

Original Article

Plantamajoside alleviates DSS-induced ulcerative colitis by modulating gut microbiota, upregulating CBS, and inhibiting NF- κ B

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ABSTRACT

Background: Plantamajoside (PMS) is a natural bioactive compound derived from medicinal, food homologous plants of the genus *Plantago*.

Purpose and Methods: This study aimed to investigate the protective effects of PMS on dextran sulfate sodium (DSS)-induced ulcerative colitis (UC) in mice and explore the associated mechanisms.

Results: We found that PMS treatment significantly alleviated UC symptoms in mice by preventing body weight loss, increasing colon length, and reducing disease activity index scores. Moreover, PMS alleviated colonic lesions, increased the number of goblet cells, upregulated the expression of intestinal barrier proteins (ZO-1, occludin, and claudin-3), and decreased the levels of pro-inflammatory factors. PMS treatment modulated the gut microbiota by increasing the relative abundance of Bacteroidota and Verrucomicrobiota and decreasing that of Firmicutes and Proteobacteria at the phylum level. At the genus level, PMS suppressed the abundance of pathogenic bacteria, such as *Turicibacter* and upregulated the abundance of *[Eubacterium]_xylanophilum_group*. Fecal microbiota transplantation experiments further confirmed that PMS treatment alleviated UC by modulating the gut microbiota. Transcriptomic analysis of colon tissues, coupled with reverse transcription-quantitative polymerase chain reaction and western blotting, showed that PMS treatment upregulated cystathionine beta-synthase (CBS) expression and inhibited NF- κ B pathway activation. In a lipopolysaccharide-induced inflammation model in RAW264.7 cells, PMS treatment inhibited the secretion of pro-inflammatory cytokines, upregulated CBS expression, and prevented NF- κ B pathway activation.

Conclusion: PMS protects against UC in mice via multiple mechanisms, including modulating the gut microbiota, increasing the expression levels of CBS, and inhibiting the NF- κ B pathway.

Introduction

Ulcerative colitis (UC) is a subtype of inflammatory bowel disease that commonly presents with abdominal pain, diarrhea, weight loss, and bloody mucus in the stool (Honap et al., 2024). The incidence and prevalence of UC have increased, making it one of the most prevalent gastrointestinal diseases globally. The mainstay treatments for UC

include 5-aminosalicylic acid (5-ASA), corticosteroids, immunosuppressants, and biologics. However, these medications could not sustain long-term remission and may cause adverse effects, including gastrointestinal discomfort, nausea, and immune dysfunction (Krugliak Cleveland et al., 2022). Therefore, the development of safe and effective drugs for UC treatment is significant.

Although the exact etiology of UC is poorly understood, researchers

Abbreviations: CBS, (Cystathionine beta-synthase); DAI, (Disease activity index); DEGs, (Differentially expressed genes); DSS, (Dextran sSulfate sSodium); FMT, (Fecal microbiota transplantation); LPS, (Lipopolysaccharide); PBS, (Phosphate-buffered saline); PMS, (Plantamajoside); SCFAs, (Short-chain fatty acids); UC, (Ulcerative colitis); 5-ASA, (5-aminosalicylic acid).

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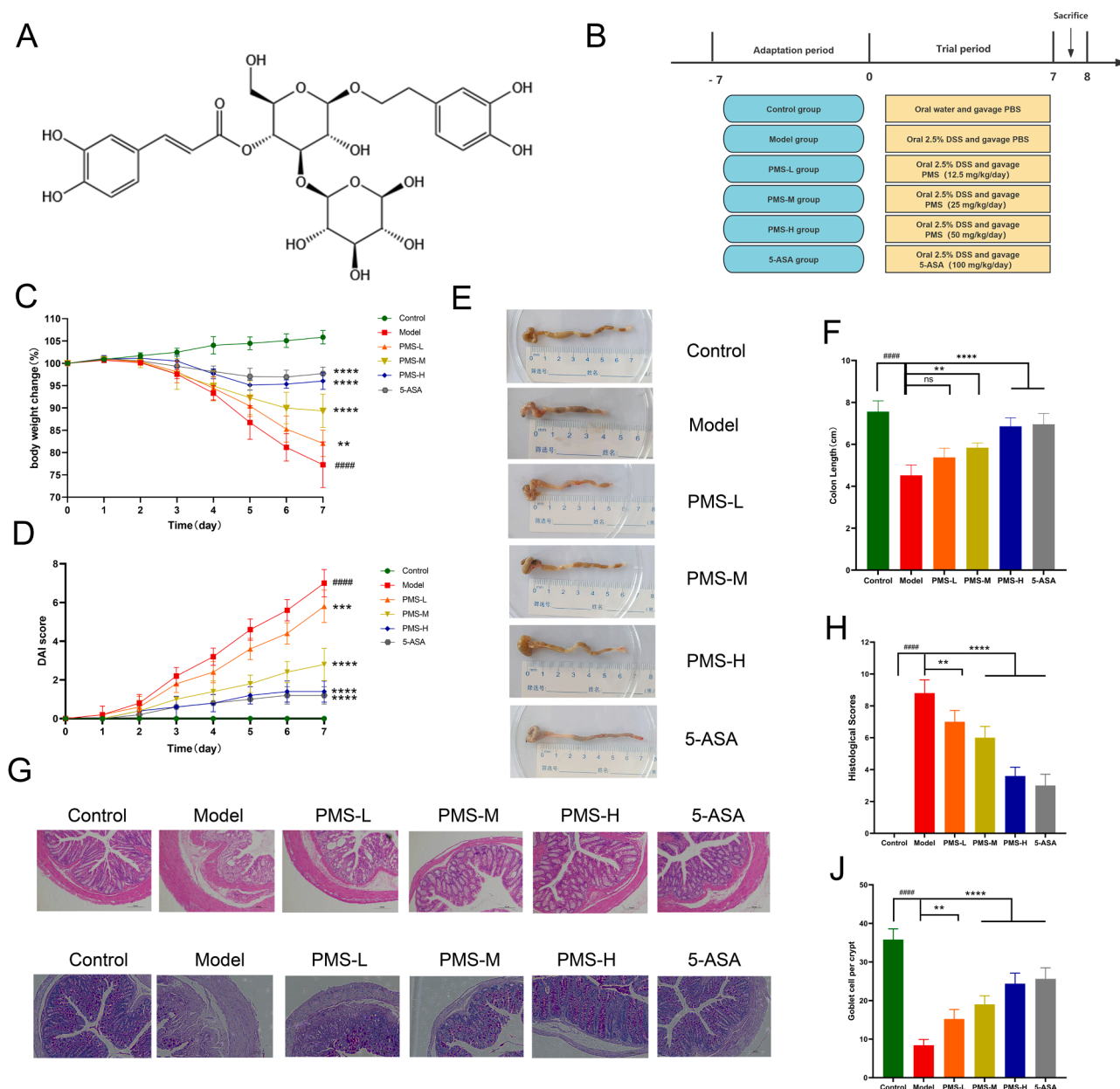


Fig. 1. Effects of PMS on DSS-induced ulcerative colitis symptoms and pathological damage in mice.

(A) Structural diagram of plantamajoside (PMS). (B) Schematic diagram of the experimental design for the treatment of dextran sulfate sodium (DSS)-induced ulcerative colitis (UC) with PMS. (C) Average body weight of the mice in each group. (D) Disease activity index (DAI) score for each group. (E) Representative images of colons in each group. (F) Colon length in each group. (G) Representative hematoxylin and eosin (H-E)-stained histological images of colon tissues in each group. (H) Pathological damage scores of colon tissues. (I) Representative periodic acid-Schiff (PAS)-stained histological images of colon tissues in each group. (J) Number of goblet cells.

All data are presented as the mean $\pm$ standard error of the mean (SEM; $n = 5$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ vs the Model group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, and #### $P < 0.0001$ vs the Control group.

have proposed that its pathogenesis may involve genetic predisposition, immune dysfunction, environmental factors, and gut microbiota dysbiosis (Sinha et al., 2022). Recent studies have revealed a strong association between UC and the gut microbiota (Zhang et al., 2024). An imbalance in the gut microbiota of patients with UC is often characterized by a reduced diversity of beneficial microbes and an increased abundance of opportunistic pathogens. The enhanced proliferation of pathogenic bacteria disrupts metabolic pathways and triggers intestinal inflammation, leading to UC.

Plantago is a genus of perennial herbs that thrive in warm and humid climates and are resistant to cold conditions. They grow in diverse environments, such as forests, mountains, and hills (Wen et al., 2023).

Plantamajoside (PMS), a phenylethanoid glycoside compound isolated from *Plantago* spp., exerts diverse biological activities, including anti-inflammatory, antioxidant, and anti-tumor effects (Zan et al., 2019). However, its therapeutic effects against UC have not been previously reported.

