Bacillus licheniformis–fermented products improve growth performance and the fecal microbiota community in broilers

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ABSTRACT This study investigated the effects of *Bacillus licheniformis*–fermented products on the growth performance and fecal microbial community of broilers. A total of 144 one-day-old male broiler chicks (Ross 308) were randomly assigned into 4 dietary treatments, with 6 replicate cages per treatment and 6 birds per cage. The dietary treatments comprised a basal diet as control, control plus 1 and 3 g/kg of *B. licheniformis*–fermented products, and control plus 10 mg/kg of enramycin. The results indicated that 3 g/kg of *B. licheniformis*–fermented products increased ($P < 0.05$) the BW and ADG of broilers relative to controls. No significant difference was observed in the growth performance of broilers fed enramycin and 3 g/kg of *B. licheniformis*–fermented products. However, principal coordinate analysis and a heatmap of species abundance indicated distinct clusters between the groups treated with enramycin and 3 g/kg of *B. licheniformis*–fermented products. The abundance of the phylum Firmicutes in feces increased ($P < 0.05$) in broilers fed 3 g/kg of *B. licheniformis*–fermented products, whereas the abundance of the phyla Verrucomicrobia and Bacteroidetes in feces decreased ($P < 0.05$) in response to treatment with 3 g/kg of *B. licheniformis*–fermented products. The abundance of the genera *Enterococcus*, *Akkermansia*, *Ruminococcus torques group*, *Faecalibacterium*, and *Parabacteroides* in feces decreased ($P < 0.05$) in broilers fed 3 g/kg of *B. licheniformis*–fermented products, whereas the abundance of the genus *Lactobacillus* in feces increased ($P < 0.05$) in response to treatment with 3 g/kg of *B. licheniformis*–fermented products. The average abundance of the genus *Lactobacillus* in feces was positively correlated with the growth performance of broilers. These results demonstrate that *B. licheniformis*–fermented products can improve the growth performance and fecal microflora composition of broilers.

Key words: *Bacillus licheniformis*, broiler, fermented product, microbiota

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INTRODUCTION

Antibiotic growth promoters (**AGP**) have been commonly used worldwide for the prophylactic treatment of infectious diseases in poultry. However, the overuse of in-feed antibiotics leads to bacterial resistance and antibiotic residues in poultry products, prompting the European Union to ban the use of antibiotics (Mehdi et al., 2018; Roth et al., 2019). However, this ban resulted in an increase in disease outbreaks in poultry in European countries (Casewell et al., 2003; Van Immerseel et al., 2004). It is therefore imperative to explore effective alternatives to AGP in infectious disease prevention in the poultry industry.

Increasing evidence suggests that probiotics can be applied as an AGP substitute (Abudabos et al., 2015, 2017). The dietary supplementation of probiotics can promote feed intake as well as nutrient digestion and absorption, thereby improving the growth performance of broilers (Lutful Kabir, 2009; Tabidi et al., 2013). Furthermore, probiotics can inhibit the growth of enteric pathogens and the development of subsequent diseases through the production of antimicrobial substances (Patterson and Burkholder, 2003; Lutful Kabir, 2009). Among *Bacillus* species, *Bacillus licheniformis* was identified from the gastrointestinal tract of broilers to exhibit antipathogenic activity (Barbosa et al., 2005). It has been demonstrated that the dietary supplementation of *B. licheniformis* improves growth performance and alleviates *Clostridium perfringens*–induced necrotic enteritis in broilers (Knap et al., 2010; Liu et al., 2012; Gong et al., 2018; Musa et al., 2019). Our previous studies have demonstrated that *B. licheniformis*–fermented products could inhibit the growth of *C. perfringens* and *Staphylococcus*

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aureus in vitro (Lin et al., 2019). Furthermore, *B. licheniformis*–fermented products mitigate *C. perfringens*–induced necrotic enteritis in broilers (Lin et al., 2019).

The gut microbiota plays an important role in utilizing nutrients, protecting against enteric pathogens, and modulating the immune system. Gut microbial diversity and composition can be modulated by diets and feed additives (Danzeisen et al., 2011). Antibiotic supplementation leads to a microbial imbalance in the gastrointestinal tract (Takesue et al., 2002). The dietary supplementation of fermented products that contain probiotics can regulate the gut microbiome and immunity, leading to improved health status and growth performance in poultry (Pan and Yu, 2014; Yan et al., 2019). However, to the best of our knowledge, no study has examined the effects of *B. licheniformis*–fermented products on improvement in the fecal microbiota of broilers. Nonetheless, it has been demonstrated that *B. licheniformis* has antimicrobial activity against pathogens through the production of antibacterial cyclic lipopeptide (Thaniyavarn et al., 2003; Lin et al., 2019). Therefore, we hypothesize that the beneficial effects of *B. licheniformis*–fermented products in broilers might be mediated by altering the gut microflora.

In the present study, we investigated the effects of different levels of *B. licheniformis*–fermented products on the growth performance and fecal microbiota of broilers.

MATERIALS AND METHODS

Preparation of B. licheniformis*–Fermented Products*

B. licheniformis was purchased from the Food Industry Research and Development Institute (ATCC 12713, Hsinchu, Taiwan). Details of the preparation of *B. licheniformis*–fermented products are provided in a previous study (Lin et al., 2019). Briefly, solid-state fermentation substrates were mixed with water in a space bag to obtain the required initial moisture content, and the mixture was autoclaved at 121°C for 30 min. The cooled substrates were inoculated with 4% (v/w) inoculum of *B. licheniformis*, mixed carefully under sterile conditions, and incubated at 30°C in a chamber with free oxygen and relative humidity above 80%. Fermented products were dried at 50°C for 2 D and homogenized through mechanical agitation. The fermented powder was then stored at 4°C before analysis. For the determination of bacteria counts in fermented products, the fermented powder was diluted serially in 0.85% NaCl, plated on tryptic soy agar (Sigma-Aldrich, St. Louis, MO), and incubated for 18 h at 30°C. Bacterial growth was counted and is expressed as colony-forming units per gram (**CFU/g**). For the determination of surfactin (*B. licheniformis*–derived antibacterial cyclic lipopeptide) content in fermented products, the fermented powder was adjusted to pH 2.0 with concentrated HCl and incubated overnight at 4°C. The precipitate was dissolved in distilled water and extracted with methanol. The mixture was shaken vigorously, and the organic phase was concentrated at reduced pressure at 40°C. The extract was further filtered using a syringe filter with a 0.22-μm membrane. Surfactin in the filtrate was identified using liquid chromatography–mass spectrometry/mass spectrometry (Figure 1). The details on the determination of surfactin from fermented products are provided in a previous study (Cheng et al., 2018). The surfactin concentration in the filtrate was measured using HPLC. *B. licheniformis* quantities and surfactin concentrations in fermented products were 3×10^{12} CFU/g and 4.7 mg/g, respectively (Table 1).

