, and then, plasma was collected after centrifugation at 3,000 × *g* for 15 min at 4°C. The plasma samples were kept at −80°C until analyses.

Table 1. Components of a base diet,[1] separated by growth phase.

Ingredient %	Starter (1–14 d)	Grower (15–28 d)	Finisher (29–35 d)
Corn ground	57.66	63.76	66.9
Soybean meal 47.5%	35.27	29.68	26.3
Soybean oil degummed	3	3	3.52
Calcium carbonate	1.41	1.38	1.49
Phosphate monocalcium	1.42	1.02	0.82
L-Lysine	0.11	0.1	0.02
Salt plain	0.48	0.46	0.48
L-Threonine 98%	0.06	0.04	0
DL-Methionine	0.24	0.21	0.12
Poultry turkey starter	0.35	0.35	0.35
Calculated Analysis			
CP %	23.4	22.8	19.2
Poultry ME kcal/kg	3,050	3,151	3,200
Calcium %	0.95	0.85	0.75
Available phosphorus %	0.50	0.44	0.36
Methionine %	0.66	0.59	0.53
Methionine + Cystine %	1.04	0.97	0.86
Lysine %	1.42	1.29	1.09
Threonine %	0.97	0.89	0.74
Na %	0.22	0.20	0.19

[1]The ration formulation was produced as per Aviagen (2018). Provided per kilogram of diet: vitamin A, 13.233 IU; vitamin D3, 6.636 IU; vitamin E, 44.1 IU; vitamin K, 4.5 mg; thiamine, 2.21 mg; riboflavin, 6.6 mg; pantothenic acid, 24.3 mg; niacin, 88.2 mg; pyridoxine, 3.31 mg; folic acid, 1.10 mg; biotin, 0.33 mg; vitamin B12, 24.8 µg; choline, 669.8 mg; iron from ferrous sulfate, 50.1 mg; copper from copper sulfate, 7.7 mg; manganese from manganese oxide, 125.1 mg; zinc from zinc oxide, 125.1 mg; iodine from ethylene diamine dihydroidide, 2.10 mg; selenium from sodium selenite, 0.30 mg.

After the blood collection, the birds were slaughtered as per the traditional Islamic Halal Method (Shahdan et al., 2016): cutting through the jugular veins, bled for 120 s, and then semiscalded at 54°C for 30 s before manual plucking. The birds were eviscerated manually, and the carcasses were washed and allowed to drain for 10 min. After draining, the right leg of each bird was separated as thigh and drumstick and stored at 3°C ± 0.5°C for 30 min, 5 h, and 24 h, by following the traditional farm fresh meat procedure.

Calcium, Phosphorus, and Magnesium Analyses Plasma concentrations of Ca, P, and Mg were measured using the commercial kits (Egyptian Company of Biotechnology, Cairo, Egypt) with a digital spectrophotometer (Cecil instrument, Cambridge, England) by following the manufacturer's instructions.

Bone Morphometric Parameter Analyses The bone morphometric parameters were measured by following the method published previously (Kocabagli, 2001). Briefly, the labeled drumsticks were immersed in boiling water (100°C) for 10 min, and then, the soft tissues and the patella were removed. Next day, the tibiotarsal length, tibiotarsal weight, and the tibiotarsal length/weight index were measured. After the measurements, tibiotarsal breaking strength was estimated at the midshaft using the compression testing (Geotechnical testing equipment; Milton Keynes Buckinghamshire, UK); then, the diaphysis diameter, thickness of the medial wall and the lateral wall, medullary canal diameter, robusticity index, and tibiotarsal index were measured using a digital caliper (Figure 1).

Medullary canal diameter = the diameter at the diaphysis − the thicknesses of the tibia (the distance from the medial and lateral walls) (Patterson et al., 1986).

The bone weight/length index = the tibia weight/the tibial length (Seedor et al., 1991).

Robusticity index = bone length/cube root of bone weight (Reisenfeld, 1972).

Tibiotarsal index = the diaphysis diameter − medullary canal diameter/diaphysis diameter × 100 (Barnet and Nordin, 1960).

