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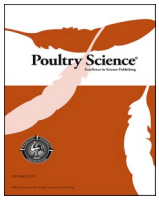

Effects of dietary lysozyme supplementation on growth performance, intestinal morphology, immune function, antioxidant capacity, and gut microbiota in broilers

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ABSTRACT

This study was conducted to investigate the effects of dietary lysozyme (LZ) on growth performance, intestinal morphology, immune function, antioxidant capacity, and gut microbiota in broiler chickens. A total of 360 one-day-old yellow-feathered broilers were randomly assigned to three dietary treatments: basal diet (CON) or supplemented with 250 mg/kg lysozyme (LZ250) or 500 mg/kg lysozyme (LZ500) for 56 days. Results showed that LZ, particularly LZ250, significantly enhanced the growth performance with the higher body weight (BW), and average daily weight gain (ADG), and the lower feed-to-gain ratio (F:G) ($P < 0.05$). Supplementation with LZ markedly enhanced the intestinal mucosal barrier by increasing the villus height and villus height-to-crypt depth ratio (VH/CD) ($P < 0.05$), and reducing the crypt depth in the ileum and jejunum ($P < 0.05$). Furthermore, LZ supplementation regulated the intestinal injury genes (*Villin, MMP3, I-FABP*) and promoted the expression of tight junction proteins *(Occludin, Claudin-1)* ($P < 0.05$). Serum immunoglobulins (IgA, IgM, IgY) were significantly increased by LZ ($P < 0.05$), while the serum inflammatory factors (IL-1β, IL-6, TNF-α) showed no significance ($P > 0.05$). In ileum mucosa, LZ500 significantly decreased TNF-α but increased IL-6 ($P < 0.05$). In addition, LZ activated the NLRP3 inflammasome via the NF-κB pathway by increasing *TLR4, MYD88, IRAK4, NF-κB, NLRP3*, and *Caspase-1* levels. Moreover, LZ enhanced antioxidant capacity, as the evidenced by increasing superoxide dismutase (SOD) and decreasing malondialdehyde (MDA) ($P < 0.05$). Lysozyme increased the volatile fatty acid (VFA) levels, with LZ250 group showing significant increases in propionic and isobutyric acids and LZ500 group in isobutyric and isovaleric acids. 16S RNA sequencing showed LZ improved microbiota abundance and diversity by upregulating *Romboutsia, Blautia*, and *Lactobacillus* ($P < 0.05$) while decreasing *Rikenellaceae* ($P < 0.05$). In summary, dietary with lysozyme improved growth performance, gut barriers, antioxidant capacity, immune functions, and optimized the intestinal microbiota, providing its potential application in poultry production.

Introduction

Antibiotics have been used for many years in animal production (Dibner and Richards, 2005). The extensive utilization of antibiotics has greatly enhanced production and promoted growth performance of livestock and poultry (Ayalew et al., 2022). However, the over use of antibiotics in animal feed may result in antibiotic residue in animal products and the selection of antibiotic-resistant microbes, which may cause harm in humans (Seal et al., 2013). In recent years, antibiotics are banned in many countries (Zhang et al., 2021b). Consequently, animal husbandry suffers a greater challenge and the increment of animal infections and the diminution of animal production are inevitable (Cheng et al., 2014). Therefore, there is an urgent need to find effective alternatives to control infectious diseases and limit the spread of resistant bacteria (Mehdi et al., 2018).

Lysozyme is a protein peptide that is distributed in animal and plant

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tissues (Ferraboschi et al., 2021). It is a 1,4-β-N-acetylmuramidase (Callewaert and Michiels, 2010), and its primary chemical structure is a single polypeptide chain with 129 amino acids including 4 pairs of cysteine (Proctor et al., 1988). The commercial source of lysozyme is obtained from poultry egg white (Bastamy et al., 2024). Lysozyme has been applied as a natural food preservative and a therapeutic drug for humans due to its extensive functions (Sava, 1996). Lysozyme has functions of antibacterial, antifungal, antiviral, immunomodulatory activities and intestinal barrier enhancement. Firstly, lysozyme has natural antibacterial activity and exerts bacteriolytic activity directly by hydrolyzing the β−1,4-glycosidic linkage between N-acetylmuramic acid and N-acetyl glucosamine of bacterial peptidoglycans in the cell wall (Ibrahim et al., 1996). Lysozyme also has antifungal capability with its mechanism of action being the agglutination effect on the cell surface to degrade important proteins and polysaccharides in the cell wall (Jiang et al., 2021). Meanwhile, lysozyme has antiviral functions by interacting with nucleic acids to alter their functions and thereby inhibit the ability of viral replication (Bergamo and Sava, 2024). Moreover, lysozyme also plays a role in immune regulation. It can induce the secretion of immunoglobulin A (IgA) through the hydrolysis of products such as muramyl dipeptide, thereby contributing to immune defense (Kawano et al., 1981). Finally, lysozyme has been shown to support gut barrier function, which can improve growth performance in animals (May et al., 2012).

Due to its antimicrobial properties and safety, lysozyme has been widely used in the food industry (Oliver and Wells, 2015). In recent years, lysozyme has been gradually applied in the livestock and poultry breeding industry and may be a potential alternative to antibiotics. For instance, dietary supplementation with lysozyme has been reported to improve growth performance, enhance intestinal morphology, and reduce *Campylobacter* shedding in 10-day-old pigs (Zou et al., 2019). Similarly, the addition of lysozyme to the diet of weaned piglets has been shown to improve growth performance, alleviate inflammation, and enhance intestinal barrier function (Wu et al., 2023b). Moreover, lysozyme has been demonstrated to promote the development of intestinal structure and function in piglets, while also enriching beneficial microorganisms in the gut microbiota (Xiong et al., 2019). However, the research on the use of lysozyme as a substitute for antibiotics in poultry is limited (Gong et al., 2017). Therefore, we aimed to study the effects of lysozyme on growth performance, intestinal morphology, immune function, antioxidant capacity, and gut microbiota of yellow-feathered broilers, and provided a scientific basis for the application of lysozyme in poultry production.

Materials and methods

Preparation of lysozyme

Lysozyme (LZ) was supplied by Zhejiang Huijia Biotechnology Co. Ltd (Huzhou, China), which was isolated from hen egg albumin using ion-exchange chromatography, and the enzyme activity was 20000 IU/mg.

Animals and experimental design

This research was conducted in accordance with the guiding principles of the Care and Use of Agricultural Animals in Research and Teaching, and was approved by the Animal Ethics Committee of Zhejiang Agriculture and Forestry University (ZAFUAC2023054) . A total of 360 yellow-feather broilers (one-day-old) were ——randomly assigned to three treatment groups: CON group (basal diet), LZ250 group (basal diet supplemented with 250 mg/kg lysozyme), and LZ500 groups (basal diet supplemented with 500 mg/kg lysozyme). Each treatment group was comprised of eight replicates, with each replicate containing 15 birds. Broiler management was carried out based on recommendations (Feng et al., 2020). The basal diet was prepared according to NRC (1994) and Chinese yellow chickens (China Agricultural Industry Standard, 2020). The formula and the concentration of nutrients are shown in Table 1. Birds were fed three times a day. Birds were raised in a temperature-controlled room which was cleaned and disinfected daily. The body weight (BW) and feed intake (FI) were recorded during the trials. To ascertain the growth performance, the average daily gain (ADG), the average daily feed intake (ADFI) and the ratio of feed to gain (F/G) were calculated.

Sample collection

On days 28 and 56 of the experiment, one bird of approximately average weight was selected from each pen after a 12-hour fast. Blood was collected from the jugular vein, allowed to precipitate at room temperature, centrifuged at 4 °C and 3000 rpm for 15 minutes, and the resulting serum was aliquoted into 1.5 ml eppendorf tubes and stored at −20 °C for subsequent biochemical analysis. The spleen, thymus, liver, and bursa of fabricius were collected and weighed. The immune organ indexes were calculated as 100 % × immune organ weight (g)/ body weight (g). Two centimeter of intestinal segments (jejunum and ileum) was collected. Mucosa samples were harvested and immediately frozen in liquid nitrogen. The contents of the cecum were extracted for microbial analysis. All samples were stored at −80 °C for later analysis.

Analysis of intestinal morphology

The jejunal and ileal specimens were fixed, dehydrated, embedded in paraffin, and sectioned into 5 μm slices. Subsequently each section was stained with hematoxylin–eosin (H&E) according to the manufacturer's instructions (Servicebio, Wuhan, China). The images were observed and acquired using a microscope. Six crypts and villi were chosen randomly in each section for detecting crypt depth and villus height under a 10 × magnification and their ratios were calculated. The villus height was determined by measuring the distance from the top of the villus to the junction between the villus and crypt. The depth of the crypt was defined as the distance between two adjacent villi. Based on these measurements, the ratio of villus height to crypt depth (VCR) was calculated by dividing the villus height by the crypt depth.

Table 1
Composition and nutrient levels of the basal diet.

Items	1 to 28 Days of Age	29 to 56 Days of Age
Ingredients	/	/
Corn	53	53
Soybean meal	24.5	16
Extruded soybean	5	3
DDGS	8	8
Rice bran	/	8
Corn gluten	/	2
Soybean oil	1.7	4.5
Limestone	1.3	1.5
Fermented soybean meal	2.5	/
Premix1	4	4
Total	100	100
Nutrient levels	/	/
Crude protein	20.3	17.2
ME(MJ/kg)	2916	3090
Crude fat	5.5	8.6
Lysine	1.19	0.96
Methionine	0.54	0.44
Methionine+Cysteine	0.89	0.74
Threonine	0.86	0.71
Tryptophan	0.23	0.2
Calcium	0.87	0.73
Total Phosphorus	0.6	0.57

1Concentrate mixture provided the following per kilogram of complete diet: 1500 IU of vitamin A; 200 IU of vitamin D3; 10 IU of vitamin E; 35 g of vitamin K; 1.5 mg of vitamin B1; 3.5 mg of vitamin B2; 3 mg of vitamin B6; 10 μg of vitamin B12; 10 mg of pantothenic acid; 30 mg of nicotinic acid; 0.15 mg of biotin; 1000 mg of choline chloride; 8 mg of copper; 60 mg of manganese; 80 mg of iron; 40 mg of zinc; 0.18 mg of iodine; 0.15 mg of selenium.

