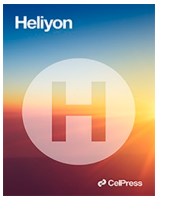
Heliyon 10 (2024) e33705

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

# Ameliorative effects of the mixed aqueous extract of Aurantii Fructus Immaturus and Magnoliae Officinalis Cortex on loperamide-induced STC mice

Ting Cai [a], Yun Dong [b], Zeyu Feng [c], Bin Cai [c,*]

[a] *Department of Nephrology, The Affiliated Wuxi People's Hospital of Nanjing Medical University, Wuxi People's Hospital, Wuxi Medical Center, Wuxi, 214000, China*
[b] *Department of Pathology, Wuxi Hospital Affiliated to Nanjing University of Chinese Medicine, Wuxi, 214071, China*
[c] *Department of Anorectal Surgery, Wuxi Hospital Affiliated to Nanjing University of Chinese Medicine, Wuxi, 214071, China*

ARTICLE INFO

*Keywords:*
*Aurantii Fructus Immaturus*
*Magnoliae officinalis Cortex*
Slow transit constipation
Aquaporin
Gut microbiota

ABSTRACT

*Aurantii fructus immaturus* (AFI) and *Magnoliae Officinalis Cortex* (MOC) have been used to treat constipation in China for thousands of years. In this study, a mouse model of slow transit constipation (STC) was established by gavage of loperamide at a dose of 10 mg/kg bw/day for seven days. Seventy-two mice were randomly allocated to six groups (control, STC model, 3 g/kg AFI + MOC, 6 g/kg AFI + MOC, 12 g/kg AFI + MOC, and mosapride). A mixed aqueous extract of AFI and MOC was administered to the STC mice at the corresponding doses from the first day of modelling. Body weight, faecal water content, gastrointestinal transit time, and intestinal propulsion rate were evaluated. Serum levels of neurotransmitters and gastrointestinal hormones, colonic expression of aquaporins (AQP), and interstitial cells of Cajal (ICC) were assessed using ELISA, immunohistochemistry, and Western blot analysis. The abundance and diversity of the gut microbiota were analysed by 16S rRNA gene sequencing. The mixed aqueous extract significantly increased faecal water content and intestinal propulsion rate and shortened gastrointestinal transit time in STC mice. Furthermore, the administration of AFI and MOC significantly decreased serum vasoactive intestinal peptide (VIP), nitric oxide (NO), and somatostatin (SS) levels and increased serum motilin (MTL) levels in STC mice. The protein expression levels of AQP3 and AQP4 in the colon tissue of STC mice significantly decreased following AFI + MOC treatment, whereas those of AQP9 significantly increased. Moreover, the AFI + MOC treatment led to an increase in the number and functionality of ICCs. In addition, the relative abundances of *Ruminococcus* and *Oscillospira* increased in response to the administration of AFI + MOC in STC mice. In conclusion, the mixed aqueous extract of AFI and MOC promoted defaecation and increased intestinal mobility in STC mice. Its mechanisms of action involve modulatory effects on neurotransmitters, gastrointestinal hormones, AQPs, and ICCs. AFI + MOC treatment also improved the diversity and abundance of the gut microbiota in STC mice, particularly short-chain fatty acid-producing bacteria, which may play an important role in its beneficial effect on constipation.

* Corresponding author.
*E-mail address:* fsyy01159@njucm.edu.cn (B. Cai).

https://doi.org/10.1016/j.heliyon.2024.e33705
Received 9 March 2024; Received in revised form 22 June 2024; Accepted 25 June 2024
Available online 27 June 2024

## 1. Introduction

Slow transit constipation (STC) based the Rome criteria for the disorders of gut-brain interaction is a clinically common functional disorder of the digestive system [1]. The clinical manifestations of STC include a prolonged decrease in stool frequency, lack of stool intention, abdominal distension, anorexia, difficulty defaecating, prolonged defaecation time, and granular or bulbous stools. The clinical management options for STC include conservative medical therapy and surgery, of which conservative treatment is used in the majority of cases. Although a variety of drugs are available to modulate gastrointestinal (GI) motility, including dopamine receptor antagonists (e.g. metoclopramide and domperidone), 5-hydroxytryptamine receptor agonists (e.g. cisapride, mosapride, itopride, and prucalopride), and macrolide antibiotics (e.g. azithromycin and erythromycin), the need for safe and effective medications to regulate GI motility and effectively treat STC remains. Chinese herbal medicine has a long history of use in the treatment of digestive system diseases, with particular efficacy demonstrated in the treatment of constipation [2].

The traditional Chinese herbal medicines, *Aurantii Fructus Immaturus* (AFI; Zhishi in Chinese) and *Magnoliae Officinalis Cortex* (MOC; Houpo in Chinese) have been documented in several classic prescriptions for constipation. AFI is the dried, unripe fruit of *Citrus aurantium*, which is widely used to relieve flatulence and improve GI digestion [3]. Modern pharmacological studies have found that AFI has a variety of activities, including anti-inflammatory, antitumour, and intestinal microbiota regulatory activities [4,5]. The water decoction of AFI has been reported to significantly enhance the frequency of isolated rabbit intestinal smooth muscle contractions. Furthermore, in silico analysis predicted that the flavonoids in AFI were the main active ingredients targeting the GI system [6]. Naringin, naringenin, and neohesperidin are the three most abundant flavonoid components in AFI [7]. MOC, the dried stem bark of *Magnolia officinalis*, is commonly used in traditional Chinese medicine (TCM) to treat abdominal distension and oedema [8]. MOC has been reported to have pharmacological effects on the digestive, nervous, cardiovascular, and cerebrovascular systems, as well as antibacterial, antitumour, analgesic, anti-inflammatory, and antioxidant effects [9]. Phytochemical studies have demonstrated that the main components of MOC are phenols, including magnolol and honokiol, which have been reported to improve gastric emptying and exhibit intestinal propulsive activity [10]. Magnolol is nontoxic, but its gastrointestinal absorption is poor, with an oral bioavailability of only 4.9 % [11]. Honokiol also has minimal toxicity; intravenous administration of honokiol showed a rapid rate of distribution and absorption with slower elimination [12]. The mixed aqueous extract of AFI and MOC has been used to treat defaecation problems in China for more than 2000 years and is now widely used as an important ingredient in TCM prescriptions for constipation treatment [13,14]. In light of the TCM theory and the findings presented above, therapeutic effects of AFI and MOC on STC can be expected.

However, the pathophysiological mechanisms underlying STC remain unclear. It is now generally accepted that the abnormal expression of neurotransmitters and GI hormones, abnormal expression of aquaporins (AQP) in intestinal epithelial cells, and reduction and dysfunction of interstitial cells of Cajal (ICC) play important roles in the pathogenesis of STC [15–17]. Peristalsis in the GI tract is caused by smooth muscle contractions, which are regulated by the enteric nervous and endocrine systems. Several neurotransmitters and GI hormones, including cholecystokinin (CCK), motilin (MTL), vasoactive intestinal peptide (VIP), and glucose-dependent insulinotropic peptide (GIP) exert regulatory effects on gut motility [18]. ICCs are mesenchymal cells found within the muscle layers of the alimentary tract. They act as mediators in the communication between the autonomic nervous system and the smooth muscles of the GI tract. Their function is to initiate and transmit slow waves in the GI smooth muscle, which is considered to be their primary physiological role [19]. AQPs are a family of water channel proteins distributed in the intestinal epithelial cells that play an important role in controlling faecal water content and are associated with the development of constipation [20]. To date, thirteen types of AQPs (AQP0–AQP12) have been identified in mammals, and several AQPs are aberrantly expressed in STC mice, including AQP3, AQP4, AQP8, and AQP9 [21,22]. Moreover, as research on the brain-gut axis has increased over the past few years, that the intricate link between the pathogenesis of constipation and gut microbiota dysbiosis has become increasingly evident [23].

In this study, the mixed aqueous extract of AFI and MOC was administered to mice with loperamide-induced STC. To verify the overall effect of the mixed aqueous extracts of AFI and MOC on STC mice, we measured the changes in body weight, faecal water content, gastrointestinal transit time, and intestinal propulsion rate. To investigate the potential mechanisms of AFI and MOC in STC treatment, we assessed the serum levels of several neurotransmitters and hormones, including VIP, nitric oxide (NO), somatostatin (SS), and MTL, the colonic expression of AQP3, AQP4, and AQP9, and the number and function of ICCs in the colon tissues of STC mice. The abundance and diversity of the gut microbiota were analysed using 16S rRNA gene sequencing. Our results confirmed that mixed aqueous extracts of AFI and MOC promoted defaecation and enhanced intestinal mobility in STC mice. Its mechanism of action involves the modulation of neurotransmitters, GI hormones, AQPs, ICCs, and gut microbiota. This study provides a scientific basis for the clinical application of AFI and MOC in the treatment of STC, and the subsequent exploration of their active components.