In the present study, we explored the therapeutic effects of PMS on UC in mice. Using 16S rRNA sequencing, the effects of PMS on the gut microbiota composition were explored and its protective effects were investigated using fecal microbiota transplantation (FMT) experiments. Transcriptomic analysis was conducted to elucidate the molecular mechanisms underlying the protective effects of PMS against UC. These findings were further validated using *in vitro* experiments, reverse

Table 1
The Disease Activity Index (DAI) Scoring.

Score	Weight loss	Fecal property	Rectal bleeding
0	0%	Normal	Normal
1	1%-5%	Soft stool	Occult blood
2	6%-10%	Mucus-like stool	Slight bleeding
3	11%-15%	Thin liquid stool	Blood in the stool
4	>15%	Watery diarrhea	Gross bleeding in the stool

transcription-quantitative polymerase chain rection (RT-qPCR), and western blotting.

Materials and Methods

Reagents

PMS was purchased from MedChemExpress (Monmouth Junction, NJ, USA), and dextran sulfate sodium (DSS) was obtained from MP Biomedicals (Santa Ana, CA, USA).

Animal experiments

All animal experiments were performed in accordance with the guidelines of the Animal Care and Use Committee of the Jilin Engineering Normal University (registration date, January 1, 2024; application number, 2024SY10106). Male C57BL/6J mice (6-weeks-old, weighing 16–20 g) were purchased from Changsheng Biotechnology Co., Ltd. (Liaoning, China). All mice were reared under controlled conditions (12 h/12 h light/dark cycle, 22 ± 2°C) and allowed free access to standard food and purified water. The mice were allowed to acclimatize to the laboratory conditions for 7 d, and were randomly divided into the following six groups: Control, Model, PMS-L (12.5 mg/kg), PMS-M (25 mg/kg), PMS-H (50 mg/kg), and 5-ASA (100 mg/kg). Mice from all groups except the Control group received a 2.5% DSS solution *ad libitum* for 7 d to induce UC. PMS (0.1 mL/10 g body weight) was administered by gavage for 7 d, whereas those in the Control and Model groups were administered phosphate-buffered saline (PBS) by gavage. At the end of the experiment, the mice were euthanized by cervical dislocation to collect colon tissues, intestinal contents, and blood samples (Fig. 1B).

Evaluation of disease activity index

The mice were monitored daily during the experiment for body

weight, fecal pattern, rectal bleeding, and the occurrence of UC. The disease activity index (DAI) was used to assess overall disease severity. The specific scoring rules are listed in Table 1.

Histopathology

Colon tissues were fixed in 4% paraformaldehyde for 24 h. Hematoxylin and eosin (H-E) and periodic acid-Schiff (PAS) staining were performed on 5 μm sections. Histological scoring was performed using H-E-stained sections. Histological damage was scored using the criteria presented in Table 2.

Immunofluorescence

Immunofluorescence staining for claudin-3, occludin, and ZO-1 was performed on mouse colon tissues. Tissue sections underwent dewaxing and antigen retrieval, after which, endogenous enzymes were blocked and nonspecific binding was blocked using serum. The tissue sections were incubated with primary antibodies overnight in a refrigerator at 4°C. They were then incubated with secondary antibodies for 1 h at 37°C. Next, a working solution of 4',6-diamidino-2-phenylindole was added dropwise to the tissues, and the nuclei were counter-stained for 5 min at 37°C. Finally, an anti-fluorescent sealer was added dropwise to the sections and were observed under a light microscope.

Cell culture and activity assessment

Raw264.7 cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C and 5% CO₂. The effect of PMS on cell activity was assessed using the Cell Counting Kit-8 assay. Raw264.7 cells (1 × 10⁵) were inoculated in 96-well plates in six replicates, and the absorbance at 450 nm was assessed using a plate reader 24 h after the addition of PMS (10, 20, 30, 40, 50, or 100 μg).

Cellular lipopolysaccharide (LPS) induction and treatment

RAW264.7 cells were seeded in six-well plates overnight, then PMS was added and after 1 h, the cells were stimulated by adding LPS (1 μg/mL). The concentration of PMS was selected based on the maximum concentration without significant cytotoxicity in the cell viability assay. The cells were subjected to the experimental conditions as follows: Control, Model (1 μg of LPS) and PMS groups (30 μg of PMS + 1 μg of LPS). The supernatant of the cell culture medium and extracted cellular RNA and proteins were collected after 24 h for RT-qPCR, and western blotting.

Table 2
The scoring of Histological damage.

Goblet cell loss	Mucosal structure destruction	Muscle thickening	Inflammatory cell infiltration	Crypt abscess formation
Non-existence	Non-existence	Normal	Normal	Normal
Existence	Existence	Mild	Mild	Mild
		Moderate	Moderate	Moderate
		Severe	Severe	Severe

Table 3
Primer sequences for RT-qPCR.

Gene	Forward(5'→3')	Reverse(5'→3')
TNF-α	GGGCCATAGAAGTATGATGAGAGGG	TCCAGAAGTCCAGGCGGT
IL-1β	GTAGTGCAAGTTGTCTAATGGAACG	GCTACCTGTGTCTTCCCGTGA
IL-6	TTGTATGAACAACGATGATGCACT	TGTTCTTCATGTACTCCAGGTAGC
Claudin-3	AACTGCGTACAAGACGAGAC	ACCAGGACACCGGTACTAA
Occludin	TTATACTCCTGCAGACCTGCATCAA	GACGGACCCTGACCACTATGAAACA
ZO-1	CACTACAGTATGACCATCTCA	TTCTCTGTTCACACAGATAAGC
CBS	GTAGAGGACTTTGCAGACTTCG	CTGTGCTCAACGAGTCTGG
β-actin	TACCACCAGACGACCTGTGTT	GAGGCTCTTTCCAGCCTTCCTT

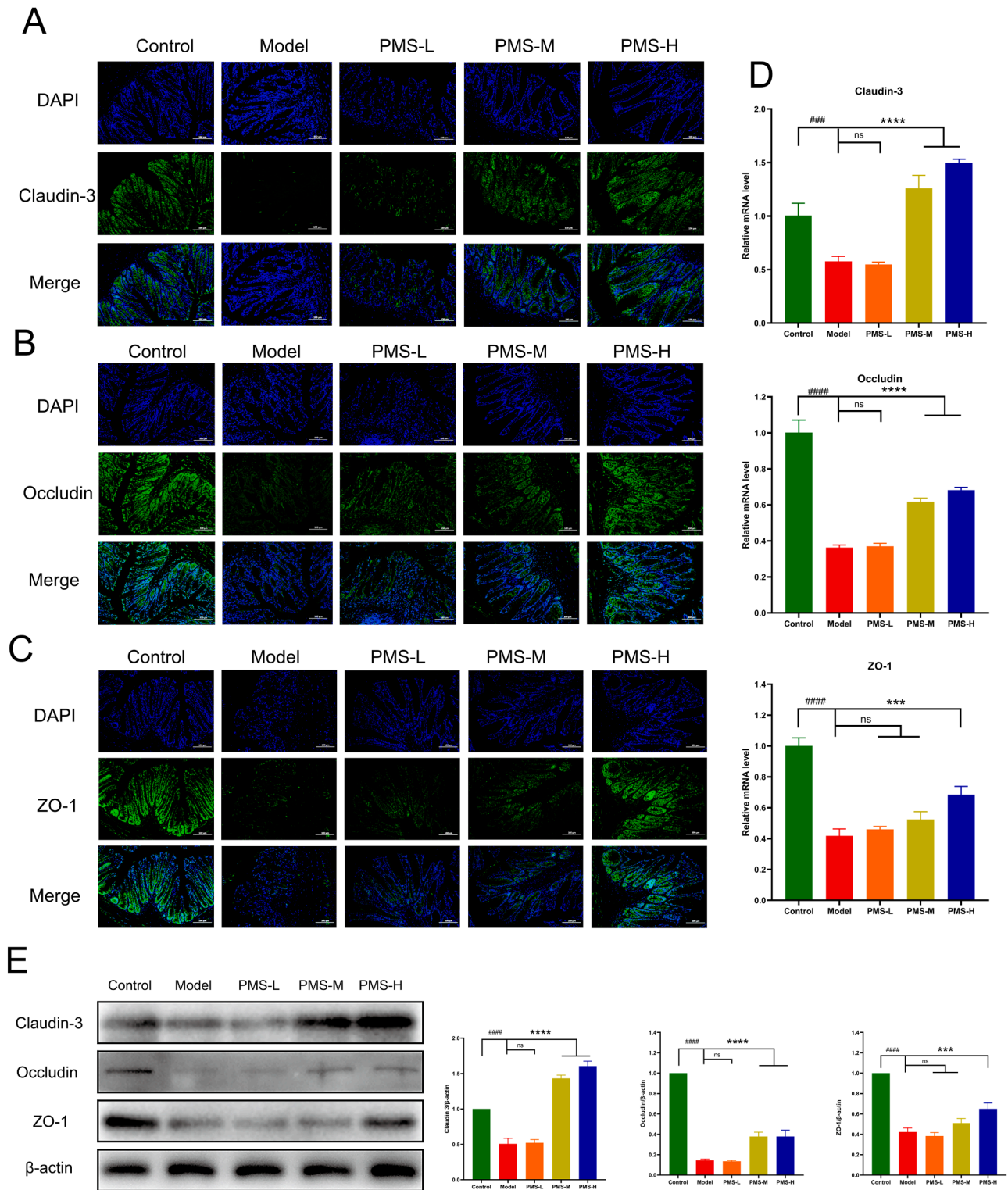


Fig. 2. Effects of PMS on the disruption of colon barrier integrity in mice with UC.

