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Rosmarinic acid prevents dextran sulfate sodium-induced ulcerative colitis in mice by enhancing the function of the intestinal barrier and preserving intestinal homeostasis

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ABSTRACT

Rosmarinic acid (RA) is a natural phenolic acid with multiple biological and pharmacological properties. However, its protective effect on ulcerative colitis (UC) remains uncertain. This study aims to explore the protective ability and potential mechanism of RA on UC, focusing on the intestinal barrier and homeostasis. UC mice model was established through the induction by dextran sulfate sodium (DSS) of 2.5%, and the progression of the UC after RA treatment was monitored using clinical manifestations, histopathological examination, and biochemical analysis. The mice's composition of intestinal flora was assessed through 16S rRNA sequencing methods, while the concentrations of short-chain fatty acids (SCFAs) and bile acids (BAs) were analyzed using targeted metabolomics. The findings demonstrated that RA could prevent a decrease in body weight, reduce the scores of disease activity index (DAI), shorten colon length, and restore the claudin-1, zonula occludens-1 (ZO-1), and occludin levels in UC mice. Furthermore, RA effectively suppressed intestinal inflammation, modulated the compositin of gut microbiota, and influenced the levels of SCFAs and BAs. Hence, RA could offer therapeutic benefits for UC mice by enhancing intestinal barrier function and preserving intestinal homeostasis. Given the availability of scientific evidence, it may serve as a preventive agent or remedy for UC.

1. Introduction

Ulcerative colitis (UC) is a type of chronic inflammatory bowel disease (IBD) that predominantly occurs in the colon and rectum, presenting clinical symptoms such as body mass loss, flank pain, diarrhea, and hematochezia[1]. This condition typically progresses over a prolonged period with frequent relapses and an elevated risk of developing colorectal carcinoma[2]. A global rise in the occurrence and spread of UC has been observed in recent years, imposing a significant burden on healthcare systems worldwide[3]. Current medications used for UC treatment, including glucocorticoids, amino salicylates, immunosuppressants, antibiotics, monoclonal antibodies against tumor necrosis factor-α (TNF-α), and other biologic agents, exhibit varying degrees of inefficacy and severe adverse reactions[4-5]. It is urgently necessary to search for safe and effective treatment options for UC.

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The development of UC is multifactorial, involving interactions among environmental, genetic, infection, immune system dysfunction, and alterations in the gut microbiota, all potentially contributing to its onset[6-7]. The dysfunction of the intestinal barrier plays a crucial role in the pathogenesis of UC[8]. The intestinal barrier refers to the collective structure and function of the intestines, which prevent harmful substances from passing through the intestinal mucosa and entering other tissues, organs, and the human body's bloodstream. It primarily consists of mechanical, chemical, immune, and biological barriers[9-10]. Therefore, safeguarding the function of the intestinal barrier and preserving intestinal homeostasis may present a promising therapeutic strategy for managing UC. Compared with synthetic drugs, natural compounds found in plants, like dietary polyphenols, offer gentler, safer, cost-effective, and environmentally friendly alternatives for managing UC, particularly in modulating the intestinal microbiota and enhancing intestinal barrier function[11-12].

Rosmarinic acid (RA), a hydroxycinnamic acid commonly found in the Labiatae and Boraginaceae families, is recognized as a high-value natural phenolic acid component. Numerous studies have reported RA's excellent bioactivities and pharmacological properties, including antioxidant, antibacterial, anti-inflammatory, and immune-regulatory functions[13-14]. In addition, RA has been shown to modulate gut microbiota, eliminate pathogenic bacteria such as *Escherichia coli*, *Salmonella*, and other enteropathogenic bacteria[15-17], and enhance the immune response by increasing interleukin-10 (IL-10) levels and reducing pro-inflammatory mediator levels such as nterleukin-1β (IL-1β), interleukin-6 (IL-6) and TNF-α[15,18]. Given the close association between the onset of UC and intestinal inflammation, RA may have significant potential for treating UC and related conditions. Current studies have indicated the beneficial effects of RA-rich extracts or products in improving UC, such as RA can treat UC by regulating murine macrophage polarization or suppressing colitis-associated tumor development by enhancing the activation of NF-κB and STAT3 through TLR4 mediation[19-20]. Wang et al.[21] and Jin et al.[22] have individually highlighted the positive impact of RA on UC through regulating gut microbial-derived metabolism and inhibition of NF-κB and STAT3 activation. Nonetheless, research on RA in ameliorating UC through the modulation of gut microbiota and enhancement of intestinal barrier function remains scarce.

Hence, given the close relationship between UC, gut microbiota, and intestinal immunity, this study hypothesized that RA holds promising therapeutic potential for managing UC and established a dextran sulfate sodium (DSS)-induced UC mice model to examine the therapeutic effects of RA on UC. The assessment involved analyzing a range of physiological and biochemical indicators, gut microbiota composition, intestinal immunity, as well as short-chain fatty acids (SCFAs) and bile acids (BAs) metabolism, with the hope of elucidating the underlying mechanisms by which RA impacts the development of UC.

2. Materials and methods

2.1 Materials and reagents

DSS (molecular weight of 36–50 kDa, lot: S0798) was purchased from MP Biomedicals, LLC (Santa Ana, CA, USA). RA was procured from Sigma-Aldrich Trading Co., Ltd. (Shanghai, China). Enzyme-linked immunosorbent assay (ELISA) kits of IL-1β, IL-6, and TNF-α were bought from Thermo Fisher Scientific (Invitrogen brand) (Shanghai, China). Polyvinylidene difluoride (PVDF) was purchased from Pall Corporation (Port Washington, New York, USA). Ultra-sensitive enhanced chemiluminescence (ECL) chemiluminescent substrate was purchased from Biosharp Life Sciences (Hefei, China).

2.2 Design of animal experiment

A total of 50 female C57BL/6 mice (n = 50; 6–8 weeks old; body weight 16–18 g) were obtained from Hunan SJA Laboratory Animal Co., Ltd. (Changsha, China). The Academic Committee of Southwest Forestry University approved animal experiments involving mice (SWFU-2018003). During the study, the mice were kept in a specific pathogen-free (SPF)-grade animal room, where the environment was maintained at 22 ℃ with a 12-h light-dark cycle. The experimental mice were allowed unrestricted access to water and food for a week to acclimatize, which aimed to normalize the gut microbiota. Following a week of acclimatization, the mice were randomly divided into 5 groups (n = 10 mice). RA was dissolved in sterilized water to a concentration of 10 mg/mL. The mice were orally administrated RA at low, medium, and high doses of 12.5 mg/kg·day (RAL), 25 mg/kg·day (RAM), and 50 mg/kg·day (RAH) for 14 days[21-22]. Experimental colitis was induced from the eighth day by adding 2.5% DSS (m/V) to autoclaved drinking water. The mice in the control (BLANK) and DSS (DSS) groups only received sterilized water as a vehicle (Fig. 1A). The water was changed every 3 days during the experiment. On the final day, the body weight, water consumption, and food intake were documented, and the disease activity index (DAI) score was measured for all groups. Blood (0.5–1.0 mL) was drawn from the eyeballs into non-anticoagulant tubes, and the mice were promptly euthanized through cervical dislocation. After dissection, colonic, liver tissues, and spleen were extracted and weighed to determine the spleen index (spleen weight/body weight), and the length of each colon was estimated. The colonic and liver tissues were preserved at –80 ℃ for later analyses.

2.3 DAI and histological assessment

Daily monitoring and recording of each mice's body weight were conducted. Fresh feces were collected for occult blood tests using fecal occult blood guaiac paper[23]. The DAI was assessed by considering the changes in body weight, stool consistency, and the presence of occult blood. The DAI was calculated using the scoring scales for weight change (0, no notable changes; 1, body weight loss 1%–5%; 2, body weight loss 5%–10%; 3, body weight loss 10%–20%; 4, more than 20% body weight loss)[24]; stool consistency (0, normal feces; 1, soft and moist feces; 2, soft but still formed; 3, watery feces), and fecal occult blood (0, negative; 1, pale blue; 2, dark blue; 3, visible blood)[25-26].

The colonic tissues were fixed, dehydrated in absolute ethanol and isopropanol[27], encased with paraffin, sectioned, and hematoxylin and eosin (H&E) staining[28]. The assessment criteria are detailed in Table 1, with a minimum score of 0 and a maximum score of 20.

Table 1
Histological assessment criteria.

Items	Symptom	Score
Depth of the ulcer	No ulcer	0
	Mucosal involvement	1
	Mucosal + submucosal involvement	2
	Penetration of muscularis propria	3
	Full thickness involvement	4
Extent of the ulcer	No ulcer	0
	Punctate	1
	Minimal	2
	Moderate	3
	Widespread	4
Presence of inflammation	None	0
	Minimal	1
	Mild	2
	Moderate	3
	Severe	4
Extent of inflammation	None	0
	Mucosal	1
	Mucosal + submucosal involvement	2
	Mucosal + submucosal + muscle penetrate	3
	Full thickness involvement	4
Location of fibrosis	None	0
	Mucosa only	1
	Mucosa + submucosa	2
	Including muscle layer	3
	Full thickness fibrosis	4

2.4 Serum sample preparation and cytokine assays

Collected the blood samples from the eye sockets and placed in 1.5 mL microcentrifuge tubes at room temperature for 1 h at the end of the experiment. Centrifuged the samples at 1 500 × *g* for 10 min at 4 ℃, collected the resulting supernates for subsequent analysis. Using an ELISA kits to quantify IL-1β, IL-6 and TNF-α concentrations according to the manufacturer's protocols.

