Comparing the potential of *Bacillus amyloliquefaciens* CGMCC18230 with antimicrobial growth promoters for growth performance, bone development, expression of phosphorus transporters, and excreta microbiome in broiler chickens

Chong Li [*,†] Huiyi Cai [*,‡] Shuzhen Li [*] Guohua Liu,[*] Xuejuan Deng,[‡] Wayne L. Bryden [§] and Aijuan Zheng[*,1]

[*]*Key Laboratory for Feed Biotechnology of the Ministry of Agriculture and Rural Affairs, Institute of Feed Research, Chinese Academy of Agriculture Sciences, Beijing 100081, China;* [†]*Precision Livestock and Nutrition Laboratory, Teaching and Research Centre (TERRA), Gembloux Agro-Bio Tech, University of Liège, Gembloux 5030, Belgium;* [‡]*National Engineering Research Center of Biological Feed, Beijing 100081, China; and* [§]*School of Agriculture and Food Sciences, University of Queensland, Gatton QLD 4343, Australia*

ABSTRACT Bone health of broiler chickens is essential for welfare and production. In this study, the probiotic *Bacillus amyloliquefaciens* (**BA**) CGMCC18230 was compared with antimicrobial growth promoters (**AGP**s) for its ability to promote growth and bone health. To address this, a total of 180 Arbor Acres (**AA**) 1-day-old, male, broiler chicks were randomly allocated into 3 treatment groups, with 6 replicates, containing 10 chicks in each replicate. The treatment groups were: control group (**CON**) fed a corn-soybean based diet; BA treatment group fed the basal diet supplemented with 2.5×10^{10} CFU/kg BA CGMCC18230; AGPs treatment group was fed the basal diet containing the antibiotics aureomycin (75 mg/kg), flavomycin (5 mg/kg) and kitasamycin (20 mg/kg). Over the 42 d experiment, broilers fed BA and AGPs diets both had higher BW, and the ADG was significantly ($P < 0.05$) higher than that of the CON group both in the grower phase (22−42 d) and overall. Moreover, with BA birds had higher ($P < 0.05$) serum concentrations of phosphorus (P, day 42) and alkaline phosphatase (**ALP**, days 21 and 42). Conversely, the content of P in excreta decreased significantly ($P < 0.05$) on days 21 and 42. Tibia bone mineralization was improved in BA, and the mRNA of P transport related genes *PiT-1,2* in the duodenum and jejunum were significantly up-regulated in the BA group than in the CON group ($P < 0.05$). 16S rRNA gene sequencing revealed that dietary BA supplementation increased the relative abundance of butyrate-producing bacteria (*Ruminococcaceae*) and polyamine-producing bacteria (*Akkermansia* and *Alistipes*), which had a positive effect on bone development. These data show that dietary supplementation of BA CGMCC18320 improves broiler growth performance and bone health similar to supplementation with AGPs through up-regulation of intestinal P transporters, microbial modulation and increase P retention. However, no significant influence of BA CGMCC18320 supplementation on the retention of Ca was found.

Keywords: bone development, phosphorus transporters, excreta microbiota, broiler, *Bacillus amyloliquefaciens* CGMCC18230

2022 Poultry Science 101:102126
https://doi.org/10.1016/j.psj.2022.102126

INTRODUCTION

Globally there has been a rapid increase in the demand for chicken meat, reflecting human population growth and affluence, and the relative cost of poultry compared to other meat (Mottet and Tempio, 2017). This has coincided with the phasing out of antimicrobial growth promoters (**AGP**s) that have been widely used to improve the production efficiency of broiler chickens (Dibner and Richards, 2005; M'Sadeq et al., 2015; Tellez and Latorre, 2017). The challenges mentioned above, have prompted a global search for alternative feed supplements to AGPs including probiotics which are used widely by the human population and are rapidly gaining acceptance as an animal feed additive (Bajagai et al., 2016; Ma et al., 2021; Shini and Bryden, 2022).

Received May 12, 2022.
Accepted August 3, 2022.

[1]Corresponding author: zhengaijuan@caas.cn

Bone mineralization is a function of calcium (**Ca**) and phosphorus (**P**) metabolism, an area of much interest to the poultry industry because the rapid weight gain of the broiler increases stress on the skeletal system, leading to susceptibility to bone disease (González-Cerón et al., 2015; Li et al., 2017). Gradually, the roles of probiotics in bone development and health are being delineated, which include an ability to reduce the incidence of lameness (Wideman et al., 2012), and modulate bone mineralization (Quach and Britton, 2017; Yan et al., 2019). *Bacillus amyloliquefaciens* (**BA**) is a probiotic species that has been extensively used in the animal feed industry. A component of BA's mode of action is the secretion of multiple extracellular enzymes and antibacterial substances, such as phytase (Schofield et al., 2016). Therefore, we predicted that BA would have an impact on bone mineralization.

The current study determined the effect of BA and AGPs on growth performance, and importantly, evaluated the impact on tibia development of broiler chickens. In addition to the physiological and biochemical analyses, the excreta microbiota was also investigated to determine possible molecular and microbial mechanisms underlying the effects of BA on broiler performance and bone biology.

MATERIALS AND METHODS

Ethics Statement

The experiment was conducted in Nankou pilot base of the Chinese Academy of Agricultural Sciences. This research were licensed by the ethical approval of the Animal Care and Use Committee of the Institute of Feed Research of the Chinese Academy of Agricultural Sciences (Statement No. AEC-CAAS-20191106, Beijing, China). In addition, the methods for animal experiments were set out by the National Institute of Animal Health and research reporting follows the guidelines of ARRIVE (Kilkenny et al., 2012).

Preparation of Bacillus Amyloliquefaciens CGMCC18230

The probiotic *Bacillus amyloliquefaciens* CGMCC18230 (viable count $\geq 5.0 \times 10^{11}$ CFU/g, powder state) was provided by Challenge Biotechnology Co., LTD (Beijing, China) in microcapsules, which were added to feed. The viable feed count of *Bacillus amyloliquefaciens* was determined using a modified protocol as described in Nikoskelainen (2003). The amount of the probiotic was determined by homogenizing 1 g of feed in 9 mL sterile phosphate-buffered saline (PBS, pH 7.2; HKM, Guangdong, China), pH 7.4. The homogenate was spread at dilutions from 10^{-1} to 10^{-10} on Luria-Bertani (**LB**) broth media (Land Bridge, CM158, Beijing, China) plates; the plates were incubated at 37°C for 48 h prior to colony counting. Following a conservative strategy, the amount of *Bacillus amyloliquefaciens* in feed was examined daily throughout the experiment in order to ensure cell viability.

Experimental Design and Bird Management

In total, 180 newly hatched, male, Arbor Acres (**AA**) broiler chickens were raised in the Nankou experimental base of the Chinese Academy of Agricultural Sciences (CAAS, Beijing, China). The chicks were randomly allocated into 3 treatment groups, with 6 replicates, containing 10 chicks in each replicate; each replicate had the same mean body weight. The experiment lasted for 42 d with 2 feeding phases, starter (1−21 d) and grower (22−42 d). The basal experimental diets (CON, control group) were formulated to meet the nutritional requirements (Table 1) of the birds as determined by National Research Council (1994) and Ministry of Agriculture of the People's Republic of China (2004). In the second treatment group, *Bacillus amyloliquefaciens* CGMCC18230 homogenate was added at 100mg/kg to the basal diets (BA group); after pelleting at 65 to 70°C, the diet was checked for the viable strain count, meet the final concentration of 2.5×10^{10} CFU/kg feed. Aureomycin (20%, 75 mg/kg), flavomycin (50%, 5 mg/kg),

Table 1. Ingredient and nutrient composition of basal broiler diets.

