Original Article

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Amelioration of gastrointestinal motility and gut dysbiosis by acupoint application of Tongfu Powder to loperamide-induced constipation in mice

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SUMMARY: Chronic constipation is one of the most common gastrointestinal disorders worldwide. It has an impact on daily life and poses a considerable economic burden. Tongfu Powder originated from the Xiaochengqi Decoction with the intent of promoting defecation. Acupoint application of Tongfu Powder has exhibited potentially beneficial effects in the treatment of constipation. However, the potential mechanisms by which acupoint application of Tongfu Powder regulates gastrointestinal motility and gut microbiota are still unclear. The current study sought to investigate the effects and underlying mechanisms of acupoint application of Tongfu Powder on loperamide-induced constipation in mice. The results demonstrated that acupoint application of Tongfu Powder significantly improved the overall defecation of constipated mice, including an increase in the number of fecal pellets, fecal weight, and water content, a decrease in gastric residual volume, and an increase in the intestinal propulsion rate. It also alleviated loperamide-induced colonic histopathological deterioration such as cellular infiltration and thinning of the muscular and mucosal layers in constipated mice. Acupoint application of Tongfu Powder significantly up-regulated the levels of interstitial cells of Cajal (ICC) markers (c-Kit and SCF) and it increased synthesis of intestinal 5-hydroxytryptamine (5-HT) and related proteins (TPH1, HTR4 and SERT). Acupoint application of Tongfu Powder promoted intestinal mucin-2 (MUC2) secretion and increased the expression of tight junction proteins (claudin-1 and occludin). 16S rRNA gene sequencing revealed that acupoint application of Tongfu Powder significantly increased the abundance of *Akkermansia muciniphila*, a bacterium known to be involved in regulating gut motility and intestinal barrier function, thereby alleviating intestinal dysfunction. In addition, it is worth noting that the therapeutic effect of Tongfu Powder acupoint application combined with lactulose is superior to that of either treatment alone. In conclusion, results revealed that acupoint application of Tongfu Powder might alleviate loperamide-induced constipation by regulating the intestinal barrier and gut microbiota.

Keywords: Chronic constipation, Tongfu powder, acupoint application, gastrointestinal motility, gut microbiota

1. Introduction

Chronic constipation is one of the most frequent gastrointestinal disorders caused by altered gastrointestinal dynamics. Its incidence is increasing due to modern lifestyles, dietary composition, and psychological changes, with a global prevalence between 10% and 15% in the population (*1*). Chronic constipation is not a life-threatening condition, but it has a great impact on the quality of life of patients, posing a serious mental and physical burden (*2*). In addition, chronic constipation can increase economic costs and result in a burden to healthcare delivery systems worldwide (*3*). Direct costs attributed to constipation-related health care in the US are estimated to be more than US$230 million per year (*4*).

Various treatment options are generally recommended by the American Gastroenterological Association, including dietary changes, lifestyle modifications, the use of fiber supplements, stool softeners, and laxatives (*5*). Unfortunately, more than 50% of patients fail to respond to these standard treatments mainly due to dissatisfaction with efficacy, safety, adverse reactions, and cost (*6*). Therefore, the treatment of chronic constipation remains a challenge. A safe and cost-effective treatment for chronic constipation needs to be identified. Nowadays, many patients with chronic constipation seek help from complementary and alternative therapies, and external treatment with traditional Chinese medicine (TCM) is their usual choice (*7*).

External treatment with TCM, as a unique traditional treatment with a long history in China, refers to a series of external treatments such as acupuncture, moxibustion, massage, and acupoint application (*8*). These external therapies were reported to be efficacious and to avoid adverse consequences such as abdominal pain, electrolyte disturbance, melanosis coli, and severe drug dependence after long-term use of laxatives (*9*). Acupoint application is a form of external TCM that is considered a non-invasive method combining acupuncture points, meridians, and Chinese herbal medicines. Chinese herbal medicines can treat or prevent diseases by permeating and stimulating skin-related acupoints (*10*). When treating constipation, several Chinese herbal medicines to promote defecation are usually the preferred choice for acupoint application.

Xiaochengqi Decoction is an ancient traditional herbal formula originating from the Treatise on Febrile Diseases (Han Dynasty China), which has been used to treat chronic constipation for thousands of years (*9*). It consists of three herbs, *Rheum officinale* Baill., *Citrus aurantium* L., and *Magnolia officinalis* Cortex. Tongfu Powder is a prescription formulated by the authors' hospital based on the Xiaochengqi Decoction and consists of five herbs, *Rheum officinale* Baill., *Citrus aurantium* L., *Magnolia officinalis* Cortex, *Areca catechu* L. and *Dryobalanops aromatica* C.F. Gaertn. The plant names have been checked with "World Flora Online (WFO)" (*http://www.worldfloraonline.org*). *Rheum officinale* Baill. extract can promote the secretion of colonic mucus and regulate the intestinal flora related to mucin secretion (*11*), while *Citrus aurantium* L. can increase colon propulsion and correct abnormal colonic slow waves to relieve chronic constipation (*12*). *Magnolia officinalis* Cortex can significantly promote gastrointestinal motility, and its main component magnolol can alleviate gastrointestinal dysfunction by up-regulating the expression of the c-kit/SCF signal pathway (*13*). Arecoline, as the main active component of *Areca catechu* L., can stimulate the contraction of rat distal colon smooth muscle *via* muscarinic (M3) receptor-mediated extracellular calcium influx and calcium pool release (*14*). It also can relieve constipation by regulating intestinal microorganisms and their metabolites and by regulating the intestinal genome (*15*). In addition, *Dryobalanops aromatica* C.F. Gaertn., as a natural transdermal enhancer, can effectively promote the absorption of herbal active ingredients through the skin (*16*).

Tongfu Powder has significant laxative effects and has been used for acupoint application to treat chronic constipation in clinics for more than ten years at the authors' hospital, but the underlying mechanisms by which acupoint application of Tongfu Powder treats chronic constipation remain unknown. A point worth noting is that gut microbiota dysbiosis is closely associated with the pathogenesis of gut dysmotility, which is receiving increasing attention (*17*). Gut microbiota dysbiosis results in inflammatory responses, an impairment in colonic epithelial integrity, and a reduction in bowel secretions, thus decreasing intestinal peristalsis and inducing constipation (*18*). Therefore, the current study aimed to explore the potential mechanisms of acupoint application of Tongfu Powder in treating chronic constipation in the context of the intestinal barrier and microbiota. The hope is that this experimental study will provide new avenues and theoretical support for the clinical practice of acupoint application of Tongfu Powder in treating chronic constipation.

2. Materials and Methods

2.1. Preparation of Chinese herbal medicines and acupoint selection for acupoint application

The Chinese herbal medicine Tongfu Powder used in this study originated from the Xiaochengqi Decoction, and its preparation was as follows: *Rheum officinale* Baill., *Citrus aurantium* L., *Magnolia officinalis* Cortex, *Areca catechu* L. and *Dryobalanops aromatica* C.F. Gaertn were ground into powder, mixed in a ratio of 3:5:5:5:1, and then made into a paste with Chinese yellow rice wine. The above drugs are all from the traditional Chinese medicine pharmacy of Shandong Provincial Hospital. Approximately 1 g of the Chinese herbal medicine ointment was placed onto a ring-shaped patch (with a diameter and thickness of about 1.5 cm and 0.5 cm, respectively) and applied to the navel center (Shenque point) of mice (Figure 1A). Shenque (RN8), located at the navel, is one of the effective acupuncture points for chronic constipation (*19*).

2.2. Chemicals

A lactulose oral solution was purchased from Abbott Biologicals B.V. Loperamide hydrochloride (L129465-1g) was purchased from Aladdin (Shanghai, China). A serotonin 5-hydroxytrytamine (5-HT) ELISA kit was purchased from KeyBio (Jinan, China). The mouse monoclonal antibody SCF (sc-13126) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas). The rabbit monoclonal antibodies C-Kit (ab256345) and TPH (ab52954) were purchased from Abcam (Cambridge, UK). Claudin-1 (CY6872) and Occludin (CY5997) were purchased from Abways (Shanghai, China). SERT (19559-1-AP), HTR-4 (21165-1-AP), and MUC2 (27675-1-AP) were purchased from PTG (Wuhan, China). GAPDH (ZB15004-HRP-100) was purchased from Servicebio (Wuhan, China).

2.3. Animals and experimental design

Forty specific-pathogen-free (SPF) male Kunming mice, aged 8 weeks and weighing 40–47 g, were purchased from Beijing Charles river Laboratory Animal Technology Co., Ltd. (Beijing, China; license No.

