Total flavone of *Abelmoschus Manihot* improves colitis by promoting growth of *Akkermansia* in mice

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Research Article

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Abstract

The total flavone of *Abelmoschus manihot* (TFA), a compound extracted from the flowers of *Abelmoschus manihot* (L.) Medic, has been widely used for the treatment of Crohn's disease, chronic glomerulonephritis and other diseases. The aim of this study was to investigate the effect of TFA on gut microbiota and intestinal barrier in dextran sulfate sodium (DSS) induced experimental colitis. C57BL/6J mice were treated with 2.5% DSS in drinking water to induce colitis. Mice were orally administrated with TFA (62.5mg/kg, 125mg/kg) or prednisone acetate (PAT, 2.5mg/kg) once daily for 7 days. Biological samples were collected for analysis of inflammation cytokines, gut microbiota and intestinal barrier integrity. TFA-H (125mg/kg) markedly attenuated DSS-induced colon shortening and histological injury in experimental colitis. The therapeutic effect was similar with PAT administration. TFA-H notably modulated the dysbiosis of gut microbiota induced by DSS and greatly enriched the *Akkermansia muciniphila* (*A. muciniphila*). Moreover, TFA-H remarkably ameliorated colonic inflammatory response and intestinal epithelial barrier dysfunction. Interestingly, TFA directly promotes the growth of *A. muciniphila* in vitro. Taken together, the results for the first time revealed that TFA, as a prebiotic of *A. muciniphila*, improved DSS-induced experimental colitis, at least partly through modulating gut microflora profile to maintain the colonic integrity and inhibit inflammation response.

Introduction

Ulcerative colitis (UC), as a chronic non-specific intestinal inflammatory disease, is clinically manifested as abdominal pain, bloody diarrhea and various degrees of systemic symptoms. In traditional Chinese medicine theory, UC is called as "changpi" and "dysentery". In recent decades, its incidence and prevalence have been continuously rising in Asia, which predominantly torments young adults[1]. Most of the people consider that the occurrence and development of UC is caused by combining the effects of intestinal barrier, gut microbiota, and mucosal immunity[2]. Researches have proved that the gut microbiota in UC patients remains dysbiosis. Disordered gut microbiota as a pathogenic factor leads to impaired intestinal barrier and promotes the onset of UC, and targeting gut microbiota can also be used as a means to treat UC.

Herbal medicine has been used for thousands of years and has a good therapeutic effect in many diseases by achieving multiple goals in clinical treatment. Although the effectiveness of herbal medicine has been confirmed, the mechanism of its action on the body is still unclear.

As a traditional Chinese medicine, Abelmoschus Manihot is often used as the main drug of Huang Kui Lian Chang Decoction in the treatment of ulcerative colitis[3]. Total flavone of Abelmoschus Manihot (TFA) is effective compound extracted from *Abelmoschus Manihot*, which removes damp heat, fire or toxicity. In our previous study, the total flavone of Abelmoschus Manihot was analyzed by HPLC (Fig. 1). The results showed that TFA comprises eight flavone glycosides, including quercetin-3-O-robinobioside, gossypetin-3-O-glucoside, quercetin-3'-O-glucoside, isoquercetin, hyperoside, myricetin, gossypetin and quercetin[4]. Animal experiments have verified that TFA has therapeutic effect on UC. However, its effect on

intestinal flora and intestinal barrier function has not been proved. Therefore, we hypothesized that TFA as a prebiotic alleviates dextran sulfate sodium (DSS) induced colitis by regulating the intestinal flora.

Our results suggested that TFA protected intestinal barrier integrity, inhibited inflammatory response and significantly improved DSS-induced colitis by regulating intestinal flora and increasing the abundance of *Akkermansia muciniphila*(*A. muciniphila*). Its therapeutic effect was similar to that of PAT, and high dose was better than low dose. As far as we know, this is the first time to research the effect of TFA on the intestinal flora in colitis and the promotion of *A. muciniphila* in vitro. It is expected that this work will provide experimental evidence for the application of TFA in UC. In addition, it may provide support for the development of potential prebiotics.

Materials And Methods

Drugs and reagents

TFA was extracted from flowers of *Abelmoschus manihot* by the Jiangsu Provincial Hospital of Traditional Chinese Medicine, Nanjing, China. *Abelmoschus Manihot* flowers was purchased from Anhui xiehecheng co.,ltd (Batch No 20092701). The source and production process of the *Abelmoschus Manihot* flowers are in accordance with Chinese Pharmacopoeia standards (2015 version). Dr Fengyu Zhu identified the *Abelmoschus Manihot* flowers in Department of Pharmacy, Jiangsu Provincial Hospital of Traditional Chinese Medicine. *Abelmoschus Manihot* flowers was immersed in 75% ethanol for 60 min. refluxed the mixture for 60 mins at 90°C，then filtered with analytical filter paper. Finally, rotary evaporation was used to evaporate the extracts under vacuum at 60°C[5]. DSS (molecular weight of 36-50 kDa) was provided by German MP Biopharmaceutical Company. Prednisone acetate tablets (PAT) was purchased from Cisen Pharmaceutical Co. Ltd. (Jining, Shandong, China). Mucin (from porcine stomach) was purchased from Sigma (USA).

Animals

Six-week-old male C57BL/6J mice were purchased from Beijing Si Pei Fu Laboratory Animal Technology Co. Ltd. The mice were raised in the laboratory of Basic Pharmacology, Affiliated Hospital of Nanjing University of Chinese Medicine (Nanjing, Jiangsu, China). Sterilized standard rodent chow food and sterilized water were not restricted during the experiment, the temperature was controlled at 23 ± 1°C, the humidity was controlled at 50 ± 5%, and the light system was set at 12 hours/day. The care and use of the animals were followed the animal welfare guidelines, and all the experimental protocols were approved by the Institutional Animal Care and Use Committee of the Nanjing University of Chinese Medicine.

Induction of colitis and treatment

As shown in Fig. 1A, colitis was induced by 2.5% DSS in the drinking water adlibitum for 7 consecutive days (days 1-7). The mice were randomly allocated after modeling. The mice were supplemented daily

with 200μL of phosphate buffered saline (vehicle), TFA (125mg/kg, 62.5mg/kg) or PAT(2.5mg/kg) by intragastric gavage for 7 consecutive days (days 8-14). Mice were sacrificed on 15 day, and the colon was obtained to measure the colon length. 1 cm of the distal colon tissues was collected for histologic examination. All samples were stored at -80℃ for further analysis.

Disease activity index (DAI)

The changes of DAI were measured using the following criteria: (1) weight loss (%), (2) stool consistency and (3) blood in feces as previously described (Table1)[6].

Table 1. Disease activity index(DAI)

Weight loss (%)	Stool consistency	Occult blood	Score
None	Normal	Negative	0
1-5	-	-	1
5-10	Loose stools	Hemoccult+	2
10-20	-	-	3
>20	Diarrhoea	Gross bleeding	4

Hematoxylin 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 hematoxylin and eosin, and pathological changes were observed with a light microscope.

