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# *Lactobacillus reuteri* alleviates LPS-induced intestinal mucosal damage by stimulating the expansion of intestinal stem cells via activation of the Wnt/β-catenin signaling pathway in broilers

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**ABSTRACT** The continuous expansion of intestinal stem cells (**ISCs**) is crucial for maintaining the renewal of the intestinal epithelium, particularly in inflammatory conditions. It remains largely unknown how the internal microbiota repair damage to the internal mucosal barrier. Hence, investigating potential anti-inflammatory probiotics from the intestinal symbolic microbes of broilers and analyzing their mechanism of action to support the intestinal mucosal barrier function can offer novel regulatory tools to alleviate broiler enteritis. In this research, we utilized in vivo broilers plus ex vivo organoids model to thoroughly examine the effectiveness of *Lactobacillus reuteri* (**LR**) in protecting the integrity of the intestinal mucosa during lipopolysaccharide-induced (**LPS**-induced) enteritis in broilers. The findings indicated that LR feeding maintained intestinal morphological and structural integrity, enhanced proliferation of intestinal epithelial cells, and inhibited cell apoptosis and inflammatory response against the deleterious effects triggered by LPS. Simultaneously, LR enhanced ISCs activity and stimulated intestinal epithelial regeneration to protect the intestinal barrier during LPS-induced injury conditions. The coculture system of LR and ileum organoids revealed that LR increased the growth of organoids and attenuated LPS-stimulated damage to organoids. Furthermore, the LPS-induced decrease in ISC activity was rescued by reactivation of Wnt/$\beta$-catenin signaling by LR ex vivo and in vivo. This research revealed that LR promoted the expansion of ISCs and intestinal epithelial cell renewal by regulating the Wnt/$\beta$-catenin signaling pathway, thereby maintaining the integrity of the intestinal mucosal barrier. This finding provided theoretical support for *lactobacillus* as a probiotic additive in livestock feed to improve intestinal inflammation and treat intestinal diseases.

**Key words:** *Lactobacillus reuteri* (LR), expansion, Wnt/$\beta$-catenin signaling, intestinal stem cells (ISCs), organoids

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## INTRODUCTION

The intestinal tract serves as a crucial site for the digestion and absorption of nutrients, while concurrently serving as the primary immune organ in avian species (Nawab et al., 2018). Maintaining optimal gut health is paramount for poultry productivity and performance (Lilburn and Loeffler, 2015; Nawab et al., 2018). However, modern intensive farming practices have presented numerous challenges to the healthy growth of broilers, such as poor feeding equipment, high stocking density, and uncomfortable temperatures have been implicated in intestinal inflammation, contributing to compromised intestinal health and reduced production efficiency in poultry (Lilburn and Loeffler, 2015; Feng et al., 2021; Gu et al., 2021). Moreover, the characteristics of modern animal production (e.g., increased feed intake and excess nutrition) can predispose broilers to infections and inflammation of the intestinal epithelium, which can trigger a breakdown in barrier function and immune balance, worsen oxidative stress and disruption of gut microbiota composition, and ultimately have a detrimental impact on the metabolism and productivity of the host (Okumura and Takeda, 2018; Zhou et al., 2022).

The integrity of the intestinal barrier function is not only essential for efficient nutrient absorption, but also serves as a defense against pathogenic bacteria and maintains the immune function of the intestinal mucosa, while



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facilitating recovery from inflammatory injury (Nii et al., 2020). Repair of the damaged intestinal mucosal barrier relies primarily on the sustained expansion of intestinal crypt stem cells, which replenish the damaged and shed intestinal epithelial cells, thereby preserving intestinal homeostasis (Hou et al., 2018). Intestinal commensal bacteria are a crucial part of the intestinal mucosal barrier, contributing significantly to the regulation of barrier function and overall host well-being. Dysregulation of the intestinal commensal flora or perturbations in its metabolites can compromise the intestinal mucosal barrier function, consequently predisposing to enteritis development (Martens et al., 2018). Therefore, under the current environment of total feed antibiotic ban and healthy breeding, exploring the potential anti-inflammatory probiotics from broiler intestinal commensal microorganisms and analyzing their mechanism of action in maintaining the intestinal mucosal barrier function will provide new regulatory tools to alleviate broiler enteritis.

*Lactobacillus*, a predominant bacterium inhabiting the animal intestinal tract, exerts multifaceted effects on intestinal health by augmenting the resistance of the mucosal barrier, bolstering mucosal cell growth and repair processes. Recent studies have reported that *Lactobacillus plantarum, Lactobacillus casei*, and *Bacillus amyloliquefaciens* H57 all enhance intestinal mucosal integrity to ameliorate intestinal inflammation (Shini et al., 2020; Xu et al., 2020; Deng et al., 2021). *Lactobacillus reuteri* is a hetero-fermentative lactic acid bacterium with excellent probiotic properties mentioned above. In the previous study, we successfully screened a strain of *Lactobacillus reuteri* with excellent probiotic properties from broiler intestines, and in vitro assays revealed that this bacterium and its prominent metabolite, reuterin, not only exhibited significant bacteriostatic effects against *Clostridium perfringens* but also attenuated the macrophage inflammatory response induced by *Clostridium perfringens* (Xu et al., 2022a, 2022b; Xu et al., 2023). Nevertheless, the precise mechanism regarding the intrinsic anti-inflammatory properties of *Lactobacillus reuteri* and its potential ability to preserve the intestinal barrier function by modulating the function of ISCs remains unclear. LPS is a strong immunogenic particle present in the outer membrane of Gram-negative bacteria, which is a major triggering factor for the inflammatory cascade in response to a Gram-negative bacteria infection. The use of LPS as a strong pro-inflammatory agent is a well-known model of inflammation applied in both in vivo and in vitro studies(Skrzypczak-Wiercioch and Salat, 2022).

Therefore, in this research, we initially evaluated the alleviating effect of *Lactobacillus reuteri* on LPS-induced enteritis in broilers through in vivo feeding experiments. Subsequently, we established an in vitro co-culture model of *Lactobacillus reuteri* with broiler intestinal organoids to explore whether *Lactobacillus reuteri* could exert an anti-inflammatory role by regulating the expansion of ISCs and facilitating the renewal of intestinal epithelial cells to uphold the function of the intestinal mucosal barrier.

## MATERIALS AND METHODS

### Ethics Statement

This research adhered to the animal welfare guidelines set by China and received approval from the Animal Care and Welfare Committee as well as the Scientific Ethical Committee of Zhejiang University (No. ZJU20220310).

### Experimental Design and Diets

In this experiment, 240 male fast-type yellow-feathered broilers at 1-day-old were subjected to random allocation into 4 groups of 6 replicates, with 10 broilers in each replicate. The trials were grouped as follows: (1) Control CON group; (2) *Lactobacillus reuteri* microencapsulated LR group; (3) LPS injection-induced enteritis model group; and (4) LPS+LR group. CON and LPS groups were fed a basal diet, LR and LPS+LR groups were supplemented with LR microencapsulated preparation 1 kg/t (containing LR $3\times10^9$ CFU/kg feed) in basal diet, LPS, and LR+LPS groups received intraperitoneal injections of 0.5 mg/kg LPS on d 22, 24, 26, and 28, respectively; and CON group and LR group received equal volumes of sterile PBS via intraperitoneal injections, and the injection dose of LPS was referred to the study conducted by (Xie et al., 2021). Pellets were prepared as basal diets following the poultry nutritional guidelines established by the National Research Council (NRC, 1994). The nutrient levels and composition of the experimental diets are delineated in Table 1. The experiment was conducted for 28 d under conventional feeding management with ad libitum feeding and watering.

### Sample Collection

On the 28th d of the experiment, 2 h after receiving the final LPS injection, 2 broilers with body weights proximal to the mean were chosen from each replicate sample for slaughter and subsequent sampling, and

**Table 1.** The nutrient levels and composition of the basal diet (% dry matter).

| Ingredients % | | Nutrient levels[c] | |
|---|---|---|---|
| Corn | 62.5 | ME (MJ/kg) | 12.22 |
| Soybean meal | 31.0 | CP | 21.09 |
| CGM[a] | 2.0 | Lys | 1.09 |
| Soybean oil | 0.5 | Met | 0.49 |
| NaCl | 0.3 | Met+Cys | 0.87 |
| $CaHPO_4$ | 1.2 | Calcium | 0.90 |
| Limestone | 1.5 | Total phosphorus | 0.58 |
| Premix[b] | 1.0 | | |

[a]CPM: Corn gluten meal

[b]The premix provided the following per kilogram of the diet: Mn 80mg, Zn 60mg,Cu 8mg,Fe 80mg,Se 0.15mg,I 0.35mg,VA 9,600IU,$VB_1$ 2.2mg, $VB_6$ 4.2mg,$VB_{12}$ 0.012mg,$VD_3$ 1500IU,VE 20mg,$VK_3$ 1mg,D-pantothenic acid 12 mg,nicotinamide 42 mg,choline 800 mg,D-biotin 0.18 mg,folic acid 1.0 mg,lysine 1.03g,methionine 2.4g.

