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Research Article

Supplemented Pulsatilla Decoction Alleviated Ulcerative Colitis by Attenuating the Pro-Inflammatory Response and Modifying Gut Microbiota in Mice

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Abstract

The present study aimed to combine *Portulaca oleracea* L with PD to compose a new formula supplemented PD (SPD), and evaluate its effect on ulcerative colitis (UC). The UC mouse model was obtained by supplementing 3.5% dextran sulfate sodium salt (DSS) orally. Subsequently, the mice were treated with PBS, sulfasalazine, high dose SPD, medium dose SPD or low dose SPD. Morphological analysis, RT-qPCR, ELISA assay, LC-MS/MS analysis and 16s rRNA sequencing were conducted to evaluate the effect of SPD on UC. DSS treatment resulted in the decreased body weight and increased DAI value of the mice, while SPD mitigated the changes of body weight and DAI value induced by UC. SPD attenuated the proinflammatory response induced by UC by down regulating the expression of proinflammatory cytokines, and alleviated the oxidative stress in the gut of the mice with UC by downregulating the MDA level and enhancing the activity of antioxidant enzymes. Additionally, SPD also enhanced the transcription of *MUC2* and *Occludin* which were inhibited by UC. Furthermore, DSS induced the dysbiosis of the colonic microbiota and relative abundance of the *Akkermansia* at genus level was higher in UC group whereas treatment with high-dose SPD restored its abundance to levels comparable to the control group. SPD demonstrated therapeutic effects on DSS-induced UC of the mice by attenuating the proinflammatory response, enhancing the antioxidant ability and key molecules related with barrier function, and modifying the gut microbiota, thereby providing a potential strategy for the treatment of ulcerative colitis.

Keywords: Gut microbiota, Supplemented pulsatilla decoction, Ulcerative colitis, Proinflammatory response

Introduction

Ulcerative colitis (UC), a subtype of inflammatory bowel disease (IBD), is characterized by chronic, recurrent, and non-specific inflammatory lesion in the mucosa of large intestine [1,2]. As a relapsing and remitting disorder, UC not only impairs the absorption of the nutrients, disrupts the integrity of barrier but also increases the risk of colorectal carcinogenesis. Clinically, patients with UC often present with symptoms such as diarrhea, abdominal pain, bloody stool, and weight loss, which can progress to colorectal cancer, disability and impaired quality of life [3].

Although the exact etiology remains elusive, genetic predisposition, environmental factors, dysregulation of immune response, and gut microbiota dysbiosis are recognized as key contributors to UC pathogenesis [3]. A hallmark of UC is inflammatory response in intestinal mucosa, where proinflammatory cytokines- including interleukin-1beta (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α) - play a significant role in disease progression [4,5]. Generation of cytokines and signal transduction initiated by cytokines were tightly regulated by intricate mechanisms. However, the pathophysiological dysregulation of gut immune response in UC resulted in imbalanced cytokines secretion, ultimately causing damage of gut mucosa [6]. Under physiological conditions, reactive oxygen species (ROS) are pivotal for cell signaling and tissue homeostasis [7]. However, in the context of inflammation in IBD, the imbalance of redox system resulted in excessive accumulation of ROS leading to cellular and molecular damages in the gut [8]. Oxidative stress is therefore considered as a key driver of the pathogenesis of UC and is closely associated with the progression of intestinal inflammation [8]. Additionally, gut microbiome formed a symbiotic relationship with the host, regulating intestinal immunity and gut function, and

its dysbiosis has been identified as a contributing factor to the pathogenesis of intestinal inflammation [9].

Currently, treatments primarily aim to alleviate symptoms of the patients, with clinical therapeutic strategies including 5-aminosalicylates, corticosteroids, thiopurines, pathway molecules inhibitors (e.g., TNF inhibitors, Janus kinase inhibitors), and probiotics [10]. Most existing therapies usually target a single mechanism, such as anti-inflammatory response or immunosuppressants [10]. Pulsatilla decoction (PD), a classical traditional Chinese medicine formula (CMF) recorded in the classical ancient Chinese book *Shang Han Treatise*, consists of four herbs: *Pulsatillae chinensis, Coptidis chinensis, Phellodendri Chinensis Cortex,* and *Fraxini Cortex*. It has long been used to treat diarrhea and IBD which alleviated UC in mouse model by reducing the tissue damage, inflammatory response and modifying the gut microbiota, and exhibits multi-target effect, low cost, and minimal side effects [11-13]. *Portulaca oleracea* L. (known as Ma Chi Xian in Chinese) is widely distributed globally, with a cold in nature and sour in taste in traditional Chinese medicine. It is traditionally used to cool the blood, stanch bleeding, antipyretic, and clear toxins [14]. Previous studies have shown that *Portulaca oleracea* L. traditional Chinese medicine compound or polysaccharides exerts therapeutic effects in UC [15,16].

Given these findings, the present study hypothesized that combining the classical PD with *Portulaca oleracea* L. (supplementary PD, SPD) may boost the therapeutic efficacy against UC. The aim of the present study is to investigate the effect of SPD on DSS-induced UC and explore its underlying mechanisms.

Material and Methods

Ethical Approval

The protocols of the trial involving animal experiments were reviewed and approved by Institutional Animal Use Committee of Henan Agricultural University (March 2nd 2022, HNND202203816), and the experiments were performed in accordance with the *Guidelines for the Care and Use of Laboratory Animals* of the Ministry of Science and Technology. The number of the animals used in the experiment is rational and operations during the invasive experiment were performed under anesthesia to minimize the pain. Animals were euthanized at the end of the experiment to collect the samples.