Litter Moisture and pH Analyses At the end of the experiment (day 35), the moisture level of litter was detected as a percentage of litter weight lost before and after drying (Toppel et al., 2019). Briefly, the litter samples (4 cm × 4 cm per sample) through the depth of bedding were collected at 5 sites/pen (the 4 corners and the middle, 30 cm away from the drinkers) across all the pens. The pooled litter samples were dried using a hot air oven at 700°C until a constant weight was reached. The moisture content % was calculated as follows: (the wet litter weight–the dried litter weight)/the wet litter weight × 100.

The sampled litter's pH was determined by following the previously published method (Toppel et al., 2019). Briefly, 5 g of pooled samples was mixed with deionized water at 1:10 dilution. After 1 h resting, the pH value of the mixture was determined using an electronic pH meter (Oakton Instruments, Singapore) calibrated with pH 4 and pH 7 buffers. Measurements were performed in triplicate, and the mean was presented.

Meat Quality Analysis

Meat pH The meat pH values at 3 different locations of both the chilled drumsticks and thighs were determined immediately at 30 min and 5 h after slaughter using directly a HI-99163 FoodCare pH Meter (Hanna instruments) that was calibrated with pH 4 and pH 7 buffers. (Pelicano et al., 2003). The mean was presented as the leg meat pH value.

Meat Color The color values of the leg meat from the 5-h postslaughter group were determined using the CIELab Color System (1976) including CIE L*(lightness) a* (redness), and b* (yellowness) (Pelicano et al., 2003). Briefly, 2 random readings were made on each leg muscle including both the drumstick and the thigh with a CR-400 Chroma Meter (Konica Minolta). The mean was calculated for each leg sample as per the American Meat Science Association color measurement guidelines (AMSA, 2012).

Meat Water-Holding Capacity Meat water-holding capacity (**WHC%**) was estimated from the meat 5 h after slaughter group. The estimation was made based on the meat weight loss (%) when a pressure is applied to the muscle (Pelicano et al., 2003). Briefly, approximately 0.5-g cube meat from the same location of each leg sample was placed between 2 filter papers, then added a glass plate at each side. A 10-kg weight was placed on the top glass plate for 5 min. The water loss was calculated as follows: the difference between the muscle weight before and after weight loaded. The results were presented as the percentage of exudate water in relation to the initial sample weight.

Meat Cooking Loss Meat cooking loss (**CL**) of the 5-h postslaughter group was determined using an oven prewarmed to 170°C by following the method published previously (Pelicano et al., 2003). Briefly, the crude leg muscle samples were weighed and put in stainless steel trays with a parchment paper, and then dried in an incubator Mfr. No. SMI2 (Thomas Scientific) for 30 min. The trays were then set inside the oven until internal temperature reached 75°C. The meat temperature was monitored using a data logger (Thomas Scientific). Cooking loss was calculated as the percent difference between the initial and final weight of the samples.

Sensory Analysis Sensory analysis was carried by following the published procedure (Pelicano et al., 2003). Briefly, the test was carried out at 24 h after slaughter. The meat samples were previously treated with 1% (w/w) of salt and then cooked in a prewarmed oven (190°C) until internal temperature reached 75°C. The samples were standardized (codification, size, and tasting temperature) and evaluated by a sensory team. A nine-point hedonic scale was applied to the following parameters: flavor (sensation of smell and taste emitted from the samples during chewing), texture (perception of the strength i.e., necessary to obtain the shearing of the samples when biting), preference (sum of all sensory

perceptions, expressing the evaluation of the quality of the product by the sensory team), and general aspect (ideation of the product). The mean of each sensory portion received from the test team was presented.

Statistical Analysis The experimental design was performed in a randomized block design with the dietary supplement as the fixed effects and pen (n = 7/treatment) considered as the experimental unit. Individual broilers and their interactions with the fixed effect were considered as a random effect. The data collected from the birds of the same pen were checked, and the averaged data were used for the statistical analysis as there was no significant difference. The overall effects of the probiotic dietary inclusion on broilers bone mass and meat quality were analyzed by one-way ANOVA (SAS Institute Inc., Cary, NC). The Tukey-Kramer test was used to compare the means when a significant difference was detected. Statically difference was set $P < 0.05$; and the results were reported as mean ± SE.

