Effects of *Lactobacillus reuteri* and its combination with gluco-oligosaccharides on improving growth performance and intestinal health of broilers

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Abstract

The ban on antibiotics as feed additives requires modern intensive farming with more comprehensive diseases control approaches. Currently, synbiotics serve as promising alternatives to enhance growth performance and improve health in the poultry industry. In this research, we investigated beneficial effects of Lactobacillus reuteri (LR) with its combination of gluco-oligosaccharides (GlcOS) supplementation on growth performance and intestinal health of broilers. A total of 900 1-day-old male Lingnan yellow-feather broilers were randomly allocated into the control group (CON group, and two experimental groups feeding basal diet supplementing LR (LR group) and its combination with GlcOS (RG group), respectively. The findings indicated beneficial effects of growth performance in experimental groups (LR and RG groups), as evidenced by decreasing the feed-to-weight ratio (F/G) in both experimental groups (P < 0.05) and increasing the average daily gain (ADG) in RG group (P < 0.05). Simultaneously, both experimental groups increased the villus height/crypt depth ratio (VH:CD) (P<0.001). Furthermore, the RG group showed increased activity of digestive enzymes (P < 0.05) and up-regulated mRNA expression of tight junction protein and transportation protein (P < 0.05), while decreased the serum levels of D-lactic acid and Diamine oxidase (DAO) (P<0.05), suggesting the improvement of the nutrient digestion and absorption, as well as the mucosal barrier integrity. Moreover, increased abundance of beneficial bacteria, including Bacteroides, Muribaculaceae and Prevotellaceae_UCG-001 (P<0.05), leading to a finely altered gut microbiome and metabolome. Collectively, the findings of this research revealed that dietary supplemented LR and its combination with GlcOS could enhance the intestinal morphology, digestion, absorption, and barrier function, improve the cecal microbiota structure and metabolic function finally achieving the effect of improving growth performance of broilers. Overall, the effect of the combination of LR and GlcOS was synergistic, providing a future alternative to antibiotics as growth promoter.

Keywords: *Lactobacillus reuteri*; Gluco-oligosaccharides; Synbiotic; Broilers; Growth performance; Intestinal health

Introduction

Globally, the multi-drug resistant (MDR) bacteria-caused deaths exceeded 4.71 million in 2021, the number of which was estimated at 10 million annually by 2050 (Naghavi et al. 2024) As the biggest antibiotics consumer in food animal industry since 2010, China has banned the antibiotics usage as growth promoter in 2020 to alleviate the antimicrobial selection pressure, contributing to less spread of MDR clones (Chen et al. 2024). However, the prevention against pathogens including Salmonella, Escherichia coli O:78 and Clostridium perfringens was weaker after the ban due to its high reliance on the antibiotics (Chen et al. 2024; Smith et al. 2024; Sun and Chen 2024). Probiotics, defined as live microorganisms which can benefit hosts when ingested sufficiently, is the most widely used, natural and safe substitutes to enhance health and growth in poultry production, among which LR, a hetero-lactic acid bacteria with superior probiotic characteristics, can regulate immune response, modulate gut microbiota, produce beneficial metabolites, restore impaired intestinal barrier function by reducing intestinal epithelial cells apoptosis via PKC-Nrf-2/HO-1 pathway activation and NF-kB pathway inhibition, and induce intestinal stem cell differentiation to Paneth cells by activating Wnt- β -catenin pathway, thus leading to better growth performance (Ding et al. 2024; Xu et al. 2023; Zhou et al. 2022). In previous studies, we screened an LR strain from broiler gut microbiota with significant bacteriostatic and anti-inflammatory effects owing to its reuterin producing ability (Ding et al. 2024; Xu et al. 2022a; Xu et al. 2022b; Xu et al. 2023).

Prebiotics refer to substrates selectively utilized by the gut microbiota bestowing a positive impact on the host health, which can promote the proliferation and/or activity of beneficial bacteria, as well as increase the generation of advantageous metabolites such as short-chain fatty acids, mostly non-digestible carbohydrates, especially short-chain oligosaccharides (Salminen et al. 2021; Sanders et al. 2019; Zeng et al. 2023). GlcOS was reported to show prebiotic potentials, including antimicrobial effect, anti-inflammation, antioxidation, and providing selective substrates for certain probiotics (Chaari et al. 2015; Hu et al. 2020). It is reported that GlcOS can activate intraepithelial natural killer (NK) cell and increase relative abundance of commensal lactic acid bacteria in young broiler chickens, contributing to improved intestinal health and growth performance in broilers (Meijerink et al. 2021).

Synbiotics can facilitate the colonization and proliferation of advantageous gut microorganisms, typically consisting of *Lactobacillus, Bifidobacterium* and *Streptococcus* as the probiotic ingredient, and oligosaccharides, fiber or inulin as the prebiotic ingredient (Krumbeck et al. 2016). Synbiotics were effective in improving antioxidant capacity, immune function, intestinal morphology and micro-ecological homeostasis, whose dual synergistic role in broilers was increasingly evident (Chen et al. 2018; Kridtayopas et al. 2019; Mounir et al. 2022; Tanner et al. 2015). Notably, LR can utilize some prebiotics like GOS as a carbon resource, providing the foundation for symbiotic development.