Representative immunofluorescence images of (A) claudin-3, (B) occludin, and (C) ZO-1 in colon tissues. (D) Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of claudin-3, occludin, and ZO-1 expression levels in colon tissues. (E) Representative bands of western blotting analysis of claudin-3, occludin, and ZO-1 in colon tissues and results of the gray-scale analysis of the corresponding proteins. β -actin served as the loading control.

All data are presented as the mean $\pm$ SEM (n = 5). * P < 0.05, ** P < 0.01, *** P < 0.001, and **** P < 0.0001 vs the Model group. # P < 0.05, ## P < 0.01, ### P < 0.001, and #### P < 0.0001 vs the Control group.

RT-qPCR

RT-qPCR was used to determine the expression levels of inflammatory factors (including *IL-1 β* , *IL-6*, and *TNF- α*), barrier proteins (claudin-3, occludin, and *ZO-1*), and *CBS* in colon tissues and cytokines (including *IL-1 β* , *IL-6*, and *TNF- α*) and *CBS* in RAW264.7 cells. The relative expression levels of the genes were calculated using the $2^{-\Delta\Delta C_t}$ method, with β -actin as the internal reference gene. Table 3 lists the primers used for RT-qPCR.

Enzyme-linked Immunosorbent Assay (ELISA)

IL-6, *IL-1 β* , and *TNF- α* levels in serum were detected using ELISAs. The test samples were sequentially added to a microtiter plate containing the antibody, and horseradish peroxidase (HRP)-labeled antibodies were bound to the test material to form an antibody-antigen-enzyme-labeled antibody complex. The plate was washed and stained with 3,3',5,5'-tetramethylbenzidine substrate, and the absorbance was measured at 450 nm.

Western blotting

Colon tissues and cells were ground and extracted using radio-immunoprecipitation assay lysis buffer, phenylmethylsulfonyl fluoride, a protease inhibitor, and phosphatase inhibitor. A bicinchoninic acid protein assay kit was used to measure the protein content and protein concentration was adjusted. The relative expression levels of proteins were measured using western blotting. Proteins were separated according to their molecular weight using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. Unoccupied sites on the membrane were blocked using a protein-free rapid blocking solution for 20 min. The membrane was incubated with the primary antibody overnight at 4°C and with an HRP-labeled secondary antibody for 1 h at 37°C. After the addition of the enhanced chemiluminescence reagent, the protein bands were visualized using a fully automated chemiluminescence analysis system. The relative optical density of the protein bands was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

16S rRNA sequencing

16S rRNA gene sequencing was performed on DNA from the feces of mice in the Control, Model, and PMS-H groups. Mouse intestinal contents were rapidly frozen and stored for subsequent detection of the gut microbiota. Total stool DNA was extracted, and the variable regions V3 and V4 of the bacterial 16S rRNA gene were amplified using the forward sequencing primer, 5'-CCTAYGGGRBGCASCAG-3', and reverse sequencing primer, 5'-GGACTACNNGGTATCTAAT-3'. Paired-end sequencing was conducted using a platform (Illumina, San Diego, CA, USA). Bioinformatics analysis was conducted using NovoMagic (Novogene, Beijing, China).

Transcriptomic analysis of colon tissue

Three mice were randomly selected from each of the Control, Model, and PMS-H groups. Total RNA was extracted from the colon tissues, RNA purity was determined using UV spectrophotometry, and RNA integrity was determined using gel electrophoresis. After mRNA was removed using oligo magnetic bead enrichment and random fragmentation, cDNA was synthesized using reverse transcriptase. The cDNA was then subjected to 2×150 bp paired-end sequencing on a Novaseq 6000 platform (Illumina). Bioinformatics analysis was conducted using NovoMagic. Raw sequencing reads were filtered to obtain clean reads, and differential gene expression analysis was performed using the R package to calculate fold change and *p*-values. Differentially expressed

genes (DEGs) were identified based on $P < 0.05$ and $|\log_2 \text{fold change [FC]}| > 2$.

FMT trial

Ten male C57BL/six donor mice were randomly divided into two groups: Control and PMS. Over 2-weeks, gavage with PBS was administered to the Control group, and PMS (50 mg/kg) was administered to the PMS group. On day 14, fresh stool samples were collected from the donor mice. The feces from each donor group were prepared into bacterial suspensions and frozen at -80°C for FMT.

Fifteen male C57BL/6J recipient mice were administered an antibiotic cocktail (Vancomycin 100 mg/kg, Neomycin sulfate 200 mg/kg, Metronidazole 200 mg/kg, and Ampicillin 200 mg/kg) for 14 d to induce gut microbiota depletion. The mice were then divided into three groups: Model, FMT-CON, and FMT-PMS. All mice received 2.5% DSS solution for 7 d. Subsequently, fecal microbiota from the Control and PMS donors were administered via gavage to the FMT-CON and -PMS groups, respectively (Fig. 6A). At the end of the experiment, the mice were euthanized by cervical dislocation to collect colon tissues.

Statistical analysis

Statistical analyses were performed using Prism 9.0 (GraphPad, San Diego, CA, USA). The Kruskal–Wallis test was used to identify differences in relative microbial taxa abundance between groups. For all other datasets, one-way analysis of variance was performed for multiple comparisons, followed by Tukey's post hoc test. Differences were considered significant when $P < 0.05$.

Results

PMS attenuated the symptoms and histological damage in mice with UC

Mouse UC models were established to explore the therapeutic effects of PMS (chemical structure shown in Fig. 1A). Mice in the Model group exhibited significant weight loss (Fig. 1C) accompanied by severe colitis, increased DAI scores (Fig. 1D), and reduced colon length (Fig. 1E–F). PMS treatment (12.5, 25, and 50 mg/kg) significantly abolished the body weight loss, decreased the DAI scores, and ameliorated DSS-induced colon shortening. These data suggested that PMS effectively alleviated DSS-induced colitis in mice. H-E staining (Fig. 1G–H) demonstrated that mice in the Control group exhibited an intact colon epithelium, normal tissue architecture, orderly glandular arrangement, and no inflammatory cell infiltration. In contrast, those in the Model group displayed disrupted mucosa, disorganized glands, and intense infiltration of inflammatory cells. PMS treatment restored intact tissue and ordered glandular structures, reduced inflammatory infiltration, significantly alleviated colon tissue damage, and improved the histopathological scores. PAS staining (Fig. 1I–J) showed that mice in the Model group exhibited a reduced number of goblet cells. After PMS treatment, the apoptosis of goblet cells was reduced.

PMS mitigated intestinal mucosal barrier damage in mice with UC

Immunofluorescence analysis of mouse colon tissue sections demonstrated that the fluorescence intensity of claudin-3, occludin, and *ZO-1* was downregulated in the Model group compared to that of the Control group, but treatment with PMS reversed these pathological changes (Fig. 2A–C). RT-qPCR and western blotting of mouse colon tissue were employed for further verification. Claudin-3, occludin, and *ZO-1* were downregulated in the Model group compared to that of the Control group, but treatment with PMS reversed these pathological changes (Fig. 3D–E).