Preparation of Supplemented Pulsatilla Decoction (SPD) Crude Extract

The supplemented pulsatilla decoction was composed of *Portulaca oleracea* L., *Pulsatillae chinensis, Coptis chinensis, Phellodendri Chinensis Cortex,* and *Fraxini Cortex*. Details of the herbs used in the present study were listed in *Table 1.* To prepare the crude extract of SPD, 2550 mL of distilled water was added to the mixed herbal materials of SPD and incubated for 30 min at room temperature. The mixture was then boiled for 30 min, and filtered through 4 layers of cotton gauze. The filtrate was collected and 2550 mL was added to the residue and boiled for an additional 30 min. The boiled mixture was filtered through 4 layers of cotton gauze and the filtrate was collected. The filtrates were combined, filtered through 8 layers of cotton gauze, and lyophilized with vacuum freeze dryer (Brocade Holding Group Limited, China) to obtain the SPD extract and was stored at -80°C.

Table 1. *Detailed information of herbs in SPD*

Chinese Name	Latin Name	Part (s) Used	Amount (g)
Ma Chi Xian	*Portulaca oleracea L.*	Stems and leaves	60
Bai Tou Weng	*Pulsatillae chinensis*	Roots	60
Huang Lian	*Coptis chinensis*	Roots and rhizomes	30
Huang Bai	*Phel lodendri Chinensis Cortex*	Epidermis	45
Qin Pi	*Fraxini Cortex*	Epidermis	60

LC-MS/MS Analysis

Supplemented pulsatilla decoction extract was diluted in double distilled water and subjected to LC-MS/MS analysis. The detection procedure was performed as previous description [17]. LC-MS/MS analysis was performed on an UHPLC system (Vanquish, Thermo Fisher Scientific) with a Waters UPLC BEH C18 column (1.7 µm 2.1*100 mm). An Q Exactive Focus mass spectrometer coupled with an Xcalibur software was employed to obtain the MS and MS/MS data based on the IDA acquisition mode. The original data of mass spectra were imported into XCMS software for processing such as retention time correction, peak identification, peak extraction, peak integration, and peak alignment. The peak information of compounds was searched through the in-house secondary mass spectrometry database provided by Shanghai BIOTREE biotech Co., Ltd.

Animals, Treatments, and Sample Collection

BABL/C male mice (6-8 weeks old, 20-24 g) were obtained from Beijing HFK Bioscience Co., LTD. Mice were housed individually in the individual ventilated cages (IVC) with free access to food and water. The housing temperature was maintained at 22°C with 50% humidity and a 12:12 light - dark cycle. The protocols of the trial were reviewed and approved by Institutional Animal Use Committee of Henan Agricultural University.

After the adaptive feeding for 7 days, 30 mice were randomly divided into 6 groups with 5 mice in each group and were treated as follows *(Fig. 2-a)*: control group (Ctrl); ulcerative colitis group (UC), where the ulcerative colitis mouse model was obtained by supplementing 3.5% dextran sulfate

sodium (DSS) in the drinking water for 7 days; sulfasalazine group (SF), where UC mice were treated with sulfasalazine (150 mg/kg) for 7 days; SPD treatment groups, where UC mice were treated either with high dose of SPD (SPDH, 17.29 g/kg), medium dose of SPD (SPDM, 8.75 g/kg) or low dose of SPD (SPDL, 4.37 g/kg) for 7 days. Sulfasalazine and different dose of SPD was given to the mice by intragastric gavage and all treatments were administered to each mouse within 1 minute. According to *Methodology of Traditional Chinese Medicine Pharmacological Research*, the adult dosage is about 1.9 g/kg per day, and the conversion coefficient of body weight for mice is 9.1. Thus, the normal gavage dosage of SPD is 9.1 x 1.9 = 17.29 g/kg, which was used as the high-dose SPD administration group. Serial dilution was performed to obtain the medium-dose (8.75 g/kg) and low-dose (4.37 g/kg) groups of the SPD. Weight of the mice and disease activity index (DAI) were evaluated according to the method descripted previously [18].

Mice were anesthetized with isoflurane and euthanized with cervical dislocation at the end of the 14th day on trial. Serum was collected by orbital venipuncture and the length of the colon was recoded. Colonic tissue about 1 cm in length was fixed in 4% formaldehyde and the remaining colon tissue was washed with 1 x PBS, fast frozen in liquid nitrogen, and then stored at -80°C. The content of the colon was sampled and stored in -80°C for microbiota analysis.

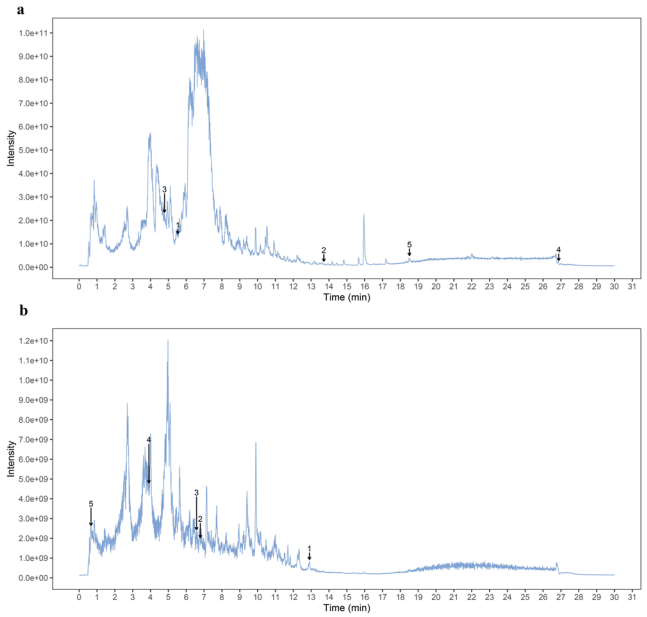

Fig 1. Representative components of SPD by UHPLC-QTOF-MS listed in *Table 1*. The total ion chromatograms (TIC) of SPD. **a-** The positive mode, **b-** the negative mode. Numbers indicate the representative compounds detected in SPD