[c]Metabolizable energy was calculated values; others were analyzed values.

0.5 cm ileal intestinal segments were collected and placed in 2.5% glutaraldehyde or 4% paraformaldehyde solution for fixation and treatment for subsequent morphological analysis. Meanwhile, mucosal samples of the above ileum were collected to assess the expression of genes associated with mucosal barrier function and to detect the concentration of biomarkers related to the inflammatory response.

## Intestinal Morphology

Ileal tissues were fixed by paraffin embedding and sections were prepared. Hematoxylin and eosin (**HE**) staining was employed on sections to assess villus structure. The villus height (**VH**) and crypt depth (**CD**) in the marked samples were subsequently assessed utilizing an image processing and analysis platform (Leica EC3, Leica, Germany), and the VH:CD was derived from these measurements. Measurements were conducted on 10 undamaged villi, and the average value was used as the assay data. Additionally, ileum samples designated for transmission electron microscopy (**TEM**) and scanning electron microscopy (**SEM**) underwent processing following the previously reported protocols (Geng et al., 2018). The digital electron micrographs were obtained using a Hitachi Model-7650 TEM (Hitachi, Tokyo, Japan) and a Hitachi SU-8010 SEM (Hitachi, Tokyo, Japan).

## Crypt Isolation and Ileum Organoid Culture

Crypts were isolated from the broiler ileum and used to establish intestinal organoids based on a previously published protocol with minor modifications (Zhou et al., 2019). The dissected intestinal samples that had been precooled were washed and then sliced into small segments. Once the supernatant was clarified, these pieces were transferred to DPBS supplemented with 2.5 mM EDTA and digested on a shaker at 4°C for 1 h. The intestinal crypt suspension underwent filtration using a 70 $\mu$m cell strainer to remove large particles, followed by centrifugation at 1200 r/min for 5 min at 4°C, and subsequent gentle decantation of the supernatant. Following that, the crypts were placed back onto 48-well plates with the same amount of Matrigel (BD Bioscience/Corning, NY) and IntestiCultOGM Human organoid growth medium (Stemcell Technologies, Vancouver, BC, Canada). After solidifying the Matrigel suspension at 37°C for 15 min, 200 $\mu$L of IntestiCultOGM Human Organoid Growth Medium was introduced into each well. The medium renewal process was conducted every 2 to 3 d thereafter. For organoid digestion and passaging, 1 mL of precooled PBS was introduced into the wells, followed by vigorous disruption of Matrigel and organoids using a pipette gun. The mixture was then moved to a centrifuge tube and spun at 4°C and 1,200 r/min for 5 min. Crypts were suspended in Matrigel and allowed to culture prior to proceeding with the next steps.

## LR Pretreatment and LPS Stimulation of Intestinal Organoids

The co-culture system involving organoids and LR was established as described previously with slight modifications (Lu et al., 2020). The LR bacteria were obtained through centrifugation and then rinsed with PBS 3 times. Afterward, the LR bacteria were suspended in organoid medium to treat intestinal organoids. After passaging, intestinal organoids were inoculated into 48-well plates and treated with LR ($10^6$ CFU/well) in antibiotic-free cultures. An inflammation model was constructed using LPS (200 $\mu$g/mL, Sigma-Aldrich) to stimulate the organoids for 24 h, as reported in earlier studies (Huang et al., 2022). The particular procedures for conducting organoid experiments included the use of CON (organoid medium), LPS (200 $\mu$g/mL for 24 h) (Wang et al., 2024), LR (LR pretreatment at $10^6$ CFU/well), LR+LPS (LR pretreatment at $10^6$ CFU/well, then adding 200 $\mu$g/mL LPS for 24 h), and HK-LR+LPS (heat-killed LR pretreatment at $10^6$ CFU/well, followed by adding 200 $\mu$g/mL LPS for 24 h). Organoids disruption efficiency and budding efficiency were calculated according to Li et al methodology (Li et al. 2019).

## Immunofluorescence Analysis

The ileum was treated with 4% paraformaldehyde, then dehydrated using ethanol, followed by embedding in paraffin and slicing into 5 $\mu$m thick sections. In addition, the intestinal organoids inoculated in the 48-well plate only need to be fixed at 4% paraformaldehyde for 30 min. After undergoing antigenic repair for 3 h, the samples were treated with 0.3% Triton X-100 for 10 min, followed by sealing with 10% BSA at room temperature for 2 h. They were then exposed to PCNA antibody (GB111402, 1:500), ZO-1 antibody (GB11195, 1:500), Claudin-1 antibody (GB15032, 1:500), Lgr5 antibody (GB112018, 1:500), and $\beta$-catenin antibody (GB111401, 1:500) overnight at 4°C. Subsequently, FITC or Cy3-conjugated antibodies (GB21303, 1:200) were applied at room temperature for 2 h, and DAPI (blue) was used to visualize the nuclei for 10 min. Finally, The LSM 510 META confocal laser microscope (Carl Zeiss Ltd, Oberkochen, Germany) was used to capture the images, which were then analyzed with Zeiss LSM800. Additionally, apoptosis quantification was conducted using the Terminal deoxynucleotidyl transferase-mediated dUTP nick labeling (**TUNEL**) assay following the manufacturer's instructions.

## Extraction of RNA and RT-qPCR Analysis

Ileal mucosa and intestinal organs were obtained for RNA extraction with TRIzol® reagent. The extracted RNA was then reverse transcribed into cDNA utilizing the Vazyme HiScript II 1st Strand cDNA Synthesis Kit. Following that, cDNA fragments were amplified with the 2X Universal SYBR qPCR Master Mix kit to assess

**Table 2.** Primers sequences for RT-qPCR.

| Gene name | Sequence | Accession number | Product length | Efficiency % |
|---|---|---|---|---|
| *β-actin* | F: GAGAAATTGTGCGTGACATCA<br>R: CCTGAACCTCTCATTGCCA | NM_205518.2 | 152 | 98.33 |
| *Caspase-3* | F: GGTCCTCAGAAAGGGGGAGC<br>R: CTCTGTATCTTGAAGCACCAACG | XM_015276122.4 | 108 | 92.16 |
| *β-catenin* | F: TGCGAATCAACCCAACAGTA<br>R: CTCACCAGCAGACATCAGGA | NM_205081.3 | 136 | 90.58 |
| *ZO-1* | F: CTTCAGGTGTTTCTCTTCCTCCTC<br>R: CTGTGGTTTCATGGCTGGATC | XM_046925214.1 | 131 | 94.07 |
| *Claudin1* | F: TGGCCACGTCATGGTATGG<br>R: AACGGGTGTGAAAGGGTCATAG | NM_001013611.2 | 62 | 96.45 |
| *TGF-β1* | F: GCCGACACGCAGTACACCAA<br>R: TGCAGGCACGGACCACCAT | NM_001318456.1 | 169 | 95.22 |
| *IL-1β* | F: ACTGGGCATCAAGGGCTA<br>R: GGTAGAAGATGAAGCGGGTC | NM_204524.2 | 131 | 96.16 |
| *IL-6* | F: ATCCTCTGTTACCAATCTGC<br>R: ACATTTTCTTTGGCGTTGAC | NM_204628.2 | 273 | 96.73 |
| *TNF-α* | F: GAGCGTTGACTTGGCTGTC<br>R: AAGCAACAACCAGCTATGCAC | NM_204267.2 | 64 | 98.62 |
| *IFN-γ* | F: AAGTCAAAGCCGCACATCAAAC<br>R: CTGGATTCTCAAGTCGTTCATCG | NM_205149.2 | 132 | 91.93 |
| *IL-10* | F: TGCTGCGCTTCTACACAGAT<br>R: TGGCTTTGCTCCTCTTCTCG | NM_001004414.4 | 159 | 93.57 |
| *PCNA* | F: CAGATGTTCCTCTCGTTGTGGA<br>R: AGCAGATCACCCCCTCAGTC | NM_204170.3 | 127 | 94.38 |
| *c-Myc* | F: CAGAGTCCAGCACAGAAGA<br>R: GAGGAGCAGCGTAGTTGTGT | NM_001030952.2 | 108 | 90.72 |
| *Bmi1* | F: ATCGTGCGGTACTTGGAGAC<br>R: TTTGAAAAGGCCCGGTACGA | NM_001007988.3 | 138 | 92.48 |
| *Lgr5* | F: TACGTCTTGCAGGAAATGGCT<br>R: GGAACCTGGCGTAGTTGGTTA | XM_046909876.1 | 106 | 95.81 |
| *Wnt3a* | F: TGTGACTCCCACCACAAAGG<br>R: GGTCCAGAATGGTCGTCCTC | NM_001081696.2 | 181 | 94.19 |
| *Axin2* | F: GGGCTGGGGAGCTTAAAAGT<br>R: TCACTATCGTTTGCGCTGGT | NM_204491.1 | 263 | 93.73 |
| *Lrp5* | F: TACTGGGTAGATGGGCGTCA<br>R: GGTCATGTGGCTGCTTCTCT | NM_001012897.2 | 118 | 92.85 |
| *Cyclin D1* | F: CTGCTCAATGACAGGGTGC<br>R: TCGGGTCTGATGGAGTTGT | NM_205381.2 | 341 | 94.82 |