RNA extraction and real-time quantitative PCR

Total RNA was extracted from intestinal mucosal samples using RNAiso reagent (ABClonal, Wuhan, China). The concentration was assessed via NanoDrop spectrophotometry (Allsheng, Hangzhou, China). Then, reverse transcription of total RNA was carried out using Hifair AdvanceFast One-step RT-gDNA Digestion SuperMix for qPCR with gDNA remover (Yeasen, Shanghai, China). The Q-PCR assay was accomplished using a CFX96 Real-Time PCR system (Bio-Rad, Hercules, USA) and Hieff qPCR SYBR Green Master Mix (Low Rox Plus) (Yeasen, Shanghai, China) according to the protocol. Primer sequences are provided in Table 2. Gene expression levels were normalized to β-actin and calculated using the 2^(-ΔΔCT) method (Livak and Schmittgen, 2001).

Analysis of immunoglobulin content by ELISA assay

Serum immunoglobulin A (IgA), immunoglobulin Y (IgY), immunoglobulin M (IgM), interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) concentrations were quantified using commercial chicken-specific IgA, IgY, IgM, IL-1β, IL-6 and TNF-α ELISA kits (Jinhengnuo, Hangzhou, China). All procedures were conducted in accordance with the instructions provided by the manufacturer.

Determination of antioxidant indicators in serum

The activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), antioxidant capacity (T-AOC) and malondialdehyde (MDA) in the serum were quantified using SOD, GSH-Px, T-AOC, and MDA assay kits (Jinhengnuo, Hangzhou, China).

Analysis of volatile fatty acids

The volatile fatty acids (VFAs) were detected using the method previously described by (Guan et al., 2024). In detail, 0.5 g of the caecal contents was weighed and placed into a 2 mL centrifuge tube. Pre-cooled ddH_2O was added at a mass-to-volume ratio of 1:3, and the sampling amount and water addition ratio were recorded. The centrifuge tube was placed on a vortex oscillator, and the contents were mixed thoroughly after thawing. The mixture was centrifuged at 4 °C and 12,000 r/min for 10 min, and the supernatant was collected. Subsequently, 25 % metaphosphate was added to the supernatant at a ratio of 5:1 (volume ratio), mixed well, and subjected to an ice bath for 30 min. Finally, the mixture was centrifuged again at 4 °C and 12,000 r/min for 10 min. The supernatant was extracted and diluted tenfold, then filtered through a 0.22 μm filter. The concentration of VFA were measured using an Agilent Technologies 7890A Network System (Agilent Technologies) equipped with a 30 m × 0.32 mm × 1.8 μm column (DB-624; Agilent Technologies).

Analysis of gut microbiota

The cecal contents were collected for 16S rDNA sequence analysis. Genomic DNA was isolated from the cecal material employing the E.Z.N.A. soil DNA extraction kit (Omega Bio-tek, Norcross, USA). Amplification of the V3-V4 hypervariable regions of bacterial 16S rRNA genes was performed with the universal primers 338F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT). The PCR products were recovered by agarose electrophoresis and purified using a DNA Gel Extraction Kit (Axygen Biosciences, Union City, USA). The final amplicon libraries were sequenced on an Illumina MiSeq system (San Diego, USA) following the standardized procedures provided by Majorbio Bio-Pharm Technology (Shanghai, China).

Operational taxonomic units (**OTUs**) were generated at a 97 % similarity threshold, and the taxonomy of each 16S rRNA gene sequence was analyzed using the Ribosomal Database Project (**RDP**) classifier algorithm against the database, at a confidence threshold of 70 %. The microbiota diversity analysis included an alpha diversity component—Shannon, Chao, and Simpson, and observed species indices—and a beta diversity component in different samples, Weighted Uniface distances were visualized by non-metric multidimensional scaling (**NMDS**). Linear discriminant analysis (LDA) effect size (LEfSe) analysis was performed.

Statistical analysis

Following the method by Zhang et al. (2021a), the effect of the LZ supplementation on the different parameters was analyzed by one-way ANOVA that was performed SPSS software (version 27.0, Chicago, USA). Differences were determined using the Tukey-Kramer test. Probability values less than 0.05 were considered statistically significant. Figures were prepared using GraphPad Prism (version 8.0, Chicago, USA).

Results

Effects of LZ on the growth performance and immune organ indexes of broilers

The effects of LZ on the growth performance of broilers are shown in Table 3. During the feeding period (1–28 days), compared to the CON group, the BW, ADG and the ADFI were significantly increased ($P < 0.05$) in the LZ250 group, the F/G was no significant difference in the LZ250 group. The BW, the ADG, and the F/G had no significant difference in the LZ500 group compared with the CON group ($P > 0.05$). During the feeding period (28–56 days), both the LZ250 and LZ500 groups exhibited a significant reduction in ADFI compared to the CON group ($P < 0.05$). The F/G of the LZ250 group was significantly lower

Table 2
Primers of the target genes.

Target genes	Accession No.	Forward primers (5′—3′)	Reverse primers (5′—3′)
β-actin	NM_205518	CCGCTCTATGAAGGCTACGC	CTCTCGGCTGTGGTGGTGAA
NLRP3	XM_046918112	GAGGTCCTCTACAGCTTGTG	ACATGATCATCTGTGTGGTG
Caspase-1	AF031351.1	GTGCTGCCGTGGAGACAACATAG	AGGAGACAGTATCAGGCGTGGAAG
IL-1β	XM_46931582	ACGTGGCAGCTTTTGAAGAT	GCGGTGGTTTTGTAACAGTG
IL-18	XM_046932263	ACGTGGCAGCTTTTGAAGAT	GCGGTGGTTTTGTAACAGTG
ZO-1	XM_046925214.1	CTAAGGGGAAGCCAACTGAT	ATTCTGAGGTGGAGGAGGGT
Occludin	XM_046904540.1	GGTTCCTCATCGTCATCCTG	TTCTTCACCCACTCCTCCAC
Claudin-1	NM_001013611.2	CCGCTGTCCTGAGCAGAGTT	TTTCCAGTGGCGATACCTAC
Villin	NM_205442.2	GGCACCAACGAGTACAACACCA	TGCAGCCCTTCCCATACCAGA
FABP	NM_001007923	AGGCTCTTGGAACCTGGAAG	CTTGGCTTCAACTCCTTCGT
MMP3	XM_046909299.1	CAAGCATTTTTGGCGCAAGC	ATGCAGCGTCAACACCAGAT
TLR4	NM_001030693.1	AGGCACCTGAGCTTTTCCTC	TACCAACGTGAGGTTGAGCC
MyD88	NM_001030962.4	TGATGCCTTCATCTGCTACTG	TCCCTCCGACACCTTCTTTCTA
IRAK-4	NM_001389294.2	AGAGTTGTGGGAACAGCAGCCTA	CGTCTACTGGTGGCAGACCTGTTA
NF-κB	NM_205129.1	GTGTGAAGAAACGGGAACTG	GGCACGGTTGTCATAGATGG

[1]NLRP3: NOD-like receptor thermal protein domain associated protein 3; ZO-1: zona occludens-1; FABP: fatty acid-binding protein; MMP3: matrix metallopeptidase 3; TLR4: toll-like receptor 4; MyD88: myeloid differentiation factor 88; IRAK-4: interleukin-1 receptor-associated kinase 4; NF-κB: nuclear factor-kappa B.

Table 3
dietary supplementation of LZ of growth performance of broilers.

Items	Control	LZ250	LZ500	*P*-value
BW(g)				
1d	35.60 ± 0.13	35.75 ± 0.08	35.50 ± 0.10	0.324
28d	677.58 ± 1.31^{b}	690.42 ± 4.56^{a}	672.67 ± 4.86^{b}	0.017
56d	1966.94 ± 38.80^{b}	2056.58 ± 12.33^{a}	1979.745 ± 32.40^{ab}	0.023
ADG(g/d)				
1-28d	22.94 ± 0.04^{b}	23.36 ± 0.14^{a}	22.76 ± 0.17^{b}	0.018
28-56d	46.09 ± 0.46	48.24 ± 0.77	46.54 ± 1.05	0.167
1-56d	33.63 ± 0.277^{b}	35.17 ± 0.23^{a}	33.9 ± 0.552^{ab}	0.027
ADFI(g/d)				
1-28d	41.92 ± 0.66^{ab}	42.67 ± 0.84^{a}	40.07 ± 0.43^{b}	0.039
28-56d	129.49 ± 0.72^{a}	122.75 ± 2.40^{b}	122.96 ± 1.54^{b}	0.02
1-56d	81.32 ± 1.16	79.91 ± 1.93	79.17 ± 0.85	0.556
F/G				
1-28d	1.73 ± 0.03	1.73 ± 0.03	1.67 ± 0.02	0.208
28-56d	2.739 ± 0.02^{a}	2.54 ± 0.06^{b}	2.64 ± 0.05^{ab}	0.052
1-56d	2.36 ± 0.10^{a}	2.22 ± 0.047^{b}	2.29 ± 0.03^{ab}	0.038

^{a,b}Mean with different superscripts in the same row differ significantly ($P < 0.05$). Abbreviations: CON, basal diet; LZ250, basal diet supplemented with 250 mg/kg LZ; LZ500, basal diet supplemented with 500 mg/kg LZ.

than that of the CON group ($P < 0.05$), while there was no significant difference between the LZ500 group and the other two groups ($P > 0.05$). During the feeding period (1-56 days), compared to the CON group, the BW and ADG of the LZ250 group were significantly higher ($P < 0.05$), and the F/G of the LZ250 group was significantly lower ($P < 0.05$); the BW, ADG, ADFI, and F/G of the LZ500 were not significant ($P > 0.05$). The effect of LZ on the immune organ index of broilers is shown in the Fig. 1. Compared with the CON group, the LZ250 group significantly reduced the spleen index of broilers ($P < 0.05$), whereas had no statistical effect on other organ index ($P > 0.05$).