Birds and Experimental Design

All experiments were performed in accordance with approved guidelines. The animal protocol was approved by the Institutional Animal Care and Use Committee of National Ilan University. One-day-old healthy male broiler chicks (Ross 308) were obtained from a local commercial hatchery. On day 1, 144 birds with an average BW of 44.30 ± 0.05 g were randomly assigned to 4 treatments (with 6 replicates of 6 birds per cage) in a completely randomized design. Broilers were reared in stainless-steel, temperature-controlled cages (190 cm × 50 cm × 35 cm). The experimental diets consisted of (1) a basal diet with no treatment as control, (2) a basal diet plus 1 g/kg of *B. licheniformis*–fermented products (3×10^9 CFU/kg of feed), (3) a basal diet plus 3 g/kg of *B. licheniformis*–fermented products (9×10^9 CFU/kg of feed), and (4) a basal diet plus 10 mg/kg of enramycin. The diets were formulated to meet or exceed the requirements of birds according to breeder recommendations (Aviagen, 2014, Table 2). Feed and water were available ad libitum throughout the experiment. The feeding program had 2 phases that spanned days 1–14 and days 15–35. The lighting and temperature program were based on breeder recommendations (Aviagen, 2014). Broilers were vaccinated by nose drop administration with combined Newcastle disease–infectious bronchitis vaccines on days 4 and 15. Their average BW, ADG, average daily feed intake, and feed conversion ratio (**FCR**) were calculated from days 1 to 35.

Blood Biochemistry Analysis

At the end of the experiment (day 35), blood samples from 2 broilers per replicate (12 birds/treatment, n = 6) were collected through cardiac puncture and separated through centrifugation at 1,500 *g* for 10 min. The serum was collected for the measurement of glucose, triglyceride, cholesterol, high-density lipoprotein, low-density lipoprotein, aspartate transaminase, alanine transaminase, alkaline phosphatase, creatine kinase, and amylase through an automatic clinical chemistry analyzer (TOSHIBA TBA-80FR NEO2, Tokyo, Japan).

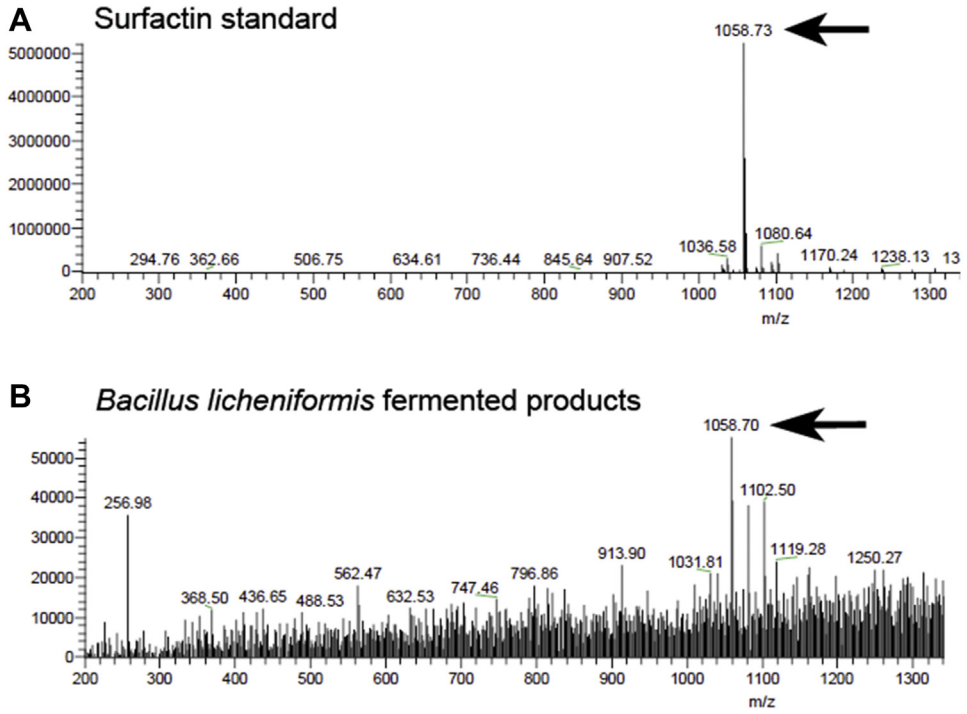

Figure 1. Liquid chromatography–mass spectrometry/mass spectrometry spectrum of *Bacillus licheniformis*–fermented products. Black arrows indicate the spectrum of surfactin in tested samples in comparison with (A) the surfactin standard and (B) *B. licheniformis*–fermented products. Three experiments were conducted, and 1 representative result is presented.

16S rRNA Sequencing and Data Processing

On day 35, feces from 2 broilers per replicate were freshly collected. 4 replicates (8 birds/treatment, n = 4) were used for fecal microbiota analysis. Total genomic DNA from feces was extracted using a Zymo-BIOMICS DNA Miniprep Kit (Zymo Research, Irvine, CA). DNA concentration and purity were assessed through 1% agarose gel electrophoresis. DNA amplicons from individual broiler samples were amplified with specific primers for the V3-V4 regions of the 16S rRNA gene through PCR. PCR products were purified using a QIAquick Gel Extraction Kit (QIAGEN, Germantown, MD). Sequencing libraries were generated using TruSeq Nano DNA Library Prep Kits (Illumina, San Diego, CA) according to manufacturer's recommendations. The library quality was assessed on a Qubit 2.0 Fluorometer (Thermo Scientific, Waltham, MA) and an Agilent Bioanalyzer 2100 system. The library was then sequenced on an Illumina MiSeq platform, and 300 bp paired-end reads were generated. Using the cluster program, sequences were clustered into operational taxonomic units (**OTU**) at 97% identity. A Venn diagram (version 1.6.17) was used to illustrate the similarities and differences between the 4 groups. Diversity analysis (alpha and beta) and phylogenetic assignment were executed using QIIME 2 (version

Table 1. Measurement of bacterial number and surfactin content in *B. licheniformis*–fermented products.

Item	Measured value
Bacteria colony (CFU/g fermented products)	3×10^{12}
Surfactin (mg/g fermented products)	4.7

Abbreviation: CFU, colony-forming unit.

Table 2. Composition of basal diets.

Item	Day 1 to 14	Day 15 to 35
Ingredient, g kg^{-1}		
Corn, yellow	554.2	607.3
Soybean meal	355.2	315.3
Fish meal	39.9	36.3
Vegetable oil	35.2	30.2
Limestone	15.2	12.7
Salt	3.0	3.0
Monocalcium phosphate	9.2	7.8
Mineral premix[1]	2.0	2.0
Vitamin premix[2]	2.0	2.0
DL-methionine	2.0	2.0
L-lysine	1.0	0.6
Choline chloride	0.5	0.5
Calculated value, g kg^{-1}		
Dry matter	88.9	88.7
CP	221.6	206.3
Analyzed calcium	10.2	8.7
Analyzed total phosphorus	6.9	6.3
Lysine	11.2	9.5
Methionine + cystine	8.5	7.6
ME, kcal/kg	3,081.1	3,057.2

[1]Supplied per kg of diet: 32 mg of Mn ($MnSO_4 \cdot H_2O$), 16 mg of Fe ($FeSO_4 \cdot 7H_2O$), 24 mg of Zn (ZnO), 2 mg of Cu ($CuSO_4 \cdot 5H_2O$), 800 μg of I (KI), 200 μg of Co ($CoSO_4$), and 60 μg of Se.

[2]Supplied per kg of diet: 1.8 mg of all-trans-retinyl acetate, 0.02 mg of cholecalciferol, 8.3 mg of alpha-tocopheryl acetate, 2.2 mg of menadione, 2 mg of pyridoxine HCl, 8 mg of cyanocobalamin, 10 mg of nicotine amid, 0.3 mg of folic acid, 20 mg of D-biotin, and 160 mg of choline chloride.

