Dietary *Lactobacillus plantarum* improves the growth performance and intestinal health of Pekin ducks

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ABSTRACT *Lactobacillus plantarum* (***LP***) contributes to the intestinal health, whereas the study about the effects of *LP* on Pekin ducks is lacking. This study aimed to investigate the effects of *LP* on growth performance and intestinal health of Pekin ducks. A total of 180 one-day-old birds were randomly allotted to 3 treatments, and ducks were fed with a basal diet (**Control**) or basal diet supplemented with 400 (**LP1**) and 800 (**LP2**) mg/kg *LP* (5×10^9 CFU/g). The animal trial lasted for 42 d. Results showed that the LP1 and LP2 treatments improved growth performance (feed conversion) of ducks during the period of 1 to 42 d. At the end of 21 d, the decreased serum levels of interleukin (**IL**)-1β, interferon (**IFN**)-γ as well as downregulated ileal mRNA expression of *IL-1*β were observed in 2 doses of *LP* group. Meanwhile, the ileal mRNA levels of major histocompatibility complex (***MHC***)*-II, IL-4, Claudin, Occludin* were upregulated with 2 doses of *LP* supplemented. In addition, both *LP* treatments increased the relative abundance of *Firmicutes* and decreased *Bacteroidetes* wherein the relative abundance of *Bacteroides fragilis* was dropped parallelly. It is worth mentioning that markedly increased secretory immunoglobulin A content in ileal mucosa was observed in the LP1 group at d 21. At the end of the trial, the levels of serum complement 3 and β-defense were elevated with 2 doses of *LP* treated. Additionally, the ileal mRNA expressions of *MHC-II, lysozyme* were upregulated, and the diversity of the flora was also improved in the LP1 and LP2 groups. In conclusion, dietary *LP* improved the growth performance and intestinal health of Pekin ducks, and 400 mg/kg *LP* seemed to work better.

Key words: *Lactobacillus plantarum*, Pekin duck, growth performance, intestinal health

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INTRODUCTION

The emergence and spread of antibiotic resistance lead to a major public health problem. Hence, antibiotics in feed have been banned (Van Boeckel et al., 2015), which however, has brought a series of negative impacts on growth performance and health status in poultry production. There is increasing urgency to find safe green feed additives for the food animal industry (Jeni et al., 2021). Probiotics offer exciting opportunities to improve growth performance and health status in livestock (Lutful Kabir, 2009), and the major mechanism could be summarized as enhancement of nutrients absorption (Yeo and Kim, 1997; Jin et al., 2000; Wang and Gu, 2010), stimulation of host immune system (Azad et al., 2018), and modulation of gut microbial composition (Angelakis, 2017; Clavijo and Florez, 2018; Guo et al., 2021).

Lactic acid bacillus is a group of important natural inhabitants in the poultry digestive tract (Reuben et al., 2019), and *Lactobacillus plantarum* (***LP***) is a member of the facultative anaerobic, heterofermentative species of *Lactobacilli* that has been widely used in animal husbandry (Seddik et al., 2017). Previous studies have described that *LP* could modulate intestinal immunity, by way of illustration, inducing swine defense peptides expression via activating TLR2 as well as the ERK1/2/JNK and c-jun/c-fos signaling pathways (Wang et al., 2019a), and promoting proliferation of intestinal lymphocytes (Mizuno et al., 2020). Studies also described that *LP* contributes to not only the growth performance but also the secretion of intestinal immunoglobulin A (**sIgA**) and serum interleukin (**IL**)-4 in broilers (Wang et al., 2015; Benbara et al., 2020). Moreover, *LP* could relieve intestinal inflammation induced by *Clostridium perfringens* and reshape the microbial composition in broilers (Gong et al., 2020). In contrast, there are few studies investigating the biological effects of *LP* on Pekin ducks. We hold an interest in exploring the

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possible beneficial effects of *LP* addition on Pekin ducks and determining whether *LP* could use as an alternative feed additive in the duck industry for the enhancement of growth performance and health status. Hence, the present study was conducted to elucidate the effects of *LP* on growth performance and intestinal health of Pekin ducks and discussed the possible correlation between gut microbiota and growth performance to propose a potential mechanism of *LP* induced effects.

MATERIALS AND METHODS

The study was conducted in accordance with the Chinese guidelines for animal welfare and approved by the Animal Ethics Committee of China Agricultural University (Beijing, China). The animal welfare number is AW10211202-2-1.

Birds Management and Experimental Design

A total of 180 Pekin ducklings (Nankou 1) at 1-day-old (**1 d**) with similar body weights (59.99 ± 0.69 g) were reared in a net rearing system at the Poultry Experiment Base of China Agricultural University (Zhuozhou, Hebei, China). Ducklings were randomly allocated to 3 treatment groups with 6 replicates per one, and 10 birds each. The nutrient levels of the basal diet were designed based on the feeding standards of ducks (NRC, 1994) (Table 1). The 3 groups were arranged as follows, Control group (fed with the corn-soybean basal diet), LP1(basic diet supplemented with 400 mg/kg *LP*), LP2 (basic diet supplemented with 800 mg/kg *LP*). The *LP* freeze-dried powder (5×10^9 CFU/g, batch No. WYRSJ1000) used in the experiment was purchased from Guangzhou Weiyuan Biotechnological Co., Ltd., China. All diets were prepared in powder form. The animal trial lasted for 6 wk and conducted in winter. All ducks were housed in an environmentally controlled house. The initial temperature of the duck shed was maintained at 35°C during the first week, and then gradually reduced until it reached 22°C. All ducks had free access to feed and water and were subjected to 24 h light throughout the whole trial.

