Akkermansia muciniphila alleviates colonic epithelial endoplasmic reticulum stress through activation of farnesoid X receptors in murine experimental colitis

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Research Article
Abstract

Background

Endoplasmic reticulum (ER) stress-related mucin depletion could be involved in the pathogenesis of inflammatory bowel disease (IBD). Akkermansia muciniphila (A. muciniphila), a symbiotic bacterium of the mucus layer, uses mucin as its sole energy source and shows potential in the treatment of colitis. However, the effects and underlying mechanisms of A. muciniphila on colonic epithelial ER stress in colitis are largely unknown.

Methods

Colitis was induced by adding 2.5% DSS in drinking water. Mice were orally administered A. muciniphila (3*10^7, 3*10^8 cfu/day) once daily for 10 days during DSS intervention. UHPLC high-resolution orbitrap mass spectrometry-based metabolomic analyses were performed on faeces. 16S rRNA sequencing were used to quantify and characterize the gut microbiota of mice and human. Colons were collected from mice and analyzed by histopathology, quantitative PCR and immunofluorescence. Colon biopsies from the patients with ulcerative colitis (UC) and controls were collected and analyzed by immunohistochemistry.

Results

Metabolite pathway enrichment analysis demonstrated that colitis-affected metabolites after A. muciniphila supplementation were mainly enriched in mineral absorption, bile secretion and protein digestion and absorption. P-hydroxyphenyl acetic acid, which showed the highest VIP scores, was significantly increased by A. muciniphila, and could cause ER stress. A. muciniphila supplementation changed the relative abundance and composition of intestinal microbiota especially a decrease in Parasutterella, which showed the potential role in bile acid maintenance. A. muciniphila supplementation protected colon shortening, histological injury, intestinal inflammation and barrier damage in wild-type (WT) mice but not in farnesoid X receptor-null (FXR^-/-) mice. Mechanistically, A. muciniphila supplementation activated FXR/SHP signaling, which directly increased X-box binding protein-1 splicing (XBP1s) and phosphorylated inositol requiring enzyme 1α (p-IRE1α) expression, and in turn formed XBP1s-SHP regulatory loop in response to ER stress. We further showed that the abundance of A. muciniphila in faeces from UC patients positively correlates with p-IRE1α expression.

Conclusions

Our results suggest that A. muciniphila supplementation alleviates DSS-induced colitis involvement of the IRE1α/XBP1 ER stress pathway via FXR/SHP axis activation.
Introduction

Ulcerative colitis (UC) is a chronic inflammatory disease in which inflammation begins in the rectum and extends proximally throughout the colon, with major symptoms including blood in the stool, diarrhea and abdominal pain[1, 2]. Although medications are the most effective way for UC treatment, only approximately 40% of patients achieve clinical remission, highlighting the need to explore new treatment strategies[3]. Akkermansia muciniphila (A. muciniphila) has been widely regarded as a promising candidate of next-generation probiotics since it promotes the integrity of the mucosal layer, thereby creating a healthy environment for intestinal epithelial cells[4, 5]. Ample studies have showed that A. muciniphila was significantly reduced in UC patients and UC animal models, and the intervention of A. muciniphila could significantly improve DSS-induced colitis in mice[6–9], however, the mechanisms underlying A. muciniphila supplementation against UC are largely unknown.

As a mucin-degrading bacterium, A. muciniphila feeds on mucin and produces specific byproducts such as acetate and propionate to feed other beneficial gut bacteria to make butyrate, providing energy source for mucus-secreting goblet cells and intestinal epithelial cells. Recent studies have revealed that aberrant mucin assembly in mice causes endoplasmic reticulum (ER) stress and spontaneous inflammation resembling UC[10]. ER is the main site of protein synthesis, folding, lipid synthesis, and carbohydrate metabolism[11]. Under ER homeostasis, binding immunoglobulin protein (BiP) proteins bind to ER stress sensors (three ER transmembrane proteins, activating transcription factor 6(ATF6)/ IRE1α/ protein kinase RNA-like ER kinase (PERK)) and keep them in an inactive state[12]. In disease states, protein misfolding and accumulation of unfolded proteins occur, BiP proteins have a higher affinity for misfolded/unfolded proteins, and the aforementioned ER sensors are released, activating ER stress, which in turn induces an unfolded protein response (unfolded protein response (UPR), also known as ER stress response)[13–15]. ER stress may contribute to the aggravation of UC by disrupting intestinal barrier function and activating intestinal inflammatory responses[16, 17]. These data reveal that the proven efficacy of A. muciniphila in colitis may be involved in modulating the mucin secretion process and ER restoring.

Gut microbiota homeostasis could be affected by bile acid signaling both directly and indirectly[18]. A. muciniphila, one of the most related commensal gut bacteria, could efficiently increase bile acid metabolism[19, 20]. Farnesoid X receptor (FXR), the main regulator of maintaining bile acid homeostasis, has been proven to be related to ER stress[21, 22]. FXR activation by betulinic acid[23], obeticholic acid[24] and GW4064[25] has been shown to improve disease phenotype from ER stress. Based on the findings, we speculate that A. muciniphila may modulate endoplasmic reticulum restoring in experimental colitis through FXR signaling.