Table 3. Effect of *B. licheniformis*–fermented products on the growth performance of broilers.

	C[1]	L[2]	H[3]	E[4]	SEM	*P*-value
BW (g/bird)						
1 D	44.28	44.30	44.33	44.28	0.05	0.56
35 D	1,600.44^{a}	1,606.00^{a}	1,799.44^{b}	1,861.44^{b}	104.48	<0.001
ADG (g/D/bird)						
1–14 D	23.18	22.37	23.59	23.94	1.21	0.20
15–35 D	58.65^{a}	59.46^{a}	67.85^{b}	70.57^{b}	4.91	0.001
1–35 D	44.46^{a}	44.62^{a}	50.15^{b}	51.92^{b}	2.99	<0.001
ADFI (g/D/bird)						
1–14 D	27.20^{a}	26.92^{a}	28.35^{a,b}	29.00^{b}	1.05	0.01
15–35 D	96.93^{a,b}	92.43^{a}	104.57^{b}	102.03^{b}	5.80	0.01
1–35 D	69.04^{a,b}	66.23^{a}	74.08^{b,c}	72.82^{c}	3.82	0.01
Feed conversion ratio						
1–14 D	1.17	1.21	1.2	1.22	0.07	0.62
15–35 D	1.66^{a}	1.56^{a,b}	1.55^{a,b}	1.45^{b}	0.13	0.08
1–35 D	1.56^{a}	1.48^{a,b}	1.48^{a,b}	1.40^{b}	0.09	0.08

$^{a–c}$Means of a row with no common superscript are significantly different ($P < 0.05$).

[1]C indicates basal diet.

[2]L indicates basal diet plus 1 g/kg of *B. licheniformis*–fermented products.

[3]H indicates basal diet plus 3 g/kg of *B. licheniformis*–fermented products.

[4]E indicates basal diet plus 10 mg/kg of enramycin.

2017.4) software and the RDP Classifier Bayesian Algorithm (http://rdp.cme.msu.edu/), respectively. The alpha diversity was analyzed by species richness estimator (Chao1 and Fisher alpha) and species evenness estimator (Shannon and Enspie). The beta diversity was analyzed using principal component analysis and principal coordinate analysis on UniFrac distance matrices (Lozupone and Knight, 2005). Color correlograms were constructed using the corrplot package in R (version 0.84).

Statistical Analysis

Data were analyzed using one-way ANOVA through the GLM procedure in SAS software (version 9.4; SAS Institute, Cary, NC). Replicates were considered to be the experimental units. The results are expressed as mean ± SEM. Means were compared using Tukey's honestly significant difference test at a significance level of $P < 0.05$. The relationship between growth performance and abundant genera in broilers of different groups was assessed by Pearson's correlation coefficient (r).

RESULTS

Effect of B. licheniformis*–Fermented Products on Growth Performance and Blood Biochemical Parameters in Broilers*

The effect of *B. licheniformis*–fermented products on the growth performance of broilers is described in Table 3. As expected, the dietary supplementation of enramycin in broilers increased ($P < 0.05$) their BW at the end of the experiment. Similar to the effects of enramycin, the dietary supplementation of 3 g/kg of *B. licheniformis*–fermented products in broilers also increased ($P < 0.05$) their BW during the entire feeding period. Broilers fed both 3 g/kg of fermented products and enramycin had higher BW gain in the growth phase (15–35 D of age) and over the trial period (days 1–35) ($P < 0.05$). Broilers fed only enramycin had a higher ($P < 0.05$) feed intake in the starter phase (1–14 D of age) and during the whole trial period (days 1–35). Although the changes in the average feed intake (**AFI**) were not statistically significant, the trends of improved and decreased feed intake were observed during the entire feeding period with the supplementation of 1 and 3 g/kg of fermented products in broilers, respectively. Broilers fed enramycin had lower feed efficiency in the growth phase (15–35 D of age) and over the trial period ($P < 0.05$). Although the changes in feed efficiency were not statistically significant, a trend of an improved FCR was observed with the supplementation of 1 and 3 g/kg of fermented products in broilers. The effect of *B. licheniformis*–fermented products on the blood biochemical parameters of broilers is described in Table 4. With the exception of creatine kinase levels, no statistically significant differences were observed in blood biochemical parameters between the groups. Relative to the control group, at 35 D of age, serum creatine kinase levels were higher ($P < 0.05$) in broilers that received basal diets that were supplemented with enramycin. In addition, serum aspartate aminotransferase and alanine aminotransferase results indicated that *B. licheniformis*–fermented products did not cause liver toxicity or injury in broilers.

Effect of B. licheniformis*–Fermented Products on Fecal Bacterial Microbiota*

The effect of *B. licheniformis*–fermented products on the fecal microbiota of broilers is presented in Table 5. After stringent quality trimming of raw data, the averages of high-quality reads from the fecal content of broilers fed only a basal diet, 1 g/kg of *B. licheniformis*–fermented products, 3 g/kg of *B. licheniformis*–fermented products, or enramycin (hereafter referred to in sequence as the "4 aforementioned groups") were 21,231, 21,218, 21,394, and 23,606, respectively. The average bacterial sequences from the fecal content in the 4 aforementioned groups were 1,141, 940, 1,003, and 666 OTU, respectively. These results indicate decreased ($P < 0.01$) bacterial diversity in the fecal content of the enramycin-treated group. Furthermore, the average numbers of OTU for the 4 aforementioned groups, as estimated using the Chao 1 estimator, were 211, 175.5, 169.25, and 88.5, respectively. Similar results were obtained from Fisher alpha analysis ($P < 0.01$); species richness was lower (relative to the control group) in the fecal content of the groups that were fed enramycin and 3 g/kg of *B. licheniformis*–fermented products. Among the groups, fecal content in the enramycin-treated group had the lowest species richness

Table 4. Effect of *B. licheniformis*–fermented products on blood biochemistry parameters in broilers on the 35th D.

	GLU	TG	CHOL	HDL	LDL	AST	ALT	ALKP	CK	AMY
	(mg/dL)	(mg/dL)	(mg/dL)	(mg/dL)	(mg/dL)	(U/L)	(U/L)	(U/L)	(U/L)	(U/L)
C[1]	293.00	33.40	99.67	81.20	8.74	203.67	3.33	6,832.40	4.198.00^{x}	374.20
L[2]	302.50	47.80	103.33	78.22	10.76	196.00	3.17	6,445.60	3.344.40^{x}	389.20
H[3]	340.67	34.40	100.17	77.74	10.06	210.50	3.33	5,727.40	5,101.20^{x,y}	437.40
E[4]	341.17	40.00	99.50	75.42	11.02	217.00	4.17	5,974.40	6,623.40^{y}	428.60
SEM	68.58	18.26	13.44	6.97	2.03	18.76	0.81	2,404.34	1,083.18	63.33
P-value	0.54	0.64	0.96	0.41	0.37	0.31	0.20	0.90	<0.01	0.41

Abbreviations: ALKP, alkaline phosphatase; ALT, alanine aminotransferase; AMY, amylase; AST, aspartate aminotransferase; CHOL, cholesterol; CK, creatine kinase; GLU, glucose; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglyceride.

$^{x–y}$Means of a column with no common superscript are significantly different ($P < 0.05$).