Effects of LZ on intestinal morphology of broiler

Fig. 2 displays the histological structure of the jejunum and ileum. Compared to the CON group, more complete villi were observed in the LZ250 and LZ500 groups (Fig. 2A, Fig. 2B). As shown in Fig. 2C, the villus length of the jejunum was significantly increased in the LZ250 groups ($P < 0.05$). The LZ250 and LZ500 groups had dramatically decreased crypt depth ($P < 0.05$), and a marked reduction occurred in the LZ250 group compared to the LZ500 group ($P < 0.05$). The villus/crypt ratio was significantly upregulated by LZ treatments, with the highest was in the LZ250 group. For the ileum, LZ supplementation significantly enhanced the villus length and villus/crypt in comparison to the CON group ($P < 0.05$) (Fig. 2D), and markedly decreased the crypt depth ($P < 0.05$) (Fig. 2D). The villus length of the LZ250 group was higher and the crypt depth was lower, compared with that of the LZ500 group ($P < 0.05$) (Fig. 2D). These results indicated that LZ could maintain the intestinal structure of broilers.

Effects of LZ on intestinal mucosal functions of broilers

Fig. 3A shows the gene expression of mucosal injury biomarkers of broilers. Compared with the CON group, the LZ500 group significantly upregulated the expression of *MMP3, Occludin* and *Claudin-1* in the ileum of broilers ($P < 0.05$), and the LZ250 group significantly upregulated the expression of *MMP3* ($P < 0.05$). The gene expression of tight junctions was displayed in Fig. 2B. When compared to the CON, LZ supplement significantly increased the expression of ileum *Occludin* and *Claudin-1* ($P < 0.05$). No significance was found in *ZO-1* by LZ treatments ($P > 0.05$). These results showed that LZ alleviated mucosal injury and enhanced tight junctions in broilers.

Effect of LZ on serum antioxidant performance of broilers

As displayed in Fig. 4, compared with the CON group, LZ supplementation significantly reduced the level of MDA ($P < 0.05$), markedly improved the levels of SOD ($P < 0.05$). There was no significant difference in the levels of T-AOC and GSH-PX among all the groups ($P > 0.05$).

Effects of LZ on immunoglobulins and cytokines of broilers

Fig. 5 shows the levels of immune factors in the serum of broilers and inflammatory factors in the serum and ileum of broilers. Compared with the CON group, the levels of immunoglobulins (IgY, IgM, and IgA) in the serum of the LZ250 and LZ500 groups were significantly increased ($P < 0.05$) (Fig. 5A). The levels of IL-1β and TNF-α in the serum of the LZ250 group and the LZ500 group were lower than those of the CON group, but there was no significant difference ($P > 0.05$) (Fig. 5B). In ileum mucosa, IL-6 was significantly increased and TNF-α was significantly decreased in the LZ500 group ($P < 0.05$) (Fig. 5C). Compared with the CON group, the LZ250 group showed a tendency of decreased IL-1β and TNF-α and increased IL-6, but there was no significance ($P < 0.05$) (Fig. 5C).

Effects of LZ on NLRP3 inflammasome and the mediated signaling pathways

The effect of LZ on the relative expression of NLRP3 inflammasome mRNA is shown in Fig. 6A. The gene expression levels of *NLRP3*

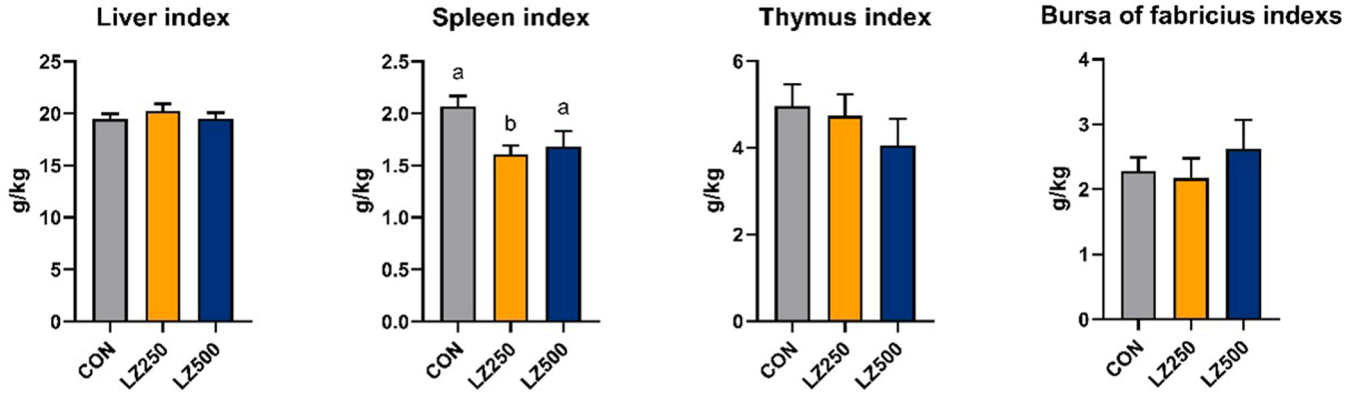

Fig. 1. Effects of lysozyme on immune organ index of broilers.
Abbreviations: CON, basal diet; LZ250, basal diet supplemented with 250 mg/kg LZ; LZ500, basal diet supplemented with 500 mg/kg LZ. Bars represent mean ± SEM (n = 6). Different lowercase letters (a, b) above bars represent significantly different means ($P < 0.05$).

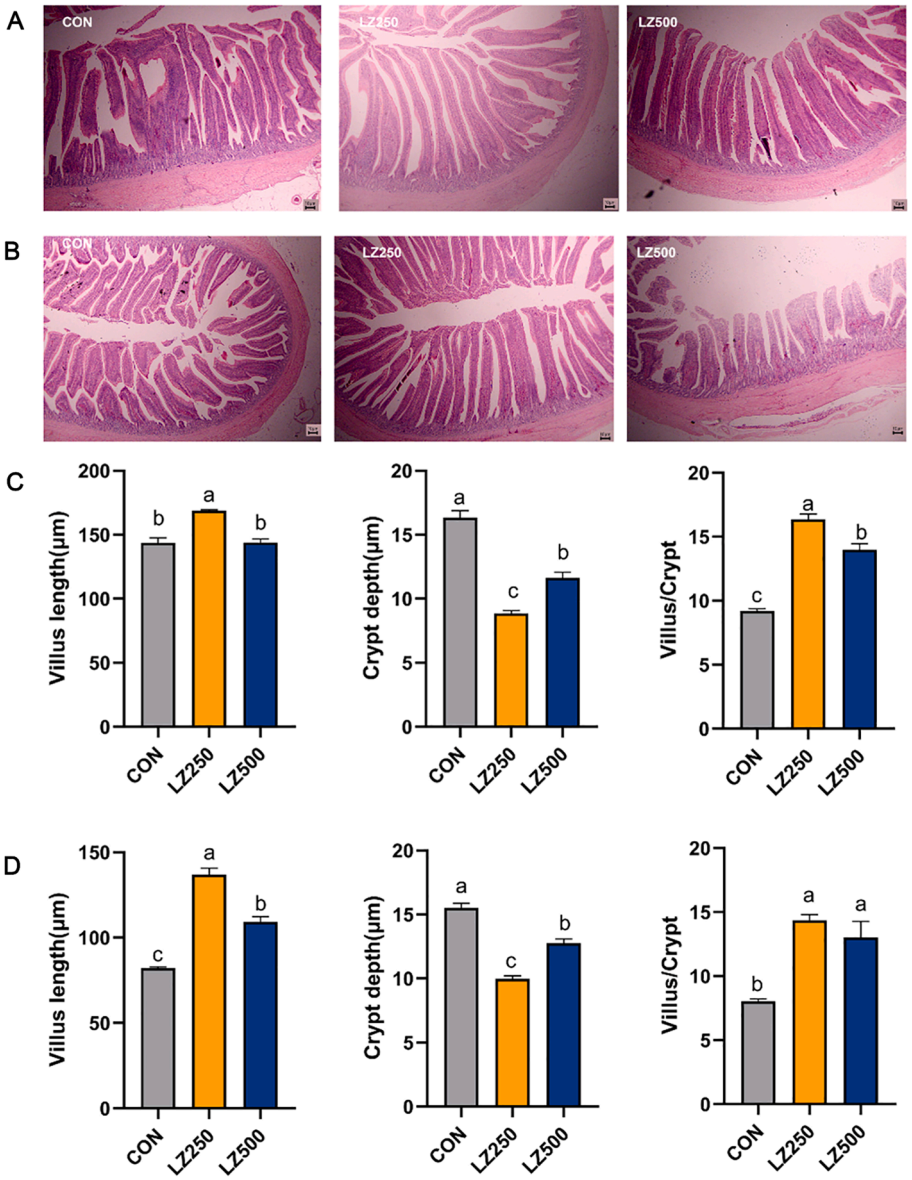

Fig. 2. Effect of LZ on intestinal morphology of broilers.
(A) and (B) Histomorphometric pictures of jejunum (A) and ileum (B) by Hematoxylin & Eosin (H&E) staining. 10 × magnification, scale bar: 10 μm. (C) and (D) Statistical analysis of villus length, crypt depth and Villus/crypt ratio of jejunum(C) and ileum (D). The villus height and crypt depth were measured from six fields in eight samples from each group.

inflammasome biomarkers, *NLRP3* and *Caspase-1*, were significantly upregulated in the ileum of the LZ250 and LZ500 groups compared to the CON group ($P < 0.05$), but there was no significant difference between the LZ250 group and the LZ500 group ($P > 0.05$). Compared with the CON group, the expression levels of *IL-1β* and *IL-18* were lower in the LZ250 group and higher in the LZ500 group, but there were no statistically significant differences ($P > 0.05$).

Fig. 6B displays the gene expression of upstream signaling pathways associated with the NLRP3 inflammasome. Compared to the CON group, the expression levels of *TLR4* and *NF-κB* were significantly elevated in the LZ500 group ($P < 0.05$). A similar upward trend was observed in the LZ250 group, but there was no significant difference ($P > 0.05$). The expression levels of *MYD88* and *IRAK4* were significantly higher in the LZ250 and LZ500 groups than in the CON group ($P < 0.05$).