Therefore, we aimed to investigate the effects of LR in combination with GlcOS on growth performance and gut health of broilers, and to preliminarily explore its underlying mechanism by analyzing the intestinal mucosal morphology, gut microbiota and metabolome to lay a theoretical foundation for practical application in production.

Materials and ethods

Ethics statement

The experimental procedures complied with the animal welfare guidelines established by China and were approved by the Animal Care and Welfare Committee as well as the Scientific Ethical Committee of Zhejiang University (No. ZJU20220310, Hangzhou, China).

Experimental design and diets

A total of 900 male, one-day-old yellow-feathered broilers were randomly distributed into 3 groups with 6 replicates of 50 each. The strain used for study is a single *Lactobacillus reuteri* strain (LR 21), previously isolated from broiler cecum contents kept in lab stock (Xu et al. 2023). Experimental treatments were as follows: basal diet (CON group), basal diet supplemented with 2500 g/t microencapsulated *Lactobacillus reuteri* (1×10^7 CFU/g feed, LR group) and basal diet supplemented 2500 g/t microencapsulated *Lactobacillus reuteri* (1×10^7 CFU/g feed) and 900 g/t GlcOS (RG group). The total of 56 day-experiment period was divided into two phases, namely early growth phase (1-21 d) and the later growth phase (22-56 d) (Bhogoju et al. 2021; Chai et al. 2023). All broilers were subjected to routine feeding management and immunization schedules. Feed and water were provided *ad libitum*. Basal diet was formulated in accordance with the guidelines of the nutritional requirements of broiler chickens as recommended in NY/T 3645-2020, with its composition and nutrient levels detailed in Table 1.

Items	1 to 21 d	22 to 56 d
Ingredients (%)		
Corn	23.55	32.53
Wheat	40.00	40.00
Soybean meal	24.50	14.20
Corn gluten meal	5.00	7.00
Soybean oil	2.55	2.22
Calcium hydrogen phosphate	0.88	0.71
Limestone	1.74	1.50
Sodium chloride	0.30	0.30
Lysine hydrochloride (70%)	0.35	0.42
DL- Methionine (99%)	0.13	0.12
Premix ¹	1.00	1.00
Total	100.00	100.00
Nutrient levels ² (%)		
ME (MJ/kg)	12.26	12.66
СР	20.95	18.45
Ca	0.99	0.83
TP	0.55	0.49
Lys	1.13	0.97
Met	0.47	0.45
Met+Cys	0.81	0.75

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

¹Supplied per kilogram of diet: vitamin A, 9,600 IU; vitamin D3, 2,700 IU; vitamin E, 36 mg; vitamin K₃, 3.0 mg; vitamin B₁, 3.0 mg; vitamin B₂, 10.5 mg; vitamin B₆, 4.2 mg; vitamin B₁₂, 0.03 mg; folic acid, 1.5 mg; nicotinamide, 60 mg; D-calcium pantothenate, 18 mg; biotin, 0.225 mg; choline chloride, 1,000 mg; Fe, 80 mg; Cu, 8.0 mg; Mn, 80 mg; Zn, 60 mg; I, 0.35 mg; Se, 0.15 mg.

² ME is calculated value, other nutrient levels are the measured values.

All ingredients were provided from the Zhejiang xinxin Feed Co., Ltd. The feed preparation process as follow: Firstly, corn flour as the adsorption carrier was put into the mixer, where it was thoroughly mixed with microencapsulated LR (prepared by the Feed Science Institution of Zhejiang University) and/or GlcOS (provided by DSM (Shanghai) Co. Ltd). Subsequently, other feed ingredients listed in Table 1 were added to the mixer and blended uniformly. Finally, the mixture undergone low-temperature conditioning (steam temperature $\leq 65^{\circ}$ C) to produce either powdered feed or pelleted feed, which was then rapidly cooled to room temperature by a cold air blower for subsequent experimental. The feed used in the early growth phase is powder form while in the late growth stage, it is pelleted.

Growth performance

Body weight and feed intake for each replicate (6 broilers) were recorded at the conclusion of each period, the ADG, ADFI, and F/G were calculated during the early growth phase (1-21 d), the later growth phase (22-56 d) and the overall period (1-56 d).

Sample collection

After the feeding trial, two broilers with body weights close to the average were randomly selected from each group per replicate for 10 mL of blood samples collection from the wing vein, ultimately sacrifice and sampling, totaling 36 broilers. 1 cm samples of duodenum, jejunum and ileum were collected and fixed in 4% paraformaldehyde solution, another 1 cm segment of jejunum was collected and fixed in 2.5% glutaraldehyde solution. The cecum was separated, and the cecal content was gathered and preserved at -80 °C for subsequent analysis of gut microbiota and metabolites.

Intestinal morphology

The intestinal segments of the duodenum, jejunum, and ileum fixed in 4% formaldehyde solution were dehydrated with alcohol, transparent with xylene, embedded in paraffin. These segments cut into 5 µm thickness, and stained with hematoxylin-eosin (HE) and periodic acid-Schiff's (PAS) staining for analysis, respectively. The HE-stained and PAS-stained sections were observed using an optical microscope (Nikon, Tokyo, Japan) to capture and documented images Image-Pro software (MediaCybernetics, Rockville, MD) was utilized to measure VH, CD, and the number of goblet cells was counted per 1 mm jejunum.

