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Beneficial effects of *Lactobacillus plantarum* on growth performance, immune status, antioxidant function and intestinal microbiota in broilers

Xiao Xiao ,[*,†,‡,§,‖] Tiantian Cui,[*,†] Songke Qin,[*,†] Tao Wang,[*,†] Jinsong Liu,[‖] Lihan Sa,[*,†] Yanping Wu,[*,†,‡,§,‖] Yifan Zhong,[*,†,‡,§] and Caimei Yang [*,†,‡,§,1]

[*]*Key Laboratory of Applied Technology on Green-Eco-Healthy Animal Husbandry of Zhejiang Province, College of Animal Science and Technology, College of Veterinary Medicine, Zhejiang A&F University, 311300, Hangzhou, China;* [†]*Zhejiang Provincial Engineering Laboratory for Animal Health and Internet Technology, College of Animal Science and Technology, College of Veterinary Medicine, Zhejiang A&F University, 311300, Hangzhou, China;* [‡]*Zhejiang International Science and Technology Cooperation Base for Veterinary Medicine and Health Management, College of Animal Science and Technology, College of Veterinary Medicine, Zhejiang A&F University, 311300, Hangzhou, China;* [§]*China-Australia Joint Laboratory for Animal Health Big Data Analytics, College of Animal Science and Technology, College of Veterinary Medicine, Zhejiang A&F University, 311300, Hangzhou, China; and* [‖]*Zhejiang Vegamax Biotechnology Co. Ltd., Anji, 313300, Huzhou, China*

ABSTRACT *Lactobacillus plantarum* (***L. plantarum***) has been globally regarded as antibiotic alternative in animal farming in the past few years. However, the potential function of *L. plantarum* in broilers has not been systemically explored. In this study, a total of 560 one-day-old yellow-feathered broilers were randomly divided into 3 groups, fed with basal diet and drank with *L. plantarum* HJZW08 (**LP**) at the concentration of 0 (**CON**), 1000 × 10^5 (**LP1000**), and 2000 × 10^5 CFU/L (**LP2000**) for 70 d. Results showed that the body weight (**BW**), average daily gain (**ADG**), average daily feed intake (**ADFI**), immunoglobulin A (**IgA**), **IgY**, and anti-inflammatory interleukin 10 (**IL-10**) were markedly improved ($P < 0.05$), while the levels of pro-inflammatory **IL-2, IL-1β, IL-6**, and tumor necrosis factor-α (**TNF-α**) in serum were decreased ($P < 0.05$) in the LP2000 group comparing with the CON group. Besides, LP treatment groups prominently increased the levels and activities of antioxidant enzymes and decreased the content of malondialdehyde (**MDA**). Additionally, the levels of isobutyric acid in the LP1000 and LP2000 groups and isovaleric acid in the LP2000 group were significantly improved. More importantly, the α-diversity and microbial structure of intestinal microbiota were pronounced altered by LP supplementation. The results showed that only the relative abundance of Actinobacteriota was significantly increased in the LP2000 group, while 6 kinds of bacteria on genus level were significantly changed. For further validation, linear discriminant analysis with effect size (**LEfSe**) plots revealed that 8 amplicon sequence variants (**ASVs**) were predominant in the CON group, while *Bacteroides* and other beneficial species such as *Lactimicrobium massiliense* (ASV4 and ASV36), *Intestinimonas butyriciproducens* (ASV71), and *Barnesiella viscericola* (ASV152 and ASV571) were enriched in the LP groups. Taken together, dietary supplementation with LP obviously enhanced the immune status, antioxidant capacity, and stabilized the cecal microbiota and SCFAs, contributing to the improvement of growth performance of broilers. Our study laid good foundation for the application of probiotic *Lactobacillus* in animal industry in the future.

Key words: *Lactobacillus plantarum*, broiler, growth performance, immune response, intestinal microbiota

2024 Poultry Science 103:104280
https://doi.org/10.1016/j.psj.2024.104280

INTRODUCTION

Over the past decades, antibiotics have been globally used for their beneficial effects on promoting growth performance, maintaining intestinal health and treating various diseases in almost all types of livestock, including poultry (Hutchings, et al., 2019; Roth, et al., 2019). However, the long-term and irrational use of antibiotics led to animal-derived products safety risks, severe environmental pollution, and the occurrence of antibiotic-resistant bacteria (Aghamohammad and Rohani, 2023; Hutchings, Truman and Wilkinson, 2019), which finally impacted human health and wellbeing. Thus, the prohibition of antibiotics has been conducted all over the

Received June 18, 2024.
Accepted August 26, 2024.

[1]Corresponding author: yangcaimei2012@163.com

world, and there is an urgent need to find potential alternatives for antibiotic (Allen, et al., 2014). Nowadays, many functional substitutes, such as probiotics, prebiotics, antimicrobial peptides, active enzymes, plant-derived essential oils, and polysaccharides, have been paid more and more attention due to their regulatory effect on intestinal integrity, barrier functions, and microbial homeostasis (Abd El-Hack, et al., 2022; Irawan, et al., 2021; Ningsih, et al., 2023; Putra, et al., 2024; Qin, et al., 2023; Yang, et al., 2019). Nonetheless, how to search suitable, effective and safe alternatives for antibiotics and the evaluation of the efficiency of these alternatives in animals still need further investigation.

Among the potential alternatives already mentioned, probiotics attracted much interest for their colonization of the gut, modulation of the intestinal flora, reported high safety and efficacy in the animal field (Neveling and Dicks, 2021; Ningsih, et al., 2023; Sanders, et al., 2019). Probiotics, first identified in 1905, have been defined by Food and Agricultural Organization (**FAO**) and World Health Organization (**WTO**) in 2014 as live microorganisms able to have beneficial effects on the host when administered in sufficient amounts (Fu, et al., 2021; Hill, et al., 2014). Typical probiotics, including *Lactobacillus, Bifidobacterium, Bacillus* spp., *Roseburia* spp., *Akkermansia* spp., *Propionibacterium* spp., and *Faecalibacterium* spp., have been shown to play regulatory roles in promoting growth performance, producing secondary metabolites, improving barrier functions, and maintaining intestinal health in livestock (Cani, et al., 2022; De Filippis, et al., 2020; Hu et al., 2023; Ningsih, et al., 2023; Qin, et al., 2023; Sanders, et al., 2019). Thus, target probiotics can be efficient substitutes for animal health and growth.

Lactobacillus plantarum (***L. plantarum***), widely used in foods for decades, has been also approved as feed additives in animals including piglets and broilers (Dell'Anno et al., 2021; Yin, et al., 2023). Previous studies have reported that *Lactobacillus* spp. can produce lactic and acetic acid as primary end-products of carbohydrate metabolism, as well as diverse and potent bacteriocins which can inhibit pathogens growth and toxins production (Sanders, et al., 2019; Seddik, et al., 2017). Acetic acid, as one type of short chain fatty acids (**SCFA**), was reported to induce ulcerative colitis in rats (Ghasemi-Pirbaluti, et al., 2017; Shahid, et al., 2022). Recently, literatures have showed that acetic acid beneficially affected host energy and substrate metabolism to control body weight (**BW**) (Hernández, et al., 2019), and protected against natural aging-related disorders in mice (Ma, et al., 2023). Moreover, accumulating evidence has revealed that different *L. plantarum* strains (live or heat-killed) alone or combined with other probiotics successfully improved the growth performance, protected barrier function, ameliorated intestinal and hepatic injury, and kept microbial balance in piglets and broilers (Deepthi, et al., 2017; Dell'Anno et al., 2021; Wang, et al., 2021; Wang, et al., 2019; Yin,et al., 2023). However, the precise mechanism and reported effect of different *L. plantarum* strains are variable.

