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ORIGINAL RESEARCH

Olive Oil Solution of Volatile Oil from *Citri Reticulatae Pericarpium Viride* Alleviates Slow-Transit Constipation via Regulating SCF/c-Kit Signaling Pathway and Intestinal Flora

Shuting Zou[1,*], Bin Xie[2,*], Zhentao An[1], Fang Li[3], Li Cui[1], Zhenhai Zhang[1], Weiquan Bu[1], Dandan He[1]

[1]Affiliated Hospital of Integrated Traditional Chinese and Western Medicine, Nanjing University of Chinese Medicine, Nanjing, Jiangsu, 210028, People's Republic of China; [2]Department of Gastroenterology, Nanjing Lishui District Hospital of Traditional Chinese Medicine, Nanjing, Jiangsu, 211200, People's Republic of China; [3]Department of Gastroenterology, Changshu Hospital Affiliated to Nanjing University of Chinese Medicine, Changshu, Jiangsu, 215500, People's Republic of China

*These authors contributed equally to this work

Correspondence: Weiquan Bu; Dandan He, Affiliated Hospital of Integrated Traditional Chinese and Western Medicine, Nanjing University of Chinese Medicine, No. 100, Cross Street, Hongshan Road, Qixia District, Nanjing, Jiangsu, 210028, People's Republic of China, Email buweiquan2008@126.com; happydandan007@163.com

Objective: The aroma of the aromatic class of traditional Chinese medicines can promote gastrointestinal peristalsis. This study aimed to explore the mechanisms by which volatile oil from *Citri Reticulatae Pericarpium Viride* (VOCRPV) alleviates slow-transit constipation (STC).

Methods: The main active ingredients in VOCRPV were determined by High-Performance Liquid Chromatography (HPLC). Due to poor stability, an olive oil solution was prepared to enhance the volatile oil's stability. A mouse model of STC was induced using loperamide hydrochloride. The mice's body weight was monitored weekly. The number of fecal pellets, fecal water content, and small intestinal propulsion rate were detected. The colon tissues were analyzed using HE staining. The serum content of gastrointestinal hormones was measured using the corresponding ELISA kit. The protein expressions of stem cell factor (SCF) and c-Kit in colon tissues were detected by Western blot and immunohistochemistry methods. The 16S rRNA gene sequencing was used to detect the intestinal flora.

Results: The contents of p-isopropyl toluene, γ-Terpinene, and d-Limonene were determined by HPLC. VOCRPV and its olive oil solution significantly enhanced body weight, increased the number of fecal pellets, improved fecal water content, and boosted small intestinal propulsion rate in mice with loperamide-induced STC, while also repairing colon mucosa damage. They also increased gastrin (Gas) and motilin (MTL) levels in treated mice, upregulated the expression of SCF and c-Kit proteins, and restored intestinal flora balance in STC mice.

Conclusion: VOCRPV could effectively alleviate STC, and olive oil enhances its therapeutic effect. VOCRPV alleviates STC by elevating Gas and MTL levels, activating the SCF/c-Kit signaling pathway, and modulating intestinal flora.

Keywords: slow-transit constipation, volatile oil from *Citri Reticulatae Pericarpium Viride*, olive oil, stem cell factor/c-Kit signaling pathway, intestinal flora

Introduction

Functional constipation (FC), a global gastrointestinal dysfunction, has recently been shown to have a significantly high prevalence.[1,2] It is estimated that 15.3% of adults suffer from FC.[3] Slow-transit constipation (STC) accounts for approximately 55% of FC cases,[4] and its main clinical manifestations include reduced intestinal motility and prolonged passage of feces through the intestine. STC occurrence has increased with the rapid modern life pace and mounting

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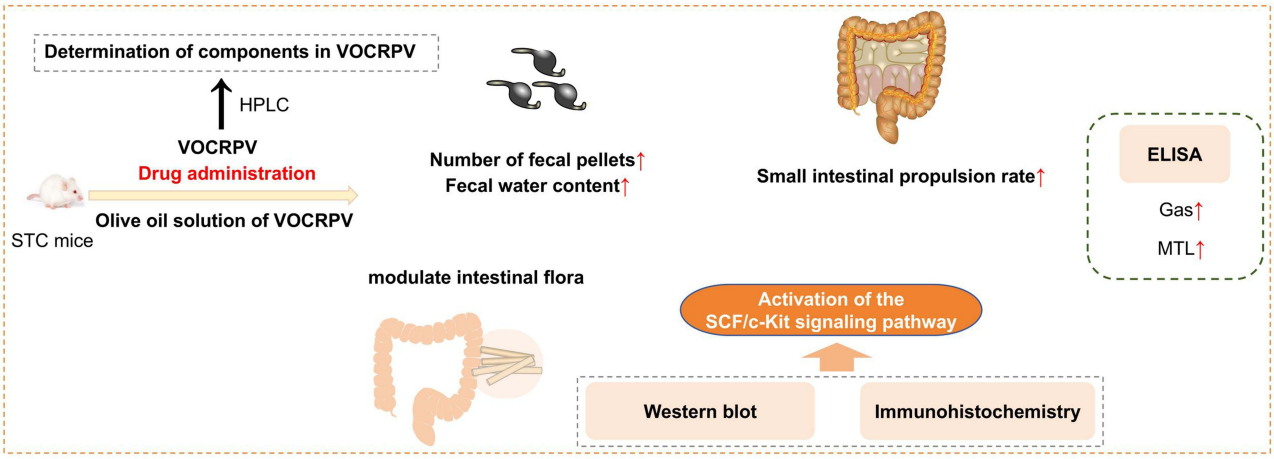
Graphical Abstract



pressures. If STC is not managed effectively, the condition gradually worsens. Patients may experience longer intervals between bowel movements, drier stools, and more difficult bowel movements and may even need to rely on medication or enemas to assist bowel movements. Prolonged constipation leads to complications, such as anal fissures, hemorrhoids, rectal prolapse, and other anorectal diseases. Due to the long-term retention of feces in the intestines, harmful bacteria increase, which can cause intestinal inflammation, gut microbiota imbalance, and other problems. In addition, STC negatively affects the patient's mental health, leading to emotional problems, such as anxiety and depression, which aggravate the symptoms of constipation and form a vicious cycle. Currently, drugs used to treat STC include volumetric laxatives (eg, methylcellulose and polycarbophil calcium), osmotic laxatives (eg, lactulose), and prokinetic drugs (eg, prucalopride and mosapride).[5–7] However, methylcellulose, polycarbophil calcium, and lactulose frequently induce bloating, while prucalopride and mosapride sometimes cause diarrhea and headache.[8–10] Some patients experience weakened or ineffective efficacy after the use of these drugs for a certain period. Therefore, the development of therapeutic drugs that are more effective in promoting intestinal transport and improving constipation symptoms with fewer side effects is urgently needed.

The aromatic class of traditional Chinese medicines has a unique position and an important role in disease treatment and is often used in the treatment of digestive system diseases, nervous system diseases, etc. Aromas of the aromatic class of traditional Chinese medicines can stimulate gastrointestinal nerves, promote gastrointestinal peristalsis, and enhance digestive function, thus improving constipation symptoms.[11] Aromas of the aromatic class of traditional Chinese medicines can also soothe emotions and relieve stress and anxiety. Patients with constipation are often affected by mental and psychological factors, thus regulating mood helps to improve constipation. Compared with some chemical drugs, the aromatic class of traditional Chinese medicines usually has relatively fewer side effects and is more suitable for long-term conditioning. *Citri Reticulatae Pericarpium Viride* (CRPV) is the dried infantile fruit or immature fruit peel of *Citrus reticulata* Blanco and its cultivars.[12] Its main active compounds include flavonoids, volatile oils, and alkaloids.[13] Among them, flavonoids have antioxidant and antitumor activities.[14,15] The study showed that the total flavonoid content of CRPV was 303.836 mg/g (total flavonoids is expressed as rutin (mg/g)).[16] d-Limonene, a major bioactive compound in the volatile oil of *Citri Reticulatae Pericarpium Viride* (VOCRPV), promotes gastrointestinal peristalsis and demonstrates antibacterial activity.[17–19] Current pharmacological research has shown that CRPV has a regulatory effect on the smooth muscles of the gastrointestinal tract and can promote the secretion of digestive juices, which helps to improve digestive function.[20] VOCRPV has the potential to treat STC, as it is one of the main pharmacodynamic components of CRPV. However, no study has been reported on the relief of STC by the VOCRPV. Owing to its volatility and poor stability,[21,22] volatile oil usually evaporates on its own at 25°C and is sensitive to environmental factors, such as air, light,

and temperature, limiting its clinical application. Olive oil, as a nutritious grease, contains natural antioxidant components, such as vitamin E and polyphenols, which protect the active components of volatile oil from oxidative damage. An olive oil solution of volatile oil can effectively reduce the contact area of volatile oil with the outside air and slow the oxidation process. In addition, VOCRPV has good solubility in olive oil.[23] Therefore, to enhance the stability of the VOCRPV, it can be prepared as an olive oil solution.

Inadequate intestinal motility is one of the most important factors in the development of STC.[24] Under normal conditions, muscle contraction and peristalsis in the intestine propel food residue through the intestine and eventually out of the body. When the intestinal tract is underpowered, the frequency of peristalsis decreases, and the peristaltic force is weakened, leading to prolonged retention of feces in the intestinal tract. This excessively absorbs water in the feces, which then becomes dry and hard, further increasing the difficulty of defecation. In addition, insufficient intestinal motility affects the function of the intestinal nervous system and impairs coordinated movement of the intestine, further aggravating constipation symptoms.

The development of STC is intimately linked to an imbalance in intestinal flora. Normal intestinal flora produces beneficial metabolites, such as short-chain fatty acids, to promote intestinal peristalsis. When the intestinal flora is dysregulated, the production of these beneficial metabolites is reduced, leading to insufficient intestinal motility and slowing fecal transmission, thus triggering STC.[25] The intestinal flora participates in the neuromodulation of gut motility by interacting with the enteric nervous system. Dysregulated intestinal flora disrupt normal neural communication and affect rhythmic contractions and peristalsis in the intestine, promoting STC formation.[26] Unhealthy intestinal flora affects the sensitivity of enteric nerves and the function of intestinal smooth muscles by causing chronic intestinal inflammation and intestinal mucosal damage, leading to intestinal transmission dysfunction and increasing the risk of STC.[27] The altered intestinal environment in patients with STC, such as prolonged intestinal passage time, further aggravates the intestinal flora imbalance, forming a vicious cycle. Dysregulated intestinal flora impair food digestion and absorption, disrupt metabolic processes, and alter the intestinal environment (eg, through pH changes and electrolyte imbalances). These alterations further compromise normal intestinal peristalsis and transport functions.[28] An imbalance in intestinal flora is closely related to the initiation and progression of STC, and these two interact to form a complex pathophysiological mechanism.

This study explored the mechanism of action of VOCRPV in alleviating STC by investigating its effects on the related indicators of STC, histopathological changes in the colon, gastrointestinal hormone levels, the stem cell factor (SCF)/c-Kit pathway, and intestinal flora.