Here we show that A. muciniphila supplementation could reshape the gut microbiota, modulate bile acid metabolism, and alleviate the colonic epithelial ER stress in DSS-induced colitis. Mechanistically, A. muciniphila supplementation stimulated the IRE1α/XBP1 pathway activation via FXR, and, in turn, formed XBP1s-SHP regulatory loop to maintain ER homeostasis. Moreover, UC patients had lower levels of XBP1s when compared with healthy controls. The richness of human gut A. muciniphila in UC patients
correlates with p-IRE1α expression. Our data demonstrate a significant effect of \( A. \) \textit{muciniphila} on experimental colitis through FXR-dependent ER stress inhibition and reveal XBP1s-SHP positive regulatory loop activation by bile acids may be protective during colitis and have therapeutic implications for IBD.

**Results**

3.1 \( A. \) \textit{muciniphila} \textit{altered faecal metabolic composition in DSS-induced mice}

Oral gavage with \( A. \) \textit{muciniphila} in DSS-induced mice was conducted since the abundance of \( A. \) \textit{muciniphila} in UC patients was lower significantly than healthy control (supplementary data 1). Faecal samples from the control, DSS, and \( A. \) \textit{muciniphila} (AKK) groups were collected and analyzed by UHPLC-QE Orbitrap/MS in the positive ion mode, which represented physiological status, pathological conditions, and intervening effects. The supervised OPLS-DA analysis was performed among the three groups. As is depicted in Fig. 1A and 1B, there were significant separations in the metabolic profiles among the three groups.

The volcano plots generated to identify the differential metabolites were shown in Fig. 1C, and there were 125 differential metabolites identified. Moreover, red dots represented the up-regulated metabolites (fold change, FC > 1), and green dots represented the down-regulated metabolites (FC < -1). Venn diagrams indicated different and shared metabolites among the three groups. Based on the KEGG pathway enrichment analyses, differential metabolites between DSS and AKK groups were predominantly enriched in mineral absorption, bile secretion and protein digestion and absorption (Fig. 4E). In the mineral absorption pathway, we found that hydrogen phosphate was significantly downregulated by \( A. \) \textit{muciniphila} \((P = 0.026)\), and hydrogen phosphate had been reported significantly associated with ER stress\([26, 27]\).

The metabolites with features of VIP > 1 and \( p < 0.05 \) were considered potential significant differential metabolites. The top 20 differential metabolites in fecal samples between the DSS and AKK groups were shown in Fig. 1F. The intervention of \( A. \) \textit{muciniphila} significantly increased the expression of P–Hydroxyphenyl Acetic Acid, Ketophenylbutazone, p-Cresol, Carnitine C2:0, and decreased the expression of 3-Formyl-2,4-dihydroxy-6-methylbenzoic acid, 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine, Lysope 18:1, Demethylphylloquinone, 2-Amino-2-deoxymannose. Among them, P–Hydroxyphenyl Acetic Acid\([28]\), and 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine with the top 3 VIP scores were closely related to the inhibition of ER stress \([29]\). The analyses results of fecal metabolomics revealed that the activation of ER stress induced by DSS was attenuated by \( A. \) \textit{muciniphila}. Moreover, \( A. \) \textit{muciniphila} might also have certain impact on bile acids under the experimental colitis setting.

3.2 \( A. \) \textit{muciniphila} \textit{supplementation modulated the structure of gut microbiota and bile acid dysmetabolism in DSS-induced mice}
To quantify and characterize the gut microbiota of mice under different conditions, 16S rRNA sequencing was utilized. The Shannon index was used as a metric to analyze differences in alpha diversity between the groups. ACE and PD-tree index was used as a metric to analyze differences in alpha diversity between the groups (Fig. 2A-B). We observed a decrease to normal level in alpha diversity in the AKK-H group compared to that in the DSS group (P < 0.05). Principal coordinates analysis (PcoA) plots were calculated from unweighted_unifrac distances to evaluate the composition of the community, and the results revealed a clear separation among the three groups (Fig. 2C). We also observed a marked separation among the three groups in the system clustering tree (Fig. 2D). The most abundant taxa at the phylum, family and genus levels are shown in Fig. 2F-H. Following DSS exposure, A. muciniphila supplementation significantly increased the relative abundance of Bacteroidota and Desulfovibrio. Notably, A. muciniphila supplementation significantly decreased the relative abundance of Parasutterella (Fig. 2E), which was found enhance the catabolism of bile acid[30].

We therefore identified a total of 27 fecal bile acids (BAs) across all treated groups using LC/QTRAP-MS. The ratio of secondary to primary BAs was lower in the colitis group compared with those in the controls and was restored to normal levels in the A. muciniphila-treated group (Fig. 2I). DSS administration markedly decreased the levels of DCA, CDCA and UDCA, which are natural agonists of FXR, and A. muciniphila treatment substantially enhanced their levels in mice (Fig. 2J). Compared with the colitis group, A. muciniphila treatment increased the levels of TDCA, TCA, TUDCA, LCA, isoLCA and HDCA. The results demonstrated that A. muciniphila treatment regulated the dysbiosis of gut microbiota as well as bile acid dysmetabolism induced by DSS.

3.3 A. muciniphila supplementation prevented DSS-induced colitis in WT mice, while this effect was lost in Fxr-null mice.