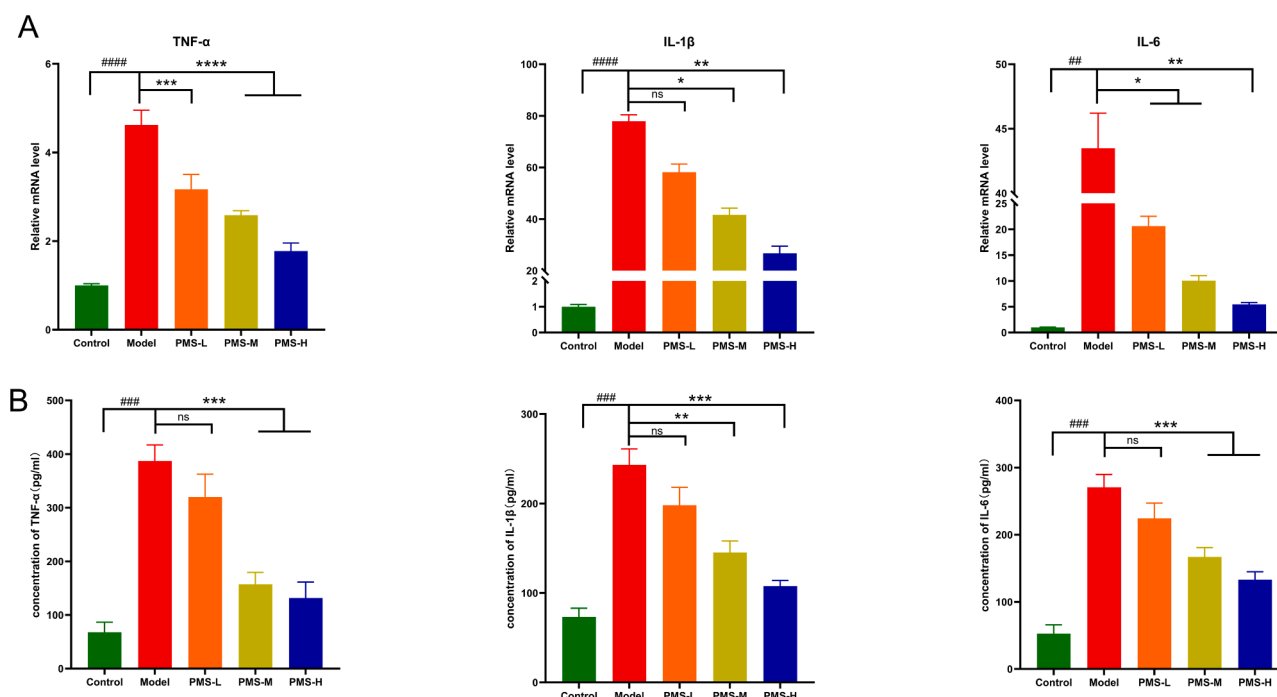


Fig. 3. Effects of PMS on inflammatory cytokine levels in colon tissues of mice with UC.

The expression levels of inflammatory cytokines (TNF-α, IL-1β, and IL-6) in colon tissues were determined using (A) RT-qPCR and (B) enzyme-linked immunosorbent assays (ELISAs).

All data are presented as the mean ± SEM (n = 5). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001 vs the Model group. #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001, and ####*P* < 0.0001 vs the Control group.

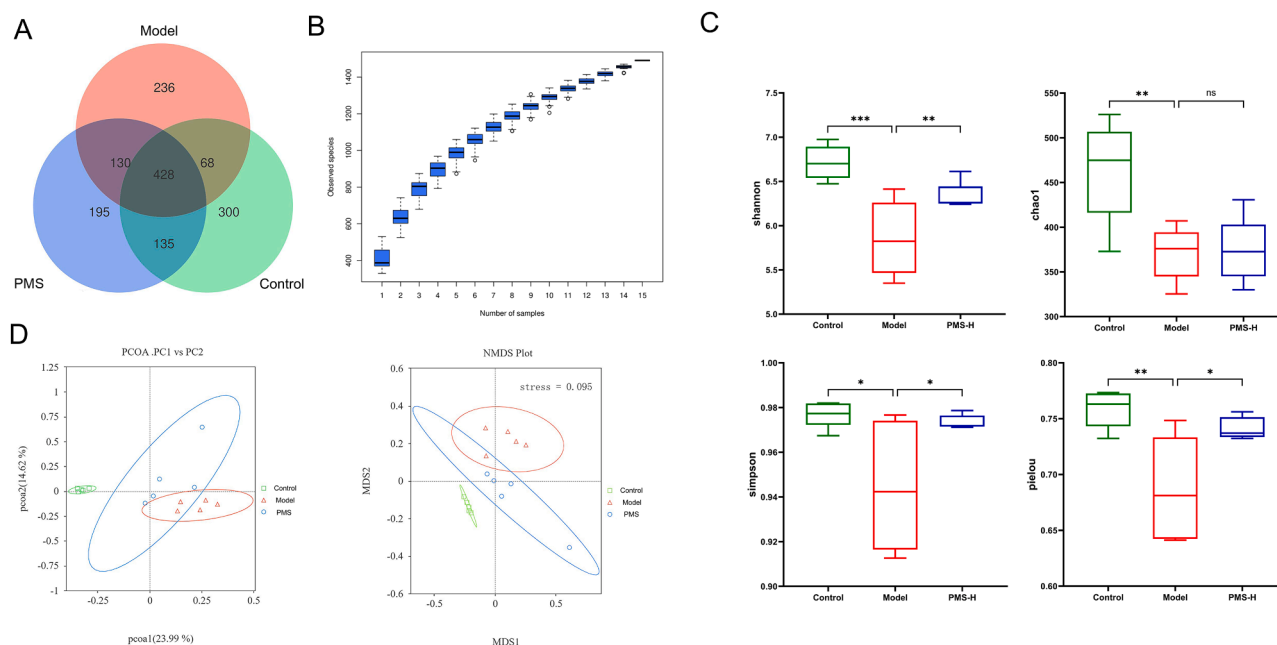


Fig. 4. Effects of PMS on gut microbiota diversity in mice with UC.

(A) Changes in the operational taxonomic units (OTUs) in each group. (B) Boxplot of species accumulation. (C) Alpha diversity analysis based on observed Shannon, Chao1, Simpson, and Pielou index values. (D) Beta diversity analysis, including principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) analysis.

All data are presented as the mean ± SEM (n = 5). **P* < 0.05 and ***P* < 0.01.

PMS reduced the inflammatory responses in mice with UC

The RT-qPCR results (Fig. 3A) demonstrated that the levels of TNF-α, IL-1β, and IL-6 in the colon were significantly higher in the Model group

than that of the Control group. PMS treatment significantly suppressed the levels of TNF-α, IL-1β, and IL-6 in a dose-dependent manner. Similarly, the ELISA results (Fig. 3B) confirmed that the serum levels of TNF-α, IL-1β, and IL-6 were higher in the Model group, but were reduced in a

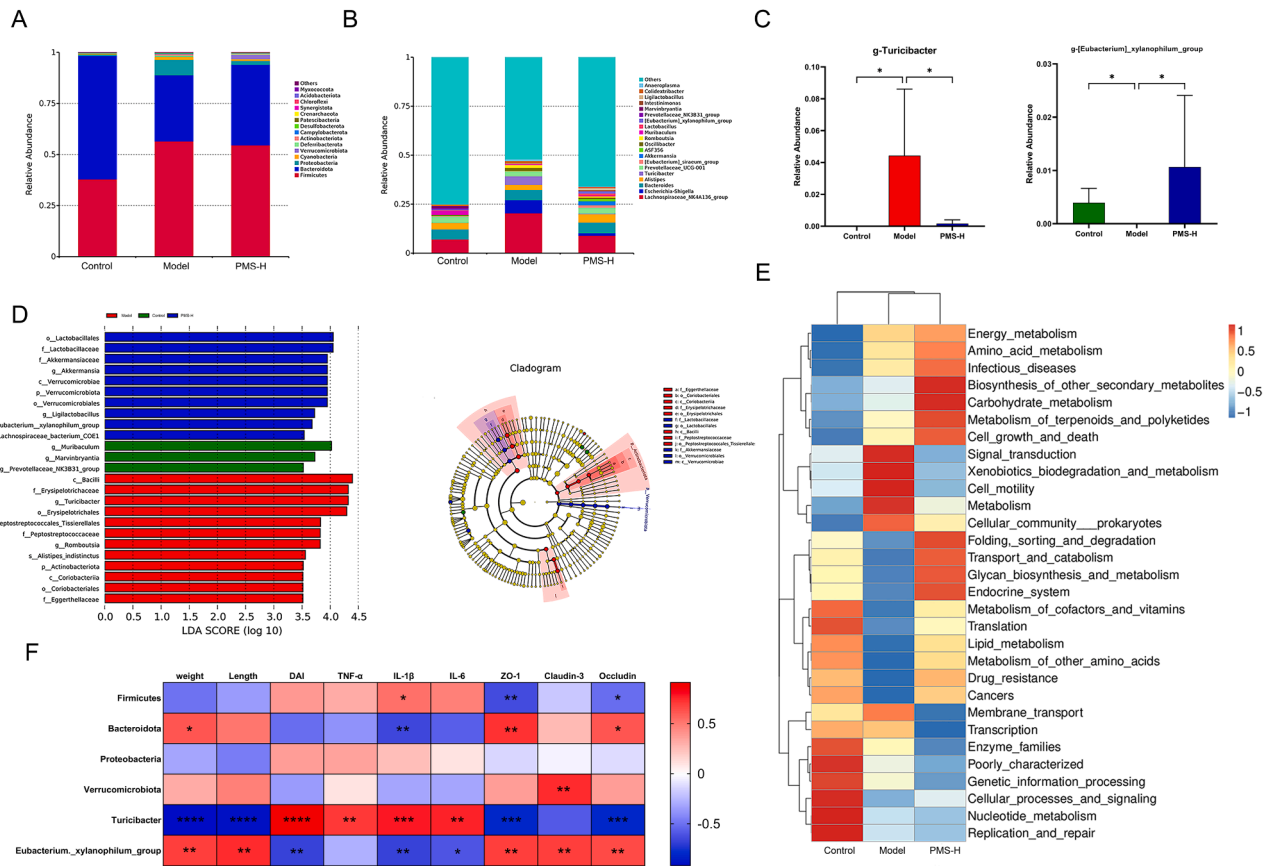


Fig. 5. Effects of PMS on gut microbiota composition in mice with UC.

