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Bacillus subtilis based probiotic improved bone mass and altered brain serotoninergic and dopaminergic systems in broiler chickens

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ABSTRACT

The aim of the study was to assess the effects of dietary supplementation of a *Bacillus subtilis* based probiotic on broiler bone health. After 43 days, probiotic fed broilers had greater bone mineralization, wall thickness, size, and weight of tibias and femurs compared to the controls. We further found that probiotic fed broilers also had higher serum calcium levels at day 14 and a trend of lower serum c-terminal telopeptide of type I collagen levels, a bone resorption indicator, at day 43. Moreover, the level of serotonin was increased in the raphe nuclei, whereas the concentrations of norepinephrine and dopamine were decreased in the hypothalamus of broilers fed probiotic at day 43. These results indicate that dietary supplementation of a *Bacillus subtilis* based probiotic improves broiler bone traits, most likely through increased calcium intestinal absorption and reduced bone resorption by inhibiting sympathetic activity via the central serotonergic system.

1. Introduction

Skeletal disorders are commonly seen in domestic poultry. Due to its negative effects on the locomotor system, resulting in impaired mobility or lameness, leg disorder in broilers is one of the most significantly welfare and productivity issues facing the poultry meat industry. Over 27.6% of broilers are estimated to exhibit poor locomotion in the United Kingdom (Knowles et al., 2008), with a range from 14.1% to 30.1% in other European countries (Sanotra, Lund, Ersboll, Petersen, & Vestergaard, 2001; Sanotra, Berg, & Lund, 2003). Genetic selection for rapid growth rate and great breast muscle deposition is likely a contributor to lameness and musculoskeletal damage (Talaty, Katanbaf, & Hester, 2009). The effects of an uneven load and overweight on developing bones result in a high incidence of leg abnormalities causing lameness (Kestin, Gordon, Su, & Sorensen, 2001). When adjusted for body weight, fast-growing broilers have both lower tibia density and lower percentage of bone ash than slow-growing broilers (Shim et al., 2012) as a consequence of less mineralization and higher porosity (Williams, Waddington, Murray, & Farquharson, 2004).

The effect of serotonin (5-HT) on bone health is depended on its sources. Serotonin is synthesized in the brain as well as in the gut, the latter produces the majority (approximately 95%) of total 5-HT in the body (El-Merahbi, Loffler, Mayer, & Sumara, 2015). Peripheral 5-HT is stored in the platelets and cannot cross over the brain-blood barrier from bloodstream to the brain (Mann et al., 1992), creating 2 separated compartments with different functions. Brain 5-HT is synthesized in the raphe nuclei and, acting as a neurotransmitter, stimulates bone formation and inhibits bone resorption, causing an increase in bone mass; whereas peripheral 5-HT, acting as a hormone, inhibits bone formation, resulting in a reduction of bone mass (Ducy and Karsenty, 2010). Gut-derived 5-HT that directly regulates bone metabolism is depended on its receptors. Specifically, when the 5-HT_{1B} receptor is blocked, bone mass, the number of osteoblasts, and bone formation are increased. Yadav et al. (2008) revealed the inhibitory effect of 5-HT on osteoblast proliferation through 5-HT_{1B} receptor, as *5-HT$_{1B}$-/-* mice displayed increased bone mass and bone osteoblast numbers. Besides, intracellular 5-HT is essential in osteoclast differentiation (Battaglino et al., 2004). Bone resorption in *Tph1-/-* mice (*Tph1*; the key enzyme for 5-HT synthesis in the gut) were markedly decreased, whereas addition of 5-HT rescued osteoclastogenesis in cultured bone cells derived from *Tph1-/-* mice (Chabbi-Achengli et al., 2012). These results may explain, at least partially, why patients treated with some antipsychotic drugs, e.g., 5-HT reuptake inhibitors, have low bone mass of their hips with a high risk of osteoporotic fractures (Gebara et al., 2014). In the brain, via binding to 5-HT_{2C} receptors on the ventromedial hypothalamic neurons, 5-HT stimulates bone mass through reduction of sympathetic activity (Yadav et al., 2009). Activated sympathetic system negatively regulate bone mass via releasing norepinephrine (NE) which binds to

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β2-adrenergic receptors expressed on osteoblasts and osteocytes (Bonnet et al., 2008; Elefteriou et al., 2005; Kajimura et al., 2011). The activated β2-adrenergic receptors on bone cells subsequently trigger a series of signaling pathways leading to inhibition of osteoblast proliferation (Fu, Patel, Bradley, Wagner, & Karsenty, 2005) and an increase in osteoclast formation (Bonnet et al., 1985, 2005; Niedermair et al., 2014).

Probiotics are live microorganisms which confer health benefits, including improvements of bone in humans and experimental animals (McCabe, Britton, & Parameswaran, 2015; Parvaneh, Jamaluddin, Karimi, & Erfani, 2014; Scholz-Ahrens et al., 2007) when administered in appropriate amounts. Probiotics have been proposed to increase skeletal health via nutrient acquisition improvement, immune regulation, and or hormonal regulation (McCabe et al., 2015; Charles, Ermann, & Aliprantis, 2015; Weaver, 2015; Ohlsson and Sjogren, 2015; Hernandez, Guss, Luna, & Goldring, 2016). Regulation of 5-HT has also been proposed to be one of the possible mechanisms involved in probiotic-based improvement of bone in humans and rodents (McCabe et al., 2015; Charles et al., 2015). However, no study has been conducted to determine the possible role of 5-HT on probiotic-associated bone improvement in broilers. The objective of the present experiment was to investigate the effect and mechanism of a *Bacillus subtilis* based probiotic on broilers' bone health. We hypothesized that probiotic supplementation would improve bone traits in broilers through regulating mineral bioavailability, 5-HT synthesis, immune cytokines, or combinations thereof.