Materials and Methods

Reagents and Chemicals

Olive oil was purchased from Macklin Biochemical Science and Technology Co., Ltd. (Shanghai, China). p-isopropyl toluene (98%), γ-Terpinene (> 95%, GC), and d-Limonene (95%) were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Loperamide hydrochloride (HPLC ≥ 98%) was obtained from Chengdu Desite Biotechnology Co., Ltd. (Chengdu, China). Mosapride (98% purity) was purchased from Guangzhou Cola Biotechnology Co., Ltd. (Guangzhou, China). The activated carbon powder and gum Arabic were obtained from Wuxi Yatai United Chemical Co., Ltd. (Wuxi, China). Gastrin (Gas) and motilin (MTL) ELISA kits were purchased from Shanghai Kaijing Biotechnology Co., Ltd. (Shanghai, China). Antibodies against SCF, c-Kit, and β-actin were obtained from Proteintech Group, Inc (Wuhan, China).

Animals

Eighty male Kunming mice of SPF grade (7–8 weeks, 20 ± 2 g) were purchased from Sipeifu (Suzhou) Biotechnology Co., Ltd. (China) with an animal quality certificate NO.202404028. The animals were housed in the Jiangsu Province Academy of Traditional Chinese Medicine's experimental animal center at room temperature (25 ± 2°C) with relative humidity (55 ± 5%). Animal procedures were conducted under the approved guidelines of the Animal Ethics Committee

of Jiangsu Provincial Academy of Chinese Medicine (Approval number: NO. AEWC-20240131-367), and followed the National Research Council Guide.

Extraction of VOCRPV and Preparation of Its Olive Oil Solution

CRPV was provided by the Nanjing Shangyuantang pharmacy (Nanjing, China) and identified by Dr. Li Cui of the Third Clinical Medical College of Nanjing University of Chinese Medicine. CRPV (300 g) was crushed and placed in a 5000 mL flask. Subsequently, 10 times the volume of water was added. After soaking for 1 h, the volatile oil tester was connected to a reflux-condensing tube. The upper end of the self-condensing tube was filled with water to fill the scale of the volatile oil detector, which overflowed into the flask. The flask was placed in an electric heating jacket, slowly heated to boiling, and kept slightly boiled for approximately 3 h until the amount of oil in the detector no longer increased and heating was stopped. After a few moments, the piston at the lower end of the detector was opened, and the water was slowly discharged to collect the volatile oil. The 1 mL of volatile oil collected was sealed and stored at 4°C. An appropriate amount of VOCRPV was mixed with olive oil and sonicated to prepare the olive oil solution of VOCRPV.

Determination of Components in VOCRPV

10mg of VOCRPV was weighed precisely, and acetonitrile was used as a solvent to prepare a sample solution of 0.2 mg/mL. An appropriate amount of each of the p-isopropyl toluene, γ-Terpinene, and d-Limonene standard substances was weighed precisely, and a mixed reference solution was prepared using acetonitrile as the solvent. Chromatographic evaluations were performed using the US Agilent 1260 high-Performance Liquid Chromatography (HPLC). The ZORBAX Extend C18 column (250 × 4.6 mm, 5 μm) was used. Acetonitrile (A) and water (B) were mobile phases. The test conditions were shown in Table 1. The content was calculated after obtaining the peak area.

STC Mouse Model Establishment, Grouping, and Administration

80 mice were randomly divided into eight groups equally: normal control group (NC), model group (Mod), mosapride group (Mos, 3 mg/kg), olive oil group (OO, 6 mg/kg), low-dose VOCRPV group (VOCRPV-LD, 100 mg/kg), high-dose VOCRPV group (VOCRPV-HD, 200 mg/kg), olive oil + low-dose VOCRPV group (OO + VOCRPV-LD, 6 mg/kg olive oil + 100 mg/kg VOCRPV), and olive oil + high-dose VOCRPV group (OO + VOCRPV-HD, 6 mg/kg olive oil + 200 mg/kg VOCRPV). To establish the STC model, a loperamide hydrochloride suspension (10 mg/kg) was intragastrically administered to the mice twice daily at 8:30–9:30 and 15:30–16:30 for 14 consecutive days. Following model establishment, the mice were intragastrically administered the corresponding drug once daily for 14 days. Equal volumes of distilled water were intragastrically administered to the NC and Mod groups. To maintain the stability of the model, according to previous studies and pre-experiments,[29] loperamide hydrochloride was administered during drug administration. The experimental dose in this study was obtained from the pre-experiments.

Detection of Body Weight, Number of Fecal Pellets, and Fecal Water Content

The mice were weighed once weekly. Feces were continuously collected for 4 h after the end of modeling and administration in each group. The number of fecal pellets was calculated, and the wet weight of the feces was weighed.

Table 1 The conditions for testing component

Component	The Concentration of Each Component in the Mixed Reference Solution	Gradient Condition
p-isopropyl toluene	0.086 mg/mL	0–5 min, 5–10% A; 5–10 min, 10–70% A; 10–40 min, 70–80% A; 40–45 min, 80–100% A
γ-Terpinene	0.085 mg/mL	
d-Limonene	0.084 mg/mL	

Notes: Other: the flow rate was 1.0 mL/min; the detection wavelength was set to 205 nm; the column temperature was 25°C; the injection volume was set at 10 μL.

Then the feces were dried, and constant weight was recorded as the dry weight of the feces. The formula used to determine the fecal water content was fecal water content (%) = (wet weight - dry weight)/wet weight × 100%. [30]

Detection of Small Intestinal Propulsion Rate

The activated charcoal powder was mixed with gum Arabic to prepare a suspension of 10% activated charcoal and 5% gum Arabic. Each mouse was given 0.3 mL of the suspension by intragastric administration, which was executed half an hour later. Subsequently, the mesentery was isolated, and the small intestine was removed and laid flat on a filter paper without additional traction. The small intestinal propulsion rate was calculated using the following formula: Small intestinal propulsion rate (%) = distance of charcoal powder propulsion in the intestine/full length of the small intestine × 100%.[31]

Sample Collection

When the last administration ended, the mice in each group were fasted overnight but allowed to drink freely. Blood samples were collected and centrifuged for 15 minutes at 4°C and 3000 ×g, and the upper layer of serum was taken. Feces were collected and frozen at −80°C. The colon tissues were collected, washed with saline, part of which were immersed in 4% paraformaldehyde fixation fluid, and the others were stored at −80°C for subsequent analysis.

HE Staining Analysis of Colon Tissue

Mouse colon tissues were fixed in 4% paraformaldehyde for 24 h, dehydrated and transparentized with gradient ethanol and xylene, dipped in wax, embedded, and cut into 5 μm slices. The cell nucleus and cytoplasm were stained with hematoxylin and eosin. Sections were dehydrated, made transparent, sealed, and observed under a microscope (Olympus IX73).

Determination of Gas and MTL in Serum

Serum Gas and MTL contents were measured by ELISA kits. A microplate reader was used to measure the absorbance, and the Gas and MTL contents in the serum of each group were calculated.

Western Blot Analysis

RIPA buffer was used to homogenize the tissues of the mouse colon, the homogenate was centrifuged, and the supernatant was collected as the total protein extract. SDS-PAGE electrophoresis was used to separate equivalent amounts of protein, which were then transferred to polyvinylidene difluoride (PVDF) membranes. Sealed membranes, the membranes were incubated with primary antibodies against SCF and c-Kit at 4°C overnight. TBST was used to wash the membranes multiple times. The membranes were incubated with secondary antibodies labeled with horseradish peroxidase (HRP) for an hour at room temperature with shaking. The membranes were washed several times with TBST. The color reaction was performed using chemiluminescent substrates, and the signals were captured using a chemiluminescent imaging system. The bands were analyzed using ImageJ software, and expression levels of each protein were calculated using β-actin. Based on the resource equation approach,[32] it was calculated that a minimum of 3 animals were needed for each group in this study. Meanwhile, the sample size of each group in the Western blot was set to 3 based on reference to related literature.[33–36]

Immunohistochemistry Analysis

Paraffin sections of the colon tissues were dewaxed in xylene and subsequently hydrated using an ethanol gradient. Antigen repair was performed by heat induction, endogenous peroxidase activity was blocked with a 3% hydrogen peroxide solution, and the sections were sealed with BSA at room temperature. Sections were incubated with primary antibodies against SCF and c-Kit overnight at 4°C and washed thrice with PBS. The sections were incubated with biotin-labeled secondary antibodies for 30 min at room temperature with shaking. The sections were washed three times with PBS. The color development reaction was performed using a DAB chromogenic agent. The reaction was monitored under a microscope until the desired staining intensity was achieved. Distilled water was used to terminate the color

development reaction. The sections were redyed using hematoxylin, dehydrated, made transparent, and sealed. A light microscope was used to view the slices, and a camera was used to capture the images. The staining results were quantitatively analyzed using the ImagePro Plus software.

16S rRNA Detection of Mouse Intestinal Flora

After the extraction of microbial DNA, the intestinal flora of the mice was detected by Illumina sequencing. PCR amplification of the highly variable region V3-V4 of the 16S rRNA gene was performed using universal primers (338F and 806R). Data analysis and information mining were performed on the Shanghai Majorbio Bio-pharm Technology Co., Ltd. platform.

Statistical Analysis

Statistical software SPSS 26.0 (IBM, Chicago, USA) was used for the analysis. The experimental results were presented as mean ± standard deviation. Experimental data ($n \geq 5$) were statistically compared using a one-way analysis of variance (ANOVA) for comparison among multiple groups, and the LSD-*t* test was used for pairwise comparison when there were overall differences. Experimental data ($n = 3$) were statistically compared using a bootstrap test. A *P*-value below 0.05 was regarded as statistically significant.

Results

The Content of Components in VOCRPV

The content of d-Limonene was 28.2%. The contents of p-isopropyl toluene and γ-Terpinene were 1.68% and 7.8%, respectively. Figure 1 shows the HPLC chromatogram.

Effects of VOCRPV and Its Olive Oil Solution on Relevant Indicators in STC Mice

As illustrated in Figure 2A–D, compared with the NC group, the body weight of mice, the number of fecal pellets within 4 h, fecal water content, and small intestinal propulsion rate in the Mod group were significantly reduced ($P < 0.01$). Compared with the Mod group, the body weight, number of fecal pellets within 4 h, fecal water content, and small intestinal propulsion rate in the VOCRPV-LD, VOCRPV-HD, OO + VOCRPV-LD, OO + VOCRPV-HD, and Mos groups were significantly higher ($P < 0.05$, $P < 0.01$; Figure 2E–H). Moreover, the number of fecal pellets within 4 h, fecal water content, and small intestinal propulsion rate in the OO + VOCRPV-LD and OO + VOCRPV-HD groups were higher than those in the VOCRPV-LD and VOCRPV-HD groups, respectively ($P < 0.05$, $P < 0.01$; Figure 2F–H). However, the fecal water content did not differ significantly between the Mos and OO + VOCRRV-HD groups. These results demonstrate that VOCRPV can effectively improve the symptoms of STC mice, and olive oil can enhance its therapeutic effect.

