Probiotics and Antimicrobial Proteins (2022) 14:947–959
https://doi.org/10.1007/s12602-022-09967-9

Lacticaseibacillus rhamnosus LS8 Ameliorates Azoxymethane/Dextran Sulfate Sodium-Induced Colitis-Associated Tumorigenesis in Mice via Regulating Gut Microbiota and Inhibiting Inflammation

Tao Wang[1] · Jiaqi Zheng[1] · Shuchen Dong[1] · Mohamedelfaieh Ismael[1] · Yuanyuan Shan[1] · Xin Wang[1] · Xin Lü[1]

Accepted: 21 June 2022 / Published online: 5 July 2022

Abstract
Gut microbiota dysbiosis may promote the process of colorectal cancer (CRC). *Lacticaseibacillus rhamnosus* LS8 (LRL) is a potential gut microbiota regulating strain because it can produce a novel antimicrobial substance (like cycloalanopine). In addition, this probiotic had an inflammation-ameliorating effect on the dextran sulfate sodium (DSS)–induced colitis mice. However, it is not known whether treatment with this probiotic could ameliorate colitis-associated CRC via regulating gut microbiota. In this study, a CRC mouse model was induced by a single intraperitoneal injection of azoxymethane (AOM, 10 mg/kg) and followed by three 7-day cycles of 2% DSS administration. Results showed that LRL could inhibit tumor formation. Moreover, LRL enhanced the gut barrier by preventing goblet cell loss and promoting the expression of ZO-1, occludin, and claudin-1. Furthermore, LRL ameliorated gut microbiota dysbiosis, which was conducive to the growth of beneficial bacteria (e.g., *Faecalibaculum* and *Akkermansia*), and further led to an increase in SCFAs and a decrease in LPS. In addition, LRL alleviated colonic inflammation by inhibiting the overexpression of TLR4/NF-κB, pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-γ, and IL-17a), and chemokines (Cxcl1, Cxcl2, Cxcl3, Cxcl5, and Cxcl7). In conclusion, LRL could alleviate CRC by regulating gut microbiota and preventing gut barrier damage and inflammation.

Keywords *Lacticaseibacillus rhamnosus* · Colorectal cancer · Inflammation · Gut barrier · Gut microbiota

Introduction

Colorectal cancer (CRC) is the third most common malignancy and the second most prevalent cause of cancer mortality worldwide [1]. In addition to the heritability of CRC, the occurrence and progression of CRC are highly related to several risk factors, such as changes in lifestyle and diet, including our daily intake of too much red meat and too little dietary fiber [2]. At present, surgery is the main treatment for CRC, but it may also cause some adverse effects, such as postsurgical trauma, systemic inflammation, and mucosal barrier damage [3]. Therefore, it is necessary to exploit some safe and effective CRC preventive and/or therapeutic strategies from natural biological sources.

The human gastrointestinal tract coexists with a large diversity of microorganisms (~ 100 trillion) [4], which play a crucial role in maintaining the health of the host, such as regulating gut immune homeostasis, affecting intestinal barrier function, and producing some beneficial or harmful metabolism [5, 6]. Therefore, changes in the gut microbiota may prevent or aggravate the development of gut-related diseases, such as colitis or even its associated CRC [7, 8]. Probiotics are an indispensable part of the intestinal microbiota and have broad application prospects in preventing or attenuating CRC. For example, *Lactobacillus acidophilus* CICC 6074 could induce the apoptosis of colon tumor cells [9]; *Lactobacillus plantarum* YYC-3 could regulate the colon tumor microenvironment [10], and *Companilactobacillus crustorum* MN047 could alleviate azoxymethane (AOM)/dextran sulfate sodium (DSS)–induced gut microbiota dysbiosis and gut barrier damage [11, 12]. In general, the CRC mitigation effects of probiotics mainly include enhancing host immune defense and gut barrier function,

✉ Xin Wang
wangxin_2018@nwsuaf.edu.cn

✉ Xin Lü
xinlu@nwsuaf.edu.cn

[1] College of Food Science and Engineering, Northwest Agriculture and Forestry University, No. 22 Xinong Road, Yangling 712100, Shaanxi, China

maintaining gut microbiota balance, and promoting tumor cell apoptosis [3, 13, 14]. Notwithstanding, the current use of probiotics as dietary supplements to ease CRC remains limited, as the species-specific effects of probiotic strains may cause different mitigation effects and mechanisms. Therefore, it is a promising study to explore new probiotic strains with anti-tumorigenesis effects and focus on their functional mechanisms.

Lacticaseibacillus rhamnosus LS8 (LRL, formerly known as *Lactobacillus rhamnosus* LS8) was previously isolated from homemade fermented milk in Xinjiang Autonomous Region, China. A novel unusual cyclic opine antimicrobial substance (cycloalanopine) produced by this probiotic could inhibit the growth of multidrug-resistant pathogens [15]. Moreover, LRL could alleviate the pathological symptoms of ulcerative colitis (UC) induced by DSS [16]. Since pathogenic microorganisms and UC may trigger intestinal tumorigenesis [5, 17], we hypothesized that LRL administration could attenuate CRC by regulating gut microbiota and ameliorating colonic inflammation. Therefore, this study aimed to assess the ameliorating effect of LRL on the AOM/DSS-induced CRC mouse model by analyzing intestinal microbiota, colonic inflammation, and intestinal permeability. The results of the present study will expand the application of LRL as a potential CRC-ameliorating dietary supplement.

Materials and Methods

Preparation of LRL

To prepare the live probiotic supplement, LRL (GenBank no. KJ152776) was cultivated in the MRS medium at 37 ℃ for 16 h. The bacterial cells were collected by centrifugation (7500 *g*, 4 ℃, 5 min), washed twice with ice-cold physiological saline solution (PSS, 0.9% NaCl solution), and resuspended in PSS with a concentration of 5×10^9 CFU/mL for subsequent gavage administration.

Animals and Treatment

Forty-five C57BL/6 male mice (6-week-old) were purchased from Hunan SJA Laboratory Animal Co. Ltd. (Changsha, Hunan, China) and divided into three groups ($n = 15$): Ctrl (healthy control mice), Model (CRC model mice), and LRL (LRL-treated CRC mice). All mice were given ad libitum access to food and water under controlled conditions (temperature 23 ± 2 ℃, relative humidity $55 \pm 5\%$, and 12-h light–dark cycles). All animal protocols were approved by the Animal Ethics Committee of Xi'an Jiaotong University (Permission no. SCXK 2018–001).

The detailed experimental scheme is shown in Fig. 1A. Briefly, after 1 week of adaptive feeding, mice in the Model and LRL groups were given a single intraperitoneal injection of AOM (10 mg/kg body weight, dissolved in PSS, Sigma-Aldrich, St. Louis, MO, USA). One week after AOM injection, the mice underwent 3 cycles of DSS (36,000–50,000 M.Wt., MP Biomedicals, Aurora, OH, USA) administration. In each cycle, mice were given 1 week of 2% DSS (w/v) in drinking water, followed by 2 weeks of normal drinking water for a recovery period. Mice in the Ctrl group were given a single intraperitoneal injection of PSS (10 mL/kg body weight) and only supplemented with normal drinking water at the same time. During weeks 5 to 18, mice in the LRL group were given intragastric administration of LRL bacterial suspension (200 μL, ~ 1×10^9 CFU) once daily, while the Ctrl and Model groups were administrated with 200 μL PSS. Finally (week 18), all mice were anesthetized with an intraperitoneal injection of xylazine and ketamine (10 and 100 mg/kg, intraperitoneal injection, Sigma-Aldrich). All mouse colon tissues were collected and dissected longitudinally for macroscopic tumor statistical analysis. The number and diameter of tumors were counted by an independent observer who was not familiar with the different treatment groups. Mouse spleen and thymus tissues were weighted and divided by their body weight to calculate organ index. During the DSS induction periods, the disease activity index (DAI), including changes in body weight, fecal occult blood, and fecal consistency, was calculated according to previously proposed criteria to assess the severity of colitis [16]. A fecal occult blood reagent kit was purchased from Nanjing Jiancheng Technology (Nanjing, Jiangsu, China).

