Original Research Article

Probiotic *Paenibacillus polymyxa* 10 and *Lactobacillus plantarum* 16 enhance growth performance of broilers by improving the intestinal health

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ABSTRACT

With the ever-growing strict prohibitions on antibiotic growth promoters (AGP) in animal production, in-feed probiotics are becoming attractive alternatives to antibiotics in the poultry industry. To investigate the effects of *Paenibacillus polymyxa* 10 and *Lactobacillus plantarum* 16 on the growth performance and intestinal health of broilers, 540 male Cobb 500 broilers of 1 d old were randomly divided into 3 groups with 6 replicates per group and 30 chicks per replicate. Broilers were fed with either a basal diet or basal diets supplemented with 1×10^8 colony-forming units (CFU)/kg *P. polymyxa* 10 (BSC10) or *L. plantarum* 16 (Lac16) for 42 d. Results showed that Lac16 treatment improved ($P < 0.05$) the growth performance (body weight and feed conversion) of broilers at the starter phase, while BSC10 treatment slightly improved ($P > 0.05$) the growth performance of the starter phase broilers. The increased villus height ($P < 0.05$) at d 14, 21 and 42 and villus height to crypt depth ratio ($P < 0.05$) at d 14 and 21 were observed in the ileum of the 2 probiotic groups. Besides, transmission electron microscopy results showed that the 2 probiotics enhanced the intestinal epithelial barrier. Both probiotic treatments up-regulated ($P < 0.05$) the mRNA expression of fatty acid binding protein 1 (*FABP1*) and sodium-dependent glucose transporters-1 (*SGLT-1*) in the ileal mucosa of broilers at d 21. In addition, BSC10 and Lac16 treatments significantly ($P < 0.05$) increased the relative abundance of short-chain fatty acids-producing bacteria, such as *Butyricicoccus pullicaecorum*, *Faecalibacterium prausnitzii*, *Lachnospira* and *Coprococcu*, and significantly ($P < 0.05$) decreased the relative abundance of enteric pathogens (*Escherichia coli*, *Bacteroides fragilis* and *Shigella sonnei*). Furthermore, the 2 probiotic treatments also increased the positive connection among the intestinal microbes and the carbohydrate metabolism-related pathways of the intestinal bacteria ($P < 0.05$), with decreasing ($P < 0.05$) nucleotides biosynthesis-related pathways of the intestinal bacteria. Overall, these results suggest that the 2 probiotics, especially Lac16, have a potential beneficial effect on the growth performance and intestinal health of starter phase broilers.

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1. Introduction

Antibiotics were first used as feed additives in poultry production in the 1940s. In-feed antibiotic growth promoters (AGP) have been shown to be effective in boosting overall performance and health of food animals by preventing gastrointestinal infectious diseases, reducing morbidity and mortality, and improving feed utilization efficiency (Broom, 2017; Teillant et al., 2015; Yang et al., 2019). However, abuse of antibiotics has resulted in serious

consequences, such as antimicrobial resistance (AMR), antibiotic residues in food animal products, and drug environmental pollution, which seriously threatens the health of animals and human consumers (Marshall and Levy, 2011; Yang et al., 2019). For this reason, quite a few countries, including China, have limited the in-feed antibiotics used for livestock animal production (Castanon, 2007; China, 2019). With the strict ban of antibiotics, there is an increasing interest in seeking green safe alternatives to antibiotics for food animal production. In past decades, multi-categories of dietary alternatives, such as direct-fed microbials (also called probiotics), prebiotics and plant extracts, have proven to be beneficial for performance and overall health of food animals; these dietary alternatives were considered as "Generally Recognized as Safe (GRAS)" alternatives to antibiotics (Buntyn et al., 2016; Cheng et al., 2014; Mehdi et al., 2018).

Intestinal microbiota plays important roles in physiology and health of the host (Caballero and Pamer, 2015; Hooper et al., 2012). It exerts considerable effects in different aspects, such as inhibiting pathogenic infection, facilitating digestion of complex plant fiber into short chain fatty acids, synthesizing essential vitamins and amino acids, regulating fat metabolism, and shaping the development of the immune system (Ahmed et al., 2016; Blander et al., 2017; Clemente et al., 2018; Frick and Autenrieth, 2013). Generally, the composition of intestinal microbiota in birds is relatively stable and resilient over time (Clemente et al., 2018). However, various environmental factors, such as feed, stresses, viruses, and drugs, especially antibiotics, may lead to intestinal dysbacteriosis and immune dysregulation in birds, which consequently compromises growth performance, bird welfare and may induce the occurrence of intestinal necrotic diseases (Isaac et al., 2017; Kernbauer et al., 2014; Prescott et al., 2016; Wang et al., 2020). Therefore, a balanced intestinal microbiota is crucial for the improvement of overall performance and quality of poultry products.

As attractive green safe alternatives to AGP, numerous studies have reported that probiotics are beneficial for growth performance and animal health through enhancing intestinal development and nutrients absorption, regulating the mucosal immune system, inhibiting intestinal pathogen colonization and infection, and reshaping intestinal microbiota (Bajagai et al., 2016; Buntyn et al., 2016). Our previous works found that 2 screened probiotics, *Paenibacillus polymyxa* BSC10 and *Lactobacillus plantarum* Lac16, had in vitro anti-*Clostridium perfringens* activities and could protect *Clostridium elegans* against *C. perfringens* infection (unpublished data). The present study further investigated the effects of *P. polymyxa* 10 and of *L. plantarum* 16 on the growth performance and intestinal health of broilers.

2. Materials and methods

All protocols of this study were carried out according to the Chinese guidelines for animal welfare and were approved by the Zhejiang University Institutional Animal Care and Use Committee (Permission number: ZJU20160416). Detailed methods are provided in Appendix.

2.1. Experimental design

Five hundred and forty male Cobb 500 broilers of 1 d old raised in XinXin Broiler Farm (Jiaxing, China) were randomly allocated into 3 groups with 6 replicates per group and 30 birds per replicate. All birds were fed basal diets formulated to meet the nutritional requirements of broilers (National Nutrition Council, 1994) for starter (d 1 to 21) and grower (d 22 to 42) periods (Table 1). A diagram of the present animal study is shown in Fig. 1. Control group: birds were fed a basal diet; BSC10 group: birds were fed a basal diet

supplemented with 1.0×10^8 colony-forming units (CFU)/kg *P. polymyxa*; Lac16 group: birds were fed a basal diet supplemented with 1.0×10^8 CFU/kg *L. plantarum* 16 for 42 d. Broilers were allowed ad libitum access to fresh water and mashed diets. Feed consumption and mortality were recorded every day and body weight was recorded on d 14, 21 and 42 of age. Dead birds were weighed to adjust estimates of body weight gain, intake and feed conversion ratio. The body weight, average daily gain (ADG), average daily feed intake (ADFI), and feed conversion rate (FCR) were calculated.

2.2. Sample collection

At 13, 20 and 41 d of age, all birds were deprived of feed but not water, for a fasting period of 12 h (overnight). At 14, 21 and 42 d of age, 2 birds from each replicate were chosen randomly, weighed and then the birds were electrically stunned, exsanguinated and scalded to enable the collection of tissues. The ileal segments were fixed in 4 % paraformaldehyde for hematoxylin and eosin (H&E) staining, fixed in 2.5 % buffered glutaraldehyde for transmission electron microscopy (TEM), and the ileal mucosa was gently scraped, snap frozen in liquid nitrogen and stored at −80 °C for quantitative reverse transcription PCR (RT-qPCR) analysis. The whole caecum of birds was sampled, snap frozen in liquid nitrogen and then stored at −80 °C for further microbial analysis.

