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Contents

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ORIGINAL ARTICLE

Basic Study

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Contents

ABOUT COVER

Editorial Board Member of *World Journal of Gastrointestinal Pharmacology and Therapeutics*, Mostafa Yakoot, MD, MSc, Chairman, Chief Doctor, Senior Researcher, Internal Medicine, Pediatrics and Hepatology, Green Clinic and Research Center, Alexandria University, Alexandria 21121, Egypt. yakoot@yahoo.com

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WJGPT mainly publishes articles reporting research results obtained in the field of gastrointestinal pharmacology and therapeutics and covering a wide range of topics including acid-related disorders, functional gastrointestinal disorders, fundamentals of gastrointestinal pharmacology, *etc.*

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ORIGINAL ARTICLE

Basic Study

Cinnamic acid regulates the intestinal microbiome and short-chain fatty acids to treat slow transit constipation

Jin-Guang Jiang, Qian Luo, Shuang-Shuang Li, Tian-Ying Tan, Kai Xiong, Tao Yang, Tian-Bao Xiao

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Jin-Guang Jiang, Qian Luo, Department of Colorectal and Anal Surgery, Suqian Hospital of Traditional Chinese Medicine, Suqian 223800, Jiangsu Province, China

Shuang-Shuang Li, Tian-Ying Tan, Kai Xiong, College of Clinical Medicine, Guizhou University of Traditional Chinese Medicine, Guiyang 550000, Guizhou Province, China

Tao Yang, Tian-Bao Xiao, Department of Colorectal and Anal Surgery, The First Affiliated Hospital of Guizhou University of Traditional Chinese Medicine, Guiyang 550000, Guizhou Province, China

Corresponding author: Tian-Bao Xiao, MD, Doctor, Department of Colorectal and Anal Surgery, The First Affiliated Hospital of Guizhou University of Traditional Chinese Medicine, No. 71 Baoshan North Road, Guiyang 550000, Guizhou Province, China.
prof_xiaotianbao@163.com

Abstract

BACKGROUND

Slow transit constipation (STC) is a disorder with delayed colonic transit. Cinnamic acid (CA) is an organic acid in natural plants, such as *Radix Scrophulariae* (Xuan Shen), with low toxicity and biological activities to modulate the intestinal microbiome.

AIM

To explore the potential effects of CA on the intestinal microbiome and the primary endogenous metabolites-short-chain fatty acids (SCFAs) and evaluate the therapeutic effects of CA in STC.

METHODS

Loperamide was applied to induce STC in mice. The treatment effects of CA on STC mice were assessed from the 24 h defecations, fecal moisture and intestinal transit rate. The enteric neurotransmitters: 5-hydroxytryptamine (5-HT) and vasoactive intestinal peptide (VIP) were determined by the enzyme-linked immunosorbent assay. Hematoxylin-eosin and Alcian blue and Periodic acid Schiff staining were used to evaluate intestinal mucosa's histopathological performance and secretory function. 16S rDNA was employed to analyze the composition and abundance of the intestinal microbiome. The SCFAs in stool samples were quantitatively detected by gas chromatography-mass spectrometry.

RESULTS
CA ameliorated the symptoms of STC and treated STC effectively. CA ameliorated the infiltration of neutrophils and lymphocytes, increased the number of goblet cells and acidic mucus secretion of the mucosa. In addition, CA significantly increased the concentration of 5-HT and reduced VIP. CA significantly improved the diversity and abundance of the beneficial microbiome. Furthermore, the production of SCFAs [including acetic acid (AA), butyric acid (BA), propionic acid (PA) and valeric acid (VA)] was significantly promoted by CA. The changed abundance of *Firmicutes, Akkermansia, Lachnoclostridium, Monoglobus, UCG.005, Paenalcaligenes, Psychrobacter* and *Acinetobacter* were involved in the production of AA, BA, PA and VA.

CONCLUSION
CA could treat STC effectively by ameliorating the composition and abundance of the intestinal microbiome to regulate the production of SCFAs.

Key Words: Slow transit constipation; Cinnamic acid; Intestinal microbiome; Short-chain fatty acids; Intestinal motility

Core Tip: Studies on the gut microbiome and its metabolites are increasingly in slow transit constipation (STC). In this study, we found that Cinnamic acid (CA) improved and treated STC effectively by ameliorating intestinal mucosa's histopathological performance and secretory function in STC mice induced by loperamide, with alpha and beta diversity significantly decreased. Meanwhile, CA ameliorated the composition and abundance of the intestinal microbiome.

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INTRODUCTION

Slow transit constipation (STC) is one of the most boresome disorders in the digestive system and characterized by delayed colonic transit, caused by either myopathy or neuropathy[1]. The severity of slow transit may be severe enough to cease spontaneous bowel movements completely. The proportion of STC in chronic idiopathic constipation was estimated at 42%[2]. STC has curtailed the quality of life, burdened psychological distress, and significantly increased the social and economic burden[3,4].

It is very hard to manage and treat STC clinically because of the unknown pathophysiologic mechanisms. Abnormalities of the enteric nervous system and neurotransmitters [such as vasoactive intestinal peptide (VIP), substance P (SP), nitric oxide synthase (NOS)], imbalance of intestinal microbiome and decreased number of interstitial cells of Cajal have been described as the slow transit colon in the STC patients[5-7]. Alterations of the intestinal microbiome in patients with chronic constipation are characterized by a relative decrease in beneficial bacteria and a parallel increase of potentially pathogenic or opportunistic microbiome[8]. Previous studies have revealed the intimate association between STC and altered abundance of the interstitial microbiome. A cross-sectional pilot study using 16S rRNA gene pyrosequencing indicated that the abundances of *Bacteroidetes* were decreased and the abundances of genera *Blautia*, *Coprococcus* and *Ruminococcus* were increased significantly in constipated patients[6]. When analyzed at the phylum level, a previous study revealed that the *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* were increased significantly in patients with chronic constipation[9]. Using culture-based methods, it was also indicated that *Faecalibacterium*, *Roseburia* and *Coprococcus* were increased significantly in constipated patients[10]. All these alterations of the intestinal microbiome influenced intestinal motility and metabolic function by changing the number of metabolites and the metabolic environment of the gut[11]. Short-chain fatty acids (SCFAs), the primary endogenous metabolites, were produced from the fermentation of undigested carbohydrates by intestinal bacteria. SCFAs could enhance the absorption of fluid and sodium absorption potentially aggravate STC symptoms[12]. A case-control study indicated that butyrate, acetate, and propionate levels were significantly lower in constipated patients[13]. Furthermore, a previous study demonstrated that the administration of SCFAs with 100-200 *mM* directly into rats

stimulated colonic motility and accelerated colonic transit[14]. In these regards, the regulation of regulating crobiome and the metabolism of SCFAs *via* interventional drugs may be essential to treat STC.

Cinnamic acid (CA) is an organic acid in natural plants, such as *Radix Scrophulariae* (Xuan Shen), that has low toxicity and with a broad spectrum of biological activities. A previous study showed that CA restrained gram-positive (*Staphylococcus aureus*) and gram-negative (*Escherichia coli*) bacteria effectively [15]. Furthermore, another study indicated that CA ameliorated lipopolysaccharide-induced inflammation and oxidative stress in mice[16]. However, a rare study clarified the therapeutic effects of CA in STC. In this study, we intend to explore the potential effects of CA on the intestinal microbiome and SCFAs and evaluate the therapeutic effects of CA in STC.

MATERIALS AND METHODS

Reagents

CA (CAS: 140-10-3) was purchased from Chroma Biotechnology Co. Ltd. (Chengdu, China) with HPLC purity ≥ 98%. Prucalopride (PRU) was purchased from Jiangsu Haosen pharmaceutical group Co., Ltd (Cat. No. H20183482). Loperamide was obtained from Xi'an Janssen Pharmaceutical Co., Ltd (Cat. No. LFJ8574). When applied to mice, all drugs were dissolved in sodium chloride injection (NS 0.9%).