the expression of specific genes. Normalization of each target gene's expression abundance was achieved relative to *β-actin* expression utilizing the $2^{-\Delta\Delta Ct}$ method, as outlined by (Livak and Schmittgen, 2001). The sequences of primers utilized are detailed in Table 2.

### Statistical Analysis

Statistical analysis involved employing 1-way ANOVA via SPSS 19.0, with Duncan's multiple range test applied for multiple comparisons, with significance set at $P < 0.05$ for detecting differences, where ** and * indicated $P$ -values of <0.01 and <0.05, respectively. Origin Pro 8.5 was used to visualize the data. Mean ± standard deviation is used to represent experimental data.

## RESULTS

### LR Protects the Intestinal Mucosa and Repairs LPS-Induced Intestinal Damage

We assessed how LR accelerates intestinal growth and improves intestinal inflammation by studying its ability to repair LPS-induced damage to the intestinal epithelium. Histological analysis revealed that the ileal villi of the LPS group exhibited severe damage, disorganization, decreased quantity and height, along with atrophic defects, increased mucosal collapse, and cell shedding. Conversely, the LPS+LR group displayed a more preserved and compact structure with no apparent surface damage, representing a notable improvement compared to the LPS group (Figure 1A-C). Compared to the LPS group, the LR+LPS group had significantly increased VH ($P < 0.05$) (Figure 1D) and reduced CD ($P < 0.01$) (Figure 1E), which led to an increased VH: CD in the ileum ($P < 0.05$) (Figure 1F). These results suggested that LR effectively ameliorated LPS-induced damage to the intestinal epithelium.

### LR Alleviates the Inflammation Triggered by the LPS Stimulation

The levels of cytokines and chemokines in the gut can notably influence the regulation and homeostasis of intestinal physiological functions. In our study, supplementation with LR significantly increased the gene expression of *IL-10* and *TGF-β1* but decreased the gene expression of *IL-1β, IL-6, TNF-α*, and *IFN-γ* after LPS stimulation ($P < 0.05$ or $P < 0.01$) (Figure 2). These results proved that supplementation with LR contributed to alleviating the inflammatory reaction triggered by LPS stimulation.

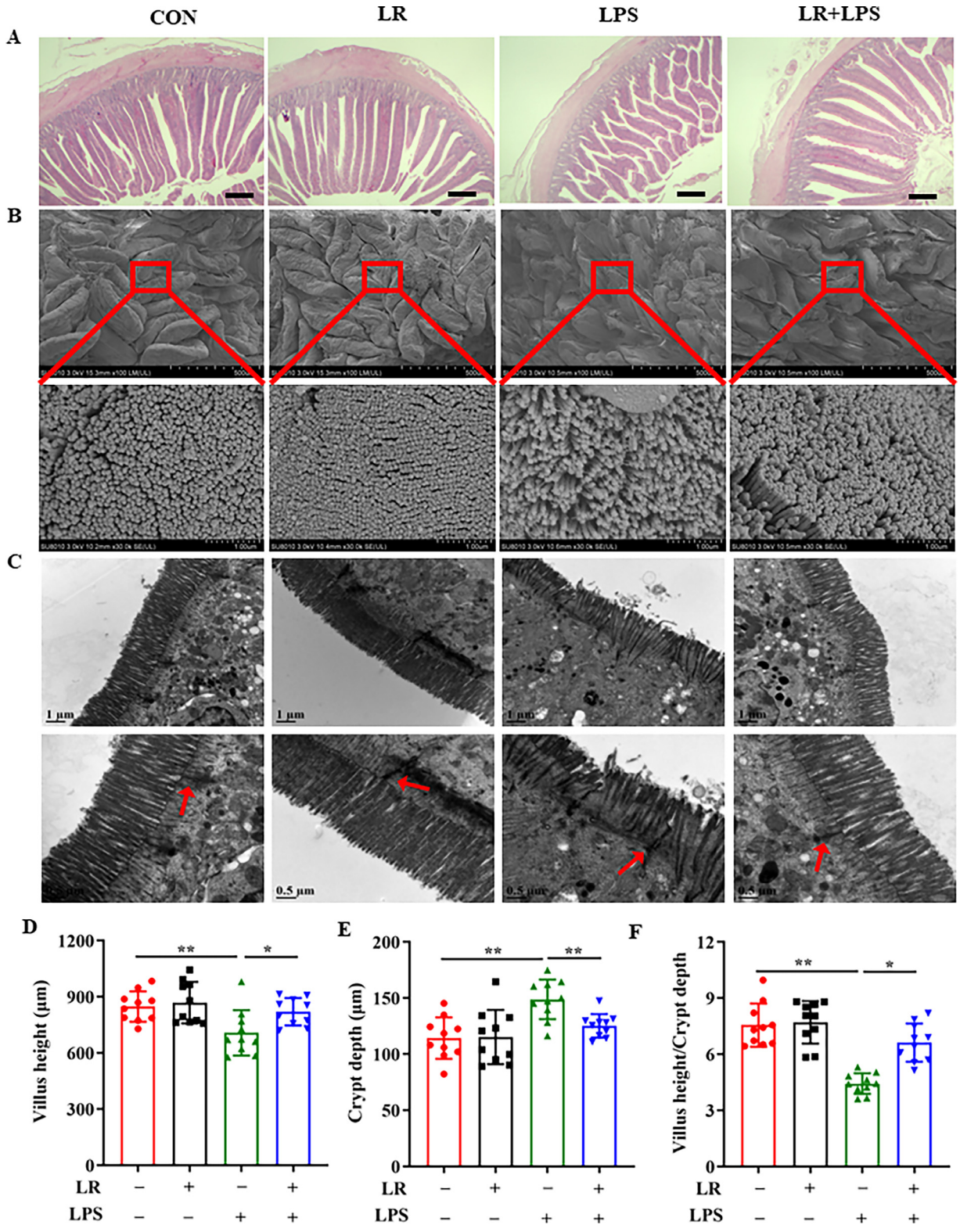


**Figure 1.** LR improves the morphology and structure of the ileum in LPS-stimulated broilers. (A) Representative H&E-stained images in the ileum (scale bar = 300 $\mu$m); (B) SEM in the ileum (×100 and ×30000); (C) TEM in the ileum (×15000 and ×30000); (D) villus height, (E) crypt depth and (F) the ratio of villus height to crypt depth. Data were presented as the mean ± SEM (n = 10). $^{*}P < 0.05$, $^{**}P < 0.01$.