Effects of VOCRPV and Its Olive Oil Solution on Pathological Histomorphological Changes of Colon in STC Mice

As depicted in Figure 3, the colonic tissues of mice in the NC group showed clear tissue structures with smooth and intact mucosal surfaces, good crypt morphology, and no inflammatory cell aggregation. However, the colonic tissues of mice in the Mod and OO groups were disorganized, with damaged mucosal layers and crypts, decreased numbers of goblet cells, and infiltration of inflammatory cells. The colonic mucosal structure of mice in all treatment groups (VOCRPV-LD, VOCRPV-HD, OO + VOCRPV-LD, OO + VOCRPV-HD, and Mos groups) was intact. In addition, inflammatory cell infiltration and crypt damage were alleviated, and there were more goblet cells.

Effects of VOCRPV and Its Olive Oil Solution on Serum Gastrointestinal Hormone Levels in STC Mice

We analyzed serum levels of gastrointestinal hormones (Figure 4). The Mod group showed a significant reduction in Gas and MTL content compared to the NC group ($P < 0.01$). However, the levels of Gas and MTL in the VOCRPV-LD,

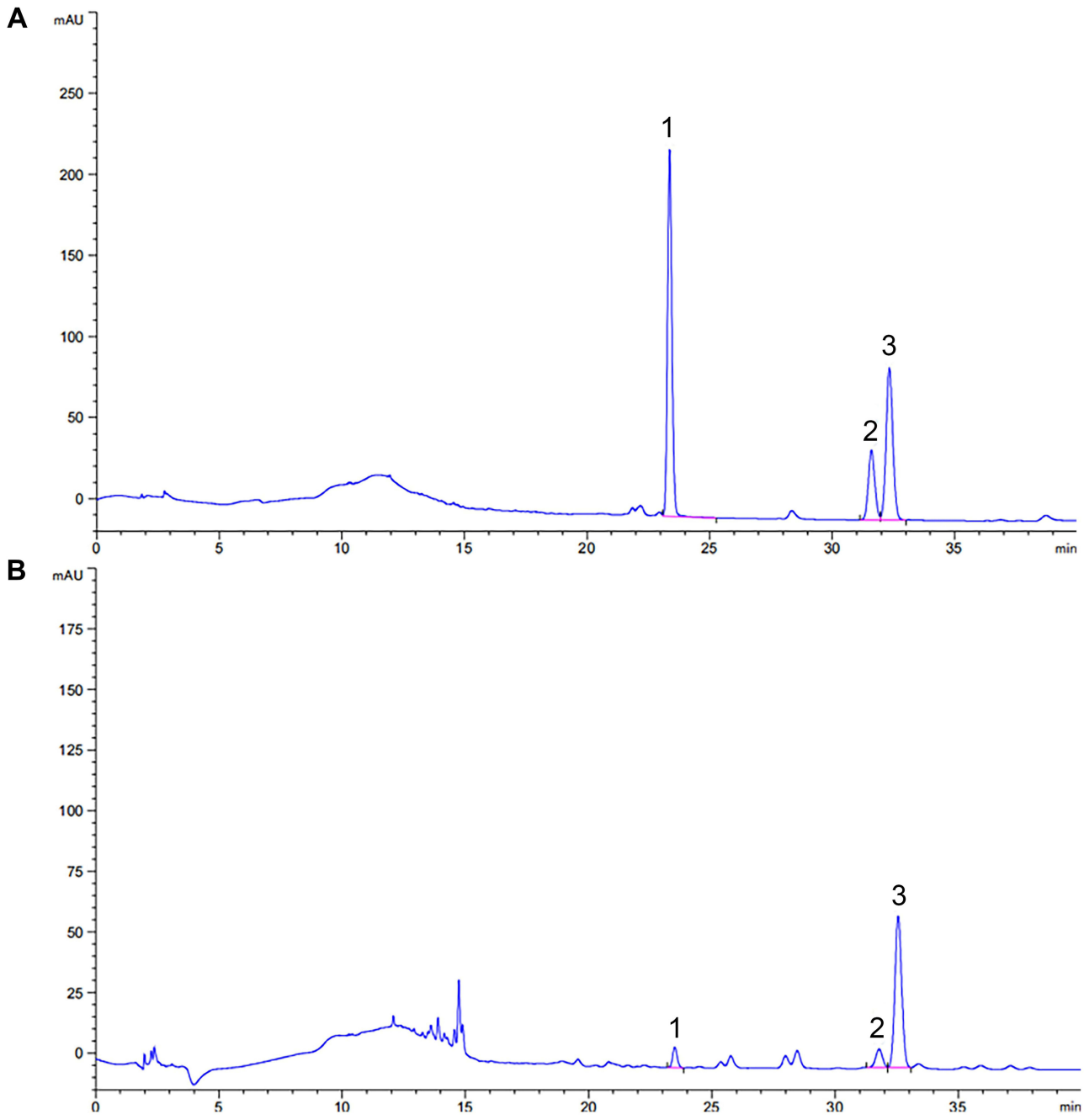


Figure 1 HPLC Chromatogram of the mixed reference solution and the sample solution. (**A**) The HPLC Chromatogram of the mixed reference solution. (**B**) The HPLC Chromatogram of the sample solution. 1. p-isopropyl toluene, 2. γ-Terpinene, 3. d-Limonene.

VOCRPV-HD, OO + VOCRPV-LD, OO + VOCRPV-HD, and Mos groups were significantly higher than those in the Mod group ($P < 0.05$, $P < 0.01$). When compared to the VOCRPV-LD and VOCRPV-HD groups, the Gas content in the OO + VOCRPV-HD group was higher ($P < 0.01$). However, there was no significant variation in Gas content between the OO + VOCRPV-HD and Mos groups. The OO + VOCRPV-LD and OO + VOCRPV-HD groups had higher MTL levels than the VOCRPV-LD and VOCRPV-HD groups ($P < 0.01$). These results indicated that the VOCRPV significantly enhanced the levels of gastrointestinal hormones (Gas and MTL) in STC mice. It is noteworthy that compared with VOCRPV, the effect of the olive oil solution of VOCRPV on gastrointestinal hormones in STC mice was more significant.

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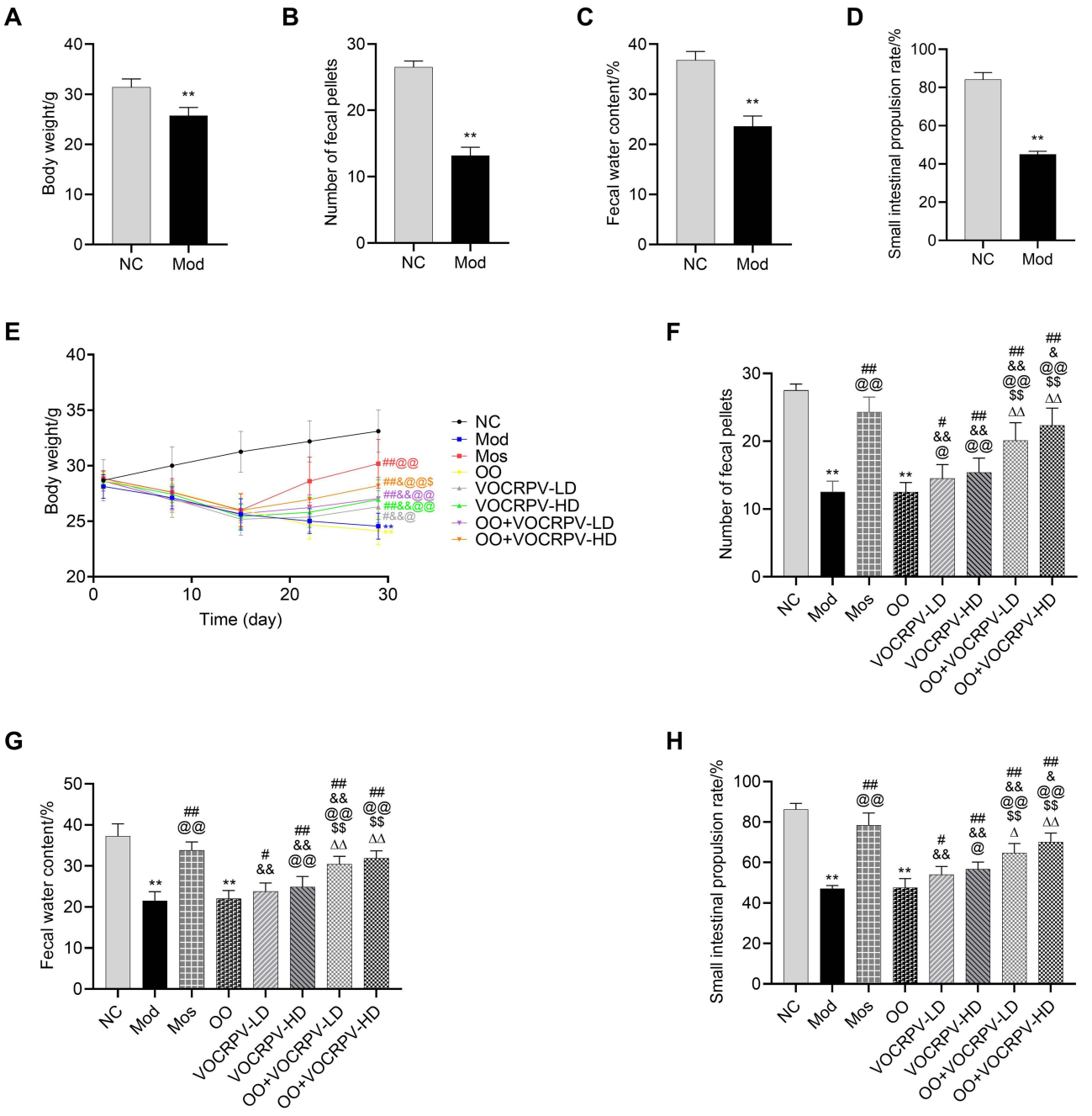

Figure 2 Effects of VOCRPV and its olive oil solution on relevant indicators in STC mice. Body weight (**A**), number of fecal pellets (**B**), fecal water content (**C**), and small intestinal propulsion rate (**D**) in the NC and Mod groups after the end of modeling. (**E**) Body weight during modeling and treatment. Number of fecal pellets (**F**), fecal water content (**G**), and small intestinal propulsion rate (**H**) after the end of treatment. Data are expressed as mean ± SD (n = 8). Each repeat was performed as a separate, independent experiment or observation. $^{**}P < 0.01$, vs NC group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs Mod group; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, vs Mos group; $^{@}P < 0.05$, $^{@@}P < 0.01$, vs OO group; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, vs VOCRPV-LD group; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$, vs VOCRPV-HD group.

Abbreviations: NC, normal control; Mod, model; Mos, mosapride; OO, olive oil; VOCRPV-LD, low-dose VOCRPV; VOCRPV-HD, high-dose VOCRPV; OO + VOCRPV-LD, olive oil + low-dose VOCRPV; OO + VOCRPV-HD, olive oil + high-dose VOCRPV.