In the current study, *L. plantarum* HJZW08 (**LP**) was isolated from cecal contents of healthy piglets by our group and used for further investigation. Our previous data have showed that LP-derived postbiotics effectively suppressed intestinal inflammation and microbial dysbiosis in *Salmonella*-infected mice (Hu et al., 2023; Wu, et al., 2023; Wu, et al., 2022). However, the effect of active LP in broilers remained unknown. Therefore, the present study was carried out to explore the regulatory effect of dietary supplementation with LP on growth performance, immune status, antioxidant capacity, and microbial homeostasis of broilers, which could provide good theoretical foundations for the application of *L. plantarum* in broiler feed in the future.

MATERIALS AND METHODS

Ethic Approval

To ensure the welfare of the animals, all experiments and animal procedures were conducted strictly according to the principles recommended by the Ethics Committee of Zhejiang Agricultural and Forestry University (Hangzhou, China, ZAFUAC2023041). All the experiments and methods were designed with the aim of minimizing animal suffering.

L. plantarum *HJZW08 Isolation and Preparation*

LP was isolated by selective deMan Rogosa Sharpe (**MRS**) medium from cecal contents of healthy piglets by Zhejiang Vegamax Biotechnology Co., Ltd. (Huzhou, China) and has been deposited in the China General Microbiological Culture Collection Center (**CGMCC**) and the deposition number is CGMCC No.23777. The bacterium stored at −80°C was recovered in MRS medium overnight at 37°C, and then expanded at 1:50 for 48 h to obtain the bacterial solution. The number of LP was measured by spread plate method at OD600 = 1.650, which indicated that the concentration of LP was 5 × 10^8 CFU/mL. The bacterium was centrifuged and the concentration was adjusted to 1 × 10^6 CFU/mL.

Experiment Design and Management

A total of 560 1-day-old yellow-feathered broilers (local commercial company) were randomly divided into 3 groups, 8 to ten replicates each group with twenty birds per replicate. The whole experiment lasted for 70 d. The drinking water was added with 0, 1000, and 2000 mL of LP (1 × 10^6 CFU/mL), and the final volume was set as 10 L. Then the drinking water containing LP was further drank by the broilers in the CON (0 CFU/L), LP1000 (1000 × 10^5 CFU/L), and LP2000 (2000 × 10^5 CFU/L), individually. All broilers were fed the basal diet. The basal diet was formulated to meet the nutritional requirements of broilers described

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

Item	Day 1−35	Day 36−70
Ingredients, %		
Corn	53.20	61.20
Soybean meal	25.10	14.10
Extruded soybean	5.00	5.00
DDGS	5.00	8.00
Soybean oil	2.30	2.00
Fermented soybean meal	2.50	0.00
Corn protein meal	2.00	3.50
Wheat middling	0.00	2.00
Limestone	1.20	1.30
$CaHPO_4$	1.70	0.90
Premix[1,2]	2.00	2.00
Total	100.00	100.00
Nutrient composition[2]		
Digestible energy, MJ/kg	12.43	12.63
Crude protein	21.03	17.53
Lysine	1.26	0.95
Methionine	0.55	0.44
Methionine + Cysteine	0.90	0.76
Threonine	0.82	0.65
Tryptophan	0.22	0.16
Ca	0.94	0.76
P	0.73	0.29

[1]Provided kilogram of diet: Vitamin A (retinyl acetate), 1,500 IU; cholecalciferol, 200 IU; vitamin E (DL-α-tocopheryl acetate), 10 IU; riboflavin, 3.5 mg; pantothenic acid, 10 mg; niacin, 30 mg; cobalamin, 10 μg; choline chloride, 1,000 mg; biotin, 0.15 mg; folic acid, 0.5 mg; thiamine, 1.5 mg; pyridoxine, 3.0 mg; Fe, 80 mg; Zn, 40 mg; Mn, 60 mg; I, 0.18 mg; Cu, 8 mg; Se, 0.15 mg; Lysine, 3,000 mg; Methionine, 2,000 mg; Threonine, 1,000 mg.

[2]Values of digestible energy were calculated from data provided by Feed Database in China (2020). Crude protein and amino acids were measured values.

by the NRC (1994), and the ingredient, chemical compositions and nutritional level used in this study were shown in Table 1. All birds were raised in cages and provided free access to feed and water. The temperature was set at 33°C at the age of 1 to 7 d and then reduced by 3°C per week to a final temperature of around 24°C. The humidity was set 60 to 65% at the age of 1 to 7 d and then 50 to 60%.

Growth Performance

In the 70 d feeding trial, BW and feed intake were measured on d 1, 35, and 70 for each replicate. The average daily gain (**ADG**), average daily feed intake (**ADFI**) and feed conversion ratio (**FCR**) were recorded per replicate for each growth phase.

Sample Collection

Samples were collected on the d 70. All birds were humanly fasted for 12 h before sampling, among which 1 broiler per replicate in 3 groups were selected randomly. Blood samples were collected using a coagulation-promoting tube, and placed at room temperature for 4 h. After that, the serum samples were obtained by centrifuging at 3,000 × *g* for 15 min, and stored at −20°C for further analysis. Then, birds were killed and rapidly dissected, the cecal contents were collected into an aseptic cryopreservation tube, immediately frozen in liquid nitrogen, and stored at −80°C for the measurement of SCFAs and intestinal microbiota.

Measurement of Immune Parameters in Serum

Several types of cytokines, including interleukin-1β (**IL-1β**; CAS: ANG-E32218C), **IL-2** (CAS: ANG-E32014C), **IL-6** (CAS: ANG-E32013C), **IL-10** (CAS: ANG-E32011C) and tumor necrosis factor-α (**TNF-α**; CAS: ANG-E32030C), and immunoglobulins (**Ig**), including **IgA** (CAS: ANG-E32004C), **IgM** (CAS: ANG-E32005C) and **IgY** (CAS: ANG-E32209C), were quantified by commercially available enzyme-linked immunosorbent assay (**ELISA**) kits (Angle Gene Bioengineering Institute, Nanjing, China) according to manufacturer's procedures.

Determination of Antioxidant Indicators in Serum

The total antioxidant capacity (**T-AOC**; A015-2-1), the activities of glutathione peroxidase (**GPX**; A005-1-2), superoxide dismutase (**SOD**; A001-3-2) and catalase (**CAT**; A007-1-1), the content of malondialdehyde (**MDA**; A003-1-2) in serum were detected according to the protocols from Angle Gene Bioengineering Institute (Nanjing, China).

Detection of Cecal SCFA

Quantification of cecal SCFAs (including acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, and isovaleric acid) was measured by gas chromatography according to our previous study (Cao, et al., 2022), with slight modifications. In brief, 0.5 g of cecal contents was suspended and thawed in 2 mL of precool sterile water. The suspension was then vortexed and centrifuged at 10,000 × *g* for 10 min at 4°C. After that, 1 mL of supernatant fluid was added and mixed with 25% phosphorous acid (m/v, 1:3) for 30 min in ice-bath. Then, the extracted samples were centrifuged at 10,000 × *g* for 10 min at 4°C, the supernatant fluid was filtered (membranes of 0.22 μm pore size) before analysis. The external standards of SCFAs used in this study were purchased from Sigma-Aldrich (USA). The standards and samples were injected into and run through an Agilent Technologies 7890B GC System and flame ionization. The contents of SCFAs were quantified by using an external standard method with a standard mixture of SCFAs.