2. Materials and methods

2.1. Birds, management, and sample collection

A total of one hundred and twenty day-old Ross 708 male broiler chicks were obtained from a commercial hatchery (Miller Poultry, Orland, IN). Chicks were weighed and placed into 24 floor pens (152 cm × 81 cm) ensuring similar average body weight across the pens. There were 5 chicks per pen resulting in a stocking density of 2462 cm^2 per broiler. Wood shavings about 5 cm thickness was used as litter. Each pen was equipped with 1 hanging feeder and drinker. Room temperature was gradually decreased from 35 °C on day 1 to 21 °C by 0.5 °C/day and maintained at 21 °C for the rest of the experimental period. The lighting program was gradually decreased from 23 light:1 dark (0100–0200 h) at 30 lx during the first 7 day of age to 20 light:4 dark (0100–0500 h) at 10 lx until 44 day of age. Pens were assigned to 2 dietary treatments with 12 replicate floor pens per treatment: regular diet and the diet mixed with 250 ppm probiotic, resulting in 1.0×10^6 spores/g of feed (Sporulin, Novus International, Inc., Saint Charles, MO, US). The probiotic consisted of 3 strains of *Bacillus subtilis*. The dose of probiotic was recommended by the company, and the regular diets were formulated as the recommendations of Aviagen (2014). Birds were fed a starter, grower, and finisher diet from 1 to 14, 15 to 28, and 29 to 44 days of age, respectively (Table 1). Feed and water were provided *ad libitum*. Prior to the experiment, all the diets were prepared and sampled for bacterial analysis to ensure the diets were mixed properly. The husbandry and the following procedures were approved by the Purdue Animal Use and Care Committee (PACUC Number: 1111000262).

At 14 days of age, 1 bird per pen (n = 12) was weighted then sedated using intravenous administration of sodium pentobarbital (30 mg/kg of body weight) followed by blood collection via cardiac puncture. A total of 8 mL blood was collected from each bird; 5 mL were placed into ice cooled EDTA-coated plasma tube and 3 mL were placed into a serum tube. The bird was euthanized immediately after bleeding by cervical dislocation. Following euthanized, the left tibia and femur were removed from the chicken and placed in individual plastic bags and kept at −20 °C until assayed. At 43 days of age, the samples of plasma, serum, and bones (n = 7 for the probiotic group and n = 8 for the control group) were collected again as previously described. The hypothalamus and raphe nuclei were additionally collected and immediately frozen on dry ice and stored at −80 °C until assayed.

Table 1
The composition of the starter, grower, and finisher diets.

	Starter	Grower	Finisher
Ingredient, %			
Corn	52	52.3	62.8
Soybean meal	40	39.1	29.7
Soy oil	3.59	4.97	4.11
Sodium chloride	0.51	0.46	0.43
DL Methionine	0.3	0.24	0.23
L-Lysine HCL	0.13	- - -	0.07
L-Threonine	0.06	- - -	- - -
Limestone	1.29	1.15	1.12
Monocalcium phosphate	1.75	1.48	1.17
Vitamin/mineral premix[a]	0.35	0.35	0.35
Calculated analyses			
Crude protein %	23.4	22.8	19.2
Poultry ME kcal/kg	3050	3151	3200
Calcium %	0.95	0.85	0.75
Available phosphorus %	0.5	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
Sodium %	0.22	0.2	0.19

[a] Provided per kilogram of diet: vitamin A, 13,233 IU; vitamin D3, 6636 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.

2.2. Bone measurements

The bone mineral density (BMD), bone mineral content (BMC), and bone area of the tibia and femur were measured using dual energy x-ray absorptiometry (Norland Medical Systems Inc., Fort Atkinson, WI, US) following a previously described procedure (Hester et al., 2013). After scanning, all the bones were boiled for 5 min to remove muscles, connective tissues, epiphyseal caps, and the fibula (Hall et al., 2003). The bones were air dried overnight at room temperature and then were weighed individually, and the length, width, and cortical bone thickness on the medial and lateral sides of each bone were determined using a digital micrometer (Coolant Proof Micrometer Series 293, Mitutoyo America Corp., Aurora, IL, US). Traditional bone density indicators of robusticity index and bone weight to length index were also calculated (Riesenfeld, 1972; Seedor, Quartuccio, & Thompson, 1991). The higher bone density was indicated by the higher weight to length index but lower robusticity index.

Latency to lie test was performed (14 broilers of probiotic group and 16 broilers of control group) at 44 days of age following the procedure descripted by Berg and Sanotra (Berg and Sanotra, 2003). Briefly, each bird was individually placed into a tub filled with 3 cm water at 28 °C. The length of time taken for the bird to sit down and touch the water was recorded. If the broiler was still standing after 600 s, the test was stopped and the data was retained for statistical analysis.

2.3. Enzyme linked immunosorbent assay (ELISA)

Commercial ELISA kits (MyBioSource, San Diego, CA, US) were used for detecting serum levels of osteocalcin (OC), a bone formation indicator, and c-terminal telopeptide of type I collagen (CTX), a bone resorption indicator. The serum calcium (Ca) and phosphate (Pi) levels were determined by using QuantiChrom kits (BioAssay Systems,

Hayward, CA) followed the manufacturer's instructions.

Plasma concentrations of 5-HT, tryptophan (TRP), interleukin (IL)-6, IL-10, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) were measured by using commercial ELISA kits (MyBioSource, San Diego, CA, US) followed the manufacturer's instructions.