2.3. Ileal morphological analysis

The H&E staining was performed as described previously with minor modification (Wu et al., 2018). Ileum samples of broilers fixed in 4 % paraformaldehyde were embedded in paraffin, sliced, dehydrated and stained with hematoxylin and eosin. Images were captured using an Olympus microsystem (Tokyo, Japan). The samples for TEM observation were processed according to the previous reported protocols (Mo et al., 2019). The ultrathin sections were cut using an LKB Nova ultramicrotome (Leica Microsystems, Buffalo

Table 1
Composition and nutrient level of the basal diet (%, as fed basis).

Item	Starter	Grower
	Day 1 to 21	Day 22 to 42
Ingredients		
Corn	60.00	66.00
Soy bean meal	28.50	24.00
Fish meal	2.00	1.00
Wheat middling	4.50	4.00
Salt	0.30	0.30
Choline chloride (50%)	0.15	0.10
CaHPO$_4$	1.20	1.00
Limestone	1.20	1.20
Zeolite power	1.20	1.40
Premix[1]	1.00	1.00
Nutrient levels[2]		
ME, MJ/kg	11.80	12.00
CP	21.01	18.80
Lys	1.12	1.08
Met	0.49	0.46
Ca	1.01	0.90
Total P	0.69	0.61
Available P	0.47	0.39

[1] Supplied per kilogram of diet: Vitamin A 25,000 IU, Vitamin D 5,000 IU, Vitamin E 12.5 IU, Vitamin K 1.25 mg, Vitamin B$_1$ 1.0 mg, Vitamin B$_2$ 8 mg, Vitamin B$_{12}$ 15 μg, pantothenic acid 250 μg, pyridoxine 9.09 mg, biotin 22.50 mg, folic acid 1.67 mg, ZnSO$_4$.H$_2$O 180.93 mg, CuSO$_4$.H$_2$O 33.18 mg, FeSO$_4$.H$_2$O 247.75 mg, MnSO$_4$.H$_2$O 248.45 mg, Ca(IO$_3$)$_2$ 85.80 mg, Na$_2$SeO$_3$ 37.60 mg.
[2] Calculated nutrient levels.
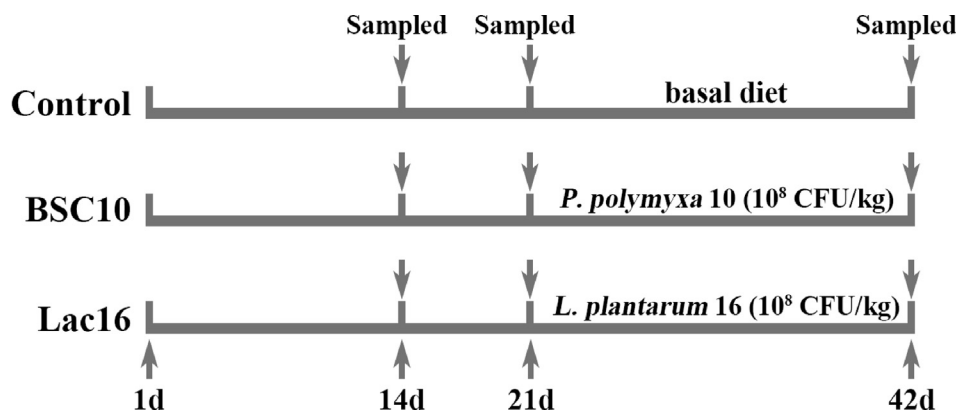
Fig. 1. Diagram of experimental design. Control: basal diet; BSC10: basal diet supplemented with *Paenibacillus polymyxa* 10 (1.0 × 10⁸ CFU/kg feed); Lac16: basal diet supplemented with *Lactobacillus plantarum* 16 (1.0 × 10⁸ CFU/kg feed).

Grove, IL) and stained with uranyl acetate. Electron micrographs of the samples were then captured by the transmission electron microscope (JEOL, Tokyo, Japan).

2.4. RT-qPCR

The protocol for RT-qPCR analysis was conducted according to the previous study (Wang et al., 2018). The RT-qPCR analysis was conducted using SYBR PremixEx TaqII (TAKARA) by the StepOne real-time PCR system (Applied Biosystems). All primer sequences for target genes are listed in Table 2, and the fold changes were calculated after normalizing to the housekeeping gene β-actin by the $2^{-\Delta\Delta Ct}$ method (Bustin et al., 2009).

2.5. Microbial analysis

Microbial genomic DNA was extracted under sterile conditions from the cecal content of broilers at 21 d of age using the TIANamp Stool DNA Kit (Tiangen, Beijing, China) according to the manufacturer's protocols. The V3 to V4 region of the 16S rRNA gene was amplified using the 341F/805R primer pairs and the sequencing was performed on an Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA). Raw sequences were filtered and clustered into operational taxonomic units (OTU) at 97% similarity by QIIME software (version 1.9.1). Bacterial OTU representative sequences were assigned to a taxonomic lineage by Ribosomal Database Project (RDP) classifier based on the Greengenes database (13_8 release).

Alpha and beta diversity were analyzed based on a subsample of a minimum number of sequences (15949) by QIIME software. Beta diversity was visualized by Principal coordinates analysis (PCoA) using the "ggplot2" package of R software. Analysis of similarities

(ANOSIM), permutational multivariate analysis of variance (PERMANOVA) and multi response permutation procedure (MRPP) analyses were calculated by "vegan" package to determine significant differences in microbial beta diversity among the 3 groups (based on the Bray–Curtis distance matrices).

Co-occurrence patterns of microbial communities in different groups were built based on significant correlations (Spearman's $R > 0.6$ and FDR-adjusted $P < 0.05$) (Jiao et al., 2016), and were visualized by Gephi software (https://gephi.org/). The topological

Table 2
Primer Sequences used for quantitative reverse transcription PCR.

Gene	Primer Sequence (5′ to 3′)	Product size, bp	Accession No.
FABP1	F: AGTACGAGCTCCAGTCCCAT	288	NM_204192
	R: TCACGGATTTCAGCCCCTTC		
SGLT-1	F: TGATTGGCTCCACTTGCTACA	243	NM_001293240
	R: TGTGGGAAGACACACCATGC		
PEPT1	F: TACGCATACTGTCACCATCA	205	NM_204365
	R: TCCTGAGAACGGACTGTAAT		
β-actin	F: TATGTGCAAGGCCGGTTTC	110	NM_205518
	R: TGTCTTTCTGGCCCATACCAA		

FABP1 = fatty acid binding protein 1; F = forward; R = reverse; *SGLT-1* = sodium-dependent glucose transporters-1; *PEPT1* = peptide transporter 1.

Table 3
Effect of probiotics on the growth performance and immune organ indexes of broilers (*n* = 6/group).