Illumina MiSeq High-Throughput Sequencing Technology and Data Analysis

Microbial composition of cecal contents in broilers were analyzed by 16S rRNA sequencing according to

our previous study (Xiao, et al., 2021). Briefly, total genomic DNA of cecal contents were extracted using a DNeasy PowerSoil Kit (QIAGEN Sciences, Inc., MD, USA) and quantified using NanoDrop2000 (Thermo Fisher Scientific, Waltham, USA) according to manufacturer's instructions. DNA purity and concentration were monitored on 1% agarose gels and then diluted. Samples were stored at 4°C during library preparation and at −20°C thereafter for longer storage. The V3-V4 hypervariable regions of the bacterial 16S rRNA genes were amplified with specific primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) resulting in amplicons of approximately ∼ 420 bp. Dual indices and Illumina sequencing adapters were attached to the V3−V4 amplicons. Subsequently, library quantification, normalization, and pooling were performed and loaded the samples for MiSeq sequencing. All reads were used to merge paired-end reads with Flash 1.2.11 and qualify with Fastp 0.19.6. Reads were assembled and chimeras removed as per data2 protocol. Taxonomy was assigned to each amplicon sequence variant (**ASV**) generated by dada2 using PECAN (version 1.0). For downstream analysis, the filtered ASV table was obtained by flatting according to the minimum number of sample sequences on Majorbio Cloud Platform and used for later analysis. Alpha diversity, including Shannon, Simpson, and chao1, were calculated to reflect the bacterial diversity and richness. Beta diversity was evaluated according to the principal component analysis (**PCA**) and the principal coordinate analysis (**PCoA**) based on Bray Curtis metrics. Dissimilarity in community structure between samples was calculated by nonmetric dimensional scaling (**NMDS**). Linear discriminant analysis with effect size (**LEfSe**) was used to identify microbial clades that were significantly changed. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (**PICRUSt**) was applied to obtain functional prediction from the 16S rRNA data. Above analysis was performed on Majorbio Cloud Platform (www.majorbio.com).

Statistical Analysis

The obtained data was represented as Mean ± SEM. GraphPad Prism version 9.0 (San Diego) was used for statistical analysis. One-way analysis of variance (**ANOVA**) and Tukey test were used to determine significant differences. $P < 0.05$ was considered statistically significant.

RESULTS

Growth Performance

The effect of LP on the growth performance of broilers was presented in Table 2. No impact of LP treatments on BW on d 1 and d 35 was recorded ($P > 0.05$), but a significant gain of BW on d 70 in LP2000 group was shown ($P < 0.05$). Compared to the CON group, LP1000 showed no effect on ADG and ADFI across all phases ($P > 0.05$), while LP2000 markedly improved ADG and ADFI from d 35 to d 70 and d 1 to d 70 ($P < 0.05$). Moreover, there were no difference of FCR among the CON and different LP supplementation groups in this study throughout all phases ($P > 0.05$).

Immune Parameters in Serum

The effect of LP on serum immunoglobulins at d 70 was listed in Figure 1A. the levels of IgA and IgY were significantly improved in the LP2000 group ($P < 0.05$), which was unchanged in the LP1000 group compared with the CON group ($P > 0.05$). Besides, both broilers of the LP1000 and LP2000 groups showed no effect on the level of IgM in contrast to the CON group ($P > 0.05$).

Table 2. Effect of LP on the growth performance of broilers.

	Treatments[1]			
Item	CON	LP1000	LP2000	*P*-value
BW, g				
d 1	31.65 ± 0.49	32.06 ± 0.48	33.13 ± 0.46	0.8032
d 35	549.20 ± 3.98	550.80 ± 6.28	559.30 ± 8.62	0.3975
d 70	1507.00 ± 33.71[b]	1561.00 ± 26.22[b]	1616.00 ± 22.25[a]	0.0342
ADG, g/d				
d 1 to 35	14.79 ± 0.12	14.82 ± 0.17	15.04 ± 0.25	0.5989
d 35 to 70	21.85 ± 9.35[b]	25.48 ± 5.37[b]	27.65 ± 5.31[a]	0.0382
d 1 to 70	21.08 ± 0.48[b]	21.84 ± 0.37[b]	22.61 ± 0.32[a]	0.0376
ADFI, g/d/broiler				
d 1 to 35	30.66 ± 0.28	30.43 ± 0.19	30.74 ± 0.41	0.7954
d 35 to 70	85.70 ± 3.17[b]	90.24 ± 2.23[b]	95.93 ± 2.02[a]	0.0310
d 1 to 70	57.42 ± 1.40[b]	59.64 ± 1.07[b]	62.50 ± 0.86[a]	0.0148
FCR				
d 1 to 35	2.08 ± 0.03	2.06 ± 0.02	2.05 ± 0.03	0.7776
d 35 to 70	3.14 ± 0.07	3.14 ± 0.04	3.16 ± 0.03	0.9439
d 1 to 70	2.73 ± 0.04	2.73 ± 0.02	2.76 ± 0.02	0.6500

[a,b]Values in the same row with the different letters were statistically significant ($P < 0.05$).

[1]CON: basal diet; LP1000: basal diet, 1000 U/L *L. plantarum* HJZW08 (LP) in the drinking water; LP2000: basal diet, 2000 U/L LP in the drinking water. (n = 8−10 replicates/treatment).Abbreviations: ADFI, average daily feed intake; ADG, average daily gain; BW, body weight; FCR, feed conversion ratio (feed: gain ratio).Data were shown as Mean ± SEM.

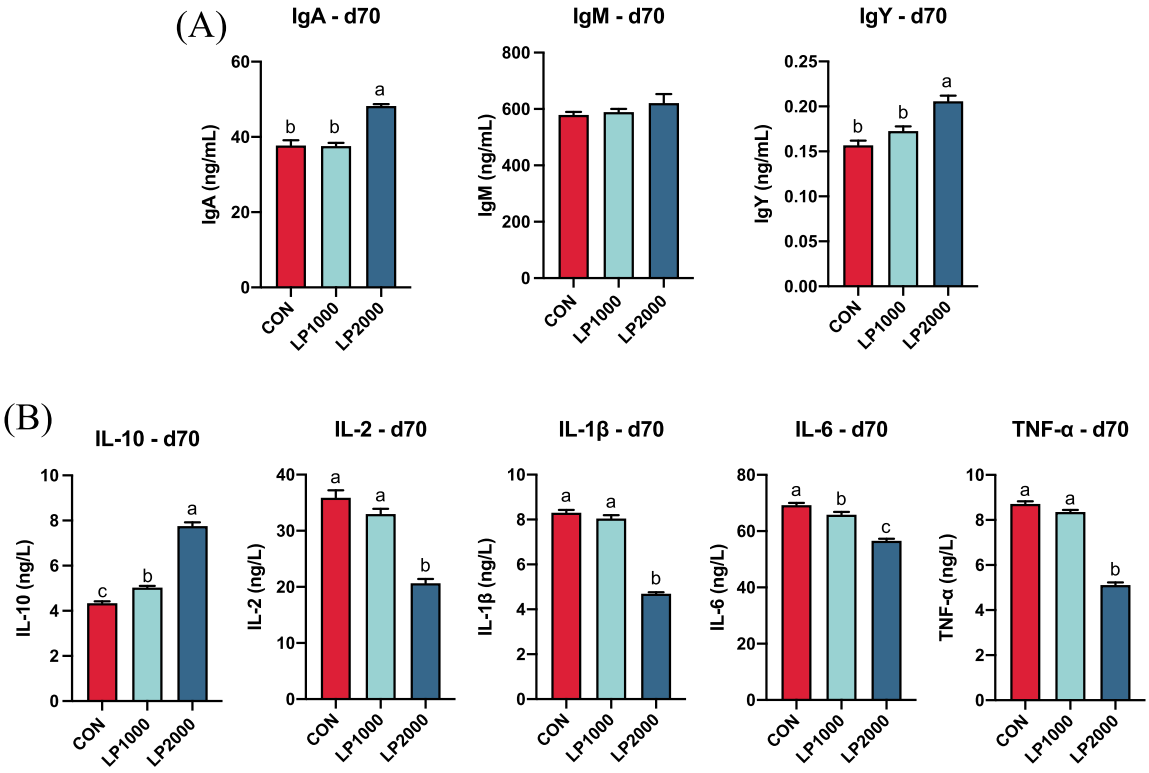

Figure 1. Immune parameters in serum at d 70. (A) The levels of immunoglobulins. (B) The levels of cytokines. a, b different letters between groups indicated statistical significance ($P < 0.05$). CON: basal diet; LP1000: basal diet, 1000 × 10^5 CFU/L *L. plantarum* HJZW08 (LP) in the drinking water; LP2000: basal diet, 2000 × 10^5 CFU/L LP in the drinking water. Ig, immunoglobulin; IL, interleukin; TNF-α, tumor necrosis factor-α.

