ShenLing BaiZhu San alleviates Ulcerative Colitis in Rats by Regulating Gut Microbiota

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Research

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Abstract

**Background:** Recent studies have suggested that Shenling Baizhu San (SLBZS), an complementary and alternative medical therapy for ulcerative colitis (UC), alleviate clinic symptoms by the improvement of biochemical criteria and restoration of the intestinal barrier function. SLBZS as a famous Chinese herbal formula has been reportedly used to treat UC, of which mechanism is unknown. This study investigated the therapeutic effects of SLBZS on restoring the gut microbiota in a UC rat model.

**Methods:** We proposed a 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced UC rat model to monitor the structural modulation of the gut microbiota. The test period was 10 days (observation for two days after modeling, treatment for 8 days by SLBZS). In this study, the level of inflammatory cytokines and activity of antioxidant enzymes in serum were ascertained by enzyme-linked immunosorbent assay (ELISA) and histological changes of colon were observed. Feces were collected for high-throughput sequencing of 16S rRNA gene.

**Results:** SLBZS partly reduced the diversity of the gut microbiota, while the abundance of that is increased. Furthermore, at the genus level, the relative abundance of short chain fatty acids (SCFA) producing bacteria including *Prevotella* and *Oscillospira* increased, while the relative abundance of harmful bacteria including *Desulfovibrio*, and *Bilophila* decreased. Additionally, SLBZS could improve the lesions of colon and significantly reduce the expression of Interleukin-6 (IL-6) and Myeloperoxidase (MPO) and increase the activities of Superoxide dismutase (SOD) and Catalase (CAT) in rats serum.

**Conclusions:** These results demonstrate that SLBZS may treat UC effectively by inhibiting inflammation, enhancing antioxidant capacity and regulating gut microbiota.

**Background**

Gut microbiota is considered to be an critical factor in deriving ulcerative colitis (UC) [1, 2, 3], which is characterized by abnormal microbiota leading to disruption of flora balance, decreasing the complexity of the intestinal microbial ecosystem [4]. At the same time, UC is thought to be caused by an imbalance between intestinal microbiota and mucosal immunity [5]. Among UC patients, the composition and functional diversity of intestinal microbiota and the stability of intestinal bacteria were reportedly destroyed [6]. Furthermore, the specific *Firmicutes* decreased, yet *Bacteroides* and facultative anaerobic bacteria increased [7].

Studies have shown that traditional Chinese medicine could obviously modulate the composition of the gut microbiota and the gut microenvironment [8, 9, 10]. Meanwhile, the gut microbiota is essential for the metabolism of traditional Chinese medicine in vivo [11, 12]. Shenling Baizhu San (SLZBS), originated from the Song Dynasty "Taiping Huimin Mixing Agent", is composed by *Panax Ginseng*, *Poria cocos*, *Atractylodes macrocephala*, *Dioscorea opposita*, *Dolichos Lablab*, *Semen Nelumbinis*, *Semen Coicis*, *Fructus Amomi*, *Platycodon grandiflorus*, and *Glycyrrhiza uralensis Fisch*, which is used for weakened of the spleen and stomach [13]. Modern pharmacological studies have revealed that many components of
SLBZS contain anti-inflammatory activities. Ginseng polysaccharides, one of the constituents of Panax Ginseng, improved intestinal metabolism and absorption of ginsenosides [14]. Furthermore, Ginsenoside Rg1, also one of the main constituents of Panax ginseng, and its metabolites could inhibit colitis [15]. 16α-hydroxytrametenolic acid from Poria cocos improved intestinal barrier function [16]. Yam polysaccharide from Dioscorea opposita reduced inflammation in the rat model of colitis induced by TNBS [17]. Moreover, it has been reported that SLBZS, by means of application of the combination, can treat UC in a significant way [13]. Besides, research shows that SLBZS could regulate the pathogenesis of UC with reduction of inflammatory cytokines, inhibition of pyroptosis and protection of colonic barrier integrity [18, 19]. Currently, there is no uniform conclusion on the effect of UC on gut microbiota, and the traditional Chinese medicine SLBZS treats UC, which has not been theoretically explained.