Loperamide-induced STC mice and grouping

The model using loperamide-induced mice with STC was previously reported[17]. Briefly, specific pathogen-free (SPF), Balb/c mice (n = 50, 25 was male and 25 was female, weight: 20 ± 2 g, age: 4-6 wk) were purchased from Beijing Huahukang Animal Breeding Center (permission No. SCXK-(jing) 2019–0008). All mice were maintained under SPF conditions in a shelter sustained facility and provided with sterile food and water. All mice were randomly divided into the control group, model group, PRU group, CA with low-dose and high-dose groups (n = 10 per group) respectively. The mice in the model group were given loperamide at a dose of 9.6 mg/kg, once per day *via* oral gavage for 4 consecutive weeks. Mice in the positive group received 0.26 mg/kg·d^{-1} PRU (the equivalent doses of the clinic). Mice in the low-dose and high-dose CA group received 40 mg/kg·d^{-1} and 80 mg/kg·d^{-1} CA respectively, according to the study of Yan *et al*[18] and Wang *et al*[19]. PRU and CA were orally administered once daily for four weeks. The serum samples, stool samples in the intestine and colon tissues were collected when all mice were sacrificed on the 29th day. All collected samples were kept at -80 °C condition and then taken for further experiments.

Assessment of 24 h defecations, fecal moisture and intestinal transit rate

At the end of experiment, all mice were housed individually in metallic cages to collect feces once an hour for 24 h, and the feces number and weight were recorded. The fecal water content was calculated after drying the feces in a desiccator at 60 °C for 12 h, according to the equation: (wet weight-dry weight)/wet weight ×100%. To evaluate the intestinal transit rate, all mice in each group were gavaged with 0.2 mL Indian ink after 2 h at the end of the last treatment. After 24 h, mice were sacrificed. The intestinal transit rate was calculated according to the equation: traveled distance of Indian ink in the intestine (cm)/full length of intestine (cm) × 100%.

Measurement of 5-HT and VIP concentration

Two main enteric neurotransmitters: 5-hydroxytryptamine (5-HT) and VIP were determined by the enzyme-linked immunosorbent assay (ELISA). The serum concentration of 5-HT and the VIP content in the colon tissue was detected by the ELISA kits. ELISA kits of 5-HT (Cat No.: JYM0433Mo) and VIP (Cat No.: JYM0436Mo) were purchased from Colorful-Gene Biotechnology Co., Ltd. (www.jymbio.com, Wuhan, China). All assays were performed rigorously according to the manufacturer's instructions. The Synergy H1 Hybrid Reader (Biotech, United States) was applied to measure the relative optical density of 5-HT and VIP spectrophotometrically at a wavelength of 450 *nm*.

Hematoxylin-eosin and Alcian blue/periodic acid-Schiff staining of colon tissue

Mice were sacrificed at the end of the experiment. Parts of the colons were fixed in 4% paraformaldehyde cleared in xylene, embedded in paraffin, and cut into 5 mm thick slices. The histopathological performance of colon tissue was stained with hematoxylin-eosin (HE). Furthermore, the mucous cells in the colon were stained with Alcian blue/periodic acid-Schiff (AB/PAS). All experiment processes were performed according to the manufacturer's instructions.

Intestinal microbiome analysis by 16S rDNA

The total DNA in stool samples was extracted with a stool DNA kit (Omega Bio-Tek, Norcross, GA, United States). Then, the V3-4 hypervariable region of the bacterial 16S rRNA gene was amplified with the universal primers, forward (5'-3'): ACTCCTACGGGAGGCAGCAG and reverse (5'-3'): GGACTA-

CHVGGGTWTCTAAT (806R). The PCR products were purified using a QIAquick Gel Extraction Kit (QIAGEN, Germany), quantified using RT-PCR, and sequenced. The deep sequencing was performed on Miseq platform. After the run, image analysis, base calling and error estimation were performed using Illumina Analysis Pipeline Version 2.6. The raw data were screened and sequences were removed if they were shorter than 200 bp, had a low-quality score (≤ 20), contained ambiguous bases or did not match primer sequences and barcode tags. Finally, the dataset analysis was performed using the online platform of Majorbio Cloud Platform (www.majorbio.com).

Quantitative detection of SCFAs by gas chromatography-mass spectrometry

The SCFAs in stool samples were quantitatively detected by gas chromatography-mass spectrometry (GC-MS). All of the seven SCFAs, including acetic acid (AA), butyric acid (BA), caproic acid (CA-1), isobutyric acid (IBA), isovaleric acid (IVA), propionic acid (PA) and valeric acid (VA), were extracted as previously described[18]. Briefly, 50 mg of stool sample was used for metabolite extraction with 400 μL methanol-acetonitrile and 30 μL L-2-chlorophenyl alanine. After homogenization and ultrasonic extraction, the samples were incubated at -20 °C for 30 min and centrifuged for 10 min at 12000 rpm at 4 °C. Finally, 20 μL of supernatant from each sample was transferred to a vial for GC-MS analysis. The condition of GC-MS referred to the previous study[19]. All samples were evaluated in duplicate.

Statistics analysis

All data were presented as mean ± standard deviation and analyzed with the SPSS software program (version 21.0). Data were presented using one-way ANOVA followed by an LSD test. $P < 0.05$ was considered statistically significant and $P < 0.01$ was highly significant. R software (version 4.0.4) and GraphPad Prism software for Windows (version 8.02; Inc., San Diego, United States) were utilized for the visible presentation of all results.

RESULTS

CA increased 24 h defecations, fecal moisture and intestinal transit rate of STC mice

The pharmacological effects of CA on STC were evaluated from the 24 h defecations, fecal moisture and intestinal transit rate aspects. As presented in Figure 1A and B, mice in the STC model group showed more feces remaining in the colon and shorter length than the control group. Meanwhile, the 24 h defecations in the STC model group was significantly decreased (Figure 1C). After treated by CA with 40 mg/kg·d^{-1} and 80 mg/kg·d^{-1}, the number of fecal remnants in the colon (Figure 1B) was significantly decreased and 24 h defecations (Figure 1C) was significantly increased when compared with the STC model group. In addition, the fecal water content also was significantly increased by the CA treatment, especially in the CA with high doses group (Figure 1D). Parallelly, the intestinal transit rate was significantly higher in the CA with high doses group compared with the STC model group (Figure 1E). Those results indicated that CA could ameliorate the symptoms of STC and treat STC effectively.

CA ameliorated the histopathological performance and secretory function of intestinal mucosa in STC mice

As presented in Figure 2A, mice in the control group showed the mucosa, muscular and goblet cells were normal. However, the model group showed that the thickness of the mucosa and muscular was significantly thinner. The mucosal integrity was compromised and chronic inflammation was observed in the mucosa, presented with a large number of eosinophilic infiltration, mainly in the lamina propria. Furthermore, the number of goblet cells was significantly reduced. Compared with the model group, the thickness of the mucosa and muscular were increased significantly, inflammatory cell infiltration was reduced, mucosa was smoother, and the structure of glandular was gradually restored and arranged more neatly in the PRU group. The CA group with low and high doses showed smoother mucosa, more intact morphology and structure of glandular (secreting mucus, lubricating the intestinal tract, and facilitating bowel movements), less infiltration of neutrophils and lymphocytes, and higher numbers of phagocytes compared to the PRU group (Figure 2A).

Then, the secretory function of goblet cells in the mucosa was tested. As presented in Figure 2B, the secretion of acidic mucus (the blue part) was significantly decreased in the model group compared with the control group. The acidic mucus secreted by the goblet cells in the PRU group increased slightly. Conversely, the secretion of acidic mucus was significantly increased in the CA group, especially in the CA with high doses group.