The effect of LP on serum inflammatory cytokines at d 70 was listed in Figure 1B. Compared with CON group, LP supplementation groups pronouncedly increased the level of anti-inflammatory IL-10 ($P < 0.05$). In addition, broilers in the LP2000 group showed significant decrease of pro-inflammatory IL-2, IL-1β, IL-6, and TNF-α ($P < 0.05$), whereas broilers in the LP1000 group only markedly reduced the level of IL-6 ($P < 0.05$) with no obvious impact on other pro-inflammatory cytokines ($P > 0.05$).

Antioxidant Capacity

The effect of LP on the serum antioxidant activities at d 70 was exhibited in Figure 2. Compared with CON group, the level of T-AOC, the activities of GPX and SOD were significantly increased, whereas the level of MDA was markedly decreased in the LP supplementation groups ($P < 0.05$). Importantly, only LP2000 obviously improved the activity of CAT ($P < 0.05$).

The Level of SCFAs in the Cecal Contents

The effect of LP on SCFAs in the cecal contents was summarized in Figure 3. At the 70 d, LP2000 significantly enhanced the concentrations of isobutyric acid and isovaleric acid ($P < 0.05$), LP1000 markedly improved the level of isobutyric acid ($P < 0.05$), with no obvious change of other metabolites such as acetic acid, propionic acid, butyric acid and valeric acid among all treatments ($P > 0.05$).

Microbial Diversity of Cecal Contents

The 16S rRNA Miseq sequences from the 24 samples (n = 8/group) of broiler cecal microbiota were used from subsequent analysis. According to the results in Figure 4A, LP supplementation showed no impact on the species diversity as indicated by Shannon and Simpson indexes ($P > 0.05$). However, LP2000 significantly improved community richness as indicated by higher Chao1 and Sobs indexes ($P < 0.05$, Figure 4B), which suggested that broilers in the LP2000 group markedly increased α-diversity of cecal microbiota at d 70. According to β-diversity results, principal component analysis (**PCA**) showed a distinction trend among the CON, LP1000, and LP2000 groups ($P = 0.183$, Figure 4C), principal coordinate analysis (**PCoA**) and nonmetric dimensional scaling (**NMDS**) based on Bray Curtis metrics showed a significant separation among 3 groups ($P = 0.015$, Figure 4D; stress = 0.19, $P = 0.015$, Figure 4E), which implied that the structure of intestinal microbiota was pronounced altered by LP supplementation.

Microbial Composition and Differential Species of Cecal Contents

Distribution tables of composition and abundance of samples in each group at the phylum and genera levels were obtained using QIIME software. The analysis results based on phylum level were shown in Figure 5,

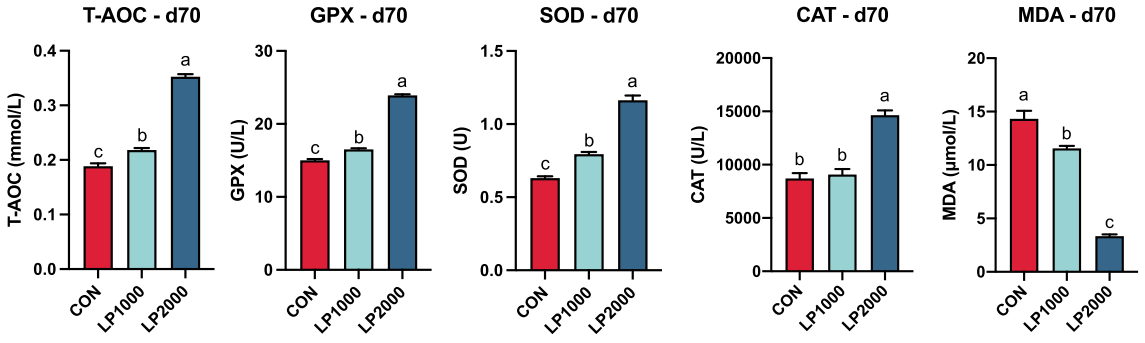

Figure 2. The antioxidant indicators in serum at d 70. a, b different letters between groups indicated statistical significance ($P < 0.05$). CON: basal diet; LP1000: basal diet, 1000 × 10^5 CFU/L *L. plantarum* HJZW08 (LP) in the drinking water; LP2000: basal diet, 2,000 × 10^5 CFU/L LP in the drinking water T-AOC, total antioxidant capacity; GPX, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde.

approximate 40% of Firmicutes and Bacteroidota were the most dominant phyla, followed by Desulfobacterota, Verrucomicrobiota and other bacteria (Figure 5A), among which only the relative abundance of Actinobacteriota was significantly increased in the LP2000 group ($P < 0.05$, Figure 5B). At the genus level, TOP15 genera, including major *Bacteroides, Prevotellaceae UCG-001*, Lachnospiraceae, *Faecalibacterium, Rikenellaceae RC9 gut group*, and other bacteria were shown in Figure 6A. Among the whole genera, 6 kinds of bacteria were significantly changed, of which *Bacteroides* and *Oscillibacter* were markedly downregulated in LP groups, others like *Paludicola, UCG-004, Megasphaera*, and *Olsenella* were dramatically increased compared to CON group ($P < 0.05$, Figure 6B). Furthermore, LEfSe plots based on ASV level showed bars representing the magnitude of abundance of differences among 3 treatment groups (Figure 7). Results indicated that 8 species, including ASV314 (*B. plebeius*), ASV15 and ASV13 (*B. barnesiae*), ASV31 (*Barnesiella intestinihominis*), ASV150 (*Clostridium porci*), ASV69 (*Kineothrix alysoides*), ASV74 (*Coprobacter fastidiosus*) and ASV678

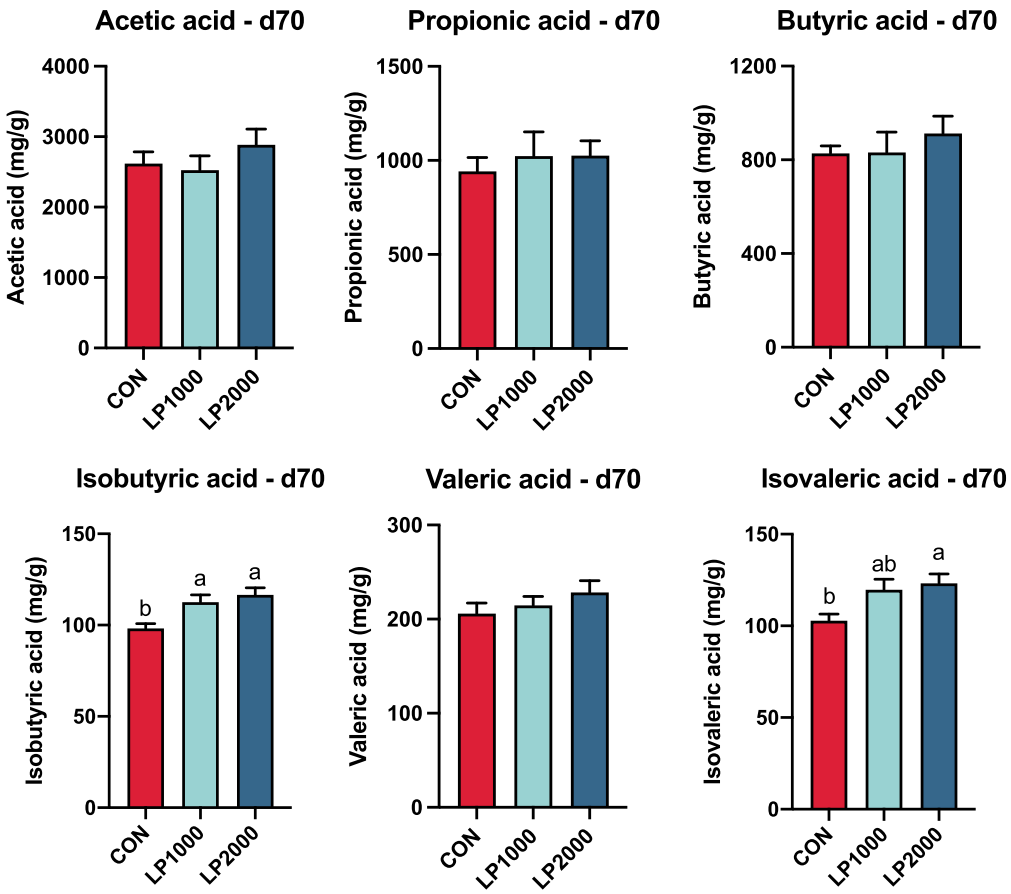

Figure 3. The levels of SCFAs in the cecal contents at d 70. a, b different letters between groups indicated statistical significance ($P < 0.05$). CON: basal diet; LP1000: basal diet, 1000 × 10^5 CFU/L *L. plantarum* HJZW08 (LP) in the drinking water; LP2000: basal diet, 2,000 × 10^5 CFU/L LP in the drinking water.