Ingredient (g/kg)	Starter (1−21 d)	Grower (22−42 d)
Corn	570.7	581.8
Soybean meal, 46%	297.6	287.5
Cotton seed meal	49.8	29.9
Soybean oil	15.0	39.6
L-Lysine	1.5	0.9
DL-Methionine	1.4	1.6
Limestone	12.6	10.2
$CaHPO_4$	19.3	16.5
NaCl	3.0	3.0
Choline chloride	2.0	2.0
Vitamin premix	0.3	0.3
Mineral premix[1]	1.0	1.0
Zeolite powder	1.7	1.7
Titanium dioxide[2]	24.0	24.0
Total	1000	1000
Nutrient concentrations[3]		
Metabolic energy (MJ/kg)	12.35	13.02
Crude protein	211.8	198.4
Calcium	10.1	8.5
Available P	4.5	4.0
Total P	6.9	6.3
Lysine	11.4	10.5
Methionine	4.9	4.8
Methionine + Cysteine	8.3	8.1
Threonine	7.7	2.2
Analyzed content		
Calcium	10.2	8.5
Total P	6.8	6.2
Calcium: Total P	1.50	1.37

[1]The premix provided the following per kilogram diet: vitamin A 10, 000 IU, vitamin D_3 2000 IU, vitamin E 10 IU, vitamin K_3 2.5 mg, vitamin B_1 1 mg, vitamin B_2 6 mg, vitamin B_3 10 mg, vitamin B_5 40 mg, vitamin B_6 3 mg, vitamin B_{11} 0.3 mg, vitamin B_{12} 0.01 mg, biotin 0.12 mg, Cu (as copper sulfate) 8 mg, Fe (as ferrous sulfate) 80 mg, Mn (as manganese sulfate) 60 mg, Zn (as zinc sulfate) 40 mg, Se (as sodium selenite) 0.15 mg, I (as potassium iodide) 0.35 mg.

[2]Prepared as 4 g of titanium dioxide mixed with 20 g of ground corn.

[3]Calculated nutrient concentrations.

and kitasamycin (50%, 20 mg/kg) were added before pelleting to the basal diet as the third experimental group (AGPs group). The AGPs content in the diet after pelleting was quantified using high-performance liquid chromatography (**HPLC**). No related antibiotics were detected in CON and BA group, and AGPs group including aureomycin (14.45 ± 0.16 mg/kg feed), flavomycin (2.43 ± 0.04 mg/kg feed) and kitasamycin (9.73 ± 0.08 mg/kg feed), respectively. All diets contained the indigestible marker, titanium dioxide (de Vries and Gerrits, 2018).

The broiler chickens were housed in an environmentally controlled facility (fiberglass feeders and plastic net floor), and had ad libitum access to feed and purified water. The lighting program was controlled to a 16 h light: 8 h dark cycle, throughout the experiment. Relative humidity was controlled at 60 to 70% during days 1 to 7, and then at 50 to 60% for the remainder of the experiment. For the first week, the ambient temperature was maintained at 33 ± 2°C and then gradually decreased to 24°C (1−2°C per/d). This temperature was maintained until the end of the study. The broilers were vaccinated against infectious diseases according to commercial practices, containing Newcastle disease vaccine (strain La Sota) and the infectious bronchitis (strain H120) on day 7, and infectious bursal disease (IBD, strain B87) on day 14; all vaccines were purchased from Harbin Pharmaceutical Group Bio-vaccine Co. Ltd. (Harbin, China). Chickens were monitored twice daily and excreta was cleared daily.

Sample Collection and Parameter Determination

In order to evaluate the growth performance parameters, body weight (**BW**) and feed consumption were measured at days 21 and 42; average daily feed intake (**ADFI**), average daily gain (**ADG**), and the feed conversion ratio (**FCR**) were calculated (g feed/g gain) for the starter (1−21 d) and grower (22−42 d) phases. If broilers died during the study, adjustments were made to the growth parameters. Mortality was recorded daily for each replicate cage and analyzed as previously described (Du and Guo, 2021).

From day 18 to 21 and day 39 to 42 of the experiment, total excreta from each replicate was collected for 3 consecutive days. The collected excreta for each replicate was thoroughly mixed and stored at −20°C, before oven drying at 65°C for 48 h to a constant weight. The diet and excreta samples were finely ground to pass through a 0.5 mm screen before analysis and dried at 105°C in an oven for 16 h for dry matter determination (Method 934.01; AOAC International, 2006). The samples were placed into a muffle furnace at 550°C for 4 h and ash percentage was determined (Liu et al., 2017). The content of Ca in ash were determined by inductively-coupled plasma emission spectrometry (Method 968.08; AOAC International, 2000), and P was determined using the vanadate-molybdate method (Method 967.17; AOAC International, 2000). The retention of Ca and P were calculated as described previously (Chung et al., 2013).

$$Retention, \% = [1 - (X_{excreta}/X_{diet}) \times (Ti_{diet}/Ti_{excreta})] \times 100$$

Where: $X_{excreta}$ and X_{diet} were the Ca or P content in the excreta and diet (g/kg), respectively, and Ti_{diet} and $Ti_{excreta}$ the titanium dioxide content in diet and excreta (g/kg), respectively.

On days 21 and 42, one broiler (close to the average BW) from each replicate was selected after a 12 h fast. Blood samples were taken (2.5 mL) from the wing vein using an anticoagulant-free vacuum test tube (5 mL), and immediately placed on ice. Serum was harvested after centrifuging at 3,000 × g for 10 min, and stored at −20°C until analyzed. Ca, P, and alkaline phosphatase (**ALP**) in serum was determined with an automatic biochemical analyzer (Hitachi 7600, Japan), using assay kits purchased from the Nanning Jiancheng Biological Engineering Institute (Jiangsu, China).

After blood sampling, the broilers were euthanized by electric stunning and immediate manual slaughter. Proximal duodenal, jejunal, and ileal tissue were collected, opened longitudinally and cleaned with sterile saline. The tissue was snap-frozen in liquid nitrogen and transferred to a −80°C freezer till analyzed for mRNA.

The 2 tibiotarsus (hereafter referred to as the tibia) bones from each bird were then removed and cleaned prior to analysis. The biomechanical strength of the right tibia bone was measured (Latorre et al., 2017), with load representing bone strength; defined as the force in grams per square millimeter of cross-sectional area (g/mm^2). The data were calculated by the software of Instron's Series IX (Norwood, MA). The left tibia bone was degreased in a soxhlet apparatus, then dried at 100°C for 24 h before ashing in ceramic crucibles for 24 h at 600°C. The contents of Ca and P in tibia were determined using the same methods as for excreta ash and the data were expressed as percent of defatted bone on a dried basis.

RT-qPCR

The total RNA from the intestinal mucosa were isolated using TRI-zol reagent (TIANGEN, Beijing, China) and reversely transcribed into complementary DNA (**cDNA**) pursuant to the manufacturer's protocol. The concentration of total RNA was determined from OD 260/280 with a spectrophotometer (Ultrospec 2100 pro, GE Healthcare), and purity measured by agarose gel electrophoresis. Then 500 ng of total RNA was reversely transcribed into cDNA using the primescript of Fast Quant RT Kit (with gDNase) (TIANGEN, Beijing, China) according to the manufacturer's protocol. qPCR was conducted using the iCycler iQ5 system following the manufacturer's instructions. The primers

Table 2. Primer sequences of broiler *NaPi-IIb, PiT-1, 2,* and *β-actin.*

Gene	Primer sequence (5'-3')	Accession number
PiT-1	F: GCTCGTGGCTTCGTTCTTG R: GACCATTTGACGCCTTTCT	XM_015297502.1
PiT-2	F: GCAGCAGATACATCAACTC R: ATTTCCACTCCACCCTC	NM_001305398·1
NaPi-IIb	F: CTGGATGCACTCCCTAGAGC R: TTATCTTTGGCACCCTCCTG	NM_204474.1
β-actin	F: GAGAAATTGTGCGTGACATCA R: CCTGAACCTCTCATTGCCA	NM_205518.1

sequences for *PiT-1, Pit-2, NaPi-IIb*, and *β-actin* were listed in Table 2. *β-actin* was used to normalize the expression of the targeted genes. The mRNA level of the relative gene was calculated using the method of $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). All the samples were analyzed in triplicate and the geometric mean of internal references.

Microbial Analysis

On day 42, excreta from one broiler per replicate in the CON and BA groups was freshly collected, snap-frozen in liquid nitrogen and stored at −80°C for further analysis. Microbial genomic DNA of the excreta were extracted under sterile conditions using the GenElute Stool DNA Isolation Kit (Sigma-Aldrich, St. Louis, MO), following the manufacturer's instructions. Subsequently, gene sequencing was implemented by Majorbio Biotech Co., Ltd (Shanghai, China). The V3 to V4 variable region of the 16S rRNA gene was amplified with universal primers 343F and 798R. The PCR products were collected and sequenced using the Illumina MiSeq platform (Illumina inc., San Diego, CA). High-quality reads were filtered and clustered into operational taxonomic units (**OTU**s) based on sequences with ≥97% similarity and then analyzed using the QIIME software (version 1.9.1). The online platform (https://cloud.majorbio.com/) of Majorbio Biotech Co., Ltd was used to analyze the reads data. In particular, alpha-diversity indices including Chao1 index, Shannon index, Coverage index, and number of OUTs were analyzed by student's t-test at OTU level. The beta-diversity analysis including the principal component analysis (**PCA**) and principal coordinate analysis (**PCoA**) (Lozupone and Knight, 2005). The 2-sided Student's t-test was employed for analysis of the relative abundance at the phylum and genus levels. Linear discriminant analysis (**LDA**) effect size (**LEfSe**) analysis was implemented using the non-parametric factoria Kruskal-Wallis rank sum test to obtain significantly different species between the CON and BA groups (Segata et al., 2011), differences between groups were assessed using the Wilcoxon rank sum test, and finally LDA was used to access the influence of each species abundance on the differences.

