Enterococcus Faecium Modulates the Gut Microflora of Broilers and Enhances Phosphorus Absorption and Utilization

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Research

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Abstract

Background: The modern broiler or meat chicken has ongoing bone health problems. Phosphorus (P) plays an important role in bone development and increased understanding of P metabolism should improve the skeletal health of broilers. *Enterococcus faecium* has been widely used as a probiotic in broiler production and shown to improve skeletal health of rats; but its effect on the bones of broilers remains unclear. This study investigated the effect of *E. faecium* on P absorption and utilization in broilers and associated changes in the gut microflora using 16S rDNA sequencing.

Results: Dietary supplementation with *E. faecium* improved P absorption through up-regulation of the expression of intestinal *NaP-IIb* mRNA, and increased the concentration of serum [alkaline phosphatase](#). These actions increased P retention and bone mineralization in *E. faecium* treated broilers. The positive effects of *E. faecium* on P metabolism were associated with changes in the structure of the intestinal microbiota. There was increased relative abundance of the following genera, *Alistipes*, *Eubacterium*, *Rikenella* and *Ruminococcaceae*, and a decrease in the relative abundance of *Faecalibacterium* and *Escherichia-Shigella*.

Conclusions: Dietary supplementation with *E. faecium* changed gut microbiota structure of broilers, increased the relative abundance of SCFA (short chain fatty acid) producing bacteria, improved intestinal P absorption, bone forming metabolic activities, and decreased P excretion. *E. faecium* facilitates increased utilisation of P in broilers.

Background

Skeletal disorders and associated welfare problems are an ongoing issue for fast growing broilers or meat chickens and a major concern throughout the global poultry industry[1, 2]. Calcium (Ca) and phosphorus (P) are the most important minerals in bone development and comprise the inorganic component of bone tissue, providing hardness and strength to the skeleton [3]. Diets deficient or imbalanced in these co-dependent minerals severely decrease growth performance and nutrient retention of broilers[4, 5]. Many studies have investigated the absorption and metabolism of Ca and P, and there is a greater understanding of the regulatory mechanisms controlling Ca metabolism than P metabolism[5]. In addition, eutrophication due to high P excretion is becoming more and more serious, which intensifies concerns about P utilization and the sustainability of broiler production[6].

The addition of probiotics to poultry diets has increased significantly in recent years as the use of antibiotic growth promoters has declined[7]. This has prompted much research into the use of new probiotic feed additives. *E. faecium*, a lactic acid bacterium and normal inhabitant in the gut, is a probiotic that can promote growth performance, reduce mortality, improve intestinal morphology, and beneficially modulate the gut microflora of broilers[8-10]. These characteristics of *E. faecium*, along with the ability to increase the efficiency of intermediary metabolism[11] and improve meat quality[12], have made it an attractive poultry feed additive. Moreover, some probiotics have shown beneficial effects on

the skeletal health of broilers[13, 14] and rodents[15]. These observations are consistent with probiotics modifying the gut environment, including the gut microflora, and/or enhancing mineral absorption. Although the mode of action(s) of probiotics are poorly understood[7], modulation of the gut microflora is likely to be important.

There is little research on the effect of *E. faecium* on bone health. However, it has been suggested that *E. faecium* can prevent whole body bone mineral density loss in arthritic rats[16]. It is therefore likely, that *E. faecium* will have some effect on the bone health of broilers. The objective of the present study was to investigate the effects of dietary *E. faecium* on performance traits, bone strength, P absorption and gut microbiota of broilers, and to explore the regulatory mechanism of *E. faecium* on P absorption and utilization in broilers.

Materials And Methods

Experimental design

A total of 120 1-day-old male, Arbor Acres (AA) broilers, were purchased from the Huadu Broiler Breeding Co. (Beijing, China), and housed in the Nankou experimental farm of the Feed Research Institute, CAAS, Beijing, China. The day-old chicks (body weight, 47.2± 0.31 g) were randomly divided into two groups, control and treatment. Each group had 6 cages (replicates) with 10 birds per cage. The chickens were reared in two stages, starter (1–21 days) and grower (22–42 days), and fed a basal (control) corn-soybean meal diet (Table 1), to which 6.75×10^9 cfu/g of *E. faecium* was added for the treatment group. Microcapsules of *E. faecium* CGMCC 2516[17, 18] (viable count $\geq 15 \times 10^{10}$ cfu/g; Challenge Group, Beijing, China) were used in this study.