2.4. High performance liquid chromatography (HPLC)

Serotonin and dopamine (DA) as well as their metabolites of the hypothalamus and raphe nuclei from the left hemisphere of the brain were analyzed using HPLC (UltiMate™ 3000 RSLCnano System, Thermo Fisher Scientific Inc., Waltham, MA, US). Each brain region was weighed and homogenized in ice-cold 0.2 M perchloric acid at a 10:1 ratio (μL of perchloric acid:mg of sample). The homogenized mixtures were centrifuged at 18,187*g* for 15 min at 4 °C. Each resultant supernatant was drawn into microcentrifuge tube and diluted 1:1 with mobile phase (MD-TM, Thermo Fisher Scientific, Waltham, MA, US). The mixtures were centrifuged again at 18,187*g* for 15 min at 4 °C. Each supernatant was draw off and filtered through a 0.2-μm polyvinylidene fluoride filter into an HPLC sample vial. The mobile phase flow rate was 0.8 mL/min. A MD-150 column (3.2 mm × 150 mm, 3 μM C18; Thermo Fisher Scientific, Waltham, MA, US) was used. The concentrations of 5-HT, TRP, 5-hydroxyindoleacetic acid (5-HIAA), DA, NE, epinephrine (EP), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were calculated from a reference curve made by using relative standards. The DOPAC/DA and the HVA/DOPAC turnover ratios were calculated as indexes of dopaminergic activities (Badruzzaman, Bapary, & Takemura, 2013; Bast, Diekamp, Thiel, Schwarting, & Gunturkun, 2002).

2.5. Radioimmunoassay (RIA)

Plasma concentrations of corticosterone were measured using a commercial ^{125}I corticosterone RIA kit (MP Biomedicals, Orangeburg, NY, US) followed a method described previously (Cheng, Dillworth, Singleton, Chen, & Muirt, 2001).

2.6. Statistical analysis

A 2-way ANOVA of the mixed model procedure of SAS 9.4 software (SAS Institute Inc., Cary, NC) was used in the data analysis for bone traits. Probiotic treatment and age of the broilers were the fixed effects. A one-way ANOVA was used to analyze the rest data with probiotic treatment as the fix effect. The experiment unit was the cage. Transformation of data was performed for normality when variances were not homogeneous (Steel, Torrie, & Dickey, 1997). Statistical trends were similar for both transformed and untransformed data; therefore, the untransformed least square means and S.E.M. were presented. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Bone traits, mineralization and lameness

To explore the effects of probiotic on bone, we measured bone traits and mineralization of tibia and femur in broilers at both 14 and 43 days of age. We demonstrated that bone traits, including bone weight, area, size and density, showed significant improvements in probiotic fed broilers ($P < 0.03$ or lower, Tables 2 and 3). Meanwhile, age was a significant factor for all the bone parameters we measured ($P < 0.0001$, Tables 2 and 3). There was age by treatment interaction for all the bone traits except for robusticity index ($P < 0.05$ or lower, Tables 2 and 3). In details, compared to controls, probiotic fed broilers had significantly heavier tibia and femur, higher weight/length index and lower robusticity index in both tibia and femur at 43 days of age. In addition, probiotic fed broiler showed significantly larger tibia and femur compared to controls at 43 days of age, as indicated by higher bone area, length and width. For bone mineralization, BMC and BMD of both tibia and femur were all higher in broilers with probiotic supplementation. However, no significant differences was found in bone traits at 12 days of age. We further assessed bone wall thickness and found that probiotic fed broilers had significantly greater tibia lateral wall thickness and femur medial wall thickness at 43 days of age ($P = 0.05$ and 0.04, respectively; Fig. 1). Broiler lameness by using latency to lie test was additionally performed at 44 days of age. Probiotic fed broilers showed a 34% more increase in the time spent standing, but this did not reach statistical significance ($P = 0.58$, Fig. 2).

3.2. Blood mineral and bone remodeling

In order to investigate how probiotic improved bone mass in broilers, we first measured serum Ca and Pi, which are components of bone in the form of hydroxyapatite. We found that serum Ca levels were higher in probiotic fed broilers at 14 but not at 43 days of age compared to controls ($P = 0.05$ and 0.64, respectively; Fig. 3A). Meanwhile, serum Pi levels were unaffected by probiotic supplementation at both ages ($P = 0.20$ and 0.47, respectively; Fig. 3B). We additionally measured OC and CTX to explore the effect of probiotic on bone remodeling. Probiotic supplementation tended to have lower bone resorption as indicated by the lower of serum CTX levels ($P = 0.08$, Fig. 4B). There were no probiotic effects on serum concentrations of OC ($P = 0.58$, Fig. 4A).

3.3. Plasma cytokines, corticosterone and serotonin

We further explored the effect of probiotic on plasma cytokines that play an important role in bone mineralization. Of the cytokines measured, neither pro-inflammatory (IFN-γ, TNF-α, and IL-6) nor anti-inflammatory (IL-10) cytokines were affected by probiotic supplementation ($P > 0.05$, Table 4). Corticosterone is another hormone that regulates bone mass. In the present study, there was no difference of plasma corticosterone levels between the groups ($P > 0.05$, Table 4). Furthermore, plasma 5-HT as well as its precursor TRP were similar between the groups ($P > 0.05$, Table 4), which is reported to reduce bone mass.

3.4. Brain 5-HT, catecholamines and their metabolites

To explore the effect of probiotic on brain monoamines, concentrations of 5-HT, catecholamines and their metabolites were measured in the raphe nuclei and hypothalamus. We revealed that compared to controls, probiotic fed broilers had higher concentrations of central 5-HT in the raphe nuclei ($P = 0.04$; Table 5), where is the major location for 5-HT synthesis in the brain. This upregulation was not found in the hypothalamus ($P > 0.05$, Table 6), and there was no any change of brain TRP concentrations in both the raphe nuclei ($P > 0.05$, Table 5) and hypothalamus ($P > 0.05$, Table 6). Hypothalamic concentrations of DA and NE were dramatically lower and accompanied by higher turnovers of DA (expressed as DOPA/DA) and DOPAC (expressed as HVA/DOPAC) in probiotic fed broilers ($P = 0.02$ or lower; Table 6). However, the concentrations of catecholamines, their metabolites, as well as turnover indices in the raphe nuclei were not affected by the probiotic supplementation ($P > 0.05$, Table 5).

4. Discussion

There are several studies conducted in broilers indicating the positive effects of probiotics on bone health (Houshmand et al., 2011; Mutus et al., 2006; Panda, Rao, Raju, & Sharma, 2006; Sadeghi, 2014; Ziaie et al., 2011). These studies focus on the measurements of the tibia, most likely due to its proneness to deformities such as valgus/varus

Table 2
The effects of probiotic and age on broiler tibia traits.