Intestinal Permeability Assessment

At the end of the entire feeding time (week 18), the mice were first fasted for 6 h, followed by intragastric administration of fluorescein isothiocyanate (FITC)-dextran (600 mg/kg body weight, 3,000–5,000 kDa, Sigma-Aldrich). After an additional 4 h of fasting, mice were euthanized and their serum samples were collected in the dark. The serum samples were diluted (1:1) with phosphate buffer saline (PBS, pH 7.4) and the fluorescence intensity of each sample was immediately measured using a Multi-Mode Microplate Reader (VictorX3, Perkin Elmer, Waltham, MA, USA) at the excitation wavelength of 485 nm and emission wavelength of 535 nm.

Histopathological Assessment

Distal colonic tissues were fixed overnight in 4% paraformaldehyde solution and then embedded in paraffin (stored at 4 ℃). Tissue sections were sliced into 5-μm thickness for pathological analysis, including hematoxylin and eosin (H&E), terminal deoxynucleotidyl transferase dUTP nick

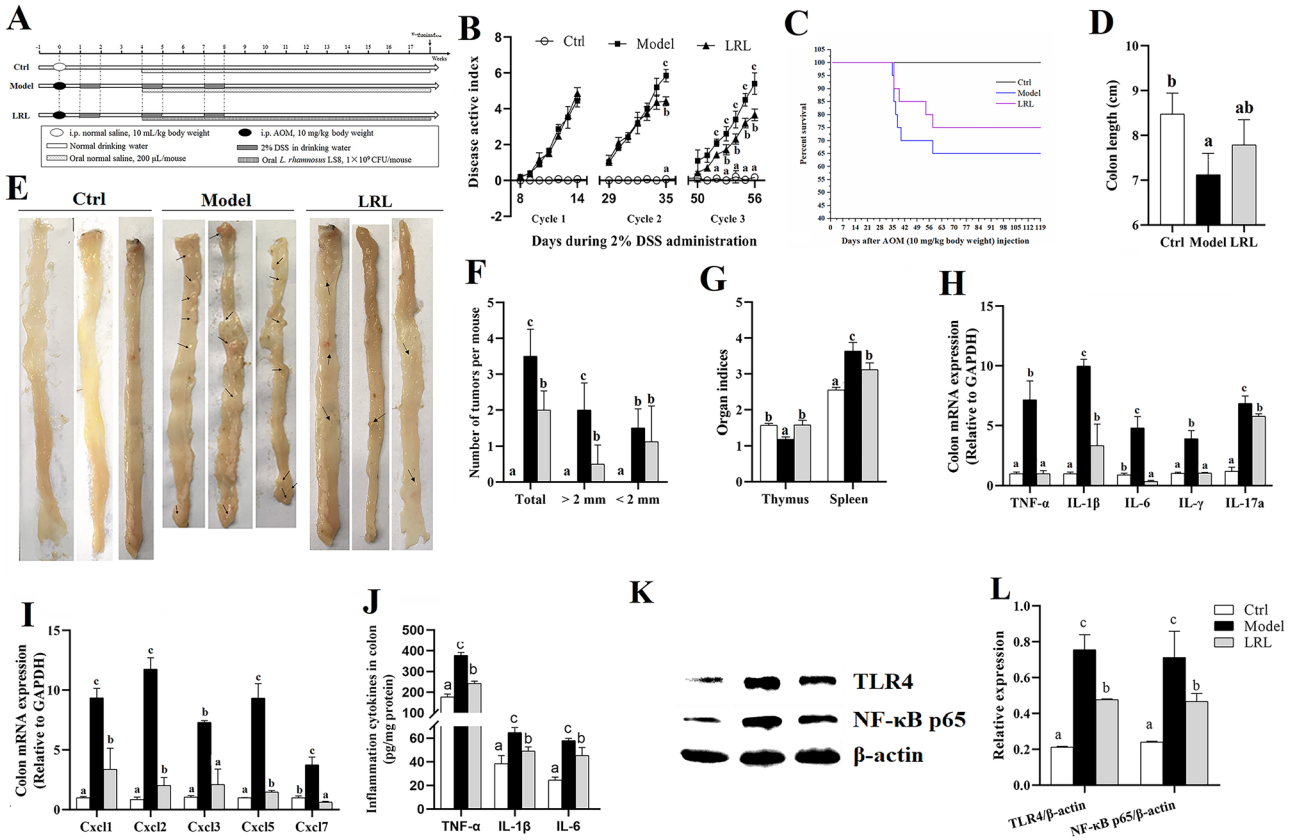

Fig. 1 Effects of LRL on the intestinal tumorigenesis in AOM/DSS-induced CRC mice. **A** The experimental protocol of LRL administration; **B** disease activity index; **C** survival rate; **D** colon length; **E** the representative macroscopic image of colonic tissues; **F** number of tumors; **G** thymus and spleen indices; **H** the mRNA levels of pro-inflammatory cytokines (*TNF-α*, *IL-1β*, *IL-6*, *IL-γ*, and *IL-17a*); **I** the mRNA levels of CXCR2 ligands chemokines (*Cxcl1*, *Cxcl2*, *Cxcl3*, *Cxcl5*, and *Cxcl7*); **J** the protein levels of inflammatory cytokines (TNF-α, IL-1β, and IL-6) measured using ELISA test kit; **K** and **L** Western blot analysis of TLR4 and NF-κB in the colonic tissue. Data in **B**, **E**–**G** ($n = 10$), **H**–**J** ($n = 5$), **K** and **L** ($n = 3$) are presented as mean ± SD, bars with different lowercase letters indicate significant differences ($p < 0.05$)

end labeling (TUNEL), and Alcian blue staining. The stained areas were photographed with an Olympus microscope (Olympus Corporation, Shinjuku, Tokyo, Japan). The pathological damage of colonic tissue was scored as described previously [18]. The TUNEL-positive cells and goblet cells were counted using Image J software (National Institutes of Health, Bethesda, MD, USA).

Biochemical Assessment

The levels of colonic pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and serum lipopolysaccharides (LPS) were tested using the ELISA test kit (Jingmei Biotech, Yancheng, Jiangsu, China). To prepare colonic tissue homogenate supernatant, mouse colonic tissue was homogenized with PBS (m/v = 1:9) and centrifuged at 5000 *g* for 5 min to remove the precipitates. The total protein concentration in mouse colonic tissue homogenate supernatant was tested using a bicinchoninic acid (BCA) protein assay kit (Zhonghuihecai Biotech, Xi'an, Shaanxi, China).

