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ORIGINAL RESEARCH

Rhoifolin Attenuates DSS-Induced Colitis in Mice by Modulating Gut Microbiota and Restoring Th17/Treg Balance

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Introduction: Rhoifolin (ROF), a flavonoid compound isolated from citrus plants, has been shown in modern research to possess a range of important biological activities, including anti-inflammatory and anti-tumor properties.

Methods: In this study, we induced ulcerative colitis (UC) in mice using dextran sulfate sodium (DSS) and treated them with ROF during the therapeutic phase.

Results: The results showed that ROF significantly alleviated the weight loss, colon shortening, and histopathological damage in the colon tissues of the mice, while also improving intestinal barrier function by restoring the expression of ZO-1 and E-cadherin. 16S rDNA sequencing analysis indicated that ROF treatment significantly altered the diversity and composition of the gut microbiota, increasing the relative abundance of *Bacteroidetes*, and *Lactobacillus*. Flow cytometry analysis revealed that ROF significantly reduced the proportion of Th17 cells in peripheral blood while increasing the proportion of Treg cells. Molecular docking analysis demonstrated that ROF could effectively bind to the Th17 cell transcription factor RORγt and the Treg cell transcription factor FOXP3, suggesting a potential regulatory effect. Further transcriptomic analysis revealed that ROF downregulated the expression of genes associated with the IL-17 signaling pathway, including IL-17A, TNF-α, NF-κB, CXCL10, and CXCL5, further supporting its anti-inflammatory mechanism by inhibiting the IL-17 pathway.

Discussion: In conclusion, we provides the first evidence that ROF alleviates DSS-induced colonic inflammation by modulating gut microbiota diversity, restoring the Th17/Treg cell balance, and inhibiting the IL-17 signaling pathway.

Keywords: rhoifolin, colitis, gut microbiota, Th17/Treg balance, IL-17 signaling pathway

Introduction

Ulcerative colitis (UC) is a chronic inflammatory disorder of the colon and rectum, causing varying degrees of impact on these organs.[1] UC manifests as a prolonged condition that is characterized by frequent relapses and remissions. The primary symptoms of UC include abdominal pain, bloody diarrhea, and weight loss.[2,3] The estimated global prevalence of UC for the year 2023 was 5 million cases.[4] The incidence of UC is on the rise, which highlights its emergence as a growing challenge for public health worldwide.[5,6]

The development of UC is primarily associated with genetic predisposition, various environmental factors, gut microbiota, and irregularities in the immune system.[7,8] Mounting evidence suggests that the dysbiosis of intestinal microbiota, which is characterized mainly by the loss of beneficial commensal bacteria, expansion of the pathogenic bacteria, and reduction in the overall biodiversity in the gut,[9] plays an important role in the pathogenesis of UC.[10,11] Recent studies have demonstrated that short-chain fatty acids (SCFAs) such as butyrate and propionate, produced by

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the gut microbiota, can induce the differentiation of regulatory T cells (Tregs), promote macrophage polarization toward the anti-inflammatory M2 phenotype, and regulate autophagy activity, thereby playing a pivotal role in maintaining immune homeostasis.[12–15] In inflammatory bowel disease, changes in the intestinal microbiota lead to dysregulated Th17/Treg cell balance, which further exacerbates the inflammatory response in the gut.[16,17] Treg cells are key regulators of inflammation and capable of suppressing various inflammatory and immune responses via the secretion of IL-10 and TGF-β.[18] Th17 cells also play important roles in various autoimmune diseases. Under inflammatory conditions, IL-6 and TGF-β drive Th17 cell differentiation via STAT3 activation, leading to the production of the inflammatory factor IL-17.[19,20] Simultaneously, STAT3 prevents Treg cell differentiation, leading to increased inflammation levels through the downregulation of FOXP3 expression.[21] Therefore, the balance between Th17 and Treg is crucial to maintain intestinal immune stability. Several studies have also indicated that gut microbiota dysregulation leads to a shift in the Th17/Treg balance toward Th17 cells, resulting in an increased risk of intestinal inflammation.[22–24]

Rhoifolin (ROF) is a flavonoid compound isolated from citrus plants.[25] Recent research has demonstrated that ROF possesses various key biological properties, including anti-inflammatory[26] and anti-tumor[27] properties. Chen et al[28] reported that ROF could alleviate arthritis symptoms in mice by modulating the NFR2/NF-κB signaling pathway. Another study confirmed that ROF reduces the secretion of the inflammatory mediator nitric oxide (NO) in RAW264.7 cells and inhibits the production of TNF-α, IL-1β, and IL-6.[29] However, despite the above-stated notable anti-inflammatory effects, no studies to date have yet explored the potential role of ROF in UC. Considering the critical roles of gut microbiota and the Th17/Treg balance in the pathogenesis of UC, the present study aimed to investigate the impact of ROF administration on the gut microbiome and Th17/Treg cell balance in mice with DSS-induced colitis to understand the protective effects of ROF against colitis.

Materials and Methods

Animals

Male C57BL/6J mice (6–8 weeks, 18–20 g), obtained from the Shanghai University of Traditional Chinese Medicine Laboratory Animal Center, were housed under specific pathogen-free (SPF) conditions at 25 ± 2°C with 60–65% humidity and a 12-hour light/dark cycle. All animal procedures were performed in compliance with the ethical guidelines approved by the Animal Experimentation Ethics Committee at the Shanghai University of Traditional Chinese Medicine Laboratory Animal Center (License NO. PZSHUTCM2303240007).

Induction of DSS Colitis and Treatment Protocol

Colitis was induced by providing 3% dextran sulfate sodium (DSS, MW 36–50 kDa, Yeason Biotech, Shanghai, China) in the drinking water.[30] The chemical structure of ROF (Yuanye Biotechnology, Shanghai, China) is shown in Figure 1A. ROF was dissolved in 1% dimethyl sulfoxide (DMSO), and studies have demonstrated that oral administration at 50 mg/kg exhibited no toxicity in animals.[31] Mice were randomly assigned to three groups (n=8): the control group, receiving regular drinking water for 14 days; the DSS group, drinking 3% DSS for 7 days followed by 7 days of regular water; and the DSS+ROF group, treated with 3% DSS and ROF (50mg/kg, oral gavage) for 7 days and then ROF only by another 7 days. Daily monitoring included scoring for stool consistency, presence of blood in stool, and body weight loss. Disease activity index (DAI) scores were calculated, and on day 15, mice were euthanized. Fresh fecal samples and colon tissues were harvested for further analysis.

