Effects of different probiotic fermented feeds on production performance and intestinal health of laying hens

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ABSTRACT The aim of this study was to investigate the effects of different probiotic fermented diets on production performance and intestinal health of laying hens. A total of 360 healthy 22-wk-age Jingfen No. 6 layers were randomly divided into 4 treatments: basal diet (**CON**); supplemented with 6% *Clostridium butyricum* fermented feed (**CB**); supplemented with 6% *Lactobacillus crispatus* fermented feed (**LC**); supplemented with 6% *Lactobacillus salivarius* fermented feed (**LS**). The experiment lasted for 8 wk. The results showed that the levels of crude fiber, β-glucan and pH of feed decreased significantly after fermentation ($P < 0.05$). Compared with CON group, the feed conversion ratio (**FCR**) was decreased significantly ($P < 0.05$), and albumen height and Haugh unit in LC group and LS group were increased significantly ($P < 0.05$). Fermented feed supplementation significantly improved villus height (**VH**) of the jejunum and the ratio of villus height to crypt depth (**VH/CD**) of the ileum ($P < 0.05$). Additionally, the VH and VH/CD of the duodenum were significantly increased in LS group ($P < 0.05$). Furthermore, the ACE and chao1 indexes in LS group were extremely significant higher than that in the other 3 groups ($P < 0.05$). In addition, compared with CON group, the abundance of *Rikenellaceae* and *Methanobacteriaceae* was significantly decreased at the family level in LC group and LS group ($P < 0.05$), while the abundance of *Ruminocaceae* was significantly higher ($P < 0.05$). Collectively, feeding *Lactobacillus salivarius* and *Lactobacillus crispatus* fermented feed improved the FCR, albumen height and Haugh unit of laying hens, and *Lactobacillus salivarius* fermented feed supplementation could improve intestinal health by ameliorating intestinal morphology, altering microbial composition and enhancing microbial community richness.

Key words: fermented feed, laying hen, production performance, intestinal health

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INTRODUCTION

With the shortage of protein feed and the rise of feed price, there is a tendency to move toward alternative or unconventional feed ingredients in the poultry industry. Some feedstuffs are high in protein, but contain a large number of anti-nutritional factors (**ANF**), such as protease inhibitors, soybean protein and oligosaccharides in soybean meal, nonstarch polysaccharides in corn, phytic acid in bran, etc. The existence of ANF limits their wide application in animal production. Fermentation has been used to improve the nutritional value of unconventional feed ingredients by lowering the crude fiber content (Skrede et al., 2003; Khempaka et al., 2014; Sugiharto et al., 2015), increasing crude protein and crude fat content (Agrahar-Murugkar and Subbulakshmi, 2006; Wang et al., 2010), and degrading the ANF (Feng et al., 2007).

Recently, fermented products have been commonly applied in poultry production. Feeding fermented feed can improve performance, antioxidant capacity and immune function of laying hens (Zhu et al., 2020). Meanwhile, adding fermented feed can improve egg weight, shell weight and shell stiffness of laying hens (Engberg et al., 2009). In addition, studies have shown that fermented diets had the potential to improve intestinal digestive function and morphology, as well as modulated the gut microbial ecosystem in poultry (Gao et al., 2009; Hu et al., 2016; Li et al., 2020a). Thus, feeding fermented feeds is beneficial to the health of poultry. The key to produce fermented feed is to select suitable bacterial strains. The lactic acid bacteria in the fermented feed can lower intestinal pH by producing organic acids and inhibit the colonization of intestinal pathogenic bacteria by producing antibacterial substances (Sugiharto and Ranjitkar, 2019). Bacillus in the

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fermented feed could promote digestion and absorption of nutrients by degrading cellulose, hemicellulose and lignin, and inhibit the growth of pathogenic bacteria by maintaining anaerobic environment in the intestinal tract (Cutting, 2011). Most of the selected strains for fermented feed were *Lactobacillus plantarum, Bacillus subtilis, Lactobacillus acidophilus,* and *Aspergillus niger* (Abdel-Latif et al., 2018; Wang et al., 2019; Li et al., 2020b; Shi et al., 2020; Zhu et al., 2020). Our team previous study has shown that adding 6% fermented feed to basal diet has the best effects on production performance and intestinal barrier function of laying hens. However, few studies have used *Lactobacillus salivarius* (***L. salivarius***), *Lactobacillus crispatus* (***L. crispatus***) and *Clostridium butyricum* (***C. butyricum***) as fermentation strains and compared the effects of different probiotic fermented feed on poultry.

The current study was therefore conducted to investigate and compare the effects of different probiotic fermented feeds on production performance, intestinal health of Jingfen No.6 laying hens and further examine associations between production performance and intestinal development.

Table 1. Composition and nutrient levels of the basal diet (air-dry basis).

Items (%, unless otherwise indicated)	CON	Experimental diets
Ingredients		
Corn	60.80	57.20
Soybean meal	22.20	21.00
Ground limestone	7.50	7.50
Wheat bran	4.00	2.80
Soybean oil	0.50	0.50
Premix[1]	5.00	5.00
Fermented feed	0.00	6.00
Total	100	100
Nutrient level[2]		
Dry matter	87.60	87.55
Metabolizable energy (MJ/kg)	12.09	12.07
Crude protein	15.99	15.92
Calcium	4.99	5.00
Total phosphorus	0.42	0.42
Available phosphorus	0.32	0.32
Lysine	0.94	0.92
Methionine	0.44	0.44

[1]The premix provided the following per kg of diets: VA 10000 IU, vitamin D_3 1800 IU, VE 10 IU, VK 10 mg, vitamin B_{12} 1.25 mg, thiamine l mg, riboflavin 4.5 mg, calcium pantothenate 50 mg, niacin 24.5 mg, pyridoxine 5 mg, biotin 1 mg, folic acid 1 mg, choline 500 mg, Mn 60 mg, I 0.4 mg, Fe 80 mg, Cu 8 mg, Se 0.3 mg, Zn 60 mg.