Table 1
Ingredient and nutrient composition of basal broiler diets

Ingredient	Starter (1–21 days) (g/kg)	Grower (22–42 days) (g/kg)
Corn	593.1	604.2
Soybean meal	298.8	288.7
Cotton seed meal	50.0	30.0
Soybean oil	15.1	39.8
L-Lys	1.5	0.9
<i>DL</i> -Met	1.4	1.6
Limestone	12.7	10.2
CaHPO ₄	19.4	16.6
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
Total	1000	1000
Nutrient levels ²⁾		
ME (MJ/kg)	12.35	13.02
CP	211.8	198.4
Ca	10.1	8.5
AP	4.5	4.0
TP	6.9	6.3
Lys	11.4	10.5
Met	4.9	4.8
Met+Cys	8.3	8.1
Thr	7.7	2.2

¹⁾ The premix provided the following per kg diet: VA 10, 000 IU, VD₃ 2000 IU, VE 10 IU, VK₃ 2.5 mg, VB₁ 1 mg, VB₂ 6 mg, VB₃ 10 mg, VB₅ 40 mg, VB₆ 3 mg, VB₁₁ 0.3 mg, VB₁₂ 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⁾ Calculated values.

Bird management

Birds were raised in accordance with the AA Broiler Management Guide. Chicks were vaccinated for Marek's Disease at day-old and for Newcastle Disease and Infectious Bronchitis at 7 days post-hatching. Room temperature was maintained at 33 °C for days 0~3 and gradually reduced to 24 °C and maintained at 24 °C till the end of the study. Photoperiod was controlled to 23 hours of light and 1 hour of darkness. Relative humidity was set at 60 %~70 % during the first week and then at 50 %~60 % for the rest of the experiment.

Sample collection and parameter determination

From day 18 to 21 and day 39 to 42 of the experiment, excreta from each replicate was collected, mixed and dried in an oven at 105 °C to a constant weight. The dried excreta was ashed in a muffle furnace at 550 °C for 4 hours. The P content of the ash samples was determined using the vanadate-molybdate method[19].

On day 21 and day 42, body weight (BW) and feed intake were measured to calculate the average daily gain (ADG), average daily feed intake (ADFI), and the ratio of feed/gain (F/G). On those days, one broiler close to the cage average body weight was randomly selected from each replicate. The chosen birds were electrically stunned, and manually slaughtered within 5 min[20]. Blood was collected from the jugular vein, and serum was obtained after centrifuging at 3000 g for 10 min at 4 °C and stored at -20 °C for further analysis. Serum alkaline phosphatase (ALP) and P were determined with a Hitachi 7600 automatic biochemical analyzer, using kits were purchased from Nanjing Jiancheng Biological Engineering Institute.

After the blood sampling on day 42, the duodenum (about 10 cm distal to the pylorus), jejunum (about 10 cm preceding the Meckel's diverticulum) and ileum (about 10 cm preceding the ileocecal junction) were separated[21], and flushed gently with saline solution. The mucosa samples were scraped with a coverslip and snap-frozen in liquid nitrogen for analysis of mRNA.

The right tibiae was cleaned and dried for determination of tibia weight and tibia breaking strength[22]. The bones were then ashed in a muffle furnace at 550 °C for 16 h[19]. After that, the tibia P content was measured using the vanadate-molybdate method.

RNA extraction, reverse transcription and real-time quantitative PCR

Total RNA was extracted using TRNzol-A+ (TIANGEN, Beijing, China). The concentration of total RNA was estimated by spectrophotometer (Ultrospec 2100 pro, GE Healthcare), and the purity was determined by

agarose gel electrophoresis. 500 ng of total RNA was reversely transcribed into cDNA using the Fast Quant RT Kit (with gDNase) (TIANGEN). qPCR was conducted using the iCycler iQ5 system. The specific primers for *NaP-Ilb*, *PiT-1*, *PiT-2*, and *β-actin* were listed in Table 2. *β-actin*, was used as internal reference gene. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method[23]. All the samples were analyzed in triplicate and operational program for qPCR strictly followed the MIQE[24].

Table 2
Primer sequences of chicken *NaP-Ilb*, *PiT-1*, *2*, and *β-actin*

Gene	Primer sequence (5'-3')	Accession number
<i>NaP-Ilb</i>	F: CTGGATGCACTCCCTAGAGC	NM_204474.1
	R: TTATCTTTGGCACCTCCTG	
<i>PiT-1</i>	F: GCTCGTGGCTTCGTTCTTG	XM_015297502.1
	R: GACCATTTGACGCCTTTCT	
<i>PiT-2</i>	F: GCAGCAGATACATCAACTC	NM_001305398.1
	R: ATTTCCACTCCACCCTC	
<i>β-actin</i>	F: GAGAAATTGTGCGTGACATCA	NM_205518.1
	R: CCTGAACCTCTCATTGCCA	

Illumina sequencing analysis

The fecal samples were collected on day 42 and snap-frozen in liquid N₂ prior to further processing. Gene sequencing (16S rDNA) was performed by OE Biotech Co., Ltd (Shanghai, China). Total genomic DNA from frozen fecal samples was isolated using the GenElute™ Stool DNA Isolation Kit (Sigma-Aldrich, St. Louis, USA), then the V3-V4 hypervariable region of the 16S rDNA genes was amplified. The PCR products were collected and sequenced using the Illumina MiSeq platform (Illumina, San Diego, CA, USA). High-quality reads were clustered into operational taxonomic units (OTUs) based on sequences with ≥ 97 % similarity and then analyzed using the QIIME platform.

