New Baitouweng Decoction Combined with Fecal Microbiota Transplantation Alleviates DSS-induced Colitis in Rats by Regulating Gut Microbiota Metabolic Homeostasis and the STAT3/NF-κB Signaling Pathway

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Research Article

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Abstract

**Aim of the study:** We aimed to elucidate the synergistic effect and potential mechanism of New Baitouweng Decoction (NBD) combined with fecal microbiota transplantation (FMT) in rats with DSS-induced ulcerative colitis (UC).

**Materials and methods:** Colitis was induced by 5% (w/v) dextran sulfate sodium (DSS) in drinking water for 7 days. NBD or NBD combined with FMT were administered to the colitis rats. Body weight and disease activity index were measured, and the colon histological change was imaged to further examine the efficacy of NBD and FMT. The specific effects of NBD on STAT3/NF-κB Signaling Pathway and gut microbiota in rats with UC were also investigated.

**Results:** The efficacy of NBD in combination with FMT was demonstrated by the lower disease activity index scores; increased tight junction proteins expression; and a lower expression of macrophage marker (F4/80) in colon tissues. NBD combined with FMT elevated the concentrations of short-chain fatty acids and inhibited activation of the JAK2/STAT3/NF-κB related proteins. Furthermore, 16SrDNA sequencing indicated that the gut microbiota in rats with UC was perturbed, in contrast to that in healthy rats. After treatment with NBD and FMT, the diversity and abundance of intestinal flora showed clear improvements. Spearman correlation analysis indicated a strong correlation between specific microbiota and fecal concentrations of acetate, propionate and butyrate.

**Conclusions:** The protective mechanism of NBD combined with FMT may be linked to regulation NF-κB/STAT3 and restoration of the intestinal flora.

Background

Ulcerative colitis (UC), a highly prevalent chronic disease, has a complex and unclear pathogenesis, but is considered to be associated with the interaction of genetic factors, the external environment, autoimmune dysfunction and imbalances in the intestinal flora [1]. Monotherapies with aminosalicylates, corticosteroids, immunosuppressive drugs and biological agents have poor efficacy and cause many adverse effects [2]. For aggressive UC, combination therapy at an early stage is the most appropriate treatment [3]. Combination treatment decreases inflammation and regulates the intestinal flora, thus significantly improving ulcerative symptoms [4, 5]. Therefore, combination treatment may be better than monotherapy.

Recent studies have demonstrated that imbalances in the intestinal microflora are a paramount factor in the initiation and progression of UC. Imbalances in the gut microbiota alter microbial diversity, and consequently disrupt symbiosis of microbiota and the host [6]. In addition, the intestinal microbiota derived metabolites could trigger mucosal immune response in colon, thereby disrupting the immune function of the intestinal mucosa and triggering UC [7]. Recently, fecal microbiota transplantation (FMT) has become a pleasurable therapeutic approach for UC. FMT could induce intestinal microecological changes in patients in whom biological and immunosuppressive agents are ineffective, thus enhancing
the therapeutic effects of other drugs. A clinical study has indicated that the clinical efficacy of mesalamine combined with probiotics in patients with mild-to-moderate UC has greater efficacy than mesalamine alone [8]. The improvements in the gut microbiota are believed to affect the regulation of nuclear factor-kappa B (NF-κB), a gene transcriptional regulator crucial in the inflammatory process [9]. When IκBα undergoes phosphorylation and degradation, the downstream NF-κB signaling pathway is activated, thus initiating target gene transcription and mRNA formation. In addition, the binding of cytokines to the transmembrane receptor induces the phosphorylation of the Janus kinase (JAK), thus stimulating signal transduction and transcriptional activator 3 (STAT3). Activated STAT3 is involved in the gene transcription and protein expression of a variety of inflammatory factors, thereby promoting the formation and persistent exacerbation of inflammation [10]. Thus, regulating JAK2/STAT3/NF-κB signaling is an effective strategy to treat UC.

