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Article

Neohesperidin Attenuates DSS-Induced Ulcerative Colitis by Inhibiting Inflammation, Reducing Intestinal Barrier Damage, and Modulating Intestinal Flora Composition

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ABSTRACT: Flavonoid natural products are emerging as a promising approach for treating Ulcerative Colitis (UC) due to their natural origin and minimal toxicity. This study investigates the effects of Neohesperidin (NEO), a natural flavonoid, on Dextran Sodium Sulfate (DSS)-induced UC in mice, focusing on the underlying molecular mechanisms. Early intervention with NEO (25 and 50 mg/kg) mitigated colon shortening, restored damaged barrier proteins, and significantly reduced the inflammatory cytokine levels. Moreover, NEO inhibited the MAPK/NF-κB signaling pathway and enhanced the levels of intestinal barrier proteins (Claudin-3 and ZO-1). Additionally, NEO increased beneficial intestinal probiotics (*S24−7* and *Lactobacillaceae*) while reducing harmful bacteria (*Erysipelotrichi*, *Enterobacteriaceae*). Fecal microbial transplantation (FMT) results demonstrated that NEO (50 mg/kg) markedly improved UC symptoms. In conclusion, early NEO intervention may alleviate DSS-induced UC by inhibiting inflammatory responses, preserving intestinal barrier integrity and modulating gut microbiota.

KEYWORDS: *NEO, UC, barrier protein, Lactobacillaceae, intestinal flora composition*

INTRODUCTION

Inflammatory Bowel Disease (IBD) is a chronic, recurrent inflammatory condition that severely impairs intestinal function.[1] The global incidence of IBD has steadily risen over the past decade.[2] Currently, the high prevalence of Ulcerative Colitis (UC) has become a significant global health burden in both developed and developing countries.[3] Long-term pharmacological interventions, including corticosteroids and immunosuppressants, are commonly used but often result in adverse effects such as osteoporosis, type 2 diabetes, and depression.[4] Recently, plant extracts, as natural bioactive compounds with anti-inflammatory properties, have garnered increased attention due to their ability to modulate gut flora composition without imposing additional physiological burdens, positioning them as promising candidates for UC treatment now and in the future.[5] Utilizing plant extracts not only deepens our understanding of UC pathogenesis but also provides a theoretical foundation for their practical application in UC therapy.

The human gastrointestinal tract hosts trillions of bacteria that play a pivotal role in disease prevention and treatment. A balanced gut microbiota is essential for maintaining gut mucosal immunity, systemic immune homeostasis, and the integrity of the gut epithelial barrier, thereby reducing the risk of pathogenic microbial invasion.[6] Modulating the gut microbiota has been identified as an effective strategy for UC treatment, making it a critical therapeutic target.[7]

Flavonoids, widely distributed in natural products, are abundant and diverse.[8] Neohesperidin (NEO), a flavonoid predominantly found in Brassicaceae family plants and the roots and peels of citrus species, exhibits minimal toxicity and side effects.[9] Numerous studies have demonstrated NEO's antioxidant, anti-inflammatory, anticancer, and immunomodulatory properties.[10] Previous research has confirmed NEO's ability to prevent colon tumors and mitigate obesity by improving gut flora composition.[11] However, the specific mechanisms and targets of NEO in the chemical prevention and treatment of UC remain unclear. Given the global rise in UC incidence and the lack of effective treatments, further investigation into NEO's therapeutic potential for UC is warranted. Therefore, this study explored the mechanisms of NEO in a DSS-induced UC model and evaluated its therapeutic efficacy.

MATERIALS AND METHODS

Animal Experimentation. The study adhered to all relevant legal and regulatory guidelines and received approval from the Animal Care and Use Committee of Jilin University, Changchun, China (approval number SY202404004). Male C57BL/6J mice (aged 6−7 weeks; 20 ± 1 g; n = 30) were used, provided by Liaoning Changsheng Biotechnology Co. The mice were acclimatized with adequate food and water for one week prior to the experimental procedures. NEO with 98% purity, sourced from Chengdu Ruifenshi Biological Co., was used in the study. Two NEO concentrations (25 and 50 mg/kg) were selected based on preliminary studies. After the acclimation period, the mice were divided into five groups: Control, DSS (2.5%), NEO (50 mg/kg), and two DSS + NEO groups (25 and 50 mg/kg). NEO

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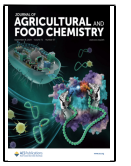

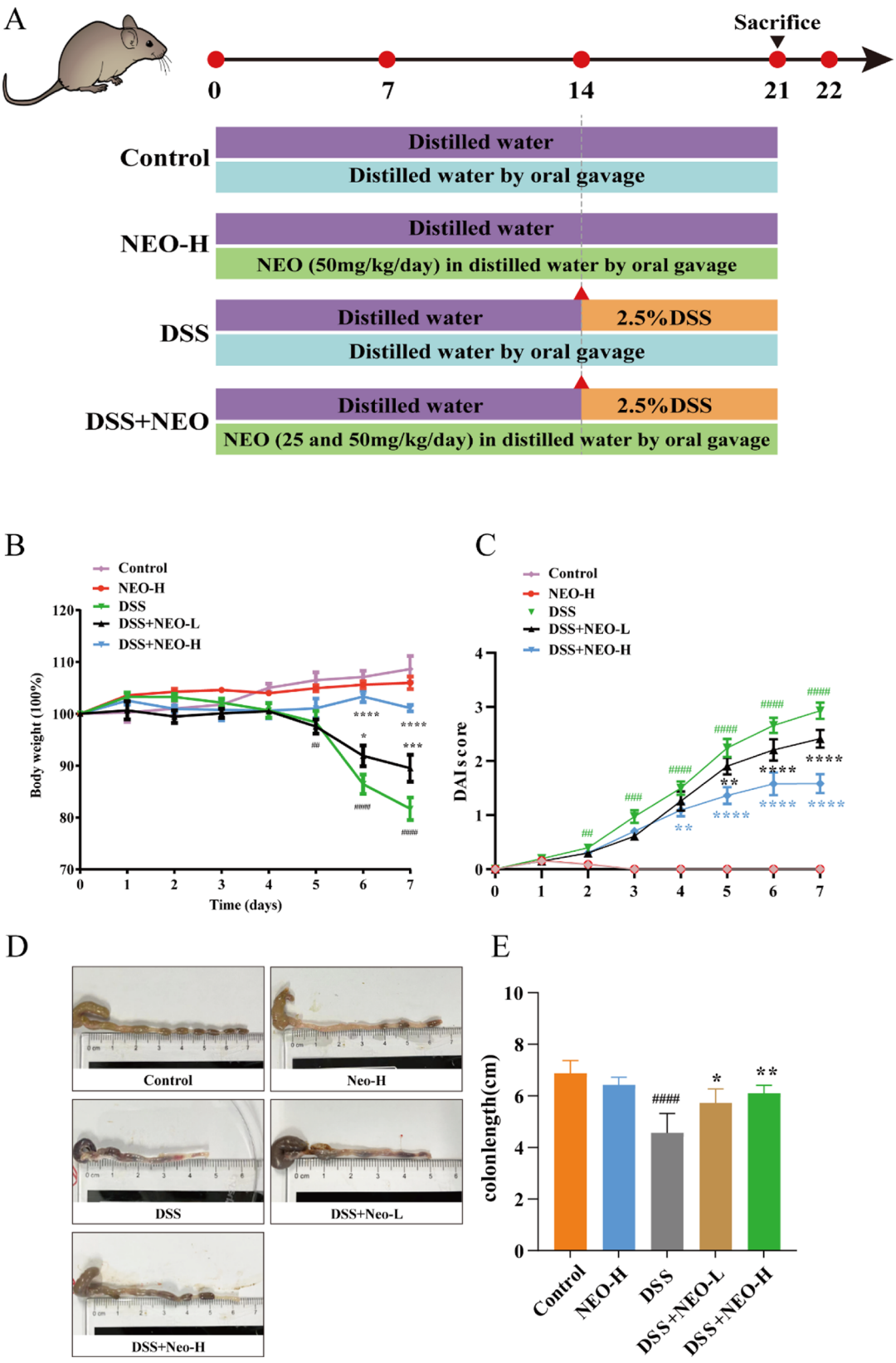

Figure 1. NEO's effect on DSS-induced UC inflammation in mice. (A) Flowchart of the experimental design. (B) Changes in body weight across groups during DSS induction. (C) DAI scores of mice in each group during DSS induction. (D) Photographs of colon length from each group during DSS induction. (E) Histograms showing colon length across groups, with corresponding analysis. Data are presented as mean ± SEM (n = 6). Significance levels: #P < 0.05, ##P < 0.01, ###P < 0.001, ####P < 0.0001 vs Control group; *P < 0.05, **P < 0.01, ****P < 0.0001 vs modeling group. H: high dose (50 mg/kg), L: low dose (25 mg/kg). CMC-Na: sodium carboxymethyl cellulose.