The jejunal samples were fixed in 2.5% glutaraldehyde at 4 °C overnight and rinsed three times with 0.1 M phosphate buffer saline (PBS). Subsequently, the samples were treated with 1% osmium for 1 h. After rinsing in PBS three times, the

samples were dehydrated with Gradient ethanol, embedded in epoxy resin, sectioned by using a LEICA EM UC7 ultratome (Leica Microsystems, Wetzlar, Germany). Finally, the sections were observed with a Hitachi H-760 TEM (Hitachi, Tokyo, Japan).

Measurements of intestinal permeability and digestive enzyme activity

Following the manufacturer's manuals, the serum concentration of D-lactic acid and DAO were determined using enzyme-linked immunosorbent assay (ELISA) (Jiangsu Enzyme Free Industrial Co., LTD., Yancheng, China).

Pre-preparation of duodenal, jejunal and ileal tissue homogenates. Firstly, the thawed duodenum, jejunum and ileum were quickly dissected, and the contents were rinsed with PBS solution, then the intestinal mucosa was scraped from 0.1 g to 0.15 g with a small blade, and the corresponding PBS solution was added in the ratio of 1:9 between the weight of the tissue (g) and the volume of PBS solution (mL), and then homogenized with a tissue homogenizer, and then centrifuged at 3,500 rpm, 10 min at 4 °C, and 10% of the tissue homogenate was obtained by taking the supernatant. The activities of amylase (AMS), lipase (LPS) and trypsin (TPS) in duodenum, jejunum and ileum were detected with the kit instructions (Nanjing Institute of Jiancheng Bioengineering, Nanjing, China).

Extraction of RNA and RT-qPCR analysis

The RNA of jejunal tissues was extracted using the Trizol kit (Nanjing Novozymes Bioscience and Technology Co., Ltd., Nanjing, China) following the

provided manuals. The RNA obtained was then transcribed reversely into complementary DNA (cDNA) with the HiScript II Q RT SuperMix Reverse Transcription Kit (Novozymes Bioscience and Technology), and subsequently stored at -20°C. The primer sequences presented in Table 2 and were synthesized by Beijing DynaPro Biotechnology (China). QRT-PCR was conducted using ChamQ Universal SYBR qPCR Master Mix (Novozymes Biotechnology) in a CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA) The relative expression of each target gene was calculated by $2^{-\Delta\Delta Ct}$ method with relative to β -actin.

Ductoing Comes		D: C (7/2)	Products		Accession
Proteins	Genes	Primer Sequence (5'-5')	length	(bp)	number
Occludin Occludin	F: GAGCCCAGACTACCAAAGCAA	68		NM_205128	
	R: GCTTGATGTGGAAGAGCTTGTTG				
ZO-1 ZO-1	F: CTTCAGGTGTTTCTCTTCCTCCTC	121			
	20-1	R: CTGTGGTTTCATGGCTGGATC	13	1	XM_046925214
Claudin-1 Claudin-1	F: TGGCCACGTCATGGTATGG	(2)		NRC 001010711	
	Claudin-1	R: AACGGGTGTGAAAGGGTCATAG	02	2	NM_001013611
MUC2 MUC2	F: GCCTGCCCAGGAAATCAAG	50		NR 001210424	
	R: CGACAAGTTTGCTGGCACAT	55	,	NM_001318434	
D T1	SI C15 A 1	F: CCCCTGAGGAGGATCACTGTT			NM_204365.2
Pep11	SLCI5AI	R: CAAAAGAGCAGCAGCAACGA	60)	
		F: TGCTGCTTTGGATTCCAGTGT		NR 6 10 10 00	
EATT3 SLC1A1	R: AGCAATGACTGTAGTGCAGAAGTAATATATG	/9		XM_424930	
B ⁰ AT SLC6A19	F: TGCGTAGGGTTTTGTGTTGG	184		XM_419056.8	
	R: AACTCCAGACTCCCACACTG				
CATI SLC7A1	F: CGAACAACAGAGGAGACAGATAA			NR 001145400 0	
	R: GGGACACAGTATGGCTTTGA	11	1	NM_001145490.2	
SGLT1 SLC5A1	F: TTAGAGAGGTTGGAGGGTATGA	11	110	NR 001002040 0	
	R: GAATCTGCTCGAGGCGTATAG	118		NM_001293240.2	
	F: AGTACATCGCGGATCTGTGC	110	NRA 207179 2		
GLU12	SLC2A2	R: TGACTTCCCCTTCGTTTCGG	110		NM_207178.2
		F: ATACCTCTGGCACTACGGGAAT	117		XXX 0406402062
FATP4 SLC27A4	SLC27A4	R: CATACATCACATCATCGGGTCT			XM_040649296.2
0	0	F: GAGAAATTGTGCGTGACATCA			NIM 205519
β -actin β -actin	p-actin	β-actin R: CCTGAACCTCTCATTGCCA	152	2	INIVI_205518

Table 2. Primer sequences for qRT- PCR

The genes encoding for PepT1, EATT3, B⁰AT, CAT1, SGLT1, GLUT2, and FATP4 are designated as SLC15A1, SLC1A1, SLC6A19, SLC7A1, SLC5A1, SLC2A2 and SLC27A4 respectively.

16s rRNA-based microbiota analysis

The cecum microbiota DNA of was extracted using TIANGEN kit following the manufacturer's manuals. Primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R

(5'-GGACTACNNGGGGTATCTAAT-3') were synthesized for V3-V4 regions amplification, then purified, quantified, and homogenized to construct a sequencing library, which was subjected to high-throughput sequencing on Illumina platform. Thea and β diversity analyses were performed, and the relative abundance and differential flora were analyzed.