Immunohistochemical staining

First, paraffin sections were dewaxed in water; antigen repair was performed, and endogenous peroxidase was blocked. The sections were blocked in serum, which was followed by primary antibody application, secondary antibody application, DAB color development, nuclear staining, dehydration and sealing. Finally, the positive expression of mucin-2(MUC2); Kruppel-like factor 4(KLF4) and zonula occludens-1(ZO-1) in the colonic mucosal epithelial cells was observed under the microscope.

Assessment of cytokine level in serum

The level of tumor necrosis factor-α(TNF-α); interleukin-1β(IL-1β) and interleukin-6(IL-6) were measured using commercial ELISA kits (Jin Yi bai Biological Technology Co. Ltd., Nanjing, China) according to the manufacturer's instructions.

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 5x PrimerScript. The primer sequences are shown in Table 2. GAPDH was used as the reference gene. The ΔΔCt method was used to compute the relative expression. The abundance of *A. muciniphila* in stool samples was quantified by quantitative PCR as described in Everard et al[7].

Table 2. Primers used in the real-time PCR assays

Gene	Primer Sequences (5’-3’)
TNF-α	
Forward	CACCACGCTCTTCTGTCTACTG
Reverse	GGGCTACAGGCTTGTCACTC
IL-1β	
Forward	CTCGTGCTGTCGGACCCAT
Reverse	GCTTGTGCTCTGCTTGTGA
IL-6	
Forward	GAGGATACCACTCCCAACAGACC
Reverse	AAGTGCATCATCGTTGTTCAT
IL18	
Forward	GTGAACCCCAGACCAGACTG
Reverse	CCTGGAACACGTTTCTGAAAGA
IL-17a	
Forward	GTTAGGGTGCTTTAGGTCC
Reverse	TAACAATGAGTTTCTGTACG
CCL2	
Forward	TGCCCTAAGGTCTTCAGCAC
Reverse	AAGGCATCACAGTCCGAGTC
MUC2	
Forward	TGCCCACCTCCTCAAAGAC
Reverse	TAGTTTCCGTTGGAACAGTGAA
KLF4	
Forward	CAGGATTCCATCCCCATCCG
Reverse	GAGAGGGGACTTGTGACTGC
ZO-1	
Forward	GGGGCCTACACTGATCAAGA
Reverse	TGGAGATGAGGCTTCTGCT
GAPDH	
Forward	AGAACATCATCCCTGCATCC CTGGGATGGAAATTGTGAGG

Reverse

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'-GGACTACHVGGG TWTCTAAT-3'). On the Illumina MiSeq platform, the extracted PCR products were analyzed 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) software was used to multichannel decode and quality control filter the fastq file output.

Bacterial strains and growth curve

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 3×10^8 colony-forming units/mice for gavage. To acquire the growth curve of *A. muciniphila*, different concentrations (1, 10, and 100 μg/mL) of TFA was added into BHI medium, and then add *A. muciniphila* suspension to make the final concentration of bacteria 10^6cfu /ml. The growth profile was evaluated by intermittently measuring absorbance at 600 nm every 5 hours[8]. Mucin was added to the final concentration of 4 g/L. TFA was dissolved in PBS. Each experiment was repeated three times.

Statistical analysis

Graphing was performed using GraphPad Prism 8 (GraphPad Software, Inc.). One-way analysis of variance was applied to compare differences between multiple groups. When only two groups were compared, Student's *t*-test was conducted. 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 as statistically significant.

Result

Effects of TFA on damage in the colon of DSS-induced colitis mice

As shown in Fig.2, the DSS group showed significant weight loss, diarrhea, hematochezia and other colitis symptoms (Fig. 2B-C). Treatment with TFA (62.5 and 125 mg/kg) significantly improved weight loss and decreased DAI score in a dose-dependent manner. As shown in Fig. 2D-E, compared to that of the control group，the colon length was markedly shortened in the DSS group (P < 0.01). By contrast, TFA-H (P <0.01) and PAT (P <0.01) were used to significantly improve the colonic shortening induced by DSS. As shown in Fig. 2F, In the control group, the colon tissue structure was intact. by contrast, the DSS group was characterized by inflammatory cell infiltration, epithelial cell destruction and mucosal thickening. Consistent with the effects on symptoms observations, compared to the DSS group，TFA-H and PAT

groups were observed to significantly restore the intestinal epithelial structure and reduce severe inflammation. These results indicated that TFA-H has an obvious protective effect on DSS-induced colitis, which was similar to that of PAT and superior to that of TFA-L.

Effects of TFA on the productions of inflammatory cytokines

Inflammatory molecules are involved in the processes that occur in colitis. To elucidate the inflammatory response in DSS-induced mice, various pro-inflammatory cytokines were measured in colon tissues in mRNA. As illustrated in Fig. 3A-F, the levels of pro-inflammatory cytokines, including TNF-α, IL-6, interferon-γ (IFN-γ), interleukin-18 (IL-18) and interleukin-17a(IL-17a) were significantly increased in DSS-induced colitis mice ($P < 0.05$ vs. CON). These elevated pro-inflammatory cytokines were all decreased by TFA in a dose-dependent manner. Chemokine ligand 2 (CCL2) was significantly increased by DSS treatment compared with those in the control group (Fig. 3F). Moreover, TFA and PAT significantly decreased CCL2 compared with the DSS group (Fig 3F). Pro-inflammatory cytokines were also measured in serum in protein level. Quantification of specific cytokines using ELISA showed the same patterns in regulation of production and secretion of pro-inflammatory cytokines (Fig. 3G-I). It was noteworthy that TFA-H (125mg/kg) exhibited pronounced effect in suppressing these inflammatory cytokines.

TFA improved the intestinal barrier integrity in DSS-induced colitis mice

To understand the effect of TFA on the intestinal barrier integrity of the mice with DSS-induced colitis, the expression of MUC2, KLF4 and ZO-1 in the colon was determined by qPCR and immunohistochemical staining. Immunohistochemical staining results showed that the content of MUC2, KLF4 and ZO-1 positive cells in DSS-induced mice was significantly lower than that in control group (Fig. 4A-C). TFA treatment significantly increased MUC2, KLF4 and ZO-1 positive cells. MUC2, KLF4 and ZO-1mRNA expression was decreased in the colon tissue of the DSS group. In contrast, the treatment of TFA-H returned the expression of MUC2, KLF4 and ZO-1 to the normal level (Fig. 4D-F). This result suggested that TFA treatment protects the epithelial barrier by recovery or even enhancement of mucus and tight junction-associated proteins in DSS-induced colitis.

TFA modulated the structure of gut microbiota

To demonstrate whether TFA-H regulated DSS-induced gut microbial dysbiosis, high-throughput sequence analysis of bacterial 16S rRNA V3-V4 region was conducted on stool samples. Principal coordinates analysis (PCoA) plots were calculated from Bray-Curtis metric distances to evaluate the composition of the community, the results reveal a clear separation between each group (Fig. 5A). The system clustering tree indicated marked differences among the four groups. TFA-H group and DSS group cluster separately,

demonstrating that treatment with TFA-H inhibited the DSS-induced gut microbiota dysbiosis in mice. (Fig. 5B).