	Weight (g)	R. weight (g/kg)	Area (cm^2)	Length (mm)	Width (mm)	W/L index (mg/mm)	R index (g,cm)	BMD (g/cm^2)	BMC (g)
Age (day)									
14	0.62	2.22	3.90	45.17	3.49	13.76	5.32	0.07	0.26
43	7.66	3.21	13.32	92.37	9.44	82.31	4.73	0.18	2.41
S.E.M.	0.22	0.08	0.30	0.67	0.13	2.03	0.05	0.00	0.06
P-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Treatment									
Control	3.54	2.58	7.88	67.38	5.89	42.53	5.14	0.11	1.13
Probiotic	4.74	2.85	9.35	70.16	7.04	53.55	4.91	0.13	1.53
S.E.M.	0.22	0.08	0.30	0.68	0.13	2.04	0.05	0.00	0.06
P-value	0.0005	0.03	0.002	0.006	< 0.0001	0.0005	0.001	< 0.0001	< 0.0001
Age x treatment									
14-Control	0.57	2.25	3.68	44.88	3.27	12.56	5.45	0.06	0.22
14-Probiotic	0.68	2.19	4.12	45.47	3.71	14.97	5.19	0.07	0.29
43-Control	6.52[b]	2.92[b]	12.07[b]	89.88[b]	8.52[b]	72.49[b]	4.82	0.17[b]	2.03[b]
43-Probiotic	8.80[a]	3.51[a]	14.57[a]	94.86[a]	10.36[a]	92.12[a]	4.63	0.19[a]	2.78[a]
S.E.M.	0.31	0.12	0.43	0.95	0.19	2.86	0.06	0.00	0.08
P-value	0.002	0.008	0.02	0.03	0.0008	0.005	0.58	0.03	0.0001

R. weight: relative weight (bone weight/body weight); W/L index: weight to length index; R index: robusticity index; BMD: bone mineral density; BMC: bone mineral content.
The data were presented as least square mean ± S.E.M. N equals to 12 for all groups at day 14, and equals to 7 for the probiotic group and 8 for the control group at day 43.
[a,b] Different superscripts indicate a significant difference ($P < 0.05$).

angulations, osteodystrophy, and dyschondroplasia, causing poor walking ability. The improved tibia traits as a result of providing probiotic supplementations include the changes of tibial weight, size, wall thickness, tibiotarsal index, ash Ca and phosphorus percentage, and breaking strength. In line with the previous findings, the current results indicated that probiotic (*Bacillus subtilis)* dietary supplementation improved both tibia and femur bone traits in broilers, but the effect was not profoundly evidenced until market age 43 days. Due to the short life cycle of broilers, providing probiotics at hatch or possibly even earlier through *in ovo* feeding prior to hatch may be essential to allow establishment and growth of beneficial microbiota, providing the necessary time for the favorable physiological effects to take effect. The improved bone traits in the probiotic group, however, did not improve the outcomes of latency to lie test, which is designed to assess lameness associated leg strength in broilers (Berg and Sanotra, 2003; Weeks et al., 2002). Probiotics, Biomin PoultryStar (*Enterococcus faecium*, *Bifidobacterium animalis*, *Pediococcus acidilactici*, and *Lactobacillus reuteri*) or Biomin IMBO (*Enterococcus faecium* DSM 3530), show promise in improving walking ability in broilers with wire-flooring housing induced bacterial chondronecrosis with osteomyelitis; and broilers fed probiotics beginning at 1 day of age have a lower incidence of lameness (Wideman et al., 2012).

Probiotics alter the composition and metabolic activity of the gut microbiota (Ohlsson et al., 2014). Intestinal integrity and microstructure, as indicated by increased villi height, absorptive area, and the secretion of the lubricant and mucin, are enhanced by probiotics (Thanh, Loh, Foo, Hair-bejo, & Azhar, 2009). A more favorable intestinal environment with optimum pH improves nutrient digestibility, retention, and absorption. For example, the organic matrix of bone would benefit from increased absorption of amino acids as building blocks for collagen and non-collagenous proteins (Guzyk, Sergiichuk, Dyakun, Yanitska, & Kuchmerovska, 2014). Likewise, bone mineralization would gain from the increased availability of plasma minerals such as Ca and Pi as well as vitamins such as cholecalciferol.

Table 3
The effects of probiotic and age on broiler femur traits.

	Weight (g)	R. weight (g/kg)	Area (cm^2)	Length (mm)	Width (mm)	W/L index (mg/mm)	R index (g,cm)	BMD (g/cm^2)	BMC (g)
Age (day)									
14	0.45	1.59	2.94	34.45	3.56	12.94	4.54	0.06	0.18
43	5.55	2.32	10.01	69.86	9.03	78.81	3.99	0.15	1.49
S.E.M.	0.17	0.06	0.24	0.57	0.12	2.03	0.05	0.00	0.03
P-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Treatment									
Control	2.48	1.83	5.97	51.17	5.76	39.42	4.38	0.10	0.71
Probiotic	3.52	2.08	6.98	53.14	6.83	52.33	4.15	0.11	0.95
S.E.M.	0.17	0.06	0.24	0.57	0.12	2.05	0.05	0.00	0.03
P-value	< 0.0001	0.009	0.0065	0.02	< 0.0001	< 0.0001	0.004	0.0002	< 0.0001
Age x treatment									
14-Control	0.41	1.63	2.79	34.33	3.31	11.93	4.64	0.06	0.16
14-Probiotic	0.48	1.54	3.10	34.57	3.81	13.95	4.44	0.06	0.20
43-Control	4.55[b]	2.03[b]	9.16[b]	68.00[b]	8.21[b]	66.91[b]	4.12	0.14[b]	1.27[b]
43-Probiotic	6.55[a]	2.61[a]	10.86[a]	71.71[a]	9.85[a]	90.72[a]	3.87	0.16[a]	1.71[a]
S.E.M.	0.23	0.09	0.34	0.80	0.17	2.87	0.07	0.00	0.04
P-value	0.0002	0.0007	0.05	0.04	0.002	0.0006	0.74	0.05	< 0.0001

R. weight: relative weight (bone weight/body weight); W/L index: weight to length index; R index: robusticity index; BMD: bone mineral density; BMC: bone mineral content.
The data were presented as least square mean ± S.E.M. N equals to 12 for all groups at day 14, and equals to 7 for the probiotic group and 8 for the control group at day 43.
[a,b] Different superscripts indicate a significant difference ($P < 0.05$).