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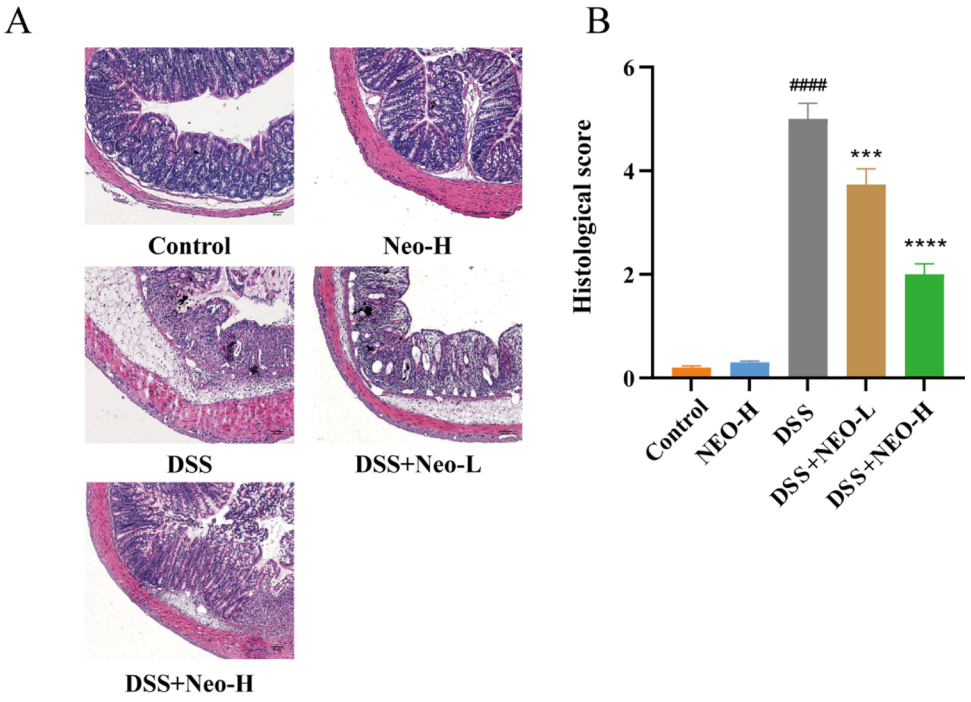

Figure 2. Pathologic changes in the colon of DSS-induced mice under NEO treatment. (A) Microscopic images of colon cross-sections at 100× magnification. (B) Histologic scores of colons in each group. Scale bar: 50 μm. Data are presented as mean ± SEM (n = 6). Significance levels: #P < 0.05, ##P < 0.01, ###P < 0.001, ####P < 0.0001 vs Control group; *P < 0.05, **P < 0.01, ****P < 0.0001 vs modeling group.

was dissolved in distilled water for administration. The experimental protocol involved administering NEO *via* gavage from day one, while DSS (2.5% w/v, obtained from MP Biomedical, California) was added to the drinking water from days 15 to 21 to induce UC in the mice. Throughout the 21-day period, the mice were monitored daily for dietary intake, body weight, and fecal physiology. On day 22, the mice were euthanized under anesthesia, and fecal and colon samples were collected and stored at −80 °C (Figure 1A).

Fecal Microbiota Transplantation (FMT). Male C57BL/6 donor mice (aged 6−7 weeks; 20 ± 1 g; n = 18) were divided into three groups: Control, PBS, and NEO treatment. In addition to a standard diet, the NEO group received a daily gavage of NEO (50 mg/kg, 200 μL), while the PBS group received an equivalent volume of distilled water for 21 days. Feces from the donor mice were collected starting on day 14. Each day, fecal samples were collected and placed in sterilized 1.5 mL EP (Eppendorf) tubes containing 200 mg of the sample. To preserve the microbial flora, the tubes were immediately stored in liquid nitrogen. The fecal suspension was prepared by mixing donor mouse feces in sterile PBS (0.1 g/mL) and centrifuging at 3000 g for 3 min, and the supernatant was administered to the corresponding recipient mice.

Male C57BL/6 recipient mice (aged 6−7 weeks; 20 ± 1 g; n = 18) were assigned to three groups: FMT-Control, FMT-PBS, and FMT-NEO treatment. After four weeks of consuming antibiotic-treated drinking water (containing ampicillin 0.2 g/L, neomycin 0.2 g/L, metronidazole 0.2 g/L, and vancomycin 0.1 g/L), the recipient mice were orally administered 200 μL of fecal bacterial suspension from the donor mice daily for 14 days. Subsequently, the mice were given DSS-containing drinking water (2.5%, w/v) to induce UC. At the conclusion of DSS treatment, all mice were euthanized, and colon tissue samples were collected for further analysis.

Analyzing Disease Activity Index (DAI). DAI is a crucial tool for evaluating the severity of the disease in mice during DSS induction. Detailed daily records of fecal traits and body weight changes are necessary throughout the experimental period. Clinical scoring, which considers fecal status, weight fluctuation, and occult blood, follows specific criteria: weight loss (0 for no loss; 1 for 1−3% loss; 2 for 3−6% loss; 3 for 6−9% loss; 4 for 9−12% loss); fecal consistency (0 for normal; 1−3 for loose stools; 4 for watery diarrhea); and rectal bleeding (0 for no bleeding; 1−3 for localized bleeding; 4 for extensive bleeding).[12,13] The combination of these criteria provides a more precise assessment of disease activity in mice.

Assessment of Tissue Damage. After fixation in 4% paraformaldehyde for 24 h, distal colon tissues were embedded in paraffin, sectioned into 5 μm thick slices, and stained with hematoxylin-eosin (H&E) following deparaffinization. Microscopic evaluation focused on histological changes, including the degree of tissue damage, such as crypt abscess formation, disruption of the physiological structure of the colon, inflammatory cell infiltration, and goblet cell loss. Specific scoring criteria included inflammation severity (0: none, 1: mild, 2: moderate, 3: severe); crypt damage (0: none, 1: slight, 2: moderate, 3: complete, 4: surface epithelium loss); and inflammation extent (0: none, 1: 1−25%, 2: 25−50%, 3: 50−75%, 4: 75−100%). These criteria enabled a more accurate characterization and evaluation of colonic tissue damage.

Measurement of Cytokine Levels by Enzyme-Linked Immunosorbent Assay (ELISA). The levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), CXC-chemokine ligand 2 (CCL2), and keratinocyte-derived chemokine (KC) in colon tissues were quantified using commercially available ELISA kits (BioLegend, San Diego, CA), strictly following the manufacturer's instructions.