The top most abundant taxa at the phylum, family and genus levels are shown in Fig. 5D–F. Compared with the control group, the abundance of *Verrucomicrobia* was lower in the DSS group, while the abundance of *Tenericutes* and *Proteobacteria* were higher, which was consistent with the previous report[9, 10]. At the genus level, the abundance of *Clostridium*, *Parabacteroides*, *Ruminococcus* and *Romboutsia* was remarkably increased, whereas *A.muciniphila* and *Lactococcus* were significantly decreased in the DSS group as compared to those of the control group ($P < 0.05$). Following treatment with TFA, the abundance of *Tenericutes* and *Proteobacteria* nearly returned to the normal level. TFA treatment significantly increased the relative abundance of *A. muciniphila*, which belonged to *Verrucomicrobia*. PCR also proved the increase of absolute abundance of *A. muciniphila* in TFA group (Fig. 5C). Microbial flora structure was favorably harmonized by treatment with TFA-H.

Furthermore, LefSe (LDA effect size) analysis was used to identify dominant flora in each group (Fig. 6). Compared with the control group the gut microbiota *Actinomycetales* (LDA=3.87) and *Ruminococcaceae* (LDA=2.973) were enriched, and there was a depletion of *Bifidobacterium* (LDA=4.29) and *A. muciniphila* (LDA=3.90) in the DSS group. TFA-H group showed significant selective enrichment of *A. muciniphila* (LDA=4.69), *Gordonibacter* (LDA=3.43) and *Erysipelatoclostridium* (LDA=3.35). PAT demonstrated a significant effect on *Bifidobacterium* (LDA=4.01), *Family_XIII_UCG_001* (LDA=2.55), and *Ruminococcaceae* (LDA=2.90). The result indicated that TFA-H alleviated the disorder of the gut microbiota in DSS-induced mice, especially increase the abundance of *A. muciniphila*.

TFA promoted *A. muciniphila* growth *in vitro*

To examine whether TFA directly promoted the growth of *A. muciniphila in vitro*, the growth curve of *A. muciniphila* was monitored in BHI medium with mucin. (Fig. 7). TFA (100 μg/mL) inhibited the growth of *A. muciniphila*, while TFA (1μg/mL or 10 μg/mL) directly promote the growth of *A. muciniphila in vitro*. When TFA (1μg/mL or 10 μg/mL) was added, *A. muciniphila* grew faster at log phase and plateaued at a much higher cell density. Thus, TFA could stimulate *A. muciniphila* growth *in vitro.*

A. muciniphila alleviated colitis in mice

In order to confirm whether *A.muciniphila* played an essential role in DSS-induced colitis. We treated DSS-induced mice daily with *A.muciniphila*（$3*10^8$） or PBS for 1week after DSS modeling. The treatment of *A.muciniphila* relieved DSS-induced colitis, which was evidenced by reduced weight loss, colon length shortening and histological damage (Fig. 8A-E). Serum and colon tissue levels of inflammatory cytokines (TNF-α, IL1β, IL6) decreased as a result of *A. muciniphila* treatment (Fig. 8F-G). *A. muciniphila* treatment

increased expression of the tight junction proteins ZO-1, MUC2 and KLF4, supporting a potential role in the regulation of intestinal barrier integrity (Fig.8H-J). To sum up, *A. muciniphila* ameliorated DSS-induced colitis, improved the macroscopic and histological damage, decreased the inflammatory cytokine, and the protected the intestinal barrier integrity.

Discussion

Studies have shown that the gut microbiota of UC patients is out of balance[11]. Metagenomic research showed that the abundance and diversity of gut microbiota of UC patients were reduced[12]. Recent studies have shown that certain dietary agents, spices, oils, and dietary phytochemicals that are consumed regularly possess beneficial effects in regulating gut microbiota[13, 14]. The aim of this study was to characterize the effects of TFA on DSS-induced colitis, mainly focusing on the composition of the gut microbiota and intestinal barrier in mice with DSS-induced colitis.

In our study, TFA relieved the symptoms of weight loss and colon length shortening in DSS-induced mice. Compared with PAT, TFA-H has advantages in weight and DAI score. In terms of colonic histopathology, DSS-induced colitis in mice exhibited serious injuries, with the loss of histological structure, disruption of the epithelial barrier, a pronounced decrease in a number of crypts, and marked infiltration of granulocytes and mononuclear cells into the mucosa and submucosa. Compared to the DSS group, TFA group were observed to effectively reduce histologic inflammation. Notably, TFA-H exhibited similar effect to PAT, and exerted superior effect to TFA-L.

Animal experiments and clinical studies have found that the increase of pro-inflammatory cytokines further damages the intestinal mucosal barrier function through the activation of the NF-κB signaling pathway. Our previous study has shown that TFA could markedly inhibit the release of the intestinal inflammatory cytokines in Crohn's disease rat induced by 2,4,6-trinitrobenzene sulfonic acid, improve intestinal inflammation and ameliorate the colitis symptoms[15]. In this study, we demonstrated supplementation of TFA significant decrease the mRNA expression of inflammatory cytokines (TNF-α, IL-6, IL-18, IL-17a, IFN-γ) and chemokines (CCL2) in colon tissue. TFA also decreased the protein expression of inflammatory factors (TNF-α, IL-6, IL-1β) in the serum. Notably, TFA-H exerted superior anti-inflammatory effect to TFA-L.

The intestinal mucosal barrier plays an important role in the pathogenesis of colitis[16]. The function of intestinal mucosal barrier mainly refers to the isolation of the intestinal lumen from the environment to prevent the invasion of bacteria and toxic substances. The intestinal mucus layer is a protective gel-like substance covering the surface of the intestinal mucosa, which is the first barrier in the intestinal lumen. A large amount of mucin synthesized and secreted by goblet cells is the most important substance that constitutes the mucus layer of the intestine[17]. The destruction of the mucus layer and the pathological changes of goblet cells are closely related to the progression of colitis[18].Our experiment showed that

DSS-induced colitis decreased the expression of KLF4 mRNA, MUC2mRNA and ZO-1mRNA in colon tissue. KLF4 is a zinc finger transcription factor expressed in the differentiated epithelial cells of the intestine, which is widely involved in the regulation of cell proliferation, differentiation and embryonic development, especially in the proliferation of goblet cells[19]. Studies have shown that goblet cells are not fully developed and the expression of mucin MUC2 is abnormal in colon tissue of KLF4$^{-/-}$ mice[20]. The number of goblet cells in the colon tissue of intestine-specific KLF4 deletion mice was significantly reduced[21]. In the mucus layer secreted by goblet cells, the highest content of intestinal epithelial cell proliferation and differentiation is mucin MUC2. MUC2 effectively blocks the pathogens in the intestinal lumen from invading intestinal epithelial cells. MUC2 also provides a habitat and nutrients for the symbiotic bacteria in the intestine. The mucus layer disappeared in the colon of MUC2$^{-/-}$ mice, and direct contact between the gut microbiota and intestinal epithelial cells triggered an inflammatory response, and eventually spontaneous colitis was formed[22, 23]. ZO-1 is crucial for connecting the individual epithelial cells and maintaining the integrity of the epithelium. The reduction of ZO-1 interrupts the assembly of tight junctions by inhibiting the recruitment of other components[24]. The destruction of the intestinal barrier integrity causes pathogens in the intestinal lumen to invade intestinal epithelial cells, increase the immune response of intestinal epithelial cells, and ultimately strengthen the intestinal inflammatory response. The treatment of TFA improve the intestinal mucosal barrier integrity by promoting the production of MUC2, KLF4 and ZO-1.

