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Regulation of intestinal health by *Lactobacillus rhamnosus* GG during fasting-induced molting in laying hens

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ABSTRACT

This study aims to investigate the regulatory effects of adding *Lactobacillus rhamnosus* GG (**LGG**) during the fasting-induced molting (**FIM**) process on the intestinal mucosal barrier and microbiota of laying hens. A total of 288 houdan chickens of 420 days of age were randomly divided into four groups, with nine replicates in each group and each replicate containing eight chickens: NC group (no LGG added); TB group (LGG was added during the pre-fasting period (**F0 period**)); TF group (LGG was added during the fasting period (**F15 period**)); TBF group (LGG was added during both the pre-fasting and fasting periods). The FIM experiment focused on four key time points: F0, F15, the 5th day (R5), and the 30th day (R30) of refeeding. At each time point, one chicken was randomly selected from each replicate, euthanized via jugular vein exsanguination, and samples of the jejunum and ileum tissues, fixed samples, and cecal contents were collected for subsequent experiments. The results show that compared with the other three groups, the TBF group exhibited significant improvements in terms of egg production rate, egg quality, and changes in the ratio of villus height to crypt depth (V/C) in the jejunum and ileum. Compared with NC group, TBF group significantly increased the activity of antioxidant enzymes in serum during fasting, enhanced of the body's immune function, and also improved the intestinal barrier function, reduced intestinal inflammation and the content of oxidase, and further enhanced the digestion and absorption ability of FIM laying hens. The 16S sequencing results indicated that compared with the NC group, the TBF group significantly increased the abundance of beneficial bacteria during the refeeding period, reduced the number of pathogenic bacteria, optimized the intestinal microbiota structure, and promoted the production of short-chain fatty acids (**SCFAs**). In conclusion, the addition of LGG during the pre-FIM and fasting periods can improve the structure of the intestinal microbiota, promote the production of SCFAs, and improve intestinal barrier function, thereby enhancing intestinal health. This study provides a theoretical foundation and reference for the development of intestinal health and nutritional strategies for FIM laying hens.

Introduction

In recent years, with fluctuating feed raw materials prices as the backdrop, extended breeding periods for laying hens and breeding chickens have become a common trend in China's poultry industry development. After poultry undergoes an egg-laying cycle, the birds' bodies deplete a substantial amount of nutrients, leading to a decline in their physical condition. Consequently, both egg production and quality start to deteriorate, culminating in the onset of natural physiological molting (Kiat, et al., 2019). Therefore, most commercial laying hens and breeding chickens are usually eliminated when the egg production rate in the first laying cycle drops to a level that is no longer profitable for the breeding company.

Molting is a natural avian behavior aids in regulating body temperature, protecting the body, and restoring reproductive functions. It typically occurs as cold seasons approach and is defined as the periodic

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shedding and renewal of feathers (Biggs, et al., 2004). In the wild, birds alternate between molting and reproduction depending on the season. Molting terminates during the period of active gonads, and gonadal secretion is suppressed during molting. During this period, feed intake is reduced, and body weight is lost by about 20-30 % (Contina, et al., 2023). Due to the prolonged natural molting period, the molting sequence and duration vary among individuals, leading to inconsistent resumption of egg-laying. As a result, the group cannot reach peak egg production simultaneously, posing significant challenges for intensive rearing and management (Garlich, et al., 1984). FIM is a comprehensive management technique applied to laying and breeding hens in the later stages of egg-laying. Through diet and environmental regulation, it stimulates and initiates the natural physiological resting process of birds, allowing them to stop laying eggs/sperm in a synchronized manner and smoothly enter a new reproductive cycle after resting. It is an important technology for the extended breeding of laying hens and breeding chickens, with broad application prospects in their production (Wang, et al., 2023). However, fasting, as a stressor, can accelerate the molting process and egg production decline in laying hens, as well as induce immune stress, leading to damage to intestinal tissue morphology, disruption of intestinal microbiota, and increased susceptibility to pathogens such as *Salmonella enteritis* (Ben-Nathan, et al., 1981). Holt et al. demonstrated that FIM can reduce cellular immune function and increase inflammatory cell infiltration in the cecum and colon due to a significant decrease in CD4+ T cell counts in laying hens, as did Alodan (Alodan and Mashaly, 1999; Holt, 1992). The homeostasis of the intestinal microbiota is crucial for poultry. During fasting, inadequate nutrition leads to a decline in intestinal immune function, a reduction in beneficial bacterial populations, and enhanced colonization by pathogenic bacteria such as *Salmonella enteritidis*, along with other associated issues (Yousaf, et al., 2009). Corrier et al. found that the concentrations of acetate and propionate in the cecum of FIM laying hens were significantly lower compared to those in normally fed laying hens, likely due to a decrease in the number of SCFAs-producing bacteria (Corrier et al., 1997).

Lactobacillus rhamnosus GG's resistance to gastric acid and bile, as well as its strong affinity for intestinal mucosal cells, has been extensively studied as a probiotic for both humans and livestock. Clinical studies have confirmed that LGG plays a crucial role in several key areas, including balancing the intestinal microbiota (Chen, et al., 2021; Shi, et al., 2020), maintaining the integrity of the intestinal barrier (Chen, et al., 2023; He, et al., 2017), regulating intestinal immune function (Zhang, et al., 2024b), enhancing production performance (Liu, et al., 2023), preventing and treating diarrhea (Wang, et al., 2019), and stimulating the innate immune system (Bai, et al., 2021). Notably, LGG can adhere to the intestinal mucosa and transiently colonize the intestinal tract (Alander, et al., 1999), suggesting its potential therapeutic application in intestinal health.

Previous research by our team found that fasting can cause oxidative damage in the intestine (jejunum and ileum) mediated by increased MDA content, decreased expression of *ZO-1, Occludin-1*, and *Claudin-1* mRNA in the intestine, resulting in increased intestinal permeability, elevated expression of inflammatory cytokines *IL-1β, IL-8*, and *NF-κB* mRNA, and a series of pathological phenomena such as villus atrophy, exfoliation of epithelial cells, and infiltration of inflammatory cells in the lamina propria. Additionally, during the fasting period, the abundance of probiotics such as *Bacteroides, Faecalibacterium, Lactobacillus*, and *Paralactobacillus* in the cecum decreased, while the abundance of harmful bacteria such as *Desulfovibrio, Lachnoclostridium*, and *Escherichia-Shigella* increased (Zhang, et al., 2024a). This is closely related to the impairment of intestinal barrier function, decreased immunity, and the development of intestinal inflammatory diseases. During the FIM process, the abundance of beneficial bacteria, such as *Lactobacillus*, decreased. We speculate that the targeted addition of LGG before or during FIM may improve the structure of the intestinal microbiota, alleviate intestinal inflammation, and promote intestinal health during FIM. Furthermore, we determined whether our research could provide a theoretical foundation and reference for the development of intestinal health and nutritional strategies for FIM laying hens.

Materials and methods

Ethics statement

To ensure the welfare of the animals, all experiments and animal procedures were conducted in strict accordance with the principles recommended by the Ethics Committee of Henan Agricultural University (Permit Number: 11-0099). All experiments and methods were designed to minimize animal suffering.

