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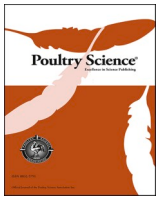

Full-Length Article

Effects of rhamnolipids on growth performance, gut barriers, antioxidant capacity, immune function, and gut microbiota in broiler chickens

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ABSTRACT

This study aimed to investigate the effects of dietary supplementation of rhamnolipids (RLS) on growth performance, gut morphology, antioxidant function, lipid metabolism, immune responses, and gut microbiota in Linnan yellow-feathered broilers. A total of 390 one-day-old broilers were randomly assigned to three groups: Control (CON), 250 mg/kg RLS (RLS250), or 500 mg/kg RLS (RLS500). The broilers were fed with basal diet, or basal diet supplemented with 250 or 500 mg/kg RLS for 56 days. Each treatment contained 10 replicates with 13 chickens per replicate. Results showed that diet supplemented with RLS250 and RLS500 markedly improved final BW and ADG on day 56. RLS reduced the CD and increased the VH/CD of jejunum and ileum. RLS significantly increased the levels of total T-AOC and GSH-Px on day 28, while the levels of T-AOC and SOD were higher than CON groups on day 56. RLS treatment also regulated the lipid metabolism in the broilers by increasing the concentration of serum HDL-C and decreased LDL-C. The levels of serum immunoglobulins including IgA, IgM and IgY in RLS250 and RLS500 groups were notably higher than those of CON groups on day 56. Meanwhile, ileum IgA and IgM in RLS500 groups were evidently higher than other two groups. RLS significantly increased the level of IL-10. RLS showed no significant effects on VFA in cecum of broilers. Results of 16S rRNA sequencing showed that RLS optimized the microbiota by lowering the relative abundance of *Anaerofilum* and *DUT089*, and regulating *g_norank_f_ Ruminococcaceae*. This study found that supplemented with RLS in diet improved the growth performance, antioxidant function, and immune function, and regulated intestinal microbiota of broilers, revealing that RLS is potential feed additive for use in animal husbandry.

Introduction

Due to the immaturity of the immune system, broilers are vulnerable to the infestation of pathogenic bacteria included *Salmonella*, and *Clostridium perfringens*, which decrease the growth performance of broilers (Meijerink et al., 2022; Awad et al., 2023; Zhang et al., 2023). Over the past decade, antibiotics has been used as health and growth promoters in chickens to protect against the infestation from pathogenic bacteria (Al-Mnaser et al., 2022). Antibiotics play an important role in increasing growth performance, raising feed utilization and reducing the mortality. However, continued use of antibiotics may lead to antimicrobial resistance in pathogenic bacteria, posing a threat to animal and human health (Hedman et al., 2020). In this context, many countries have limited or banned the use of antibiotics in feed due to the drug resistance and residues in livestock product. Nevertheless, in the poultry industry, decreasing the use of antibiotics increased the incidence of important disease and poultry which reared on a large scale system are more likely to develop microbial infections (Caly et al., 2015; Rafiq et al., 2021). One study showed in the conventional system, broilers fed with no antibiotics suffered from necrotizing enteritis (NE), while 27 % of drug-free broilers had clinical NE and 49 % had subclinical NE, which causing great harm to the poultry industry (Gaucher et al., 2015). Therefore, it is urgent to search for alternatives to antibiotics in poultry industry (Zhu et al., 2021).

Recent studies showed that glycolipids can be as potential antibiotic alternatives in animal industry (Plouguerne et al., 2014). Glycolipids are

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Table 1
Ingredients and nutrient levels of the basal diets (Air dry basis, %).

Items	
Ingredients (%)	
Corn	54.40
Soybean meal	23.60
Extruded soybean	5.00
Rice distiller's grains	5.00
Soybean oil	2.20
Limestone	1.30
Fermented soybean meal	2.50
High grade corn gluten meal	2.00
[1]Premix[1]	4.00
Total	100.00
Nutrient levels	
ME (kcal/kg)	2983.00
CP	20.40
Lys	1.18
Met	0.55
Met+Cyst	0.90
Trp	0.22
Tyr	0.88
Ca	0.86
TP	0.59

[1] Premix provided per kilogram of diet: vitamin A (trans-retinyl acetate), 1 500 IU; vitamin D_3 (cholecalciferol), 200 IU; vitamin E (all-rac-α-tocopherol acetate), 10 IU; menadione, 35 g; thiamin, 1.5 mg; riboflavin, 3.5 mg; pyridoxine·HCl, 3 mg; vitamin B12 (cobalamine), 10 μg; Pantothenic acid, 10 mg; niacin, 30 mg; biotin, 0.15 mg; choline chloride, 1 000 mg; Fe, 80 mg; Cu, 8 mg; Mn, 60 mg; Zn, 40 mg; Se, 0.15 mg; I, 0.18 mg.

glycosyl derivatives of lipids which exist in all living organisms and driving diverse biological functions (Thakur et al., 2021; Jala et al., 2022). Rhamnolipids (RLS) are kinds of glycolipids that are composed of one or two rhamnose molecules and one or two fatty acid alkyl chains by a beta-glycosidic bond (Majik et al., 2013). RLS are mainly produced by *Pseudomonas aeruginosa*, and the biosynthesis pathway of rhamnoids in *Pseudomonas aeruginosa* mainly includes biosynthesis of the fatty acid moiety, biosynthesis of the rhamnose moiety, and the enzymatic dimerization between the fatty acid moiety and the rhamnose moiety (Chen et al., 2017). RLS have excellent thermal and chemical stability, which help to maintain their characteristics in production and transportation (Karnwal et al., 2023). Accumulating studies have proved that RLS have a wide range of applications. They can be as a kind of biosurfactant used in petroleum industry and environmental remediation to reduce the surface tension and improve the emulsification due to their properties of both ydrophobic and hydrophilic moieties emulsification (Gong et al., 2021). Meanwhile, nontoxicity and high biodegradability make sure that RLS apply in pharmaceutical industry and processing industry (Karnwal et al., 2023). RLS can be used as antitumor drug. For instance, Di-rhamnolipids produced from PAB189 impeded the proliferation of breast cancer cell line MCF-7, recognized the cytoskeleton of phagocytic/non-phagocytic cells and changed their morphology (Thanomsub et al., 2007). As biosurfactant, RLS are applied in cosmetics and detergent producing industries due to the characteristic of reducing the surface tension (Mao et al., 2015).

The US Environmental Protection Agency (EPA) and FDA have confirmed RLS as a generally recognized as safe (GRAS). RLS have a broad spectrum of antibacterial activity and can be used as an alternative to prevent contamination of food-borne pathogens in the food industry (Maier and Soberon-Chavez, 2000). RLS have the ability to inhibit the growth of gram-positive and gram-negative bacteria, including *Staphylococcus spp., Enterococcus spp.* and *Escherichia spp.* (Benincasa et al., 2004; Diaz et al., 2016; de Freitas et al., 2019). Furthermore, RLS also have broad-spectrum antifungal activity including Alternaria alternata fungus and dimorphic fungi (Crouzet et al., 2020). Previous research in our lab has carried out several animal tests on mice, rats and pigs. These results had demonstrated that RLS are safe, no toxic feed additives on animals and have the functions of promoting growth and improving animal immunity, indicating RLS can be used as a potential feed additive to replace antibiotics. Therefore, this study aimed to evaluate the beneficial effects of RLS on broiler chickens.