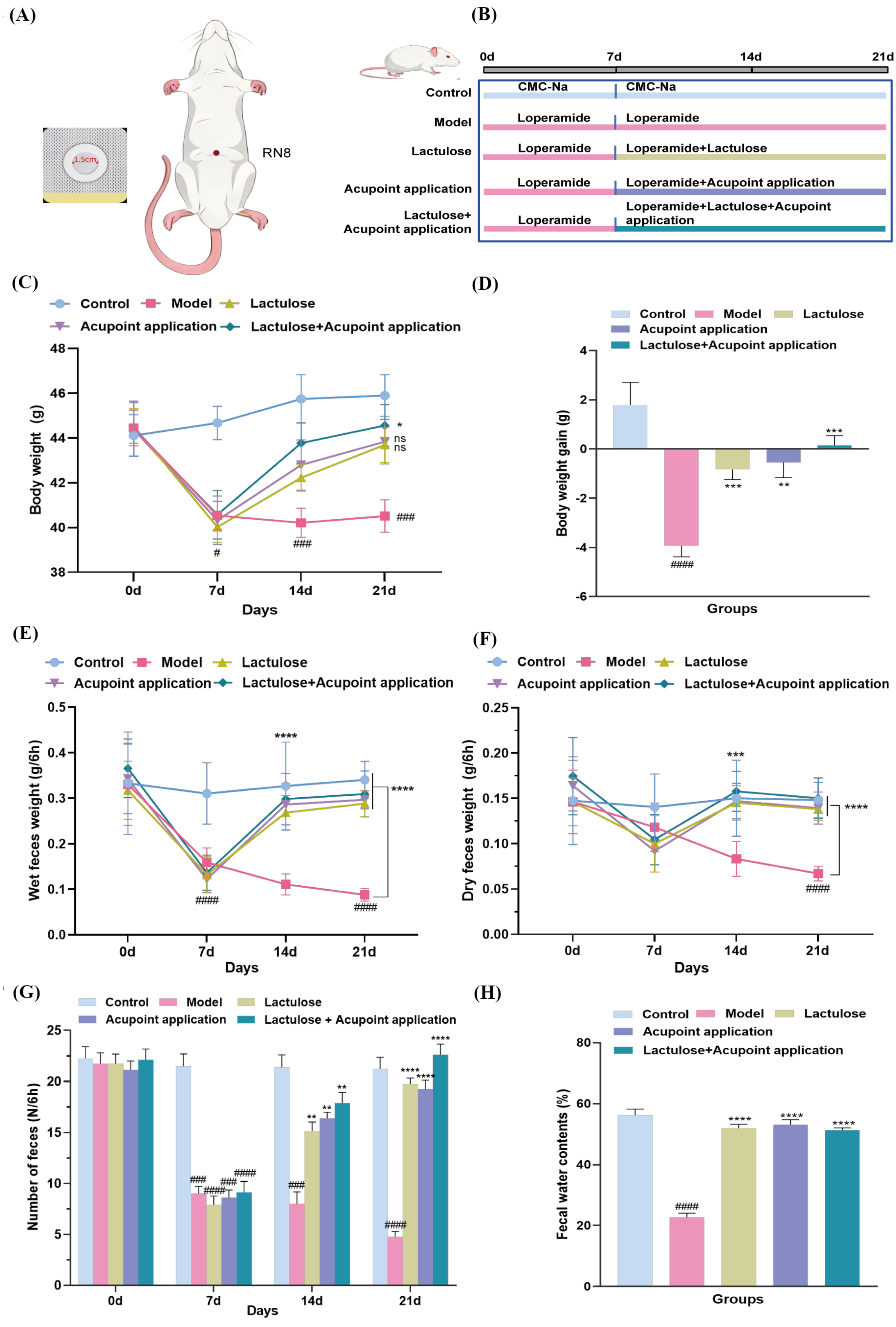

Figure 1. Study setup and effects of acupoint application on physiological parameters in constipated mice. (**A**) Tool for acupoint application and map of the abdominal Shenque acupoint (RN8) in mice. (**B**) Schematic diagram of the experimental design. (**C**) Body weight. (**D**) Body weight gain on the 21st day of the experiment compared to that on the 1st day. (**E-H**) Changes in the fecal wet weight, dry weight, pellet number, and water content within 6 hours. Data were expressed as the mean ± SEM (n = 8). $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, and $^{\#\#\#\#}p < 0.0001$ *vs.* the control group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ *vs.* the model group; ns, not significant.

SCXK(Jing)2021-0011). The mice were housed in an environment with a room temperature ranging from 20 to 24°C, a humidity level of 50 to 70%, and a 12-hour light/dark cycle. They were provided with free access to standard laboratory food and water. All procedures followed the management guidelines set forth by the Experimental Animal Ethics Committee of Shandong Provincial Hospital affiliated with Shandong First Medical University (Ethics No. 2022-096). In addition, all methods used in this study are in accordance with the ARRIVE Guidelines.

After one week of adaptive feeding, the mice were randomly divided into five groups based on their body weight with 8 mice in each group: the control group (Control), the constipation model control group (Model), the lactulose group (Lactulose), the acupoint application of Tongfu Powder group (Acupoint application), and the group treated with a combination of lactulose and acupoint application (Lactulose+Acupoint application).

A mouse model of constipation was established by slightly modifying the method used in a previous study (*20*). Because loperamide is insoluble in water, it is suspended in 0.5% sodium carboxymethyl cellulose (CMC-Na) for subsequent administration by gavage. During the experiment, from day 1 to day 21, all mice (except the control group) were given 0.2 mL of loperamide (10 mg/kg) by gavage at 8 o'clock every day to induce and maintain constipation. Mice in the control group were given the same dose of 0.5% CMC-Na. After the constipation model was successfully established, from the 8th day to the 21st day, at 10 o 'clock every day, the mice in the three treatment groups received the following treatment: the lactulose group was given 0.2 mL of a lactulose solution (3.9 mL/kg), the acupoint application group was treated with herbal medicines for 6 hours, and the combined treatment group was treated with both lactulose and acupoint application. The timeline for each treatment group is depicted in Figure 1B.

2.4. Determination of body weight and defecation

On the 0th and 7th day, the weight of each mouse, the number and weight of fecal pellets within 6 hours, and the moisture content of feces were recorded. To calculate the moisture content, the fresh feces of mice were dried in a ventilated oven at 60°C until a constant weight was obtained. Then, the following formula was used to determine the fecal moisture content: Fecal moisture content (%) = (difference between wet weight and dry weight) / wet weight × 100%. These indicators were used to evaluate the defecation of mice and determine whether the constipation model was successfully established. On days 14 and 21, the body weight and feces of all mice were recorded again to evaluate the defecation of mice.

In order to evaluate the gastrointestinal transport capacity of mice, 0.4 mL of a black semi-solid paste (consisting of water, CMC-Na, milk powder, sugar, starch, and an ink mixture) was administered intragastrically after a 12-hour fasting period following the last administration. After 20 minutes of intragastric administration, the mice were immediately sacrificed. The gastrointestinal transit rate was calculated by determining the percentage of the distance the ink in the semi-solid paste moved in relation to the length of the entire small intestine. In addition, the stomach of mice was weighed to evaluate the gastric emptying of mice.

2.5. Tissue staining

After the mice were sacrificed, the colon tissue was gently shaken in pre-cooled PBS to remove the intestinal contents. A portion of the colon was preserved in liquid nitrogen for subsequent experiments, while the other part was fixed in a 4% paraformaldehyde fixed solution. The colon tissue of mice was fixed for 48 hours, embedded in paraffin, and cut into 4-μm sections for section staining and immunohistochemistry. Hematoxylin-eosin staining (H&E) was used to evaluate histopathological changes, and Alcian blue (AB) staining was used to evaluate the amount of colonic mucus. Finally, the histopathological morphology was observed with an optical microscope and quantified using the software Image J 1.54d.

2.6. Immunohistochemical (IHC) staining

To analyze the expression of MUC2 protein, IHC staining was used as mentioned in our previous studies (*21*). The colon tissue slices were dewaxed and hydrated and then soaked in 0.3% methanol hydrogen peroxide for 30 minutes to block endogenous peroxidase activity. Next, the slices were incubated overnight at 4°C with primary antibody MUC2 (1:1,000), followed by 1 hour of incubation at room temperature with the anti-rabbit secondary antibody. Staining was visualized using the DAB horseradish peroxidase chromogenic kit (Wuhan Service Biotechnology Co., Wuhan, China), and samples were counterstained with hematoxylin. The expression of MUC2 was detected and photographed using the 3D HISTECH Digital Pathology System. The average optical density (MOD) was measured using the graphic analysis software Image J. All IHC staining was independently evaluated by two researchers, and any discrepancies were resolved through group discussion.

2.7. Enzyme-linked immunosorbent assay (ELISA)

To assess the level of the neurotransmitter 5-HT, blood was collected from the eyeballs of anesthetized mice using a sterile tube. The blood was then left at room temperature for 2 hours before being centrifuged at 3,500 rpm at 4°C for 15 minutes to obtain serum samples. All serum samples were packaged and stored in a −80°C refrigerator. The level of 5-HT in serum was determined using an ELISA kit, as mentioned above (*22*).

Table 1. Sequences of primers used for amplification and sequencing

Primer	Sequence (5'-3')	
	Forward	Reverse
C-kit	GACCCGACGCAACTTCCTTAT	TGGCAGCATCCGACTTAATCAA
SCF	TCTGCGGGAATCCTGTGACT	CGGCGACATAGTTGAGGGTTAT
Claudin-1	AGCACCGGGCAGATACAGT	GCCAATTACCATCAAGGCTCG
Occludin	CTCCTCCAATGGCAAAGTGAATG	GTCATCCACACTCAAGGTCAGA
Gapdh	TGCACCACCAACTGCTTAG	GATGCAGGGATGATGTTC

2.8. Real-time quantitative PCR (RT-qPCR)

The expression of mRNA of several genes was detected with RT-qPCR as described previously (*23*). Total RNA was extracted from mouse colon tissue using the TRIzol reagent (Exact Biology, Hunan, China), and 1 μg of RNA was reverse-transcribed into complementary DNA (cDNA) according to the instructions for AbScript III RT Master Mix (ABclonal Technology, Wuhan, China). Then, SYBR Green (ABclonal Technology, Wuhan, China) was used as a fluorescent marker, and 2 μL cDNA and corresponding upstream and downstream primers (10 μmol/L) were added to the 20 μL reaction system and amplified using the Fluorescence Quantitative PCR Detection System (Bioer, Hangzhou, China). The sequences of amplification primers (GAPDH, SCF, c-kit, claudin-1, and occludin) are shown in Table 1. The amplification protocol was as follows: initiation at 37°C for 2 m, 95°C for 3 m, one cycle each, and then amplification at 95°C for 40 cycles for 5 s and 60°C for 30 s. GAPDH was used as a reference gene, and the relative expression of the target gene was calculated according to the $2^{-\Delta\Delta Ct}$ method.

2.9. Western blotting (WB)

As mentioned previously (*24*), the expression of several proteins was detected with WB. After extracting total protein from colon tissue, proteins with different molecular weights were separated using gel electrophoresis. After the WB membrane was sealed in QuickBlock™ for 15 minutes, it was incubated with primary antibodies at 4°C overnight, including c-kit (1:1,000), SCF (1:2,000), HTR4 (1:1,000), Tph1 (1:1,000), and SERT (1:1,000). Then, the membrane was incubated with the secondary antibody for 1 h at room temperature. Finally, the protein bands were detected using enhanced chemiluminescence.

2.10. 16S rDNA sequencing of intestinal bacteria

DNA was extracted from the intestinal contents of mice using a CTAB kit. The V3-V4 variable region was amplified using barcode primers (341F (5'-CCTACGGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGGTATCTAATCC-3')). PCR products were purified using AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA) and quantified using Qubit (*Invitrogen*, USA). The purified PCR products were evaluated using the Agilent 2100 Bioanalyzer (Agilent, USA) and Illumina (Kapa Biosciences, Woburn, MA, USA) library quantification kits. The qualified library concentration should be above 2 nM. After gradient dilution of the qualified sequencing libraries (Index sequence is not repeatable), they were mixed in the corresponding proportion according to the required sequencing amount and denatured with NaOH to a single strand for sequencing. The NovaSeq 6000 sequencing instrument was used for 2 × 250 bp double-ended sequencing, and the corresponding reagent was NovaSeq 6000 SP Reagent Kit (500 cycles). After sequencing, all data were analyzed.