Animals and experimental design

The experimental animals were provided by the Poultry Germplasm Resource Factory at Henan Agricultural University, Yuanyang County, Henan Province. The study was conducted from July 2023 to October 2023, with the temperature in the chicken house maintained at 18-25°C and the humidity at 60 %-70 %. A total of 288 houdan chickens of 420 days of age were randomly divided into four groups, with nine replicates in each group and each replicate containing eight chickens: NC group (no LGG instillation, 1 mL of saline per day from days 1 to 12 prior to fasting and 1 mL of saline per day from days 4 to 15 during the fasting period); TB group (1 mL of LGG with an effective viable bacterial count greater than 1×10^9 CFU/mL was instilled daily from days 1 to 12 prior to fasting, and 1 mL of saline was instilled daily from days 4 to 15 during the fasting period); TF group (1 mL of saline daily from days 1 to 12 prior to fasting; 1 mL of LGG with an effective viable count greater than 1×10^9 CFU/mL daily from days 4 to 15 during the fasting period); TBF group (1 mL of LGG with an effective viable count greater than 1×10^9 CFU/mL was instilled daily from days 1 to 12 prior to fasting; and 1 mL of LGG with an effective viable count greater than 1×10^9 CFU/mL was instilled daily from days 4 to 15 during the fasting period).The LGG (ATCC53103) used in this experiment was provided by BeiNa Bio (BNCC, Xinyang, China).The acclimatization period was 3 days, and the experimental period (12 days pre-fasting, 15 days fasting, and 30 days refeeding) lasted 57 days. The FIM process (Liang, et al., 2024) consisted of a pre-fasting period of 12 days (ad libitum feeding and 16 h of light per day), a fasting period of 15 days (the first three days were food and water fasting, and the remaining 12 days were food and water fasting with 8 h of light per day), and a refeeding period of 30 days (with a gradual increase in feed intake and light exposure by 0.5 h per day until 16 h). Monitor changes in body weight and egg production rate, and stop fasting early when weight loss reaches 25-30 %. The composition and nutrient levels of the basal diets are detailed in Table 1.

Sample collection

Chickens were euthanized by jugular vein bloodletting 1 day before fasting (F0), 15 days of fasting (F15), 5 days after resumption of feeding (R5), and 30 days after resumption of feeding (R30), respectively. Serum was collected, and fixed samples of the jejunum and ileum were separately collected and preserved in centrifuge tubes containing 4 % paraformaldehyde for subsequent section preparation. Tissue samples of the jejunum, ileum, and cecal contents were collected, placed in 1.5 mL sterile, enzyme-free tubes, quick-frozen in liquid nitrogen, and stored in a -80°C refrigerator.

Determination of egg quality

At R30, 30 eggs were randomly collected from each group for egg quality testing to evaluate the differences in egg quality between the groups. Egg quality determination included egg shape index, eggshell thickness, eggshell strength, egg weight, yolk color, Haugh unit, and

Table 1
Composition and nutritional standards for basic diets (air-dried basis).

Ingredients	Contents%	Nutrient components[b]	Contents%
Corn		Metabolizable	10.87
	62.5	Energy (MJ/Kg)	
Soybean meal	25.5	Crude Protein	15.50
Oil powder	0.8	Lysine	0.70
Limestone	9.2	Methionine	0.32
Premix[a]	2	Methionine+Cysteine	0.56
		Threonine	0.50
		Calcium	3.50
		Total Phosphorus	0.60
		Available Phosphorus	0.32
Total	100.0		

Note: a Premix provided the following per kilogram of diet: vitamin A, 518,320 IU; vitamin D, 3,100,026 IU; 25-hydroxyvitamin D3, 3,137 μg; vitamin E, 4,550 IU; vitamin K3, 272.8 mg; vitamin B1, 136.4 mg; vitamin B2, 750.2 mg; vitamin B6, 272.8 mg; vitamin B12, 1.4 mg; D-biotin, 16.4 mg; D-pantothenic acid, 818.4 mg; folate, 136.4 mg; niacinamide, 2,728 mg; choline chloride, 25,000 mg; Fe, 3,000 mg; Cu, 454.0 mg; Mn, 5,454 mg; Zn, 4,770 mg; I, 91.0 mg; Se, 13.6 mg; methionine, 55,000 mg; no more than 10 % moisture.
b Nutrient levels were calculated values.

albumen height measurements. The egg shape index (long axis/short axis) was measured using vernier calipers. Eggshell thickness was measured with an eggshell thickness gauge (ETG-1601A; Nanjing, China). Eggshell strength was measured using an eggshell strength meter (EFG-0-503; Tokyo, Japan). Egg weight, yolk color, Haugh unit, and albumen height were measured using an automatic egg-quality analyzer (EMT-5200; Tokyo, Japan).

Determination of intestinal morphology

Tissue samples from the jejunum and ileum were embedded in paraffin and cut into 5 μm thick sections using a rotary microtome. The sections were stained with hematoxylin and eosin (H&E). Ten villi with a complete shape and straight direction in each tissue section were selected, and the villus height and crypt depth were measured using Motic DSAssistant Lite software. The mean values were used as the measurement value for the sample, and V/C was calculated.

Determination of antioxidant indicators in serum

The levels of T-AOC, SOD, MDA, CAT, and GSH-PX in the serum samples were measured using biochemical kits purchased from Jiangsu Meimian Industrial Co., Ltd. (Jiangsu, China). All experimental steps were performed according to the instructions provided with the kits.

Measurement of immunity performance indicators in serum

The levels of immunoglobulin A (**IgA**), immunoglobulin G (**IgG**), and immunoglobulin M (**IgM**) in the serum samples were measured using ELISA kits purchased from Jiangsu Meimian Industrial Co., Ltd. (Jiangsu, China). All experimental steps were performed according to the instructions provided with the kits.

Quantitative real-time PCR

Total RNA was extracted from jejunum and ileum tissue using TRIzol reagent (Vazyme, Nanjing, China). RNA concentration was measured using a NanoDrop ND2000 spectrophotometer (NanoDrop Products, Wilmington, NC, USA), following the manufacturer's instructions. High-quality RNA samples (concentration $>$ 500 ng/μL, OD: 260/280 $>$ 1.8, OD: 260/230 $>$ 2.0) were used for reverse transcription. RNA was reverse transcribed into cDNA using a PrimeScript RT Reagent Kit (Vazyme, Nanjing, China). Real-time quantitative PCR (qRT-PCR) was performed to analyze the mRNA levels of *IL-6, IL-8, IL-1β, TGF-β3, ZO-1, Claudin-1, Occludin-1, CDK1, LGR5, MYC*, and *Bmi-1* using the $2^{-\Delta\Delta CT}$ method. qRT-PCR was conducted using an SYBR Premix Ex Taq II kit (Vazyme, Nanjing, China). The system comprised 10 μL of the following components: 0.5 μL of upstream primer, 0.5 μL of downstream primer, 1 μL of cDNA, 3 μL of sterile double-distilled water, and 5 μL of ChamQ Universal SYBR qPCR Master Mix (2×). The primer sequences are shown in Table 2.

DNA extraction and 16S rDNA sequencing analysis

The 16S rDNA gene of the intestinal microbiota was sequenced by Genedenovo Biotechnology Ltd. (Guangzhou, China) using high-throughput sequencing technology. Microbial DNA was extracted using HiPure Soil DNA Kits (Magen, Guangzhou, China) according to the manufacturer's protocols. The V3-V4 regions of the 16S rRNA gene were amplified by PCR using primers 341F 5'-CCTACGGGNGGCWGCAG and 806R 3'-GGACTACHVGGGTATCTAAT. Amplicons were evaluated with 2 % agarose gels and purified using AMPure XP Beads (Beckman, CA, USA) according to the manufacturer's instructions. Sequencing libraries were generated using the Illumina DNA Prep Kit (Illumina, CA, USA) following the manufacturer's recommendations. The library quality was

Table 2
Primer sequences for RT-qPCR.