Item	Treatments[1]			SEM	*P*-value
	Control	BSC10	Lac16		
Body weight, g					
Day 1	47.89	47.82	48.07	0.49	0.34
Day 14	344.29	357.14	355.24	4.76	0.10
Day 21	637.88[b]	659.85[ab]	679.55[a]	9.67	0.003
Day 42	2,030.87	2,069.44	2,071.30	24.28	0.47
Average daily gain, g/d					
Day 1 to 14	22.80	23.79	23.63	0.34	0.07
Day 14 to 21	41.94[b]	43.24[ab]	46.33[a]	1.12	0.001
Day 1 to 21	28.09[b]	29.14[ab]	30.07[a]	0.47	0.02
Day 21 to 42	69.65	70.48	69.59	1.01	0.85
Day 1 to 42	48.11	49.09	49.1	0.56	0.46
Average daily feed intake, g/d					
Day 1 to 14	39.30	39.45	40.37	0.46	0.22
Day 14 to 21	75.81	76.24	74.62	0.65	0.19
Day 1 to 21	52.14	52.27	52.45	0.33	0.84
Day 21 to 42	150.86	148.35	151.15	1.71	0.57
Day 1 to 42	104.43	103.54	104.01	1.60	0.94
Feed conversion rate					
Day 1 to 14	1.72	1.66	1.71	0.03	0.28
Day 14 to 21	1.81[a]	1.76[a]	1.61[b]	0.06	0.01
Day 1 to 21	1.86[a]	1.79[ab]	1.74[b]	0.04	0.04
Day 21 to 42	2.17	2.10	2.17	0.03	0.27
Day 1 to 42	2.16	2.10	2.11	0.05	0.76
Spleen index					
Day 14	0.86	0.68	0.92	0.08	0.054
Day 21	0.85	0.96	1.01	0.14	0.67
Day 42	1.30	1.14	0.92	0.14	0.12
Bursa of fabrieius index					
Day 14	1.97	1.95	2.15	0.17	0.63
Day 21	1.87	2.49	2.21	0.28	0.27
Day 42	1.67	1.85	1.62	0.23	0.76

[a, b] Mean values within a row with no common superscript differ significantly (*P* < 0.05).
[1] Control: control group; BSC10: basal diet supplemented with *Paenibacillus polymyxa* 10 (1.0 × 10⁸ CFU/kg feed); Lac16: basal diet supplemented with *Lactobacillus plantarum* 16 (1.0 × 10⁸ CFU/kg feed).

Table 4
Effect of probiotics on the mucosal structure of ileum in broilers (n = 3/group).

Item	Treatments[1]			SEM	P-value
	Control	BSC10	Lac16		
Day 14					
Villus height, μm	394.25[b]	486.76[a]	481.80[a]	27.01	<0.001
Crypt depth, μm	87.37	86.42	85.51	1.98	0.84
Villus height:Crypt depth ratio	4.52[b]	5.64[a]	5.63[a]	0.33	<0.001
Day 21					
Villus height, μm	467.47[b]	546.38[a]	559.19[a]	25.88	<0.001
Crypt depth, μm	95.70	94.97	96.80	1.84	0.82
Villus height:Crypt depth ratio	4.89[b]	5.77[a]	5.78[a]	0.29	0.012
Day 42					
Villus height, μm	565.48[b]	626.32[a]	639.92[a]	21.75	0.005
Crypt depth, μm	119.89	124.44	125.70	2.53	0.26
Villus height:Crypt depth ratio	4.72	5.03	5.09	0.14	0.11

[a, b] Mean values within a row with no common superscript differ significantly (P < 0.05).
[1] Control: control group; BSC10: basal diet supplemented with *Paenibacillus polymyxa* 10 (1.0 × 10^8 CFU/kg feed); Lac16: basal diet supplemented with *Lactobacillus plantarum* 16 (1.0 × 10^8 CFU/kg feed).

properties of co-occurrence network were also calculated to describe the complex patterns of the interrelationships.

The metagenome predictions based on 16S rRNA gene sequencing data were analyzed by the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 software (PICRUSt2, v.2.3.0_b) with default scripts "picrust2_pipeline.py" (Douglas et al., 2020). The OTU table and representative sequences subsampled at a minimum number of sequences (15949) were selected for the functional abundances generation based on MetaCyc database. The predicted pathway abundances were then analyzed and visualized by statistical analysis of taxonomic and functional profiles (STAMP) software with a 2-sided Welch's t-test (Parks et al., 2014).

2.6. Statistical analysis

The rest of the data were analyzed by one-way analysis of variance (ANOVA) and the contrast of means was performed using Tukey's multiple range test by SPSS software (SPSS Inc., Chicago, IL, USA). Results were expressed as means with standard error of mean (SEM). The graphs were visualized using Origin 8.5 (OriginLab, Berkeley, CA, USA).
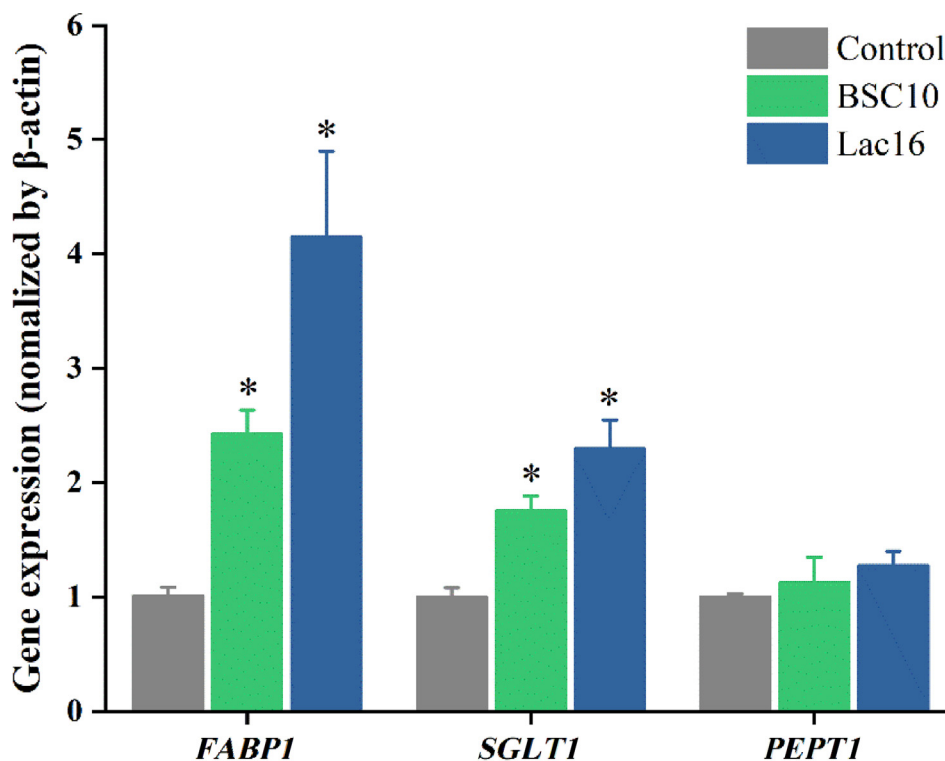
3. Results