Real-Time PCR Analysis

The total RNA from colonic tissues was extracted using the AG RNAex Pro Reagent (Accurate Biology, Changsha, Hunan, China). The quality (A260/A280 = 1.8–2.1) and concentration of extracted RNA were analyzed using the NanoDrop One (Thermo Fisher Scientific, Wilmington, DE, USA). The FastKing RT Kit (with gDNase, Tiangen Biotech, Beijing, China) was utilized to synthesize cDNA. The CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with SYBR Green BioEasy Master Mix (Bioer Biotech, Hangzhou, Zhejiang, China) was used to perform the PCR amplification and detection. Primer sequences are given in Table S1. The mRNA level was normalized with GAPDH and calculated according to the $2^{-\Delta\Delta Ct}$ method.

Short-Chain Fatty Acid (SCFA) Analysis

To obtain the fecal homogenate supernatant, mouse fecal samples were homogenized with distilled water (m/v = 1:10) and centrifuged at 10,000 *g* for 10 min to remove the solid feces. The fecal supernatant was acidified with 50% H_2SO_4 (v/v = 5:1) for 5 min, and then extracted with diethyl ether (v/v = 1:1) at 4 ℃. Before gas chromatography (GC) analysis, the organic phase was collected by centrifugation (10,000 *g*, 10 min) and filtered through a 0.22 μm nylon filter (EMD Millipore Corp., Billerica, MA, USA). The detailed GC analytical procedure was according to the previous method [19].

Intestinal Microbiota Analysis

The total bacterial DNA from the mouse fecal sample was extracted using the PowerSoil DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA). The V3-V4 region of the bacterial 16S rRNA gene was amplified by PCR with a universal primer pair (forward primer, 5′-ACTCCTACGGGAGGCAGCA-3′; reverse primer, 5′-GGACTACHVGGGTWTCTAAT-3′). The purified and pooled PCR products were then subjected to high-throughput sequencing on an Illumina HiSeq 2500 platform. All sequencing data were analyzed at the BMK Cloud platform (http://www.biocloud.net/). High-quality reads were annotated using the Ribosomal Database Project (RDP) Classifier (version 2.2) based on the SILVA database (version 123) and clustered into the same operational taxonomic unit (OTU) with the similarity threshold of ≥ 97%. Alpha-diversity of gut microbiota was analyzed using Mothur (version 1.30) at the OTU level. Principal coordinate analysis (PCoA) based on Bray–Curtis analysis was analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) software. The specific phylotypes of different groups were analyzed using the linear discriminant analysis (LDA) effect size (LEfSe) method.

Western Blot Analysis

The expressions of TLR4 and NF-κB were analyzed using the Western blot as described previously [16]. The primary antibodies (β-actin, Catalog No. WL01372; NF-κB p65, Catalog No. WL01980; TLR4, Catalog No. WL00196) and HRP-conjugated secondary antibody (Catalog No. WLA024) were purchased from Wanleibio, Shenyang, Liaoning, China.

Statistical Analysis

Data were presented as the mean ± standard deviation (SD). Significant differences among different groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's test for multiple comparisons. A p value less than 0.05 was considered statistically significant. The Pearson's correlation coefficients between gut microbiota at genus level and CRC parameters or between SCFAs levels and CRC parameters were analyzed using the R language (version 4.1.0).

Results

Effects of LRL on the Intestinal Tumorigenesis in AOM/DSS-Induced CRC Mice

CRC may be driven by a long-term intestinal inflammatory response. To study the effect of LRL on the severity of DSS-induced inflammation, the DAI score was calculated during the administration of 2% DSS. In the second DSS induction cycle, DSS induction and probiotic intervention were initiated simultaneously. The results showed that compared with the model group, the remission effect of LRL intervention on DAI was not significant (Fig. 1B), which was mainly because the LRL intervention time was too short, and its potential beneficial functions were not fully exerted. However, in the third DSS induction cycle, LRL had been administered by gavage for 3 weeks, so LRL could exert more of its probiotic function at this time, thus significantly alleviating the rise of DAI (Fig. 1B). Compared with the CRC model mice, LRL intervention reduced the mortality of mice from 35 to 25% (Fig. 1C) and increased colon length by 9.21% (Fig. 1D). The abnormality of immune organ indexes, such as increased spleen index and decreased thymus index, may be the signs of inflammation, which could also be significantly attenuated in the LRL group (Fig. 1G). Furthermore, the mRNA or protein levels of pro-inflammatory cytokines (e.g., TNF-α, IL-1β, IL-6, IL-γ, and IL-17a) and C-X-C motif receptor 2 (CXCR2) ligands chemokines (e.g., Cxcl1, Cxcl2, Cxcl3, Cxcl5, and Cxcl7) were significantly increased in the AOM/DSS-induced CRC mice but significantly alleviated in the LRL-treated mice (Fig. 1H–J). In addition, the results of Western blot analyses showed that treatment with LRL significantly ameliorated the over-activation of TLR4/NF-κB caused by AOM/DSS (Fig. 1K, L). Based on these results, AOM/DSS-induced colonic inflammation was established and LRL supplementation could significantly attenuate it.

Compared with the AOM/DSS-induced CRC mice, there were fewer adenomas in the LRL group (Fig. 1E). Moreover, the number of total tumors and large tumors (diameter > 2 mm) in the LRL-treated mice was also significantly lower than that in the CRC model mice (Fig. 1F). Furthermore, the histological analysis based on H&E staining suggested that compared with the Model group, mice in the LRL-treated group had fewer pathological damage signs, including significant remission of inflammatory infiltration and crypt damage (Fig. 2A, C). In addition, TUNEL

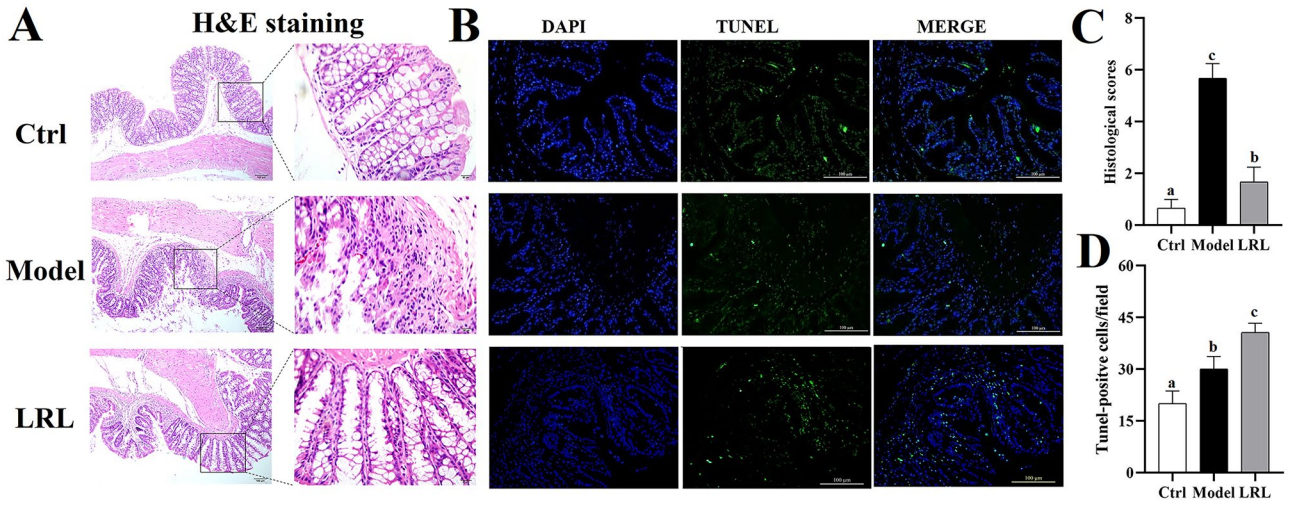

Fig. 2 Effects of LRL on the colonic histopathology in AOM/DSS-induced CRC mice. The representative images of **A** H&E staining and **B** TUNEL staining; **C** histopathological score based on H&E staining; **D** TUNEL-positive cell numbers based on TUNEL staining. Data in **C** and **D** ($n = 5$) are presented as mean ± SD, bars with different lowercase letters indicate significant differences ($p < 0.05$)

staining showed that the number of TUNEL-positive cells in the Model group was significantly less than that in the LRL-treated mice (Fig. 2B, D), suggesting that administration with LRL could promote the apoptosis of tumor cells. Taken together, it can be deduced that LRL supplementation had an anti-tumorigenesis effect on the AOM/DSS-induced CRC mice.