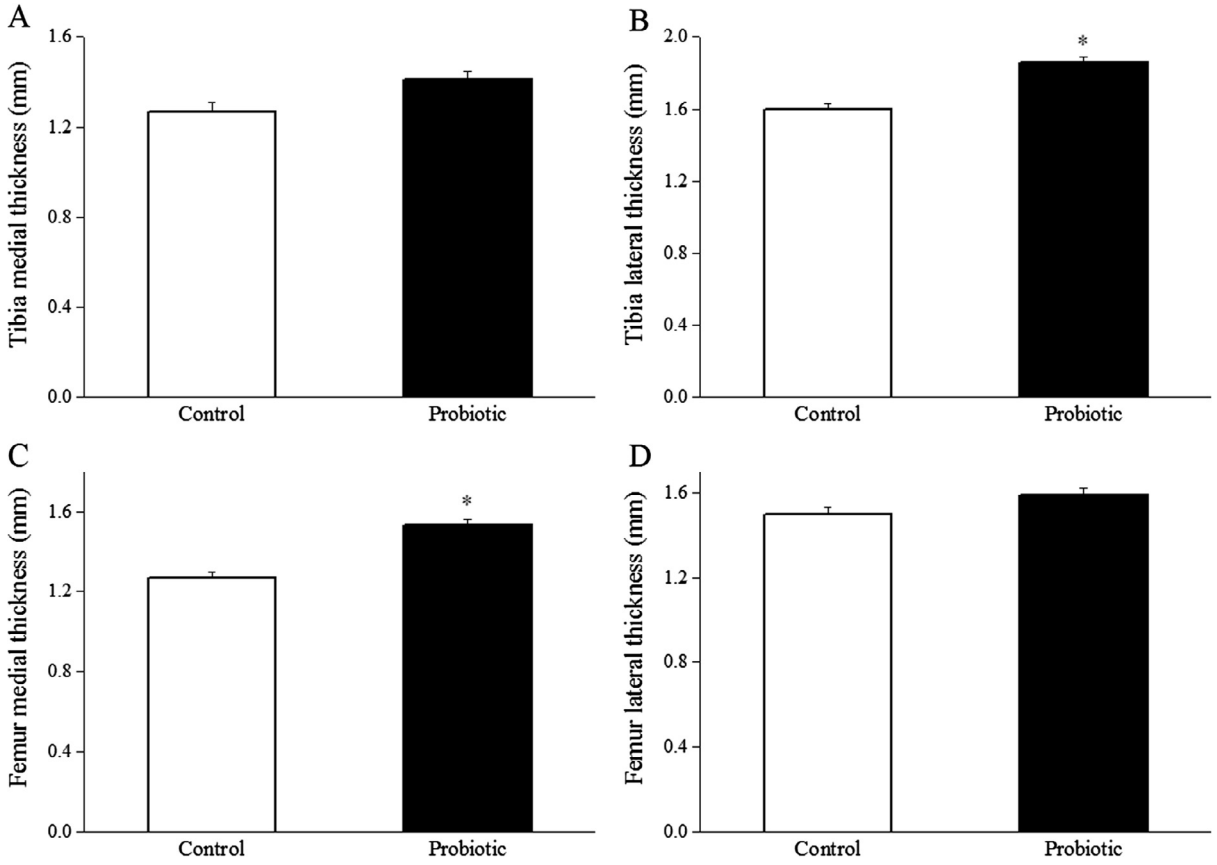

Fig. 1. Effects of probiotic supplementation on bone wall thickness of broilers at 43 days of age. A, tibia medial thickness; B, tibia lateral thickness; C, femur medial thickness; and D, femur lateral thickness. The data were presented as least square mean ± S.E.M. N equals to 7 for the probiotic group and 8 for the control group. *Different from control, $P < 0.05$.

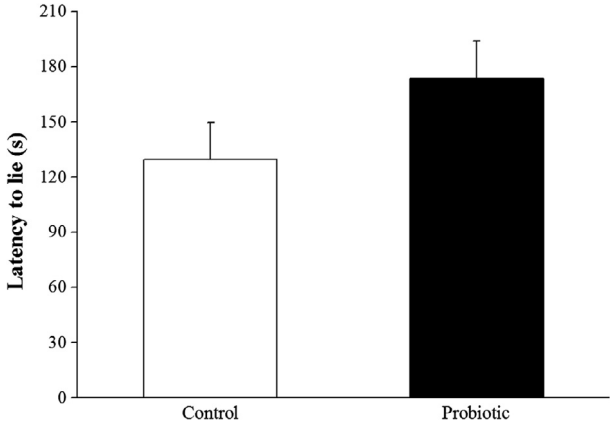

Fig. 2. Effects of probiotic supplementation on latency to lie test of broilers at 44 days of age. The data were presented as least square mean ± S.E.M. N equals to 7 for the probiotic group and 8 for the control group.