## *LR Guards Against the Intestinal Barrier Dysfunction Caused by the LPS Stimulation*

We assessed the integrity of the intestinal barrier in the ileal epithelium, which is crucial for maintaining balance in the intestinal environment. Our findings revealed that the LPS-stimulated reduced distribution of Claudin-1 and ZO-1 in the ileal epithelium was remarkably reversed by LR supplementation (Figure 3A-B). Simultaneously, the fluorescent signal intensities of Claudin-1 and ZO-1, consistent with gene expression, were both significantly improved in the ileum after LR supplementation ($P < 0.05$ or $P < 0.01$) (Figure 3C-F). These results indicated that LR

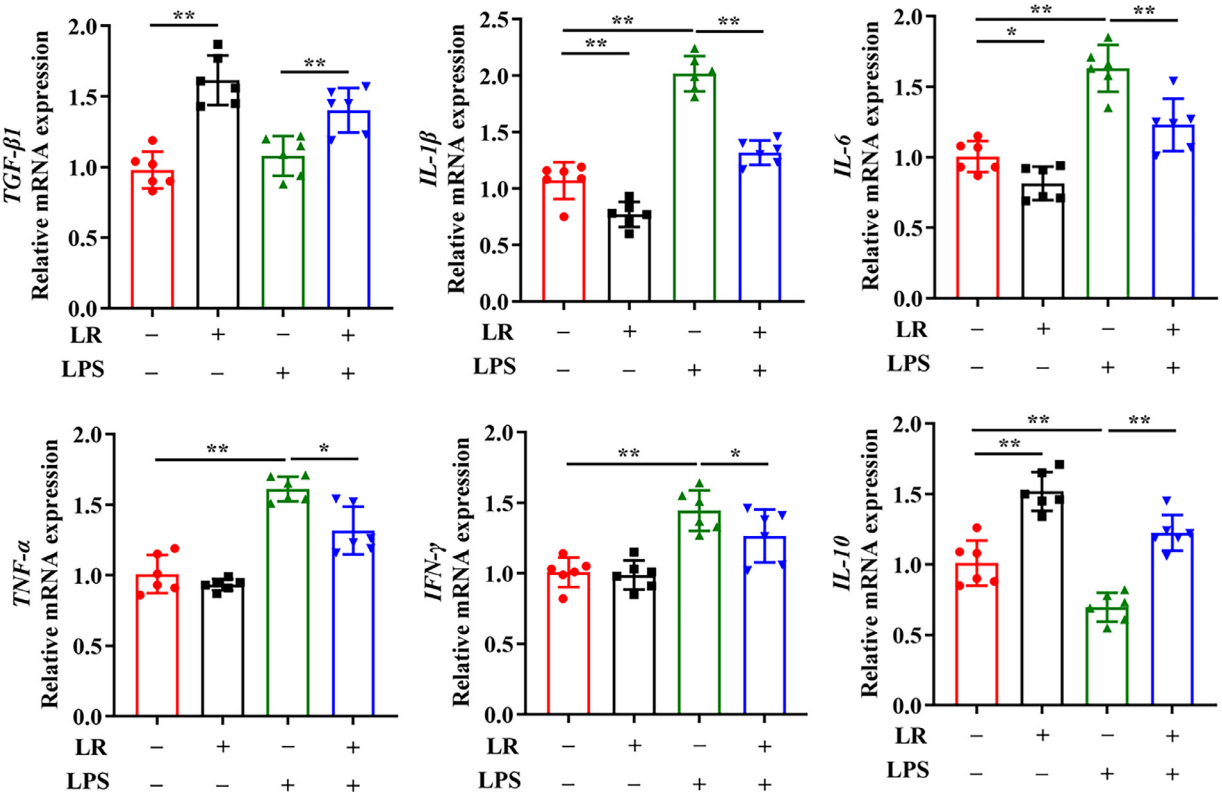


**Figure 2.** LR inhibited inflammation in the ileum caused by LPS injection. Relative mRNA expression of *TGF-β1* and typical inflammatory cytokines (*IFN-γ, TNF-α, IL-6, IL-1β, IL-10*) analyzed by RT-qPCR in the ileum. Data were presented as the mean ± SEM (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$.

alleviated the LPS-induced reduction of tight junction protein expression in the ileal epithelium.

## LR Attenuates Intestinal Epithelial Damage Caused by LPS Stimulation by Promoting Epithelial Cell Proliferation

Cell proliferation and apoptosis are conducive to intestinal epithelial cell regeneration and renewal to maintain intestinal epithelial barrier function. The findings indicated a notable rise in the quantity of PCNA cells within the ileal crypts after LR supplementation ($P < 0.01$) (Figure 4A, C). Correspondingly, treatment with LR significantly increased gene expression of *PCNA* in the ileum ($P < 0.05$ or $P < 0.01$) (Figure 4D). Conversely, the number of TUNEL apoptotic cells and the expression of the apoptosis-associated gene *Caspase-3* in the ileum was significantly suppressed after LR supplementation ($P < 0.05$ or $P < 0.01$) (Figure 4B, E-F). These findings suggested that LR alleviated LPS-induced intestinal epithelial injury by improving the proliferation and apoptosis of the epithelial cell.

## LR Increases ISCs Activity and Activates the Wnt/β-Catenin Signaling Pathway

Activation of the Wnt/β-catenin signaling pathway is beneficial to maintain the activity of Lgr5 ISCs to bolster the integrity of the intestinal epithelial barrier. Our findings demonstrated that LPS stimulation remarkably reduced the fluorescence signal intensity of β-catenin and Lgr5, which was effectively alleviated by LR supplementation ($P < 0.01$) (Figure 5A-C, E). Correspondingly, compared to the LPS stimulation group, the LR supplementation group remarkably elevated the relative expression of *Lgr5, β-catenin, Wnt3a, c-Myc, Axin2, Lrp5, Bmi1,* and *Cyclin D1* mRNA in the ileum ($P < 0.05$ or $P < 0.01$) (Fig. 5D, F-G). These findings suggested that LR stimulated the Wnt/β-catenin pathway and increased ISC function.

## LR Promotes the Growth of Intestinal Organoids and Alleviates the Organoid Damage Induced by LPS Stimulation

To explore the repair mechanism of LR on LPS-induced intestinal epithelial damage and barrier function in vitro, we established the LR and organoid co-culture model (Figure 6A). The isolated ileal crypts were co-cultured with organoid medium resuspended LR strain of around $10^6$ CFU/well in Matrigel and the medium being changed every 2 to 3 d. The results showed that the intestinal organoids grew well from 1 to 5 d after co-culture with LR, and the organoids started to grow rapidly and bud on the third d (Figure 6B). The organoids cultured to the third d were stimulated with 200 μg/mL of LPS for 24 h. The findings indicated that the organoids were severely damaged and apoptotic, which was manifested by the shedding of villous crypts and the accumulation of numerous deceased cells in the