Traditional Chinese medicine has become a common alternative treatment for UC because of its flexibility and low toxic side effect. Baitouweng decoction containing *Pulsatilla chinensis* (Bunge) Regel (Baitouweng), *Phellodendron chinense* C. K. Schneid. (Huangbai), *Fraxinus chinensis* Roxb. (Qinpi) and *Coptis chinensis* Franch. (Huanglian), a classical traditional Chinese medicine prescription, originated from “Shang Han Lun” written by Zhang Zhongjing [11]. Several studies have shown that Baitouweng decoction has a variety of effects including anti-diarrhoeal, anti-inflammatory and regulation of intestinal microbiota [12]. However, the development of UC is complex and related to depression and oxidative stress [13, 14]. Previous experiments have concluded that Baitouweng decoction is less than ideal for treating UC [11]. Therefore, based on clinical experience, we added several herbs to Baitouweng decoction to form New Baitouweng Decoction (NBD) for better treatment of UC. NBD has been demonstrated their efficacy in our previous clinical efficacy assessment [15]. Also, our previous study has indicated that a NBD-related herbal granule produced by the Affiliated Hospital of Nanjing University of Chinese Medicine improves experimental colitis by restoring dendritic cell-mediated Th17/Treg balance [16]. NBD, known as Qingchang Huashi Recipe, has been shown to inhibit the inflammatory response of HT-29 cells and macrophage chemotaxis and to reduce NF-κB activation [17]. In addition, both *Aucklandia lappa* and *Paeonia lactiflora* existing in NBD, were with anti-depressant and anti-inflammatory properties, which have been shown to have anti-ulcer effect [18, 19]. *Paeonia lactiflora Pall* and *Coptis chinensis* could alleviate UC through downregulating the NF-κB/STAT3 signaling pathway [20, 21].

We therefore hypothesized that NBD combined with FMT might improve UC through regulating immunity and the intestinal flora, and might have better effects than either treatment alone. We explored the effect of NBD administration in combination with FMT in a DSS-induced colitis model. Our study validated the possible mechanism of NBD in combination with FMT in DSS-induced UC by examining the JAK2/STAT3/NF-κB signaling pathway. Moreover, we used 16 SrDNA sequencing technology to investigate the structure of gut microbiota in colitis rats under combination therapy.

**Materials And Methods**

**Animals**
Male Sprague–Dawley rats (weight, 180–220 g) were purchased from Qinglongshan Animal Breeding Farm, Jiangning District, Nanjing, China [license No: SYXK (Su) 2018-0049]. The rats were housed in an air-conditioned room (temperature 24–25°C, humidity 60%–65%) under a 12 h light/dark cycle. Animal experiments were approved by the Ethics Committee of Zhangjiagang TCM Hospital Affiliated with Nanjing University of Chinese Medicine. Ethics No.: AEWC-20201201.

Preparation of NBD

The components of NBD were listed in Table 1. All crude medicines were purchased from the Affiliated Hospital of Nanjing University of Chinese Medicine and identified by Professor Cao Yuan. The above materials were boiled in water (1630 mL) for 1 h after soaking for 1 h. Dregs of the decoction were boiled in boiling water (1304 mL) for 1 h and filtered. The filtrate was combined and concentrated into 1 g crude drug/ml and then stored at 4°C.

Table 1

The components of NBD.

| Chinese Name | Latin Name               | Parts used in medicine | Proportion |
|--------------|--------------------------|------------------------|------------|
| Baitouweng   | *Pulsatilla chinensis* (Bunge) Regel | Root                   | 10         |
| Huangbai     | *Phellodendron chinense* C. K. Schneid | Cortex                | 10         |
| Qinpi        | *Fraxinus chinensis* Roxb. | Cortex                | 15         |
| Huanglian    | *Coptis chinensis* Franch. | Root                   | 3          |
| Baishao      | *Paeonia lactiflora* Pall. | Root                   | 15         |
| Chishao      | *Paeonia veitchii* Lynch  | Root                   | 15         |
| Danggui      | *Angelica sinensis* (Oliv.) Diels | Root                   | 10         |
| Mudanpi      | *Paeonia suffruticosa* Andrews | Root cortex           | 10         |
| Zicao        | Lithospermum erythrorhizon Siebold & Zucc. | Root                   | 15         |
| Diyu         | *Sanguisorba officinalis* L. | Root                   | 15         |
| Xianhecao    | *Agrimonia pilosa* Ledeb. | Herba                  | 30         |
| Muxiang      | Aucklandia lappa DC.      | Root                   | 10         |
| Gancao       | *Glycyrrhiza uralensis* Fisch. | Root and rhizome      | 5          |

Analysis of Components in NBD
Concentrated NBD extract was mixed with methanol and then centrifuged at 18,000 g for 10 min. The collected supernatant was subjected to LC-HR-MS analysis. The mobile phase consisted of 0.05% formic acid–water (phase A) and 0.05% formic acid–acetonitrile (phase B). The gradient elution program was as follows: 0–3.2 min, 10% B; 3.2–36 min, 10–90% B; 36–39.1 min, 90% B; 39.1–40 min, 90–10% B; and 40–42.1 min, 10% B.