[2]Crude protein, calcium and total phosphorus were measured values, and the rest were calculated values.

MATERIALS AND METHODS

Experimental Design

All experimental procedures were approved by the Animal Care and Use Committee of Northwest A&F University, Yangling, China. *C. butyricum* (viable count 2×10^8 CFU/g) was provided by Guangdong Dazenong Biotechnology Co. Ltd, *L. crispatus* (viable count 1×10^5 CFU/g) was provided by Digestive Tract and Mammary Gland Biology Laboratory of College of Animal Science and Technology of Northwest A&F University, and *L. salivarius* (viable count 1×10^8 CFU/g) from chickens was provided by Poultry Healthy Breeding Innovation Team of College of Animal Science and Technology of Northwest A&F University.

A total of 360 22-wk-age Jingfen No. 6 laying hens were randomly divided into 4 groups with 6 replicates in each group and 15 laying hens in each replicate. Four dietary treatments were as follows: a basal diet (**CON**), the basal diet supplemented with 6% *C. butyricum* fermented feed (**CB**), the basal diet supplemented with 6% *L. crispatus* fermented feed (**LC**), the basal diet supplemented with 6% *L. salivarius* fermented feed (**LS**). The experimental period was 8 wk. The corn-soybean meal basal diet fed to laying hens was formulated to meet recommended nutrient requirements (NRC, 1994). The composition and nutritional level of the basic diet and experimental diets are shown in Table 1.

Bird Management

This study was conducted in the Demonstration Farm of Nonresistance Breeding of Chunmanyuan Layers in Tongchuan District of Shaanxi Province. During the entire experimental period, diets and water were available ad libitum. Chickens were given natural light and artificial light for a period of 16 h a day. The temperature in the room was maintained at 15°C to 22°C. At the beginning of the trial, the laying rate of chickens was 87.62 ± 2.67 %. At the end of the experiment, one healthy layer was randomly selected from each replicate and euthanized by exsanguination. The intestinal segments (the duodenum, jejunum, ileum and cecum) were taken out for the determination of follow-up indexes.

Fermented Feed Preparation

The composition of fermentation substrate is 60% corn, 20% soybean meal and 20% wheat bran. Firstly, adding freeze-dried powder of *C. butyricum, L. crispatus* and *L. salivarius* to warm water respectively, and then adding glucose with twice the mass of powder to stir evenly to obtain diluted bacterium solution. Secondly, the diluted bacterium solution was added into the fermentation substrate, which was mixed and supplemented with sterile water to achieve 30% moisture content. Thirdly, putting the mixed diets into the self-sealing bag and pressurizing to drain the air in the bag. Sealing the bag to make it ferment naturally at 20 ± 5°C for 5 d. Finally, the basic diet was mixed with 6% fermented feed to obtain the mixed diets as the experimental diets. The fermented feed was designed according to the weekly feed intake and prepared once a week. The routine nutritional components, viable count, pH and ANF of feed were determined. At the beginning of the experiment, feed samples were analyzed to establish the crude protein (CP; AOAC #984.13), crude fiber (CF; AOAC #978.10) and ether extract (EE; AOAC

#2003.05) contents according to AOAC International guidelines. The viable colonies were enumerated by the plate dilution colony counting method. To determine pH value, 4 g of fermented and unfermented mixed feed were dissolved in 40 mL distilled water. The pH value of the supernatant was measured with pH meter (Shanghai Russell Technology Co., Ltd, Shanghai, China) after centrifuging at 4000 × g for 5 min. The β-glucan, Trypsin inhibitor and phytic acid in fermented and unfermented mixture were tested using a commercial ELISA kit (Shanghai Enzyme-linked Biotechnology Co., Ltd, Shanghai, China).

Production Performance and Egg Quality

Egg production and weight for each replicate were recorded every day. The feed consumption of layers was recorded by week. The average daily feed intake (**ADFI**), average egg weight, laying rate and FCR of layers were calculated from wk 1 to wk 8. The ADFI was calculated according to total of feed intake divided by 8 wk experimental period, and FCR is calculated as follows: FCR = feed intake/egg mass. All calculations regarding ADFI and FCR were done on the basis of DM.

A total of 144 eggs (6 eggs per replicate) were selected to evaluate egg quality at the end of the trial period. The albumen height, yolk color and Haugh unit were determined by an Egg Analyzer (EMT-5200, Robotmation, Tokyo, Japan). The eggshell thickness and strength were measured by eggshell thickness tester (ETG-1601A, Robotmation, Tokyo, Japan) and eggshell strength tester (EFG-0503, Robotmation, Tokyo, Japan), respectively. The length and width of eggs were measured by vernier caliper. Egg shape index was calculated using the following formula: Egg shape index = length/width.

Intestinal Morphology

At the end of the trial period, 24 laying hens (one bird per replication) were selected to collect the duodenum, jejunum, and ileum. Taking about 2 to 3 cm from the duodenum, jejunum and ileum, rinsing out the intestinal chyme gently with syringe containing 0.9% physiological saline, and fixing it in a 10 mL centrifuge tube containing 4% paraformaldehyde solution. Histological slides were prepared and sectioned at 5 μm thickness of each intestinal sample, which were mounted onto glass slides and stained with Hematoxylin & Eosin (HE). Villus height and crypt depth were kept at room temperature until microscopic assessment of mucosal morphology. The histologic sections were examined with an Olympus BX51 polarizing light microscope (Olympus Co., Tokyo, Japan). Villus height and crypt depth were measured using Olympus cellSens Entry software. Villus height was determined as the distance between the tip of the villi and the villus-crypt junction. Crypt depth was measured as the distance of the invagination between 2 adjacent villi.