RESULTS

Leg Health Indicators

The probiotic effects on leg health indicators are presented in Table 2. The gait, FPD, HB, and leg straightness scores were not affected by the probiotic supplementation regardless of dose levels ($P > 0.05$), but the standing time during the latency-to-lie test was increased in 0.5X group compared with control group ($P < 0.05$), and 0.25X group was at intermediate (0.5X, 0.25X, and control; 86.57, 68.71, and 14.14 s, respectively).

Tibial Morphometric Parameters and Strength

The effect of the probiotic supplement on the tibial morphometric parameters and strength are presented in Table 3. Compared with controls, the tibial length, weight, and strength were significantly higher in the probiotic-fed groups, especially, in the 0.5X birds ($P < 0.001$, 0.05, and 0.01, respectively). There were no treatment effects on the lateral and medial wall thickness, diaphysis and medullary canal diameter, and tibiotarsal and robusticity indexes regardless of probiotic supplement levels ($P > 0.05$).

Calcium, Phosphorus, and Magnesium

The effect of the probiotic on the plasma concentrations of Ca, P, and Mg is presented in Table 4. There were dose-related treatment effects on the measured minerals. Compared with controls, the 0.5X group had the highest plasma concentrations of Ca ($P < 0.01$) and P ($P < 0.01$), while 0.25X group were of intermediate ($P > 0.05$). There were no differences in plasma Mg concentrations between treatments ($P > 0.05$).

Litter Moisture and pH

There were no significant differences in the litter moisture (51.91%, control; 35.05%, 0.25X; and 55.82%, 0.5X) and pH values (8.39, control; 8.32, 0.25X; and 8.13, 0.5X) between treatments ($P > 0.05$).

Meat Quality

The effect of the probiotic on the general sensory analysis outcomes and preference are presented in Table 5. There was a treatment effect on meat taste. Compared with controls, the leg meat from probiotic fed broilers had better outcomes in the general sensory analysis (flavor, texture, preference, and general aspect) at 5 h after slaughter ($P < 0.001$, 0.05, 0.001, and 0.01, respectively).

The effect of the probiotic on leg meat pH, color, WHC %, and CL is presented in Table 6. The pH values were

Table 2. Effect of dietary supplementation of probiotic (CLOSTAT) on leg health profile in broiler chickens.

Treatment	Control	0.25X	0.5X	*P*-value
Latency to lie[1]	14.14^{b} ± 18.82	68.71^{a,b} ± 18.82	86.57^{a} ± 18.82	0.0355
Gait score[2]				
0	71.42 ± 13.8	42.85 ± 13.8	71.42 ± 13.8	0.1101
1	28.57 ± 13.47	40.00 ± 13.47	28.57 ± 13.47	0.4184
2	0.00 ± 5.47	14.28 ± 5.47	0.00 ± 5.47	0.1318
Foot pad dermatitis[2]				
0	42.86 ± 13.57	85.71 ± 13.57	48.57 ± 13.57	0.0785
1	57.14 ± 13.57	14.28 ± 13.57	51.43 ± 13.57	0.0785
2	–	–	–	–
Hock burn[2]				
0	71.43 ± 14.35	34.29 ± 14.35	28.57 ± 14.35	0.0995
1	28.57 ± 14.35	65.71 ± 14.35	71.43 ± 14.35	0.0995
2	–	–	–	–
Leg straightness[2]				
0	100.00 ± 6.93	88.57 ± 6.93	85.71 ± 6.93	0.3274
1	0.00 ± 6.93	11.42 ± 6.93	14.28 ± 6.93	0.3274

Least square means with different superscripts in the same row differ significantly ($P < 0.05$. n = 7).

[1]Latency-to-lie data were collected from 14 birds per treatment.

[2]The data were collected from 35 birds per treatment.