Effects of LRL on the Intestinal Barrier Integrity in AOM/DSS-Induced CRC Mice

To assess whether LRL could ameliorate the damage of the gut barrier caused by AOM/DSS, the intestinal permeability was assessed using FITC-dextran. Results showed that compared with the CRC model mice, the concentration of serum FITC-dextran was significantly reduced in the LRL-treated mice (Fig. 3C). Furthermore, Alcian blue staining of colonic tissue suggested that LRL administration could significantly attenuate AOM/DSS-induced goblet cell loss (Fig. 3A, B). Furthermore, supplementation with this probiotic could also significantly reverse AOM/DSS-induced decrease in the gene expression of some tight junction–related proteins, such as claudin-1, occludin, and ZO-1 (Fig. 3D). These results indicated that treatment with LRL could ameliorate AOM/DSS-induced gut barrier damage.

Effects of LRL on the SCFAs and LPS Levels in AOM/DSS-Induced CRC Mice

Fecal SCFAs, mainly derived from gut microbiota, showed multiple beneficial effects on CRC. Results showed that compared with the Ctrl group, except for valeric acid, the other SCFAs (including acetic acid, propionic acid, isobutyric, butyric acid, and isovaleric) were all significantly reduced in the AOM/DSS-induced CRC model mice. However, these adverse changes were all significantly ameliorated in the LRL-treated mice (Fig. 4A, B). An abnormal level of serum LPS was not only a sign of gut microbiota disturbance but also one of the causes of inflammation. In this study, treatment with LRL significantly prevented AOM/DSS-induced elevation of serum LPS (Fig. 4C).

In addition, the relationship between these intestinal microbiota–derived substances (LPS and SCFAs) and CRC parameters is shown in Fig. 4D. The results indicated that serum LPS was significantly positively correlated with the parameters that may aggravate the development of CRC, including inflammation (TNF-α, IL-1 β, IL-6, and CXCR2 levels), intestinal permeability, DAI, total tumor number, and histological score, but significantly negatively correlated with the parameters that may ameliorate the development of CRC, including colon length and gut barrier (goblet cells, ZO-1, occludin, and claudin-1). On the contrary, SCFAs, especially butyric acid, were significantly negatively correlated with the parameters that may aggravate CRC, but partly significantly positively correlated with the parameters (colon length, goblet cells, and levels of gut barrier-related proteins) that may ameliorate CRC.

Effects of LRL on the Intestinal Microbiota in AOM/DSS-Induced CRC Mice

For the analysis of gut microbiota composition, a total of 1,091,818 available reads (Ctrl 398,572, AOM 305,435, and LRL 387,811) were obtained from 45 samples and 4127

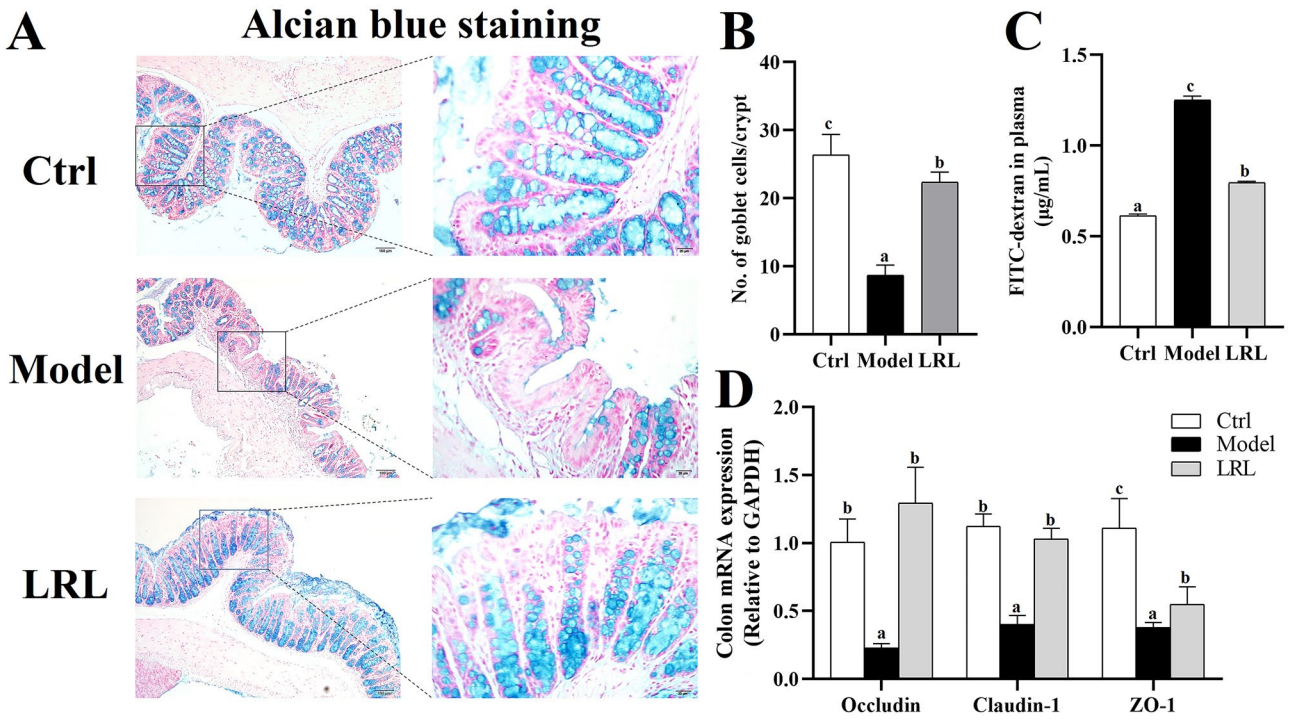

Fig. 3 Effects of LRL on the intestinal integrity in AOM/DSS-induced CRC mice. **A** Representative images of Alcian blue staining; **B** goblet cell numbers based on Alcian blue staining; **C** serum FITC level; **D** the mRNA levels of *occludin*, *claudin-1*, and *ZO-1* in mouse colonic tissues. Data in **B**–**D** ($n=5$) are presented as mean ± SD, bars with different lowercase letters indicate significant differences ($p<0.05$)