The previous study showed that the composition of the intestinal flora is altered in UC patients or in DSS-treated mice [25]. Besides, the bacteria associated with the mucosa increase the mucus layer thickness and promote the repair of intestinal barrier[26]. Several studies focused on the clinical improvement of DSS-induced colitis by using probiotics and antibiotics in order to modulate the commensal microbiota[27].Dietary flavone could contribute to the maintenance of intestinal health by preserving the gut microbial balance through the stimulation of the growth of beneficial bacteria, and the inhibition of pathogenic bacteria[28]. We found that the gut microbiota of mice with DSS was substantially changed during TFA-H or PAT treatment. In our study, lower abundance of *A. muciniphila*, *Bifidobacterium* and higher abundance of *Actinomyces*,*Escherichia coli* and *Proteobacteria* were observed in DSS group as compared with those of the control group, which was consistent with previous reports. Studies have shown that the abundance of *Actinomycetes* in UC patients is significantly increased, and *Actinomyces* was also significantly increased in intramucosal carcinomas[29]. *Escherichia coli*, as a kind of conditional pathogenic bacteria, causes the disease to worsen by destroying the intestinal barrier integrity[30]. The treatment of TFA-H reversed the intestinal dysbacteriosis caused by DSS. In addition, the abundance of *A. muciniphila* in TFA-H was significantly increased.

Our previous study found that in patients with UC, the abundance of *A. muciniphila* was significantly reduced. This is consistent with the results reported in the literature[31–33]. A prospective study unequivocally showed that 3 months administration of *A. muciniphila* (10^{10} cfu/day) was safe and verified the feasibility and tolerance of *A. muciniphila* supplementation in humans. In our study, 1 week

treatment of *A. muciniphila* ($3*10^8$cfu/mice/day) improved the colitis induced by DSS. The treatment of *A. muciniphila* significantly reduces the expression of inflammatory cytokines (TNF-α, IL-6, IL-1β), and protects the intestinal barrier by increasing the thickness of the mucus layer and the expression of tight junction proteins. Studies have shown that *A. muciniphila* is a protein-degrading anaerobic bacterium attached to the mucus layer of the intestine. It can promote the metabolism of the mucous layer, thereby creating a healthy environment for intestinal epithelial cells[34, 35]. In vitro experimental studies have shown that *A. muciniphila* enhanced the integrity of the intestinal epithelium and repair the damaged intestinal mucosal barrier. This may be related to the metabolites of *A. muciniphila*. *A. muciniphila* maintains the homeostasis of the intestinal epithelium and inhibits the immune response of intestinal epithelial cells by degrading the host's intestinal mucus into short-chain fatty acids[36]. Previous study has shown that *A. muciniphila*, its outer membrane protein Amuc_1100 and the extracellular vesicles derived from *A. muciniphila* could protect the progression of DSS-induced colitis and maintaining the integrity of the intestinal mucosal barrier. The mechanism of *A. muciniphila* to relieve colitis may be related to the interaction of Amuc_1100 with Toll-like receptor 2. Extracellular vesicles derived from *A. muciniphila* promote tight junction expression by activating AMPK[37, 38]. A study certified *A. muciniphila* promotes intestinal inflammation in germ-free $IL10^{-/-}$ mice model of IBD[39]. However, a following research showed *A. muciniphila* was examined in gnotobiotic $IL10^{-/-}$ mice, and it did not promote intestinal inflammation[40]. *A. muciniphila* plays a regulatory role in maintaining intestinal barrier integrity, host metabolism and other biological functions[41, 42].

In vivo experiment, PCR confirmed the increase ($p < 0.01$) of absolute abundance of *A. muciniphila* in mice treated with TFA-H (Fig. 4C). *In vitro* experiment, low concentration of TFA (1 μg/mL and 10μg/mL) promote the growth of *A. muciniphila. A. muciniphila* grew faster at the logarithmic phase and at a higher cell density in platform period. TFA may improve the intestinal barrier to relieve colitis through its prebiotic effect on *A. muciniphila.*

Conclusion

The study showed that TFA treatment improved the colitis caused by DSS, at least partly through modulating gut microbiota and restoring the integrity of the intestinal barrier. Our study further suggested that the TFA administration to promote the growth of *A. muciniphila*, which may be in association with this protective effect. We reveal that TFA as a prebiotic of *A. muciniphila* effectively treats colitis.

Declarations

Authors' contribution

Investigation and methodology were completed by GPS and YGC. TC and RW contributed reagents/ materials/ analytical tools. MMX, DZ and FJ assessed the data for potential analysis. FB wrote an original draft, while QW and JYZ review the draft.

Conflicts of interest

There are no conflicts of interest to declare

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Statement

We confirm that all methods in our study are reported in accordance with ARRIVE guidelines.

