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Classical famous prescription of Jichuan decoction improved loperamide-induced slow transit constipation in rats through the cAMP/PKA/AQPs signaling pathway and maintained inflammatory/intestinal flora homeostasis

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ABSTRACT

Introduction: Jichuan decoction (JCD) is a well-known traditional Chinese medicinal formula that moistens the intestines and is widely used for the treatment of constipation in China. However, its effects and mechanisms in alleviating slow transit constipation (STC) in vivo remain unclear. We attempted to demonstrate the effect of JCD, with and without essential oil (VO), on intestinal transit and its underlying molecular mechanisms in rats with loperamide-induced STC.

Materials and methods: Water consumption, body weight, fecal water content, time to first melena excretion, and intestinal transit ratio of the animals were measured. 5-Hydroxytryptamine (5-HT), substance P (SP), vasoactive intestinal peptide (VIP), and interleukin-6 (IL-6) levels in the sera of rats were evaluated using ELISA. Hematoxylin and eosin and Periodic Acid–Schiff staining were used to determine intestinal tissue histology, while quantitative real-time PCR, western blotting, and immunohistochemical analysis were used to assess the relative expression levels of cAMP/PKA/AQPs pathway- and inflammation-related proteins. 16 S rDNA sequence analysis of rat feces was used to determine the diversity and abundance of the intestinal flora.

Results: The JCD groups showed reduced time to first melena excretion and expression of VIP and IL-6. The JCD groups, specifically JCD + VO groups, showed increased fecal water content, intestinal transit rate, and SP expression. Further, these groups showed improved histological characteristics of the colon, with no significant change in the index of immune organs or

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morphological characteristics of other organs. In addition, a significant decrease in the activation of the cAMP/PKA/AQPs signaling pathway in the colon tissue was observed in these groups, specifically the JCD + VO groups. Moreover, treatment with JCD, with or without VO, down-regulated the expression of inflammatory factors and enriched the diversity of intestinal flora as evidenced by polymorphism analysis and the contents of *Bacteroides*, *Lactobacillus*, and *Erysipelas*, with the JCD + VO groups showing better therapeutic outcomes.
Conclusion: JCD improved loperamide-induced STC, and co-administration with VO exhibited better activity than sole JCD therapy. JCD may improve STC by inhibiting the cAMP/PKA/AQPs signaling pathway and maintaining inflammatory/intestinal flora homeostasis.

Abbreviations

Adenylate cyclases ACs
Aquaporins AQPs
B-cell lymphoma-extra-large Bcl-xl
Cyclooxygenase-2 COX-2
Cyclic adenosine monophosphate cAMP
Gas chromatography GC
High-performance liquid chromatography HPLC
Hematoxylin and eosin HE
5-Hydroxytryptamine 5-HT
Inducible nitric oxide synthase iNOS
Interleukin-6 IL-6
Jichuan decoction JCD
Operational taxonomic units OTUs
Periodic acid–Schiff PAS
Protein kinase A PKA
Vasoactive intestinal peptide VIP
Volatile oil VO
Substance P SP
Slow transit constipation STC
Sprague Dawley SD
Standard deviation S.D
Traditional Chinese medicine TCM

1. Introduction

Constipation is a common and complex clinical symptom. With changes in dietary structures and the influence of psychological and social factors, the incidence of constipation tends to increase [1]. It affects many professionals and elderly people, with a serious impact on their quality of life [2]. People with constipation are prone to intestinal flora disorders, and long-term constipation is closely related to acute cardiovascular and cerebrovascular diseases, dementia, and colorectal cancer [3]. Slow transit constipation (STC) is a relatively common disease with many causes and poor treatment outcomes [4]. Its pathogenesis remains unclear, and has become a challenge for clinical treatment [5].

Normally, the colon absorbs water and electrolytes, forms feces, and secretes an appropriate amount of mucus to lubricate the intestines, thereby facilitating rapid excretion of feces [6]. However, if the colon absorbs too much water from the stool, it can cause the stool to dry and harden, leading to constipation [7]. Aquaporins (AQPs), mainly distributed in digestive system tissues, are involved in water metabolism and balance [8]. Of the 13 AQPs identified in mammals, studies have found that AQP3 and AQP4 are closely related to the transport of water in the colon tissue [9]. G protein induces and activates adenylate cyclase, which increases the content of cAMP and induces the activation of protein kinase A (PKA), thereby regulating the expression level of AQPs and increasing water reabsorption [10,11].

The gut is a complex network of bacteria, fungi, and viruses, with bacteria constituting the largest proportion [12]. In healthy individuals, *Firmicutes* and *Bacteroidetes* are the dominant flora in the gut, accounting for approximately 90 % of the gut microbial system, including *Bacteroides*, *Bifidobacterium*, and *Peptostreptococcus* [13]. Oxygen-consuming bacteria, such as *Enterococcus* and *Streptococcus* are less common [14]. The intestinal flora absorbs nutrients by decomposing food; participates in immunity, metabolism, and other reactions; and maintains homeostasis in the host. However, when the intestinal flora is unbalanced, it leads to the occurrence of various diseases, including tumor formation [15]. Inflammatory factors play an important role in regulating body functions and

maintaining homeostasis. Interleukin-6 (IL-6) can induce the production of anti-apoptotic factors, B-cell lymphoma-extra-large (Bcl-xl), and Bcl-2, leading to abnormal accumulation of T cells in the intestinal mucosa, further aggravating the inflammatory response [16]. IL-17 is an inflammatory factor that is secreted by Th17 cells. When the intestinal mucosa is damaged, the expression of IL-17 and IL-22 is significantly upregulated, protecting the intestinal mucosa by regulating the interactions between intestinal bacteria and immune cells [17]. The intestinal flora secretes a variety of enzymes to prevent the invasion of pathogenic bacteria and ensure normal functioning of the immune system [18]. However, when the intestinal flora is unbalanced, it often leads to a reduction in beneficial bacteria and the proliferation of pathogenic bacteria, resulting in an increase in the secretion of intestinal G bacteria, such as endotoxins, which activate the inflammatory cascade in the body, leading to increased secretion of inflammatory factors and triggering an inflammatory response [15]. Therefore, the interconnection between the type of intestinal flora and the secretion of inflammatory factors has become a research hotspot.

Studies have shown that therapeutic outcomes of STC are unsatisfactory in clinical practice owing to various problems such as inadequate drug efficacy, weak specificity, adverse reactions, and limited treatment range [19]. With the use of chemical drugs, which generally involve symptomatic treatment, constipation symptoms often recur after drug withdrawal; therefore, some patients must undergo colectomy. Traditional Chinese medicine (TCM) is characterized by mildness, fewer side effects, and multiple targets, highlighting its unique advantages in the treatment of constipation [20,21]. In TCM, STC is attributed to a deficiency in kidney-yang and Jing-Jin, and treatment should focus on warming the kidney and nourishing the essence, as well as moistening and relaxing the intestines. Jichuan decoction (JCD) is a TCM prescription recorded in *Jingyue Quanshu*, written by Zhang Jingyue during the Ming Dynasty. It is composed of six types of TCMs: *Angelica sinensis* (Oliv.) Diels, *Achyranthes bidentata* Bl., *Cistanche deserticola* Y. C. Ma, *Alisma orientalis* (Sam.) Juzep., *Cimicifuga foetida* L., and *Citrus aurantium* L. JCD has been shown to have a good therapeutic effect on constipation and other diseases in long-term clinical practice [22,23]; however, its mechanism of action remains unclear and needs to be further explored. In this study, we established a loperamide-induced STC rat model to explore the effects and mechanisms of action of JCD on STC. The Application Data Requirements for Ancient Classical Chinese Medicine Preparation and their Material Standards (Draft), compiled by the Chinese State Food and Drug Administration, recommends that research on classical famous prescriptions requires the preparation of a reference sample (in the form of a soup, extract, or freeze-dried powder), which is then made into granules through extraction, concentration, drying, granulation, and other processes. During these processes, the reference sample contains volatile components; however, the final granules are condensed under a negative-pressure environment, thus removing the volatile components. Therefore, we evaluated the efficacy of different JCD dosages with and without volatile oils. This study provides a basis for further clinical experiments on managing SCT.