Western Blot (WB). The collected colon tissues were homogenized in precooled protein lysis buffer (Beyotime Biotechnology, Shanghai, China). Total protein was extracted by centrifugation at 12,000 rpm for 15 min at 4 °C, and the supernatant was collected. Protein concentration was measured using a BCA protein assay kit. Protein samples were then prepared, separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto activated poly(vinylidene difluoride) (PVDF) membranes. After a 2 h blocking procedure, the membranes were incubated with primary antibodies at 4 °C for 12 h. Following this, the membranes were washed four times with TBS-T buffer, 15 min per wash, and subsequently incubated at room temperature for 1 h with horseradish peroxidase (HRP)-labeled secondary antibodies. After another series of four washes, immunoreactive protein bands were detected by using an electrochemiluminescence (ECL) detection kit. Protein band density was quantified with ImageJ software and

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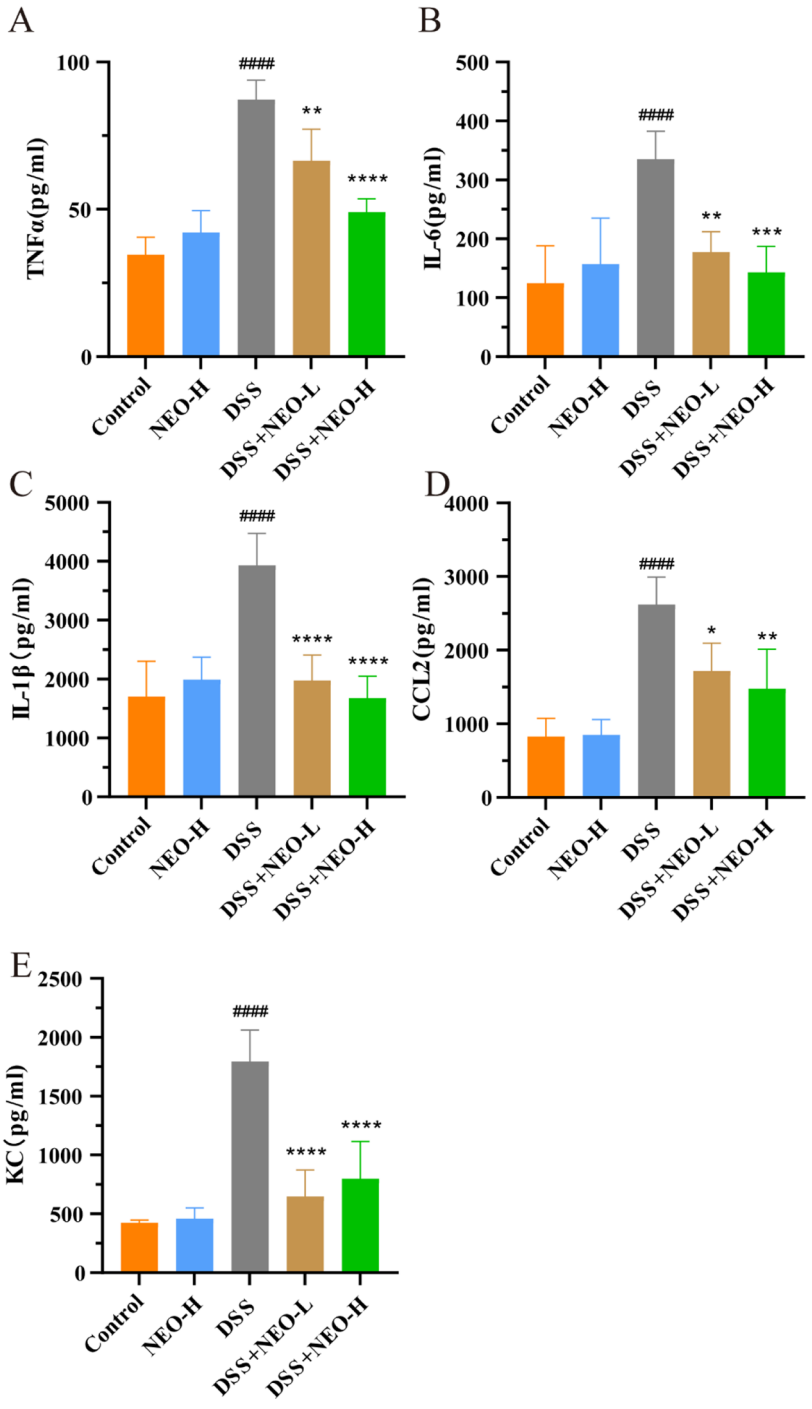

Figure 3. NEO's effect on inflammatory factors in DSS-induced UC mice. (A) TNF-α levels in colonic tissues of each group. (B) IL-6 levels in colon tissues of each group. (C) IL-1β levels in colonic tissues of each group. (D) CCL2 levels in colon tissues of each group. (E) KC levels in colon tissues of each group. Data are presented as mean ± SEM (n = 6). Significance levels: #P < 0.05, ##P < 0.01, ###P < 0.001, ####P < 0.0001 vs Control group; *P < 0.05, **P < 0.01, ****P < 0.0001 vs modeling group.

normalized against β-actin. The primary antibodies used in this experiment included anti-p-p38 (Proteintech, 28796-1-AP), anti-p-38 (Proteintech, 14064-1-AP), anti-p-JNK (Cell Signaling Technology, 9251), anti-JNK (Cell Signaling Technology, 9252), anti-p-ERK (Proteintech, 28733-1-AP), anti-ERK (Proteintech, 11257-1-AP), anti-p-p65 (Cell Signaling Technology, 3033), anti-p-65 (Cell Signaling Technology, 8242), anti-p-IκB (Proteintech, 82349-1-AP), anti-IκB (Proteintech, 10268-1-AP), antibeta-actin (Servicebio, GB15003-100), anti-ZO-1 (Proteintech, 21773-1-AP), and anti-Claudin-3 (Abcam, ab15102). Antibody dilutions were prepared by using BF06085 (Suzhou Biodragon Immunotechnologies Co., Ltd.).

Immunofluorescence Staining and FITC-Dextran. Following ether solution degreasing, cells were dehydrated using a gradient ethanol series, then boiled in citrate buffer to enhance antigen retrieval, and washed three times with PBS. Blocking was conducted with 5% donkey serum for 60 min at room temperature, followed by three PBS washes. Primary antibodies Claudin-3 (1:200; Wanleibio, WL00910, Shenyang, China) and ZO-1 (1:200; Wanleibio, WL03419, Shenyang, China) were incubated overnight. After three additional PBS washes, the sections were incubated with goat antirabbit IgG (1:1000, Invitrogen) diluted in 5% donkey serum for 60 min at room temperature. Finally, after three PBS washes, the

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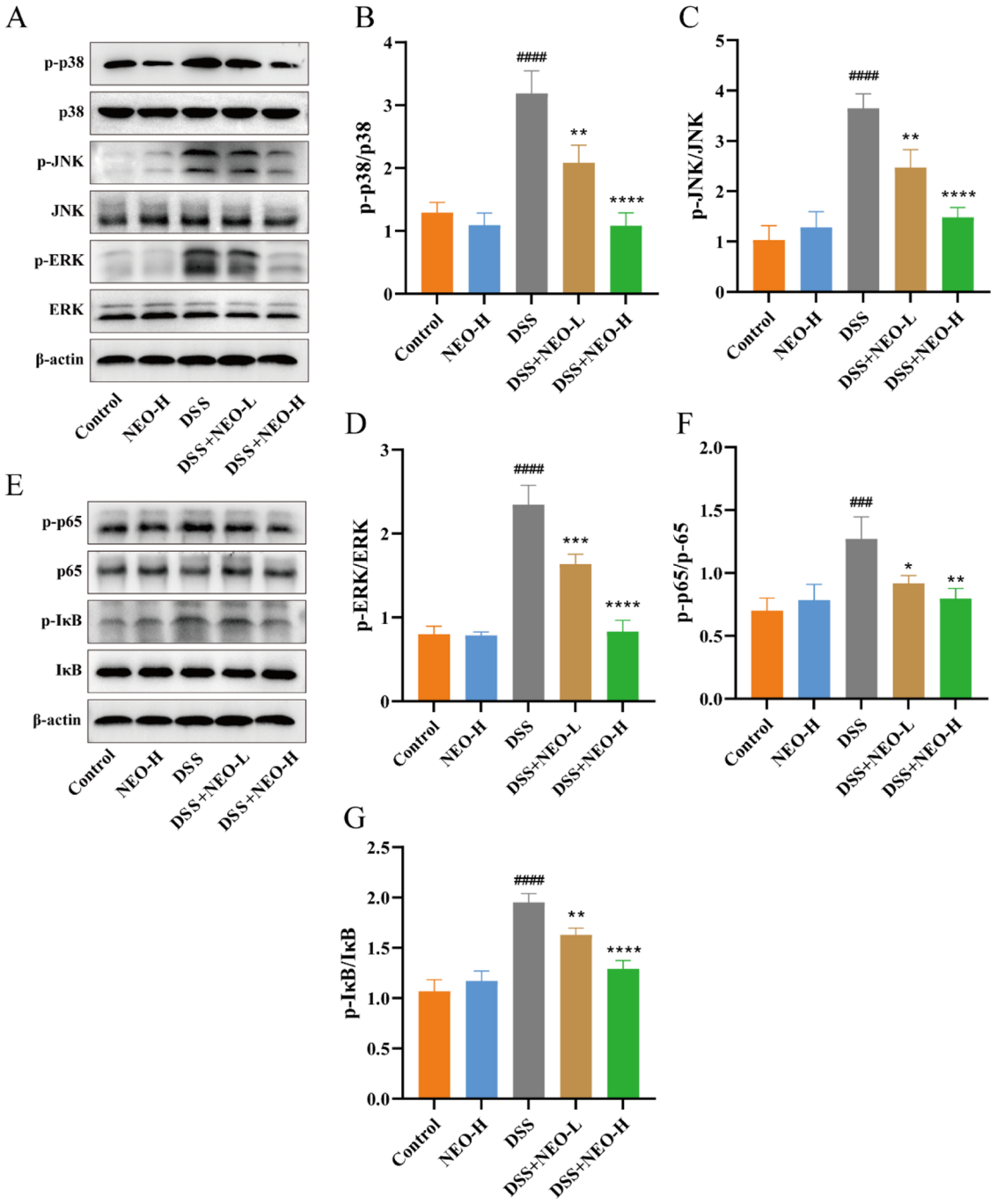