**Preparation of Fecal Microbiota Transplantation**

Fresh feces from healthy rats were sent to Nanjing Hilshou Biotechnology Co., Ltd. to make capsules. The capsules were 2.7 mm in diameter and dissolved only in the colon. Each capsule contained approximately 15 mg of extract with a bacterial content of 109 cfu/g. Fecal bacteria capsules were cryopreserved at -80°C.

**Induction of the Colitis Model in Rats and Treatment with NBD and FMT**

All rats were randomly divided into five groups: control group (Ctrl), DSS group (DSS), DSS+NBD group (NBD, 17 g/kg), DSS+FMT group (FMT, 0.5 g/kg) and DSS+NBD+FMT group (NBD, 17 g/kg; FMT, 0.5 g/kg). Except for the Ctrl group, which was given water, rats in the other four groups were given 5% DSS (Yeasen Biotech Co., Ltd., Shanghai, China) for 7 days to establish the UC model and then were then drunk with water for another 3 days. The DSS+NBD group and DSS+FMT group were treated with NBD and FMT from the second day of DSS intervention. The combination group was given a combination of FMT and NBD from day 1 to day 10. All rats were anesthetized by inhalation of 3% isoflurane and sacrificed by cervical dislocation. After anesthetic euthanasia, the colons were removed, rinsed with PBS and measured. The colons and feces were cryopreserved at -80°C until further analysis.

**Assessment of the DAI Score**

During the experiment, daily body weight, stool consistency and the presence of occult blood in feces were documented to illustrate the overall status of the rats [22]. Specifically, five levels of weight loss were recorded, from 0 to 4, indicating no weight loss, <5% loss, 5–10% loss, 10%–20%, and >20% loss, respectively. Diarrhea was graded on a scale of 0, 2 and 4, indicating normal stools, loose stools and watery diarrhea, respectively. Similarly, hematochezia was quantified with scores of 0, 2 and 4, representing no bleeding, slight bleeding and gross bleeding, respectively.

**Histology Analysis**

Colon sections (approximately 1 cm) were immediately fixed in 10% buffered formalin after being rinsed with ice-cold PBS. Then colon sections were subjected to H&E staining and then examined in a light microscopic to assess histological changes in pathological sections, and the Chiu standard grading was performed in **Supplementary Table S1** [23].

**Quantitative Real-Time PCR (qRT-PCR)**
Total RNA was extracted from the colon tissues with RNA isolater (Vazyme BioTech Co., Ltd, Nanjing, China) and then subjected to reverse transcription. The cDNA was then used to perform qPCR with SYBR Green I chimeric fluorescence. A CFX Connect RT-PCR Detection System (Bio-Rad, Hercules, CA, USA) was used to conduct qPCR. Gene expression analysis was performed by using the $2^{-\Delta\Delta Ct}$ method. Primer sequences are shown in Supplementary Table S2.

**Western Blot Analysis**

Colonic tissues were completely homogenized with RIPA lysis buffer. Next, the denaturated proteins were separated with SDS-polyacrylamide gel electrophoresis and electro-blotted onto a PVDF membrane. The membranes were submerged for 2 h in blocking solution before incubation with the antibodies against GAPDH (60004-1-lg, Proteintech); occludin (13409-1-AP, Proteintech); ZO-1(21773-1-AP, Proteintech); STAT3 (AP0366, Bioworld); p-STAT3 (AP0248, Bioworld); NF-κB p65 (66535-1-lg, Proteintech); p-NF-κB p65 (#3033S, Cell Signaling Technology); JAK2 (ET1607-35, HUABIO); and p-JAK2 (ET1607-34, HUABIO). Then, all PVDF membranes were incubated with secondary antibodies, bands were exposed with enhanced ECL solution and analyzed in Image J software. (The blots cut prior to hybridization with antibodies were presented the cropped blots in the manuscript).

**Immunofluorescence Analysis**

First, colonic tissues dewaxed, rinsed and sectioned. The sections were incubated with various primary antibodies. After sections were incubated with secondary antibodies for 1 h, 4′,6-diamidino-2-phenylindole (DAPI) staining solution (Beyotime Biotechnology, Shanghai, China) was used. Finally, confocal images were acquired with a LEICA STELLARIS confocal microscope (Leica Camera AG, Germany).