Statistical analysis

The statistical analyses were performed using SPSS 17.0. The data were statistically analyzed by one-way or two-way ANOVA, and the F-test was used for factor significance. For the indexes with significant main effect, Duncan’s method was used to compare the mean values among groups. A *P*-value less than 0.05 was considered significant.

Results

The birds grew normally and remained in good health throughout the experiment.

Growth performance

The effects of dietary *E. faecium* on growth performance of broilers is shown in Table 3. In the starter stage (1–21 days), dietary supplementation with *E. faecium* decreased ($P<0.05$) ADFI of broilers, but did not affect ($P>0.05$) BW, ADG and F/G. In the grower stage (22–42 days), *E. faecium* groups showed a tendency to numerically increase BW and ADG, and to decrease ADFI and F/G of broilers.

Table 3
Dietary *E. faecium* supplementation and broiler growth performance

Treatment	BW(g)	ADG(g/d)	ADFI(g/d)	F/G
Starter (1–21 days)				
Control	771	38.6	51 ^a	1.37
<i>E. faecium</i>	793	39.6	45 ^b	1.25
Pooled SEM	12.50	0.48	1.00	0.028
<i>P</i> Value	0.088	0.453	0.011	0.219
Grower (22–42 days)				
Control	2207	73.7	153	2.12
<i>E. faecium</i>	2262	76.4	152	2.07
Pooled SEM	30.70	1.84	2.89	0.033
<i>P</i> Value	0.067	0.085	0.081	0.632

^{a,b,c} Mean values with unlike superscript letters within the same list are significantly different ($P<0.05$).

Ash and P of excreta, serum P and ALP concentrations

Supplementation with *E. faecium* did no effect excreta ash content during either growth stage nor P excreta content of starter chicks but did decrease ($P<0.05$) P excretion in the grower stage (Table 4). No difference in serum P concentration was observed between the two groups of broilers. However, serum ALP increased significantly ($P<0.05$) in both the starter and grower stages when compared to the control group.

Table 4

Dietary *E. faecium* supplementation and excreta ash and P content, serum P concentration, and alkaline phosphatase (ALP) of broilers

Treatment	Ash (g/kg excreta)	P (g/kg excreta)	P (mmol/L)	ALP (U/L)
Starter(1–21 days)				
Control	148.6	12.4	1.50	3291 ^b
<i>E. faecium</i>	151.1	13.1	1.64	4099 ^a
Pooled SEM	1.7	0.2	0.03	145
P Value	0.516	0.226	0.163	0.002
Grower (22–42 days)				
Control	155.8	13.8 ^a	1.47	1843 ^b
<i>E. faecium</i>	156.8	12.8 ^b	1.52	2787 ^a
Pooled SEM	0.10	0.02	0.03	161
P Value	0.220	0.038	0.753	0.001

^{a,b,c} Mean values with unlike superscript letters within the same list are significantly different ($P < 0.05$).

P and ash of bone, tibia strength

In the starter stage, P and ash content of bone was not influenced by *E. faecium* supplementation (Table 5) but in the grower stage, the probiotic significantly ($P < 0.05$) increased both parameters. While tibia strength was not affected in the starter or grower stage.

Table 5. Dietary *E. faecium* supplementation on P and ash content of bone, ash of bone and tibia strength of broilers

Treatment	P of bone, %	Ash of bone, %	Tibia strength, g
Starter (1–21 days)			
Control	8.54	53.51	14795
<i>E. faecium</i>	8.80	54.25	13408
Pooled SEM	0.11	0.32	1125
<i>P</i> Value	0.102	0.298	0.109
Grower (22–42 days)			
Control	8.34 ^b	52.16 ^b	24822
<i>E. faecium</i>	10.99 ^a	56.32 ^a	19010
Pooled SEM	0.38	1.18	1282
<i>P</i> Value	0.001	0.003	0.109

^{a,b,c} Mean values with unlike superscript letters within the same list are significantly different ($P < 0.05$).

Type IIb sodium-dependent phosphate cotransporter (*NaP-IIb*) and type III sodium-dependent phosphate cotransporter-1, 2 (*PiT-1, 2*) mRNA expressions in the duodenum, jejunum and ileum of broilers

Dietary supplementation of *E. faecium* significantly increased ($P < 0.05$) *NaP-IIb* mRNA expression levels in the duodenum, jejunum, and ileum (Table 6). However, *PiT-1* and *PiT-2* mRNA expressions were not affected in the duodenum and jejunum, but increased in the ileum of the *E. faecium* treated group.