H&E Staining Assay

Slides were prepared by the method described previously [19]. Briefly, the colon tissue fixed in formaldehyde were embedded in paraffin after dehydrated with different concentrations of ethanol and stained with H&E. Slides were visualized with a light microscope and captured using a high-resolution digital camera (Nikon Digital Sight DS-Fi1; Nikon Corporation, Minato-ku, Tokyo, Japan).

RNA Extraction, Synthesis of cDNA and Real-Time Quantitative PCR (RT-qPCR)

Total RNA was extracted from the colon tissue using RNA isolater Total RNA Extraction Reagent (Vazyme, Nanjing, Jiangsu, China) following the manufacturer's instruction. Concentrations of the isolated RNA were determined by NanoDrop2000 (Thermo Fisher Scientific Inc., Waltham, MA, United States). RNA was then reverse transcribed to cDNA using HiScript II Q RT SuperMix for qPCR kit (Vazyme, Nanjing, Jiangsu, China) according to the manufacturer's instructions. Relative gene expression of *MUC2*, and *Occludin* were assessed by RT-qPCR assay using a ChamQ SYBR qPCR Master Mix Kit (Vazyme) according to the manufacturer's instruction. RT-qPCR was performed on a Quantitative Real-Time PCR instrument (Analytik jena, Jena, Germany) and the reaction conditions were as follow: 95°C for 30 s; 95°C 10 s→60°C 30 s, 40 cycles; 95°C 15 s→60°C 60 s→95°C 15 s. Relative gene expression was analyzed by normalizing to GAPDH (an internal control) which has been tested by a previous report [20]. Data (CT value) obtained from RT-qPCR was analyzed by the $2^{-\Delta\Delta Ct}$ calculation method as previously described [21]. Primers were commercially synthetized by Sangon Biotech (Shanghai) Co., Ltd., and the information of primers used in the present study was listed in *Table 2*.

Detection of MDA, SOD, CAT, and GSH

Enzymatic activity of superoxide dismutase (SOD) in the serum of the mice were measured by Total Superoxide Dismutase Assay Kit (Beyotime Biotechnology,

Table 2. *Information of primers used in the present study*

Gene Name	Gene ID	Primer sequence (5'-3')	Product Length (bp)
MUC2	NM_023566.4	Forward: AGGGCTCGGAACTCCAGAAA Reverse: CCAGGGAATCGGTAGACATCG	106
Occludin	NM_001360538.1	Forward: ACGGACCCTGACCACTATGA Reverse: TCAGCAGCAGCCATGTACTC	260
GAPDH	NM_001411843.1	Forward: TGGAGAAACCTGCCAAGTATGA Reverse: TGGAAGAATGGGAGTTGCTGT	135

Shanghai,China) following the instructions. Enzymatic activity of catalase (CAT) and reduced glutathione (GSH), and the level of malondialdehyde (MDA) in serum of mice were detected with the kit purchased from Nanjing Jiancheng Biological Engineering Research Institute (Nanjing, China) according to the manufacturer's instructions. Detail information of the kits was presented in *Table 3*.

Enzyme-Linked Immunosorbent Assay (ELISA)

Protein levels of IL-1β, IL-6 and TNF-α in colonic tissue was measured by the kits obtained from Jiangsu Meimian Industrial Co., Ltd (Yancheng, China) according to the manufacturer's instructions. All experiments were replicated twice and data were calculated and normalized as the method described in the manufacturer's instruction. Detail information of the kits were listed in *Table 3*.

Gut Microbiota Analysis

Total genome DNA from samples was extracted using CTAB method. PCR amplification of the bacterial 16S rRNA genes 16S V4/16S V3/ 16S V3-V4 region was performed using the specific primers. Sequencing libraries were generated usingTruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit at 2.0 Fluorometer (Thermo Scientific, USA) and Agilent Bioanalyzer 2 1 0 0 system. At last, the library was sequenced on an Illumina NovaSeq platform and 250 bp paired-end reads were generated at Novogene Biotech Co., Ltd. (Beijing, China).

After the sequencing data were spliced and quality-controlled, the optimized sequence was obtained. Based on the optimized sequence, operational taxonomic unit (OTU) clustering was performed by UPARSE ((Uparse v7.0. 1001, *http://drive5. com/uparse/)* at 97% similarity, and taxonomic information of OTU representative sequences was obtained for subsequent bioinformatics analysis. Relative abundance of top 10 species at phylum level and top 20 species at genus level was analyzed and depicted with perl-SVG software (NovoGene, Beijing, China). The linear discriminant analysis (LDA) effect size (LEfSe) method was used to find species with significant differences in abundance between groups. All analyses were performed on the NovoMagic cloud platform *(https://magic.novogene.com)*.