As shown in Fig. 3, the DSS group showed significant weight loss, diarrhoea, haematochezia and other colitis symptoms. Compared to that of the control group, the colon length was markedly shortened in the DSS group, and the colon tissue was characterized by inflammatory cell infiltration, epithelial cell destruction and mucosal thickening both in WT mice and FXR−/− mice. AKK-H was used to significantly improve weight loss, DAI score, colonic shortening and restored intestinal epithelial structure induced by DSS in WT mice. Consistent with the symptom observations, A. muciniphila treatment did not contribute to weight loss, DAI score, colonic shortening and the reduction of severe intestinal epithelial damage in FXR−/− mice. These results indicated that AKK-H has an obvious protective effect on DSS-induced colitis in WT mice but not in FXR−/− mice.

To elucidate the inflammatory response in DSS-induced mice, various proinflammatory cytokines were measured in colon tissues at the mRNA level. The levels of proinflammatory cytokines, including interleukin6 (IL6) and interleukin1β (IL1β), were increased in DSS-induced colitis mice. Quantification of specific cytokines using ELISA showed the same patterns in the regulation of production and secretion of pro-inflammatory cytokines. These elevated proinflammatory cytokines were all decreased by AKK in WT mice not in FXR−/− mice.
3.4 A. muciniphila supplementation FXR-dependently maintained the intestinal barrier integrity in DSS-induced mice.

To understand the effect of A. muciniphila on the intestinal barrier integrity of the mice with DSS-induced colitis, intestinal permeability was measured using dextran-FITC as a tracer. The expression of MUC2 in the colon was determined by immunohistochemical staining. Due to mucin produced by goblet cells, we examined the colon in mice from different groups and counted the number of goblet cells by PAS/AB staining. As shown in Fig. 4, serum dextran-FITC levels were significantly higher in colitis mice. After treatment of A. muciniphila, the levels of dextran-FITC in serum was decreased in WT mice, but not in FXR⁻/⁻ mice. Immunohistochemical staining results showed that the content of MUC2-positive cells in DSS-induced mice was significantly lower than that in the control group. PAS/AB staining results revealed the number of goblet cells was significantly decreased in DSS mice compared with that in control mice. A. muciniphila supplementation significantly enhanced the expression of MUC2 and increased the number of goblet cells and MUC2-positive cells in each villus. While, in FXR⁻/⁻ mice, the recovery of the DSS-induced goblet cells injury mediated by A. muciniphila was largely suppressed. Meanwhile, the effects of A. muciniphila on the expression of MUC2 were also blocked in FXR-deficient mice.

3.5 A. muciniphila supplementation FXR-dependently attenuated colonic ER Stress in DSS-induced mice.

In order to observe the effect of DSS on ER stress in colon and the role of A. muciniphila in regulating ER stress, some markers relevant to ER stress response were examined respectively using qPCR and immunofluorescence assay. PCR analysis showed the gene levels of XBP1s was significantly decreased after 10 days DSS exposure (p < 0.05) in WT mice. However, the elevation of XBP1s were significantly increased by A. muciniphila (p < 0.05). Meanwhile, the results of immunofluorescence suggested that A. muciniphila could increase the expression of p-IRE1α and decrease C/EBP homologus protein (CHOP) induced by DSS, indicating A. muciniphila alleviated DSS-induced ER stress in colon tissues of mice. However, the activating effects of A. muciniphila on the expression of XBP1s, p-IRE1α were blocked in FXR-deficient mice. These data indicated that the effects of A. muciniphila on colitis were involved in the IRE1α/XBP1 ER stress pathway, which was mediated by FXR.

3.6 Colonic SHP was upregulated sharply by A. muciniphila in DSS-induced colitis

Recently, it had been reported that SHP, the target gene of FXR, regulated the protein stability of the XBP1s and acted as modulator of ER stress[31]. In order to explore whether A. muciniphila treatment could affect SHP, colonic SHP expression in each group was measured. qPCR results showed that DSS significantly reduced the colonic mRNA expression of Shp in mice, and the intervention of A. muciniphila increased the Shp mRNA level with statistical significance. The results of WB showed that the protein level of SHP in the colon tissue of mice in the DSS group was significantly decreased, and the treatment of A. muciniphila significantly increased the protein level of SHP, which was consistent with the mRNA trend. Immunohistochemical results showed that compared with the control group, the positive expression of SHP in the colon tissue of the mice in the DSS group was reduced, which was reversed by the treatment
of *A. muciniphila*. These results demonstrated that *A. muciniphila* increased colonic SHP expression sharply in experimental colitis.

### 3.7 Correlation between the abundance of *A. muciniphila* and ER stress markers in UC patient’s colon biopsies

Immunohistochemical analysis were performed to examine ER stress markers in biopsy samples from the colon of controls and patients with UC. Our results showed that the expression of XBP1s was significantly decreased in UC patients, while other endoplasmic reticulum stress indicators (CHOP, IRE1, p-IRE1) had no significant difference. We then collected stool from UC patients and analyzed the *A. muciniphila* abundance. Correlation analysis showed that the abundance of *A. muciniphila* was only positively correlated with the colonic expression of p-IRE1.