2.11. Statistical analyses

The software GraphPad Prism 8.0 (GraphPad Software, LA Jolla, CA, USA) was used to perform statistical analysis, and the results were expressed as the mean ± SEM. One-way analysis of variance (ANOVA) was used to determine the differences between more than two groups, and comparisons between two groups were further analyzed using Tukey's multiple comparisons test. For all results, statistical significance was indicated by a p value < 0.05.

3. Results

3.1. Effects of acupoint application on improving defecation in constipated mice

Throughout the experiment, the mice exhibited a sluggish response, reduced activity, yellow and dull fur, anorexia, and other symptoms after administration of loperamide. Their feces were dry, short, hard, and light yellow, with undigested food visible in them. However, mice in the control group were active and had white, shiny fur and wet, sticky feces that were slightly larger, softer, and darker.

As shown in Figure 1C-1D, on the 7th day of loperamide treatment, loperamide significantly inhibited the weight gain of mice compared to the control group, and the number of fecal pellets, dry and wet weight, and fecal water content of mice decreased significantly (Figure 1E-1H). This indicates that the constipation model has been successfully replicated. After two weeks of treatment, the weight of mice in the lactulose group, acupoint application group, and lactulose + acupoint application group increased, and defecation gradually recovered compared to that in the model group.

In addition, the small intestine propulsion rate in the three intervention groups improved significantly compared to that in the model group, and the gastric residual weight decreased significantly (Figures 2A-2C). Moreover, there were no significant differences in the intestinal propulsion rate and gastric residual weight between the acupoint application group and the lactulose group ($p > 0.05$). Compared to the lactulose group or the acupoint application group, the lactulose + acupoint application group had a significantly higher intestinal propulsion rate ($p < 0.05$). These results show that the acupoint application of Tongfu Powder alleviated the symptoms of constipation in mice and maintained the healthy state of the gastrointestinal tract by increasing the amount and moisture content of feces and accelerating gastrointestinal peristalsis. The efficacy of the lactulose group was similar to that of the acupoint application group, whereas the combination therapy exhibited superior effects.

3.2. Effects of acupoint application on reducing colon tissue damage

To evaluate the effects of acupoint application of Tongfu Powder on the intestinal composition of constipated mice, H&E staining was used. Compared to the control group, staining in the model group indicated that loperamide caused a decrease in the number and disordered arrangement of goblet cells in the colon of mice, edema in the submucosa, destruction of the crypt structure, and thinning of muscle and mucosal layers (Figure 3A). Fortunately, the damaged colonic structures in the lactulose group, acupoint application

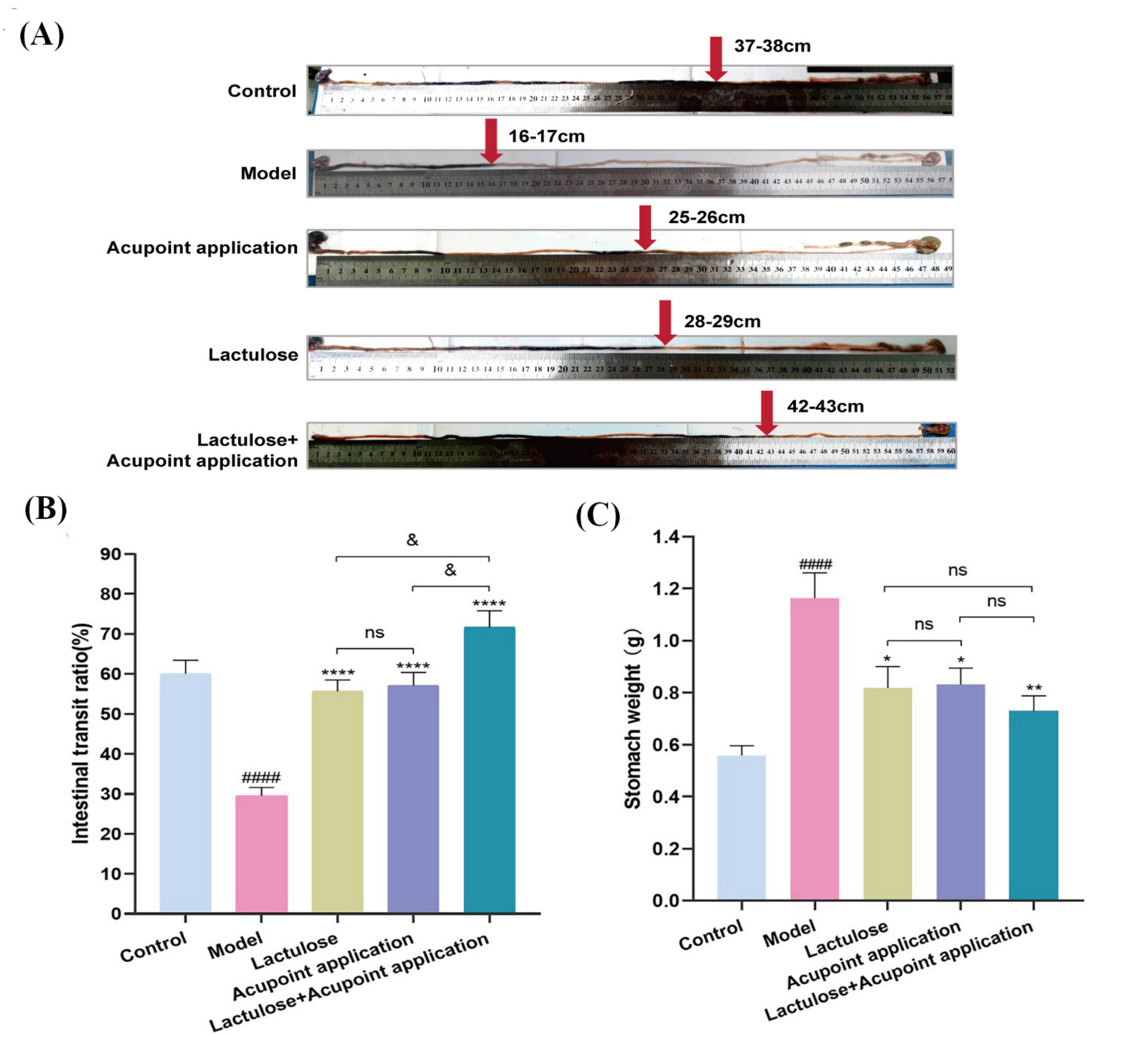

Figure 2. Effects of acupoint application on small intestinal propulsion in constipated mice. (**A**) The propulsive distance of ink in semi-solid nutrients in the intestinal tract. (**B**) Intestinal transit rates. (**C**) Gastric residue. Data were expressed as the mean ± SEM ($n = 8$). $^{####}p < 0.0001$ *vs.* the control group; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{****}p < 0.0001$ *vs.* the model group; $^{\&}p < 0.05$ *vs.* the lactulose + acupoint application group; ns, not significant.

group, and lactulose + acupoint application group improved, and especially the thickness of the muscle and mucous membrane, which was significantly thicker than that in the model group (Figures 3B-3C). Moreover, there were no significant differences in the thickness of colonic muscle and mucosa between the acupoint application group and the lactulose group ($p > 0.05$). In addition, the thickness of the colon muscle and mucosa in the lactulose + acupoint application group increased compared to that in the lactulose group or the acupoint application group, but the difference was not statistically significant ($p > 0.05$). These results indicated that the acupoint application of Tongfu Powder significantly reversed the damage caused by loperamide to colon histology. In addition, the effect of acupoint application on colon recovery is not inferior to lactulose, and the combined therapy showed superior efficacy.

3.3. Effects of acupoint application on the SCF/c-kit signaling pathway

Interstitial cells of Cajal (ICC) can regulate gastrointestinal motility, and SCF/c-kit is essential for the growth and development of ICC (*25*). Therefore, SCF/c-kit signaling pathway-related proteins were examined to evaluate the mechanism of acupoint application of Tongfu Powder in treating constipation. RT-qPCR indicated that acupoint application increased the levels of c-kit and SCF genes in constipated mice (Figure 3D). WB also indicated that, the levels of c-kit and SCF increased in the three treatment groups compared to the model group (Figures 3E-3F). The most significant increase was noted in the lactulose + acupoint application group ($p < 0.0001$ and $p < 0.001$). In addition, there were no significant differences in expression of the c-kit and SCF gene and protein in the acupoint application group compared to those in the lactulose group ($p > 0.05$). These results indicated that acupoint application with Tongfu Powder regulated intestinal motility by modulating the expression of c-kit and SCF in colonic tissue. Moreover, the effect of acupoint application on