Histopathological Analysis

At the study's endpoint, after deep anesthesia, the distal colon was excised, fixed in 4% paraformaldehyde, and processed for histopathological examination. Tissues were dehydrated in an alcohol gradient, embedded in paraffin, and cut into 4 μm sections. Hematoxylin and eosin (HE) staining was performed, and the inflammation severity, crypt damage, and colon involvement were scored from 0 to 14, based on previously described methods.[32]

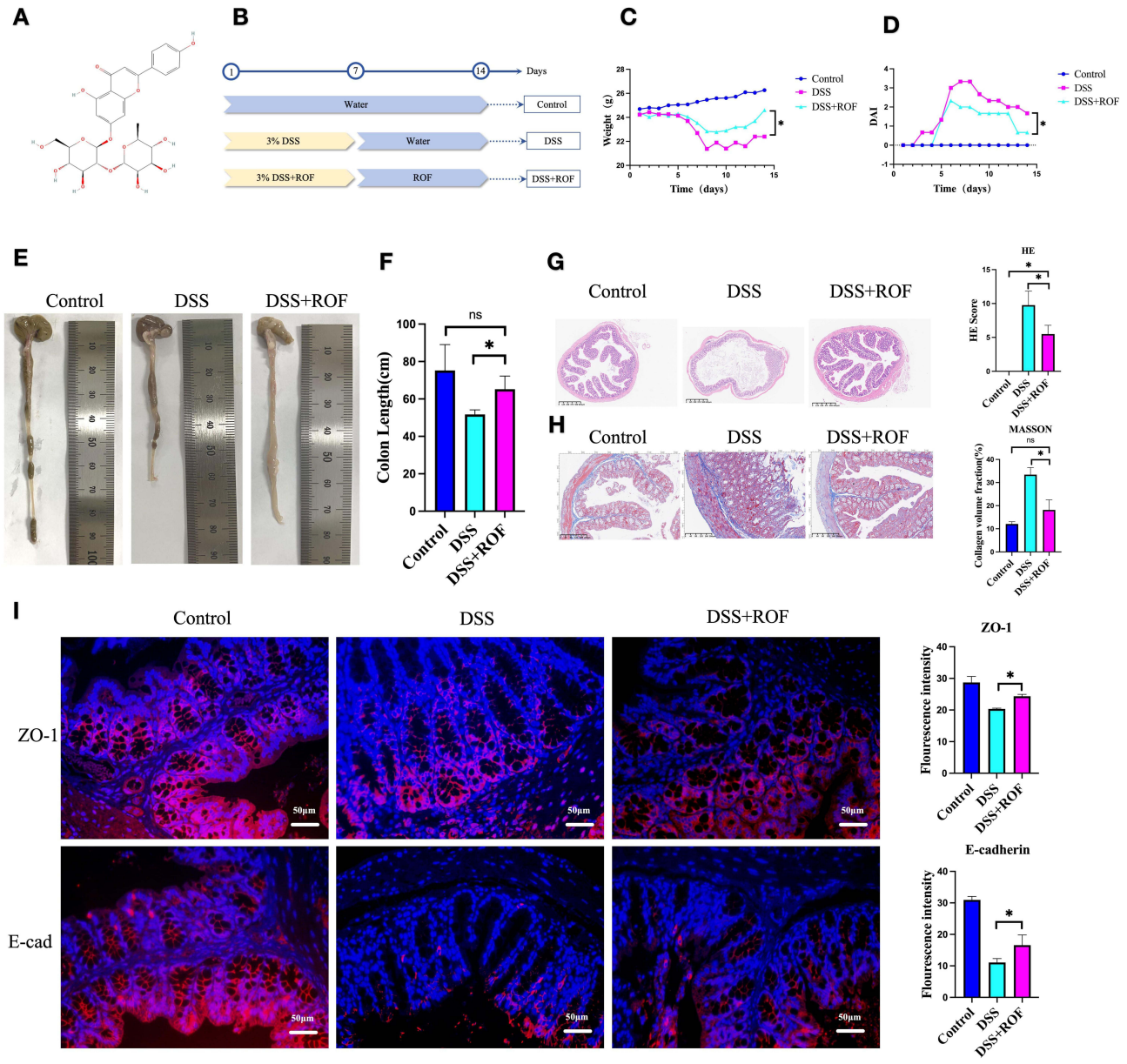

Figure 1 ROF treatment ameliorates DSS colitis in mice. (**A**) The chemical structure of ROF. (**B**) Animal experimental protocol of this study. (**C**) The body weight, (**D**) DAI score throughout the entire duration of the study and (**E** and **F**) colonic length were measured. (**G**) Representative HE staining of colon sections from the mice in three different groups, and HE scores were measured. Scale bar: 125 μm. (**H**) Representative MASSON staining of colon sections from the mice in three different groups, and Collagen volume fraction were measured. Scale bar: 40 μm.(**I**) Representative immunofluorescence of colon sections from the mice in three different groups and quantitative analysis. Scale bar: 50 μm. *$P < 0.05$.

Masson's Trichrome Staining

Colon sections were stained with Masson's trichrome to assess collagen deposition. The collagen fibers, nuclei, and background were stained blue, black, and red, respectively. Fibrosis levels were quantified using ImageJ software.

Fecal Genomic DNA Extraction and 16S rRNA Sequencing

The composition of the gut microbiota was analyzed using 16S rRNA gene sequencing. Total DNA was extracted from fresh fecal samples using the PF Mag-Bind Stool DNA Kit (Omega Bio-tek, GA, USA). DNA quality was assessed by electrophoresis and spectrophotometry, and stored at −80°C until further processing. The V3-V4 region of the 16S rRNA gene was amplified with primers 341F and 806R,[33] followed by PCR and sequencing on an Illumina PE250 platform.

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Sequence quality was evaluated and processed using fastp and UPARSE software, clustering sequences into OTUs at a 97% similarity threshold. The α-diversity and β-diversity were calculated using QIIME v1.8.0, and significant taxa differences were identified using the LDA effect size method. The 16S rRNA sequencing was completed by Hangzhou Guangke Ande Biotechnology Co., Ltd.

Establishment of cDNA Sequencing Libraries and High-Throughput RNA-Seq

Total RNA was extracted using the Total RNA Extractor(Trizol) kit (B511311, Sangon, China). According to their experimental procedures, a random fragment sequencing library was constructed using a VAHTSTM mRNA-seq V2 Library Prep Kit. Nucleic acid cleaving reagents were added, and the mRNA was randomly disrupted into short segments in a shaking incubator. First-strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second strand cDNA synthesis was subsequently performed using DNA polymerase I and RNase H. After adenylation of 3' ends of DNA fragments, adaptor was ligated to prepare for library. Finally, the cDNA was used for PCR amplification to obtain a sequencing library. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system and was subjected to high throughput sequencing using an Illumina HiSeq™ 2000 Sequencer after passing quality control. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed on HIPLOT PRO (https://hiplot.com.cn/).

Flow Cytometric Analysis

Flow cytometry was used to assess Th17 and Treg cell populations from peripheral blood lymphocytes (PBL). For Th17 cells, cells were stained with anti-CD4, anti-Rorγt, and anti-IL17A antibodies (ThermoFisher, MA, USA). For Treg cells, cells were stained with anti-CD4, anti-CD25, and anti-Foxp3 antibodies (ThermoFisher, MA, USA). After permeabilization, cells were analyzed using a flow cytometer and data analyzed with CellQuest software.