Table 3. Effect of dietary supplementation of probiotic (CLOSTAT) on the tibial morphometric parameters and strength in broiler chickens.

Treatment[1]	Control	0.25X	0.5X	*P* Value
Morphometric parameters				
Tibial length (cm)	9.50[b] ± 0.12	10.72[a] ± 0.12	10.50[a] ± 0.12	0.0001
Tibial weight (g)	16.04[b] ± 1.31	20.02[a,b] ± 1.31	21.37[a] ± 1.31	0.0257
Lateral wall thickness (cm)	0.15 ± 0.01	0.15 ± 0.01	0.17 ± 0.01	0.0658
Medial wall thickness (cm)	0.32 ± 0.09	0.39 ± 0.09	0.23 ± 0.09	0.4869
Diaphysis diameter (cm)	1.25 ± 0.31	1.51 ± 0.31	0.95 ± 0.31	0.4589
Medullary canal diameter (cm)	0.77 ± 0.22	0.96 ± 0.22	0.53 ± 0.22	0.4135
Tibiotarsal index	0.003 ± 0.0003	0.004 ± 0.0003	0.004 ± 0.0003	0.1036
Robusticity index	2.47 ± 0.05	2.47 ± 0.05	2.31 ± 0.05	0.0891
Bone strength				
Tibial strength (kN/s)	0.28[b] ± 0.04	0.39[b] ± 0.04	0.55[a] ± 0.04	0.0016

Least square means with different superscripts in the same row differ significantly ($P < 0.05$. n = 7).
[1]The data were collected from 14 birds per treatment.

reduced in the meat from the probiotic-fed birds at both 30 min and 5 h after slaughter than those of controls ($P < 0.001$ and $P < 0.01$). In addition, compared with controls, the lightness, redness, and yellowness of leg meat were significantly higher in the probiotic-supplemented birds ($P < 0.01$, 0.01, and 0.001, respectively). Water-holding capacity of leg meat was increased in the probiotic-fed birds regardless of probiotic levels compared with controls ($P < 0.001$); consequently, the meat CL was reduced in the probiotic-fed groups although without statistic significant ($P > 0.05$).

DISCUSSION

Leg disorders, including lameness, impair broiler growth, health, and welfare as well as meat quality (Reiter and Bessei, 2009). In addition, compared with slow-growing broilers, fast-growing broilers have lower percentage of bone ash and mineral density (Shim et al., 2012). Recent findings showed that increased colonization of the beneficial bacteria in the gastrointestinal tract improves broiler health and leg disorders (Sobczak et al., 2018; Yan et al., 2018), indicating that gut microbiota play an important role in maintaining hosts' biological homeostasis. These results have brought great enthusiasm for the use of commensal bacteria, such as probiotics and synbiotics, as dietary supplements to improve skeletal health and meat quality in broiler chickens.

The direct link between the probiotic dietary additives and improved bone health and welfare of broilers has been established (Scholz-Ahrens et al., 2007; McCabe et al., 2015). Supporting the hypothesis, the current results suggested that the dietary probiotic supplement, *B. subtills*, improves musculoskeletal health profiles in broilers, evidenced by increased tibial strength, length, and weight with greater availability of blood minerals (Ca and P) and stood much longer during the latency-to-lie test compared with controls, especially in 0.5X probiotic-fed broilers. Similar to the current findings, Yan et al. (2018) reported that a synbiotic-supplemented diet improved several bone parameters including the time spent during the latency-to-lie test as well as gait score, bone density, and bone mineral content in heat-stressed broilers. The synbiotic consisted of a prebiotic (fructooligosaccharides) and a probiotic mixture of 4 microbial strains (*Enterococcus faecium, Pediococcus acidilactici, Bifidobacterium animalis,* and *Lactobacillus reuteri*). The improvement in measured bone mass and related parameters could be attributed to the probiotic effect on the mineral and nutrient resorptions in the intestines. Absence of the differences in the scores of gait, FPD, and HB as well as leg straightness could be linked to the differences in the rearing time and related final BW between the present study and the commercial meat production: 35 d vs. 42 d or longer and 2.3 kg vs. 3.0 kg or heavier (Mohammed et al., 2018).