2,4,6-trinitrobenzene sulfonic acid (TNBS) used to establish UC model is a common modelling method [20]. In this study, TNBS was used to induce the UC model to evaluate the efficacy and safety of SLBZS in the treatment of UC. And then fecal samples were collected after 7-day to identify the change of structure and diversity of the gut microbiota in response to the SLBZS treatment for the alleviation of UC. Furthermore, the levels of serum inflammatory factors and activity of antioxidant enzymes were measured, and the pathological changes of colon in UC rats were observed.

Materials And Methods

Preparation of TNBS and SLBZS

TNBS was purchased from Sigma-Aldrich Co., Ltd.. TNBS is dissolved in 50% ethanol solution to prepare 5% TNBS ethanol solution when used. The traditional Chinese medicine prescription SLBZS, purchased from Beijing Tongrentang (Lot no:16101034), comprises of Panax Ginseng, Poria cocos, Atractylodes macrocephala, Dioscorea opposita, Dolichos Lablab, Semen Nelumbinis, Semen Coicis, Fructus Amomi, Platycodon grandiflorus, and Glycyrrhiza uralensis Fisch, and is dissolved in distilled water when used.

Animal

80 g-100 g SD male rats, purchased from the Center of Experimental Animals of Southern Medical University (approval number:SCXK 2016-0041), and the rats were housed in plastic cages with the ambient temperature controlled at 22 °C – 24 °C, light for 12 hours and free drinking water and food. The bottom horn mesh and white paper make it easy to observe the stool specimens. Replace the padding every day during the test. All the experimental procedures of this study were approved by the Animal Ethics Committee of the South China Agricultural University (Guangzhou).

In this experiment, after 5 days of adaptive feeding, 40 male rats were randomly divided into normal control group (CON), model group (TNBS), low dose group (TNBS-L), medium dose group (TNBS-M) and high dose group (TNBS-H) (n = 8). With TNBS using in model building, rats were weighed one day ahead of modeling and would fast except water in 12 hours before modeling. Anesthetized with isoflurane, except in CON group, rats were given an enema with 2.5 ml/kg TNBS according to their weigh to induce
UC, while rats in CON group were given an enema with an equal volume of physiological saline. The test period was 10 days (observation for two days after modeling, treatment for 8 days). After modeling, rats in CON group and TNBS group were intragastrically administered with 2 ml of physiological saline, 2 ml (0.1 g) SLBZS for rats in TNBS-L group, 2 ml (0.2 g) of SLBZS for rats in group TNBS-M and 2 ml (0.3 g) of SLBZS for rats in TNBS-H group. On the 9th day, feces were frozen immediately with liquid nitrogen and stored at -70 °C. On the last day of the experiment, the abdominal aorta of anesthetized rats were dissected to collect blood samples, which were then separated for serum.

16S rRNA gene sequence analysis of gut microbiota in fecal samples

The total DNA of the feces was extracted using the TIANamp STool DNA Kit (BEIJING, DP328). The extracted DNA was determined by 0.8% agarose gel electrophoresis, quantitatively analyzed by an ultraviolet spectrophotometer. And then, selected DNA was amplified by 16S rRNA V3-V4 region. The 16S rRNA V3-V4 region-specific primer for PCR amplification was 338F (5-barcode + ACTCCTACGGGAGGCAGCA-3’), 806R (5’-GGACTACHVGGGTWTCTAAT-3’). The PCR reaction system (25 µL) was as follows: 0.25 µL Q5 high-fidelity DNA polymerase PCR, 5 µL Reaction Buffe (5×), 5 µL High GC Buffer (5×), 2 µL ntp (10 mM), 2 µL of template DNA, 1 µL of each primer, 8.75 µL of double distilled water. The PCR reaction conditions were 98 °C for 30 s initially, followed by 25 cycles of denaturation at 98 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 30 s. The PCR amplification products were identified by electrophoresis, and then the amplified products were recovered and purified using the Axygen DNA Gel Recovery and Purification Kit. The products were sequenced on the Illumina MiSeq sequencing platform.