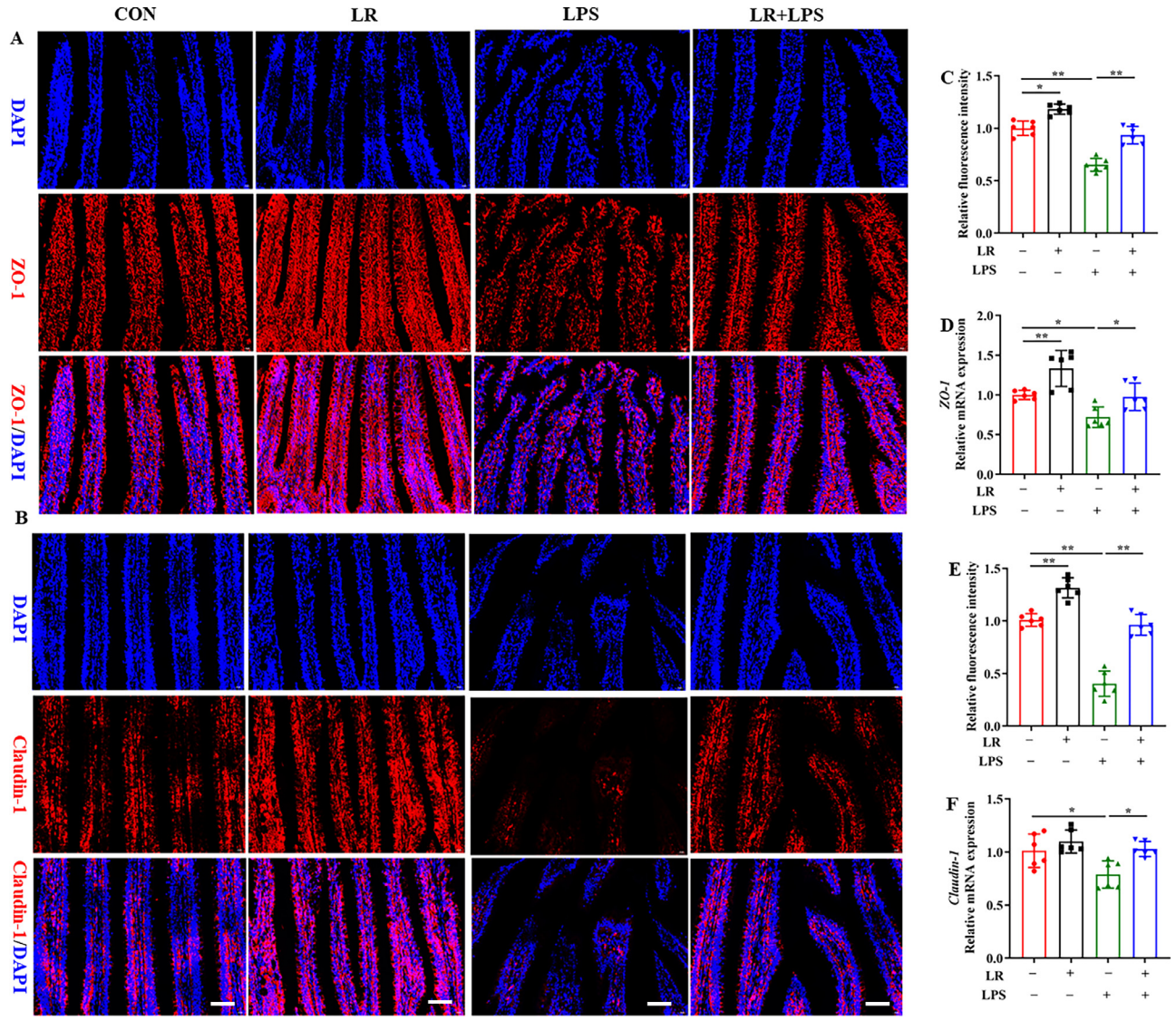


**Figure 3.** LR enhanced the integrity of the intestinal epithelium in LPS-stimulated broilers. (A, B) Images showing immunohistochemistry (IHC) staining with ZO-1 (A) and Claudin-1 (B) antibodies in the ileum (scale bar, 50 $\mu$m); (C, E) The relative fluorescence intensity of ZO-1 (C) and Claudin-1 (E) based on the images shown, expressed as the multiple of CON group; (D, F) The gene expression of *ZO-1* (D) and *Claudin-1* (F) analyzed by RT-qPCR in the ileum. Data were presented as the mean ± SEM (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$.

center of the crypts, resulting in a notable decline in budding frequency and a rise in the disruption rate of intestinal organoids (Figure 7). In contrast, LR-pretreated organoids showed relatively intact morphology, and the budding rate of crypts was significantly increased. On the contrary, severe damage and apoptosis of intestinal organoids also occurred in the HK-LR group (Figure 7). The aforementioned findings suggested that administering live LR was successful in reducing the intestinal organoid damage caused by LPS.

### *LR Protects Against LPS-Induced ISC Injury Ex Vivo by Activating Wnt/β-Catenin Signaling Pathway*

Immunofluorescence or RT-qPCR was used to detect alterations in Wnt/β-catenin signaling in intestinal organoids to confirm the role of Wnt/β-catenin signaling in regulating ISC activity and reducing LPS-induced inflammatory damage. The findings revealed that the LPS-induced reduction in immunofluorescence signal intensity of β-catenin and Lgr5 in intestinal organoids was significantly reversed by LR pretreatment ($P < 0.01$) (Figure 8A-C, E). In line with this, the activity of genes linked to the Wnt/β-catenin signaling, including *Lgr5* and *β-catenin*, as well as downstream *Wnt3a, c-Myc, Axin2, Lrp5, Bmi1* and *Cyclin D1* in the intestinal organoids significantly inhibited after LPS stimulation, which was mitigated by LR pretreatment ($P < 0.05$ or $P < 0.01$) (Figure 8D, F-G). The findings provided additional confirmation that the activation of the Wnt/β-catenin pathway promoted the expansion of ISCs, helping to mitigate the damage caused by LPS in the intestine.

## DISCUSSION

*Lactobacillus*, a predominant bacterium inhabiting the animal intestinal tract, is crucial for promoting intestinal

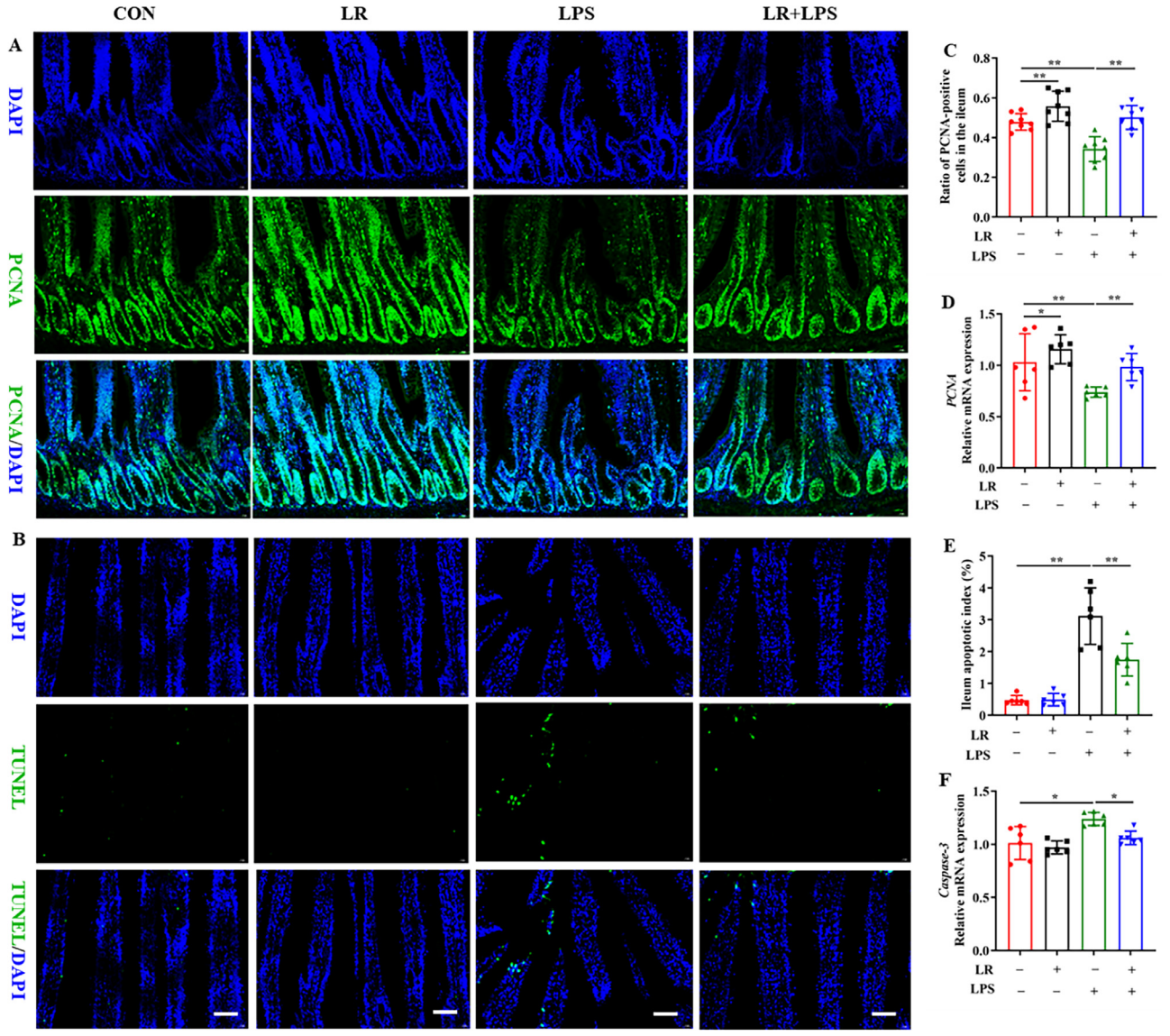


**Figure 4.** LR enhances the proliferation and apoptosis of intestinal cells in LPS-stimulated broilers. (A, B) Images showing IHC staining with PCNA (A) antibodies and TUNEL (B) in the ileum (scale bar, 50 $\mu$m); (C, E) Quantification of PCNA (C) and TUNEL (E) positive cells; (D, F) Gene expression analysis of *PCNA* (D) and *Caspase-3* (F) using RT-qPCR in the ileum. Data were presented as the mean ± SEM (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$.

health by strengthening the mucosal barrier and supporting the regeneration of mucosal cells (Suez et al., 2019; Shao et al., 2022). Recent studies have found that *Lactobacilli* stimulated the expansion of ISCs, controlled the repair and replacement of intestinal epithelial cells to uphold the intestinal barrier function, and thus exert an anti-inflammatory effect (Hou et al., 2018; Lu et al., 2020). In this research, we have shown that LR remarkably mitigated intestinal inflammation and bolstered the epithelial barrier in LPS-challenged broilers. These beneficial effects were mediated via activation of the Wnt/$\beta$-catenin signaling pathway, facilitating the expansion of Lgr5+ ISCs. In vitro co-culture experiments of LR and organoids further elucidated the ability of LR to stimulate the regeneration and proliferation of ISCs through activation of the Wnt/$\beta$-catenin pathway. These findings suggested that LR acted directly on ISCs by activating the Wnt/$\beta$-catenin signaling pathway, thereby promoting the repair of damaged intestinal epithelium.