Immunofluorescence

For immunofluorescence, colon tissue sections were fixed, permeabilized, and blocked before incubation with primary antibodies against ZO-1 (1:200, Servicebio, Wuhan, China) and E-cadherin (1:500, Servicebio, Wuhan, China). Isotype-specific secondary antibodies were used, and DAPI stained the nuclei. Images were captured under a fluorescence microscope.

RNA Extraction and qRT-PCR

Total RNA was extracted from colon tissues using TRIzol reagent (Invitrogen, CA, USA). After DNase treatment, RNA concentration was measured, and cDNA was synthesized using the ReverTra Ace-α First Strand cDNA Synthesis Kit (Toyobo). Quantitative PCR was performed on a RealPlex4 system (Eppendorf, Hamburg, Germany) using SYBR Green Real-Time PCR Master Mix (Toyobo, Shanghai, China). The relative gene expression was calculated using the 2-ΔΔCt method, with 18S rRNA as the internal control.

Western Blot Analysis

Colon tissue proteins were extracted using 2x loading lysis buffer, separated by SDS-PAGE, and transferred to PVDF membranes (EMD Millipore, MA, USA). Membranes were blocked with 5% skim milk and incubated with primary antibodies overnight at 4°C. After washing, membranes were incubated with secondary antibodies for 1 hour. Protein signals were detected using an enhanced chemiluminescence kit (Yamei, Shanghai, China).

Statistical Analysis

Data analysis was performed using GraphPad Prism 7.0 software (GraphPad Software Inc., San Diego, USA). For comparisons between two groups, Student's *t*-test was used. Data are presented as mean ± SD, with statistical significance indicated as $*p < 0.05$, and NS for no significant difference.

Results

ROF Alleviates DSS-Induced Colitis and Restores the Intestinal Barrier

Mice were administered a 3% solution of DSS for 7 days to establish the UC model, followed by administering normal water for 7 days. The DSS + ROF group of mice were then treated with ROF via gavage (Figure 1B), daily for 15 days (from Day 0 to Day 14). It was observed that compared to the DSS group, the ROF treatment group underwent a significant reduction in weight loss (Figure 1C) and notable alleviation in colon shortening (Figure 1E and F). Next, the Disease Activity Index (DAI) was assessed based on stool consistency, rectal bleeding, and body weight loss. The DAI values determined for the different groups of rats also revealed that ROF administration significantly improved the inflammation associated with DSS-induced colitis (Figure 1D). The histological analysis of the colon, conducted using HE staining, revealed that DSS treatment led to crypt loss, inflammatory cell infiltration, severe mucosal damage, and elevated histological scores. The DSS + ROF group, on the other hand, exhibited relatively intact colon structures, less mucosal damage, and lower histological scores (Figure 1G). Masson's trichrome staining analysis revealed strong collagen deposition in the colon of DSS-treated mice, and this deposition level was significantly reduced upon ROF treatment (Figure 1H). Intestinal barrier integrity plays a key role in the pathogenesis of colitis, and was, therefore, studied using immunofluorescence. The results revealed that the expressions of ZO-1 and E-cadherin (E-cad) in the intestinal mucosa were significantly decreased in the model group and were markedly restored following ROF treatment (Figure 1I). The above findings suggested that ROF significantly alleviated the severity of DSS-induced colitis and improved the function of the intestinal barrier.

ROF Modulates the Gut Microbiota in DSS-Induced Colitis Mice

A 16S rDNA sequencing analysis was conducted for the fecal bacterial genomes from control, DSS, and DSS + ROF groups to determine whether ROF treatment altered the gut microbiota. Uparse (version 11) software was employed to cluster the sequences into operational taxonomic units (OTUs) based on 97% sequence similarity. The analysis revealed similar numbers of OTUs for the three groups (Figure 2A). The Venn diagram of the OTUs of each group revealed 396 overlapping OTUs (Figure 2B). The representative sequences of the OTUs were aligned with a microbial reference database, with each OTU assigned to a species, to calculate the community composition for each sample. Phylum-level analysis indicated that ROF treatment led to an increase in the relative abundances of *Bacteroidota, Firmicutes*, and *Proteobacteria* and a decrease in the relative abundances of *Actinobacteriota* and *Verrucomicrobiota* (Figure 2C and Figure 1S). At the genus level, the relative abundances of *Bacteroides, Lactobacillus*, and *Alistipes* were significantly higher in the DSS + ROF group compared to the DSS group, while the relative abundances of *Akkermansia* and *Bifidobacterium* were lower in the former (Figure 2D and Figure 2S). At the species level, the relative abundances of *Bacteroides acidifaciens, Lactobacillus murinus*, and *Lactobacillus johnsonii* were higher in the DSS + ROF group compared to the DSS group, while *uncultured bacterium g_norank_f_Muribaculaceae, Akkermansia muciniphila*, and *Bifidobacterium pseudolongum* were observed to have reduced abundances in the former (Figure 2E and Figure 3S).

Clustering analysis was performed next to determine the microbial diversity, which revealed that the major contributors to microbial diversity in the ROF treatment group (Figure 3A and B) were microorganisms from *Bacteroides, Lactobacillus, Romboutsia*, and *Muribaculum* (all had increased abundances), along with *norank_f_Eubacterium_coprostanoligenes_group, Candidatus_saccharimonas*, and *Acetatifactor* (abundances were decreased for all of these).

In the subsequent α-diversity analysis, the rank abundance curve was flat, suggesting a highly consistent species composition across the samples (Figure 4A). The rarefaction curve was smooth, indicating sufficient sequencing depth and reliability of the data analysis (Figure 4B). The Shannon index showed no significant change following ROF intervention, suggesting that ROF may restore microecological balance by restructuring the microbial community rather than increasing species richness. (Figure 4C).

The β-diversity analysis was conducted next, which included principal component analysis (PCA), principal coordinate analysis (PCoA), and non-metric multidimensional scaling (NMDS). The results of these analyses revealed distinct