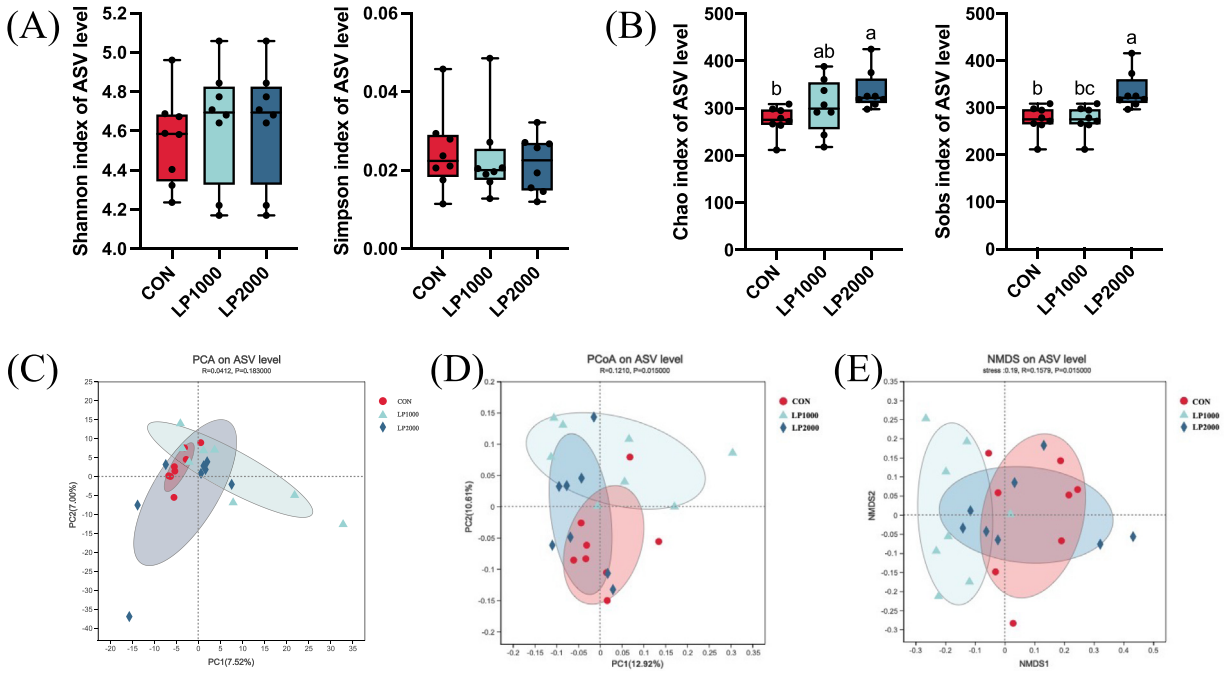

Figure 4. Microbial diversity of the cecal contents at d 70. (A) Shannon and Simpson indexes. (B) Chao and Sobs indexes. (C) Principal component analysis (PCA) plot. (D) Principal coordinate analysis (PCoA) plot based on Bray Curtis metrics. (E) Nonmetric dimensional scaling (NMDS) plot based on Bray Curtis metrics. a, b different letters between groups indicated statistical significance ($P < 0.05$). CON: basal diet; LP1000: basal diet, 1000 × 10^5 CFU/L *L. plantarum* HJZW08 (LP) in the drinking water; LP2000: basal diet, 2000 × 10^5 CFU/L LP in the drinking water.

(*Phascolarctobacterium faecium*) were predominant in CON group (Figure 7). In LP-treated groups, Bacteroides such as *B. salanitronis* (ASV14 and ASV57), *B. uniformis* (ASV459 and ASV458), and *B. plebeius* (ASV310, ASV311 and ASV91), other beneficial species such as *Lactimicrobium massiliense* (ASV4 and ASV36), *Intestinimonas butyriciproducens* (ASV71), and *Barnesiella viscericola* (ASV152 and ASV571) were enriched (Figure 7).

Functional Prediction of Microbiota

Change of microbial composition and structure are closely related to functional alteration of microbes. Thus, Kyoto Encyclopedia of Genes and Genomes (**KEGG**) analysis was further used to predict the altered pathways in our study. Results showed that the 6 pathways in level 1, including Metabolism, Genetic Information Processing, Environmental Information Processing, Cellular Processes, Human Diseases, and Organismal System, were unchanged among the 3 groups (Figure 8A). The relative abundance of pathways in level 2 of the TOP3 pathways in level 1 were further analyzed. No significant differences were observed in level 2 of "Metabolism" and "Environmental Information Processing" pathways in level 1 (data not shown). However, among pathways in level 2 of "Genetic Information Processing" pathway in level 1, the "Translation" and "Replication and repair" pathways were significantly improved by LP supplementation (Figure 8B), indicating the functional shifts of microbiota induced by LP in our study.

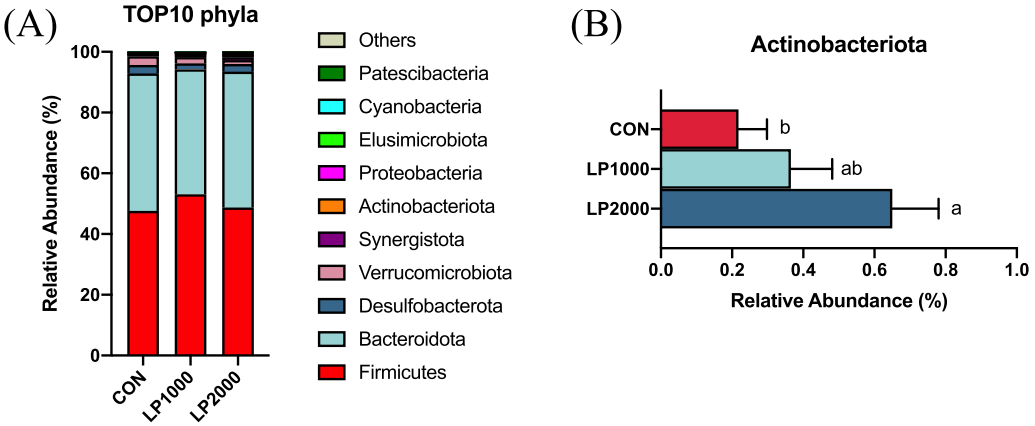

Figure 5. Relative abundance of ceca microbial composition. (A) The TOP10 phyla. (B) The differential phyla. a, b different letters between groups indicated statistical significance ($P < 0.05$). CON: basal diet; LP1000: basal diet, 1000 × 10^5 CFU/L *L. plantarum* HJZW08 (LP) in the drinking water; LP2000: basal diet, 2000 × 10^5 CFU/L LP in the drinking water.