CA increased the concentration of 5-HT and reduced VIP

As presented in Table 1 and Figure 3A, the serum concentration of 5-HT was significantly decreased in the STC model group, which was significantly lower than the control group ($P < 0.01$). In the PRU group, 5-HT concentration was significantly increased, even higher than the control group. In the CA group with low and high doses, 5-HT concentration was significantly increased compared with the STC

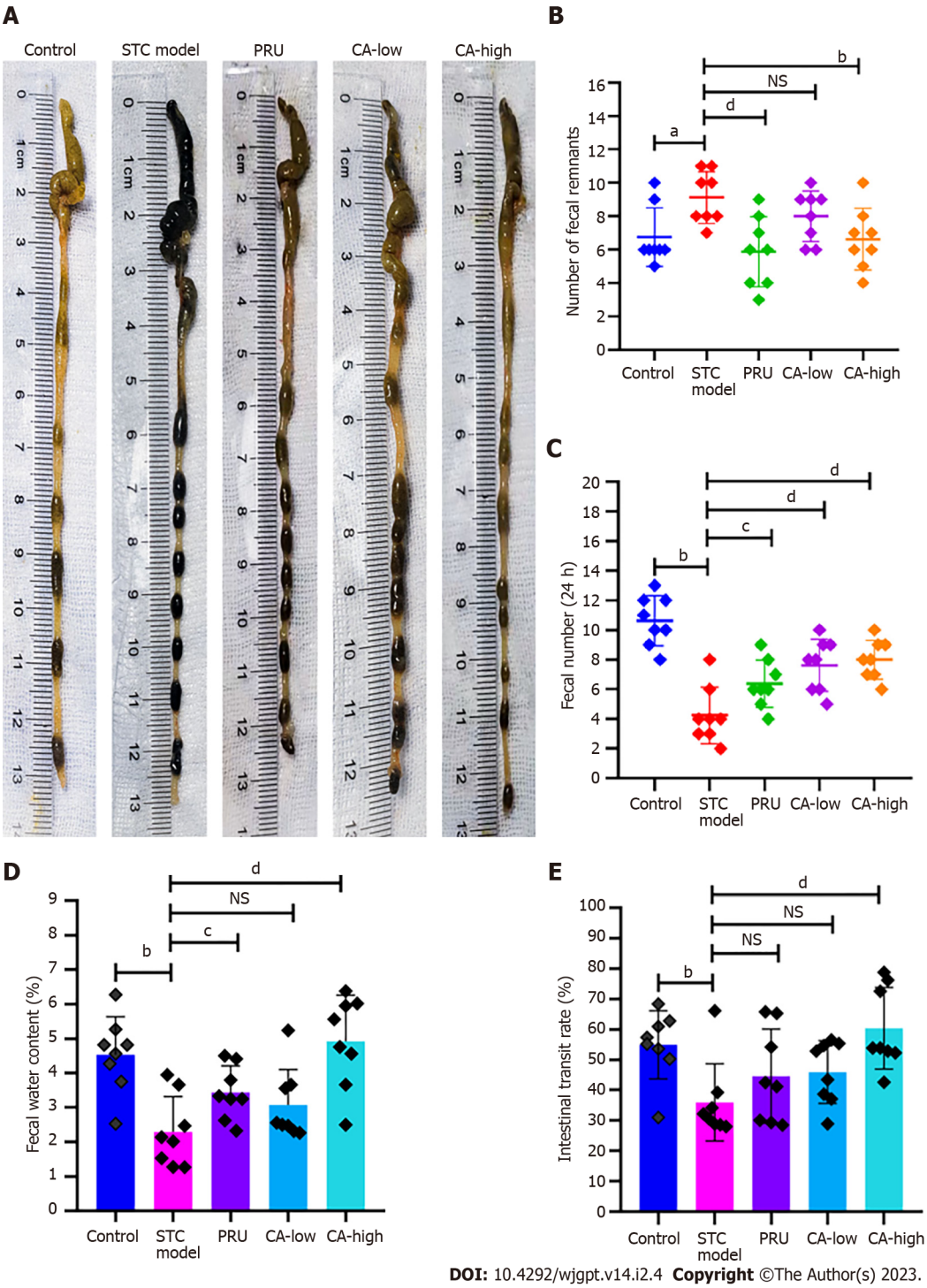

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Figure 1 Pharmacological effects of cinnamic acid on slow transit constipation mice. A: Number of fecal remnants in the colon of mice in each group (n = 8); B: Number of fecal remnants in the colon of mice in each group (n = 8); C: 24 h defecations of mice in each group (n = 8); D: Fecal water content of mice in each group (n = 8); E: Intestinal transit rate of mice in each group (n = 8). $^{a}P < 0.05$ and $^{b}P < 0.01$ versus the control group; $^{c}P < 0.05$ and $^{d}P < 0.01$ versus the slow transit constipation model group. NS: Not significant. PRU: Prucalopride; CA-low: Cinnamic acid with low dose; CA-high: Cinnamic acid with high dose.

model group ($P < 0.01$, Table 1 and Figure 3A). On the contrary, the VIP concentration in the colon tissue was increased significantly in the STC model group (Table 1 and Figure 1B). After treated by CA with low and high doses, the content of VIP was significantly decreased compared with the STC model group ($P < 0.01$, Table 1 and Figure 3B).

CA improved the alpha diversity of intestinal microbiome in STC mice

The Shannon index, one of the diversity indices for estimating microbial diversity, was applied to evaluate the alpha diversity of the intestinal microbiome in different groups.

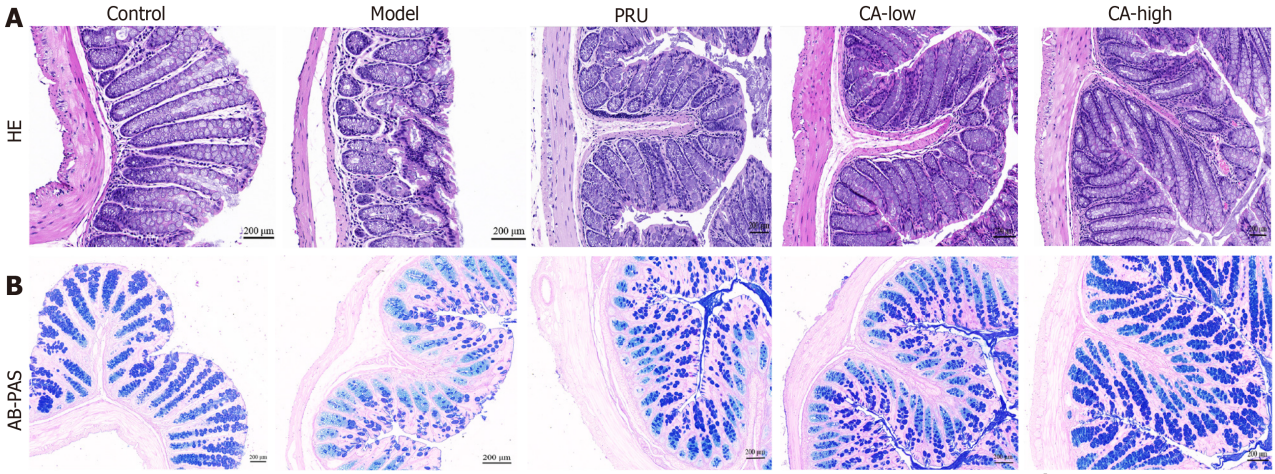

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Figure 2 Hematoxylin-eosin and Alcian blue/periodic acid-Schiff staining of each group of mice. A: HE staining of each group of mice; B: Alcian blue/periodic acid-Schiff staining of each group of mice. PRU: Prucalopride; CA-low: Cinnamic acid with low dose; CA-high: Cinnamic acid with high dose.