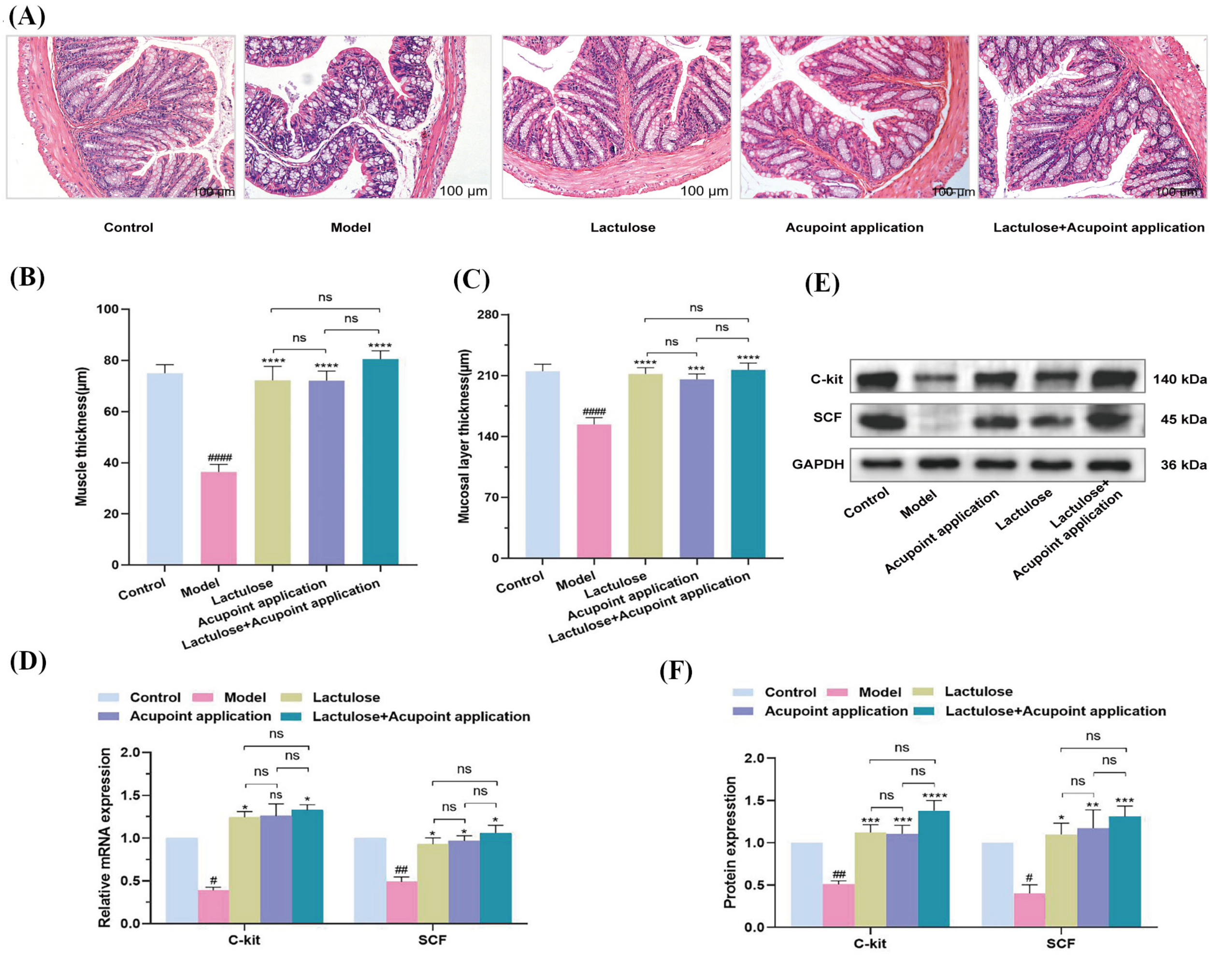

Figure 3. Effects of acupoint application on intestinal tissue damage and the SCF/c-kit signaling pathway in constipated mice. (**A**) Pathological features of colon tissue detected with H&E staining (100× magnification). (**B**) Thickness of colon muscle. (**C**) Thickness of colonic mucosa. (**D**) Expression of c-kit and SCF mRNA detected with RT-qPCR. The levels of C-Kit and SCF protein according to WB (**E**) and quantified using Image J 1.54d (**F**). Data were expressed as the mean ± SEM ($n = 8$). $^{\#}p < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}p < 0.001$, and $^{\#\#\#\#}p < 0.0001$ *vs.* the control group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ *vs.* the model group; ns, not significant.

colon recovery was not inferior to that of lactulose, and the combination of lactulose and acupoint application appeared to offer additional advantages.

3.4. Effects of acupoint application on the 5-hydroxytryptamine (5-HT) signaling pathway

To further study the mechanism of acupoint application of Tongfu Powder on intestinal peristalsis, 5-HT signaling was analyzed using ELISA and the expression of 5-HT-related proteins was analyzed using WB. As shown in Figure 4, acupoint application of Tongfu Powder significantly increased the serum levels of 5-HT, up-regulated the expression of TPH1 and HTR4, and decreased the expression of SERT compared to levels in the model group. In addition, there were no significant differences in the expression of 5-HT and 5-HT-related proteins between the acupoint application group and the lactulose group ($p > 0.05$). Expression of 5-HT and 5-HT-related proteins tended to improve in the lactulose + acupoint application group compared to the lactulose group or acupoint application group, but the difference was not statistically significant ($p > 0.05$). These results indicate that acupoint application of Tongfu Powder regulated intestinal motility *via* modulation of the 5-HT signaling pathway. Furthermore, the regulatory effect of acupoint application on colonic function was not inferior to that of lactulose, and the combined treatment was superior to either monotherapy alone.

3.5. Effects of acupoint application on the function of the colonic mucus barrier

The colonic mucus layer is reported to play a crucial role in alleviating constipation (*26*). Therefore, the content of the acidic mucus layer in the colon was detected using AB staining (Figure 5A). The quantified image of the blue stained area in Figure 5B indicates that, in comparison to the control group, the blue stained area of the colonic mucus diminished significantly in the model group. However, the blue stained area increased significantly in the lactulose group, acupoint application group, and lactulose + acupoint application group, with the most pronounced increase in the lactulose + acupoint application group. In addition, the level of mucin2 (MUC2), which is involved in the production of colonic mucus, was also examined. Findings revealed that the immunohistochemical detection and image quantification of MUC2 exhibited a similar trend as AB staining (Figures 5C-5D), which further validated the results of

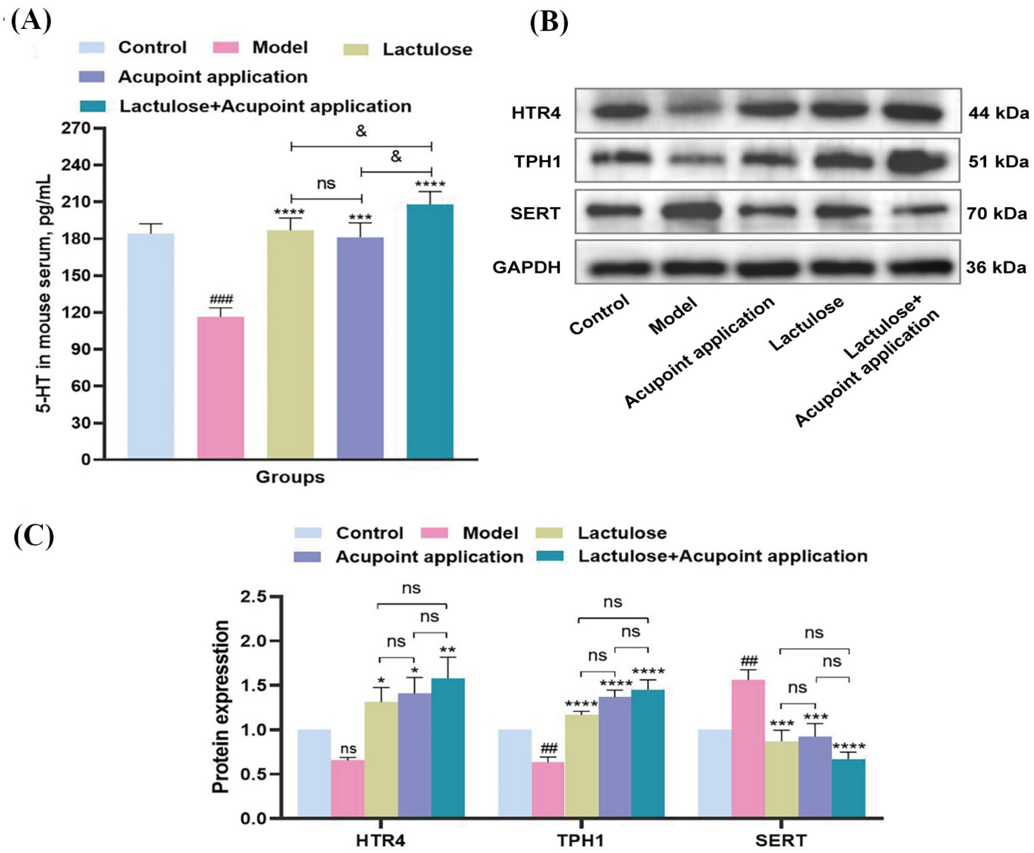

Figure 4. Effects of acupoint application on the 5-hydroxytryptamine (5-HT) signaling pathway. (**A**) The levels of 5-HT in serum measured with ELISA. The levels of HTR4, TPH1, and SERT protein according to WB (**B**) and quantified using Image J 1.48 (**C**). Data were expressed as the mean ± SEM ($n = 6$). $^{\#\#}P < 0.01$, and $^{\#\#\#}p < 0.001$ *vs.* the control group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ *vs.* the model group; $^{\&}p < 0.05$ *vs.* the lactulose + acupoint application group; ns, not significant.

AB staining.

In addition, there were no significant differences in the blue stained area and the proportion of MUC2 expression in the acupoint application group compared to the lactulose group ($p > 0.05$). Compared to the acupoint application group and the lactulose group, the proportion of the blue stained area in the lactulose + acupoint application group increased significantly ($p < 0.05$). These results indicate that acupoint application of Tongfu Powder improved the dry and hard feces of constipated mice by increasing the production of colonic mucus, that the increase in colonic mucus as a result of acupoint application was not inferior to lactulose, and that the combined treatment has advantages.