2. Materials and methods

2.1. Chemicals and reagents

The Chinese herbs used in JCD are listed in Table 1. Qirong Runchang oral liquid was purchased from Beijing Huarun High-Tech Natural Medicine Co., Ltd. (Beijing, China). Interleukin-6 (IL-6) ELISA kits were acquired from Wuhan ABclonal Biotechnology Co., Ltd. (Hubei, China). 5-Hydroxytryptamine (5-HT), substance P (SP), and vasoactive intestinal peptide (VIP) ELISA kits were acquired from Shanghai Jianglai Biotechnology Co., Ltd. (Shanghai, China). TRIzol™ reagent was obtained from Sangon Biotech (Shanghai, China). HiScript 1st Strand cDNA Synthesis Kit and ChamQ Universal SYBR qPCR Master Mix were obtained from Nanjing Vazyme Biotechnology Co., Ltd. (Jiangsu, China). Tissue lysates were obtained from Solar Bio Science and Technology Co., Ltd. (Beijing, China). iNOS and GAPDH antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). The cyclooxygenase-2 (COX-2) antibody was obtained from Beyotime Institute of Biotechnology (Shanghai, China). Antibodies against AQP3, AQP4, and PKA were purchased from Wuhan ABclonal Biotechnology Co. Ltd. (Hubei, China).

Table 1
Components of Jichuan decoction.

Chinese name	Botanical name	Part used	Weight (g)	Place of origin	Batch number	Manufacturer
Danggui	*Angelica sinensis* (Oliv.) Diels	Roots	14.92	Dingxi, Ganasu	210,511	Bozhou Yonggang Decoction Pieces Co., Ltd.
Niuxi	*Achyranthes bidentata* Bl.	Roots	7.46	Jiaozuo, Henan	210,519	Bozhou Yonggang Decoction Pieces Co., Ltd.
Roucongrong	*Cistanche deserticola* Y. C. Ma	stems	9.33	Alxa League, Inner Mongolia	210,404	Bozhou Yonggang Decoction Pieces Co., Ltd.
Zexie	*Alisma orientalis* (Sam.) Juzep.	stems	5.60	Jiangxi	K202107001	Guangdong Hexiang Pharmaceutical Co., Ltd.
Shengma	*Cimicifuga foetida* L.	stems	2.64	Dongbei	HX21K01	Guangdong Hexiang Pharmaceutical Co., Ltd.
zhiqiao	*Citrus aurantium* L.	fruits	3.73	Yiyang, Hunan	210,225	Bozhou Yonggang Decoction Pieces Co., Ltd.

