Effects of rhamnolipids on growth performance and intestinal health parameters in Linnan yellow broilers

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ABSTRACT This study determined the effects of dietary supplementation of rhamnolipids (**RLS**) on the growth performance, gut morphology, immune function, intestinal volatile fatty acid, and microflora community in Linnan yellow broilers. A total of 480 1-day-old broiler chicks were randomly assigned to groups for supplementation with one of the following for 56 d: no supplement (control), 30 mg/kg bacitracin (**ANT**), 500 mg/kg RLS, or 1,000 mg/kg RLS (**RLS2**). The RLS2 diet was found to improve the final BW and ADG on day 56. The RLS diet reduced jejunal crypt depth, increased jejunal villus length, and increased serum IgA, IgM, IgY, IL-1β, IL-6, and tumor necrosis factor-alpha (TNF-α) levels. The RLS broilers had higher cecum concentrations of acetic acid, propionic acid, butyrate, isobutyric acid, valerate, and isovalerate. High-throughput sequencing indicated that RLS affected microbial quantity and diversity in the cecum. Bacterial richness was higher in the RLS broilers than the ANT broilers. The RLS broilers had higher relative abundances of *Megasphaera hypermegale* and *Lachnospiraceae bacterium 19gly4* on day 28 and *Clostridium spiroforme* and *Alistipes obesi* on day 56. These results suggest that RLS supplementation improves growth performance, benefits the intestinal villus morphology, regulates host immune function, and raises intestinal volatile fatty acid content and the relative abundance of the gut microbiota in broiler chickens.

Key words: rhamnolipid, growth performance, intestinal health parameter, broiler

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INTRODUCTION

The commercial poultry industry consumes a wide range of antibiotics for disease prevention and growth promotion (Subedi et al., 2018). However, antibiotics abuse can lead to the development of antibiotic resistance and pose potential risks to the environment, public health, and food safety; the banning of antibiotics in feed has already begun (Vadalasetty et al., 2018; Suphoronski et al., 2019). Such bans have led to increased interest in alternatives for use in the livestock industry. Green and safe alternatives are increasingly already used in the breeding industry, as well as being a hot topic in research and development.

Rhamnolipids (**RLS**) are biosurfactants produced mainly by the pathogenic gram-negative bacterium *Pseudomonas aeruginosa*. Currently, they are approved for use in food products, cosmetics, and pharmaceuticals by the US Environmental Protection Agency (Soberón-Chávez et al., 2011). With respect to their pharmaceutical and therapeutic applications, RLS are nontoxic and have antimicrobial properties against pathogens such as *Staphylococcus aureus* and *Listeria monocytogenes* (Chen et al., 2017). The US Environmental Protection Agency exempted RLS from toxicity testing in poultry feed in 2004, suggesting that they could be used as feed additives. The RLS have antibacterial and antifungal properties (Haba et al., 2003; Benincasa et al., 2004). Studies have shown that RLS can affect the secretion of immune-related cytokines and stimulate the release of a large number of immune factors (Shryock et al., 1984; Kharazmi et al., 1989; König et al., 1992; Bedard et al., 1993; Piljac and Piljac, 1995).

The RLS have been generally applied in oil, the environment, cosmetics, food, and agriculture (Varvaresou and Lakovou, 2015; Mnif and Ghribi, 2016; Wolf et al., 2018; Li et al., 2019; Sancheti et al., 2019). However,

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there is no research on the application of RLS in livestock and poultry. The present study aimed to assess the effects of RLS on the growth performance, gut morphology, immune function, intestinal volatile fatty acids (**VFA**), and cecal microbiota in broiler chickens, aiming to demonstrate the potential of RLS as an antibiotic substitute for the poultry industry.

MATERIALS AND METHODS

Animals and Dietary Treatments

A total of 480 1-day-old broiler chickens were randomly assigned to 4 treatments. Each treatment group consisted of 8 pens, each containing 15 broilers. A basic diet designed to meet the nutritional requirements recommend by the NRC (1994) and Nutrient Requirements of Yellow-Feather Broiler (NY/T 33, 2004, China) and without any antibiotics (Table 1) was fed to all groups as follows: no supplement (**NCO**), 30 mg/kg bacitracin (**ANT**), 500 mg/kg RLS (**RLS1**), and 1,000 mg/kg RLS (**RLS2**) (Liu et al., 2020). The bacitracin and RLS were provided by Zhejiang Vegamax Biological Technology Co., Ltd. China. The RLS was absorbed onto silica to form a uniformly dispersed powdery solid. Rhamnolipids accounts for 50% of the compound formed by RLS and silica. The dose of RLS we added is its actual dose. The relevant food and fresh water were supplied ad libitum. This study was conducted as per the recommendations on the protection and utilization of laboratory animals of the Institutional Animal Care and Use Committee of Zhejiang Agricultural and Forestry University. The agreement was approved by the Ethics Committee of Zhejiang Agricultural and Forestry University, Hangzhou, China (SYXKzhe2016-087).

Growth Performance

Broilers BW (g) was measured on day 1, 28, and 56. The ADG (g/day), ADFI (g/day), and feed:gain (g/g) were directly calculated from the collected data.

Sample Collection

On day 28 and 56, eight broilers (1 broiler for every replicate) were chosen by average BW from the same treatment and slaughtered. Each blood sample was taken from the carotid artery and centrifuged (3,000 × *g*, 10 min) at 4°C. The serum was then separated and instantly stored at −80°C for further tests.