OTUs were identified with a 97% similarity cutoff (data not shown). The results of the Shannon and Rarefaction curves showed that most bacterial diversity was captured in all samples (Fig. 5A). The results of the Shannon, Simpson, ACE, and Chao indexes indicated that the alpha diversity of gut microbiota had no significant difference among all groups (data not shown). The results of PCoA based on Bray–Curtis analysis indicated that the samples in the CRC model mice were separated from the healthy Ctrl mice, while it was attenuated in the LRL-treated mice (Fig. 5B). Therefore, LRL could regulate the change of gut microbiota caused by AOM/DSS. On the phylum-level analysis, compared with the Ctrl group, AOM/DSS induction caused an increase in Acteroidetes, Patescibacteria, and Tenericutes, but a decrease in Actinobacteria and Verrucomicrobia. However, LRL administration not only prevented these changes but also facilitated the enrichment of Verrucomicrobia and Proteobacteria (Fig. 5C). In the genus-level analysis (top 30), 12 genera had significant differences among different groups (Fig. 5D–F). Briefly, compared with the healthy Ctrl group, 6 genera (*Lactobacillus*, *Bifidobacterium*, *Dubosiella*, *Akkermansia*, *Lachnospiraceae_NK4A136_group*, and *Faecalibaculum*) were decreased and 6 genera (*Ruminococcaceae_UCG-014*, *Turicibacter*, *Candidatus_Saccharimonas*, *Coriobacteriaceae_UCG-002*, *Bacteroides*, and *uncultured_bacterium_o_Mollicutes_RF39*) were increased in the AOM/DSS-induced CRC model mice. However, except for a slight reversal (no significant differences) in the abundances of *Lactobacillus*, *Bifidobacterium*, *Dubosiella*, and *Akkermansia*, the other changes mentioned above were all significantly reversed in the LRL-treated mice (Fig. 5E, F).

To further exploit the specific phylotypes in the different groups, LEfSe analysis was performed from the phylum to the genus level. Results suggested that the number of specific significant genera in the Ctrl, Model, and LRL groups were 2 (*Lactobacillus* and *Bifidobacterium*), 2 (*Coriobacteriaceae_UCG_002* and *unculture_bacterium_o_Mollicutes_RF39*), and 3 (*Odoribacter*, *Faecalibaculum*, and *Akkermansia*), respectively (Fig. 6A, B).

Relationship Between Gut Microbiota and CRC Parameters

A heatmap of Pearson's correlation was performed to investigate the potential relationship between the gut microbiota at the genus level and CRC parameters (Fig. 7). The clustering results showed that the top 30 gut microbiota were divided into three groups. Except for the genera of *Ruminococcaceae_UCG-013* and *Parabacteroides*, the

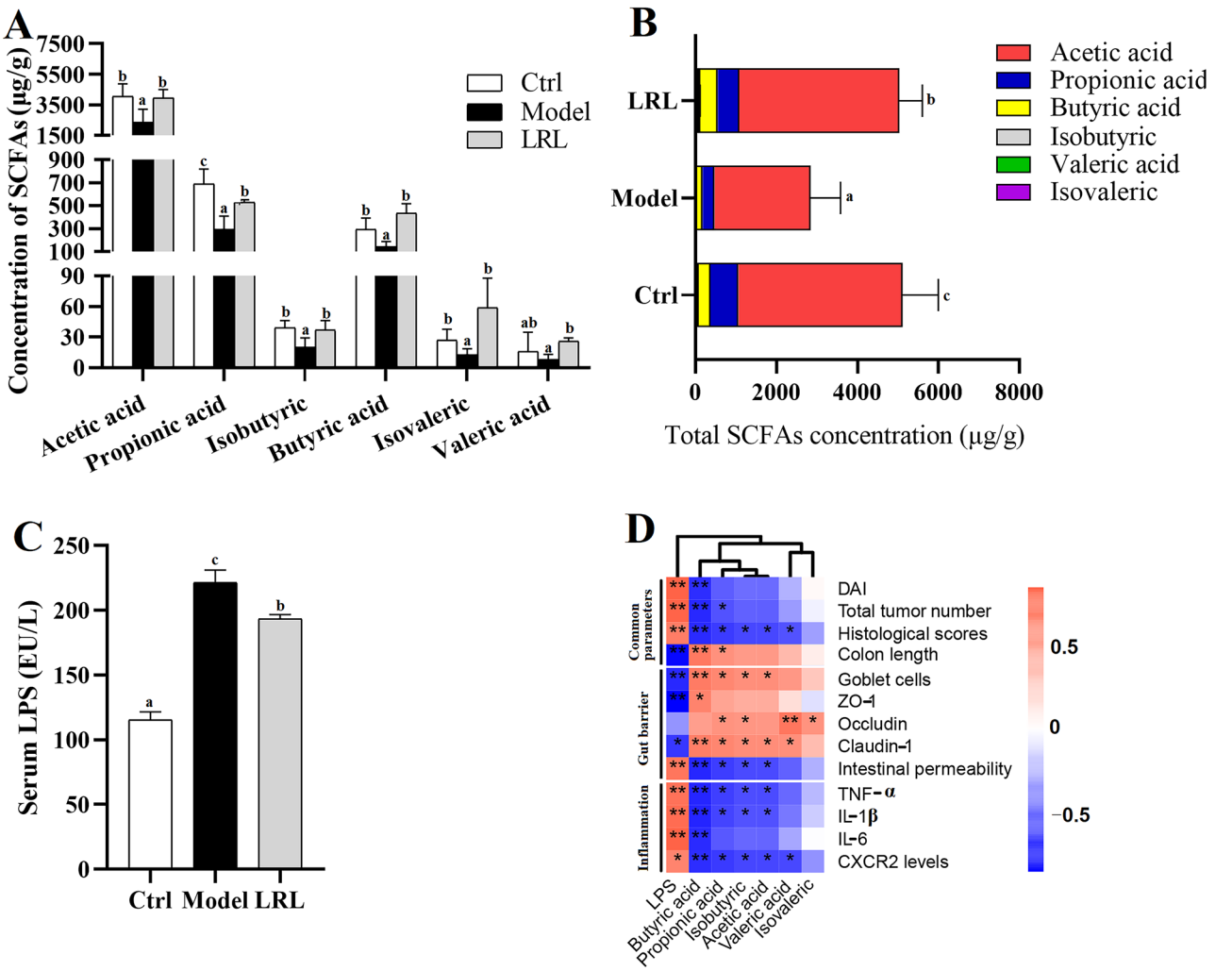

Fig. 4 Effects of LRL on the fecal SCFAs and serum LPS concentration in AOM/DSS-induced CRC mice. **A** Individual and **B** total SCFAs levels; **C** serum LPS; **D** the relationship between the gut bacteria-derived substances (LPS and SCFAs) and CRC-related parameters (DAI is the data from the last DSS induction; intestinal permeability is the data of serum FITC-dextran level; CXCR2 levels are the total levels of Cxcl1, Cxcl2, Cxcl3, Cxcl5, and Cxcl7). Data in **A**–**C** ($n = 5$) are presented as mean ± SD, bars with different lowercase letters indicate significant differences ($p < 0.05$)

other genera in group I were partially positively correlated with the parameters that might aggravate CRC (e.g., DAI, total tumor number, histological scores, intestinal permeability, TNF-α, IL-1β, IL-6, CXCR2 levels, and LPS), but negatively correlated with parameters that might attenuate CRC (e.g., colon length, goblet cells, ZO-1, occludin, claudin-1, and SCFAs). On the contrary, the most genera in group III, including *Akkermansia*, *Lactobacillus*, *Bifidobacterium*, *Dubosiella*, and *Faecalibaculum*, were partly positively correlated with the parameters that might ameliorate CRC, but partly negatively correlated with the parameters that might aggravate CRC. Compared with groups I and III, the genera in group II had no significant correlation with CRC parameters.