### Discussion

In the past few years, the impact of the gut microbiota on IBD has received extensive attention. Dysbiosis of gut microbiota or related metabolic dysregulation affects the occurrence and development of IBD. Studies from both animal models and clinical trials have shown that *A. muciniphila* are significantly reduced in the UC disease state[32, 33]. Our previous study showed that *A. muciniphila* could markedly inhibit the release of intestinal inflammatory cytokines in colitis mice induced by DSS, improve intestinal inflammation and ameliorate colitis symptoms[34], but the effective dose and potential mechanism of action are not clear.

*A. muciniphila* may promote the metabolism of the mucus layer by increasing the production of MUC2 to maintain the health of intestinal epithelial cells and the integrity of the barrier[35]. *A. muciniphila* has also been shown to repair the intestinal barrier in vitro[36]. Consistent with the previous study, the application of *A. muciniphila* can dose-dependently inhibit inflammation and increase the expression of MUC2 in experimental colitis. KEGG enrichment analysis in mice faeces showed that the differential metabolites were significantly enriched in the mineral absorption pathway before and after *A. muciniphila* treatment, and the level of hydrogen phosphate increased in the DSS group, while *A. muciniphila* treatment could significantly decrease the level of hydrogen phosphate (P < 0.05). It has reported that high levels of hydrogen phosphate promote the occurrence of ER stress[37]. Through 16sRNA sequencing, we found that the application of *A. muciniphila* could reduce the F/B ratio in the model group and adjust the intestinal flora homeostasis, in which *Parasutterella* was significantly reduced after administration. Studies have shown that this genus is closely related to bile acid metabolism. The intervention of *Parasutterella* can significantly reduce the content of DCA and LCA, inhibit the expression of FXR and its downstream target genes SHP and FGF15, and reduce the levels of bile acid transporters OSTβ and IBABP[30]. Since the application of *A. muciniphila* significantly reduced the abundance of *Parasutterella*, we further examined the changes in faecal bile acids in mice before and after *A. muciniphila* intervention, and the results showed that *A. muciniphila* could significantly increase the content of DCA, LCA, TCA, TDCA, HDCA, and TUDCA. Previous studies have shown that DCA, LCA, and TCA in the gut can activate
FXR, and tauroursodeoxycholic acid (TUDCA) is a clinically available ER stress inhibitor[38]. These findings suggest that FXR activation plays important roles in maintaining ER homeostasis in colitis, and this effect may be involved in the anti-colitis effect of A. muciniphila.

We detected the related indexes of ER stress in the colon tissues of each group, and the results showed that the application of A. muciniphila increased p-IRE and XBP1s expression. These results suggest that A. muciniphila may inhibit the anti-inflammatory response and regulate the integrity of the intestinal barrier by activating IRE1α/XBP1 signaling. Recently, the role of the IRE1α/XBP1 signaling pathway in UC has received more attention[39]. IRE1 is a transmembrane protein with protein kinase and endoribonuclease activities. Unfolded proteins in the ER stimulate IRE1 phosphorylation, which activates endoribonuclease activity. Upon activation, IRE1 splices the substrate precursor XBP1 mRNA intron, producing an active XBP1 protein. XBP1 protein controls the transcription of a group of UPR target genes, including protein disulfide isomerase (PDI)[40], and regulates the expression of CHOP, which is involved in the occurrence and development of UC by inducing apoptosis through a mitochondria-dependent pathway. p-IRE1 can also catalyze the signaling pathway mediated by JNK and caspase-12 through TRAF2 to promote apoptosis[41, 42], which are essential for maintaining ER function. It is worth noting that XBP1s deficiency had been identified as susceptibility factors in both murine colitis model and human IBD[43]. Intestinal inflammation can originate from XBP1s abnormalities in IECs, thus linking cell-specific ER stress to the induction of organ-specific inflammation. Consistent with this, in the current study, decreased XBP1s expression were found in the colon biopsies from UC patients compared with healthy control. In addition, among the UC patients, the abundance of A. muciniphila was positively correlated with the colonic expression of p-IRE1. Combined with the above results, it indicates that A. muciniphila can alleviate the symptoms of DSS-induced colitis in mice by inhibiting ER stress through the IRE1α/XBP1 signaling pathway.

Previous studies have shown that activation of FXR can suppress intestinal inflammation, improve colon shortening and weight loss in colitis mice. In this study, the intervention of A. muciniphila changed the size and composition of the bile acid pool in experimental colitis, impacting the response to pharmacologic FXR activation. To verify the critical role of FXR in A. muciniphila treatment of UC and its regulation of ER stress, we conducted DSS-induced colitis on the basis of FXR−/− mice and used A. muciniphila for intervention treatment. The results showed that after the deletion of FXR, the therapeutic effect of A. muciniphila was significantly weakened, the symptoms such as weight loss, blood in the stool and diarrhea in mice could not be improved, and the colon length and pathological conditions were not alleviated. In terms of the expression of pro-inflammatory factors, in FXR-deficient colitis mice, the application of A. muciniphila could not inhibit the expression of IL-1β and IL-6 in colon tissue and serum. In FXR−/− colitis mice, the effects of A. muciniphila on enhancing the mucus layer and increasing the expression of MUC2 were largely suppressed. At the same time, the activating effects of A. muciniphila on the expression of XBP1s and p-IRE were also abolished in FXR-deficient mice. These results were in line with the findings that FXR signaling activated the hepatic XBP1 pathway in mice, which was mediated at least in part by SHP[44]. As expected, we found A. muciniphila induced colonic SHP
expression sharply in DSS-induced mice. It was demonstrated that the positive regulatory loop between XBP1s and SHP governs ER stress in the pancreas[45]. Therefore, A. muciniphila supplementation might regulate ER-associated degradation to maintain ER homeostasis through FXR/SHP axis in experimental colitis. Of note, the increase of A. muciniphila concentration may exacerbate the effects of the positive feedback loop, resulting in excessive degradation of ER and breaking the ER balance. In the future, it will be worth investigating the proper concentration range of A. muciniphila that could modulate ER homeostasis in normal colon physiology and during colitis.