Table 6. Dietary *E. faecium* supplementation and *NaP-IIb* and *PiT-1, 2* mRNA expression in small intestinal segments of broilers

Treatment	<i>NaP-1b</i>	<i>PiT-1</i>	<i>PiT-2</i>
Duodenum			
Control	1.000 ^b	5.861	3.969
<i>E. faecium</i>	2.335 ^a	6.106	4.547
Pooled SEM	0.32	0.06	0.13
<i>P</i> Value	0.042	0.256	0.833
Jejunum			
Control	3.93 ^b	3.703	1.791
<i>E. faecium</i>	11.291 ^a	10.176	3.705
Pooled SEM	0.06	1.30	0.80
<i>P</i> Value	0.014	0.590	0.664
Ileum			
Control	1.117 ^b	0.893 ^b	1.111 ^a
<i>E. faecium</i>	4.265 ^a	8.87 ^a	3.523 ^{ab}
Pooled SEM	0.88	0.36	0.61
<i>P</i> Value	0.001	0.008	0.073

^{a,b} Mean values with unlike superscript letters within the same list are significantly different ($P < 0.05$).

Gut microbiota analysis

As revealed by principal component analysis (PCA), dietary supplementation with *E. faecium* changed the structure of the gut microbiota of broilers (Figure 1a). In addition, *E. faecium* led to a significant increase in the observed species and Simpson indices with respect to the control values (Figures 1b, c), suggesting that *E. faecium* exerted stronger positive effects on the α diversity of the gut microbiota of broilers.

The gut microbiota composition at phylum and genus levels is shown in Figure 2. The predominant phyla were *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Epsilonbacteraeota*, *Actinobacteria*, and *Tenericutes*, representing 59.8%, 35.0%, 4.25%, 0.49%, 0.21% and 0.20% of the total sequences, respectively (Fig. 2a). There are no significant differences in phylum level between *E. faecium* treated and control birds. Compared to the control group, a higher abundance of *Bacteroidetes* and a lower abundance of *Proteobacteria* were observed in the *E. faecium* treated group. The genus level analysis revealed that *E. faecium* mainly increased the relative abundance of *Alistipes*, *Eubacterium*, *Rikenella*, and

Ruminococcaceae, while the the relative abundance of *Faecalibacterium* and *Escherichia-Shigella* was decreased (Fig. 2b).

We compared the gut microbiota of the two experimental groups using linear discriminant analysis effect size (LEfSe) to identify the specific microbiota linked to *E. faecium* treatment. *Peptoclostridium*, *Ruminococcaceae*, *Papillibacter*, and *Eubacterium* were more abundant in the *E. faecium* groups (Figures 3a, b).

Discussion

As has been shown in other studies [9, 25], addition of a probiotic to broiler diets under ideal experimental conditions, does not always improve broiler performance as occurred in this study. However in other studies using various probiotic strains, significant improvement in growth performance of broilers has been demonstrated [26-29]. The different outcomes in broiler growth performance are a response to many factors including the probiotic strain used and the experimental conditions. Studies have shown that probiotic supplementation successfully overcame subclinical exposure to necrotic enteritis but was without effect with unchallenged birds[30].

Bone metabolism largely mediates P and Ca metabolism, which are closely related but differ in relation to the endocrine control of absorption and renal reabsorption/excretion[31]. Dietary P is absorbed and accumulated in the small intestinal mucosa, then released into the blood gradually [32], where concentrations are maintained by homeostasis [33], as was evident in the current study. Previous studies in rats, humans and chickens have suggested that probiotics could promote intestinal absorption of P[34-37]. In this study *E. faecium* increased the P content of bone. This result reflects increased P accretion in bone, where the bone-forming cells, osteoblasts, are responsible for the deposition of bone matrix [38]. The concentration of ALP in serum, a marker of osteoblast activity, is usually elevated when bone formation rates are increased[39]. In the current study, ALP levels in serum of the *E. faecium* group were significantly higher than the control in both starter and grower stages resulting in greater retention of P.

The increase in P deposition reflects both increased absorption and reduced excretion. The decreased concentration of P in excreta may reflect both enhanced intestinal absorption and renal reabsorption. Much of the intestinal absorption of P is accomplished by the sodium-dependent transporter *NaP-IIb*, which is primarily expressed in the duodenum of broilers and is considered to be the most important P transporter in the small intestine[40]. In our study, mRNA expression levels of *NaP-IIb* were increased in the small intestine of *E. faecium* treated broilers; indicating that increased P absorption occurred in this study. The elevation of *NaP-IIb* expression levels in the *E. faecium* group may have resulted from increased available P in the intestine. The expression of *NaP-IIb* mRNA increases when the level of dietary P increases and is concentration dependent[41]. Some probiotics produce phytase[42], which would enhance phytate digestibility, releasing P for absorption. Perhaps *E. faecium* produces a phytase or facilitates increased phytase activity by the intestinal microbiota.