Gene	Sequence of nucleotide	Gene ID	Product length (bp)
IL-1β	F 5- CTGCCTGCAGAAGAAGCCT -3′ R 5- TGTCAGCAAAGTCCCTGCTC -3′	NM_204524.2	164
IL-6	F 5- AAATGTCCAGCAGCCAGACT -3′ R 5- CTTTCGGAGCGGCCTTCATA -3′	NM_204628.2	130
IL-8	F 5- GGCTGGAGCAAAAGGTATGG -3′ R 5- AGCAGTGGGATCCAGACACA -3′	NM_205018.2	196
TGF-β3	F 5- GACTGGACAGTTGATGCGGA -3′ R 5- AATTCTTCAGGCCAGTCGGG -3′	NM_205454.2	161
ZO-1	F 5- GGTACGAGACCGAAGTGCAA -3′ R 5- ATATGTGGCTTGCCAACCGT -3′	NM_001301025.3	100
Claudin-1	F 5- AGCCTGGCTTAACTGAGTGT -3′ R 5- TGCTAGCCGTTGTAGCTGTA -3′	NM_001013611.2	100
Occludin-1	F 5- CCGTGGAGTCCAGTGATGAG -3′ R 5- TCGAACTCCTGCTTGTAGCG -3′	XM_046904540.1	123
CDK1	F 5- CCGCTGCGGGAAGGTAT -3′ R 5- TCTTAACACGCGAACGATCCA -3′	NM_205314.2	130
LGR5	F 5- TCAATACCTGAGCGTGCGTT -3′ R 5- TGTGAGTGTCAAACTCTCCAGAC -3′	XM_046909876.1	159
MYC	F 5- CAGCAGCGACTCGGAAGAAGAA -3′ R 5- ATGTTGACGTGACACCGCTT -3′	NM_001030952.2	179
BCL2	F 5- CAACGGAGGATGGGATGCC -3′ R 5- CTTATGTCCAAGATAAGCG -3′	NM_205339.3	145
GAPDH	F 5- GAACATCATCCCAGCGTCCA -3′ R 5- CGGCAGGTCAGGTCAACAAC -3′	NM_205518	110

assessed using ABI StepOnePlus Real-Time PCR System (Life Technologies, Foster City, USA). Finally, 2 × 250 bp paired-end reads were generated by sequencing on the Novaseq 6000 platform.

Short-chain fatty acid concentration determinations

First, take 0.2 g of cecal content and dilute it 10 times with deionized water. Then, use an ultrasonic cleaner to sonicate for 30 min, followed by centrifugation at 10,000 rpm for 10 min. Take 100 μL of the supernatant in a centrifuge tube, then add 900 μL of deionized water to further dilute the sample 10-fold. Finally, filter the sample through a 0.22 μm microporous membrane, and use the ion chromatograph (Thermo SCIENTIFIC DIONEX ICS-5000+) to determine the concentration of SCFAs.

Statistical analysis

The experimental data were organized using Excel 2021, and one-way ANOVA was performed using SPSS 26.0 (SPSS, Illinois, USA) followed by Tukey's HSD test. GraphPad Prism 8 (GraphPad Software, California, USA) was used for plotting. Data are shown as the means ± SEM. "*" means $P < 0.05$, "* *" means $P < 0.01$, "* * *" means $P < 0.001$. The data with different superscript letters (a, b, c) were significantly different ($P < 0.05$).

Results

Lactobacillus rhamnosus GG improve production performance

During the FIM process, the body weight of each group dropped most rapidly at F3, and the weight loss rate reached its maximum at F15 (Fig. 1A). After resuming feeding, the body weight recovered rapidly, and the recovery rate reached its maximum at R24, reaching approximately 96.5 % of the initial body weight. The body weight remained relatively stable in subsequent periods (Fig. 1B), and there was minimal difference in body weight changes among the four groups. The effect of adding LGG during FIM on the mortality rate is shown in Table 3. The mortality rate of each group was similar during the pre-fasting period, but the mortality rate of the control group during the fasting period was significantly higher than that of the three treatment groups. No chicken deaths were recorded in the treatment groups during the recovery period.

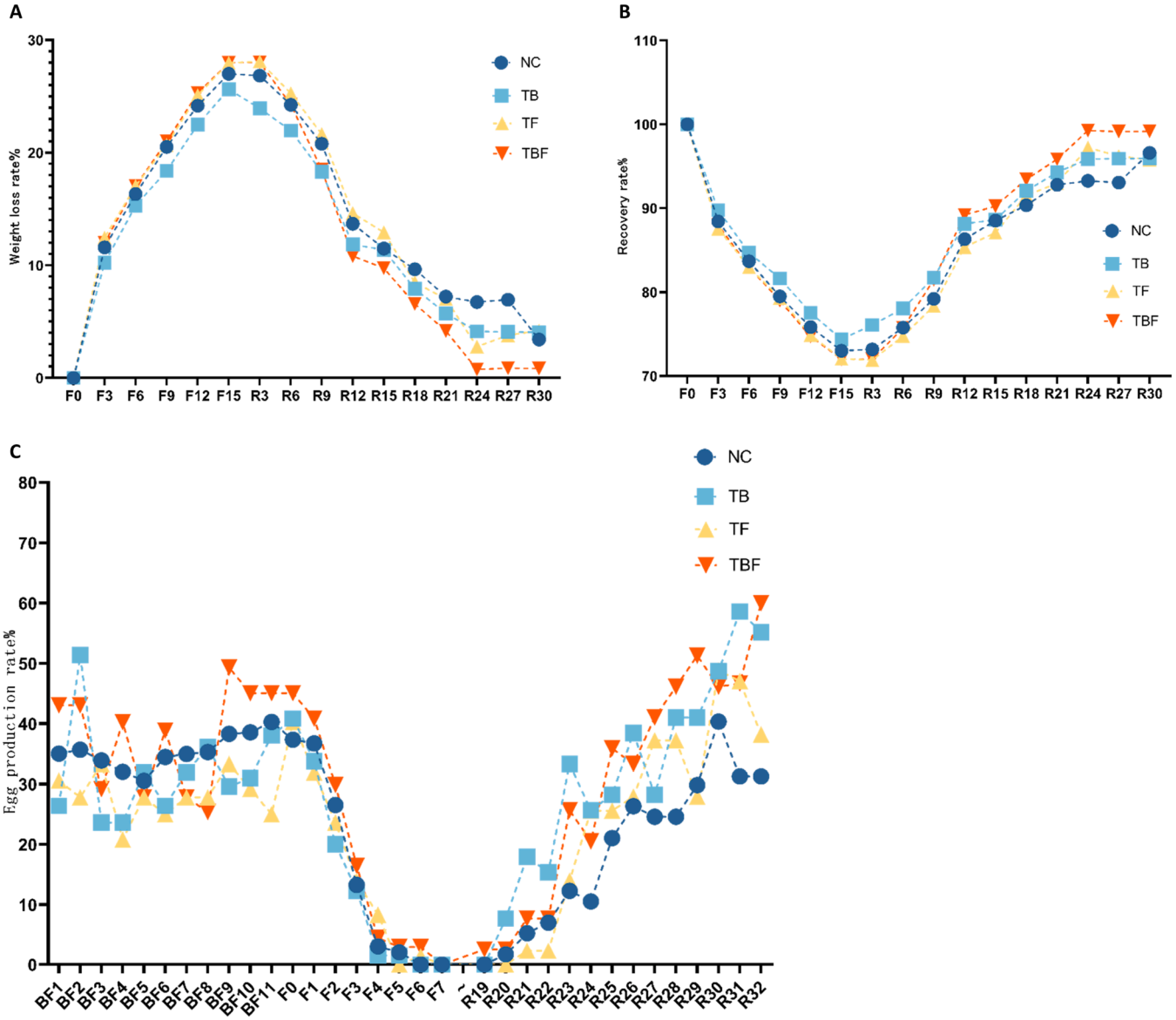

Fig. 1. Effect of LGG on the production performance of laying hens during the FIM process. (A) Weight loss rate; (B) Recovery rate; (C) Egg production rate.

Table 3
Effect of LGG on the mortality rate during the FIM process.

Items		NC	TB	TF	TBF
Mortality rate %	Adaptation period	0	1.38	1.38	1.38
	Fasting period	8.33	0	1.38	1.38
	Recovery period	6.94	0	0	0

NC: no LGG added; TB: LGG was added during the pre-fasting period (F0 period); TF: LGG was added during the fasting period (F15 period); TBF: LGG was added during both the pre-fasting and fasting periods.

The initial egg-laying rate was approximately 30 % in each group, and egg laying ceased at F7 and resumed at R19. The TBF group began laying eggs earlier than the NC group, and at R32 (Fig. 1C), the egg laying rates of the TB and TBF groups were about 25 % higher than those of the NC group. The effects of adding LGG during FIM on egg quality are shown in Table 4. During the R30 period, the eggshell weight of the TF group was significantly higher than that of the TB group; the albumen height of the TBF group was significantly higher than that of the other three groups, and the yolk color of the TF group was significantly lighter ($P < 0.05$). In summary, adding LGG during the FIM process can improve egg quality to some extent.