## 2. Materials and methods

### 2.1. Preparation of aqueous extract

AFI was purchased from Suzhou Tianling Chinese Medicine Decoction Pieces Co., Ltd. (Batch No. 210906). MOC was purchased from Jiangsu Yabang Chinese Medicine Decoction Pieces Co., Ltd. (Batch No. 21110801). The mixed aqueous extract of AFI and MOC (1:1 mass ratio) was prepared using a traditional aqueous extraction method [24]. The detailed steps were as follows: The dried crude herbs of AFI and MOC were pulverised, and then 30 g of each herb was weighed and thoroughly mixed. The mixed powder was decocted in distilled water (500 mL) for 2 h and then passed through a 2 mm stainless-steel sieve to retain most of the solids. Subsequently, the remaining liquid was sterile-filtered (0.22 μm) and made up to 50 mL with distilled water to achieve a final

concentration of 1.2 mg/mL. The aqueous extract was stored at 4 °C. Before use, the aqueous extract was dissolved in distilled water to obtain the desired concentrations.

### 2.2. Constituent analysis of aqueous extract

The constituent analysis of the mixed aqueous extract of AFI and MOC was conducted using ultra-high-performance liquid chromatography with mass spectrometry (UHPLC-MS). After centrifugation at 13,000 rpm for 15 min, 400 μL of the supernatant was dried and redissolved in 200 μL of 70 % methanol. Chromatographic separation was performed using an Exion LC system (AB Sciex, USA). A Waters Acquity UHPLC HSS T3 column (2.1 × 150 mm, 1.8 μm) was used at a temperature of 35 °C. Mobile phase A was water with 0.1 % formic acid (v/v) and mobile phase B was acetonitrile [25]. The gradient was optimised as follows: 0–5 min from 3 % to 8 % B, 5–11 min from 8 % to 30 % B, 11–20 min from 30 % to 80 % B, 20–21 min from 80 % to 95 % B, and 21–27 min at 95 % B, then returned to the initial ratio of 3 % B, and maintained for an additional 10 min for re-equilibration. The injection volume for all samples was 2 μL. An X500B Q-TOF mass spectrometer (AB Sciex, USA) equipped with an electrospray ionisation source (Turbo Ionspray) was used for high-resolution detection. MS detection was performed in both the negative and positive ion modes. The mass spectrometer parameters were as follows: gas1 and gas2, 55 psi; curtain gas, 35 psi; heat block temperature, 550 °C; ion spray voltage, −4.5 kV in negative mode and 5.5 kV in positive mode; declustering potential, 60 V; collision energy, ±35 V; and the collision energy spread was ±15 V. The mass range was set to *m/z* 100–1250.

### 2.3. Animals

Seventy-two male C57BL/6 mice, aged 6–8 weeks and weighing 20 ± 2 g, were purchased from Cavans Experimental Animal Co. (Changzhou, China; licence number: SCXK (Su) 2021-0010). All mice were housed in a barrier-level environment at the Wuxi People's Hospital Laboratory Animal Centre. The temperature and humidity of the housing environment were maintained at 24–26 °C and 50–70 %, respectively, with a 12-h light/12-h dark cycle. All experimental procedures were approved by the Ethics Committee of Wuxi Hospital Affiliated to Nanjing University of Chinese Medicine and conducted in accordance with the 3R principles for the use of laboratory animals.

### 2.4. Study design

According to a report by Nair and Jacob [26], the dose conversion ratio between humans and mice is 1:9. In clinical practice, the recommended dosage for both AFI and MOC is 10 g/day for a 60-kg man. Consequently, the dosage levels for mice were determined to be 3, 6, and 12 g/kg bw/day, corresponding to the low-, medium-, and high-dose categories, respectively. Mosapride is a prokinetic serotonin receptor 4 (5-HT4) agonist that is used to treat constipation by enhancing GI motility [27]. The STC model was established by administering loperamide (Xian Janssen Pharmaceutical Ltd., China) via gavage at a dose of 10 mg/kg bw/day, as previously reported [28]. Seventy-two mice were randomly divided into six groups. The treatment groups were as follows: control, STC model, 3 g/kg AFI + MOC, 6 g/kg AFI + MOC, 12 g/kg AFI + MOC, and mosapride (positive control). After one week of acclimatisation, mice in all groups, except the control group, were administered loperamide by gavage for seven consecutive days. From the first day of modelling, the three AFI + MOC groups were intragastrically administered the appropriate dose of the mixed aqueous extract of AFI and MOC once per day. The mice in the mosapride group were administered mosapride (Guangdong Anno Pharmaceutical Ltd. China) intragastrically at a dose of 2.5 mg/kg bw/day, and the mice in the control and STC model groups received equal amounts of saline.

### 2.5. General condition observation and weight measurement

The general condition of the mice was observed daily, including assessment of their mental state, activity levels, drinking water consumption, and stool characteristics. The weights of mice were recorded daily at fixed times.

### 2.6. Measurement of faecal water content, GI transit time, and intestinal propulsion rate

On the seventh day after modelling, 36 mice (six per group) were randomly selected from each group and individually housed in cages with free access to food and water. Following the methods of a previous study [29], faecal samples were collected within 6 h and then dried in an oven at 60 °C for 12 h. The formula for calculating faecal water content was as follows: faecal water content (%) = (faecal weight before drying - faecal weight after drying)/faecal weight before drying × 100 %. Following the completion of faecal collection, all of the above mice were administered 200 μL of carbon solution via gavage, after which they were permitted to resume their normal food and water intake. The carbon solution was prepared in accordance with previously published methods and comprised 5 % carbon and 10 % gum Arabic [30]. GI transit time was defined as the interval between the administration of the carbon solution and the passage of the first black stool. On the eighth day after modelling, the mice were subjected to a 12-h fast with access only to water. Subsequently, 200 μL of the above carbon solution was gavaged. After 20 min, the mice were euthanised by cervical dislocation and the small intestine from the pylorus to the ileocecal valve was removed as quickly and completely as possible. The total length of the small intestine and propulsive distance of the carbon solution in the intestine were measured in a tension-free state. The intestinal propulsion rate was calculated using the following formula [31]: Intestinal propulsion rate = carbon solution propulsion distance/total length of the small intestine × 100 %.

### 2.7. Sample collection

On the eighth day following modelling, the remaining mice (six per group) were subjected to a 12-h fast. After isoflurane anaesthesia, blood was collected via cardiac puncture. Subsequently, the mice were sacrificed via cervical dislocation. The caecal contents were then collected, snap-frozen, and stored at −80 °C. Colonic tissues were collected and divided into two portions. One portion was immediately frozen at −80 °C, whereas the remaining portion was fixed with a 4 % paraformaldehyde solution. The collected blood was centrifuged at 4 °C and 3000×*g* for 10 min to obtain serum, which was subsequently stored at −20 °C for further testing.

### 2.8. Immunohistochemistry

After a 48-h incubation period in a 4 % paraformaldehyde solution, the colon tissue was embedded in paraffin and sectioned at a thickness of 4 μm. Colon tissue sections were dewaxed, incubated with 3 % hydrogen peroxide to inactivate endogenous peroxidase, and sealed with 5 % bovine serum albumin (Gibco, USA) for 1 h. The sections were incubated overnight at 4 °C with an anti-*c*-Kit antibody (1:3000, ab256345, Abcam, UK) and then incubated at 37 °C with a secondary antibody (1:100, sc-2357, Santa Cruz, USA). The colour reaction was developed using a 3,3′-diaminobenzidine solution and counterstained with haematoxylin. Sections were observed using an Olympus CX-43 microscope (Olympus, Tokyo, Japan).

### 2.9. Enzyme-linked immunosorbent assay

The frozen serum was gradually warmed for dissolution. Serum levels of NO, SS, VIP, and MTL were quantified using enzyme-linked immunosorbent assay (ELISA) kits (Shanghai Enzyme-Linked Biotechnology Co., Ltd., China) following the manufacturer's instructions. The absorbance was measured at 450 nm using a microplate reader (BioTek, USA).

### 2.10. Western blot analysis

Freshly frozen colon tissues were homogenised in radioimmunoprecipitation assay (RIPA) buffer (Beyotime Biotechnology, China) containing 1 % protease inhibitor (Beyotime Biotechnology, China) and 1 % phosphatase inhibitor (Beyotime Biotechnology, China). The homogenate was centrifuged at 4 °C and 12,000×*g* for 20 min, after which the protein supernatant was collected. Protein concentration was measured using a BCA Protein Assay Kit (Pierce, USA). The protein samples were mixed with 5 × loading buffer (Beyotime Biotechnology, China), boiled at 95–100 °C for 10 min, and then stored at −20 °C for further detection. Protein (30 μg) was loaded onto a 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Beyotime Biotechnology, China) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, UK). After blocking with the QuickBlock blocking buffer (Beyotime Biotechnology, China) for 1 h, the membranes were subsequently incubated overnight with primary antibodies, including anti-AQP3 (1:500, AF5222, Affinity Biosciences, China), anti-AQP4 (1:1000, ab259318, Abcam, UK), anti-AQP9 (1:100, sc-74409, Santa Cruz, USA), anti-stem cell factor (SCF) (1:200, sc-13126, Santa Cruz, USA), anti-*c*-Kit (1:1000, ab256345, Abcam, UK), and anti-α-tubulin (1:200, sc-5286, Santa Cruz, USA), all at 4 °C on a shaking table to ensure uniformity of incubation. After three washes with Tris-buffered saline with Tween-20 (TBST), the membranes were incubated with the appropriate secondary antibodies, namely anti-mouse IgG antibody (1:1000, 7076, CST, USA) and anti-rabbit IgG antibody (1:2000, sc-2357, Santa Cruz, USA), for 1 h at room temperature. Protein bands were visualised using a ChemiScope 3500 Mini Imaging System (Clinx Science Instruments, China).