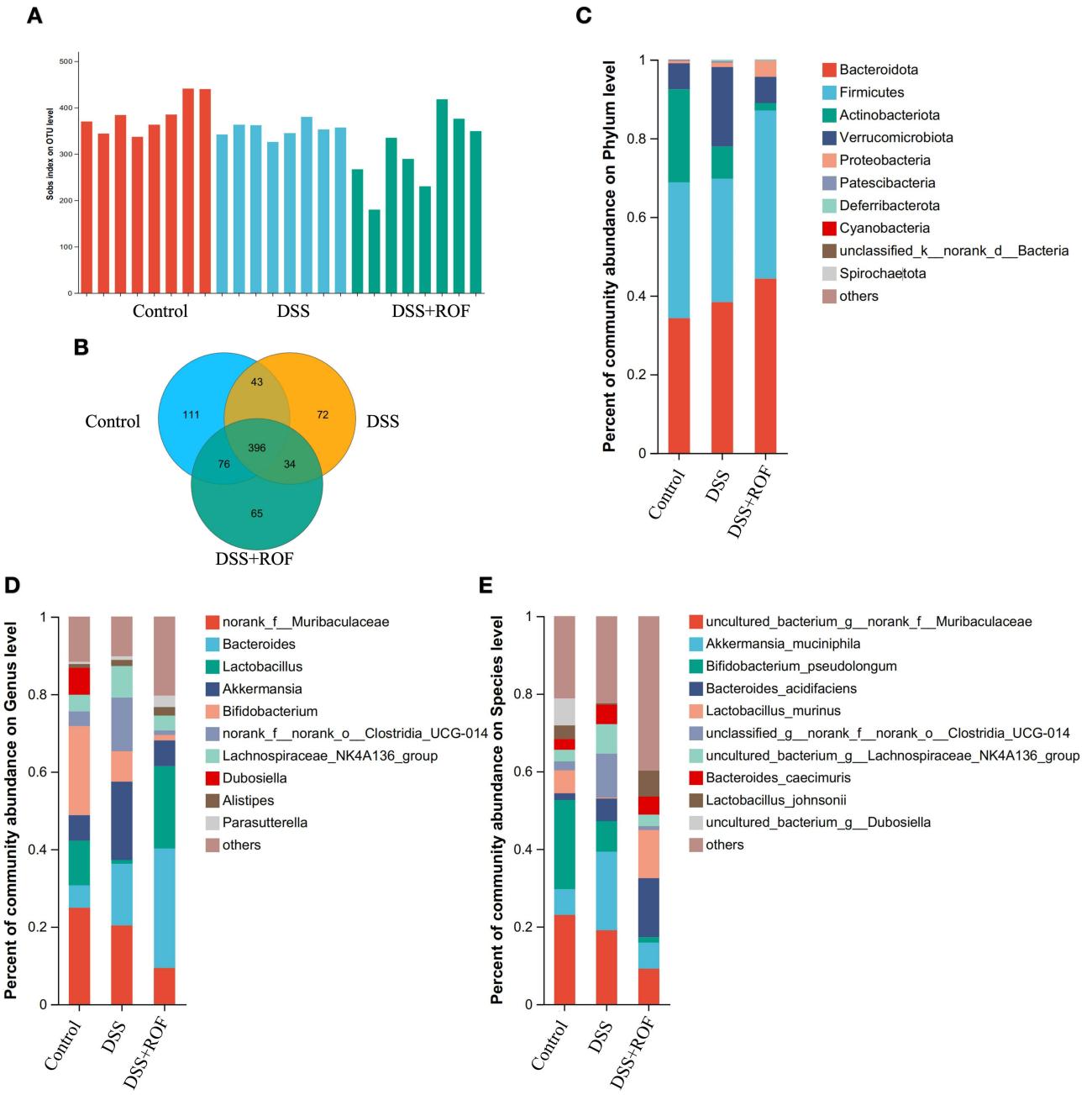

Figure 2 Analysis of OTUs. (**A**) Number of OTUs in each group. (**B**) Venn diagram of the three groups. (**C**) Gut microbiota clustering and species distribution on Phylum level, (**D**) Genus level and (**E**) Species level.

differences in microbial communities among the ROF + DSS, DSS, and control groups. In other words, the microbial populations in these groups formed distinct communities (Figure 4D–F).

According to the results of hierarchical clustering analysis conducted using the unweighted pair-group method with arithmetic mean (UPGMA), the intestinal microbiota in the ROF treatment group and DSS group exhibited lower homology, which indicated different genetic backgrounds (Figure 4G).

Further, the key biomarkers of the gut microbiota were identified using Linear Discriminant Analysis (LDA) Effect Size. An LDA score of 4.0 was identified as a significant biomarker. The dendrogram and the LDA score distribution revealed that the abundances of *s_Bacteroides, o_Lactobacillates, p_Firmicutes, p_Proteobacteria, c_Gammaproteobacteria, f_Sutterellaceae, o_Burkholderiales, f_Peptostreptococcaceae, s_Romboutsia_ilealis,*

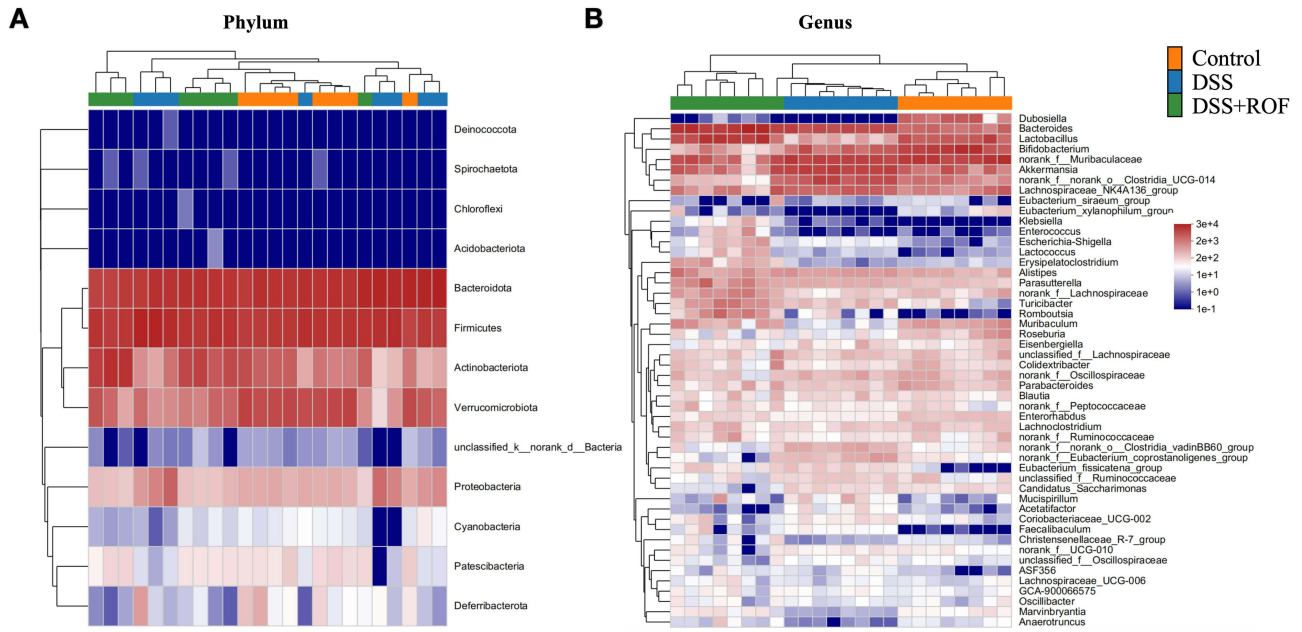

Figure 3 Microbial diversity clustering. (**A**) Phylum-level analysis; the relative abundance of Actinobacteria and Verrucomicrobia in the DSS+ROF group was significantly higher than DSS group. (**B**) Genus-level analysis; the relative abundance of Lactobacillus, Enterococcus, Turicibacter and Romboutsia in the DSS+ROF group was significantly higher than DSS group.

g_Romboutsia, and *g_Turicibacter* were significantly higher in the DSS + ROF group than in the control group, indicating that these microbial communities were specific to the DSS + ROF group. In the DSS group, *s_Akkermansiaceae, p_Verrucomicrobiota, c_Clostridia, g_Lachnospiraceae_NK4A136_group*, and *g_Bacteroides_caecimuris* were the unique microbial communities. In the control group, *p_Actinobacteriota, o_Bifidobacteriales, f_Muribaculaceae, g_Dubosiella*, and *o_Erysipelotrichales* were the unique microbial communities (Figure 5A–C).