Statistics Analysis

Data except for body weight and DAI value were analyzed via one-way ANOVA with Dengken's post hoc test, using SPSS Statistics 26.0 for Windows (IBM Corp., Armonk, NY). Data of body weight and DAI value were analyzed with general liner model by SPSS Statistics 26.0. Normality distribution of variables were assessed using the Shapiro-Wilk test. Results were presented as mean ± standard error of the mean (SEM). The GraphPad Prism software 8.3.0 (Boston, Massachusetts USA) was used to display the data graphically. Significance was set at $P<0.05$.

Results

The Chemical Components of SPD

Representative chemical components were detected by UHPLC-QTOF-MS and the components were labeled with number in the total ion chromatograms (TIC) by the positive ESI+ *(Fig. 1-a)* or negative ESI− mode *(Fig. 1-b)*. Detail information of the representative chemical such as Hyoscyamine, Quinic acid and Salicylic acid. components labeled in the TIC were listed in *Table 4*.

Effect of SPD on Body Weight (BW) and DAI Value of the Mice

As shown in *Fig. 2-b,* BW of mice treated with DSS decreased dramatically from the 5th day compared with that of control group. The lowest BW of the mice in groups treated with DSS were observed on the 8th day. The weight gain was observed after the removal of DSS (on the 9th day on trial). Body weight of the mice in SF and SPDH group were higher relative to that in UC group from the 10th day till the end of the trail. Body weight of mice in SPDM group were higher relative to that of UC group from the 12th day till the 14th day.

As shown in *Fig. 2-c*, the highest DAI value was observed in mice of all groups treated with DSS on the 6th day of the trial. DAI value in mice of SF and SPDH groups was lower relative to the mice in UC groups from the 7th day till the end of the experiment. DAI value in mice of SPDL and SPDM group was lower from the 10th day till the end of the experiment.

Table 3. *Information of kits used in the present study*

Name of Kit	Catalog Number	Inter-assay CV	Intra-assay CV	Assay Range
Catalase (CAT)	A007-1-1	4.94	1.9	0.2-24.8 U/mL
Malondialdehyde (MDA)	A003-1-2	4.11	3.5	0-113.0 nmol/mL
Reduced glutathione (GSH)	A006-2-1	3.86	1.2	0.3-147.1 mg GSH/L
Mouse tumor necrosis α (TNF-α)	MM-0132M1	<12%	<10%	25 ng/L - 800 ng/L
Mouse interleukin 6 (IL-6)	MM-0163M1	<12%	<10%	3 pg/mL - 120 pg/mL
Mouse interleukin 1β (IL-1β)	MM-0040M1	<10%	<10%	3.75 pg/mL -120 pg/mL

Table 4. Representative bioactive compounds in SPD

NO.	Name	Formula	Class	RT (min)	Intensity
1	Hyoscyamine	C17H23NO3	Alkaloids	287.2	2514433851.0
2	Quinic acid	C7H12O6	Phenolic acids	39.9	1043744763.5
3	6,7-Dihydroxycoumarin	C9H6O4	Phenylpropanoids	234.9	505557176.2
4	Salicylic acid	C7H6O3	Phenols	394.7	367475974.3
5	Isokobusone	C14H22O2	Alcohol	774.2	221537844.1
6	7,8-dihydroxy-6-methoxychromen-2-one	C10H8O5	Phenylpropanoids	332.2	144166554.2
7	Betaine	C5H11NO2	Alkaloid	1612.8	71955250.4
8	Rosmarinic acid	C18H16O8	Phenylpropanoids	407.3	32940559.2
9	Neoandrographolide	C26H40O8	Terpenoids	1110.8	22078150.8
10	Cafestol	C20H28O3	Terpenoids	822.9	18558173.1

Effect of SPD on Colon Length and Histopathological Changes of the Colon

As shown in *Fig. 2-d,e,* colon length (CL) of the mice in UC group (6.90±0.089 cm) was significantly shorter than that of the mice in control group (8.34±0.081 cm, P<0.05). Colon length of mice in SPDM (7.54±0.068 cm, P <0.05) and SPDH (8.00±0.45 cm, P<0.05) group was longer compared with CL of mice in UC group (6.90±0.089 cm).