Osteoblasts synthesize and release OC, as an indicator of bone formation, to facilitate bone building and mineralizing. Another role for OC is to assist with maintaining Ca homeostasis indirectly through the recruitment of osteoclasts and osteoblasts (Hoang, Sicheri, Howard, & Yang, 2003). Although in the current study circulating levels of Ca increased only in 14-d-old broilers but not in 43-d-old broilers and OC concentrations at 43 days of age were unaffected, it is hypothesized that bone sequestered the increased availability of intestinal Ca and Pi for building its hydroxyapatite matrix as evidenced by the dramatic increased BMD in 43-d-old broilers fed probiotics. As a result of increased bone mineral accrual, circulating concentrations of Ca and Pi may remain at consistent levels even though probiotics most likely enhance mineral absorption at the level of the intestines. Increased solubility and absorption of minerals have been associated with probiotic-induced bone health benefits (Scholz-Ahrens et al., 2007). *Bacillus subtilis* enhances utilization of Ca, most likely due to the increase in lactic acid production from proliferating *Lactobacilli* (Guo, Li, Lu, Piao, & Chen, 2006; Hosoi, Ametani, Kiuchi, & Kaminogawa, 2000).

Besides increased availability of minerals and nutrients needed for bone formation, other pathways, such as reduced bone resorption, may also be involved in increasing bone mass found in probiotic fed broilers. In clinic, serum CTX has been used as a biomarker to evaluate bone turnover in patients with diabetes- and obesity-associated bone damage as that circulating concentrations of CTX are positively correlated to osteoclastic activity (Biver, 2012). This specific peptide is cleaved from collagen by osteoclasts during bone resorption, so a lower level of CTX suggests reduced osteoclast activity (Florencio-Silva, Sasso, Sasso-Cerri, Simoes, & Cerri, 2015). In the current study, bone resorption may have been inhibited or reduced in the probiotic fed broilers as suggested by the lower serum CTX levels compared to the controls.

In rodents, one of the proposed mechanisms of gut microbiota regulating bone mass is through the down regulation of pro-inflammatory cytokines via the gut-blood-bone axis (Sjogren et al., 2012), as that pro-inflammatory cytokines involve in osteoclast formation (de Vries, Yousovich, Schoenmaker, Scheres, & Everts, 2016; Yokota et al., 2014). For example, reduced TNF-α mRNA levels in the jejunum and ileum were accompanied by increased trabecular bone mass in healthy male mice fed *Lactobacillus reuteri* for 4 weeks (McCabe, Irwin, Schaefer, & Britton, 2013). On the other hand, cytokines such as INF-γ and IL-10, as anti-inflammatory cytokine, inhibit osteoclastogenesis (Pappalardo & Thompson, 2013; Takayanagi et al., 2000; Zhang et al., 2014). In the

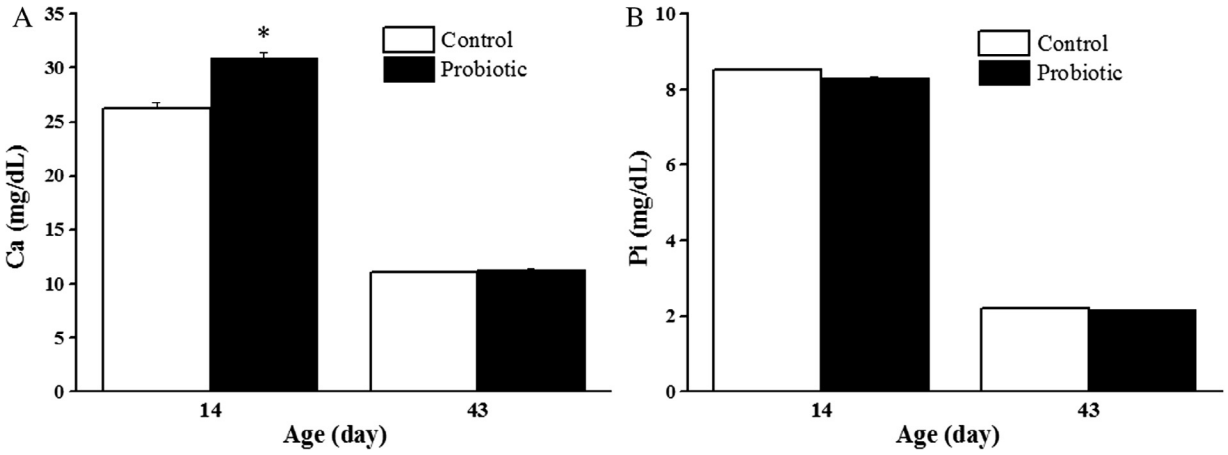

Fig. 3. Effects of probiotic supplementation on serum Ca (A) and Pi (B) of broilers at different ages. The data were presented as least square mean ± S.E.M. N equals to 12 for all groups at day 14, and equals to 7 for the probiotic group and 8 for the control group at day 43. *Different from control at the same age, $P < 0.05$.

current study, plasma levels of IFN-γ, IL-10, IL-6, and TNF-α in the probiotic fed broilers were similar to those in the control group, indicating that the bone promoting effect of probiotic may not be through the regulation of systemic inflammation. Limited studies have been conducted on the effect of *Bacillus subtilis* on systemic immune cytokine levels with some studies focused on the local effects such as gut and spleen cytokine expression. Dietary inclusion of a probiotic contains 3 *Bacillus subtilis* strains (Enviva Pro, Danisco Animal Nutrition, UK) for 28 days did not affect mRNA expression of IFN-γ and IL-10 in the gut (pooled jejunal and ileal samples) of Ross 708 broilers (Lee, Lillehoj, Jang, & Lee, 2014). In contrast, reduced ileal IL-6 as well as splenic IL-6 and IL-10 transcripts in Ross 308 broilers were found after using the same probiotic product for 22 d, suggesting a role for the probiotic in suppressing inflammation (Waititu et al., 2014). Ducks also showed cytokine responses after consuming a single-strain of *Bacillus subtilis* for 63 d; specifically, jejunal IFN-γ expression was higher but ileal IL-10 expression was lower (Xing et al., 2015). Unfortunately, bone traits were not measured in these studies.