On day 28 and 56, eight broilers per treatment were selected for sacrifice. After slaughter, the jejunum segment (1 cm) located 5 cm behind the duodenum was collected and immediately fixed in a fresh 4% paraformaldehyde solution at room temperature. The cecal contents were gathered and immediately stored at −80°C for VFA analysis and high-throughput sequencing.

Serum Parameter Analysis

The concentrations of the serum factors IgA, IgM, IgY, IL-1β, IL-6, and TNF-α were estimated using a multifunction microplate reader and kits purchased from the Huamei Biological Engineering Research Institute, Wuhan, China. The experiment was conducted on the basis of the operating guide.

Jejunum Morphologic Analysis

The jejunum was fixed with 10% formaldehyde solution, routinely sampled, dehydrated, paraffin-embedded, sliced (4 μm), and stained with hematoxylin–eosin, then observed, photographed, and described with an optical microscope (Eclipse Ci with DS-FI2 camera; Nikon, Japan). The villus height and crypt depth were measured on 8 visual fields from each gut sample, and the villus height/crypt depth ratio calculated for each treatment group.

Volatile Fatty Acid Analysis

The VFA concentrations were measured using the gas chromatography procedure of Zhou et al. (2019). Briefly, 1 g cecal content was mixed with 6% phosphorous acid (w/v, 1:3) and injected into a GC7890 Network System (Agilent Technologies) equipped with a 30 m × 0.25 mm × 0.25 μm column (HP-FFAP, Agilent Technologies) for flame ionization (Yang et al., 2020).

16S rRNA Sequencing of Microflora in Cecum Contents

Cecal content samples were used for the intestinal flora analysis. The V4 region of the 16S rRNA gene was explored using an Illumina-HiSeq platform (Novogene Bioinformatics Technology Co., Ltd., Beijing, China). Shortly, using Quantitative Insights into Microbial Ecology (http://qiime.org/) and Uparse (https://drive5.com/uparse/) software, we gained 97% similarity between taxa and Ribosomal Database Project classifiers. The operational taxonomic unit (**OTU**) clustering and species classification analysis was based on valid data. Species annotations were made for each clustered OTU sequence to obtain the relative species information and species-based abundance distribution. Simultaneous, alpha diversity (Shannon index) calculations were used to obtain species richness and evenness and Venn diagram analysis to identify general and specific OTU in the different groups. The OTU were also aligned with multiple sequences to structure a phylogenetic tree. Principal component analysis and unweighted pair group method with arithmetic mean cluster trees in MetaStat were used to explore and display the differences in community structure between different groups.

Table 1. Composition and nutrient levels of the basal diet.

Items	Ages (day) 1–28	28–56
Ingredients (air-dry basis, %)		
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	
Premix[1]	4	4
Total	100.00	100.00
Nutrient levels		
ME (kcal/kg)	2,916	3,090
CP (%)	20.3	17.2
Lysine (%)	1.19	0.96
Methionine + cysteine (%)	0.89	0.74
Calcium (%)	0.87	0.73
Total phosphorus (%)	0.6	0.57

[1]Concentrate mixture provided the following per kilogram of complete diet: 1,500 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; 1,000 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.

Statistical Analysis

Data processing and analysis were performed with Microsoft Excel and SPSS 21.0 software (SPSS Inc.). Plotting was accomplished using Prism software (GraphPad Software Inc.). Data were analyzed with 1-way ANOVA and least significant difference experiments at $P < 0.05$.

RESULTS

Growth Performance

The RLS2 broilers had higher BW ($P < 0.01$) and ADG than those in the NCO and ANT groups on day 56 (Table 2). The effect of the RLS1 was not significant in any of BW and ADG on day 28 and 56. The RLS had no significant effect on ADFI, compared with the control and antibiotic groups on day 28 and 56 ($P > 0.05$). The feed:gain in broilers fed ANT and RLS2 diets were lower ($P < 0.05$) than those fed NCO or RLS1 between day 1 and 28.

Jejunal Morphology

The effects of dietary supplementation with RLS on jejunal morphology are shown in Figure 1. Compared with NCO and bacitracin (ANT) broilers, RLS1 and RLS2 diets resulted in shorter jejunal crypts ($P < 0.05$), longer jejunal villi ($P < 0.05$), and higher villus height/crypt depth ratio ($P < 0.05$) on day 28 and 56. Especially in the high-dose RLS group, the effect was more pronounced.

Serum Ig

The effects of RLS on serum Ig are displayed in Table 3. Compared with the NCO and ANT broilers, RLS1 and RLS2 broilers showed higher concentrations of IgA ($P < 0.05$) on day 28. Significant differences in IgA ($P > 0.05$) were not detected on day 56. Compared with the NCO broilers, RLS2 broilers showed higher serum levels of IgM ($P < 0.05$) on day 28. The RLS broilers showed higher serum levels of IgY ($P < 0.05$) on day 28 and 56.

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

Items	Treatments NCO	ANT	RLS1	RLS2	SEM	*P*-value
BW, g						
1 d	35.36	35.28	35.33	35.32	0.13	0.997
28 d	491.90	517.10	507.95	520.07	8.05	0.631
56 d	1,501.26[b]	1,547.25[b]	1,517.19[b]	1,598.64[a]	10.77	0.002
ADG, (g/day)						
1–28 d	16.31	17.21	16.88	17.31	0.29	0.628
29–56 d	36.05	36.79	36.04	38.52	0.42	0.108
1–56 d	26.18[b]	27.00[b]	26.46[b]	27.92[a]	0.19	0.002
ADFI, g						
1–28 d	35.23	35.62	35.79	35.83	0.22	0.212
29–56 d	91.21	92.34	91.18	96.69	1.05	0.169
1–56 d	63.09	64.26	63.50	66.45	1.06	0.101
F: G, (g/g)						
1–28 d	2.16[a]	2.07[b]	2.12[a]	2.07[b]	0.01	0.002
29–56 d	2.53	2.51	2.53	2.51	0.02	0.980
1–56 d	2.41	2.38	2.40	2.38	0.01	0.914

[a,b]Mean with different superscripts in the same row differ significantly ($P < 0.05$).