Statistical Analysis

The data were analyzed by a one-factor ANOVA procedure of SPSS19.0 software package for Windows (SPSS Inc., Chicago, IL) and indexes were expressed as means with standard error of mean (**SEM**). Significant differences between groups were separated using Duncan's multiple range test. A *P*-value less than 0.05 was set as statistically significant. The graphs were designed using GraphPad Prism 5 Project (GraphPad Software Inc., San Diego, CA) and Origin 8.5 (Origin Lab, Berkeley, CA).

RESULTS

Growth Performance

The birds grew normally and had a low mortality rate of 2.4% that was not related to the dietary treatments (data not shown). Growth performance during the study is shown in Table 3. On day 21 the broilers fed BA and AGPs had a higher ($P < 0.05$) BW than the CON group. The ADG of the BA and AGPs groups was significantly ($P < 0.05$) higher than the CON group in the grower phase (22−42 d) and overall. The ADFI for these groups was also higher that the CON group in the grower phase (22−42 d), but the difference was not significant ($P = 0.091$). The FCR of the AGPs group was superior ($P < 0.05$) to the CON group during both the grower phase and overall. Furthermore, the overall FCR of the BA group was numerically superior to the CON group but the difference did not reach significance.

Retention of Ca and P

Excreta concentrations and retention of Ca and P are shown in Table 4. Neither Ca concentration nor retention was effected by diet. In contrast, supplementing the diet with BA or AGPs decreased ($P < 0.05$) P excretion, and this was reflected in increased ($P < 0.05$) retention of P.

Table 3. Growth performance of broiler chickens fed a basal diet (CON), or the basal diet supplemented with either *Bacillus amyloliquefaciens* (BA), or antibiotic growth promoters (AGPs).

		Dietary treatment				
Parameter	Days	CON	BA	AGPs	SEM	*P-value*
BW, g	0	45.4	45.2	45.6	0.32	0.742
	21	771	794	757	13.14	0.053
	42	2281[b]	2350[a]	2391[a]	29.58	0.039
ADG, g/(bird-d)	1-21	34.1	35.3	33.2	0.67	0.531
	22-42	71.7[b]	74.3[a]	76.2[a]	1.51	0.024
	1-42	53.0[b]	54.9[a]	55.5[a]	1.04	0.037
ADFI, g/(bird-d)	1-21	51.0	49.2	48.5	1.68	0.107
	22-42	163.9	166.0	165.6	1.59	0.091
	1-42	106.1	107.4	107.9	1.98	0.281
FCR, g/g	1-21	1.50	1.40	1.41	0.05	0.154
	22-42	2.27[a]	2.23[a]	2.16[b]	0.03	0.015
	1-42	2.01[a]	1.95[ab]	1.92[b]	0.01	0.039

$n = 6$.

BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; F/G, feed/gain. CON group, basal diet in control group; BA group, basal diet + 2.5×10^{10} CFU/kg *Bacillus amyloliquefaciens*; AGPs group, basal diet+ aureomycin 75 mg/kg + flavomycin 5 mg/kg + Kitasamycin 20 mg/kg.

[a,b]In the same row, values with different small letter superscripts mean significant difference ($P < 0.05$).

Table 4. Retention of Ca and P in broiler chickens fed a basal diet (CON), or the basal diet supplemented with either *Bacillus amyloliquefaciens* (BA), or antibiotic growth promoters (AGPs).

Item	Dietary treatment			SEM	*P*-Value
	CON	BA	AGPs		
Day 21					
Excreta Ca, g/kg DM	25.58	24.24	24.93	0.208	0.657
Excreta P, g/kg DM	13.81[a]	12.47[b]	12.57[b]	0.170	0.001
Retention of Ca, %	60.61	62.71	61.51	1.071	0.455
Retention of P, %	49.73[b]	50.98[a]	50.78[a]	0.224	0.034
Day 42					
Excreta Ca, g/kg DM	24.73	24.01	23.98	0.153	0.211
Excreta P, g/kg DM	13.12[a]	12.37[b]	12.91[ab]	0.165	0.047
Retention of Ca, %	62.73	64.58	63.24	0.103	0.067
Retention of P, %	46.58[b]	48.72[a]	47.19[b]	0.478	0.017

$n = 6$.

CON group, basal diet in control group; BA group, basal diet + 2.5×10^{10} CFU/kg *Bacillus amyloliquefaciens*; AGPs group, basal diet+ aureomycin 75 mg/kg + flavomycin 5 mg/kg + Kitasamycin 20 mg/kg.

[a,b]In the same row, values with different small letter superscripts mean significant difference ($P < 0.05$).

Concentrations of Ca, P, and ALP in Serum

As shown in Table 5, all groups had comparable serum concentrations of Ca on days 21 and 42. Serum ALP content was higher ($P < 0.05$) in the BA and AGPs group than the CON group on day 21. Interestingly, the serum P and ALP of BA broilers were both higher ($P < 0.05$) than the other groups on day 42.

Tibia Bone Strength and Mineralization

Tibia breaking strength and mineral content on days 21 and 42 are summarized in Figure 1. On day 21, no significant differences were observed in the tibia strength and bone ash among treatments. Tibia ash concentrations of P and Ca were both elevated in the BA and AGPs groups relative to birds on the control diet, although not reaching statistical significance ($P = 0.074$ and $P = 0.061$, respectively). Supplementation with BA significantly ($P < 0.05$) increased tibia breaking strength on day 42, along with tibia ash Ca and P concentrations compared to birds receiving the basal diet. A similar, but non-significant trend in bone ash content was noted.

Table 5. Serum concentrations of Ca, P and ALP broiler chickens fed a basal diet (CON), or the basal diet supplemented with either *Bacillus amyloliquefaciens* (BA), or antibiotic growth promoters (AGPs).

Parameter	Dietary treatment			SEM	*P*-value
	CON	BA	AGPs		
Day 21					
Ca, mmol/L	1.96	2.27	2.19	0.056	0.052
P, mmol/L	1.50	1.55	1.60	0.031	0.453
ALP,[1] U/L	3292[b]	3866[a]	4252[a]	155	0.016
Day 42					
Ca, mmol/L	2.41	2.42	2.39	0.023	0.890
P, mmol/L	1.47[b]	1.56[a]	1.48[b]	0.017	0.028
ALP,[1] U/L	1543[b]	2346[a]	1894[b]	110	0.006

$n = 6$.

[1]ALP, alkaline phosphatase. CON group, basal diet in control group; BA group, basal diet + 2.5×10^{10} CFU/kg *Bacillus amyloliquefaciens*; AGPs group, basal diet+ aureomycin 75 mg/kg + flavomycin 5 mg/kg + Kitasamycin 20 mg/kg.

[a,b]In the same row, values with different small letter superscripts mean significant difference ($P < 0.05$).

Gene Expression of Intestinal P Transporters

The mRNA expression of *NaPi-IIb* and *PiT*-1, 2 genes, involved in P transport in different intestinal segments were examined on day 42 and the results appear in Figure 2. Supplementation with BA significantly ($P < 0.05$) up-regulated expression of *PiT-1,2* mRNA in the duodenum and jejunum. However, no effect of BA on the expression of *NaPi-IIb* was found. The AGPs up-regulated *NaPi-IIb* in all sections when compared to BA supplementation and control birds, with the most noticeable change in the ileum. The AGPs also up-regulated the expression of *PiT-1* and *PiT-2* in the ileum ($P < 0.05$), to a greater extent that the 2 other treatments but this was not observed in the duodenum or jejunum.