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Figures

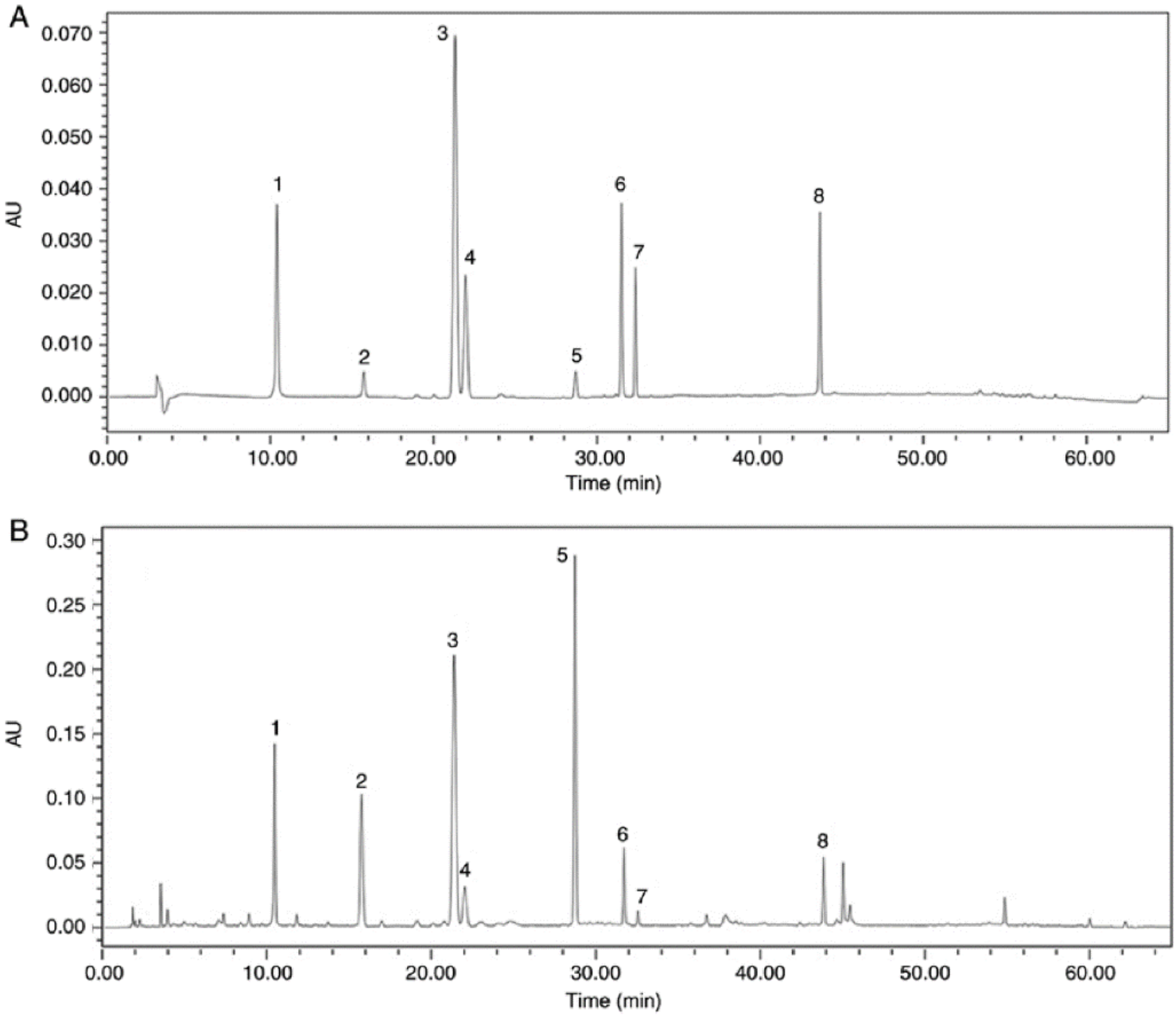

Figure 1

Chromatographic analyses of standards materials to TFA by high pressure liquid chromatography. (A) HPLC chromatograms of standards (1, quercetin 3 O robinobioside, 2, gossypetin 3 O glucoside, 3, quercetin 3' O glucoside, 4, isoquercetin, 5, hyperoside, 6, myricetin, 7, gossypetin and 8, quercetin) and (B) TFA. TFA, total flavone of Abelmoschus manihot L. Medic.

Figure1

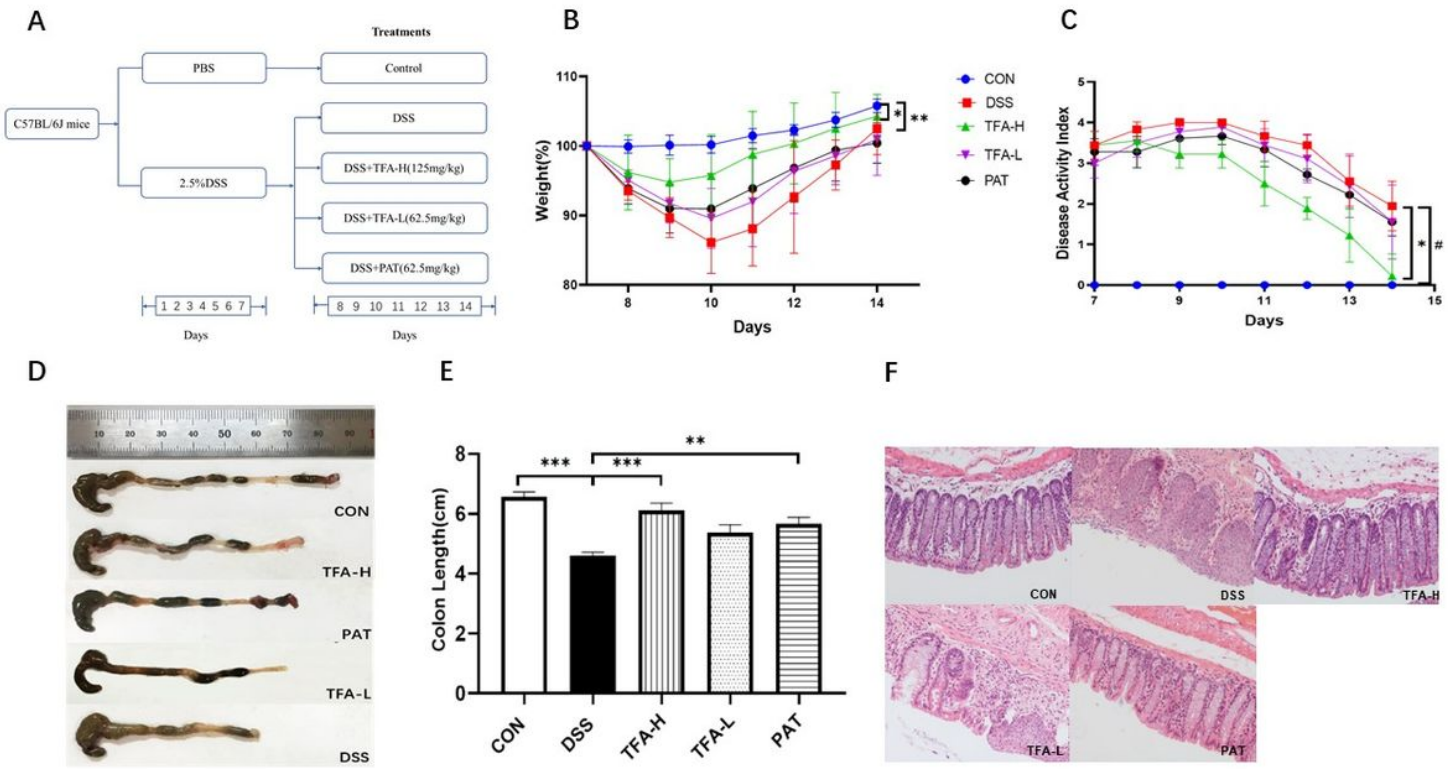

Figure 2

TFA ameliorated the symptoms of DSS-induced colitis in mice. (A) Schematic diagram of the experimental design. (B) Weight changes are expressed as the mean change from the starting weight from day 7 to 14. (C) DAI score from day 7 to 14. (D) Macroscopic appearances of colon tissues. (E) The lengths of colon. Data are presented as the mean ± S.E.M and n = 6/group. *P < 0.05, **P < 0.01, ***P < 0.001, #P<0.0001. (F) Histological changes (H&E staining images of colonic sections at original magnification 200×)