Cecal Microflora

Total genome DNA from cecal contents was extracted using SDS method. DNA concentration and purity were monitored on 1% agarose gels. Then amplicon generation was performed to obtain the PCR products. Mixing same volume of 1X loading buffer (contained SYB green) with PCR products and operating electrophoresis on 2% agarose gel for detection. Samples with bright main strip between 400 and 450 bp were chosen for further experiments. Then, mixture PCR products were purified with GeneJET Gel Extraction Kit (Thermo Scientific, Shanghai, China). The library was sequenced on an Illumina HiSeq platform and 250 bp paired-end reads were generated.

Paired-end reads from the original DNA fragments were merged by using FLASH. Paired-end reads were assigned to each sample according to the unique barcodes. Sequences were analyzed using QIIME (Quantitative Insights into Microbial Ecology), and in-house Perl scripts were used to analyze alpha and beta diversity. First, reads were filtered by QIIME quality filters. Then using pick_de_novo_otus.py to pick operational taxonomic units (**OTUs**) by making OTU table. Sequences with ≥97% similarity were assigned to the same OTUs. Picking a representative sequence for each OTU and using the RDP classifier to annotate taxonomic information for each representative sequence. In order to compute alpha diversity, we rarified the OTU table and calculated Chao1, ACE, Shannon and Simpson indexes. QIIME calculated both weighted and unweighted unifrac, which are phylogenetic measures of beta diversity. We used weighted unifrac for Principal Coordinate Analysis (**PCoA**) and Nonmetric multidimensional scaling (**NMDS**). To mine deeper data of microbial diversity of the differences between the samples, significance test was conducted with some statistical analysis methods, such as T-test and LEfSe.

Statistical Analysis

Datas of feed and production performance of laying hens were analyzed by one-way ANOVA and Duncan's multiple range tests for multiple comparisons using SPSS 26.0 (SPSS Inc., Chicago, IL). Results were presented as means with standard error of the mean (SEM). A value of $P < 0.05$ was considered statistically significant.

16S rRNA gene sequences of 12 samples (3 laying hens per replication) were analyzed using QIIME software package (Quantitative Insights Into Microbial Ecology). Differences in the abundance of phylum and family and microbial alpha-diversity were assessed by the Wilcoxon rank-sum test and considered significant at $P < 0.05$. Mann-Whitney U-test was used to assess statistical significance of measures derived from alpha diversity metrics. QIIME calculated weighted unifrac for PCoA and NMDS of beta diversity. LEfSe method with an alpha value of 0.05 for the Kruskale-Wallis test among classes was applied.

Table 2. The changes of the nutrient composition of feed before and after fermentation.

Item	Unfermented feed	CB	LC	LS	SEM	*P*-value
Crude protein (%)	14.06	13.22	13.67	13.63	0.127	0.132
Moisture (%)	34.55	34.74	34.51	34.51	0.103	0.862
Crude fat (%)	1.65	1.41	1.48	1.11	0.101	0.310
Crude fiber (%)	4.45[a]	3.82[b]	3.54[bc]	3.37[c]	0.119	<0.001
β-glucan (pg/mL)	202.38[a]	194.36[b]	193.11[bc]	188.60[c]	1.488	0.001
Trypsin inhibitor (ug/g)	220.36	214.76	215.43	212.33	1.664	0.414
Phytic acid (%)	0.46	0.42	0.40	0.41	0.008	0.075
pH	6.43[a]	4.97[b]	4.85[c]	4.81[c]	0.203	<0.001

[a-c]Means within each row with different superscripts are statistically significantly different ($P < 0.05$).

Table 3. Effects of fermented feed on production performance and egg quality of laying hens.

Item	CON	CB	LC	LS	SEM	*P*-value
Production[1]						
Laying rate (%)	88.83	91.33	91.50	90.17	0.005	0.130
ADFI (g)	120.90	119.57	113.55	118.00	1.126	0.100
ADFI (g DM/hen.d)	106.15	104.92	99.64	103.54	0.990	0.097
Average egg weight(g)	53.23	53.58	53.59	56.16	0.503	0.135
FCR	2.27[a]	2.23[ab]	2.12[bc]	2.11[c]	0.024	0.016
FCR (g DM/g egg mass)	1.99[a]	1.96[ab]	1.86[bc]	1.85[c]	0.024	0.016
Egg quality[2]						
Egg shape index	0.79	0.78	0.79	0.78	0.004	0.775
Eggshell thickness(mm)	0.36	0.38	0.35	0.37	3.910	0.877
Eggshell strength(N/m^2)	41.99	45.01	46.11	41.62	1.854	0.524
Albumen height (mm)	8.78[b]	8.93[b]	9.07[ab]	9.37[a]	0.072	0.017
Yolk color	6.93	6.97	6.87	6.70	0.184	0.964
Haugh unit	94.40[c]	95.55[bc]	96.50[ab]	97.58[a]	0.356	0.004

[a-c]Means within each row with different superscripts are statistically significantly different ($P < 0.05$).
[1]n = 6 replicates (15 birds/replicate) per treatment.
[2]Means were calculated using 6 replicates (6 eggs/replicate) per treatment.

Table 4. Effects of fermented feed on intestinal morphology of laying hens[1].