Abbreviations: ANT, basal diet supplemented with 30 mg/kg bacitracin; F:G, feed:gain; NCO, basal diet provided as control; RLS, rhamnolipid; RLS1, basal diet supplemented with 500 mg/kg RLS; RLS2, basal diet supplemented with 1,000 mg/kg RLS.

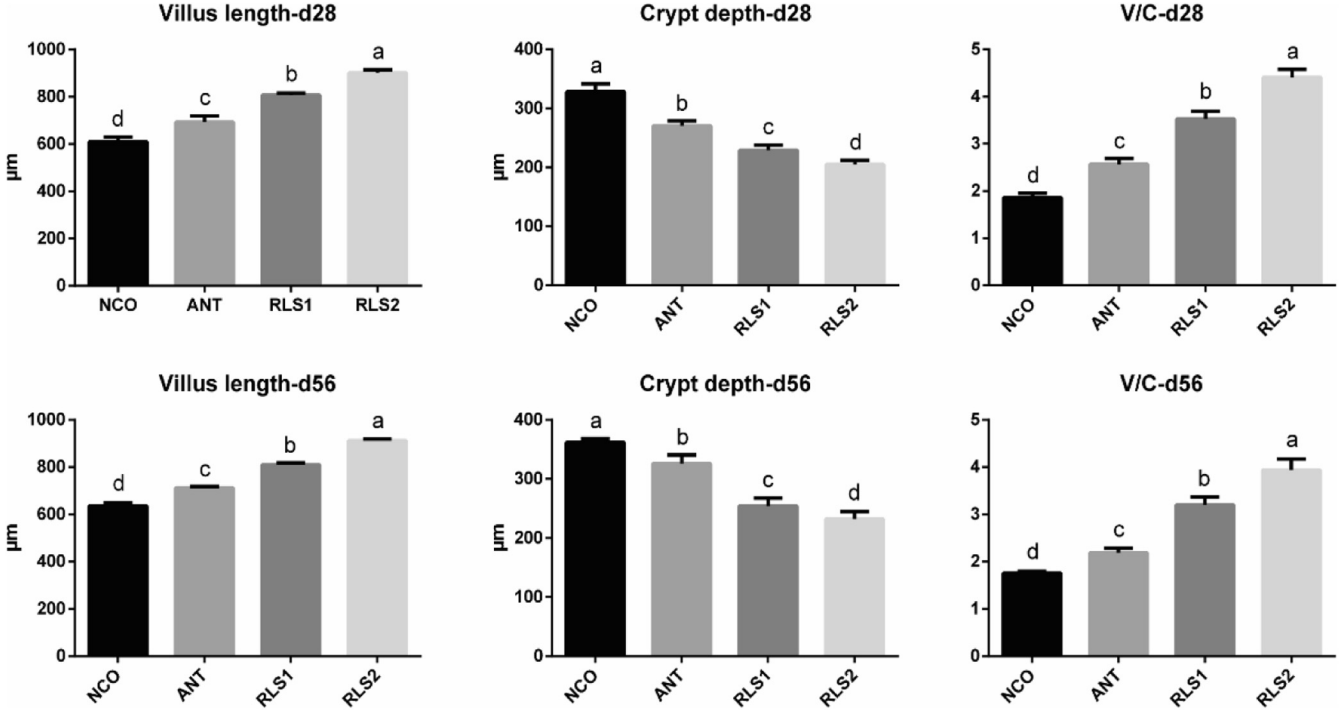

Figure 1. Effect of the RLS on jejunum villus of broilers. A represents the villus length in the jejunum. B represents the crypt depth in the jejunum. C represents the ratio of the villus length and crypt depth. NCO represents the control broilers; ANT represents the broilers supplemented with 30 mg/kg bacitracin; RLS1 represents the broilers supplemented with 500 mg/kg RLS; RLS2 represents the broilers supplemented with 1,000 mg/kg RLS. Different lowercase letters indicate a significant difference ($P < 0.05$). Values means n = 8 for the analysis of the jejunum form. Data are means ± SD. Abbrevaition: RLS, rhamnolipid.

Serum Inflammatory Factors

The effects of RLS on serum inflammatory factors are shown in Figure 2. Compared with the bacitracin broilers, RLS1 and RLS2 broilers showed higher levels of IL-1β and TNF-α on day 28 and 56 ($P < 0.05$). Furthermore, RLS2 broilers showed higher serum levels of IL-6 on day 56 ($P < 0.05$).

Cecal VFA

The VFA concentrations in cecal contents are displayed in Figure 3. The levels of acetic acid and isovalerate in RLS1 broilers were higher ($P < 0.05$) than in NCO and ANT broilers on day 28. The acetic acid, isobutyric acid, and isovalerate levels in RLS2 chickens were higher ($P < 0.05$) than in control and bacitracin chickens on day 28. The concentrations of propionic and isobutyric acids in RLS1 and RLS2 broilers were higher ($P < 0.05$) than in NCO and ANT chickens on day 56. The acetic acid and butyrate levels in RLS1 broilers were higher ($P < 0.05$) than in NCO and ANT broilers on day 56.