In conclusion, we showed that A. muciniphila supplementation caused the activation of the FXR/SHP axis by changing the size and composition of bile acid pool in experimental colitis. The anti-colitis effect of A. muciniphila was exerted by maintaining ER stress homeostasis via FXR-mediated positive regulatory loop of XBP1s and SHP (Fig. 8). Our data suggested that A. muciniphila supplementation in appropriate dose might extend the therapeutic benefit for the treatment of UC.

**Materials And Methods**

**Drugs and reagents**

Dextran sulfate sodium salt (DSS, 36–50 kDa) was purchased from MP Biomedicals (CA, USA). Mucin (from porcine stomach) and FITC-dextran (3–5 kDa) was purchased from Sigma-Aldrich (St. Louis, MO, USA). TRIzol reagent was purchased from Life Technologies Inc.(Grand Island, NY, USA).

**Bacterial strains**

A. muciniphila strain ATCC was cultured in brain heart infusion (BHI) medium in tubes at 37°C in anaerobic chamber. A. muciniphila were collected in log phase and diluted with sterile phosphate-buffered saline (PBS) to 3*10^7 and 3 × 10^8 colony-forming units/mice for gavage. A. muciniphila freshly prepared every day for gavage.

**Animal Experiments**

The care and use of the animals followed the animal welfare guidelines, and all the experimental protocols were approved by the Institutional Animal Care and Use Committee of Nanjing University of Chinese Medicine. The mice were housed in a specific pathogen-free environment controlled for temperature and light (23 ± 1°C, 12 h light/dark cycle), and humidity (45–65%). Male 6- to 8-week-old wild-type, Fxr-null mice were on a C57BL/6J background. Fxr-null mice were obtained from BRL Medicine Inc., Shanghai. Wild-type or Fxr-null mice were randomly divided into four groups: control group, DSS group, AKK-L group and AKK-H group. Mice in the DSS group, AKK-L group and AKK-H group were treated with 2.5% of DSS in their drinking water for 10 days, respectively. The mice were supplemented daily with 200 µL of phosphate buffered saline (vehicle), AKK-L (3*10^7 CFU/mice) or AKK-H (3*10^8 CFU/mice) by intragastric gavage for 10 consecutive days. Mice were sacrificed by cervical dislocation on the 11th day,
and the colon was obtained to measure the colon length. One centimeter of distal colon tissue was collected for histologic examination. The remaining intestinal tube was cut longitudinally, the intestinal mucosa was quickly scraped with a glass slide, and the tube was stored at -80°C for RNA extraction. The blood samples were also collected, set at room temperature for 30 minutes and then centrifuged at 3500 rpm for 10 minutes at 4°C to obtain serum.

**Disease Activity Index (Dai)**

The changes in DAI were measured using the following criteria: (1) weight loss (%), (2) stool consistency and (3) blood in faeces as previously described (Table 1)[46].

<table>
<thead>
<tr>
<th>Weight loss (%)</th>
<th>Stool consistency</th>
<th>Occult blood</th>
<th>Score</th>
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<td>Normal</td>
<td>Negative</td>
<td>0</td>
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<tr>
<td>1–5</td>
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<td>5–10</td>
<td>Loose stools</td>
<td>Haemoccult+</td>
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<td>10–20</td>
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<td>3</td>
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<td>&gt; 20</td>
<td>Diarrhoea</td>
<td>Gross bleeding</td>
<td>4</td>
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**Analysis Of Fecal Metabolomics Using Uhplc -q Exactive Orbitrap-mass Spectrometry**

Sample extraction process. Remove the sample from the −80 refrigerator and thaw it on ice, the whole process is performed on ice, weigh the sample 20 mg (± 1 mg), add 70% methanol 400µl, vortex for 3 min. Sonicate in the ice water bath for 10 min, remove the sample vortex for 1 min, and let stand in the −20°C refrigerator for 30 min. Centrifuge at 12000 r/min for 10 min at 4°C, take 300 µl of supernatant, centrifuge the supernatant at 4°C, centrifuge at 12000 r/min for 3 min, take 200 µl of supernatant for analysis.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses were performed using a UHPLC system (1290, Agilent Technologies, Hilden, Germany) with a UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 µm) coupled to a Q Exactive benchtop Orbitrap mass spectrometer (Orbitrap MS; Thermo, Waltham, MA, USA).