Item	CON	CB	LC	LS	SEM	*P*-value
Duodenum						
VH (μm)	975.37[b]	981.49[b]	983.31[b]	1023.35[a]	6.327	0.007
CD (μm)	159.70	156.62	153.27	158.76	1.024	0.066
VH / CD	6.11[b]	6.27[ab]	6.42[a]	6.45[a]	0.048	0.037
Jejunum						
VH (μm)	1038.93[b]	1073.1[a]	1047.93[ab]	1074.03[a]	5.434	0.033
CD (μm)	158.50[b]	158.63[b]	152.58[a]	155.36[a]	0.889	0.042
VH/CD	6.55	6.77	6.87	6.92	0.052	0.073
Ileum						
VH (μm)	985.78	1024.64	1004.30	1017.09	6.153	0.089
CD (μm)	138.46	136.04	133.27	133.88	0.768	0.056
VH/CD	7.12[b]	7.53[a]	7.54[a]	7.60[a]	0.068	0.032

[a-c]Means within each row with different superscripts are statistically significantly different ($P < 0.05$).
[1]Six replicates per treatment.

RESULTS

Fermented Feed Characteristics

As shown in Table 2, compared to CON diet, there was no significant difference in crude protein, crude fat and water content of the feed after fermentation; however, crude fiber content and pH value were significantly reduced ($P < 0.05$). Additionally, in CB, LC and LS groups, the β-glucan content in feed was decreased significantly ($P < 0.05$), which was helpful to improve the nutritional value of feed and enhance the absorption efficiency of feed in gastrointestinal tract. Moreover, the number of viable bacteria in CB group, LC group and LS group reached 1.32×10^6 CFU/g, 1.79×10^7 CFU/g, 1.98×10^7 CFU/g, respectively. The contents of crude fiber, β-glucan and phytic acid in L. salivarius fermented feed were significantly higher than those in unfermented feed and C. butyricum fermented feed ($P < 0.05$).

Production Performance and Egg Quality

As presented in Table 3, compared with CON group, the FCR in LC group and LS group decreased significantly ($P < 0.05$). ADFI of laying hens fed with fermented feed tended to decrease ($P = 0.097$). There was no significant difference in the laying rate and average egg weight among the groups. In terms of egg quality, the albumen height in LS group was significantly higher than that of CON group ($P < 0.05$). The Haugh unit in LC group and LS group was significantly higher than that of CON group ($P < 0.05$).

Intestinal Morphology

As shown in Table 4, compared with CON group, VH and VH/CD of the duodenum, VH of the jejunum and VH/CD of the ileum in LS group were significantly increased ($P < 0.05$), while the CD of the jejunum was significantly decreased ($P < 0.05$). The VH/CD of the duodenum and ileum in LC group was significantly higher than that in CON group ($P < 0.05$), and the CD of the jejunum in LC group was significantly lower than that in CON group ($P < 0.05$). The VH of the jejunum in CB group was significantly higher than that in the CON group ($P < 0.05$).

Cecal Microflora

The results showed that there were 36 phyla, 53 classes, 104 orders, 171 families, 300 genera and 192 species. Within the 4 groups, 2196 operational taxonomic units (OTUs) were observed, of which 101, 69, 74 and 994 unique elements were unique in the CON, CB, LC and LS groups, respectively (Figure 1). At the phylum level, *Firmicutes* and *Bacteroidetes* were the most

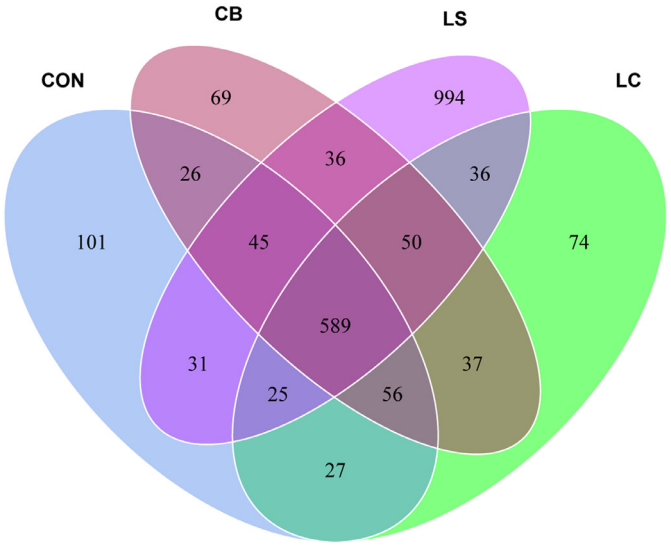

Figure 1. Venn diagram depicts OTUs in CON, CB, LS and LC groups.

predominant phyla in the microbial composition of 4 groups. The results also demonstrated that the relative abundance of Euryarchaeota in the LC group (0.69%) and LS group (0.21%) was significantly decreased ($P < 0.05$) than that in the CON group (3.99%) (Figure 2A). At the family level (Figure 2B), *Bacteroides* was the dominant family in 4 groups, and it had a higher abundance in LC group compared with the CON group (36.69% vs. 27.48%, $P > 0.05$). The abundance of *Methanobacteriaceae* in LC group (0.48%) and LS group (0.18%) was significantly decreased ($P < 0.05$) than that in the CON group (3.86%). Compared with the CON group (10.00%), the relative abundance of *Ruminococcaceae* in CB group (16.92%), LC group (14.88%) and LS group (17.36%) was higher ($P < 0.05$). The richness estimators (ACE and Chao 1) (Figures 3A and B) of bacteria in LS group were found to be extremely significantly higher than that in the other 3 groups ($P < 0.01$). In comparison with the CON group, the Shannon indexes of bacteria in CB and LS groups were significantly increased ($P < 0.05$, Figure 3C). Based on PCoA and NMDS analyses, the microbiota of cecum samples was remarkably differentiated in the 4 groups (Figure 4). Based on the LEfSe analysis, we found that the species with significant differences, indicated by an LDA score greater than 3.0, which reflected the degree of influence of a species with a significant difference between the groups (Figure 5). In the CON group, *Faecalibacterium* (genus), *Alloprevotella* (genus) and *unidentified Actinobacteria* (class) were identified as potential biomarkers (Figure 5). It was increased of the abundance of many microbial taxa such as *Euryarchaeota* (phylum), *Methanobrevibacter* (genus), *Barnesiella* (genus), etc. in the CB group. Feeding *L. crispatus* fermented feed enriched the amount of *Bacteroides-sp-Marseille-P3108* at the species level and increased *Mailhella* and *Shuttleworthia* at the genus level. Compared to the other 3 groups, the structure of the cecal microbiota was more diverse changes in the LS group, and there were 26 bacterial taxa including