Effects of LZ on VFA in cecal contents of broilers

Fig. 7 shows the effects of LZ on VFA levels in cecal contents of broilers. Compared to the CON group, the LZ250 group significantly increased the level of propionic acid ($P < 0.05$), the LZ500 group significantly increased the content of isovaleric acid ($P < 0.05$), and both the LZ250 and LZ500 groups showed significantly higher levels of isobutyric acid ($P < 0.05$). The contents of acetic acid, butyric acid, and valeric acid showed an increasing trend in LZ250, but there was no significant difference ($P > 0.05$). Similar trends were also observed in the contents of butyric acid and valeric acid by LZ treatment ($P > 0.05$). On the contrary, the concentration of acetic acid and butyric acid showed a reduced trend in the LZ500 group ($P > 0.05$).

Effects of LZ on cecal microbial diversity of broilers

The LZ250 group was selected for 16 s analysis. Microbial analysis was conducted based on the ASVs table. As illustrated in Fig. 8A, the Venn diagram revealed a total of 5,697 and 5,925 OTUs in the CON and LZ groups, respectively. Fig. 8C shows the parameters representing alpha diversity. Compared to the CON group, LZ supplementation

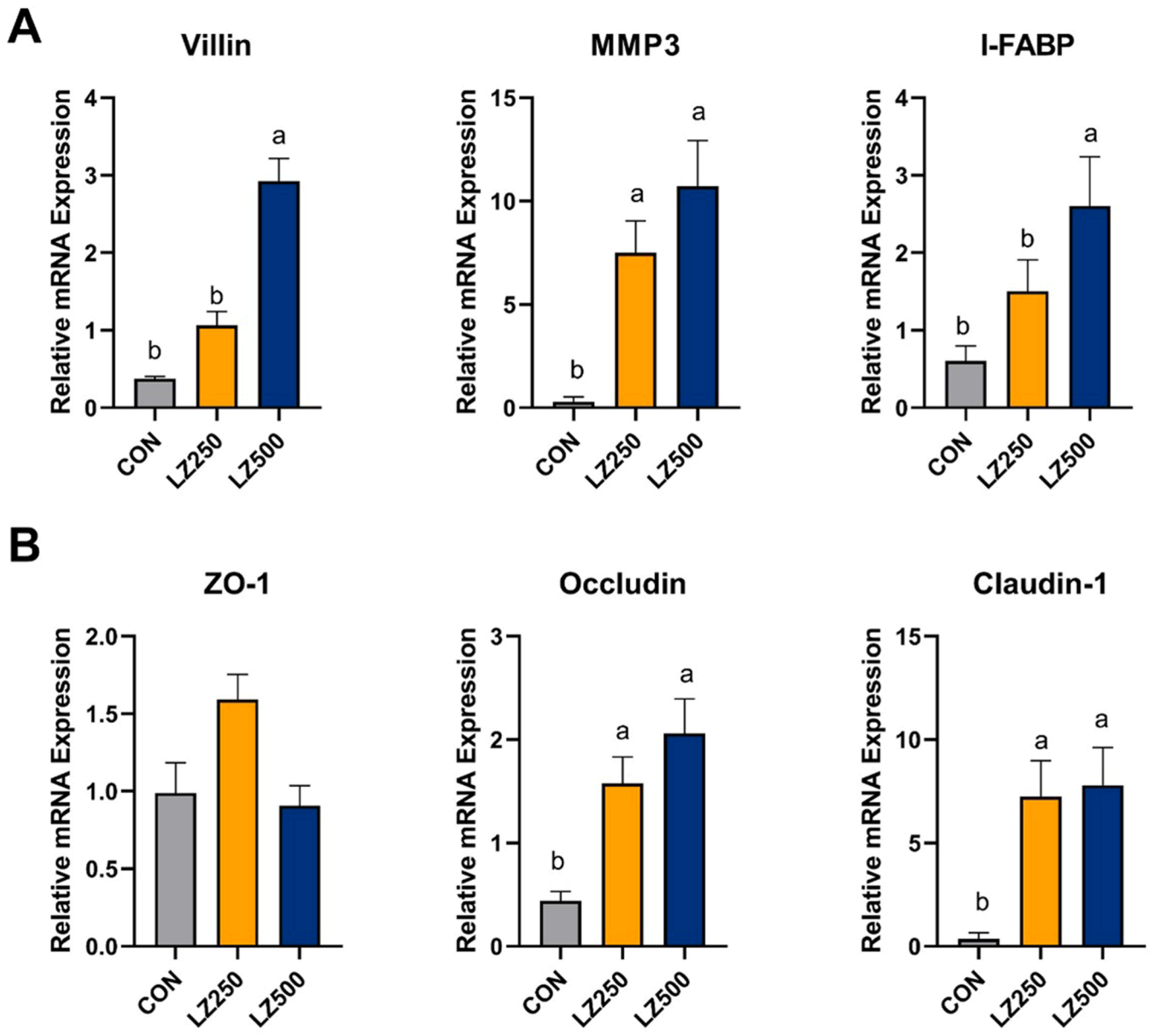

Fig. 3. Effect of LZ on intestinal mucosal functions of broilers.
The relative gene expression levels of ZO-1, Occludin, Claudin-1, Villin, MMP3, I-FABP in ileum.

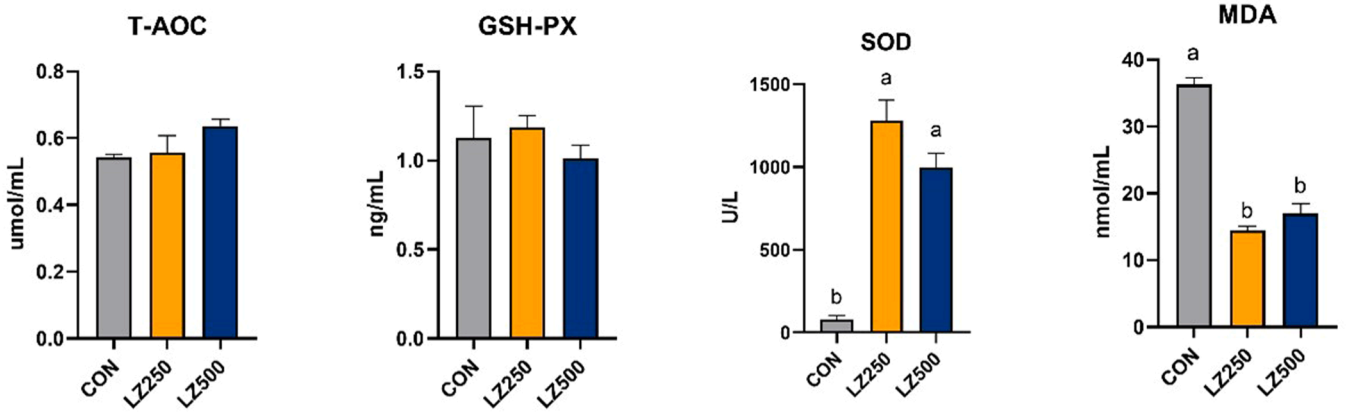

Fig. 4. Effects of LZ on antioxidant functions of broilers.
Total antioxidant capacity (T-AOC); Glutathione peroxidase (GSH-Px); Superoxide dismutase (SOD); Malondialdehyde (MDA).

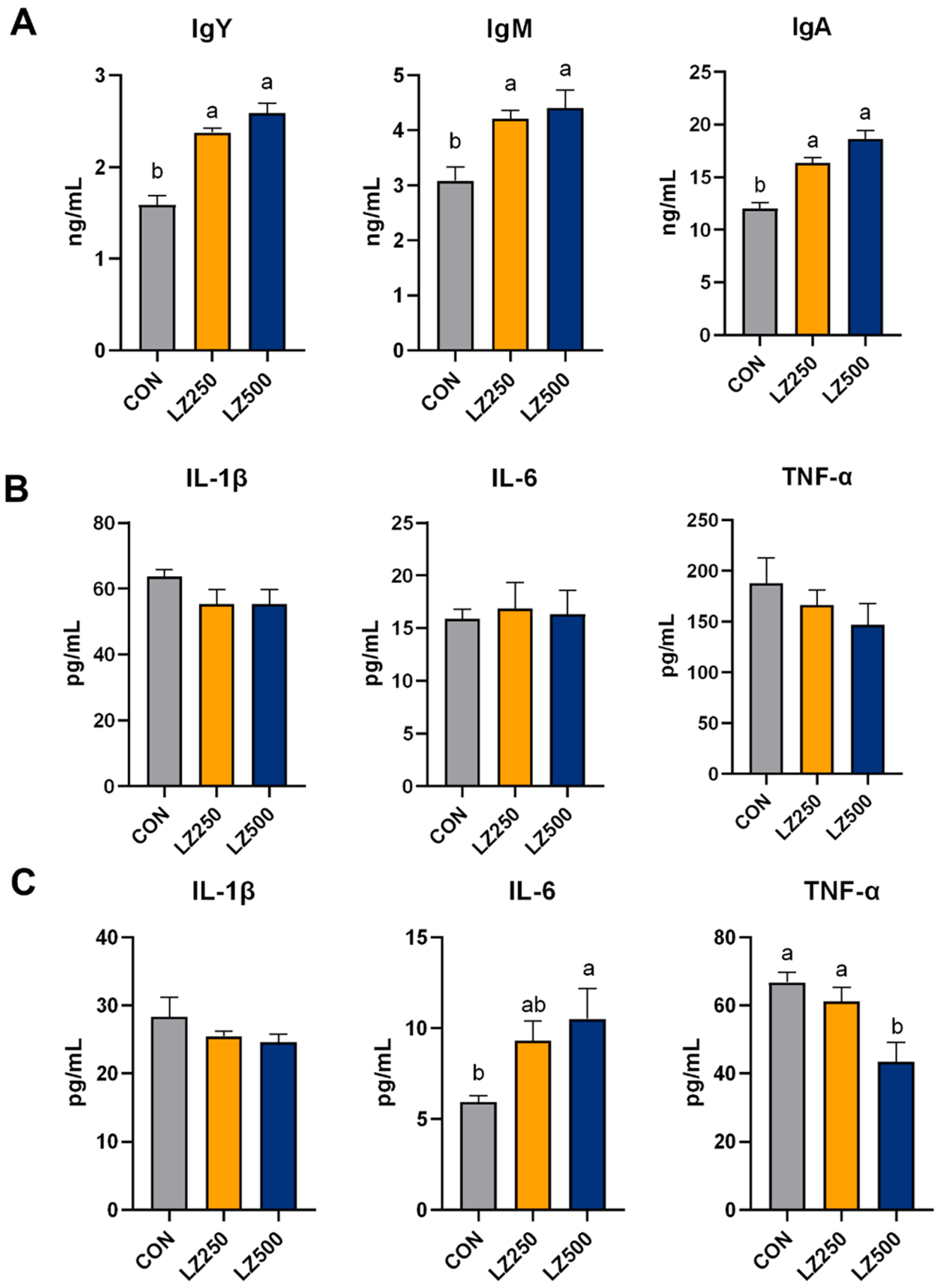

Fig. 5. Effects of LZ on immunoglobulins and inflammatory factors of broilers.
(A) Effect of dietary supplementation of LZ on serum immunoglobulins in broilers. (B) Effect of dietary supplementation of LZ on serum inflammatory factors in broilers. (C) Effect of dietary supplementation of LZ on ileal mucosa inflammatory factors in broilers.