The mobile phase A comprised of 0.1% formic acid in water for positive ionization mode, and 5 mmol/L ammonium acetate in water for negative ionization mode, and the mobile phase B comprised of acetonitrile. The elution gradient was set as follows: 0 min, 1% B; 1 min, 1% B; 8 min, 99% B; 10 min, 99% B; 10.1 min, 1% B; and 12 min, 1% B. The flow rate was set to 0.5 mL/min and the injection volume was 2
µL. The Q Exactive mass spectrometer was used because of its ability to acquire MS/MS spectra on an information-dependent acquisition (IDA) mode during the LC-MS/MS experiment. The data acquisition software (Xcalibur 4.0.27, Thermo) continuously evaluated the full scan survey MS data in the IDA-based mode because it collects and triggers acquisition of MS/MS spectra depending on the preselected criteria. Electrospray ionization (ESI) source conditions were set as follows: sheath gas flow rate was 45 Arb; Aux gas flow rate was 15 Arb. capillary temperature was 320 ºC, full Ms resolution was 70000, MS/MS resolution was 17500, collision energy was 20/40/60 eV in normalized collisional energy model, spray voltage was 3.8 kV (positive mode) or -3.1 kV (negative mode).

Full MS raw data files including retention time alignment, peak detection, and peak matching were converted to mzML format using ProteoWizard, and processed using R package XCMS. Afterwards, the data files were filtered based on the following criterion: sample numbers with metabolites that were less than 50% of all sample numbers in a group. Subsequently, normalization to an internal standard for each sample was conducted, and missing values were replaced by half of the minimum value observed in the dataset by default. The preprocessing results generated a data matrix comprising retention times (RTs), mass-to-charge ratio (m/z) values, and peak intensity.

16s Rdna Gene High-throughput Sequencing

The V3-V4 variable region of the bacterial 16S rRNA gene was amplified by F338 (5’-ACTCCTACGGGAGGCAGCA-3’) and R806 (5’-GGACTACHVGGGTWTCTAAT-3’). On the Illumina MiSeq platform, the extracted PCR products were analysed by isomolecular 250-bp double-terminal sequencing. The original pyrophosphate sequence was uploaded to the NCBI Data Center database SRA (Sequence Read Archive). High-quality sequence merge overlaps generated fastq files. QIIME (version 1.9.1, https://qiime.org/) software was used to multichannel decode and quality control filter the fastq file output. All sequencing and bioinformatics analysis were performed using the Omicsmart online platform (http://www.omicsmart.com).

Bile Acid Analysis

All of the bile acids (BAs) standards were synthesized by Metabo-Profile lab or obtained from Steraloids Inc. (Newport, RI, USA) and TRC Chemicals (Toronto, ON, Canada). All the BAs and Isotopic internal standards were accurately weighed and prepared in methanol solution to obtain individual stock solution at a concentration of 5.0 mM. Appropriate amount of each bile acids stock solution was mixed and stepwise diluted in bile-acid-free matrix (BAFM, serum or uric) at the concentration of 2500, 500, 250, 50, 10, 2.5 and 1.0 nM to prepare standard solutions. Additionally, appropriate amount of each bile acids stock solution was mixed and stepwise diluted in BAFM at the concentration of 1500, 150 and 5 nM (High, Medium and Low) to prepare quality control samples. Internal standard was added into all standard solutions and quality control samples to monitor data quality and to adjust for the matrix effect. All standard solutions and quality control samples were prepared to have the same concentration Internal
standard within (with GCA-d4, TCA-d4, TCDCA-d9, UDCA-d4, CA-d4, GCDCA-d4, GDCA-d4, DCA-d4, LCA-d4 and β-CA-d5 all at the concentration of 150 nM).

**Haematoxylin And Eosin(H&E) Staining**

Distal colon specimens were fixed for 48 h in 4% formalin after mice were sacrificed. Then, the distal colon specimens were paraffin-embedded. Finally, the sections were segmented and stained with H&E, and pathological changes were observed with a light microscope.

**Periodic Acid-Schiff and Alcian Blue Staining** (PAS/AB).

After deparaffinization and rehydration, the sections were stained with PAS/AB. The goblet cells are blue. The number of goblet cells was counted using Image J software and expressed as positive cells per villus.

**Immunohistochemical Staining**

First, paraffin sections were dewaxed in water; antigen repair was performed, and endogenous peroxidase was blocked. The sections were blocked in serum (same source as secondary antibody), which was followed by primary antibody application, secondary antibody application, DAB colour development, nuclear staining, dehydration and sealing. Finally, the positive expression of mucin-2 (MUC2) in colonic mucosal epithelial cells was observed under a microscope[47].

**Intestinal Permeability**

Intestinal permeability was determined by the fluorescein isothiocyanate conjugated dextran (FITC-dextran) assay. Mice were fasted overnight and were administered FITC-dextran (2.5 mg/mice) by enema 4 h before blood collection. The concentration of FITC was determined by spectrophotofluorometry (490/525 nm).

**Assessment Of Cytokine Level In Serum**

The level of interleukin-1β (IL-1β) and interleukin-6 (IL-6) were measured using commercial ELISA kits according to the manufacturer’s instructions.

**Immunofluorescence Staining**

Immunostaining was performed with the standard protocol using antibodies targeting CHOP, XBP1s, IRE1, p-IRE1. The samples were incubated overnight with the primary antibodies at 4°C, rinsed with PBS, and then probed with the Cy3-conjugated secondary antibody (1:1000, Cat #ab6939; Abcam) for 1 h at 37°C.
in the dark. After counterstaining with DAPI, the samples were observed using laser scanning confocal microscopy (Leica, Wetzlar, Germany).