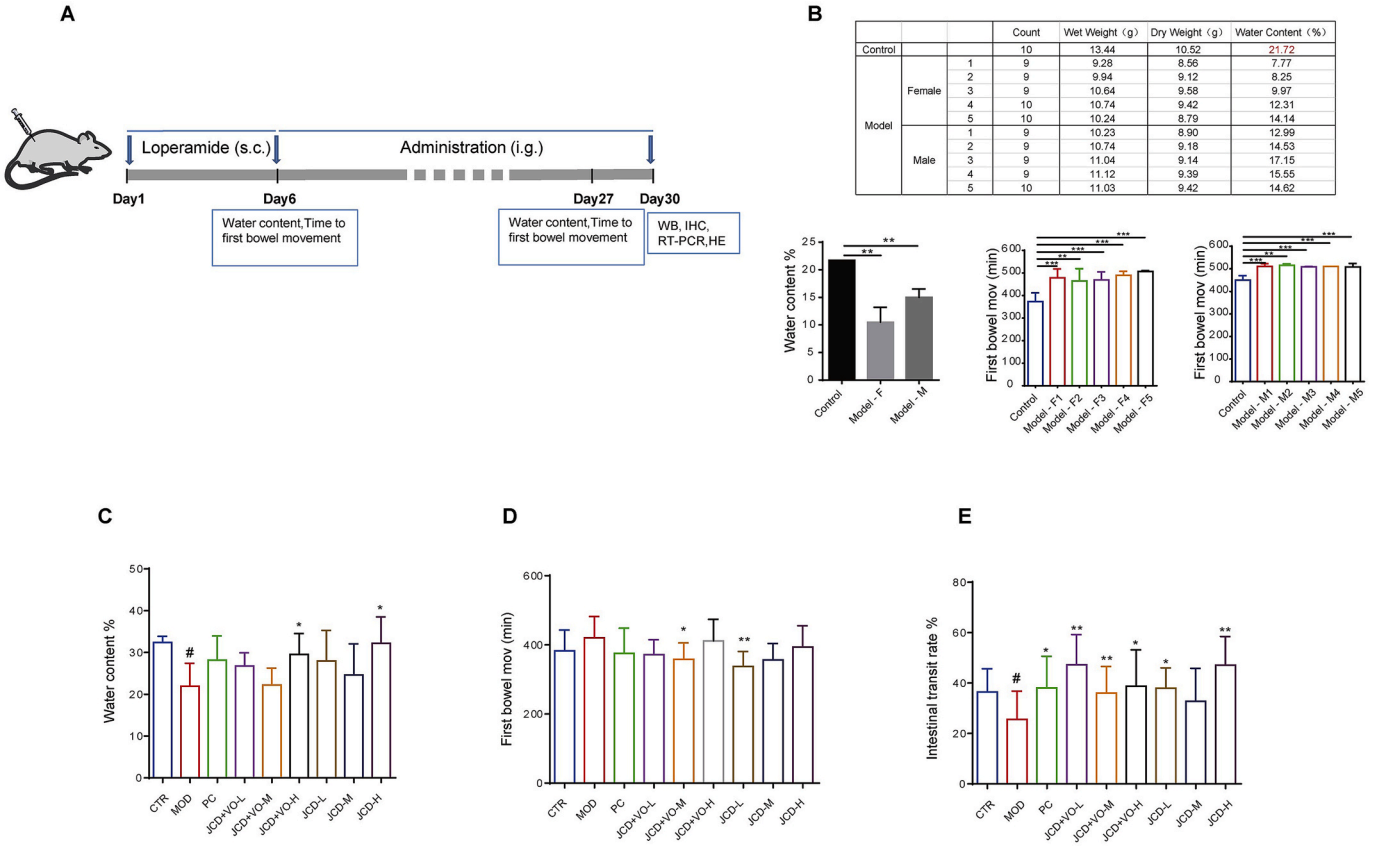

Fig. 1. Effects of JCD on fecal excretion and intestinal transit time in rats with loperamide-induced STC. (A) Schematic illustration of the experimental design; (B) On the 6th day of modeling, the water content of feces and the time to first melena excretion were determined in each group of rats; (C) Fecal water content testing; (D) The first melena excretion time experiment; (E) Measurement of intestinal transit ratio. CTR, control group; MOD, model group (constipation group); PC, rats treated with Qirong Runchang oral liquid (4.8 mL/kg/d); (JCD + VO)-L, rats treated with low-dose JCD + VO (1.8 g/kg/d); (JCD + VO)-M, rats treated with medium-dose JCD + VO (3.6 g/kg/d); (JCD + VO)–H, rats treated with high-dose JCD + VO (7.2 g/kg/d); JCD-L, rats treated with low-dose JCD (1.8 g/kg/d); JCD-M, rats treated with medium-dose JCD (3.6 g/kg/d); JCD-H, rats treated with high-dose JCD (7.2 g/kg/d). Results are expressed as mean ± SD. $^{\#}p < 0.05$ compared with the control group, $^{*}p < 0.05$ compared with the model group, $^{**}p < 0.01$ compared with the model group, $^{***}p < 0.001$ compared with the model group.

2.2. Preparation of JCD

To investigate the effect of volatile oil (VO) in JCD, JCD with and without VO (JCD + VO and JCD, respectively) were prepared. After soaking for 30 min, the six Chinese herbs from JCD were boiled at 100 °C for 24 min in 300 mL of water. When 150 mL of the decocted liquid remained, it was filtered through a double layer of medical gauze, and the filtrate was freeze-dried at −80 °C to obtain JCD + VO. Simultaneously, JCD was refluxed for 1 h with six times the amount of water after soaking for 30 min. The filtrate was then concentrated at 60 °C to an extract with a relative density of 1.20–1.25, filtered, and dried. The chemical differences between JCD and JCD + VO were assessed using a Waters Alliance e2695 HPLC system with a 2998 PDA detector (Waters, Milford, USA) and SHIMADZU GC-2010 plus (FID) (Shimadzu Co., Kyoto, Japan).

High-performance liquid chromatography (HPLC): chromatographic separation was performed using a Waters XSelect HHS T3 C_{18} column (column number: 25–438; 250 mm × 4.6 mm; 5 μm; flow rate: 1.2 mL/min; column temperature: 35 °C; wavelength: 210 nm; sample intake: 10 μL). The aqueous phase was a mixture of acetonitrile (A) and water (B; 0.1 % phosphoric acid), and the gradient elution procedure was set as follows: 0–5 min, 1–5% A; 5–15 min, 5–12 % A; 15–35 min, 12–14 % A; 35–45 min, 14–15 % A; 45–55 min, 15–18 % A; 55–70 min, 18–22 % A; 70–75 min, 22–35 % A; 75–85 min, 35–66 % A; 85–90 min, 66–66 % A; and 90–95 min, 66–1% A.

Gas chromatography (GC): chromatographic conditions: Agilent HP-5 capillary column (30 m × 0.32 mm × 0.25 μm); sample inlet temperature: 220 °C; column chamber temperature: initial temperature 60 °C; hold for 5 min, 10 °C/min to 220 °C, hold for 5 min; carrier gas: nitrogen; flow rate = 1.5 mL/min; split ratio = 30:1.

The HPLC and GC chromatograms of JCD and JCD + VO are shown in Fig. S1.

2.3. Animals and experimental groups

Male and female Sprague Dawley (SD) rats (100 ± 10.7 g, license number: SYXK (Yue) 20,200,230 and 20,200,051) were purchased from Zhuhai Beston Biotechnology Co., Ltd. (Guangdong, China). The feed was processed at the Guangdong Provincial Medical Laboratory Animal Center. All animals were kept at the SPF animal center (12 h daylight cycle, 18–22 °C). The rats were provided with free access to their daily diet and drinking water. After acclimation for 1 week, all rats in the control group were injected with saline, whereas the other groups were injected subcutaneously with loperamide (1.5 mg/kg) in saline twice a day at 09:00 and 18:00 on days 1–6 of the experiment (Fig. 1A). The rats were divided into model, positive (Qirong Runchang oral liquid), JCD + VO low-dose, JCD + VO medium-dose, JCD + VO high-dose, JCD low-dose, JCD medium-dose, and JCD high-dose groups. The drug groups were treated with 1.8, 3.6, and 7.2 g/kg JCD, with or without VO in saline, once daily by gavage from days 7–30, as described previously. The positive control group was treated with 4.8 mL/kg Qirong Runchang oral liquid. The rats in the control and model groups were administered the same amount of normal saline by gavage. At the end of the treatment, abdominal aortic blood was collected and the rats were sacrificed. After sacrifice, the liver tissues of the animals were harvested and weighed, and liver indices were calculated (liver index = liver weight/body weight × 100).

All animal experiments were approved by the Institutional Animal Care and Use Committee of Shenzhen People's Hospital (approval document no. LL-KT-2021797).

2.4. Feeding conditions and body weight

The water consumption of the animals was measured during the experiment. In addition, body weight was measured using an automatic electronic balance.

2.5. Fecal water content testing

After the final administration of treatment, fresh feces excreted by the rats in each group were collected and weighed and then placed in an oven set at 150 °C for 15 min for drying and weighing. The ratio of dry to wet weight of feces was calculated as follows: mass after baking/mass before baking × 100 %.

2.6. Determination of the first melena excretion time

After the last treatment gavage, the rats were fasted for 12 h, and 3 mL of activated carbon suspension (100 g/L) was administered to each animal. The time taken to excrete the first melena was recorded.

2.7. Measurement of intestinal transit ratio

After the final administration of treatment, all rats were fasted for 24 h, but allowed free access to water. Each rat was infused with 1 mL charcoal powder. After 30 min, all rats were sacrificed and the whole intestine from the rectum to the pylorus was removed. Subsequently, the full length of the intestine and propelling distance of the charcoal powder were measured. The intestinal transport rate (%) was calculated using the following formula: intestinal transit rate (%) = propelling distance of the charcoal powder/length of the intestine × 100 %.

2.8. Detection of 5-HT, SP, VIP, and IL-6 in rat serum by ELISA

After the final administration of treatment, 1 mL of 10 % sodium pentobarbital was intraperitoneally injected into each rat. B lood was collected from the abdominal aorta and serum was isolated. The levels of 5-HT, SP, VIP, and IL-6 in sera of the rats were determined according to the instructions of the corresponding ELISA kit.

2.9. Quantitative real-time PCR

Total RNA was extracted using TRIzol™ reagent according to the manufacturer's protocol. The HiScript 1st Strand cDNA Synthesis Kit was used to synthesize cDNA via reverse transcription. Quantitative real-time PCR was performed using the ChamQ Universal SYBR qPCR Master Mix. GAPDH was used as a reference gene, and the fold changes of gene expression were calculated by relative quantification ($2^{-\Delta\Delta Ct}$) [24]. The primers used for qPCR were follows: PKA forward 5′-GAGCAGGAGAGCGTGAAAGA-3′, reverse 5′-CGGTGCCAAGGGTCTTGATT-3'; AQP3 forward 5′-CACCCCATAAAAGCC CCCTT-3′, reverse 5′-TGCTGAGTCCAAAGCAGAGG-3'; IL-6 forward 5′-AGAGACTTCCAGCCAGTTGC-3′, reverse 5′-AGTCTCCTCTCCGGACT TGT-3'; IL-1β forward 5′-GCACAGTTCCCCAACTGGTA-3′, reverse 5′-ACACGGGTTCCATGGTGAAG-3'; and GAPDH forward 5′-AGACAGCCGC ATCTTCTTGT-3′, reverse 5′-TACGGCCAAATCCGTTCACA-3'.