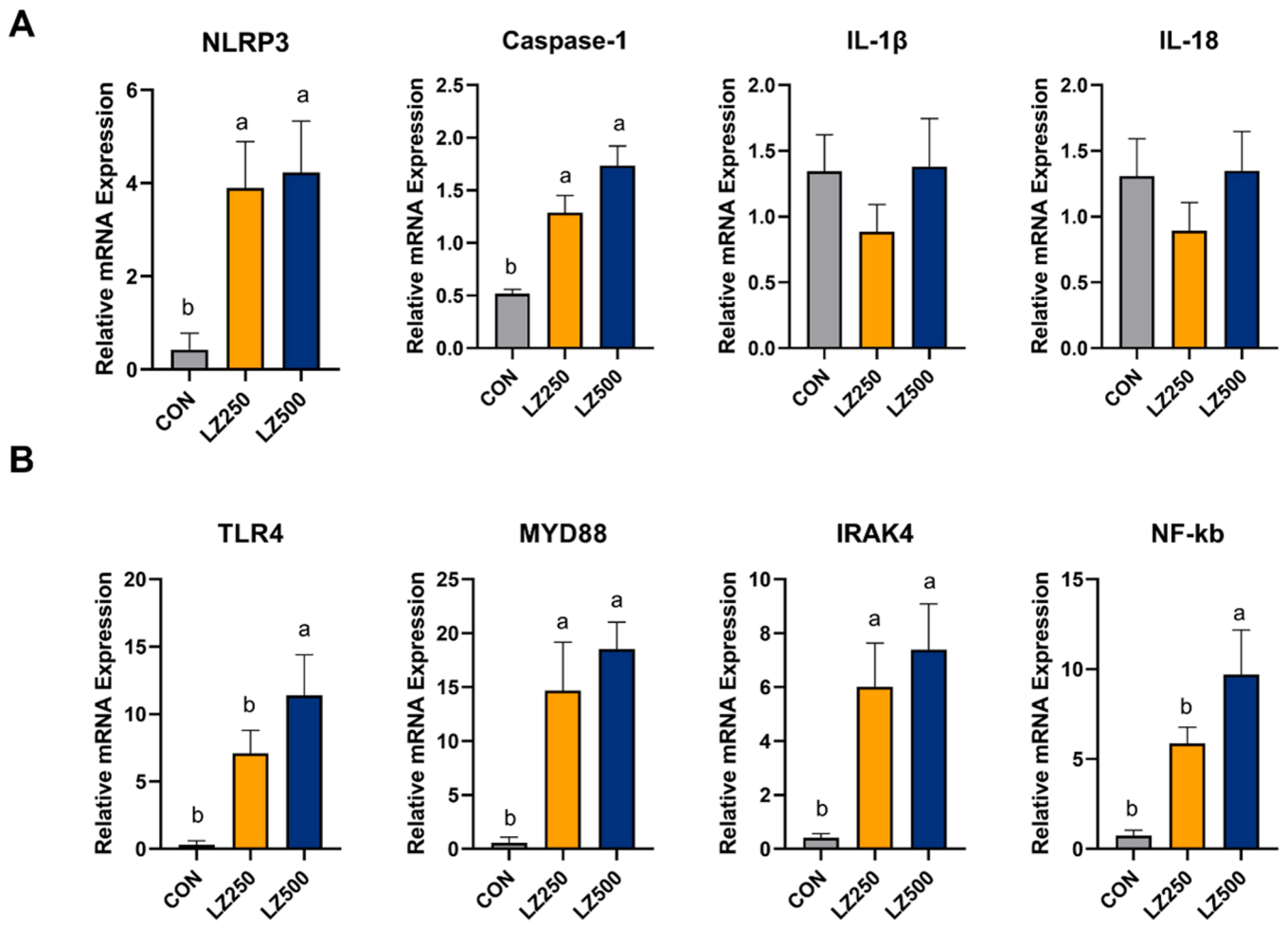

Fig. 6. Effect of LZ on NLRP3 inflammasome and the mediated signaling pathways.
(A)The relative gene expression levels of *NLRP3, Caspase-1, IL-1β* and *IL-18* in ileum. (B) The relative gene expression levels of *TLR4, MYD88, IRAK4* and *NF-κB* in ileum.

significantly increased the ACE and Sob indexes ($P < 0.05$) while reducing the Simpson and Coverage indexes ($P < 0.05$). Additionally, LZ supplementation had a trend to increase Chao, Shannon indexes, but with no significance ($P > 0.05$). For beta diversity analysis, an NMDS plot was utilized, as shown in Fig. 8B. The results indicated a clear separation in the distribution of samples between the CON and LZ groups, indicated that LZ supplementation significantly changed the microbial composition ($P < 0.05$).

Effects of LZ on cecal microbial composition of broilers

To further investigate the effect of LZ on microbial composition, the dominant bacteria at phylum, genus and species levels were showed in Fig. 9. The top phylum was *Firmicutes*, followed by *Bacteroidota* and *Actinobacteriota* 2 group (Fig. 9A). Supplementation with LZ increased *Firmicutes* and decreased the abundance of *Bacteroidota* at the phylum level. As shown in Fig. 9B, at the genus level, LZ supplementation decreased the number of *Rikenellaceae_RC9_gut_group* and *Bacteroides* (Fig. 9B). The top species was *uncultured_bacterium_g_ Rikenellaceae_RC9_gut_ group,* followed by *unclassified_f_ Lachnospiraceae* and *unclassified_g_Blautia* among 2 groups (Fig. 9C). Specifically, Fig. 9D shows that LZ treatment enriched intestinal microorganisms of broilers. At the genus level, LZ treatment significantly reduced the abundance of *Rikenellaceae_RC9_gut_group* and significantly increased the abundance of intestinal beneficial bacteria *Blautia, Romboutsia, Lactobacillus, Subdoligranulum, Turicibacter, Butyricimonas*, and the unnamed genus *norank_o_Clostridia_UCG-014*.

The method of LEfSe was applied to explore the relative richness ($P < 0.05$, LDA > 3) of the cecal microbiota of broilers (Fig. 10). Results showed that dietary LZ modified the cecal microbial composition. The LZ group exhibited enrichment of several bacterial taxa, including the phylum *Firmicutes*; the classes *Clostridia and Bacilli*; the genus *Romboutsia*; the order *Lactobacillales*; and the genus *Lactobacillus*.

Discussion

The poultry industry is one of the largest food industries in the world (Chowdhury and Morey, 2019), and the level of poultry consumption continues to rise. Poultry products play an important role in people's daily lives. The abuse of antibiotics in broiler production will not only lead to bacterial resistance but also lead to drug residues in animal products (Ronquillo and Hernandez, 2017), which will harm human health. Therefore, producing feeds without antibiotics is the trend in the poultry industry today around the world (Haque et al., 2020). Lysozyme is a natural antimicrobial protein considered to be an important component of the innate immune system (Liu et al., 2010). It can take part in the natural defence mechanism in Gram-positive bacteria (Thammasirirak et al., 2010), and perhaps indirectly affect Gram-negative bacteria (Wells et al., 2015). Furthermore, Ginsburg (2002) found that lysozyme has many other biological functions, including regulation of inflammatory response, antiviral and antitumor activities. Therefore, lysozyme is one of the most promising antibiotic