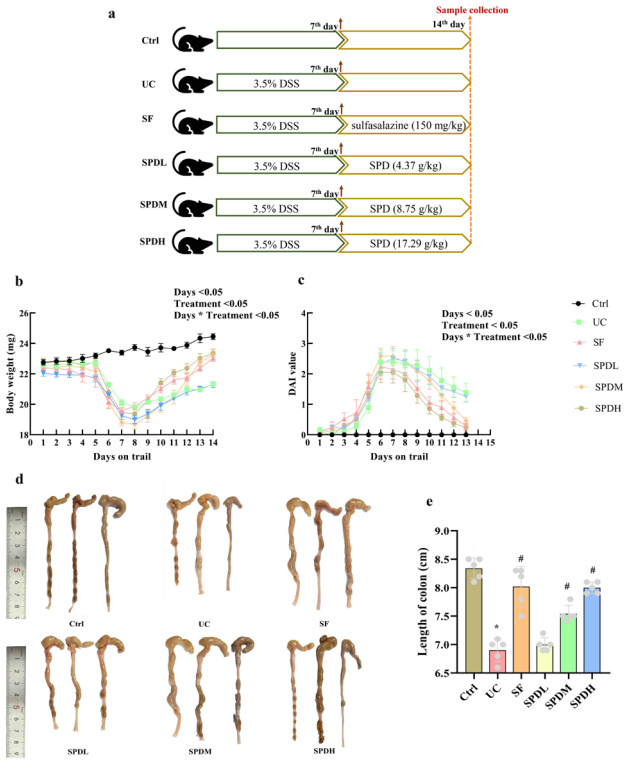

Fig 2. Effect of SPD on body weight, DAI index, and length of colon in DSS-induced UC mice. **a-** schematic diagram of the treatments, **b-** Body weight changes, P<0.05 indicates a significant difference, n=5, **c-** disease activity index (DAI), P<0.05 indicates a significant difference, n=5, **d-** Representative photos of colon, e- colon length in different groups. All data were expressed with mean ± SEM, * indicates significant change vs Ctrl group, P<0.05; # indicates significant change vs UC group, P<0.05. Ctrl, control group; UC, ulcerative colitis group; SF, sulfasalazine group; SPDL, low dose of SPD; SPDM, medium dose of SPD; SPDH, high dose of SPD

As depicted in *Fig. 3*, severe submucosal edema was observed in colon of mice in UC group compared with that in control group. Additionally, severe inflammatory cell infiltration in the mucosal layer and submucosa layer, intestinal gland atrophy and lysis was also observed in the colon of mice in UC group compared with that of control group. While the submucosal edema, inflammatory cell infiltration, and intestinal gland atrophy observed in the colon of the mice in UC group were dramatically decreased with the treatment of SF, SPDM and SPDH *(Fig. 3)*.

SPD Ameliorated UC by Inhibiting Proinflammatory Cytokine Expression and Enhancing Barrier Function

As shown in *Fig. 4-a,c*, protein concentration of IL-1β, IL-6 and TNF-α in colon tissue of the mice were significantly higher in UC group compared with that of Ctrl group (P<0.05). Protein concentration of IL-1β and IL-6 in colon tissue of the mice in SF, SPDL, SPDM, and SPDH groups were lower relative to

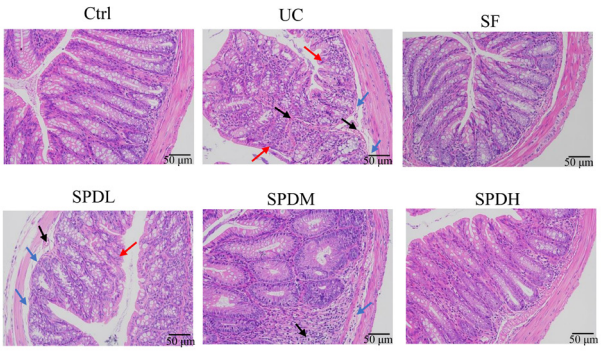

Fig 3. Representative sections from each group stained with H&E. Sections of different group were prepared and stained with H&E. The magnification was 200x and the scale bar was 50 μm. The red arrow indicates the atrophy and lysis of the intestinal glands. The black arrow represents inflammatory cell infiltration. The blue arrow represents tissue edema. Ctrl, control group; UC, ulcerative colitis group; SF, sulfasalazine group; SPDL, low dose of SPD; SPDM, medium dose of SPD; SPDH, high dose of SPD

that of UC group (P<0.05). Protein concentration of TNF-α in colon tissue of the mice in SF, SPDL and SPDH groups were lower compared with that of UC group (P<0.05).

Transcription of *mucin 2 (MUC2)* and *Occludin* was significantly lower in colon tissue of the mice in UC group compared with that of Ctrl group *(Fig. 4-d,e,* P<0.05). While transcription of MUC2 and Occludin was higher in colon tissue of the mice in SF, SPDM and SPDH groups compared with that of UC group *(Fig. 4-d,e,* P<0.05).

SPD Ameliorated the Oxidative Stress in Colon Induced by UC

Concentration of MDA in peripheral blood of mice in UC group was significantly higher compared with that of Ctrl group *(Fig. 5-a,* P<0.05). While the level of MDA decreased in peripheral blood of the mice in SF and SPDL, SPDM, and SPDH compared with that of UC group *(Fig. 5-a,* P<0.05). The enzymatic activity of GSH, SOD and CAT was lower in peripheral blood of mice in UC group compared with that of Ctrl group *(Fig. 5-b,d,* P<0.05). The enzymatic activity of GSH was higher in peripheral blood of the mice in SF, SPDM and SPDH groups compared with that in UC group *(Fig. 5-b,* P<0.05). The enzymatic activity of SOD was higher in peripheral blood of the mice in SF, SPDL, SPDM and SPDH groups relative to that of UC group *(Fig. 5-c,* P<0.05). The enzymatic activity of CAT in peripheral blood of the mice in SF and SPDH groups was higher compared with that of UC group (P<0.05).

SPD Ameliorated UC by Modulating the Microbiota in the Colon

As depicted in *Fig. 6-a,* the relative abundance of Proteobacteria was higher in the colon of the mice in UC group compared with that of the Ctrl group, while the abundance was decreased in response to SF and high dose of SPD treatment at phylum level *(Fig. 6-a)*. At genus level abundance of *Bacteroides*, *Parabacteroides* and *Prevotellaceae_UCG-001* was higher in the colon of the mice in UC group compared with that of the Ctrl group, but the abundance of these genus was lower in SF and SPDH group *(Fig. 6-b)*.