[1]C indicates basal diet.

[2]L indicates basal diet plus 1 g/kg of *B. licheniformis*–fermented products.

[3]H indicates basal diet plus 3 g/kg of *B. licheniformis*–fermented products.

[4]E indicates basal diet plus 10 mg/kg of enramycin.

($P < 0.01$). Furthermore, Shannon and Enspie analysis indicated that fecal species evenness was lower ($P < 0.01$) in the groups treated with 1 and 3 g/kg of *B. licheniformis*–fermented products relative to the control group. Among the groups, fecal content in the enramycin-treated group had the lowest species evenness ($P < 0.01$). The Venn diagram illustrated a greater overlap (80 OTU, core) that was shared by 4 of the plotted groups (Figure 2). In total, 153, 96, 123, and 47 unique OTU were discovered in the 4 aforementioned groups, respectively. Specifically, 54 OTU were discovered in both the control group and the group treated with 1 g/kg of *B. licheniformis*–fermented products; 47 OTU were discovered in both the control group and group treated with 3 g/kg of *B. licheniformis*–fermented products. By contrast, 10 OTU were discovered in both the control group and enramycin-treated group. Principal component analysis conducted to examine the functional distinction of microbiota revealed statistically significant discrimination among the groups (PC1, 66.47%; PC2, 13.72%; PC3, 9.94%; Figure 3A). Principal coordinate analysis based on a weighted UniFrac metric indicated that the microbiota of fecal samples was clearly differentiated among the groups (PC1, 75.96%; PC2, 13.92%; PC3, 5.74%; Figure 3B). Similar results were also observed from principal coordinate analysis based on an unweighted UniFrac metric (PC1, 32.55%; PC2, 14.05%; PC3, 11.5%; Figure 3C). Beta diversity analysis based on weighted and unweighted UniFrac metrics also indicated that the microbiota of fecal samples were clearly differentiated (Figures 4A and 4B).

Table 5. Sample information, microbial diversity, and sequence abundance in the fecal contents of broilers.

	Effective reads	Number of OTU	Chao1	Fisher alpha	Shannon	Enspie
C[1]	21,231.25	1,141.00^{x}	211.00^{x}	30.32^{x}	3.41^{x}	5.88^{x}
L[2]	21,218.00	940.25^{x}	175.50^{x,y}	24.31^{x,y}	2.67^{y}	3.39^{y}
H[3]	21,393.50	1,003.00^{x}	169.25^{y}	23.77^{y}	2.49^{y}	2.72^{y}
E[4]	23,606.00	666.00^{y}	88.50^{z}	11.06^{z}	2.07^{z}	2.58^{y}
SEM	1,980.70	118.45	18.65	3.07	0.18	0.60
P-value	0.25	<0.001	<0.001	<0.001	<0.001	<0.001

$^{x–z}$Means of a column with no common superscript are significantly different ($P < 0.05$).

Abbreviation: OTU, operational taxonomic unit.

[1]C indicates basal diet.

[2]L indicates basal diet plus 1 g/kg of *B. licheniformis*–fermented products.

[3]H indicates basal diet plus 3 g/kg of *B. licheniformis*–fermented products.

[4]E indicates basal diet plus 10 mg/kg of enramycin.

Effects of B. licheniformis*–Fermented Products on Fecal Bacterial Taxonomic Composition*

The effect of *B. licheniformis*–fermented products on the bacterial taxonomy in the fecal contents of broilers is described in Table 6. Relative to the control group, at the phylum level, the abundance of the phylum Firmicutes was higher ($P < 0.05$) in the group treated with *B. licheniformis*–fermented products. No significant differences were observed in the abundance of the phylum Firmicutes between the control and enramycin-treated groups. The proportions of the phyla Verrucomicrobia and Bacteroidetes in feces were lower ($P < 0.05$) in broilers of the 3 treatment groups than in the control group. The abundance of the phylum Proteobacteria was higher ($P < 0.05$) in the groups treated with enramycin and 1 g/kg of *B. licheniformis*–fermented products relative to the control group. Regarding the abundance of the phylum Proteobacteria, no significant difference was found between the control group and the group treated with 3 g/kg of *B. licheniformis*–fermented products. Relative to the control group, at the class level, the proportions of the Bacilli class were higher ($P < 0.05$) in the feces of broilers fed basal diets that were supplemented with *B. licheniformis*–fermented products. The feces of broilers fed enramycin had the highest abundance of the Bacilli class ($P < 0.05$), and relative to the control group, the feces of broilers fed enramycin had a lower ($P < 0.05$) abundance of the Clostridia class. Regarding the abundance of the Clostridia class, no significant differences were observed between the control group and the groups treated with *B. licheniformis*–fermented products. The proportions of the Verrucomicrobiae and Bacteroidia classes were lower ($P < 0.05$) in the feces from the 3 treatment groups

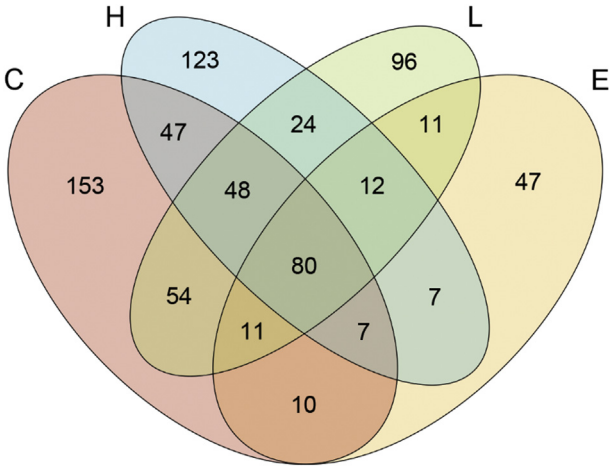

Figure 2. Operational taxonomic unit distribution and composition analysis of fecal content. Venn diagram of the operational taxonomic unit (OTU) distribution of the fecal contents. Each ellipse represents one group. The overlapping regions between the ellipses represent the OTU that is shared between the following: basal diet as the control (C), basal diet plus 1 g/kg of *B. licheniformis*–fermented products (L), basal diet plus 3 g/kg of *B. licheniformis*–fermented products (H), and basal diet plus 10 mg/kg of enramycin (E) (n = 4). The value of each region represents the number of OTUs corresponding to the region.