Lactobacillus rhamnosus GG Improve the intestinal morphology

The effect of adding LGG during FIM on intestinal morphology is shown in Fig. 2. The tissue morphology of the jejunum and ileum was intact, with neatly aligned and compactly arranged lamina propria during the pre-fasting period. Compared with the NC group, there was a significant difference in villus height as well as V/C in the jejunum of the TB and TBF groups ($P < 0.05$), but no significant change was observed in the ileum (Fig. 2A). Inflammatory cell infiltration in the lamina propria was more severe during the fasting period, and there was extensive apical shedding of villi. However, this condition improved in the TBF group compared with the NC group, and the villus height and V/C were significantly increased in the jejunum ($P < 0.05$), but the difference was not significant in the ileum (Fig. 2B). The tissue structure was relatively intact at the beginning of refeeding (R5), and although inflammatory cell infiltration occurred, it was less severe compared to the fasting period. Compared with the NC group, the TBF group showed the best recovery, with a significant increase in villus height and a significant decrease in crypt depth in the jejunum ($P < 0.05$). However, there was no significant difference in the ileum (Fig. 2C). The jejunum and ileum tissues were morphologically intact, with neatly aligned and compact lamina propria at the end of refeeding (R30). Compared with the NC group, V/C was significantly higher only in the TF group ($P < 0.05$), and there was no significant change in the other two groups (Fig. 2D). The addition of LGG during FIM may improve intestinal absorption by increasing jejunal villus height and decreasing crypt depth, thereby ameliorating intestinal damage caused by fasting.

Table 4
Effect of LGG on egg quality during the FIM process.

Indexs	NC	TB	TF	TBF	*P*-value
Average egg weight (g)	51.54 ± 5.11	50.11 ± 5.97	52.26 ± 6.22	50.8 ± 3.99	0.451
Egg shape index, %	1.33 ± 0.06	1.34 ± 0.08	1.36 ± 0.07	1.36 ± 0.08	0.448
Eggshell thickness (mm)	0.33 ± 0.04	0.34 ± 0.06	0.33 ± 0.06	0.33 ± 0.05	0.695
Eggshell strength (N)	35.48 ± 8.29	37.27 ± 7.76	38.22 ± 8.08	35.84 ± 7.18	0.503
Eggshell weight (g)	6.84 ± 0.84^{ab}	6.65 ± 0.72^{b}	7.09 ± 0.84^{a}	6.74 ± 0.72^{ab}	<0.05
Albumen height (mm)	4.64 ± 0.92^{b}	4.5 ± 0.83^{b}	4.6 ± 0.7^{b}	5.33 ± 1.80^{a}	0.334
Yolk color score	8.8 ± 0.83^{a}	8.78 ± 0.63^{a}	8.22 ± 0.92^{b}	8.79 ± 0.76^{a}	<0.05
Haugh units	67.36 ± 9.22	65.84 ± 10.01	67.27 ± 8.77	65.23 ± 12.04	0.806

The data with different superscript letters (a, b, c) were significantly different ($P < 0.05$). NC: no LGG added; TB: LGG was added during the pre-fasting period (F0 period); TF: LGG was added during the fasting period (F15 period); TBF: LGG was added during both the pre-fasting and fasting periods.

Lactobacillus rhamnosus GG enhance the antioxidant level

The effects of adding LGG on serum oxidation and antioxidant levels during the FIM process are presented in Fig. 3. During the FIM process, the activity of antioxidant enzymes SOD, CAT, and GSH-PX in the NC group first decreased and then increased, with activity significantly lower during the fasting period. Notably, compared with the NC group, the activity of the antioxidant enzymes T-AOC, SOD, CAT, and GSH-PX in the TBF group was significantly increased ($P < 0.05$), and the content of MDA was significantly lower ($P < 0.05$). These data suggest that LGG, on the one hand, reduces oxidative damage caused by fasting by lowering MDA content during FIM, and on the other hand, mitigates stress caused by fasting by increasing antioxidant enzyme activity.

Lactobacillus rhamnosus GG enhance the immune level

The effect of adding LGG during FIM on serum immune levels is shown in Fig. 4. The levels of IgA, IgG, and IgM in the NC group decreased to some extent during FIM, whereas the levels of IgA, IgG, and IgM in the TBF group were significantly higher during FIM compared to the NC group ($P < 0.05$). The results indicated that adding LGG during the FIM process could improve the body's immune function.

Lactobacillus rhamnosus GG improves the chemical barrier in the intestinal tract

The effects of adding LGG during FIM on inflammatory factors, tight junction proteins, genes related to proliferation and apoptosis are shown in Fig. 5. Compared with the NC group, during the pre-fasting period, the pro-inflammatory factor *IL-6* was significantly reduced in both the jejunum and ileum ($P < 0.05$). However, only the tight junction protein *Claudin-1* and the proliferation-promoting genes *LGR5, MYC*, and the apoptosis suppressor gene *BCL2* were significantly increased in the jejunum ($P < 0.05$), whereas only *CDK1* was significantly increased in the ileum ($P < 0.05$) (Fig. 5A–C). During the fasting period, *IL-6* and *IL-8* in the jejunum were significantly reduced ($P < 0.05$), and Occludin-1 was significantly increased in both the jejunum and ileum in the TBF group ($P < 0.05$) (Fig. 5A and B). During the refeeding period, *IL-1β* was significantly decreased ($P < 0.05$), and *Occludin-1* and *MYC* were significantly increased in both the jejunum and ileum of the TBF group ($P < 0.05$). However, *TGF-β3, ZO-1, CDK1, LGR5*, and *BCL2* were significantly elevated only in the jejunum ($P < 0.05$) (Fig. 5A–C). These results suggested that adding LGG during the FIM process could enhance intestinal barrier function, reduce intestinal inflammation, and further improve the digestion and absorption capacity of FIM laying hens.

Lactobacillus rhamnosus GG improves the structure of intestinal microorganisms

To investigate the effects of LGG on the intestinal microbiota during the FIM process in laying hens, we analyzed the composition of the cecal microbiota using 16S rDNA gene sequencing. Compared with the NC group, at the phylum level, the abundance of *Firmicutes* in the TBF group increased during the fasting period; during the recovery period, the abundance of *Bacteroidetes* and *Firmicutes* increased, and the abundance of *Desulfuromonadia* decreased ($P < 0.05$) (Fig. 6A). At the genus level, the abundance of beneficial bacteria, such as *Bacteroides, Rikenellaceae_RC9_intestinal_group*, and *Synergistes*, significantly increased during