### 2.11. 16S rRNA gene-based microbial community analysis

Total genomic DNA was extracted from the mouse caecal contents using an Omega Soil DNA Kit (M5635-02; Omega Bio-Tek, USA) in accordance with the manufacturer's instructions. The V3–V4 regions of the 16S rRNA genes were amplified by polymerase chain reaction (PCR) using the primers 338F and 806R. The PCR amplicons were purified using VAHTSTM DNA Clean Beads (N411-01, Vazyme, China) and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (P7589, Invitrogen, USA). Subsequently, the amplicons were sequenced on an Illumina NovaSeq platform using a NovaSeq 6000 SP Reagent Kit (500 cycles). The read length was 2 × 250 bp. Microbiome bioinformatics analysis was conducted using QIIME2 software (V2019.4) and R package (V3.2.0). The raw sequences were filtered, denoised, and merged, and chimaeras were removed using the DADA2 plugin [32]. Non-singleton amplicon sequence variants (ASV) were aligned using MAFFT [33] and were used to construct a phylogeny using Fasttree2 [34]. Alpha diversity analysis (Chao1, observed species, Shannon, and Simpson) was performed to analyse the species diversity and richness of each sample. Beta diversity analysis (PCoA) was performed to investigate the structural variation in microbial communities across samples. Linear discriminant analysis (LDA) effect size (LEfSe) assessment was performed to detect differentially abundant taxa across the groups.

### 2.12. Statistical analysis

All data are presented as mean ± standard deviation (SD). Statistical differences between multiple groups were analysed using one-way analysis of variance (ANOVA). Statistical differences between two independent groups were analysed using Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

## 3. Results

### 3.1. Constituent analysis of AFI and MOC aqueous extract

The chemical composition of the mixed aqueous extract of AFI and MOC was analysed using UHPLC-MS. A total of 62 components were identified, including 24 flavonoids, 9 alkaloids, 5 lignans, 5 limonoids, 6 phenylethanoid glycosides, 5 phenylpropanoids, and 8 other components. A total of 34 compounds were derived from AFI and 29 from MOC. The chromatograms of the labelled base peaks in negative and positive ion modes are shown in Fig. S1. Detailed information on these compounds is provided in Table S1.

### 3.2. Effects of AFI + MOC treatment on body weight, faecal water content, GI transit time, and intestinal propulsion rate in STC mice

In this study, all the mice survived until sacrifice. The body weight of mice in the STC model group was significantly decreased ($P < 0.05$) on day 7 after modelling compared to baseline (day 0). Furthermore, the body weights of the mice in the three AFI + MOC dose groups, as well as in the mosapride group, demonstrated a declining trend following modelling (Fig. 1A). Faecal water content was significantly lower in the STC model group than in the control group ($P < 0.01$). Faecal water content was significantly increased (all $P < 0.01$) in the 6 g/kg AFI + MOC, 12 g/kg AFI + MOC, and mosapride groups compared to that in the STC model group (Fig. 1B). The GI transit time in the STC model group was significantly longer than that in the control group ($P < 0.001$). GI transit time was significantly reduced (both $P < 0.01$) in the 6 g/kg AFI + MOC and 12 g/kg AFI + MOC groups compared with that in the STC model group (Fig. 1C). The intestinal propulsion rate in the STC model group was significantly lower than that in the control group ($P < 0.001$). The intestinal propulsion rates were significantly increased (all $P < 0.05$) in the 3 g/kg AFI + MOC, 6 g/kg AFI + MOC, 12 g/kg AFI + MOC, and mosapride groups compared to the STC model group (Fig. 1D). These results demonstrate that the administration of AFI + MOC was efficacious in promoting defaecation and improving intestinal motility in STC mice.

### 3.3. Effects of AFI + MOC treatment on serum VIP, SS, NO, and MTL in STC mice

The serum concentrations of VIP, SS, and NO were significantly higher (all $P < 0.001$) in the STC model group, and the serum concentrations of MTL were significantly lower than those in the control group. However, the serum concentrations of VIP, SS, and NO were significantly decreased (all $P < 0.01$) in the 3 g/kg AFI + MOC, 6 g/kg AFI + MOC, 12 g/kg AFI + MOC, and mosapride groups compared to those in the STC model group, whereas the serum concentrations of MTL were significantly increased ($P < 0.01$)

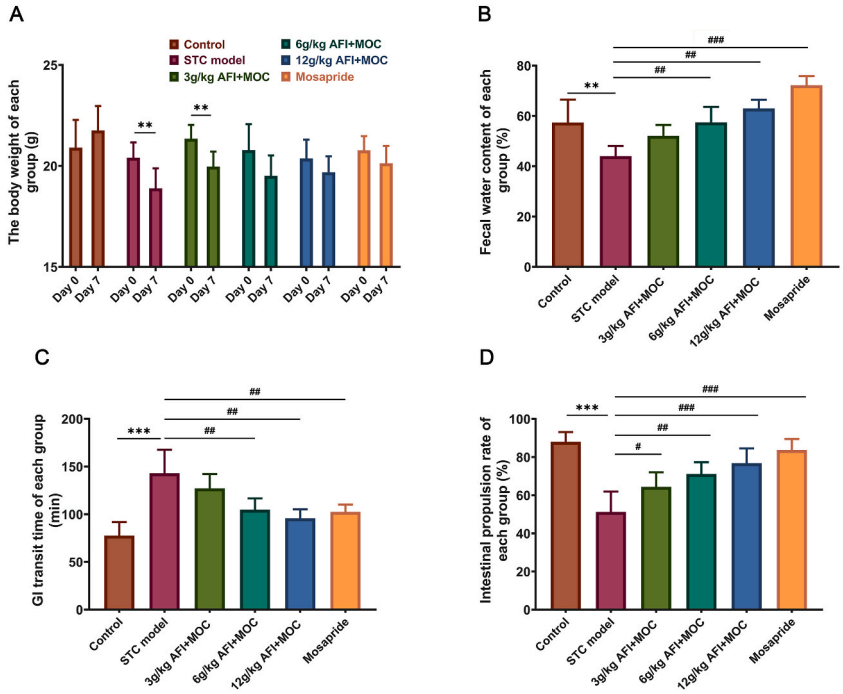



**Fig. 1.** Effects of AFI + MOC treatment on body weight, faecal water content, GI transit time, and intestinal propulsion rate in STC mice. (A) The body weight of mice in each group on day 0 and day 7 (**$P < 0.01$). (B) The faecal water content of mice in each group. (C) The GI transit time of mice in each group. (D) The intestinal propulsion rate of mice in each group. **$P < 0.01$, ***$P < 0.001$ *vs.* control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* STC model group. AFI: *Aurantii Fructus Immaturus*; MOC: *Magnoliae Officinalis Cortex*; GI: gastrointestinal; STC: slow transit constipation.

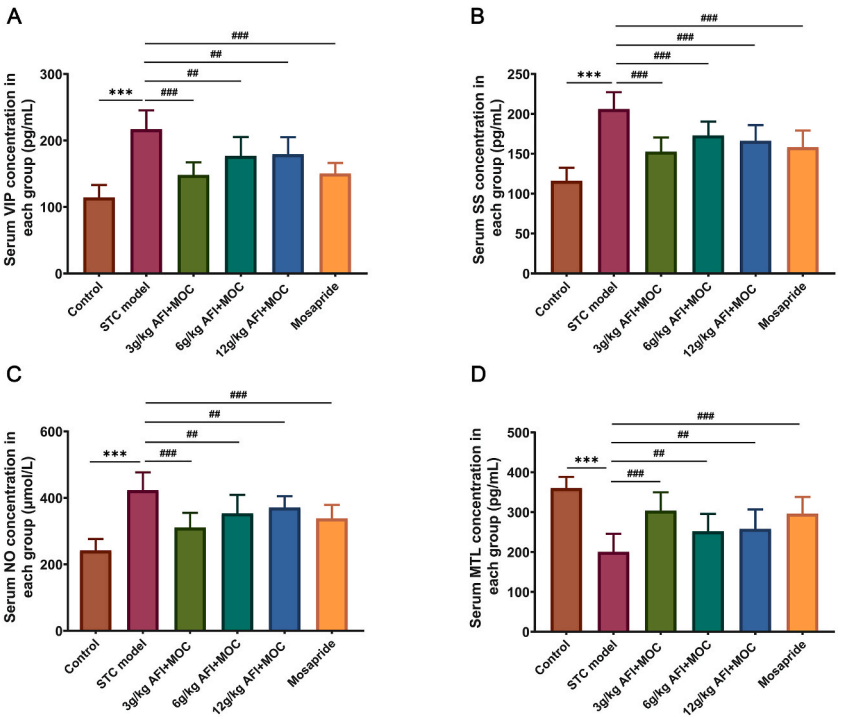



**Fig. 2.** Effects of AFI + MOC treatment on the serum concentrations of VIP, SS, NO, and MTL in STC mice. Serum concentrations of VIP (A), SS (B), NO (C), and MTL (D) in each group. $^{***}P < 0.001$ *vs.* control group; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* STC model group. AFI: *Aurantii Fructus Immaturus*; MOC: *Magnoliae Officinalis Cortex*; STC: slow transit constipation; VIP: vasoactive intestinal peptide; SS: somatostatin; NO: nitric oxide; MTL: motilin.