Figure 4. NEO's effect on MAPK/NF-κB pathway activation in DSS-induced UC mice. (A) Western blot analysis of p-p38, p38, p-JNK, JNK, p-ERK, ERK, and β-actin levels in colonic tissues of each group. (B−D) Grayscale analysis histograms of relevant MAPK pathway proteins. (E) Western blot analysis of p-p65, p65, p-IκB, IκB, and β-actin levels in colonic tissues of each group. (F, G) Grayscale analysis histograms of corresponding NF-κB pathway proteins. Data are presented as mean ± SEM (n = 6). Significance levels: #P < 0.05, ##P < 0.01, ###P < 0.001, ####P < 0.0001 vs Control group; *P < 0.05, **P < 0.01, ****P < 0.0001 vs modeling group.

nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Mice were fasted for 12 h and then administered FITC-dextran (50 mg/kg). After four h, peripheral blood was collected and left to stand for 30 min. Serum was obtained by centrifugation at 900 g for 10 min at 4 °C. The fluorescence intensity was measured to calculate the FITC-dextran concentration in the serum.

Gut Flora Sequence. Bacterial 16S rRNA sequencing of fecal samples was conducted using the Personalbio Genescloud platform (Shanghai Paysono Biotech, China). Nonrepetitive sequences were clustered into operational taxonomic units (OTUs) based on 97% similarity. From the OTU data, α-diversity and β-diversity indices were calculated to evaluate microbial diversity within and between samples. β-diversity and structural variations in intestinal flora across sample groups were analyzed using principal coordinate analysis (PCoA) with the weighted UniFrac distance algorithm and nonmetric multidimensional scaling (NMDS) with the Bray−Curtis distance algorithm. Additionally, taxonomic unit abundance at the family and genus levels was quantified and compared across groups. Raw Illumina sequencing data have been deposited in the NCBI Sequence Read Archive (BioProject ID: PRJNA1141397).

Statistical Analysis. In this study, data are expressed as mean ± SEM, with each measurement conducted in at least three independent

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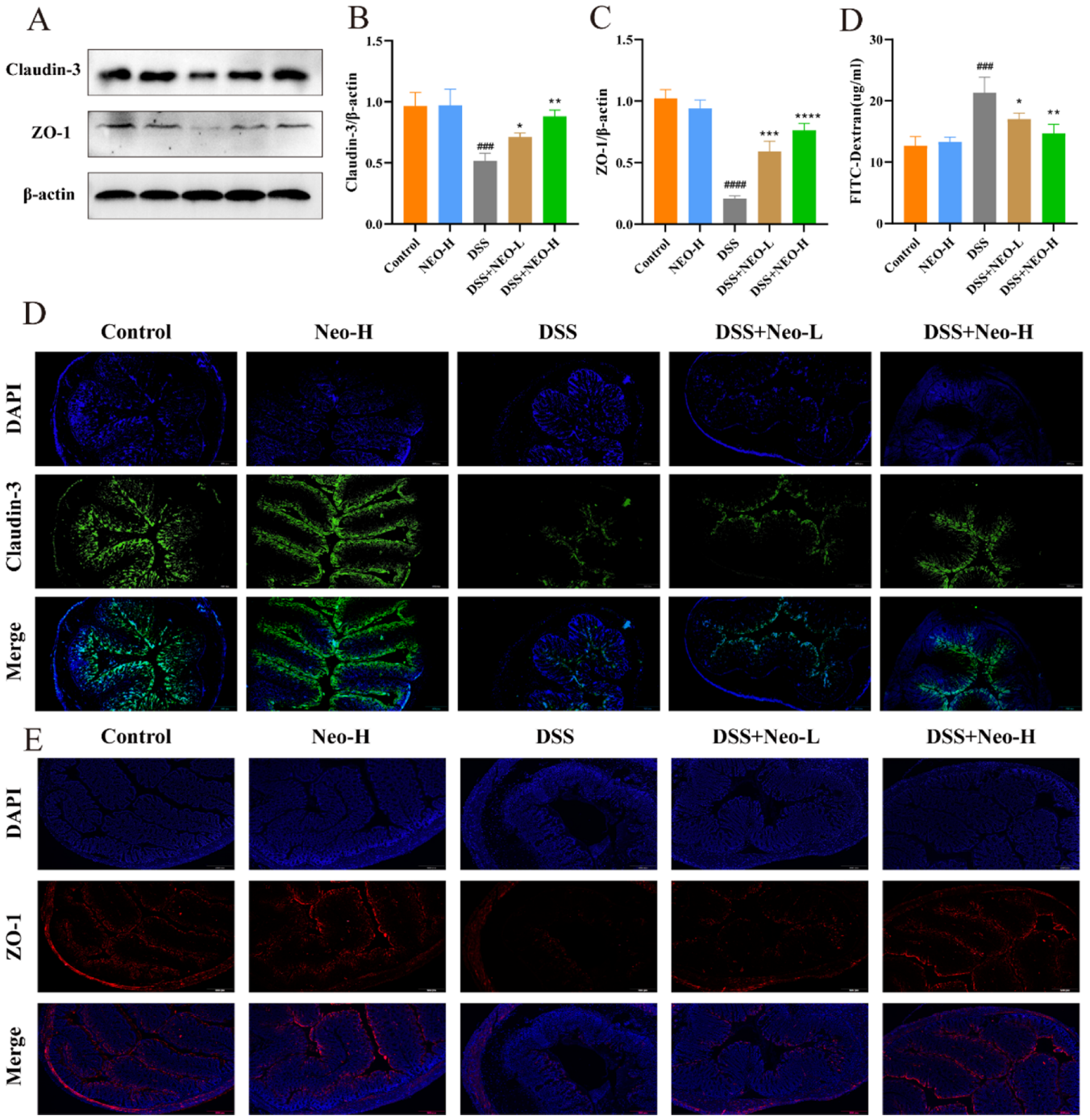

Figure 5. NEO's effect on colonic epithelial barrier integrity in DSS-induced UC mice. (A) Western blot analysis of the barrier proteins Claudin-3 and ZO-1 in colonic tissues of each group. (B, C) Grayscale analysis histograms of the corresponding barrier proteins. (D) Serum FITC-dextran levels. (E, F) Immunofluorescence staining of barrier proteins ZO-1 and Claudin-3 in colonic tissues. Scale bar: 200 μm. Data are presented as mean ± SEM (n = 6). Significance levels: #P < 0.05, ##P < 0.01, ###P < 0.001, ####P < 0.0001 vs Control group; *P < 0.05, **P < 0.01, ****P < 0.0001 vs modeling group..

replicates. Statistical analysis was performed using SPSS 21.0 (SPSS, Chicago) and GraphPad Prism 8.0. Differences between groups were evaluated using one-way ANOVA followed by Tukey's multiple comparisons test (P < 0.05).