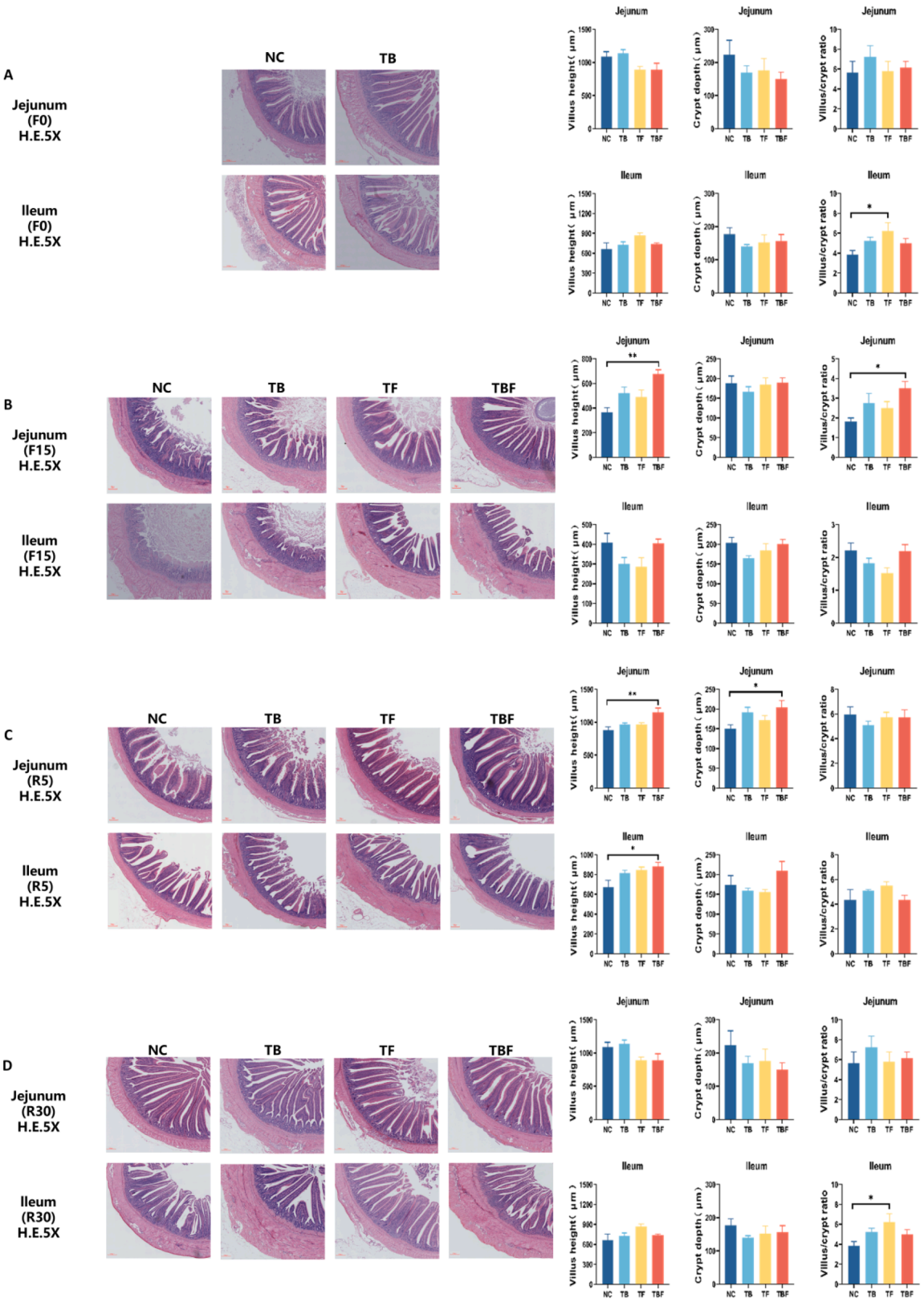

Fig. 2. Effect of LGG on the morphology of the jejunum and ileum in laying hens during the FIM process. (A) Representative images of hematoxylin and eosin staining of jejunal and ileal sections before fasting; (B) Representative images of hematoxylin and eosin staining of jejunal and ileal sections during fasting; (C) Representative images of hematoxylin and eosin staining of jejunal and ileal sections at the beginning of refeeding; (D) Representative images of hematoxylin and eosin staining of jejunal and ileal sections at the end of refeeding. Scale bar = 200 μm. Villus height and crypt depth in the jejunum and ileum of laying hens from different groups were quantified. Data are mean ± SEM. "*" means $P < 0.05$, "**" means $P < 0.01$, "***" means $P < 0.001$.

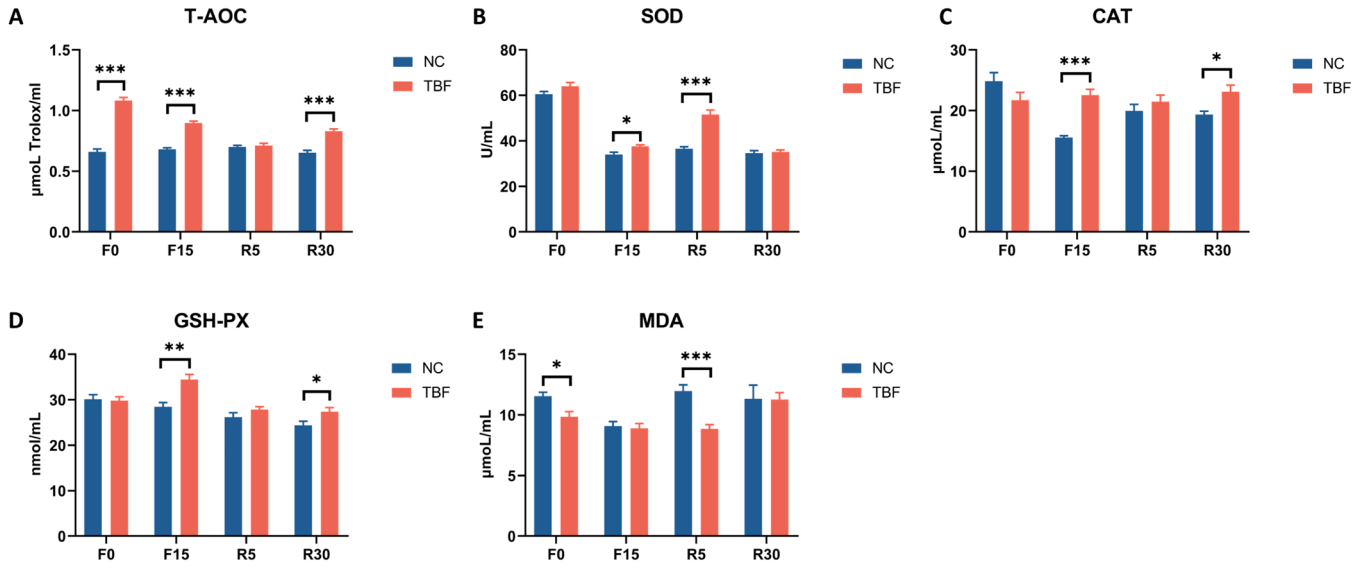

Fig. 3. Effect of LGG on oxidative and antioxidant levels in the jejunum of laying hens during the FIM process. (A) T-AOC; (B) SOD; (C) CAT; (D) GSH-PX; (E) MDA. Data are mean ± SEM. "*" means $P < 0.05$, "**" means $P < 0.01$, "***" means $P < 0.001$.

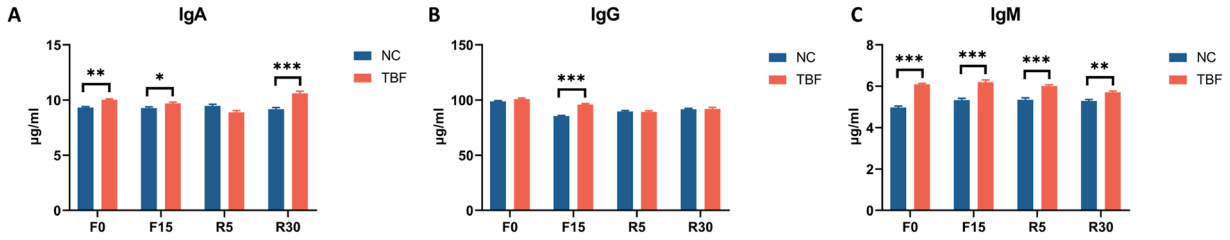

Fig. 4. Effect of LGG on serum immune parameters in laying hens during the FIM process. (A) IgA; (B) IgG; (C) IgM. Data are mean ± SEM. "*" means $P < 0.05$, "**" means $P < 0.01$, "***" means $P < 0.001$.

the refeeding period, while the abundance of harmful bacteria, such as *Desulfovibrio, Ruminococcus_torques_group*, and *Escherichia-Shigella*, was significantly decreased ($P < 0.05$) (Fig. 6B and C). Alpha diversity analyses showed that the Shannon, Simpson, and Chao1 indices in the TBF group were significantly different from those in the NC group during the pre-fasting period, and there were no significant changes in either group during the other periods. Furthermore, the Simpson index did not show significant differences in any of the three periods ($P < 0.05$) (Fig. 6D–G). Beta diversity analysis revealed that, compared with the NC group, there were significant differences in the bacterial community structure during the refeeding period ($P < 0.05$) (Fig. 6H). We compared the differential microbiota between the two groups using Welch's t-test. At the genus level, *Mucispirillum* and *Pseudoflavonifractor* were significantly increased during the pre-fasting period compared to the NC group. *Veillonella* was significantly increased, and *Monoglobus* was significantly decreased during the fasting period. However, *Bacteroides* significantly increased, and *Alistipes, Erysipelatoclostridium, Desulfovibrio, Chlamydia*, and *Bilophila* significantly decreased during the refeeding period ($P < 0.05$) (Fig. 6I). These data suggest that FIM alters the structure of the cecal microbiota in laying hens, and that the addition of LGG can improve intestinal health by increasing beneficial microbes.