**Quantitative Real-time Polymerase Chain Reaction (Qpcr)**

Total RNA was extracted from colon tissues using TRIzol reagent, and the concentration of RNA was measured and then reverse transcribed according to the manufacturer's instructions using a HiScript 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). cDNA was used for qPCR using SYBR Green Master Mix (Service, Wuhan, China) on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Relative amounts of mRNA were calculated using the $2^{-\Delta\Delta CT}$ method, and GAPDH served as the housekeeping gene. The primer sequences are shown in Table 2.
Table 2
Primers used in the real-time PCR assays

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences (5'-3')</th>
</tr>
</thead>
</table>
| IL-6     | Forward: TAGTCCTTCTACCCCAATTTCC  
          | Reverse: TGGTCTCTTAGCCTCTTCC |
| GAPDH    | Forward: GGTGAAGGTGCAGTGAGTG  
          | Reverse: CTCGCTCTGGGAAGATGGT |
| IL-1β    | Forward: ACCTTCAGGATGAGGACATGA  
          | Reverse: CTAATGGGAACGGTCACACCA |
| CHOP     | Forward: GAATAACAGGCGGAACCTGA  
          | Reverse: GGACGCAGGTCAAGAGTAG  
          |
| Bip      | Forward: CAAGAACCAACTCAGTTCA  
          | Reverse: CTTCCTCAACATACGCTCAG |
| PERK     | Forward: TCTGCACAAGGCTGACTTC  
          | Reverse: AGCAGCAGGAAACAGAAGCA |
| ATF6     | Forward: GTGAAGCAGGCTCAGACGA  
          | Reverse: GTGCCTTCCAGGGTTCAT |
| XBP1s    | Forward: GGTCTGCGAGGTCCCAGGAG  
          | Reverse: GAAAGGGAGGCTGGTAAGGAAC |
| SHP      | Forward: GGCCTCTCTACCCTCAGATACAT  
          | Reverse: TGTCACGTCTCCCATGATAGG |

Wb Analysis

Total protein was extracted from colon tissues using radioimmunoprecipitation assay (RIPA) lysis buffer and quantitated using a BCA protein assay kit. The concentration of extracted protein was quantified by bicinchoninic acid (BCA) protein assay kit (Beyotime). An equal amount of protein sample was loaded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and then transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). After the membrane was blocked with skimmed milk, it was incubated with primary antibodies against SHP (invitrogen, PA5-116825, 1: 2000) and GAPDH (Immunoway, YM3029, 1:10000). The next day, secondary antibodies conjugated with Horseradish peroxidase were probed for 2–3 h. Protein signals were detected with enhanced ECL chemiluminescence reagent based on the manufacturer's instructions.
**Human Samples Collection**

Participants were recruited from the Jiangsu Province Hospital of Chinese Medicine. This study was approved by the Institutional Review Board of the Affiliated Hospital of Nanjing University of Chinese Medicine, with written informed consents obtained from all participants. We collected fecal samples from 35 UC patients and 26 healthy subjects (health control, HC), and detected the abundance of *A. muciniphila* by quantitative PCR. Colon biopsy samples from 8 of these UC patients and 8 of these HC were immunofluorescence stained.

**Statistical analysis**

Graphing was performed using GraphPad Prism (version 9.0, https://www.graphpad.com). One-way ANOVA analysis of variance was applied to compare differences between multiple groups. When only two groups were compared, Student’s *t*-test was conducted. Non-parametric, were tested by the Mann-Whitney test. A value of $P < 0.05$ indicated that the difference was statistically significant. All plots are shown as the mean ± standard error of the mean (S.E.M). $P < 0.05$ was considered statistically significant.

**Abbreviations**
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>qPCR</td>
<td>Real-time Quantitative PCR</td>
</tr>
<tr>
<td>HE</td>
<td>Hematoxylin-Eosin staining</td>
</tr>
<tr>
<td>PAS-AB</td>
<td>Periodic acid Schiff -Alcian blue stain</td>
</tr>
<tr>
<td>MUC2</td>
<td>Mucin-2</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin- 6</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative colitis</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>FXR</td>
<td>farnesoid X receptor</td>
</tr>
<tr>
<td>DSS</td>
<td>Dextran sulfate sodium salt</td>
</tr>
<tr>
<td>DAI</td>
<td>Disease active index</td>
</tr>
<tr>
<td>UHPLC</td>
<td>ultra-high performance Liquid Chromatography</td>
</tr>
<tr>
<td>BAs</td>
<td>Bile acids</td>
</tr>
<tr>
<td>CDCA</td>
<td>Chenodeoxycholic acid</td>
</tr>
<tr>
<td>CA</td>
<td>Cholic acid</td>
</tr>
<tr>
<td>DCA</td>
<td>Deoxycholic acid</td>
</tr>
<tr>
<td>LCA</td>
<td>Lithocholic acid</td>
</tr>
<tr>
<td>TCA</td>
<td>Sodium salt hydrate</td>
</tr>
<tr>
<td>TUDCA</td>
<td>Tauroursodeoxycholic acid</td>
</tr>
<tr>
<td>TDCA</td>
<td>Sodium taurodeoxycholate hydrate</td>
</tr>
<tr>
<td>HDCA</td>
<td>Hyodeoxycholic acid</td>
</tr>
<tr>
<td>Iso-LCA</td>
<td>Isolithocholic acid</td>
</tr>
<tr>
<td>CHOP</td>
<td>C/EBP homologus protein</td>
</tr>
<tr>
<td>BiP</td>
<td>Binding immunoglobulin protein</td>
</tr>
<tr>
<td>ATF6</td>
<td>Activating transcription factor 6</td>
</tr>
<tr>
<td>IRE1α</td>
<td>Inositol requiring enzyme 1α</td>
</tr>
<tr>
<td>PERK</td>
<td>Protein kinase RNA-like ER kinase</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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</table>
Declarations