Growth Performance and Sampling

At the end of 21 and 42 d, all birds were weighed on an empty stomach, and the feed consumed of each pen was monitored concurrently. Average daily gain (**ADG**), average daily feed intake (**ADFI**), and the feed-to-gain ratio (**F/G**) were calculated for the periods of 1 to 21, and 1 to 42 d. After that, twelve birds with uniform weight from each treatment (2 birds per replicate) were randomly selected to harvest blood from jugular vein, and then slaughtered after anesthesia (sodium pentobarbitone, 50 mg/kg BW) to obtain liver, spleen, bursa of fabricius, thymus, intestinal tissue, and cecal chyme. Blood samples were collected into tubes without anticoagulant. The blood collection tubes were left at rest of room temperature at a tilt. After the serum was precipitated, it was centrifuged at 3,000 rpm for 15 min at 4°C, and the serum was separated and stored at −80°C for immune parameters test. The mass index of liver, spleen, bursa of fabricius, and thymus were calculated according to the formula: organ index (%) = organ weight (g) / duck live weight (g) × 100%. The ileal segments were collected and snap frozen in liquid nitrogen and then stored at −80°C for RT-qPCR. The mucosa was gently

Table 1. Test diet composition and nutrition level (air-dry basis).

Ingredients	Contents (%) 1−21 d	Contents (%) 22−42 d	Nutritional parameters	Levels[3] 1−21 d	Levels[3] 22−42 d
Corn	56.00	60.24	ME MC/kg	12.31	12.53
Soybean meal	32.69	24.67	Crude protein %	19.52	16.83
Wheat middling	5.00	9.00	Lysine%	1.12	0.87
Soybean oil	2.10	1.80	Methionine%	0.46	0.39
Phytases	0.02	0.02	Calcium %	0.88	0.89
Dicalcium phosphate	1.00	1.60	Available phosphorus %	0.29	0.39
Limestone powder	1.50	1.20	Total phosphorus %	0.54	0.62
DL-Methionine	0.15	0.12	Methonine+Cysteine	0.79	0.69
L-Lysine	0.20	0.10			
Vitamin premix[1]	0.02	0.02			
Trace element premix[2]	0.20	0.20			
NaCl	0.35	0.30			
Choline chloride (50%)	0.24	0.20			
Ethoxyquin (33%)	0.03	0.03			
Maifanite	0.50	0.50			
Total	100	100			

[1]Vitamin premix (provided per kilogram of feed) the following substances: vitamin A, 12,500 IU; vitamin D_3 3,500 IU; vitamin E 20 IU;vitamin K_3, 2.65 mg; vitamin B_1, 2 mg; vitamin B_2, 6 mg; vitamin B_{12}, 0.025 mg; vitamin E, 30 IU; biotin, 0.0325 mg; folic acid, 12 mg; pantothenic acid, 50 mg; niacin, 50 mg.

[2]Trace element premix (provided per kilogram of feed) the following substances: copper, 6 mg; zinc, 40 mg; iron, 80 mg; manganese, 100 mg; selenium, 0.15 mg; iodine, 0.35 mg.

[3]The levels of nutritional parameters are calculated. According to the previous studies (added into the basal diet at 10^8 CFU/kg) and the recommended dose by the manufacturer (800 mg/kg, added into the basal diet at 4×10^8 CFU/kg), the full dose treatment (800 mg/kg) was set up and the half-dose treatment (400 mg/kg) was also set up to test whether it is able to confer beneficial effects equally (Wu, et al., 2019; Xu, et al., 2020).

Table 2. List of gene primer sequences.

Gene name[a]		Prime sequence (5′-3′)	Accession number
IL-1β	F	GCTACACCCGCTCACAGTCCTT	XM_038166868.1
	R	GCCTCACTTTCTGGCTGGATG	
IL-2	F	GCCAAGAGCTGACCAACTTC	AF294323
	R	ATCGCCCACACTAAGAGCAT	
IL-4	F	CAATGAGACAGGCACCGACAT	XM_027469096.2
	R	GCTACTCGTTGGAGGGTTCTG	
IL-6	F	TACCCAGAAATCCCTCCTCACA	XM_027450925.2
	R	AATAGCGAACAGCCCTCACG	
IL-10	F	GAACGAGAACGGCATCTACAAG	NM_001310368.1
	R	TCCTCCTCTTCATCAGCAAGTATT	
IFN-γ	F	ACTGGCTTGAAAATCCAACG	NM_001310417.1
	R	GGAGACTGGCTCCTTTTCCT	
LYZ	F	TAACACGCAGGCTACAAACCG	XM_005008880.2
	R	TTCCATCGCTGACAATCCTCTT	
MHC-II	F	CCACCTTTACCAGCTTCGAG	AY905539
	R	CCGTTCTTCATCCAGGTGAT	
ZO-1	F	TACGCCTGTGAAGAATGCAG	XM 013104939.1
	R	GGAGTGTGTGGTGTTTGCTTT	
Claudin-3	F	GGCGTCATCTTCCTGCTCTC	XM_005015884.4
	R	GCTCCCTCTTCTGCGATTCAA	
Occludin	F	CAGGATGTGGCAGAGGAATACAA	XM 013109403.1
	R	CCTTGTCGTAGTCGCTCACCAT	
GAPDH	F	GTAGTGAAGGCTGCTGCTGAT	XM_038180584.1
	R	AGGTGGAGGAATGGCTGTCA	

[a]Abbreviations: *IL-1β, IL-2, IL-4, IL-6* and *IL-10*, interleukin 1*β*, IL-2, IL-4, IL-6 and IL-10, *IFN-γ*, interferon *γ*, *LYZ*, lysozyme, *MHC-II*, major histocompatibility complex II, *Claudin-3, Occludin* and *ZO-1* belong to tight junction proteins.

scraped from the remaining ileum. One decigram of ileal mucosa sample was homogenized with 900 μL of 0.9% sterile normal saline on ice and then centrifuged at 3,000 rpm and 4°C for 15 min to aspirate the supernatant for sIgA test. The whole cecal chyme of birds were aseptically sampled, quickly frozen in liquid nitrogen and then stored at −80°C for further 16 s sequencing analysis.

Immune Status

The levels of complement 3 (**C3**), *β*-defensin, lysozyme (**LYZ**), IL-4, IL-1*β*, and interferon (**IFN**)-*γ* in the serum, along with the sIgA content of ileum mucosa were determined according to the manufacturer's instruction (Jiancheng Bioengineering Institute, Nanjing, China). The concentration of serum IgA, IgG, and IgM were measured using the Elisa kits strictly following manufacturer's protocols (Solarbio Bioengineering Institute, Beijing, China). The BCA protein quantification kit (Cwbio, Beijing, China) was used to evaluate the total protein concentration of ileal supernatant according to the manufacturer's instructions.