2.5 Western blot analysis

Colonic tissues were homogenised in RIPA lysis buffer supplemented with protease inhibitors. The protein level was assessed using a BCA protein assay. Separated the protein (40 μg) on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred onto the polyvinylidene difluoride (PVDF) membranes (Pall Co., Port Washington, NY, USA). The membranes were blocked with 5% non-fat dry milk in TBS-T to prevent nonspecific binding, followed by overnight incubation at 4 ℃ with primary antibodies targeting claudin-1, zonula occludens-1 (ZO-1) and occludin. After 3 washes with TBS-T, the membranes were then treated at room temperature for 1 h with the respective HRP-conjugated secondary antibodies, then subjected to 5 extra TBS-T washes. Protein signals were detected using the ultra-sensitive ECL chemiluminescent substrate (Biosharp, Life Sciences, Hefei, China), visualized on a ChemiDoc MP Imaging System (Bio-Rad, Laboratories, Hercules, CA, USA), and analyzed via ImageJ software (version 1.53a; National Institutes of Health, Bethesda, MD, USA).

2.6 Gut microbiota analysis

DNA extraction from the colonic contents of the mice was carried out utilizing a commercial DNA extraction kit. (Cat. No12888-100, QIAGEN, GmbH, Hilden, Germany) according to the operating instructions. The purity and concentration of the DNA were subsequently evaluated through agarose gel electrophoresis. Genomic DNA served as a template for primary PCR amplification, and the resultant primary amplification product was subsequently purified and utilized as a DNA template for secondary amplification. The secondary amplification product was subsequently purified, and quantification was performed using a Qubit. Equal amounts of samples were combined according to the concentration of PCR products and then clustered the sequences into operational taxonomic units (OTU) using V search (version 2.4.2) software based on 97% similarity.

2.7 SCFAs analysis

SCFAs in liver samples were analyzed using a gas chromatography-mass spectrometry (GC-MS). Thawed the liver samples obtained from mice on ice, transferred into centrifuge tube for 2 mL, and suspended in 900 μL of 5% phosphoric acid. Centrifuged subsequently the samples at 14 000 × *g* for 10 min. In addition, the standard products of acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid and caproic acid were weighed and prepared with ethyl acetate into 8 mixed standard concentrations gradient (0.1, 0.5, 1.0, 5.0, 10.0, 20.0, 50.0, 100.0 μg/mL). A 600 μL aliquot of the organic phase and standards was taken and added 500 μmol/L 4-methyl-valeric acid as the internal standard. Separated the samples on capillary column (Agilent DB-WAX, 30 m × 0.25 mm × 0.25 μm), and an Agilent 7890A/5975C GC-MS system was employed for analysis. Using MSD Chemstation software, the retention times and peak areas were extracted for generating a standard curve and calculating the SCFAs content in the samples.

2.8 BAs analysis

Analyzed the BAs of mice's liver samples through a liquid chromatography-triple quadrupole-mass spectrometry system (LC-QQQ-MS) operating with multiple reaction monitoring (MRM). 30 mg of liver tissue were weighed and add 100 μL pre-cooled ultra-pure water to homogenate. Then, 500 μL pre-cooled methanol and 10 μL internal standard were added to the sample. After vortexing, the sample was incubated at –20 ℃ for 20 min. Centrifuged the sample at 14 000 × *g*, 4 ℃ for 15 min, and then collected the supernatant liquor and vacuum dried. For mass spectrometric detection, the sample was redissolved in water:methanol (1:1, *V/V*) and centrifuged. The ion pairs were detected using MRM mode. Peak chromatographic areas and retention times were analyzed with Multiquanta software (AB SCIEX, Framingham, USA) to identify the primary and secondary BAs.

2.9 Statistical analysis

For animal experiments, we used 10 mice per group, and experiments were repeated at least 3 times. The results were presented as means ± standard deviations (SD). Data analysis was carried out using Microsoft Excel, Origin 2022, GraphPad Prism 9.5, and IBM

SPSS Statistics V.26.0. Significance of the data was assessed with Duncan's test at a significance level of 0.05. Nonparametric test was analyzed by Kruskal-Wallis test and Wilcox test. Principal component analysis (PCA), heatmap generation, and correlation analysis were utilized the R-language package (R 4.1.3).

3. Results and discussion

3.1 RA prevented DSS-induced colitis symptoms and clinical disease activity

UC is an idiopathic inflammation distinguished by clinical presentations like diarrhea, weight loss, and blood in the stool[29]. Studies have demonstrated that mice with UC may exhibit symptoms mimicking UC, including diarrhea, weight loss, shortened colon length, increased colon weight, and other clinical indicators[30-32]. Compared to the BLANK group, the DSS treatment group exhibited a significant increase in body weight loss and DAI score, along with a shortened colon length, a reduced colon length-to-weight ratio, and an elevated spleen-to-body weight ratio (Fig. 1). These collective alterations in physiological and immunological parameters confirm the successful establishment of a mice model of DSS-induced colitis. After day 3, all mice with 2.5% DSS supplementation experienced significant body weight loss compared to the BLANK group ($P < 0.05$, Fig. 1B). Whereas RA significantly prevented reductions in weight in UC mice, with RAH treatment showing the most pronounced effect, in contrast to the DSS group. There are no notable differences in body weight loss between the groups administered by the RAL and DSS groups.

The DAI is commonly employed in clinical practice to assess the severity of UC symptoms[33-35], including body weight loss, fecal consistency, and occult blood[36-37]. As illustrated in Fig. 1C,

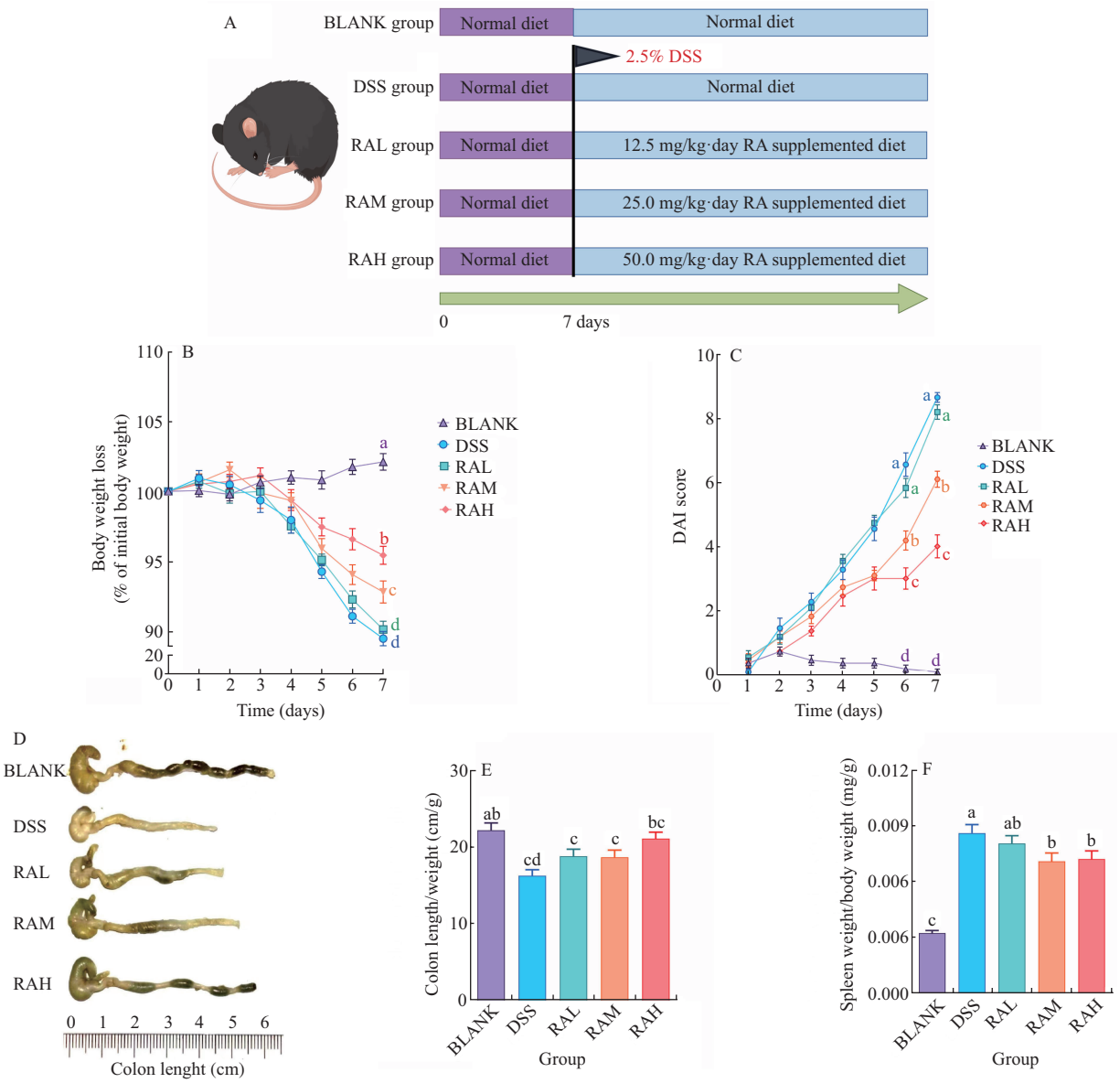

Fig. 1 RA prevented DSS-induced colitis symptoms and clinical disease activity. (A) Experimental design (by Figdraw (https://www.figdraw.com)). (B) Body weight loss. (C) DAI score. (D) Representative images of colons from mice. (E) Colon length/colon weight. (F) Spleen index. Data are presented as means ± SD. $n = 10$. Different lowercase letters represent significant differences among the groups ($P < 0.05$).

compared with the BLANK group, the scores of DAI in the DSS, RAL, RAM, and RAH groups increased obviously ($P < 0.05$) from the 3rd day onward. Significantly, starting from day 5, the DAI scores of the RA treatment groups were lower than those in the DSS group. The RA's administration has exhibited dose-dependently reduced DAI values, suggesting the prevention of UC clinical characteristics.