Cecal Microbial Community

The abundance and diversity of cecal microorganisms were obtained using data from 16S rRNA high-throughput sequencing on day 28. The composition of

Table 3. Effects of dietary supplementation of RLS of serum Ig in broilers.

	Treatments					
Items	NCO	ANT	RLS1	RLS2	SEM	*P*-value
IgA, (mg/L)						
28 d	872.63^{c}	1,144.33^{c}	2,557.02^{b}	3,868.16^{a}	382.92	<0.001
56 d	602.36	948.40	898.81	775.87	62.59	0.211
IgM, (mg/L)						
28 d	4.55^{c}	10.87^{b}	12.19^{b}	21.78^{a}	1.88	<0.001
56 d	26.70^{c}	43.98^{a}	32.53^{b}	32.84^{b}	2.00	<0.001
IgY, (mg/L)						
28 d	272.33^{c}	447.00^{b}	527.02^{a}	548.68^{a}	33.80	<0.001
56 d	573.71^{c}	824.23^{b}	1,014.12^{a}	930.50^{a,b}	52.10	<0.001

^{a,b,c}Mean with different superscripts in the same row differ significantly ($P < 0.05$).

Abbreviations: ANT, basal diet supplemented with 30 mg/kg bacitracin; NCO, basal diet provided as control; RLS, rhamnolipid; RLS1, basal diet supplemented with 500 mg/kg RLS; RLS2, basal diet supplemented with 1,000 mg/kg RLS.

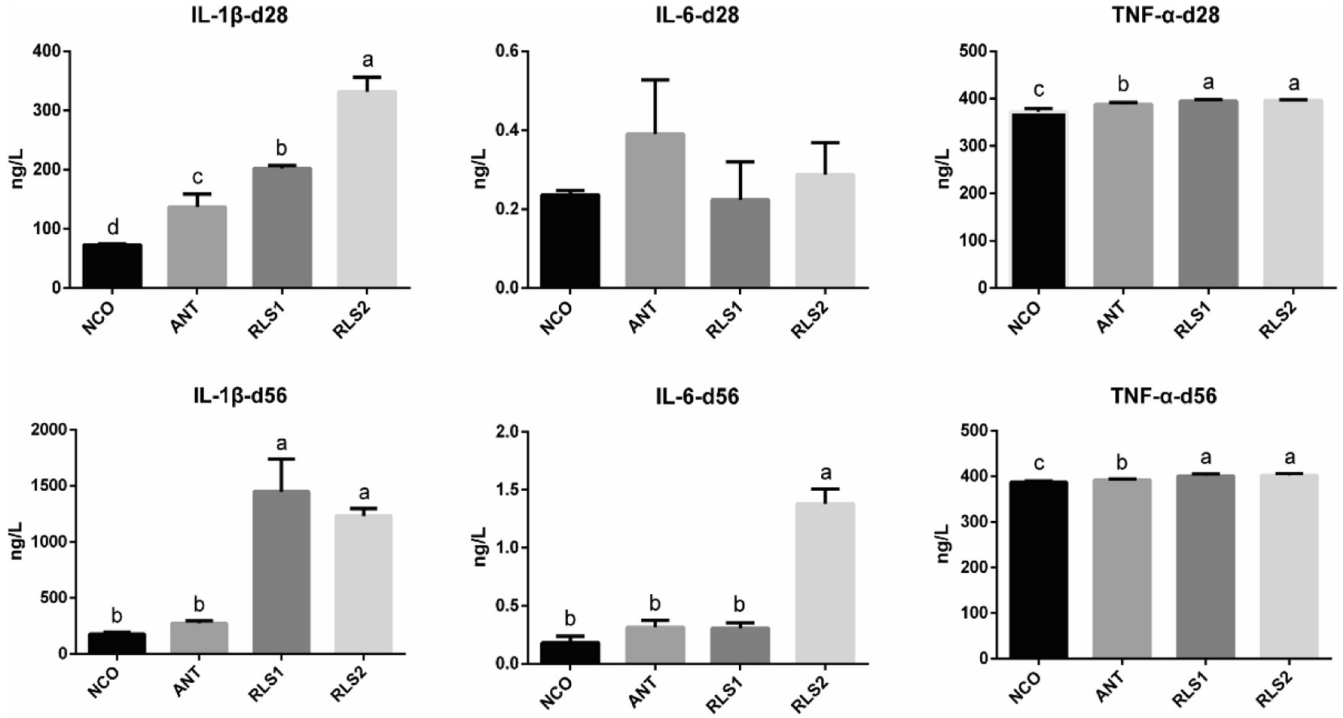

Figure 2. Effect of RLS on the serum IL-1β, IL-6 and TNF-αin broilers. NCO represents the control broilers on day 28 and 56, respectively; ANT represents the broilers supplemented with 30 mg/kg bacitracin on day 28 and 56, respectively; RLS1 represents the broilers supplemented with 500 mg/kg RLS on day 28 and 56, respectively; RLS2 represents the broilers supplemented with 1,000 mg/kg RLS on day 28 and 56, respectively. Diverse lowercase letters show significant differences between treatments ($P < 0.05$). Values means n = 8 for the analysis of serum inflammatory. Data are means ± SD. Abbrevaition: RLS, rhamnolipid.