RESULTS

NEO Reduces the Severity of DSS-Induced UC in Mice. The impact of NEO on UC-related symptoms was assessed by monitoring changes in body weight, DAI scores, and colon length, which are key indicators of UC severity.[13] Experimental results revealed a significant weight loss in DSS-treated mice, while early NEO intervention notably restored body weight (Figure 1B). The DAI scores of DSS-treated mice were markedly higher compared to the Control group; however, pretreatment with NEO significantly reduced these scores (Figure 1C). Additionally, the colon length, which was significantly shortened in the DSS-treated group, was substantially preserved with NEO pretreatment (Figure 1D,E). These results suggest that NEO pretreatment may

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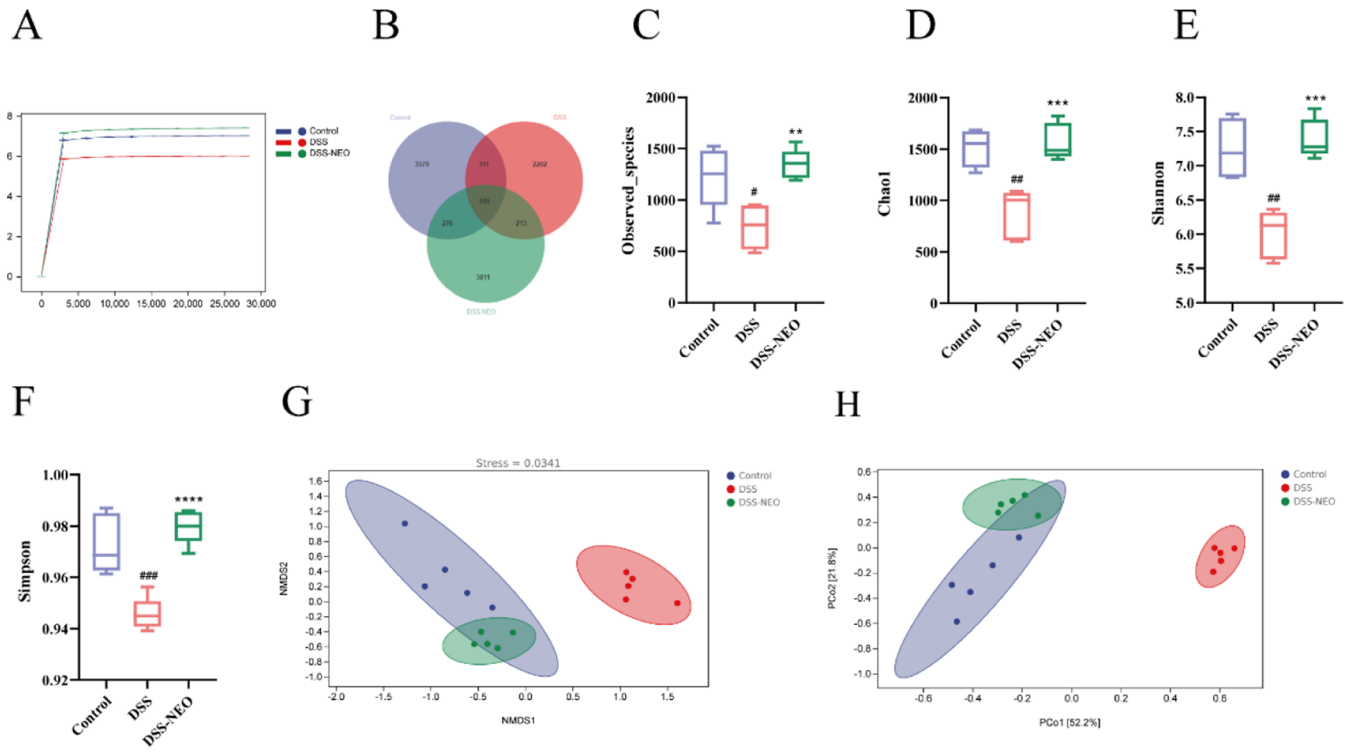

Figure 6. NEO's effect on the structure and composition of intestinal flora in DSS-induced UC mice. (A) Sparse curve of gut flora in the α diversity analysis; (B) ASV/OTUs shared by corresponding groups, indicated by overlapping zones between color blocks; (C) Observed_species index from the α diversity analysis; (D) Chao1 index from the α diversity analysis; (E) Simpson index from the α diversity analysis; (F) Shannon index from the α diversity analysis; (G) NMDS analysis of colonic intestinal flora using the unweighted Unifrac algorithm; (H) NMDS analysis of intestinal flora using the Bray−Curtis algorithm. OTU: Operational Taxonomic Unit. Data are presented as mean ± SEM (n = 6). Significance levels: #P < 0.05, ##P < 0.01, ###P < 0.001, ####P < 0.0001 vs Control group; *P < 0.05, **P < 0.01, ****P < 0.0001 vs modeling group.

play a role in mitigating the severity of DSS-induced UC symptoms.

Effect of NEO on Histopathologic Changes in the Colon of DSS-Induced Mice. To further investigate the protective effects of NEO on DSS-induced UC, histological changes in the colon were examined.[14] Results showed that DSS-induced UC in mice led to submucosal edema, extensive inflammatory cell infiltration, and crypt structure disruption. In contrast, mice pretreated with NEO exhibited significantly reduced crypt disruption and epithelial damage (Figure 2A). Moreover, histologic scores were significantly lower in the NEO-pretreated group compared to those in the DSS group (Figure 2B). These findings suggest that NEO pretreatment may mitigate the intestinal tissue damage induced by DSS in mice.

DSS-Induced UC Inflammation in Mice was Alleviated by NEO. To evaluate the impact of NEO on inflammatory symptoms, key molecules such as pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and chemokines (CCL2, KC) were measured in colon tissue.[15] Experimental data revealed that DSS-treated mice exhibited significantly elevated expression of these cytokines compared to that of the Control group. However, NEO pretreatment significantly reduced their expression levels (Figure 3A−C). A similar pattern was observed for the chemokines (Figure 3D,E), demonstrating NEO's effectiveness in alleviating DSS-induced inflammation in UC.

NEO Reduces the Symptoms of UC by Inhibiting DSS-Induced Mitogen-Activated Protein Kinase (MAPK) Pathways and the Activation of Nuclear Factor (NF-κB). The MAPK/NF-κB signaling pathways are central to UC-related inflammation.[16] It was hypothesized that NEO might inhibit UC in this pathway. Results indicated that DSS-induced mouse colon tissues showed significantly increased protein levels of p-p38, p-ERK1/2, p-JNK1/2 (Figure 4A−D), and p-p65 and p-IκB of the NF-κB pathway (Figure 4E−G). NEO pretreatment, particularly in the DSS + NEO-H (50 mg/kg) group, markedly reduced these protein levels. These results suggest that NEO mitigates colonic inflammation in mice, potentially by inhibiting the activation of the MAPK/NF-κB pathways.