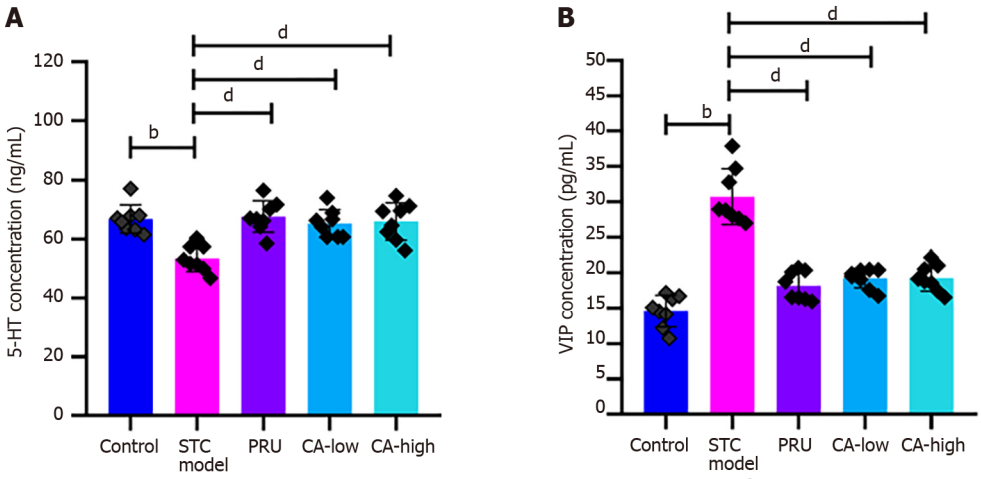

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Figure 3 Effects of Cinnamic acid on the concentration of 5-HT and vasoactive intestinal peptide. A: Serum concentration of 5-hydroxytryptamine in each group of mice (n = 8); B: Content of vasoactive intestinal peptide in the colon of mice in each group (n = 8). $^{b}P < 0.01$ versus the control group; $^{d}P < 0.01$ versus the slow transit constipation model group. PRU: Prucalopride; CA-low: Cinnamic acid with low dose; CA-high: Cinnamic acid with high dose.

The higher the Shannon index, the greater the community's diversity in the intestinal microbiome. As presented in Figure 4A and B, the Shannon index was significantly decreased at the operational taxonomic units (OTU) level compared with the control group ($P < 0.05$) and the CA with doses group ($P < 0.01$). In addition, the Shannon index also decreased significantly in the model group at the phylum (Figure 4C and D) and genus level (Figure 4E and F). After CA treatment, the Shannon index was upgraded significantly. Altogether, these results indicated that CA could improve the alpha diversity of the intestinal microbiome in STC mice.

CA increased the beta diversity of intestinal microbiome in STC mice

Principal coordinates analysis (PCoA) of Bray-Curtis distance matrices was carried out for beta diversity determination among different groups. As shown in Figure 5A, evident separation of the microbiome was observed on the two-dimensional PCoA plots among different groups. The microbiome separated significantly from the control and CA in the model group with the high doses group. The Venn plots (Figure 5B) indicated the co-species number in all groups was 506. In the model group, the species number was lower than in the remaining four groups (the control, PRU, CA-low and CA-high groups). A hierarchical clustering analysis at the OUT (Figure 5C), phylum (Figure 5D) and genus level (Figure 5E) showed significant differences between each group. The CA-treated samples were clustered separately from the model group but close to the control group, indicating that CA could alleviate the distribution of species in microbial beta diversity.