Discussion

A growing body of literature suggested that the intestinal microbiota of patients with CRC was different from that of healthy individuals and long-term chronic intestinal inflammation might trigger colitis-associated tumorigenesis [5, 17]. Therefore, preventing the imbalance of gut microbiota and suppressing colonic inflammation were promising strategies to prevent and/or ameliorate colitis-related CRC. LRL was a potential gut microbiota-regulating probiotic as it can produce a novel effective antipathogenic substance: cycloalanopine [15]. Furthermore, LRL could ameliorate the inflammatory response

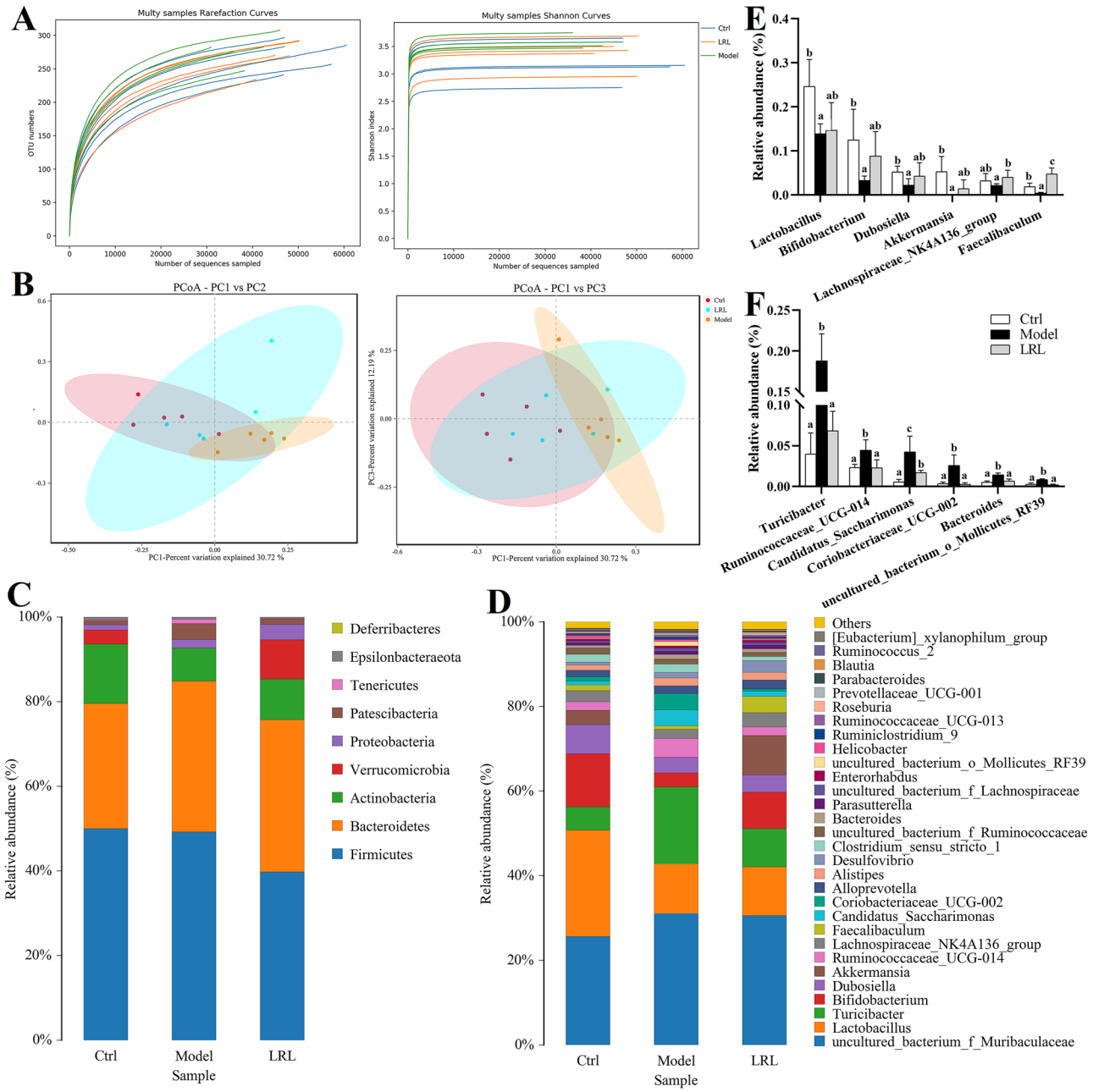

Fig. 5 Effects of LRL on the gut microbiota in AOM/DSS-induced CRC mice. **A** Rarefaction and Shannon curves; **B** PCoA results based on Bray–Curtis analysis; the compositions of gut microbiota at **C** phylum and **D** genus levels; **E** and **F** the relative abundance of 12 genera that had significant differences among different groups; data are presented as mean ± SD ($n = 5$), bars with different lowercase letters indicate significant differences ($p < 0.05$)

in the DSS-induced UC mice [16]. However, it was not clear whether LRL supplementation could attenuate UC-related CRC via regulating gut microbiota and inhibiting colonic inflammation. Therefore, the present study aimed to investigate the anti-carcinogenic effect of LRL on the AOM/DSS-induced CRC mice. In this study, compared with the CRC model mice, the total and large (diameter > 2 mm) tumor numbers were significantly decreased in the LRL-treated mice (Fig. 1E, F), indicating that LRL could ameliorate the development of CRC. This deduction was also proved by the histopathological results, including ameliorated crypt damage and more TUNEL-positive cells in the LRL supplementation mice (Fig. 2A, B). In the present study, it should be emphasized that LRL intervention initiated after the end of the first DSS induction cycle rather than before the induction of colitis was closer