Effects of VOCRPV and Its Olive Oil Solution on SCF/c-Kit Signaling Pathway

As indicated in Figure 5, Western blot results showed that the protein expression of SCF and c-Kit in the Mod group was significantly lower than that in the NC group ($P < 0.01$). Compared with the Mod group, the protein expression of SCF and c-Kit in the VOCRPV-LD, VOCRPV-HD, OO + VOCRPV-LD, and OO + VOCRPV-HD groups was significantly higher ($P < 0.05$, $P < 0.01$). The protein expression levels of SCF and c-Kit in the OO + VOCRPV-LD and OO +

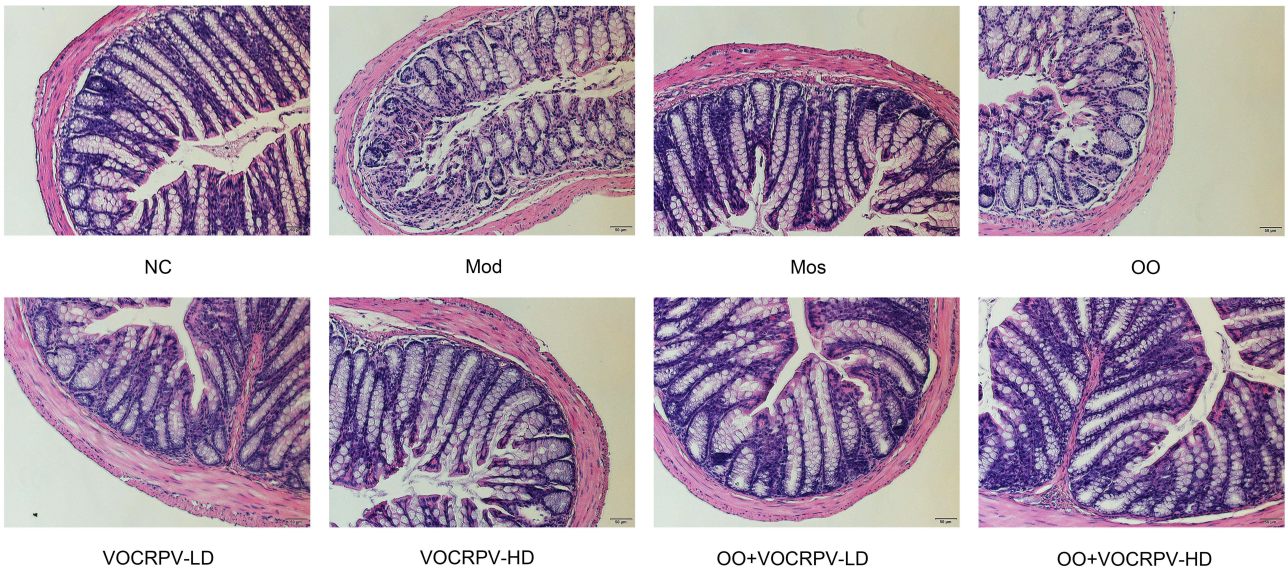

Figure 3 Effects of VOCRPV and its olive oil solution on pathological histomorphological changes of colon in STC mice (×200).
Abbreviations: NC, normal control; Mod, model; Mos, mosapride; OO, olive oil; VOCRPV-LD, low-dose VOCRPV; VOCRPV-HD, high-dose VOCRPV; OO + VOCRPV-LD, olive oil + low-dose VOCRPV; OO + VOCRPV-HD, olive oil + high-dose VOCRPV.

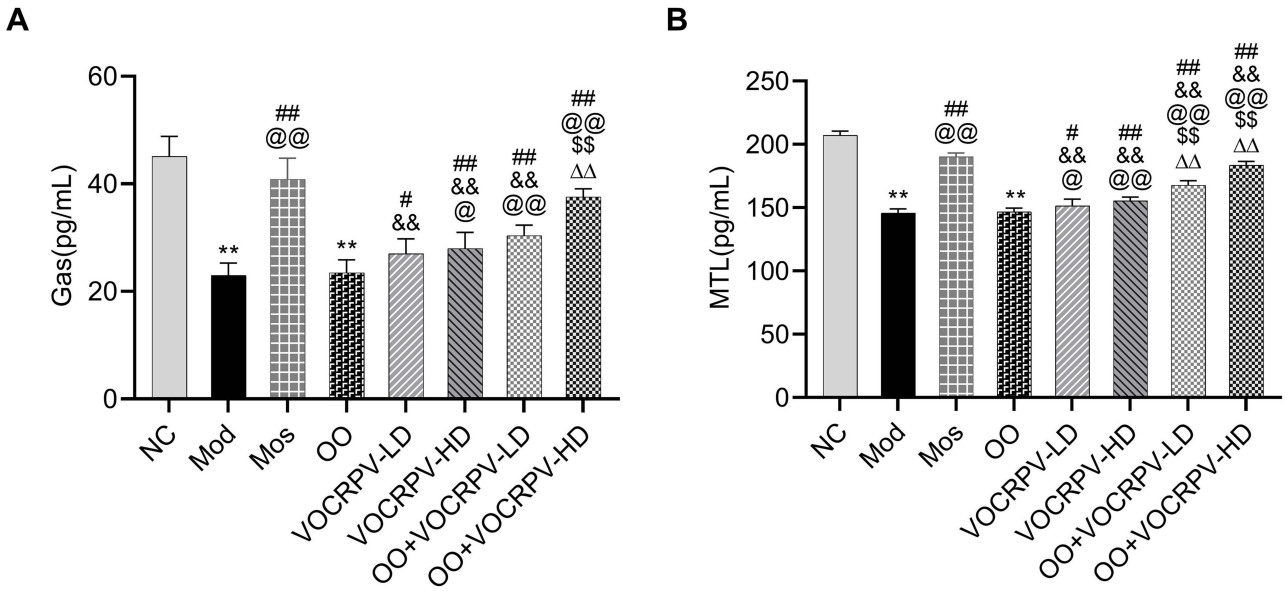

Figure 4 Effects of VOCRPV and its olive oil solution on serum gastrointestinal hormone levels in STC mice. (**A**) Serum Gas level. (**B**) Serum MTL level. Data are expressed as mean ± SD (n = 5). Each repeat was performed as a separate, independent experiment or observation. $^{**}P < 0.01$, vs NC group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs Mod group; $^{\&\&}P < 0.01$, vs Mos group; $^{@}P < 0.05$, $^{@@}P < 0.01$, vs OO group; $^{\$\$}P < 0.01$, vs VOCRPV-LD group; $^{\Delta\Delta}P < 0.01$, vs VOCRPV-HD group.
Abbreviations: Gas, gastrin; MTL, motilin; NC, normal control; Mod, model; Mos, mosapride; OO, olive oil; VOCRPV-LD, low-dose VOCRPV; VOCRPV-HD, high-dose VOCRPV; OO + VOCRPV-LD, olive oil + low-dose VOCRPV; OO + VOCRPV-HD, olive oil + high-dose VOCRPV.

VOCRPV-HD groups were higher than those in the VOCRPV-LD and VOCRPV-HD groups ($P < 0.01$). As indicated in Figure 6, immunohistochemistry results showed that the Mod group exhibited a significant decrease in SCF and c-Kit protein expression levels compared to the NC group ($P < 0.01$). However, the VOCRPV-HD, OO + VOCRPV-LD, and OO + VOCRPV-HD groups had higher SCF and c-Kit protein expression levels compared to the Mod group. ($P < 0.05$, $P < 0.01$). The protein expression of c-Kit in the OO + VOCRPV-HD group was higher than that in the VOCRPV-LD group ($P < 0.05$). The above results indicate VOCRPV could activate the SCF/c-Kit signaling pathway to improve STC, and olive oil increased the effect of VOCRPV on the SCF/c-Kit signaling pathway.

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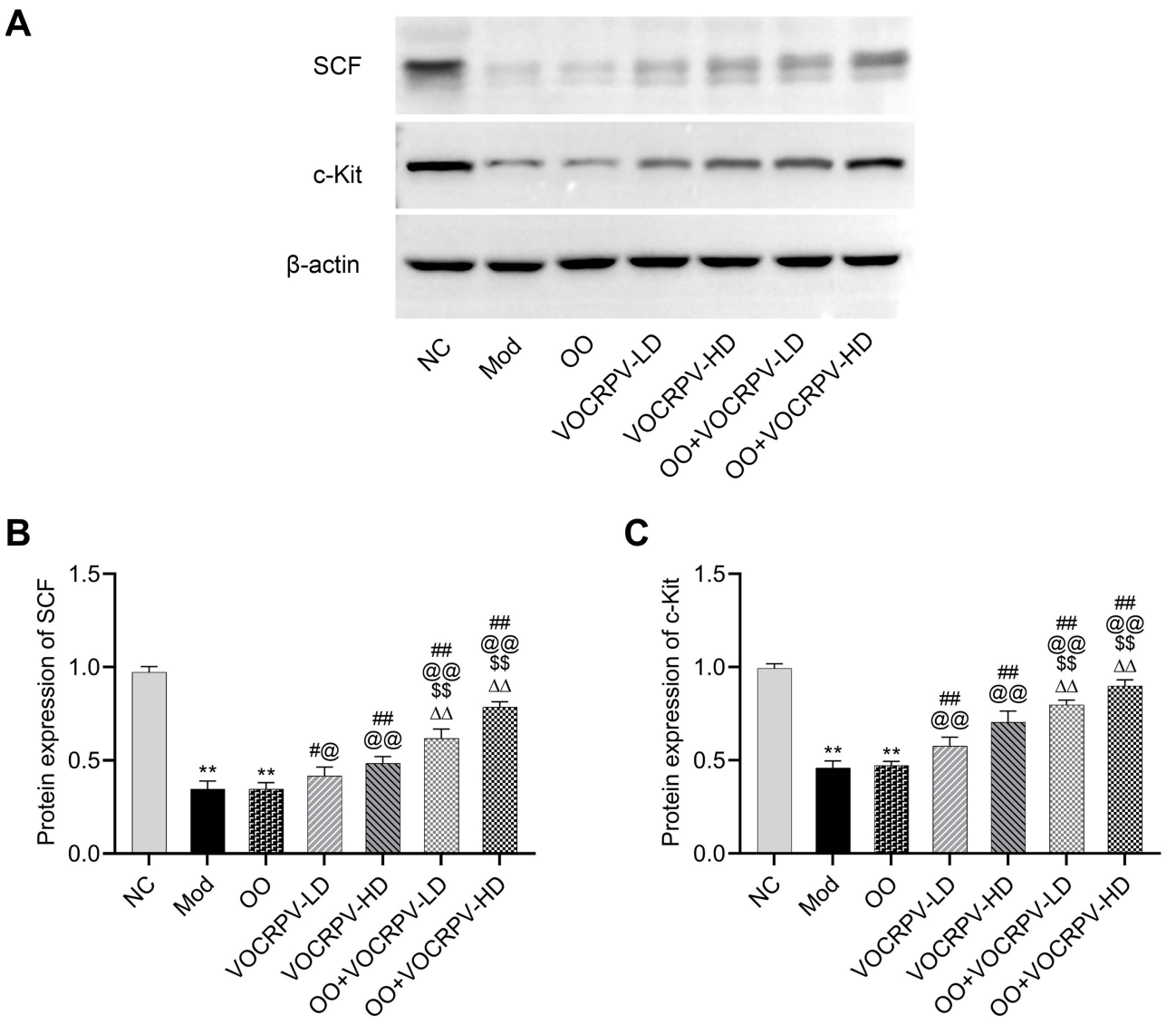

Figure 5 Western blot detection of SCF and c-Kit protein expression in the colon tissues of mice. (**A**) Detection of SCF and c-Kit protein expression by Western blot. (**B**) Quantitative analysis of SCF protein expression. (**C**) Quantitative analysis of c-Kit protein expression. Data are expressed as mean ± SD (n = 3). Each repeat was performed as a separate, independent experiment or observation. $^{**}P < 0.01$, vs NC group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs Mod group; $^{@}P < 0.05$, $^{@@}P < 0.01$, vs OO group; $^{\$\$}P < 0.01$, vs VOCRPV-LD group; $^{\Delta\Delta}P < 0.01$, vs VOCRPV-HD group.