The VH, CD, and VH:CD serve as key indicators for evaluating the nutrient absorption efficacy of the small intestine (Saadatmand and Toghyani, 2019). Increased villus height typically correlates with improved digestion and absorption in the small intestine. A higher villi-to-crypt ratio indicates a larger area for absorption, requiring fewer intestinal epithelial cells for renewal and leaving more cells for digesting and absorbing nutrients (Qin et al., 2019; Upadhaya et al., 2020; Wang et al., 2021). Our results showed that LPS-stimulated broilers displayed pronounced villi damage, characterized by disorganization, diminished number and height, along with atrophic features, mucosal collapse, and increased cell detachment. Conversely, LPS+LR-treated broilers exhibited minor impairments. Moreover, the increased VH and VH:CD in the LR group revealed that supplementation with microencapsulated LR promoted intestinal development, resulting in a more developed intestinal structure conducive to enhanced nutrient absorption.

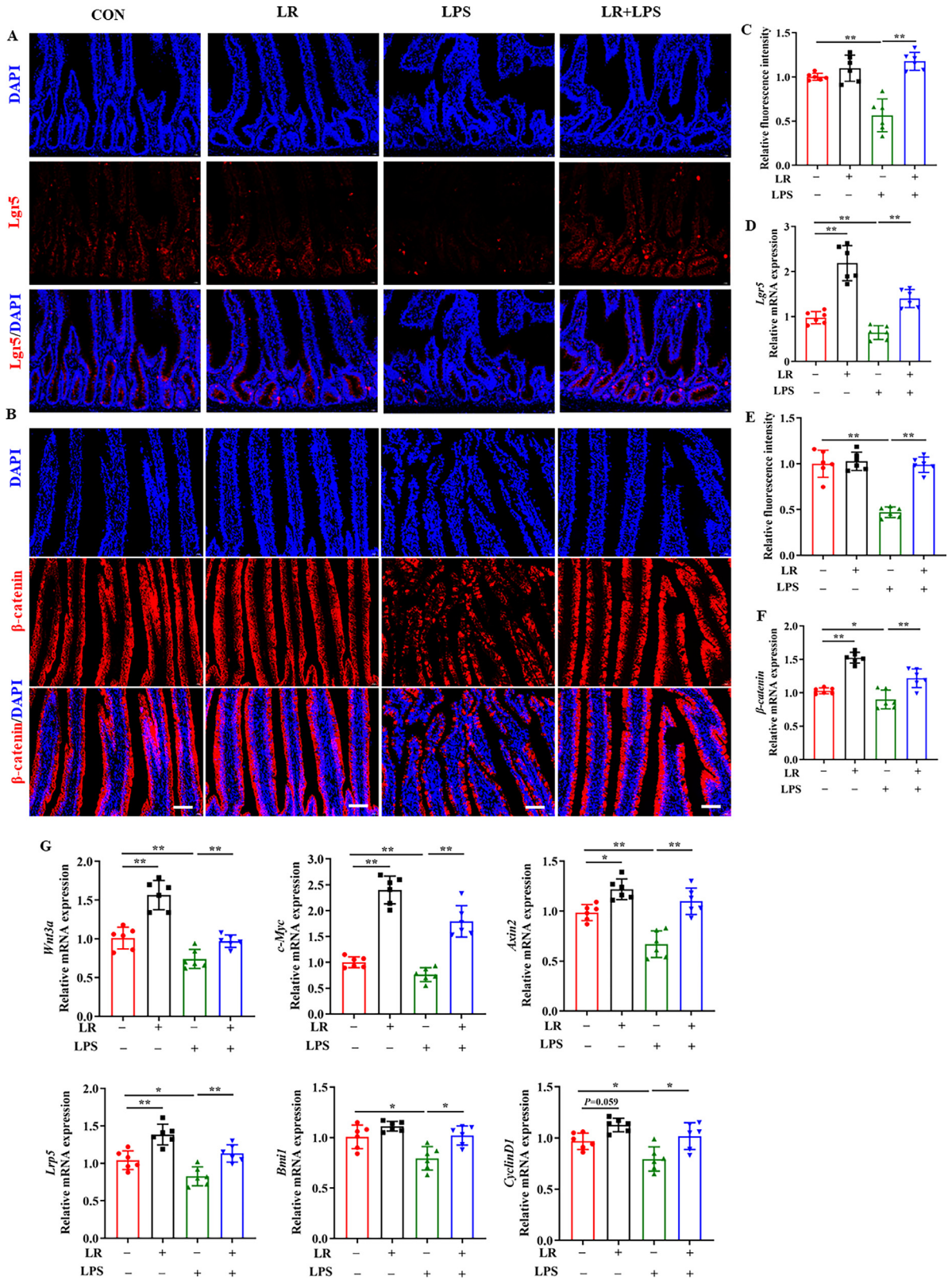


**Figure 5.** LR increases ISC activity and activates the Wnt/$\beta$-catenin signaling pathway in LPS-stimulated broilers. (A, B) Images showing IHC staining with Lgr5 (A) and $\beta$-catenin (B) antibodies in the ileum (scale bar, 50 $\mu$m); (C, E) The relative fluorescence intensity of Lgr5 (C) and $\beta$-catenin (E) based on the images shown, expressed as the multiple of CON group; (D, F, G) The gene expression of *Lgr5, β-catenin, Wnt3a, c-Myc, Axin2, Lrp5, Bmi1* and *Cyclin D1* analyzed by RT-qPCR in the ileum. Data were presented as the mean ± SEM (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$.

The intestinal barrier serves as a crucial defense mechanism, preserving intestinal homeostasis by impeding the ingress of pathogens, toxins, and antigens into mucosal tissues (Martin-Venegas et al., 2006; Turner et al., 2009). Tight junctions, integral to the mechanical integrity of the intestinal mucosa, regulate barrier function by facilitating nutrient absorption while preventing the translocation of pathogenic agents and bacteria from

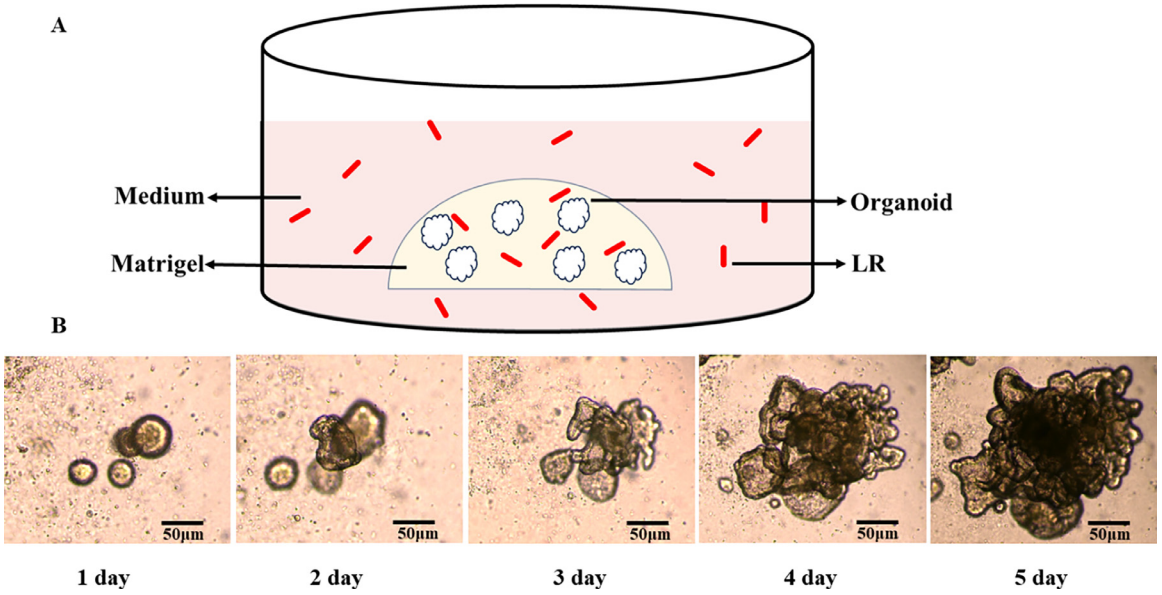


**Figure 6.** The establishment of a co-cultured system with intestinal organoids and LR. (A). The co-culture system of LR and organoids; (B). The progression of organoids from 1 to 5 d was monitored using a light microscope (scale bar, 50 $\mu$m).