3.6. Effects of acupoint application on the function of the colonic epithelial barrier

To investigate the impact of acupoint application of Tongfu Powder on the colonic epithelial barrier in constipated mice, the expression of mRNA of the tight junction proteins Occludin and Claudin-1 was analyzed using RT-qPCR and WB. After acupoint application of Tongfu Powder, the expression of Claudin-1 mRNA and protein increased significantly, and the increase in Claudin-1 was more pronounced in the lactulose + acupoint application group ($p < 0.05$, Figures 5E-5G). Moreover, the expression of Occludin mRNA and protein in the treatment groups was up-regulated compared to

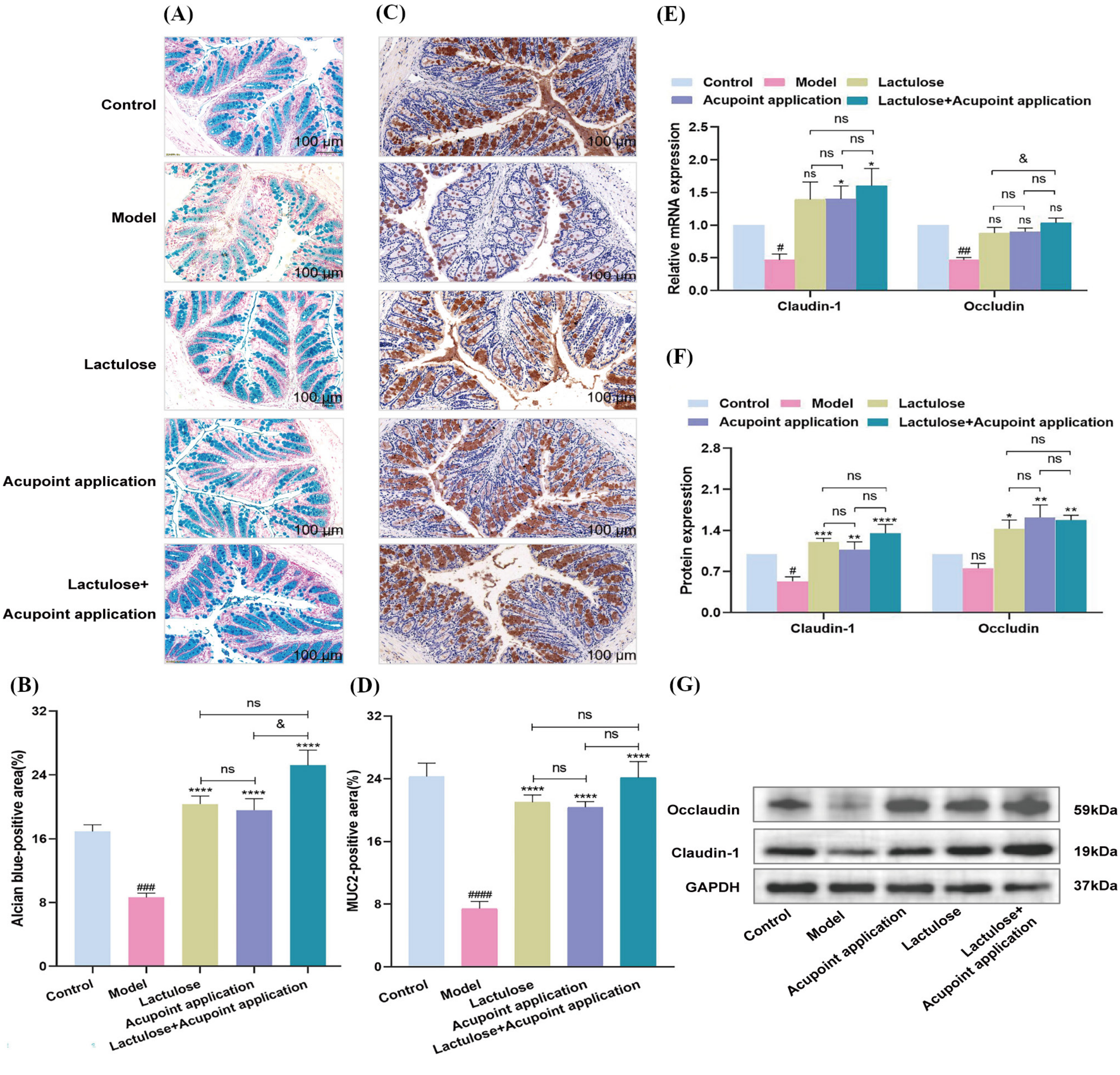

Figure 5. Effects of acupoint application on the function of the colonic mucus and epithelial barriers. The amount of colonic mucus evaluated with AB staining (100× magnification) (**A**) and quantified using Image J 1.48 (**B**). The levels of MUC2 protein detected with IHC staining (100× magnification) (**C**) and quantified using Image J 1.48 (**D**). (**E**) Expression of Occludin and Claudin-1 mRNA detected with RT-qPCR. The levels of Occludin and Claudin-1 protein according to WB (**G**) and quantified using Image J 1.48 (**F**). Data were expressed as the mean ± SEM ($n = 6$). $^{\#}P < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, and $^{\#\#\#\#}p < 0.0001$ *vs.* the control group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ *vs.* the model group; $^{\&}p < 0.05$ *vs.* the lactulose + acupoint application group; ns, not significant.

that in the model group ($p < 0.05$). This suggested that acupoint application of Tongfu Powder restored the barrier function of colon tissue by up-regulating the expression of the connective proteins Claudin-1 and Occludin and that the combined effect is superior to either treatment alone.

3.7. Effects of acupoint application of Tongfu Powder on the abundance and diversity of the intestinal microbiota in constipated mice

To explore the potential role of the intestinal microbiota in the treatment of constipation by acupoint application of Tongfu Powder, the V3-V4 region of the 16S rDNA gene was sequenced. Results yielded 2,080,127 pieces of original data, and the effective data after quality control accounted for 100% of the original data, indicating that sequencing was highly effective. After sequencing, the final ASV feature table and feature sequence were analyzed and annotated. By comparing the bacterial sequences (Figure 6A), one can intuitively see the number of common and unique ASVs in each group. A point worth noting is that the number of ASVs in the model group was significantly smaller than that in other groups. The α diversity reflects the intra-group diversity of microbial samples, and the Goods coverage dilution curve (rarefaction curve) indicates the rate of microbial coverage and changes in species. When the curves of all samples reached the plateau period and the value approached one, this indicated that the sequencing quantity was reasonable and sufficient (Figure 6B). The Chao1 index and observed species index reflected the number of microbial species (Figures 6C-6D). The Shannon index and Simpson index were used to estimate the species diversity in the microbial sample group (Figures 6E-6F). Results indicated that the number and diversity of microbial species in the model group decreased significantly ($p < 0.01$, compared to the control group), while the change in the Alpha diversity index caused by loperamide was effectively reversed in the acupoint application group ($p < 0.01$, compared to the model group).

The β diversity explains the inter-group diversity of microbial samples, which was mainly evaluated by principal component analysis (PCA, Figure 6G), principal coordinates analysis (PCoA, Figure 6H), and clustering analysis (UPGMA, Figure 6I). Results indicated that the similarity within each group and the difference between groups was obvious, and the model group and control group were relatively separate. The three treatment groups were close to the control group. These results indicate that acupoint application of Tongfu Powder has a certain restorative effect on the decrease in intestinal microbiota abundance and diversity in mice with constipation induced by loperamide.

3.8. Effects of acupoint application on reconfiguring the composition of the gut microbiota in constipated mice

The impact of acupoint application on the composition of the intestinal microbiota in mice was investigated at the phylum, family, and genus levels. In the species relative abundance cluster diagram (Figures 7A, 7D, 7I), the distance between the control group and the lactulose group, acupoint application group, and lactulose + acupoint application group was similar, as was the abundance of each species, but they obviously differed from the model group.

The top 30 species were analyzed at the phylum and genus levels. At the phylum level, the main bacteria found in mouse feces were Firmicutes, Bacteroidetes, Proteobacteria, and Verrucomicrobiota. At the phylum level, the proportion of Firmicutes, Proteobacteria, and Bacteroideta decreased in the model group compared to the control group, while the proportion of Verrucomicrobiota increased. These changes in the microbiota were reversed in the treatment groups (Figures 7B-7C). At the family level, loperamide reduced the population size of Lachnospiraceae, Muribaculaceae, Prevotellaceae, Bacteroidaceae, Bifidobacteriaceae, Family_XI, and Veillonellaceae (Figures 7E-7G). The population size of Akkermansiaceae increased (Figure 7H). However, recovery of the abundance of these species was evident to varying degrees in the treatment groups. At the genus level, the model group had a significant increase in the abundance of *Akkermansia* compared to the control group, while the abundance of *Alloprevotella*, *Oscillibacter*, *Helicobacter*, *Anaerotruncus*, *Acetatifactor*, *Clostridium*, *Muribaculum*, and *Bacteroides* decreased. In contrast, recovery of their abundance was evident to different degrees in the treatment groups (Figures 7J-7N). The abundance of *Acetatifactor*, *Clostridium*, *Muribaculum*, and *Bacteroides* increased significantly in the lactulose + acupoint application group ($p < 0.01$). Acupoint application may regulate the intestinal microecology by increasing the abundance of potentially beneficial bacteria and reducing the abundance of potentially harmful bacteria, thus promoting gastrointestinal peristalsis.

3.9. Prediction of the function of the intestinal microbiota in constipated mice

PICRUSt2 is a bioinformatic tool designed to predict the functional potential of gut microbiota based on 16S rRNA gene sequencing data. STAMP analysis and the KEGG database were used to select the top 20 statistically significant functions to display in the form of pictures. As shown in Figure 8A, the histogram on the left represents the differences between groups, with different colors indicating different groups. The higher the column, the higher the abundance ratio, while the dot graph on the right shows the abundance of different