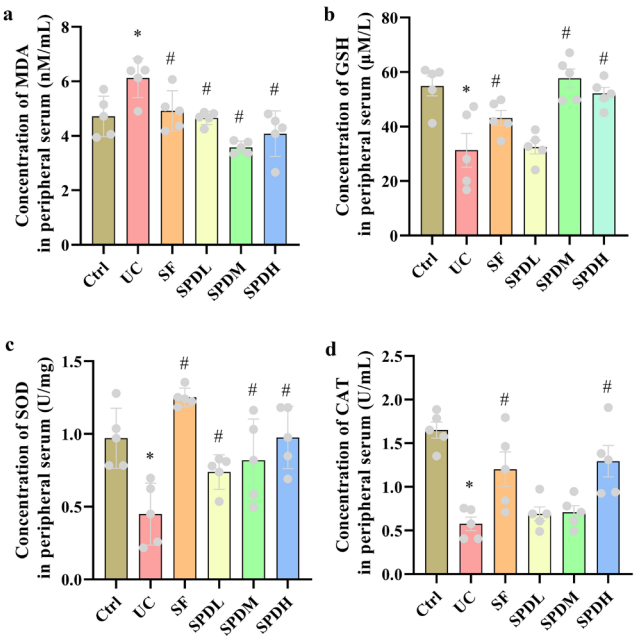

Fig 5. Effect of SPD on antioxidation index. Concentration of **(a)** MDA, **(b)** GSH, **(c)** SOD, and **(d)** CAT in peripheral blood of mice in different groups. All data were expressed with mean ± SEM. * indicates significant change vs Ctrl group, P<0.05; # indicates significant change vs UC group, P<0.05, n=5. Ctrl, control group; UC, ulcerative colitis group; SF, sulfasalazine group; SPDL, low dose of SPD; SPDM, medium dose of SPD; SPDH, high dose of SPD

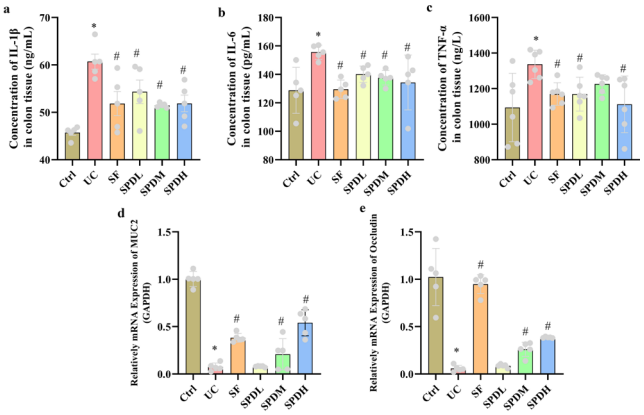

Fig 4. Effect of SPD on the level of proinflammatory cytokines and transcription of barrier proteins. Protein level of **(a)** IL-1β, **(b)** IL-6, **(c)** TNF-α and relative mRNA expression of **(d)** MUC2 and **(e)** Occludin in colon tissue of different groups. All data were expressed with mean ± SEM. * indicates significant change vs Ctrl group, P<0.05; # indicates significant change vs UC group, P<0.05, n=5. Ctrl, control group; UC, ulcerative colitis group; SF, sulfasalazine group; SPDL, low dose of SPD; SPDM, medium dose of SPD; SPDH, high dose of SPD

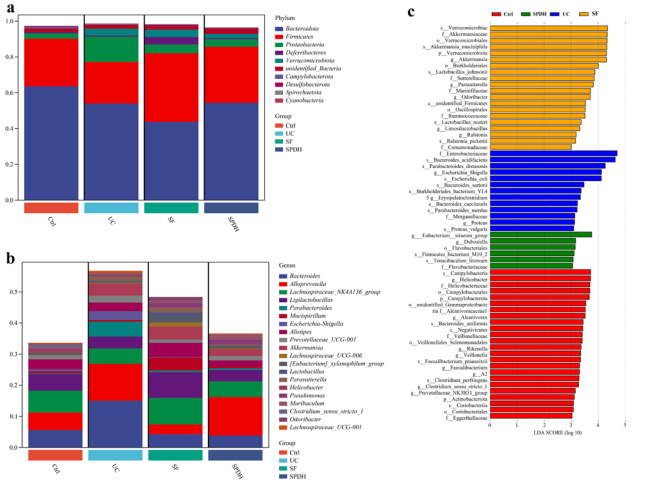

Fig 6. Effect of SPD on microbiota of mice with UC. **a**- Top 10 percent of community abundance of microbiota at phylum level, **b**- Top 20 percent of community abundance of microbiota at genus level, **c**- The LEfSe analysis of microbial abundance among groups, and LDA scores of bacterial taxa that were significantly enriched in each group (LDA score >3). Ctrl, control group; UC, ulcerative colitis group; SF, sulfasalazine group; SPDH, high dose of SPD

The representative species were further evaluated by LEfSe analysis. As presented in *Fig. 6-c,* genus *Helicobacter*, phylum Campylobacterota and other 22 species were significantly enriched in colon of the mice in Ctrl group. Genus *Escherichia_Shigella*, family *Enterobacteriaceae* and other 11 species were significantly enriched in colon of the mice in UC group. Phylum *Verrucomicrobiota,* genus *Akkermansia* and other 18 species were enriched in SF group and 6 species at genus level including: *Eubacterium__siraeum_group*, *Dubosiella*, *Flavobacteriales*, *Firmicutes_bacterium_M10_2*, *Tenacibaculum_litoreum* and *Flavobacteriaceae* were significantly enriched in colon of the mice in SPDH group.