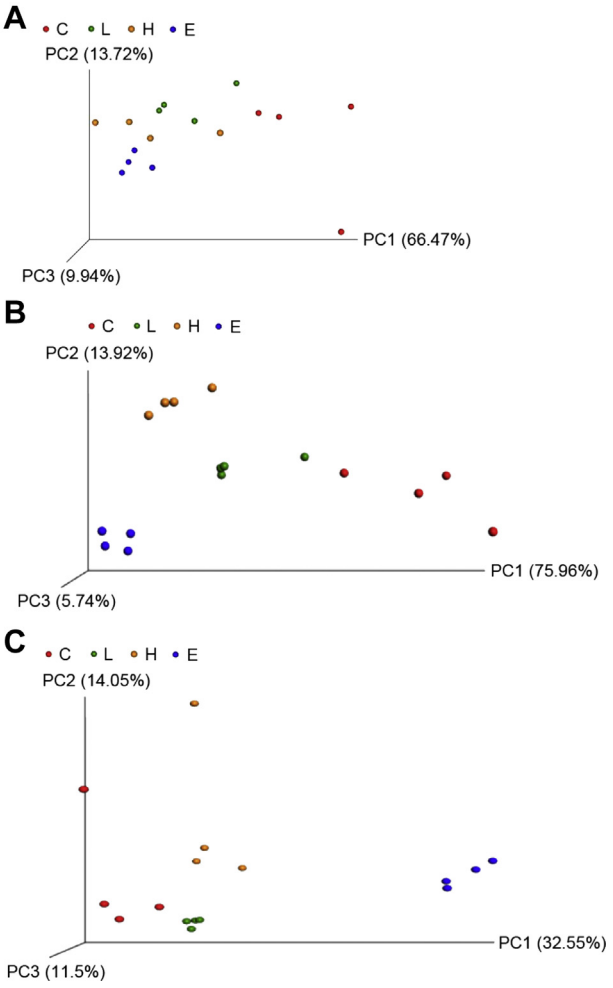

Figure 3. Comparison of the bacterial communities of the fecal contents by advanced analysis. (A) Principal component analysis plots of the fecal contents of basal diet as the control (C), basal diet plus 1 g/kg of *B. licheniformis*–fermented products (L), basal diet plus 3 g/kg of *B. licheniformis*–fermented products (H), and basal diet plus 10 mg/kg of enramycin (E) (n = 4). Principal coordinate analysis of (B) weighted UniFrac and (C) unweighted UniFrac distance of the fecal bacterial communities from C, L, H, and E (n = 4).

than those from the control group. The abundance of the Gammaproteobacteria class was higher ($P < 0.05$) in the groups treated with enramycin and 1 g/kg of *B. licheniformis*–fermented products relative to the control group. No significant difference was found in the abundance of the Gammaproteobacteria class between the control group and the group treated with 3 g/kg of *B. licheniformis*–fermented products. At the order level, the proportions of the Lactobacillales order were higher ($P < 0.05$) in the feces of broilers fed basal diets that were supplemented with *B. licheniformis*–fermented products relative to those that only received basal diets. The feces of the enramycin-treated group had the lowest and highest (both $P < 0.05$) abundance of the Clostridiales and Lactobacillales orders, respectively. The abundance of the Verrucomicrobiales and Bacteroidales orders was lower ($P < 0.05$) in broilers of the 3 treatment groups relative to the control group. Relative to the control group, the abundance of the Enterobacteriales order was higher ($P < 0.05$) in the groups treated with enramycin and 1 g/kg of *B. licheniformis*–fermented products. Regarding the abundance of the Enterobacteriales order, no significant difference was observed between the control group and the group treated with 3 g/kg of *B. licheniformis*–fermented products. At the family level, the proportions of the Lactobacillaceae family were higher in the 3 treatment groups ($P < 0.05$) than in the control group. The feces of broilers fed enramycin or 3 g/kg of *B. licheniformis*–fermented products had the highest ($P < 0.05$) abundance of the Lactobacillaceae family. The abundance of the Peptostreptococcaceae family in the feces of the enramycin-treated group was the lowest ($P < 0.05$). The proportions of the Enterococcaceae family in the 3 treatment groups were lower ($P < 0.05$) than those in the control group. The feces of broilers fed 3 g/kg of *B. licheniformis*–fermented products had the lowest ($P < 0.05$) abundance of the Enterococcaceae family. Relative to the control group, the abundance of the Lachnospiraceae family was lower ($P < 0.05$) in the groups that were treated with enramycin and 1 g/kg of *B. licheniformis*–fermented products. The proportions of the Akkermansiaceae, Ruminococcaceae, and Tannerellaceae families in the 3 treatment groups were lower ($P < 0.05$) than those in the control group. Relative to the control group, the abundance of the Enterobacteriaceae family was higher ($P < 0.05$) in the groups that were treated with enramycin and 1 g/kg of *B. licheniformis*–fermented products. The abundance of the Enterobacteriaceae family was the highest ($P < 0.05$) in the feces of broilers fed enramycin. At the genus level, the proportions of the *Lactobacillus* genus in the 3 treatment groups were higher ($P < 0.05$) than those in the control group. The abundance of the *Lactobacillus* genus was the highest ($P < 0.05$) in the feces of broilers fed enramycin or 3 g/kg of *B.*

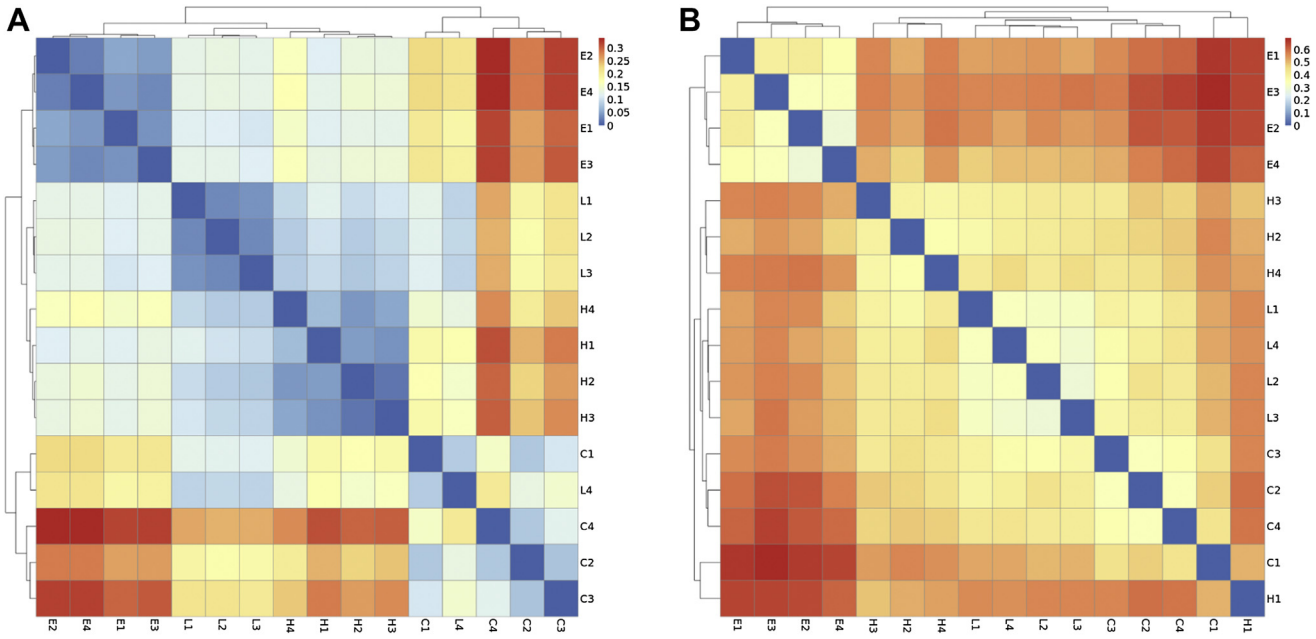

Figure 4. Comparative analysis of the fecal contents across the samples. (A) The beta diversity index of the fecal contents from the basal diet as control (C), basal diet plus 1 g/kg of *B. licheniformis*–fermented products (L), basal diet plus 3 g/kg of *B. licheniformis*–fermented products (H), and basal diet plus 10 mg/kg of enramycin (E) based on weighted UniFrac metrics (n = 4). (B) The beta diversity index of the fecal contents from C, L, H, and E based on unweighted UniFrac metrics (n = 4).