**Targeted SCFA Quantitative Analysis**

Short-chain fatty acid concentrations (acetate, propionate, butyrate, isobutyrate, valerate and isovalerate) in rat feces were determined by using previously reported methods [24]. Briefly, fecal samples (approximately 50 mg) were added proportionally to 0.5 ml 50% acetonitrile (10 µl solvent per 1 mg stool) and vortexed until fully mixed. The samples were centrifuged at 18,000 g at 4°C for 5 min. The supernatant (30 µL) was combined with 180 mM 3-nitrophenylhydrazine in 50% acetonitrile △30 µL△ and 100 mM EDC-6% pyridine solution △30 µL△. The mixture was reacted at 40°C and maintained for 30 min before centrifuged at 12,000 g at 4°C for 10 min. Then, 50 µL supernatant collected was diluted to 100 µL with 50% aqueous acetonitrile for LC-MS/MS analysis.

**16 SrDNA Sequencing and Sequencing Data Analysis**

The DNA extracts of colon contents were detected and examined. Then DNA samples were sent to Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China) for 16 SrDNA sequencing. The microbial diversity detection was performed on the V3-V4 hypervariable region of the 16SrDNA of the bacteria.
sequencing was performed with the PE300 sequencing strategy on the Illumina MiSeq platform (Illumina, San Diego, USA).

The SILVA (version 138) database was used to compare the bacterial diversity. Observed taxonomic unit (OTU) clustering of non-repeat sequences was performed with Uparse (version 7.0.1090) software, according to 97% similarity, and chimeras were removed. According to the silva138/16s_bacteria species classification database, OTU representative sequences of 97% similar levels were compared with the RDP classifier Bayesian algorithm. With the UniFrac algorithm, principal coordinate analysis (PCoA) was performed to compare the beta diversity of species community among samples. Classifications of bacteria among different groups at the phylum and genus levels were compared using the Kruskal-Wallis rank sum test. The dominant bacterial communities with statistical differences were analyzed using line discriminant analysis (LDA) effect size (LEfSe).

**Statistical Analysis**

All results are expressed as the mean and standard error of mean. GraphPad Prism 8.0.2 software was used to analyze all data. One-way analysis of variance was used to analyze the differences between groups. P-values less than 0.05 were considered to indicate statistical significance, reported as follows: *P*<0.05, **P**<0.01 and ***P**<0.001.

**Results**

**Identification of the Main Constituents of NBD**

*Fig. 1* shows the total ion chromatography of NBD in positive and negative ion modes. A total of 78 components were identified from NBD, including 26 terpenoids, 10 flavonoids, 15 alkaloids, six coumarin components, 12 phenolic components, six organic acids, one naphthoquinone component and two other components. In addition, the identified main chemical components of NBD with analyzed retention times, precise molecular ion peaks and secondary mass spectrometry cleavage fragments are shown in [Supplementary Table S3](#).

**NBD Combined with FMT Alleviated Colonic Injury in DSS-Induced Colitis in Rats**

The body weights of the rats with UC were clearly lower than those of the rats in the Ctrl group (*P*<0.001), whereas the body weights of the rats with NBD combined with FMT treatment were significantly closer to those of the rats in the Ctrl group (*P*<0.001) (*Fig. 2A*). Furthermore, the DAI score was also markedly decreased by treatment (*P*<0.001) (*Fig. 2B*). The colon length in DSS-induced rats with UC was shorter than that in rats in the Ctrl group, but NBD combined with FMT prevented the DSS-induced colon shortening (*P*<0.01) (*Fig. 2C,D*). Notably, three treatments were effective in alleviating the symptoms of colitis in rats. NBD combined with FMT showed the most significant efficacy. Although the pathological staining results of the colon tissues indicated that rats with colitis in the DSS group had symptoms of disruption of epithelial tissues, mucosal layer incompleteness and massive infiltration of
inflammatory cells; all these symptoms were markedly ameliorated by NBD and FMT treatment (Fig. 2E). Together, our data indicated that NBD combined with FMT in alleviating DSS-stimulated colitis is more effective than either treatment alone.

**NBD Combined with FMT Up-regulated Tight Junction Protein Expression**

We measured the representative tight junction proteins and found that the mRNA expression of ZO-1 and occludin were diminished in the DSS group, but combination treatment reversed this response ($P<0.001$ or $P<0.01$) (Fig. 3A,B). Additionally, the protein expression levels of occludin and ZO-1 decreased in the colon of UC rats but were upregulated by NBD combined with FMT ($P<0.01$ or $P<0.05$) (Fig. 3C). Interestingly, no significant differences were observed between the NBD group or the FMT group versus the DSS group. Moreover, immunofluorescence staining indicated that DSS-induced colitis downregulated the protein expression of ZO-1 and occludin in the colon ($P<0.01$ or $P<0.05$) (Fig. 3D). However, NBD combined with FMT restored the expression levels of these proteins.