2.10. Western blot analysis

After treatment, cells were lysed on ice for 30 min in RIPA buffer containing protease and phosphatase inhibitors. Nuclear and cytosolic extracts were prepared using NEPER™ nuclear and cytoplasmic extraction reagents, according to the manufacturer's instructions. Equivalent amounts of protein were separated on 8%–12 % sodium dodecyl sulfate-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. After blocking with 5 % nonfat milk for 1 h, the membranes were probed with primary antibodies and then with peroxidase-conjugated secondary antibodies. Target proteins were visualized using an immunoblotting chemiluminescence reagent [25].

2.11. Hematoxylin and eosin and Periodic Acid–Schiff staining

Tissues were fixed in neutral formalin for 24 h, dehydrated, embedded in paraffin, cut into 4 mm slices, and stained with hematoxylin and eosin (HE) and Periodic Acid–Schiff (PAS).

2.12. Immunohistochemical analysis

Tissue samples were embedded in paraffin and sectioned into 4 mm slides for immunohistochemical analysis. This was performed using a ready-to-use UltraSensitiveTM *S*–P immunohistochemistry hypersensitivity kit (Maxim Integrated, San Jose, CA, USA) following the manufacturer's protocols. Briefly, the tissue sections were deparaffinized, rehydrated, and heat-fixed with an antigen retrieval solution. The sections were subsequently incubated with primary antibodies (AQP3 antibody [1:100], AQP4 antibody [1:100], PKA antibody [1:100], and iNOS antibody [1:400]), followed by incubation with horseradish peroxidase–labeled secondary antibody. Diaminobenzidine was used for colorization [26].

2.13. 16 S rDNA sequencing of rat feces

The colonic feces of rats in each group were collected and stored in a refrigerator at −80 °C. 16 S rDNA detection of the samples was performed by Shenzhen Huada Gene Co., Ltd. (Shenzhen, Guangdong province, China). We used 30 ng of qualified genomic DNA samples and the corresponding fusion primers to configure the PCR system, set the corresponding PCR parameters for PCR amplification, purified the PCR amplification products, and constructed a library. The fragment range and concentration of the library were determined using an Agilent 2100 Bioanalyzer. The qualified libraries were sequenced on a suitable platform (HiSeq/MiSeq) according to the size of the insert. The off-machine data were filtered to discount low-quality reads, and the remaining high-quality clean data were used for subsequent analysis. Reads were spliced into tags through the overlap relationship between reads, tags were aggregated into given similarity operational taxonomic units (OTUs), and then the OTUs were compared with the database to perform species annotation. Based on the OTUs and species annotation results, sample species complexity analysis and difference analysis between the groups were performed.

2.14. Statistical analysis

All data are presented as mean ± standard deviation (S.D.). Differences between groups were compared using the Student's t-test or one-way analysis of variance (ANOVA) using GraphPad Prism 7. Statistical significance was set at $p < 0.05$.