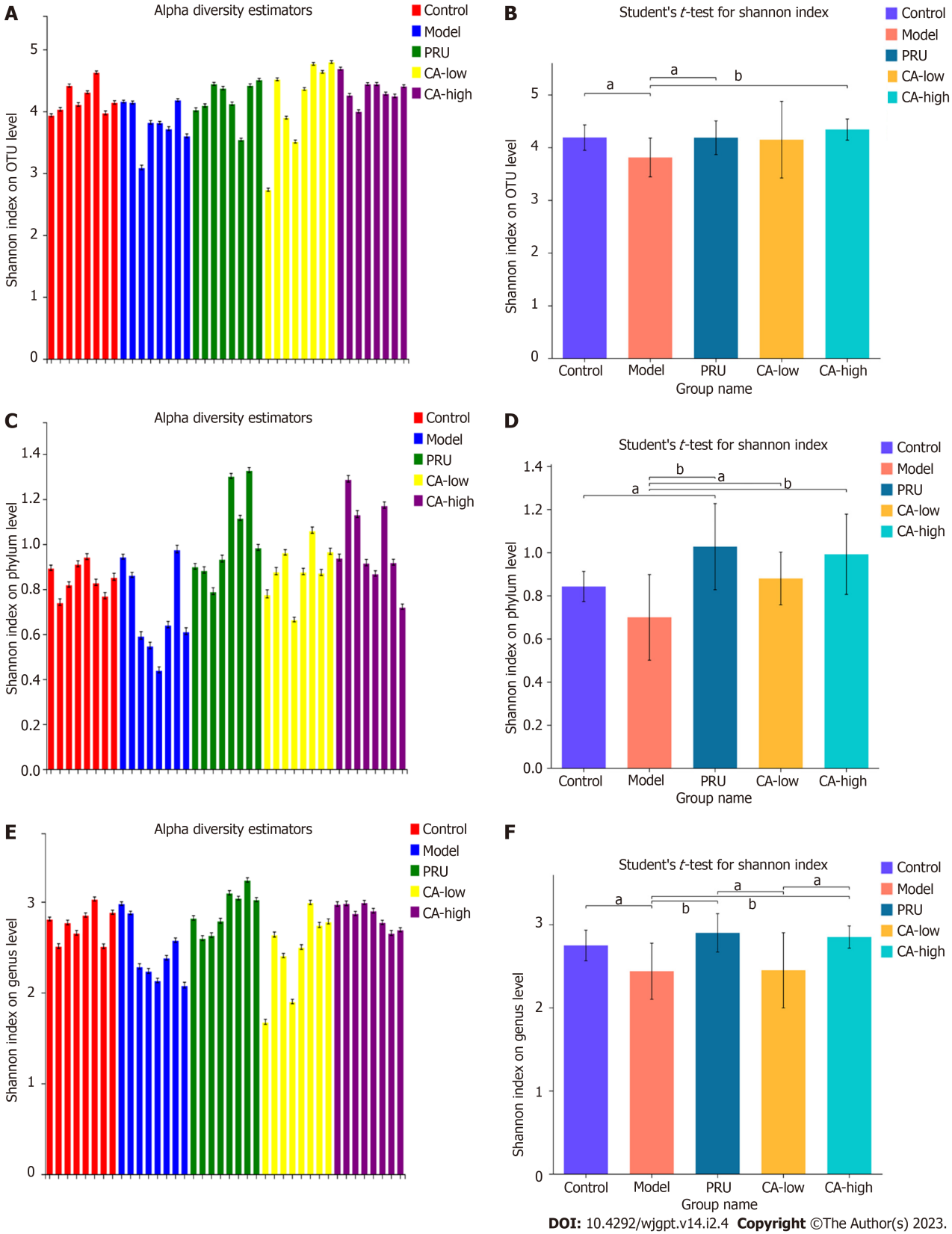

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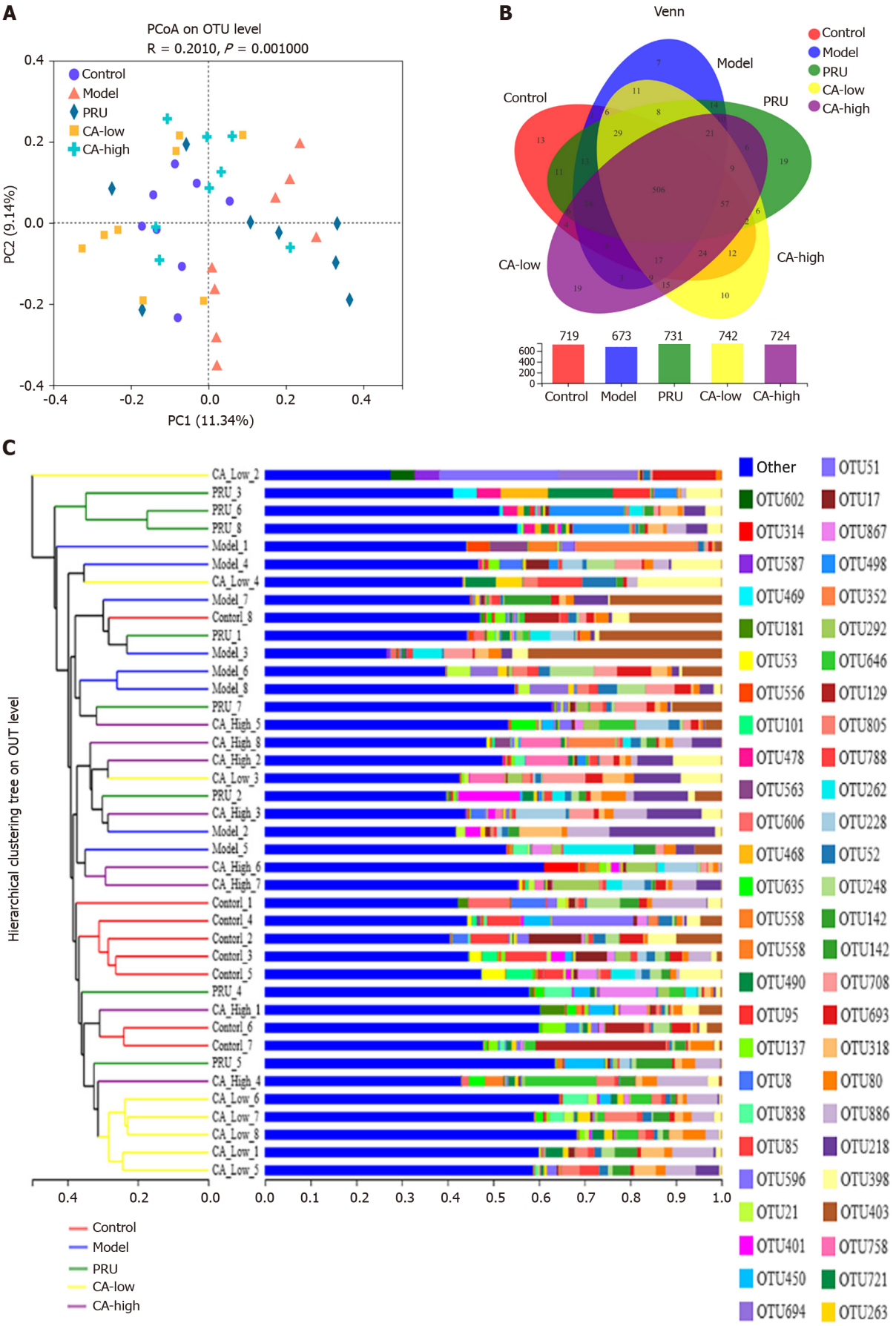
Figure 4 Alpha diversity of intestinal microbiome in each group of mice (*n* = 8). A: Shannon index evaluation of each group of mice at the OTU level; B: Difference test of Shannon index at the OTU level; C: Shannon index evaluation of each group of mice at the phylum level; D: Difference test of Shannon index at the phylum level; E: Shannon index evaluation of each group mice at the genus level; F: Difference test of Shannon index at the genus level; $^{a}P < 0.05$ and $^{b}P < 0.01$. PRU: Prucalopride; CA-low: CA with low dose; CA-high: CA with high dose.

CA promoted the composition and abundance of the intestinal microbiome in STC mice

The species difference analysis was conducted to identify the specific microbiome in different groups. The linear discriminant analysis effect size (LefSe) (Figure 6A) based on the linear discriminant analysis


A
PCoA on OTU level
R = 0.2010, P = 0.001000
Control
Model
PRU
CA-low
CA-high
PC2 (9.14%)
PC1 (11.34%)
B
Venn
Control
Model
PRU
CA-low
CA-high
719
673
731
742
724
C
Hierarchical clustering tree on OUT level
Other
OTU602
OTU314
OTU587
OTU469
OTU181
OTU53
OTU556
OTU101
OTU478
OTU563
OTU606
OTU468
OTU635
OTU558
OTU558
OTU490
OTU95
OTU137
OTU8
OTU838
OTU85
OTU596
OTU21
OTU401
OTU450
OTU694
OTU51
OTU17
OTU867
OTU498
OTU352
OTU292
OTU646
OTU129
OTU805
OTU788
OTU262
OTU228
OTU52
OTU248
OTU142
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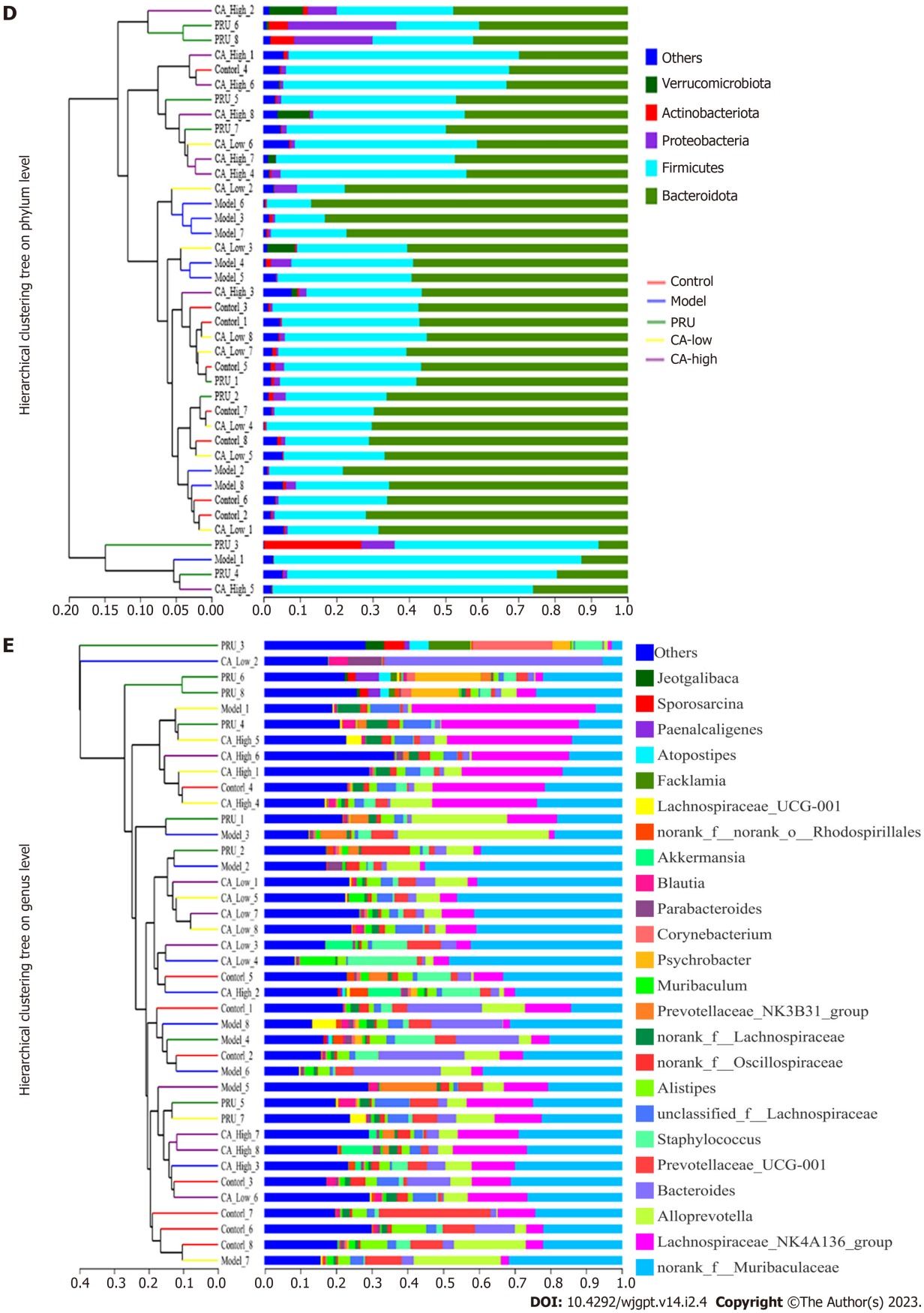