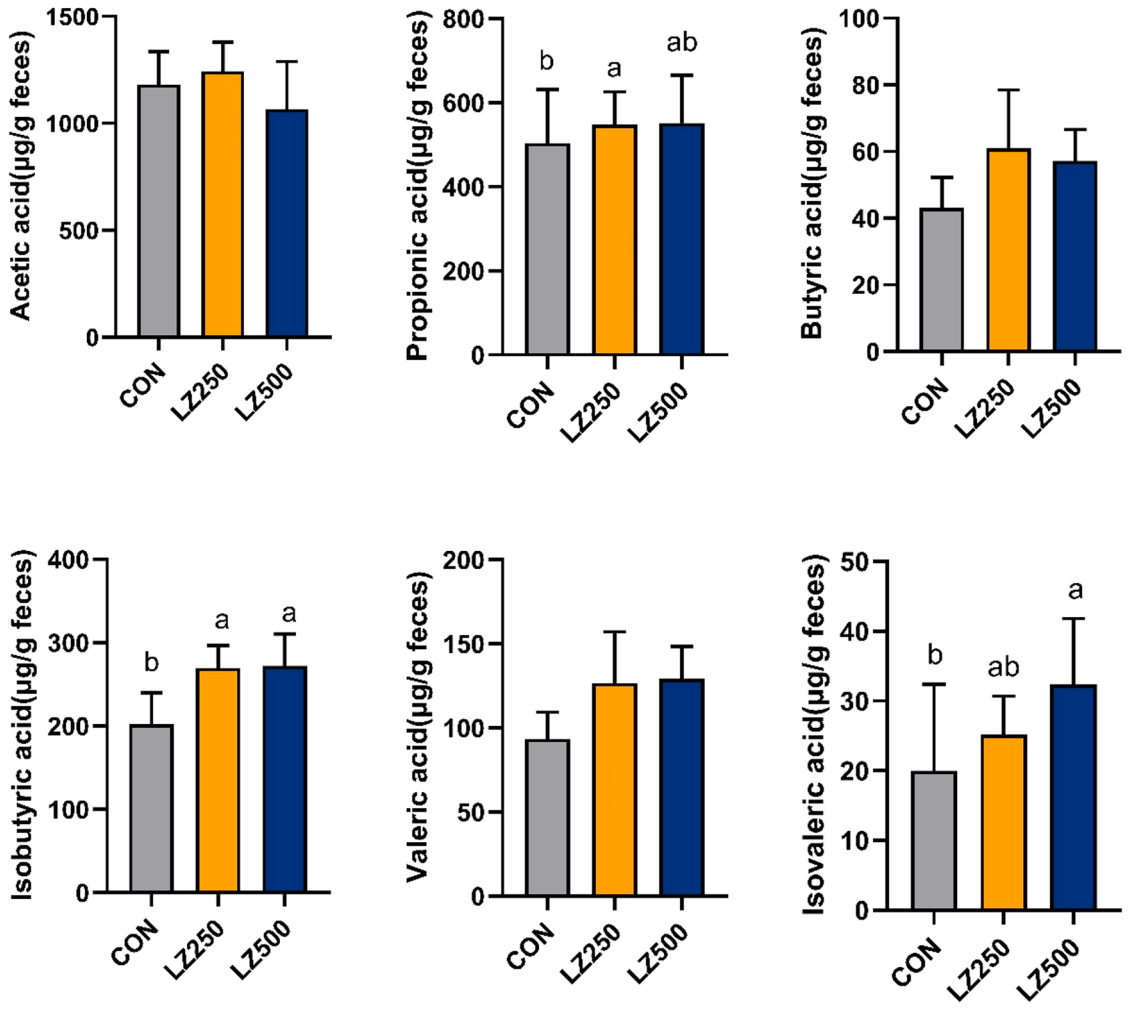

Fig. 7. Effects of LZ on VFA contents in cecal contents of broilers.
Abbreviations: CON, basal diet; LZ250, basal diet supplemented with 250 mg/kg LZ; LZ500, basal diet supplemented with 500 mg/kg LZ. Bars represent mean ± SEM (n = 6). Different lowercase letters (a, b) above bars represent significantly different means ($P < 0.05$).

alternatives (Ferraboschi et al., 2021). In our study, we found that lysozyme was effective in improving the growth performance of broilers, reducing inflammation, enhancing immune function, and improving intestinal health.

Our research indicated that LZ enhanced the growth performance of broilers, and LZ250 showed a greater effect than LZ500. The LZ250 group improved growth performance by increasing the BW, ADG, and reducing the F:G ratio during the feeding phase. These results align with previous research by Zhou et al. (2020) who reported that dietary lysozyme supplementation improved both ADG and gain-to-feed ratio (G:F) in pigs ($P < 0.05$). Similarly, Dawood et al. (2024) found that, compared with 3 g/kg lysozyme supplementation in the diet of Nile tilapia (Oreochromis niloticus), 1.85-2.05 g/kg lysozyme enhanced growth performance, protein efficiency ratio, and protein productivity value while reducing the feed conversion ratio of tilapia. The above results indicate that the effect of additives shows a dose effect. Excessive doses may have adverse effects, and the optimal effect can be achieved at an appropriate dose.

We evaluated the effect of LZ on the morphology of ileum and jejunum. The morphology of the villus and crypt is closely linked to the health of the intestine and its nutrient absorption capacity (Zhang et al., 2022). It is widely recognized that longer intestinal villi are associated with enhanced nutrient absorption efficiency. We found that the LZ250 group significantly increased the intestinal villus length and V/C ratio, and significantly decreased the crypt depth in jejunum and ileum of broilers. Consistently, it was reported that feeding lysozyme reduced intestinal damage and increased villus length of weaned piglets (Xiong et al., 2019). Similarly, the study of Khan et al. (2017) showed that adding Moringa oleifera leaf powder to broilers could increase the villus height of jejunal ileum in broilers. The above results indicate that the addition of lysozyme can improve the intestinal epithelial structure of broilers and promote the digestion and absorption of nutrients.

Furthermore, LZ improved the functions of intestinal mucosal barrier. The injury and slow growth as well as the barrier function reducing of intestinal mucosa were probably associated with some genes such as *Villin, I-FABP*, and *MMP3* (Wu et al., 2023a). *Villin* is one kind of actin binding protein and a marker of villus cell differentiation, which conduce to prop up the microfilaments of the microvilli of the mucosal villus (Chantret et al., 1988). The increase of *Villin* in the LZ250 and LZ500 groups indicates a protective role in epithelial cells. The Intestinal Fatty Acid-Binding Protein participates in the uptake of long-chain fatty acids from digesta in the small intestine into intestinal epithelial cells (Prows et al., 1995). The *I-FABP* levels in the LZ supplement group were higher than the CON group. This increase may be related to an increase in certain short-chain fatty acids in the gut, such as acetic acid, isobutyric acid, and isovaleric acid. *MMP3* is an important member of the *MMP* family, which is mainly found in epithelial cells, stromal fibroblasts and macrophages, and can provide great support for the normal physiological function of cells (Sternlicht et al., 1999). The expression level of *MMP3* in LZ250 and LZ500 groups was significantly higher than

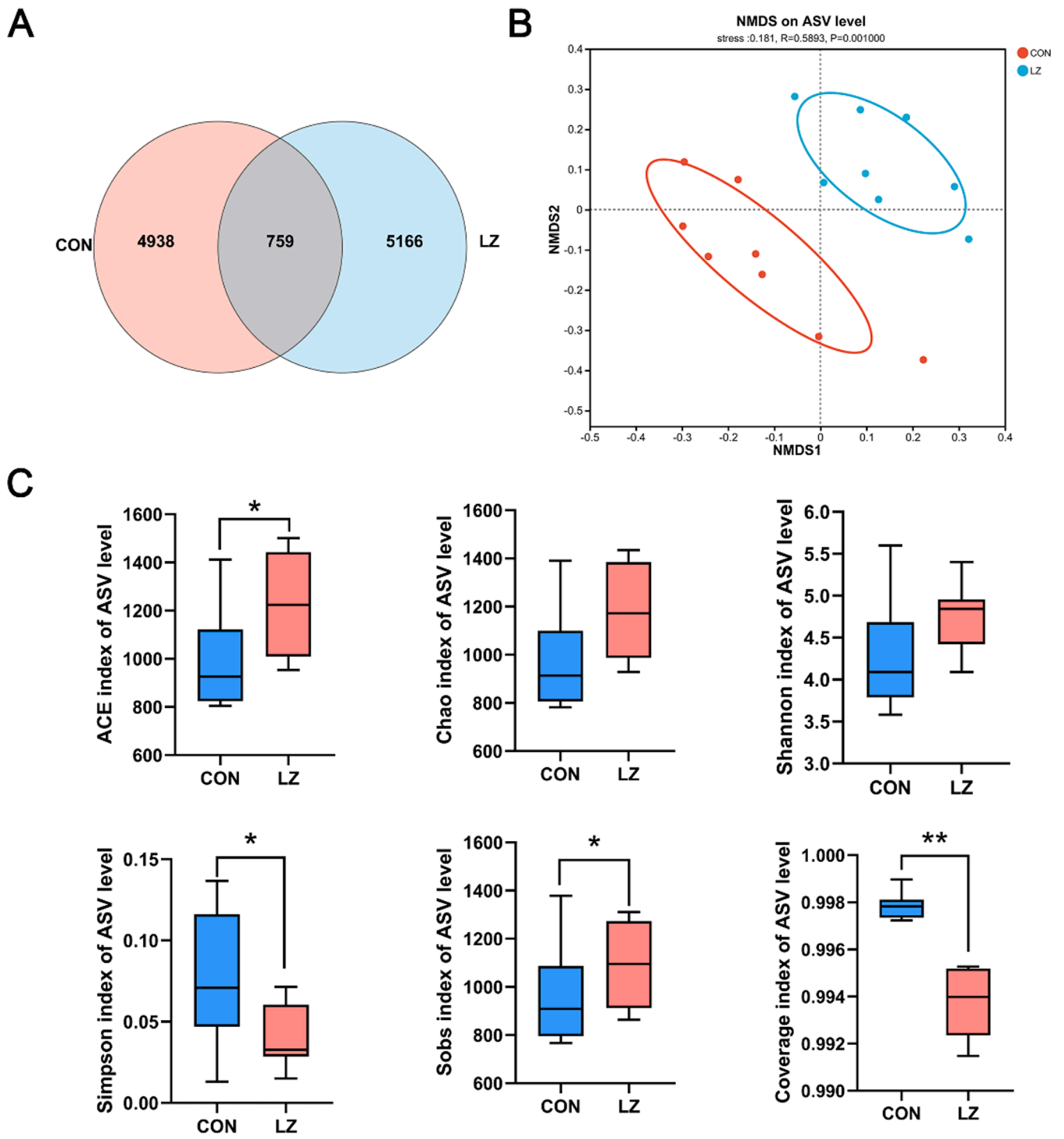

Fig. 8. Effects of LZ on microbial diversity in cecum of broilers.
(A) The Venn diagram summarizing the numbers of common and unique OTUs in the microflora community in cecal contents of broilers. (B) The non-metric multidimensional scaling analysis (NMDS) reflecting species beta diversity within and between groups. (C) The shannon index, chao index, simpson reflecting species alpha diversity between groups.

the CON group, indicating that LZ promoted intestinal health of broilers. Impairment of tight junctions causes disruption of the intestinal barrier and increases permeability, leading to diffusion of pathogens, antigens and endotoxins from the intestinal lumen into the blood (Zhang et al., 2012). *ZO-1, Occludin* and *Claudin-1* are key tight junction proteins that maintain gut barrier function (Gao et al., 2023). We found that the expression of *Occludin* and *Claudin-1* was higher in the LZ250 and LZ500 groups than in the CON group. Similar to our results, the study by Emami et al. (2019) found that expression of *Claudin-3* was higher in the probiotic-supplemented birds. In conclusion, LZ supplementation enhanced the mucosal barrier of broilers.