In the current study, the structure of ceecal microbiota was changed in the *E. faecium* group compared with the control group. *E. faecium* treatment resulted in a dramatic elevation in observed species and

Simpson indices, indicated that the richness and diversity of the microbiota was significantly increased. Previous studies have shown that inclusion of *E. faecium* in broiler diets beneficially alters the gut microflora[8, 43]. Our results demonstrate that dietary *E. faecium* can modulate the intestinal flora of broilers as indicated by 16S rDNA-based analysis. To be specific, relative abundance of genera, including *Alistipes*, *Rikenella*, *Eubacterium* and *Ruminococcaceae* were up-regulated in the *E. faecium* group. *Alistipes*, initially isolated from human gut, is a strict anaerobe that produces succinic acid as the principal metabolic end-product of glucose fermentation[44, 45]. Mice studies suggest that members of this genus affect host physiology, including sites distal to the gastrointestinal tract[46]. *Rikenella* has been isolated from faeces and caeca of a variety of animals including chickens. It is an obligate anaerobe, and yields propionic and succinic acids together with moderate amounts of acetic acid from glucose fermentation[47]. The other two up-regulated genera, *Eubacterium* and *Ruminococcaceae*, are generally associated with increased butyrate production[48], which is an important energy substrate for intestinal enterocytes. In general, the up-regulated genera in the *E. faecium* group all are associated with increased short chain fatty acids (SCFA) production. SCFA production, followed by a decrease in gut pH would help mineral absorption via increased solubilization[49]. Increased circulating concentrations of SCFA can interact with the skeleton to directly inhibit bone resorptive osteoclast differentiation and to activate bone forming osteoblasts, thus increasing bone mass and preventing bone loss[50, 51].

In conclusion, regardless of the mechanism(s), *E. faecium* facilitates increased utilisation of P in broilers. Dietary supplementation with *E. faecium* increased the relative abundance of SCFA producing bacteria, improved intestinal absorption of P, and bone forming metabolic activities, and decreased P excretion. Further research is required to more clearly define the metabolic actions of probiotics to permit their strategic use. The results of this study are, however, another illustration of the benefits of supplementing poultry diets with probiotics.

Abbreviations

P: Phosphorus; *E. faecium*: *Enterococcus faecium*; SCFA: Short chain fatty acid; Ca: Calcium; AA: Arbor Acres; BW: Body weight; ADG: Average daily gain; ADFI: Average daily feed intake; F/G: Ratio of feed/gain; ALP: Alkaline phosphatase; NaP- b : Type IIb sodium-dependent phosphate cotransporter; PiT-1, 2: Type III sodium-dependent phosphate cotransporter-1, 2; PCA: Principal component analysis; LEfSe: Linear discriminant analysis effect size; LDA: linear discriminant analysis.

Declarations

Ethics approval

The care and use of the chicks used in this experiment were approved by the Animal Care and Use Committee of the Feed Research Institute of the Chinese Academy of Agricultural Sciences (CAAS).

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

Wayne L. Bryden and Aijuan Zheng contributed to the conception and design of the work. Huiyi Cai and Guohua Liu contributed to the design of the work. Weiwei Wang and Anrong Zhang executed the experiments. Zhimin Chen, Wenhuan Chang, Aijuan Zheng and Xuejuan Deng contributed to the analysis and interpretation of the data. Weiwei Wang and Aijuan Zheng drafted the manuscript. Weiwei Wang, Wayne L. Bryden and Aijuan Zheng contributed to the final approval of the version for publication. All the authors read and approved the final manuscript.

References

1. Rath NC, Huff GR, Huff WE, Balog JM. Factors regulating bone maturity and strength in poultry. *Poult Sci.* 2000;79(7):1024-1032. <https://doi.org/10.1093/ps/79.7.1024>.
2. Fleming, Robert H. Nutritional factors affecting poultry bone health. *Proceedings of the Nutrition Society.* 2008; 67(2),177-183. <https://doi.org/10.1017/s0029665108007015>.
3. Shapiro R, Heaney RP. Co-dependence of calcium and phosphorus for growth and bone development under conditions of varying deficiency. *Bone.* 2003;32(5):532-540.
4. Gautier AE, Walk CL, Dilger RN. Influence of dietary calcium concentrations and the calcium-to-non-phytate phosphorus ratio on growth performance, bone characteristics, and digestibility in broilers. *Poult Sci.* 2017;96(8):2795-2803.
5. Li X, Zhang D, Bryden WL. Calcium and phosphorus metabolism and nutrition of poultry: Are current diets formulated in excess? *Animal Production Science.* 2017;57(11):2304-2310.