ROF Restored the Th17/Treg Cell Balance in DSS-Induced Colitis Mice

The Th17/Treg cell balance plays a pivotal role in maintaining mucosal integrity and mitigating inflammation in colitis.[34] Therapeutic agents were demonstrated to modulate the Th17/Treg cell balance by regulating gut microbiota and thereby alleviating colonic inflammation in mice.[35] In the present study, ROF treatment reduced the number of Th17 cells in the peripheral blood of UC mice (Figure 6A), while significantly increasing the proportion of Treg cells (Figure 6B). The molecular docking analysis revealed that ROF could effectively bind to the transcription factors RORγt and FOXP3 specific to Th17 cells and Treg cells, respectively, with the corresponding binding energies of –10.7 kcal/mol and –7.9 kcal/mol (Figure 6C and D). In the subsequent quantitative reverse transcription PCR (qRT-PCR) analysis, ROF treatment was observed to downregulate the mRNA expression of RORγt, while significantly upregulating the mRNA expression of FOXP3 (Figure 6E). Western blotting results confirmed these observed changes at the protein level as well (Figure 6F). Therefore, it was concluded that ROF reprograms the Th17/Treg cell balance, thereby alleviating colonic inflammation in UC mice and preserving intestinal immune homeostasis.

ROF Regulated IL-17 Signaling Pathway in DSS-Induced Colitis Mice

In order to gain deeper insights into the mechanism underlying the therapeutic effects of ROF in UC, a transcriptomic sequencing analysis was conducted using the colonic tissues from the control, DSS, and DSS + ROF groups. Venn diagram comparisons revealed 1125 differentially expressed genes (DEGs) between the control and DSS groups and 913 DEGs between the DSS and DSS + ROF groups, with 454 shared DEGs (Figure 7A). The heatmap of these shared DEGs

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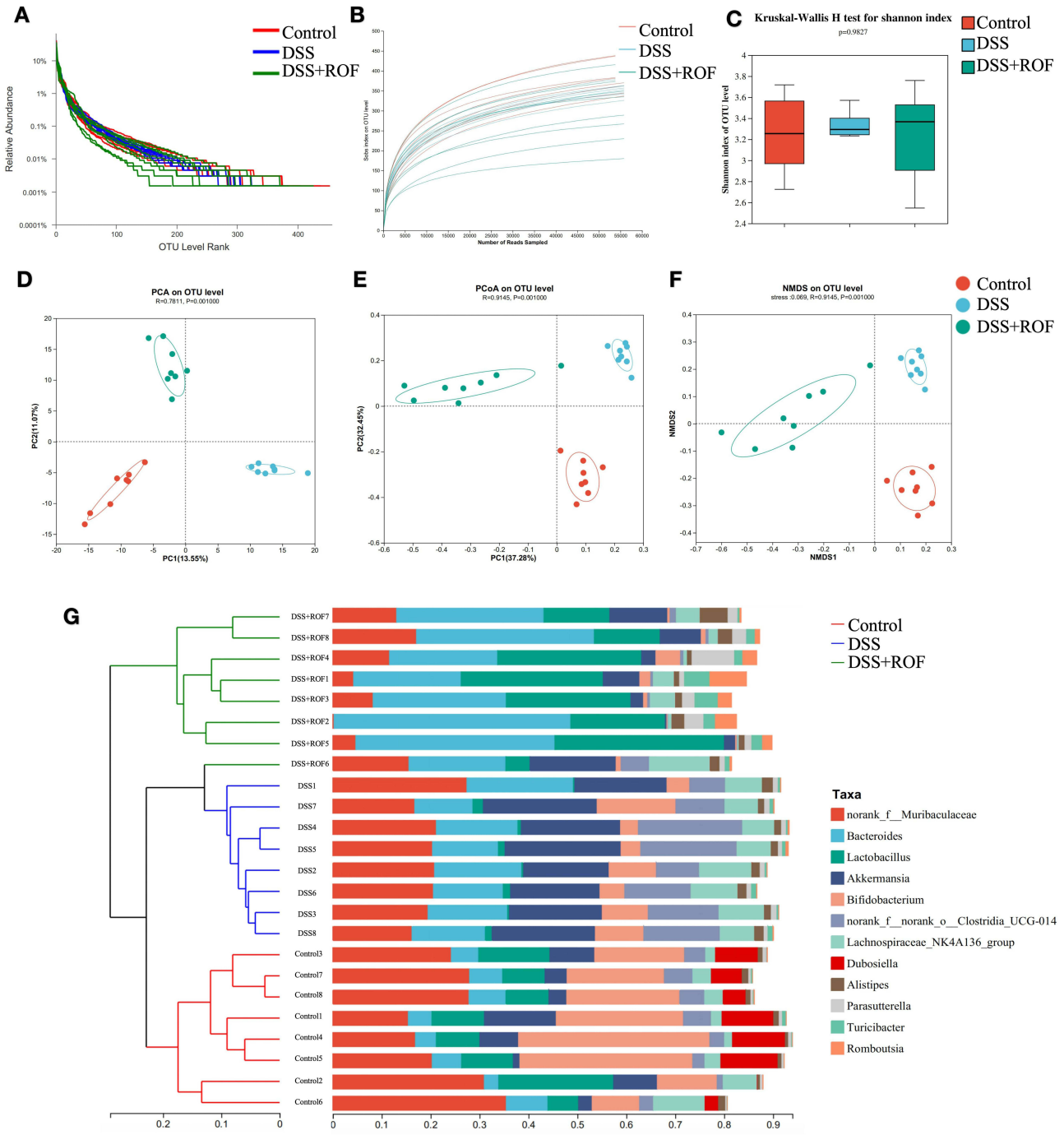

Figure 4 α- and β-diversity analysis. (**A**) As evident, α-diversity analysis showed that the rank abundance curve was flat, indicative of a high degree of uniformity in species composition. (**B**) The rarefaction curve appeared smooth. (**C**) Shannon index. (**D**) PCA, (**E**) PCoA, and (**F**) NMDS analysis. (**G**) Clustering and histogram combination.

revealed that ROF treatment could restore the expression levels of certain genes in the DSS group to levels similar to those in the control group (Figure 7B).