Microbial Diversity and Community in Excreta

Comparisons of alpha and beta microbial diversity indices are shown in Figure 3. The results revealed that the Chao1 and Shannon indexes of OTU level were not significantly different between the CON and BA groups (Figure 3A and B). The coverage index for every sample was greater than 0.999, indicating that the subsequent analyses were not affected by biases in sequencing depth (Figure 3C). Furthermore, a trend of increase in the number of OTUs was observed in the BA group compared with the CON group (Figure 3D). The β-diversity analysis was shown in Figure 3E and F, the results of PCA and PCoA showed that these 2 groups demonstrated no significant differences at the OTU level. In the stacked bar graphs were created to show the different OTUs at the level of Phylum and Genus (Figure 3G and H), results indicating the *Firmicutes, Actinobacteria, Proteobacteria*, and *Bacteroidetes* dominated the microbiomes of both the CON and BA chickens at the phylum level, but there were differences in abundance. At the genus level, *Candidatus_Arthromitus, Streptococcus, Bifidobacterium*, and *Rothia* were the most predominant genera in the excreta microbiota communities of chickens. Additionally, LEfSe analysis (LDA > 2) revealed the significant differences in microbiota structure between the CON group and the BA group (Figure 4A). Adopting the 2-sided Student's t-test, we found that *Bacteroidetes, Deinococcus-Thermus*, and *Verrucomicrobia* at the phylum level were more abundant ($P < 0.05$) in the BA group than in the CON group (Figure 4B). At the genus level (Figure 4C), 15 bacteria were significantly different, the relative abundance of *Acinetobacter, Muribaculaceae, Ruminococcaceae, Cupriavidus, Vagococcus, Akkermansia, Blautia*, and *Alistipes* were notably elevated in the BA group ($P < 0.05$).

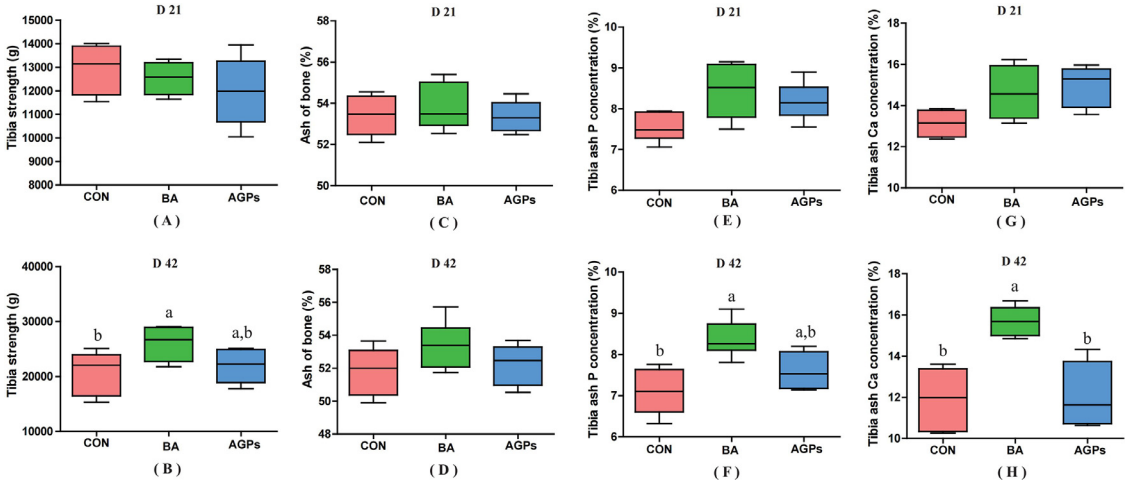

Figure 1. Tibia bone strength and mineralisation of broiler chickens fed a basal diet (CON), or the basal diet supplemented with either *Bacillus amyloliquefaciens* (BA), or antibiotic growth promoters (AGPs). Data are indicated as means ± SEM ($n = 6$). (A) and (B) tibia strength on days 21 and 42, respectively; (C) and (D) bone ash content on days 21 and 42, respectively; (E) and (F) tibia P content on days 21 and 42, respectively; (G) and (H) tibia Ca content on days 21 and 42, respectively. [a,b]Values, for the same parameter and day, with different superscripts are significantly different ($P < 0.05$).

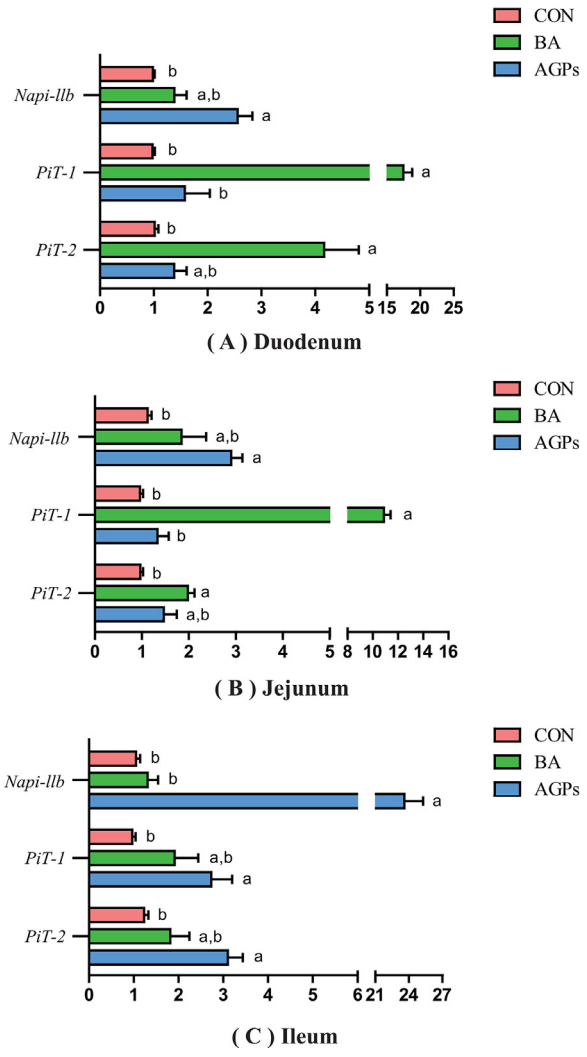

Figure 2. *NaPi-IIb* and *PiT-1, 2* gene mRNA expression in different intestinal segments of broiler chickens fed a basal diet (CON), or the basal diet supplemented with either *Bacillus amyloliquefaciens* (BA) or antibiotic growth promoters (AGPs) on day 42. Data are indicated as means ± SEM ($n = 6$). (A) duodenum; (B) jejunum; (C) ileum. [a,b]Values for each intestinal segment with different superscripts are significantly different ($P < 0.05$).

DISCUSSION

For many years AGPs have sustained animal and poultry productivity through maintaining gut health by reducing inflammation (Niewold, 2007; Mountzouris et al., 2019). However, with the curtailing of AGPs use, many strategies have been examined to fill this void and probiotics have received much attention. In most studies that evaluate probiotics, AGPs are not included, thus making it difficult to determine the relative importance of probiotics in substituting for AGPs. Both AGPs and a probiotic were included in this study to allow comparison of bird performance under the same conditions. Moreover, we were interested in the role of these feed additives beyond growth, especially bone metabolism.

In the current study, birds consuming BA and AGPs had similar final BW and utilised feed with similar efficiency. This has been observed previously (Lei et al., 2015). Other studies have also shown positive effects of BA on broiler growth performance (Luan et al., 2019; Hong et al., 2021). The improvement of growth parameters by a probiotic is complex and involves a number of possible mechanisms (Paulina and Katarzyna, 2018; Shini and Bryden, 2022), including enhanced intestinal integrity that is facilitated by BA (Shini et al., 2020, 2021). It has also been shown that BA may contribute to improved growth through regulation of immunity by increasing the expression of genes involved ileal mucosal immunity (Ahmed et al., 2014), and genes regulating the adaptive immune response, such as B and T cells lymphocyte activation (Luise et al., 2019).