RNA Extraction and RT-PCR

Total RNA was isolated using Trizol (Invitrogen Life Technologies, Carlsbad, CA) according to the method described by Fan et al. (2018), and then RNA was reverse-transcribed using the PrimeScript RT reagent Kit with gDNA Eraser (Takara Biotechnology, Dalian, China). qPCR was conducted with the Applied Biosystems 7500 Fast Real-Time PCR System (Foster City, CA) using the TB Green Premix Ex Taq kit (Takara Biotechnology). Amplification was carried out with the following profile: 2 min at 50°C, 10 min at 95°C, then 40 cycles of 95°C for 15 s, 60°C for 1 min, and a final extension at 72°C for 10 min. Results were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**) and calculated using the method described by Fu et al. (2010). The RT-PCR analysis of genes expression was performed using primers listed in Table 2.

Cecal Microbiota Analysis

Total DNA of cecal chyme was extracted using QIAamp DNA Stool Mini Kit (Qiagen Company, Germany). Samples were quantified by NanoDrop ND-2000C (Thermo fisher Scientific, Waltham, MA), and purity was assessed by 1% agarose gel electrophoresis. 16SrRNA gene V3-V4 region universal primers 338 F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806 R (5′-GGACTACHVGGGTWTCTAAT-3′) were used to amplify bacterial DNA. The PCR products were purified, quantified, and homogenized to construct a sequencing library, and then sequenced using Illumina HiSeq 2500 by Baimaike Company. Qiime software (Qiime2-2019.7, Nature Biotechnology, New York, NY) was used to analyze alpha and beta diversity. Subsequently, beta diversity was visualized by Principal Coordinates Analysis (**PCoA**) diagram using R software (Version 2.15.3), and the microbial composition in different groups was clarified by analyzing the bacteria at a variety of taxonomic levels. Analysis of similarities (**ANOSIM**) and permutational analysis of variance

Table 3. Effect of *LP* on growth performance of Pekin ducks[1].

Item	Treatments[2]			SEM	*P*-value
	Control	LP1	LP2		
1 to 21 d					
BW(g)	963.66[b]	994.87[ab]	1,030.24[a]	9.36	< 0.01
ADG(g)	43.05[b]	44.52[ab]	46.2[a]	0.44	< 0.01
ADFI(g)	82.9	84.02	87.13	1.25	0.38
F/G	1.93	1.89	1.89	0.03	0.80
1 to 42 d					
BW(g)	2,875.09	2,913.47	2,890.17	16.2	0.65
ADG(g)	57.69	58.73	58.22	0.45	0.67
ADFI(g)	190.81	179.11	181.11	2.55	0.26
F/G	3.31[a]	3.05[b]	3.11[b]	0.04	0.03

[a,b]Means with different superscript differ significantly ($P < 0.05$) within a same row.
[1]n = 6/group.
[2]Control group (fed with the corn-soybean basal diet), LP1 (basic diet supplemented with 400 mg/kg *LP*), LP2 (basic diet supplemented with 800 mg/kg *LP*).

(**PERMANOVA**) were employed to further determine the significant differences in beta-diversity among 3 groups.

Statistical Analysis

Statistical analysis was conducted using the SPSS 26.0 software (SPSS Inc., Chicago, IL). The data were analyzed by one-way ANOVA with the Duncan's multiple comparisons tests. Results were reported as means with SEM. Pearson correlation analysis was employed to analyze the correlation between the relative abundance of bacteria with growth performance and serum immune parameters. Value of $P < 0.05$ was considered significant. Graphpad prism 8.0 software (GraphPad Software Inc., San Diego, CA) was used to graph the data.

Table 4. Effect of *LP* on organ indices of Pekin ducks[1].

Item	Treatments[2]			SEM	*P*-value
	Control	LP1	LP2		
21 d					
Liver (%)	56.63	57.32	56.44	0.87	0.92
Spleen (%)	1.12	1.00	1.02	0.03	0.23
Bursa of fabricius (%)	1.34	1.13	1.08	0.05	0.11
Thymus (%)	3.29[ab]	3.57[a]	3.04[b]	0.08	0.01
42 d					
Liver (%)	37.74	41.09	39.04	0.64	0.09
Spleen (%)	0.6	0.68	0.72	0.02	0.11
Bursa of fabricius (%)	0.92[b]	1.14[a]	0.90[b]	0.04	0.03
Thymus (%)	1.94[b]	2.24[a]	2.38[a]	0.06	< 0.01

[a,b]Means with different superscript differ significantly ($P < 0.05$) within a same row.
[1]n = 12/group.
[2]Control group (fed with the corn-soybean basal diet), LP1(basic diet supplemented with 400 mg/kg *LP*), LP2 (basic diet supplemented with 800 mg/kg *LP*).

RESULTS

Growth Performance and Organ Index

During the periods of 1 to 42 d, the F/G in the LP1 and LP2 groups were decreased ($P < 0.05$) compared to the control group (Table 3). Besides, there were greater BW and ADG values of the starter phase (1−21 d) ducks in the LP2 group. Thymus index was heightened ($P < 0.01$) in 2 doses of *LP* supplemented groups at 42 d (Table 4). Moreover, bursa of fabricius index was significantly improved ($P < 0.05$) and liver index was slightly improved ($P = 0.094$) in the LP1 treatment. Thus, *LP* could improve growth performance and organ development of Pekin ducks.