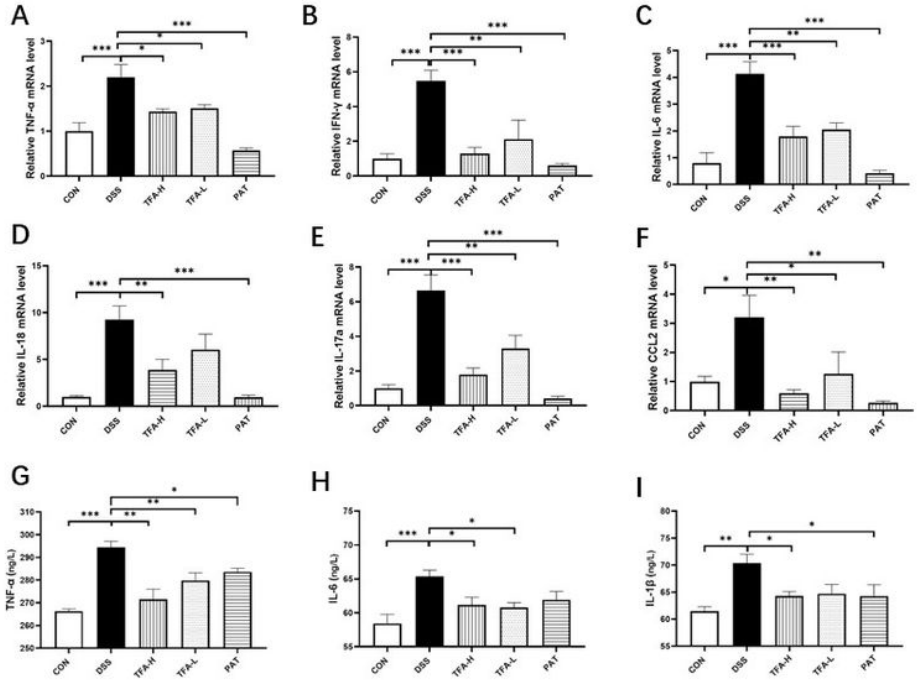

Figure 3

Inflammatory cytokines of the mouse colon by treatment of DSS and administration of TFA and PAT. (A-F) mRNA quantification of pro-inflammatory cytokines (TNF-α, IL-6, IFN-γ, IL-18 and IL-17a) and Chemokines CCL2 using Real Time RT-PCR. (G-I) Determination of protein productions of the inflammatory cytokines using ELISA. The data present the mean ± SEM and n = 5/group. *P < 0.05, **P < 0.01, ***P < 0.001, #P<0.0001.

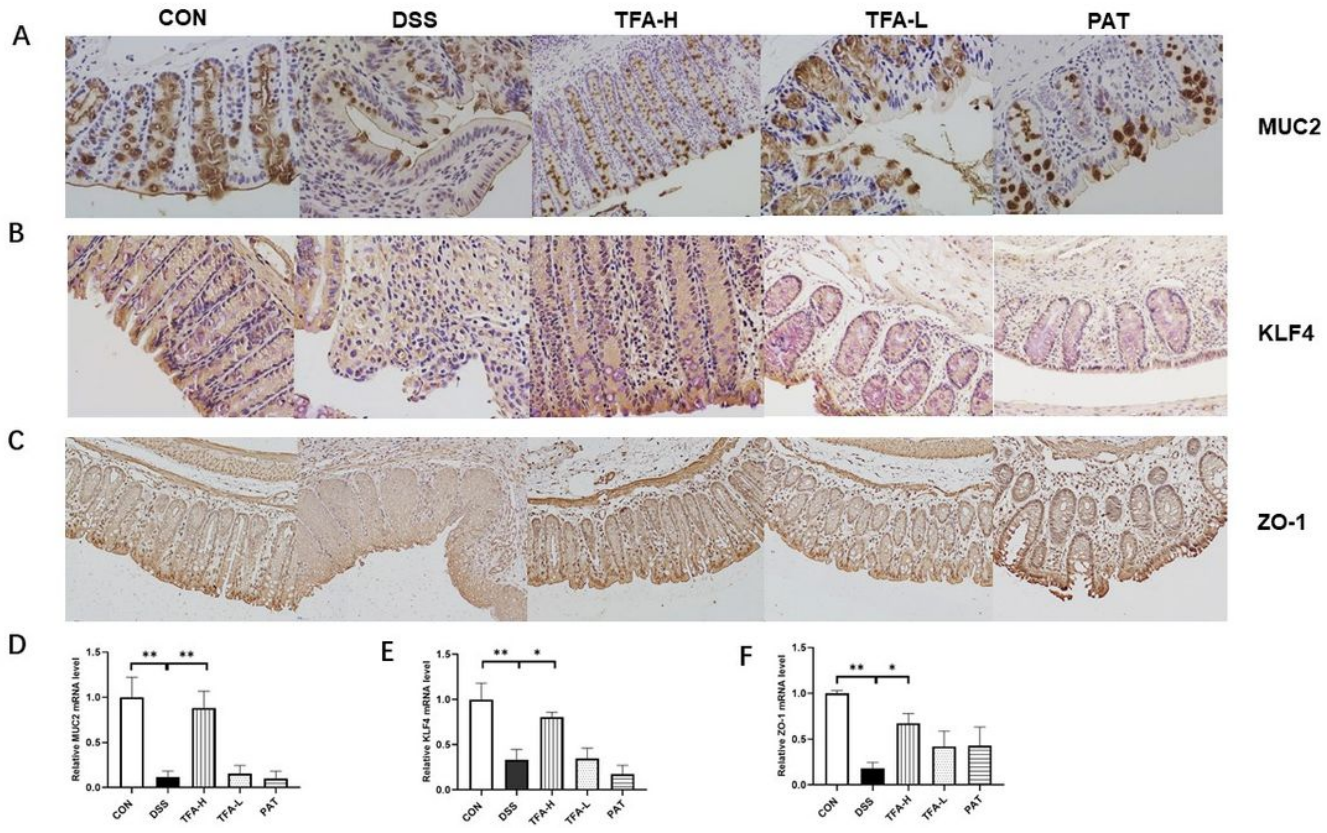

Figure 4

Recovery of mucus- and tight junction-associated proteins from the colons of the DSS-induced colitis mice by TFA and PAT. (A) The number of colonic MUC2 positive cells in DSS-treated mice. Representative images (×200 magnification, scale bar 50 μm). (B) The number of colonic KLF4 positive cells in DSS-treated mice. Representative images (×200 magnification, scale bar 50 μm). (C) The number of colonic ZO-1 positive cells in DSS-treated mice. Representative images (×200 magnification, scale bar 50 μm). (D) mRNA quantification of MUC2. (E)mRNA quantification of KLF4. (F) mRNA quantification of ZO-1.