cecum flora is shown in the Venn diagram in Figure 4A. A total of 1123 OTU were shared among the 4 treatment groups. The NCO, ANT, RS1, and RS2 broilers had 32, 27, 157, and 58 unique OTU, respectively. Both unweighted pair group method with arithmetic mean cluster tree and principal component analysis manifested that the ANT and RLS2 broilers' microflora were more similar (Figures 4B and 4C). The alpha diversity (Shannon index) was higher in both the ANT and RLS2 broilers than in the NCO group (Figure 4D). Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Tenericutes were the primary bacterial phyla (Figure 4E). The relative abundance of Synergistetes in the RLS2 group was obviously higher ($P < 0.05$) than that in the NCO and ANT groups (Figure 4H). At the genus level, *Faecalibacterium*, *Barnesiella*, *Bacteroides*, *Megamonas*, and *Alistipes* were the main genera in 4 treatment groups (Figure 4F). The relative abundance of *Megasphaera* in the RLS2 group was significantly higher ($P < 0.05$) than that in the NCO and bacitracin groups (Figure 4I). At the species level, we discovered that *Bacteroides sp.*, *Clostridiales sp.*, *bacterium ic1379*, *Intestinimonas timonensis*, *Escherichia coli*, *Lactobacillus salivarius*, and *Firmicutes bacterium CAG:822* were the dominant species in all samples (Figure 4G). *Megamonas hypermegale* was more abundant in the RLS2 group ($P < 0.05$) than in the NCO group (Figure 4J).

The cecal microbiota composition at 56 d is displayed in the Venn diagram in Figure 5A. A total of 1356 OTU were shared among the 4 treatment groups. The NCO, ANT, RLS1, and RLS2 broilers had 98, 38, 22, and 122 unique OTU, respectively. Both unweighted pair group method with arithmetic mean cluster tree and principal component analysis showed that the NCO and RLS1 broilers' microflora were more similar (Figures 5B and 5C). The alpha diversity (Shannon index) was visibly higher in the RLS2 broilers than in the ANT group ($P < 0.05$) (Figure 5D). Bacteroidetes, Firmicutes, Verrucomicrobia, Tenericutes, and Fusobacteria were the primary bacterial phyla (Figure 5E). The relative abundance of Melainabacteria in the RLS2 group was clearly higher ($P < 0.05$) than that in the ANT group (Figure 5H). At the genus level, the results showed that *Bacteroides*, *Parabacteroides*, *Akkermansia*, *Megamonas*, and *Fusobacterium* were the main genera in all samples (Figure 5F). The relative abundance of *Faecalibacterium* in the RLS2 group was distinctly higher ($P < 0.05$) than that in ANT group (Figure 5I). At the species level, we observed that *Bacteroides sp.*, *F. bacterium CAG:822*, *Candidatus saccharibacteria bacterium UB2123*, and *Clostridium sp.* were the dominant species in all samples (Figure 5G). *Lachnoclostridium phocaeense* ($P < 0.05$) and *Clostridium spiroforme* ($P < 0.01$) had lower abundances in the RLS2 group than in the NCO group (Figures 5J and 5K). *Alistipes obesi* was more abundant in the RLS1 and RLS2 groups ($P < 0.05$) than in the NCO group (Figure 5L).

DISCUSSION

Current antibiotic alternatives are predominantly antimicrobial peptides, probiotics, organic acids, and