Ca and P are important factors in cellular metabolism and pivotal to bone mineralization and strength. The metabolism of both minerals is tightly controlled to maintain homeostasis (Proszkowiec-Weglarz and Angel, 2013; Li et al., 2017). In the current study bone mineralization was increased when both BA and AGPs were added to the diet as indicated by ALP activity. ALP it

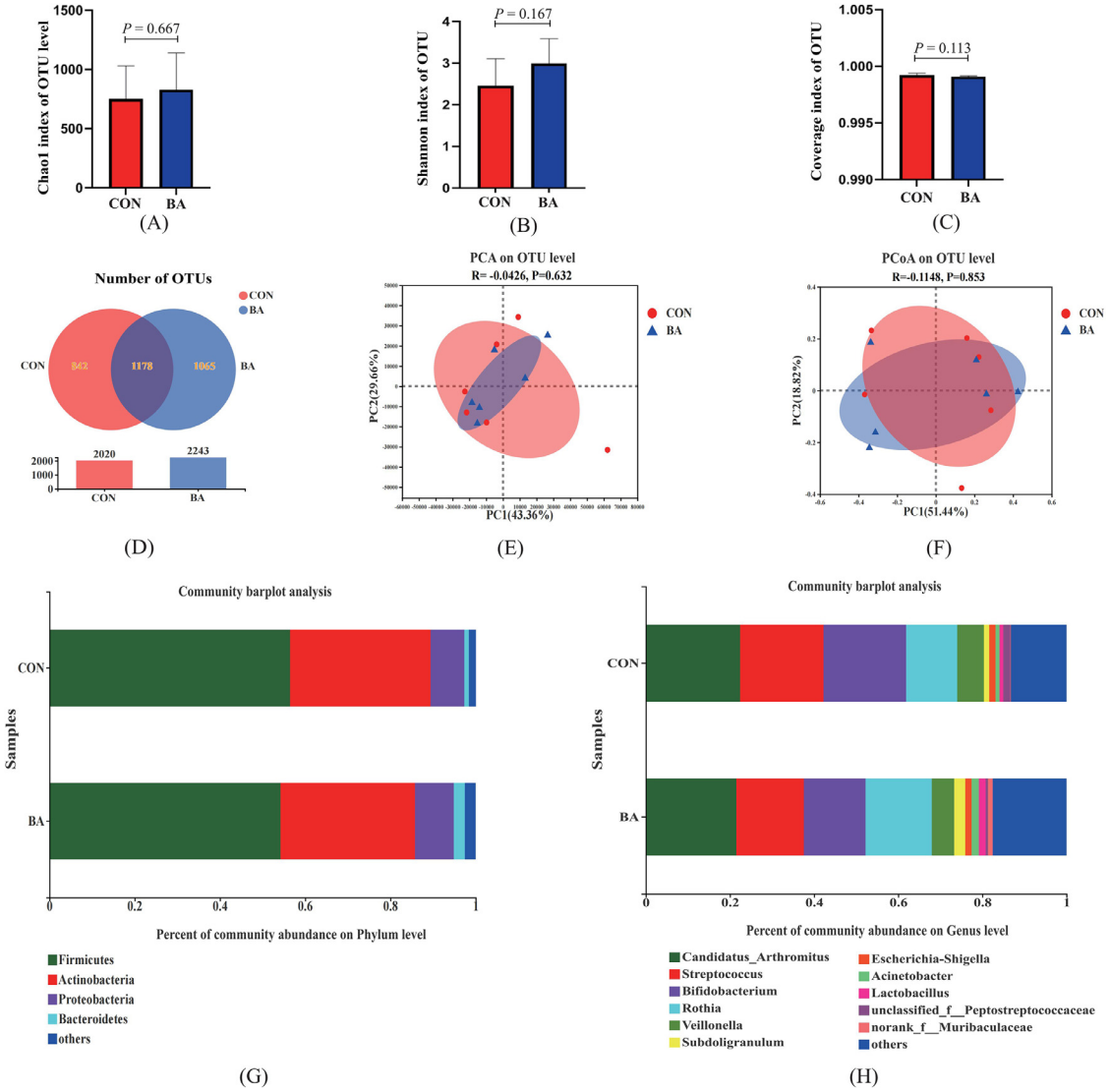

Figure 3. The diversity of the microbial community in excreta of broilers fed a basal diet (CON), or the basal diet supplemented with either *Bacillus amyloliquefaciens* (BA) on day 42. Data are indicated as means ± SEM ($n = 6$). (A) Chao1 index of OUT level; (B) Shannon index of OUT level; (C) Coverage index of OUT level; (D) Number of OTUs; (E) & (F) β-diversity was estimated by the PCA and PCoA on OUT level, respectively; (G) & (H) The relative abundance of bacteria at the phylumand level and genus level, respectively.

is a marker of skeletal mineralization in birds (Tilgar et al., 2008) and as expected, circulating ALP values were greater at 21 d of age reflecting greater bone development by the rapidly growing broiler during the initial weeks posthatching. Circulating concentrations of Ca and P were unaffected, except for a significant increase in P concentration in birds supplemented with BA at 42 d of age. This increase in serum P concentration coincided with decreased P excretion but increased P retention; no changes with Ca were observed. These changes in mineral metabolism were reflected in increased tibia bone strength and mineralization. Supplementing broiler diets with BA significantly increased bone strength on day 42, reflecting significant increases in tibia Ca and P content when compared to birds supplemented with AGPs. Research conducted by Bielke et al. (2017) indicated that bone density and strength were impacted positively by the gastrointestinal microbiome (Bielke et al., 2017), and Latorre et al. (2017) also demonstrated that *Bacillus spp.* increase tibial mineralization of broilers by modulation of the cecal microbial community through a probiotic role (Latorre et al., 2017). Studies in laying hens, demonstrated that dietary supplementation with *Bacillus* increased tibia bone P concentration and improved bone quality, presumably through enhanced P intestinal absorption (Ciurescu et al., 2020). Interestingly, we found no effect of BA supplementation on serum Ca level, excreta Ca concentration and Ca retention. In previous studies, some lactic acid bacteria promoted Ca absorption by producing large amounts of acid metabolites, *L. salivarius* UCC 118, stimulated Ca uptake by enterocytes in *in vitro* models, and *L. rhamnosus* GG was shown to stimulate bone improvement in estrogen-deficient mice (Gatej et al., 2018; Zakłos-Szyda et al., 2020). However, in this study, we did not find that BA affected Ca metabolism.

The small intestine is the main tissue for P absorption in poultry and absorption occurs in the different segments in this organ (Wasserman and Taylor, 1973). Throughout the small intestine P absorption is

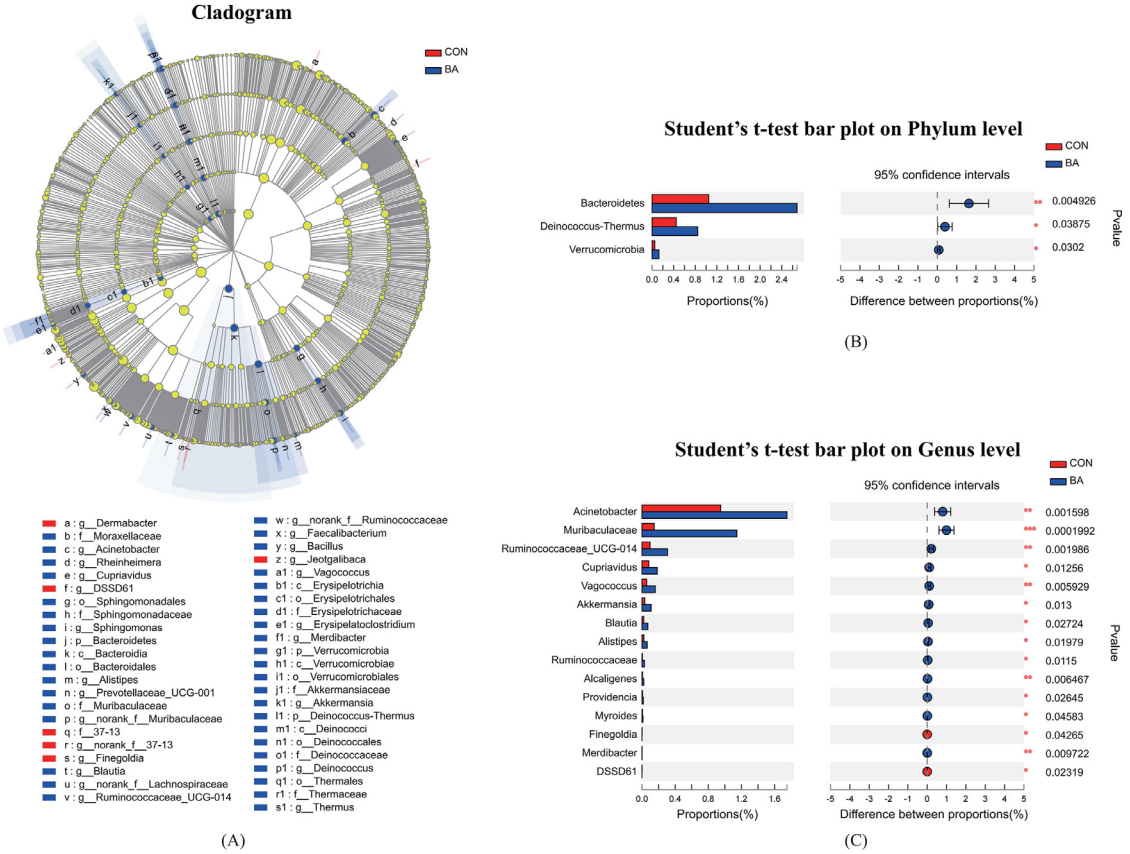

Figure 4. The abundance of the microbial community in excreta of broilers fed a basal diet (CON), or the basal diet supplemented with either *Bacillus amyloliquefaciens* (BA) on day 42. Data are indicated as means ± SEM ($n = 6$). (A) Cladogram of LEfSe multilevel species difference discriminant analysis (LDA > 2), different color nodes indicate microbial communities that are significantly enriched in the corresponding groups and significantly different between groups; (B) & (C) Comparative analysis of the relative abundance of bacteria at the phylum and genus level, respectively.