licheniformis–fermented products. The abundance of the *Romboutsia* genus was the lowest ($P < 0.05$) in the feces of broilers fed enramycin. The abundance of the *Enterococcus* genus in the 3 treatment groups was lower ($P < 0.05$) than that in the control group and was the lowest ($P < 0.05$) in the feces of broilers fed 3 g/kg of *B. licheniformis*–fermented products. The proportions of the genera *Akkermansia*, *Ruminococcus torques* group, *Faecalibacterium*, and *Parabacteroides* in the 3 treatment groups were lower ($P < 0.05$) than those in the control group. The abundance of the unclassified *Lachnospiraceae* genus was the highest ($P < 0.05$) in the feces of broilers fed 3 g/kg of *B. licheniformis*–fermented products. The abundance of the *Escherichia-Shigella* genus in the groups that were treated with enramycin and 1 g/kg of *B. licheniformis*–fermented products was higher ($P < 0.05$) than that in the control group. The abundance of the *Escherichia-Shigella* genus was the highest in the feces of broilers fed enramycin ($P < 0.05$). The proportions of the unclassified Peptostreptococcaceae genus in the groups treated with *B. licheniformis*–fermented products were higher ($P < 0.05$) than those in the control group; this proportion was the highest ($P < 0.05$) in the feces of broilers fed 3 g/kg of *B. licheniformis*–fermented products. An overview of the taxonomy at the genus level is also presented in Figure 5A. Based on the heatmap of the 35 most abundant genera, 2 distinct clusters were observed between the control and enramycin-treated groups (Figure 5B). The group treated with 3 g/kg of *B. licheniformis*–fermented products formed another cluster. In addition, bacterial community clusters were partially shared between the control group and the group treated with 1 g/kg of *B. licheniformis*–fermented products. Among the 35 most abundant genera, 6 genera (*Parabacteroides*, *CHKCI001*, *Butyricicoccus*, *GCA-900066575*, *R. torques* group, and *Intestinimonas*) were more abundant in the control group, and 3 genera (*Lachnospiraceae FE2018 group*, unclassified *Peptostreptococcaceae*, and *Turicibacter*) were enriched in only the groups treated with *B. licheniformis*–fermented products. Two genera (unclassified *Lachnospiraceae* and *Anaerostipes*) were the most abundant in the group treated with 3 g/kg of *B. licheniformis*–fermented products.

Association Between the Growth Performance and Average Abundance of the Genera

The results of correlation analysis between growth performance and the abundant genera in the broilers of different groups are presented in Figure 6. The average abundance of the genera *Lactobacillus* and *Escherichia-Shigella* was positively correlated with BW, ADG, and AFI, whereas the genera of *Romboutsia*, *Enterococcus*, *Akkermansia*, *R. torques* group, *Faecalibacterium*, and *Parabacteroides* were negatively correlated with these 3 variables. In addition, the average abundance of the genera *Romboutsia*, *Enterococcus*, *Akkermansia*, *R. torques group*, *Faecalibacterium*, and *Parabacteroides* was positively correlated with FCR, whereas the genera of *Lactobacillus* and *Escherichia-Shigella* were negatively correlated with FCR.

DISCUSSION

In this study, we demonstrated that 3 g/kg of *B. licheniformis*–fermented products improved the BW and ADG of broilers. Principal coordinate analysis and

Table 6. Bacterial taxonomy within the fecal contents of broilers.

	Relative abundance (%)					
	C[1]	L[2]	H[3]	E[4]	SEM	*P*-value
Phylum						
Firmicutes	87.87[a]	93.63[b]	97.42[b]	90.59[a]	1.99	<0.001
Verrucomicrobia	6.67[a]	0.90[b]	0.05[b]	0.01[b]	1.15	<0.001
Bacteroidetes	4.02[a]	0.86[b]	0.09[b]	0.07[b]	0.79	<0.001
Proteobacteria	1.29[a]	4.52[b]	2.35[a]	9.27[c]	0.87	<0.001
Class						
Bacilli	54.77[a]	65.22[b]	67.57[b]	78.65[c]	3.61	<0.001
Clostridia	32.60[a]	27.31[a]	28.57[a]	11.87[b]	2.53	<0.001
Verrucomicrobiae	6.67[a]	0.90[b]	0.05[b]	0.01[b]	1.15	<0.001
Bacteroidia	4.02[a]	0.86[b]	0.09[b]	0.07[b]	0.79	<0.001
Gammaproteobacteria	1.05[a]	4.49[b]	2.11[a]	9.27[c]	0.85	<0.001
Order						
Lactobacillales	54.61[a]	65.14[b]	67.54[b]	78.56[c]	3.64	<0.001
Clostridiales	32.60[a]	27.31[a]	28.57[a]	11.87[b]	2.53	<0.001
Verrucomicrobiales	6.67[a]	0.90[b]	0.04[b]	0.01[b]	1.15	<0.001
Bacteroidales	4.02[a]	0.86[b]	0.09[b]	0.07[b]	0.79	<0.001
Enterobacteriales	1.05[a]	4.49[b]	2.11[a]	9.27[c]	0.85	<0.001
Family						
Lactobacillaceae	35.66[a]	53.46[b]	66.15[c]	68.80[c]	5.18	<0.001
Peptostreptococcaceae	19.03[a]	21.96[a]	19.62[a]	10.74[b]	1.84	<0.001
Enterococcaceae	18.92[a]	11.54[b]	1.37[c]	9.69[b]	3.00	<0.001
Lachnospiraceae	8.04[a]	3.65[b]	7.72[a]	0.87[b]	1.75	<0.001
Akkermansiaceae	6.67[a]	0.90[b]	0.04[b]	0.01[b]	1.15	<0.001
Ruminococcaceae	4.97[a]	1.15[b]	0.99[b]	0.06[b]	0.84	<0.001
Tannerellaceae	2.88[a]	0.21[b]	0.04[b]	0.03[b]	0.54	<0.001
Enterobacteriaceae	1.05[a]	4.49[b]	2.11[a]	9.27[c]	0.85	<0.001
Genus						
Lactobacillus	35.66[a]	53.46[b]	66.15[c]	68.80[c]	5.18	<0.001
Romboutsia	18.73[a]	20.76[a]	16.77[a]	10.65[b]	1.89	<0.001
Enterococcus	18.92[a]	11.54[b]	1.37[c]	9.69[b]	3.00	<0.001
Akkermansia	6.67[a]	0.90[b]	0.05[b]	0.01[b]	1.15	<0.001
Ruminococcus torques group	3.87[a]	0.70[b]	0.78[b]	0.50[b]	0.60	<0.001
Faecalibacterium	3.74[a]	0.71[b]	0.12[b]	0.03[b]	0.61	<0.001
Parabacteroides	2.88[a]	0.21[b]	0.04[b]	0.03[b]	0.54	<0.001
Lachnospiraceae_ unclassified	1.34[a]	1.64[ab]	4.53[b]	0.21[a]	1.35	<0.001
Escherichia-Shigella	1.05[a]	4.48[b]	2.10[a]	9.12[c]	0.86	<0.001
Peptostreptococcaceae_ unclassified	0.25[a]	1.16[b]	2.82[c]	0.09[a]	0.40	<0.001

[a–c]Means of a row with no common superscript are significantly different ($P < 0.05$).
[1]C indicates basal diet.
[2]L indicates basal diet plus 1 g/kg of *B. licheniformis*–fermented products.
[3]H indicates basal diet plus 3 g/kg of *B. licheniformis*–fermented products.
[4]E indicates basal diet plus 10 mg/kg of enramycin.

the heatmap of species abundance indicated distinct clusters between the groups treated with enramycin and 3 g/kg of *B. licheniformis*–fermented products. The abundance of the phylum Firmicutes was higher in the fecal content of the group treated with 3 g/kg of *B. licheniformis*–fermented products. The abundance of the *Lactobacillus* genus was higher in the fecal content of the group treated with 3 g/kg of *B. licheniformis*–fermented products. Furthermore, the average abundance of the *Lactobacillus* genus was positively correlated with growth performance.