Materials and methods

Animals and dietary treatments

All experimental design and procedures were strictly followed the Guidelines for the Care and Use of Laboratory Animals of Zhejiang Agriculture and Forestry University (ZAFUAC202473). Broiler management followed Aviagen's recommendations. The basal diet was formulated according to NRC standards, and the formula and nutrient concentration are shown in Table 1(NRC, 1994).

A total of 390 one-day-old male Linnan yellow-feathered broilers were randomly divided into three groups with 10 replicates per group and 13 broilers per replicate. The birds were fed as follows: basal diet with no supplement (CON), basal diet with 250 mg/kg RLS (RLS250) and basal diet with 500 mg/kg RLS (RLS500). The RLS used in this experiment were provided by Zhejiang Vegamax biological Technology Co. Ltd. (Hangzhou, China). The feeding period lasted for 56 days. During the trial, the chickens were kept in self-feeding, self-replenishing 3-layer cages with unlimited feed and water. At the first week, the temperature of the cage was controlled at 35 ±1 °C and reduced by 3 °C per week until the temperature reached 26 ± 1 °C. In the first week, the light exposure in the cage was 24 h, which was reduced by 2 h per week to 18 h, and maintained for 18 h until the end of the experiment.

Sample collection

On day 28 and day 56, one broiler chicken was taken from 10 repetitions in each groups randomly. After weighing, blood samples were collected from the jugular vein of each broiler. The serum was separated by centrifuging at 3000 g for 10 min at 4 °C and stored at -80 °C for subsequent analysis. Humanely killed the broilers by cervical dislocation after collecting the blood sample. The liver, jejunum, ileum, cecal contents were isolated, immediately freezed in the dry ice, stored at -80 °C. Moreover, part of jejunum and ileum were taken, gently washed with PBS, fixed in 4 % formaldehyde and stored at 4 °C.

Growth performance

Growth performance of broilers was evaluated by body weight (BW), average daily gain (ADG), average daily feed intake (ADFI) and the feed conversion ratio (FCR). After fasted for 2 h on the day 28 and 56, the BW and feed intake of broilers were measured, and the ADG, ADFI and FCR of the broilers were calculated.

Organ index

The immune organ such liver, spleen, thymus and bursa of fabricius were collected and weighted, the immune organ indexes were calculated as follows: immune organ weight (g) / BW (g) *100.

Intestinal morphological analysis

Jejunum and ileum tissue samples were immobilized in 10 % formalin buffered saline for at least 24 h. And the sample were dehydrated, impregnated with xylene, embedded with paraffin, sliced, and stained with hematoxylin and eosin (H&E). The structures were observed by optical microscope. Villus height (VH) and Crypt depth (CD) were measured at 10 visual fields of each intestinal sample in each group and the villus height/crypt depth radio (VH/CD) was calculated

Table 2
Effects of dietary supplementation of RLS of growth performance in broilers.

Items	CON	RLS250	RLS500	P-value
BW(g)				
1d	31.72±0.037	31.69±0.051	31.76±0.030	0.461
28d	702.76±2.410	741.30±21.602	736.16±18.539	0.233
56d	1942.28 ±4.954[b]	2041.69 ±6.754[a]	2052.54 ±27.593[a]	<0.001
ADG (g/d)				
1-28d	23.97±0.087	25.34±0.772	25.16±0.661	0.232
28-56d	44.27±0.240	46.44±0.992	47.01±1.023	0.079
1-56d	34.12±0.089[b]	35.89±0.120[a]	36.09±0.493[a]	<0.001
ADFI(g/d)				
1-28d	40.82±0.445[b]	42.80±0.347[a]	42.93±0.181[a]	<0.001
28-56d	100.39±1.994	102.16±1.202	102.96±1.974	0.584
1-56d	70.60±1.194	72.48±0.720	72.94±1.046	0.251
FCR				
1-28d	1.63±0.019	1.63±0.060	1.64±0.040	0.975
28-56d	2.28±0.030	2.22±0.031	2.21±0.027	0.199
1-56d	2.08±0.026	2.03±0.012	2.03±0.019	0.189

[a,b] Mean with different superscripts in the same row differ significantly ($P < 0.05$). All values are expressed as mean ± SEM (n = 13 chickens/group/time-point) Abbreviations: CON, basal diet; RLS250, basal diet supplemented with 250 mg/kg RLS; RLS500, basal diet supplemented with 500 mg/kg RLS.

Serum parameter analysis

The biochemical parameters including the total antioxidant capacity (T-AOC), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), malondialdehyde (MDA), immunoglobulin (Ig) A, IgY, IgM, interleukin (IL)-1β, IL-6, IL- 10, and tumor necrosis factor-α (TNF-α) were assayed using specific ELISA kits from jinhengnuo (Hangzhou, China).

According to the manufacturer's instructions, the lipid metabolism parameters such as total cholesterol (TC), triglyceride (TG), low density lipoprotein cholesterol (LDL-C) and heigh density lipoprotein cholesterol (HDL-C) were measured using assay kits from Nanjing Jiancheng Bioengineering Institute(Nanjing,Jiangsu, China).

Analysis of volatile fatty acids (VFA)

Following the previous study (Zhou et al., 2019), Headspace Sampling Gas Chromatography (Agilent Technologies, Beijing, China) was used to measure cecal VFA concentration. 1 g cecal content was mixed with 1 mL dd H_2O. After centrifugation, supernatant was dissolved into 25 % metaphosphoric acid (w/v, 5:1; Aladdin, Shanghai, China). VFA concentration analysis was analyzed by Agilent 1890 Network System equipped with a 30 m × 0.25 mm × 0.25 μm column and flame ionization detector.

16S rRNA Sequencing of microflora in cecum contents

Microbial DNA was extracted from cecal contents by using the E.Z.N.A. Soil DNA Kit (Omega Bio-tek, Norcross, GA) according to the manufacturer's protocols. The Illumina-Hiseq platform was used to explore the V4 region of the 16S rRNA gene. The hypervariable regions V3-V4 of the bacteria 16S rRNA gene were amplified with primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′);806R (5′-GGACTACHVGGGTWTCTAAT-3′) by T100 Thermal Cycler PCR system (BIO-RAD, Hercules, CA). The PCR products were extracted from 2 % agarose gel, purified according to the manufacturer's instructions (Yuhua, Shanghai, China), and quantified using Qubit 4.0 (Thermo Fisher Scientific, Waltham, MA). The resulting sequences were quality filtered with fastp (0.19.6) and merged with FLASH (v1.2.11). Taxonomic assignment of OTUs was performed using the Naive bayes consensus taxonomy classifier implemented in Qiime2 and the SILVA 16S. The RDP classification algorithm was used to analyze each gene sequence, and the confidence threshold was 70 %. The α diversity was determined by Mothur1.30.2. The β diversity was calculated using QIIME1.9.1, based on unweighted UniFrac distance, and displayed by principal coordinate analysis. Microbiota composition was performed using tax_summary and the R package (version 3.3.1). STAMP software (version 2.1.3) was used to detect the difference of microbiota composition in each group.