(Fig. 2A–D).

### 3.4. Effects of AFI + MOC treatment on ICCs in STC mice

The number of ICCs in intestinal tissue was assessed by immunostaining for c-Kit. As illustrated in Fig. 3A, the number of c-Kit-positive cells in the colonic muscle layer was lower in the STC model group than in the control group. The number of c-Kit-positive cells was higher in the 3 g/kg AFI + MOC, 6 g/kg AFI + MOC, 12 g/kg AFI + MOC, and mosapride groups than in the STC model group. Western blot analysis demonstrated that the protein expression of c-Kit and SCF was significantly decreased (both $P < 0.001$) in the STC model group compared to the control group and that of c-Kit and SCF was significantly increased (all $P < 0.05$) in the 3 g/kg AFI + MOC, 6 g/kg AFI + MOC, 12 g/kg AFI + MOC, and mosapride groups compared to the STC model group (Fig. 3B–D).

### 3.5. Effects of AFI + MOC treatment on the expression of AQP3, AQP4, and AQP9 proteins in colon tissues of STC mice

The protein expression levels of AQP3 and AQP4 were significantly increased (both $P < 0.05$), and AQP9 expression was significantly decreased ($P < 0.05$) in the colon tissues of the STC model group compared to those in the control group. The protein expression levels of AQP3 and AQP4 were significantly decreased (all $P < 0.05$) in the colon tissues of the 6 g/kg AFI + MOC, 12 g/kg AFI + MOC, and mosapride groups compared to those in the STC model group. The protein expression level of AQP9 was significantly increased (both $P < 0.05$) in the 6 g/kg AFI + MOC and 12 g/kg AFI + MOC groups compared to the STC model group (Fig. 4A–D).

### 3.6. Effect of AFI + MOC treatment on the gut microbiota in STC mice

The caecal contents of mice in the control, STC model, and 12 g/kg AFI + MOC groups were used for 16S rRNA gene-based microbial community analysis. The ASV numbers in the control, STC, and 12 g/kg AFI + MOC groups were 8,176, 3,828, and 6,540, respectively (Fig. 5A). Alpha diversity analysis (Chao1 and Observed species) demonstrated that species diversity was significantly lower (both $P < 0.05$) in the STC model group than that in the control group (Fig. 5B). Alpha diversity analysis (Shannon and Simpson) demonstrated that species diversity was significantly higher (both $P < 0.05$) in the 12 g/kg AFI + MOC group than in the STC model group (Fig. 5B). Beta diversity analysis (PCoA) demonstrated that the overall taxonomic composition of the control and 12 g/kg AFI + MOC groups exhibited structural proximity to each other, but both were distinct from the STC model group (Fig. 5C). Differences in the gut microbiota profiles across the groups were analysed at different taxonomic levels. The results demonstrated that, at the phylum level, Firmicutes constituted the largest proportion, and its relative abundance was higher in the STC model group than in the control

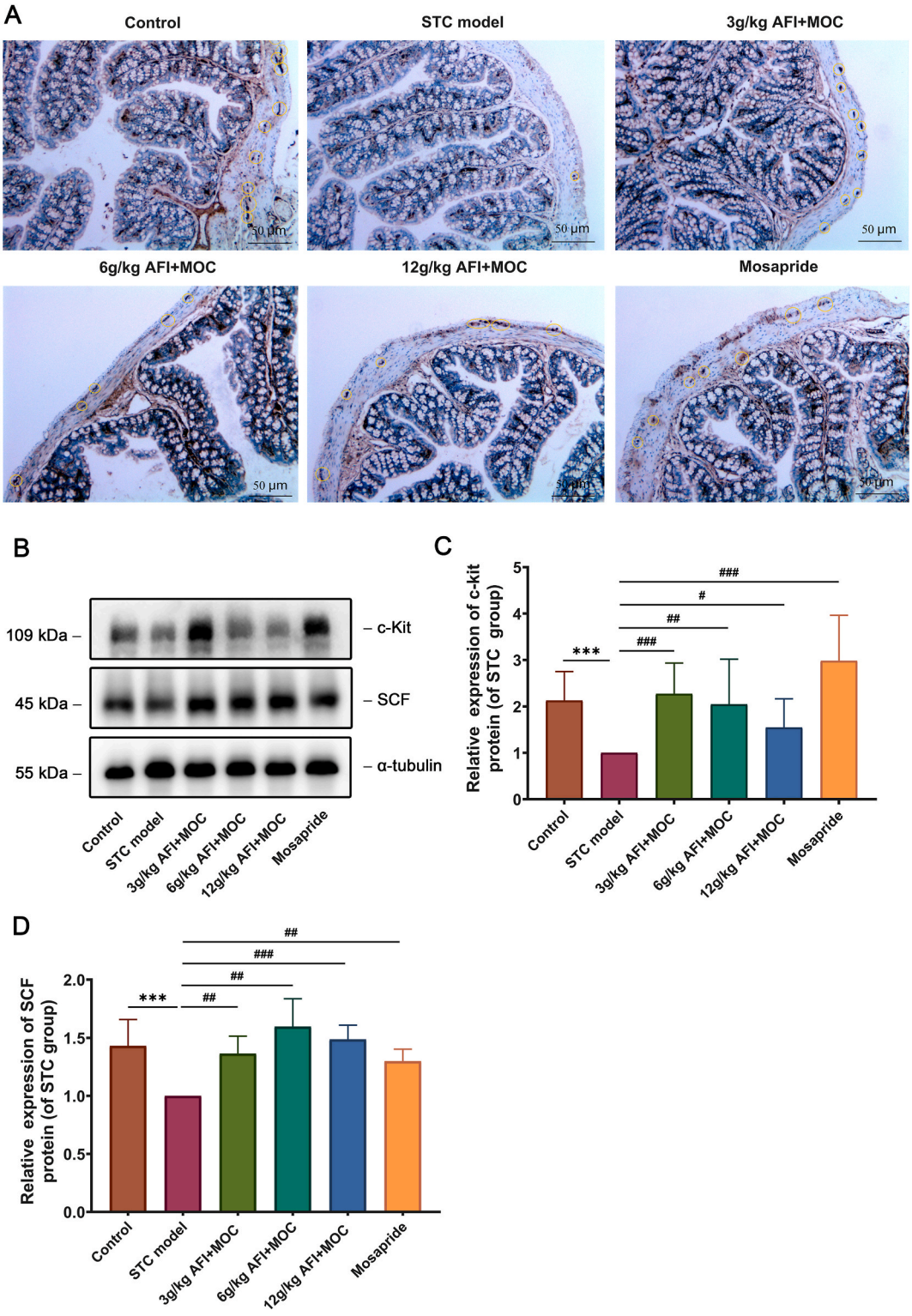



**Fig. 3.** Effects of AFI + MOC treatment on the ICCs in STC mice. (A) Immunostaining for c-Kit in the control, STC model, 3 g/kg AFI + MOC, 6 g/kg AFI + MOC, 12 g/kg AFI + MOC, and mosapride groups (100 × ). (B) The expression levels of c-Kit, SCF, and α-Tubulin proteins in the colon of each group were detected by Western blot analysis. (C) Relative protein expression of c-Kit in each group. (D) Relative expression of SCF protein in each group. $^{***}P < 0.001$ *vs.* control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* STC model group. AFI: *Aurantii Fructus Immaturus*; MOC: *Magnoliae Officinalis Cortex*; ICC: interstitial cells of Cajal; STC: slow transit constipation; SCF: stem cell factor.

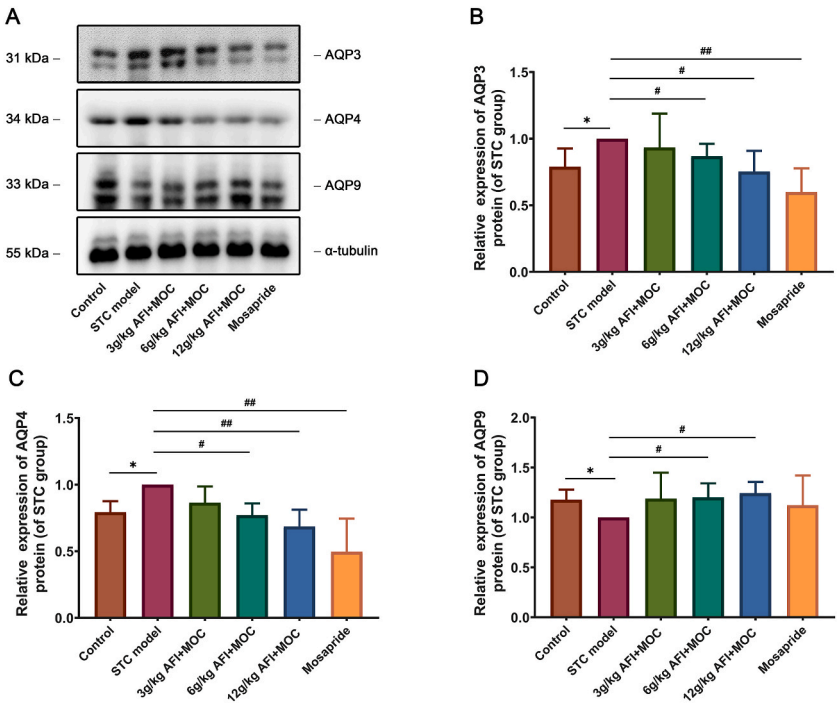



**Fig. 4.** Effects of AFI + MOC treatment on the protein expression of AQP3, AQP4, and AQP9 in the colon of STC mice. (A) The expression levels of AQP3, AQP4, AQP9, and α-Tubulin proteins in the colon of each group were detected by Western blot analysis. Relative protein expression of AQP3 (B), AQP4 (C), and AQP9 (D) in each group. $^{*}P < 0.05$ *vs*. control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs*. STC model group. AFI: *Aurantii Fructus Immaturus*; MOC: *Magnoliae Officinalis Cortex*; AQP: aquaporins; STC: slow transit constipation.