Colon shortening is regarded as a typical sign of inflammation in the course of colitis[38-39] and is, therefore, frequently used as a morphologic parameter to indicate the degree of inflammation. As illustrated in Figs. 1D and E, the colon length significantly decreased in the DSS group compared to the BLANK group. At the same time, there was a substantial increase in the RA-treated group compared to the DSS group. Notably, the RAH group showed a colon length close to the BLANK group with normal value. In addition, the spleen plays a crucial role in regulating inflammatory and immune responses. Once inflammation develops, severe inflammatory responses originating from the inflamed colon can impact the periphery, resulting in spleen enlargement[40-41]. Hence, the spleen index (spleen weight/body weight) will increase with the severity of colitis. A more severe colitis corresponds to a higher spleen coefficient[42]. As shown in Fig. 1F, the spleen coefficient was markedly increased in the DSS group compared to the BLANK group, whereas decreased in the RA-treated groups.

Overall, the colonic tissue damage in DSS-induced colitis mice was attenuated meaningfully by RA treatment. The studied data suggested that RA may have a protective effect against UC in mice.

3.2 *RA prevented colonic tissue injuries*

Colitis is a disease characterized by a pathological lesion in the colon's tissue. As H&E staining shown in Fig. 2A, in the BLANK group, the colonic tissues of the proximal, middle, and distal regions showed normal morphology, with crypts displaying well-defined villi and abundant goblet cells, with an absence of inflammatory cell infiltration. Compared to the BLANK group, the colonic tissues of mice in the DSS group exhibited severe ulceration, infiltration of inflammatory cells, depletion of goblet cells, crypt distortion, and pronounced submucosa edema, consistent with the disease profile of UC. Interestingly, RA significantly prevented colitis-associated morphological alterations by restoring populations of crypts and goblet cells and reducing infiltration of inflammatory. Although the morphology of colonic tissues in the RAL group improved, the RAM and RAH groups showed a more significant protective effect. Previous studies have indicated that goblet cells can promote mucus secretion to form a protective mucus layer and protect the intestinal barrier[43-44]. The alcian blue staining results indicated that DSS-induced colitis in mice reduced the number of colonic goblet cells and colonic mucus. In contrast, RA treatment markedly increased them in a dose-dependent manner (Fig. 2B). Histological score assessment confirmed the protection of RA on colon damage that DSS-induced (Figs. 2C-E, $P < 0.05$). Overall, these results demonstrated that RA effectively protected against colonic injuries induced by DSS.

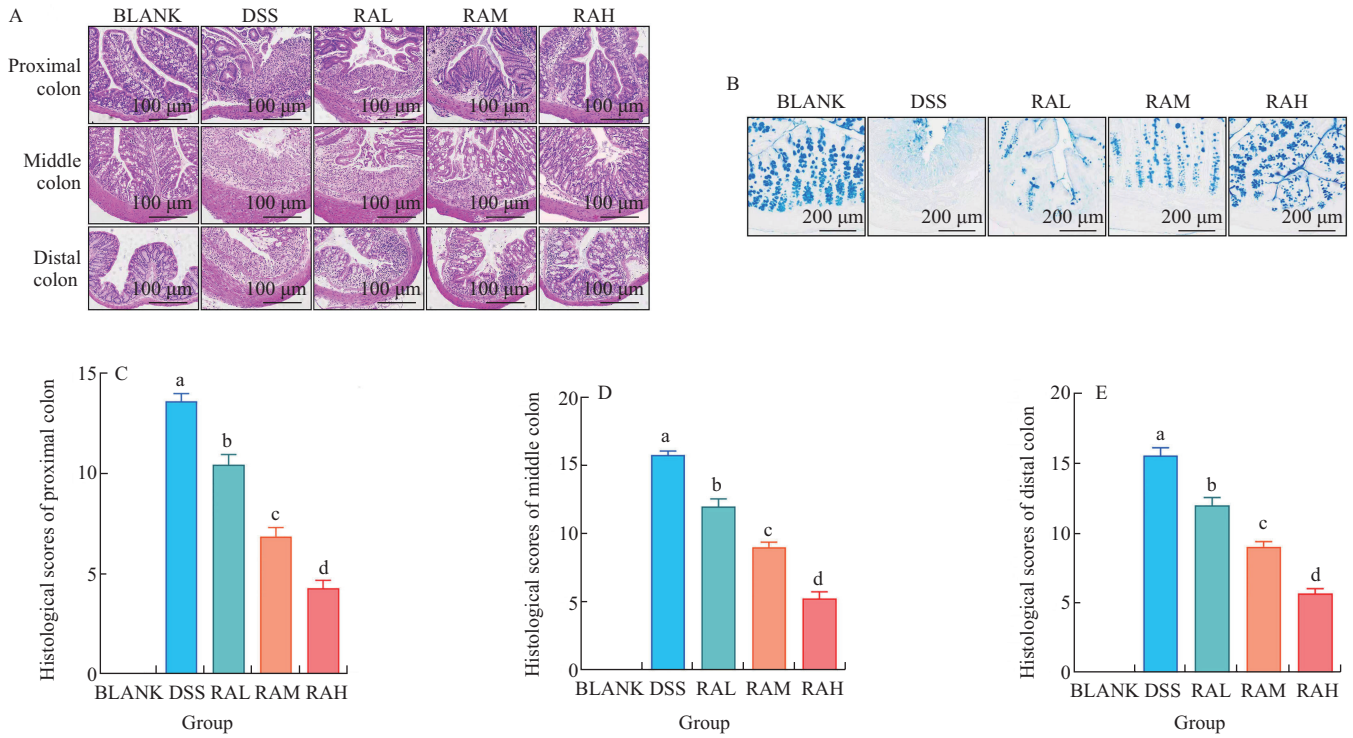

Fig. 2 RA prevented colonic tissue injuries in DSS-induced colitis mice and enhanced intestinal barrier structure. (A) Representative photographs of H&E staining of the proximal, middle and distal colon tissues. (B) Representative photographs of Alcian blue staining of the proximal, middle and distal colon tissues. (C-E) Histological scores. Data are presented as means ± SD. $n = 10$. Different lowercase letters represent significant differences among the groups ($P < 0.05$).

3.3 RA inhibited the overproduction of inflammatory factors and enhanced intestinal barrier structure

During the inflammatory response process, inflammatory factors serve as crucial immune mediators, and their alterations significantly influence the onset and intensity of colitis induced by DSS[45-46]. As typical inflammatory markers, IL-1β, IL-6, and TNF-α reflect colitis's severity[47]. Therefore, the expression levels of these typical inflammatory factors were measured to determine the influence of 3 RA doses (low, medium, and high) in the inflammatory mechanism of colitis. The results revealed that the DSS group exhibited elevated levels of IL-1β, IL-6, and TNF-α, which were 6.5-, 8.7-, and 4.2-fold of the BLANK group, respectively. However, IL-1β, IL-6, and TNF-α levels decreased significantly in contrast to the DSS group in the treatments with different dosages of RA ($P < 0.05$, Fig. 3). Interestingly, the high-dose RA group (50 mg/kg) exhibited a dramatic reduction in IL-1β compared to the RAM and RAL groups, with a 4.7-fold decrease ($P < 0.05$, Fig. 3A). In contrast, no significant differences were observed among the 3 doses of RA in the inhibition of IL-6 and TNF-α. This analysis showed that a high dose of RA (50 mg/kg) had a greater inhibitory effect on inflammatory factors.

Furthermore, the intestinal barrier is a protective mechanism against damage from harmful intestinal bacteria, and its dysfunction is an important pathological indicator of UC. Hence, the reduction in tight junction protein expression is a common feature in the pathophysiology of UC[48]. ZO-1 and members of the occludin family are the classical intercellular tight junction proteins that serve a vital function in regulating mucosal permeability and enhancing intestinal barrier integrity. Therefore, Western blot analysis was used to visualize the tight junction protein expression. The expressions of ZO-1, occludin, and claudin-1 were significantly reduced ($P < 0.05$) compared to the BLANK group in DSS-induced colitis mice, indicating a weakening of the intestinal barrier. In contrast, the expression of ZO-1 increased markedly in the RAH group, while the RAM group showed significant improvement in regulating the quantities of occludin and claudin-1 (Figs. 3D-G, $P < 0.05$). Therefore, it suggested that RA supplementation effectively prevented the disease severity and enhanced intestinal barrier function in mice with DSS-induced colitis.