Oxidative stress has been hypothesized to negatively impact animal growth performance (Iqbal et al., 2005). To evaluate antioxidant capacity, researchers commonly measure enzymes such as SOD and GSH-Px (Fu et al., 2019). Among these, SOD and GSH-Px are critical enzymes that scavenge free radicals (Sahin et al., 2016). In the present

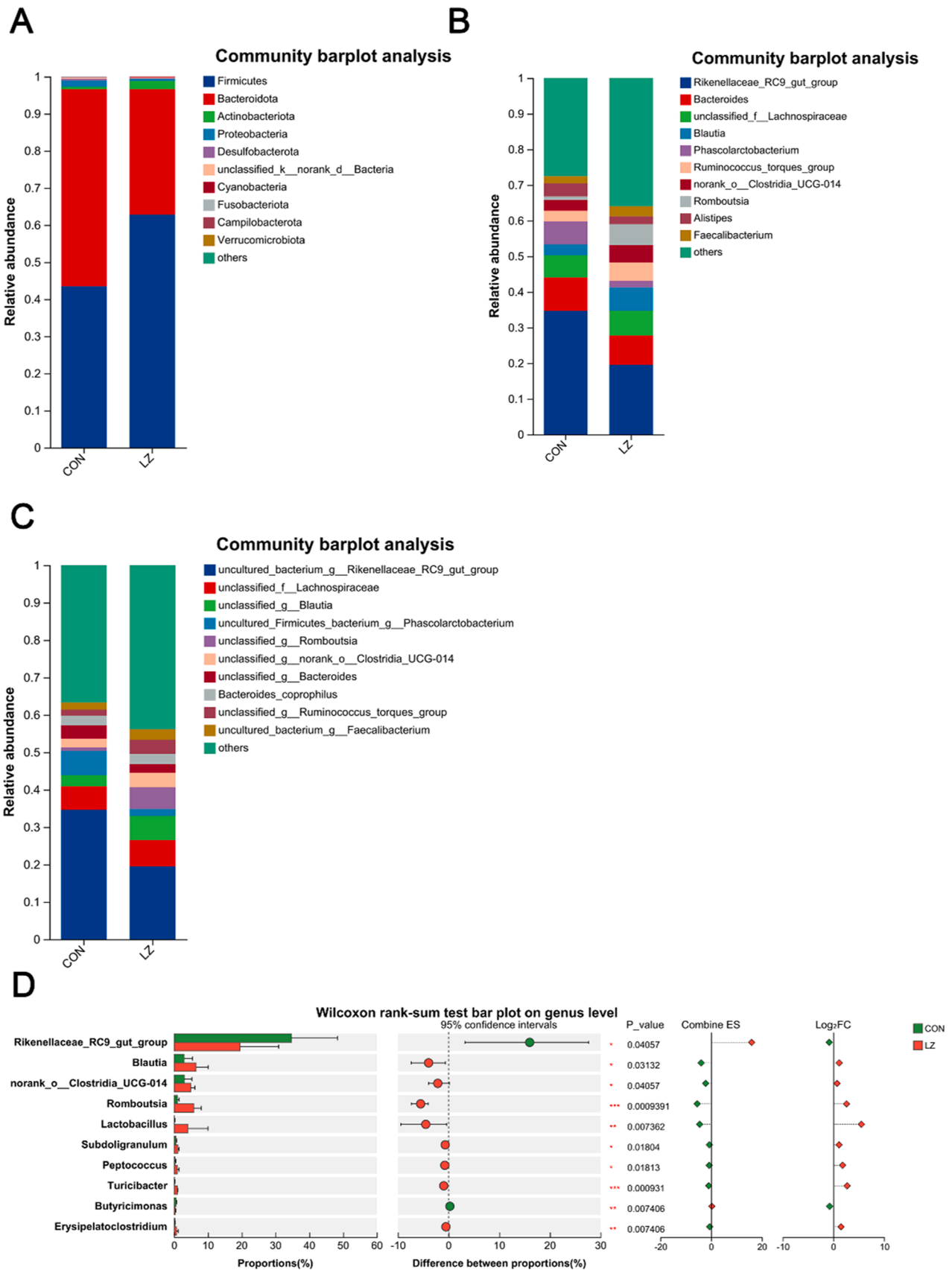

Fig. 9. Effect of LZ on microflora structure of broilers.
(A) phyla level of flora structure; (B) The level of flora structure and genus; (C) Species level of flora structure (D) Comparative analysis of intestinal flora level between two groups of broiler chickens by LZ.

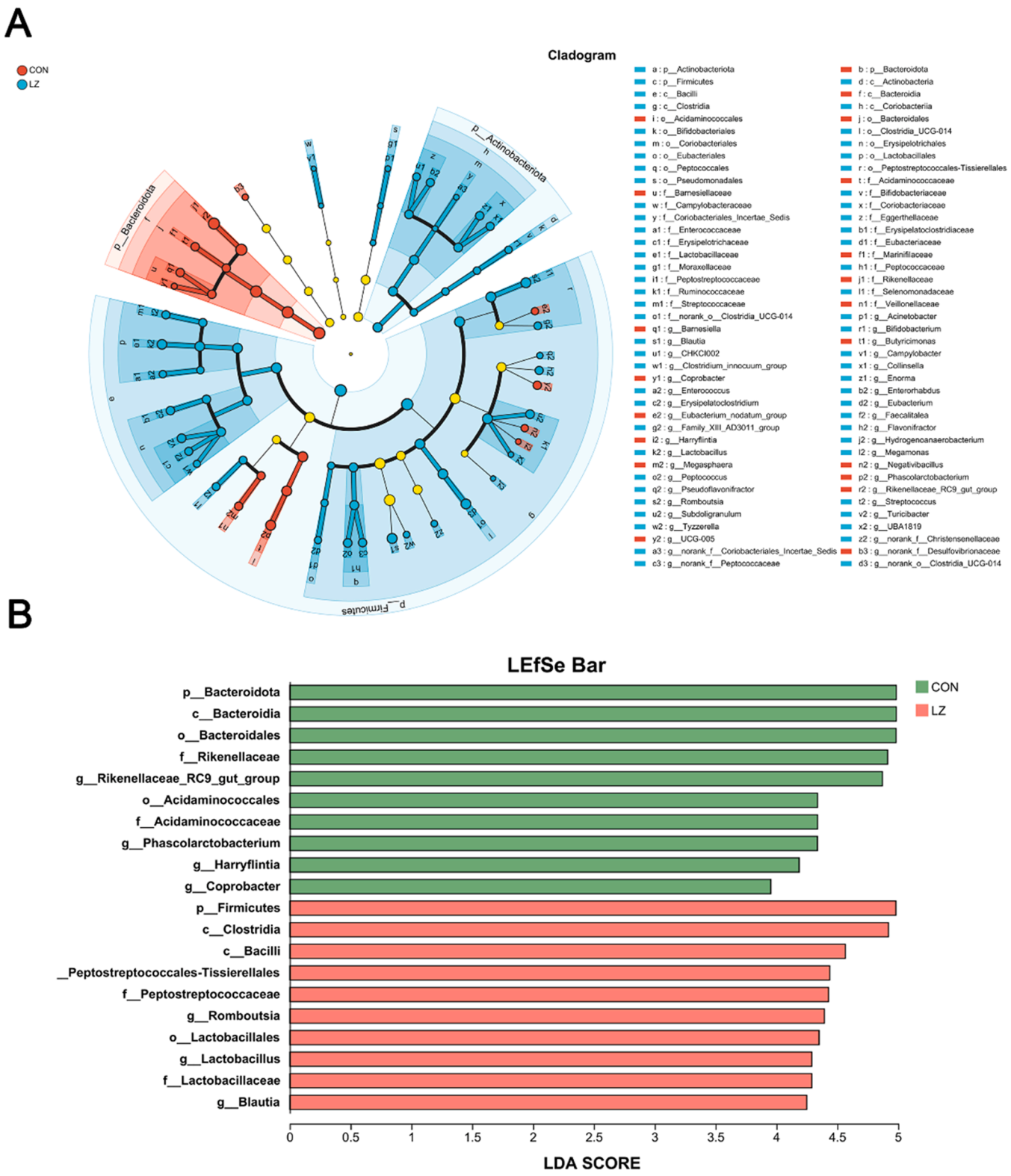

Fig. 10. Linear discrimination analysis (LDA) coupled with effect size (LEfSe) analysis of the cecal microbial community of broilers in the CON and LZ groups. (**A**) Cladogram showing microbial species with significant differences among the two treatment groups. Red and blue represent different groups. Species classification at the phylum, class, order, family, and genus levels are displayed from inner to outer layers. The red and blue nodes represent microbial species in the phylogenetic tree that play important roles in the CON and LZ groups, respectively. Yellow nodes represent no significant difference between species. (**B**) Significantly different species with an LDA score greater than the estimated value (default score = 3). The length of the histogram represents the LDA score of different species in the two groups.

study, dietary LZ supplementation improved the antioxidant status of broilers, primarily through increased SOD activity. Similar to the results of this study, Bai et al. (2017) demonstrated that supplementation of probiotics Bacillus subtilis fmbJ in broilers increased SOD and GSH-Px concentrations in serum and liver. Malondialdehyde (MDA) is a byproduct of lipid peroxidation and serves as a biomarker for oxidative stress, with elevated serum MDA levels indicating free radical-induced damage (Min et al., 2018). Our findings revealed a significant reduction in serum MDA levels in broilers fed LZ-supplemented diets. This aligns with previous studies reporting similar decreases in MDA levels in quails supplemented with lysozyme (Hassan et al., 2023; Sindaye et al., 2023). In conclusion, LZ increased serum antioxidant capacity of broilers.