Immunity and Physical Barrier

Desirable feed efficiency relies heavily on a healthy gut that is reflected in intestinal physical barrier and immunological barrier functions. Apart from intestinal immunity, systemic immunity would also be activated by probiotic via various cytokine cascade reactions. Thus, systemic immune status (Figure 1), and immune and physical barriers of intestine (Figure 2) were further investigated. The results indicated that serum levels of IL-1β, IFN-γ and the ileal mRNA levels of *IL-1β, IL-6* were significantly decreased ($P < 0.01$) in 2 doses of *LP* treatments at 21 d (Figures 1A and 2A). Meanwhile, the mRNA expressions of *major histocompatibility complex* (***MHC***)*-II, IL-4* in ileum were upregulated ($P < 0.01$) by 2 doses of *LP* treatments. In addition, markedly increased sIgA content ($P < 0.05$) in ileal mucosa was observed in the LP1 group at 21 d. In 42-day-old ducks, the serum levels of C3, β-defense, and ileal expressions of *MHC-II, LYZ, IL-2* were significantly higher ($P < 0.05$) in two doses of *LP* groups (Figures 1B and 2B). Moreover, the serum level of IgG and ileal expressions of *IL-1β, IL-4* were significantly elevated ($P < 0.05$) in the LP1 group. LP2 treatment significantly upregulated the ileal expressions of *IL-6, IL-10* compared to the control group.

To further delineate the effect of *LP* on intestinal physical barrier function, the expressions of tight junction (**TJ**) protein-related genes were examined (Figure 2C). The results showed that, compared with the control group, the mRNA expressions of *occludin* and *claudin-3* in ileum were upregulated ($P < 0.01$) by 2 doses *LP* treatments at 21 d, and no effects

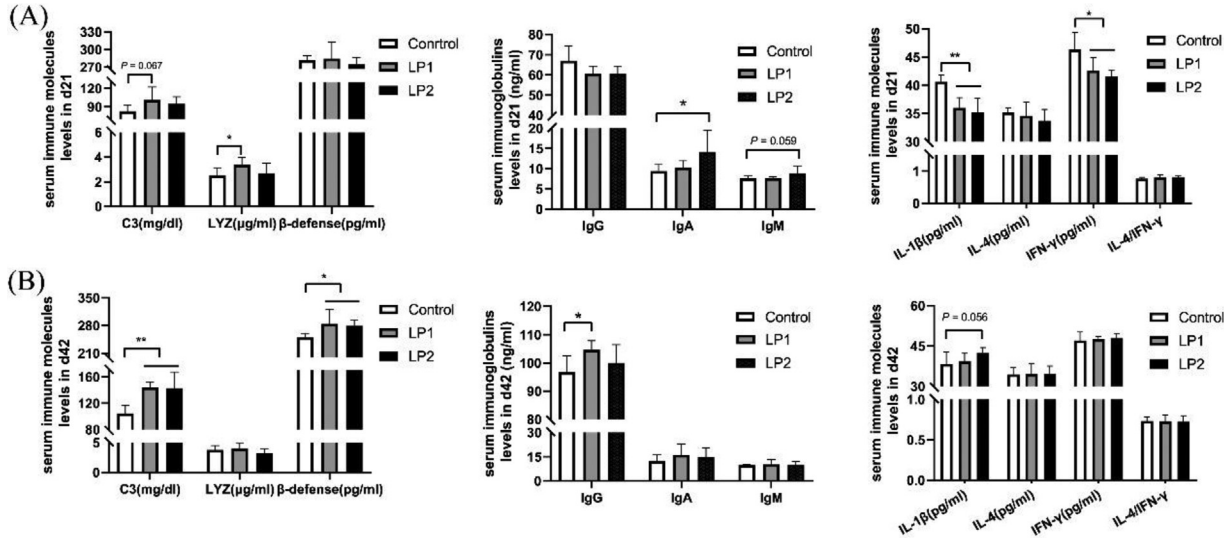

Figure 1. Effects of *LP* on immune parameters of Pekin ducks. The serum levels of immune parameters were determined at 21 d (A), 42 d (B), respectively. Results are means ± SD (n = 8/group). *: $P < 0.05$; **: $P < 0.01$.