facilitated by transcellular active transport mechanisms or diffusion through paracellular routes (Berndt and Kumar, 2009; Hu et al., 2018). Liu et al. (2016) demonstrated that the duodenum was the major segment for P absorption in broiler chickens and was facilitated by a carrier mediated process. However, a nonsaturated diffusion process was evident in the jejunum and ileum (Liu et al., 2016). Type-IIb sodium-dependent phosphate cotransporter (*NaPi-IIb*) is a carrier protein involved in P absorption; it is Na^+-dependent and preferentially transports divalent P (HPO_4^{2-}) (Levi et al., 2019). Inorganic Phosphate Transporter 1, 2 (*PiT-1,2*) belong to the type III Na-Pi cotransport carriers (Aniteli et al., 2018). Most of these studies were carried out in mammals where it has been shown that *PiT-1* is highly expressed along the brush border membrane throughout the small intestine of the rat, with highest expression in the ileum (Giral et al., 2009). Moreover, *PiT-2* is mostly expressed in the crypt-villus axis epithelial cells within the mouse small intestine (Bai et al., 2000). Both proteins are housekeeping proteins widely distributed in several tissues, and belong to the type-III sodium-phosphate cotransporters (Collins et al., 2004). Dietary supplementation with BA significantly increased the expression of *PiT-1, 2* in the duodenum and jejunum while the changes in the ileum were not significant (Figure 5). However, BA had no effect on the expression of *NaPi-IIb* in the current experiment. Although the role of *PiT-1/2* in intestinal P transport is not fully understood, previous studies have shown that expression was regulated by several factors, including P concentration (Virkki et al., 2007). Extracellular enzymes and especially phytase is secreted by BA, could catalyze the hydrolysis of phytic acid in feed grain and increase P concentration in the digestive tract (Lee et al., 2008). *In vitro* studies of smooth muscle cells, when exposed to elevated concentrations of P, the *PiT-1/2* cotransporter will act as a P-sensor, facilitating its transport into cells (Giachelli,

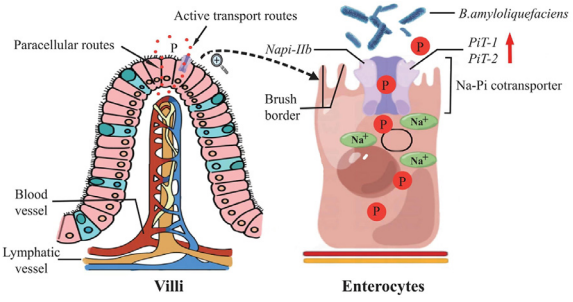

Figure 5. Schematic representation of possible mechanisms contributing to P absorption. Uptake of P by epithelial cells in the intestinal tract from digesta (transcellular active transport mechanisms and diffusion through paracellular routes) and PiT-1/2 represent the most likely locus for BA function in active transport of P.

2003). This may explains why P absorption in the anterior and middle sections of the small intestine (relatively high P concentration) is greater than in the posterior section (relatively low P concentration), indicating that *PiT-1/2* are possible sites for BA promotion of P absorption by intestinal epithelial cells. Taken together, BA modulated bone health by impacting mineral acquisition though upregulation of transporter gene expression and deposition in the tibia; in agreement with previous research (Lavoie et al., 2017). Interestingly, AGPs supplementation had little effect on the expression of transporters in the duodenum and jejunum but caused the most significant upregulation of all transporters in the ileum. It appears that both BA and AGPs can increase intestinal P absorption and presumably deposition in the tibia.

There is evidence that dietary supplementation with probiotics has a positive effect in regulation of the gut microbiota (Wang et al., 2016; Gao et al., 2017; Qiu et al., 2021). In an attempt to better understand this, we analyzed the microbiota community of excreta. Although the intestinal microflora were not directly monitored, a number of studies have shown a good correlation between intestinal and excreta microbiotas of broilers (Stanley et al., 2015; Andreani et al., 2020). The analysis of abundance in the excreta microbial community showed that BA induced differentially enriched bacteria at different taxonomic levels. At the phylum level, there was an increased abundance of *Bacteroidetes* and *Verrucomicrobia* in BA supplemented birds. The functions of *Bacteroidetes* closely associated with carbohydrate, protein, and fiber metabolism, and several studies have reported that inflammatory bowel problems are linked to a reduction of *Bacteroidetes* (Rajilić-Stojanović et al., 2011; Huo et al., 2014). *Verrucomicrobia* is known to improve glucose metabolism in animals. Moreover, the increase in the count of *Verrucomicrobia* could contributed to the depletion of pathogenic microorganism, such as *Escherichia* and *Shigella* (Turnbaugh et al., 2006; Shin et al., 2014). At the genus level, *Muribaculaceae, Ruminococcaceae, Akkermansia*, and *Alistipes* species were greatly enriched in the BA group. The abundance of *Muribaculaceae* correlates with increased production of short-chain fatty acids (Smith et al., 2019). *Ruminococcaceae* represents an important family of butyrate-producing bacteria, and as a member of the *Clostridia* class, has been implicated as having important roles in gastrointestinal health in animals (Schoster et al., 2017). Butyrate could regulate bone anabolism via Treg cell-mediated regulation of $CD8^+$ T cell Wnt10b production (Tyagi et al., 2018). In addition to producing butyrate, *Ruminococcus* also secretes extracellular digestive enzymes, contributing to digestibility of nutrients and growth performance (Saburi et al., 2010). *Akkermansia* and *Alitsipes*, may be the main contributors to the polyamine biosynthesis *in vivo*. The increased polyamines could mediate enhanced osteoblast activity and have a positive effect on reducing osteoporosis and increasing bone strength (Chevalier et al., 2020). In addition, bacteria can also regulate skeleton health by neuroendocrine signaling pathways inducing intestinal cells to produce endocrine factors such as oestrogen-like molecules, serotonin, and incretin that act as signals for skeletal cells (Ramsey and Isales, 2017). It is possible that BA is modulating some of these mechanisms but this requires much further study.

CONCLUSION

The present study demonstrated that dietary supplementation with BA provides similar efficacy as AGPs for the promotion of growth performance and bone development of broiler chickens. These responses may be associated with mechanisms involved in the up-regulation of intestinal P transporters, microbial modulation and increased P retention. These results are encouraging and suggesting that probiotics have an important role in the post-AGP era. The stage is set for the design of microbiota-based interventions to promote broiler productivity. In so doing, probiotics will improve skeletal health and bird welfare.

ACKNOWLEDGMENTS

Our research team would like to thank the "China Agriculture Research System of MOF and MARA" (No. CARS-41) and China Scholarship Council (CSC) for supporting this research.

Availability of Data and Material: The raw amplicon sequencing data from this study is available in the NCBI Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra/) with the BioProject identifier PRJNA860046.

DISCLOSURES

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

Ahmed, S. T., M. M. Islam, H. S. Mun, H. J. Sim, Y. J. Kim, and C. J. Yang. 2014. Effects of Bacillus amyloliquefaciens as a probiotic strain on growth performance, cecal microflora, and fecal noxious gas emissions of broiler chickens. Poult. Sci. 93:1963–1971.

Andreani, N. A., C. J. Donaldson, and M. Goddard. 2020. A reasonable correlation between cloacal and cecal microbiomes in broiler chickens. Poult. Sci. 99:6062–6070.

Aniteli, T. M., F. R. de Siqueira, L. M. dos Reis, W. V. Dominguez, E. M. C. de Oliveira, P. Castelucci, R. M. A. Moysés, and V. Jorgetti. 2018. Effect of variations in dietary Pi intake on intestinal Pi transporters (NaPi-IIb, PiT-1, and PiT-2) and phosphate-regulating factors (PTH, FGF-23, and MEPE). Pflugers Arch. Eur. J. Physiol. 470:623–632.