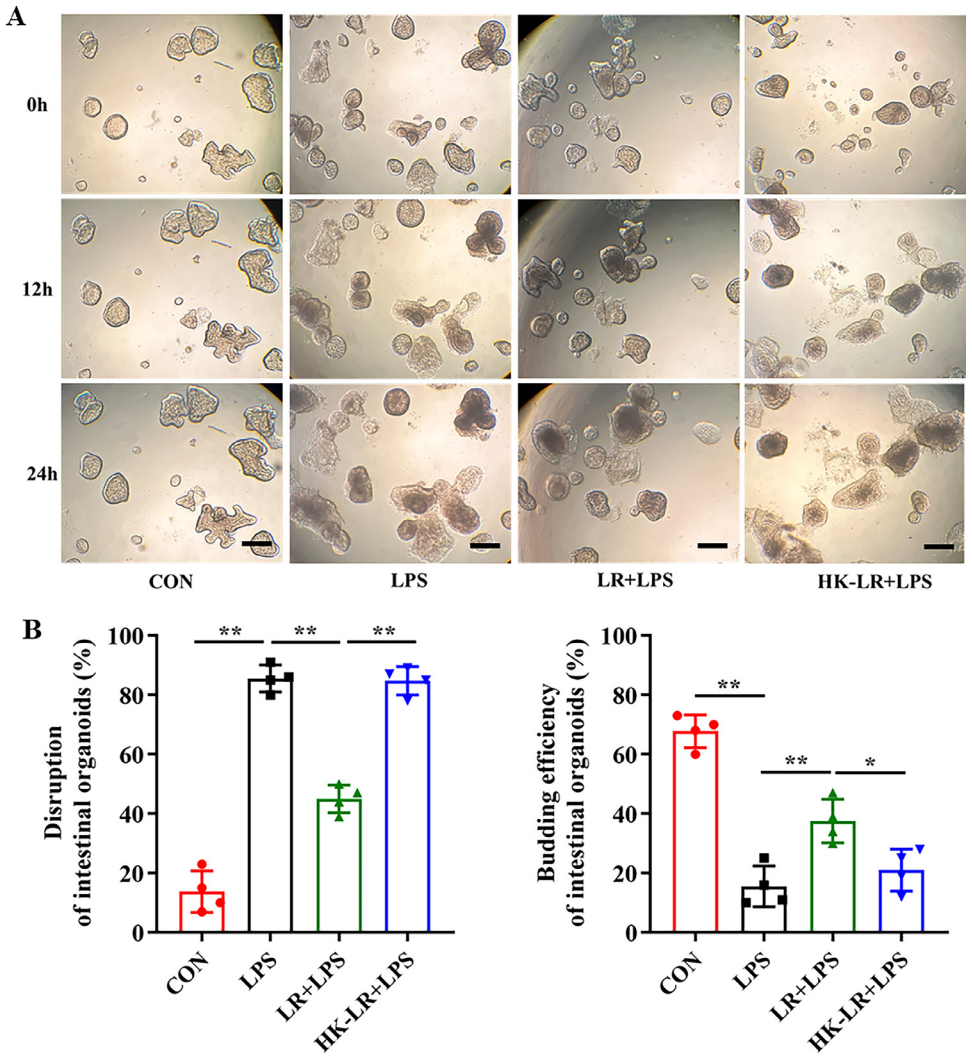


**Figure 7.** LR enhances the regeneration of intestinal organoids following injury induced by LPS. (A) Organoids were exposed to LPS (200 $\mu$g /ml) overnight with or without LR and HK-LR ($1\times10^6$ CFU per well) (scale bar = 100 $\mu$m); (B) The disruption and budding efficiency of intestinal organoids were quantified per well; Data were presented as the mean ± SEM (n = 4). $^*P < 0.05$, $^{**}P < 0.01$.

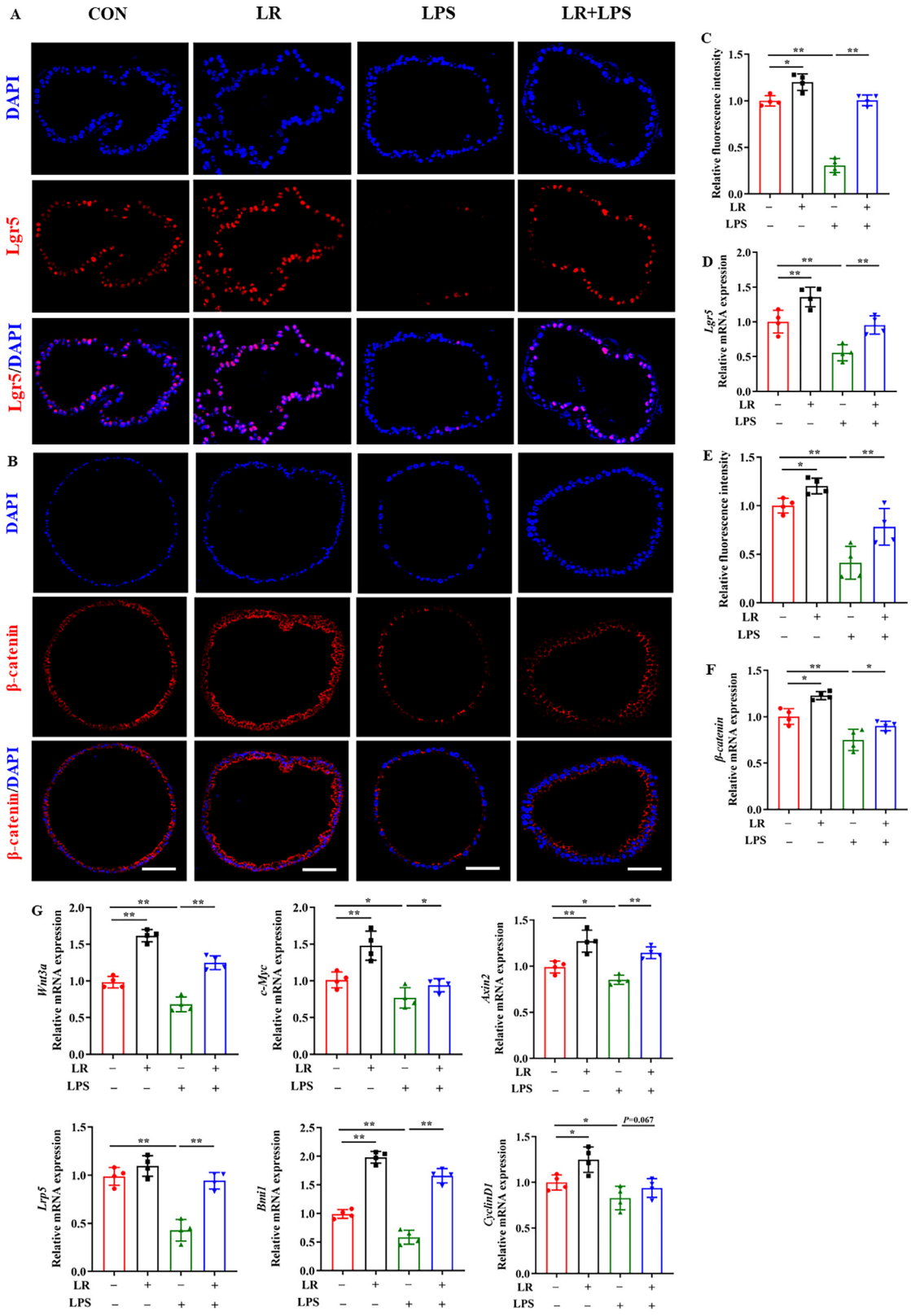


**Figure 8.** LR protects against LPS-induced ISC injury ex vivo. (A, B) Images showing IHC staining with Lgr5 (A) and $\beta$-catenin (B) antibodies in the ileum (scale bar, 50 $\mu$m); (C, E) The relative fluorescence intensity of Lgr5 (C) and $\beta$-catenin (E) based on the images shown, expressed as the multiple of CON group; (D, F, G) The gene expression of *Lgr5, β-catenin, Wnt3a, c-Myc, Axin2, Lrp5, Bmi1* and *Cyclin D1* analyzed by RT-qPCR in intestinal organoids. Data were presented as the mean ± SEM (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$.

the intestinal lumen into the internal milieu, and mainly tight junction proteins include ZO-1, Occludin, and Claudin (Runkle and Mu, 2013; Chelakkot et al., 2018). Prior research has shown that LPS decreases the expression of tight junction proteins, compromising barrier function (He et al., 2022). Our findings revealed that LPS stimulation resulted in decreased fluorescence and gene expression levels of both ZO-1 and Claudin-1 in the

ileal mucosa, which was alleviated by LR supplementation, suggesting that LPS impaired the intestinal barrier function and increased the penetration of hazardous substances contained in foods or feeds, and also revealing that LR alleviated LPS-stimulation-induced inflammation response by bolstering the intestinal epithelial barrier function.