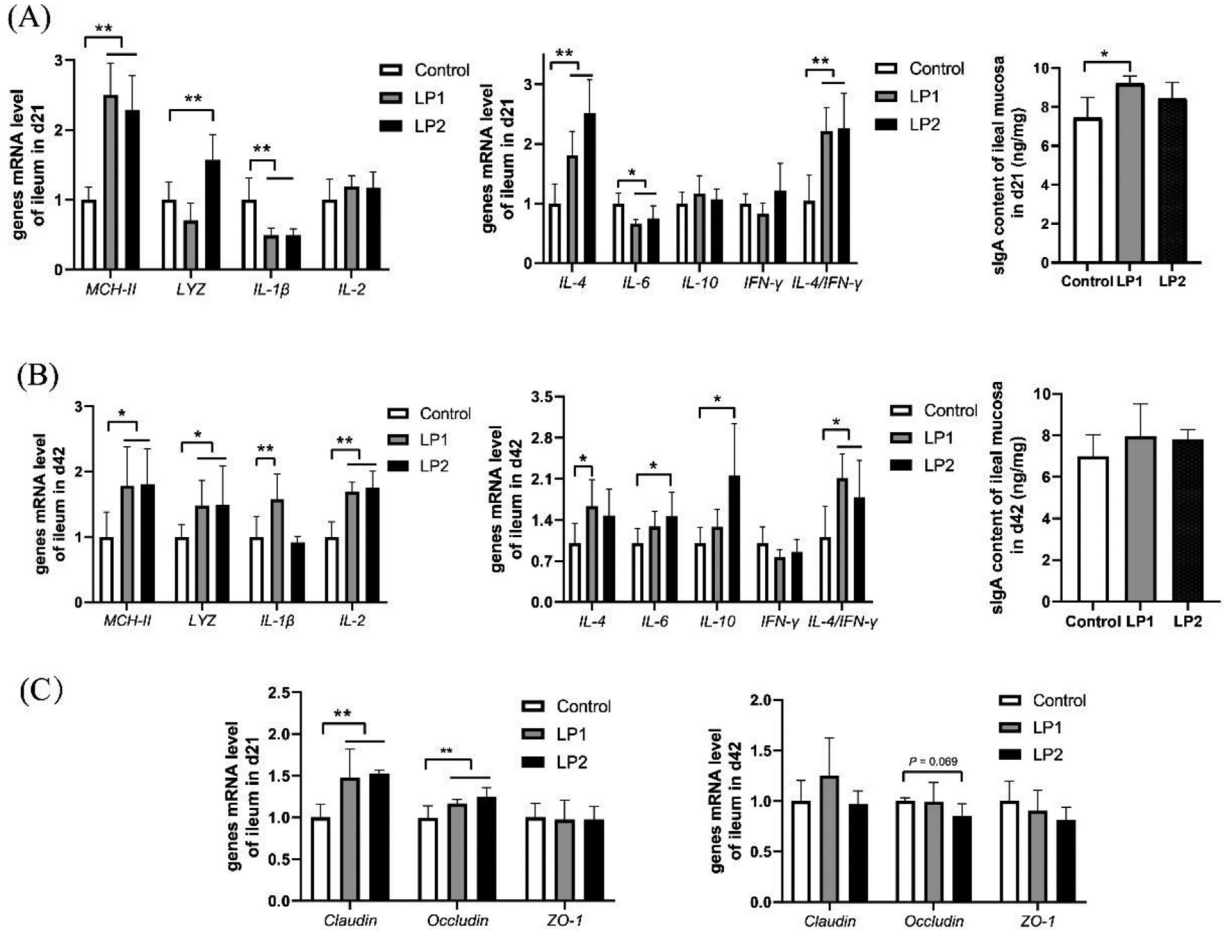

Figure 2. Effects of *LP* on immune and physical barriers in ileum of Pekin ducks. The ileal mRNA levels of immune parameters and sIgA content in ileal mucosa were measured at 21 d (A), 42 d (B), respectively. (C) The ileal expressions of TJ-related genes. Results are means ± SD (n = 8/group). *: $P < 0.05$; **: $P < 0.01$.

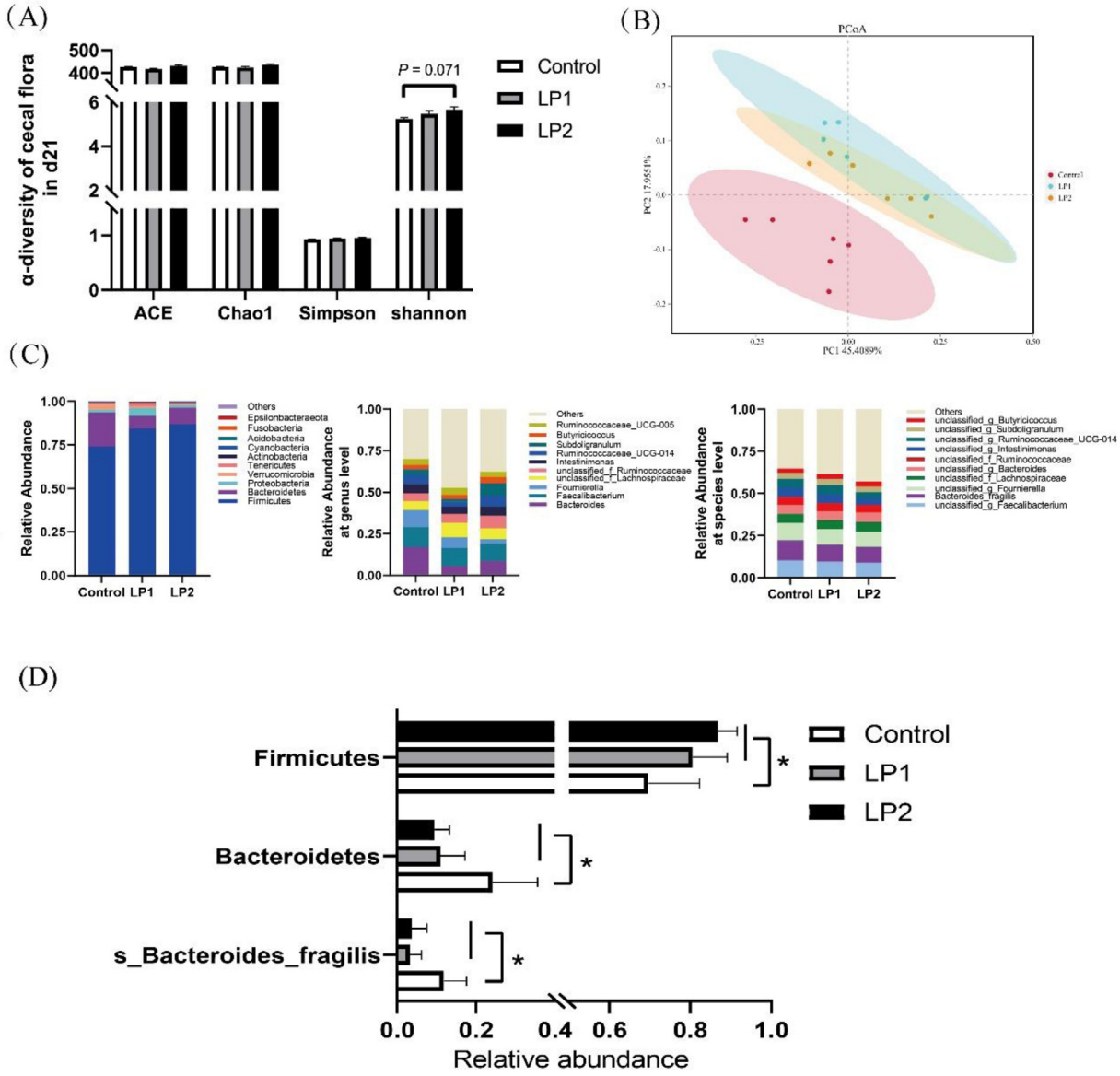

Figure 3. Effects of *LP* on density and composition of cecal flora of Pekin ducks at 21 d. (A) α-density. (B) PCoA of cecal flora. (C) the relative abundance of microbial composition at phylum, genus, and species level. (D) bacteria with significant difference among different groups, s_ means species level. Results are means ± SD (n = 6/group). *: $P < 0.05$.

were found at 42 d. Collectively, *LP* could facilitate systemic immunity and intestinal barrier function of Pekin ducks.