Bioinformatics and statistical Analysis

QIIME was used for Operational Taxonomic Unit (OTU) classification and identification [21, 22]. Using R software to draw rarefaction curve, and calculate Alpha diversity index, including Chao1 estimator, Shannon diversity index. The Principal Component Analysis (PCA) and weighted and unweighted Nonmetric Multidimensional Scaling (NMDS) analysis based on UniFrac were carried out for community composition structure at genus level by R software [23, 24]. According to the statistics of the relative abundance of two levels of taxonomy, two levels of the phylum and the genus are analyzed. All data obtained in this study were processed statistically and divergence were presented as means ± SE. Analysis of variance was used for multiple comparison. SPSS Statistics 20.0 for Windows was used and P < 0.05 was considered to be significant differences.

ELISA Test for IL-6, MPO, SOD and CAT after treatment

Interleukin-6 (IL-6), Myeloperoxidase (MPO), Superoxide dismutase (SOD) and Catalase (CAT) levels of serum were determined using ELISA kit according to the manufacturer's protocol (Shanghai Meilian Biological Technology Co., Ltd., Shanghai, China; Nanjing Jiancheng Bioengineering Inst., Nanjing, China).
Histological observation of colon

The collected colon tissue was fixed in 10% formalin, dehydrated with different concentrations of ethanol, embedded in paraffin, stained with hematoxylin and eosin (HE) and cleaned by PBS, incubated in proteinase K and TUNEL solution, labeled by DAPI (TUNEL), sliced under a fluorescence microscope and imaged.

Result

Histological Changes of colon tissue in each group after treatment

Diarrhea, slightly rectal prolapse, and slightly colonic swelling were observed in TNBS-induced rats. After administration of SLBZS, the lesions of colon in UC rats was improved. The details are shown in Supplementary materials (Fig. S1). The results illustrated that there was normal histological feature in CON group, but a large number of infiltratedly inflammatory cells and fuzzy structure of each layer in TNBS group and the structure of each layer of TNBS-H group was clearer and more integrated compare to TNBS group, TNBS-L group and TNBS-M group (Fig. 1a). More apoptotic cells in TNBS group compared to SLBZS group (Fig. 1b). SLBZS reversed these changes.

Change of the structure of the whole intestinal microbiota of each group after treatment

After high-throughput sequencing, 1707839 effective sequences were obtained, including 307951 in CON group, 302534 in TNBS group, 383561 in TNBS-L group, 377902 in TNBS-M group and 335891 in TNBS-H group. The QIIME software performed OTU partitioning on these sequences, which were based on 97% sequence similarity. Beta diversity analysis including PCA and and weighted and unweighted NMDS based on unifrac were used to analyze the similarity of the gut microbiota among different samples. PCA and NMDS analysis revealed that (Fig. 2a-c) the structure of gut microbiota in TNBS group differed from CON group. However, after administration of SLBZS, the structure of intestinal microbiota in SLBZS group was similar to CON group, particularly in TNBS-M and TNBS-H group, which proved that administration of SLBZS could restore the intestinal structure of UC rats. The Chao1 and Shannon curves (Fig. 2d,e) indicated that the curve tended to be flat when the sequencing depth was greater than 15000, proving that the sequencing depth was sufficient to reflect the species diversity and basically contained all species in the sample. The Alpha diversity index (Fig. 2f,g) showed that shannon diversity index in TNBS-L group was lower than CON group and TNBS group (P < 0.05) and chao1 estimator in SLBZS group was higher than CON group and TNBS group (P > 0.05).

Taxonomic composition of communities at phylum and genus levels after treatment
The three typical microbiota at the phylum level were *Firmicutes*, *Bacteroidetes* and *Proteobacteria* (Fig. 3a). SLBZS treatment could increase the relative abundance of *Firmicutes* and *Proteobacteria* and reduce *Bacteroidetes* in UC rats (Fig. 3b). At the genus level (Fig. 3c), 6 of 110 genera were typically different after SLBZS treatment. In TNBS-L and TNBS-H group, the relative abundance of *Prevotella* increased to normal level, but *Bilophila*, *Bacteroides* and *Helicobacter* decreased compared to TNBS group. In TNBS-M group, the relative abundance of SLBZS *Oscillospira* was close to normal level (Fig. 3d).