AOAC International. 2006. Official Methods of Analysis. 18th AOAC Int., Gaithersburg, MD.

AOAC International. 2000. Official Methods of Analysis. 17th AOAC Int., Arlington, VA.

Bai, L., J. F. Collins, F. K. Ghishan, J. F. Collins, and F. K. Ghishan. 2000. Cloning and characterization of a type III

Na-dependent phosphate cotransporter from mouse intestine. Am. J. Physiol. Physiol. 279:1135–1143.
Bajagai, Y. S., A. V Klieve, P. J. Dart, and W. L. Bryden. 2016. Probiotics in Animal Nutrition: Production, Impact and Regulation. FAO. http://www.fao.org/3/a-i5933e.pdf.
Berndt, T., and R. Kumar. 2009. Novel mechanisms in the regulation of phosphorus homeostasis. Physiology 24:17–25.
Bielke, L. R., B. M. Hargis, and J. D. Latorre. 2017. Impact of enteric health and mucosal permeability on skeletal health and lameness in poultry. Adv. Exp. Med. Biol. 1033:185–197.
Chevalier, C., S. Kieser, M. Çolakoğlu, N. Hadadi, J. Brun, D. Rigo, N. Suárez-Zamorano, M. Spiljar, S. Fabbiano, B. Busse, J. Ivanišević, A. Macpherson, N. Bonnet, and M. Trajkovski. 2020. Warmth prevents bone loss through the gut microbiota. Cell Metab. 32:575–590.e7.
Chung, T. K., S. M. Rutherfurd, D. V. Thomas, and P. J. Moughan. 2013. Effect of two microbial phytases on mineral availability and retention and bone mineral density in low-phosphorus diets for broilers. Br. Poult. Sci. 54:362–373.
Ciurescu, G., M. Dumitru, A. Gheorghe, A. E. Untea, and R. Drăghici. 2020. Effect of Bacillus subtilis on growth performance, bone mineralization, and bacterial population of broilers fed with different protein sources. Poult. Sci. 99:5960–5971.
Collins, J. F., L. Bai, and F. K. Ghishan. 2004. The SLC20 family of proteins: dual functions as sodium-phosphate cotransporters and viral receptors. Pflugers Arch. Eur. J. Physiol. 447:647–652.
Dibner, J. J., and J. D. Richards. 2005. Antibiotic growth promoters in agriculture: History and mode of action. Poult. Sci. 84:634–643.
Du, E., and Y. Guo. 2021. Dietary supplementation of essential oils and lysozyme reduces mortality and improves intestinal integrity of broiler chickens with necrotic enteritis. Anim. Sci. J. 92:e13499.
Gao, P., C. Ma, Z. Sun, L. Wang, S. Huang, X. Su, J. Xu, and H. Zhang. 2017. Feed-additive probiotics accelerate yet antibiotics delay intestinal microbiota maturation in broiler chicken. Microbiome. 5:91.
Gatej, S. M., V. Marino, R. Bright, T. R. Fitzsimmons, N. Gully, P. Zilm, R. J. Gibson, S. Edwards, and P. M. Bartold. 2018. Probiotic Lactobacillus rhamnosus GG prevents alveolar bone loss in a mouse model of experimental periodontitis. J. Clin. Periodontol. 45:204–212.
Giachelli, C. M. 2003. Vascular calcification : in vitro evidence for the role of inorganic phosphate. J. Am. Soc. Nephrol. 14:300–304.
Giral, H., Y. Caldas, E. Sutherland, P. Wilson, S. Breusegem, N. Barry, J. Blaine, T. Jiang, X. X. Wang, M. Levi, N. Barry, J. Blaine, T. Jiang, W. Xx, and L. M. Regulation. 2009. Regulation of rat intestinal Na-dependent phosphate transporters by dietary phosphate. Am. J. Physiol. Physiol. 297:1466–1475.
González-Cerón, F., R. Rekaya, and S. E. Aggrey. 2015. Genetic relationship between leg problems and bone quality traits in a random mating broiler population. Poult. Sci. 94:1787–1790.
Hong, Y., Y. Cheng, L. Guan, Z. Zhou, X. Li, D. Shi, and Y. Xiao. 2021. Bacillus amyloliquefaciens tl downregulates the ileal expression of genes involved in immune responses in broiler chickens to improve growth performance. Microorganisms. 9:1–21.
Hu, Y., X. Liao, Q. Wen, L. Lu, L. Zhang, and X. Luo. 2018. Phosphorus absorption and gene expression levels of related transporters in the small intestine of broilers. Br. J. Nutr. 119:1346–1354.
Huo, W., W. Zhu, and S. Mao. 2014. Impact of subacute ruminal acidosis on the diversity of liquid and solid-associated bacteria in the rumen of goats. World J. Microbiol. Biotechnol. 30:669–680.
Kilkenny, C., W. J. Browne, I. Cuthi, M. Emerson, and D. G. Altman. 2012. Improving bioscience research reporting: the ARRIVE Guidelines for Reporting Animal Research. Vet. Clin. Pathol. 41:27–31.
Latorre, J. D., X. Hernandez-Velasco, J. L. Vicente, R. Wolfenden, B. M. Hargis, and G. Tellez. 2017. Effects of the inclusion of a Bacillus direct-fed microbial on performance parameters, bone quality, recovered gut microflora, and intestinal morphology in broilers consuming a grower diet containing corn distillers dried grains with solubles. Poult. Sci. 96:2728–2735.
Lavoie, B., J. B. Lian, and G. M. Mawe. 2017. Regulation of bone metabolism by serotonin. Adv. Exp. Med. Biol. 1033:35–46.
Lee, Y. J., B. K. Kim, B. H. Lee, K. I. Jo, N. K. Lee, C. H. Chung, Y. C. Lee, and J. W. Lee. 2008. Purification and characterization of cellulase produced by Bacillus amyoliquefaciens DL-3 utilizing rice hull. Bioresour. Technol. 99:378–386.
Lei, X., X. Piao, Y. Ru, H. Zhang, A. Péron, and H. Zhang. 2015. Effect of Bacillus amyloliquefaciens-based direct-fed microbial on performance, nutrient utilization, intestinal morphology and cecal microflora in broiler chickens. Asian-Australasian J. Anim. Sci. 28:239–246.
Levi, M., E. Gratton, I. C. Forster, N. Hernando, C. A. Wagner, J. Biber, V. Sorribas, and H. Murer. 2019. Mechanisms of phosphate transport. Nat. Rev. Nephrol. 15:482–500.
Li, X., D. Zhang, and W. L. Bryden. 2017. Calcium and phosphorus metabolism and nutrition of poultry: are current diets formulated in excess? Anim. Prod. Sci. 57:2304–2310.
Liu, S. B., Y. X. Hu, X. D. Liao, L. Lu, S. F. Li, L. Y. Zhang, H. Z. Tan, L. Yang, H. Q. Suo, and X. G. Luo. 2016. Kinetics of phosphorus absorption in ligated small intestinal segments of broilers. J. Anim. Sci. 94:3312–3320.
Liu, S. B., X. D. Liao, L. Lu, S. F. Li, L. Wang, L. Y. Zhang, Y. Jiang, and X. G. Luo. 2017. Dietary non-phytate phosphorus requirement of broilers fed a conventional corn-soybean meal diet from 1 to 21 d of age. Poult. Sci. 96:151–159.
Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods. 25:402–408.
Lozupone, C., and R. Knight. 2005. UniFrac: a new phylogenetic method for comparing microbial communities. Appl. Environ. Microbiol. 71:8228–8235.
Luan, S. J., Y. B. Sun, Y. Wang, R. N. Sa, and H. F. Zhang. 2019. Bacillus amyloliquefaciens spray improves the growth performance, immune status, and respiratory mucosal barrier in broiler chickens. Poult. Sci. 98:1403–1409.
Luise, D., M. Bertocchi, V. Motta, C. Salvarani, P. Bosi, A. Luppi, F. Fanelli, M. Mazzoni, I. Archetti, G. Maiorano, B. K. K. Nielsen, and P. Trevisi. 2019. Bacillus sp. probiotic supplementation diminish the Escherichia coli F4ac infection in susceptible weaned pigs by influencing the intestinal immune response, intestinal microbiota and blood metabolomics. J. Anim. Sci. Biotechnol. 10:74.
Ma, J., S. Mahfuz, J. Wang, and X. Piao. 2021. Effect of dietary supplementation with mixed organic acids on immune function, antioxidative characteristics, digestive enzymes activity, and intestinal health in broiler chickens. Front. Nutr. 8:1–15.
Ministry of Agriculture of the People's Republic of China. 2004. Nutrient Requirements of Chinese Feeding Standard of Chicken (GB, NY/T 33-2004). Agriculture Standard Press, Beijing, China.
Mottet, A., and G. Tempio. 2017. Global poultry production: Current state and future outlook and challenges. Worlds. Poult. Sci. J. 73:245–256.
Mountzouris, K. C., I. Palamidi, V. Paraskeuas, E. Griela, and K. Fegeros. 2019. Dietary probiotic form modulates broiler gut microbiota indices and expression of gut barrier genes including essential components for gut homeostasis. J. Anim. Physiol. Anim. Nutr. (Berl). 103:1143–1159.
M'Sadeq, S. A., S. Wu, R. A. Swick, and M. Choct. 2015. Towards the control of necrotic enteritis in broiler chickens with in-feed antibiotics phasing-out worldwide. Anim. Nutr. 1:1–11.
National Research Council. 1994. Nutrient Requirements of Poultry. 9th National Academy Press, Washington, DC.
Niewold, T. A. 2007. The nonantibiotic anti-inflammatory effect of antimicrobial growth promoters, the real mode of action? A hypothesis. Poult. Sci. 86:605–609.
Nikoskelainen, S., A. C. Ouwehand, G. Bylund, S. Salminen, and E. M. Lilius. 2003. Immune enhancement in rainbow trout (Oncorhynchus mykiss) by potential probiotic bacteria (Lactobacillus rhamnosus). Fish Shellfish Immunol. 15:443–452.
Paulina, M., and Ś. Katarzyna. 2018. The role of probiotics, prebiotics and synbiotics in animal nutrition. Gut Pathog 10:1–20.
Proszkowiec-Weglarz, M., and R. Angel. 2013. Calcium and phosphorus metabolism in broilers: effect of homeostatic mechanism on calcium and phosphorus digestibility. J. Appl. Poult. Res. 22:609–627.
Qiu, K., C. L. Li, J. Wang, G. H. Qi, J. Gao, H. J. Zhang, and S. G. Wu. 2021. Effects of dietary supplementation with bacillus subtilis, as an alternative to antibiotics, on growth performance, serum immunity, and intestinal health in broiler chickens. Front. Nutr. 8:1–13.