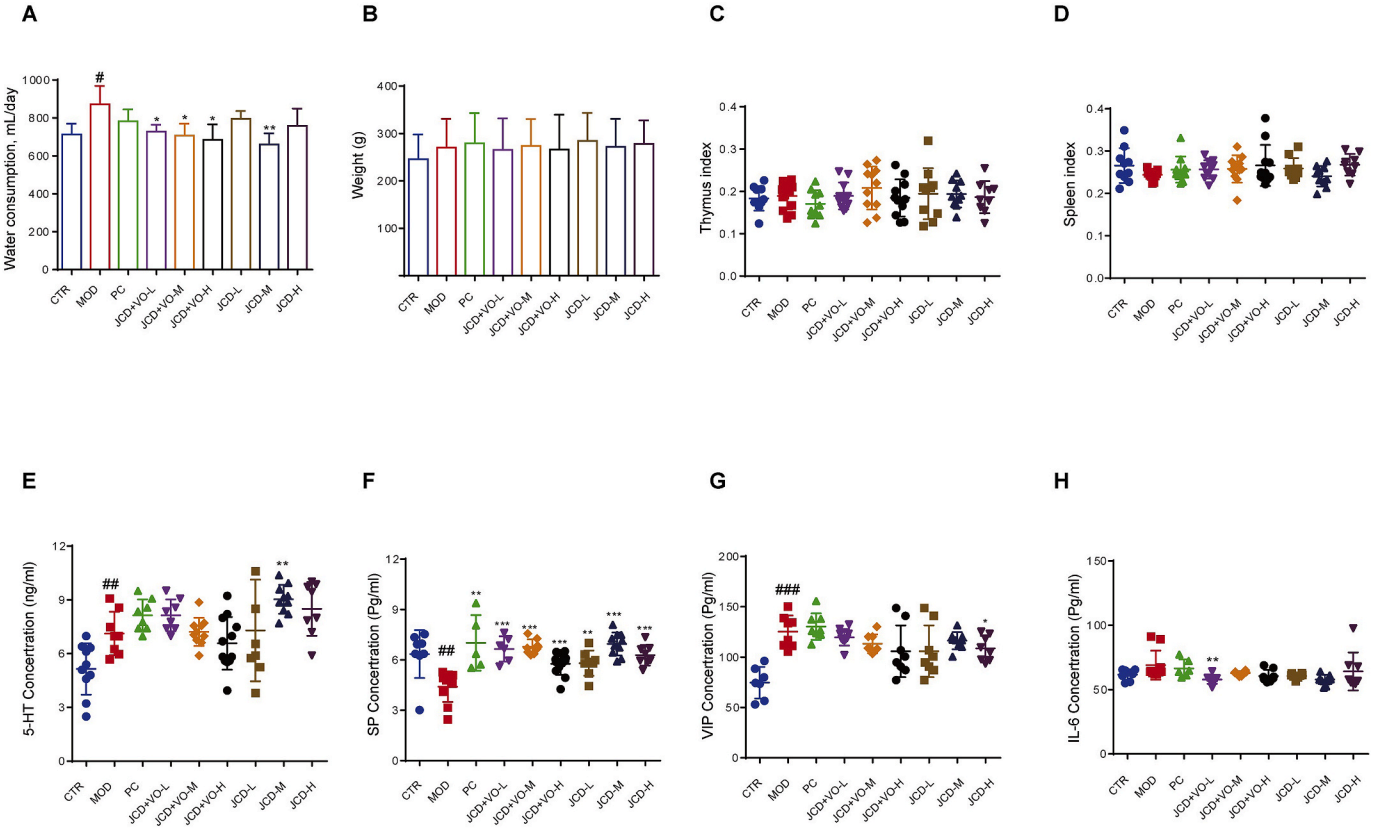

Fig. 2. Effects of JCD on immunity, nerves, and inflammation in rats with loperamide-induced STC. (A) Water consumption was measured immediately using a measuring cylinder. (B) Body weights were measured using an electric balance. (C–D) Thymus and spleen indexes were calculated as the ratio of the thymus or spleen weight to the body weight. (E) 5-HT, (F) SP, (G) VIP, and (H) IL-6 levels in sera of all rats were measured using ELISA. CTR, the control group; MOD, the model group; PC, rats treated with Qirong Runchang oral liquid (4.8 mL/kg/d); (JCD + VO)-L, rats treated with low-dose JCD + VO (1.8 g/kg/d); (JCD + VO)-M, rats treated with medium-dose JCD + VO (3.6 g/kg/d); (JCD + VO)–H, rats treated with high-dose JCD + VO (7.2 g/kg/d); JCD-L, rats treated with low-dose JCD (1.8 g/kg/d); JCD-M, rats treated with medium-dose JCD (3.6 g/kg/d); JCD-H, rats treated with high-dose JCD (7.2 g/kg/d). Results are expressed as mean ± SD. $^{\#}p < 0.05$ compared with the control group, $^{\#\#}p < 0.01$ compared with the control group, $^{\#\#\#}p < 0.001$ compared with the control group, $^{*}p < 0.05$ compared with model group, $^{**}p < 0.01$ compared with the model group, $^{***}p < 0.001$ compared with the model group.

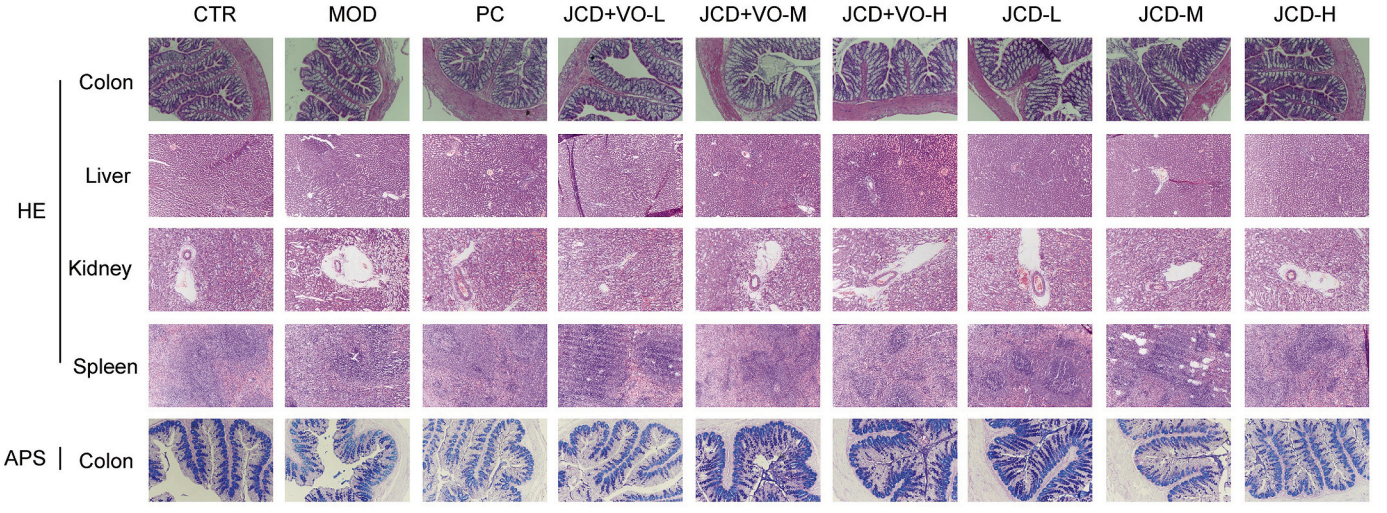

Fig. 3. Effects of JCD on the histological characteristics of rats with loperamide-induced STC. HE staining results of the colon, liver, kidney, spleen, and APS staining results of the colon of all rats. CTR, control group; MOD, model group (constipation group); PC, rats treated with Qirong Runchang oral liquid (4.8 mL/kg/d); (JCD + VO)-L, rats treated with low-dose JCD + VO (1.8 g/kg/d); (JCD + VO)-M, rats treated with medium-dose JCD + VO (3.6 g/kg/d); (JCD + VO)–H, rats treated with high-dose JCD + VO (7.2 g/kg/d); JCD-L, rats treated with low-dose JCD (1.8 g/kg/d); JCD-M, rats treated with medium-dose JCD (3.6 g/kg/d); JCD-H, rats treated with high-dose JCD (7.2 g/kg/d).