**ELISA test for serum inflammatory factors and antioxidant enzymes after treatment**

The ELISA test (Fig. 4) showed that low dose of SLBZS treatment could significantly reduce the level of IL-6 ($P < 0.05$) and SLBZS treatment could reduce the heightened activity of MPO induced by UC ($P < 0.05$). The SOD activity of TNBS-L and TNBS-M group was elevated compared to TNBS group ($P > 0.05$). The CAT activity of TNBS-L and TNBS-H group was elevated compared to TNBS group ($P > 0.05$).

**Discussion**

Ulcerative colitis is a chronic inflammatory disease of colon with unclear mechanism. Generally, it has been believed that its pathogenesis involves the defect of epithelial barrier defects, dysregulated immune responses, and the disorder of intestinal microbiota. In TNBS-induced ulcerative colitis, UC rats are represented by diarrhea, ulceration of colon tissue, increase of IL-6 level and enhancement of MPO activity in serum [25, 26]. In this study, all UC model rats demonstrated clinical symptoms of diarrhea. Concurrently, overexpression of inflammatory factors and the disturbance of gut microbiota existed in UC model rats, indicating the UC model was successfully established in this study.

Colonic epithelial cells and mucosal barrier are strongly related to the pathogenesis of UC. By inhibiting the apoptosis of colonic epithelial cells, mucosal ulceration and mucosal epithelial cell damage in UC rats can be improved [27]. As a famous formula for 900 years, SLBZS has been widely used in the treatment of gastrointestinal diseases. It’s has been reported that SLBZS might exhibit ameliorating effects against diarrhea by modulations on intestinal absorption function as well as mucosal ultra structure [28]. From the pathological section of the colon and the change of the colon in each group of the experiment, high dose of SLBZS for UC had a certain recovery effect on the intestinal villi detachment, the over all structural damage of the colon, inflammatory cell infiltration and apoptosis induction, which was beneficial to regulate the reabsorption capacity and mucosal barrier of the colon. The level of inflammatory factors and activities of antioxidant enzymes in rats serum were also ascertained. Previous studies delineated that overexpression of IL-6 could lead to a continuous inflammatory response and in turn promote inflammatory bowel disease [29]. IL-6 can promote inflammation by activating multiple target cells, including antigen-presenting cells and T cells [30]. MPO is mainly located in aniline blue particles in neutrophils [31], which reflects the inflammatory state to some extent. Studies have found that reactive oxygen species (ROS) is closely related to UC colon mucosal tissue damage [32]. Although
low level of ROS are necessary for some physiological processes, excessive ROS are produced in UC patients [33]. SOD and CAT can remove ROS, prevent lipid peroxidation and maintain the stability of cell membrane. In our study, we observed that low dose of SLBZS treatment could decrease the level of IL-6 and MPO (P < 0.05) and increase the activities of SOD and CAT (P > 0.05) while all dose SLBZS treatment could significantly decreased the level of MPO (P < 0.05) compared to TNBS group. Our study proved that SLBZS could treat UC by inhibiting inflammation and improving antioxidant capacity.