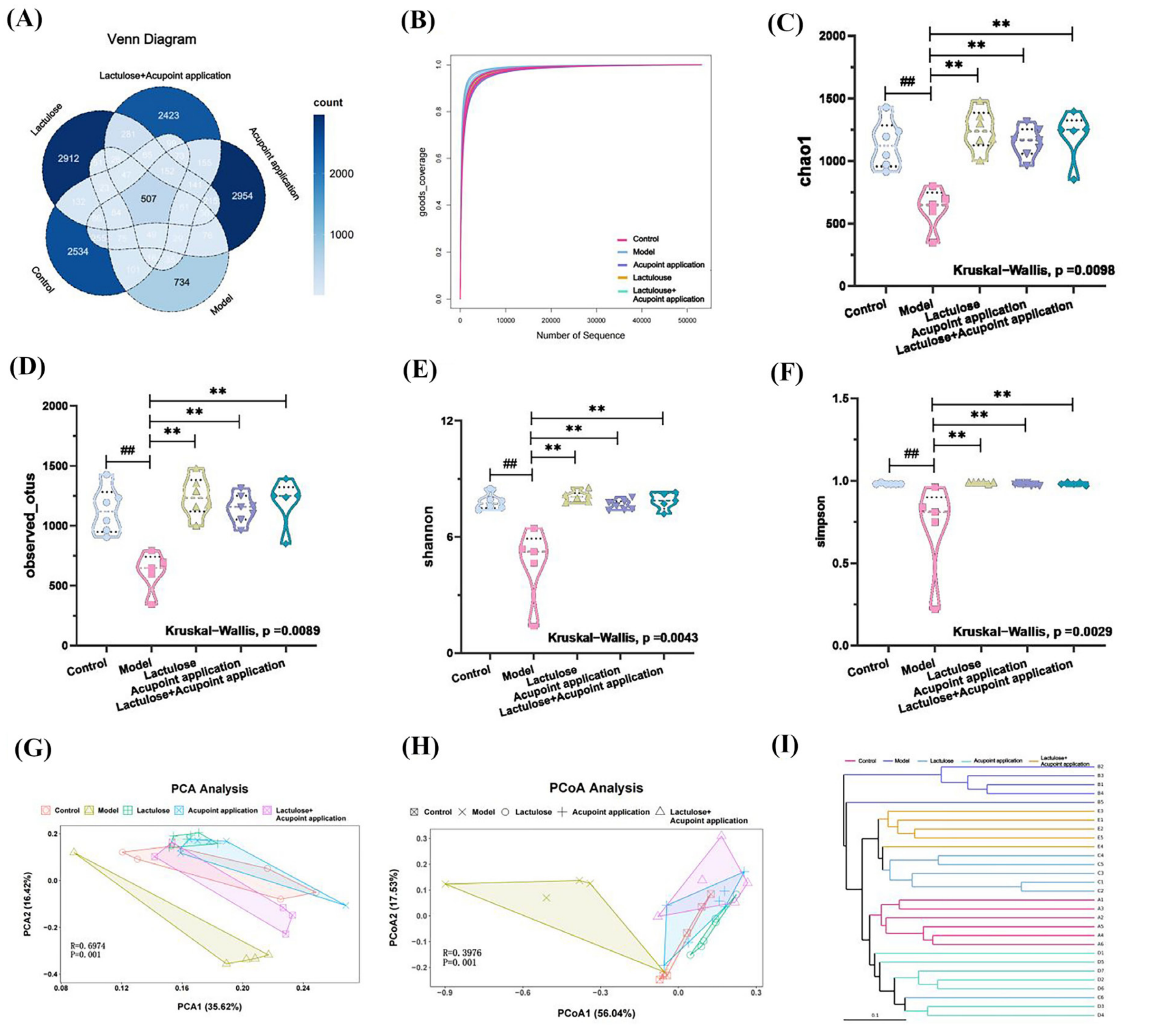

Figure 6. Effects of acupoint application on the abundance and diversity of the gut microbiota in constipated mice. (**A**) Venn diagram of characteristic sequence ASVs. (**B**) The Goods _coverage rarefaction curve. (**C**) The Chao1 index. (D) The observed species index. (**E**) The Shannon index. (**F**) The Simpson index. (**G**) PCA analysis. (**H**) PCoA analysis. (**I**) Clustering analysis. Data were expressed as the mean ± SEM (*n* = 5-7). $^{##}p$ < 0.01 *vs.* the control group; $^{**}p$ < 0.01 *vs.* the model group.

items. If the abundance of an item is higher in the control group than in the model group, the dot will be displayed on the right and marked with the color of the control group. Otherwise, it will be marked on the left with the color of the model group. The middle dotted line represents the 95% confidence interval. The farther a point is from this line, the greater the difference. The right axis shows the *p* value of the difference, with values increasing from bottom to top. The results of microbial function prediction indicated that the constipation model group had decreased levels of restriction enzymes, taurine and hypotaurine metabolism, primary and secondary bile acid biosynthesis, and limonene and pinene degradation compared to the control group. After the application of Tongfu Powder at the acupoint (Figure 8B), however, the metabolic pathways that were predicted to decrease instead increased. In addition, the signaling pathways were analyzed (Figure 8C), which revealed that acupoint application stimulated mannan degradation, heme biosynthesis, CMP− legion salt biosynthesis I, and L-glutamic acid degradation V while inhibiting pyrimidine deoxynucleotide phosphorylation.

3.10. Prediction of the bacterial phenotypes in constipated mice as a result of acupoint application

The tool BugBase was used to predict the bacterial phenotypes of intestinal microorganisms. The levels of aerobic bacteria, mobile elements, biofilm formation, Gram-negative bacteria, and stress tolerance increased in the model group compared to levels in the control group (Figures 9A, 9C, 9D, 9E, and 9H), while the levels of anaerobic bacteria, Gram-positive bacteria, and potentially pathogenic bacteria tended to decrease (Figure 9B, 9F, and 9G). These parameters returned to normal in the lactulose group, acupoint application group, and

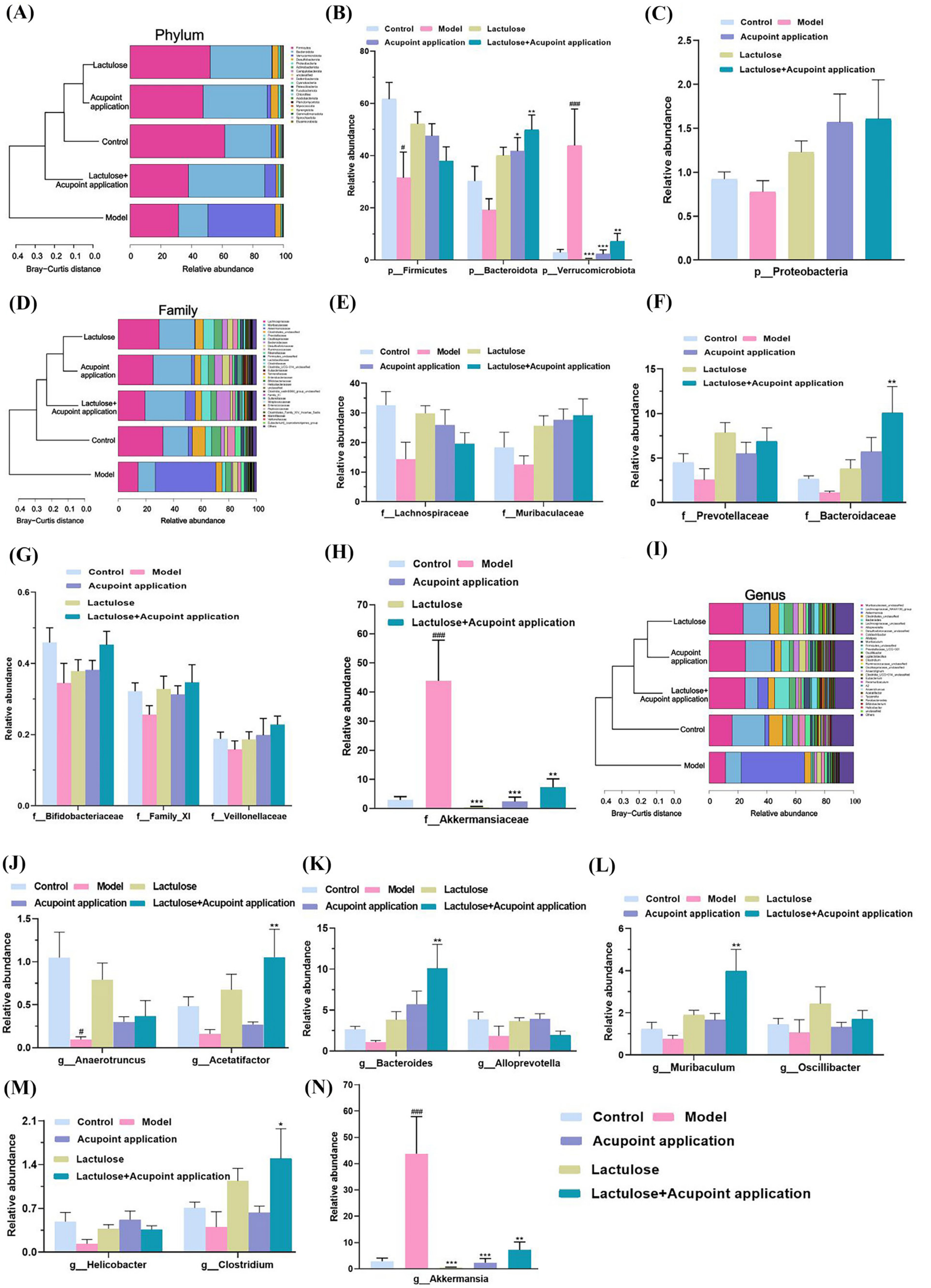

Figure 7. Effects of acupoint application on reconfiguring the composition of the gut microbiota in constipated mice. The differences in bacteria among each group are distinguished by a cluster diagram and histogram at the phylum (**A**), family (**D**), and genus (**I**) levels. (**B-C**) Relative abundance of the gut microbiota at the phylum, level. (**E-H**) Relative abundance of the gut microbiota at the family level. (**J-N**) Relative abundance of the gut microbiota at the genus level. Data were expressed as the mean ± SEM (n = 5-7). $^{\#}p < 0.05$, and $^{\#\#\#}p < 0.001$ *vs.* the control group; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ *vs.* the model group.