3.1. Growth performance

As shown in Table 3, Lac16 treatment significantly (P < 0.05) increased the body weight of broilers at 21 d of age and their feed conversion during the periods of 1 to 21 d and 14 to 21 d of age,
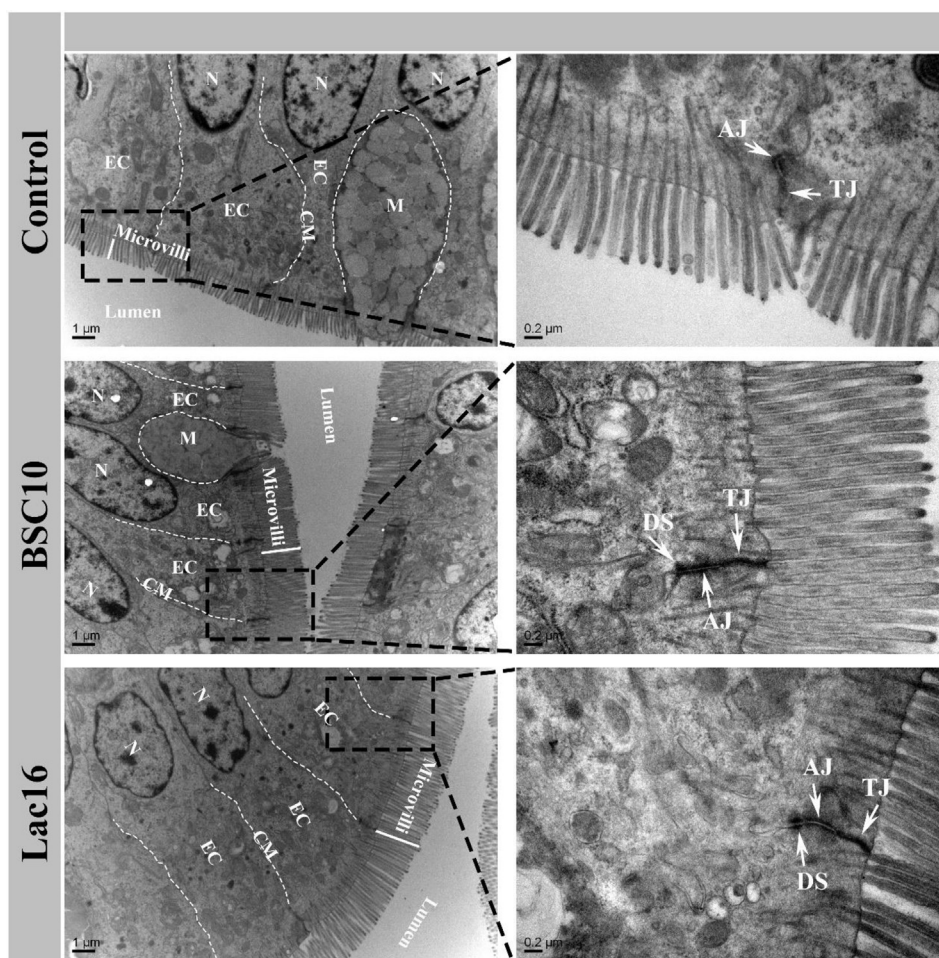
Fig. 2. Transmission electron micrographs of the ileal microvilli in broilers at 21 d of age. EC = epithelial cell; N = nucleus; M = microfold cell; CM = cell membrane; TJ = tight junction; AJ = adherens junction; DS = desmosomes. Control: control group; BSC10: basal diet supplemented with *Paenibacillus polymyxa* 10 (1.0 × 10^8 CFU/kg feed); Lac16: basal diet supplemented with *Lactobacillus plantarum* 16 (1.0 × 10^8 CFU/kg feed).

Fig. 3. Gene expression of nutritional absorption related receptors in ileal mucosa of broilers at 21 d of age. Results are means ± standard error of mean ($n = 6$/group). *FABP1* = fatty acid binding protein 1; *SGLT-1* = sodium-dependent glucose transporters-1; *PEPT1* = peptide transporter 1. Control: control group; BSC10: basal diet supplemented with *Paenibacillus polymyxa* 10 (1.0×10^8 CFU/kg feed); Lac16: basal diet supplemented with *Lactobacillus plantarum* 16 (1.0×10^8 CFU/kg feed). *: significant difference compared with control ($P < 0.05$).

while BSC10 slightly improved ($P > 0.05$) the body weight and feed conversion of the starter phase (1 to 21 d) broilers. BSC10 or Lac16 had no effect ($P > 0.05$) on the immune organ index (spleen and bursa of fabricius).

3.2. Mucosal structure and transmission electron micrograph of ileum

H&E staining results showed that BSC10 or Lac16 supplementation in the diet significantly ($P < 0.05$) increased the villus height of broilers at 14, 21 and 42 d of age and the villus height to crypt depth ratio (VCR) at 14 and 21 d of age ($P < 0.05$), but had no effect ($P > 0.05$) on the crypt depth (Table 4).

According to the results of growth performance and mucosal structure, the effect of BSC10 and Lac16 on broilers at 21 d of age was further investigated. TEM results showed that, compared with the control group, the ileum of the broilers fed with BSC10 or Lac16 showed ordered arrangement and higher microvillus, longer tight junction (TJ) and adherens junction (AJ) and darker desmosomes (DS) (Fig. 2).

3.3. Gene expression of nutritional absorption related-receptors

The gene expression of nutritional absorption related receptors was analyzed by RT-qPCR (Fig. 3). The results indicated that BSC10 or Lac16 treatments significantly ($P < 0.05$) up-regulated the mRNA expression of fatty acid binding protein 1 (*FABP1*) and sodium-dependent glucose transporters-1 (*SGLT-1*) in the ileal mucosa of broilers at 21 d of age, but had no effect on the peptide transporter 1 (*PEPT1*) mRNA expression ($P > 0.05$).

3.4. Intestinal microbiota

Intestinal microbiota plays crucial roles in maintaining gut homeostasis. The results showed that BSC10 or Lac16 treatments had no effect ($P > 0.05$) on the alpha diversities of the intestinal microbiota in broilers at 21 d of age (Table 5). Principal coordinates analysis (PCoA) of intestinal microbiota based on Bray—Curtis distance revealed that there were no significant differences for the bacterial communities among the 3 groups (Fig. 4), which was further confirmed by ANOSIM, PERMANOVA and MRPP analysis (Table 6). The results of the intestinal bacterial compositions showed that BSC10 or Lac16 treatments significantly ($P < 0.05$) increased the relative abundance of *Butyricicoccus pullicaecorum*, *Faecalibacterium prausnitzii*, and *Lachnospira* (Fig. 5). In addition, Lac16 supplementation also significantly ($P < 0.05$) increased the relative abundance of *Coprococcu*. Accordingly, BSC10 or Lac 16 treatments significantly ($P < 0.05$) decreased the relative abundance of enteric pathogenic microorganisms, such as *Escherichia coli*, *Bacteroides fragilis* and *Shigella sonnei* (Fig. 5).

Table 5
Alpha diversity analysis of intestinal microbiota in broilers at 21 d of age ($n = 5$/group).

Item	Treatments[1]			SEM	*P*-value
	Control	BSC10	Lac16		
Observed species	795.00	767.00	855.40	58.98	0.61
Ace	1,118.18	1,081.81	1,175.26	72.68	0.71
Chao1	1,090.99	1,057.90	1,156.94	72.41	0.68
Simpson	0.93	0.96	0.98	0.02	0.51