LC-MS cecal metabolomics analysis

Metabolites extraction and LC-MS/MS analysis of cecal contents were as previously described (Yi et al. 2022). Briefly, after separation of the sample on an HSS T3 column, the mass spectrometric data were acquired in the Data Dependent Acquisition (DDA) mode. The LC-MS raw data was imported into the Progenesis QI (Waters Corporation, Milford, USA) software for preprocessing. The metabolites were identified by searching through databases including Human Metabolome Database (HMDB, <u>http://www.hmdb.ca/</u>) and Metlin (<u>https://metlin.scripps.edu/</u>). The R package 'ropls' (Version 1.6.2) was used to conduct principal component analysis (PCA) and orthogonal least partial squares discriminant analysis (OPLS-DA). The significantly different metabolites were identified based on the variable importance in the projection (VIP) scores from the OPLS-DA model and *p*-value derived from the t-test, t with criteria set at VIP > 1 and P < 0.05. Finally, metabolic pathway annotation of these significant differential metabolite was performed by Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https: //www.kegg.jp/kegg/pathway.html) to identify the differential metabolites implicated in major pathways, with a significance threshold of P < 0.05.

Statistical analysis

The experimental data were expressed as mean \pm standard deviation (Mean \pm SD), and then One-Way ANOVA analysis of variance (ANOVA) was carried out using SPSS 20.0 statistical software, with Duncan's multiple range test employed for multiple comparisons. *P* < 0.05 was considered indicative of a significant difference. Significance was shown as * *P* < 0.05, ***P* < 0.01, ****P* < 0.001. Figures were

plotted by GraphPad Prism 7.0 software, while comparison without significance was not shown for better presentation.

Results

Growth performance

The effects of LR and its combination with GlcOS on growth performance of broilers across the early growth phase, the later growth phase, and the overall period are shown in Table 3. During the early growth phase, no significant differences were observed in all performance indicators among all groups (P > 0.05). During the later growth phase, compared with the CON group, the effect of LR and RG dietary supplementation was significant in ADG and F/G (P < 0.01), and RG supplementation significantly increased FW (P < 0.05). During the overall period, LR group exhibited significant decrease in F/G (P < 0.05), while RG supplementation both decreased F/G (P < 0.05) and increased ADG (P < 0.05), with no significance in ADFI (P > 0.05) difference among groups, which strongly testify the synergistic benefits between LR and GlcOS during the later growth phase and overall period.

Items	CON	LR	RG	<i>P</i> -value
Day1-21				
Dayl IW (g)	34.50±0.52	34.43±0.38	34.50±0.59	0.966
Day21 FW (g)	523.03±6.88	516.98±8.92	509.63±13.07	0.098
ADFI (g/d)	38.32±1.33	38.03±0.56	38.12±0.72	0.858
ADG (g/d)	23.26±0.33	22.98±0.42	22.62±0.60	0.092
F/G	1.65±0.04	1.66 ± 0.04	1.68 ± 0.02	0.128
Day22-56				
Day56 FW (g)	2155.66±27.52 ^b	2220.54±69.31 ^{ab}	2258.20±47.98 ^a	0.025

Table 3. Effects of LR and its combination with GlcOS on growth performance of broilers

ADFI (g/d)	112.92±2.26	113.80±3.30	115.55±3.16	0.386
ADG (g/d)	$45.34{\pm}0.82^{b}$	47.33±1.74 ^a	48.50±1.47 ^a	0.012
F/G	$2.49{\pm}0.04^{a}$	2.41 ± 0.05^{b}	$2.38{\pm}0.03^{b}$	0.004
Day1-56				
ADFI (g/d)	86.93±1.72	87.19±2.23	88 47+2 03	0 451
			00.17±2.05	0.101
ADG (g/d)	37.88±0.49 ^b	39.04±1.23 ^{ab}	39.71±0.86 ^a	0.024

IW, initial weight; FW, final weight.

Values were presented as mean \pm SD of 6 replicates.

^{a, b} Values in a same row with different superscript letters are significantly different.

Intestinal morphology and barrier function of intestine

The intestinal morphology and barrier function of the CON, LR and RG groups are shown in Figure 1. In comparison to the CON group, dietary LR and RG significantly increased VH (P < 0.05) and decreased CD (P < 0.001), leading to an increased VH:CD (P < 0.001) (Figure 1A-C), and displayed more compact intestinal structure (Figure 1D). The microvilli in jejunum of LR and RG groups were better-arranged (Figure 1E), and the number of goblet cells in the LR and RG groups significantly more than CON group (P < 0.001) (Figure 1F, G).

In addition, dietary LR and RG significantly reduced the serum concentrations of DAO (Figure 1H) and D-lactic acid (Figure 1I), while mRNA levels of Occludin and Mucin 2 (MUC2) in the jejunum were notably increased in the RG group (P < 0.05) (Figure 1J). These results suggested that the synergistic effect of LR and GlcOS could improve the intestinal morphological structure and enhance the intestinal barrier function.