Acknowledgements

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Authors’ contribution

The investigation and methodology were completed by QW and YGC. QZ, XMY, DZ and YWD contributed reagents/materials/analytical tools. ZHL, HB and CC assessed the data for potential analysis. FB wrote an original draft, while WZ, YT and FJ reviewed the draft.

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Availability of data and materials

All data is contained within the main manuscript and supplemental files.

Ethics approval and consent to participate

All experimental procedures were conducted in compliance with the institutional guidelines for the care and use of laboratory animals in China and approved by the Institutional Animal Care and Use Committee of Nanjing University of Chinese Medicine(2020DW-30-01). Animal welfare and experimental protocols were strictly in accordance with the guidelines for the care and use of laboratory animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


Figures
Figure 1

*A. muciniphila* altered faecal metabolic composition in DSS-induced mice. (A-B) Score plot of OPLS-DA analysis comparing the metabolome profile among three groups. (A) ESI+ mode and (B) ESI–mode. (C) Volcanic plots of faecal metabolic profiling. (D) VENN diagram. (E) Top 20 of the KEGG pathway enrichment analysis of differential metabolites between DSS and AKK groups. (F) Differential metabolites between DSS and AKK groups.
Figure 2

*A. muciniphila* modulated the structure of gut microbiota and bile acid dysmetabolism in experimental colitis. (A) PD-TREE index. (B) ACE index. (C) pCoA score plot based on unweighted Unifrac metrics. (D) Cluster analysis based on Bray-Curtis metric distances. (E) Relative abundance of taxa in different groups. Data are shown as the mean ± SEM. ∗P < 0.05. (F–H) Relative abundance of taxa at the phylum (F), family (G) and genus (H) levels. (I) Ratios of second/primary BAs in feces. (J) Relative abundance of the significantly altered BAs from different groups. Data are shown as the mean ± SEM. ∗P < 0.05, versus DSS group.
Figure 3

*A. muciniphila* supplementation ameliorated the symptoms of DSS-induced colitis in mice. (A) DAI score *P* < 0.05. (B) colon weight/length. (C) the lengths of colon, data are presented as the mean ± S.E.M. *P* < 0.05, **P* < 0.01, ***P* < 0.001. (D) weight changes. (E) Macroscopic appearances of colon tissues. (F) Histological changes (H&E staining images of colonic sections at original magnification 200×) (G-H) mRNA quantification of pro-inflammatory cytokines (il1β and il6). (I-J) Determination of protein
productions of the inflammatory cytokines using ELISA (IL-1β and IL-6). The data present the mean ± SEM and n = 5/group. *P < 0.05, **P < 0.01, ***P < 0.001.

**Figure 4**

Effects of *A. muciniphila* on the intestinal barrier integrity in DSS-treated mice. (A) Representative images showing the number of colonic MUC2 positive cells. (×200 magnification, scale bar 50 μm). (B) Representative pictures showing that colonic specimens stained with PAS/AB (magnification ×200, scale bar: 50 μm). (C) The numbers of MUC2-positive cells in each villus (D). Numbers of goblet cells in each villus. *p < 0.05, **p < 0.01. (E) Epithelial permeability of FITC-dextran.
Figure 5

*A. muciniphila* supplementation FXR-dependently attenuated colonic ER Stress in DSS-induced colitis mice. Sections of colonic tissues were immunostained with DAPI and antibodies and then observed under 200× fluorescence microscope. (A) CHOP and XBP1s immunofluorescence staining of representative colon tissue. (B) IRE1 and p-IRE1 immunofluorescence staining of representative colon tissue. (C-I) mRNA quantification of ER stress markers (PERK, ATF6, Bip, XBP1s and CHOP).
Figure 6

Colonic SHP was upregulated by *A. muciniphila* in DSS-induced colitis. (A) Immunohistochemistry staining for SHP (×200 magnification, scale bar 50 μm). (B) mRNA quantification of Shp. (C) Representative immunoblots of SHP proteins in colonic tissues.
Figure 7

Correlation between the abundance of *A. muciniphila* and ER stress markers in UC patient's colon biopsies. Sections of colonic tissues were immunostained with DAPI and antibodies and then observed under 200× fluorescence microscope. (A) CHOP and XBP1s immunofluorescence staining of representative colon tissue. (B) IRE1 and p-IRE1 immunofluorescence staining of representative colon tissue. (C) Immunohistochemical quantitative analysis. (D-G) Correlation analysis.
Figure 8

*Akkermansia muciniphila* alleviates colonic epithelial ER stress through activation of FXR/SHP axis in experimental colitis.

**Supplementary Files**

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- supplementfig.tif