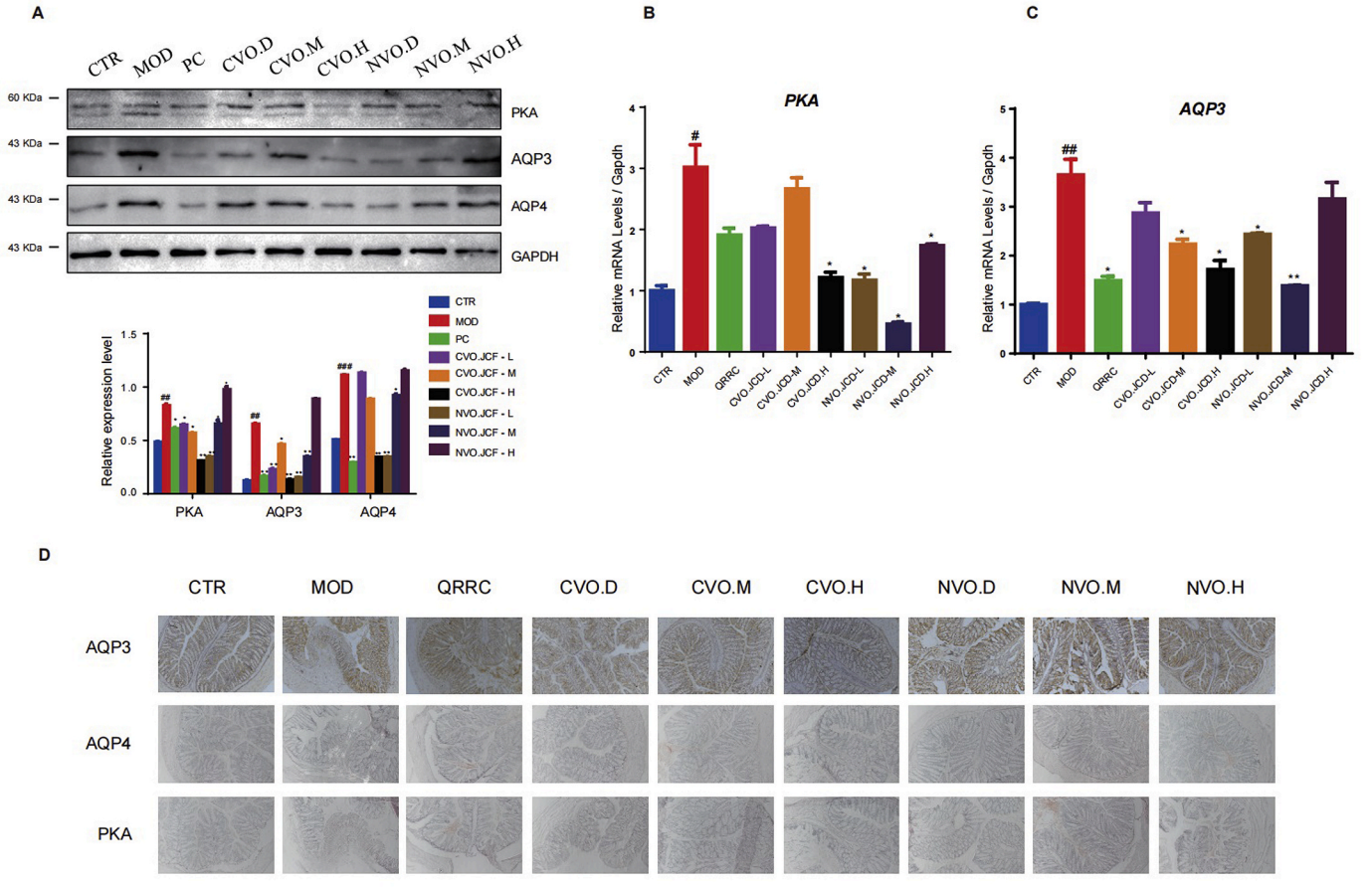

Fig. 4. Effects of JCD on the cAMP/PKA/AQPs pathway in rats with loperamide-induced STC. (A) The protein expressions of AQP3, AQP4, and PKA in the colon were detected by western blotting. The mRNA levels of PKA (B) and AQP3 (C) in the colon were detected by qRT-PCR. (D) Immunohistochemical analysis of AQP3, AQP4, and PKA. CTR, the control group; MOD, the model group; PC, rats treated with Qirong Runchang oral liquid (4.8 mL/kg/d); (JCD + VO)-L, rats treated with low-dose JCD + VO (1.8 g/kg/d); (JCD + VO)-M, rats treated with medium-dose JCD + VO (3.6 g/kg/d); (JCD + VO)–H, rats treated with high-dose JCD + VO (7.2 g/kg/d); JCD-L, rats treated with low-dose JCD (1.8 g/kg/d); JCD-M, rats treated with medium-dose JCD (3.6 g/kg/d); JCD-H, rats treated with high-dose JCD (7.2 g/kg/d). Results are expressed as mean ± SD. $^{\#}p < 0.05$ compared with the control group, $^{\#\#}p < 0.01$ compared with the control group, $^{\#\#\#}p < 0.001$ compared with the control group, $^{*}p < 0.05$ compared with model group, and $^{**}p < 0.01$ compared with the model group.

3. Results

3.1. Effects of JCD on fecal excretion and intestinal transit rate in rats with loperamide-induced STC

To explore the specific mechanism of action of different JCD preparations, with or without VO, against STC, a rat model of STC induced by loperamide was established. The specific model development process is shown in Fig. 1A. Fecal water content in the model group was lower than that in the control group, and the first melena was excreted later than in the control group (Fig. 1B). These results indicate that the model was successfully established. Next, the effect of JCD on STC was studied. Compared with the control group, the water content in rat feces was significantly reduced when loperamide was administered (Fig. 1C), and the water content was markedly improved in the other drug-treated groups, especially in the (JCD + VO)–H and JCD-H groups ($p < 0.01$) (Fig. 1C). In addition, defecation time increased in the model group (Fig. 1D). Compared with the model group, the drug-treated groups showed a reduction in the time to the first melena excretion, especially the (JCD + VO)-M and JCD-L groups, with the effect of the JCD-L group being better than that of the other groups (Fig. 1D). Furthermore, the intestinal transit rate reduced by loperamide was significantly increased in different groups, with (JCD + VO)-L and JCD-H showing the best activity (Fig. 1E). Overall, JCD with and without VO alleviated loperamide-induced STC in rats, while both treatments showed little difference in improvement on loperamide-induced STC.

3.2. Effects of JCD on immunity, nerves, and inflammation in rats with loperamide-induced STC

The effect of JCD on water intake was investigated in rats with loperamide-induced STC. Compared with the control group, water intake was significantly increased in the model group and relatively reduced in the drug-treated groups, especially in (JCD + VO)-L, -M, –H, and JCD-M (Fig. 2A). The thymus and spleen are the main immune organs crucial for maintaining normal body function [27]. The experimental results showed that there were no significant differences in body weight, thymus index, or spleen index among the different groups (Fig. 2B–D), indicating that loperamide, Qirong Runchang oral liquid (the positive control), and JCD had no obvious toxic effects. As an inhibitory neurotransmitter, 5-HT may be involved in the regulation of physiological functions, such as nociception, sleep, and body temperature. SP is a neuropeptide that is widely distributed in nerve fibers and is the main neurotransmitter used by the body to transmit pain. SP distributed in the gastrointestinal tract can directly act on intestinal smooth muscles, promote peristalsis, and accelerate the excretion of intestinal contents. VIP is an inhibitory neurotransmitter that dilates the gastrointestinal blood vessels, relaxes smooth muscles, and reduces visceral resistance [28–30]. Herein, the content of 5-HT in the model group was higher than that in the control group. However, compared with the model group, the drug-treated groups showed no significant reduction in 5-HT, with the JCD-M group exhibiting an increased expression of 5-HT (Fig. 2E). The SP content in the model group was lower than that in the normal group, and all the drug-treated groups showed an increase in SP compared with the model group, especially the (JCD + VO)-L group (Fig. 2F). Owing to the antagonistic relationship between VIP and SP, the VIP content showed a roughly opposite tendency to that of SP in the different groups; however, compared to the model group, the JCD-H group showed a significant decrease in VIP concentration (Fig. 2G). In addition, the secretion of the inflammatory factor IL-6 in the sera of the animals was measured. The results showed that the release of IL-6 was increased in the model group, the secretion of IL-6 in the other groups showed a downward trend, and (JCD + VO)-L treatment significantly downregulated IL-6 secretion compared to the model group (Fig. 2H). These data suggest that JCD could improve the symptoms of STC induced by loperamide in rats.

3.3. Effects of JCD on the histological characteristics of rats with loperamide-induced STC

The colon, liver, spleen, and kidneys of each animal in all groups were collected and embedded in paraffin. HE staining was performed to investigate the effect of JCD on the histological features of rats with STC. As depicted in the colon tissue, the muscle layer thickness and number of goblet cells in the model group were significantly lower than those in the control group (Fig. 3). Moreover, these histological features improved after treatment with the positive control drug or different concentrations of JCD (Fig. 3). However, there were no significant differences in the morphological characteristics of other organs (liver, spleen, and kidney) among the different groups (Fig. 3).