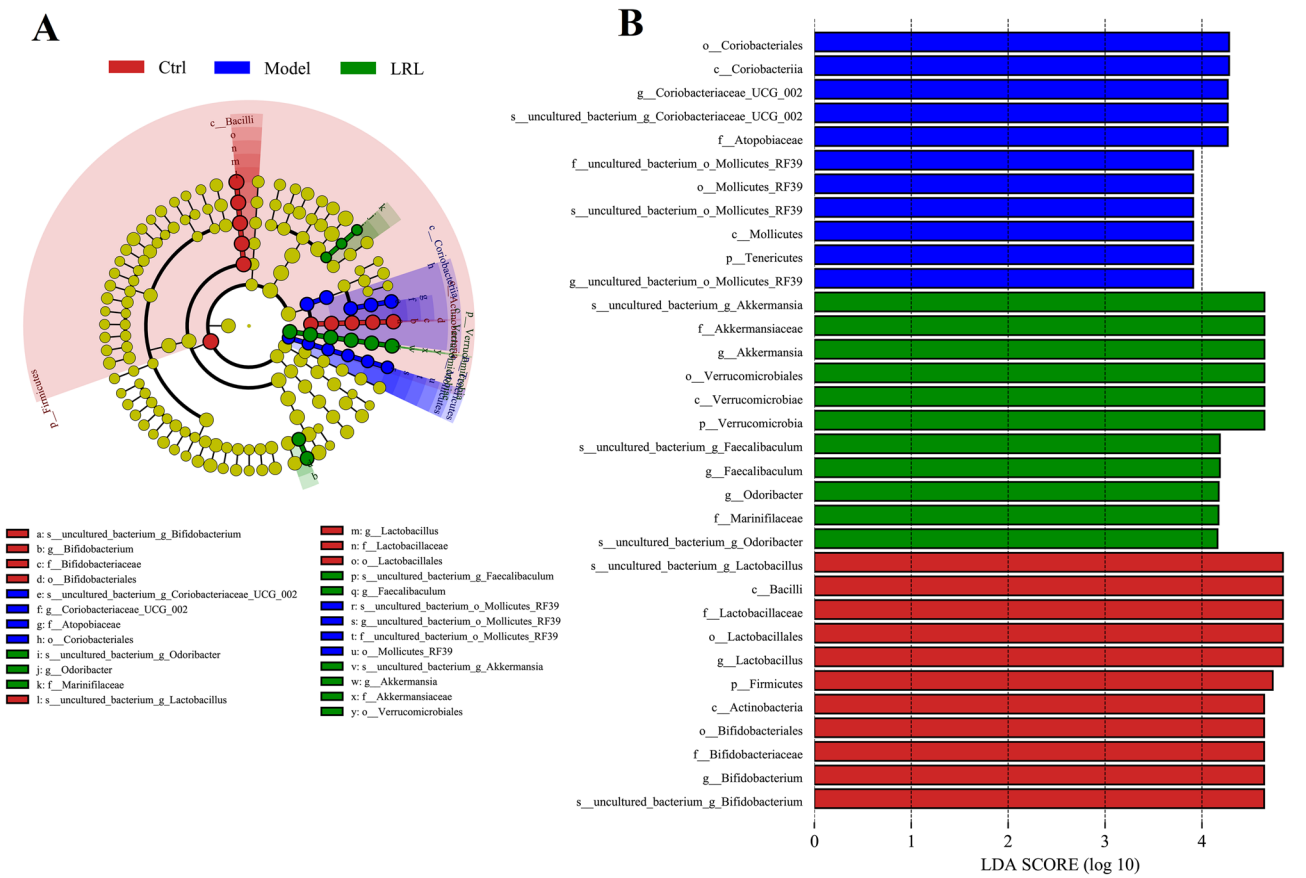

Fig. 6 Effects of LRL on the specific intestinal microbiota phylotypes in AOM/DSS-induced CRC mice. Only the taxa with LDA score > 3.5 are shown. **A** LEfSe cladogram (the size of the circle shows the relative abundance of the taxa and yellow dots indicate no statistical significance); **B** LEfSe score plot

to assessing the therapeutic effect of LRL on CRC rather than just the preventive effect. In addition, some probiotics have also been reported to have the ability to adsorb carcinogens. Therefore, starting LRL intervention 4 weeks after AOM injection could rule out the possibility that LRL might alleviate AOM/DSS-induced CRC by adsorbing and removing AOM.

Long-term chronic inflammation was known to predispose individuals to cancer, and the presence of inflammatory bowel disease might increase the risk of CRC [4, 20]. Therefore, inhibition of colonic inflammatory response was considered an effective strategy to ameliorate or prevent colitis-related CRC. The high expression of CXCR2 chemokines in the inflamed intestine was responsible for the recruitment of granulocytic myeloid-derived suppressor cells into the intestinal mucosa and its knockout significantly inhibited AOM/DSS-induced colorectal tumorigenesis [21]. LRL supplementation was shown to significantly down-regulate the expression of some CXCR2 ligands (e.g., *Cxcl1*, *Cxcl2*, *Cxcl3*, *Cxcl5*, and *Cxcl7*) caused by AOM/DSS (Fig. 1I). Corresponding to these results, the high levels of pro-inflammatory cytokines (e.g., TNF-α, IL-1β, IL-6, IL-γ, and IL-17a) caused by AOM/DSS were also suppressed in the LRL-treated mice (Fig. 1H, J). Therefore, the anti-CRC effect of LRL may be partly due to its anti-inflammatory properties.

Patients with CRC are often accompanied by intestinal barrier dysfunction [22]. The intestinal barrier is essential for maintaining intestinal health, preventing gut microbiota translocation and leakage of intestinal substances. Intestinal inflammation and pathogenic microorganisms infection may disrupt intestinal barrier function, which may further lead to increased intestinal permeability, intestinal bacteria translocation, and immune activation [23, 24]. In the present study, LRL supplementation significantly ameliorated the increase of intestinal permeability caused by AOM/DSS, which was mainly manifested by preventing goblet cell loss and up-regulating tight junction-associated protein expressions, such as ZO-1, occludin, and claudin-1 (Fig. 3A, D). Therefore, it can be speculated that LRL administration could enhance the intestinal barrier, prevent gut bacteria translocation, and further alleviate inflammatory responses.

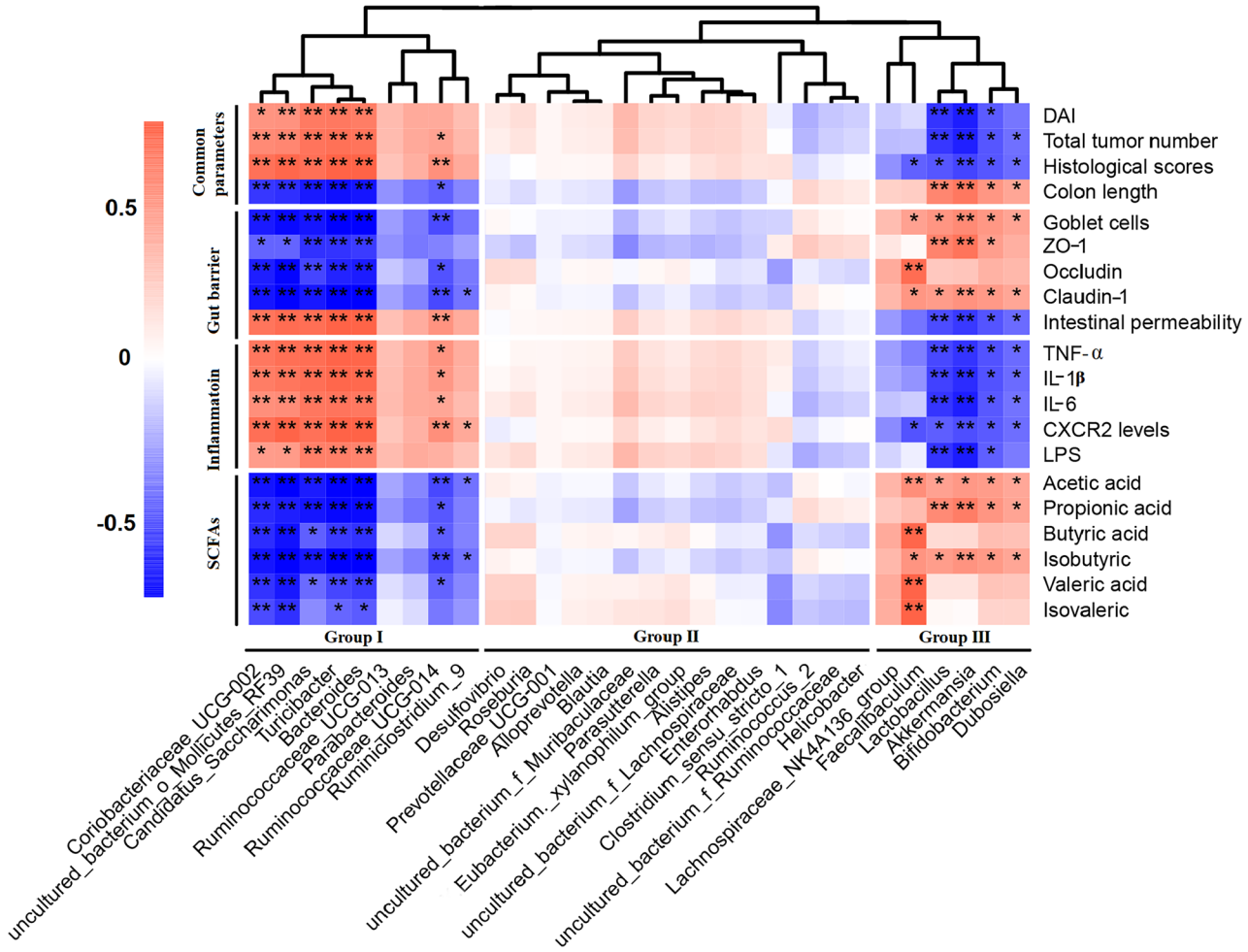

Fig. 7 Correlation analysis between gut microbiota and CRC parameters. DAI is the data from the last DSS induction; intestinal permeability is the data of serum FITC-dextran level; CXCR2 levels are the total levels of Cxcl1, Cxcl2, Cxcl3, Cxcl5, and Cxcl7