**NBD Combined with FMT Inhibited the Inflammatory Cytokines in DSS-Stimulated Colitis**

To understand the anti-inflammatory effects of NBD and FMT in rats with colitis, we measured the gene expression of pro-inflammatory cytokines and enzymes by PCR. Higher TNF-α, IL-1β, IL-6, iNOS and COX-2 levels were found in the colitic rats than rats in Ctrl group, but were dramatically downregulated by co-treatment with NBD and FMT (Fig. 4A-E). Macrophage infiltration is considered a parameter for evaluating the severity of UC [25]. NBD combined with FMT significantly decreased the percentage of F4/80$^+$ cells (Fig. 4F).

**NBD Combined with FMT Suppressed the JAK2/STAT3/NF-κB Signaling Pathway in Vivo**

NF-κB regulates the expression of targeted inflammatory mediators, and JAK/STAT activity plays a crucial role in the pathogenesis of UC. We used protein blot analysis to investigate whether NBD combined with FMT might have therapeutic effects via the JAK2/STAT3/NF-κB signaling pathway. As shown in Fig. 5A, DSS-stimulated colitis rats showed a clear trend of upregulation of p-NF-κB p65, p-STAT3 and p-JAK2 proteins, all of which were downregulated in rats treated with NBD combined with FMT ($P<0.001$ or $P<0.01$). However, no significant differences were observed in the expression of JAK2, STAT3 and NF-κB p65 proteins between the DSS group and the other two groups ($P>0.05$).

**Composition Analysis of the Gut Microbiota**

The substantial separation of the Ctrl and DSS group on the two-dimensional coordinate map indicated some differences in the microbial structures between the groups, whereas the distance between samples indicated better reproducibility within the groups (Fig. 6A). The within-group reproducibility was poor in the NBD, FMT and combination group. However, the distance between the FMT group and the combination group was relatively close, and the two groups were distant from the DSS group. The bacterial composition of the FMT group was closest to that of the Ctrl group, followed by the
The composition of the intestinal microflora in rats changed after treatment.

To further understand the effects of NBD combined with FMT on the gut microbiota, we investigated the composition of the gut microbiota at two taxonomic levels. The microbial community stacking histogram indicated that the fecal microorganisms in rats primarily comprised *Firmicutes*, *Bacteroidetes* and *Actinobacteria* at the phylum level (Fig. 6B). DSS intervention resulted in a clear decrease in the relative abundance of *Actinobacteria* and *Patescibacteria* ($P<0.001$ or $P<0.05$) (Fig. 6C,D). After FMT treatment, their relative abundance was significantly reversed. NBD combined with FMT upregulated the relative abundance of *Actinobacteria* and *Patescibacteria* but showed no statistical difference ($P>0.05$).

Furthermore, NBD decreased the relative abundance of *Proteobacteria* below that in the DSS group ($P=0.127$) (Fig. 6E). The genus-level intestinal flora was mainly composed of *Lactobacillus*, *unclassified_f__Lachnospiraceae*, *Romboutsia* and *Allobaculum* (Fig. 6F). Among these, the relative abundance of *Lactobacillus* was low in the DSS group ($P<0.001$) (Fig. 6G). NBD, FMT and combination therapy alleviated the inhibition of the above bacterial genera ($P<0.001$ or $P<0.05$). In addition, the relative abundance of *Allobaculum* increased in the DSS group but decreased after treatment with FMT ($P<0.01$) and NBD combined with FMT ($P=0.084$) (Fig. 6H). Notably, the effect of FMT was better than that of combination treatment. Compared with that in rats with colitis, the relative abundance of *Akkermansia* was greater in the group with NBD combined with FMT ($P=0.099$) (Fig. 6I).