Abnormal microbial composition and reduced complexity of intestinal microbial ecosystem are common features of ulcerative colitis [34]. To monitor the structural modulation of the gut microbiota during UC treatment with SLBZS, high-throughput sequencing analysis of 16S rRNA genes was performed in our study. As reflected by the chao1 estimator and shannon diversity index, we found that SLBZS treatment reduced the shannon diversity index but increased the chao1 estimator, suggesting SLBZS treated UC by reducing the diversity of gut microbiota while increasing its richness. PCA and NMDS showed that SLBZS treatment could change the structure and composition of the microbiota, and the structure of the microbiota can be closer to the normal state than the TNBS group. In order to analyze the further difference in the structure of gut microbiota after treatment, this study also carried out a comparative analysis of the gut microbiota at the phylum and genus levels of each group. We found that the relative abundance of Bilophila, Desulfovibrio and Bacteroides decreased in TNBS-L and TNBS-H compared to TNBS group while Oscillospira and Helicobacter increased in TNBS-M and Prevotella increased in TNBS-L and TNBS-H group. Prevotella and Oscillospira are short chain fatty acid (SCFA) producing bacteria [35, 36], and SCFA, important nutrients of colon mucosal, is capable of colon cell proliferation and mucosal growth [37]. Undigested dietary fiber, protein and peptides can be fermented through gut microbiota in the cecum and colon, resulting in the generation of SCFA. SCFA can induce intestinal epithelial cells to secret IL-18, antimicrobial peptide, mucin and upregulate the expression of tight junction to regulate the integrity of intestinal barrier [38]. Meanwhile, SCFA can induce neutrophil migration and enhance phagocytosis [39]. Bacteroides is involved in metabolism and nutrient absorption in vivo [40], but promotes inflammation in inflammatory bowel disease [41]. Both Desulfovibrio and Bilophila are conditional pathogens, creating H₂S in combination with H₂ by sulfuric acid or sulfur-containing compounds, which has an important relationship with the inflammatory state of the intestinal epithelium (such as UC) [42]. Studies have shown that the body with Helicobacter removed is more susceptible to colitis than the untreated group, suggesting that Helicobacter has potential protective effects on colitis patients [43]. Therefore, these result further indicated that the amelioration of UC using SLBZS may be mediated by the enrichment of beneficial bacteria to product SCFA for protection of colon mucosa and a reduction in bacteria, such as Bacteroides, Desulfovibrio and Bilophila, to inhibit inflammation.

Conclusions

In summary, our study proves that gut microbiota structurally remodeled in UC rats after administration of SLBZS, additionally with increase of beneficial bacteria, such as Prevotella and Oscillospira, and reduction of harmful bacteria like Desulfovibrio and Bilophila. In addition to remodeling the structure of
gut microbiota, SLBZS can inhibit inflammation and enhance antioxidant capacity. But how SLBZS-mediated changes in the gut microbiota contribute to the improvement of UC need further study.

**Abbreviations**

SLBZS: Shenling Baizhu San; UC: ulcerative colitis; TNBS: 2,4,6-trinitrobenzene sulfonic acid; ELISA: enzyme-linked immunosorbent assay; SCFA: short chain fatty acids; OTU: Operational Taxonomic Unit; IL-6: Interleukin-6; MPO: Myeloperoxidase; SOD: Superoxide dismutase; CAT: Catalase; PCA: Principal Component Analysis; NMDS: Nonmetric Multidimensional Scaling.

**Declarations**

**Acknowledgements**

Not applicable.

**Authors’ contributions**

DG, SZ, and CL jointly participated in the experimental design in this study. DG, SZ, LY, YT, XC, DS and SG performed relative experiments. DG and SZ wrote the original manuscript. All authors edited the manuscript. LY and XC contributed to sampling and data analysis. All authors contributed to the revision of this manuscript.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

All the experimental protocols involving handling and euthanasia of rats were approved by the Animal Ethics Committee of the South China Agricultural University.

**Consent to publish**
The authors declare that they consent to publish this manuscript.

**Competing interests**

The authors declare that they have no competing interests.

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Figures
Figure 1

Histological changes of colon. (a) HE staining (scale bar is 100 μm). (b) TUNEL staining (scale bar is 100 μm)
Figure 2

The effect on the intestinal microbiota structure. (a) PCA analysis; (b) Unweighted NMDS analysis; (c) weighted NMDS analysis; (d) Shannon curves; (e) Chao1 curves; (f) Shannon diversity index of each group; (g) Chao1 estimator of each group. a-cBars in the same index marked without the same letters differ significantly (P<0.05)
Figure 3

Taxonomic composition of communities. (a) Histogram of microbial composition at phylum level in each group. (b) Relative abundance of bacteria at the phylum level. (c) Histogram of microbial composition at genus level in each group. (d) Relative abundance of bacteria at the genus level. a-cBars in the same index marked without the same letters differ significantly (P<0.05)
Figure 4

ELISA test for serum inflammatory factors and antioxidant factors after treatment. IL-6, MPO, SOD, CAT levels in UC cats. a-bBars in the same index marked without the same letters differ significantly (P<0.05)

Supplementary Files

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