Bone mineralization plays a vital role in maintaining skeletal health and preventing leg disorders, which can be evaluated by several methods, such as bone weight, breaking strength, and various morphometric measurements including the robusticity and tibiotarsal indexes (Barnet and Nordin, 1960; Onyango et al., 2003). In the present study, the tibial length, weight, and strength were significantly higher in the 0.5X group in comparison with control group, while 0.25X group was at intermediate. Similar to our results, several studies have reported that dietary probiotic supplements, such as

Table 4. Effect of dietary supplementation of probiotic (CLOSTAT) on plasma concentrations of calcium, phosphorus, and magnesium in broiler chickens.

Treatment[1]	Control	0.25X	0.5X	*P*-value
Calcium (mg/dl)	7.49[b] ± 0.22	8.19[a,b] ± 0.22	8.66[a] ± 0.22	0.0048
Phosphorus (mg/dl)	4.95[b] ± 0.19	5.45[b] ± 0.19	6.16[a] ± 0.19	0.0010
Magnesium (mg/dl)	2.12 ± 0.02	2.14 ± 0.02	2.18 ± 0.02	0.0776

Least square means with different superscripts in the same row differ significantly ($P < 0.05$. n = 7).
[1]The data were collected from 14 birds per treatment.

Table 5. Effect of dietary supplementation of probiotic (CLOSTAT) on the general sensory of broiler leg meat.

Treatment[1]	Control	0.25X	0.5X	*P*-value
Flavor	$6.40^{b} \pm 0.29$	$8.40^{a} \pm 0.29$	$8.20^{a} \pm 0.29$	0.0007
Texture	$6.80^{b} \pm 0.24$	$8.00^{a} \pm 0.24$	$7.80^{a} \pm 0.24$	0.0102
Preference	$6.40^{b} \pm 0.29$	$8.40^{a} \pm 0.29$	$8.20^{a} \pm 0.29$	0.0007
General aspect	$6.40^{b} \pm 0.23$	$8.20^{a} \pm 0.23$	$8.60^{a} \pm 0.23$	0.001

Least square means with different superscripts in the same row differ significantly ($P < 0.05$, n = 7).
[1]The data were collected from 14 birds per treatment.

B. licheniformis and *B. subtilis,* increase bone strength and growth in broilers (Plavnick and Scott, 1980; Mutus et al., 2006). The beneficial effect of probiotics on tibial bone growth and strength may be attributed to the increased diet digestibility and resorption of Ca and P by the beneficial bacteria in the gut, which, in turn, increases availability of serum Ca and P for bone formatting and or remodeling (Rizzoli and Biver, 2020). In addition, *B. subtills* may be similar to some other probiotics, such as *Lactobacillus,* with estrogen-like functions in hydrolyzing the glycoside bonds of food starches to increase bioavailability of minerals (Chiang and Pan, 2011; Ng et al., 2016). The hypothesis will be examined in the future studies.

Litter is a mixture of bedding material (such as wood shaving), spilled feed, manure, and feathers. Litter quality greatly affects bird health and welfare (Granquist et al., 2019). The two major factors influencing the litter conditions are moisture and pH, and wet litter serves as a medium for growing of a variety of pathogenic bacteria (Abdel–Mohsein and Mahmoud, 2015). In addition, wet litter is a critical factor associated with FPD and HB in broilers as well as other farm animals (Taira et al., 2014). In the present study, the litter moisture and pH values were not affected by treatments. The similar results have been reported in several previous studies (Da Cruz et al., 2013; Mahardhika et al., 2019). The reasons of the lack of treatment effects are unclear but could be related to the shorter rearing period used in the current experience. In general, litter quality is affected by bird slaughter age as well as group size and density (Kyvsgaard et al., 2013; Cengiz et al., 2018). Compared with a high age at slaughter (42 d or longer) used in commercial poultry meat production, the short rearing time (35 d) may cause less influence on litter quality. This hypothesis will be investigated in a following up study.