Eight microbial species at genus level were found significantly modified in colon of the mice in Ctrl, UC, SF and SPDH groups *(Fig. 7-a)*. Furthermore, relative abundance of genus *Akkermansia* was significantly higher in in colon of the mice in UC and SF groups compared with that of Ctrl group *(Fig. 7-b,* P<0.05). While relative abundance of genus Akkermansia was lower in in colon of the mice in SPDH group compared with that of UC group *(Fig. 7-b,* P=0.2) and relative abundance of Ak*kermansia* in colon of the mice in SPDH group was similar compared with that of Ctrl group *(Fig. 7-b,* P>0.05).

Correlation Analysis Between Microbiota and Expression of Proinflammatory Cytokines, Antioxidant Enzymes and Tight-Junction Molecules

The results showed that the abundance of Bacteroidota was negatively associated with the level of IL-1β and positively associated with the level of CAT at phylum level *(Fig. 8-a)*. The level of IL-1β was positively associated

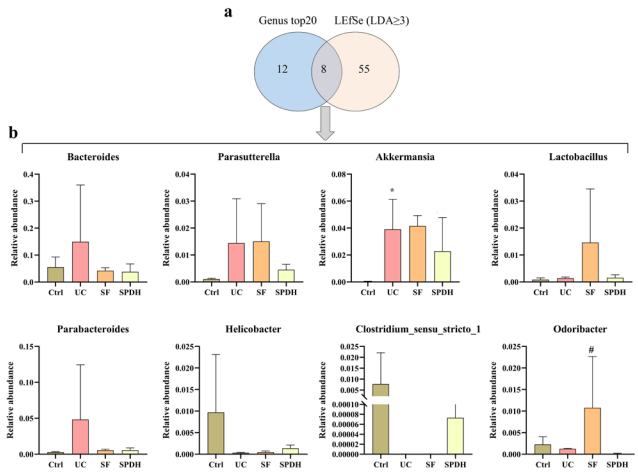

Fig 7. Differential gene screening and differential analysis. **a**- Venn Diagrams for LEfSe analysis and Random Forest Analysis, **b**- Relative abundance of differential microorganisms in different groups. All data were expressed with mean ± SEM. * indicates significant change vs Ctrl group, P<0.05; # indicates significant change vs UC group, P<0.05, P<0.05, n=4. Ctrl, control group; UC, ulcerative colitis group; SF, sulfasalazine group; SPDH, high dose of SPD

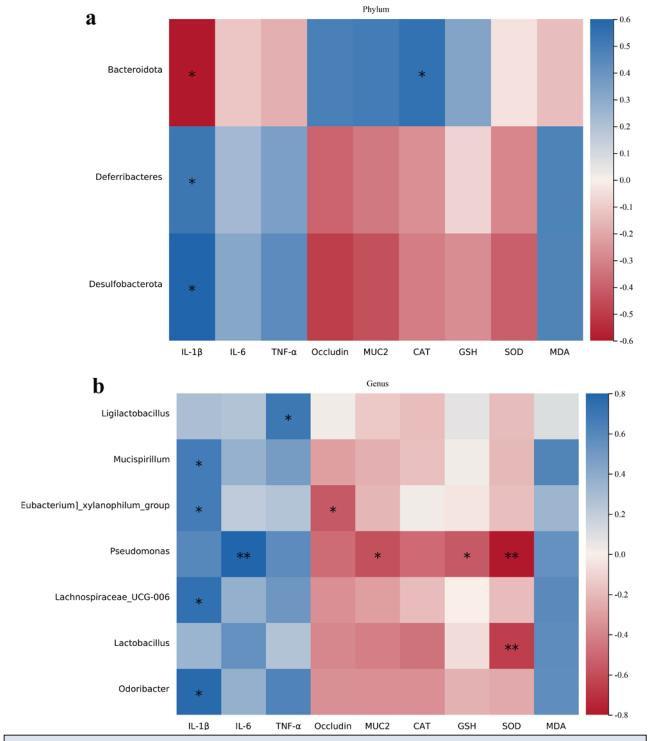

Fig 8. Spearman correlation analysis between intestinal microflora and inflammatory cytokines, barrier molecules and antioxidant enzymes. **a**- Phylum level, **b**- Genus level. Red color indicates negative correlation and bule color indicates positive correlation. * P<0.05, ** P<0.01, n=4

with abundance of Deferribacteres and Desulfobacterota at phylum level *(Fig. 8-a)*. The level of IL-1β was positively associated with abundance of *Odoribacter, Lachnospiraceae_UCG-006*, *Eubacterium]_xyanophilum_group* and *Mucispirillum* at genus level *(Fig. 8-b)*. The level of TNF-α was positively associated with the abundance of *Ligilactobacillus* at genus level *(Fig. 8-b)*. The abundance of *Pseudomonas* was positively associated with the level of IL-6, but was negatively associated with the level of *MUC2*, GSH and SOD at genus level *(Fig. 8-b)*. Relative mRNA level of *Occludin* was negatively associated with the abundance of *Eubacterium] _ xyanophilum_group* at genus level *(Fig. 8-b)*.

Discussion

Ulcerative colitis is a recurrent and relapsing disease, and its etiology is still not fully elucidated. Consequently, extensive studies have focus on developing novel therapeutic strategies for UC. Clinical manifestations, such as diarrhea, hematochezia, and weight loss are always observed in animals with UC [22]. In the present study, SF and SPD at different doses alleviated the lesions (increased DAI value and weight loss) induced by DSS. These findings confirm the therapeutic potential of SPD against UC. Subsequently, the present study investigated the underlying mechanisms of the therapeutic potential of SPD against UC.