**LEfSe Analysis of the Intestinal Flora in Rats**

LEfSe is a statistical tool enabling the identification of taxa that may distinguish groups on the basis of biostatistical differences. Microorganisms with higher abundance were identified by LDA values greater than 2.0 as the criterion. As shown in our results, 27 taxa were identified in DSS-treated rats (Fig. 7A). A total of 17, 25 and 5 taxa were identified from rats with colitis treated with NBD, FMT, and NBD combined with FMT, respectively. According to the analysis results, *g__Allobaculumthe* and *g__Escherichia-Shigella* may be the key bacterial types responsible for the imbalance in the gut flora in the DSS group (Fig. 7B). The difference in the degree of contribution of *g__UCG-005*, *g__Blautia*, *g__Alloprevotell*, etc., was higher in the NBD group, and *g__Lactobacillus*, *g__Romboutsia*, *g__Lachnospiraceae_UCG-006*, *p__Actinobacteriota*, *g__Lachnospiraceae_NK4A136_group*, etc. were relatively prominent in the FMT group. Furthermore, *g__Clostridium_sensu_stricto_1*, *g__norank_f__UCG-010*, *f__Clostridiaceae*, etc. in the NBD combined with FMT group were significantly distinct from the microbiota in the other groups. In general, the diversity and composition of the gut microbiota were significantly transformed after the three different treatments.

**NBD Combined with FMT Increased SCFAs in Rats with Ulcerative Colitis**

SCFAs, metabolites of the gut microbiota, provide the primary energy source for colonocytes and play a role in intestinal homeostasis by inhibiting inflammation [26]. To confirm whether NBD in combination with FMT might affect SCFA metabolism, we determined the amounts of SCFAs in the colonic contents. The SCFA profiles of the DSS group were completely different from those of treatment groups: the
content of acetate, butyrate, propionate and valerate acids were significantly lower in the rats with UC than the Ctrl group (P<0.001) (Fig. 8A-D). Combination treatment markedly upregulated the content of butyrate, propionate and acetate above that in rats with DSS-induced colitis (P<0.001 or P<0.05). Furthermore, the content of acetate was clearly higher in the combination group than the NBD and FMT groups (P<0.01). The valerate, isovalerate and isobutyrate concentrations were slightly higher in the combination group than the DSS group, but were not statistically significantly different (P>0.05) (Fig. 8D-F).

Correlation heatmap analysis was used to determine the potential relationship between altered gut microflora and SCFAs. The result of the correlation heatmap analysis revealed a link between the gut microbiota and SCFAs (Fig. 8G). At the genus level, acetate, propionate and butyrate were positively correlated with *Lactobacillus*, *norank_f__Lachnospiraceae*, *Enterorhabdus* and *Roseburia*, but were negatively associated with *Subdoligranulum*, *Shuttleworthia*, *unclassified_f__Ruminococcaceae*, *Allobaculum* and *Faecalibacterium*.

**Discussion**

To seek better treatments for UC, therapies such as traditional Chinese medicine, FMT and combination treatment are increasingly being used as adjuvant treatments. NBD, a clinical prescription used in clinical practice in China, is highly effective in light-to-moderate active UC. In addition, a variety of combination treatments have been demonstrated to be superior to monotherapy [27, 28]. Our results suggested that NBD in combination with FMT may have more significant therapeutic effects on colitis than either treatment alone. The body weight, DAI score and the shortening of the colon length were alleviated by NBD and FMT treatment. NBD and FMT mitigated the effects of DSS-induced colitis and may be used to prevent IBD in patients. The combination therapy performed better than the single treatments alone.

The intestinal microbiota plays a pivotal role in maintaining gastrointestinal health. Imbalances in the intestinal microflora can promote the development of UC, thus affecting the renewal of intestinal epithelial cells, intestinal peristalsis and metabolism, and mucosal and immune function [29, 30]. Therefore, investigating the roles of gut microflora in the pathogenesis of UC is crucial, to find effective ways to restore the intestinal microbial balance and alleviate UC. In our results, the diversity of the gut microbiota community was significantly diminished in rats with DSS-induced colitis. The PCoA beta diversity revealed that the gut microbiota of colitic rats was clearly separate from that of rats in the Ctrl group, in agreement with a previous report [31]. The human gut microbiota is mainly assigned to four phyla. Rats have similar dominant bacteria to humans. The *Firmicutes* and *Bacteroidetes* phyla are the dominant microbiota, followed by the *Proteobacteria* and *Actinobacteria* phyla. [32]. It was reported that the relative abundance of *Actinobacteria* significantly decreases with DSS [33]. *Actinobacteria*, which are gram positive anaerobic bacteria, maintain intestinal homeostasis [34]. In our results, the abundance of *Actinobacteria* remarkably reduced in the colitic rats, but increased in the FMT group. Meanwhile, FMT increased the relative abundance of the beneficial bacteria *Patescibacteria*. In our study, *Escherichia-Shigella* were the dominant bacteria in the DSS group. *Shigella*, a genus in the *Enterobacteriaceae* family,
is a crucial factor in increasing intestinal permeability and a major pathogen causing intestinal infection [35, 36]. Moreover, FMT treatment increased the abundance of *Lactobacillus* genera. *Lactobacillus*, characterized as a common probiotic, can enhance immunity, regulate the intestinal flora, and have anti-cancer and anti-diarrhea effects [37]. One study has shown that *Lactobacillus reuteri* ameliorates DSS-induced colitis in rats by increasing the colonic mucus thickness [38]. *Akkermansia* which belongs to the genus *Verruca*, interacts closely with hosts: it regulates mucus homeostasis of the host, and influences intestinal inflammation and immune system function [39]. All our gut microbiota results suggested that NBD, FMT and combination treatment maintained the gut microbial balance by interfering with different physiological processes through the gut flora. Notably, of the three treatments, FMT had the most significant role in the intestinal microbiota in UC, and was followed by NBD combined with FMT; NBD alone had the least clear role in influencing the intestinal microbiota.