Lactobacillus rhamnosus GG increase the concentration of SCFAs in the cecum

In this study, the concentrations of acetate, propionate, butyrate, valerate, isobutyrate, and isovalerate in the cecal contents of FIM-treated laying hens were analyzed. The results showed that the concentrations of valerate, isobutyrate, and isovalerate in the TBF group increased significantly during fasting, compared to the NC group ($P < 0.05$) (Fig. 7D–F). However, there were no significant changes in the concentrations of acetate, propionate, and butyrate during fasting (Fig. 7A–C). These data suggest that adding LGG to the FIM process improves the fasting-induced reduction in SCFAs.

Discussion

FIM can enable laying hens and breeding chickens in the late stage of production to re-enter a new laying cycle after egg production has ceased. It not only effectively extends the service life of laying hens and breeding chickens, and reduces the cost of purchasing chicks, but also saves time and feed in chicken breeding. This can bring significant benefits to breeding companies. However, during the FIM process, immune stress may be induced in laying hens, which can lead to a significant increase in susceptibility to *Salmonella enterica*, and the mortality rate of the flock may also increase significantly (Buyse, et al., 2023; Webster, 2003; Zhang, et al., 2024a). Therefore, improving intestinal health is critical during the fasting period. LGG is a probiotic that naturally occurs in the human intestines and vagina. It plays an important role in maintaining digestive health, enhancing immunity, reducing allergic symptoms, and improving vaginal health (Capurso, 2019). During the FIM process, although the addition of LGG had no significant effect on body weight changes, it increased the egg production rate and reduced chicken mortality, thereby improving egg quality to some extent. Weight change is a key indicator during the FIM process. Gongruttananun et al. found that the weight loss of hens subjected to forced molting using tapioca starch was approximately 30.13 %, which was higher than the weight loss in the group that was fed only tapioca

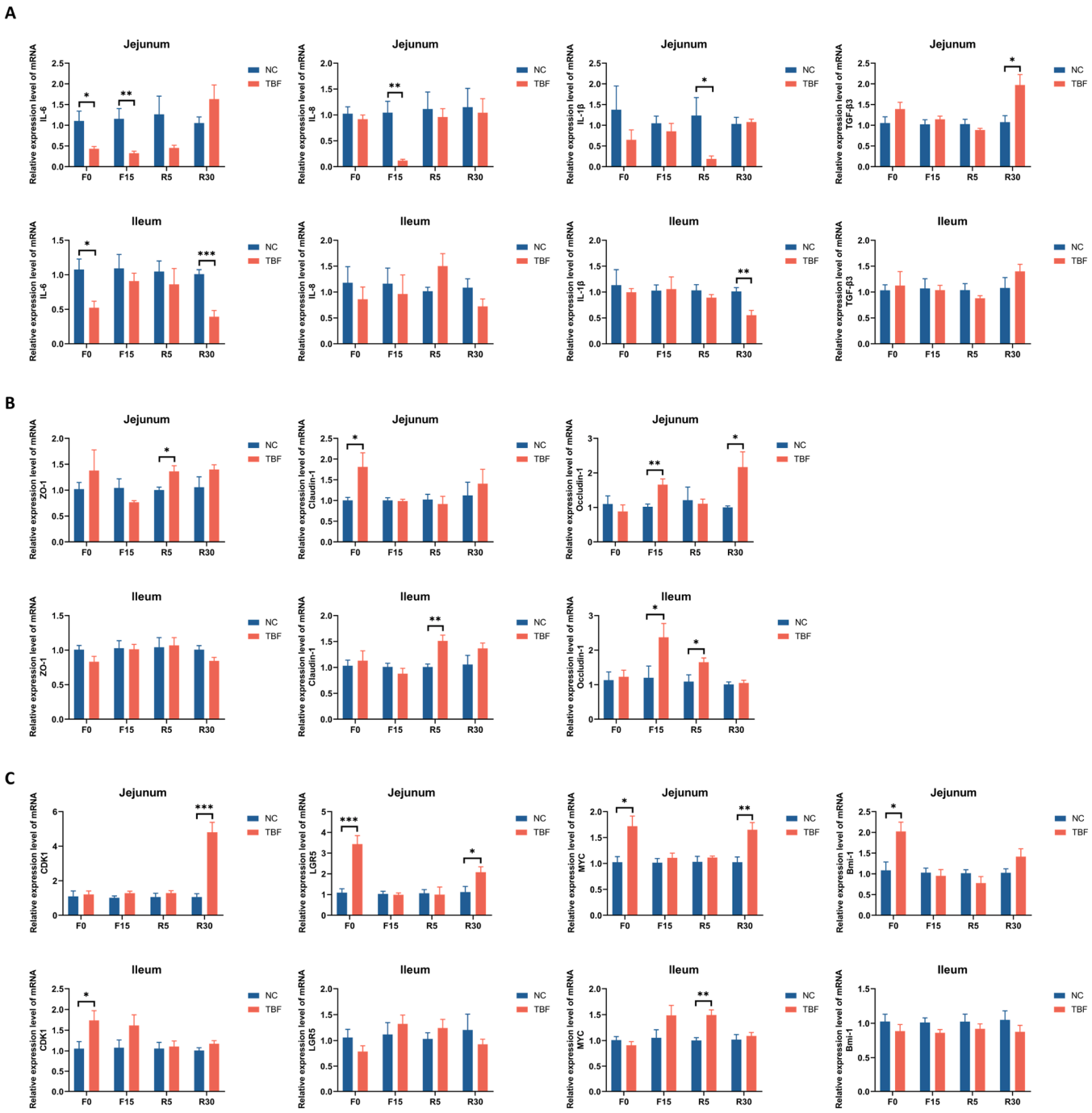

Fig. 5. Effects of LGG on inflammatory factors, tight junction proteins, and proliferation-apoptosis-related genes in laying hens during the FIM process. (A) Expression of inflammatory factors during the FIM process; (B) Expression of tight junction proteins during the FIM process; (C) Expression of genes related to proliferation and apoptosis during the FIM process. Data are mean ± SEM. "*" means $P < 0.05$, "**" means $P < 0.01$, "***" means $P < 0.001$.

starch for 3 weeks (25.23 %) (Gongruttananun et al., 2017). Furthermore, another study pointed out that weight loss is related to laying performance after molting, with a weight loss of about 27 % to 31 % leading to the optimal performance after molting (Baker, et al., 1983). Liu et al. (2023) found that laying hens with LGG added to their diet exhibited significant reductions in feed conversion rates over the 31-45-day period, and eggshell strength and thickness also increased significantly. In summary, LGG can improve egg production and quality to some degree, as well as reduce the mortality rate.

Intestinal morphology can significantly influence the growth performance of FIM laying hens. Both Xiyan Kou et al. and Samiullah Khan et al. have demonstrated that the microbiota plays a significant role in maintaining intestinal morphology and influencing overall performance in laying hens (Khan, et al., 2020; Kou, et al., 2024). Therefore, we hypothesize that LGG may improve the growth performance of FIM laying hens by enhancing intestinal function (Zou, et al., 2019). Our results indicate that the addition of LGG can increase villus height in the jejunum and decrease crypt depth during the fasting period. Therefore, LGG may enhance nutrient absorption and utilization in the small intestine, ultimately improving the growth performance of FIM laying hens.