The colorectum is the most gut microbiota-exposed region of the human gastrointestinal tract, and it is both the CRC formation site and the colonization site of gut microbiota. Therefore, colon tumorigenesis is more likely to be related to gut microbiota than other cancer diseases. The cross-talk between intestinal microbiota and the host's immune systems could play essential roles in controlling intestinal homeostasis and inflammatory response and further affecting tumor formation [25]. Gavage of fecal microbiota from patients with CRC to germ-free mice could cause gut carcinogenesis [26]. Supplementation with probiotics has been proved to be an effective method to ameliorate CRC via maintaining the balance of gut microbiota [10–12]. Compared with the Ctrl group, AOM/DSS induction caused an increase in some harmful bacteria (e.g., *Candidatus_Saccharimonas*, *Turicibacter*, and *Bacteroides*, Fig. 5D, F), which were related to the high risk of CRC [27–29]. On the contrary, treatment with LRL could not only prevent these adverse alterations but also significantly enhance the relative abundance of some beneficial bacteria abundance (e.g., SCFAs-producing bacteria *Lachnospiraceae_NK4A136_group* and *Faecalibaculum*, Fig. 5D–F). The modulating effect of LRL on the gut microbiota might be due to its potential ability to inhibit the colonization or even growth of harmful bacteria by competing for co-receptors and nutrients, or by producing antibacterial substances (e.g., organic acid and cycloalanopine).

Corresponding to the high abundances of SCFAs-producing bacteria in the LRL-treated mice, the levels of total SCFAs in the LRL-treated mice were also significantly increased (Fig. 4A, B). As the typical beneficial metabolites of intestinal microbiota, SCFAs (especially butyric acid) played important roles in ameliorating CRC [30], including inhibiting inflammation and histone deacetylases [31, 32], maintaining colonic epithelial health [33], inhibiting microbial pathogens [34], and regulating cell growth and differentiation [35]. Consistent with the high levels of SCFAs in the LRL-treated mice, the CRC-related pathological parameters, such as inflammation,

gut barrier, and tumor cell apoptosis were also significantly improved compared with the AOM/DSS-induced CRC model mice. In this study, although the changes in the levels of SCFAs were mainly caused by the changes in the gut microbiota, SCFAs could also adversely affect the balance of the gut microbiota. In general, SCFAs could reduce the pH environment of the intestinal lumen, thereby affecting the structure of bacterial cell membrane units, such as proteins and phospholipids, which could further affect the permeability of the cell membrane and lead to the leakage of intracellular metabolites. Additionally, SCFAs could also penetrate bacterial cells and adversely affected intracellular activities such as DNA replication and protein synthesis, and ultimately lead to bacterial cell death. As another bacteria-derived substance, LPS was the primary activator of TLR4, showing the highest level in AOM/DSS-induced CRC model mice (Fig. 4C). LPS could trigger a precancerous inflammatory milieu to cause the development of tumors by promoting the accumulation of monocyte-like macrophages [36]. Furthermore, the over-expression of TLR4/NF-κB in the colonic tissue was an important pathway to facilitate colitis-associated CRC [37]. In the present study, LRL supplementation inhibited the increase of serum LPS (Fig. 4C) and the over-activation of TLR4/NF-κB caused by AOM/DSS (Fig. 1K, L), which was consistent with the reduced inflammatory response and alleviated tumor formation in the LRL group (Fig. 1F, H–J). Therefore, treatment with LRL in the colitis-associated CRC mice could ameliorate the imbalance of gut microbiota, which was conducive to the growth of beneficial bacteria and inhibited the growth of harmful bacteria, thereby increasing SCFAs levels and reducing LPS levels.

Taken together, based on the analyses of colonic inflammation, intestinal integrity, and gut microbiota, LRL supplementation regulated gut microbiota, as evidenced by increasing the relative abundances of beneficial bacteria (e.g., SCFAs-producing bacteria, *Lachnospiraceae_NK4A136_group*, and *Faecalibaculum*), but decreasing the relative abundances of harmful bacteria (such as proinflammatory or LPS-producing bacteria, *Candidatus_Saccharimonas*, *Turicibacter*, and *Bacteroides*), which in turn increased levels of gut microbiota-derived anti-inflammatory substances (like SCFAs) and decreased levels of gut microbiota-derived pro-inflammatory substances (like LPS). Moreover, treatment with LRL could also alleviate the damage of the intestinal barrier by preventing goblet cell loss and promoting tight junction–related protein expression (e.g., claudin-1, occludin, and ZO-1), which could in turn prevent intestinal bacterial translocation and immune activation. Under the regulation of gut microbiota and the strengthening of the gut barrier, the colonic inflammatory response was ameliorated via inhibiting intestinal pathogenic bacteria or LPS-activated TLR4/NF-κB pathway. Therefore, it could deduce that the CRC ameliorating effect of LRL was mainly attributed to the inhibition of the TLR4/NF-κB pathway. Furthermore, LRL also showed a potential role in promoting tumor cell apoptosis, which was demonstrated by more TUNEL-positive cells in LRL-treated mice than in CRC model mice. Nonetheless, the role of LRL in promoting tumor cell apoptosis remains to be further studied. In addition, it should be emphasized that probiotics are only a food ingredient or dietary supplement compared to drugs used to treat CRC, so it is not reasonable to supplement LRL in the diet only after a diagnosis of CRC. Furthermore, LRL is an exogenous probiotic (isolated from the traditional fermented food rather than the human gastrointestinal tract), which may be difficult to achieve long-term colonization in our gastrointestinal tract with a short period of dietary intervention to exert its potential function. Therefore, it is necessary to use LRL as a common dietary supplement or starter to develop a series of daily products, such as yogurt, so that we can take it for a long time through our daily diet to achieve its potential function in preventing or ameliorating CRC.

Conclusion

This study demonstrated that LRL could ameliorate AOM/DSS-induced CRC via regulating gut microbiota, strengthening gut barrier, and alleviating colonic inflammation. These results may promote the use of LRL as a dietary supplement to mitigate colitis-associated CRC. Although LRL has a certain CRC-ameliorating effect, the molecular mechanism of this strain on intestinal microbiota and CRC also needs to be further studied.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12602-022-09967-9.

Funding This research was supported by the National Natural Science Foundation of China (No. 32001652), Keypoint Research and Invention Program of Shaanxi Province (NO. 2021ZDLNY05-06), and Chinese Universities Scientific Fund (NO. 2452018062).

Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing Interests The authors declare no competing interests.

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