SCFAs, important microbial metabolites, have anti-tumor and anti-inflammatory activity, and play essential roles in ameliorating enteritis, regulating the immune system, promoting intestinal epithelial barrier function and enhancing defense [40]. Therefore, the amounts of SCFAs were determined in this study. NBD and FMT increased the content of SCFAs in the intestinal tract in rats with UC to levels greater than those in the DSS group. Acetate, generated by probiotic *Lactobacillus*, can maintain the barrier function of the intestinal epithelium and protect the host from lethal infection [41]. Meanwhile, propionate and butyrate maintain intestinal health and delay the development of colitis [42]. Spearman correlation analysis showed that acetic acid, butyric acid and propionic acid were positively associated with *Lactobacillus, Roseburia* and *Enterorhabdus*. Our results revealed that *Lactobacillus, Roseburia* and *Enterorhabdus* were associated with the increase in SCFAs with NBD and FMT treatment, in agreement with findings from previous reports [43, 44].

The development of colitis is known to increase the production of inflammatory cytokines and proinflammatory enzymes [9]. As expected, in our study, NBD combined with FMT decreased the mRNA expression of TNF-α, IL-1β, IL-6, iNOS and COX-2, thus indicating anti-inflammatory activity in UC. Macrophages, which are the major source of many inflammatory cytokines, spread in tissues throughout the body and are important effector cells of the innate immune system [45]. Tissue macrophages in IBD can significantly increase, thus leading to persistent inflammation. Immunofluorescence indicated that the presence of NBD in combination with FMT significantly inhibited the number of DSS-induced colon macrophages. NBD in combination with FMT inhibited macrophage activation, exerting anti-inflammatory activity.

NF-κB releases pro-inflammatory cytokines and induces proinflammatory enzymes [46]. We found that NBD combined with FMT also inhibited the phosphorylation of NF-κB p65 in the DSS-stimulated colon. Consequently, we hypothesized that the combination of NBD and FMT downregulated the expression of inflammatory cytokines and enzymes in DSS-stimulated colitis, a response potentially associated with inhibition of NF-κB activation. Furthermore, IL-6 activates STAT3, thus leading to elevated levels of anti-apoptotic factors induced by downstream STAT3 [47]. Inhibition of STAT3 ameliorates DSS-induced damage due to colon inflammation by downregulating pro-inflammatory cytokines. STAT3 is a key
signaling molecule downstream of the JAK substrates and cytokine receptors, and JAK2/STAT3 signals are the major pathway for transcription factors involved in the pro-inflammatory cytokine response [48]. An important finding in our study was the decreased phosphorylation of JAK2/STAT3 after NBD combined with FMT treatment, thus suggesting that inhibition of the JAK/STAT pathway might be involved in the anti-colitis effect of combination therapy. These findings suggested that NBD in combination with FMT effectively inhibited JAK2/STAT3/NF-κB activation in the experimental UC model. Previous evidence has suggested that, the release of proinflammatory cytokines affects the expression of tight junction proteins [43]. Subsequently, the altered expression of tight junction proteins enhances intestinal permeability, thus leading to an inflammatory cascade [49].

Tight junction proteins are important for function gut barrier and permeability [50]. Accumulating evidence indicates that dysfunctioned intestinal mucosal barrier may lead to the progression of colitis. In our study, DSS-induced UC significantly decreased the colonic occludin and/or ZO-1 expression, but the levels of these proteins significantly increased after NBD and FMT co-treatment. The results suggested that treatment with NBD combined with FMT ameliorated colitis by promoting the repair of intestinal mucosa.