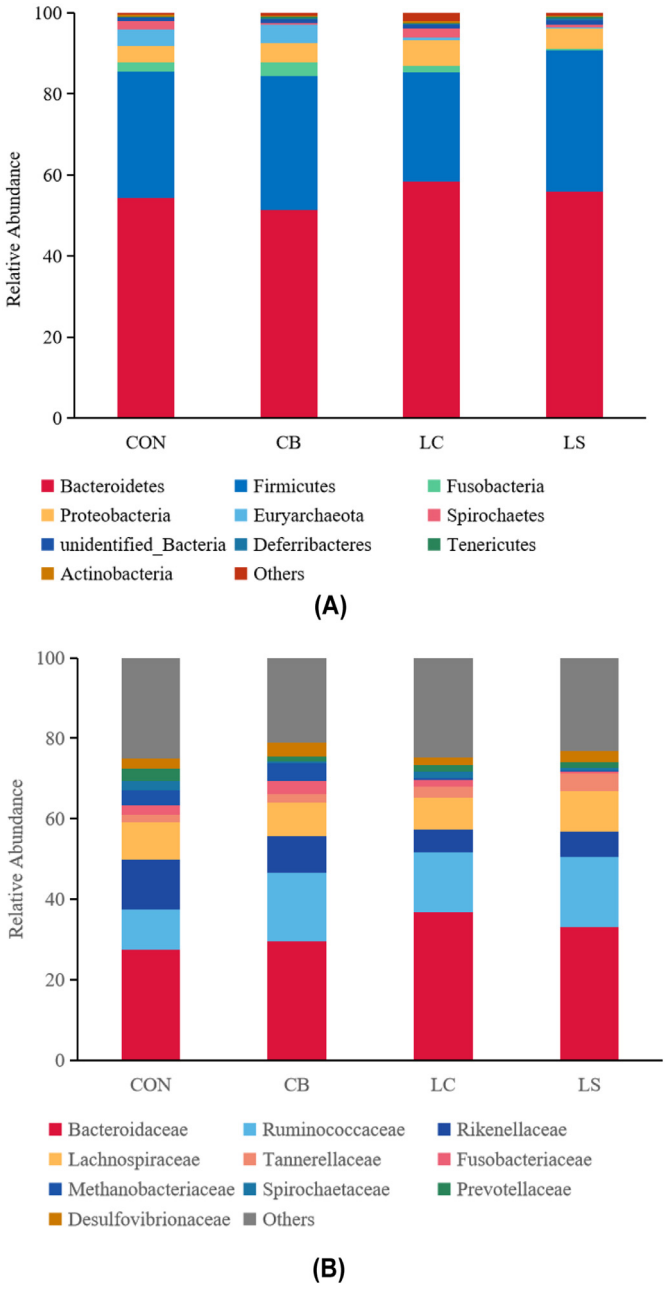

Figure 2. Analysis of the microbial composition in 4 groups(n = 3 birds/group). (A) Top 10 bacterial phyla of the cecum in CON, CB, LC, and LS groups; (B) Top 10 bacterial families of the cecum in CON, CB, LC, and LS groups.

Gammaproteobacteria (class), *Brachyspira* (genus), *Romboutsia* (genus), etc., with distinct relative abundances.

DISCUSSION

Fermented Feed Characteristics

In the current study, we compared the chemical composition of different probiotic fermented feeds. Microbial fermentation is considered economically viable processing technique to improve the nutritional quality of feed (Guo et al., 2020; Li et al., 2020b; Lin and Lee, 2020). The crude protein content of fermented feed was increased while the crude fiber content was decreased