3.4. Effects of JCD on the cAMP/PKA pathway in rats with loperamide-induced STC

Aquaporins (AQPs) play an important role in water transport, and AQP3 and AQP4 are particularly important for water transport in the colon. Activation of the cAMP/PKA signaling pathway can induce the expression of AQPs, and abnormal expression of AQPs in the intestine can lead to disturbances in the absorption and secretion of water in the colon, causing constipation [10,11]. To explore the specific mechanism by which JCD improves the symptoms of loperamide-induced STC in rats, proteins related to the cAMP/PKA signaling pathway were measured in all groups. As shown in Fig. 4A–C, compared to the control group, the protein levels of PKA, AQP3, and AQP4, as well as the mRNA levels of PKA and AQP3, in the colon tissue of the model group were significantly increased. This suggests that the cAMP/PKA signaling pathway was activated by loperamide. AQP3 and AQP4 can enhance the body's reabsorption of water, leading to metabolic disorders in the colon tissue, resulting in constipation. In this study, the increased protein and mRNA levels of PKA, AQP3, and AQP4 in the colon tissue were significantly inhibited after treatment with the positive control drug and JCD, with or without volatile oil (Fig. 4A–C). Similar results were observed using immunohistochemistry (Fig. 4D). All the above data indicate that JCD may regulate the expression of AQP3 and AQP4 through the cAMP/PKA signaling pathway, thereby correcting intestinal metabolic disorders and playing a therapeutic role in functional STC. However, JCD with or without volatile oil had little

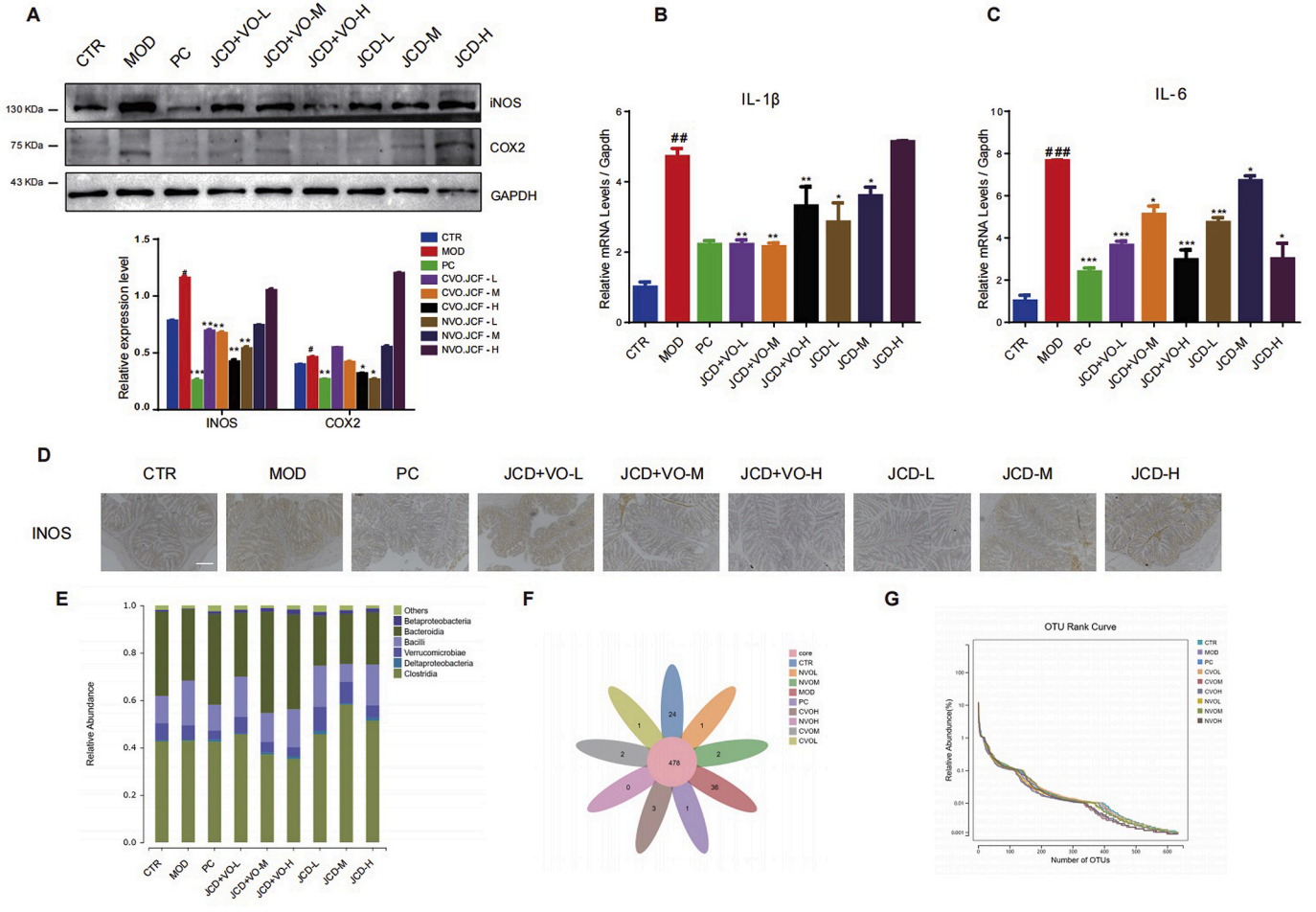

Fig. 5. Effects of JCD on the inflammatory pathway and intestinal flora in rats with loperamide-induced STC. (A) Protein levels of iNOS and COX2 in the colon were detected by western blotting. The mRNA levels of IL-1β (B) and IL-6 (C) in the colon were detected by RT-PCR. (D) The immunohistochemical analysis of iNOS. (E–G) 16 S rDNA sequencing and bioinformatics analysis of rat fecal samples. CTR, the control group; MOD, the model group; PC, rats treated with Qirong Runchang oral liquid (4.8 mL/kg/d); (JCD + VO)-L, rats treated with low-dose JCD + VO (1.8 g/kg/d); (JCD + VO)-M, rats treated with medium-dose JCD + VO (3.6 g/kg/d); (JCD + VO)–H, rats treated with high-dose JCD + VO (7.2 g/kg/d); JCD-L, rats treated with low-dose JCD (1.8 g/kg/d); JCD-M, rats treated with medium-dose JCD (3.6 g/kg/d); JCD-H, rats treated with high-dose JCD (7.2 g/kg/d). Results are expressed as mean ± SD. $^{\#}p < 0.05$ compared with the control group, $^{\#\#}p < 0.01$ compared with the control group, $^{\#\#\#}p < 0.001$ compared with the control group, $^{*}p < 0.05$ compared with model group, $^{**}p < 0.01$ compared with the model group, and $^{***}p < 0.001$ compared with the model group.

effect on the cAMP/PKA signaling pathway.

3.5. Effects of JCD on the inflammatory pathway and intestinal flora in rats with loperamide-induced STC

Studies have shown that constipation can easily lead to damage to the barrier function of the intestinal mucosa and produce a variety of inflammatory factors, resulting in pathological damage to the intestinal mucosal structure [31,32]. Therefore, we investigated the changes in inflammatory factors in the colon. As an inhibitory transmitter, NO synthesis or increased release can induce excessive relaxation of smooth muscles, leading to the inhibition of colonic propulsive contractions, which may contribute to the slowing of colonic transit [33]. Studies have shown that iNOS immunoreactivity is significantly enhanced in the sigmoid myenteric plexus of animals with STC compared to that in the control group. Consistent with this, the protein levels of iNOS and COX2 and the mRNA levels of IL-1β and IL-6 in the colon tissue of the model group were significantly higher than those in the control group (Fig. 5A–C), indicating that inflammation in the colon increased significantly in the model group. When the inflammatory signaling pathway in the colon tissue is activated, a variety of inflammatory factors are secreted, leading to impairment of the barrier function of the intestinal mucosa and metabolic disorders, thereby causing constipation. Compared with the model group, the protein levels of iNOS and COX2, as well as the mRNA levels of IL-1β and IL-6 in the colon tissue of the rats were significantly decreased in the positive control and JCD groups. Expression of the inflammation-related protein iNOS was further validated by immunohistochemistry (Fig. 5D).