6. Li X, Zhang D, Yang TY, Bryden WL. Phosphorus bioavailability: A key aspect for conserving this critical animal feed resource with reference to broiler nutrition. *Agriculture*. 2016;6(2):25.
7. Bajagal YS, Klieve AV, Dart PJ, Bryden WL. Probiotics in animal nutrition: Production, impact and regulation. In: Harinder PSM, editors. *FAO Animal Production and Health Paper No. 179*. Rome. 2016. <http://www.fao.org/3/a-i5933e.pdf>.
8. Cao GT, Zeng XF, Chen A, Zhou L, Zhang L, Xiao YP, et al. Effects of a probiotic, *Enterococcus faecium*, on growth performance, intestinal morphology, immune response, and cecal microflora in broiler chickens challenged with *Escherichia coli* *Poult Sci*. 2013;92(11):2949-2955.
9. Luo JJ, Zheng AJ, Meng K, Chang WH, Bai YG, Li K, et al. Proteome changes in the intestinal mucosa of broiler (*Gallus gallus*) activated by probiotic *Enterococcus faecium*. *Journal of Proteomics*. 2013;91:226-241. <https://doi.org/10.1016/j.jprot.2013.07.017>.
10. Huang LQ, Luo LQ, Zhang YR, Wang Z, Xia ZF. Effects of the dietary probiotic, *Enterococcus faecium* NCIMB11181, on the intestinal barrier and system immune status in *Escherichia coli* 078-challenged broiler chickens. *Probiotics and Antimicrobial Proteins*. 2019;11(3):946-956. <https://doi.org/10.1007/s12602-018-9434-7>.
11. Zheng AJ, Luo JJ, Meng K, Li JK, Bryden WL, Chang WH, et al. Probiotic (*Enterococcus faecium*) induced responses of the hepatic proteome improves metabolic efficiency of broiler chickens (*Gallus gallus*). *BMC Genomics*. 2016;17: 89. <https://doi.org/10.1186/s12864-016-2371-5>.
12. Zheng AJ, Luo JJ, Meng K, Li JK, Zhang S, Li K, et al. Proteome changes underpin improved meat quality and yield of chickens (*Gallus gallus*) fed the probiotic *Enterococcus faecium*. *BMC Genomics*. 2014;15: <https://doi.org/10.1186/1471-2164-15-1167>.
13. Yan FF, Murugesan GR, Cheng HW. Effects of probiotic supplementation on performance traits, bone mineralization, cecal microbial composition, cytokines and corticosterone in laying hens. *Animal*. 2018;13(1):33-41. <https://doi.org/10.1017/S175173111800109X>.
14. Yan FF, Mohammed A, Murugesan R, Cheng HW. Effects of a dietary synbiotic inclusion on bone health in broilers subjected to cyclic heat stress episodes. *Poult Sci*. 2018;98(3):1083-1089.
15. Hernandez CJ, Guss JD, Luna M, Goldring SR. Links between the microbiome and bone. *Journal of Bone and Mineral Research*. 2016;31(9):1638-1646. <https://doi.org/10.1002/jbmr.2887>.
16. McCabe LR, Britton RA, Parameswaran N. Prebiotic and probiotic regulation of bone health: Role of the intestine and its microbiome. *Current Osteoporosis Reports*. 2015;13(6):363-371. <https://doi.org/10.1007/s11914-015-0292-x>.
17. Wang TT, Li AK, Tao HH, Yi JM. Study on the production technology and properties of microencapsulated *Enterococcus faecium*. *Journal of Northwest A&F University*. 2009;37(12):51-62.
18. Wang TT, Li AK, Yi JM, Tao HH, Wang Y. Screening, identification and characterization of *Enterococcus faecium* for feed use. *Journal of the Chinese Cereals and Oils Association*. 2010;25(3):89-93,97. (in Chinese)
19. Liu S B, Liao XD, Lu L, Li SF, Wang L, Zhang LY, et al. Dietary non-phytate phosphorus requirement of broilers fed a conventional corn-soybean meal diet from 1 to 21 d of age. *Poult Sci*. 2016;96(1):151-