Relative abundance of components of the gut microbiota at the (A) phylum and (B) genus level in each group. (C) Differences in the relative abundance of specific bacteria. (D) Linear discriminant analysis (LDA) effect size (LEfSe) (LDA >3.5, $P < 0.05$). (E) Tax4fun functional annotation clustering heatmap, where red and blue represent higher and lower relative abundance of functions, respectively, in each group. (F) Correlations among gut microbiota abundance, pro-inflammatory mediator levels, colitis index values, and tight junction protein levels based on Spearman's rank correlation analysis. The red color represents a positive correlation, and the blue color represents a negative correlation.

All data are presented as the mean $\pm$ SEM ($n = 5$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

dose-dependent manner by PMS treatment.

Microbial diversity analysis

To investigate the effect of PMS on the gut microbiota composition of mice with UC, 16S rRNA gene sequencing was conducted on DNA extracted from fecal samples collected from the Control, Model, and PMS-H groups. The relative distribution of bacterial operational taxonomic units (OTUs) among the groups is presented as a Venn diagram (Fig. 4A). Notably, 430 shared OTUs were identified among the three groups, with 300, 262, and 182 unique OTUs in the Control, Model, and PMS-H groups, respectively. The species accumulation boxplot demonstrated a sufficient sample size and convincing data analysis (Fig. 4B). Alpha diversity analysis revealed that the Chao1 (species richness), Shannon (diversity), Simpson (diversity), and Pielou (evenness) index values were significantly decreased in the Model group relative to that of the Control group. However, PMS treatment significantly restored the Shannon, Simpson, and Pielou index values (Fig. 4C), demonstrating that microbial diversity and evenness were markedly improved. Further the analysis of beta diversity revealed clustering of the gut microbiota composition within each group and clear separation between the groups (Fig. 4D).

PMS modulated the gut microbiota composition in mice with UC

At the phylum level, the gut microbiota mainly comprised

Bacteroidota, Firmicutes, Proteobacteria, and Verrucomicrobiota. Notably, compared with the Control group, the Model group showed an increased abundance of Firmicutes and Proteobacteria, but a decreased abundance of Bacteroidota and Verrucomicrobiota. PMS treatment restored the relative abundance of Bacteroidota and Verrucomicrobiota and decreased the abundance of Firmicutes and Proteobacteria (Fig. 5A). At the genus level, compared with the Control group, the Model group showed increased abundance of *Turicibacter* and decreased abundance of *Eubacterium_xylanophilum_group*. The changes induced by the UC model were alleviated by PMS treatment (Fig. 5B–C). Linear discriminant analysis effect size identified *Ligilactobacillus*, *Akkermansia*, and *Eubacterium_xylanophilum_group* as the dominant taxa in the PMS-H group, and *Turicibacter* and *Romboutsia* as the dominant taxa in the Model group (Fig. 5D). Tax4Fun analysis revealed that signal transduction, biodegradation, cell motility, and metabolism were elevated in the model group, but reduced in the PMS-H group. Conversely, functions such as folding, classification and degradation, transport and catabolism, vitamin metabolism, lipid metabolism, and amino acid metabolism decreased significantly in the model group, whereas these functions were improved in the PMS-H group (Fig. 5E). Spearman's rank correlation analysis suggested that *Turicibacter* abundance was positively correlated with inflammatory factor levels and the DAI score, and negatively correlated with body weight, colon length, and barrier protein levels in mice. Additionally, *Eubacterium_xylanophilum_group* showed a trend in the opposite direction to *Turicibacter*.

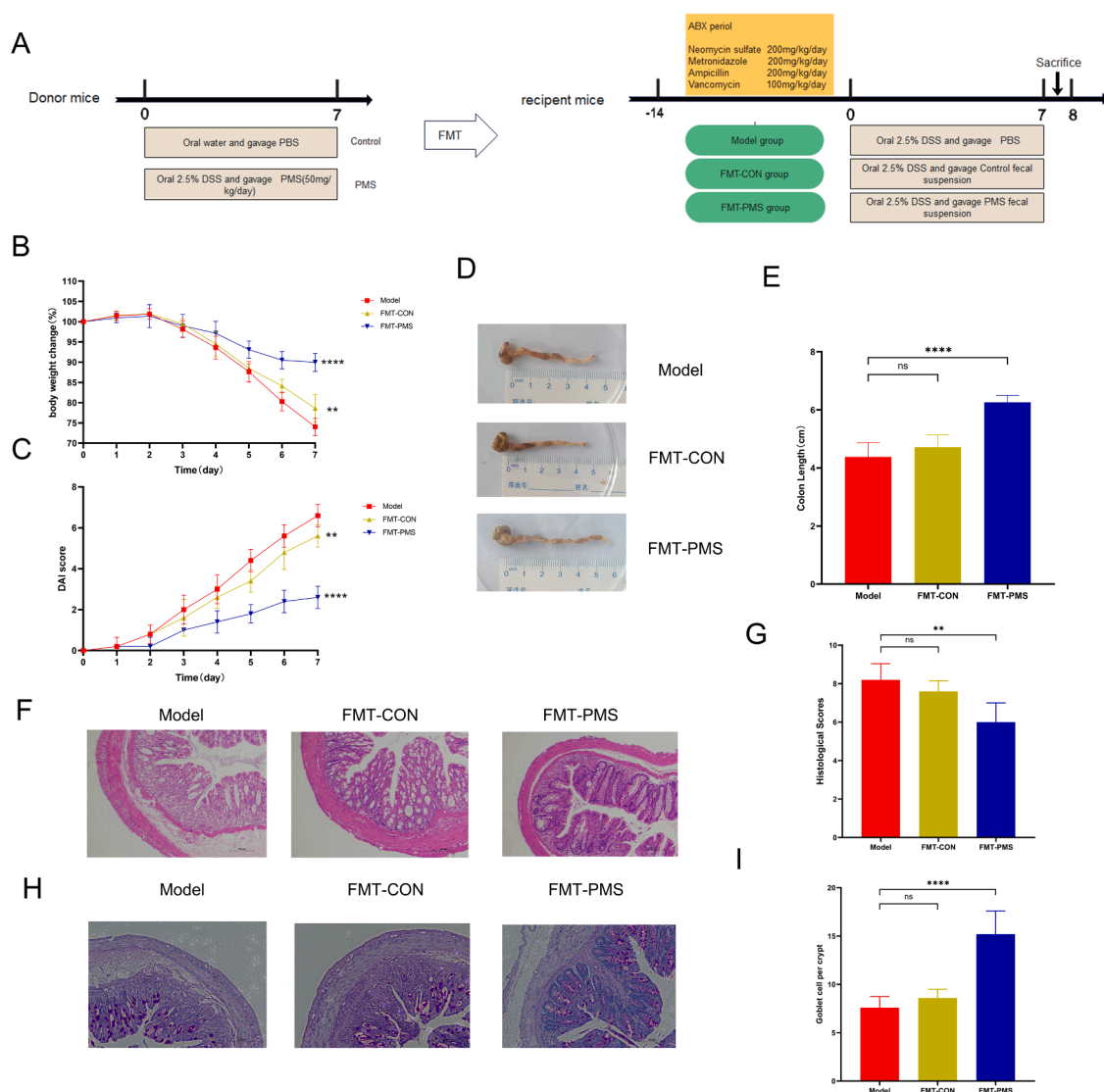


Fig. 6. Effects of FMT on symptoms and pathology in mice with UC.