In conclusion, we found that NBD, FMT and combination treatment had therapeutic effects in rats with UC. The protective mechanism may be linked to regulation NF-κB/JAK2/STAT3 and restoration of the intestinal flora.

Declarations

Acknowledgements

Not applicable.

Authors’ contributions

XG and ZM conceived of and designed the experiments and wrote the manuscript; YW and YY performed the experiments; TY and YX analyzed the data. All authors read and approved the final manuscript.

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Availability of data and materials

The raw data supporting the conclusions of this article will be made available by the authors upon request. The sequencing data used in this study are stored in NCBI SRA database (SUB11837168).
Ethics approval and consent to participate

All experiments were performed in accordance with relevant guidelines and regulations. The animal experiment was reviewed and approved by the Ethics Committee of Zhangjiagang TCM Hospital Affiliated to Nanjing University of Chinese Medicine (Ethics No.: AEWC-20201201). All methods are reported in compliance with ARRIVE guidelines for the reporting of animal experiments.

Competing interests

The authors declare no competing financial interests.

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Figures

Figure 1
Base peak ion flow chromatogram of the major constituents of NBD. (A) Negative ion mode. (B) Positive ion mode.

**Figure 2**

NBD combined with FMT attenuated DSS-stimulated UC. (A) Body weight loss, (B) DAI score, (C,D) shortened colon length, (E) H&E staining and histological colitis score. Data are expressed as mean±SEM, *P<0.05, **P<0.01, ***P<0.001, n=6.

**Figure 3**

NBD combined with FMT up-regulated tight junction protein expression in colitic rats. (A,B) The expression of occludin (A) and ZO-1 (B) mRNA was down-regulated in the DSS group but up-regulated by NBD combined with FMT (n=6). (C) Western blotting indicated that NBD combined with FMT elevated the protein expression of occludin and ZO-1 beyond that in DSS-induced rats with UC, n=3. (D) Representative images of DAPI (blue), occludin and ZO-1 immunostaining (red) in colonic sections (×200 magnification). Data are expressed as mean±SEM, *P<0.05, **P<0.01, ***P<0.001.

**Figure 4**

NBD combined with FMT inhibited inflammatory cytokines in rats with DSS-induced colitis. (A-E) NBD combined with FMT downregulates IL-6 (A), IL-1β (B), TNF-α (C), COX-2 (D) and iNOS (E) mRNA in colonic tissue of rats with UC. (F) Immunohistochemical staining showing F4/80 expression in diseased colons. Data are expressed as mean±SEM, *P<0.05, **P<0.01, ***P<0.001.

**Figure 5**

NBD combined with FMT suppressed the activity of the JAK2/STAT3/NF-κB pathway in rats with DSS-induced colitis. (A) Western blot images of p-JAK2, JAK2, p-STAT3, STAT3, p-NF-κB, NF-κB and GAPDH. The p-JAK2/JAK2, p-STAT3/STAT3 and p-NF-κB/NF-κB ratios are presented, n=3. Data are expressed as mean±SEM, *P<0.05, **P<0.01, ***P<0.001.
Figure 6

Composition analysis of the intestinal microbiota. (A) PCoA of gut microbiota communities on the basis of OTU levels, n = 5. (B) Bacterial compositions in each group of rats at the phylum level, n = 5. (C-E) Relative abundance of Actinobacteria (C), Patescibacteria (D) and Proteobacteria (E), n = 5. (F) Bacterial compositions in each group of rats at the genus level, n = 5. (G-I) Relative abundance of Lactobacillus (G), Allobaculum (H) and Akkermansia (I), n = 5.

Figure 7

(A,B) Differences in dominant microorganisms among four groups, on the basis of a cladogram (A) and distribution histogram (B), with an LDA score larger than 2.0 as the criterion, n = 5.

Figure 8

NBD combined with FMT increased SCFAs in the colonic feces of rats with colitis to strengthen the intestinal microbial balance. (A-F) The amounts of SCFAs, including acetate (A), propionate (B), butyrate (C), valerate (D), isobutyrate (E) and isovalerate (F), as detected by LC-MS, n = 6. Data are expressed as mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001. (G) Heatmap showing Spearman correlations between several fecal SCFAs with significant differences and bacterial genera with a coefficient less than 0.05.

Supplementary Files

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