group. The relative abundance of Firmicutes was lower in the 12 g/kg AFI + MOC group than in the STC model group (Fig. 5D). At the class level, Clostridia, Bacteroidia, and Erysipelotrichi collectively accounted for >75 % of all microbial species. The relative abundances of Clostridia and Bacteroidia were lower, whereas that of Erysipelotrichi was higher, in the STC model group than in the control group. The relative abundance of Clostridia and Bacteroidia increased, whereas that of Erysipelotrichi decreased in the 12 g/kg AFI + MOC group compared to the STC model group (Fig. 5E). At the order level, the microbiota community was primarily composed of Clostridiales, Bacteroidales, Erysipelotrichales, Lactobacillales, and Desulfovibrionales, and their relative abundance was influenced by the administration of AFI and MOC (Fig. 5F).

Fig. 6A shows the top 50 average abundances at the genus level. We found that the relative abundance of *Ruminococcus* and *Oscillospira* was significantly lower in the STC model group than in the control group, whereas they were markedly upregulated by AFI + MOC treatment (Fig. 6B and C). In addition, the relative abundances of *Allobaculum* and *Turicibacter* were significantly higher in the STC model group than in the control group, whereas they were markedly downregulated by AFI + MOC treatment (Fig. 6D and E). These results indicate that the mixed aqueous extract of AFI and MOC ameliorated the gut microbiota imbalance in STC mice to exert its beneficial effects.

## 4. Discussion

The pathophysiological mechanisms of STC are complex and involve numerous factors, including defects or dysfunction of the central nervous system and enteric nervous system, aberrant secretion of neurotransmitters and GI hormones, aberrant expression of AQPs in the intestinal epithelium, reduction and dysfunction of ICCs, and alterations in the gut microbiota and associated metabolites [35] Chinese herbal medicine has been used to treat constipation for thousands of years in China. Two of the most commonly prescribed medications for constipation, AFI and MOC, have been documented in numerous classic prescriptions [13,14]. In our study, the administration of a mixed aqueous extract of AFI and MOC resulted in a significant increase in faecal water content and intestinal propulsion rate in STC mice. Furthermore, the GI transit time in STC mice was significantly shortened after AFI + MOC treatment. These results indicated that the mixed aqueous extract of AFI and MOC had ameliorative effects on STC. Therefore, we investigated the mechanisms underlying their beneficial effects on STC.

Neurotransmitters and GI hormones regulate intestinal muscle contraction and peristalsis. Alterations in these factors are strongly correlated with the onset of constipation [36]. Neurotransmitters and GI hormones are typically classified as excitatory or inhibitory, based on their distinct physiological functions. Many studies have demonstrated that the levels of VIP, NO, and SS are elevated in loperamide-induced STC models, which is consistent with our findings [30,37,38]. VIP is an important inhibitory neurotransmitter in the enteric nervous system. It exerts its physiological effects by binding to its specific receptors, including vasoactive intestinal peptide

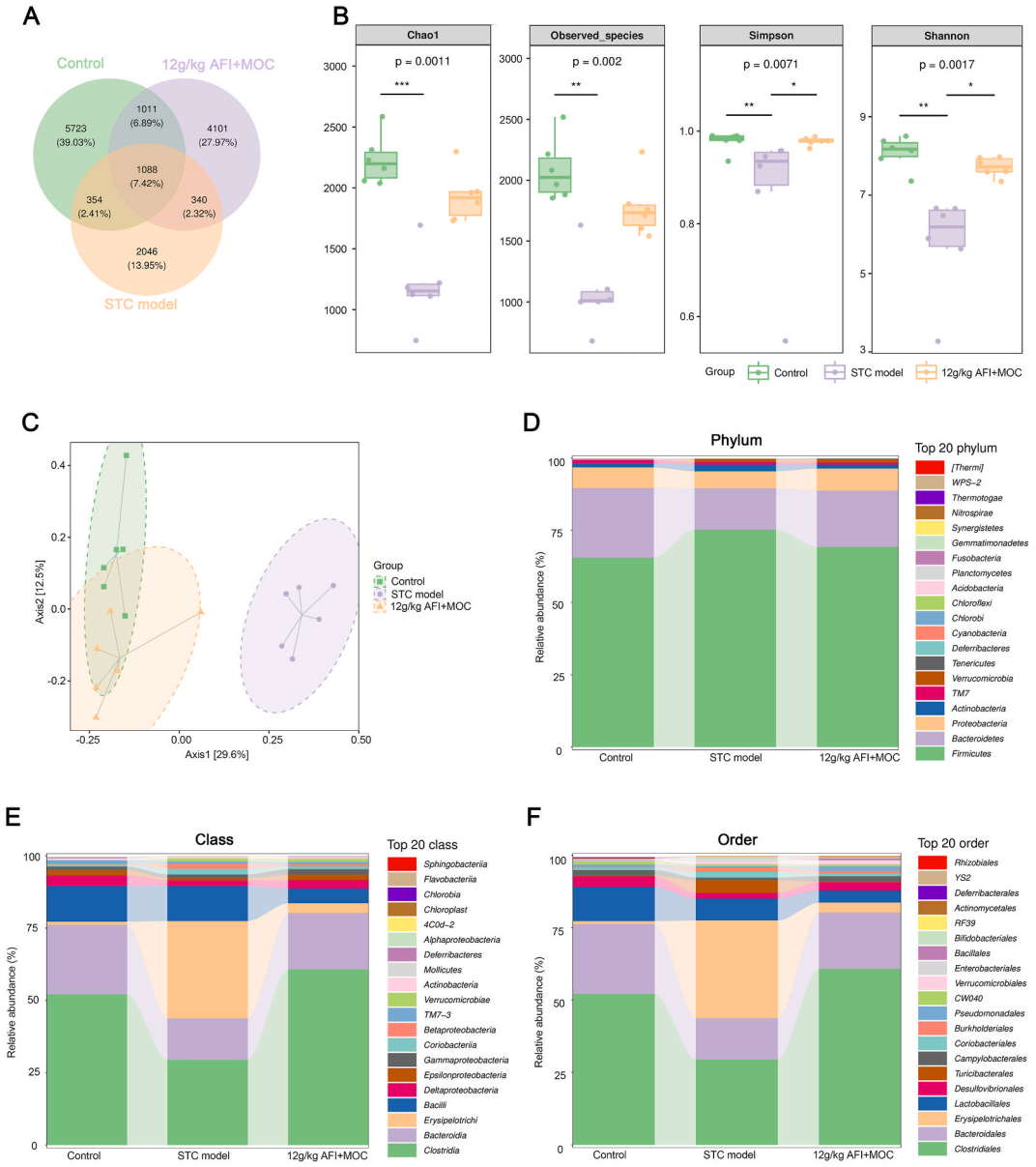



**Fig. 5.** Effect of AFI + MOC treatment on the gut microbiota in STC mice. (A) The ASV numbers in the control, STC model, and 12 g/kg AFI + MOC groups. (B) Alpha diversity analysis (Chao1, Observed species, Shannon, and Simpson). (C) Beta diversity analysis (PCoA). The gut microbiota profiles in the control, STC model, and 12 g/kg AFI + MOC groups at the phylum level (D), class level (E), and order level (F). AFI: *Aurantii Fructus Immaturus*; MOC: *Magnoliae Officinalis Cortex*; STC: slow transit constipation; ASV: amplicon sequence variants.

receptor 1 (VPAC1) and VPAC2, which can relax the GI smooth muscle and reduce intestinal peristalsis [39]. NO is another important inhibitory neurotransmitter catalysed by nitric oxide synthase (NOS) [40]. NO increases intracellular cyclic guanylate (cGMP) levels by activating soluble guanylate cyclase (sGC), which in turn activates protein kinase G (PKG), leading to calcium reabsorption, smooth muscle relaxation, and intestinal motility [41]. In addition, co-localisation of VIP and NOS has been observed in myenteric neurones of the GI tract in several species. It has been hypothesised that VIP may induce the release of NO, and NO may promote the secretion of VIP [42]. SS is an inhibitory hormone secreted by D cells in enteric neurones and exhibits a potent inhibitory effect on GI hormone release and GI motility [36]. MTL is an important GI hormone that can promote GI motility and increase the contractility and tone of the GI tract [43]. The results of our study indicated that the mixed aqueous extract of AFI and MOC significantly decreased the levels of serum VIP, NO, and SS and increased serum MTL levels in loperamide-induced STC mice, indicating that AFI + MOC treatment has a modulatory effect on neurotransmitter and GI hormone secretion in STC mice.