(A) Schematic diagram of the fecal microbiota transplantation (FMT) experimental design. (B) Changes in average body weight of the mice. (C) Changes in the disease activity index (DAI) values of the mice. (D) Representative images of colons in the treatment group. (E) Changes in colon length. (F) Representative H-E-stained histological images of colon tissues in each group. (G) Pathological damage scores of colon tissues. (H) Representative PAS-stained histological images of colon tissues in each group. (I) Number of goblet cells.

All data are presented as the mean $\pm$ SEM (n = 5). *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

FMT alleviated the symptoms and histological damage in mice with UC

To further confirm the role of the gut microbiota in the therapeutic effects of PMS on UC, FMT experiments were performed. The FMT-PMS group experienced less weight loss (Fig. 6B), lower DAI scores (Fig. 6C), and reduced colon shortening than the Model group (Fig. 6D–E). Analysis of the H-E staining results showed that the FMT-PMS group had significantly reduced histopathological damage to the colon compared to that of the Model group (Fig. 6F–G). PAS staining revealed that the FMT-PMS group effectively maintained the abundance of goblet cells in the colonic epithelium (Fig. 6H–I). However, compared to the Model group, the FMT-CON group only showed reduced weight loss and lower DAI scores, with no significant differences in colon length, histological score, or goblet cell numbers.

Transcriptomic analysis of the effects of PMS treatment in mice with UC

To elucidate the mechanisms underlying the therapeutic effects of

PMS against UC, transcriptomic sequencing of colon tissues was performed to explore changes in gene expression. DEGs ($P < 0.05$, $|\log_2FC| > 2$) exhibited close clustering of individual samples within each group and a clear separation between the groups (Fig. 7A). Compared with the Control group, 1,319 DEGs were detected in the Model group, of which 592 were upregulated and 727 were downregulated. Compared with the Model group, 1,060 DEGs were identified in the PMS-H group, of which 549 were upregulated and 511 were downregulated (Fig. 7B). Overall, 385 DEGs upregulated and 338 DEGs downregulated in the Model group compared with that of the Control and PMS-H groups (Fig. 7C). Gene Ontology (GO) enrichment analysis of these 723 DEGs revealed that they were significantly enriched in pathways associated with leukocyte migration, cytokine activity, and receptor regulator activity (Fig. 7D). Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis indicated significant enrichment of cytokine-cytokine receptor interactions, IL-17, TNF- α , and NF- κ B signaling pathways in pathways associated with inflammation (Fig. 7E). Among the identified DEGs, *CBS* was most significantly differentially expressed between the PMS-H and

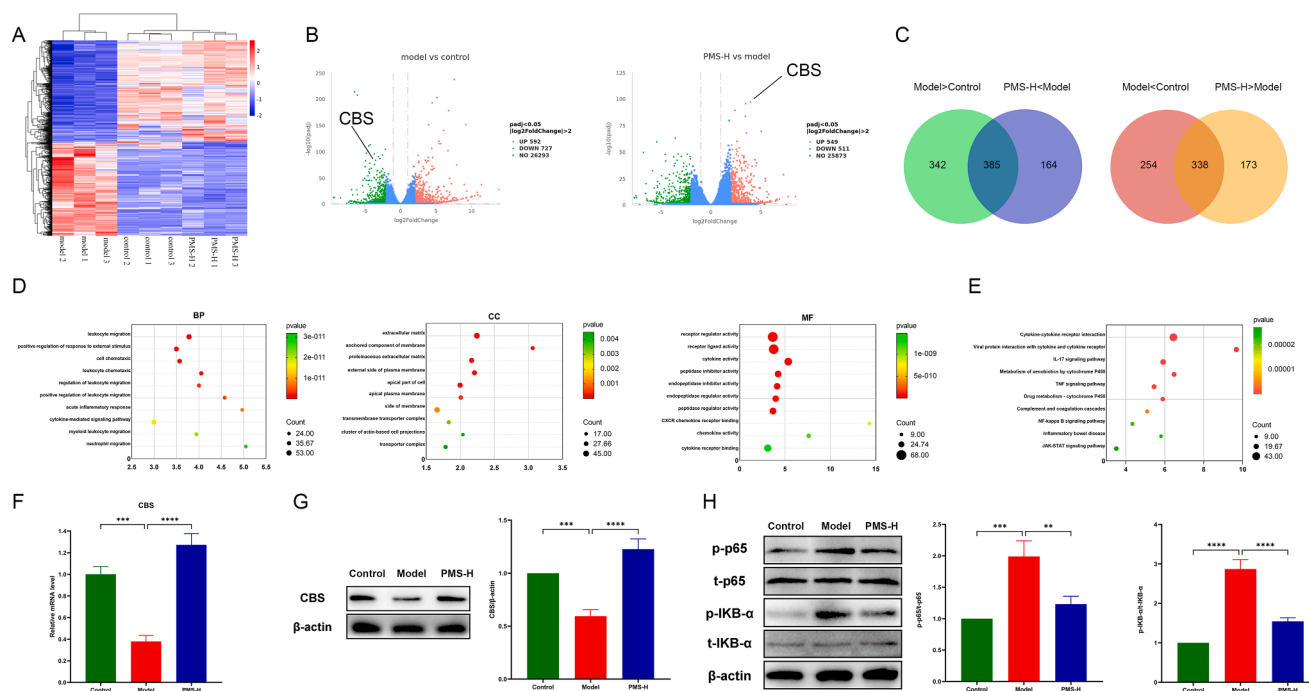


Fig. 7. Transcriptomic analysis of colons in mice with UC treated with PMS.

(A) Clustering heatmap of differentially expressed genes (DEGs). (B) Volcano plot of DEGs. The red dots represent upregulated genes, the green dots represent downregulated genes, and the blue dots represent genes with no significant change. (C) Venn diagram of DEGs. (D) Gene Ontology (GO) analysis of the biological process (BP), cellular component (CC), and molecular function (MF) categories. (E) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. (F) RT-qPCR analysis of CBS expression levels in colon tissues. (G) Representative bands of western blotting analysis of CBS in colon tissues and the results of the gray-scale analysis of the corresponding proteins. β -actin served as the loading control. (H) Representative bands of western blotting analysis of p65, p-p65, I κ B, and p-I κ B in colon tissues and the results of the gray-scale analysis of the corresponding proteins. β -actin served as the loading control. All data are presented as the mean $\pm$ SEM ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

Model groups, suggesting that it may contribute to the modulation of the therapeutic effects of PMS. RT-qPCR and western blotting of colon tissue samples demonstrated that CBS expression levels were significantly lower in the Model group than that of the Control group, whereas PMS treatment significantly upregulated CBS expression levels (Fig. 7F–G). Western blotting of colon tissue further confirmed that the levels of NF- κ B pathway proteins, including phosphorylated NF- κ Bp65 and I κ B, were significantly elevated in the Model group relative to that of the Control group; however, PMS treatment suppressed these changes (Fig. 7H).

PMS inhibited LPS-induced inflammation in macrophages

Cell viability tests of PMS-treated RAW264.7 macrophages demonstrated no toxicity when applied in the dose range of 10–30 μ g/mL (Fig. 8A). As shown by RT-qPCR analysis, PMS strongly alleviated the LPS-induced increase in the levels of pro-inflammatory cytokines *TNF- α* , *IL-1 β* , and *IL-6* (Fig. 8B). RT-qPCR and western blotting validation demonstrated that CBS expression levels were significantly lower in the Model group compared to that of the Control group, whereas PMS treatment significantly upregulated CBS expression levels (Fig. 8C–D). Further analysis of proteins involved in the NF- κ B signaling pathway in RAW264.7 cells revealed that PMS significantly inhibited the LPS-induced phosphorylation of NF- κ Bp65 and I κ B (Fig. 8E).

Discussion

Recently, significant progress has been made in understanding the pathogenesis and treatment of UC. However, the prolonged use of biologics carries risks such as nausea, vomiting, abdominal pain, and even infections. Moreover, the high cost of biologics imposes a substantial financial burden on the patients. Therefore, the development of

efficient, safe, and cost-effective drugs for the treatment of UC is of great importance.

Natural bioactive compounds have emerged as key sources of novel therapies for UC. Several studies have demonstrated the anti-UC potential of these compounds. For instance, syringaresinol exerted therapeutic effects against UC through its anti-inflammatory properties, ability to repair the colonic mucosal barrier, and modulation of the gut microbiota (Liu et al., 2024). Similarly, resveratrol reduces intestinal inflammation by inhibiting protein kinase phosphorylation, thereby effectively treating UC (Erol Doğan et al., 2024).