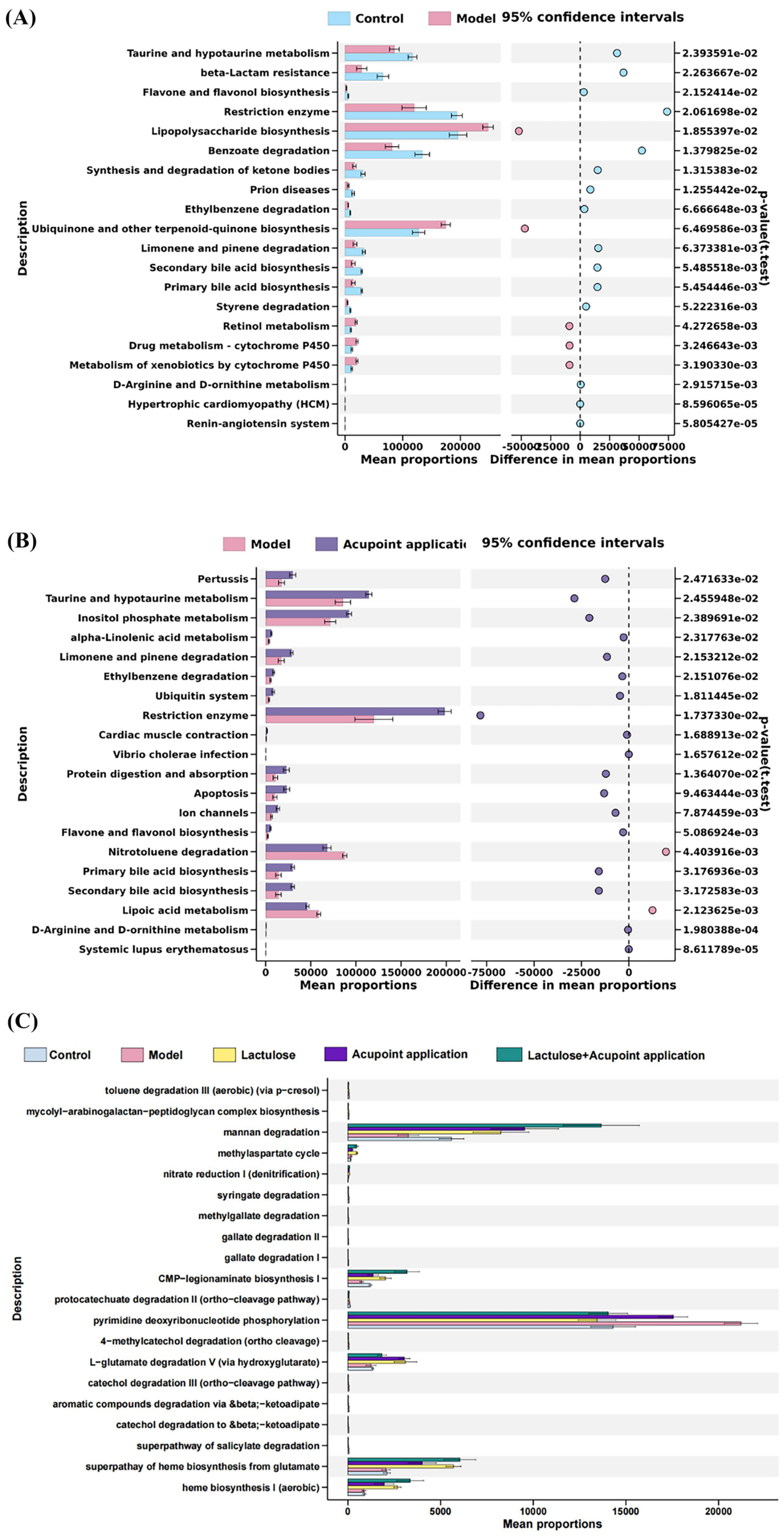

Figure 8. Prediction of the function of acupoint application in intestinal microbiota in constipated mice (n = 5-7). (**A**) Comparison between the control group and model group. (**B**) Comparison between the acupoint application group and model group. (**C**) Prediction of signal path function. STAMP analysis and the KEGG database were used to predict PICRUSt function, and the difference was analyzed when $p < 0.05$.

lactulose + acupoint application group. A point worth noting is that the number of beneficial bacteria decreased in the model group, while it increased in the three treatment groups. This phenomenon may be related to the significant increase in Akkermansia spp., which is widely considered a beneficial microbiota, in the model group. These results confirmed that the microbiota plays an important role in the treatment of constipation through acupoint application of Tongfu Powder, and they provide a new direction for future research.

4. Discussion

Acupoint application of Chinese herbal medicines, as one of the characteristic forms of Chinese medicine treatment, has been widely used clinically. Clinical studies demonstrated that applying *Rheum officinale* Baill. to the Shenque acupoint significantly improved the fecal characteristics and increased the frequency of defecation within 24 hours in patients with chronic constipation (*27*). A meta-analysis revealed that acupoint application of Chinese herbal medicines not only relieves constipation symptoms, shortens defecation time, promotes spontaneous complete defecation, and reduces rates of recurrence but also enhances the quality of life for patients (*28*). The current study explored the potential mechanisms of acupoint application of Tongfu Powder in treating chronic constipation in the context of the intestinal barrier and microbiota.

In this study, loperamide-induced constipated mice were used to investigate the alleviation of constipation by acupoint application. After 21 days of loperamide, mice exhibited a decrease in body weight, food intake, fecal water content, and a longer colonic transit time. However, treatment with acupoint application or lactulose significantly improved the overall defecation of constipated mice, including an increase in the number of fecal pellets, fecal weight, and water content, a decrease in gastric residue, and a significant increase in the intestinal propulsion rate (Figures 1-2). Acupoint application or lactulose also alleviated loperamide-induced colonic inflammation and histopathological deterioration such as cellular infiltration and thinning of the muscular and mucosal layers in constipated mice (Figure 3). Notably, the group treated with acupoint application and lactulose displayed the most obvious effect. These results suggest that acupoint application significantly restored gastrointestinal motility by increasing fecal moisture, promoting colonic transit, and alleviating colonic inflammation. Notably, the combined therapy of acupoint application and lactulose conferred additional advantages.

As pacemaker cells of the gastrointestinal tract, ICC can regulate gastrointestinal motility (*29*). A decrease in or damage to ICC in the gastrointestinal tract promotes the development and progression of constipation (*30*). c-kit is a specific marker of ICC. The combination of c-kit and its ligand SCF play an important role in maintaining

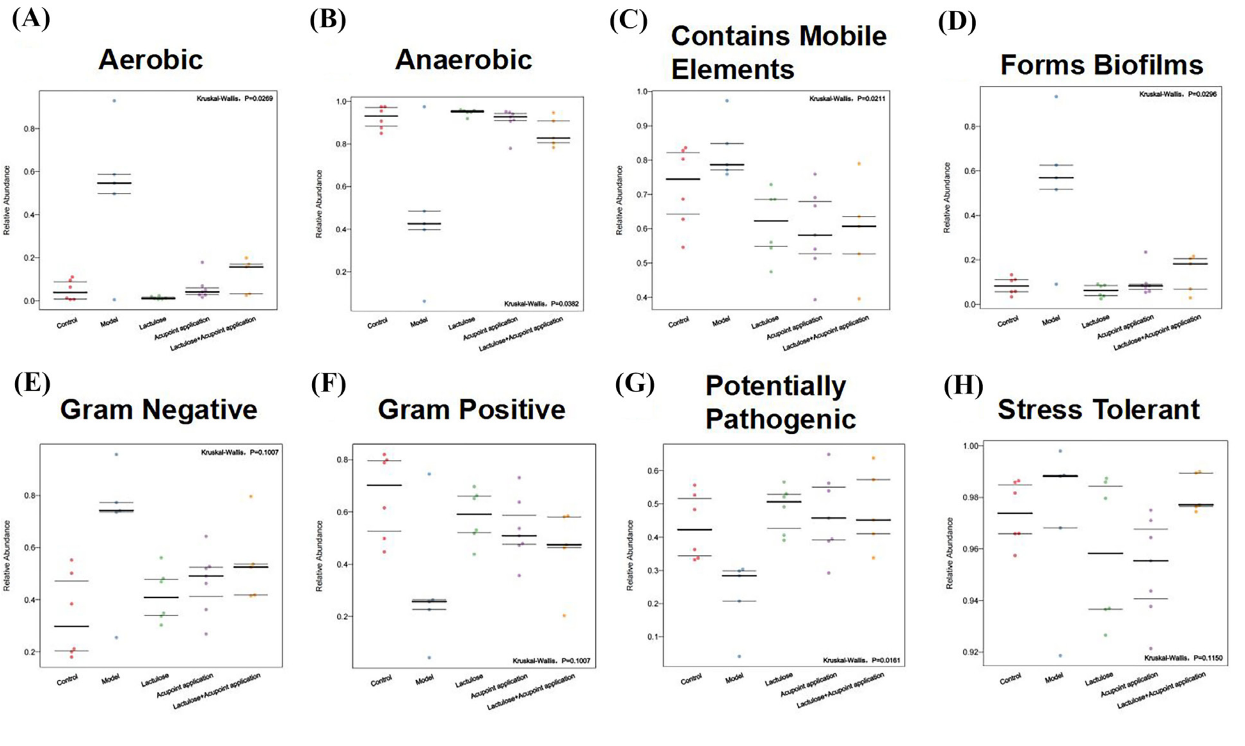

Figure 9. Prediction of the bacterial phenotypes of the intestinal microbiota in constipated mice as a result of acupoint application (*n* = 5-7). (**A**) Aerobic. (**B**) Anaerobic. (**C**) Contains Mobile Elements. (**D**) Forms Biofilms. (**E**) Gram-negative. (**F**) Gram-positive. (**G**) Potentially Pathogenic. (**H**) Stress Tolerant.

the differentiation, development, and maintenance of ICC (*25*). Damage to ICC can be reduced by up-regulating the levels of SCF and c-kit in colon tissue, thereby improving the contractility of colon tissue (*31*). The current results indicated that the levels of c-kit and SCF protein expression were up-regulated in the intestinal tissues of the acupoint application and lactulose treatment groups (Figure 3). Notably, the group treated with acupoint application and lactulose displayed the most obvious effect. Therefore, acupoint application might alleviate constipation by up-regulating the c-kit/SCF signaling pathway to increase the number of ICC, thus enhancing gastrointestinal motility.

5-Hydroxytryptamine (5-HT) is a brain neurotransmitter mostly secreted by enterochromaffin (EC) cells (*32*). It is an important sensor in gastrointestinal motility. The synthesis and release of 5-HT can enhance gastrointestinal peristalsis and alleviate constipation. 5-HTR4 as a receptor of 5-HT, is expressed in the colonic mucosa, and when activated, promotes propulsive motility and attenuates visceral hypersensitivity (*33*). The current results indicated that 5-HT and 5-HTR4 were significantly stimulated especially by the combination of acupoint application and lactulose, which played an important role in the improvement of intestinal motility (Figure 4). Tryptophan hydroxylase 1 (TPH-1) is a member of the amino acid hydroxylase family and a rate-limiting enzyme for the synthesis of 5-HT. TPH-1 is specific and is present in low levels in tissues, so it is usually used as a specific marker of 5-HT neurons and a differentiating feature of 5-HT neurons (*34*). As illustrated in Figure 4, combined therapy with acupoint application and lactulose exhibited superior advantages, which can effectively promote the expression of TPH-1 and play an important role in the synthesis of 5-HT. Serotonin transporter (SERT) is a trans-membrane transporter with a high affinity for 5-HT, which re-uptakes excessive 5-HT to terminate its physiological effects and is involved in regulating gastrointestinal motility (*35*). The current results indicated that the abnormal increase in SERT expression in the model group may have caused a large amount of 5-HT transfer, resulting in poor intestinal motility. However, SERT expression may be inhibited after acupoint application or/and lactulose, and the accumulation of 5-HT near the intestine may have increased, thus alleviated the poor intestinal motility of constipated mice. On the whole, acupoint application restores the 5-HT signaling pathway by regulating the process of 5-HT synthesis, binding to receptors, and inactivation, thereby alleviating constipation symptoms. Therefore, the 5-HT signaling pathway may be an important target for acupoint application in the treatment of constipation.