Abbreviations: SCF, stem cell factor; NC, normal control; Mod, model; OO, olive oil; VOCRPV-LD, low-dose VOCRPV; VOCRPV-HD, high-dose VOCRPV; OO + VOCRPV-LD, olive oil + low-dose VOCRPV; OO + VOCRPV-HD, olive oil + high-dose VOCRPV.

VOCRPV and Its Olive Oil Solution Influenced the Alpha- and Beta-Diversity of Intestinal Flora

The Sobs index reflects the actual number of OTUs (operational taxonomic units) in the intestinal flora of the sample, and the Shannon index reflects the diversity of the community. The coverage index is primarily used to detect species coverage by sequencing. As shown in Figure 7, the Sobs and Shannon indices of the Mod group showed significant reduction compared to the NC group ($P < 0.01$), indicating that the diversity and richness in the Mod group were reduced. Compared with the Mod group, the Sobs and Shannon indices in the VOCRPV-HD, OO + VOCRPV-LD, and OO + VOCRPV-HD groups were remarkably higher ($P < 0.05$, $P < 0.01$). In addition, the OO + VOCRPV-LD group had a higher Sobs index than the VOCRPV-LD group ($P < 0.05$). The OO+VOCRPV-HD group demonstrated a higher Sobs index than the VOCRPV-LD and VOCRPV-HD groups ($P < 0.01$) and a higher Shannon index than the VOCRPV-LD group ($P < 0.01$). Each group had a coverage index greater than 0.9, suggesting that sequencing had wide coverage of the

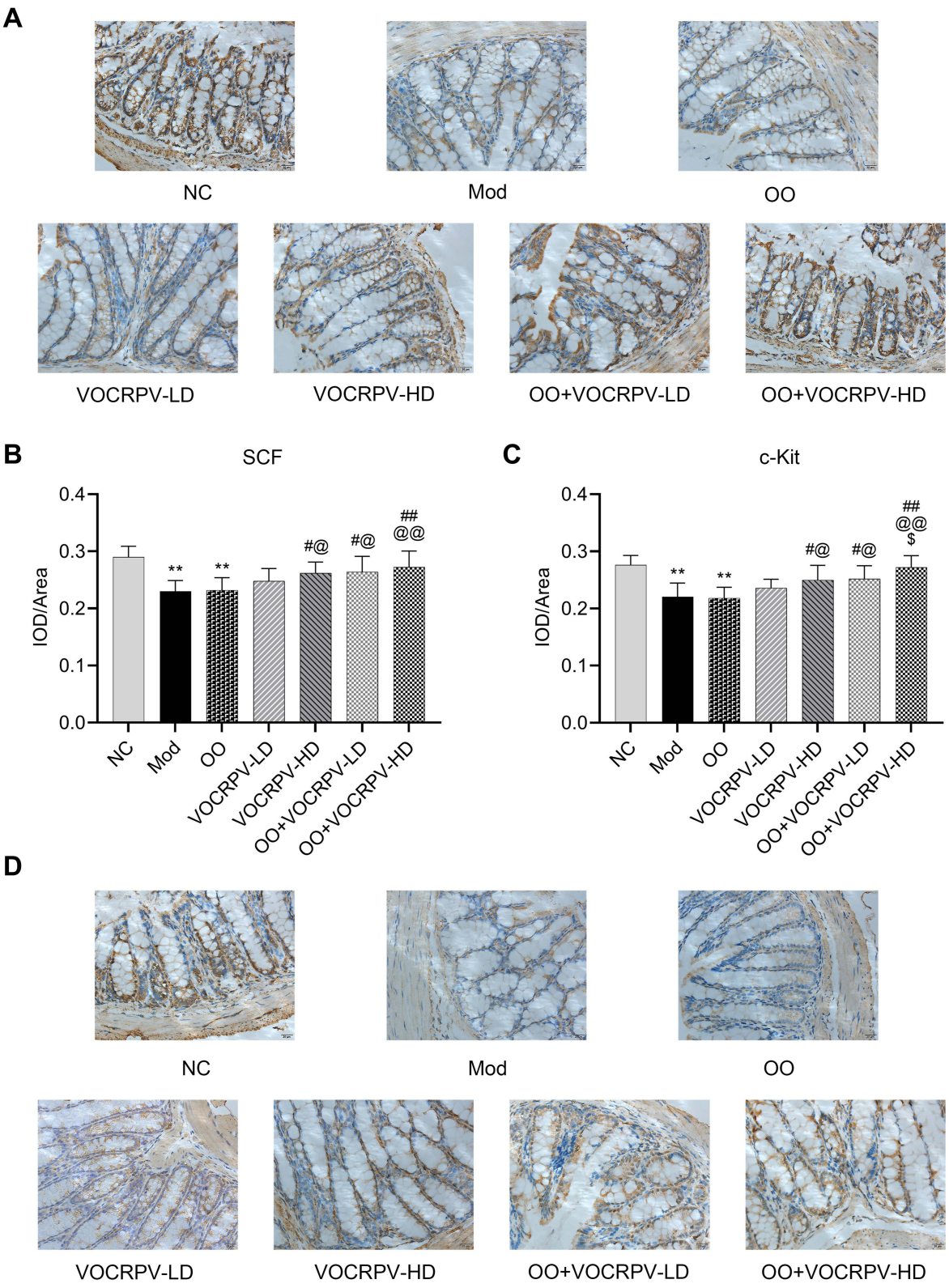

Figure 6 Immunohistochemistry detection of SCF and c-Kit expression in the colon tissues of mice. (**A**) The level of SCF protein detected by immunohistochemistry analysis (×400). (**B**) Quantitative analysis of SCF protein. (**C**) Quantitative analysis of c-Kit protein. (**D**) The level of c-Kit protein detected by immunohistochemistry analysis (×400). Data are expressed as mean ± SD (n = 5). Each repeat was performed as a separate, independent experiment or observation. $^{**}P < 0.01$, vs NC group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs Mod group; $^{@}P < 0.05$, $^{@@}P < 0.01$, vs OO group; $^{\$}P < 0.05$, vs VOCRPV-LD group.

Abbreviations: SCF, stem cell factor; NC, normal control; Mod, model; OO, olive oil; VOCRPV-LD, low-dose VOCRPV; VOCRPV-HD, high-dose VOCRPV; OO + VOCRPV-LD, olive oil + low-dose VOCRPV; OO + VOCRPV-HD, olive oil + high-dose VOCRPV.

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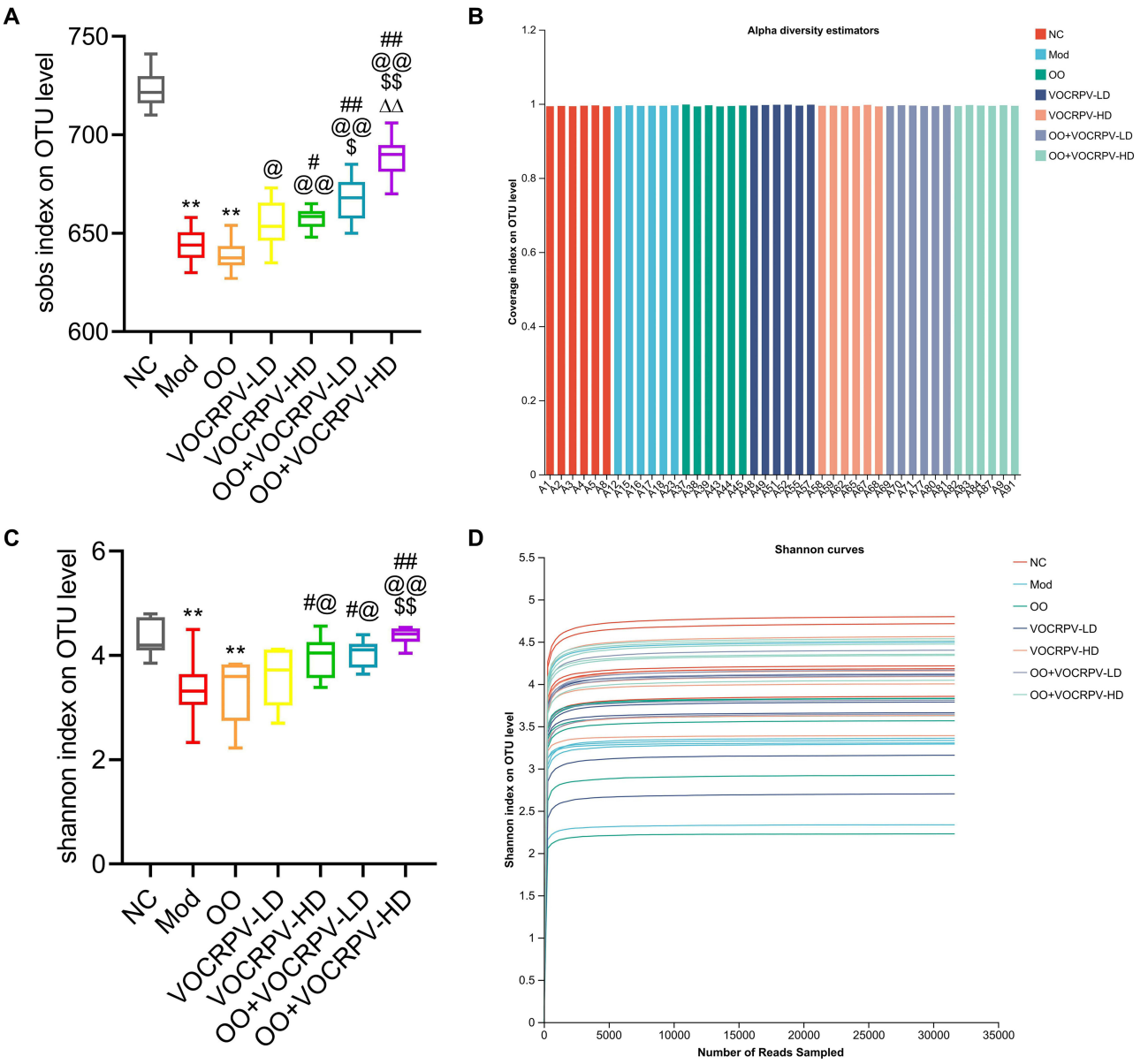

Figure 7 Effects of VOCRPV and its olive oil solution on alpha diversity in STC mice. (**A**) The Sobs index. (**B**) The coverage index. (**C**) The Shannon index. (**D**) Shannon curves. Data are expressed as mean ± SD (n = 6). Each repeat was performed as a separate, independent experiment or observation. $^{**}P < 0.01$, vs NC group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs Mod group; $^{@}P < 0.05$, $^{@@}P < 0.01$, vs OO group; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, vs VOCRPV-LD group; $^{\Delta\Delta}P < 0.01$, vs VOCRPV-HD group.