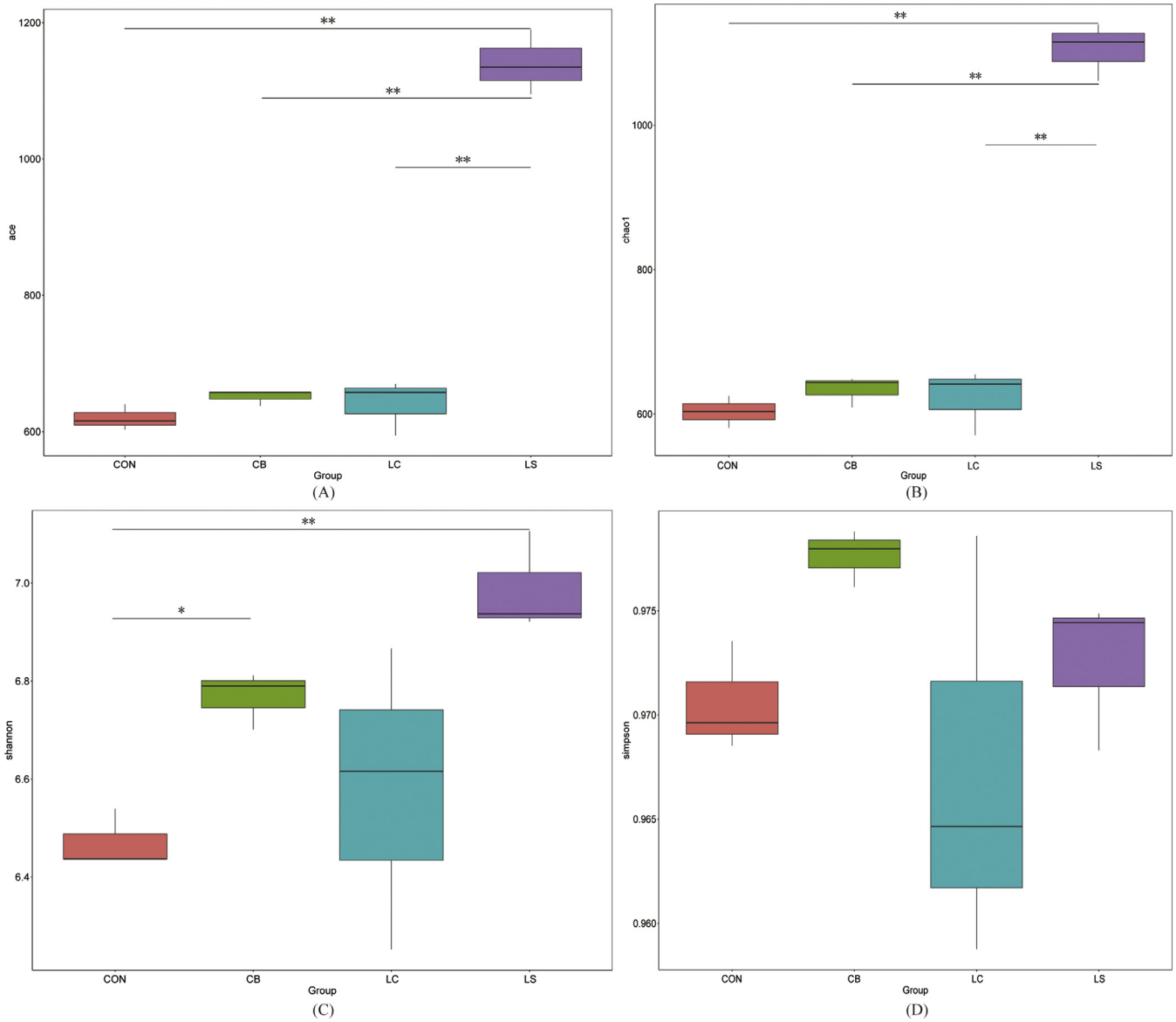

Figure 3. Alpha-diversity of cecum microbiota in 4 groups (n = 3 birds/group). Box plots indicate microbiome diversity differences of (A) ACE diversity, (B) Chao1 diversity, (C) Shannon diversity, and (D) Simpson diversity in CON, CB, LC, and LS groups. *Indicates $P < 0.05$, **Indicates $P < 0.01$.

(Khempaka et al., 2014; Sugiharto et al., 2015). It has been reported the crude protein of fermented feed was significantly improved (Engberg et al., 2009). In this study, the results were similar to previous studies, but not completely consistent. Compared with the CON group, the crude fiber contents of the 3 experimental groups were significantly reduced, especially in the LS group. However, the crude protein contents of feed decreased slightly after fermentation. In addition, we confirmed that the pH of feed was significantly reduced by the production of organic acids after probiotic fermentation, which agrees with previous studies (Engberg et al., 2009; Canibe and Jensen, 2012; Ranjitkar and Engberg, 2016). Furthermore, several studies have described that microbial fermentation could reduce the content of ANF to improve its nutritional characteristics (Feng et al., 2007; Sokrab et al., 2014; Medeiros et al., 2018). Similar to other studies, the level of β-glucan in experimental groups was decreased significantly, especially in LS group, and the contents of phytic acid and Trypsin inhibitor in the 3 experimental groups were lower than that in the CON group, which is beneficial to improve the digestibility of nutrients (Jazi et al., 2017; Drażbo et al., 2019).

Production Performance and Egg Quality

The beneficial effects of fermented products on growth performance of chickens have been demonstrated (Jazi et al., 2017; Drażbo et al., 2019; Guo et al., 2020; Cheng et al., 2021). The hens fed with fermented feed had better FCR (g DM/g egg mass) (Engberg et al., 2009). Relative to CON group, *L. crispatus* and *L. salivarius* fermented feed supplementation significantly decreased the FCR, and slightly enhanced laying rate in laying hens. Also, ADFI of laying hens fed with fermented feed tended to decrease, especially in the LC