Interaction between cytokines and tight junction proteins might exacerbate UC. Pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α, are highly expressed in UC, which might alter the structure and injure the mucosal intestinal barrier. Our results revealed elevated IL-1β, IL-6, and TNF-α concentrations in the DSS group. Meanwhile, ZO-1, occludin, and claudin-1 levels were reduced (Fig. 3). The IL-1β, IL-6, and TNF-α expression showed inverse correlations with ZO-1, occludin, and claudin-1. Of considerable interest, the intervention of RA elevated the levels of claudin-1, occludin, and ZO-1 expression and mitigated the overexpression of pro-inflammatory cytokines.

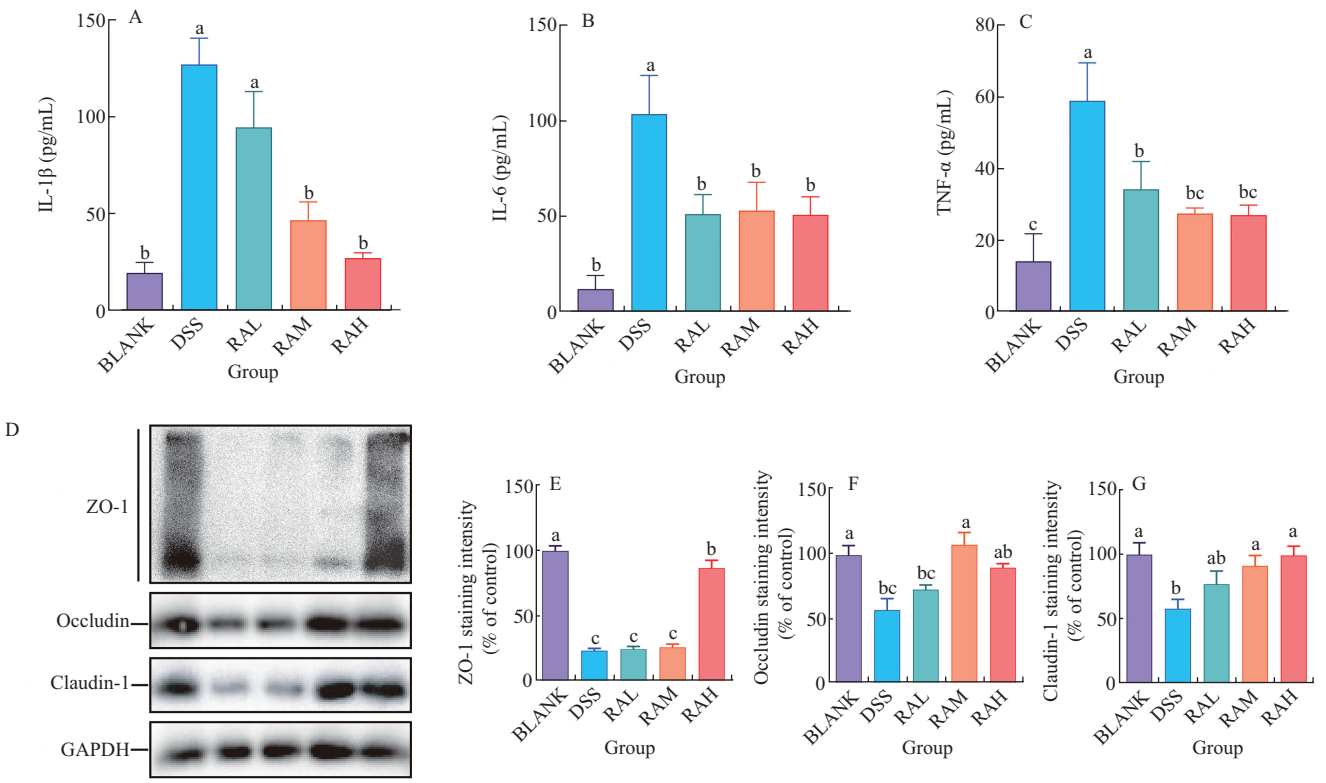

Fig. 3 RA inhibited the overproduction of inflammatory factors caused by DSS. (A) Expression levels of IL-1β. (B) Expression levels of IL-6. (C) Expression levels of TNF-α. (D-G) Expression levels of ZO-1, occludin, and claudin-1. Data are expressed as means ± SD. $n = 10$. Different lowercase letters represent significant differences among the groups ($P < 0.05$).

3.4 RA improved the gut microbiota diversity

Research has shown that alterations in microbial community structure and diversity may be associated with the extent of colonic inflammation, and intestinal dysbiosis is a particularly prominent symptom in colitis[49]. Therefore, the infection of RA on the gut microbial diversity in all experimental mice was determined using 16S rRNA gene-sequencing analysis. As shown in Figs. 4A and B, sequences with high similarity (97%) were gathered into one OTU, and the common and unique OTU numbers between different experimental groups were analyzed, represented by an upset plot and Venn diagrams. As illustrated in Figs. 4A-B, there were 461 OTU species shared between groups, and the numbers of OTU unique to the BLANK, DSS, RAL, RAM, and RAH groups were 870, 785, 727, 823, and 830, respectively. The number of OTUs in the DSS group was significantly lower than that of the BLANK group and the number of OTUs in the RA-treated groups recovered somewhat.

Furthermore, the composition of gut microbiota in each group was evaluated based on the resulting OTU species. α-Diversity, which reflects species abundance and diversity, is greater in healthy groups than in those with intestinal conditions. Therefore, within-sample diversity (α-diversity) was calculated using the Shannon index, Chao1 index, and other metrics to estimate gut microbiota richness and evenness. A decrease in gut microbiota and diversity was noted in DSS-induced colitis compared with the BLANK group, indicating a successful establishment of the UC model. Notably, RA administration prevented the decrease in microbiota abundance and diversity induced by DSS (Figs. 4C-F). Microbiota analysis revealed that RA-treated groups displayed increased gut microbiota richness, as evidenced by a higher Chao1 index and Shannon index in contrast to the DSS group (Figs. 4C-D). The PD_whole_tree index data showed a significant decrease after DSS ingestion, with a recovery trend observed following RA treatment. Notably, 50 mg/kg RA (RAH group) significantly reduced the DSS-induced decrease of gut microbiota diversity ($P < 0.05$, Fig. 4F).

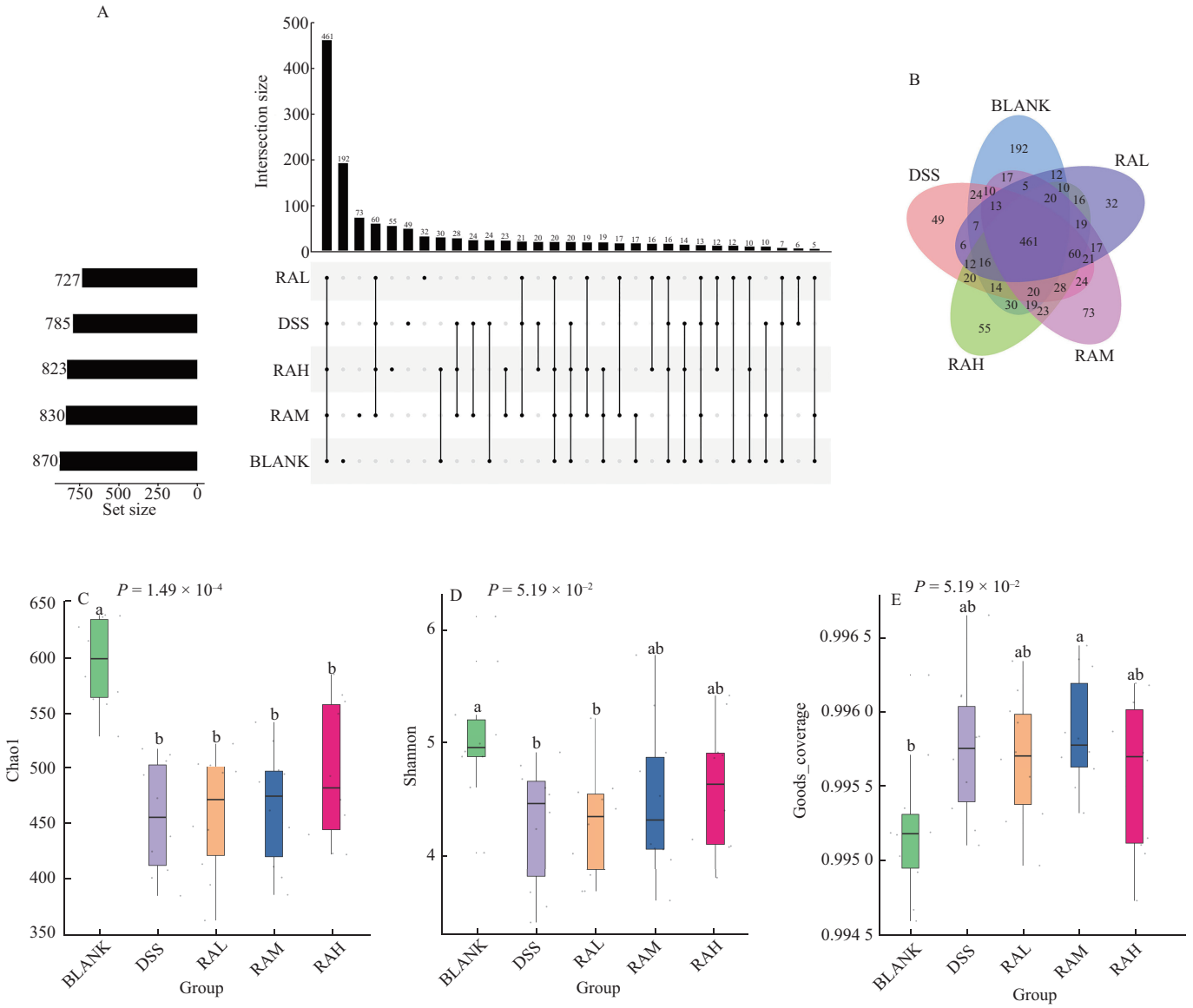

Fig. 4 RA improved the gut microbiota diversity in colitis mice. (A) Upset diagram. (B) Venn diagram. (C) Chao1 index analysis. (D) Shannon index analysis. (E) Goods coverage index analysis. (F) PD_whole_tree index analysis. (G) PCA at the OTU level in 2D plot. (H) PCA at the OTU level in 3D plot. Data are expressed as means ± SEM. $n = 10$. Different lowercase letters represent significant differences among the groups ($P < 0.05$).