NEO Maintains the Intestinal Barrier Integrity in DSS-Induced Mice. A key pathological feature of UC is the compromised integrity of the intestinal barrier, with TJ proteins playing a critical role in maintaining cell gap junctions and regulating intestinal mucosal permeability.[17] Previous studies have shown a negative correlation between TJ protein expression levels and UC severity.[18] TJ proteins, primarily composed of ZOs and Claudins, are downregulated by inflammatory factors such as IL-6, IL-1β, and TNF-α, leading to increased intestinal permeability and exacerbated barrier dysfunction.[19] To investigate this, the expression levels of two barrier proteins, ZO-1 and Claudin-3, were examined in the colonic tissues of mice. Western blot analysis revealed a significant reduction in Claudin-3 and ZO-1 levels in the DSS-treated group compared to that in the Control group. However, NEO pretreatment substantially enhanced the expression of these proteins (Figure 5A−C). Immunofluorescence staining results further corroborated these findings (Figure 5E, F). From the perspective of gut barrier function,

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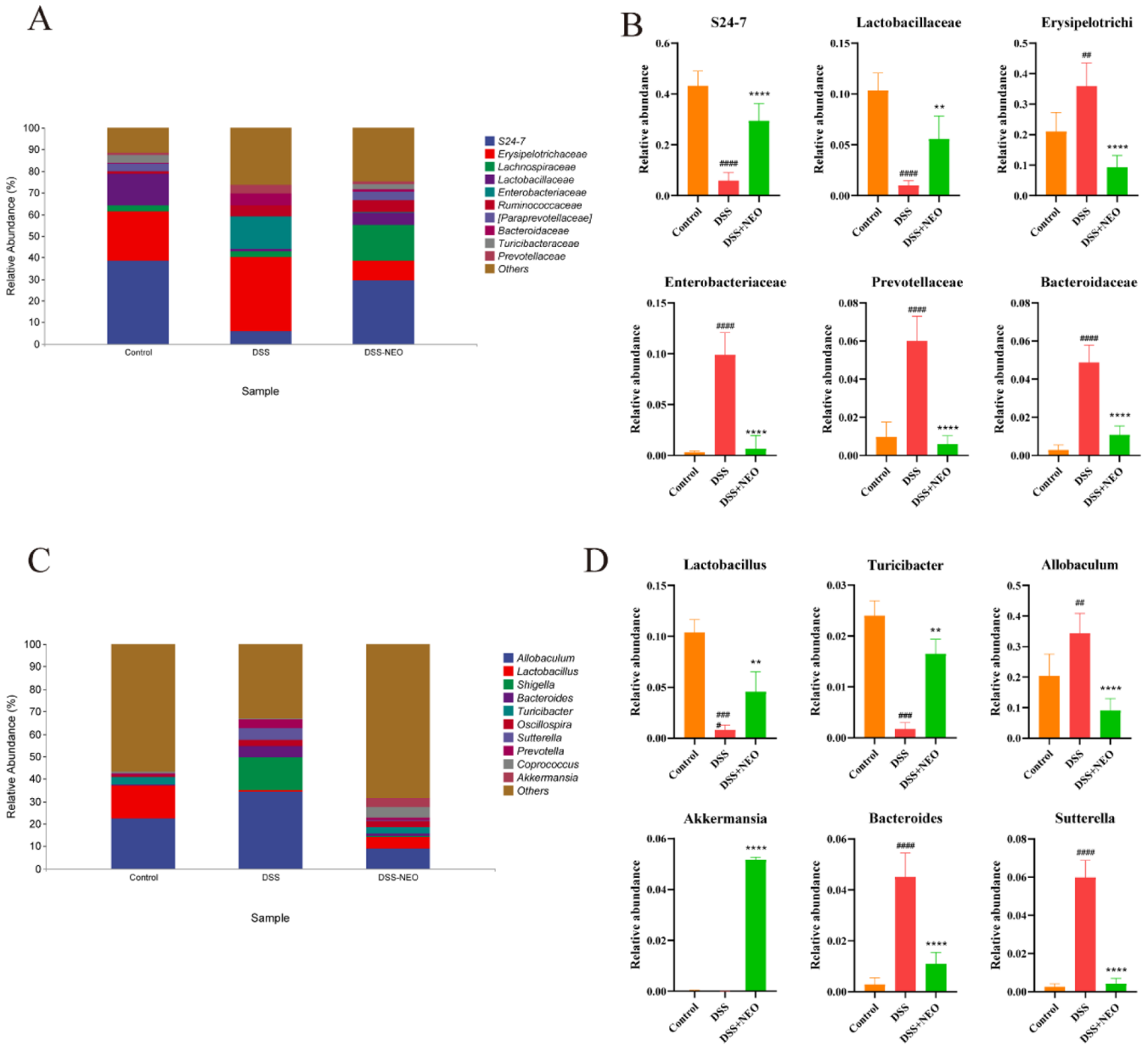

Figure 7. NEO's effect on the richness of intestinal flora in the colons of DSS-induced UC mice. (A) Abundance of colonic intestinal flora composition at the family level in each group. (B) Variations in family level richness of colonic intestinal flora composition among the groups. (C) Quantity and composition of intestinal flora at the genus level in each group. (D) Variations in genus-level richness of colonic intestinal flora composition among the groups. Data are presented as mean ± SEM (n = 6). Significance levels: #P < 0.05, ##P < 0.01, ###P < 0.001, ####P < 0.0001 vs Control group; *P < 0.05, **P < 0.01, ****P < 0.0001 vs modeling group.

serum FITC-glucan levels were elevated in DSS-induced UC mice. NEO treatment significantly reduced this elevation (Figure 5D), indicating that NEO may help mitigate UC-related damage by preserving the intestinal barrier integrity. These results suggest that NEO may protect against DSS-induced UC by enhancing TJ protein expression and thereby improving intestinal barrier integrity.

NEO Modifies the Composition of the Intestinal Flora in Mice with DSS-Induced UC. Numerous studies have identified dysbiosis of the intestinal flora as a key pathogenic factor in UC.[20] To explore whether NEO's anti-inflammatory effects are linked to its influence on gut microbiota composition, 16S rRNA sequencing was conducted. α diversity analysis showed that the rarefaction curves plateaued, indicating sufficient sequencing depth to capture the diversity within the samples (Figure 6A). The Venn diagram revealed 3602, 2227, and 3215 unique OTUs in the DSS+NEO-H (50 mg/kg), Control, and DSS groups, respectively (Figure 6B). Analysis of the α Diversity Index, including Observed_species, Chao 1, Shannon, and Simpson indices,[21] indicated a decrease in these indices in the DSS group, while the DSS + NEO-H group exhibited significant increases, suggesting that NEO enhances the richness and diversity of the intestinal microbiota (Figure 6C−F). β diversity indices (PCoA and NMDS)[22] further highlighted significant alterations in the intestinal flora of DSS-induced mice, which were mitigated by NEO treatment (Figure 6G,H). These results suggest that NEO may regulate the gut microbiota in DSS-induced UC.

Further analysis of microbial composition at the family and genus levels (Figure 7A,C)[23] revealed that NEO significantly

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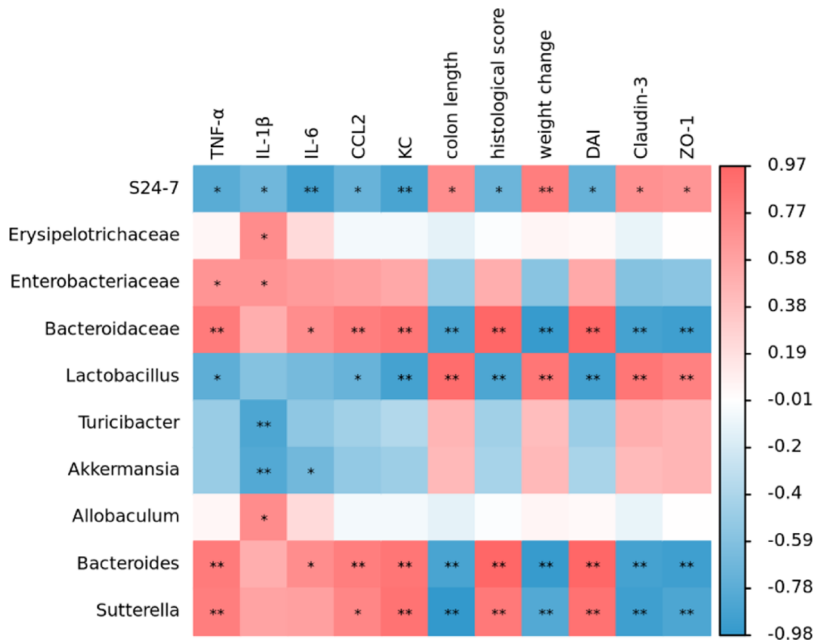

Figure 8. Spearman rank correlation analysis between gut microbiota, inflammatory markers, chemokines, DAI score, and tight junctions. Positive correlations are indicated in red, while negative correlations are shown in blue. Significance levels: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

reduced the relative abundance of *Erysipelotrichi*, *Enterobacteriaceae*, *Prevotellaceae*, and *Bacteroidaceae* in DSS-treated mice while increasing the relative abundance of *S24−7* and *Lactobacillaceae* at the family level (Figure 7B). At the genus level, NEO decreased the abundance of *Allobaculum*, *Bacteroides*, and *Sutterella*, while increasing the abundance of *Lactobacillus*, *Turicibacter*, and *Akkermansia* (Figure 7D). These data suggest that NEO may alleviate DSS-induced UC by modulating specific gut microbiota.