Figure4

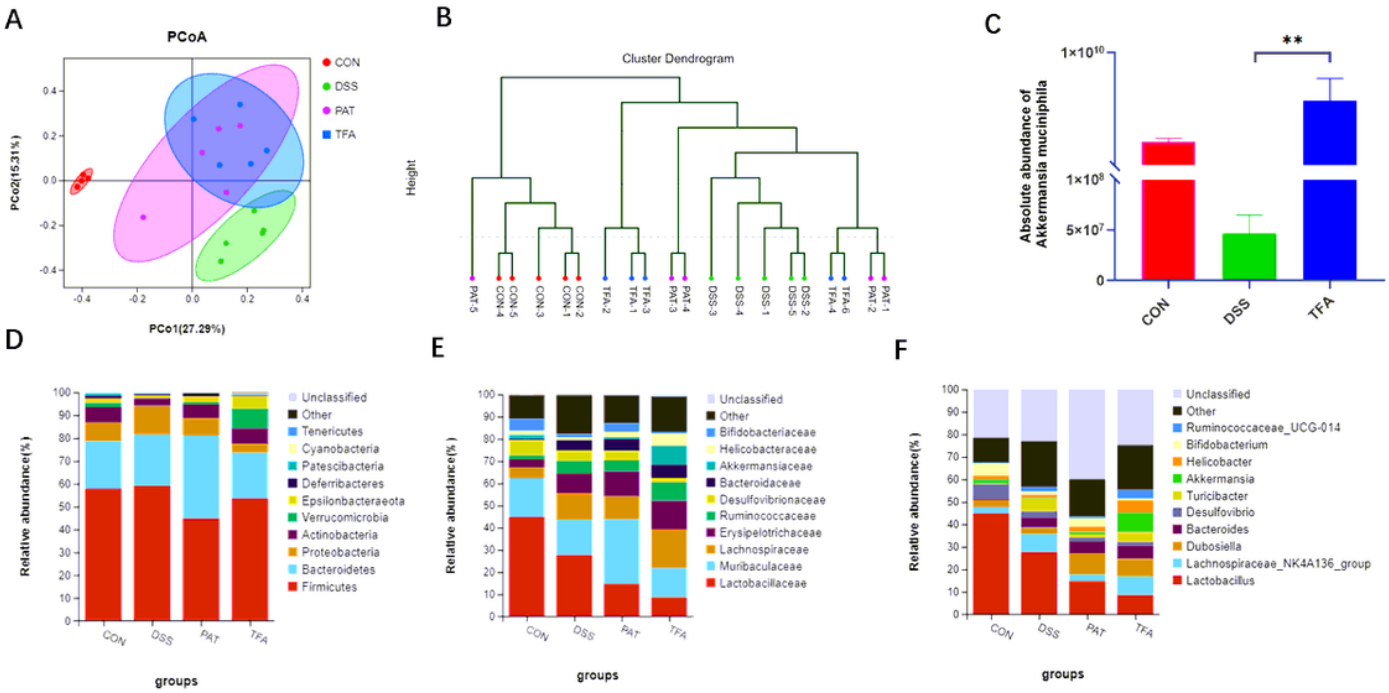

Figure 5

The composition of the gut microbiota in treatment of DSS and administration of TFA and PAT. (A) PCoA plot of OTU data. (B) Cluster analysis based on Bray-Curtis metric distances. (C) Absolute abundance of Akkermansia muciniphila in fecal content by qPCR. **P < 0.01. (D-F) Relative abundance of taxa at the phylum (D), family (E) and genus (F) levels.

Figure5

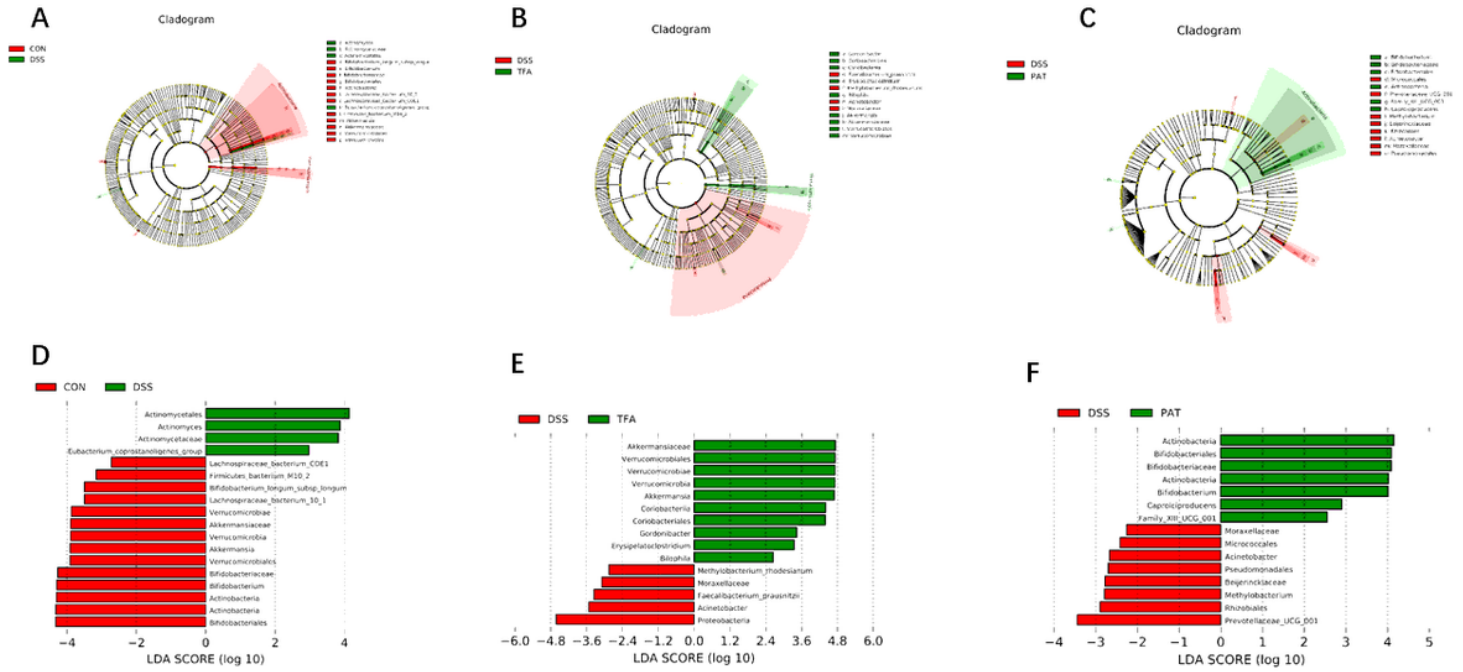

Figure 6

Characteristics of microbial community composition using LEfSe analysis. (A) LEfSe cladogram represents the taxa enriched in the DSS group (Green) and control group (Red). (B) Discriminative biomarkers with an LDA score >2 between the DSS group (Green) and control group (Red). (C) LEfSe cladogram represents taxa enriched in the DSS group and TFA group. (D) Discriminative biomarkers with an LDA score >2 between the DSS group (Red) and TFA group (Green). (E) LEfSe cladogram represents taxa enriched in the DSS group and PAT group. (F) Discriminative biomarkers with an LDA score >2 between the DSS group (Red) and PAT group (Green).