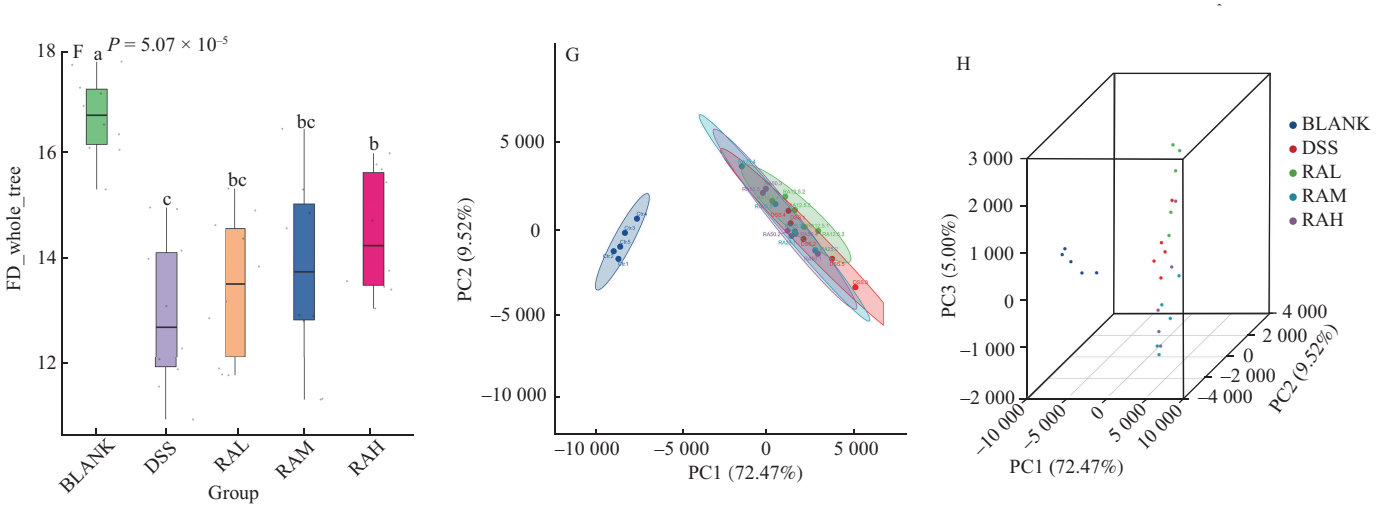

Fig. 4 (*Continued*)

Bacterial β-diversity, assessed through PCA, illustrates the comparative similarity of gut microbiota structure across various groups. The results suggested a significant distinction between the BLANK group and the remaining groups (Figs. 4G-H, $P < 0.05$), indicating that DSS treatment has a significant effect on intestinal microbiota diversity in mice. Notably, the different doses of RA treatment groups and the DSS group are not clearly separated on the PCA plot, indicating that the effects of RA do not fully reverse these alterations. This finding is consistent with the previous results concerning α-diversity (Figs. 4C-F).

Overall, the data indicated that RA prevented and restored the decreased gut microbiota diversity induced by the DSS, which could partially explain its inhibitory effect on colitis.

3.5 *RA altered the composition of gut microbiota*

According to relevant studies, UC is thought to be implicated in an imbalance of gut microbiota, marked by a rise in pathogenic bacteria and a decline in beneficial species[49-50]. To gain insight into the precise impact of RA on the gut microbiota, the colonic bacteria regulated by RA were examined by assessing the relative quantity of the key taxa across the group. Figs. 5A and B visualize the predominant microbiota among the groups through linear discriminant analysis effect size (LEfSe). It was found that the abundance of pathogenic and potentially pathogenic bacteria, such as Deferribacteres, Epsilonbacteraeota and *Turicibacter*, were upregulated in the DSS group. Notably, *Mucispirillum*, a genus from the Deferribacteres phylum,

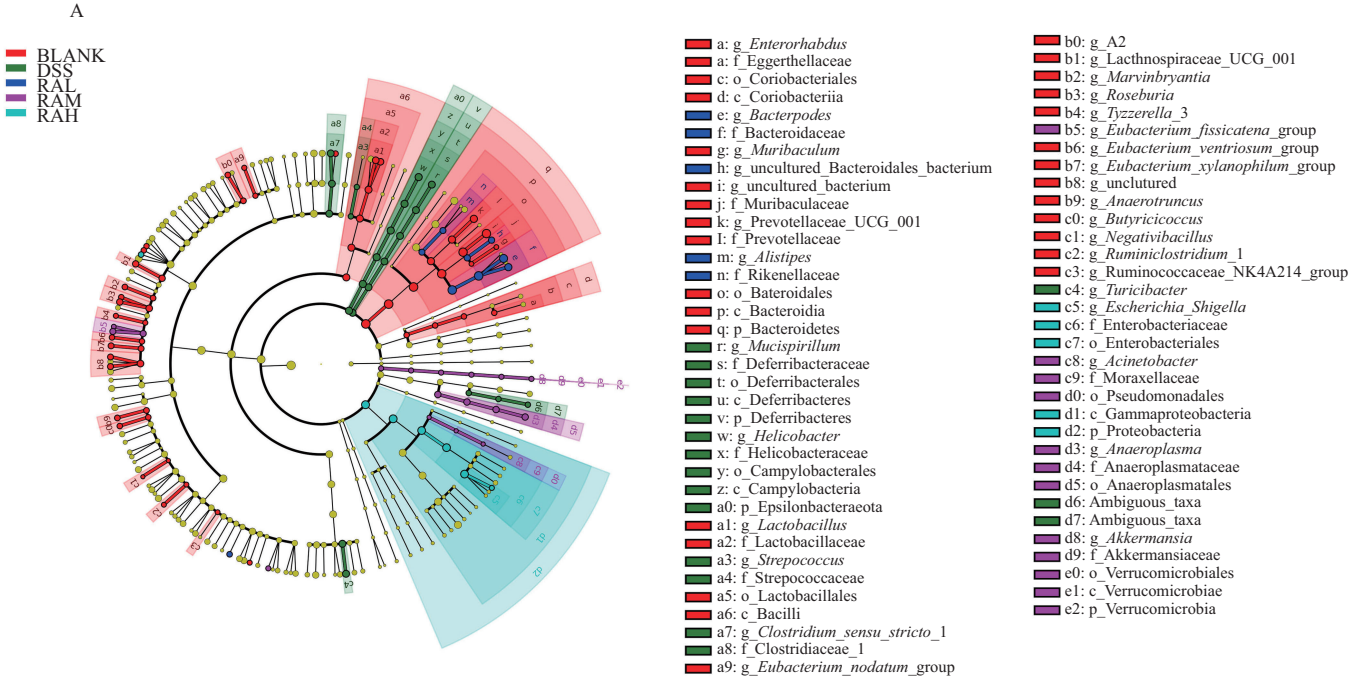

Fig. 5 RA altered the composition of the gut microbiota in the colitis mice. (A-B) LEfSe analysis of microbiota. Taxonomic cladogram obtained from analysis of 16S sequence and LDA scores of the differentially abundant taxa. (C) Relative abundance of the gut microbiota at the phylum level. (D) Relative abundance of the gut microbiota at the family level. (E) Relative abundance of the gut microbiota at the genus level.