Correlation between Intestinal Flora, Colitis Symptoms, Inflammation, and TJ Integrity. To explore the correlation between intestinal flora and UC, Spearman's analysis was employed to assess the relationship between intestinal flora and inflammatory factors. The analysis revealed that *Bacteroidaceae*, *Bacteroides*, and *Sutterella* exhibited significant negative correlations with body weight, colon length, and intestinal barrier proteins, while showing significant positive correlations with inflammatory factors and DAI score (Figure 8). These results suggest that NEO's preventive effect on DSS-induced enteritis in mice may be linked to its ability to modulate intestinal flora.

Fecal Microbiota Transplantation (FMT) Diminishes Colonic Inflammation and Augments Intestinal Barrier Function. To investigate the impact of intestinal flora changes on UC, fecal samples from three groups of mice (Control, PBS, NEO) were collected and transplanted into pseudogerm-free (PGF) recipient mice induced by compound antibiotics (Figure 9A). The FMT-NEO group exhibited significantly less weight loss (Figure 9B) and marked improvement in DAI scores (Figure 9C) compared with the FMT-PBS group. Additionally, colon shortening was effectively mitigated in the FMT-NEO group (Figure 9E,F). Pathological indicators, including intestinal crypt damage and inflammatory cell infiltration, were significantly reduced in the FMT-NEO group (Figure 9D), as were histological scores (Figure 9G). Pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 were substantially decreased in the FMT-NEO group compared to the FMT-PBS group, indicating reduced secretion of these cytokines (Figure 9H−J).[24] Furthermore, the expression levels of intestinal barrier proteins, including ZO-1 and Claudin-3, were significantly elevated in the FMT-NEO group, as confirmed by Western blot analysis (Figure 10A−C). These results suggest that NEO may alleviate UC by modulating the intestinal flora.

DISCUSSION

This study investigated the effects of NEO on DSS-induced UC in mice, revealing that NEO effectively mitigates mucosal barrier damage and significantly improves UC symptoms. Further analysis indicated that UC development is closely linked to the overactivation of MAPK/NF-κB inflammatory pathways, with NEO reducing inflammation by inhibiting these pathways. Additionally, NEO modulates the intestinal flora, increasing the abundance of anti-inflammatory bacteria and offering further protection. This study is the first to highlight the potential role of NEO in ameliorating the intestinal inflammatory response.

In a DSS-induced UC mouse model, pathological features such as submucosal edema, inflammatory cell infiltration, and crypt structure destruction were observed.[25] NEO pretreatment alleviated these symptoms, reducing body weight loss and colon shortening. Furthermore, NEO pretreatment significantly reduced DAI and histopathological scores, suggesting that preadministration of NEO may protect against tissue damage in DSS-induced UC.

The severity of UC is closely tied to the integrity of the intestinal barrier.[26] Damage to this barrier is a hallmark of UC pathophysiology, as impaired intestinal function allows pathogens and toxins to penetrate the intestinal wall, exacerbating inflammation.[27] Tight junctions in the intestinal epithelium are essential for preventing harmful substances from crossing the intestinal barrier, making their integrity a key indicator of intestinal permeability. The structure of TJs,

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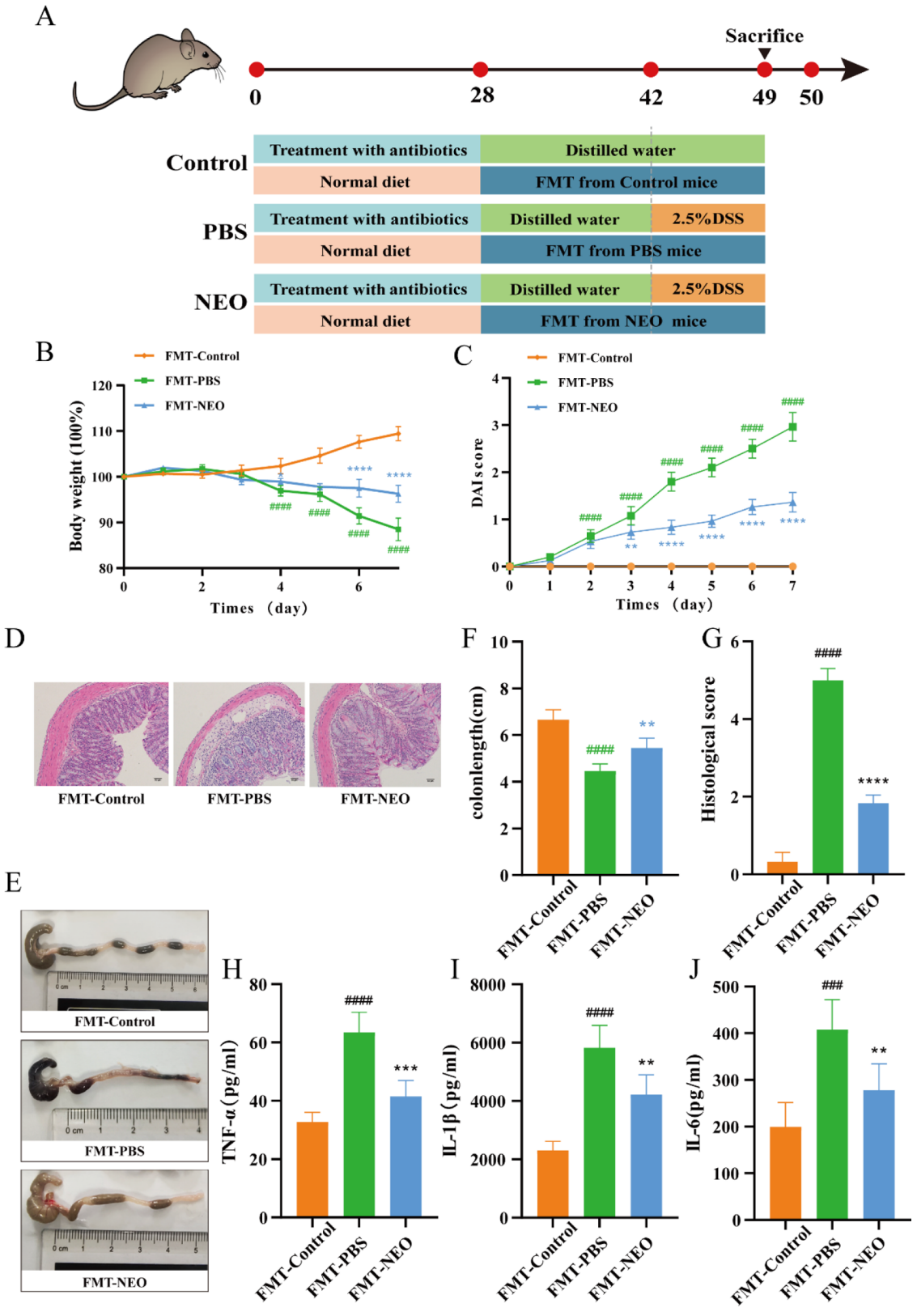

Figure 9. Effect of FMT on DSS-induced UC in mice. (A) Diagram of the FMT experimental course. (B) Changes in body weight across groups during DSS induction. (C) Changes in DAI scores across groups during DSS induction. (D) HE staining of colon sections in each group. (E) Images of colon length in each group. (F) Histogram of colon length analysis in each group. (G) Histologic scores across groups. (H–J) Histograms showing levels of colonic inflammatory factors TNF-α, IL-1β, and IL-6 in each group. Data are presented as mean ± SEM (n = 6). Significance levels: #P < 0.05, ##P < 0.01, ###P < 0.001, ####P < 0.0001 vs Control group; *P < 0.05, **P < 0.01, ****P < 0.0001 vs modeling group.