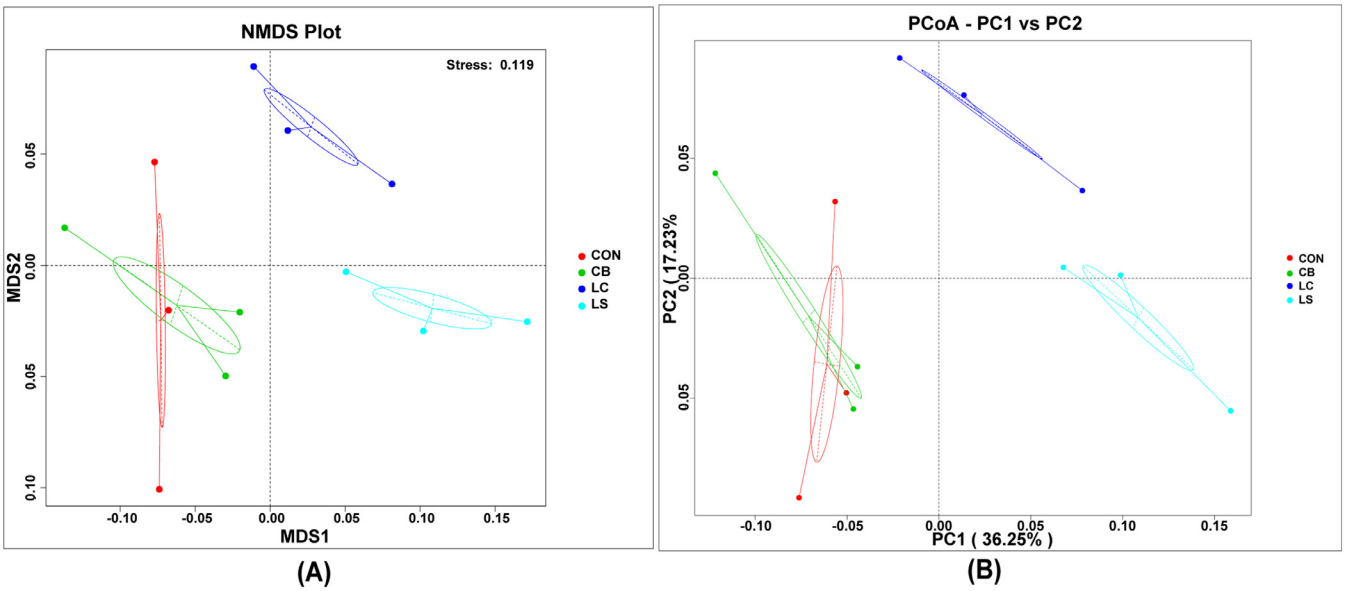

Figure 4. Beta-diversity analysis of cecum microbiota in 4 groups (n = 3 birds/group). (A) NMDS results in CON, CB, LC, and LS groups; (B) PCoA analysis in CON, CB, LC, and LS groups.

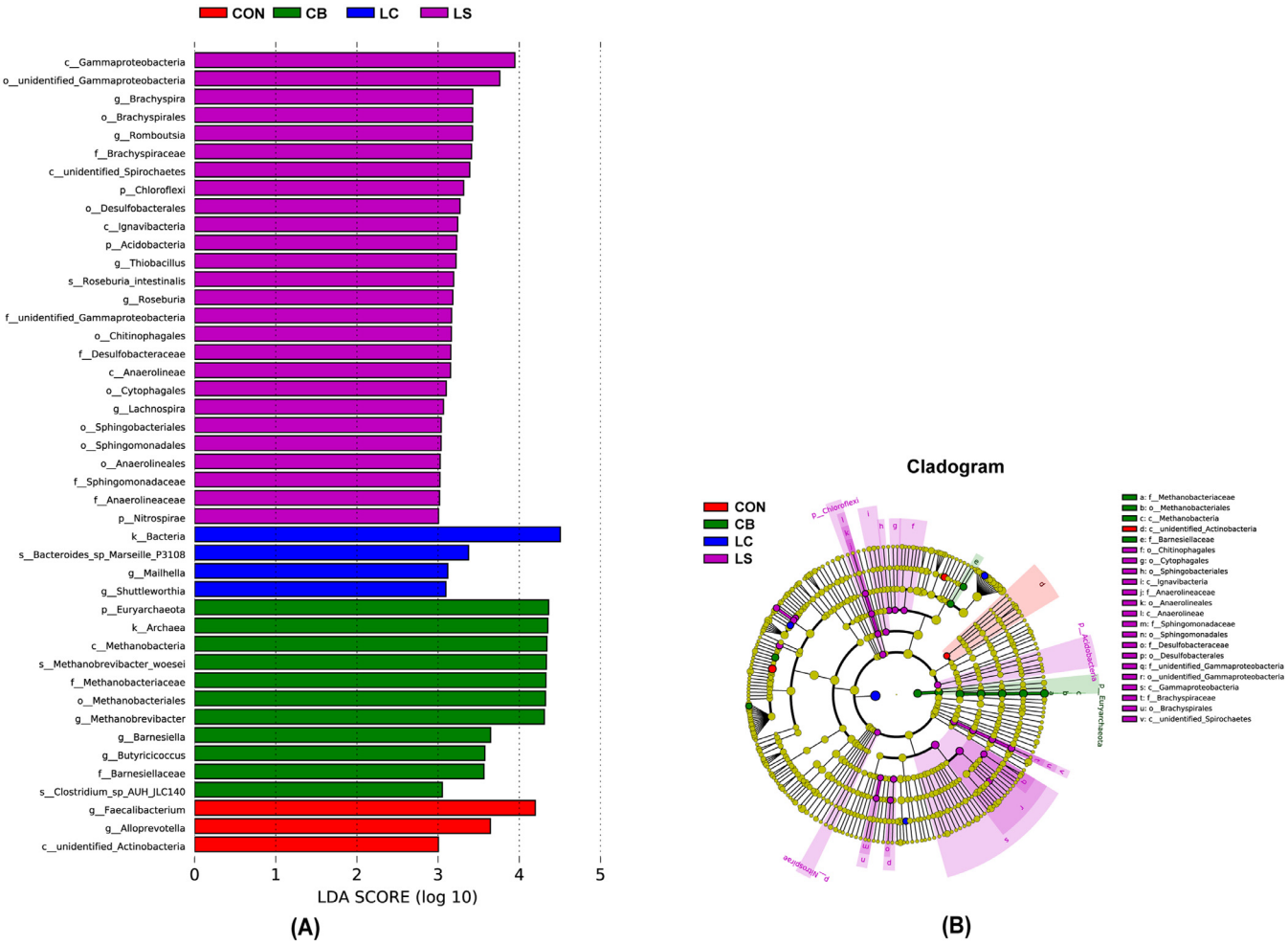

Figure 5. LEfSe and LDA analyses based on OTUs characterized the microbiomes of 4 groups. (A) LDA scores show the significant bacterial differences in the (log LDA >3.0; n = 3 birds/group). (B) Cladogram using the LEfSe method shows the phylogenetic distribution of the colonic microbes associated with the CON group (red), CB group (green), LC group (blue) and LS group (purple).