Figure 7

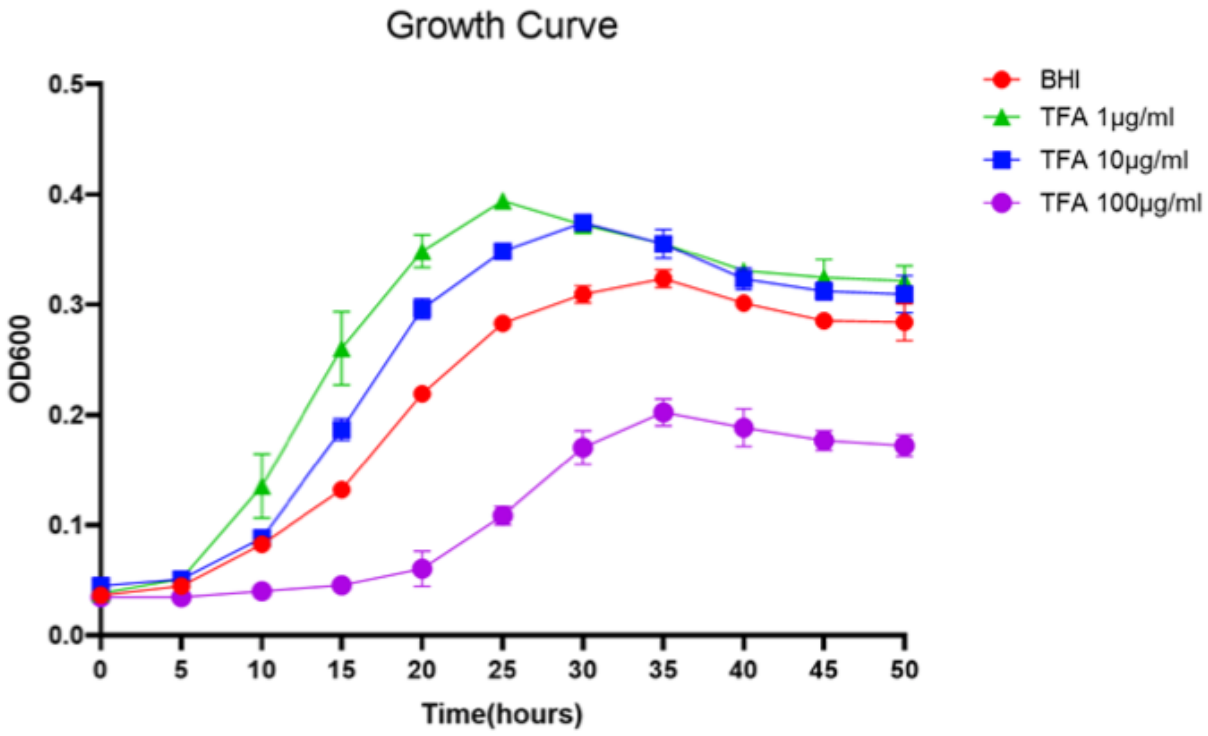

Figure 7

Growth curves of Akkermansia muciniphila in different conditions.

Figure8

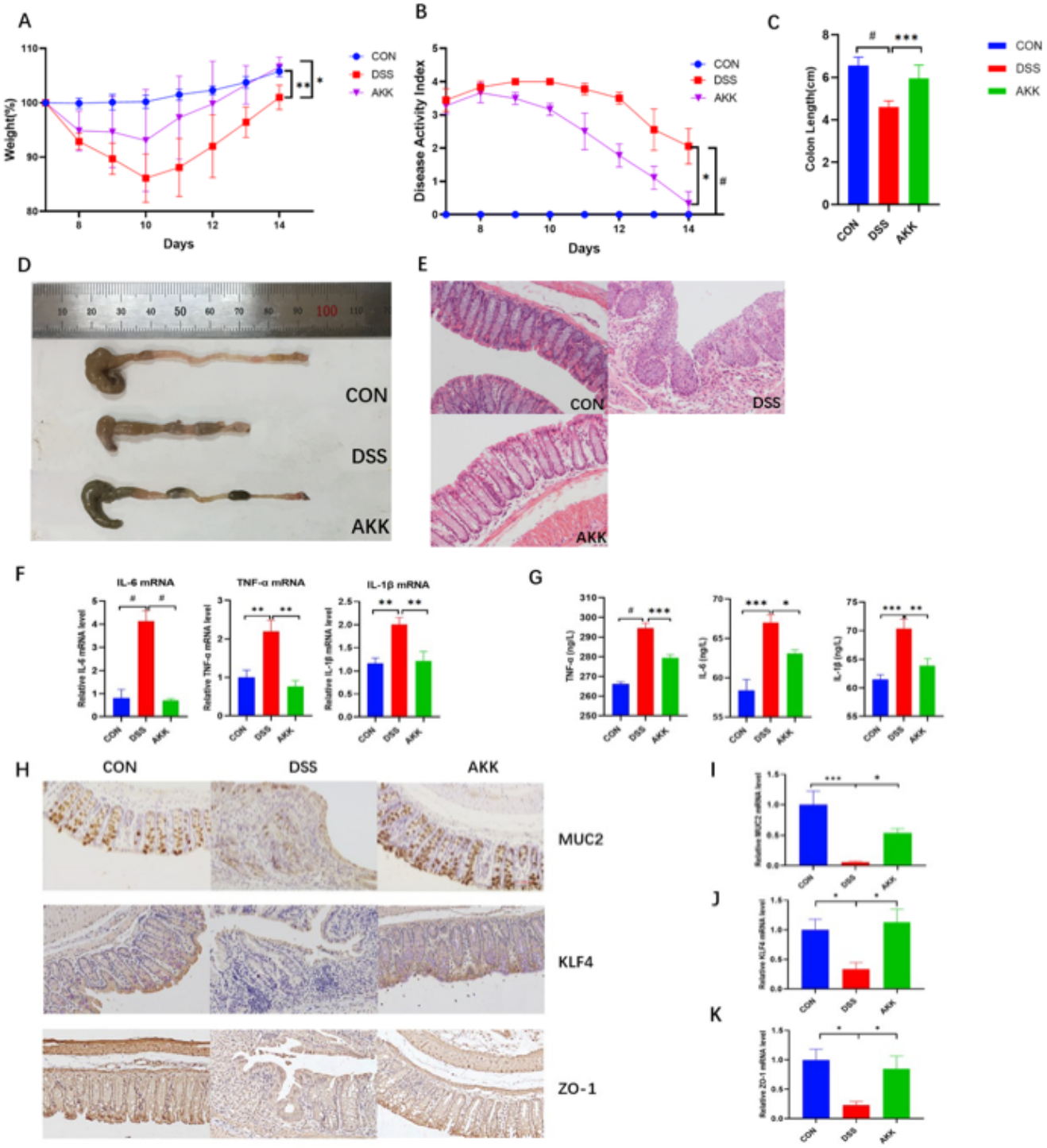

Figure 8

AKK ameliorated the symptoms of DSS-induced colitis in mice. (A) Daily bodyweight changes from day 7 to 14. (B) DAI score from day 7 to 14. (C) The lengths of colon. Data are presented as the mean ± S.E.M. *P < 0.05, **P < 0.01, ***P < 0.001, #P<0.0001 (D) Macroscopic appearances of colon tissues. (E) Histological changes. (F) mRNA quantification of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β using Real Time RT-PCR. (G)Determination of protein productions of the inflammatory cytokines using ELISA.

The data present the mean ± S.E.M. Asterisks denote significance vs. DSS group by one-way ANOVA (*p <0.05, **p <0.01, ***p <0.001). (H) The number of colonic MUC2, KLF4, ZO-1positive cells in DSS-treated mice. Representative images (×200 magnification, scale bar 50 μm). (I-K) mRNA quantification of MUC2, KLF4 and ZO-1.