In order to further explore the potential biological functions of ROF, GO and KEGG analyses were conducted using the downregulated genes (Log2 fold change ≤ −1.5) determined in the DSS + ROF vs DSS comparative analysis. The GO analysis revealed that genes related to humoral immune response, leukocyte migration, neutrophil migration, and chemokine-mediated signaling pathways exhibited the most significant differential expression (Figure 7C). In regard to cellular components (CC), immunoglobulin complex and immunoglobulin complex were the ones most significantly

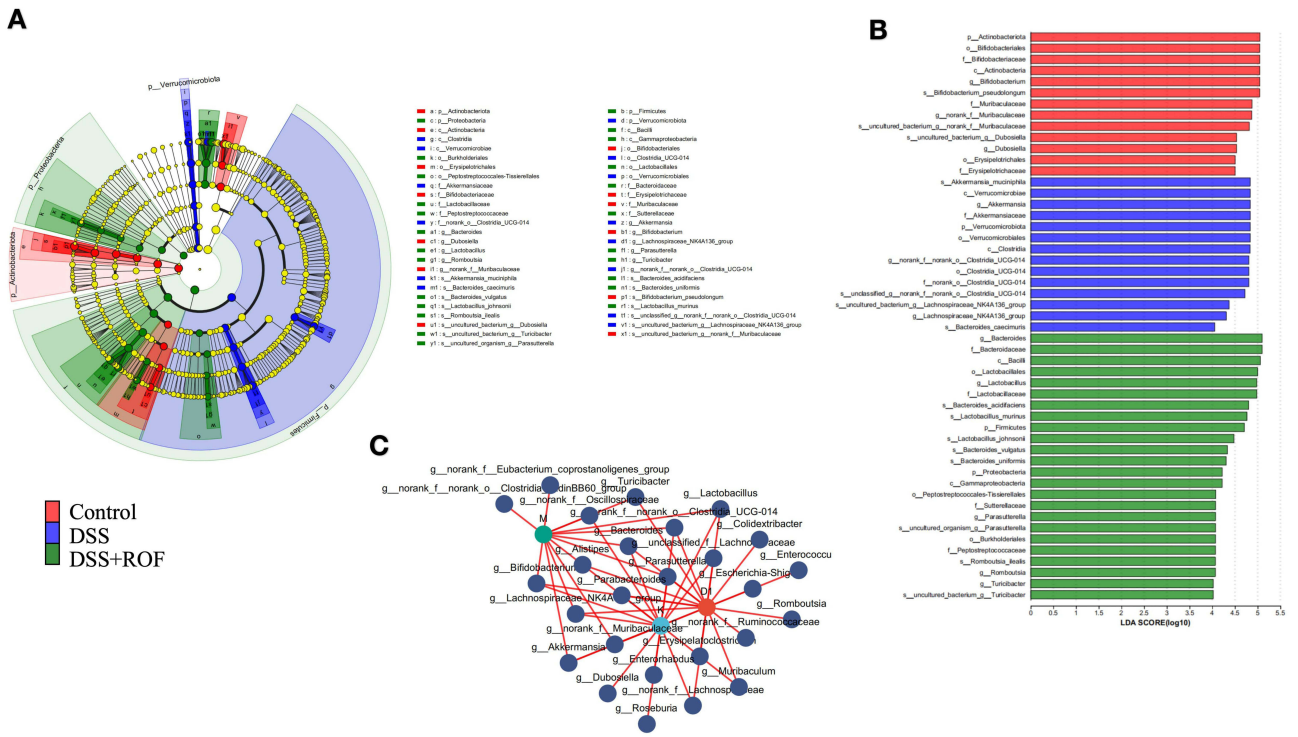

Figure 5 Significant difference analysis. (**A**) Species annotation was visualized using KRONA. (**B**) Value distribution histogram of line discriminant analysis effect size. (**C**) Species network at the genus level.

altered (Figure 7D). Among the molecular functions (MF), cytokine activity, chemokine activity, cytokine receptor binding, and chemokine receptor binding were the most significantly altered (Figure 7E). According to the KEGG pathway analysis, the IL-17 signaling pathway exhibited the most significant differential expression (Figure 7F). The above results collectively suggested that ROF modulates the expression of multiple genes and the associated signaling pathways in the colonic tissues of mice.

The above findings were validated by performing qRT-PCR to determine the mRNA expression levels of the genes involved in the IL-17 signaling pathway, including IL17A, TNF-α, NF-κb, CXCL10, and CXCL5. It was observed that the expression levels of these genes were significantly reduced in the DSS + ROF group (Figure 7G), which was consistent with the results of the transcriptomic analysis.

Discussion

UC is a refractory chronic inflammatory bowel disease with a complex pathogenesis. Several studies have demonstrated that the onset of UC is closely related to gut dysbiosis and abnormal immune responses.[36–38] In the present study, ROF was observed to significantly alleviate DSS-induced colitis, which manifested as a reduction in weight loss, DAI scores, colon shortening, HE scores, and collagen volume index. This alleviation of colitis was achieved by restoring the expressions of ZO-1 and E-cad. The results of 16S sequencing also demonstrated that ROF alleviated colitis by modulating the abundance and diversity of gut microbiota. The flow cytometry analysis of peripheral lymphocytes revealed that ROF significantly reduced the proportion of Th17 cells and increased the proportion of Treg cells, resulting in reduced secretion of inflammatory factors such as IL-17. In addition, the transcriptomic sequencing performed to identify the potential targets of ROF in UC treatment revealed that the differentially expressed genes were primarily enriched in the IL-17 signaling pathway. This finding suggested that ROF probably alleviated intestinal inflammation by inhibiting this signaling pathway. Accordingly, it was inferred that ROF improves colonic inflammation in UC by modulating gut microbiota and abnormal immune responses, based on which ROF is recommended as a potential therapeutic drug for UC.