[1] Control: control group; BSC10: basal diet supplemented with *Paenibacillus polymyxa* 10 (1.0×10^8 CFU/kg feed); Lac16: basal diet supplemented with *Lactobacillus plantarum* 16 (1.0×10^8 CFU/kg feed).
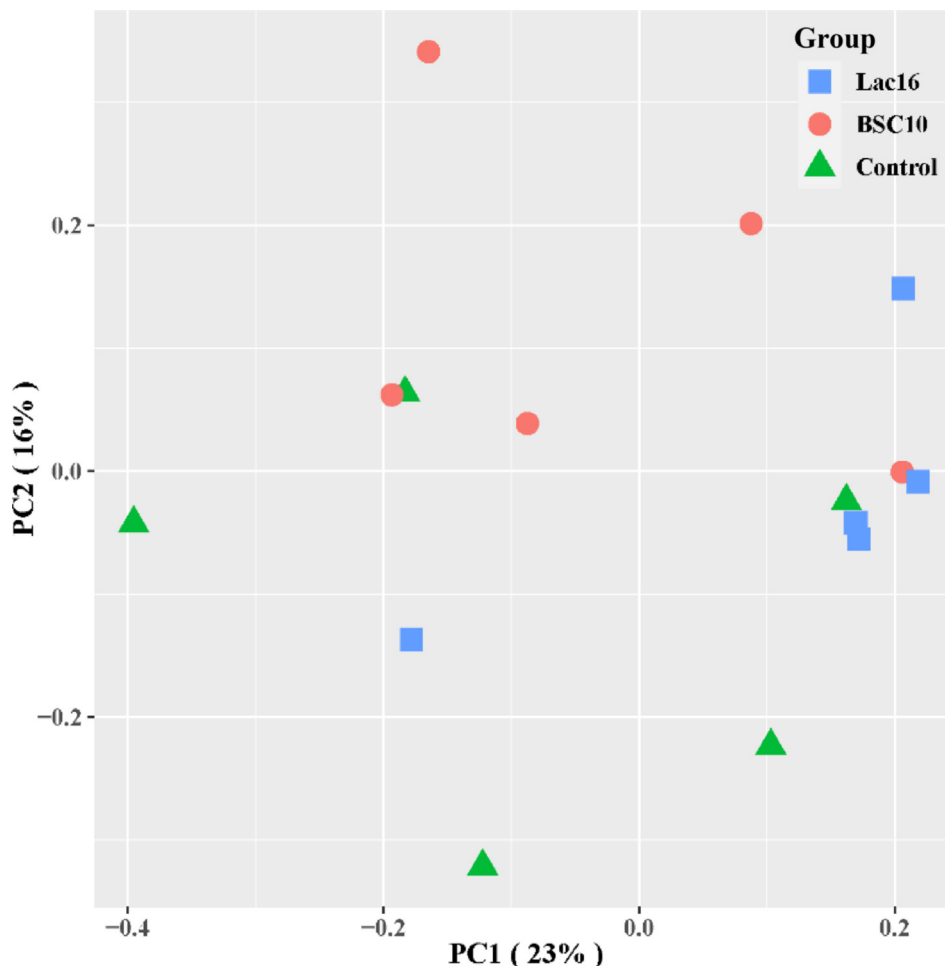
Fig. 4. Principal coordinates analysis (PCoA) of microbial communities among groups based on Bray–Curtis distance ($n = 5$/group). Control: control group; BSC10: basal diet supplemented with *Paenibacillus polymyxa* 10 (1.0×10^8 CFU/kg feed); Lac16: basal diet supplemented with *Lactobacillus plantarum* 16 (1.0×10^8 CFU/kg feed).

Table 6
ANOSIM, PERMANOVA and MRPP analysis of microbial diversity among different treatments ($n = 4$/group).

Item[1]	ANOSIM		PERMANOVA		MRPP	
	R	P-value	R^2	P-value	A	P-value
Treatment	0.212	0.047	0.200	0.044	0.035	0.059
Control vs. BSC10	0.176	0.139	0.155	0.122	0.023	0.121
Control vs. Lac16	0.200	0.084	0.158	0.082	0.029	0.084

ANOSIM = analysis of similarities; PERMANOVA = permutational multivariate analysis of variance; MRPP = multi response permutation procedure.

[1] Control: control group; BSC10: basal diet supplemented with *Paenibacillus polymyxa* 10 (1.0×10^8 CFU/kg feed); Lac16: basal diet supplemented with *Lactobacillus plantarum* 16 (1.0×10^8 CFU/kg feed).

PICRUSt2 metagenome prediction results showed that BSC10 treatment significantly ($P < 0.05$) decreased 3 metabolic pathways (pyrimidine deoxyribonucleotides biosynthesis from CTP, pyrimidine deoxyribonucleotides de novo biosynthesis IV and ADP-L-glycero-β-D-manno-heptose biosynthesis), whereas it significantly ($P < 0.05$) increased the pentose phosphate pathway and sucrose degradation III (sucrose invertase) (Fig. 6). Lac16 treatment significantly ($P < 0.05$) increased 14 predicted pathway abundances of the intestinal bacteria, including degradative superpathways (hexuronide and hexuronate, N-acetylglucosamine, N-acetylmannosamine and N-acetylneuraminate, β-D-glucuronide and D-glucuronate,

glucose and xylose), degradative pathways (4-deoxy-L-threo-hex-4-enopyranuronate, D-galacturonate, D-fructuronate, allantoin degradation to glyoxylate III, sucrose degradation III (sucrose invertase)), biosynthesis pathways (GDP-D-glycero-α-D-manno-heptose, dTDP-N-acetylthomosamine), pentose phosphate pathway, formaldehyde assimilation II (RuMP Cycle), and formaldehyde oxidation I (Fig. 6). Additionally, pathways associated with pyrimidine deoxyribonucleotides biosynthesis and palmitate biosynthesis were less active in Lac16 group ($P < 0.05$).

To investigate the co-occurrence patterns of intestinal microbes in the groups, 3 networks were constructed based on the OTU level (Fig. 7 and Table 7). It was found that the microbial networks of BSC10 and Lac16 groups had more edges than those in the control group, which was roughly at the same nodes (approximately 1,320). The values of average degree (AD) and graph density (GD) in BSC10 and Lac16 groups were higher than those in the control group. The modularity values of the co-occurrence networks in all groups were higher than 0.4. Additionally, the negative correlation of the network in the control group was more than those in the BSC10 and Lac16 groups.

4. Discussion

Probiotics have been considered as an attractive alternative to in-feed antibiotics for their unique functions, including preventing intestinal infectious diseases, enhancing overall health and
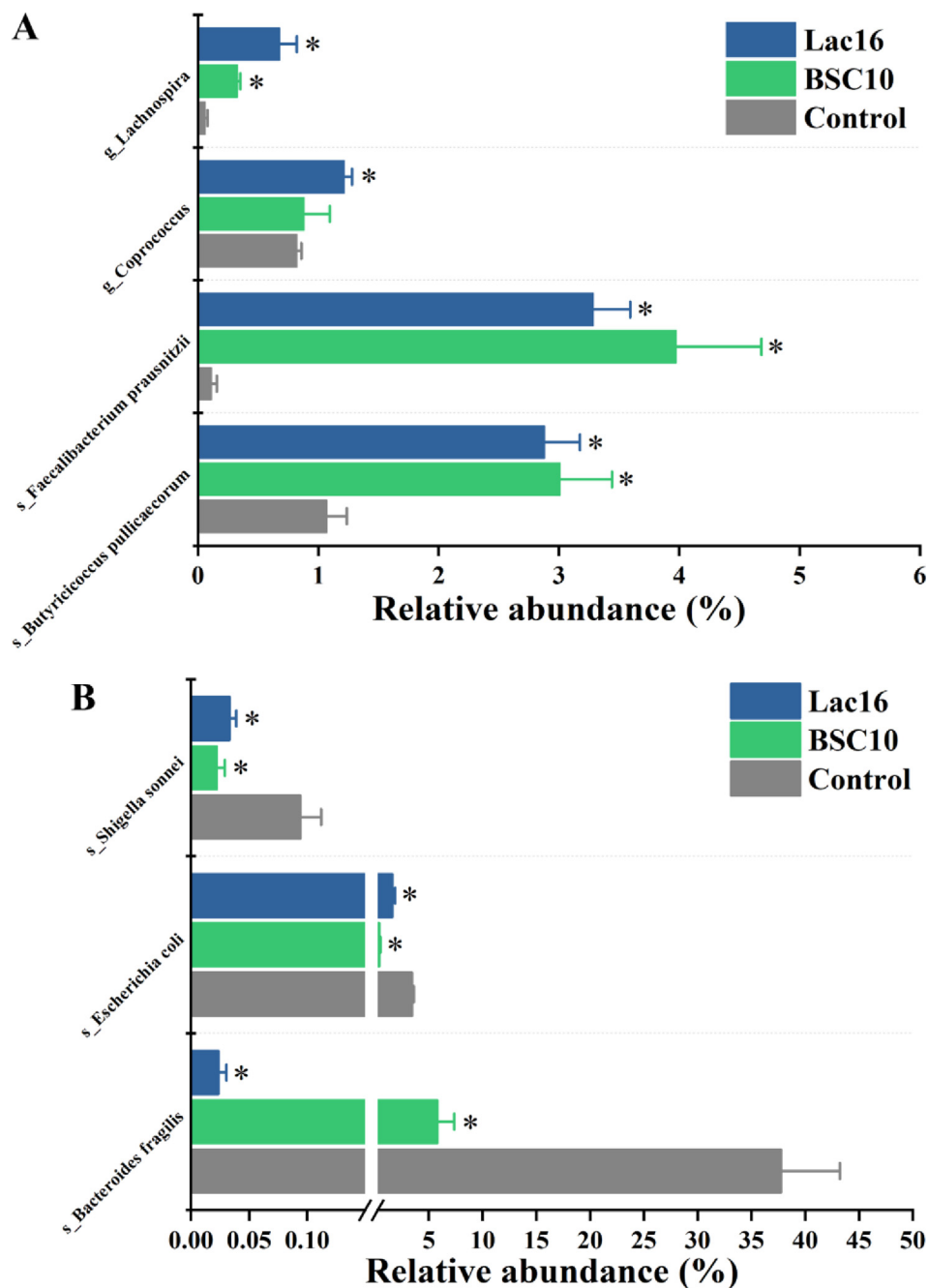
Fig. 5. Comparison of the microbial communities of broilers at 21 d of age among different treatments. (A) Short-chain fatty acids-producing bacteria; (B) enteric pathogens. Results are means ± standard error of mean (n = 5/group). *: P < 0.05 compared with Control. Control: control group; BSC10: basal diet supplemented with *Paenibacillus polymyxa* 10 (1.0 × 10⁸ CFU/kg feed); Lac16: basal diet supplemented with *Lactobacillus plantarum* 16 (1.0 × 10⁸ CFU/kg feed). g_ = genus; s_ = species.