The production of various epithelial cells driven by ISCs is integral to the maintenance of the intestinal epithelial barrier, and the proliferation and apoptosis of ISCs are essential for maintaining intestinal homeostasis (Gunther et al., 2013; Zhou et al., 2020a). LPS exposure exacerbated the apoptosis of ISCs and inhibited the proliferation of intestinal mucosal epithelial cells (Li et al. 2018; Xiao et al., 2018). In broiler chickens, intraperitoneal LPS injection triggers pro-inflammatory cascades, culminating in excessive ISC apoptosis (Xie et al., 2019). Furthermore, the LPS challenge induced a notable increase in TUNEL-positive cells in the ileum, whereas microencapsulated LR supplementation mitigated LPS-induced apoptosis of ileal epithelial cells. PCNA serves as an endogenous marker of cellular replication and is indicative of proliferative activity (Goldsworthy et al., 1993). The research showed that microencapsulated LR supplementation increased *PCNA* gene expression in the ileum, indicating that LR enhanced the regeneration of intestinal epithelial cells by boosting their proliferation, ultimately improving intestinal damage caused by LPS stimulation.

Prior research has elucidated that the dynamic modulation of the intestinal tight junction barrier by cytokines, particularly those with anti-inflammatory properties like TGF-$\beta$1 and IL-10, as well as pro-inflammatory cytokines such as IFN-$\gamma$, TNF-$\alpha$, IL-1$\beta$, and IL-6, is critical in immune stress responses. These mediators contribute to the pathological perturbation of the intestinal barrier, fostering increased permeability and systemic inflammation (Jacobi et al., 2006; Lee et al., 2015). In our investigation, LR supplementation attenuated the elevated expression of pro-inflammatory cytokines triggered by LPS. Moreover, microencapsulated LR notably augmented the relative expression of anti-inflammatory cytokines in LPS-induced enteritis in broilers. TGF-$\beta$1 exerts crucial effects on epithelial

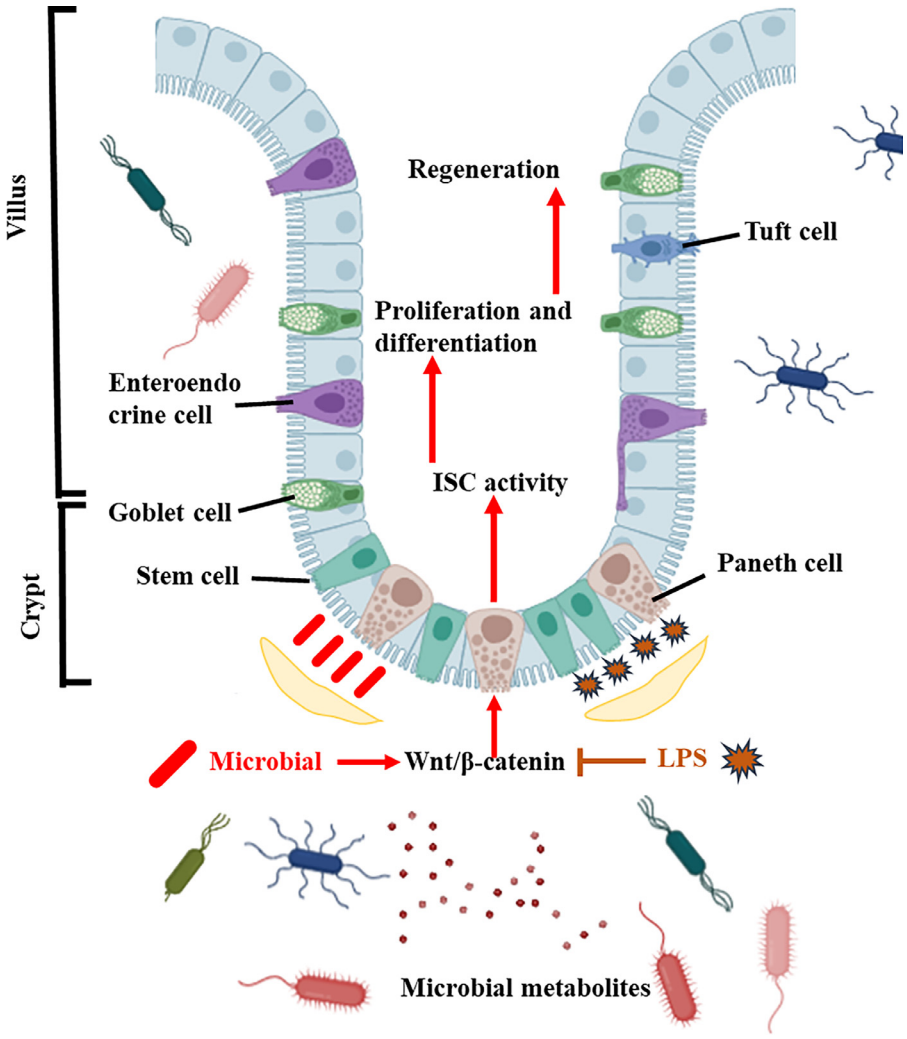


**Figure 9.** LR regulates ISC activity by reactivating the Wnt/$\beta$-catenin signaling pathway to attenuate LPS-induced intestinal epithelial injury. LPS exposure disrupts intestinal epithelial integrity by suppressing the expansion of ISCs and the activity of Wnt/$\beta$-catenin signaling. LR intervention enhanced ISCs proliferation and differentiation by reinstating Wnt/$\beta$-catenin signaling, consequently fostering intestinal epithelial regeneration.

restitution, cellular differentiation, and dampening of inflammatory cascades, with its deficiency associated with immune dysregulation and heightened inflammatory responses (Mei and Xu, 2005; Kotlarz et al., 2018). The heightened expression of TGF-$\beta$1 corresponded to increased recovery of the intestinal epithelium and diminished inflammatory cytokines levels in broilers. In general, our findings indicated that LR mitigates the vulnerability of broiler ileum to disruption of epithelial integrity and inflammatory responses induced by LPS.

Repair of intestinal damage requires preservation of ISCs stemness, as ISCs possess the capability to differentiate into various intestinal epithelial cell types. ISCs are located in crypts within the specific Wnt microenvironment and are critical for protecting the integrity of the intestinal epithelium as well as responding sensitively to external stimuli (Zhou et al., 2020b). The initiation of Wnt/$\beta$-catenin signaling enhances the function of ISCs during injury, while Bmi1+ stem cells restore the stem cell population following the ablation of Lgr5+ stem cells (Li et al., 2014; Metcalfe et al., 2014). Previous research on animals has demonstrated that probiotics can help heal inflammation in the intestines by triggering the Wnt/$\beta$-catenin signaling pathway (Lu et al., 2020; Wu et al., 2020). In our research, LR significantly increased fluorescence levels of $\beta$-catenin and Lgr5 as well as gene expression of Wnt/$\beta$-catenin target genes in enteritis broilers. This finding may help to elucidate the molecular mechanisms by which LR maintains intestinal barrier function. To further explore the role of LR on the intestinal mucosa, we established an intestinal organoid co-culture model in vitro by simulating the morphology of intestinal mucosa in vivo, concentrating on the effect of LR on intestinal epithelial regeneration. The findings indicated successful development of the intestinal organoids through co-culturing with LR. LR pretreatment under 200 ug/mL LPS stimulation not only reduced organoid damage but also enhanced levels of $\beta$-catenin and Lgr5 fluorescence, along with the expression of downstream target genes associated with the Wnt/$\beta$-catenin pathway, aligning with in vivo findings. These findings indicated that LR facilitated the proliferation of ISCs by maintaining the stability of the Wnt/$\beta$-catenin signaling pathway, thereby maintaining the stability of intestinal epithelium in the state of intestinal inflammation.

## CONCLUSIONS

In summary, our research demonstrated the efficacy of LR in mitigating LPS-induced enteritis in broilers, concurrently ameliorating intestinal epithelial injury and fortifying barrier function. The underlying mechanism was that LR improved the expansion of ISCs and renewal of intestinal epithelial cells through the regulation of the Wnt/$\beta$-catenin signaling pathway, thereby maintaining the integrity of the intestinal mucosal barrier (Figure 9). These findings provided theoretical support for *lactobacillus* as a probiotic additive in livestock feed to improve intestinal inflammation and treat intestinal diseases.

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## DISCLOSURES

The authors declare no competing interests.

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