A previous study demonstrated that the supplementation of *B. licheniformis* in drinking water improved growth parameters (BW, ADG, and FCR) of broilers (Liu et al., 2012). In another study, the dietary supplementation of *B. licheniformis* improved the BW and daily weight gain of broilers (Gong et al., 2018). In this study, we also demonstrated that *B. licheniformis*–fermented products were able to increase the BW and daily weight gain of broilers. A previous study showed that *B. licheniformis* promoted the activities of trypsin, amylase, lipase, and total protease in the duodenal contents of broilers (Gong et al., 2018). Our previous study demonstrated that *B. licheniformis*–fermented products inhibited the growth of *C. perfringens* and *S. aureus* in vitro (Lin et al., 2019). Furthermore, *B. licheniformis* can improve growth performance and alleviate *C. perfringens*–induced necrotic enteritis in broilers (Knap et al., 2010; Musa et al., 2019; Lin et al., 2019). The major difference between the present study and other studies is the formula of *B. licheniformis*. In this study, we treated broilers with *B. licheniformis*–fermented products. *B. licheniformis*–fermented products not only contain live microorganisms but also have *B. licheniformis*–derived antibacterial cyclic lipopeptide. Therefore, we hypothesize that the efficiency of *B. licheniformis*–fermented products on improvement of growth performance in broilers may be different compared with other studies. Taken together, the results indicate that the dietary supplementation of *B. licheniformis* and *B. licheniformis*–fermented products has beneficial effects on the nutrient utilization and

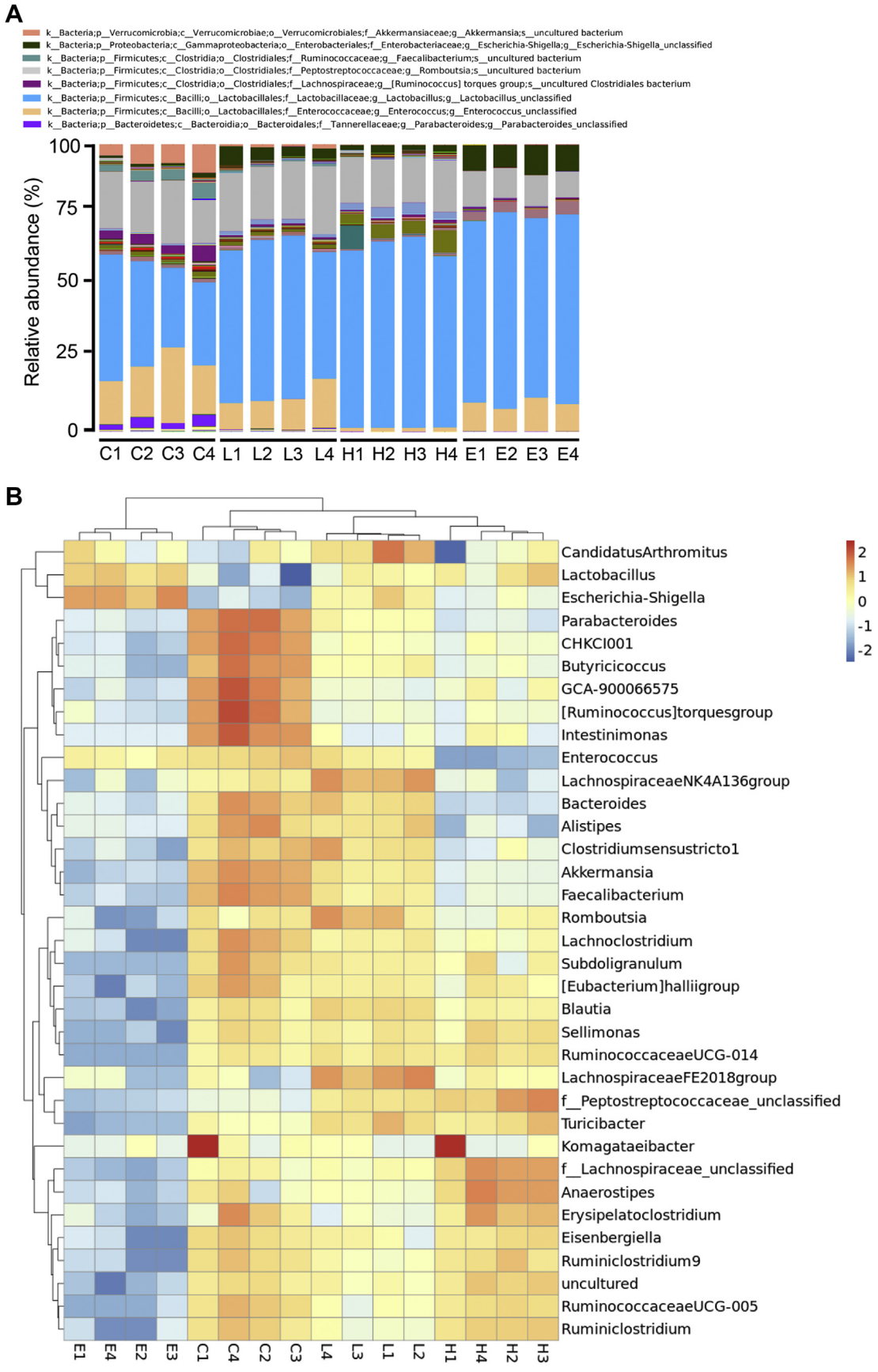

Figure 5. Bacterial taxonomic composition analysis of fecal content. (A) Genus-level composition of the microbiome from fecal content. Composition of major taxonomic groups at genus levels in samples collected from the basal diet as control (C), basal diet plus 1 g/kg of *B. licheniformis*–fermented products (L), basal diet plus 3 g/kg of *B. licheniformis*–fermented products (H), and basal diet plus 10 mg/kg of enramycin (E) (n = 4). (B) Heatmap of species abundance of the microbiome from fecal content. Abundance distribution of dominant 35 genera (Y-axis) across all samples (X-axis) were displayed in the species abundance heatmap (n = 4). Values are normalized by Z-score.