performance of poultry, and improving the quality of poultry products (Al-Khalaifah, 2018; Buntyn et al., 2016; Mehdi et al., 2018). The driving force for the increased interest in dietary probiotics is to reduce or eliminate the use of in-feed antibiotics in food animal production. Numerous studies have reported that dietary supplementation with probiotics could improve the growth performance of broilers by improving intestinal health (Forte et al., 2018; Park et al., 2020; Ramlucken et al., 2020). The present study showed that the 2 selected probiotics, *P. polymyxa* BSC10 and *L. plantarum* Lac16, are also beneficial for improving the growth performance of broiler chickens. Dietary supplementation with *L. plantarum* Lac16 significantly improved the growth performance of broilers at the early phase, as indicated by the increased body weight and feed

conversion. However, some other studies reported that dietary supplementation with probiotics had no effect on the growth performance of broilers, which might be due to the strains of probiotics, supplemented dosage, delivery methods, age and genotype of birds, and environmental conditions (Atela et al., 2019; Bai et al., 2017; Pender et al., 2017). The improved growth performance of broilers in the present study may be related to the beneficial changes in intestinal health regulated by dietary intervention, host mucosa, and intestinal microbes (Forte et al., 2018; Jacquier et al., 2019).

The intestinal epithelial integrity serves as a physical barrier against enteric pathogen invasion and is responsible for nutrient absorption and waste secretion (Turner, 2009). Longer villi and villus height to crypt depth ratio are important indicators of
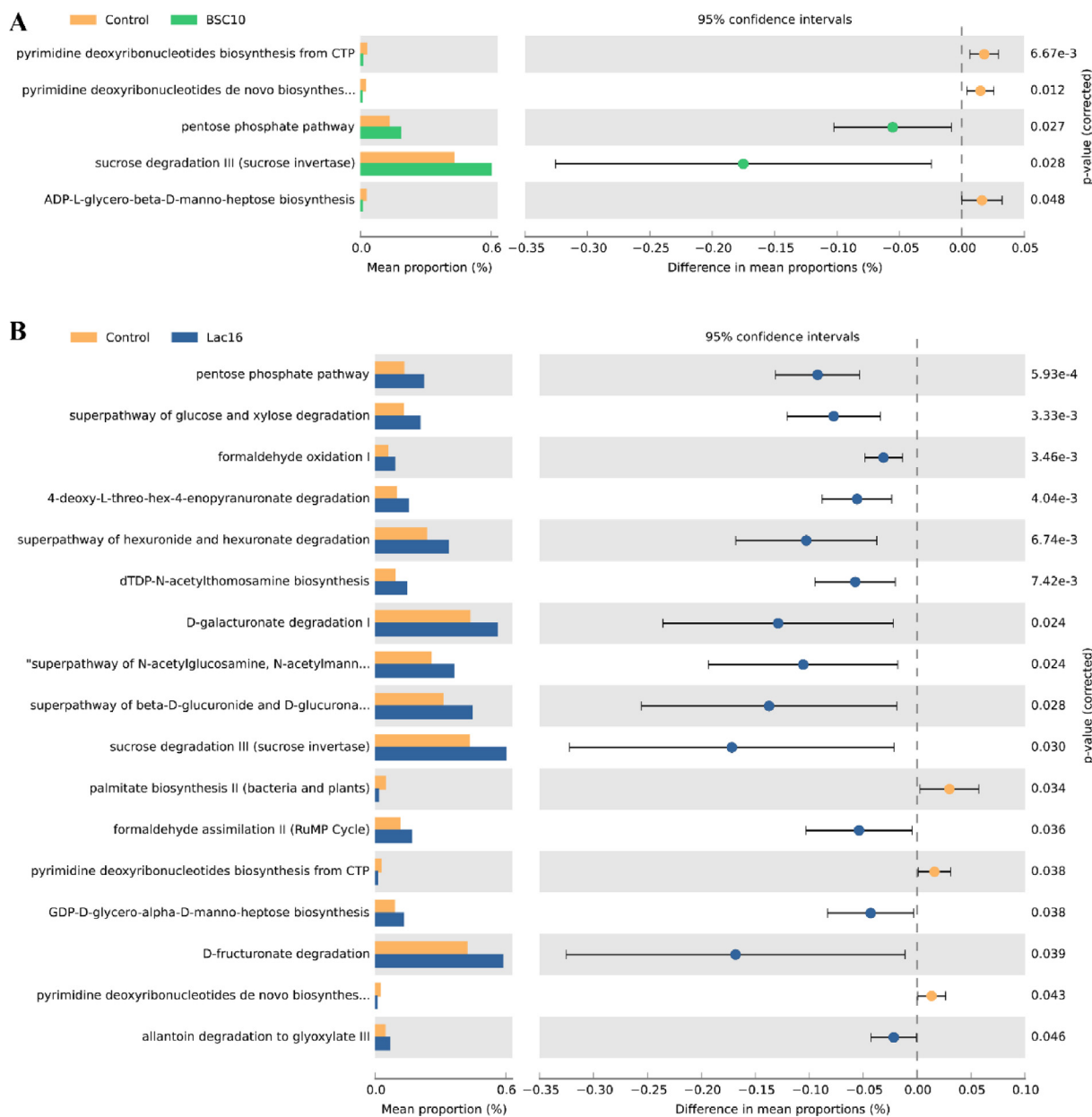
Fig. 6. Comparison of predicted pathway abundances between the groups by statistical analysis of taxonomic and functional profiles (STAMP) (A) Control versus BSC10; (B) Control versus Lac16. Control: control group; BSC10: basal diet supplemented with *Paenibacillus polymyxa* 10 (1.0×10^8 CFU/kg feed); Lac16: basal diet supplemented with *Lactobacillus plantarum* 16 (1.0×10^8 CFU/kg feed).