Statistical analysis

SPSS 22.0 software was used to conduct one-way ANOVA to analyze the influence of RLS addition on each index (SPSS Inc.). Using the Tukey-Kramer test determined the differences and $P < 0.05$ were considered statistically significant.

Results

Growth performance

As indicated in Table 2, the BW of broilers from RLS250 group and

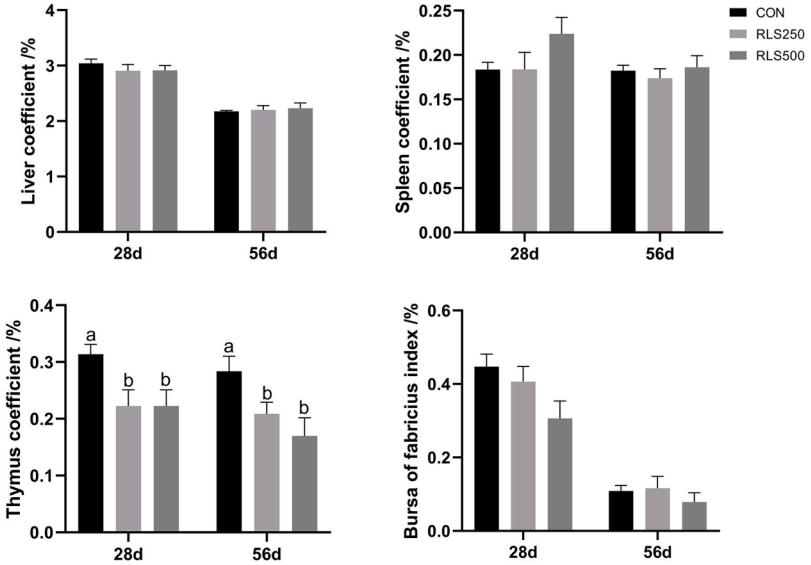

Fig. 1. Effects of dietary supplementation of RLS on organ index in broilers on day 28 and 56.
Abbreviations: CON, basal diet; RLS250, basal diet supplemented with 250 mg/kg RLS; RLS500, basal diet supplemented with 500 mg/kg RLS. Bars represent mean ± SEM (n = 6). Different lowercase letters (a, b) above bars represent significantly different means ($P < 0.05$).

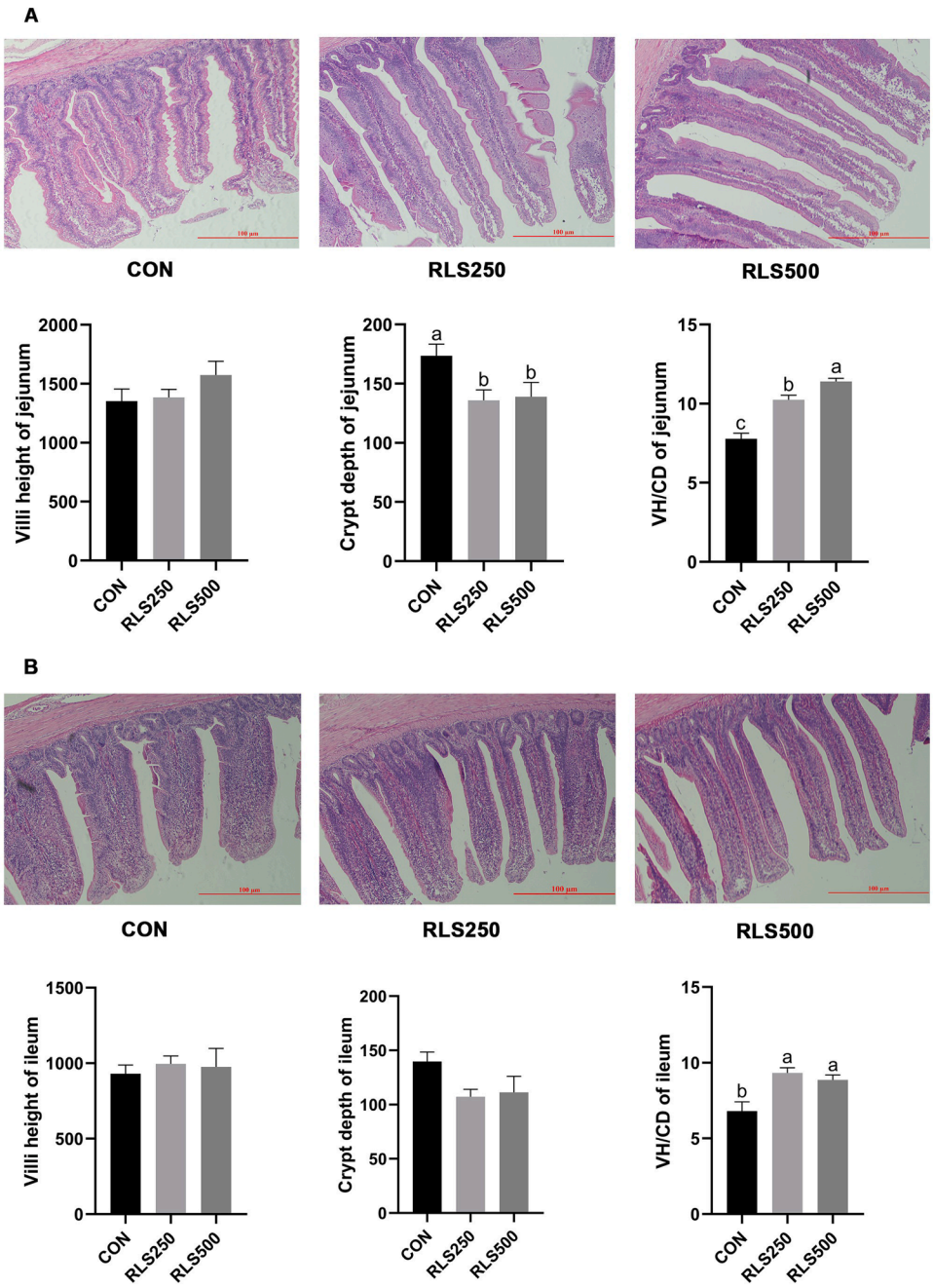

Fig. 2. Effect of the RLS on jejunum villus and ileum villus of broilers.
(A) Effect of the RLS on jejunum villus of broilers. (B) Effect of the RLS on ileum villus of broilers. Abbreviations: CON, basal diet; RLS250, basal diet supplemented with 250 mg/kg RLS; RLS500, basal diet supplemented with 500 mg/kg RLS. Bars represent mean ± SEM (n = 6). Different lowercase letters (a, b) above bars represent significantly different means ($P < 0.05$).

RLS500 group were significantly higher than those in CON groups on day 56 ($P < 0.01$), and showed no significance on day 28 ($P < 0.05$). The ADG of RLS treatment broilers on day 1-56 significantly higher than ADG of CON group ($P < 0.01$). RLS remarkedly increased the ADFI at the period of day1-28 compared with CON ($P < 0.05$). RLS had no noticeably effect on FCR on day 1-56 ($P > 0.05$).