Plantago spp. are medicinal, food homologous plants. In traditional Chinese medicine, they are known to have expectorant, antidiarrheal, diuretic, hypoglycemic, immunomodulatory, antioxidant, and hypolipidemic effects. As the main active ingredient of *Plantago* major, PMS comprises two sugar groups, a caffeic acid portion and hydroxytyrosol fragment. It belongs to the phenylethanolic glycoside class of compounds, and has various anti-oxidative stress, anti-inflammatory, and anti-tumor effects, playing roles in the treatment of disparate diseases, such as osteoarthritis and cardiac muscle injury. PMS demonstrates favorable pharmacokinetics (Li et al., 2014) and minimal toxicity (Park et al., 2007) in preclinical models. In a two-week toxicity study in healthy C57BL/6 mice (50 mg/kg PMS), hematological parameters and liver function markers remained within normal ranges, as shown in Supplementary Table S1. PMS treatment significantly ameliorated weight loss, colon shortening, elevated DAI scores in mice with UC in a dose-dependent manner, and reversed the changes in the intestinal epithelial barrier and intestinal flora. Thus, PMS is an effective drug for the treatment of UC.

Gut barrier disruption is a key factor in the progression of UC. Tight junction proteins, such as occludin, claudin-3, and ZO-1, are the main factors that maintain the intestinal epithelial barrier. The restoration of

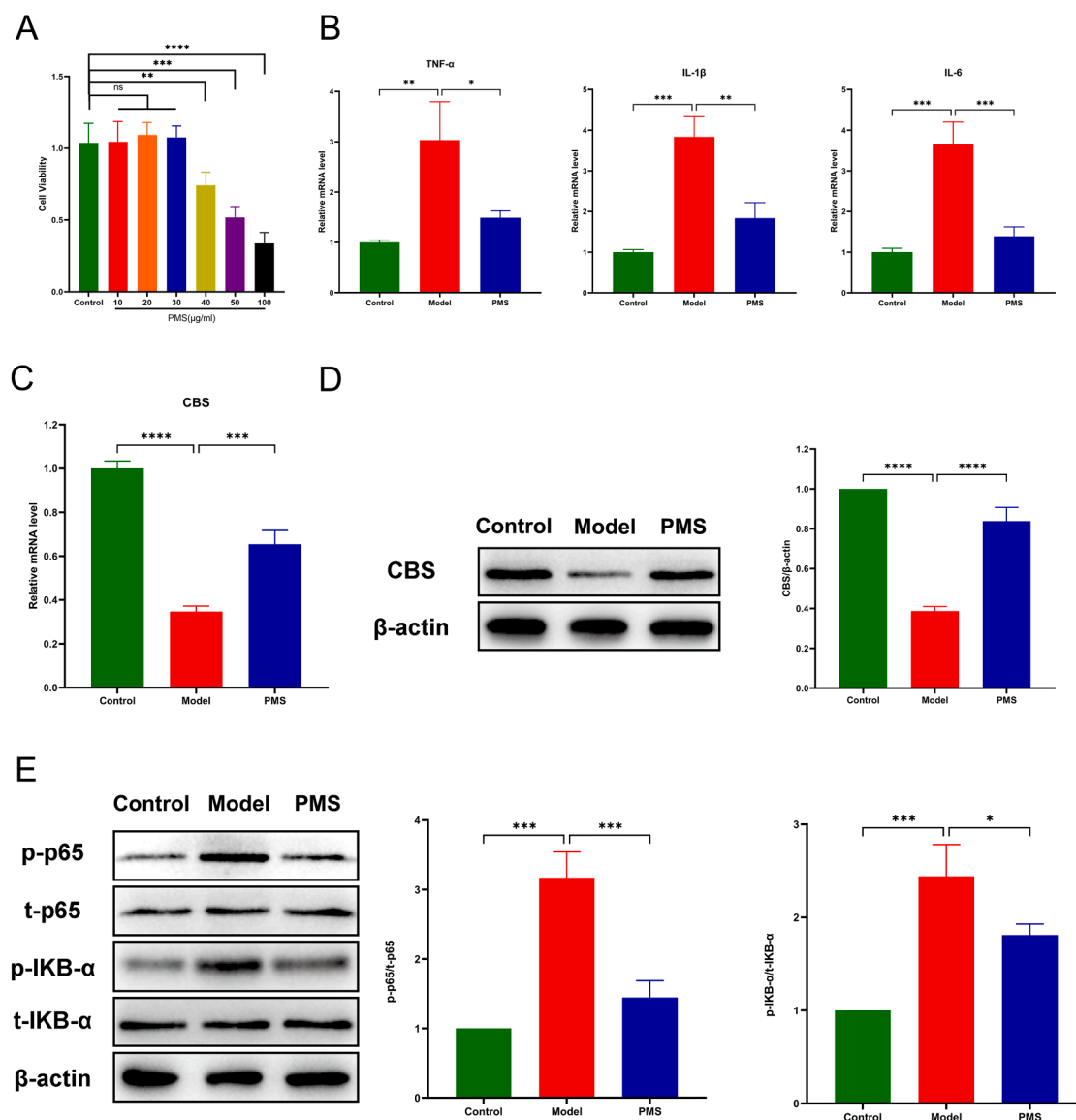


Fig. 8. PMS inhibited the production of pro-inflammatory cytokines and regulated CBS and NF-κB signaling in LPS-treated RAW264.7 cells. (A) Effects of gradient concentrations of PMS on the viability of RAW264.7 cells. (B) RT-qPCR analysis of the expression levels of inflammatory cytokines (*TNF-α*, *IL-1β*, and *IL-6*) in cells. (C) RT-qPCR analysis of *CBS* expression levels in cells. (D) Representative bands of western blotting analysis of CBS in cells and the results of the gray-scale analysis of the corresponding proteins. β-actin served as the loading control. (E) Representative bands of western blotting analysis of p65, p-p65, IκB, and p-IκB in cells and the results of the gray-scale analysis of the corresponding proteins. β-actin served as the loading control. All data are expressed as the mean ± SEM (n = 3). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

intestinal tight junctions is closely related to intestinal mucosal healing in patients with UC, and is a key indicator of the effectiveness of drug therapy (Kuo et al., 2021). PMS was found to effectively upregulate the expression of tight junction proteins in mice with UC and significantly ameliorated the increased intestinal permeability in the Model group, as evidenced by the immunofluorescence staining, RT-qPCR, and western blotting results. An increasing number of studies have identified tight junction proteins as novel targets for the prevention of UC. Qu et al. (2021) found that intestinal occludin and ZO-1 expression levels were reduced in mice with UC, and the administration of kaempferol restored their expression levels. Yang et al. (2024) found that pine conjugoside was effective at alleviating colonic injury; inhibiting inflammatory factor release; and restoring the tight junction proteins occludin, claudin-3, and ZO-1. PMS attenuated intestinal barrier damage in mice with UC, and the mechanism may be related to the inhibition of intestinal epithelial cell apoptosis and increased expression levels of tight junction proteins.

Compared with the Control group, the Model group showed increased abundance of intestinal Firmicutes and Proteobacteria along with decreased abundance of Bacteroidota and Verrucomicrobiota, but PMS treatment restored the relative abundance of Bacteroidota and Verrucomicrobiota and decreased the abundance of Firmicutes and Proteobacteria. Caffeic acid supplementation has previously been shown to alleviate the symptoms of colitis in mice, decrease the ratio of Firmicutes and Proteobacteria, and increase the abundance of Verrucomicrobiota (Zhang et al., 2016), which corresponds with our results. Members of Bacteroidota are involved in polysaccharide, bile acid, and steroid metabolism as well as produce β-glycoside hydrolase, which is negatively correlated with the symptoms of UC (Chen et al., 2024). The relationship between Firmicutes and UC is controversial, and some Firmicutes members promote intestinal inflammation through the capsule (Zhang et al., 2022). Some studies have also reported that some Firmicutes members can produce short-chain fatty acids (SCFAs), which have anti-inflammatory and lipid-lowering functions (Louis and Flint,