We speculated that oxidative damage in the intestinal might led to morphological changes, so we tested the levels of oxidation and antioxidants in the serum of laying hens. Fasting, as an external stress factor,

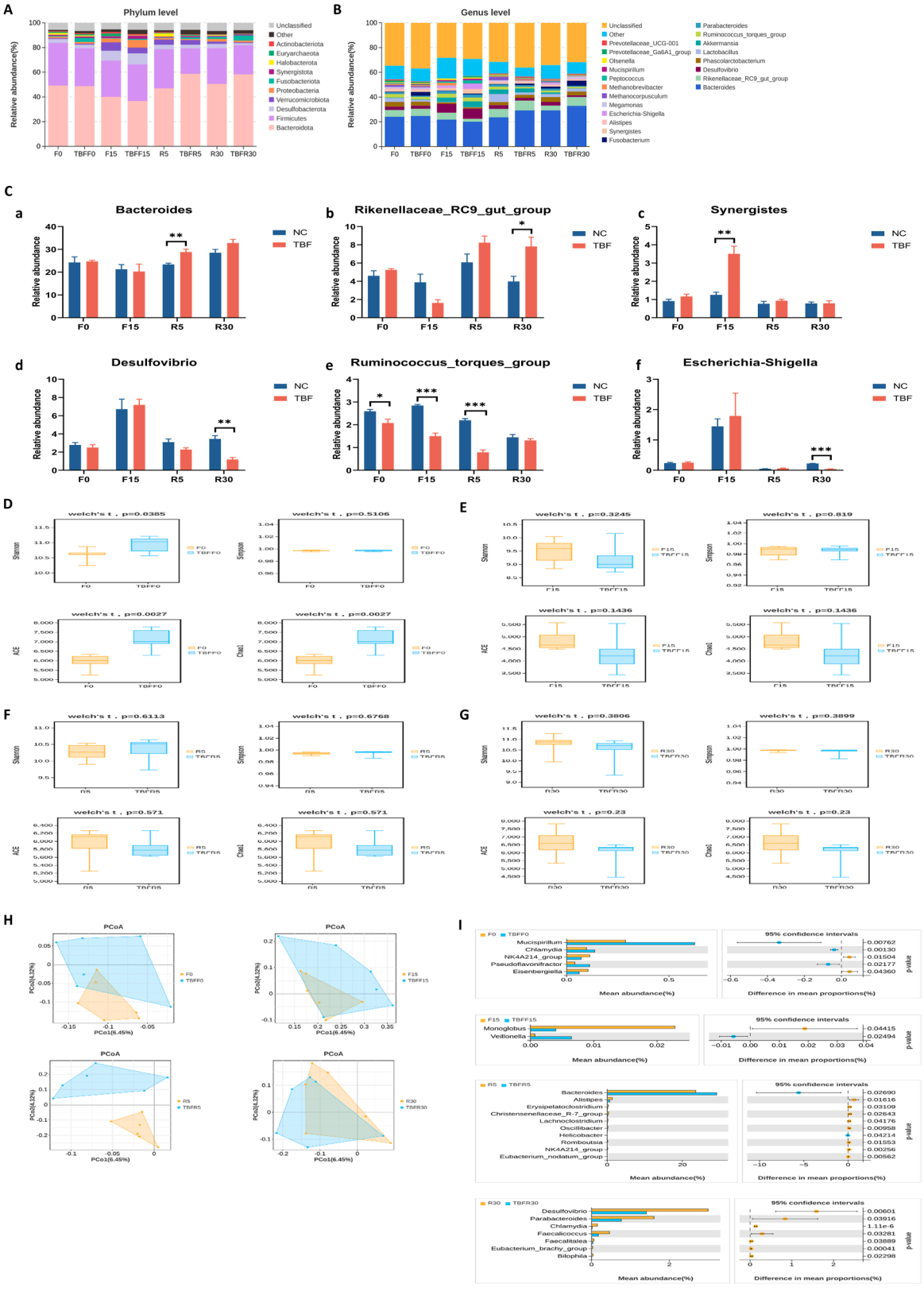

Fig. 6. Effect of LGG on the cecal microbiota of laying hens during the FIM process. (A) Relative abundance of cecal microbiota at the phylum level; (B) Relative abundance of cecal microbiota at the genus level; (C) Relative abundance of beneficial and harmful bacteria at the genus level (top 3) in different groups; (D-G) Diversity and richness indices of cecal microbiota in each group. Principal coordinate analysis (PCoA) plot of the cecal microbiota composition at the operational taxonomic unit (OTU) level from different groups. (H) Points with different colors represent the centroids of each group. The closer the points, the more similar the gut microbiota structure; (I) Differences in the relative abundance of cecal microbiota at the genus level between groups at different time points. Data are mean ± SEM. "*" means $P < 0.05$, "**" means $P < 0.01$, "***" means $P < 0.001$.

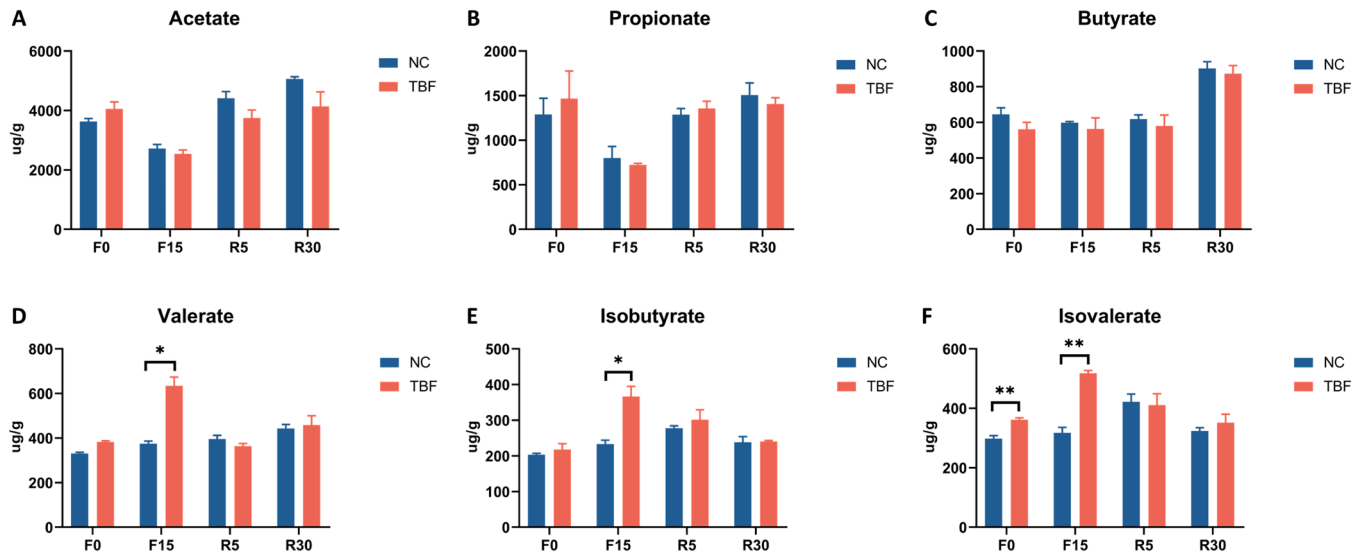

Fig. 7. Effect of LGG on cecal SCFAs in laying hens during the FIM process. (A) Acetate; (B) Propionate; (C) Butyrate; (D) Valerate; (E) Isobutyrate; (F) Isovalerate. Data are mean ± SEM. "*" means $P < 0.05$, "**" means $P < 0.01$, "***" means $P < 0.001$.

can induce changes in the body's antioxidant levels, similar to cold and heat stress (Li, et al., 2020). Oxidation and antioxidant levels are used to evaluate the body's ability to resist oxidative stress (Munteanu and Apetrei, 2021). We found that antioxidant enzyme activities in the serum of laying hens decreased initially and then increased during the fasting period, indicating that oxidative stress occurred at the onset of fasting and was alleviated by the resumption of feeding. This is consistent with findings from a previous study (Lei, et al., 2023). Additionally, adding LGG can reduce the levels of MDA and enhance the activities of T-AOC, SOD, CAT, and GSH-PX, thereby mitigating oxidative damage during the fasting period.

IgA, IgG, and IgM are crucial components of the immune system (Schroeder and Cavacini, 2010), and different types of immunoglobulins play distinct roles in immune defense, working together to maintain health by recognizing and binding to specific antigens and triggering immune effector functions. This study found that the levels of IgA, IgG, and IgM in serum decreased during the fasting period, indicating a reduction in immune function and a weakened ability to resist external risks. Similar phenomena have been observed by other researchers as well (Lei, et al., 2023). Notably, the addition of LGG during the FIM process significantly increased the serum levels of IgA, IgG, and IgM during the fasting period.