Organ index

As shown in Fig. 1, supplementation of RLS in broiler diet significantly reduced the thymus coefficient on day 28 and 56 ($P < 0.05$), whereas had no statistical effect on other organ index ($P > 0.05$).

Intestinal morphological

The effected of RLS treatment on intestinal morphology are shown in Fig. 2. On day 56, supplement with RLS in diet shortened CD of jejunum ($P < 0.05$) and significantly increased the VH/CD of jejunum compared with CON group ($P < 0.05$). RLS also increased the VH/CD of ileum on day 56 ($P < 0.05$).

Serum antioxidant indexes and lipid metabolism

RLS had no statistical significance on MDA level ($P > 0.05$). RLS significantly increased T-AOC activity on day 28 and 56 ($P< 0.05$). Chickens fed with RLS displayed higher SOD activity in serum compared

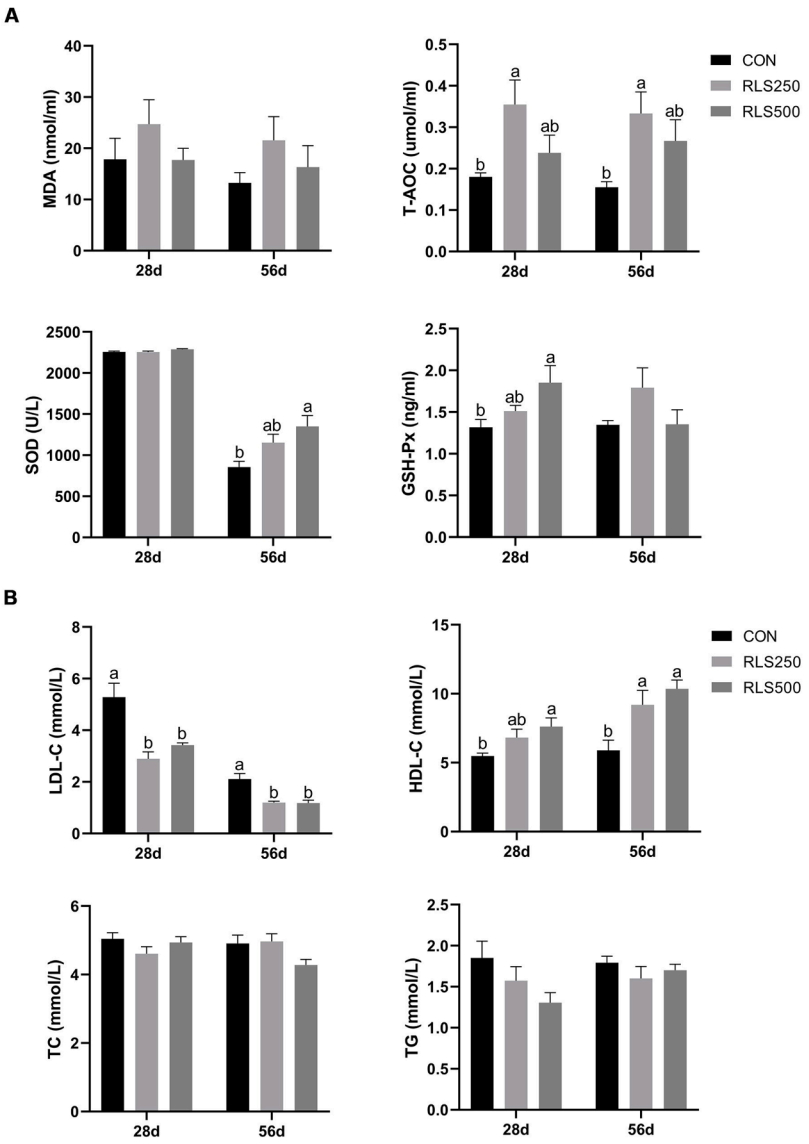

Fig. 3. Effect of dietary supplementation of RLS on serum antioxidant indexes and lipid metabolism parameters of broilers on day 28 and 56.
(A) Effect of dietary supplementation of RLS on serum antioxidant indexes of broilers on day 28 and 56. (B) Effect of dietary supplementation of RLS on serum lipid metabolism parameters of broilers on day 28 and 56. Abbreviations: CON, basal diet; RLS250, basal diet supplemented with 250 mg/kg RLS; RLS500, basal diet supplemented with 500 mg/kg RLS. Bars represent mean ± SEM (n = 6). Different lowercase letters (a, b) above bars represent significantly different means ($P < 0.05$).

to CON ($P< 0.05$) on day 56. And the increase of SOD increased with the addition of RLS in diets. RLS250 group had the highest activity compared with CON group and RLS500 group. In addition, RLS also remarkably increased the serum GSH-Px levels on day 28 ($P < 0.05$). We further detected the parameters of lipid metabolism, as shown in Fig. 3B, RLS treatment induced a remarkably increase in HDL-C ($P < 0.05$) and a significantly decrease in LDL-C ($P < 0.05$). Although serum TG level had reduced with the increase addition of RLS on day 28 but there are no significant difference among the three group ($P > 0.05$).

Immunoglobulin (Ig)

The effected of RLS treatment on the levels of serum and ileum Igs were shown in Fig. 4. Serum IgA, IgG and IgM contents of RLS treatment groups were markedly higher than that of CON group on day 56 ($P < 0.05$). Ileum Igs had different trends compared with serum Igs. On day 28, ileum IgA of RLS250 group had increased but the difference was not significant compared with the CON group ($P > 0.05$). And on day 56, the ileum IgA level of RLS500 group was significantly higher than CON group and RLS250 group ($P < 0.05$). Similarly, ileum IgM level of RLS500 group on day 56 was significantly higher than that of CON group ($P < 0.05$), and there was no statistical difference in ileum IgY ($P > 0.05$).

Inflammatory cytokines

As shown in Fig. 5, compared with CON group, RLS250 group had significantly higher serum IL-10 on day 56 ($P < 0.05$). Beyond that, supplemented with RLS had no significant difference in other serum immune factors ($P > 0.05$). On day 28, RLS treatment significantly reduced ileum IL-1 ($P < 0.05$). RLS reduced the level of ileum IL-6 but there was no statistical significance ($P > 0.05$). Supplemented with 250 mg/kg RLS could increase ileum IL-10 level ($P < 0.05$), while RLS500 group decreased the level of ileum IL-10. On day 56, the ileum IL-1and IL-6 level of RLS250 group lower than the level of CON group but there had no significant influence ($P > 0.05$).