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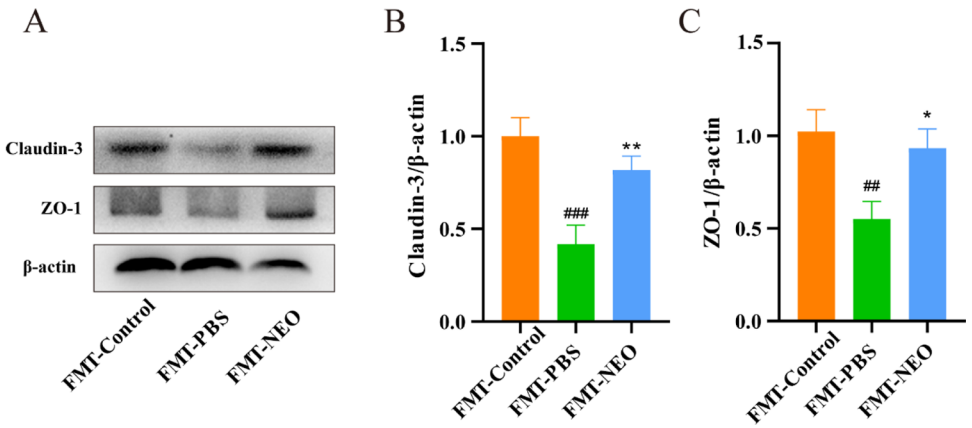

Figure 10. Effect of FMT on colonic epithelial barrier integrity in DSS-induced mice. (A) Western blot analysis of Claudin-3 and ZO-1 concentrations in the colons of each group. (B-C) Histograms corresponding to the grayscale analysis of barrier proteins. Data are presented as mean ± SEM ($n = 6$). Significance levels: #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$, ####$P < 0.0001$ vs Control group; *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$ vs modeling group. NEO attenuated MAPK/NF-κB pathway signaling, enhanced the expression of intestinal barrier proteins (Claudin-3 and ZO-1), restored damaged barrier integrity, and significantly lowered inflammatory cytokine levels. Additionally, NEO modulated the gut microbiota, increased the abundance of probiotics (Allobaculum, Bacteroides, and Sutterella), and alleviated UC symptoms.

largely composed of cytoplasmic scaffolding proteins such as ZOs and Claudins, plays a vital role in maintaining intestinal integrity.[28] In this study, ZO-1 and Claudin-3 levels were significantly reduced in the DSS-induced UC model, consistent with previous findings on intestinal barrier disruption. However, NEO preadministration restored ZO-1 and Claudin-3 levels, suggesting that NEO may mitigate UC severity by preserving the intestinal barrier integrity.

Damage to tight junctions can increase intestinal permeability, allowing harmful substances and metabolites, such as lipopolysaccharide (LPS), produced by intestinal flora, to cross the compromised intestinal barrier. This breach can trigger abnormal mucosal immunity and exacerbate the inflammatory response.[29] During this process, immune cells infiltrate the colonic mucosa, secreting inflammatory factors such as TNF-α, IL-6, and IL-1β.[30] In this study, DSS-induced UC mice exhibited elevated levels of these inflammatory mediators, while NEO preadministration significantly reduced their levels or activity, aligning with previous findings. Additionally, NEO alleviated UC through the modulation of inflammatory pathways at the molecular level. The MAPK/NF-κB pathway is essential for regulating pro-inflammatory and anti-inflammatory responses, with DSS or LPS stimulation activating this pathway and contributing to UC pathogenesis.[31] In this study, DSS-induced phosphorylation of MAPK/NF-κB proteins was significantly elevated in the colons of UC mice, but this increase was markedly inhibited by NEO preadministration. These results suggest that NEO preadministration may suppress inflammatory signal transduction and reduce the release of inflammatory factors, thereby alleviating UC.

Extensive research has established a strong link between various diseases and imbalances in gut microbiota.[32] The disruption of intestinal flora is a key factor in the pathogenesis of UC. Harmful bacteria in the gut, such as Enterobacteriaceae and Bacteroides, produce metabolites like LPS and enterotoxins,[33] which can activate the MAPK pathway, exacerbating UC. Reducing these harmful bacteria has been shown to alleviate UC severity.[34] Beneficial gut bacteria, or probiotics, inhibit the growth of harmful microbes, strengthen intestinal epithelial cells, reduce intestinal permeability, and protect the intestinal barrier. Modulating gut microbiota is therefore considered an effective strategy for preventing and treating UC.[35] In the present study, gut microbiota analysis revealed significantly lower bacterial abundance in the DSS group compared to the Control group.[36] However, NEO preadministration restored α diversity. PCoA and NMDS analyses indicated a marked difference in gut microbiota between the DSS and Control groups, which was mitigated by high-dose NEO pretreatment, suggesting NEO's potential in correcting gut microbiota imbalance.

At the family level, NEO treatment significantly increased the abundance of *S24−7* and *Lactobacillaceae* in the colons of DSS-induced mice. *Lactobacillaceae*, a key probiotic, alleviates enteritis symptoms through various mechanisms.[37,38] S24−7, known for its carbohydrate-degrading capabilities, also plays a positive role in reducing intestinal inflammation.[39] Conversely, NEO significantly reduced the relative abundance of *Erysipelotrichi*, *Enterobacteriaceae*, *Prevotellaceae*, and *Bacteroidaceae*, which is associated with increased UC severity. *Erysipelotrichi*, known to trigger inflammatory responses, and *Enterobacteriaceae*, which promote inflammation and correlate with UC severity, were notably diminished. Additionally, a reduction in *Prevotellaceae* has been linked to decreased UC severity.[40] These results suggest that NEO's ability to alleviate UC may be closely related to its modulation of gut microbiota at the family level.

At the genus level, NEO treatment significantly increased the populations of *Lactobacillus*, *Turicibacter*, and *Akkermansia* in UC mice. Lactobacillus, a well-known probiotic, has been extensively documented for its efficacy in alleviating UC symptoms when appropriately supplemented. *Turicibacter* can form a protective biofilm on the surface of intestinal epithelial cells, shielding them from harmful substances.[41] *Akkermansia*, a newly identified probiotic, modulates immune responses in the intestinal tract and mesenteric lymph nodes, thereby reducing intestinal inflammation.[42] Conversely, in DSS-induced UC mice, the abundance of *Allobaculum*, *Bacteroides*, and *Sutterella* was elevated, but NEO treatment effectively inhibited this increase. These results suggest that NEO preadministration modulates the gut microbiota in UC-affected animals by increasing beneficial bacteria and decreasing harmful ones, leading to reduced intestinal LPS levels and inhibition of the

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MAPK signaling pathway. This modulation of gut microbiota composition by NEO may be closely linked to its therapeutic efficacy in preventing UC.

Furthermore, FMT experiments showed that administering feces from NEO-treated mice to recipient mice reduced intestinal barrier damage, lowered inflammatory cytokine levels, and alleviated colonic inflammation, suggesting that fecal transplantation may mitigate UC-related damage.

This study is the first to demonstrate that NEO preadministration may inhibit the overactivation of the MAPK/NF-κB pathway, thereby attenuating DSS-induced colonic inflammation and restoring the damaged intestinal barrier. Additionally, NEO enhanced the gut microbial diversity in UC mice, significantly altering the composition at the family level by increasing the abundance of probiotics and reducing harmful bacteria. These findings suggest that NEO could be used as a food additive to mitigate the adverse effects of UC.

■ ASSOCIATED CONTENT

Data Availability Statement

All relevant data about this research can be requested from the corresponding author.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.4c04433.

Complete WB bands for MAPK/NF-κB pathway and complete bands for barrier proteins (PDF)

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Author Contributions

[§]T.J. and Z.S. contributed equally to this work. T.J., Z.S., D.Q., J.C., T.L., S.F. and G.H. designed the experiments. T.J., Z.S., D.Q., J.C., and T.L. carried out the experiments. T.J. and G.H. analyzed the sequencing data. T.J., Z.S., D.Q., S.F., and G.H. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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