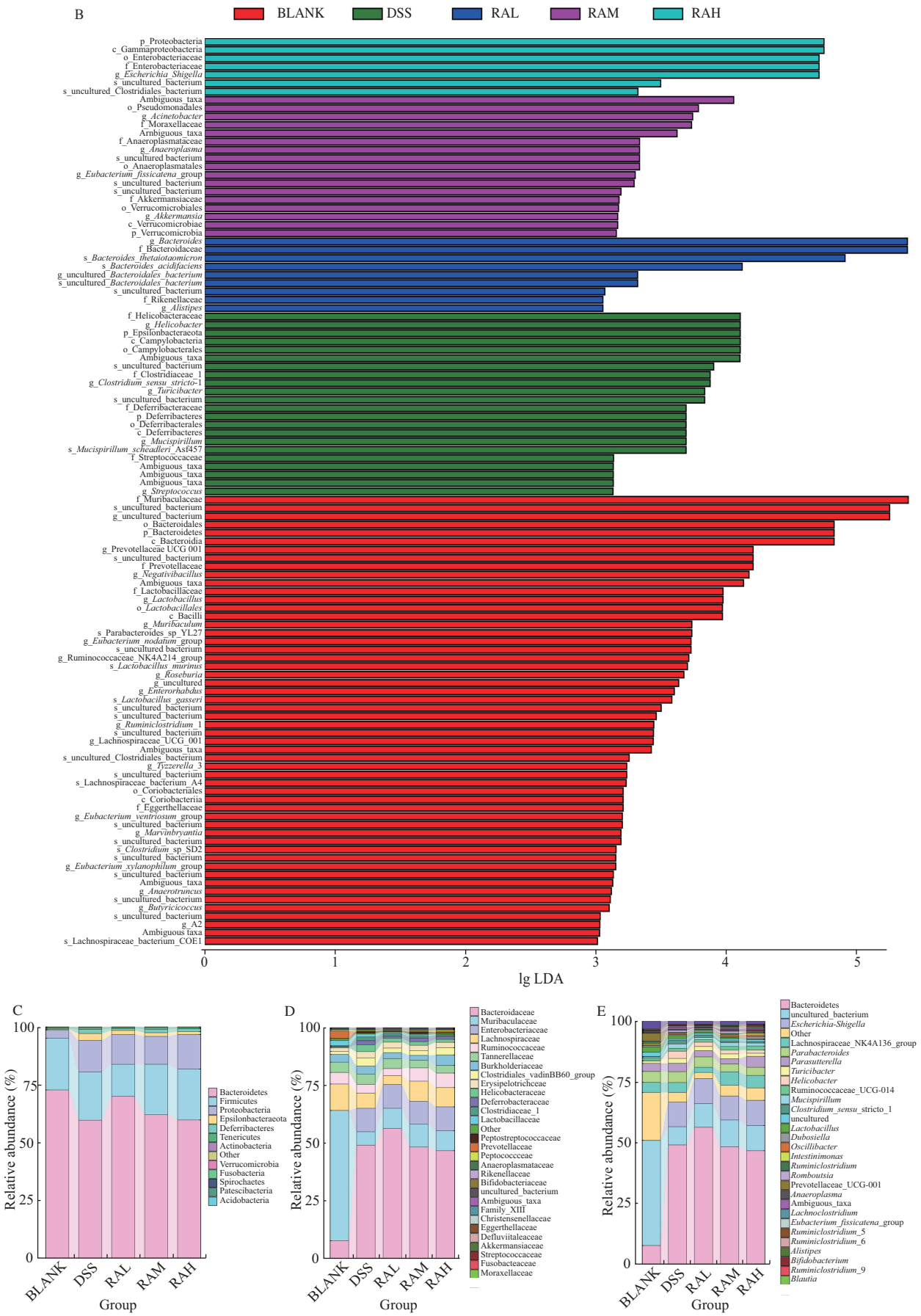

Fig. 5 (*Continued*)

and *Helicobacter*, from the Epsilonbacteraeota phylum, were the dominant strains in the DSS group. *Mucispirillum* is a pathogenic symbiotic bacterium that can induce colitis, while *Helicobacter* is a commensal intestinal pathogenic that might contribute to chronic inflammation and ulcers. Additionally, Akkermansiaceae, Bacteroidetes, Firmicutes, and Prevotellaceae were the predominant microbiota in the RA-treated groups.

Next, the relative abundance of microbial composition was compared among different groups at the phylum, family, and genus levels to explore how RA influences the microbial structure of mice with colitis. As shown in Fig. 5C, the phyla in the gut microbiota included Bacteroidetes, Proteobacteria, Firmicutes, Epsilonbacteraeota, Deferribacteres, Tenericutes, and Actinobacteria. Notably, the proportional quantity of Proteobacteria and Epsilonbacteraeota increased in the DSS group, while Bacteroidetes decreased. Conversely, RA significantly inhibited the abnormal proliferation of Epsilonbacteraeota and Proteobacteria in UC mice and the extent of Bacteroidota improved. In addition, the copiousness of Firmicutes declined in the DSS and RAL groups, while it enhanced in the RAM and RAH groups in relation to the BLANK group. Overall, it could be speculated that a low dose of RA (12.5 mg/kg) might have an inhibitory effect against Firmicutes, while medium-dose and high doses of RA could improve the abundance of Firmicutes. Therefore, an appropriate concentration of RA may increase the abundance of beneficial bacteria, which could partially explain its inhibitory effect against colitis.

Fig. 5D shows that the gut microbiota in the experimental mice was predominantly comprised of Bacteroidaceae, Muribaculaceae, Enterobacteriaceae, Lachnospiraceae, Ruminococcaceae, and Erysipelotrichaceae at the family level. Compared with the other groups, the abundance of Enterobacteriaceae and Erysipelotrichaceae was significantly higher in the DSS group, whereas Muribaculaceae, Lachnospiraceae, and Ruminococcaceae were markedly suppressed. Muribaculaceae is a part of Bacteroidetes, and Lachnospiraceae and Ruminococcaceae belong to Firmicutes, which might explain the declination of Bacteroidetes and Firmicutes in the DSS group.

Intriguingly, RA suppressed the anomalous proliferation of these bacteria. The abundance of Muribaculaceae, Lachnospiraceae, and Ruminococcaceae was significantly upregulated after RA supplementation, while there was no significant regulation of Bacteroidaceae and Enterobacteriaceae. Some studies have revealed that Muribaculaceae can reduce inflammation and alleviate gut mucosal inflammation[51]. Meanwhile, Lachnospiraceae and Ruminococcaceae are probiotics that increase the production of SCFAs and exert anti-inflammatory properties by decreasing the expression of inflammatory markers. Therefore, it is speculated that the modulation of specific microbial compositions by RA has contributed to its inhibitory effect on colonic inflammation.

Further analysis was conducted at the genus level (Fig. 5E), indicating a significant increase in the abundance of *Escherichia-Shigella* in the DSS group compared to the BLANK group, with no significant effect of RA on its abundance. However, RA treatment significantly enhanced the abundance of Lachnospiraceae_NK4A136-group, which belongs to Lachnospiraceae, potentially contributing to this increase. Lachnospiraceae_NK4A136-group is widely regarded as a promising probiotic, capable of producing the SCFAs butyrate, and intestinal inflammation was negatively correlated with its abundance. Interestingly, the abundance of *Parabacteroides* in BLANK and DSS groups showed no significant difference. A low dose of RA (12.5 mg/kg) reduced its abundance, whereas the abundance increased in the RAM and RAH groups. *Parabacteroides*, a core component of the human gut microbiome, is advantageous for its recognized effects on IBD and colorectal cancer. Furthermore, several studies have suggested that *Parabacteroides* could restore intestinal barrier integrity, participate in BAs conversion, and increase the content of secondary BAs such as lithocholic acid (LCA) and ursodeoxycholic acid (UDCA). Through this analysis, it was found that RA altered the structure of the gut microbiota by decreasing harmful bacteria and increasing beneficial bacteria, thereby alleviating DSS-induced colonic inflammation.

3.6 RA recovered the production of SCFAs

SCFAs are metabolites byproducts of gut microbial fermentation that can modulate the intestinal microbiota and have both bowel function-improving and anti-inflammatory properties. They can also function as a direct energy source for the proliferation of intestinal epithelial cells and goblet cells, thereby playing a significant role in the regeneration of impaired colonic tissues and goblet cells in mice with UC[52-53]. Therefore, SCFAs production is strongly correlated with colitis. Our findings showed that the concentrations of acetic acid, butyric acid, propionic acid, hexanoic acid, valeric acid, isovaleric acid, isobutyric acid, and total SCFAs were significantly reduced in the DSS group compared with the BLANK group (Fig. 6), which is aligned with the SCFAs change patterns observed in UC patients as reported in prior studies[54]. RA treatment efficiently restored SCFAs production in colitic mice, especially propionic acid and butyric acid. Notably, the middle-dose RA significantly increased propionic acid and butyric acid to levels similar to those in the healthy BLANK group (Figs. 6B-C). According to previous studies, SCFAs, particularly butyrate, are essential for providing energy to intestinal epithelial cells and promoting their growth[54]. Additionally, RA was shown to boost the prevalence of bacteria that produce SCFAs, such as Lachnospiraceae and Ruminococcaceae[55-56], and our experimental results also substantiate that RA effectively restored the abundance of microbiota producing SCFAs, indicating that the regulatory function of RA on the gut microbiome in colitis mice may influence the concentrations of SCFAs.