Quach, D., and R. A. Britton. 2017. Gut microbiota and bone health. Adv. Exp. Med. Biol. 1033:47–58.

Rajilić-Stojanović, M., E. Biagi, H. G. H. J. Heilig, K. Kajander, R. A. Kekkonen, S. Tims, and W. M. De Vos. 2011. Global and deep molecular analysis of microbiota signatures in fecal samples from patients with irritable bowel syndrome. Gastroenterology 141:1792–1801.

Ramsey, W., and C. M. Isales. 2017. Intestinal incretins and the regulation of bone physiology. Adv. Exp. Med. Biol. 1033:13–33.

Saburi, W., T. Yamamoto, H. Taguchi, S. Hamada, and H. Matsui. 2010. Practical preparation of epilactose produced with cellobiose 2-epimerase from Ruminococcus albus NE1. Biosci. Biotechnol. Biochem. 74:1736–1737.

Schofield, B. J., A. Skarshewski, N. Lachner, D. Ouwerkerk, A. V Klieve, P. Dart, and P. Hugenholtz. 2016. Near complete genome sequence of the animal feed probiotic, Bacillus amyloliquefaciens H57. Stand. Genomic Sci. 11:1–7.

Schoster, A., H. R. Staempfli, L. G. Guardabassi, M. Jalali, and J. S. Weese. 2017. Comparison of the fecal bacterial microbiota of healthy and diarrheic foals at two and four weeks of life. BMC Vet. Res. 13.

Segata, N., J. Izard, L. Waldron, D. Gevers, L. Miropolsky, W. S. Garrett, and C. Huttenhower. 2011. Metagenomic biomarker discovery and explanation. Genome Biol. 12:1–18.

Shin, N. R., J. C. Lee, H. Y. Lee, M. S. Kim, T. W. Whon, M. S. Lee, and J. W. Bae. 2014. An increase in the Akkermansia spp. population induced by metformin treatment improves glucose homeostasis in diet-induced obese mice. Gut 63:727–735.

Shini, S., and W. L. Bryden. 2022. Probiotics and gut health: linking gut homeostasis and poultry productivity. Anim. Prod. Sci. 62:1090–1112.

Shini, S., R. C. Acland, and W. L. Bryden. 2021. Avian intestinal ultrastructure changes provide insight into the pathogenesis of enteric infections and probiotic mode of action. Sci. Rep. 11:167.

Shini, S., D. Zhang, R. C. Aland, X. Li, P. J. Dart, M. J. Callaghan, R. E. Speight, and W. L. Bryden. 2020. Probiotic Bacillus amyloliquefaciens H57 ameliorates subclinical necrotic enteritis in broiler chicks by maintaining intestinal mucosal integrity and improving feed efficiency. Poult. Sci. 99:4278–4293.

Smith, B. J., R. A. Miller, A. C. Ericsson, D. C. Harrison, R. Strong, and T. M. Schmidt. 2019. Changes in the gut microbiome and fermentation products concurrent with enhanced longevity in acarbose-treated mice. BMC Microbiol. 19:1–16.

Stanley, D., M. S. Geier, H. Chen, R. J. Hughes, and R. J. Moore. 2015. Comparison of fecal and cecal microbiotas reveals qualitative similarities but quantitative differences. BMC Microbiol. 15:1–11.

Tellez, G., and J. D. Latorre. 2017. Editorial: alternatives to antimicrobial growth promoters and their impact in gut microbiota, health and disease. Front. Vet. Sci. 4:196.

Tilgar, V., P. Kilgas, A. Viitak, and S. J. Reynolds. 2008. The rate of bone mineralization in birds is directly related to alkaline phosphatase activity. Physiol. Biochem. Zool. 81:106–111.

Turnbaugh, P. J., R. E. Ley, M. A. Mahowald, V. Magrini, E. R. Mardis, and J. I. Gordon. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444:1027–1031.

Tyagi, A. M., M. Yu, T. M. Darby, C. Vaccaro, J. Y. Li, J. A. Owens, E. Hsu, J. Adams, M. N. Weitzmann, R. M. Jones, and R. Pacifici. 2018. The microbial metabolite butyrate stimulates bone formation via T regulatory cell-mediated regulation of WNT10B expression. Immunity 49:1116–1131.e7.

Virkki, L. V, J. Biber, H. Murer, and I. C. Forster. 2007. Phosphate transporters : a tale of two solute carrier families. Am. J. Physiol. Physiol. 293:643–654.

de Vries S. and Gerrits W.J.J., The use of Tracers or Markers in Digestion Studies, In Feed Evaluation Science, Moughan P. & Hendriks W.H., 2018, Wageningen Academic Publishers; Wageningen, The Netherlands, 275−294.

Wang, Y. B., W. Du, A. K. Fu, X. P. Zhang, Y. Huang, K. H. Lee, K. Yu, W. F. Li, and Y. L. Li. 2016. Intestinal microbiota and oral administration of Enterococcus faecium associated with the growth performance of new-born piglets. Benef. Microbes. 7:529–538.

Wasserman, R. H., and A. N. Taylor. 1973. Intestinal absorption of phosphate in the chick: effect of vitamin D and other parameters. J. Nutr. 103:586–599.

Wideman, R. F., K. R. Hamal, J. M. Stark, J. Blankenship, H. Lester, K. N. Mitchell, G. Lorenzoni, and I. Pevzner. 2012. A wire-flooring model1 for inducing lameness in broilers: evaluation of probiotics as a prophylactic treatment. Poult. Sci. 91:870–883.

Yan, F. F., G. R. Murugesan, and H. W. Cheng. 2019. Effects of probiotic supplementation on performance traits, bone mineralization, cecal microbial composition, cytokines and corticosterone in laying hens. Animal. 13:33–41.

Zakłos-Szyda, M., A. Nowak, N. Pietrzyk, and A. Podsędek. 2020. Viburnum opulus L. juice phenolic compounds influence osteogenic differentiation in human osteosarcoma saos-2 cells. Int. J. Mol. Sci. 21:1–26.