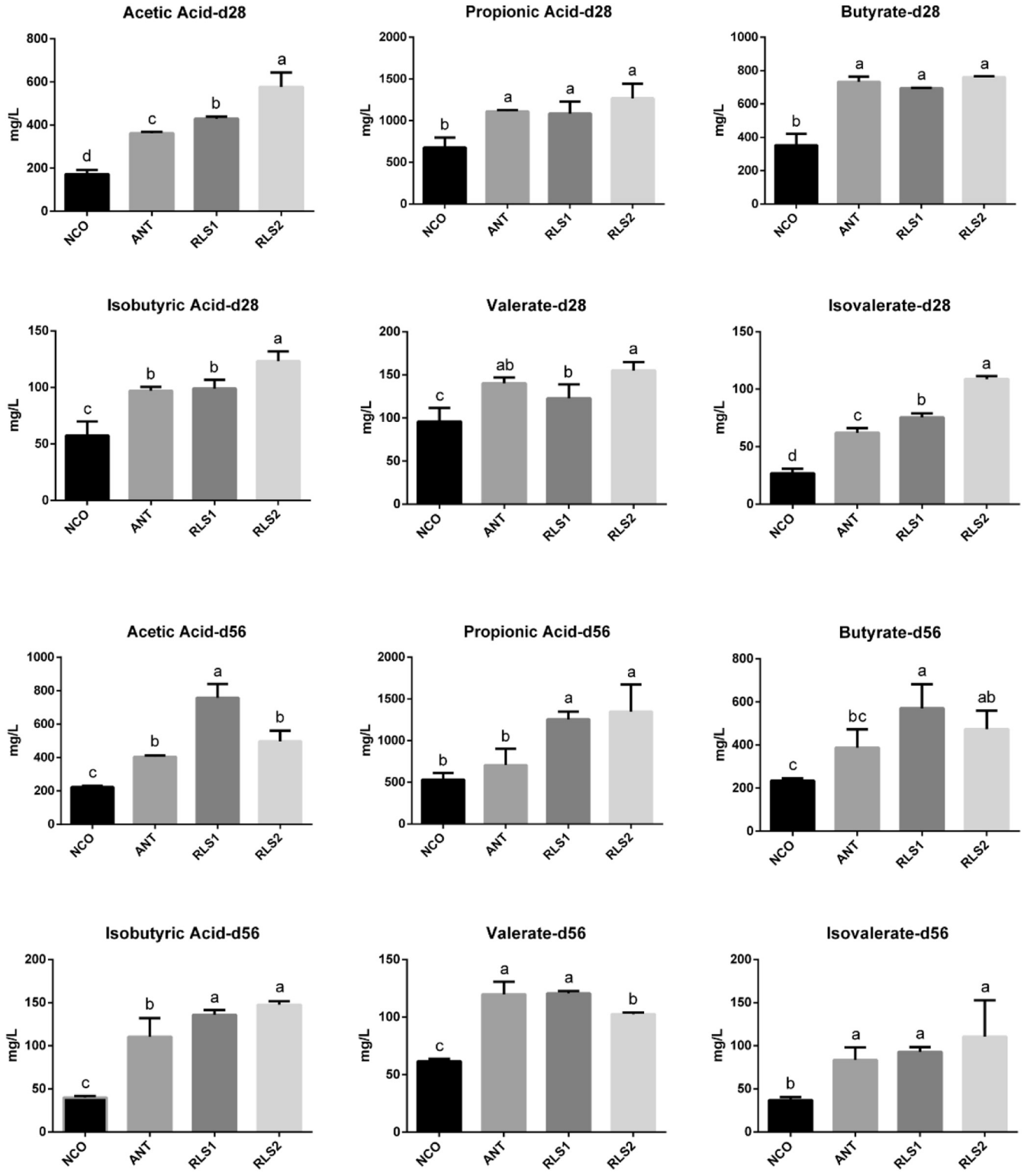

Figure 3. Effect of the RLS on the VFA in cecal content of broilers. NCO represents the control broilers; ANT represents the broilers supplemented with 30 mg/kg bacitracin; RLS1 represents the broilers supplemented with 500 mg/kg RLS; RLS2 represents the broilers supplemented with 1,000 mg/kg RLS. Diverse lowercase letters show significant differences between treatments ($P < 0.05$). Values means n = 6 for the analysis of VFA. Data are means ± SD. Abbrevaitions: RLS, rhamnolipid; VFA, volatile fatty acid.

vaccines, in relation to livestock (Song and Lee, 2014). In recent decades, RLS have attracted a lot of attention owing to their antimicrobial, antiviral, and immunomodulatory activities, which make them a promising antibiotic substitute (Liu et al., 2014; Kristoffersen et al., 2018).

Van Boeckel et al. (2015) reported that antibiotics are widely used to promote the animal's growth rate. Our results showed that 1,000 mg/kg RLS significantly increased growth performance compared with antibiotics on day 56. However, compared with antibiotics, 500 mg/kg RLS did not significantly improve growth