To further investigate the role of LGG in intestinal injury in FIM laying hens, we examined the expression of inflammatory factors and tight junction proteins in the jejunum and ileum. The normal function of *IL-1β, IL-8*, and *NF-kB* is essential for maintaining intestinal homeostasis and defense mechanisms. Dysregulation of these cytokines can lead to the development of inflammatory diseases and activation of immune responses in the organism (Aggeletopoulou, et al., 2024; Hai Ping, et al., 2016; Mukherjee, et al., 2024; Rius, et al., 2008). We found that after the addition of LGG during FIM, *IL-6* and *IL-8* in the jejunum were significantly reduced during the fasting period, while *TGF-β3* was significantly increased compared with the NC group during the refeeding period. In the ileum, only *IL-1β* and *IL-6* were significantly reduced during the refeeding period. Tight junction proteins play a crucial role in the intestine by maintaining the integrity of the intestinal epithelial barrier. These proteins include Claudin, Occludin, and Zonula occludens (**ZO**), which seal the gaps between cells, regulate the permeability of substances, and prevent the invasion of harmful substances (Horowitz, et al., 2023). We observed that, in the TBF group supplemented with LGG, the expression levels of *ZO-1, Claudin-1*, and *Occludin-1* increased significantly during the FIM process compared to the NC group. However, in the ileum, only the expression levels of *Claudin-1* and *Occludin-1* were significantly increased compared to the NC group. Hao Zhang et al. found that, during the fasting period, the expression levels of inflammatory factors *IL-8* and *IL-1β* increased in the jejunum and ileum of laying hens, while the expression levels of tight junction proteins *Occludin-1* and *ZO-1* decreased. After refeeding, these indicators returned to pre-fasting levels (Zhang, et al., 2024a).

Proliferative genes play an important role in the rapid renewal of the intestinal epithelial cell layer to maintain homeostasis by controlling cell cycle progression and the proliferative state of the cells (Chelakkot, et al., 2018). The role of apoptotic genes in different cell types is multifaceted, as they are involved in regulating the cell life cycle, including cell proliferation, differentiation, and clearance of damaged or unnecessary cells (D'Arcy, 2019; Elmore, 2007). After the addition of LGG, we found that during the refeeding period, the expression of genes *CDK1, LGR5*, and *MYC*, which promote cell proliferation, was significantly increased, and the expression of the gene *BCL2*, which suppresses cell apoptosis, was also significantly increased. However, in the ileum, only the expression levels of *CDK1* and *MYC* were significantly altered. Both Lien Verboom et al. and Chaithanya Chelakkot et al. found that apoptotic genes may increase and proliferative genes may decrease in intestinal epithelial cells during fasting, thereby affecting the integrity of the intestinal barrier (Chelakkot, et al., 2018; Verboom, et al., 2023). Interestingly, the results of our study show that LGG can improve intestinal barrier function, consistent with previous research findings (Orlando, et al., 2018). In summary, LGG may reduce intestinal permeability and repair the damaged intestinal barrier by decreasing the inflammatory response in the intestines and promoting the proliferation and differentiation of intestinal epithelial cells. This helps reduce intestinal villi atrophy, improve intestinal absorption capacity and immune function, and accelerate intestinal recovery.

The intestinal microbiota plays an important role in maintaining intestinal barrier function and immune homeostasis (Aguilera, et al., 2015; Cani, 2016). Dysbiosis of the microbiota is closely associated with intestinal inflammation, and these changes are classified as diseases (Rooks and Garrett, 2016). Previous studies have shown that FIM strongly influences the composition and structure of the intestinal microbiota (Zhang, et al., 2024a). Compared with the NC group, at the phylum level, the abundance of *Firmicutes* in the TBF group increased during the fasting period. During the refeeding period, the abundance of both *Bacteroidetes* and *Firmicutes* increased, while the abundance of *Desulfuromonadia* decreased. *Firmicutes* and *Bacteroidetes* can produce

SCFAs, such as lactate and acetate, through fermentation (Fan and Pedersen, 2021; Gibiino, et al., 2018). Additionally, they regulate bile acid metabolism, jointly promote the balance of intestinal microbiota, and play a significant role in host health and disease (Gibiino, et al., 2018; Pushpass, et al., 2022). We suggest that LGG can increase the abundance of *Firmicutes* and *Bacteroidetes*, thereby improving intestinal health. At the genus level, the abundance of beneficial bacteria, such as *Bacteroides, Rikenellaceae_RC9_intestinal_group*, and *Synergistes*, significantly increased during the recovery period, while the abundance of harmful bacteria, such as *Desulfovibrio, Ruminococcus_torques_group*, and *Escherichia-Shigella*, significantly decreased. *Bacteroides* contribute to the breakdown of complex polysaccharides in food to produce SCFAs, such as acetate, propionate, and butyrate (Shin, et al., 2024). These SCFAs play an important role in maintaining the balance of the intestinal microbiota, improving intestinal permeability, reducing inflammatory responses, and participating in various important metabolic activities in the human colon (Gibiino, et al., 2018; Soto-Martin, et al., 2020; Zafar and Saier, 2021). *Desulfovibrio* is a common harmful bacterium that can produce hydrogen sulfide (H_2S), which may be toxic to intestinal epithelial cells and lead to gastrointestinal diseases (Singh, et al., 2023; Zhou, et al., 2024). *Escherichia-Shigella* is a pathogen known to infect intestinal epithelial cells, causing the release of pro-inflammatory factors and leading to intestinal inflammation (Li, et al., 2022). Dawei Chen et al. found that LGG improved the composition of the intestinal microbiota by increasing the abundance of beneficial microbiota, such as *Bifidobacteria* and *Lactobacilli*, while decreasing the abundance of harmful microbiota, such as *Enterobacteriaceae* and Phylum *Mimosoideae*, which is consistent with our findings (Chen, et al., 2015). Other studies have found that different strains of LGG can affect IgA levels and IgA-microbiota interactions in the intestines of healthy mice in a strain-specific manner (Mei, et al., 2022). Qi et al. found that LGG maintains intestinal health by producing L-ornithine to regulate the intestinal microbiota and enhance intestinal barrier function (Qi, et al., 2019). In addition, *Bacteroides* and *Faecalibacterium* are producers of SCFAs (Holmstrøm, et al., 2004; Peron, et al., 2021; Xu, et al., 2021; Zheng, et al., 2020), and SCFAs produced by the intestinal microbiota are the main source of energy for enterocytes. Increasing evidence suggests that SCFAs regulate intestinal inflammation and improve intestinal barrier function (Aho, et al., 2021; Amabebe, et al., 2020). In fact, the levels of valerate, isobutyrate, and isovalerate were significantly higher in the TBF group during the fasting period compared to the NC group. This may be attributed to the supplementation of LGG, which increases the relative abundance of bacteria that produce SCFAs, such as *Bacteroides*. Consequently, LGG may alleviate intestinal inflammation and improve intestinal barrier function by reshaping the structure of the intestinal microbiota. In summary, the addition of LGG during the FIM process mitigates the dysbiosis of the intestinal microbiota caused by fasting and promotes the production of SCFAs, thereby enhancing intestinal health.

Conclusion

During the FIM process, adding LGG helps repair Intestinal damage due to fasting in laying hens, improves antioxidant and immune functions, enhances intestinal barrier function, and reduces inflammation. Additionally, LGG increases the abundance of beneficial bacteria, decreases the number of harmful bacteria, optimizes the structure of the intestinal microbiota, and promotes the production of SCFAs, thereby contributing to improve intestinal health, particularly when administered to laying hens before and during fasting.

Author contributions

Ruirui Jiang created the trial protocol for the study. Zhixuan Luo was responsible for data analysis and drafting. Yujie Gong and Qiang Li provided guidance for the trial design and data analysis. Mengyan Zhang, Jun Zhang, Hao Zhang, Qingduo Zeng and Yidan Zhu were responsible for the validation of the experiments. Yujie Guo, Donghua Li, Yadong Tian, and Xiangtao Kang critically revised the manuscript for important intellectual content. All authors approved the final manuscript.

Disclosure statement

The authors have nothing to disclose.

Declaration of competing interest

The authors declare no conflict of interest.

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