Cecal Microbiota

As a biological barrier of intestine, gut microbiota plays essential roles in maintaining intestinal homeostasis and nutrient utilization. The results revealed that there was no significant difference ($P > 0.05$) in the α-diversity of the cecal microbiota among three groups at 21 d (Figure 3A). By contrast, PCoA displayed the different clusters of microbial communities between the *LP* treatment groups and control group (Figure 3B), which was further demonstrated by ANOSIM and PERMANOVA (Table 5) Figure 3C displayed the microbial composition in different groups at 21 d. Specifically, at the phylum level, 2 doses of *LP* supplementation significantly increased ($P < 0.05$) the relative abundance of *Firmicutes* and decreased ($P < 0.05$) *Bacteroidetes* (Figure 3D). At the species level, the relative abundance of *Bacteroides fragilis* was significantly diminished ($P < 0.05$) in *LP* groups. At 42 d, the LP1 and LP2 groups had significantly higher ($P < 0.05$) Shannon diversity index values than the control group (Figure 4A). Besides, LP1 tended to improve Simpson index ($P = 0.059$), and PCoA showed significant differences in cecal flora structure between the LP1 and control treatments (Figure 4B). The microbial composition in different groups at 42 d was depicted in Figure 4C. Moreover, the LP1 group exhibited significant ($P < 0.05$) increases in the relative abundance of butyrate-producing bacteria, such as *unclassified_f_Lachnospiraceae, Ruminococcaceae_UCG-005* and *Intestinimonas* (Figure 4D). Thus, optimum dose of *LP* supplementation could enhance α-diversity and the relative abundances of beneficial microorganisms.

Table 5. ANOSIM and PERMANOVA analysis of β-diversity between control and LP treatments[1].

	ANOSIM		PERMANOVA	
Group[2]	*R*-value	*P*-value	R^2-value	*P*-value
21 d				
Control-LP1	0.413	0.005	0.304	0.003
Control-LP2	0.446	0.005	0.319	0.004
42 d				
Control-LP1	0.389	0.001	0.261	0.001
Control-LP2	−0.041	0.738	0.094	0.427

[1]n = 6/group.
[2]Control group (fed with the corn-soybean basal diet), LP1(basic diet supplemented with 400 mg/kg *LP*), LP2 (basic diet supplemented with 800 mg/kg *LP*).

Correlation Analysis

Pearson correlation analysis was employed to assess the interactions between cecal microbiota with growth performance and serum immune parameters. It appeared that *Firmicutes* had a significant positive correlation with ADG as well as significant negative correlations with F/G and serum IL-1β at 21 d, which was completely opposite to the effects of *Bacteroidetes* (Figure 5A). Likewise, positive correlations were found between *Bacteroides fragilis* and serum pro-inflammatory cytokines (IL-1β, IFN-γ). At 42 d of age, *unclassified_f_Lachnospiraceae* was positively correlated with serum level of IgG, and *Ruminococcaceae_UCG-005* was positively correlated with serum IL-4 (Figure 5B). Results validated that *LP* probably relied on the