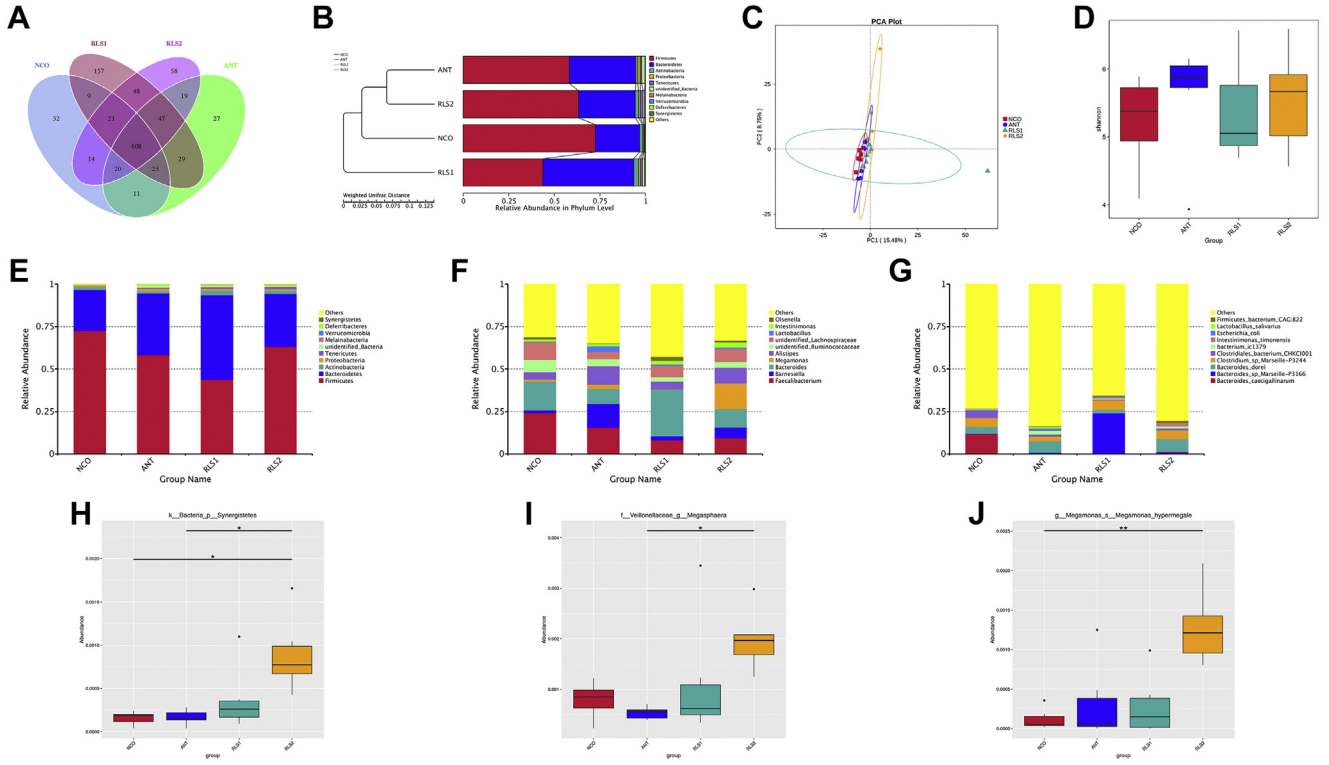

Figure 4. Summary of microbial community in cecal contents of broilers on day 28. (A) Venn diagram. (B) UPGMA cluster tree. (C) Principle component analysis. (D) Shannon index. (E–G) The top 10 taxa by relative abundance (E: phylum; F: genus; G: species). (H–J) Species with significant inter-group differences (H: phylum; I: genus; J: species). Broiler basal diet supplementation: NCO, none; ANT, 30 mg/kg bacitracin; RLS1, 500 mg/kg rhamnolipids; and RLS2, 1,000 mg/kg rhamnolipids. OTU, operational taxonomic unit. Broilers were regarded as the experimental units, n = 6 per treatment. Significant differences: * at $P < 0.05$, ** at $P < 0.01$. Abbreviation: UPGMA, unweighted pair group method with arithmetic mean.

performance on day 28 and 56. This indicated that the effect of RLS on the growth performance of broilers was dose-related; further study is needed to identify the ideal dose. Cao et al. (2019) reported that the intestinal epithelium acts as a protective barrier and has a positive effect on nutrient absorption; its morphology is used to assess gut development and function. Our data showed that both 500 mg/kg and 1,000 mg/kg dietary RLS enhanced the intestinal development of broilers by lower crypt depth, longer villi, and a higher intestinal gland ratio in broilers on day 28 and 56. Wu et al. (2018) reported that villus height and crypt depth directly mirror the function of the intestinal tract. Xu et al. (2012) covered that the villus height-to-crypt depth ratio is a crucial parameter for estimation of the absorption capacity of the small intestine. Long et al. (2018) reported that longer intestinal villi can strengthen the contact between the intestine and nutrients and improve digestion and absorption. Li et al. (2015) reported that a larger ratio of villus height to crypt depth reflects a higher capacity for digestion and absorption. This suggests that RLS promotes the digestion and absorption of nutrients by increasing the villus length.

The animal immune response is closely associated with Ig. Bian et al. (2016) found that IgA is associated with mucosal immunity and IgM correlates with acute infection. Haese et al. (2015) reported that IgY is the primary serum Ig of birds and the functional equivalent of mammalian IgG. Wang et al. (2019a) reported that IgY is the key circulating antibody found in broilers and that specific IgY production can be achieved by immunizing laying hens with foreign pathogens, which reduces an immune response leading to high levels of IgY antibodies concentrated in the egg yolk. Piljac and Piljac (1995) found that RLS is an immunomodulator, which can regulate many immune cells. We found that adding RLS resulted in higher IgA, IgM, and IgY levels in broilers. IL-1β, IL-6, and TNF-α are cytokines involved in regulating the inflammatory response (Chen et al., 2019). Bédard et al. (1993) showed that RLS can stimulate human nasal epithelial cells to release a large number of immune factors, such as IL-8, granulocyte-macrophage colony-stimulating factor, and IL-6. We found that RLS1 and RLS2 birds had higher levels of serum IL-1β and IL-6, and lower levels of TNF-α, than control birds on day 28 and 56. These results indicate that RLS could enhance the immune function in broilers.

The VFA are the main end product of bacterial metabolism in the large intestine (Tang et al., 2018). Their presence in the cecum and colonic contents indicate good intestinal health and microbial activity, and they can provide maintenance energy (Bindelle et al., 2009; Beckers et al., 2017). Yadav et al. (2019) reported that acetate, propionate, and butyrate are beneficial to host