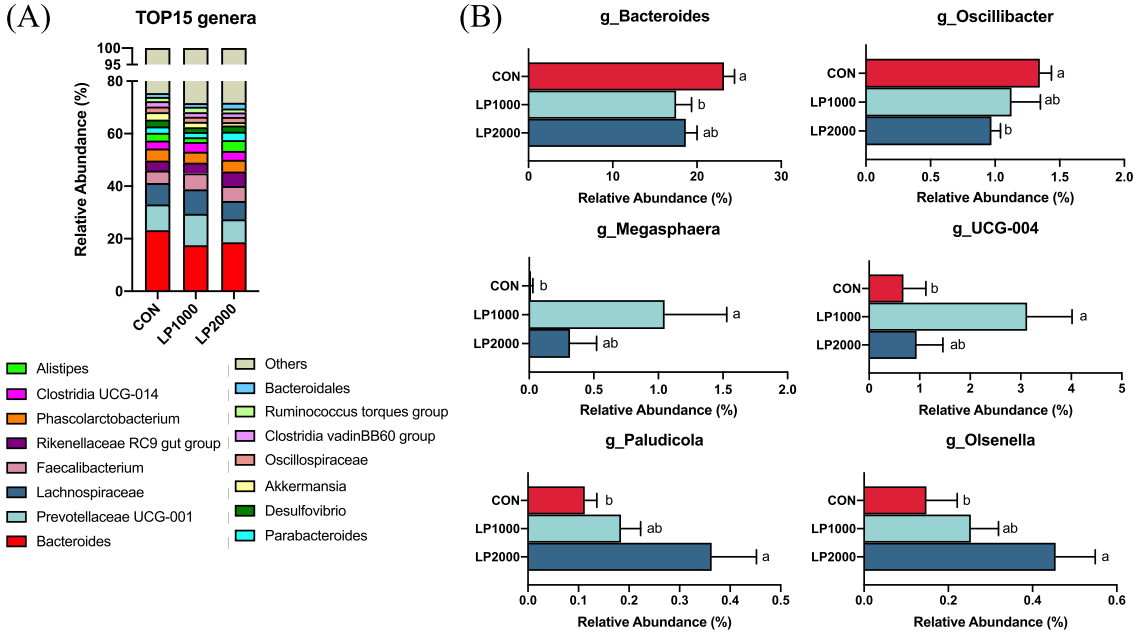

Figure 6. Relative abundance of ceca microbial composition. (A) The TOP15 genera. (B) The differential genera. a, b different letters between groups indicated statistical significance ($P < 0.05$). CON: basal diet; LP1000: basal diet, 1000 × 10^5 CFU/L *L. plantarum* HJZW08 (LP) in the drinking water; LP2000: basal diet, 2000 × 10^5 CFU/L LP in the drinking water.

DISCUSSION

Antibiotics, as an effective growth promotor and agent for treating infectious diseases in the past few decades, led to residues in foods, severe environmental pollution, and antibiotic resistance due to long-term and irrational overuse nowadays (Aghamohammad and Rohani, 2023; Hutchings, Truman and Wilkinson, 2019; Roth, et al., 2019). Moreover, the prohibition of antibiotics in animal farming have been carried out worldwide including China (Allen, et al., 2014; Fu, et al., 2021). Thus, finding safe and efficient antibiotic substitutes is urgent and important. In this study, *L. plantarum* HJZW08 (**LP**), a type of probiotic with permission as

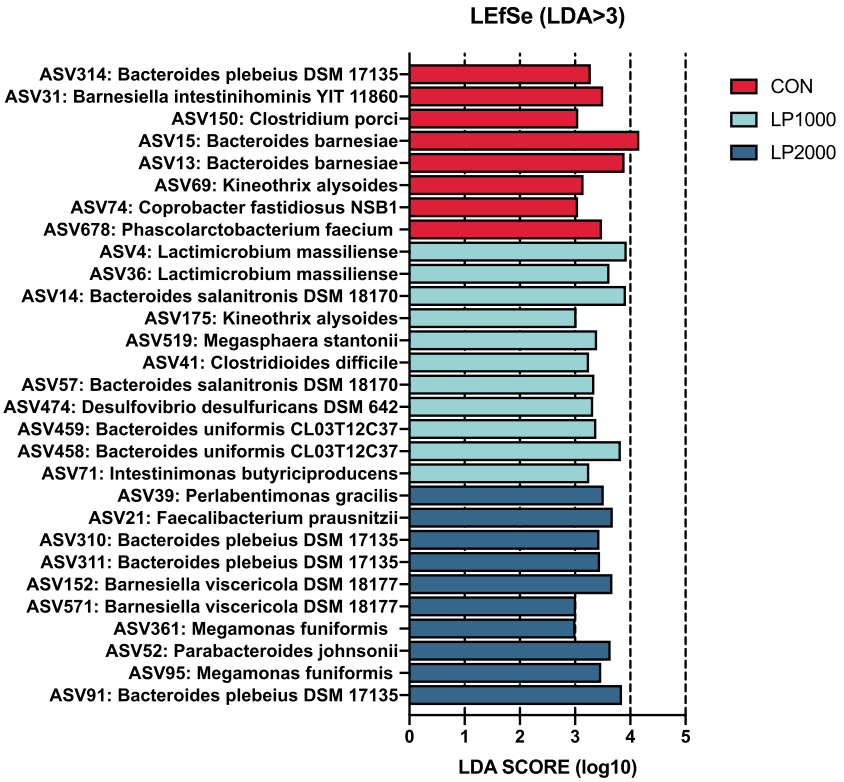

Figure 7. LEfSe linear discriminant analysis (LDA) score based on ASV level, LDA score higher than 3 indicates a higher relative abundance. CON: basal diet; LP1000: basal diet, 1000 × 10^5 CFU/L *L. plantarum* HJZW08 (LP) in the drinking water; LP2000: basal diet, 2000 × 10^5 CFU/L LP in the drinking water.

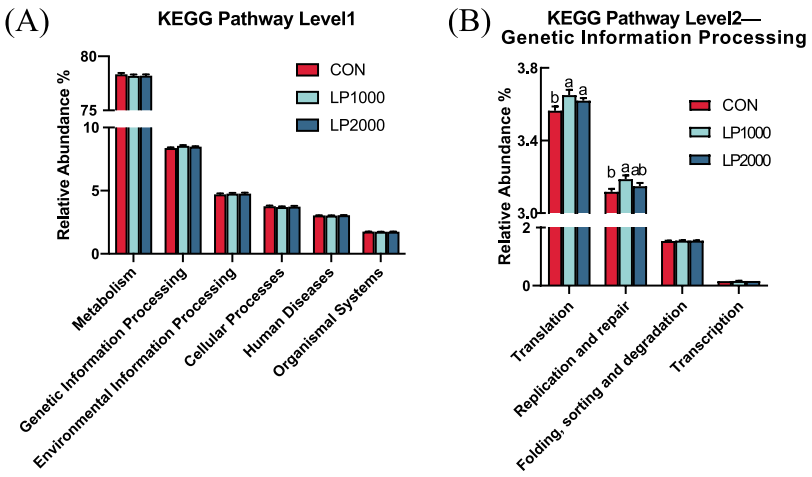

Figure 8. Functional prediction analysis of microbiota in the cecal contents of broilers. (A) Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation of pathway level 1. (B) KEGG annotation of pathways in level 2 of Genetic Information Processing in level 1. a, b different letters between groups indicated statistical significance ($P < 0.05$). CON: basal diet; LP1000: basal diet, 1,000 × 10^5 CFU/L *L. plantarum* HJZW08 (LP) in the drinking water; LP2000: basal diet, 2000 × 10^5 CFU/L LP in the drinking water.

feed additives in animals, was isolated by our lab and used to investigate its effect on broilers. Our research demonstrated that LP supplementation significantly improved growth performance, immune and antioxidant functions, enhanced the contents of SCFAs, and stabilized the cecal microbial homeostasis of broilers, especially the beneficial effect of LP2000 group.