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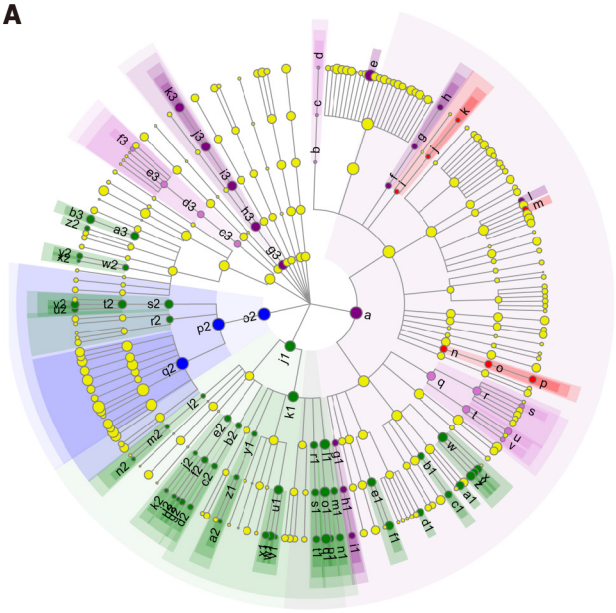
Figure 5 Beta diversity of intestinal microbiome in each group of mice (*n* = 8). A: PcoA plot at the operational taxonomic units (OTU) level; B: Venn plot at the OTU level; C: Hierarchical clustering tree at the OTU level; D: Hierarchical clustering tree at the phylum level; E: Hierarchical clustering tree at the genus level; PRU: Prucalopride; CA-low: Cinnamic acid with low dose; CA-high: Cinnamic acid with high dose.

A



a : p__Firmicutes
b : o__Eubacteriales
c : f__Anaerofustaceae
d : g__Anaerofustis
e : g__Lachnospiraceae_NK4A136_group
f : o__unclassified_c__Clostridia
g : f__unclassified_c__Clostridia
h : g__unclassified_c__Clostridia
i : o__norank_c__Clostridia
j : f__norank_o__norank_c__Clostridia
k : g__norank_f__norank_o__norank_c__Clostridia
l : g__UCG-005
m : g__NK4A214_group
n : o__Monoglobales
o : f__Monoglobaceae
p : g__Monoglobus
q : o__Erysipelotrichales
r : f__Erysipelotrichaceae
s : g__unclassified_f__Erysipelotrichaceae
t : f__Erysipelatoclostridiaceae
u : g__Erysipelatoclostridium
v : g__Candidatus_Stoquefichus
w : f__Aerococcaceae
x : g__Aerosphaera
y : g__Aerococcus
z : g__Facklamia
a1 : g__Jeotgalibaca
b1 : f__Enterococcaceae
c1 : g__Enterococcus
d1 : g__Bacillus
e1 : f__Planococcaceae
f1 : g__Sporosarcina
g1 : o__Mycoplasmatales
h1 : f__Mycoplasmataceae
i1 : g__Mycoplasma
j1 : p__Proteobacteria
k1 : c__Gammaproteobacteria
l1 : o__Pseudomonadales
m1 : f__Pseudomonadaceae
n1 : g__Pseudomonas
o1 : f__Moraxellaceae
p1 : g__Acinetobacter
q1 : g__Psychrobacter
r1 : o__Aeromonadales
s1 : f Aeromonadaceae
t1 : g__Oceanisphaera
u1 : f__Alcaligenaceae
v1 : g__Alcaligenes
w1 : g__Paenalcaligenes
x1 : g__Oligella
y1 : o__Xanthomonadales
z1 : f__Xanthomonadaceae
a2 : g__Stenotrophomonas
b2 : o__Caulobacterales
c2 : f__Caulobacteraceae
d2 : g__Brevundimonas
e2 : o__Rhizobiales
f2 : f__Rhizobiaceae
g2 : g__Paenochrobactrum
h2 : g__unclassified_f__Rhizobiaceae
i2 : g__Pseudochrobactrum
j2 : f__Devosiaceae
k2 : g__Devosia
l2 : o__Rhodobacterales
m2 : f__Rhodobacteraceae
n2 : g__Paracoccus
o2 : p__Bacteroidota
p2 : c__Bacteroidia
q2 : o__Bacteroidales
r2 : o__Flavobacteriales
s2 : o__Sphingobacteriales
t2 : f__Sphingobacteriaceae
u2 : g__Sphingobacterium
v2 : g__unclassified_f__Sphingobacter
w2 : f__Microbacteriaceae
x2 : g__Microbacterium
y2 : g__Leucobacter
z2 : g__Glutamicibacter
a3 : f__Demequinaceae
b3 : g__norank_f__Demequinaceae
c3 : c__Coriobacteriia
d3 : o__Coriobacteriales
e3 : f__Eggerthellaceae
f3 : g__unclassified_f__Eggerthellacea
g3 : p__Verrucomicrobiota
h3 : c__Verrucomicrobiae
i3 : o__Verrucomicrobiales
j3 : f__Akkermansiaceae
k3 : g__Akkermansia

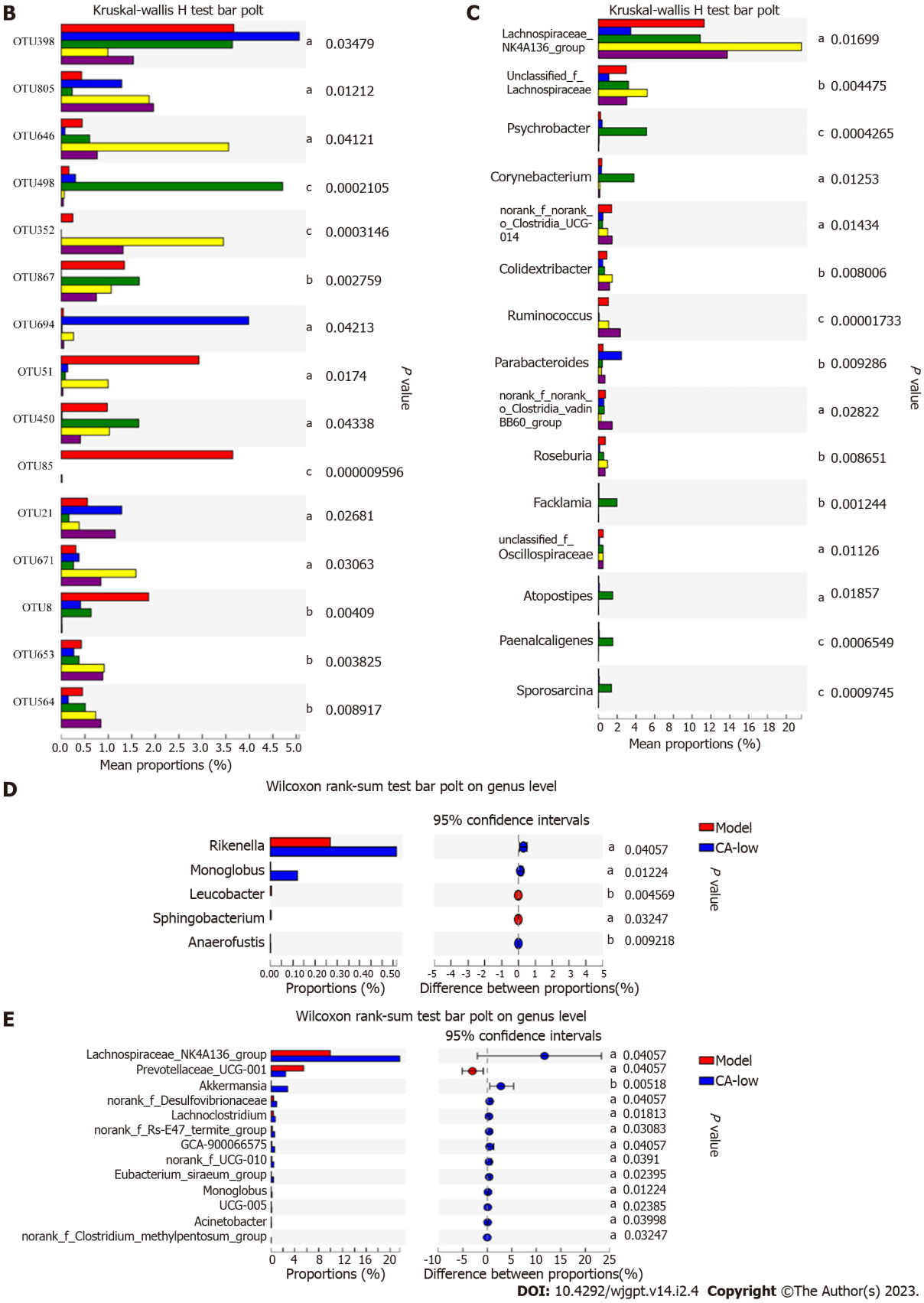


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Figure 6 The composition and abundance of the intestinal microbiome in each group of mice (*n* = 8). A: LefSe analysis of the significantly differential microbiome in each group; B: Differential comparison between multiple groups at the operational taxonomic units level; C: Differential comparison between multiple groups at the genus level; D: Differential comparison between model and CA-low group at the genus level; E: Differential comparison between model and CA-high group at the genus level. $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{e}P < 0.001$. PRU: Prucalopride; CA-low: Cinnamic acid with low dose; CA-high: Cinnamic acid with high dose.