ICCs are a type of interstitial cells widely distributed in the muscular layer of the GI tract. They are considered pacemaker cells of GI motility because of their function in initiating and transmitting slow waves of GI smooth muscle [19]. The distribution of ICCs

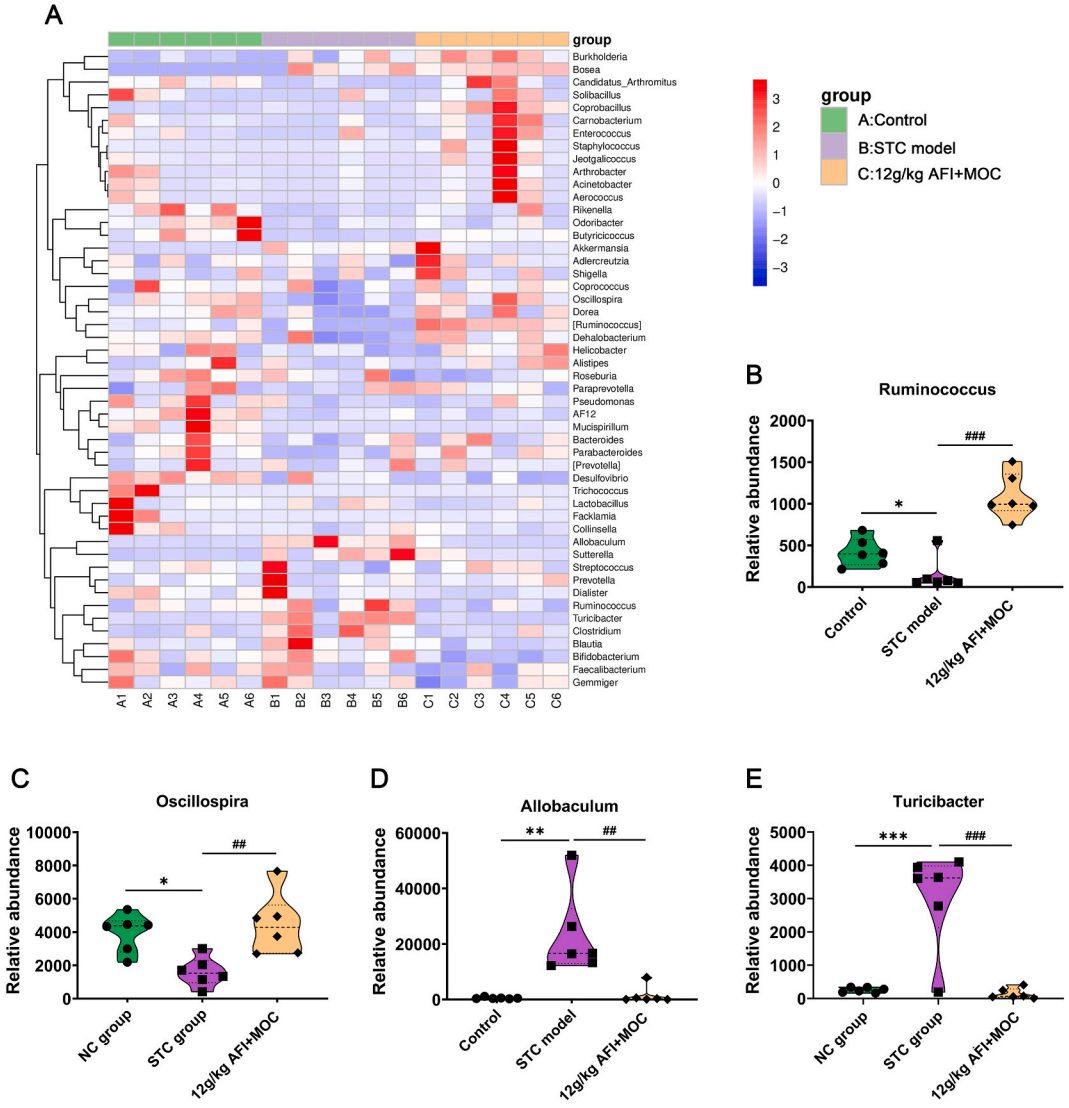



**Fig. 6.** Effect of AFI + MOC treatment on the gut microbiota at the genus level. (A) The top 50 average abundances at the genus level. The relative abundance of *Ruminococcus* (B), *Oscillospira* (C), *Allobaculum* (D), and *Turicibacter* (E) in the control, STC model, and 12 g/kg AFI + MOC groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* control group; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* STC model group. AFI: *Aurantii Fructus Immaturus*; MOC: *Magnoliae Officinalis Cortex*; STC: slow transit constipation.

throughout the human colon has been reported to be relatively uniform and the number and volume of ICCs in all segments of the colon were significantly lower in patients with STC than in healthy controls [15,44]. ICCs are typically identified by the expression of c-Kit, which binds to the extracellular ligand, SCF. When SCF dimerises, the structure of the c-Kit monomer changes, leading to homopolymerization and automatic phosphorylation of amino residues on the cell membrane. The activation of the SCF/c-Kit pathway plays a critical role in ICC survival and function of ICCs [30]. An *in vivo* study reported that the blockade of c-Kit resulted in the disappearance of ICCs and loss of slow-wave activity in the intestines of mice. In contrast, topical application of SCF restored the number of ICCs and the electrical rhythm of the intestine [45]. Our findings demonstrate that the number of ICCs and protein expression of c-Kit and SCF in the colon were significantly elevated following AFI + MOC treatment in STC mice, suggesting that the mixed aqueous extract of AFI and MOC may enhance intestinal transport function by increasing the number and functionality of ICCs.

AQPs are a family of membrane channel proteins that facilitate selective transport of water across membranes and play a pivotal role in human water metabolism [16]. Constipation is usually associated with abnormal water metabolism in the intestine, which is manifested by reduced water content in the faeces. In the present study, loperamide administration resulted in a reduction in faecal water content in STC mice, accompanied by an increase in AQP3 and AQP4 protein expression, and a decrease in AQP9 protein expression in the colon. Several studies have reported that AQP3 is highly expressed in the human colonic villous epithelial cells. Furthermore, AQP3 immunostaining in these cells was more intense in the apical membranes than basolateral membranes [46].

Several studies have demonstrated that a decrease in faecal water content is associated with an increase in colonic AQP3 protein expression in constipation models. These findings suggest that AQP3 plays an important role in regulating intestinal water transport and faecal water content [36,47]. AQP4 is present in the basement membranes of crypts and epithelial cells [48]. Wang et al. observed that patients with higher AQP4 expression were more likely to have dry and hard stools [49]. Consequently, they proposed that increased AQP4 expression may accelerate colonic water absorption, which may be a pivotal event in STC pathogenesis. AQP9 is an aquaglyceroporin with broad permeability to water molecules and various neutral solute molecules, including glycerol, urea, polyols, purines, and pyrimidines [50]. AQP9 is involved in colonic mucosal secretions and plays a role in mucosal protection, bowel lubrication, and defaecation. Reduced AQP9 expression may result in decreased mucus secretion by goblet cells, which can lead to constipation [21]. Our findings indicate that the administration of a mixed aqueous extract of AFI and MOC resulted in a reduction in AQP3 and AQP4 expression and an increase in AQP9 expression in the colon of STC mice, which contributed to an increase in faecal water content, thereby promoting defaecation.

Over the past decade, many studies using 16S rRNA-based gut microbiota assays have demonstrated a strong association between gut microbiota and disease onset [51–54]. Our results demonstrated that the administration of AFI + MOC to STC mice led to an increase in the relative abundances of *Ruminococcus* and *Oscillospira*. *Ruminococcus* is one of the most important participants in the utilisation and degradation of carbohydrates and other indigestible plant fibres in the gut [55,56]. As an anaerobic bacterium, *Ruminococcus* plays a pivotal role in the production of short-chain fatty acids (SCFAs), including butyrate, acetate, and propionate [57]. SCFAs serve not only as primary energy sources for colonic epithelial cells, but also regulate their physiological functions [58]. Recent studies have demonstrated that SCFAs are involved in the pathogenesis of constipation by affecting intestinal motility [23]. Furthermore, SCFAs can stimulate G protein-coupled receptors (GPCRs), including GPR42, which was recently identified as a functional GPCR that modulates $Ca^{2+}$ channel flux [59]. SCFAs can also influence the synthesis of 5-HT, thus affecting colonic motility [60]. Soret et al. demonstrated that butyrate enhanced the excitability of choline acetyltransferase-positive neurones in a monocarboxylate transporter 2-dependent manner, thereby improving colonic transit [61]. *Oscillospira* also produces SCFAs and previous research has suggested that it can produce butyrate and propionate [62]. Furthermore, *Oscillospira* stimulates mucus production by cupped cells, maintains the integrity of intestinal epithelial cells, and mitigates inflammatory damage to colonic tissues [63]. Consequently, we suggest that the regulatory effects of AFI and MOC on the aforementioned SCFA-producing bacteria in the gut may be related to their therapeutic effects on STC; however, this requires further investigation.

## 5. Conclusion

In conclusion, the results demonstrated that the mixed aqueous extract of AFI and MOC promoted defaecation and improved intestinal mobility in STC mice. Its mechanism of action involves the regulation of neurotransmitters, GI hormones, AQPs, and ICCs. Furthermore, AFI + MOC treatment improved the diversity and abundance of the gut microbiota, particularly SCFA-producing bacteria, in STC mice. The results of our study confirm the therapeutic effects of the mixed aqueous extract of AFI and MOC on STC and provide a scientific basis for the clinical application of AFI and MOC in the treatment of STC.