As the huge demand and consumption for meat in our country, growth performance is the first and most pivotal marker for animal feeding, especially for piglets and broilers. Nonetheless, there were controversial data regarding the effect of probiotics on growth performance of broilers previously. For instance, various probiotics individually or mixture, such as *Clostridium, Enterococcus, Bacillus, Bifidobacterium* and *Lactobacillus*, significantly improved growth performance of broilers (Dang, et al., 2024; Liu, et al., 2023; Ningsih, et al., 2023; Qin, et al., 2023; Wang, et al., 2021; Yin, et al., 2023; Zhao, et al., 2013). In contrast, other studies indicated that probiotics supplementation, such as *E. faecium* and *B. animalis*, showed no effect on BW, ADG, ADFI, and FCR during the whole period in broilers (Hernández-Granados, et al., 2022; Zhao, et al., 2013). In our study, LP2000 significantly improved BW at d 70, ADG and ADFI from d 35 to d 70 and d 1 to d 70 compared with the CON group, while FCR were unchanged during the whole period, which was in line with others studies (Dang, et al., 2024; Zhao, et al., 2013). Another report had inferred that compound probiotics (*L. casei, L. acidophilus* and *Bifidobacterium*) improved BW at d 42, ADFI during d 1-21, ADG and ADFI during 22 to 42, reduced FCR during 22 to 42 (Liu, et al., 2023). Moreover, other study also indicated that *L. plantarum* 16 supplementation for 42 d significantly increased the BW of broilers at 21 d of age and FCR during the periods of 1 to 21 d and 14 to 21 d of age, with no impact on ADFI during the whole experiment (Wang, et al., 2021), while *L. plantarum* GX17 in the drinking water for 42 d increased ADFI and F/G, with no impact on ADG of yellow-feathered broiler chickens (Yin, et al., 2023), which was partially similar to our results. Another study showed that *L. plantarum* supplementation for 70 d in broilers showed no effect on growth performance (Song, et al., 2022). The inconsistent results between our study and others might resulted from the different breed of broilers, the concentration of probiotics and the various strains of *L. plantarum.* However, the potential mechanism of LP improving growth performance needed further investigation.

Besides, immunoglobulins (IgA, IgM, and IgY) are essential regulators of immune functions, which protected against various viruses, bacteria, and robust stimuli (Qin, et al., 2023). Cytokines, including anti-inflammatory IL-10, and pro-inflammatory IL-2, IL-1β, IL-6, and TNF-α, are commonly regarded as immunomodulatory factors participating in the occurrence and development of inflammation and biomarkers for systemic defense responses (Song et al., 2019). Apart from immune organ indexes, the levels of immunoglobulins and various cytokines are also common indicators for evaluating the immune status of host. Qin et al. (Qin, et al., 2023) found that *Bacillus licheniformis* supplementation significantly increased the levels of IgY, IgA, and IL-10, reduced the contents of TNF-α, IL-2, IL-1β, and IL-6, but with no impact on IgM and IL-18 in serum of broilers. One study demonstrated that *L. plantarum* GX17 (1.5×10^9 CFU/each) in the drinking water for 42 d had no significant effect on IgY, IgA, IgM of yellow-feathered broilers (Yin, et al., 2023). In contrary, another study reported that lower concentration of *L. plantarum* (1×10^5 CFU/kg) supplementation for 70 d in broilers showed no impact on the levels of Igs (IgA, IgM, and IgY) and pro-inflammatory cytokines (IL-2 and IFN-γ), while higher concentration of *L. plantarum* (1×10^8 CFU/kg) significantly improved the serum IgA and IgG, with no impact on IgM, IL-2 and IFN-γ (Song, et al., 2022). These findings suggested that the concentration of probiotics affected the immune status of broilers to some extents. Similarly, our study reflected that lower concentration of LP (LP1000) showed no

impact on serum IgA, IgM and IgY, whereas higher concentration of LP (LP2000) markedly increased the levels of IgA and IgY, with no effect on IgM. However, Wang et al. reported that *L. plantarum* PFM105 (2×10^7 CFU/g) supplementation for 21 d increased the serum IgM, but showed no in impact on serum IgA and IgY in piglets (Wang, et al., 2019). In addition, LP supplementation groups pronouncedly increased the level of anti-inflammatory IL-10, which was in line with others (Wang, et al., 2019; Wu, et al., 2018). Furthermore, only the level of IL-6 was markedly reduced in the LP1000 group, while all pro-inflammatory IL-2, IL-1β, IL-6, and TNF-α in our study was pronouncedly dropped in the LP2000 group. However, another study focused on the effect of LP on piglets indicated no impact of LP on serum IL-6 (Wang, et al., 2019). Collectively, our data exhibited that LP supplementation significantly improved the immune functions of broilers to further enhance the growth performance, the possible reason why differences between our results and others might due to different animals and breeds, concentrations and types of probiotics, and the experimental period.

Oxidative stress is a common process in life, and could produce various ROS in bodies. Antioxidant enzymes, mainly including GPX, SOD, CAT, and T-AOC, act as the first line to eliminate the oxidative damage upon stress and stimuli, and the by-products (MDA) of oxidation reactions were accumulated during the injury conditions (Bai, et al., 2018). Consistent with our study, it has been reported that dietary *L. plantarum* at 10^9 CFU/mL showed higher antioxidant capacity in mice (Ge, et al., 2021). In the current study, we also measured the antioxidant enzymes activities and the contents of MDA in serum, and the results indicated that the level of T-AOC, the activities of GPX, CAT and SOD were significantly increased, whereas the level of MDA was markedly decreased in the LP supplementation groups compared with those in the CON group. Our previous studies also showed that dietary probiotics could improve the whole antioxidant status in broilers and piglets (Qin, et al., 2023; Yu, et al., 2022a; Yu, et al., 2022b; Zhang, et al., 2021), which had been reported by other researches (Bai, et al., 2018; Khattab, et al., 2021; Liu, et al., 2022; Mengistu, et al., 2021; Zhang, et al., 2020). However, another study suggested that *L. plantarum* supplementation (10^5, 10^6, 10^7, 10^8 cfu/kg) for 70 d showed no impact on GPX, T-SOD, and MDA in Daheng broilers (Song, et al., 2022), the inconsistent results with ours may due to broilers breeds, *L. plantarum* species and concentration. The above findings indicated that LP supplementation could enhance the antioxidant capacity of broilers, and further study is still demanded on this specific mechanism and the interaction between antioxidant capacity and growth performance induced by probiotics.

Robust change of microbiota and derived metabolites, such as SCFAs, are closely related to intestinal health conditions and the balance of gut microbiome is indispensable for maintain host homeostasis (Dang, et al., 2024; Liu, et al., 2023; Ma, et al., 2023; Wang, et al., 2019). SCFAs, including acetic, propionic, butyric, valeric, isobutyric, and isovaleric acids, are the main metabolites produced by microbial fermentation of complex carbohydrates and/or dietary fiber in the colon and play key effects on the gut (Tan, et al., 2023). Previous studies have suggested that SCFAs can maintain gut epithelial integrity, increase immune cells numbers and functions, decreased expressions of numerous pro-inflammatory cytokines, alter microbial balance to modulate intestinal health (Dalile, et al., 2019; Tan, Macia and Mackay, 2023; Wang, et al., 2023). Moreover, probiotics have been reported to change the contents of SCFAs. For example, *B. licheniformis* supplementation significantly increased the levels of isobutyric acid and isovaleric acid in cecal samples of broilers (Qin, et al., 2023). Similarly, our study also showed that LP supplementation significantly enhanced the concentrations of isobutyric acid and isovaleric acid, with no obvious change of other SCFAs. However, another study showed that *L. plantarum* PFM105 (2×10^7 CFU/g) supplementation for 21 d markedly increased the levels of acetic acid, butyric acid, and total SCFAs, with no significant difference in the colonic content concentrations of propionic, isobutyric, valeric, and isovaleric acids in piglets (Wang, et al., 2019). Previous findings suggested that SCFAs could stimulate the release of glucagon-like peptide 1 (**GLP-1**) and peptide YY (**PYY**), which were involved in appetite and food intake regulation (Dalile, et al., 2019; Zanchi, et al., 2017). Thus, LP supplementation increased the ADFI partially due to the increased SCFAs, the concrete interactions between isobutyric and isovaleric acid and growth performance still needs further investigation.