group. In addition, the average egg weight was the heaviest in LS group. These results supported that supplying fermented feed could slightly promote production of poultry by decreasing the FCR. Egg quality is also one of the main indexes to evaluate the performance of laying hens. The Haugh unit, a parameter of egg protein quality, was determined by the height of thick albumen and egg weight (Shi et al., 2020). Song et al. (2019) found that diets supplemented with fermented feed had significant effects on eggshell strength and Haugh unit. In this study, there was no significant difference in egg yolk color, eggshell thickness, eggshell strength, and egg shape index among the treatment groups. In LS group and LC group, the Haugh unit was significantly higher than that in CON group. The results were similar to those observed by Zheng et al. (2012) with dietary supplementation of Chlorella vulgaris CBT fermented feed in laying hens. In addition, Forbes (2003) reported hens receiving fermented feed had a greater body weight and lower DM intake, which may be attributable to an improved nutrient digestibility. In this study, feeding fermented diets with *C. butyricum, L. crispatus* and *L. salivarius* could improve the ADFI, FCR and Haugh unit of laying hens, and the effect of *L. salivarius* and *L. crispatus* is better than that of *C. butyricum.* The improvement of production performance of laying hens fed probiotics fermented feed may be due to the decrease of crude fiber, β-glucan and pH during microbial fermentation, as well as the improvement of feed nutrients.

Intestinal Morphology

It is generally believed that intestinal mucosa can protect the sterile internal environment from harmful lumen contents and resist harmful pathogens, which plays a key role in the digestion and absorption of food nutrition (Sugiharto et al., 2015). VH and CD are important indicators of intestinal morphology for the estimation of absorptive capacity of the small intestine in chickens (Dong et al., 2016). VH/CD is positively correlated with the absorption ability of nutrients by animals (Chuang et al., 2020). The small intestine is the most important organ for digestion. The massive research showed that feeding fermented feed to chickens can improve the VH of intestine (Feng et al., 2007; Missotten et al., 2013; Wang et al., 2019; Li et al., 2020b). Likewise, The VH and VH/CD of the duodenum of chickens which were fed fermented feed were significantly higher than those of the control group (Wang et al., 2019). In this study, VH of the small intestine in LS group was higher than that in the other 3 groups, and the layers in LS group presented higher FCR, which showed that FCR of laying hens may be in line with VH of the small intestine. VH/CD of the duodenum and ileum in LC group and LS group were higher than those in the CON group, which indicated that basal diet supplemented with *L. crispatus* and *L. salivarius* fermented feed can promote the gut development by improving the absorption function of the small intestine.

Cecal Microflora

The intestinal microbiota plays a vital role in maintaining host health, and it has become a research highlight in recent years (Yeoman et al., 2012). Previous study has shown that feeding fermented feed was in favor of modulating the composition of intestinal bacteria and maintained the healthy gastrointestinal ecosystem due to preventing excessive inflammatory responses against pathogens in the intestine (Missotten et al., 2013). According to the data of intestinal microflora in birds and mammals, *Firmicutes* and *Bacteroides* are the main intestinal phyla (Kohl, 2012). In our research, *Bacteroidetes* was the most abundant phylum in the cecum (CON 54.31%, CB 51.31%, LC 58.3%, and LS 55.88%). The second most abundant phylum in the cecum was *Firmicutes*. Feeding fermented feed did not change major bacterial species in the cecum of laying hens. Interestingly, at the phylum level, feeding *L. crispatus* fermented feed led to a reduced abundance of cecum *Firmicutes*, with an increased abundance of cecum *Bacteroidetes* and *Proteobacteria*, which may be due to reducing of the abundance of *Firmicum* to increase the abundance of other phyla in cecum, this finding was also reported by Shi et al. (2020). A relatively lower proportion of *Euryarchaeota* in the LS group was detected in current study. *Euryarchaeota* contains *Methanobacteriaceae,* which is usually found in animal intestines. Methane produced by *Methanobacteriaceae* can cause DNA damage in mammals and endanger animal health. It is speculated that the decrease of *Methanogenicae* may be beneficial to the growth and development of animals. Compared with CON group, the relative abundance of *Ruminococcaceae* in the cecum was significantly increased in the other 3 groups. *Ruminococcaceae* was mainly responsible for the degradation of a variety of polysaccharides and fibers, producing short chain fatty acids (SCFAs), which was believed to be conducive to the maintenance of intestinal health (Hooda et al., 2012). *Rikenellaceae*, another family with increased abundance in the cecum of laying hens, was previously reported from fecal samples and digestive tracts of a wide range of animals. There are no direct information members of the *Rikenellaceae* association with disease. It was reported that FCR can be improved by regulating nutrient digestibility and intestinal morphology, which may be attributed to the alteration of microbial composition and the increase of bacterial diversity (Zhou et al., 2021). The richer the diversity of microbial species, the more diverse the functional response and the better intestinal health, resulting in improving the production performance of laying hens (Zhang et al., 2019). In the current study, the ACE and chao1 indexes in LS group were extremely significant higher than that in the other 3 groups and the Shannon index in LS group was extremely significant higher than that in the CON group, resulting in improving FCR of laying hens. The results of PCoA and NMDS analysis showed that the microbial community structure of LC and LS group was clearly separated from that of CON

group, suggesting that the cecal flora of the 2 groups was significantly different from that of CON group. Meanwhile, there were 11, 4, 26 potential biomarkers with significant difference in CB group, LC group and LS group, respectively, further studies are needed to investigate the roles of these biomarkers in regulating gut development of laying hens.

In summary, we identified that feeding *Lactobacillus salivarius* and *Lactobacillus crispatus* fermented feed improved the FCR, Albumen height and Haugh unit of laying hens, and Lactobacillus salivarius fermented feed supplementation could improve intestinal health by ameliorating intestinal morphology, altering microbial composition and enhancing microbial community richness.

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DISCLOSURES

No potential conflict of interest was reported by the authors.

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