## Funding

This work was supported by National Natural Science Foundation of China (No. 82205110), Natural Science Foundation of Jiangsu Province (No. BK20210067), Top Talent Support Program for Young and Middle-aged People of Wuxi Health Committee (No. BJ2023066).

## Ethics declarations

This study was reviewed and approved by the Ethics Committee of Wuxi Hospital Affiliated to Nanjing University of Chinese Medicine (approval number: WXSBBJRC2020040202).

## Data availability statement

Raw sequencing reads and metadata are available from NCBI Short Read Archive accession SRP405566. Data included and/or cited in the article are available upon request from the corresponding author.

## CRediT authorship contribution statement

**Ting Cai:** Writing – original draft, Investigation, Funding acquisition, Data curation. **Yun Dong:** Methodology, Investigation. **Zeyu Feng:** Methodology. **Bin Cai:** Writing – review & editing, Writing – original draft, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33705.

## References


[1] D.A. Drossman, W.L. Hasler, Rome IV-functional GI disorders: disorders of gut-brain interaction, Gastroenterology 150 (6) (2016) 1257–1261.
[2] Y. Wen, Y. Zhan, S. Tang, F. Liu, R. Wu, P. Kong, Q. Li, X. Tang, Zhizhu decoction alleviates slow transit constipation by regulating aryl hydrocarbon receptor through gut microbiota, Pharm. Biol. 61 (1) (2023) 111–124.
[3] Y. Bai, Y. Zheng, W. Pang, W. Peng, H. Wu, H. Yao, P. Li, W. Deng, J. Cheng, W. Su, Identification and comparison of constituents of Aurantii fructus and Aurantii fructus Immaturus by UFLC-DAD-triple TOF-MS/MS, Molecules 23 (4) (2018) 803.
[4] S.Y. Chen, Q.Y. Zhou, L. Chen, X. Liao, R. Li, T. Xie, The Aurantii Fructus Immaturus flavonoid extract alleviates inflammation and modulate gut microbiota in DSS-induced colitis mice, Front. Nutr. 9 (2022) 1013899.
[5] A. Park, Y. Yang, Y. Lee, H. Jung, T.D. Kim, J.Y. Noh, S. Lee, S.R. Yoon, Aurantii Fructus Immaturus enhances natural killer cytolytic activity and anticancer efficacy in vitro and in vivo, Front. Med. 9 (2022) 973681.
[6] W. Tan, Y. Li, Y. Wang, Z. Zhang, T. Wang, Q. Zhou, X. Wang, Anti-coagulative and gastrointestinal motility regulative activities of Fructus Aurantii Immaturus and its effective fractions, Biomed. Pharmacother. 90 (2017) 244–252.
[7] C. Wang, Y. Pan, G. Fan, Y. Chai, Y. Wu, Application of an efficient strategy based on MAE, HPLC-DAD-MS/MS and HSCCC for the rapid extraction, identification, separation and purification of flavonoids from Fructus Aurantii Immaturus, Biomed. Chromatogr. 24 (3) (2010) 235–244.
[8] R. Yan, W. Wang, J. Guo, H. Liu, J. Zhang, B. Yang, Studies on the alkaloids of the bark of Magnolia officinalis: isolation and on-line analysis by HPLC-ESI-MSn, Molecules 18 (7) (2013) 7739–7750.
[9] H. Luo, H. Wu, X. Yu, X. Zhang, Y. Lu, J. Fan, L. Tang, Z. Wang, A review of the phytochemistry and pharmacological activities of Magnoliae officinalis cortex, J. Ethnopharmacol. 236 (2019) 412–442.
[10] W.W. Zhang, Y. Li, X.Q. Wang, F. Tian, H. Cao, M.W. Wang, Q.S. Sun, Effects of magnolol and honokiol derived from traditional Chinese herbal remedies on gastrointestinal movement, World J. Gastroenterol. 11 (28) (2005) 4414–4418.
[11] Y. Lin, Y. Li, Y. Zeng, B. Tian, X. Qu, Q. Yuan, Y. Song, Pharmacology, toxicity, bioavailability, and formulation of magnolol: an update, Front. Pharmacol. 12 (2021) 632767.
[12] F. Khatoon, S. Ali, V. Kumar, A.M. Elasbali, H.H. Alhassan, S.H. Alharethi, A. Islam, M.I. Hassan, Pharmacological features, health benefits and clinical implications of honokiol, J. Biomol. Struct. Dyn. 41 (15) (2023) 7511–7533.
[13] S. Zhang, H.Y. Chen, B. Shao, L. Lao, Effect of herbs on postoperative intestinal obstruction: a case report, Alternative Ther. Health Med. 27 (5) (2021) 74–76.
[14] B. Yang, F.Y. Xu, H.J. Sun, Z. Zou, X.Y. Shi, C.Q. Ling, L. Tang, Da-cheng-qi decoction, a traditional Chinese herbal formula, for intestinal obstruction: systematic review and meta-analysis, Afr. J. Tradit. Complement, Altern. Med. 11 (4) (2014) 101–119.
[15] T. Wedel, J. Spiegler, S. Soellner, U.J. Roblick, T.H. Schiedeck, H.P. Bruch, H.J. Krammer, Enteric nerves and interstitial cells of Cajal are altered in patients with slow-transit constipation and megacolon, Gastroenterology 123 (5) (2002) 1459–1467.
[16] N. Ikarashi, R. Kon, K. Sugiyama, Aquaporins in the colon as a new therapeutic target in diarrhea and constipation, Int. J. Mol. Sci. 17 (7) (2016) 1172.
[17] J. Tillou, V. Poylin, Functional disorders: slow-transit constipation, Clin. Colon Rectal Surg. 30 (1) (2017) 76–86.
[18] A. Furgala, K. Ciesielczyk, M. Przybylska-Felus, K. Jablonski, K. Gil, M. Zwolinska-Wcislo, Postprandial effect of gastrointestinal hormones and gastric activity in patients with irritable bowel syndrome, Sci. Rep. 13 (1) (2023) 9420.
[19] T. Ordog, S.M. Ward, K.M. Sanders, Interstitial cells of cajal generate electrical slow waves in the murine stomach, J. Physiol. 518 (Pt 1) (1999) 257–269.
[20] N. Ikarashi, R. Kon, K. Sugiyama, Aquaporins in the colon as a new therapeutic target in diarrhea and constipation, Int. J. Mol. Sci. 17 (7) (2016) 1172.
[21] R. Yi, P. Peng, J. Zhang, M. Du, L. Lan, Y. Qian, J. Zhou, X. Zhao, Lactobacillus plantarum CQPC02-fermented soybean milk improves loperamide-induced constipation in mice, J. Med. Food 22 (12) (2019) 1208–1221.
[22] Y. Wang, H. Jiang, L. Wang, H. Gan, X. Xiao, L. Huang, W. Li, Z. Li, Luteolin ameliorates loperamide-induced functional constipation in mice, Braz. J. Med. Biol. Res. 56 (2023) e12466.
[23] S. Zhang, R. Wang, D. Li, L. Zhao, L. Zhu, Role of gut microbiota in functional constipation, Gastroenterol. Rep. 9 (5) (2021) 392–401.
[24] Q. Cai, J. Wei, W. Zhao, S. Shi, Y. Zhang, R. Wei, Y. Zhang, W. Li, Q. Wang, Toxicity of Evodiae fructus on rat liver mitochondria: the role of oxidative stress and mitochondrial permeability transition, Molecules 19 (12) (2014) 21168–22182.
[25] A.A.M.M. Thoufic, N.S.D. Soorya, L.S. Sajitha, N. Sagnik, S. Vino, Targeting NF-κB pathway for the anti-inflammatory potential of Bhadradarvadi kashayam on stimulated RAW 264.7 macrophages, Heliyon 9 (8) (2023) e19270.
[26] A.B. Nair, S. Jacob, A simple practice guide for dose conversion between animals and human, J. Basic Clin. Pharm. 7 (2) (2016) 27–31.
[27] A. Inui, T. Yoshikawa, R. Nagai, N. Yoshida, T. Ito, Effects of mosapride citrate, a 5-HT4 receptor agonist, on colonic motility in conscious Guinea pigs, Jpn. J. Pharmacol. 90 (4) (2002) 313–320.
[28] Y. Liang, Y. Wang, P. Wen, Y. Chen, D. Ouyang, D. Wang, B. Zhang, J. Deng, Y. Chen, Y. Sun, H. Wang, The anti-constipation effects of raffino-oligosaccharide on gut function in mice using neurotransmitter analyses, 16S rRNA sequencing and targeted screening, Molecules 27 (7) (2022) 2235.
[29] H. Wu, Y. Chen, B. Huang, Y. Yu, S. Zhao, J. Liu, Z. Jia, H. Xiao, Aster tataricus alleviates constipation by antagonizing the binding of acetylcholine to muscarinic receptor and inhibiting Ca(2+) influx, Biomed. Pharmacother. 133 (2021) 111005.
[30] Y. Liu, L. Yang, C. Bi, K. Tang, B. Zhang, Nostoc sphaeroides kutz polysaccharide improved constipation and promoted intestinal motility in rats, evid. Based. Complement, Alternat. Med. 2021 (2021) 5596531.
[31] G.X. Cai, B.Y. Liu, J. Yi, X.M. Chen, F.L. Liu, Simotang enhances gastrointestinal motility, motilin and cholecystokinin expression in chronically stressed mice, World J. Gastroenterol. 17 (12) (2011) 1594–1599.
[32] B.J. Callahan, P.J. McMurdie, M.J. Rosen, A.W. Han, A.J. Johnson, S.P. Holmes, DADA2: high-resolution sample inference from Illumina amplicon data, Nat. Methods 13 (7) (2016) 581–583.
[33] K. Katoh, K. Misawa, K. Kuma, T. Miyata, MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform, Nucleic Acids Res. 30 (14) (2002) 3059–3066.
[34] M.N. Price, P.S. Dehal, A.P. Arkin, FastTree: computing large minimum evolution trees with profiles instead of a distance matrix, Mol. Biol. Evol. 26 (7) (2009) 1641–1650.
[35] Y. Huang, Y. Guo, X. Li, Y. Xiao, Z. Wang, L. Song, Z. Ren, Effects of lactiplantibacillus plantarum GUANKE on diphenoxylate-induced slow transit constipation and gut microbiota in mice, Nutrients 15 (17) (2023) 3741.
[36] W. Liu, A. Zhi, The potential of Quercetin to protect against loperamide-induced constipation in rats, Food, Sci. Nutr. 9 (6) (2021) 3297–3307.
[37] R. Jia, H.J. Xing, X.P. Zhang, X. Zhang, Y.Z. Du, L.J. Pan, J. Xu, C.S. Jia, [Effects of different acupuncture and moxibustion methods on plasma NO, NOS and VIP contents and the expression of colonic VIP protein in functional constipation rats], Zhen Ci Yan Jiu 42 (1) (2017) 50–55.
[38] T. Li, M. Hu, C. Jiang, D. Zhang, M. Gao, J. Xia, M. Miao, G. Shi, H. Li, J. Zhang, Z. Yin, Laxative effect and mechanism of Tiantian Capsule on loperamide-induced constipation in rats, J. Ethnopharmacol. 266 (2021) 113411.
[39] M. Iwasaki, Y. Akiba, J.D. Kaunitz, Recent advances in vasoactive intestinal peptide physiology and pathophysiology: focus on the gastrointestinal system, F1000Res 8 (2019) F1000.