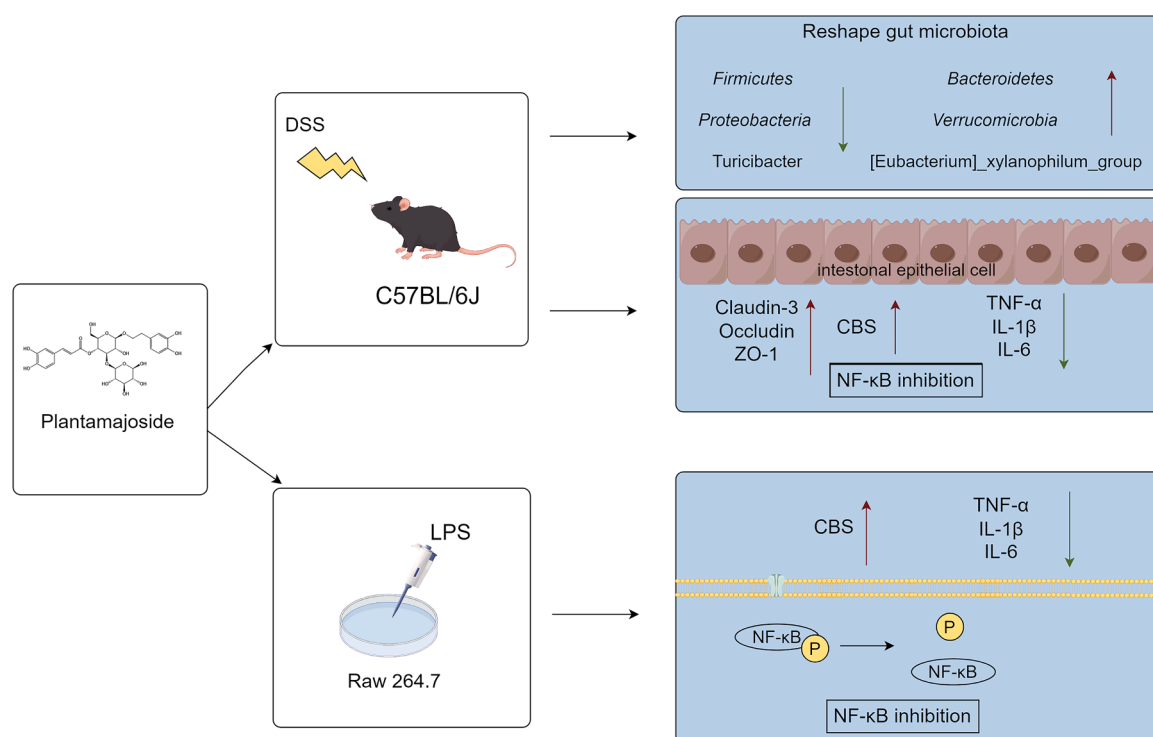


Fig. 9. Schematic of the effects and mechanisms of plantamajoside bioactivity in mice with DSS-induced UC and LPS-treated RAW264.7 cells. This figure was created using figdraw.com.

2017). Proteobacteria includes various conditionally pathogenic bacteria, such as *Escherichiaceae* and *Shigella* spp. The outer membrane is mainly composed of LPSs, and the increase in the abundance of intestinal Proteobacteria in the Model group reflected the energy imbalance of the host, which is positively correlated with susceptibility to UC (Rizzatti et al., 2017). The increase in the relative abundance of Verrucomicrobiota in the PMS-H group was mainly due to *Akkermansia*, which degrades mucus proteins and enhances the integrity of the intestinal epithelium by remodeling the mucus layer and attenuating immune response to ameliorate colitis (Zhang et al., 2019).

Turicibacter spp. are considered pro-inflammatory bacteria, and they typically exhibit a high abundance in patients with UC. A high abundance of *Turicibacter* has been found to exacerbate intestinal damage and lead to serious complications in animal models of UC (Dong et al., 2022). *Turicibacter* is associated with the development of colorectal cancer by potential mechanisms that include the promotion of chronic inflammation, DNA damage, and production of bioactive oncogenic metabolites (Chung et al., 2021). Herein, *Turicibacter* was enriched in the Model group, whereas PMS inhibited this increase in abundance. *[Eubacterium]_xylanophilum_group* is a group of bacteria that metabolizes SCFAs and contributes to intestinal health (Liu et al., 2024). SCFAs are important intestinal flora molecular mediators that affect the immune function of the host, enhance the barrier function of intestinal epithelial cells, and modulate the host mucosal immune system (Ratajczak et al., 2019). In the present study, PMS significantly increased the abundance of *[Eubacterium]_xylanophilum_group* in mice with UC, which is consistent with the findings of Liu et al. (2023), who showed that the administration of *Rhodiola rosea* glycosides in mice with UC improved its symptoms by increasing the relative abundance of *[Eubacterium]_xylanophilum_group* and butyric acid content. To further investigate the effects of changes in the structure and abundance of the intestinal flora in mice with UC following PMS treatment, we performed Tax4Fun analysis and found that the intestinal flora of mice in the PMS-H group were primarily enriched in vitamin metabolism, lipid metabolism, and amino acid metabolism. Many studies have shown that altering the levels of gut

flora metabolites is a potential treatment for UC. Tea polyphenols can increase the abundance of bacteria associated with SCFAs and levels of the metabolites, propionic and butyric acids, in mice with colitis, thereby inhibiting the production of inflammatory factors in the intestine and restoring tight junction protein levels (Wu et al., 2021). Ginsenoside Rg1 alleviates UC by modulating the gut microbiota and influencing key metabolic pathways, such as tryptophan metabolism; valine, leucine, and isoleucine biosynthesis; and vitamin B6 metabolism (Cheng et al., 2022). Therefore, PMS may play a therapeutic role in UC by regulating metabolites produced by the intestinal flora. Spearman's correlation analysis suggested that the abundance of *Turicibacter* was positively correlated with inflammatory factor levels and DAI score, as well as negatively correlated with body weight, colon length, and barrier protein levels in mice, whereas the correlations with the abundance of *[Eubacterium]_xylanophilum_group* trended in the opposite direction. This further illustrated that an imbalance in the intestinal flora is correlated with UC, and PMS improve colitis by optimizing the structure and species composition of the intestinal flora.

CBS is an important enzyme for endogenous H₂S production (Omorou et al., 2022). Using *in vivo* and *in vitro* experiments, we verified that CBS mRNA and protein expression levels were significantly reduced in the Model group compared to that of the Control group, and PMS treatment significantly increased CBS mRNA and protein expression levels. Reduced CBS expression levels in mice with UC suggests that CBS is involved in UC pathogenesis. An increasing number of studies demonstrated the therapeutic role of the CBS-H₂S axis in UC, as H₂S promotes mucosal repair and accelerates the healing of gastrointestinal ulcers, in addition to attenuating the inflammatory response, and its anti-inflammatory mechanism of action includes inhibiting leukocyte recruitment, inhibiting the expression of various pro-inflammatory cytokines, promoting neutrophil apoptosis, and inhibiting the activity of peroxidases (Zuhra et al., 2020). One study reported that, in a murine UC model, the inhibition of CBS expression reduced the endogenous H₂S levels, aggravating the colon inflammation and mucosal damage (Chen et al., 2019). Similarly, in another study, the expression levels of

pro-inflammatory cytokines, such as IL-1 β , IL-6, and TNF- α , were elevated in mice with UC, and exogenous H₂S provided by NaHS significantly down-regulated the levels of the abovementioned inflammatory markers and improved the intestinal barrier integrity by up-regulating the expression levels of the tight junction proteins ZO-1 and occludin (Zhao et al., 2016).

PMS inhibited pro-inflammatory cytokine production by suppressing the LPS-induced activation of the NF- κ B signaling pathway in murine model of acute lung injury and RAW264.7 cells (Wu et al., 2016). This is consistent with our *in vivo* and *in vitro* data showing that DSS or LPS activated NF- κ B signaling, which was significantly inhibited by PMS treatment. Recent studies have highlighted the role of H₂S in modulating inflammatory responses through the inhibition of NF- κ B activation. Supplementation with NaHS effectively suppressed the NF- κ B signaling pathway and downstream NLRP3 inflammasome activation (Chen et al., 2021). In a related investigation, Éva Sikura et al. (2020) investigated the role of endogenous H₂S by silencing the expression of CBS, revealed that the reduction in endogenous H₂S levels promotes NF- κ B activation. Therefore, PMS may protect the intestines from inflammation by regulating CBS-H₂S to inhibit the NF- κ B signaling pathway. This suggests a new therapeutic strategy for UC.

In summary, the findings of this study demonstrated that PMS alleviates UC symptoms in mice, maintains intestinal barrier integrity, and reduces inflammation. PMS is a promising therapeutic agent for UC, and its effects are mediated through modulating the gut microbiota composition, upregulating CBS expression levels, and inhibiting NF- κ B signaling activation (Fig. 9). Further studies are required to elucidate the detailed mechanisms whereby PMS upregulates CBS and induces microbiota-mediated metabolic changes.

CRediT authorship contribution statement

Yongheng Jia: Writing – original draft, Investigation, Formal analysis, Data curation. **Xianjun Liu:** Writing – review & editing, Writing – original draft, Investigation. **Xinyi Gao:** Investigation. **Siyuan Yin:** Investigation. **Kun Wu:** Investigation. **Xianglong Meng:** Investigation. **Hui Ren:** Investigation. **Jiawei Liu:** Investigation. **Zijing Liu:** Investigation. **Hao Li:** Investigation. **Yang Jiang:** Writing – review & editing, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: <https://www.ncbi.nlm.nih.gov/sra/PRJNA120297>, <https://www.ncbi.nlm.nih.gov/sra/PRJNA1204481>.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.phymed.2025.156827](https://doi.org/10.1016/j.phymed.2025.156827).

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