159. <https://doi.org/10.3382/ps/pew212>.
20. Zhang L, Yue HY, Zhang HJ, Xu L, Wu HJ, Yan HJ, et al. Transport stress in broilers: I. Blood metabolism, glycolytic potential, and meat quality. *Poult Sci*. 2009;88(10):2033-2041.
21. Hu YX, Liao XD, Wen Q, Lu L, Zhang LY, Luo XG. Phosphorus absorption and gene expression levels of related transporters in the small intestine of broilers. *British Journal of Nutrition*. 2018;119(12):1346-1354. <https://doi.org/10.1017/S0007114518000934>.
22. Frost TJ, Roland DA. Research note: Current methods used in determination and evaluation of tibia strength: A correlation study involving birds fed various levels of cholecalciferol. *Poult Sci*. 1991;70(7):1640-1643. <https://doi.org/10.3382/ps.0701640>.
23. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ Methods. 2001;25(4):402-408.
24. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett JF, Kubista M, et al. The MIQE guidelines: Minimum information for publication of quantitative Real-Time PCR experiments. *Clinical Chemistry*. 2009;55(4):611-622.
25. Shewita R, Elkatcha M, Soltan M, Sedeik M. Impact of using *Enterococcus faecium* as a probiotic in broiler diets. *Alexandria Journal of Veterinary Sciences*. 2016;51(1):102-113.
26. Shokryazdan P, Jahromi MF, Liang JB, Ramasamy K, Sieo CC, Ho YW. Effects of a *Lactobacillus salivarius* mixture on performance, intestinal health and serum lipids of broiler chickens. *Plos One*. 2017;12(5):e0175959. <https://doi.org/10.1371/journal.pone.0175959>.
27. Zhang L, Zhang LL, Zhan XA, Zeng XF, Zhou L, Cao GT, et al. Effects of dietary supplementation of probiotic, *Clostridium butyricum*, on growth performance, immune response, intestinal barrier function, and digestive enzyme activity in broiler chickens challenged with *Escherichia coli* *Journal of Animal Science and Biotechnology*. 2016;7(1):3.
28. Gadde U, Oh ST, Lee YS, Davis E, Zimmerman N, Rehberger T, et al. The effects of direct-fed microbial supplementation, as an alternative to antibiotics, on growth performance, intestinal immune status, and epithelial barrier gene expression in broiler chickens. *Probiotics & Antimicrobial Proteins*. 2017;9:397-405. <https://doi.org/10.1007/s12602-017-9275-9>.
29. Abdel-Hafeez HM, Saleh ESE, Tawfeek SS, Youssef IMI, Abdeldaim ASA. Effects of probiotic, prebiotic, and synbiotic with and without feed restriction on performance, hematological indices and carcass characteristics of broiler chickens. *Asian Australasian Journal of Animal Sciences*. 2016;30(5):672-682. <https://doi.org/10.5713/ajas.16.0535>.
30. Khalique A, Zeng D, Shoaib M, Wang H, Qing XD, Rajput DS, et al. Probiotics mitigating subclinical necrotic enteritis (SNE) as potential alternatives to antibiotics in poultry. *AMB Express*. 2020;10,50. <https://doi.org/10.1186/s13568-020-00989-6>.
31. Li X, Zhang D, Bryden, WL. Calcium and phosphorus metabolism and nutrition of poultry: Are current diets formulated in excess? *Animal Production Science*. 2017;57: 2304-2310. <https://doi.org/10.1071/AN17389>.

32. Wasserman RH, Taylor AN. Intestinal absorption of phosphate in the chick: Effect of vitamin D3 and other parameters. *Journal of Nutrition*. 1973;103(4):586-599. <https://doi.org/10.1093/jn/103.4.586>.
33. Proszkowiec-Weglarz M, Angel R. Calcium and phosphorus metabolism in broilers: Effect of homeostatic; mechanism on calcium and phosphorus digestibility. 2013;22(3):609-627. <https://doi.org/10.3382/japr.2012-00743>.
34. Pérez-Conesa D, López G, Ros G. Effect of probiotic prebiotic and synbiotic follow-up infant formulas on large intestine morphology and bone mineralisation in rats. *Journal of the Science of Food & Agriculture*. 2007;87(6):1059-1068. <https://doi.org/10.1002/jsfa.2812>.
35. Rodrigues FC, Castro ASB, Rodrigues VC, Fernandes SA, Fontes EAF, Oliveira TTD, et al. Yacon flour and *Bifidobacterium longum* modulate bone health in rats. *Journal of Medicinal Food*. 2012;15(7):664-670. <https://doi.org/1089/jmf.2011.0296>.
36. Narva M, Nevala R, Poussa T, Korpela R. The effect of *Lactobacillus helveticus* fermented milk on acute changes in calcium metabolism in postmenopausal women. *European Journal of Nutrition*. 2004;43(2):61-68. <https://doi.org/10.1007/s00394-004-0441-y>.
37. Mutu R, Kocabagli N, Alp M, Acar N, Eren M, Gezen SS. The effect of dietary probiotic supplementation on tibial bone characteristics and strength in broilers. *Poult Sci*. 2006;85(9):1621. <https://doi.org/10.1093/ps/85.9.1621>.
38. Gong Y, Slee RB, Fukai N, Rawadi G, Roman-Roman S, Reginato AM, et al. LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. *Cell*. 2001;107(4):513-23. [https://doi.org/10.1016/s0092-8674\(01\)00571-2](https://doi.org/10.1016/s0092-8674(01)00571-2).
39. Rawadi G, Vayssière B, Dunn F, Baron R, Romanroman S. BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a *Wnt* autocrine loop. *Journal of Bone & Mineral Research*. 2003;18(10):1842-1853. <https://doi.org/10.1359/jbmr.2003.18.10.1842>.
40. Liu SB, Hu YX, Liao XD, Lu L, Li SF, Zhang LY, et al. Kinetics of phosphorus absorption in ligated small intestinal segments of broilers. *Journal of Animal Science*. 2016;94(8):3312. <https://doi.org/10.2527/jas.2016-0430>.
41. Hu YX, Liao XD, Wen Q, Lu L, Zhang LY, Luo XG. Phosphorus absorption and gene expression levels of related transporters in the small intestine of broilers. *British Journal of Nutrition*. 2018;119(12):1346-1354. <https://doi.org/10.1017/S0007114518000934>.
42. Andrabi ST, Bhat B, Gupta M, Bajaj BK. Phytase-producing potential and other functional attributes of lactic acid bacteria isolates for prospective probiotic applications. *Probiotics and Antimicrobial Proteins*. 2016;8(3):121–129. <https://doi.org/10.1007/s12602-016-9220-3>.
43. Gheisar MM, Hosseindoust A, Kim IH. Effects of dietary *Enterococcus faecium* on growth performance, carcass characteristics, faecal microbiota, and blood profile in broilers. *Veterinárni Medicína*. 2016;61(1):28-34. <https://doi.org/10.17221/8680-VETMED>.
44. Song YL, Kononen E, Rautio M, Liu CX, Bryk A, Eerola E, et al. *Alistipes onderdonkii* nov. and *Alistipes shahii* sp. nov., of human origin. *International Journal of Systematic & Evolutionary Microbiology*. 2006;56(8):1985-1990. <https://doi.org/10.1099/ijs.0.64318-0>.