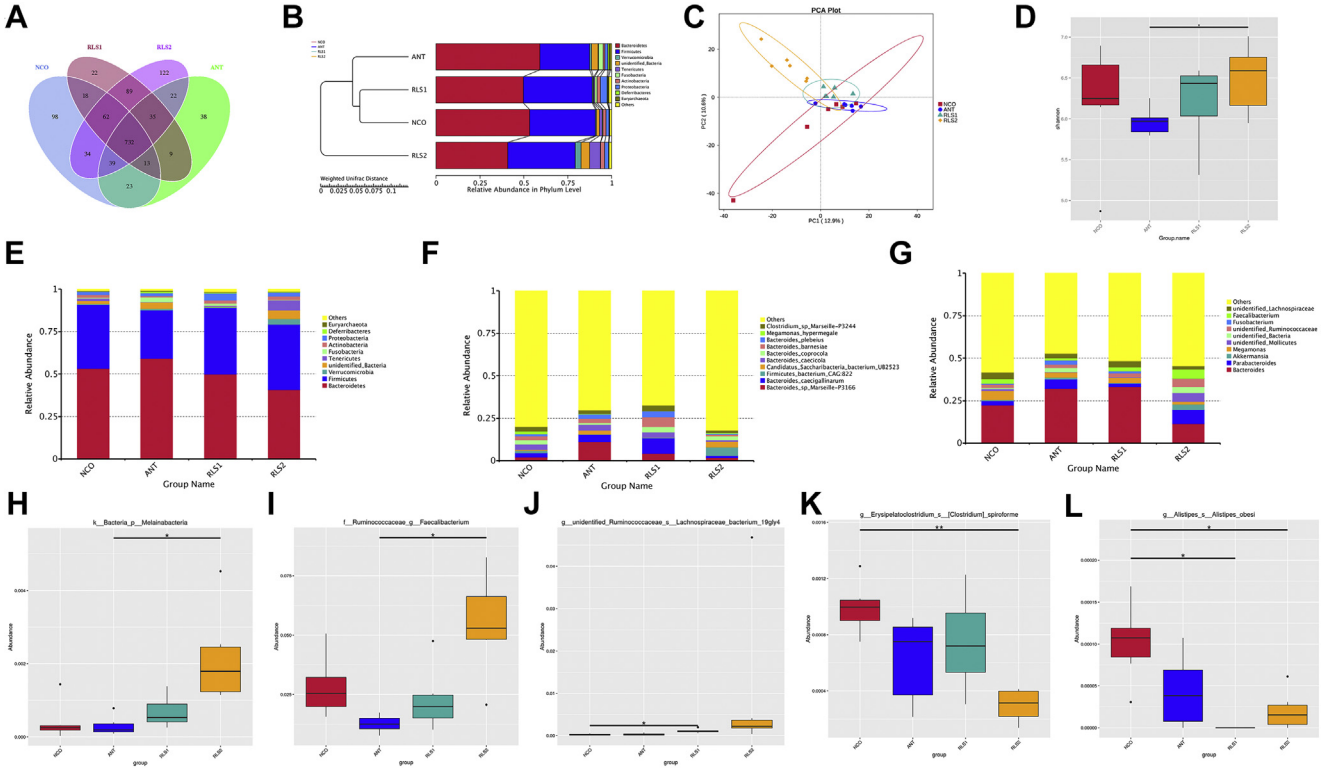

Figure 5. Summary of microbial community in cecal contents of broilers on day 56. (A) Venn diagram (B) UPGMA cluster tree. (C) Principal component analysis. (D) Shannon index. (E–G) The top 10 taxa by relative abundance (E: phylum; F: genus; G: species). (H–L) Species with significant inter-group differences (H: phylum; I: genus; J, K, L: species). Broiler basal diet supplementation: NCO, none; ANT, 30 mg/kg bacitracin; RLS1, 500 mg/kg rhamnolipids; and RLS2, 1,000 mg/kg rhamnolipids. OTU, operational taxonomic unit. Broilers were regarded as the experimental units, n = 6 per treatment. Significant differences: * at $P < 0.05$, ** at $P < 0.01$. Abbreviation: UPGMA, unweighted pair group method with arithmetic mean

in various ways, from providing energy to boosting beneficial bacteria and immune system. In the recent research, chickens supplemented with RLS tended to have obviously higher level of acetic, propionic, butyric, and isobutyric acids, as well as valerate and isovalerate. This resulted from larger and more diverse microbial populations in the broilers' ceca, which in turn likely improved their growth performance.

The complex intestinal microbiota is associated with gut health and disease (Wang et al., 2019d). Intestinal microflora is closely related to the occurrence of enteritis (Wang et al., 2018, 2019b). Recurrent, excessive use of antibiotics changes the intestinal flora, leading to bacterial resistance (Prasada et al., 2019). In the present study, Firmicutes and Bacteroidetes were the primary bacterial phyla on day 28 and 56, which is consistent with the results of previous studies showing that these were the top 2 phyla in both cecal and colonic digesta (Wang et al., 2019c). At the genus level, we discovered that the level of *Megasphaera* in RLS chicks was markedly higher than that in the bacitracin group on day 28. This genus can convert lactate to butyrate (Kamke et al., 2016). In addition, we observed that the level of *Faecalibacterium* in RLS broilers was significantly higher than in the ANT group on day 56; abundant *Faecalibacterium* has been associated with a healthy gut status (Li et al., 2019) and mediation of anti-inflammatory effects (Kiernan et al., 2019). Ye et al. (2019) reported that *Faecalibacterium* fermentation produces mainly short-chain fatty acids such as butyrate. At the species level, our results showed that *M. hypermegale* was the advantaged bacterial species in RLS-fed broilers on day 28. Scupham et al. (2010) reported that *M. hypermegale* is a large (up to 15 μm long), obligately anaerobic species that requires fermentable sugars and produces acetic and propionic acids. In addition, we observed that the level of *Lachnospiraceae bacterium 19gly4* in the RLS1 group was markedly higher than that in the NCO group on day 56; this family contains genera that are classified as butyrate producers (Barthel et al., 2003). In the present trials, we also found that the levels of *C. spiroforme* and *A. obesi* in the RLS2 group were significantly lower than that in the NCO group on day. Songer (1996) reported that *C. spiroforme* is closely ralated to diarrhea in weaned rabbits. Moschen et al. (2016) found that *Alistipes* induces intestinal inflammation by taking advantage of the gut poison secreted by intestinal bacteria. Thus, supplementation of RLS into the diets of broilers can reduce the presence of some harmful bacteria.

In conclusion, dietary supplementation with RLS improved growth performance and benefited the intestinal villus morphology in broiler chickens, as well as possibly regulating host immune function. It enhanced the level of VFA in colon contents, raised the relative abundance of the gut microbiota of the broilers, and promoted the proliferation of beneficial bacteria.

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DISCLOSURES

All authors of this manuscript warrant that this manuscript has not been published or presented elsewhere in part or in entirety and is not under consideration by another journal. The study design was approved by the appropriate ethics review board. We have read and understood your journal's policies, and we believe that neither the manuscript nor the study violates any of these. There are no conflicts of interest to declare.

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