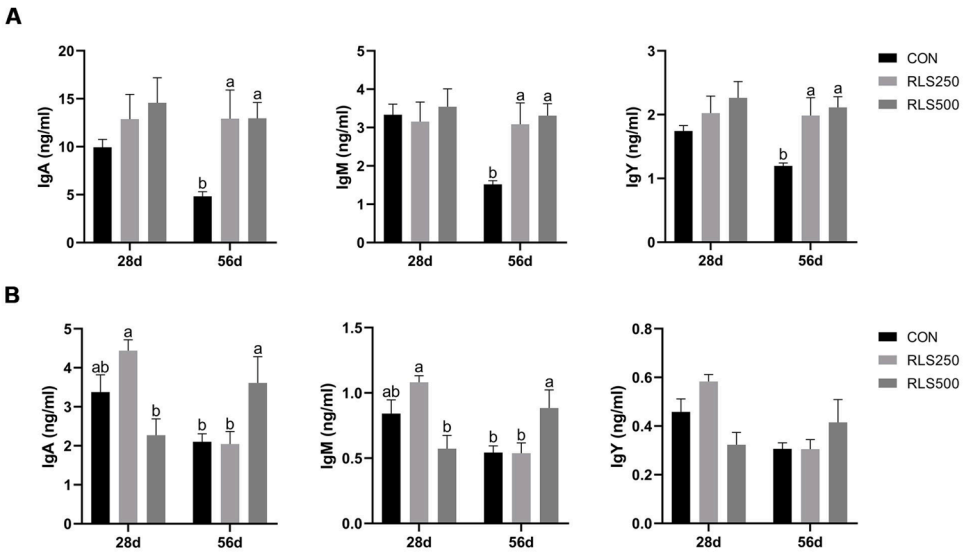

Fig. 4. Levels of Ig in the serum and ileum of broiler chickens given experimental diets on day 28 and 56.
(A) Serum Ig level of broiler chickens given experimental diets on day 28 and 56. (B) Ileum Ig level of broiler chickens given experimental diets on day 28 and 56. Abbreviations: CON, basal diet; RLS250, basal diet supplemented with 250 mg/kg RLS; RLS500, basal diet supplemented with 500 mg/kg RLS. Bars represent mean ± SEM (n = 6). Different lowercase letters (a, b) above bars represent significantly different means ($P < 0.05$).

Volatile fatty acid

The effected of RLS treatment on cecal VFA was shown in Fig. 6. Compared with CON group, supplemented RLS in diet could affect the concentration of cecal short-chain fatty acids. At low concentration, feed RLS decreased the content of acetic acid and butyric acid on day 56 ($P < 0.05$). This trend could be reversed after increasing the amount of supplementation. Whenever on day 28 or day 56, there had no significant influence on propnonic acid, Isobutyric acid, lsovaleric acid and valeric acid ($P > 0.05$).

Cecal microbial community

The abundance and diversity of the broilers cecal microorganisms were obtained using data from 16S rRNA highthroughput sequencing on day 56. Fig. 7 showed that a total of 518 OTUs were shared among the 3 treatments. There were 2200 unique OTUs in the CON groups, RLS250 group and RLS500 group had 2081 OTUs and 2138 OTUs respectively. Alpha diversity in RLS groups had no difference compared with CON group. Fig. 8 showed the TOP3 dominant phyla were *Bacteroidota, Firmicutes* and *Actinobacteriota* in the three groups. The relative abundance of *Proteobacteria* in the RLS250 group was significantly lower than CON group ($P < 0.05$). *Bacteroides, Alistipes, Prevotellaceae_NK3B31_group, unclassified_f_Lachnospiraceae, Romboutsia, Blautia, Faecalibacterium* and *Ruminococcus_torques_group* were the main genera a in the three groups at the genus level. The relative abundance of *DTU089* and *Anaerofilum* were obviously reduced after supplementing RLS in diet ($P < 0.05$). In addition, compared with CON group, RLS treatment could change the relative abundance of *g_norank_f_Ruminococcaceae*, decreased when supplemented in small amounts and increased in large amounts. At the species level, The *g_Rikenellaceae_RC9_gut_group* were only found in RLS500 group . The relative abundance of *s_unclassified_g_DTU089, s_unclassified_g_Oscillibacter, s_unclassified_g_norank_f_Eubacterium_coprostanoligenes_group, s_ unclassified _g_Lachnoclostridium*, and *s_uncultured_ bacterium_g_ Anaerofilum* were decreased with the addition of RLS ($P < 0.05$).

Disscussion

Chicken is one of most valuable domesticated animals in the world, and providing 30 % of meat products for human which plays a big part in global food security (Tan et al., 2024). Furthermore, with the increasing interest of consumers in healthy diet, antibiotic-free chicken consumption is increasing annually (Petracci and Cavani, 2012). Therefore, it is urgent to develop the nature and safe alternatives to antibiotics to improve the growth performance and meat quality of chickens. In our study, adding RLS to the feed significantly improved the growth performance, lipid metabolism, antioxidant indexes, immune function and regulate gut microbiota of broilers. The results showed RLS had the potential to be used as an alternative antibiotic as a promising feed additive in poultry production.

Our date showed that RLS increased the BW of the broilers on 56d, and ADG were also increased, which indicated RLS had the ability to promote growth performance of broilers. Similar to ours, studies have reported that adding glycolipids feed additives sophorolipid in broiler diets improved lipid digestion and absorption, and promote the growth performance of broiler chickens (Kwak et al., 2022).

Our results showed RLS improved gut barrier by decreasing CD of jejunum and ileum and improving the VH/CD. Study showed that VH and CD reflect the intestinal tract immediately and VH/CD is an important parameter for estimating the capacity of the intestine absorption (Wu et al., 2018; Cao et al., 2019). Longer villi help broilers to absorb nutrients and VH/CD reflect the strength of intestinal function. One study showed the birds fed with Sophorolipid had higher VH and VH/CD than CON group, enhancing villus turnover balance (Kwak et al., 2021). Our study revealed that RLS improved the capacity of intestinal absorptive, thereby improving growth performance of broilers.

Broilers are continuously subjected to oxidative stress because of different stress conditions, and with the onset of oxidative stress, metabolic disorders would contribute to the decrease of growth performance, diseases and even die (Lee et al., 2019; Oke et al., 2024). T-AOC is contented series of enzymes and other complex bio-molecules which can scavenge the free radical (Ghiselli et al., 2000). This study showed RLS significantly enhanced T-AOC in serum, which evidenced RLS played an important role in improving the activities of antioxidant enzyme. Antioxidant enzymes, include the SOD, which convert superperoxide anions (O^{2-}) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2); GSH-Px, which play an important roles in the system of free radicals inactivation, protect cell from oxidative stress and oxidant-induced regulate cell death (Bakhshalinejad et al., 2018; Chen