Corticosterone is the major avian glucocorticoid, which has biphasic effects on bone development and health (Mak, Shao, Dunstan, Seibel, & Zhou, 2009). A normal level of endogenous glucocorticoids promotes the differentiation of mesenchymal progenitor cells to the osteoblast lineage rather than to the adipocyte and chondrocyte lineages through regulating Wnt signaling (Zhou, Mak, Zheng, Dunstan, & Seibel, 2008). In contrast, excessive glucocorticoids, especially when administered exogenously, profoundly suppress bone formation by inhibiting osteoblast differentiation and inducing osteoblast apoptosis (Bellido & Hill Gallant, 2014). High levels of glucocorticoids transiently promote bone resorption, probably by stimulating the production of receptor activator of nuclear factor kappa-B ligand (RANKL) but inhibits osteoclastogenesis in the long-term (Henneicke, Gasparini, Brennan-Speranza, Zhou, & Seibel, 2014). In the current study, plasma corticosterone levels between the probiotic and control groups were similar to the level (2.28 ng/ml) reported from unstressed broilers (Quinteiro-Filho et al., 2010). The broilers were raised under normal management condition without subjected to any known stressor, so it is not surprised that plasma corticosterone levels were unaffected by the probiotic treatment. It has been hypothesized that under stressful conditions, the long bones of broilers fed probiotics as compared to controls would show

Table 4
The effects of probiotic on plasma measured parameters of 43-day-old broilers.

	Control	Probiotic	S.E.M.	*P*-value
IFN-γ (pg/ml)	49.67	46.77	0.93	0.43
TNF-α(pg/ml)	384.85	400.68	2.69	0.15
IL-6 (pg/ml)	11.25	10.25	0.37	0.50
IL-10 (pg/ml)	62.69	56.51	1.01	0.14
5-HT (ng/ml)	46.00	40.54	1.71	0.43
TRP (μmol/ml)	101.57	108.82	5.97	0.76
Corticosterone (ng/ml)	2.58	1.83	0.15	0.23

IFN-γ: interferon-γ; TNF-α: tumor necrosis factor-α; IL-6: interleukin-6; IL-10: interleukin-10; 5-HT: serotonin; TRP: tryptophan.
Values are least square means. N equals to 7 for the probiotic group and 8 for the control group.

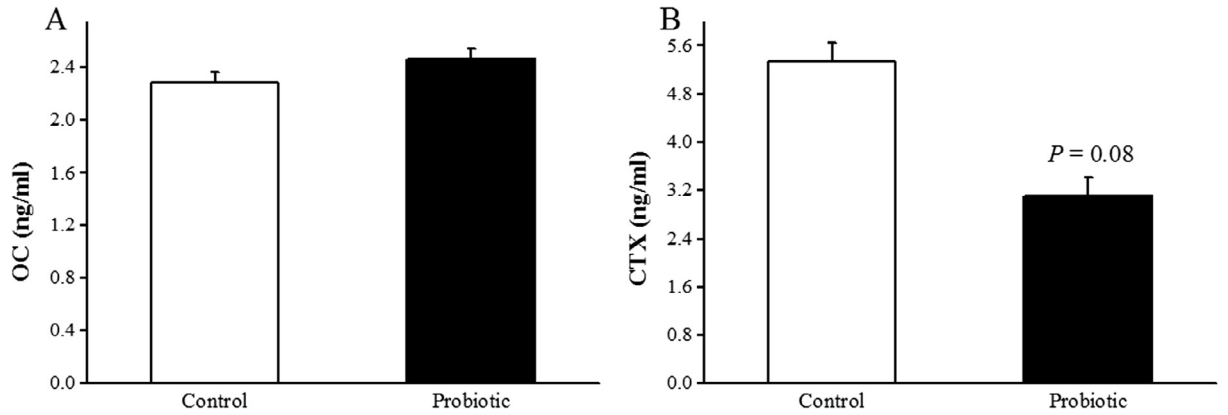

Fig. 4. Effects of probiotic supplementation on bone modeling of broilers at 43 days of age. A, osteocalcin (OC), a bone formation indicator and B, c-terminal telopeptide of type I collagen (CTX), a bone resorption indicator. The data were presented as least square mean ± S.E.M. N equals to 7 for the probiotic group and 8 for the control group.

Table 5
The effects of probiotic on catecholamines, serotonin, and respective metabolites in the raphe nuclei of 43-day-old broilers.

	Control	Probiotic	S.E.M.	*P*-value
Catecholamine system				
DA (ng/g)	118.16	111.44	1.79	0.35
NE (ng/g)	1097.12	1123.97	22.82	0.77
EP (ng/g)	182.36	296.88	24.24	0.24
DOPAC (ng/g)	58.54	58.19	1.14	0.94
HVA (ng/g)	154.83	151.14	1.29	0.47
DOPAC/DA	0.50	0.52	0.007	0.42
HVA/DOPAC	1.32	1.37	0.02	0.53
5-HT system				
TRP (ng/g)	5299.45	5423.97	58.68	0.59
5HT (ng/g)	452.60	523.97	8.19	0.04
5HIAA (ng/g)	362.31	387.34	8.42	0.46
5HIAA/5HT	0.82	0.74	0.02	0.42

DA: dopamine; NE: norepinephrine; EP: epinephrine; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: homovanillic acid; TRP: tryptophan; 5-HT: 5-hydroxytryptamine; 5-HIAA: 5-hydroxyindoleacetic acid.
Values are least square means. N equals to 7 for the probiotic group and 8 for the control group.

Table 6
The effects of probiotic on catecholamines, serotonin, and respective metabolites in the hypothalamus of 43-day-old broilers.