Intestinal digestion and absorption

Figure 2 shows the activity of intestinal digestive enzymes and expression of jejunal transporter genes. In terms of small intestinal digestive function, LPS and TPS activity was significantly improved by both LR and RG additives, in addition to AMY

in RG group (P < 0.05). Meanwhile, the TPS and AMS activity in the ileum in the RG group were notably higher than CON group (P < 0.05).

In terms of small intestinal absorption function, the mRNA expression of EAAT3, B^0AT , and SGLT1 significantly increased in the RG group, revealing that the digestive and absorptive functions of the small intestine could be enhanced by simultaneous supplementation of LR and GlcOS as growth promoter.

Cecum microbiota analysis

The Venn diagrams showed that all groups shared 640 operational taxonomic units (OUTs), and there were 232, 229, and 175 unique OUTs in the CON, LR, and RG group, respectively (Figure 3A). In regard to α -diversity, the Chao, Shannon, and observed species indices, were not notably different among groups, but community richness measured by the Simpson index of the RG group was significantly lower than the CON group (P < 0.05), indicating higher microbial diversity of the RG group (Figure 3B). Beta-diversity, conducted through the principal coordinate analysis (PCoA) revealed that the bacterial communities of groups formed distinct clusters, with the microbiota structure in both LR and RG groups deviating from the CON group (Figure 3C), which is also indicated by analysis of similarities (ANOISM) (Figure 3D).

As shown in Figure 4, the microbial community composition of the three groups was different at the phylum, family, and genus levels. *Bacteroidota* and *Firmicutes* were the top 2 most abundant phyla in each group, accounting for more than 80% of all microorganisms (Figure 4A). The relative abundance of *Desulfobacterota* increased significantly (Figure 4B) (P < 0.01). Meanwhile, the ratio of *Bacteroidetes* to *Firmicutes* was higher in the LR and RG groups, though without significance, still indicating the evident benefits of LR and RG on gut microbiota. At the family level, the cecum microbiota of the CON group was dominated by *Ruminococcaceae, Barnesiellaceae, Rikenellaceae* and *Lachnospiraceae*. *Bacteroidaceae, Ruminococcaceae,* while *Rikenellaceae* and *Lachnospiraceae* were majority in the LR

and RG groups (Figure 5A). The higher relative abundances of *Bacteroidaceae* (P < P0.05), Prevotellaceae (P < 0.05), and Desulfovibrionaceae (P < 0.01) were observed in the LR and RG groups (Figure 5C, E, G), while the relative abundances of Barnesiellaceae (P < 0.001) and norank_o_Clostridia_UGG-014 (P < 0.01) showed a significant decrease (Figure 5B, F). At the genus level, Barnesiella, Alistipes and norank_f_norank_o_Clostridia_UCG-014 dominated the microbiota in the CON group, Bacteroides, Faecalibacterium, Alistipes and norank_f_Muribaculaceae dominated in the LR group, and Bacteroides, Alistipes, Prevotellaceae_UCG-001 and norank f Muribaculaceae dominated in the RG group (Figure 6A). Compared with the CON group, the relative abundances of *Bacteroides* (P < 0.05) and *Prevotellaceae_UCG-001* (P < 0.05) in the LR and RG groups showed significant increase (Figure 6B, E), while the relative abundances of *Barnesiella* (P < 0.001) and $norank_f_norank_o_Clostridia_UCG-014$ (P < 0.01) showed a significant decrease (Figure 6D, F). In addition, the relative abundance of Ruminococcus_torques_group (P < 0.05) in the RG group was significantly higher compared to the LR group (Figure 6G).

The deviations of microbial communities among groups were screened based on LEfSe analysis (P < 0.05 and linear discriminant analysis score, LDA score > 4). It was found that genera *Bacteroides, norank_f_Clostridium_methylpentosum_group* and *Desulfovibrio* were enriched in the LR group, and *Prevotellaceae_UCG-001, norank_f_Muribaculaceae* and *Ruminococcus_torques_group* were enriched in the RG group, but not in the CON group (Figure 7).

Cecum metabolomic analysis

To investigate the effect of LR and its combination with RG supplementation on cecal microbiota, the cecal metabolite concentrations in the three groups were examined by LC-MS. Multivariate analysis including PCA and OPLS-DA was conducted between groups, before which the OPLS-DA, permutation test (n = 200) was applied to assure the good robustness of the model (Figure 8A). The PCA score

plot showed that the positive and negative ion differential metabolites in the LR and RG groups were significantly separated from the CON group (Figure 8B). Further analysis using OPLS-DA revealed specific differences among the three groups. The results demonstrated that the positive and negative ion metabolites were separated obviously between the CON group and experimental groups, with their respective clustering into groups without overlap (Figure 8B), suggesting that significantly different metabolites in cecum.

P-values were statistically analyzed by *t*-test, and VIP values were statistically analyzed by OPLS-DA to screen the differential metabolites between the CON, LR and RG groups. The criteria for determining differential metabolism are P < 0.05 and VIP > 1, and up/down regulation difference multiples > 1. According to the results, there were 539 identified differential metabolites in the LR group (337 up-regulated and 202 down-regulated) (Figure 9A) and 637 differential metabolites in the RG group (350 up-regulated and 287 down-regulated) (Figure 9B). Annotation of these differential metabolites between groups were mainly lipids and lipid-like molecules, organic acids, and organic oxygen compounds (Figure 9C-D). Furthermore, the differential metabolites were imported into the KEGG database to screen the top 30 significantly enriched metabolic pathways. Different metabolites between the CON and LR groups were mainly enriched in choline metabolism in cancer, axon regeneration, GnRH secretion, and dopaminergic synapse (Figure 10A). While biosynthesis of cofactors, alanine, aspartate, glutamate, glycerophospholipid, tyrosine and tryptophan metabolism pathways were most enriched in the differences between the CON and RG groups (Figure 10B).