In general, leg disorders (such as lameness) lead to considerable physical and biochemical alterations in the skeletal muscle. Commercial fast-growing broilers have a great mean muscle fiber cross-sectional area and a large amount of glycolytic fibers (Dransfield and Sosnicki, 1999). These histologic and biochemical characteristics of muscle fibers are directly linked to meat quality. Modification of diets has become a management strategy for improvement of meat quality in broilers (Delles et al., 2016; Tavaniello et al., 2018). For example, several studies have reported that dietary supplementation of probiotics such as *Clostridium butyricum* (Yang et al., 2010) and *Saccharomyces cerevisiae* (Zhang et al., 2015) in broilers enhances meat attributes, such as general sensory characters, color, pH, CL%, and WHC%. In the present study, the leg muscle lightness (L*), redness (a*), and yellowness (b*) were significantly increased in the probiotic-supplemented groups compared with controls. Similar findings (Wideman et al., 2016; Khan et al., 2018) and conflicting results (Haščík et al., 2015; Stęczny and Kokoszynski, 2019) have been reported previously. The improvement in the leg muscle color revealed in the present study may attribute similarly to the findings in pigs (Han et al., 2020). In that study, the dietary supplementation of the wheat bran significantly increased the muscle myoglobin contents. Myoglobin, as a heme iron–containing protein, gives meat color, consequently increasing the meat shelf-life (Min et al., 2008). Therefore, dietary supplementation of the probiotic in broilers has a positive impact on meat quality by which it affects the consumer acquisitions.

Meat quality is also affected by its pH values during the biochemical reactions, rigor mortis, during the conversion of muscle to meat after slaughter (Sanudo, 1992). Previous studies have reported that the ultimate pH range in broiler meat (breast) is of 5.9 to 6.2 at 15 min after slaughter, while the values at $\leq$ 5.8 are considered pate, soft, and exudative meat, and $\geq$6.3 was for dark, firm, and dry meat (Ristic and Damme, 2013). At 3 h after slaughter, the ultimate pH value is

Table 6. Effect of dietary supplementation of probiotic (CLOSTAT) on the pH, color, water-holding capacity (WHC%), and cooking loss (CL) of broiler leg muscle.

Treatment[1]	Control	0.25X	0.5X	*P*-value
pH				
30 min after slaughter	$6.53^{a} \pm 0.04$	$6.31^{b} \pm 0.04$	$6.20^{b} \pm 0.04$	0.0008
5 h after slaughter	$6.14^{a} \pm 0.09$	$5.66^{b} \pm 0.09$	$5.62^{b} \pm 0.09$	0.0048
Color				
Lightness (L*)	$49.66^{b} \pm 1.22$	$54.36^{a} \pm 1.22$	$56.54^{a} \pm 1.22$	0.0055
Redness (a*)	$17.46^{b} \pm 0.48$	$19.33^{a} \pm 0.48$	$20.72^{a} \pm 0.48$	0.0016
Yellowness (b*)	$7.58^{b} \pm 0.16$	$8.46^{a} \pm 0.16$	$8.98^{a} \pm 0.16$	0.0001
WHC%	$75.20^{b} \pm 1.97$	$89.00^{a} \pm 1.97$	$95.20^{a} \pm 1.97$	0.0001
CL	26.89 ± 3.07	18.04 ± 3.07	15.75 ± 3.07	0.0564

Least square means with different superscripts in the same row differ significantly ($P < 0.05$, n = 7).
[1]The data were collected from 14 birds per treatment.

in the range of 5.7–6.1; while pate, soft, and exudative meat is < 5.7; and dark, firm, and dry meat is > 6.1 (Lesiow et al., 2009). Castellini et al. (2002) recorded the postmortem pH fell from the initiative value 6.18 to ultimate value 5.96 in drumstick muscle at 24 h after slaughter. In the present study, the meat pH values of probiotic-fed broilers are similar to the reported ultimate pH values at both 30 min and 5 h after slaughter. Our findings suggest that the probiotic dietary supplementation in broilers reduces the time needed to reach the ultimate pH range during the rigor mortis process, especially in the 0.5X group. In contrast with the current findings, Pelicano et al. (2003) reported that probiotics ($1.6 \times 10^9 - 10^{10}$ CFU/g *B. subtilis* with or without *B. licheniformis* or *L. reuteri* and *S. cerevisiae* with or without *L. reuteri*) in broilers had no significant effects on pH value of breast muscle at 5 h after slaughter. The current findings could be attributed to the probiotic's functions in inhibiting the subcutaneous and intramuscular fat degradation, by which it influences both the meat pH, color, and taste (Karaoglu et al., 2004; Abreu et al., 2019).