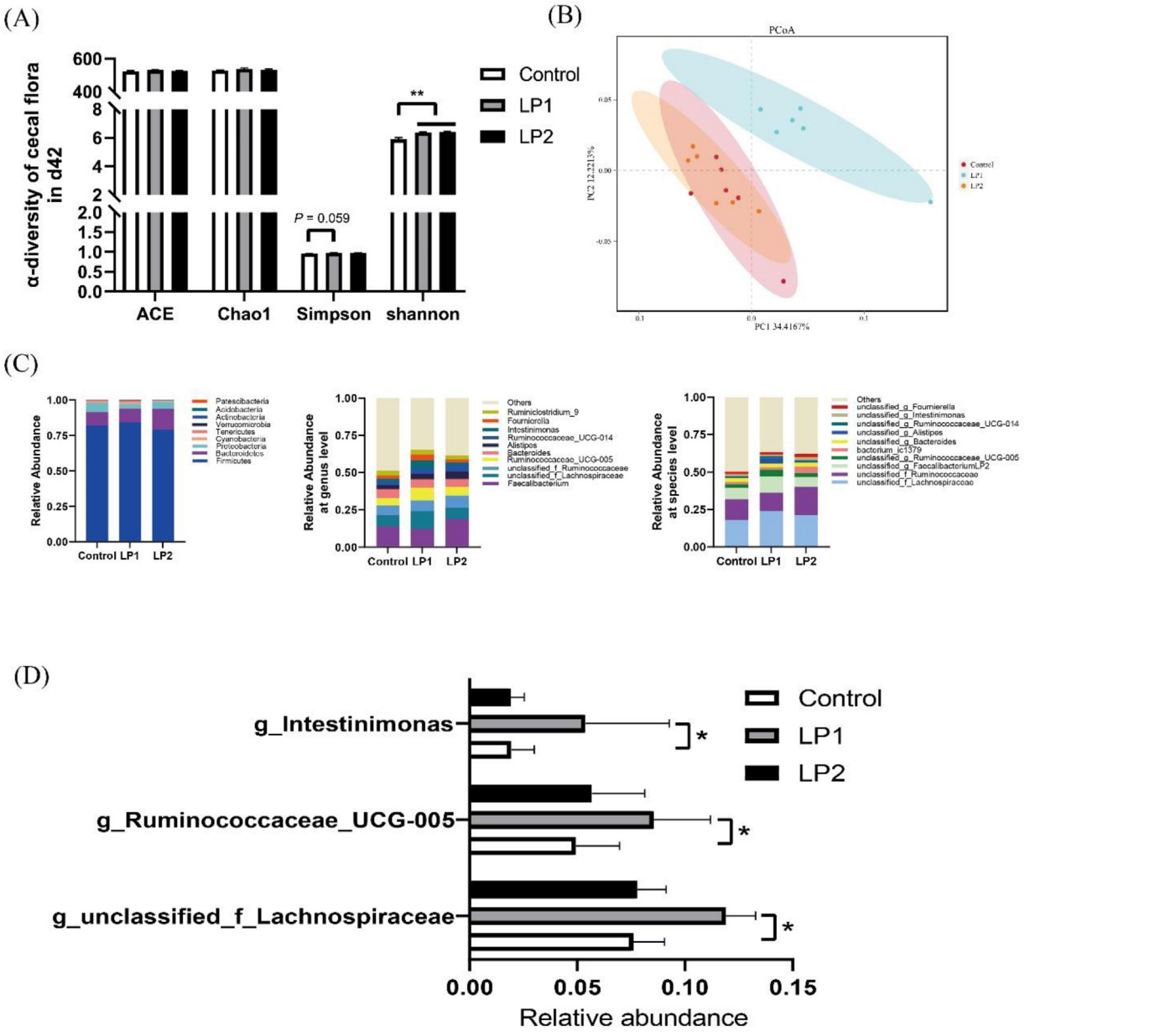

Figure 4. Effects of *LP* on density and composition of cecal flora of Pekin ducks at 42 d. (A) α-density. (B) PCoA of cecal flora. (C) the relative abundance of microbial composition at phylum, genus, and species level. (D) bacteria with significant difference among different groups, g_ means genus level. Results are means ± SD (n = 6/group). *: $P < 0.05$; **: $P < 0.01$.

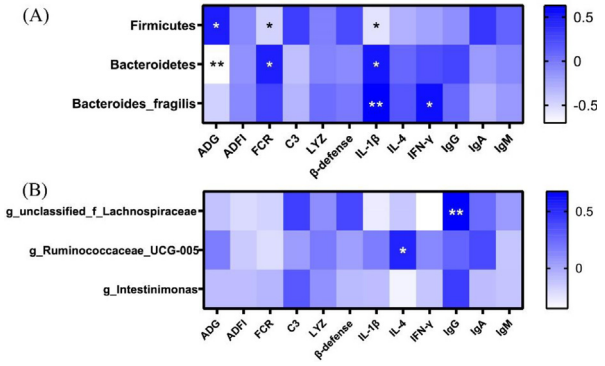

Figure 5. Pearson correlation analysis between cecal microbiota with growth performance and serum immune parameters. Correlation analysis at 21 d (A) and 42 d (B), respectively. Blue means positive correlations, while white means negative correlations. *: $P < 0.05$; **: $P < 0.01$.

changes of microbiota to improve growth performance and immune function.

DISCUSSION

Intestinal health is essential for the health status of animals and the efficient utilization of dietary nutrients (Ducatelle et al., 2018). Immunity, TJ proteins and microbiota of intestine must hold tight to ensure a healthy gut (Castoldi et al., 2015). Numerous studies have proved that dietary administration with probiotics confers a good health benefit and promotes growth performance of poultry by improving its intestinal health (Yadav and Jha, 2019; Park et al., 2020; Wang et al., 2021). The present study showed that the dietary 400 and 800 mg/kg *LP* improved growth performance as indicated by the decreased F/G of ducks. Similarly, previous studies have reported that supplementation with *LP* enhanced growth performance of chicken (Peng et al., 2016; Trabelsi et al., 2016). On the contrary, Wang et al. (2019b) and Deepthi et al. (2017) confirmed that administered with *LP* (10^9 CFU/mL, laboratory-isolated) daily by oral gavage does not affect growth performance of broilers Vineetha et al. (2017). presented that dietary supplementation with *LP* LGFCP4 isolate (10^8 CFU/g, NCBI Accession no: KM199683) does not affect growth performance of broilers, either. The variable results might be due to differences in the adding methods, probiotic strains, experimental environment, and animal models. Besides, our investigation also indicated that 400 mg/kg *LP* stimulated the development of immune organs such as thymus and bursa of fabricius of Pekin ducks. Similarly, previous studies have shown that *LP* slightly increases thymus index in egg-laying chickens infected with *Clostridium perfringens* (Xu et al., 2020), and enhances thymus and bursa of fabricius indices of chicks (Duan et al., 2021). The ameliorated growth performance and organ indices of ducks in the present study may have relation with the beneficial changes in immunity, TJ proteins, and microbiota, which are categorized as intestinal health.

C3, β-defense, and LYZ are essential innate immune molecules. Complement is a cardinal effector that facilitates the MHC class II expression of antigen-presenting cells (Sandor et al., 2009). β-defense and LYZ exhibit broad-spectrum antimicrobial activities, that function as a first line of defense (Cuperus et al., 2013). In the current study, two doses of *LP* administration increased the serum C3, β-defense and expressions of *LYZ, MHC-II* in ileum, and the evaluated serum LYZ was only observed in 400 mg/kg *LP* group. These results indicated that *LP* may have a positive effect on the innate immune function of Pekin ducks to against invading pathogens. Mizuno et al. (2020) confirmed the role of lipoteichoic acid from *LP* in amelioration of the innate immune function triggered by TLR3 activation in mice. Nonetheless, further research is needed to elucidate potential underlying mechanisms in poultry.

Cytokines are important markers of cellular immune response. Th1 cells produce IL-1β, IL-2, and IFN-γ, which promote inflammation and cytophagocytosis (Sadeyen et al., 2004; Olivares-Zavaleta et al., 2011; Wu et al., 2013). The cytokines IL-4, IL-10 and transforming growth factor (**TGF**)-β are produced by Th2 cells, which assist antibody synthesis and inhibit inflammation. Our data confirmed that dietary supplementation with 2 doses of *LP* diminished Th1 cytokines (serum levels of IL-1β, IFN-γ and ileal expressions of *IL-1β, IL-6*), whereas upregulated ileal expression of Th2 cytokine *IL-4* at 21 d, which suggested that *LP* induced Th0 cells to differentiate into Th2 cells at the starter phase of the ducks. At the end of the trail, *LP* induced expressions of both Th1 and Th2 cytokines, which indicated that *LP* stimulated both Th1 and Th2 responses at the later stage of the ducks. Similarly, Wang et al. (2015) presented that *LP* generates both Th1- and Th2-cytokines of chickens. Wu et al. (2019) showed a significant elevation of ileal mucosa *IL-10, IL-6, IFN-γ* gene expressions in broilers. Co-expression of pro-inflammatory and anti-inflammatory cytokines maintains immune homeostasis in the body (O'Garra and Vieira, 2007). Th1 cells are characterized by IFN-γ production, whereas Th2 cells produce IL-4, and hence IFN-γ/IL-4 ratio is widely used to evaluate immune homeostasis (Koarada et al., 2002). The present study found that the ileal expression of *IFN-γ/ IL-4* ratio in *LP* treated ducks was significantly reduced compared to controls, confirming that *LP* tilted the balance toward anti-inflammation in ileal. Nevertheless, there was no difference in serum IFN-γ/ IL-4 ratio among 3 groups, which illuminated that *LP* maintained the whole-body immune homeostasis. Meanwhile, ileal content of sIgA and serum concentration of IgG were increased in the 400 mg/kg *LP* group, which was consistent with the promotion of Th2 differentiation in ileal by *LP* supplementation. As the largest immune organ, intestine acts as a vital site for initiating immune response (Xiao et al., 2019). Appropriate immunomodulatory efficacy contributes to defense against invasive pathogens, yet excessive activation of the gut immune system incurs competition for nutrition (Liao and