The normal operation of the intestine relies not only on its peristalsis ability but also on the complete intestinal mucosal barriers. The barriers consist of the mucus barrier, epithelial barrier, and biological barrier (*i.e.*, gut microbiota). Results suggested that the colonic mucosal barrier is closely related to intestinal microbiota. Acupoint application can alleviate functional constipation by promoting intestinal peristalsis, increasing mucus secretion, and regulating the abundance and composition of the intestinal microbiota.

The mucus barrier is a layer of mucus that covers the outside of intestinal cells, and its main component is mucin 2 (MUC2). The intestinal mucus layer is synthesized and secreted by goblet cells, which not only maintain intestinal homeostasis but also play a role in lubricating the intestine (*36*). When the intestinal mucus layer decreases, it can lead to a large number of harmful substances and bacteria invading the intestinal crypt and intestinal epithelium, causing various intestinal diseases. It can also make the surface of stool dry and difficult to discharge. In order to verify the effect of acupoint application on the mucus barrier, the level of colonic acidic mucus and mucin MUC2 was measured. As shown in Figure 5, the stained area of the colonic mucus layer and the level of MUC2 protein increased significantly after acupoint application or lactulose treatment compared to that in the model group. Notably, the group treated with acupoint application and lactulose displayed the most obvious effect. These results suggest that acupoint application greatly increased the quantity of acidic mucus on the surface of the colonic mucosa, thus alleviating the symptoms of dry stool in mice with constipation.

The complete and continuous epithelial barrier is also the basic intestinal mucosal barrier that protects the colon. Tight junctions are the core structure of the epithelial barrier, in which Occludin, Claudin, and other proteins together constitute the main body of tight junctions. The integrity of the tight junction structure is conducive to the closure of intercellular space and plays an important role in regulating cell permeability and preventing harmful substances from invading the intestinal cavity (*37*). The current results indicated that the tight junction barrier of the intestinal mucosa was significantly damaged in the model group with the decreased expression of Occludin and Claudin-1 (Figure 5). However, combined treatment with acupoint application and lactulose increased the expression of Occludin and Claudin-1 mRNA and protein in the colon tissue of constipated mice, repaired the tight junction, and restored the intestinal mucosal barrier. Acupoint application combined with lactulose is superior to monotherapy in promoting defecation function and reducing colonic tissue injury in constipated mice.

The intestinal microbiota is another important component of the intestinal mucosal barrier. Mounting evidence indicates that constipation is closely related to a disorder of the intestinal microbiota (*38*). As shown in Figure 6, Chao1, observed species, and the Shannon and Simpson indexes showed that acupoint application reversed the decrease in the number and abundance of intestinal microbial communities in constipated mice. Therefore, the mechanism of acupoint application in the treatment of constipation is also inseparable from the regulation of the intestinal microbiota.

To further study the effect of acupoint application on the bacterial community at the phylum level, the differences in the composition of the bacterial community were analyzed. Short-chain fatty acids (SCFAs) are intestinal microbial metabolites that promote intestinal motility and relieve constipation (*39*). In the current study, Lachnospiraceae, Bifidobacteriaceae, and Prevotellaceae decreased significantly in the model group compared to the control group, and these are common bacteria that produce SCFAs. The difficulty in defecation in the model group may be related to the reduction in SCFA-producing bacteria. Fortunately, the abundance of these bacteria increased to varying degrees after acupoint application or lactulose (Figure 7). Moreover, the effect of acupoint application combined with lactulose is superior to either treatment alone.

Akkermansia muciniphila (Akk) is a mucin-degrading bacterium belonging to the phylum Verrucomicrobiota. It colonizes the mucous layer of the gastrointestinal tract and has the ability to degrade mucin. When Akk grows excessively, it may cause excessive degradation of the mucin layer in the intestine, leading to an increase in intestinal permeability and leakage. This can increase the likelihood of toxins, bacterial lipopolysaccharides, and even pathogenic bacteria entering the bloodstream and increasing the risk of intestinal diseases (*40*). In an experiment involving 16S sequencing of fecal samples from constipated mice, researchers found a correlation between proinflammatory metabolites and Akk (*41*). This may be related to the destruction of the mucus barrier due to excessive Akk degradation of mucus protein in the feces of constipated mice. In the current study, there was a significant increase in Akk in the intestinal microbiota of constipated mice but a significant decrease after acupoint application of Tongfu Powder and/or lactulose treatment ($p < 0.01$). This finding is consistent with the results of previous research (*42,43*), suggesting that acupoint application may alter the composition and metabolism of the intestinal microbiota by regulating the number of Akk, thus alleviating constipation symptoms. However, some studies have shown that Akk is a beneficial bacterium in the intestine that helps maintain intestinal homeostasis (*44,45*). This seems to contradict the current results. In order to solve this mystery, further research is needed to reveal the complex role of Akk in the intestine and its relationship to constipation and other diseases. In other words, Akk, as a unique mucus-degrading bacterium, plays an important role in intestinal health. It participates in maintaining intestinal homeostasis by degrading mucin, but it may also contribute to intestinal diseases under certain conditions. Therefore, the biological characteristics of Akk and its

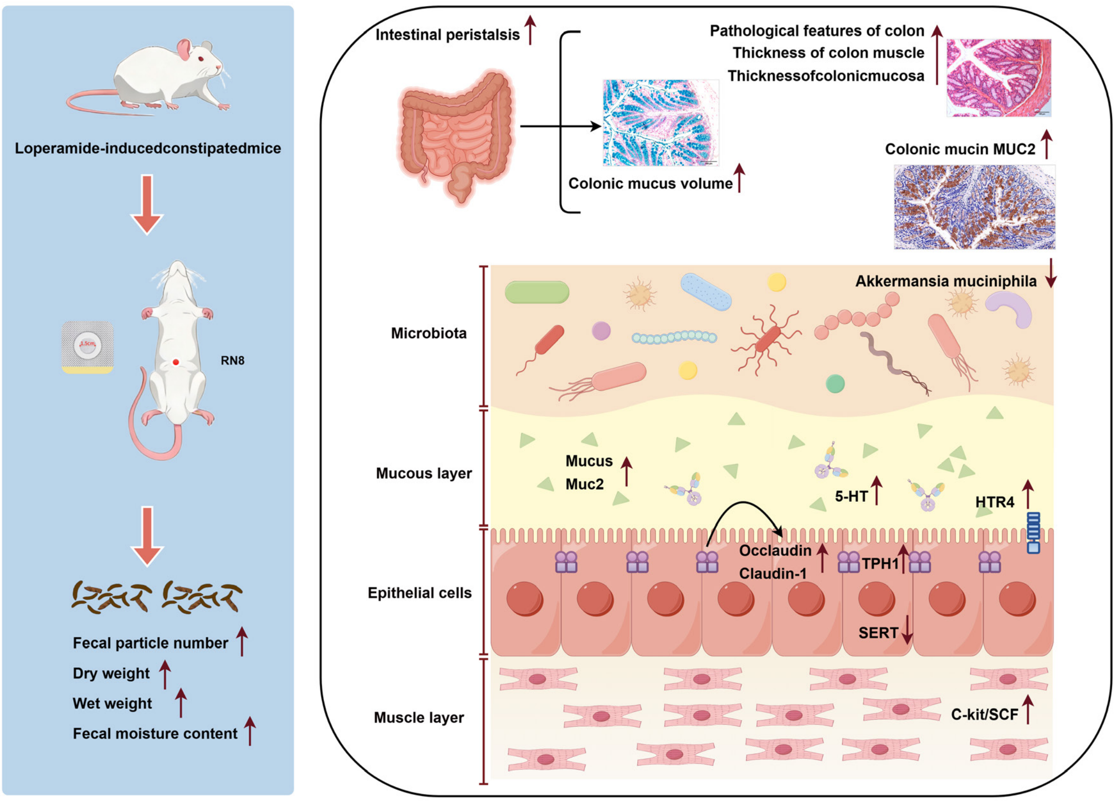

Figure 10. Acupoint application of Tongfu Powder might alleviate loperamide-induced constipation by regulating the intestinal barrier and gut microbiota.

mechanism of action in the intestine need to be studied further in order to provide new ideas and methods for preventing and treating intestinal diseases.

Apart from analyzing the composition of species in the intestinal microbiota, this study also predicted the function and bacterial phenotypes of the intestinal microbiota. As shown in Figure 8 and Figure 9, restriction enzymes, taurine and hypotaurine metabolism, primary and secondary bile acid biosynthesis, and limonene and pinene degradation are the main factors affecting the ecological imbalance of the intestinal microbiota. At the same time, acupoint application may play a role by stimulating mannan degradation, heme biosynthesis, CMP-legionaminate acid biosynthesis I, and L-glutamic acid degradation V signal pathways and inhibiting pyrimidine deoxynucleotide phosphorylation. In addition, the prediction of bacterial phenotypes revealed that the increase in aerobic bacteria, mobile genetic elements, biofilm-forming bacteria, Gram-negative bacteria, and stress-tolerant bacteria and the decrease in anaerobic bacteria and Gram-positive bacteria are related to the development of constipation. A point worth noting is that mobile genetic elements and biofilm-forming bacteria have been widely studied in many intestinal diseases due to their potential pathogenicity and their impact on the human immune system. The presence of these characteristics not only enhances the adaptability of bacteria to the external environment but also mediates their toxicity to the host, thus exacerbating constipation symptoms (*46*). However, the finding that acupoint application can reverse these abnormal bacterial phenotypes and restore them to normal levels is encouraging.

5. Conclusions

In summary, the current study demonstrated that acupoint application of Tongfu Powder acted to counter constipation as it effectively restored gastrointestinal motility by increasing fecal moisture, promoting colonic transit, and relieving colonic inflammation. The potential mechanisms of acupoint application might be related to the regulation of the intestinal barrier and gut microbiota. It significantly increased the number of ICC and enhanced intestinal 5-HT synthesis. Acupoint application of Tongfu Powder also promoted MUC2 secretion, increased the expression of tight junction proteins (Claudin-1 and Occludin), and improved the composition of the gut microbiota (Figure 10). In addition, the laxative effect of Tongfu Powder acupoint application was not inferior to that of lactulose, and the combination therapy produced a superior effect. Therefore, this study provides a reliable theoretical foundation for the clinical application of acupoint application, which may serve as a promising alternative therapy for constipation.

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