Inflammation and immune dysregulation played key roles in the pathogenesis of UC, particularly involving cytokines and their regulatory pathways [5,23]. The present study shows that SPD treatment attenuated the UC-induced elevation of proinflammatory cytokines, including IL-1β, IL-6, and TNF-α. Elevated proinflammatory cytokines trigger immune cells activation, infiltration, and the initiation of inflammatory cascades [24]. Consistent with this, morphological analysis revealed reduced immune cell infiltration and tissue damage in SPD-treated groups, confirming that SPD mitigates intestinal inflammation by suppressing proinflammatory cytokine expression.

Intestinal barrier function of the gut is critical for the gut homeostasis, comprising physical barriers such as the mucus layer (composed of mucins secreted by goblet cells) and intercellular junctional complexes linking adjacent epithelial cells [25]. Mucin 2 is the major structural components of the intestinal mucus layer [26]. Occludin, a tetraspan integral membrane protein, contributes to the stability of tight junctions (TJs) [27]. The upregulation of *MUC2* and *Occludin* mRNA levels in SPD group suggested that SPD might ameliorated UC by enhancing the intestinal barrier function. However, further study is still needed to analyze the protein expression of MUC2 and occludin to validate these transcriptional changes which is the limitation of the present study.

Oxidative stress is well-recognized a pathogenic factor in UC and excessive generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) during chronic inflammation induces tissue damage [28]. Previous study shows that DSS treatment significantly reduces the enzymatic activity of CAT, GSH and SOD in mouse colon tissue [29], which is in line with results of the present study. The body's antioxidant defense system comprises three layers: small antioxidants molecular (e.g., uric acid, glutathione), antioxidant enzymes and damage-repairing enzymes [30]. Antioxidant enzymes exert protective effects by detoxifying ROS/RNS into less reactive metabolites. Superoxide dismutases and CAT are key antioxidant enzymes responsible for scavenging superoxide (O^{2-}) and hydrogen peroxide (H_2O_2), respectively [31,32]. So, down regulation of SOD, CAT and GSH activity leads to ROS accumulation, inducing damage of biologically important molecules (e.g., lipids) or cells. In the present study, SPD inhibited DSS-induced elevation of MDA and reverse the reduction in the activity of CAT, GSH and SOD, indicating that SPD ameliorated UC by enhancing antioxidant enzymes activity and mitigating oxidative stress. Furthermore, mass spectrometry analysis of SPD identified major bioactive components -including quinic acid, 7,8-dihydroxy-6-methoxychromen-2-one (fraxetin) and salicylic acid- all of which had been reported to possess anti-inflammatory and antioxidant properties [33,34]. These effective components in SPD could contribute to the therapeutic efficacy of SPD against UC.

Dysbiosis of gut microbiota is a well-established key factor in the pathogenesis of UC [35,36]. Previous study has shown that increased abundance of phylum Proteobacteria DSS-induce UC mice [22], which was also observed in the present study. Notably, high-dose SPD treatment reduced the DSS-induced overabundance of phylum Proteobacteria, which is closely associated with inflammatory diseases [37]. Genus *Bacteroides* is a commensal gut bacterium, while overabundance of *Bacteroides* was associated with increased degradation of intestinal mucus and compromised barrier function [38]. Genus *Parabacteroides*, a group of gram-negative anaerobic bacteria that colonizes the gastrointestinal tract of various species, has also been implicated in the pathogenesis of IBD [39]. In the present study, high-dose SPD reduced the DSS-induced elevation of genus *Parabacteroides* and *Bacteroides* abundances, which may contribute to its protective effects on intestinal barrier function and UC alleviation. Additionally, DSS-induced mice exhibited an increase in relative abundance of genus *Akkermansia*, genus *Akkermansia* could born inside the GI tract and possessed the ability to degrade mucin [40], while high-dose SPD reversed this increase. Collectively, these results demonstrate that SPD alleviated UC by modulating gut microbiota dysbiosis.

Correlation analysis revealed that pathogenic bacteria species was positively correlated with proinflammatory cytokines level and negatively correlated with the antioxidant enzymes activity and key intestine barrier molecules *(MUC2, occludin)*. These findings suggest tight crosstalk between gut microbiota alterations, inflammatory responses, antioxidant capacity, and intestinal barrier function in UC. However, the underlying detailed mechanisms required further investigation. Oxidative stress is reported to interrelated with inflammation. Decreased antioxidant enzyme activity results in the accumulation of ROS which promotes the proinflammatory response by modulating NF-κB signaling [41,42]. A limitation of the present study is the absence of treatment group of traditional PD group alone and *Portulaca oleracea L* alone, which would have enabled evaluation of whether their combination could synergistically enhance the therapeutic efficacy against UC.

In conclusion, SPD meliorated the DSS-induced UC by suppressing the expression of proinflammatory cytokines (IL-1β, IL-6 and TNF-α) and enhancing the activities of antioxidant enzymes (SOD, GSH and CAT), and the expression of key intestinal barrier molecules *(MUC2* and *Occludin)* and modulating gut microbiota dysbiosis.

Declarations

Availability of Data and Materials: The authors declare that the data and materials are available on request from the corresponding author (L. F.).

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Ethical Approval: The protocols of the trial involving animal experiments were reviewed and approved by Institutional Animal Use Committee of Henan Agricultural University (March 2nd 2022, HNND202203816).

Conflict of Interests: The authors declare no conflict of interest.

Declaration of Generative Artificial Intelligence (AI): The authors declare that the article, tables and figures were not written/created by AI and AI-assisted Technologies.

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