[40] G. Nilanjan, C. Leena, B. Hiranmoy, V. Jayalakshmi, N. Sagnik, D. Abhijit, K. Tanushree, R.P. Hemachandra, K. Ramesh, D. Saikat, Exploring the complex relationship between diabetes and cardiovascular complications: understanding diabetic cardiomyopathy and promising therapies, Biomedicines 11 (4) (2023) 1126.
[41] K.M. Sanders, S.M. Ward, Nitric oxide and its role as a non-adrenergic, non-cholinergic inhibitory neurotransmitter in the gastrointestinal tract, Br. J. Pharmacol. 176 (2) (2019) 212–227.
[42] L.A. Van Geldre, R.A. Lefebvre, Interaction of NO and VIP in gastrointestinal smooth muscle relaxation, Curr. Pharm. Des. 10 (20) (2004) 2483–2497.
[43] E. Deloose, W. Verbeure, I. Depoortere, J. Tack, Motilin: from gastric motility stimulation to hunger signalling, Nat. Rev. Endocrinol. 15 (4) (2019) 238–250.
[44] G.L. Lyford, C.L. He, E. Soffer, T.L. Hull, S.A. Strong, A.J. Senagore, L.J. Burgart, T. Young-Fadok, J.H. Szurszewski, G. Farrugia, Pan-colonic decrease in interstitial cells of Cajal in patients with slow transit constipation, Gut 51 (4) (2002) 496–501.
[45] W. Tong, H. Jia, L. Zhang, C. Li, T.J. Ridolfi, B. Liu, Exogenous stem cell factor improves interstitial cells of Cajal restoration after blockade of c-kit signaling pathway, Scand. J. Gastroentero. 45 (7–8) (2010) 844–851.
[46] C. Silberstein, A. Kierbel, G. Amodeo, E. Zotta, F. Bigi, D. Berkowski, C. Ibarra, Functional characterization and localization of AQP3 in the human colon, Braz. J. Med. Biol. Res. 32 (10) (1999) 1303–1313.
[47] L. Cong, L.W. Duan, W.P. Su, S. Hao, D.F. Li, Efficacy of high specific volume polysaccharide - a new type of dietary fiber - on molecular mechanism of intestinal water metabolism in rats with constipation, Med. Sci. Monit. 25 (2019) 5028–5035.
[48] T. Matsuzaki, Y. Tajika, A. Ablimit, T. Aoki, H. Hagiwara, K. Takata, Aquaporins in the digestive system, Med. Electron. Microsc. 37 (2) (2004) 71–80.
[49] X.J. Wang, W.T. Yuan, J.M. Song, Z.Y. Zhang, [Expression and significance of aquaporin 4 in the colonic mucosa of patients with slow transit constipation], Zhonghua Wei Chang Wai Ke Za Zhi 13 (6) (2010) 445–447.
[50] H. Tsukaguchi, C. Shayakul, U.V. Berger, B. Mackenzie, S. Devidas, W.B. Guggino, A.N. van Hoek, M.A. Hediger, Molecular characterization of a broad selectivity neutral solute channel, J. Biol. Chem. 273 (38) (1998) 24737–24743.
[51] S.E. Kim, S.C. Choi, K.S. Park, M.I. Park, J.E. Shin, T.H. Lee, K.W. Jung, H.S. Koo, S.J. Myung, Change of fecal flora and effectiveness of the short-term VSL#3 probiotic treatment in patients with functional constipation, J. Neurogastroenterol. Motil. 21 (1) (2015) 111–120.
[52] I.L. Khalif, E.M. Quigley, E.A. Konovitch, I.D. Maximova, Alterations in the colonic flora and intestinal permeability and evidence of immune activation in chronic constipation, Dig. Liver, Dis 37 (11) (2005) 838–849.
[53] Y. Li, S. Long, Q. Liu, H. Ma, J. Li, W. Xiaoqing, J. Yuan, M. Li, B. Hou, Gut microbiota is involved in the alleviation of loperamide-induced constipation by honey supplementation in mice, Food Sci. Nutr. 8 (8) (2020) 4388–4398.
[54] H. Perumalsamy, S.R. Balusamy, J. Sukweenadhi, S. Nag, D. MubarakAli, M.E.A. Farh, H. Vijay, S. Rahimi, A comprehensive review on Moringa oleifera nanoparticles: importance of polyphenols in nanoparticle synthesis, nanoparticle efficacy and their applications, J. Nanobiotechnology 22 (1) (2024), 71-71.
[55] Z. Zhu, L. Zhu, L. Jiang, Dynamic regulation of gut Clostridium-derived short-chain fatty acids, Trends, Biotechnol. 40 (3) (2022) 266–270.
[56] N. Jiao, S.S. Baker, C.A. Nugent, M. Tsompana, L. Cai, Y. Wang, M.J. Buck, R.J. Genco, R.D. Baker, R. Zhu, L. Zhu, Gut microbiome may contribute to insulin resistance and systemic inflammation in obese rodents: a meta-analysis, Physiol. Genomics. 50 (4) (2018) 244–254.
[57] N.T. Baxter, A.W. Schmidt, A. Venkataraman, K.S. Kim, C. Waldron, T.M. Schmidt, Dynamics of human gut microbiota and short-chain fatty acids in response to dietary interventions with three fermentable fibers, mBio 10 (1) (2019).
[58] P.D. Kusumo, H. Maulahela, A.P. Utari, I.S. Surono, A. Soebandrio, M. Abdullah, Probiotic Lactobacillus plantarum IS 10506 supplementation increase SCFA of women with functional constipation, Iran, J. Microbiol. 11 (5) (2019) 389–396.
[59] C. Martin-Gallausiaux, L. Marinelli, H.M. Blottiere, P. Larraufie, N. Lapaque, SCFA: mechanisms and functional importance in the gut, Proc. Nutr. Soc. 80 (1) (2021) 37–49.
[60] H. Fukui, X. Xu, H. Miwa, Role of gut microbiota-gut hormone Axis in the pathophysiology of functional gastrointestinal disorders, J. Neurogastroenterol. Motil. 24 (3) (2018) 367–386.
[61] R. Soret, J. Chevalier, P. De Coppet, G. Poupeau, P. Derkinderen, J.P. Segain, M. Neunlist, Short-chain fatty acids regulate the enteric neurons and control gastrointestinal motility in rats, Gastroenterology 138 (5) (2010) 1772–1782.
[62] S.C. Chang, M.H. Shen, C.Y. Liu, C.M. Pu, J.M. Hu, C.J. Huang, A gut butyrate-producing bacterium Butyricicoccus pullicaecorum regulates short-chain fatty acid transporter and receptor to reduce the progression of 1,2-dimethylhydrazine-associated colorectal cancer, Oncol. Lett. 20 (6) (2020) 327.
[63] J. Yang, Y. Li, Z. Wen, W. Liu, L. Meng, H. Huang, Oscillospira - a candidate for the next-generation probiotics, Gut Microb. 13 (1) (2021) 1987783.