3.7 RA regulated BA metabolism

BAs are the end products of hepatic cholesterol catabolism, produced synergistically by cholesterol and gut microbes. Previous studies have indicated that the gut microbiota can affect the metabolism of BAs and alter their composition[57]. BAs are considered important signaling factors and regulators, significantly impacting on regulating inflammatory response regulation and directly associated with intestinal inflammation[58]. Furthermore, research has suggested that the disturbance in BA homeostasis could be a distinctive characteristic of UC. Hence, the investigation of BAs alterations in DSS-induced UC mice and the regulatory impact of RA on BAs expression hold crucial implications for the therapeutic

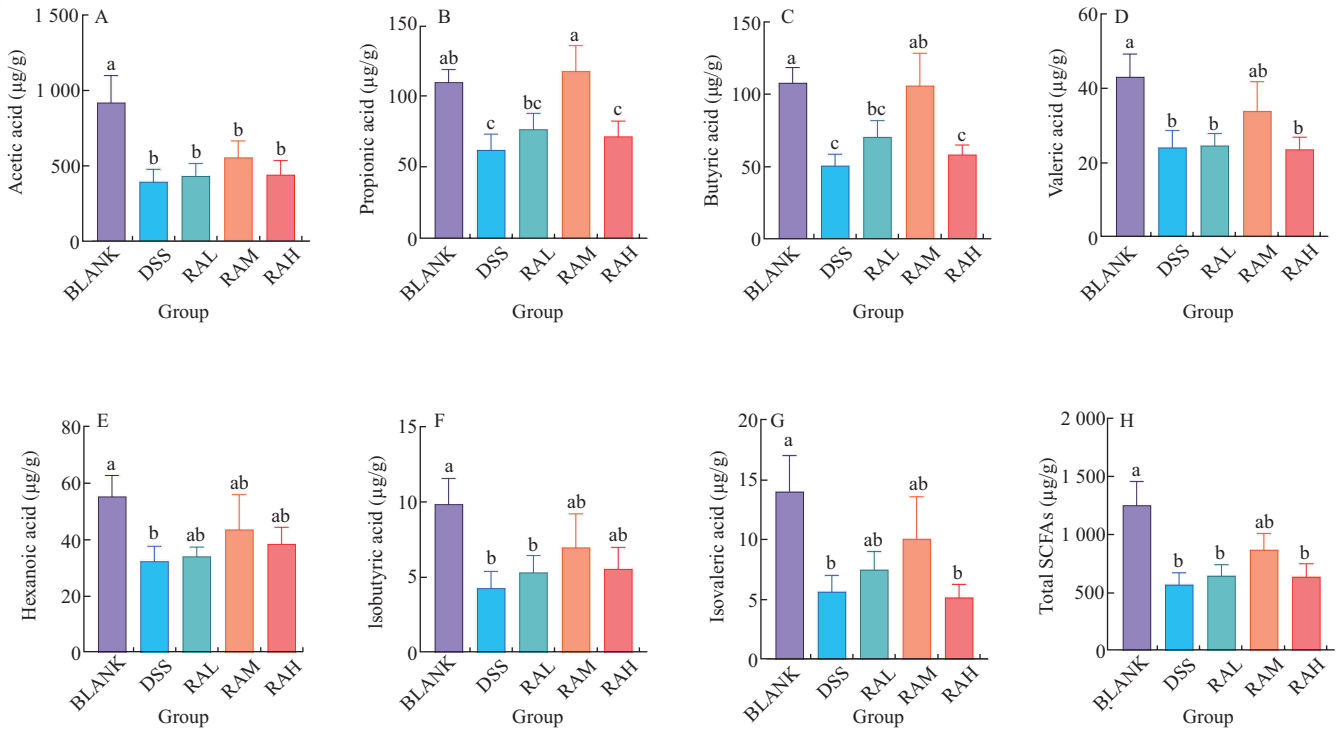

Fig. 6 RA recovered the production of SCFAs in colitis mice. Levels of (A) acetic acid, (B) propionic acid, (C) butyric acid, (D) valeric acid, (E) hexanoic acid, (F) isobutyric acid, (G) isovaleric acid, and (H) total SCFAs in the mice liver. Data are expressed as means ± SD. $n = 10$. Different lowercase letters represent significant differences among the groups ($P < 0.05$).

strategies against UC. DSS treatment markedly decreased total hepatic BAs synthesis compared with the BLANK group (Fig. 7A, $P < 0.05$). For most BAs, their levels in various RA treatment groups were more closely resembled those in the DSS group (Fig. 7B). In comparison to the BLANK group, the expression of cholic acid (CA), taurocholic acid (TCA) and tauromuricholic acid (TMCA) in the RAH group were higher. In contrast, the levels of hyodeoxycholic acid (HDCA), deoxycholic acid (DCA), LCA, and α-Muricholic acid (α-MCA) experienced a significant decrease in the DSS group ($P < 0.05$). Among them, LCA and DCA typically represent the predominant secondary BAs in the intestines, and treatment with RA partly reinstated their expression. As shown in Fig. 7C, 28 BAs were identified from the liver of experimental mice, and more primary BAs and fewer secondary BAs were found in the DSS group, which is similar to previous research reports[59]. Furthermore, Studies have shown that dysregulation of the gut microbiota in a murine colitis model can result in a scarcity of secondary BAs, consequently triggering inflammatory responses in the intestines[60]. However, the abnormal trends of these BAs were reversed by medium-dose RA (25 mg/kg). Based on these results, it is presumed that RA might prevent colitis by regulating BAs dysmetabolism.

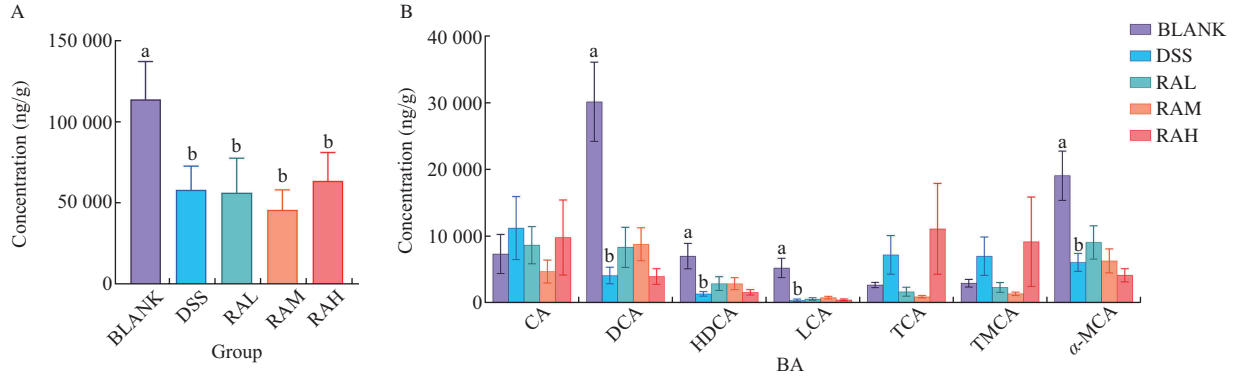

Fig. 7 RA regulated BAs metabolism of mice. (A) Concentration of total BAs. (B) Concentrations of CA, DCA, HDCA, LCA, TCA, TMCA and α-MCA in liver. (C) Relative abundance of individual BAs in liver. Data are expressed as means ± SD. $n = 10$. Different lowercase letters represent significant differences among the groups ($P < 0.05$). THDCA: taurohyodeoxycholic acid; TUDCA: taurourso-deoxycholic acid; TCDCA: taurochenodeoxycholic acid; TDCA: taurodeoxycholic acid; GDCA: glycochenodeoxycholic acid; GCA: glycocholic acid.

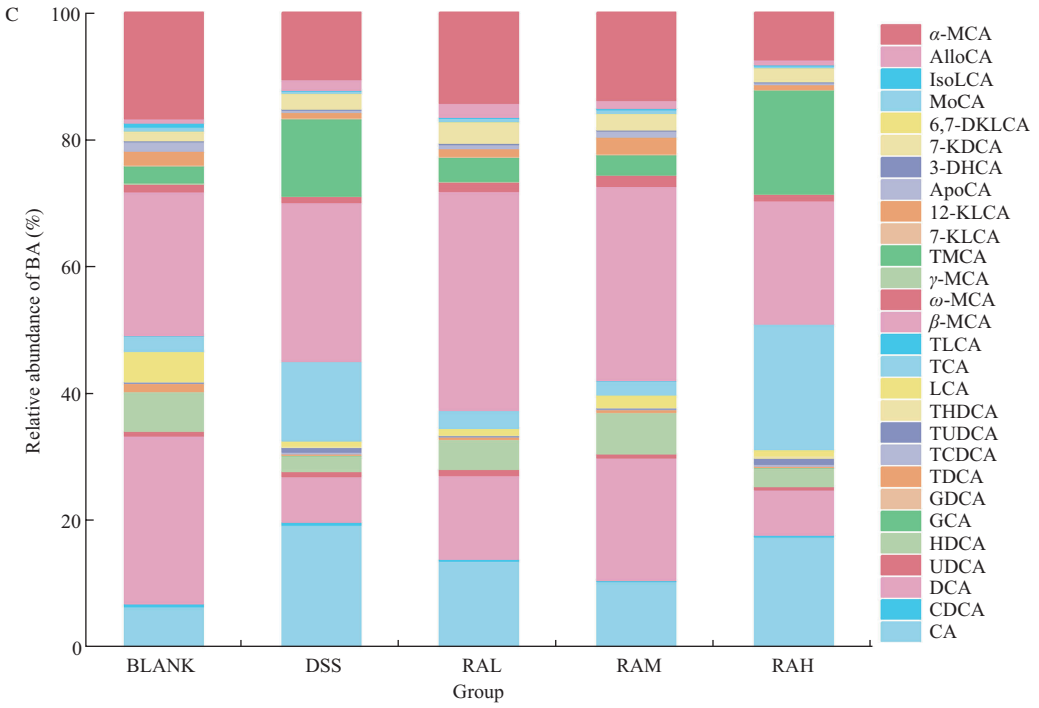

Fig. 7 (*Continued*)