Probiotic strains can interact with the gut bacteria by competition for nutrients, antagonism, cross-feeding ad support of microbial stability (Sanders, et al., 2019). In addition, many probiotic strains produced organic acids and bacteriocins to further inhibit the growth of other microbes. *Lactobacillus* can produce lactic and acetic acid, but not butyrate, to lower the intestinal pH and discourage growth of pathogenic bacteria (Aoudia, et al., 2016). In humans and animals, microbial dysbiosis can cause a range of acute and chronic diseases. Thus, the balance and stability of microbiota plays a pivotal role in regulating growth, development and health of intestine. α-diversity is commonly used to reflect community richness and species diversity, which is indicated by Chao1, Sobs, Shannon, and Simpson indexes (Xiao, et al., 2023). In our study, LP supplementation significantly improved community richness as indicated by higher Chao1and Sobs indexes, with no impact on the species diversity as indicated by unchanged Shannon and Simpson indexes. In line with our results, dietary supplementation of probiotics mixture (*B. subtilis, C. butyricum*, and *E. faecalis*) also showed no impact on Shannon and Simpson diversity of broilers (Dang et al., 2024). Whereas another study showed that compound probiotics (*L. casei, L. acidophilus*, and *Bifidobacterium*) treatment reduced species abundance in the gut

flora (Liu, et al., 2023). In contrary to our results, Wang et al. (Wang, et al., 2021) found that *L. plantarum* supplementation for 21 d had no effect on the observed species, Ace, Chao1, Simpson indexes, while Song et al. (Song, et al., 2022) suggested that *L. plantarum* supplementation for 70 d significantly reduced Shannon and Chao1 indexes. The reason may be that long-term and high-concentration of probiotics competitively absorbed nutrient to inhibit the survival of the original microbes to some extent. β-diversity was used to evaluate the microbial structure of samples, which was usually presented as PCA, PCoA, NMDS plots. In the current study, the compositional dissimilarity of intestinal microbiota, accessed by PCA, PCoA, and NMDS, was observed in the LP group compared with the CON group. Similarly, other study also showed that the compositional structure of microbiota can be easily distinguished between the CON and compound probiotics groups as accessed by PCA plot (Liu, et al., 2023). Surprisingly, related articles focused on species belonging to *L. plantarum* showed that no effect on beta diversity of the intestinal microbiota was observed by *L. plantarum* supplementation in broilers for 21 d or 70 d (Song, et al., 2022; Wang, et al., 2021), which was controversial with our study. The possible reason might be due to different stage and breed of broilers, the various strains even the same genus, and the intestinal or fecal samples.

Furthermore, Firmicutes and Bacteroidota were the most dominant phyla in the cecal contents, which was also found in others' studies (Deepthi, et al., 2017; Xiao, et al., 2021). Actinobacteriota, as one of the 4 major phyla participating in the maintenance of gut homeostasis (Binda, et al., 2018), was significantly increased in the LP2000 group in our study, indicating the beneficial effect of *L. plantarum* supplementation. In addition, our results showed that the relative abundances of *Bacteroides* and *Oscillibacter* were markedly downregulated in LP groups, whereas the abundances of *Paludicola, UCG-004, Megasphaera*, and *Olsenella* were dramatically increased compared to CON group at the genus level. *Bacteroides*, which can metabolize polysaccharides and oligosaccharides to provide nutrition, SCFAs and vitamins for the host and other intestinal microbial residents, showed beneficial and detrimental effects on the body due to the specific organismal and the potential interactions (Zafar and Saier, 2021). In our study, some *Bacteroides* including *B. plebeius* and *B. barnesiae* were enriched in the CON group, while *B. salanitronis, B. uniformis,* and *B. plebeius* were enriched in the LP-treated groups, indicating the positive regulatory effects of probiotics on intestinal microbiota (Zafar and Saier, 2021). Previous study also showed that *B. bacterium* and *Gallinaccum* were enriched in the *L. plantarum* (10^8 cfu/kg) treated group, while *Facealibacterium* and *Funiformis* were enriched in the CON group (Song, et al., 2022). *L. plantarum* 16 treatments significantly increased the relative abundance of *Butyricicoccus pullicaecorum, Faecalibacterium prausnitzii*, Lachnospira, and Coprococcus, but decreased the relative abundance of enteric pathogenic microorganisms, such as *Escherichia coli, B. fragilis* and *Shigella* (Wang, et al., 2021). Moreover, our study showed that the compositional change of SCFAs-producing species such as *Intestinimonas butyriciproducens* and *Bacteroides*, may partially explained the increased SCFAs in the cecal samples.

Overall, we declared the promoting effect of LP (2 dosages) in broilers from the perspective of growth performance, immune functions, antioxidant capacity, cecal SCFAs and microbial homeostasis. However, the concrete mechanism of probiotics on intestinal health and their future application in animals need further evaluation. Besides, which species or mixed species take the core effect still remains vague and needs more investigation.

CONCLUSIONS

On the basis of above results, we can conclude that dietary supplementation with LP at higher concentration (2000 × 10^5 CFU/L) significantly improved the growth performance of broilers as indicated by increased BW at d 70, ADG and ADFI from d 35 to d 70 and d 1 to d 70, while the FCR was not affected by LP addition at 2 dosages. Moreover, higher concentration of LP markedly promoted the immune functions and antioxidant capacity. Importantly, only the levels of isobutyric acid and isovaleric acid were pronouncedly elevated in LP treatment groups, especially in LP2000 group. Finally, the α-diversity was improved and the structure of intestinal microbiota was pronounced altered by LP supplementation. In detail, compared with the CON group, the relative abundances of p_ Actinobacteriota, *Paludicola, Olsenella, UCG-004*, and *Megasphaera* were increased, whereas the relative abundances of *Bacteroide and Oscillibacter* were deceased in the LP treatment groups. Functional analysis indicated that "Translation" and "Replication and repair" pathways were significantly improved by LP supplementation. Collectively, supplemented with LP in the drinking water showed better beneficial effect on host health of broilers.

ACKNOWLEDGMENTS

Thanks for the kindly help of all participants in this project in Zhejiang Agricultural and Forestry University. This project was funded by the Postdoctoral Science Foundation of China (2023M733150), the National Natural Science Foundation of China (32202687), Research and Development Project of Zhejiang Agricultural and Forestry University (2023LFR016), and the Student Research Training Programs of Zhejiang Agricultural and Forestry University (2023KX086).

Author contributions: Conceptualization, Xiao Xiao; Data curation, Xiao Xiao, Tiantian Cui, and Songke Qin; Formal analysis, Xiao Xiao and Tiantian Cui; Funding acquisition, Xiao Xiao and Caimei Yang; Investigation, Tiantian Cui, Tao Wang, Jinsong Liu, Lihan

Sa, Yanping Wu and Yifan Zhong; Methodology, Tiantian Cui, Songke Qin, Yanping Wu and Yifan Zhong; Project administration, Caimei Yang; Resources, Jinsong Liu; Software, Caimei Yang; Supervision, Xiao Xiao and Caimei Yang; Validation, Tiantian Cui and Songke Qin; Visualization, Lihan Sa and Yanping Wu; Writing—original draft, Xiao Xiao; Writing—review and editing, Xiao Xiao and Caimei Yang. All authors have read and agreed to the published version of the manuscript.

Data availability statement: The data presented in this manuscript are available upon request from the corresponding author. The 16S high-throughput sequencing data produced in this study was deposited in the NCBI Sequence Read Archive (SRA) database (accession number: PRJNA1144785).

DISCLOSURES

The authors declare no conflict of interest.

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