In order to visualize the differences in metabolite patterns more comprehensively, hierarchical clustering analysis was performed based on the top 50 different metabolites screened in the LR and CON groups, and the RG and CON groups, and the results are shown in Figure 11. Tryptophanamide, alanyllysine, 11-hydroxy-9-tridecadienoic acid, dodecanedioic acid and 6-hydroxynicotinic acid were significantly up-regulated in the LR group compared with the CON group, while

lysophospholipids were significantly down-regulated. Substances such as indole-3-carboxylic acid, dodecanedioic acid, 11-hydroxy-9-tridecenoic acid and tryptophanamide were significantly up-regulated in the RG group compared with those in the CON group, while lysophospholipids, oxoglutaric acid and acetophenone were significantly down-regulated.

Correlation analysis between cecum microbiota and metabolomics

To further investigate the relationships between cecal microbiota and metabolites, Spearman correlation analysis was performed in the three groups (the CON and LR groups, the CON and RG groups). The relative abundance of Bacteroides was positively correlated with tryptophanamide, alanyllysine, 11-hydroxy-9-tridecadienoic acid, dodecanedioic acid, 6-hydroxynicotinic acid, and negatively correlated with lyso-phosphatidylcholine (Lyso-PC) and lyso-phosphatidylethanolamine (Lyso-PE). Meanwhile, the relative abundance of Bacteroides Prevotellaceae_UCG-001, norank_f_Muribaculaceae and Ruminococcus_torques_group were positively correlated with indole-3-carboxylic acid. dodecanedioic acid. 11-hydroxy-9-tridecenoic acid, tryptophan amide. In addition, the relative abundance of Barnesiella and norank_o__Clostridia_UCG-014 were positively correlated with the common differential metabolites lysophospholipids, oxoglutaric acid and acetophenone.

Discussion

After the ban on antibiotics as feed additives, synbiotics is ideal alternatives for the promoting growth performance. Previous research has shown that LR and its combination of oligofructose could improve the growth performance in poultry, owing to LR abilities to produce bacteriocins and organic acids, and GlcOS catabolized and metabolized to provide suitable substrates for probiotics, inhibiting the adhesion of pathogenic bacteria on the intestine, as shown the in analogous effect of *Bacillus subtilis*, xylo-oligosaccharide and mannan oligosaccharides combination on broilers (Bhogoju et al. 2021; Min et al. 2016; Quigley 2019; Song et al. 2022; Sureshkumar et al. 2022; Xu et al. 2022b). Our investigation demonstrated that a dietary LR and GlcOS could significantly improve the ADG and decrease the F/G of broilers, the combination of which is even more efficient than solitary LR, aligning with the findings of our previous studies, showing an analogous effect (Ding et al. 2024; Xu et al. 2022a; Xu et al. 2022b; Xu et al. 2023).

One of the underlying mechanism of probiotics as growth promoters can be attributed to the improved intestinal morphology and barrier function, meaning enhancement of gut health and nutrients absorption (Paiva et al. 2014; Wang et al. 2021b). In this study, dietary LR and RG improved the mucosal morphology of the small intestine, showing as higher VH, VH:CD and better-arranged microvilli, with RG demonstrating even superior effect than LR, assembling the effect of synbiotics containing LR and fructo-oligosaccharides in broilers (Jiang et al. 2020). Besides, our findings revealed that dietary LR and RG significantly increased the number of goblet cells in the jejunum of broilers, contributing to enhancing the intestinal mucosal barrier integrity and function, in accordance to previous studies (Figueroa-Lozano et al. 2020; Pelaseyed et al. 2014). In terms of barrier function, indigestible metabolite D-lactic acid, fermented by gut microbiota, and DAO, an intestinal intracellular enzyme, are normally precluded by intact mucosal barrier from serum, serving as markers of intestinal epithelium permeability, the serum level of which are significantly reduced by LR and RG. Tight junction proteins, such as ZO-1, Occludin, and Claudin-1 and mucus barrier protein MUC2 constitutes a crucial element of the intestinal in resistance to pathogens intrusion (Chelakkot et al. 2018; Cornick et al. 2019; Murray et al. 1993; Tsunooka et al. 2006; Van Itallie and Anderson 2014). In our research, LR and RG supplementation markedly increased the mRNA expression of Occludin and MUC2, suggesting that LR and RG supplementation provided a solid intestinal barrier of tight junction and mucin secretion by goblet cells.