45. Rautio M, Eerola E, Nen-Tunkelrott MVI, Molitoris D, Lawson PA, Collins MD, et al. Reclassification of *Bacteroides putredinis* (Weinberg et al., 1937) in a new genus *Alistipes* nov., as *Alistipes putredinis* comb. nov., and description of *Alistipes finegoldii* sp. nov., from human sources. *Systematic & Applied Microbiology*. 2003;26(2):182-188. <https://doi.org/10.1078/072320203322346029>.
46. McIntosh CM, Chen LQ, Shaiber A, Eren AM, Alegre M. Gut microbes contribute to variation in solid organ transplant outcomes in mice. *Microbiome*. 2018;6:96. <https://doi.org/10.1186/s40168-018-0474-8>.
47. Collins MD, Shah HN, Mitsuoka T. Reclassification of *Bacteroides microfusus* (Kaneuchi and Mitsuoka) in a new genus *Rikenella*, as *Rikenella microfusus* nov. *Systematic & Applied Microbiology*. 1985;6(1):79-81. [https://doi.org/10.1016/S0723-2020\(85\)80015-1](https://doi.org/10.1016/S0723-2020(85)80015-1).
48. Chen DF, Jin DC, Huang SM, Wu JY, Xu MQ, Liu TY, et al. *Clostridium butyricum*, a butyrate-producing probiotic, inhibits intestinal tumor development through modulating *Wnt* signaling and gut microbiota. *Cancer letters*. 2020;469:456-467. <https://doi.org/10.1016/j.canlet.2019.11.019>.
49. Kolsoom P, Rosita J, Golgis K, Reza E. Effect of probiotics supplementation on bone mineral content and bone mass density. *The scientific world journal*. 2014;2014:595962. <https://doi.org/10.1155/2014/595962>.
50. Lucas S, Omata Y, Hofmann J, Bottcher M, Ijazovic A, Sarter K, et al. Short-chain fatty acids regulate systemic bone mass and protect from pathological bone loss. *Nature Communications*. 2018;9(1):55. <https://doi.org/10.1038/s41467-017-02490-4>.
51. Iwami K, Moriyama T. Effects of short chain fatty acid, sodium butyrate, on osteoblastic cells and osteoclastic cells. *Int J Biochem*. 1993;25(11):1631-1635. [https://doi.org/10.1016/0020-711X\(93\)90522-G](https://doi.org/10.1016/0020-711X(93)90522-G).

Figures

Figure 1

E. faecium modulated gut microbiota of broilers. (A) PCA score indicated difference in gut microbiota structure. (B, C) Observed species and Simpson diversity indexes of the gut microbiota. m1, control. m3, *E. faecium*. ns, not significant. * $P < 0.05$.

B

Figure 2

The gut microbiota composition is shown at a phylum level (A) and genus level (B). m1, control. m3, E. faecium.

Figure 3

The taxonomic cladogram (A) and the LDA (linear discriminant analysis) score (B) obtained from LEfSe analysis of gut microbiota in different groups. m1, control. m3, E. faecium diet.