The three major sensory properties (texture, flavor, and general aspect) interfered with the meat quality are the most important factors for consumer perceptions of meat choice (Liu and Stouffer, 1995; Gray et al., 1996). The present study showed that the probiotic supplementation improves the sensory properties of the leg meat. Similarly, several studies have revealed that dietary probiotic supplements contained *Lactobacillus acidophilus* and *Streptococcus cerevisiae* (Khan et al., 2018) or *B. licheniformis* and *B. subtilis* (Jensen and Jensen, 1992) improve broiler meat quality and sensory properties after cooling for 5 d. In contrast, other studies have reported there were no effects on breast meat sensory properties from broilers fed diets contained *Lactobacillus* spp., *Bifidobacterium* spp., *Lactococcus* spp., *Streptococcus thermophilus, B. subtilis, Rhodopseudomonas* spp. and *S. cerevisiae* yeast (Stęczny and Kokoszynski, 2019) as well as *E. faecium* with or without avoparcin (Loddi et al., 2000). The reasons for improved sensory properties of the leg meat of probiotic-fed broilers are unclear but could be related to the probiotic involving in lipid metabolism by preventing the storage-induced oxidative stress (Bai et al., 2016), leading to a high ratio between unsaturated fatty acids and saturated fatty acids (Endo and Nakano, 1999; Wang et al., 2017). In addition, it could be associated with the mechanisms previously reported (Zhu et al., 2009). In that study, the probiotic, *Lactobacillus*, played a role in conversing muscle fat to favorable fat and improved visual appearance through increased xanthophyll accumulation in the soft tissues of broilers' thigh muscles. However, this hypothesis needs to be examined in the future studies.

Water-holding capacity and CL are the 2 major meat quality factors closely related to its tenderness. Tenderness is one of the most important sensory characteristics of meat (Pelicano et al., 2003). The current outcomes reveal that the probiotic diet increases WHC% but trend decreases CL in broiler leg meat, by which it enhances retaining the meat moisture and related tenderness. Similarly, Park and Kim (2014) reported that a dietary probiotic supplement (*B. subtilis* B2A) improved WHC % in breast meat of grower chicks. The current and previous findings that increased meat WHC% could be another benefit of probiotic dietary supplements as that meat WHC% is influenced by the amount of muscle proteins (Filho et al., 2017). In addition, probiotic-fed broilers may have improved intramuscular fat content in the leg muscle. To support the hypothesis, a previous study reported that probiotic (*C. butyricum*) increases the levels of omega-3 fatty acids, especially docosahexaenoic acid and eicosapentaenoic acid, in broiler breast muscle. The changes in the fatty acid composition are associated with improved WHC% and contributed to meat tenderness (Yang et al., 2010).

CONCLUSION

The current results suggest that the probiotic supplement, especially at the 0.5 g/kg level, improves bone mass and meat quality in broilers. Compared with controls, probiotic-fed broilers stood much longer during the latency-to-lie test and had higher tibial physical parameters (length, weight, and strength) and blood mineral (Ca and P) concentrations. Probiotics also had favorable effects on both leg meat color and WHC% with a trend for less CL, resulting in greater sensory properties. The probiotic also enhanced meat pH to reach ultimate values at both 0.5 and 5 h after slaughtering. Overall, the current findings indicate that feeding probiotics could be a management strategy for improvement of broiler skeletal health and meat quality to meet growing demand for poultry meat products.

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DISCLOSURES

The authors declare no conflicts of interest.

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