healthy gastrointestinal tract (GIT), which would provide more surface area for absorption of available nutrients (Liu et al., 2020; Poloni et al., 2020). In this research, significantly increased villus height (at 14, 21 and 42 d of age) and villus height to crypt depth ratio (at 14 and 21 d of age) were observed in the ileum of broilers fed with the 2 screened probiotics, which may contribute to greater nutrient absorption from the feed during intestinal passage (Ramlucken et al., 2020). The present study also found that the intestinal epithelial junction was enhanced in the increased ileal villus of broilers in the 2 probiotic groups evidenced by the ordered and higher microvillus, the longer TJ, the enhanced AJ and the DS. These results above indicate that dietary supplementation with the probiotics is beneficial for the development of intestinal mucosa and enhancement of the intestinal epithelial integrity of broilers.

The intestinal nutrient transporters play a crucial important role in the nutrient absorption and utilization of food animals (Pu et al., 2020). *SGLT-1*, a membrane transporter, mediates the uptake of glucose from the intestinal tract across the brush-border membrane into intestinal enterocytes (Gorboulev et al., 2012). *FABP1* is involved in the intracellular free fatty acids (FFA) trafficking and eventually promotes intestinal nutrient absorption (Musso et al., 2011). In the present study, the 2 screened dietary probiotics significantly increased the mRNA expression of *FABP1* and *SGLT-1* in the ileal mucosa of broilers at 21 d of age, which resulted in better transport and absorption of nutrients.

The intestinal microbiota is co-evolved with the host, forming the microorganisms with a stable intestinal microenvironment which provide the host a broad range of bio-functions, such as digestion of complex dietary carbohydrates, production of
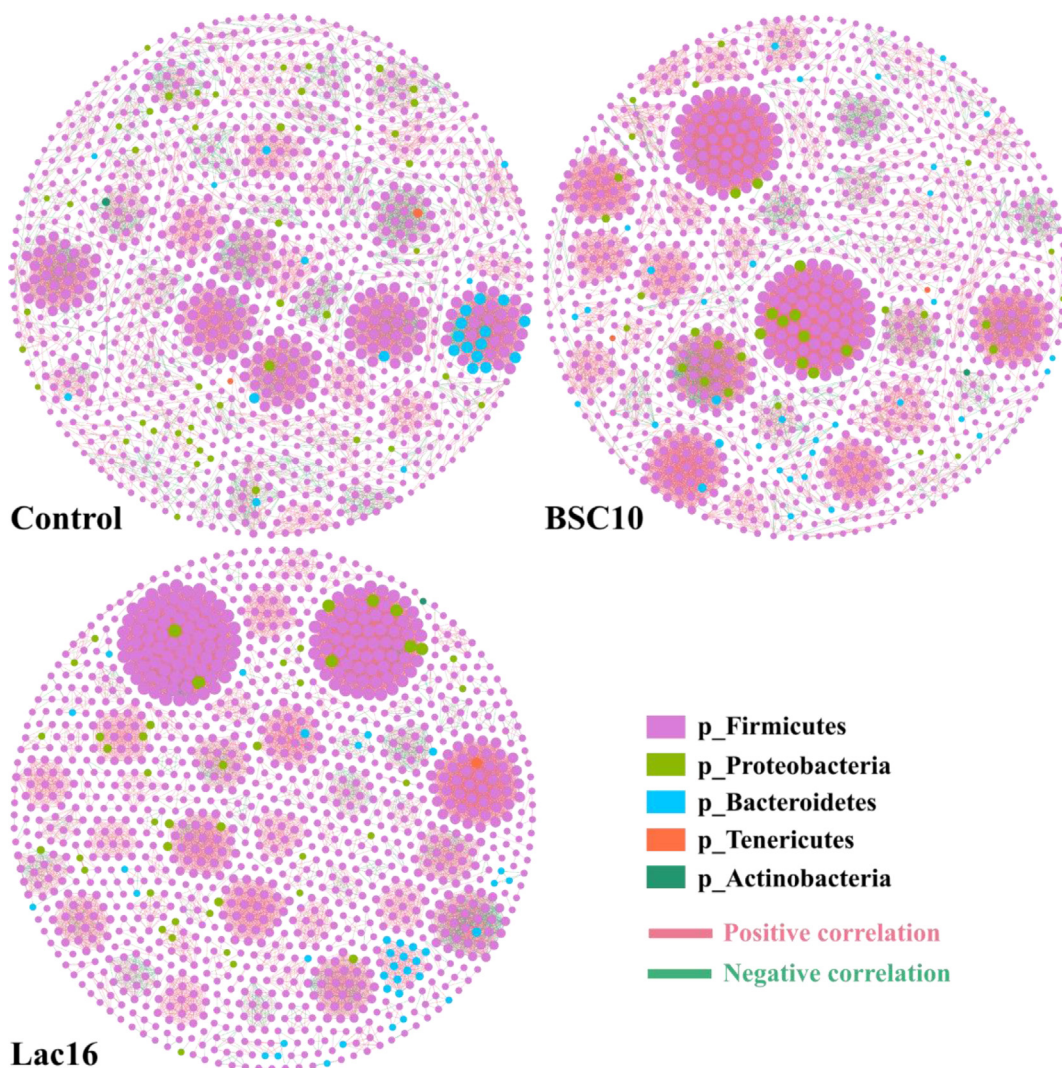
Fig. 7. Co-occurrence networks of microbial communities at operational taxonomic unit (OUT) level. A connection stands for a very strong (Spearman's $R > 0.6$) and significant (FDR-adjusted $P < 0.05$) correlation. The size of each node is proportional to the relative abundance; the thickness of each connection between 2 nodes (edge) is proportional to the value of Spearman's correlation coefficients. Red lines represent significant positive correlations and green lines denote negative correlations. Control: control group; BSC10: basal diet supplemented with *Paenibacillus polymyxa* 10 (1.0×10^8 CFU/kg feed); Lac16: basal diet supplemented with *Lactobacillus plantarum* 16 (1.0×10^8 CFU/kg feed).

absorbable nutrients and vitamins, defense against pathogenic infection, and maintenance of the intestinal homeostasis (Koh et al., 2016; Valdes et al., 2018). The 2 selected probiotics have no effects on the alpha and beta diversities, consistent with previous studies (Wang et al., 2019; Wang et al., 2016), indicating that no significant changes occurred in the microbial diversity of broilers. There are

Table 7
The topological properties of co-occurrence network.