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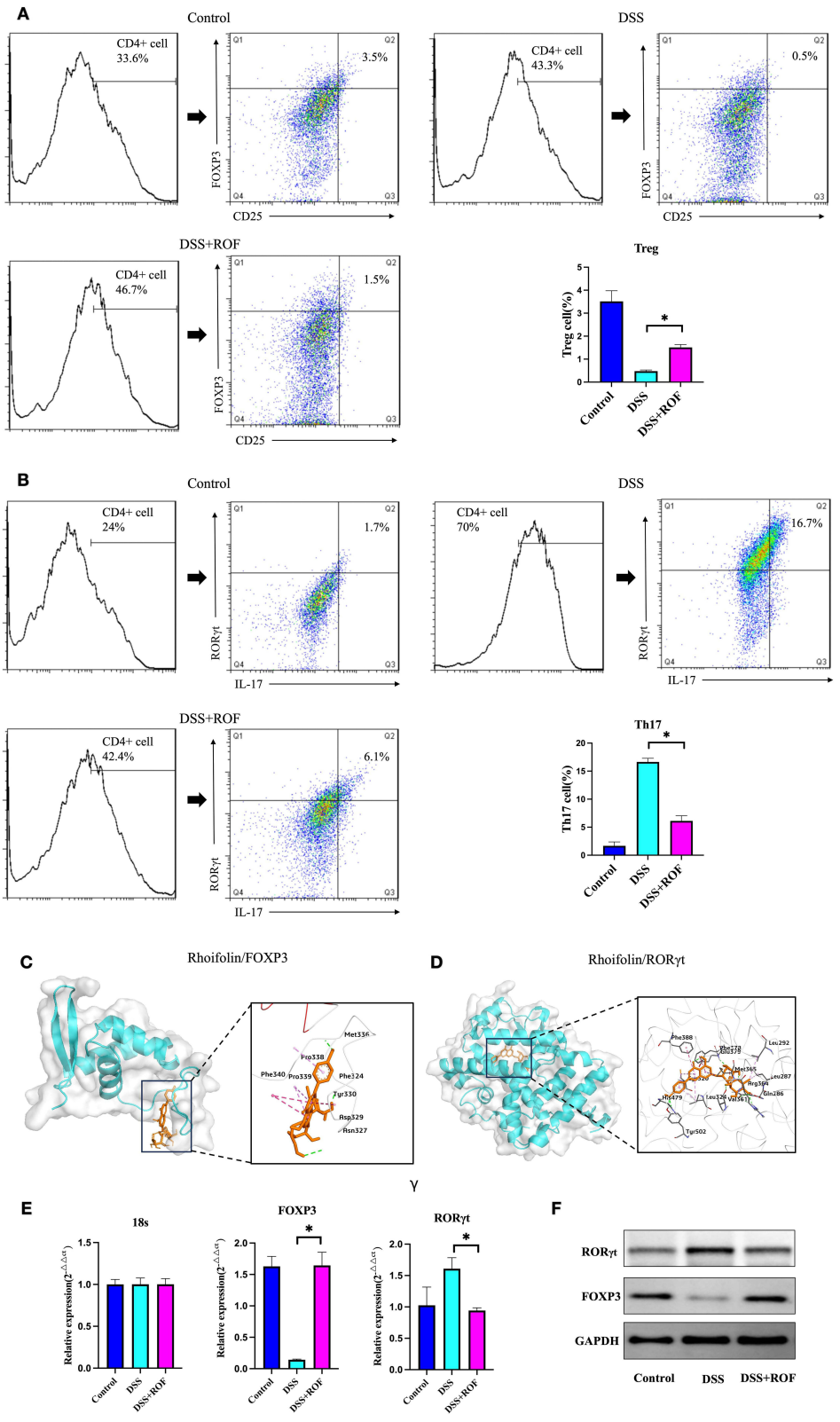

Figure 6 ROF treatment ameliorates the Th17/Treg balance in the peripheral blood. Relative to DSS group, (**A**) proportion of CD4+/CD25+/Foxp3+ cells were significantly higher than DSS group and (**B**) he proportion of CD4+/Roryt+/IL17A+ cells were significantly lower than DSS group. Molecular docking of (**C**) Rhoifolin/Foxp3 and (**D**) Rhoifolin/Roryt. (**E**) qRT-PCR analysis and (**F**) WB analysis of Foxp3 and Roryt. *$P < 0.05$.

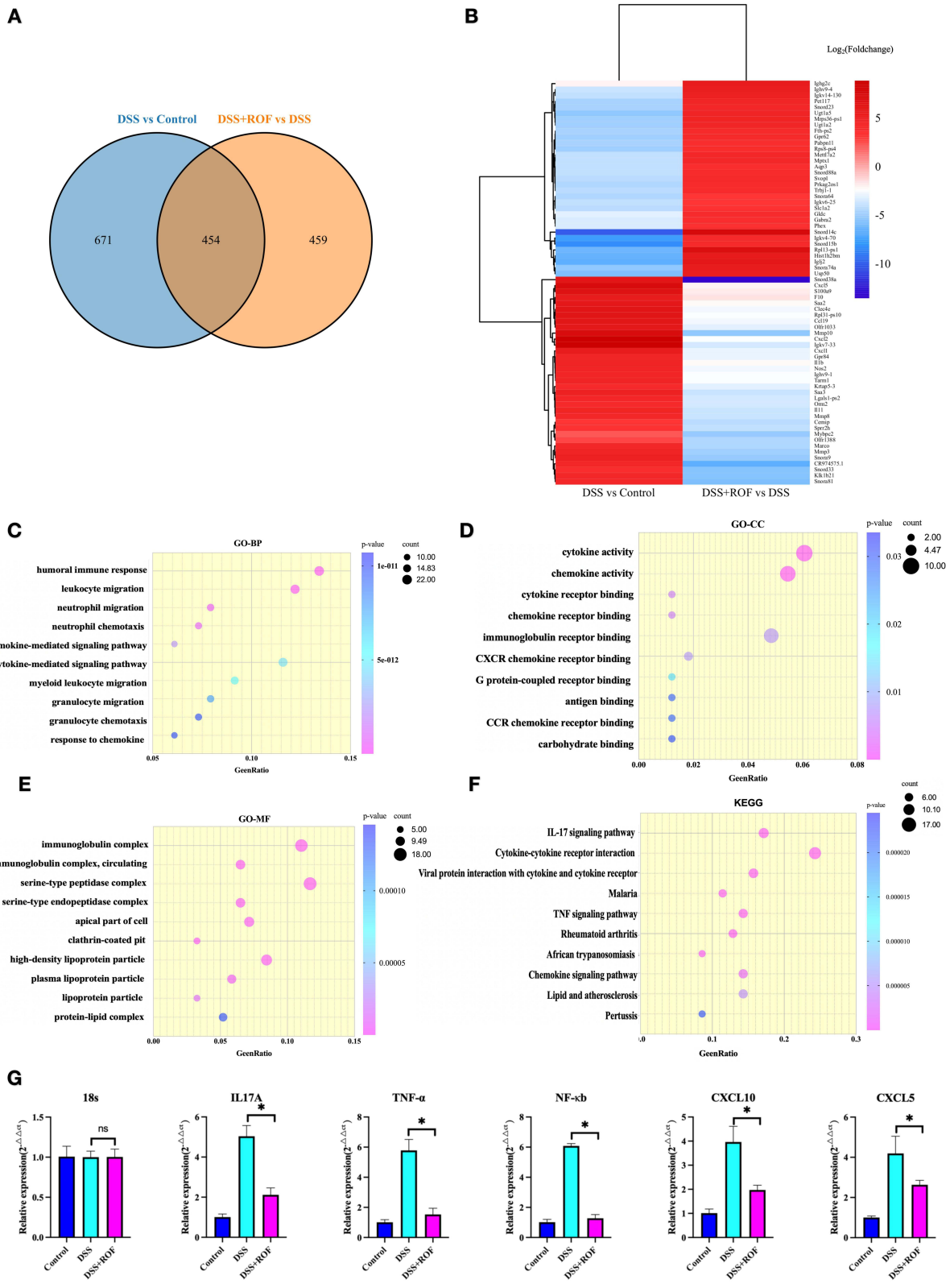

Figure 7 Transcriptomic sequencing analysis of colonic tissues. (**A**) Venn diagram of DEGs. (**B**) The heatmap of common DEGs. (**C**–**E**) GO and (**F**) KEGG analysis on the downregulated genes (Log2 fold change ≤ −1.5) in the DSS+ROF vs DSS comparison. (**G**) qRT-PCR analysis of the IL-17 signaling pathway. *$P < 0.05$.