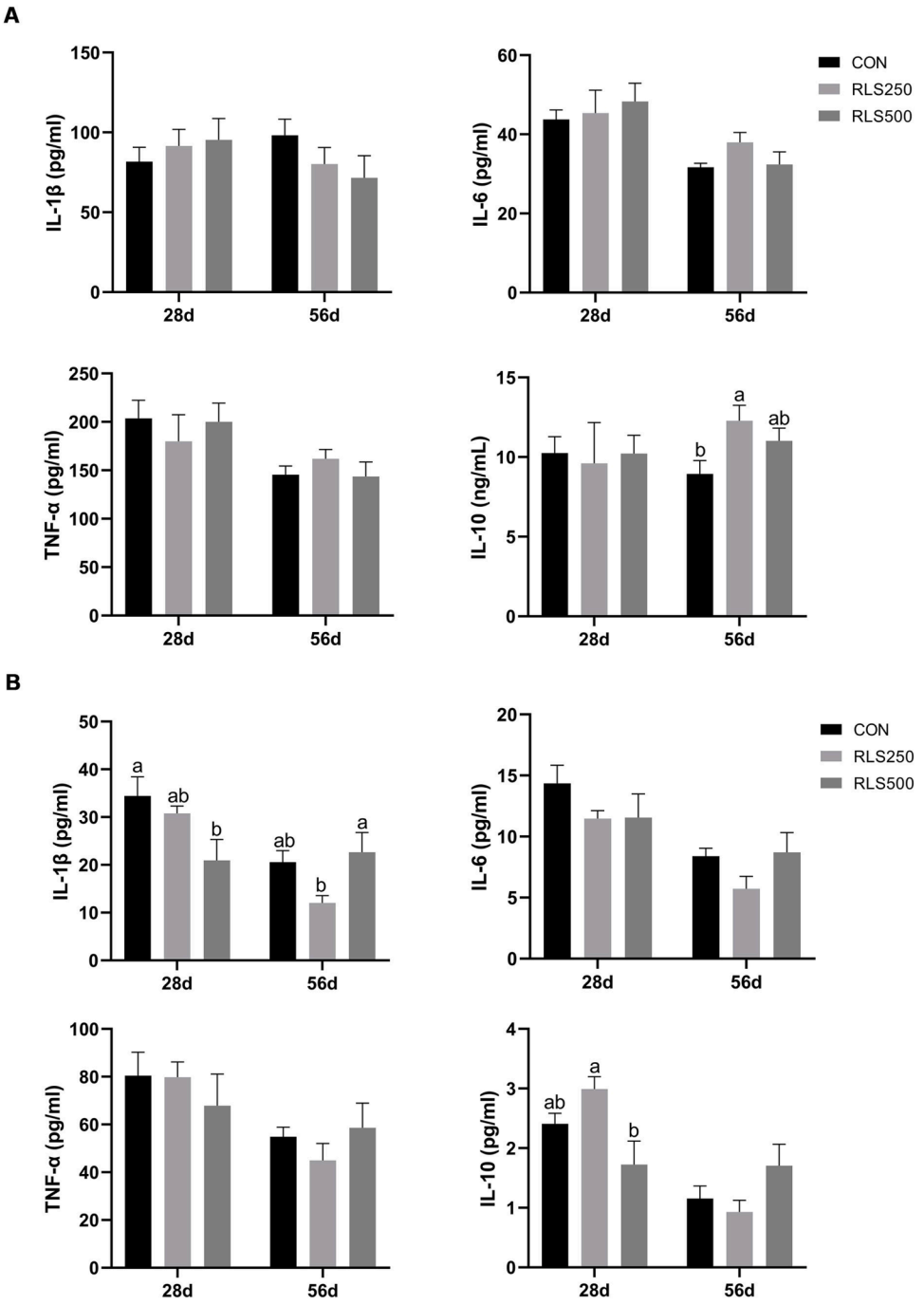

Fig. 5. Effect of dietary supplementation of RLS on serum and ileum inflammatory factors in broilers on day28 and 56.
(A) Effect of dietary supplementation of RLS on serum inflammatory factors on day28 and 56. (B) Effect of dietary supplementation of RLS on ileum inflammatory factors on day28 and 56. Abbreviations: CON, basal diet; RLS250, basal diet supplemented with 250 mg/kg RLS; RLS500, basal diet supplemented with 500 mg/kg RLS. Bars represent mean ± SEM (n = 6). Different lowercase letters (a, b) above bars represent significantly different means ($P < 0.05$).

et al., 2020). Our results exhibited that supplement RLS in diet enhanced serum activities of SOD and GSH-Px. Previous research demonstrated RLS had effective antioxidant activity as bio-surfactant (Ji et al., 2023). *In vitro* experiments, the antioxidant activity of RLS was due to the neutralization of free radicals by electron transfer and was related to the concentration of unsaturated fatty acids (Mouafo et al., 2021). In cherry tomato, RLS treatment could increase the SOD level and CAT level, which quenched the high ROS (Yan et al., 2016). Moreover, that study found RLS have the ability that promote convert the oxidized glutathione into glutathione, and intracellular ROS was decreased by this method. Therefore, we found that RLS significantly increased the activities of antioxidant enzymes, indicating it exerted a strong antioxidant capacity in broilers.

Adipose tissue plays an important role in energy-metabolism balance in organism and about 90 % body fat synthesis is mainly sited in the liver in the broilers (Ma et al., 2017; Nematbakhsh et al., 2021). Follow that, the fat transfer to adipose tissue from liver (Desert et al., 2018). Endogenous TG from feed carbohydrates are released in to blood as very low density lipoproteins (VLDL) and translate into LDL, which was related to the lipid accumulation in the vessel cells (Cui et al., 2018). HDL transport the free TC and the lipoproteins to the liver, playing a big part in removing the cholesterol and protecting the Dyslipidemia (Acton