Item	Treatments[1]		
	Control	BSC10	Lac16
Nodes	1323	1318	1332
Edges	7685	12,786	12,963
Average degree	11.618	19.402	19.464
Graph density	0.009	0.015	0.015
Modularity	0.958	0.897	0.895
Positive correlation, %	78.72	92.51	92.11
Negative correlation, %	21.28	7.49	7.89

[1] Control: control group; BSC10: basal diet supplemented with *Paenibacillus polymyxa* 10 (1.0×10^8 CFU/kg feed); Lac16: basal diet supplemented with *Lactobacillus plantarum* 16 (1.0×10^8 CFU/kg feed).

also quite a number of studies that reported that dietary probiotics have a positive effect in facilitating the abundance of beneficial bacteria and reducing the colonization of potential zoonotic pathogens in the gastrointestinal tract of broilers (Eeckhaut et al., 2016; Peng et al., 2016). The present study showed that the 2 screened probiotics significantly increased the relative abundance of short-chain fatty acids (SCFA)-producing bacteria, such as *B. pullicaecorum*, *F. prausnitzii*, *Lachnospira* and *Coprococcu*, which can ferment diverse plant polysaccharides or dietary fiber into SCFA, especially butyrate (Cornick and Stanton, 2015; Eeckhaut et al., 2008; Martin et al., 2017; Valles-Colomer et al., 2019). It has been proven that SCFA are not only an important energy source, but also play important roles in balancing intestinal homeostasis, inhibiting growth and colonization of pathogens, and promoting proliferation and differentiation of intestinal epithelium, etc (Koh et al., 2016; Shabani et al., 2019). Interestingly, both BSC10 and Lac 16 treatments significantly decreased the relative abundance of zoonotic enteric pathogens, such as *E. coli*, *B. fragilis* and *S. sonnei*, indicating that the 2 screened probiotics have a potential beneficial role in inhibiting colonization of enteric pathogens in broilers and contamination of pathogens in poultry products. Moreover, the
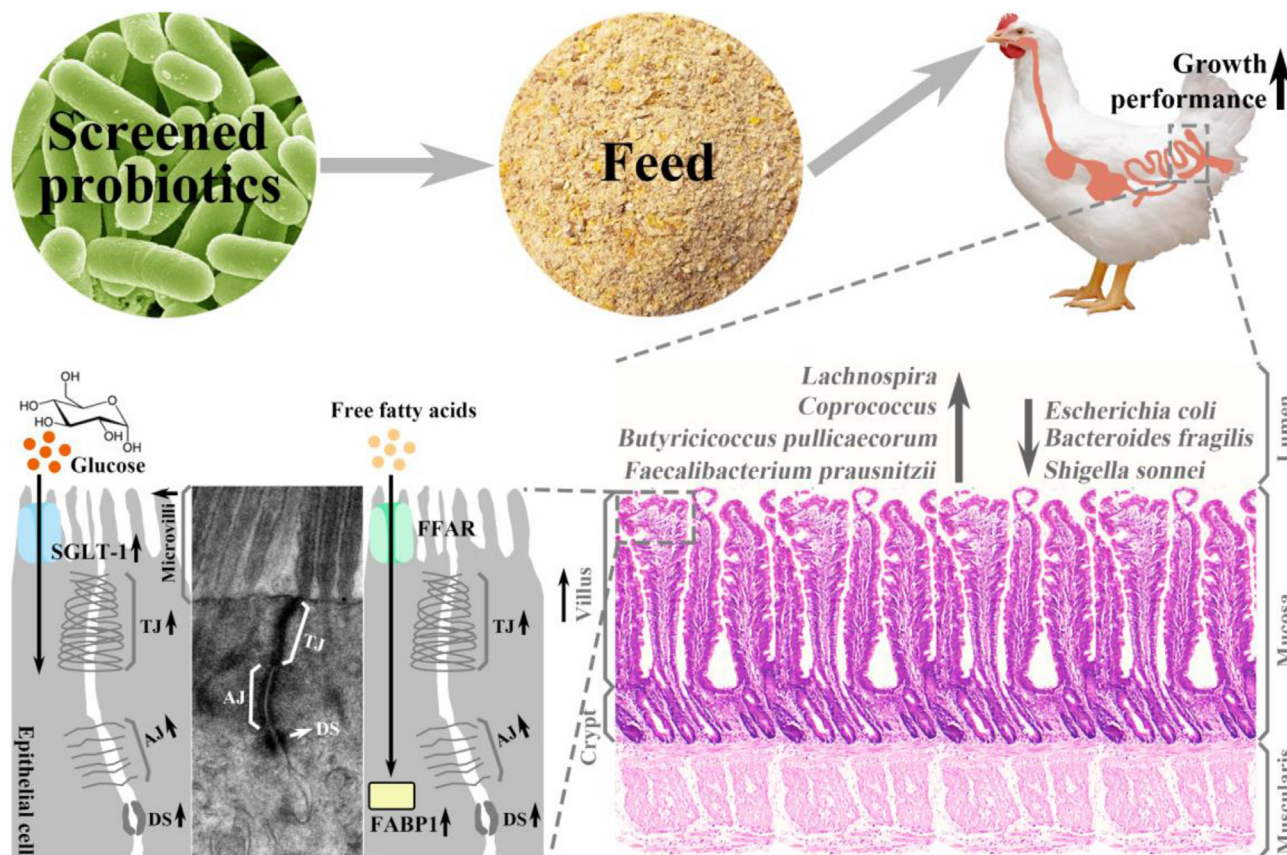
Fig. 8. Graphical summary of the effect of *Paenibacillus polymyxa* 10 and *Lactobacillus plantarum* 16 on the growth performance and intestinal health of broilers. Dietary *P. polymyxa* 10 and *L. plantarum* 16 are beneficial for the growth performance and intestinal health of broilers by improving the intestinal histomorphology, epithelial barrier, nutrient absorption and increasing the abundance of SCFA-producing bacteria. *FFAR* = free fatty acid receptors; *FABP1* = fatty acid binding protein 1; *SGLT-1* = sodium-dependent glucose transporters-1; TJ = tight junction; AJ = adherens junction; DS = desmosomes.

metagenome predictions results showed that the 2 screened probiotics, especially Lac16, could significantly increase the carbohydrate metabolism-related pathways while significantly decreasing the nucleotides biosynthesis-related pathways of the intestinal microbes, which would eventually promote the digestion of feed into absorbable nutrients and weaken the growth of microbes. Overall, this is beneficial for improving the feed utilization efficiency. The improved growth performance of broilers observed in this study may also be mediated by the increased number of SCFA-producing bacteria and carbohydrate metabolism-related pathways of the intestinal microbes.

Finally, co-occurrence patterns of intestinal microbes were employed to investigate the microbial interactions. The present results showed that the modularity values of the microbial co-occurrence networks in all groups were higher than 0.4, suggesting that these bacterial networks had a modular structure (Newman, 2006). The values of average degree and graph density in BSC10 and Lac16 groups were higher than those in the control group, suggesting that the 2 screened probiotics treatments increased the interaction among the intestinal microbes (Jiao et al., 2020). Additionally, a negative correlation of the microbial networks in the control group was more than those in BSC10 and Lac16 groups, which could be interpreted as a reduction in competitive relationships within intestinal microbes (Fan et al., 2018).

5. Conclusion

In conclusion, the present study demonstrates that the 2 screened probiotics, especially *L. plantarum* 16, have a potential beneficial effect on the growth performance and intestinal health of the starter phase broilers by improving the intestinal histomorphology, epithelial barrier, and nutrient absorption and increasing the abundance of SCFA-producing bacteria (Fig. 8). There are still calls for further investigation of the protective effect of the 2 screened probiotics against *C. perfringens* infection in broilers.

Author contributions

Weifen Li and **Dongyou Yu**: Conceptualization and Supervision. **Baikui Wang**: Data Curation, Microbial analysis, Writing - Original Draft & Review & Editing. **Baikui Wang** and **Li Gong**: conducted the animal experiments. **Li Tang**, **Yuanhao Zhou** and **Zihan Zeng** assisted with the experiments. **Qi Wang** and **Peng Zou** assisted in the manuscript preparation.

Data availability statement

Raw sequences have been deposited in the Genome Sequence Archive (GSA) of the BIG Data Center (https://bigd.big.ac.cn/gsa/) under accession number PRJCA004271/CRA003849.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2021.03.008.

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