Previous studies have shown that STC affects the secretion of intestinal inflammatory factors. The close link between gut microbiota imbalance and secretion of inflammatory factors has become a current research hotspot. Therefore, we aimed to verify whether intestinal microbiota disorders were also involved in STC. 16 S rDNA sequencing was performed on fecal samples from rats in each group. According to the obtained OUTs and the associated species, a corresponding distribution map of the intestinal flora of each sample at the family level was obtained. The intestinal flora of the rats in each group mainly included *Bacteroidales*, *Lachnospiraceae*, *Ruminococcacae*, *Lactobacillaceae*, *Helicobacteraceae*, *Erysipelotrichaceae*, *Prevotellaceae*, *Porphyromonadaceae,* and other unknown bacteria. Compared with the model group, the proportions of *Bacteroidetes*, *Lactobacillus*, and *Erysipelas* were higher in the intestinal flora of the control and drug-treated groups, while those of *Lachnospira*, *Verrucobacterium*, and *Helicobacter* were lower (Fig. 5E–G). STC was associated with the uneven distribution of intestinal flora, leading to metabolic disorders in the intestinal tract. Overall, JCD may increase the abundance of *Bacteroides*, *Lactobacillus*, *Red Toona*, and *Erysipelas* in rats with loperamide-induced STC by regulating the release of inflammatory factors, increasing the diversity of intestinal flora species, and repairing intestinal tract flora disorders.

4. Discussion

STC is a common form of functional constipation. It is characterized by slow peristalsis of the colon and delayed excretion of intestinal contents, thus seriously affecting the quality of life of patients. Loperamide can inhibit intestinal smooth muscle contraction, reduce intestinal peristalsis, and decrease fecal water content in experimental animals, indicating that it can be used to establish animal models of STC [34]. A meta-analysis showed that the efficacy of TCM in the treatment of functional STC was positive, and TCM exhibited obvious advantages over chemical drugs. For example, *Chrysanthemum morifolium* polysaccharides significantly increased the water content of stool pellets and the small intestine propulsion rate, restored the pathological state of colon tissue, and promoted peristalsis of the intestine. *C. morifolium* polysaccharides improved the structure of intestinal microorganisms by regulating the abundance of beneficial pathogenic bacteria [35]. *M. halliana polysaccharides* significantly increased the weight of feces, colon moisture content, and levels of motilin, gastrin, and SP and significantly decreased the levels of somatostatin [36]. JCD is derived from Zhang Jingyue's *Jingyue Quanshu*. It warms the kidneys, nourishes the essence, moistens the intestines, relaxes the bowels, and treats constipation and yang deficiency. Several clinical studies have shown that JCD is effective in treating chronic functional constipation [37]. To determine whether JCD could be used for the treatment of STC, we evaluated water consumption, body weight, fecal parameters, intestinal transit rate, serum index content (5-HT, SP, VIP, and IL-6), and colon histological characteristics in rats with loperamide-induced STC. Following JCD treatment, the time to first melena excretion in rats was significantly reduced, the percentage of fecal water content and intestinal transit were increased, SP was significantly increased, VIP was significantly decreased, and IL-6 was decreased. The histological characteristics of the colon in the drug-treated groups improved, whereas the morphological characteristics of other organs (e.g., liver, spleen, and kidney) were not significantly different. Mucosal goblet cells and mucus secretion in the colon tissue recovered to different degrees compared to the model group. JCD, with or without VO, significantly affected these indices.

As a neuropeptide, VIP binds to G protein–coupled transmembrane receptors and activates adenylate cyclases (ACs) to promote the release of cAMP, which is important in cells for maintenance of physiological functions. cAMP can bind to the regulatory subunits of PKA, thereby promoting the phosphorylation of the latter [38,39]. AQPs are the main downstream target proteins of the VIP/-cAMP/PKA pathway and play an important role in regulating water metabolism in cells [40]. In our study, we found that the VIP content in the JCD-H-treated group was significantly decreased and the SP content in all JCD- and *P*C-treated groups was dramatically increased compared to that in the model group. To clarify whether JCD improved loperamide-induced STC symptoms in rats through the VIP/cAMP/PKA/AQPs signaling pathway, the expression levels of related proteins were determined. The data showed that compared to the control group, the expression of cAMP/PKA/AQPs pathway-related genes in the colon of the model group was significantly increased, suggesting that the cAMP/PKA/AQPs signaling pathway was activated by loperamide. Increased expression of AQP3 and AQP4 enhances the body's reabsorption of water, leading to metabolic disorders in the colon and STC. In this study, we found that JCD may downregulate the protein and mRNA levels of AQP3 and AQP4 through the VIP/cAMP/PKA signaling pathway

and improve intestinal metabolic disorders; however, JCD with or without VO had little effect on the expression of the related proteins.

Intestinal flora dysbiosis is an important cause of STC. The intestinal microbiota and their metabolites are involved in the pathogenesis and pathology of STC in various ways, such as disturbing intestinal digestion and absorption, gastrointestinal dynamics and secretion, intestinal immune system activation and inflammation, and the gut-brain axis [41]. The development of STC is closely related to an imbalance in the intestinal flora. STC is prone to damage the intestinal mucosal barrier function and produce a variety of inflammatory factors, resulting in pathological damage to the intestinal mucosa [42]. The balance between secreted inflammatory factors and composition of the gut microbiota can regulate intestinal homeostasis. In our study, it was found that, compared with the model group, the inflammatory factors showed a significant downward trend, and JCD with OV exhibited better anti-inflammatory activity than JCD alone. In addition, JCD increased the content of *Bacteroides*, *Lactobacillus*, *Red Toona*, and *Erysipelas* in rats with loperamide-induced STC by regulating the release of inflammatory factors, increasing the diversity of intestinal flora species, and repairing the intestinal tract flora disorder, with co-administration of JCD and OV having better efficacy than sole JCD. Although inflammation and gut microbiota have been confirmed to be involved in the process by which JCD improves loperamide-induced STC, the specific mechanism by which JCD regulates inflammation and gut microbiota remains to be explored further.

5. Conclusion

Taken together, we verified that JCD exhibits excellent activity against loperamide-induced STC, and treatment with JCD plus OV exhibits better activity than JCD alone. JCD may improve STC by inhibiting the cAMP/PKA/AQPs signaling pathway and maintaining inflammatory/intestinal flora homeostasis. These findings provide new evidence that JCD plays an indispensable role in the treatment of STC and warrants further investigation.

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CRediT authorship contribution statement

Lina Lin: Investigation, Methodology. **Yuanyuan Jiang:** Investigation, Methodology. **Pengfei Lin:** Investigation, Methodology. **Lanlan Ge:** Data curation, Formal analysis. **Haoqiang Wan:** Data curation, Formal analysis. **Shuwen Dai:** Data curation, Formal analysis. **Runjing Zhang:** Project administration, Resources, Software, Supervision, Validation, Visualization. **Jie Yao:** Project administration, Resources, Software, Supervision, Validation, Visualization. **Xiaobin Zeng:** Conceptualization, Writing – original draft, Writing – review & editing, Funding acquisition. **Ying Peng:** Conceptualization, Writing – original draft, Writing – review & editing.

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

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