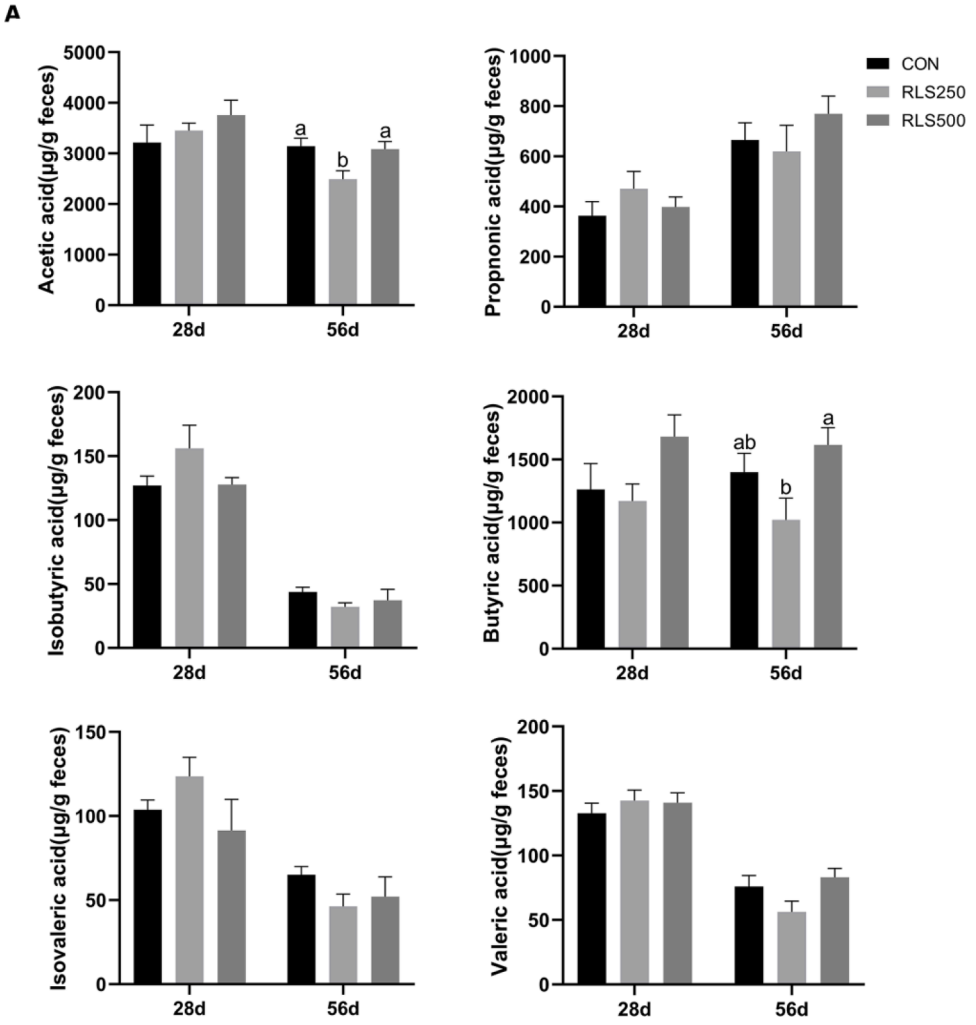

Fig. 6. Levels of volatile fatty acid in cecum contents of broiler chickens diets at day 28 and 56.
Abbreviations: CON, basal diet; RLS250, basal diet supplemented with 250 mg/kg RLS; RLS500, basal diet supplemented with 500 mg/kg RLS. Bars represent mean ± SEM (n = 6). Different lowercase letters (a, b) above bars represent significantly different means ($P < 0.05$).

et al., 1996). In our previous research, we found that RLS had the ability that improved the serum lipid metabolism of rat by reducing levels of TC, TG, and LDL-C and increasing the HDL-C and NEFA levels (Zhang et al., 2022). In our study, fed with RLS decreased the level of LDL and made the level of HDL higher, contributing to maintain the blood lipid balance and protecting the broilers from the disease of lipid metabolism.

Igs are associated with animal immune closely, which showed antibody activities bind specifically to the antigen (Zhang et al., 2021). The concentration of Igs can reflect the immune status of animals. Different Ig types make perform different immune functions in body. IgA belong among the major proteins found on mucosal surfaces of the intestinal tract and have the ability to protect mucosal surfaces (Bi et al., 2020). IgM plays important role in acute infection in broilers (Wang et al., 2023). High level of IgM contributes to protect broilers from pathogens. IgY, a kind of important circulating antibody found in birds, responsible for immune response function. IgY is used for prevention and treatment, detecting and neutralizing pathogens without activating the host's own immune system (Lee et al., 2021). RLS increased the level of IgA, IgM and IgY, detected and neutralized pathogens, improved the immune function of broilers mucosal surfaces, and ultimately boosted the immune performance of chickens. Inflammatory cytokines lead to inflammatory reactions, and could be classified as pro-inflammatory (IL-1β, IL-6, TNF-α) and anti-inflammatory (IL-10) (Zhang and An, 2007). Inflammatory responses protect the body by removing harmful physical or chemical stimuli, but excessive inflammation disturb the balance of immune function, leading to other inflammatory diseases (Pawelec et al., 2014). Previous studies reported that RLS activated the immune cell improving secretion of TNF-α (Andra et al., 2006). In this study, RLS improved the secretion of IL-1β, which helped chicken protect from harmful physical or chemical stimuli. IL-10 can ensure that protect host from over-exuberant responses to pathogens and microbiota. Our result showed RLS improved broilers secretion of IL-10 on day 56. Our study showed RLS treatment significantly increased the level of Igs and IL-10, decreased the level of IL-1, had the effect of promoting immune function and protecting the body from excessive inflammatory.

Gut microbiota plays an important role in maintaining gut homeostasis, and gut microbial. VFA are considered as tools which maintained gut homeostasis (Lee and Zhu, 2020). VFA provide maintenance energy for intestinal epithelial cells, lower the gut PH and inhibit the growth of certain harmful microorganisms by creating an environment which unsuitable for harmful bacteria (Lan et al., 2021). In our study, supplement RLS in diet had the tendency to promote the concentration of butyric acid in cecum, but showed no significance. Dietary RLS had no significant effect on cecal microbiota diversity of broilers, but significantly altered the composition of cecal microbiota. In genus level, RLS obviously lowered the relative abundance of *norank_f_Ruminococcaceae, DTU089* and *Anaerofilum*. *Anaerofilum* is a genus belonging to *norank_f_Ruminococcaceae*, which have ability to produce VFA for host (Wu