Probiotics promoting growth attribute not only to the improved intestinal morphology and barrier, but also more active digestive enzymes and higher nutrient transportation efficiency (Zhang et al. 2022). It can be concluded from our results that probiotics applied can directly secrete digestive enzymes based on the fact that RG feed additives increased the activities of AMS, LPS, and TPS in the jejunum, as well as the TPS and AMS activities in the ileum. Moreover, functional oligosaccharides can provide energy for the intestinal tract continuously, reduce the loss of digestive enzymes, and indirectly improve the activity of digestive enzymes (Zhang et al. 2016). Nutrients transportation efficiency via transporter carriers is crucial for absorption and utilization. Glucose absorption is predominantly mediated by the Na⁺-dependent glucose transporter carrier-1 (SGLT1) (encoded by SLC5A1) (Breves et al. 2007). PepT1 (encoded by SLC15A1) is primarily responsible for the transport of small peptides, the primary digestion products of proteins, while amino acids are absorbed and transported mainly through EAAT3 (encoded by SLC1A1) and B^0AT (encoded by SLC6A19) (Bröer 2009; Daniel 2004; Kanai and Hediger 2004; Mann et al. 2003). The RG group showed significantly increased mRNA levels of SGLT1, EAAT3, B⁰AT and in the jejunum of broilers, which indicated that RG could promote the absorption of amino acids and glucose and thus improve the growth performance of broiler chickens.

The gut microbiota plays a pivotal role in broilers gastrointestinal health, the dysbiosis of which can be relieved by feed supplementation of synbiotics to maintain balanced structure (Graham and Xavier 2020; Peng et al. 2021; Schirmer et al. 2019; Zheng et al. 2020). The RG group exhibited significant efficacy in augmenting the α -diversity of the intestinal microbiota in broilers, leading to more diverse gut microbiota and stable microbial equilibrium. Our results showed LR and RG induced relative abundance in immunity-regulating Ruminococcaceae higher and Lachnospiraceae, Short chain fatty acid producing, Rikenellaceae, Inhibiting the production of proinflammatory lipid peroxidation metabolites Desulfobacterota, butyrate-producing Bacteroidaceae, carbohydrates and proteins-degrading Prevotellaceae, but lower abundance of pro-inflammatory and intestinal barrier impairing Barnesiella and norank_f_norank_o_Clostridia_UCG-014 showed a significant decrease in the LR and RG groups (Guo et al. 2021; Li et al. 2022; Liu et al. 2022; Shang et al. 2018; Song et al. 2023; Sun et al. 2024; Walker et al. 2003; Wang et al. 2021a; Zhang et al. 2023; Zhao et al. 2017). In summary, our results indicate that dietary LR and RG can promote the probiotics colonization and suppressing the propagation of harmful bacteria.

The alterations in the intestinal microbiota structure would inevitably modulate intestinal metabolism and regulate host health. In this study, a cecal metabolomics analysis of broilers revealed significant changes in enrichment in cofactor biosynthesis, amino acid metabolism, glycerophospholipid and choline metabolism, suggesting their potential as targets for the health-promoting effects of LR and RG on broilers. Cofactor biosynthesis plays a significant role in a variety of life activities of the organism such as energy metabolism, DNA and protein synthesis, and immune function (Magnúsdóttir et al. 2015; Peterson et al. 2020). The findings above demonstrated a marked elevation in the levels of the cofactor metabolites flavin adenine dinucleotide (FAD) and 4-hydroxybenzoic acid in the RG group. As the active form of riboflavin in vivo, FAD orchestrates the regulation of redox reactions, the tricarboxylic acid (TCA) cycle, and reactive oxygen species (ROS), thereby mitigating oxidative stress (Powers 2003). Moreover, 4-hydroxybenzoic acid stimulates Nrf2 signaling to curtail apoptosis and mitigate inflammation associated with intestinal injury (Han et al. 2021). In contrast, RG supplementation significantly decreased tyramine levels, attributing to alleviated intestinal stress (Jacobs et al. 2023). Moreover, alanine, aspartate, and glutamate metabolism are integral to the biosynthesis of the endogenous antioxidant glutathione. Aspartate undergoes citric acid cycle transforming to glutamate, an essential amino acid constituent of glutathione (Brand and Chappell 1974). Research indicates that these metabolites contribute to energy providing in the intestinal tract, protecting intestinal mucosa, mitigate mucosal injury induced by bacterial endotoxins, delay lymphocyte apoptosis, and facilitate cellular proliferation (Wang et al. 2015). In the cecum of the RG group, the levels of alanine, aspartate, and glutamate were significantly higher than the CON group. Recent studies have uncovered the critical roles of microbial tryptophan (Trp) metabolites in mediating colonization resistance against significant fungal and bacterial enteric pathogens. In general agreement with the results of the former studies, dietary RG elevated the levels of the Trp metabolite, indole-3-carboxylic acid, which activate the AhR signaling pathway and enhance T cell production of interleukin-17 (IL-17) to promote antimicrobial peptide production and enhance barrier function (Fang et al. 2022; Montgomery et al. 2022). A microbial Trp metabolite indole-3-aldehyde contributes to hydrocarbon receptor (AhR)-dependent- IL-22 transcription that helps to protect against Candida albicans infection (Zelante et al. 2013). Prior research indicated that Trp metabolite (including indole-3-aldehyde, and indole-3-ethanol) provide resistance indole-3-pyruvate, against enterohemorrhagic E. coli and Citrobacter rodentium, further confirming the intimate association between altered cecal microbiota and corresponding metabolite profiles due to the dietary inclusion of RG(Scott et al. 2024). In our research, the relative abundance of advantageous bacteria in the LR and RG group positively associated with a significant increase in metabolites, however negatively correlated with the increase in metabolites such as lysophospholipids, oxoglutaric acid and acetophenone. The robust correlation between gut microflora and metabolites suggests that significant changes may improve the structure of the intestinal microbiota and modulate intestinal metabolism, and ultimately enhance the growth performance of broilers.