Immune organs serve as primary sites for immune cell proliferation, development, differentiation, and antibody secretion (Sun et al., 2023). In avian species, the bursa of Fabricius, thymus, and spleen are critical immune organs that play essential roles in immune regulation (Guang et al., 2011). The immune organ index, which reflects the relative weight of these organs, is a key indicator for evaluating immune function. In this study, dietary supplementation with LZ improved the bursa of fabricius index in broilers, suggesting enhanced immune capacity. Serum immunoglobulins, including IgA, IgG (avian IgY), and IgM, are vital biomarkers of immune status (Wang et al., 2018). These immunoglobulins, produced by B cells, are critical parameters for assessing humoral immunity, as they play essential roles in immune defense and resistance against infections (Balan et al., 2019; Carlier et al., 2016; Zhang et al., 2017). Lysozyme is a key modulator of innate immunity and has been shown to mitigate intestinal inflammation (Long et al., 2016; Patel and Kuyucak, 2017; Ragland et al., 2017). Our results demonstrated that LZ supplementation significantly increased IgY levels. Furthermore, serum IgA and IgM concentrations were markedly higher in the LZ group compared to the CON group. An analogous result of increased globulin levels was obtained by documented rabbits fed LZ-supplemented diets (El-Deep et al., 2021). Consistent with our result, Xu et al. (2022) observed that dietary supplementation of coated lysozyme significantly increased serum IgM concentration in pigs. Our finding indicates that adding LZ to the diet improves the immune function of broilers.

In the immune and inflammatory response, cytokines play an important role (Praveena et al., 2010). Tumor necrosis factor-alpha and interleukin-1 beta are the important pro-inflammatory cytokines, which regulate the host immunity against multiple pathogens through immune cell differentiation, proliferation, apoptosis and NO production (Lee et al., 2013). However, excessive and long-term production of pro-inflammatory cytokines may reduce feed intake and increase energy expenditure, thus reducing the performance of livestock (Kwak et al., 2021). Tagashira et al. (2019) found that lysozyme suppresses production of TNF-α in mouse hyperinflammatory macrophages. Consistent with our research, the levels of TNF- α in serum and ileum mucosa were reduced after LZ supplementation, which indicates that LZ consumption decreased pro-inflammatory cytokines of broilers and improved immune efficiency. Furthermore, our results indicated that higher lysozyme levels, particularly at 500 mg/kg, increased the concentration of IL-6 in ileal mucosa. This is similar to the results of Zhou et al. (2020), where broilers activated an inflammatory response under ammonia exposure and increased serum IL-6. This suggests that LZ may inhibit the growth of harmful bacteria in the intestinal tract and exert immunomodulatory effects. However, further research is needed to fully elucidate the underlying mechanisms.

We further studied the expression of inflammatory pathway related genes in the ileal mucosa of broilers. The inflammasome, a multimeric protein complex consisting of nucleotide binding oligomerization domain-like receptor, *ASC*, and *Caspase-1* was shown to be important in the maturation and secretion of IL-1β and related family members (Wen et al., 2013; Zhou et al., 2011). In particular, the NLRP3 inflammasome has been extensively studied (Leemans et al., 2011). Upon detecting cellular stress, NLRP3 recruits ASC and procaspase-1, which results in caspase-1 activation and processing of cytoplasmic targets, including the proinflammatory cytokines IL-1β and IL-18 (Zhou et al., 2011). Differently with the mammals, ASC is not expressed in chickens (Shi et al., 2022, 2023). The results indicated that the addition of LZ significantly increased the concentrations of *NLRP3* and *Caspase-1*, suggesting that LZ treatment activated the NLRP3 inflammasome to a certain extent. However, the changes in the expression levels of IL-1β and IL-18 were not completely synchronous with them. In the LZ250 group, the expression levels of IL-1β and IL-18 showed a decreasing trend, while in the LZ500 group, they showed an increasing trend, but neither achieved a statistically significant difference. According to previous research, the moderate activation of the NLRP3 inflammasome enhances the body's resistance to potential pathogens and maintains the balance of intestinal microbiota (Bauer et al., 2012; Menu and Vince, 2011). Moreover, the pre-activation of inflammatosomes rapidly enhances the poultry response ability to pathogens. The levels of IL-1β and IL-18 did not increase simultaneously, possibly due to the rate-limiting effect of Caspase-1 activation (Bauernfeind et al., 2009). Nuclear factor kappa-B is the upstream signaling pathway to trigger NLRP3 inflammasome. We found that LZ markedly increased the levels of *MYD88* and *IRAK4*. Activation of TLR4 can activate NF-κB, cause the synthesis and secretion of proinflammatory cytokines, and further amplify the inflammatory response (Liu et al., 2022). Lysozyme at 500mg/kg significantly upregulated the levels of *TLR4* and NF-κB. The activation of the NF-κB pathway further activates the NLRP3 inflammasome pathway, enhancing the immunity of broilers.

Intestinal volatile fatty acids play a crucial role in nutrient metabolism, immune regulation, and the modulation of gut microbiota (Chambers et al., 2015). Our study found that LZ supplementation significantly increased VFA levels. These results are consistent with previous findings reporting elevated VFA levels following mixed organic acids administration (Ma et al., 2021). Specific analysis of our results showed that compared with CON group, adding LZ significantly increased the levels of propionic acid, isobutyric acid and isovaleric acid. Similarly, Lan et al. (2021) observed increased acetic acid, propionic acid, butyric acid, valeric acid, and isovaleric acid in response to α-glyceryl monolaurate supplementation in 28 days of broilers. In contrast, Xu et al. (2022) in pigs reported no significant change in isobutyric acid levels in the colon contents of different treatment groups after the addition of lysozyme, although valeric acid levels were significantly higher in specific groups. The variations may be due to differences in LZ dosage and animal feeding protocols. In summary, lysozyme increase VFA levels in the intestine of broilers under certain conditions.

Finally, we demonstrated that LZ regulate the intestinal microflora of broilers and increase the abundance of beneficial bacteria. The gut microbiota makes an important contribution to the health of the host, participating in nutrient absorption, defense against pathogenic bacteria, and regulation of immune function (Cheng et al., 2019; Heiss and Olofsson, 2018; Pickard et al., 2017). The cecum is the site of most abundant and concentrated intestinal flora (Xu et al., 2021). Our results obtained from NMDS conducted on cecal content revealed a degree of diversity discrepancy in cecal microbiota. Similar outcomes were achieved in studies by Zou et al. (2019). From the intestinal flora Sobs index and Ace index of broilers showed that lysozyme formed intestines the abundance of bacteria is higher, and it is more beneficial to body health and immune system regulation in diversification. In addition, in the present study, alpha diversity analysis showed lysozyme significantly decreased the indices of microbiota community diversity. This is similar to the effect of lysozyme on weaned piglets, and studies by Xu et al. (2022) show that the addition of lysozyme improved the intestinal microflora of weaned piglets and increased the abundance of beneficial bacteria. *Firmicutes* are a group of gram-negative anaerobic organisms that can colonize in chicken cecals, and involve in the breakdown of otherwise indigestible (by the host) polysaccharides such as resistant

starch and cellulose (Allen and Stanton, 2014; Stanley et al., 2012). Our results show that the most dominant phylum was *Firmicutes*, followed by *Bacteroidota* and *Actinobacteriota*. The results are consistent with those of a previous report by Shi et al. (2019). Previous studies have shown that *Firmicutes, Bacteroidota*, and *Actinobacteria* were the dominant flora of broilers cecum microbiome (Xiao et al., 2017), which is agreement with our study. This result indicates that LZ supplementation enhances the absorption of nutrients in the intestines of broilers. At the genus level, we found that the abundance of *Lactobacillus* and *Romboutsia* increased and *Rikenellaceae_RC9_gut_group* deceased in the LZ group. The increase in *Lactobacillus* and *Romboutsia* was consistent with the results of Goel et al. (2022). While the decrease in the abundance of *Rikenellaceae_RC9_gut_group* is consistent with the results of Wu et al. (2022). *Lactobacillus* is a beneficial commensal for humans and animals, which could inhibit pathogenic bacteria and is highly related to host feed efficiency (Kobierecka et al., 2017; Yan et al., 2017). *Romboutsia* is a short-chain fatty acid-producing bacterium, positively correlated with indicators of body weight and serum lipids. Short chain fatty acids play crucial roles in maintaining intestinal physiology. They help preserve the integrity of the intestinal mucosa and protect the intestine from inflammatory damage (Bartolomaeus et al., 2019; Wang et al., 2022). *Rikenellaceae_RC9_gut_group* was involved in structural carbohydrate degradation (Si et al., 2021). However, it also has been proven that an increase in the abundance of *Rikenellaceae_RC9_gut_group* was positively associated with intestinal permeability and oxidative stress, subsequently impairing the intestinal barrier function and stimulating gut inflammation (Shen et al., 2022). The increase in the abundance of *Lactobacillus* and *Romboutsia* and the decrease in the abundance of *Rikenellaceae_RC9_gut_group* indicate that the balance of intestinal flora in broilers has been improved. Meanwhile, it enhances the absorption rate of nutrients by broilers, improves antioxidant function and reduces the occurrence of inflammation. Therefore, supplementing LZ in diet enhanced the richness of the intestinal flora, increased the abundance of beneficial bacteria, and reduced the abundance of harmful bacteria, thereby improving the growth performance and health status of broilers.

Conclusion

In conclusion, supplemented with LZ improved growth performance, gut barriers, antioxidant capacity, immune functions, and regulated intestinal flora, increased the abundance of intestinal beneficial bacteria and volatile fatty acid content.

CRediT authorship contribution statement

Liuyi Chen: Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Wenwen Peng:** Visualization. **Tao Wang:** Investigation. **Shiyue Ma:** Methodology. **Xiuxi Wang:** Software. **Bing Dai:** Validation. **Ruiqiang Zhang:** Funding acquisition, Project administration, Resources. **Caimei Yang:** Conceptualization, Project administration, Resources, Supervision. **Yanping Wu:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Disclosures

On behalf of all authors, we declare the following financial and personal relationships that may lead to a potential conflict of interest:

None of the authors have any competing interests to declare, including but not limited to employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, grants, or other funding.

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