	Control	Probiotic	S.E.M.	*P*-value
Catecholamine system				
DA (ng/g)	354.70	267.35	8.17	0.02
NE (ng/g)	2138.45	1541.95	60.96	0.03
EP (ng/g)	362.30	268.76	12.95	0.08
DOPAC (ng/g)	95.55	83.72	1.81	0.11
HVA (ng/g)	233.09	212.51	4.92	0.30
DOPAC/DA	0.27	0.32	0.003	0.004
HVA/DOPAC	0.66	0.81	0.009	0.001
5-HT system				
TRP (ng/g)	4463.87	4235.27	92.14	0.53
5HT (ng/g)	1055.00	934.25	25.35	0.24
5HIAA (ng/g)	234.43	216.04	4.50	0.31
5HIAA/5HT	0.22	0.24	0.004	0.41

DA: dopamine; NE: norepinephrine; EP: epinephrine; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: homovanillic acid; TRP: tryptophan; 5-HT: 5-hydroxytryptamine; 5-HIAA: 5-hydroxyindoleacetic acid.
Values are least square means. N equals to 7 for the probiotic group and 8 for the control group.

improved bone mineralization and strength because of reduced circulating levels of corticosterone. The hypothesis will be test in future studies.

Previous research reveals that brain 5-HT stimulates bone formation and inhibits bone resorption, whereas peripheral 5-HT inhibits bone formation (Ducy & Karsenty, 2010). In the current study, the concentriations of periperal 5-HT were not different between probiotic fed broilers and controls; however, the central 5-HT concentrations in the raphe nuclei but not in the hypothalamus were higher in probiotic fed broilers. In supporting the previous hypothesis, the higher levels of 5-HT in the raphe nuclei of probiotic fed broilers suggest that brain serotonin may have played a role in bone mass accural by stimulating bone formation and inhibiting resorption. The raphe nuclei locates in the brainstem, and their main function is to synthesize and release 5-HT to the remaining part of the brain (Tork, 1990). The hypothalamus, one of the serotonergic projects targeted regions, receives extremely dense serotonergic inputs (Heym & Gladfelter, 1982; Martin, DeLorenzo, Ho, Humbertson, & Waltzer, 1985).

Central catecholamines, as regulators of bone metabolism, mediate bone development and remodelation (Dimitri & Rosen, 2017). In mice, as long as normal feed intake and body weight were maintained, blocking sympathetic activty increases bone mass (Gordeladze & Reseland, 2003). Both NE and DA concentrations in the hypothalamus but not in the raphe nuclei were lower in broilers fed probiotic. The hypothalamus is the site where 5-HT is proposed to play an important role in bone regulation via binding to its specific 5-HT_{2C} receptors to modulate sympathetic tone (Yadav et al., 2009) and the hypothalamic–pituitary–adrenal axis (Heisler et al., 2007). The NE releasing neurons are located in the locus coeruleus, a nucleus in the pons of the brainstem. Both NE and 5-HT neurons terminates densely distribute in the hypothalamus, suggesting an interaction between the 5-HT and NE neurons (Tian, Eaton, Goudreau, Lookingland, & Moore, 1993). The lower levels of hypothalamic NE and DA, with a trend of lower levels of hypothalamic EP, in the probiotic fed broilers suggests that serotonergic neurons topically inhibit central noradrenergic neuron activities in the hypothalamus (Tian et al., 1993). The lower NE bioactivity through higher raphe nuclei 5-HT concentrations in the probiotic fed broilers could be related to improved bone traits in the femur and tibia. The effects of overexpression of NE on bone remodeling, with a net increase in bone resorption, can be blocked by the β-adrenergic antagonist propranolol in humans (Yirmiya et al., 2006). However, the hypothesis in poultry needs to be further examined.

In the current study, a higher DA turnover rate in the probiotic fed broilers was also observed as indicated by greater DOPAC/DA and HVA/DOPAC ratios in the hypothalamus but not in the raphe nuclei. The hypothalamus is an integrated sensing system, receiving dense inputs from the mesocorticolimbic DA system (Quarta & Smolders, 2014), and contains dopaminergic neurons in both the periventricular nucleus and arcuate nucleus (Lerant, Herman, & Freeman, 1996). Our results suggest an enhanced activation of catecholaminergic neurons in the hypothalamus. Metabolism of DA involves several pathways. Dopamine can be degraded into inactive metabolites such as DOPAC via monoamine oxidase and then to HVA via catechol-O-methyltransferase, or can be used as a precursor for synthesizing NE by the enzyme dopamine beta-hydroxylase (Meiser, Weindl, & Hiller, 2013; Saylor, Reid, & Lunte, 2015). The current data may suggest that DA metabolism in the hypothalamus of probiotic fed broilers was shifted away from NE production to degradation. The main neurotransmitter in the sympathetic nervous system is NE; and its synthetic neurons innervating numerous organs including bone (Elefteriou, Campbell, & Ma, 2014). Sympathetic nerve fibers as well as adrenergic β2 receptors are present in bone (Duncan & Edouard, 1977; Fan, Bouwense, Crawford, & Xiao, 2010), suggesting that sympathetic neurons that release NE could regulate bone homeostasis through binding to its β2 receptors (Bonnet, Pierroz, & Ferrari, 2008). When stimulated, the β2 receptors expressed on osteoblasts and osteoclasts enhance the production of bone remodeling regulatory factors, such as IL-6, RANKL, and prostaglandin E_2 (Aitken, Landao-Bassonga, Ralston, & Idris, 2009; Elefteriou et al., 2005; Kondo & Togari, 2003; Wang et al., 2015), which subsequently inhibits bone formation and promote bone resorption. Altogether, the mechanism of the regulation of NE was not examined in this study, and it could be similar in mammals (Koed & Linnet, 2000) that dietary inclusion of probiotic up-regulates the synthesis of 5-HT in the raphe nuclei, which is then released within the terminal areas of the hypothalamus, leading to lower NE synthesis in the probiotic fed broilers. This reduced sympathetic outflow in turn contribute to reduced bone resorption.

In conclusion, dietary supplementation of *Bacillus subtilis* based probiotic conferred an improvement in broiler bone traits perhaps due to increased intestinal absorption of nutrients such as Ca. Another possible mechanism involved in bone mass accrual in probiotic fed broilers is the higher brain 5-HT function in inhibiting bone resorption via lowering sympathetic activity. The results indicate that dietary probiotic supplementation has the potential as a management strategy for improving skeletal health and welfare in broilers.

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Conflict of interest

None.

Ethics statement

I have read and adhere to the Publishing Ethics.

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