Abbreviations: OTU, operational taxonomic units; NC, normal control; Mod, model; OO, olive oil; VOCRPV-LD, low-dose VOCRPV; VOCRPV-HD, high-dose VOCRPV; OO + VOCRPV-LD, olive oil + low-dose VOCRPV; OO + VOCRPV-HD, olive oil + high-dose VOCRPV.

species. These results indicated that VOCRPV could increase the species diversity and richness of STC mice, and olive oil increased the effect of VOCRPV on species diversity and richness in STC mice. Whether there was enough data available for sequencing was determined using the Shannon index. Rarefaction curve tended to be flat, indicating that most microorganisms could be detected by sequencing.

Beta diversity analysis verifies the differences or similarities in the species among the different groups. Principal coordinates analysis (PCoA) and non-metric multidimensional scaling (NMDS) analyses were performed using the Bray-Curtis distance at the OTU level to determine the effect of VOCRPV on beta diversity in STC mice. In the PCoA and NMDS analysis plots, the species composition of the Mod group differed from that of the NC group (Figure 8A and B). The VOCRPV-LD, VOCRPV-HD, OO + VOCRPV-LD, and OO + VOCRPV-HD groups deviated from the Mod group

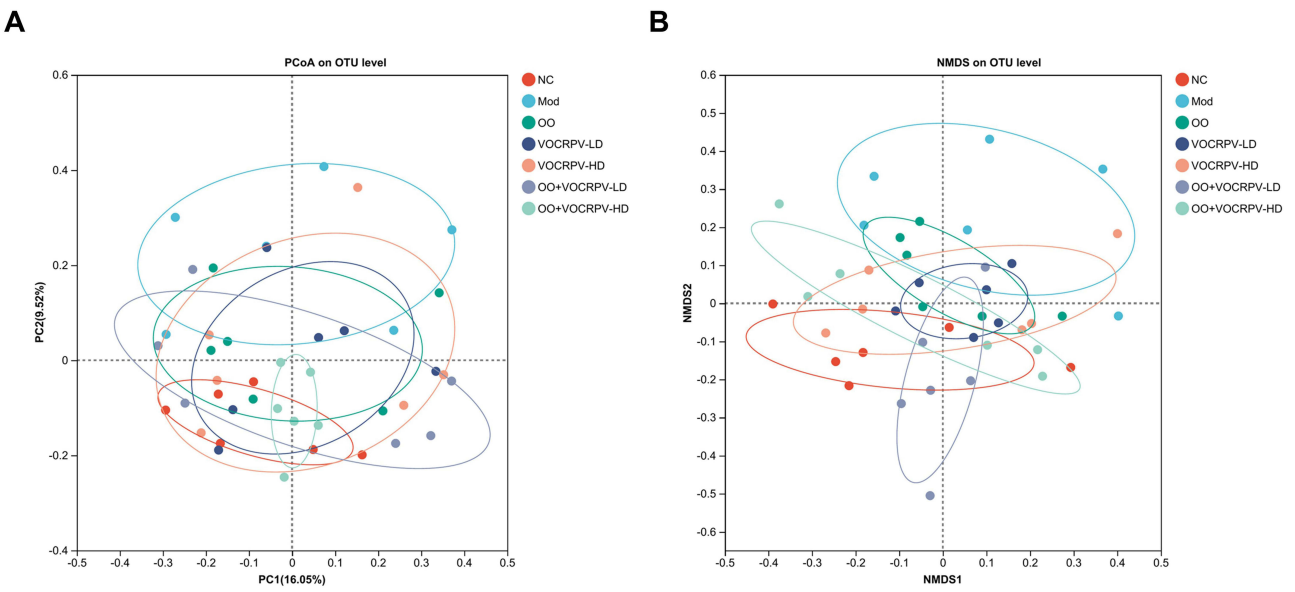

Figure 8 Effects of VOCRPV and its olive oil solution on beta diversity in STC mice. (**A**) PCoA analysis. (**B**) NMDS analysis.
Abbreviations: PCoA, Principal co-ordinates analysis; NMDS, non-metric multidimensional scaling; NC, normal control; Mod, model; OO, olive oil; VOCRPV-LD, low-dose VOCRPV; VOCRPV-HD, high-dose VOCRPV; OO + VOCRPV-LD, olive oil + low-dose VOCRPV; OO + VOCRPV-HD, olive oil + high-dose VOCRPV.

to a certain extent and tended toward the NC group (Figure 8A and B). This indicates that VOCRPV and its olive oil solution can improve beta diversity in STC mice.

VOCRPV and Its Olive Oil Solution Influenced the Composition and Abundance of Intestinal Flora

The number of common and unique OTUs in various groups can be distinguished using the Venn diagram. The number of unique OTUs of the NC, Mod, OO, VOCRPV-LD, VOCRPV-HD, OO + VOCRPV-LD, and OO + VOCRPV-HD groups was 495, 151, 213, 293, 339, 193, and 353, respectively, the number of common OTUs was 511 (Figure 9A).

At the phylum level, species with an abundance percentage of less than 0.01 were combined, and six major bacterial phyla were screened, and the species composition of each group is shown in Figure 9B. *Bacteroidota* and *Firmicutes* were the two major bacterial phyla, followed by *Campilobacterota, Verrucomicrobia, Proteobacteria*, and *Desulfobacterota*. As shown in Figure 9C and D, compared with the NC group, the abundance of *Firmicutes* in the Mod group was remarkably decreased ($P < 0.01$), and the abundance of *Bacteroidota* in the Mod group was significantly increased ($P < 0.01$). However, compared with the Mod group, the abundance of *Firmicutes* in the VOCRPV-HD, OO + VOCRPV-LD, and OO + VOCRPV-HD groups was significantly increased ($P < 0.01$), and the abundance of *Bacteroidota* in the VOCRPV-HD and OO + VOCRPV-HD groups was markedly decreased ($P < 0.01$). In addition, the abundance of *Firmicutes* in the OO + VOCRPV-LD and OO + VOCRPV-HD groups was higher than that in the VOCRPV-LD group, respectively ($P < 0.05$, $P < 0.01$).

At the genus level, twenty-nine major bacterial genera were screened, and the species composition of each group is shown in Figure 10A. Figure 10B displays the community heatmap analysis at the genus level for each group. As shown in Figure 11, loperamide hydrochloride significantly decreased the abundance of *Alloprevotella, Lactobacillus, unclassified_f__Lachnospiraceae*, and *Parabacteroides* ($P < 0.05$, $P < 0.01$) and significantly increased the abundance of *Bacteroides* and *Lachnospiraceae_NK4A136_group* ($P < 0.01$). However, when the STC mice were treated with VOCRPV-LD, VOCRPV-HD, OO + VOCRPV-LD, and OO + VOCRPV-HD, the abundance of *Alloprevotella* was significantly increased ($P < 0.01$), and the abundance of *Lachnospiraceae_NK4A136_group* was significantly decreased ($P < 0.01$). After treatment with VOCRPV-LD, VOCRPV-HD, and OO + VOCRPV-LD, the abundance of *Parabacteroides* was markedly increased ($P < 0.05$, $P < 0.01$). After treatment with VOCRPV-HD, OO + VOCRPV-

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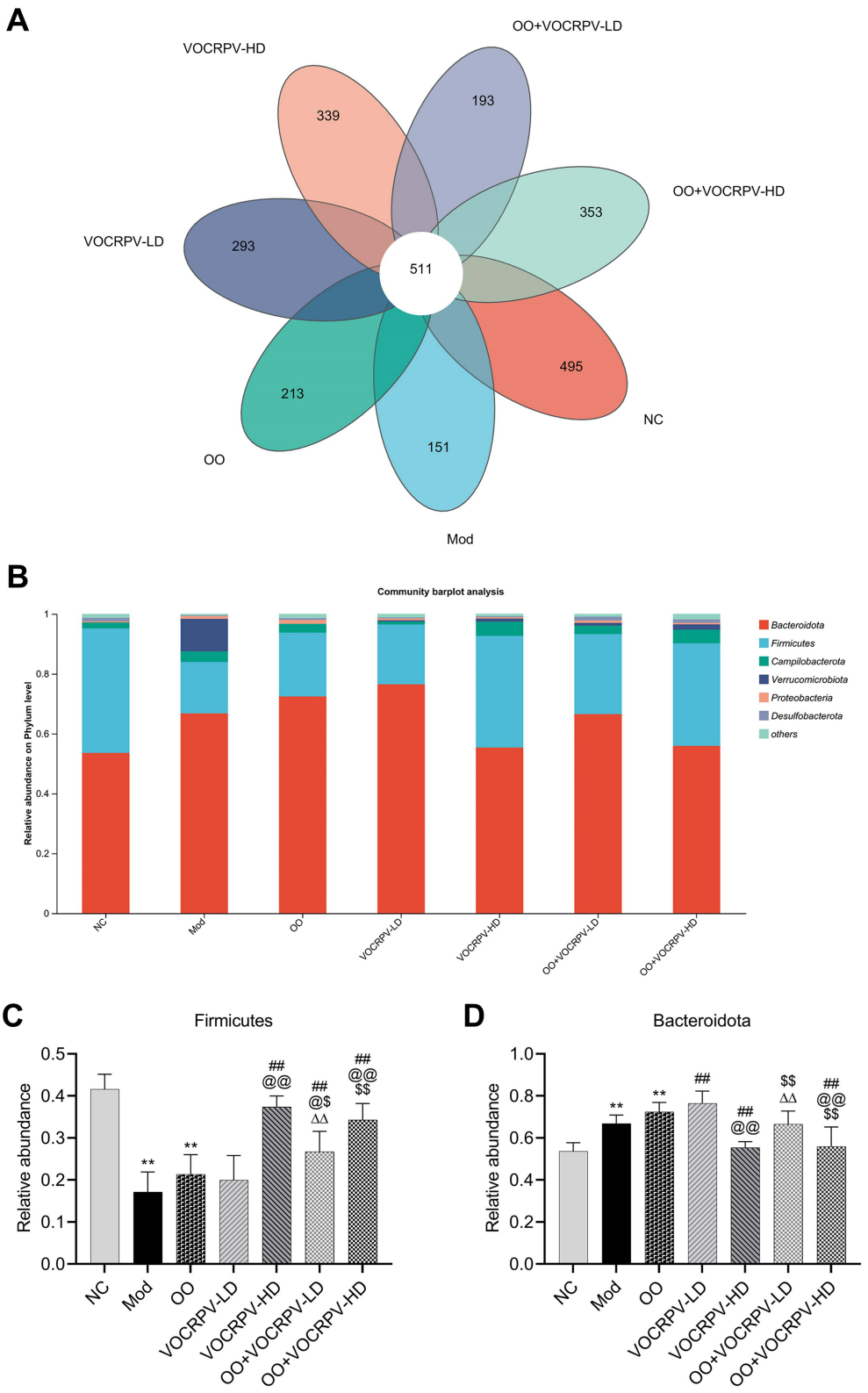

Figure 9 Venn diagram and community composition on the phylum Level. (**A**) Venn diagram. (**B**) Community barplot analysis on the phylum level. (**C**) Relative abundance of *Firmicutes*. (**D**) Relative abundance of *Bacteroidota*. Data are expressed as mean ± SD (n = 6). Each repeat was performed as a separate, independent experiment or observation. $^{**}P < 0.01$, vs NC group; $^{\#\#}P < 0.01$, vs Mod group; $^{@}P < 0.05$, $^{@@}P < 0.01$, vs OO group; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, vs VOCRPV-LD group; $^{\Delta\Delta}P < 0.01$, vs VOCRPV-HD group.