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Tight junction (TJ) proteins cover the intestinal epithelial surface and form a strong defense system within the host. ZO-1 is a representative tight junction protein and an essential structural component of the epithelial cells, which plays a key role in barrier functions.[39,40] E-cad is a calcium-dependent cell adhesion molecule located on the basolateral membrane, along the crypts. E-cad plays an important role in the development and maintenance of epithelial tissues.[41] The present study revealed that ROF significantly increased the expressions of ZO-1 and E-cad, thereby maintaining intestinal barrier homeostasis.

Gut microbiota plays an important physiological role in nutrition metabolism by preventing pathogen colonization and maintaining host immune homeostasis.[42–44] In the present study, ROF was observed to significantly increase the diversity of the gut microbiota in UC mice. When the microbiota composition was investigated at the phylum level, the abundances of *Bacteroidota* and *Firmicutes* were observed to be increased after ROF treatment. Previous studies have reported *Bacteroidota* and *Firmicutes* as the dominant phyla in the gut microbiota of healthy populations.[45] *Bacteroidota* produces most of the acetate and propionate, while *Firmicutes* are considered the main producers of butyrate. Acetate, propionate, and butyrate are important anti-inflammatory bacterial metabolites that regulate gut PH, promote mucus production, and provide energy to epithelial cells to enhance the immune function of the intestinal mucosa.[46,47] At the genus level, the abundances of *Bacteroides* and *Lactobacillus* were significantly increased upon ROF treatment. Similarly, at the species level, the abundances of *Bacteroides acidifaciens, Lactobacillus murinus*, and *Lactobacillus johnsonii* were increased after ROF treatment, which was consistent with the phylum-level and genus-level results. Certain studies have reported that supplementation of *Lactobacillus johnsonii* significantly alleviates DSS-induced colitis in UC mice, possibly by activating the CD206+ macrophages via the TLR1/2-STAT3 pathway, thereby promoting the secretion of the anti-inflammatory factor IL-10.[48] *Lactobacillus murinus* reportedly promotes macrophage IL-10 release via TLR2 to alleviate intestinal ischemia-reperfusion injury.[49] In addition, *Lactobacillus murinus* and *Lactobacillus johnsonii* have been reported to significantly reduce the proportion of Th17 cells in the host and increase the proportion of Treg cells, thereby reducing inflammation.[50,51] In the present study, although the abundance of probiotics such as *Akkermansia* and *Bifidobacterium* was observed to be decreased after ROF intervention, this decrease was not significant. This finding was also consistent with the branch diagrams and LDA scores. Accordingly, it was concluded that ROF exerts a regulatory effect on the DSS-induced gut microbiota dysbiosis in mice and regulates the Th17/Treg cell balance in UC mice via *Lactobacillus murinus* and *Lactobacillus johnsonii*, thereby alleviating colitis.

Targeting the Th17/Treg cell balance is an effective strategy for treating UC.[52] Zhong et al[53] reported that Astragaloside IV could effectively prevent and alleviate DSS-induced colitis in UC mice by reshaping Th17/Treg cell homeostasis and reducing oxidative stress. Xiao et al[54] reported that Kuijieling decoction could inhibit the expressions of IL-6R and IL-23R and reduce the production of RORγt, thereby inhibiting Th17 cell differentiation, while upregulating SMAD3 and FOXP3 to promote Treg cell differentiation. This suggests that Kuijieling decoction might improve colitis by regulating the Th17/Treg cell balance. The flow cytometry analysis conducted in the present study revealed that ROF intervention could significantly reduce the number of Th17 cells in the peripheral blood of UC mice while increasing the proportion of Treg cells. The results of WB and qPCR analyses also revealed a decrease in RORγt expression and an increase in FOXP3 expression. Molecular docking also indicated that ROF could bind well to the target genes, indicating that ROF alleviates DSS-induced colitis in UC mice by modulating the Th17/Treg cell balance. Consistent with previous studies, ROF can effectively suppress Th1/Th17 cell polarization by modulating the JAK2/JAK3/STAT1/STAT3 signaling pathway, thereby inhibiting the release of inflammatory cytokines such as TNF-α, IFN-γ, IL-2, and IL-17.[55]

Next, GO and KEGG analyses were performed using the downregulated genes in the DSS + ROF vs DSS comparative analysis. In regard to biological processes (BP), the evaluated genes were primarily enriched in humoral immune response, leukocyte migration, and neutrophil migration, all of which are closely related to colitis development. Several existing UC immunotherapies target leukocyte migration.[56–59] Accordingly, it was inferred that ROF probably reduces the abnormal immune response in UC mice. KEGG analysis revealed the IL-17 signaling pathway as the most significantly enriched signaling pathway. IL-17 was the first reported member of the novel inflammatory cytokine family, and its expression is significantly increased in IBD,[60] in which IL-7 activates the NF-κB signaling pathway. The key genes involved in the IL-17 signaling pathway include those related to pro-inflammatory cytokines, chemokines, matrix metalloproteinases, and inflammatory effectors.[61] Studies have demonstrated that the IL-17 signaling pathway is

significantly upregulated in colitis and that targeting this pathway could facilitate maintaining intestinal homeostasis and preventing excessive inflammatory response.[62] The decrease in the proportion of Th17 cells would certainly reduce IL-17 production. Therefore, the significant enrichment of the IL-17 signaling pathway revealed in the KEGG analysis further corroborated that ROF intervention could markedly inhibit this pathway. Moreover, the qRT-PCR results revealed that ROF significantly downregulated the mRNA expression levels of IL-17A, TNF-α, NF-κB, CXCL10, and CXCL5 in the IL-17 signaling pathway. These findings were consistent with previous reports, which stated that ROF could reduce the expressions of inflammatory factors such as TNF-α and NF-κB.[28,29] CXCL10 is a CXC chemokine that enhances the production of TH1 cells.[63] Previous studies have demonstrated that CXCL10 recruits lymphocytes to colonic tissues, leading to colitis.[64,65] ROF may alleviate colitis by reducing CXCL10 expression, which is consistent with the results of the GO analysis conducted in the present study, according to which leukocyte migration and cytokine activity were reduced. Our findings further support that ROF alleviates inflammation and modulates immune responses.[31,55,66]

This study has several limitations that warrant consideration. First, the lack of dose-response analysis (multiple ROF concentrations) limits our ability to determine whether its therapeutic effects are dose-dependent. Second, fecal microbiota transplantation (FMT) experiments were not conducted to establish a causal relationship between ROF-mediated gut microbiota modulation and colitis alleviation. Finally, metabolomic profiling of microbial-derived metabolites (eg, SCFAs, bile acids) was omitted, which could further elucidate the mechanistic link between microbiota restructuring and immune regulation.

Conclusion

The present study pioneers in demonstrating that ROF may alleviate DSS-induced colitis by modulating the diversity of gut microbiota in mice with colitis, thereby restoring the Th17/Treg cell balance and inhibiting the expression of the IL-17 signaling pathway. These findings would serve as a basis for further research on UC treatment, which could focus on exploring the mechanism through which ROF regulates the gut microbiota.

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Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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