3.8 Correlation between the gut microbiota and colitis

It is widely known that UC is strongly associated with dysbiosis of the gut microbiota. Thus, the adjustment of gut microbiota disorders has surfaced as a promising approach for managing intestinal inflammation[61]. Dietary interventions for treating UC have gained increasing recognition in recent years. Polyphenols are renowned for their potent anti-inflammatory and antioxidant properties[16,62]. While the impacts of polyphenols on UC and gut microbiota have been previously examined, it is essential to consider that polyphenols sourced from different origins may exhibit diverse effects on inflammatory bowel disease (IBD) and gut microbiota due to differences in their constituents. Previous reports have indicated that the gut microbiota, along with their metabolites, such as SCFAs, and BAs, plays a cardinal role in maintaining intestinal balance and have been identified as an essential molecule in signaling[48,63]. For the identification of gut microbiota linked to colitis parameters, such as inflammatory cytokines, tight junction, SCFAs, and BAs, relevant correlation analyses were conducted (Fig. 8). It was found that specific microbiota were negatively correlated with tight junction proteins, SCFAs, and BAs, positively correlated with inflammatory cytokines. For example, at the phylum level, Proteobacteria, Deferribacteres, Epsilonbacteraeota, and Tenericutes were negatively correlated with ZO-1, occludin, claudin-1, SCFAs, and BAs, and positively correlated with IL-1β, IL-6, and TNF-α ($P < 0.05$). Additionally, at the family and genus levels, Enterobacteriaceae and Ruminococcaceae_UCG-014 were negatively correlated with tight junction proteins, SCFAs, and BAs, and positively correlated with IL-1β ($P < 0.05$).

Many studies have indicated a common association between probiotics and the expression levels of tight junction proteins, inflammatory cytokines, SCFAs, and BAs[64-65]. Our results showed that RA treatment significantly increased the richness of beneficial bacteria while markedly decreasing the abundance of pathogenic bacteria. For example, Muribaculaceae, Ruminococcaceae, and Lachnospiraceae were diminished in the DSS group but were remarkably upregulated in the RA-treated groups. Of those, at the family level, Muribaculaceae and Prevotellaceae showed a positive correlation with acetic acid, butyric acid, valeric acid, DCA, LCA, *α*-MCA, and ZO-1, and negatively correlated with IL-1β and IL-6 (Fig. 8A). At the genus level, acetic acid, total SCFA DCA, LCA, and α-MCA showed a significant positive correlation with Prevotellaceae_UCG-001, while all SCFAs, DCA, HDCA, LCA, and *α*-MCA of BAs were positively correlated with *Turicibacter* (Fig. 8B). In addition, we found that the abundance of the Lachnospiraceae_NK4A136-group was significantly elevated by RA treatment. Lachnospiraceae_NK4A136-group can generate butyrate acid, which not only acts as an energy substrate for cellular metabolism in the colon epithelium but also suppresses the inflammation response by binding to specific proteins. What's more, it is well-known that Prevotellaceae_UCG-001 and *Turicibacter* are probiotics that increase the production of metabolites such as SCFAs, which are essential for gastrointestinal health. Hence, RA not only suppressed the response of inflammation by reestablishing the integrity of the intestinal barrier and decreasing the overexpression of inflammatory cytokines through enhancing the levels of ZO-1, occluding, and claudin-1 but also acted on specific microorganisms that can affect SCFAs and BAs metabolism, either directly, indirectly, or both.

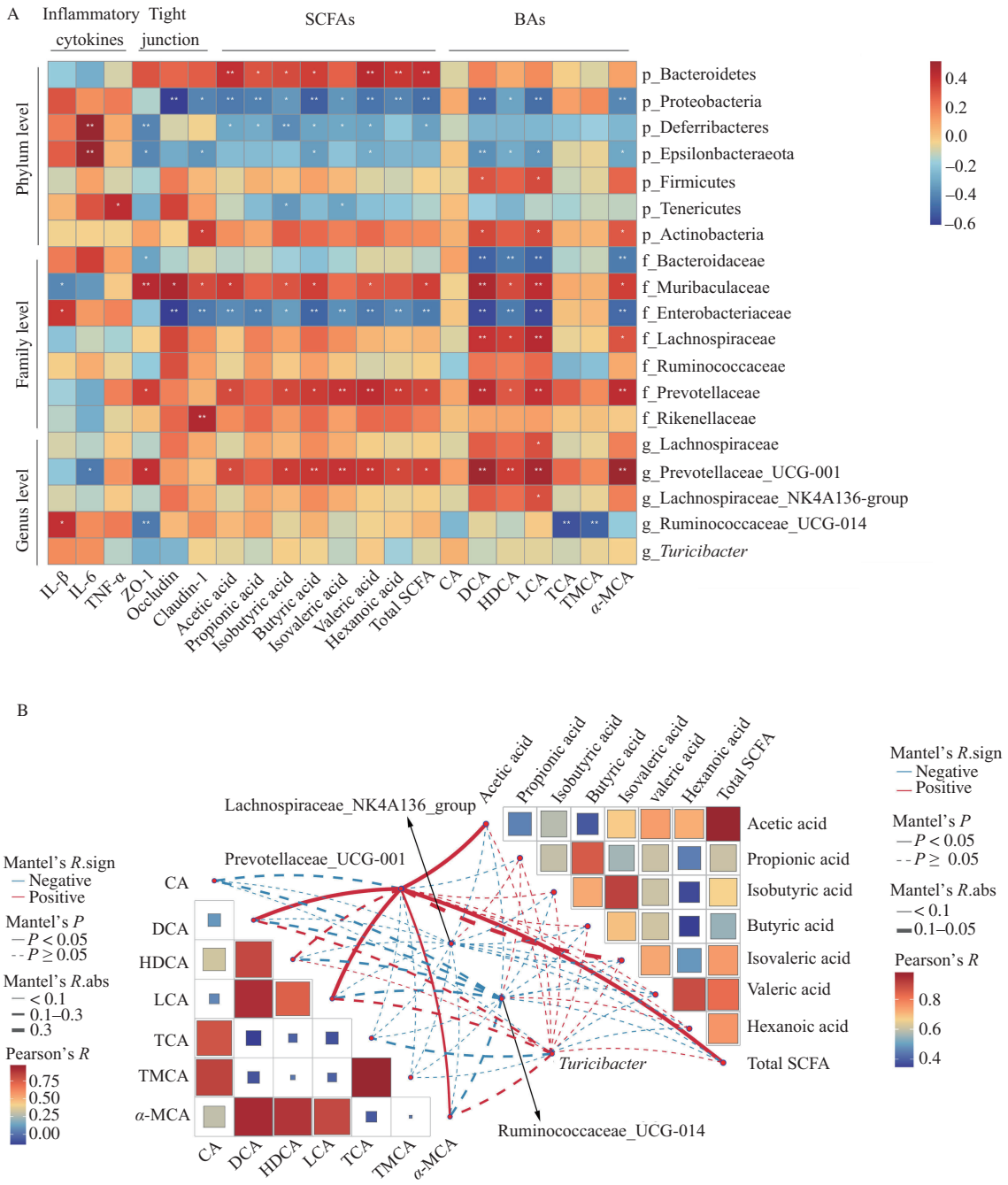

Fig. 8 Correlation between gut microbiota and the associated indicators of colitis. (A) Spearman correlation heatmap. *$P < 0.01$, **$P < 0.001$. (B) Correlation analysis between BAs, SCFAs, and specific microorganism with significant differences.

4. Conclusion

The present study exhibited that RA could prevent symptoms associated with DSS-induced UC mice through various direct and indirect mechanisms. It showed an excellent restorative effect on typical symptoms in DSS-induced colitis mice, such as body weight loss, colon length shortening, and splenomegaly. Furthermore, it lowers the overexpression of pro-inflammatory cytokines to suppress the inflammation response and enhances tight junction protein expression. Notably, this phenolic acid remodeled the gut microbiota structure and improved the synthesis of BAs and SCFAs. Further analysis reveals that treatment with RA significantly increases the abundance of beneficial gut bacteria, including Muribaculaceae, Lachnospiraceae, and Ruminococcaceae. These bacteria positively correlate with tight junction proteins, SCFAs, and secondary BAs, such as DCA and LCA, while exhibiting a negative correlation with inflammatory cytokines. Hence, we hypothesize that RA can modulate the expression levels of tight junction proteins, SCFAs, and BAs, as well as inhibit the expression of inflammatory factors, by regulating the richness and diversity of gut microbiota, thereby maintaining intestinal homeostasis to exert its therapeutic effect on UC.

Overall, this work highlighted the potential and prospects of RA as a preventive or therapeutic agent for UC. While this study primarily examines how RA ameliorates intestinal inflammation

in mice by modulating gut microbiota and enhancing the intestinal barrier, future work is warranted. Translational steps should include validation in germ-free mice, fecal microbiota transplantation studies, and the integration of clinical data to fully elucidate RA's therapeutic mechanisms against UC.

Ethics statement

The study was approved by the Academic Committee of Southwest Forestry University (ethical approval number SWFU-2018003).

Conflict of interest

The authors declare that we have no known financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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