showed the abundance of *p__Bacteroidota.c__Bacteroidia.o__Bacteroidales* was significantly increased in the model group. The abundance of *p__Proteobacteria.c__Gammaproteobacteria.o__Pseudomonadales. f__Moraxellaceae.g__Acinetobacter was* significantly increased in the PRU group. After being treated by CA, the abundance of *p__Firmicutes.c__Clostridia.o__Eubacteriales* was increased significantly in the low doses of CA group. In addition, the abundance of *p__Firmicutes.c__Clostridia* and *p__Verrucomicrobiota. c__Verrucomicrobiae. o__Verrucomicrobiales* were increased significantly in the high doses of CA group (Figure 6A). In terms of bacterial composition at the OTU level, OTU 398 and OTU 694 were significantly increased in the model group than in the remaining four groups. After being treated by CA, OTU646, OTU 352, OTU 671 and OTU 653 were significantly increased (Figure 6B). Then, we identified the specific microbiome at the genus level. Overall, the significantly statistically significant species were *norank_f__Muribaculaceae*, *Colidextribacter*, *Ruminococcus*, *Lachnospiraceae_NK4A136_group*, *Alloprevotella*, *Bacteroides* and *Prevotellaceae_UCG-001* (Figure 6C). When compared the model group with the low doses of CA group, the *g_Rikenella, g_Monoglobus and g_Anaerofustis were* significantly increased (Figure 6D). When compared with the high doses of CA group, the *Lachnospiraceae_NK4A136_group, g_Akkermansia, norank_f__Desulfovibrionaceae, g_Lachnoclostridium, g_Monoglobus* and *g_Acinetobacter* were increased significantly (Figure 6E).

CA changed the phenotypes and functions of the intestinal microbiome in STC mice

The BugBase was applied to predict the phenotypes based on the relative abundance of samples in different groups. Results indicated the phenotypes of stress-tolerant, aerobic, containing mobile elements and potentially pathogenic were predicted in different groups. The phenotypes of potentially pathogenic were increased significantly in the model group. Conversely, the phenotypes of aerobic and containing mobile elements were significantly increased in the high doses of CA group (Figure 7A). Then, the PICRUSt package of 16S amplification sequencing results was used to predict the biological functions. Results revealed that the functions of energy production and conversion, amino acid transport and metabolism, carbohydrate transport and metabolism, transcription were significantly improved by CA treatment (Figure 7B). Conclusively, CA mainly changed the phenotypes of aerobic and containing mobile elements and improved the biological functions of energy production and conversion, amino acid transport, and metabolism to regulate intestinal microbiome diversity.

CA upregulated the content of SCFAs via the intestinal microbiome in STC mice

A previous study indicated that the level of SCFAs in the stool could be involved in STC development [20-22]. Thus, the SCFAs in stool samples were quantitatively detected by GC-MS. The comparison between multiple groups showed that the content of SCFAs was decreased in the model group (Figure 8A). After being treated by CA, the content of AA and BA was increased (Figure 8A). The comparison between the model group and the low doses of CA group showed that the content of SCFAs was not increasing except for the BA (Figure 8B). However, the content of AA, BA and VA was increased in the high doses of CA group (Figure 8C). Then, the correlation between the dominant microbiome and SCFAs was analyzed by Spearman methods. Results indicated all content of SCFAs were negatively correlated with *g_Parabacteroides* and positively correlated with *g_Corynebacterium* (Figure 8D). The AA level was significantly decreased with the higher abundance of *g_Parabacteroides* ($P < 0.05$). The level of PA was significantly increased with the higher abundance of *g_Paenalcaligenes* ($P < 0.01$, Figure 8D) and *g_Psychrobacter* ($P < 0.05$, Figure 8D). In addition, the *g_Rikendlla* regulated by low doses of CA significantly increased the level of AA (Figure 8E). In the high doses of CA, most SCFAs levels were increased with the specific microbiome regulated by high doses of CA. The level of BA and VA was significantly increased with *g_UCG.005* ($P < 0.05$, Figure 8F), but the level of CA-1 was significantly decreased with *g__norank_f__Rs-E47_termite_group* ($P < 0.01$, Figure 8F). All mentioned results identified that CA could ameliorate the composition and abundance of the intestinal microbiome to regulate the content and production of SCFAs in STC mice.

DISCUSSION

There is increasing evidence indicating that the alterations of the intestinal microbiome and its metabolites are the pivotal pathophysiologic mechanism of STC. This study found that the alpha and beta diversity were significantly decreased in the STC mice induced by loperamide. In addition, the abundance of pathogenic or opportunistic bacteria, such as Bacteroides, and the phenotypes of potentially pathogenic were increased significantly in the STC mice. Meanwhile, the SCFAs, including AA, BA, IBA and VA, were decreased significantly in the STC mice compared with the normal control mice. Subsequently, we found that the organic acid: CA improved the symptoms of STC and treated STC effectively. Furthermore, CA ameliorated intestinal mucosa's histopathological performance and secretory function in STC mice. CA, especially with high dose (80 mg/kg·d^{-1}) also improved the alpha and beta diversity of the intestinal microbiome and significantly promoted *Firmicutes*' composition and abundance, *Verrucomicrobiota*, *Ruminococcus*, *Akkermansia*, *Lachnoclostridium Monoglobus* and *Acinetobacter* . Meanwhile, CA upregulated the level of AA, BA and VA *via* ameliorating the composition and