Abbreviations: NC, normal control; Mod, model; OO, olive oil; VOCRPV-LD, low-dose VOCRPV; VOCRPV-HD, high-dose VOCRPV; OO + VOCRPV-LD, olive oil + low-dose VOCRPV; OO + VOCRPV-HD, olive oil + high-dose VOCRPV.

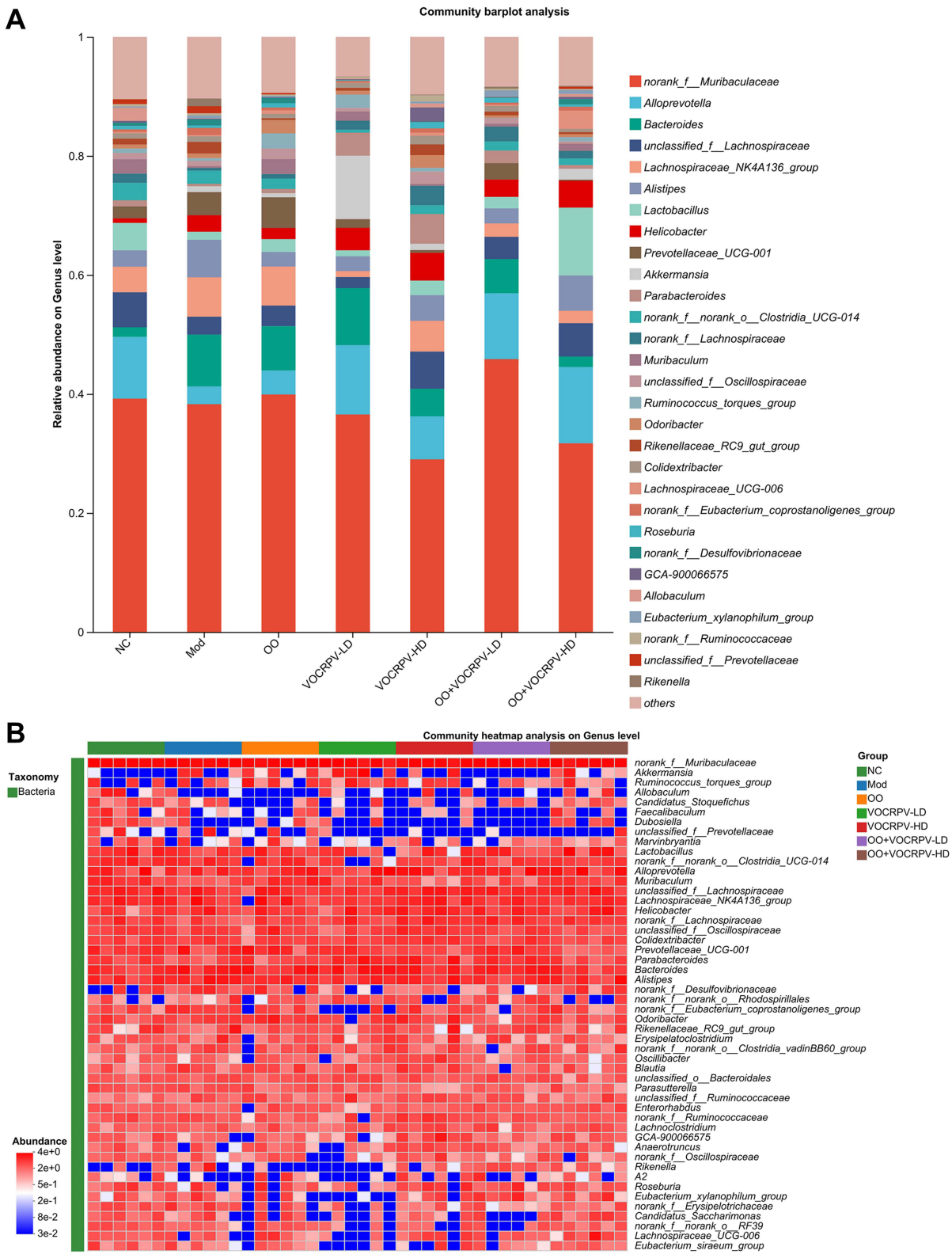

Figure 10 The community barplot analysis and heatmap analysis on the genus Level. (**A**) Community barplot analysis. (**B**) Community heatmap analysis.
Abbreviations: NC, normal control; Mod, model; OO, olive oil; VOCRPV-LD, low-dose VOCRPV; VOCRPV-HD, high-dose VOCRPV; OO + VOCRPV-LD, olive oil + low-dose VOCRPV; OO + VOCRPV-HD, olive oil + high-dose VOCRPV.

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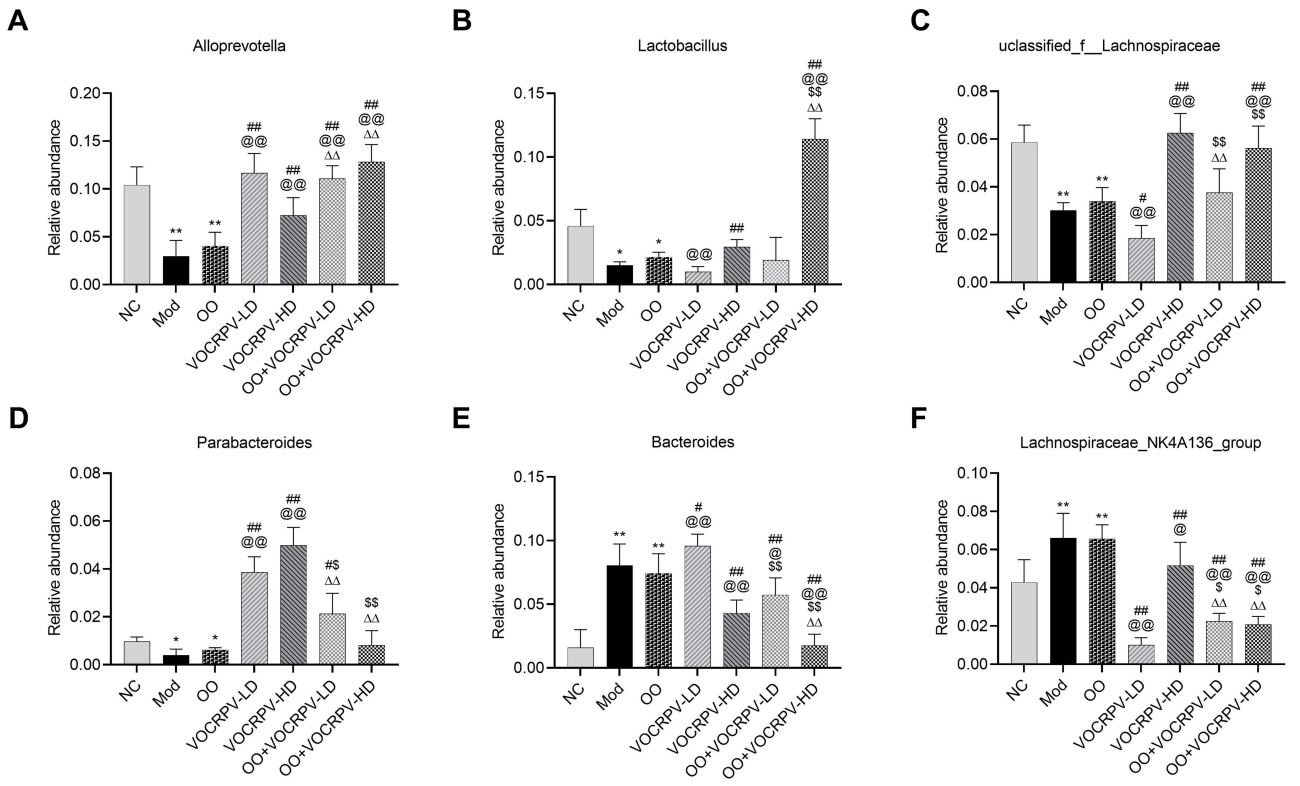

Figure 11 Effects of VOCRPV and its olive oil solution on the specific intestinal microbiota abundances on the genus level. (**A**) Relative abundance of *Alloprevotella*. (**B**) Relative abundance of *Lactobacillus*. (**C**) Relative abundance of *unclassified_f__Lachnospiraceae*. (**D**) Relative abundance of *Parabacteroides*. (**E**) Relative abundance of *Bacteroides*. (**F**) Relative abundance of *Lachnospiraceae_NK4A136_group*. Data are expressed as mean ± SD (n = 6). Each repeat was performed as a separate, independent experiment or observation. $^{*}P < 0.05$, $^{**}P < 0.01$, vs NC group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs Mod group; $^{@}P < 0.05$, $^{@@}P < 0.01$, vs OO group; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, vs VOCRPV-LD group; $^{\Delta\Delta}P < 0.01$, vs VOCRPV-HD group.

Abbreviations: NC, normal control; Mod, model; OO, olive oil; VOCRPV-LD, low-dose VOCRPV; VOCRPV-HD, high-dose VOCRPV; OO + VOCRPV-LD, olive oil + low-dose VOCRPV; OO + VOCRPV-HD, olive oil + high-dose VOCRPV.