Conclusions

In summary, dietary supplementation with LR and RG significantly enhances the intestine integrity, digestion and absorption, microbiota structure and metabolic activity of broiler chickens, thereby facilitating intestine health and growth performance, providing RG as a potential antibiotics alternative in poultry industry. However, the experiment was carried out under controlled conditions, the evidence supporting the field application of which is still lacking. Moreover, synergistic mechanisms between LR and GlcOS in immune regulation remained unexplored.

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Author Contributions

Conceptualization, X.Z. and A.F.; Methodology, R.T., J.C. and S.L.; Software, R.T., S.L. and J.C.; Validation, R.T., J.Z. and B.C; Formal analysis, X.D. and Y.X.; Data curation, R.T. and S.L.; Writing-original draft, R.T.; Writing -review &editing, R.T. and J.C. Visualization, R.T., J.C. and S.L.; Supervision, X.Z.; Project administration, X.Z. and A.F.; Funding acquisition, X.Z. A.F. and Y.X. All authors have read and agreed to the published version of the paper.

Competing Interests

The authors declare no competing interests.

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Figure 1. The effects of LR and its combination with GlcOS on intestinal morphology and barrier function of intestine. VH (A), CD (B), VH: CD (C), morphological microstructure (D) of duodenum, jejunum and ileum (40 ×). (E) Jejunum microvillus microstructure under transmission electron microscope (TEM). (F) PAS staining of jejunum (100 ×). (G) The number of goblet cells in the jejunum. Serum level of DAO (H) and D-lactic acid (I). (J) mRNA expression of jejunal tight junction genes. Data were presented as mean with SD (n=6). Significance was shown as * P < 0.05, **P <0.01, ***P < 0.001.



Figure 2. The effects of LR and its combination with GlcOS on intestinal digestion and absorption function. AMY (A), LPS (B) and TPS activity (C) of duodenum, jejunum and ileum. (D) mRNA expression of jejunal transporter genes. Data were mean with SD (n=6). Significance was shown as *P < 0.05, **P < 0.01, ***P < 0.001.



Figure 3. The effects of LR and its combination with GlcOS on structure of the cecum microbiota. (A) Venn diagram of OTUS. (B) Alpha-diversity analysis based on indices of the Chao, Shannon, Simpson, and observed species indices. (C) Beta-diversity analysis based on PCoA. (D) Beta-diversity analysis based on ANOISM. Data were mean with SD (n=6). Significance was shown as *P < 0.05.



Figure 4. The effects of LR and its combination with GlcOS on composition of the cecum microbiota at the phylum level. (A) Relative abundance of microbiota at the phylum level. (B) The relative abundances of *Desulfobacterota* in all groups. Significance was shown as * P < 0.05, **P < 0.01, ***P < 0.001.



Figure 5. The effects of LR and its combination with GlcOS on composition of the cecum microbiota at the family level. (A) Abundance of the microbial composition at the family level. The relative abundances of *Barnesiellaceae* (B), *Bacteroidaceae*

(C), Lachnospiraceae (D), Prevotellaceae (E), norank_o_Clostridia_UGG-014 (F) and Desulfovibrionaceae (G) in all groups. Significance was shown as *P < 0.05, **P < 0.01, ***P < 0.001.



Figure 6. The effects of LR and its combination with GlcOS on composition of the cecum microbiota at the genus level. (A) Abundance of the microbial composition at the genus level. The relative abundances of *Bacteroides* (B), Alistipes (C), *Barnesiella* (D), *Prevotellaceae_UCG-001* (E), *norank_f_norank_o_Clostridia_UCG-014* (F) and *Ruminococcus_torques_group* (G) in all groups. Significance was shown as *P < 0.05, **P < 0.01, ***P < 0.001.



Figure 7. LEfSe multilevel species discriminant analysis of cecum microbiota.



Bacterial taxa with LDA score > 4 are selected as biomarker taxa.

Figure 8. (A) OPLS-DA score plot and permutation test of broiler cecum metabolites. OPLS-DA plot and permutation test of the CON and LR groups in positive ion mode (A1) and in negative ion mode (A2). OPLS-DA plot and permutation test of the CON and RG groups in positive ion mode (A3) and in negative ion mode (A4). OPLS-DA plot and permutation test of the LR and RG groups in positive ion mode (A5) and in negative ion mode (A6). PCA score plot of broiler cecum metabolites in positive ion mode (B1) and in negative ion mode (B2).



Figure 9. Volcano plot and classification of different metabolites between groups. Differential volcano plot between CON and LR groups (A) and between CON and RG groups (B). Classification of differential metabolites between CON and LR groups (C) and between CON and RG groups (D).



Figure 10. Top 30 most enrichment pathways based on metabolites difference between the CON and LR groups (A) and between the CON and RG groups (B). The size of the bubbles in the figure represents the number of the pathway enriched, and the color of the bubbles indicate the magnitude of enrichment significance based on P-values.



Figure 11. (A) Hierachical clustering analysis of the differential metabolite in the LR and CON groups. (B) Hierachical clustering analysis of the differential metabolite in the RG and CON groups.



Figure 12. Correlations between significantly altered cecal microbiota and metabolite between the CON and LR groups (A), and between the CON and RG groups (B). Significance was shown as *P < 0.05, **P < 0.01, ***P < 0.001.