Nyachoti, 2017). Collectively, our data indicated that *LP* could improve systemic immunity and intestinal immune barrier function. Concurrently, *LP* may possess anti-inflammatory potential in the gut of healthy ducks to avoid losses in the efficiency of digestive function.

TJs are components of intracellular adhesion complexes that have a critical role in the maintenance of barrier integrity, including claudin-3, occludin, and ZO-1, which seal the paracellular space between the cells and tightly prevent the transportation of toxic substances from entering the systemic circulation (Chelakkot et al., 2018; Zeisel et al., 2019). We found that mRNA expression levels of *claudin-3, occludin* in ileum were upregulated by *LP* treatments, which is similar to the previous studies showing that supplementation with *LP* increases mRNA level of *claudin* (Xu et al., 2020), and exhibits longer TJ and adherens junction in ileum of broilers (Wang et al., 2021). Consequently, the optimum dose of *LP* could improve intestinal physical barrier function of Pekin ducks.

A species-rich gut ecosystem is more vigorous against environmental influences. Accordingly, diversity seems to be a capable metric of a "healthy gut" (Valdes et al., 2018). In the experiments described here, ducks fed 400 mg/kg and 800 mg/kg *LP* inclusion diets showed increased Shannon index, which indicated that *LP* may increase the α-diversity of cecal microbiota. Gut microbes play an integral role in host feed utilization efficiency and maintenance of the intestinal homeostasis (Pan and Yu, 2014). Dietary *LP* could modulate the abundance of beneficial bacteria and potential pathogens in the gut of broilers (Shen et al., 2014; Wu et al., 2018; Duan et al., 2021). Our results revealed that supplementation of 400, 800 mg/kg *LP* increased the relative abundance of *Firmicutes* and decreased *Bacteroidetes*, which have a positive effect in facilitating growth performance and negative impact in serum proinflammatory factor IL-1β. Similarly, Guo et al. (2021) explained that the increase of *Firmicutes* population could restore intestinal homeostasis of *Clostridium perfringens* infected chickens, which was positively correlated with body weight gain and improvement of immune function. Research in both mice and humans has reported that increased fecal *Firmicutes* level is associated with improvement of energy efficiency (Turnbaugh et al., 2006; Jumpertz et al., 2011). Carbohydrate fermentation by *Firmicutes* results in the production of a pool of short-chain fatty acids that act as both immunomodulators and energy sources. By contrast, the increased fecal *Bacteroidetes* content linked to the reduced nutrient absorption. *Firmicutes*/*Bacteroidetes* (**F**/**B**) ratio plays a critical role in the optimum physiological and nutritional status of the host (Oakley et al., 2014; Grond et al., 2018). The present study appeared that *LP* may improve growth performance and immune function by regulating the relative abundance of *Firmicutes* and *Bacteroidetes*.

The present study also found that the relative abundance of butyrate-producing bacteria, such as *unclassified_f_Lachnospiraceae, Ruminococcaceae_UCG-005*, and *Intestinimonas* were increased in the 400 mg/kg *LP* group, which were positively correlated with the serum levels of IgG and IL-4. As a member of the short-chain fatty acids, butyrate prevents proliferation of the pathogenic microorganisms and exerts anti-inflammatory activities (Leeson et al., 2005). Interestingly, both doses of *LP* treatments decreased the relative abundance of *Bacteroides fragilis*, an intestinal commensal that can result in bacteremia and disseminated abscesses when it leaks into the bloodstream or surrounding tissue (Wexler, 2007). Consistently, PCoA plot also showed separation of bacterial communities between *LP* group and control group. *LP, unclassified_f_Lachnospiraceae, Ruminococcaceae_UCG-005* and *Intestinimonas* belong to *Firmicutes*, which could produce lactic acid and butyric acid to reduce intestinal pH so that inhibit the growth of Gram-negative bacteria. *Bacteroides* and *Bacteroides fragilis* belong to Gram-negative bacteria. The supplementation of *LP* led to the increase of the relative abundance of *Firmicutes* and butyric acid producing bacteria and the decrease of *Bacteroides* and *Bacteroides fragilis*. Interestingly, antagonism has been identified to improve rather than reduce microbial diversity (Garcia-Bayona and Comstock, 2018), which might be the reason for the increased α-diversity in the *LP* groups. To sum up, dietary *LP* may enhance the feed efficiency and secretion of immune molecules by reshaping the microbiota of Pekin ducks.

The molecular mechanism of immunologic modulation and other relevant "omics" associated with gut microbiota warrant further study. Future research will focus on the mechanisms by the protective effect of *LP* on Pekin ducks with disease.

CONCLUSIONS

Our study demonstrated that supplementation of *LP* has great potential for improvement of growth performance and intestinal health of Pekin ducks by improving host immunity and intestinal barriers and increasing the relative abundance of beneficial bacteria. Based on the aforementioned results and cost considerations, the recommended dose of *LP* in Pekin ducks is 400 mg/kg.

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Author contributions: Keying An and Zhaofei Xia: Conceptualization. Keying An and Wenwen Gao: Conducted the animal experiments. Keying An: Data Curation, Writing. Peng Li Assisted with the experiments and manuscript preparation. Lei Li: Resources.

DISCLOSURES

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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