LD, and OO + VOCRPV-HD, the abundance of *Bacteroides* was significantly decreased ($P < 0.01$). Treatment with VOCRPV-HD and OO + VOCRPV-HD significantly increased the abundance of *Lactobacillus* and *unclassified_f__Lachnospiraceae* ($P < 0.01$). Additionally, the abundance of *Alloprevotella* in the OO + VOCRPV-LD and OO + VOCRPV-HD groups was higher than that in the VOCRPV-HD group, the abundance of *Lachnospiraceae_NK4A136_group* in the OO + VOCRPV-LD and OO + VOCRPV-HD groups was lower than that in the VOCRPV-HD group, the abundance of *Lactobacillus* in the OO + VOCRPV-HD group was higher than that in the VOCRPV-HD group, the abundance of *Bacteroides* in the OO + VOCRPV-HD group was lower than that in the VOCRPV-HD group, and they all were significant ($P < 0.01$). Therefore, VOCRPV and its olive oil solution could modulate the composition and abundance of intestinal flora.

Discussion

Gastrointestinal hormones play a role in STC, mainly by affecting the peristalsis, contraction, and secretion of the gastrointestinal tract. Imbalance and abnormal regulation of gastrointestinal hormones are important factors in the development of STC. Gas and MTL are common excitatory gastrointestinal hormones that are crucial for regulating the digestive, absorptive, and motor functions of the gastrointestinal tract.[37] Under normal conditions, MTL stimulates periodic migratory compound movements in the gastrointestinal tract, promotes gastric emptying and peristalsis of the small intestine and colon, and accelerates transmission of intestinal contents.[38] MTL secretion is often reduced in patients with STC. This leads to the weakening of gastrointestinal peristalsis, so the speed of fecal transmission in the intestine is slowed, the fecal retention time is prolonged, and water is over-absorbed, thus aggravating the symptoms of constipation.[39] Gas stimulates gastric acid secretion and enhances peristalsis in the gastrointestinal tract, promoting the

transmission of the intestinal contents. However, patients with STC exhibit abnormal Gas secretion or an altered sensitivity to Gas receptors. It has been found that Gas levels are reduced in patients with STC, which results in diminished promotion of gastrointestinal motility and is thus detrimental to the normal transmission of feces.[40] Gas also regulates the function of the intestinal nerves and smooth muscles. Disturbance of these regulatory mechanisms can impair intestinal transport function, which promotes STC development. Yao et al found that Gas and MTL levels were lower in the STC group than in the normal control group.[41] Yan et al found that the Gas and MTL levels in STC rats receiving drug intervention were higher than in STC rats.[42] In this study, we found that mice in the Mod group had lower Gas and MTL levels than mice in the NC group, consistent with previous reports. However, VOCRPV and its olive oil solution elevated the Gas and MTL content in STC mice.

SCF is a cytokine that can be soluble or membrane-bound. c-Kit is a receptor for tyrosine kinase. The c-Kit receptor dimerizes and becomes autophosphorylated when SCF binds to it, which activates downstream signal transduction pathways.[43] The SCF/c-Kit signaling pathway affects the development, proliferation, and contractile function of intestinal smooth muscle cells. In STC, abnormalities in the SCF/c-Kit signaling pathway lead to smooth muscle dysfunction, weakening intestinal peristalsis and slowing fecal transmission speed. Abnormalities in the SCF/c-Kit signaling pathway also result in an imbalance in innervation, which affects intestinal motility regulation and contributes to STC. Interstitial cells of Cajal (ICCs) are a unique class of interstitial cells located between the enteric nervous system (ENS) and smooth muscle cells.[44] As a slow-wave pacemaker in the gastrointestinal tract,[45] ICC plays a key role in modulating the transmission of nerve signals from the ENS to smooth muscle cells. There is evidence of ICC apoptosis in the colons of numerous patients with STC.[46] Disturbances in the SCF/c-Kit signaling pathway may lead to reduced numbers, morphological changes, or functional abnormalities of the ICC, affecting rhythmic contractions of the intestinal tract and triggering STC.[47] Liu et al investigated the SCF/c-Kit signaling pathway using Western blotting and RT-PCR, and the findings demonstrated that the STC model group had lower levels of SCF and c-Kit protein and mRNA expression than the control group.[48] Zheng et al found that the expression of SCF and c-Kit in the colonic tissues of STC rats was reduced compared to that in the normal control group.[46] Similarly, Western blot and immunohistochemistry results showed that the expression levels of SCF and c-Kit in the Mod group were significantly lower than those in the NC group. However, VOCRPV increased the protein expression of SCF and c-Kit in the STC mice. Therefore, VOCRPV could promote peristalsis by activating the SCF/c-Kit signaling pathway.

Intestinal flora plays an important role in the occurrence and development of STC. Alpha diversity analysis showed that the richness and diversity of the microbial community was lower in the Mod group than in the NC group but increased after the intervention with VOCRPV and its olive oil solution. Beta diversity analysis showed that the community composition of the Mod group differed significantly from that of the NC group, and VOCRPV and its olive oil solution improved the beta diversity of STC mice. The *Firmicutes*/*Bacteroidota* (F/B) value is usually considered directly related to the composition of healthy intestinal flora.[49] *Bacteroidota* abundance increased, and *Firmicutes* abundance decreased in the STC model group, as reported.[50] Consistent with the above results, in this study, the abundance of *Bacteroidota* increased and the abundance of *Firmicutes* decreased in the Mod group compared with the NC group. The F/B value in the Mod group decreased, and intestinal homeostasis was disturbed. Treatment with VOCRPV and its olive oil solution modulated this value. *Lactobacillus* is an important intestinal probiotic in the human body that inhibits the growth of harmful bacteria and maintains the stability and balance of the intestinal microbial community.[41] Zhang et al found that, after intervention with the microbial ecological agent PEP0401 to constipated mice, the abundance of *Lactobacillus* increased.[51] *Alloprevotella*, an important part of the intestinal microbial community, interacts with other flora to maintain the stability and diversity of the intestinal flora and produces several metabolites, such as short-chain fatty acids, which are beneficial to intestinal health. Zeyue et al found that the abundance of *Alloprevotella* in STC mice was reduced compared with that in the blank group.[52] High levels of *Bacteroides* were prevalent in fecal samples from mice and humans with constipation and were negatively correlated with colonic transport rate.[11] Disorders in the intestinal microbiota are key factors in the development of constipation, and one of the main features is an increased abundance of *Bacteroides*.[53] Jiang et al found that loperamide-induced STC mice showed a significant increase in the abundance of *Bacteroides* compared with normal mice.[30] The *Lachnospiraceae_NK4A136_group* is an indicator of intestinal flora imbalance. A higher abundance of the

Lachnospiraceae_NK4A136_ group was observed in the severe intestinal imbalance.[54] Tuohongerbieke et al also found that the abundance of the *Lachnospiraceae_NK4A136_group* in the STC group was higher.[55] The *unclassified__f__Lachnospiraceae* have been shown to contribute to butyric acid production and are associated with the suppression of intestinal disorders, such as irritable bowel syndrome.[56] Wang et al found that the *unclassified_f__Lachnospiraceae* abundance of STC mice was lower than that in control mice.[11] *Parabacteroides* are gram-negative anaerobic bacteria that promote host health by modulating the immune system, alleviating inflammation, regulating host metabolism, and secreting metabolites.[57,58] Zhang et al found that a 4-week probiotic intervention increased the abundance of *Parabacteroides* in constipated mice.[59] These findings are in line with the results of this study, compared with the NC group, the abundance of *Alloprevotella, Lactobacillus, unclassified_f__Lachnospiraceae*, and *Parabacteroides* in the Mod group was significantly decreased, and the abundance of *Bacteroides* and *Lachnospiraceae_NK4A136_group* in the Mod group was significantly increased. However, VOCRPV and its olive oil solution increased the abundance of *Alloprevotella, Lactobacillus, unclassified_f__Lachnospiraceae*, and *Parabacteroides* in STC mice and decreased the abundance of *Bacteroides* and *Lachnospiraceae_NK4A136_group* in STC mice. Thus, modulation of the composition and abundance of intestinal flora by the VOCRPV and its olive oil solution is one of the factors that effectively alleviates STC.

CRPV is a widely used herbal medicine with low toxicity.[60] The volatile oil, which consists mainly of lipophilic components and terpenoids, is considered safe and is commonly used in the treatment of diseases.[61] Limonene, which has a flavor, is one of the main components of the VOCRPV, which has low toxicity to humans,[62] is considered safe, and is commonly used as a food additive. After oral administration, limonene is rapidly metabolized in humans.[63] Compared to standard treatments like methylcellulose/lactulose (causing bloating) and prucalopride /mosapride (causing diarrhea/ headaches), VOCPRV demonstrates superior safety. The results of this study show that the fecal water content did not differ significantly between the Mos and OO + VOCRRV-HD groups. The colonic mucosal structure of mice in all treatment groups (VOCRPV-LD, VOCRPV-HD, OO + VOCRPV-LD, OO + VOCRPV-HD, and Mos groups) was intact. In addition, there was no significant variation in Gas content between the OO + VOCRPV-HD and Mos groups. Experimental design during the study covers all aspects of the disorder and ensures the reliability of results. To enhance clinical applicability, VOCRPV can be made into enteric-coated soft capsules for the daily treatment of STC patients. This study provides the first investigation into both the therapeutic efficacy and underlying mechanisms of VOCRPV in STC. Pharmacokinetic and clinical trials will be conducted in the future to determine the optimal dose (recommended pediatric initiation at 1/3-1/2 adult dose to prevent overdose).

Conclusion

VOCRPV significantly enhanced body weight, increased the number of fecal pellets, improved fecal water content, and boosted small intestinal propulsion rate in mice with loperamide-induced STC. Additionally, it mitigated the extent of pathological damage in the colon. Olive oil enhances the therapeutic effects of VOCRPV on STC. The mechanism by which VOCRPV alleviates STC is related to upregulation of gastrointestinal hormones (Gas and MTL), activation of the SCF/c-Kit signaling pathway, and regulation of intestinal flora. This study provides an experimental basis for future development and application of VOCRPV.

Abbreviations

FC, functional constipation; STC, slow-transit constipation; CRPV, *Citri Reticulatae Pericarpium Viride*; HPLC, High-Performance Liquid Chromatography; VOCRPV, volatile oil from *Citri Reticulatae Pericarpium Viride*; SCF, stem cell factor; Gas, gastrin; MTL, motilin; PVDF, polyvinylidene difluoride; HRP, horseradish peroxidase; ANOVA, analysis of variance; OTU, operational taxonomic units; PCoA, Principal co-ordinates analysis; NMDS, non-metric multidimensional scaling; ICCs, Interstitial cells of Cajal; ENS, enteric nervous system; NC, normal control; Mod, model; Mos, mosapride; OO, olive oil; VOCRPV-LD, low-dose volatile oil of *Citri Reticulatae Pericarpium Viride*; VOCRPV-HD, high-dose volatile oil of *Citri Reticulatae Pericarpium Viride*; OO + VOCRPV-LD, olive oil + low-dose volatile oil of *Citri Reticulatae Pericarpium Viride*; OO + VOCRPV-HD, olive oil + high-dose volatile oil of *Citri Reticulatae Pericarpium Viride*.

Consent for Publication

All authors agreed to the publication of this manuscript.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare no conflicts of interest in this work.

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