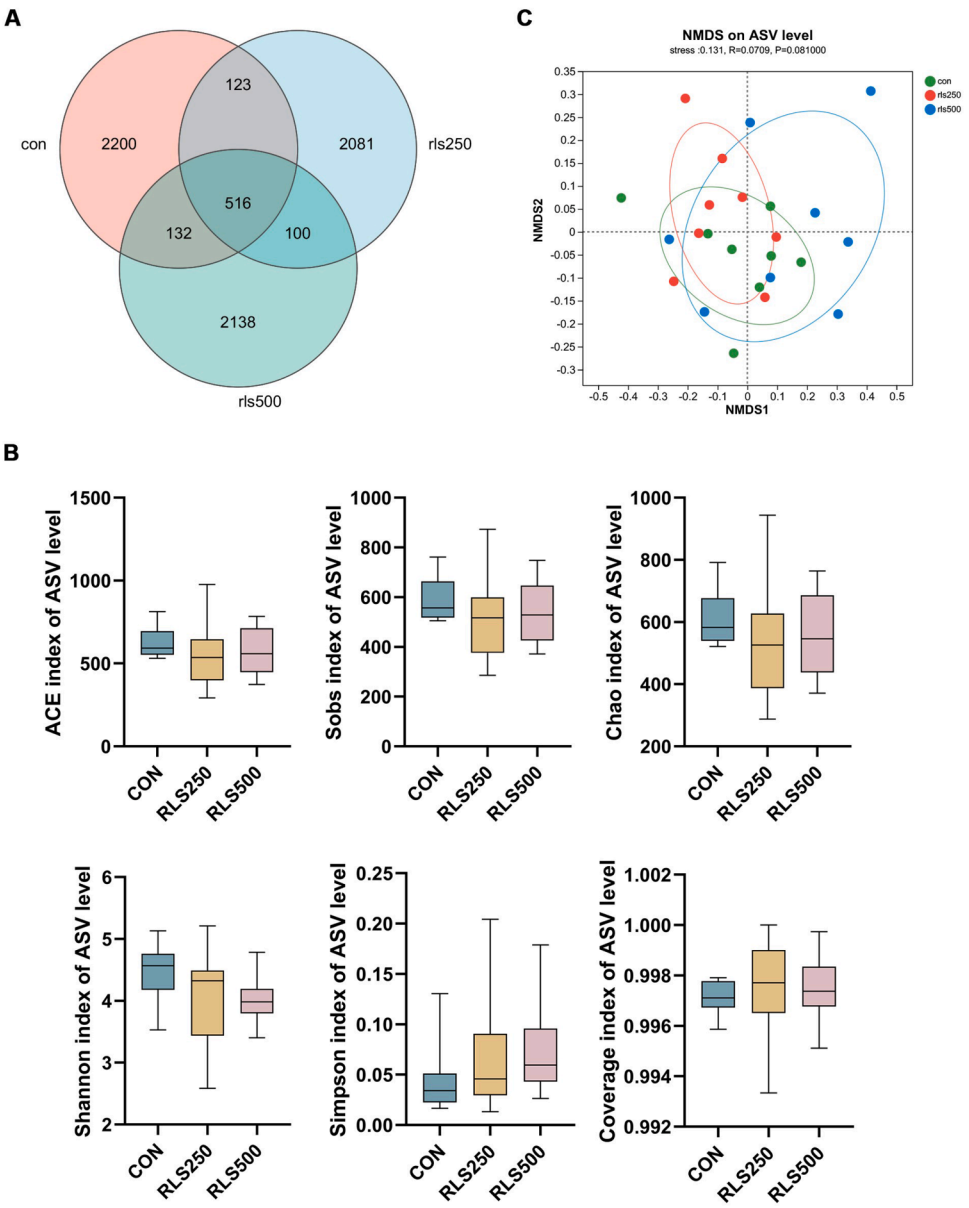

Fig. 7. Venn diagram, Alpha diversity and beta diversity of gut microbiota.
(A) Venn diagram. (B) Alpha diversity (C) non-metric multidimensional scaling analysis.Abbreviations: CON, basal diet; RLS250, basal diet supplemented with 250 mg/kg RLS; RLS500, basal diet supplemented with 500 mg/kg RLS. Bars represent mean ± SEM (n = 6). Different lowercase letters (a, b) above bars represent significantly different means ($P < 0.05$).

et al., 2022). This result could explain to some extent the decrease of VFA in cecum after RLS supplementation. But *Ruminococcaceae* family was found to be associated with non-small cell lung cancer (NSCLC) (Zheng et al., 2020). And in other study, genus *DTU089* had similarly association with NSCLC (Tesolato et al., 2024). Thus the reduction of *Ruminococcaceae* and *DTU089* had a better effect on broilers. In species level, RLS significantly reduced the relative abundance of *s_unclassified_g_Oscillibacter, s_unclassified_g_Lachnoclostridium*, and *unclassified_g_norank_Eubacterium_coprostanoligenes* while the relative abundance of *uncultured_bacterium_g_Rikenellaceae_RC9_gut_group* was increasing. The *Oscillibacter* is appeared as the top potential causal microbes (Newman et al., 2023). One study showed *Oscillibacter* promotes insulin resistance by increasing metabolic damage from mac rophages in fat tissue (Li et al., 2022). Other study showed an association of *Eubacterium_coprostanoligenes* with host lipid metabolism, which effected dyslipidemia through sphingosine (Wei et al., 2021). *Rikenellaceae_RC9_gut_group* is proved to play important roles in lipid metabolism by producing butyrate acid, increasing AMPK activity and regulating lipid deposition traits (Ahmad et al., 2020; Liu et al., 2023). Our results found RLS decreased the level of LDL and increased the level of HDL could be affected by *Eubacterium_ coprostanoligenes*, and regulated broilers lipid metabolism by *Rikenellaceae_RC9_ gut_group*. A recent research indicated that the level of *g_Lachnoclostridium* is negatively associated with antioxidant and immune capacity (Liu et al., 2022). The antigens or metabolic products derived from *g_Lachnoclostridium* could enhance T helper (Th) 1 and Th2 cells (Berer et al., 2018), whose excessive responses could lead to the inflammatory or autoimmune diseases (Sun et al., 2022). Therefore, supplement RLS in diet reduced the relative abundance of *g_Lachnoclostridium*, which might contribute to promoting immune function. RLS have the ability to reduce the relative abundance of harmful bacteria and promote probiotics growth.

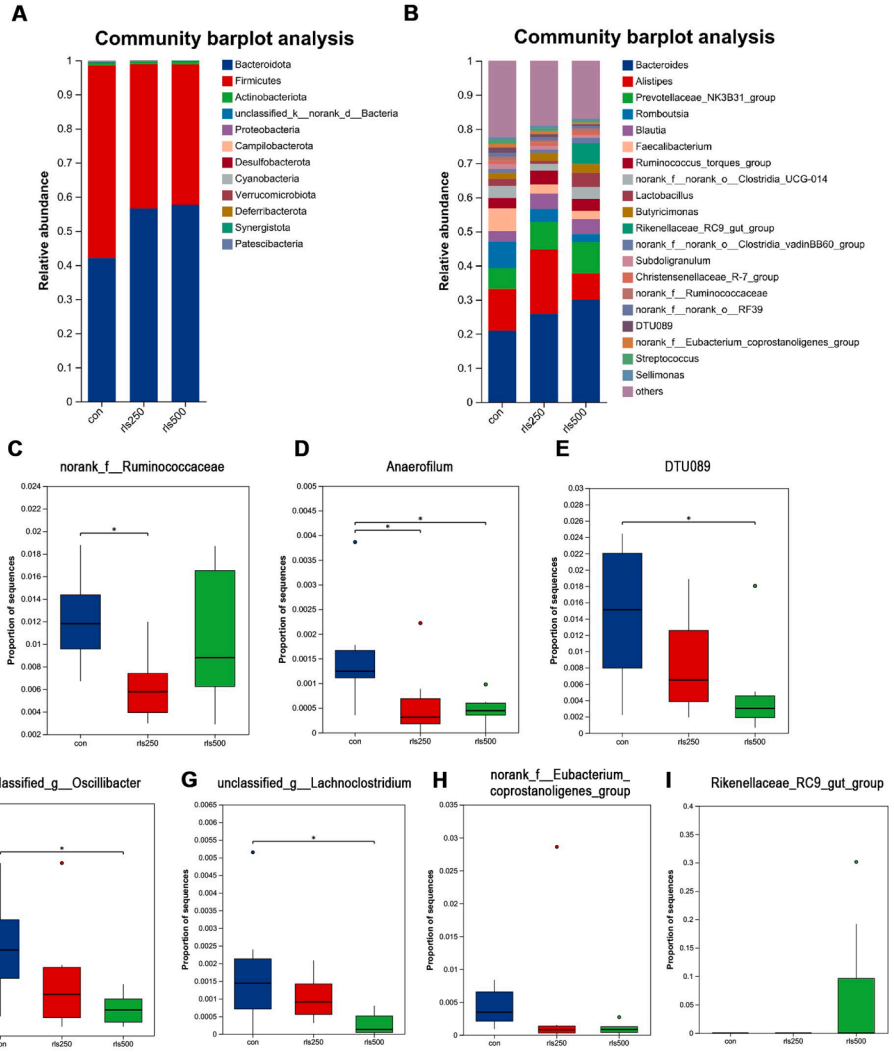

Fig. 8. Effect of Rhamnolipids on intestinal flora composition.
(A-B)The top 20 taxa by relative abundance(A:genus;B:species)(C-I) Species with significant inter-group differences (C, D, E: genus; F, G, H, I: species). Abbreviations: CON, basal diet; RLS250, basal diet supplemented with 250 mg/kg RLS; RLS500, basal diet supplemented with 500 mg/kg RLS. Bars represent mean ± SEM (n = 6). Different lowercase letters (a, b) above bars represent significantly different means ($P < 0.05$).

Conclusion

Supplement RLS improved growth performance and benefited the intestinal villus morphology in broiler chickens, as well as regulating host immune function and antioxidant capacity. It raised the relative abundance of beneficial bacteria in the cecum and promoted the proliferation of beneficial bacteria.

Declaration of competing interest

All authors declare no conflict of interests.

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