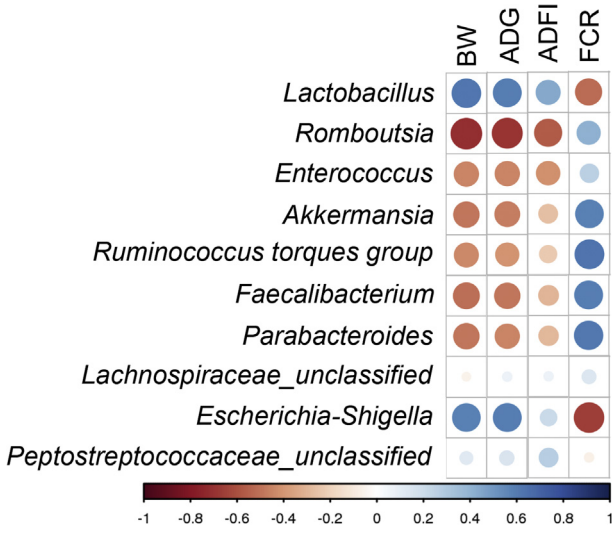

Figure 6. Correlation analysis between growth performance and abundant genera in broilers of different groups. Circle sizes and color intensity represent the magnitude of correlation. Blue circle represents positive correlations; red circle represents negative correlations. Abbreviation: FCR, feed conversion ratio.

competitive exclusion of pathogens from the intestine, thus improving growth performance in broilers. Whether the beneficial effects of *B. licheniformis*–fermented products on growth performance of broilers are different from those of only *B. licheniformis* remains to be elucidated.

The dietary supplementation of probiotics promotes the growth of beneficial bacteria and thus ensures a healthier intestinal system, thereby improving the growth performance of broilers (Pan and Yu, 2014; Yan et al., 2019; Cheng et al., 2019). Several studies have demonstrated that the fecal microbiome is associated with the growth performance of broilers (Singh et al., 2012; Hou et al., 2016; Díaz-Sánchez et al., 2019). A previous study showed that relative to broilers with a high FCR, the abundance of the phyla Firmicutes and Bacteroidetes in the feces was higher and lower, respectively, in broilers with a low FCR. (Singh et al., 2012). In our study, we observed a similar result in broilers with a low FCR (of 1 and 3 g/kg of *B. licheniformis*–fermented products). In humans, a study suggested that the phylum Firmicutes in the feces is more abundant in obese than in lean individuals, and the opposite is true for the phylum Bacteroidetes (Ley et al., 2006). Furthermore, in broilers, the phylum Firmicutes in the feces is more abundant in the fat line than in the lean line, and the opposite is true for the phylum Bacteroidetes (Hou et al., 2016). In our study, the abundance of the phylum Firmicutes in the feces was also higher in broilers with greater BW in the groups treated with enramycin and 3 g/kg of *B. licheniformis*–fermented products. Although the experimental design and *Bacillus* species strain are completely different among these studies, it still can provide an insight into the relationship between growth parameters and fecal microbiota. Taken together, these findings suggest that the dietary supplementation of *B. licheniformis*–fermented products increases the Firmicutes/Bacteroidetes ratio in the feces, which in turn enhances the growth performance of broilers.

A previous study reported that at the genus level, the proportion of the *Lactobacillus* genus was higher in broilers with a low FCR compared with broilers with a high FCR (Singh et al., 2012). Similarly, we observed that the abundance of the *Lactobacillus* genus in the feces was increased in broilers with a low FCR in the groups treated with enramycin and 3 g/kg of *B. licheniformis*–fermented products. Our correlation analysis between the other growth parameters (BW, ADG, and ADFI) and *Lactobacillus* content also provided similar findings. No significantly improved growth performance was observed with the supplementation of 1 g/kg of fermented products, although the group treated with 1 g/kg of *B. licheniformis*–fermented products had a higher *Lactobacillus* content relative to the control group. A previous study discovered that growth performance was improved after the supplementation of *Lactobacillus* species in broiler diets (Kalavathy et al., 2003). In another study, the dietary supplementation of *Lactobacillus* species inhibited the abundance of the *R. torques* group genus in the cecum of broilers (De Cesare et al., 2017). The *R. torques* group genus is known to degrade mucin in the gastrointestinal tract and is associated with gastrointestinal diseases (Malinen et al., 2010; De Cesare et al., 2017). In our study, we observed that the abundance of the *R. torques* group genus in the feces was reduced by our treatment with *B. licheniformis*–fermented products. It has been reported that the microbiome is different between fecal and cecal contents in broilers (Oakley and Kogut, 2016). The fecal microbiota are qualitatively similar to cecal microbiota but quantitatively different in broilers (Stanley et al., 2015). These results imply that the abundance of the *Lactobacillus* genus in the gastrointestinal tract is a significant factor in the growth performance of broilers. Whether fecal and cecal contents show similar microbiota in response to *B. licheniformis*–fermented product treatment needs to be investigated further.

Antibiotic supplementation results in a microbial imbalance in the gastrointestinal tract, which is associated with the pathogenesis of several metabolic and inflammatory diseases in humans (Takesue et al., 2002; Carding et al., 2015). In previous studies, an anticoccidial drug in combination with AGP treatment reduced the richness and diversity of the cecal microbiota in broilers (Danzeisen et al., 2011), and the diversity of the cecal microbiota of broilers was attenuated in response to enramycin treatment (Costa et al., 2017). In our study, we demonstrated that the supplementation of enramycin in the diet had the greatest effect on the richness and diversity of the fecal microbiota of broilers. By contrast, the supplementation of 3 g/kg of *B. licheniformis*–fermented products had a relatively low impact on the richness and diversity of fecal microbiota of broilers. More importantly, our results demonstrated a distinct bacterial community cluster between the groups treated with enramycin and 3 g/kg of *B. licheniformis*–fermented products.

According to a previous study, enramycin, a linear-ring peptide, primarily inhibits gram-positive bacteria by destroying bacterial cell walls (Kawakami et al., 1971). In another study, *B. licheniformis* was identified from the gastrointestinal tract of broilers to exhibit antipathogenic activity through the production of antibacterial cyclic lipopeptide (Thaniyavarn et al., 2003; Barbosa et al., 2005). Cyclic lipopeptides, such as surfactin, contribute to detergent-like activity and cause disruption that solubilizes the membrane of bacteria (Carrillo et al., 2003). In the present study, the abundance of some bacteria was increased by our treatment with *B. licheniformis*–fermented products. The group treated with 3 g/kg of *B. licheniformis*–fermented products had the lowest abundance of the *Enterococcus* genus and the highest abundance of the genera unclassified *Lachnospiraceae* and unclassified *Peptostreptococcaceae*. The *Enterococcus* genus has been related to human diseases, and strains that are resistant to antibiotic therapies have become a public health concern (Murray, 1998). The Lachnospiraceae and Peptostreptococcaceae families can degrade starch and nonstarch polysaccharides, thereby producing organic acids (Hang et al., 2012; Biddle et al., 2013). Therefore, the differential antimicrobial mechanisms between enramycin and *B. licheniformis*–derived surfactin may result in a distinct microbiome in broilers. The altered microbiome in broilers treated with 3 g/kg of *B. licheniformis*–fermented products may have contributed to their improved growth performance. The detailed interaction between *B. licheniformis*–derived surfactin and gut bacteria, such as *Enterococcus*, requires further investigation.

In conclusion, 3 g/kg of *B. licheniformis*–fermented products potentially improves growth performance and regulates the fecal microbiota of broilers. A distinct bacterial community cluster was found between the group treated with 3 g/kg of *B. licheniformis*–fermented products and the enramycin-treated group. These findings provide valuable insights into how enramycin and *B. licheniformis*–fermented products differentially affect the fecal microbiota of broilers. With regard to the future of microbiota manipulation in enhancing growth performance, these products are a promising alternative to antibiotics.

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