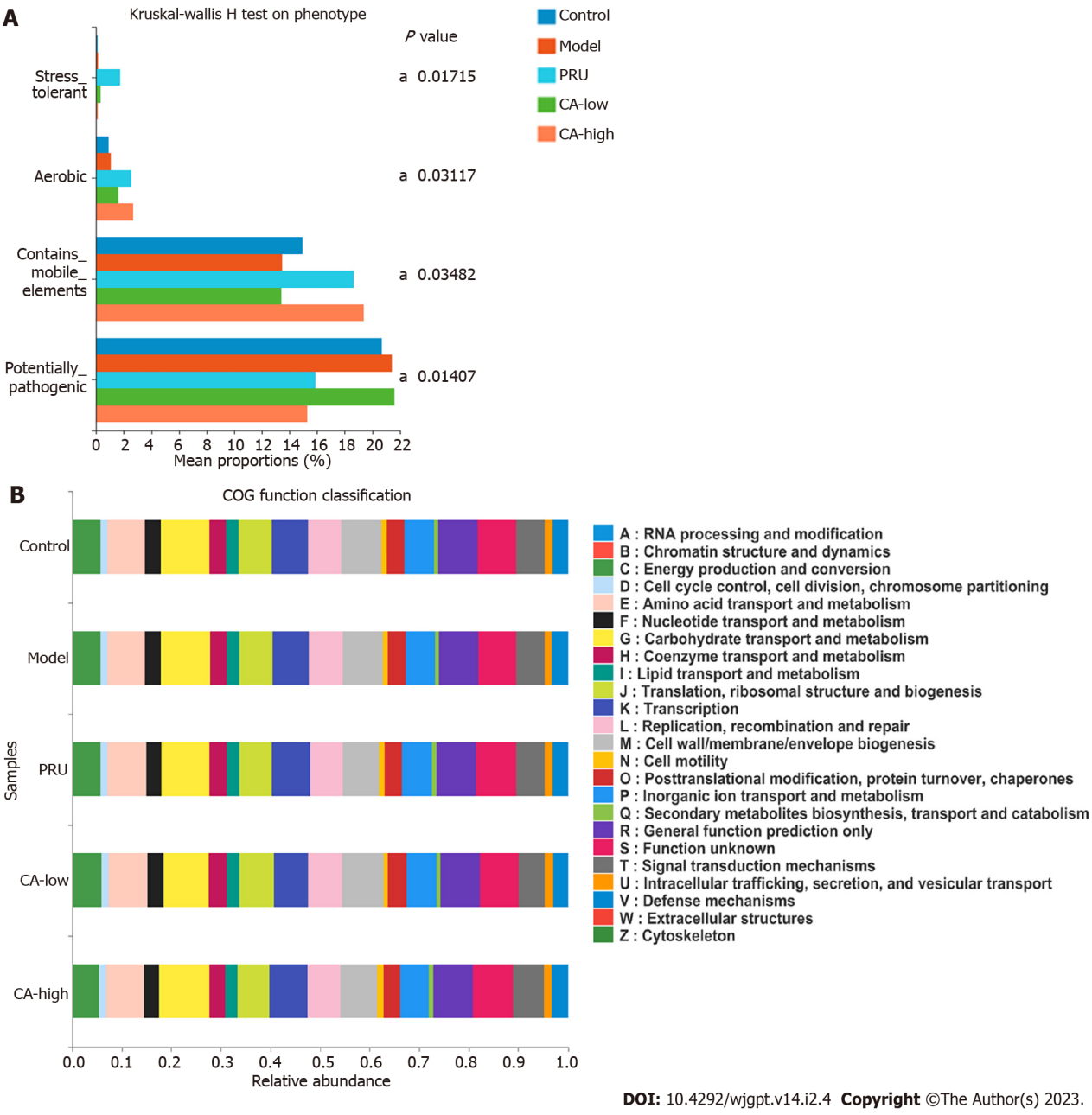

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Figure 7 Prediction of phenotypes and biological functions for the significantly differential microbiome in each group. A: Prediction of phenotypes for the significantly differential microbiome in each group; B: Prediction of biological functions for the significantly differential microbiome in each group. $^{a}P < 0.05$. PRU: Prucalopride; CA-low: Cinnamic acid with low dose; CA-high: Cinnamic acid with high dose.

abundance of the intestinal microbiome.

There has been increasing study regarding the direct association between the intestinal microbiome and gut motility and constipation. A recent study in germ-free mice (without gastrointestinal microbiota) showed that the colon transit time and gastric emptying were prolonged compared with the wild-type mice[23]. The colonization of *L.acidophilus*, *Bifidobacterium*, or *Clostridium tabificum* into germ-free rats accelerated the gut transit time and small-bowel migrating motor complexes. However, the colonization of *E. coli* significantly inhibited intestinal myoelectric activity[24]. In a murine study, the administration of loperamide significantly increased the abundance of *Bacteroides* and *Firmicutes*, and decreased the abundance of *Lachnospiraceae*. Consequently, the colonic contractility was significantly decreased and prolonged colon transit time[25]. In addition, in the loperamide-induced mice with STC, dysbiosis was also observed in intestinal bacteria. The abundance of *Bacteroidetes* was decreased and the *Firmicutes* and *Proteobacteria* increased significantly[26]. On the contrary, a decreasing abundance of *Clostridiales* and *Lactobacillales* and a significantly increasing in *Bacteroidales* abundance was noted in the loperamide-induced constipation rats[27]. Our study also found that the diversity and composition of the intestinal microbiome were dysbiotic, identifying the association between intestinal bacteria dysbiosis and the development of STC. Based on previous studies[28,29], we speculated that CA was

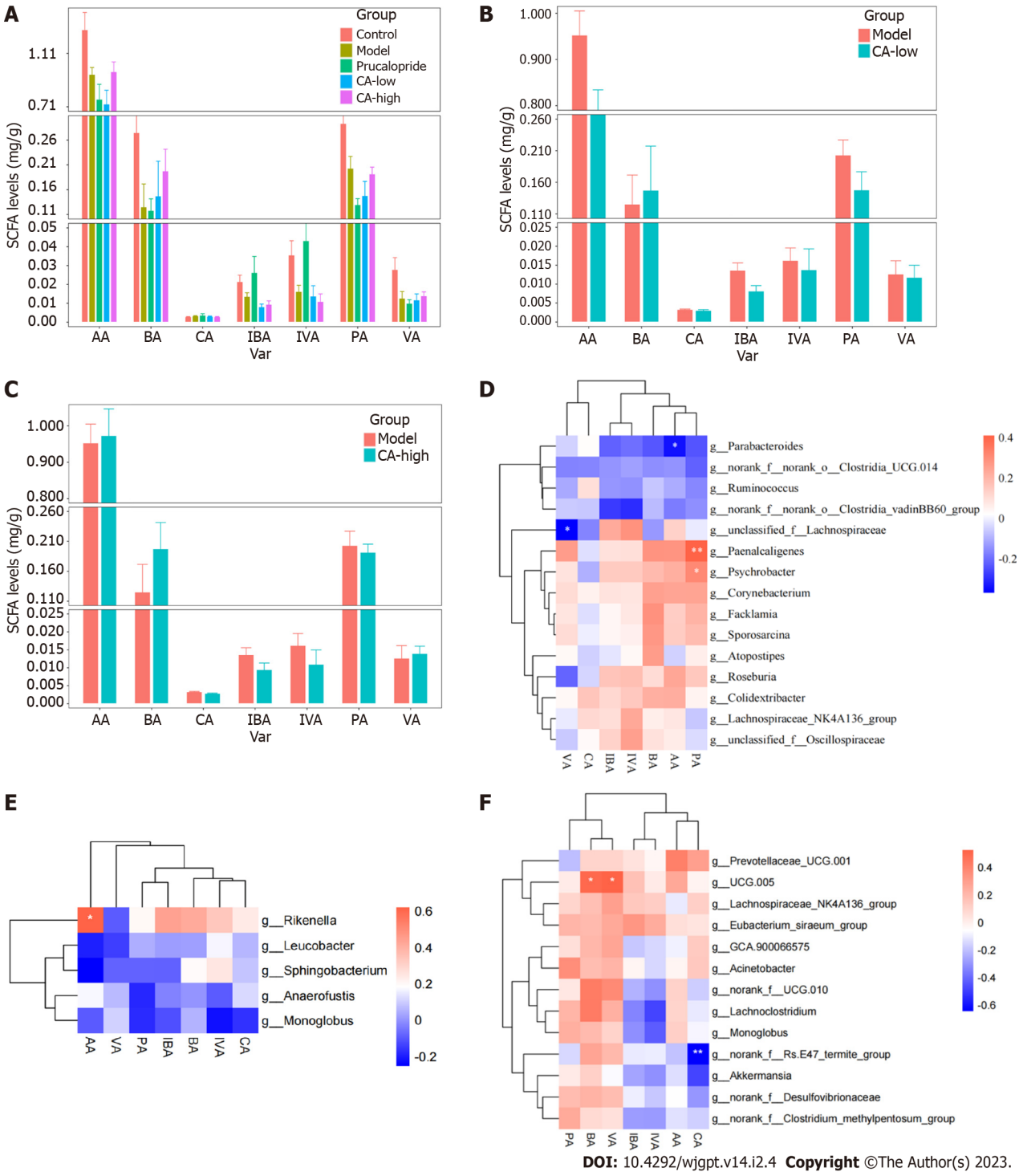

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Figure 8 Quantitative analysis of short-chain fatty acids and their correlation with the dominant microbiome (*n* = 8). A: Short-chain fatty acids (SCFAs) level in each group; B: SCFAs level in model and CA-low group; C: SCFAs level in model and CA-high group; D: Heatmap for the correlation between SCFAs and differential microbiome in each group; E: Heatmap for the correlation between SCFAs and differential microbiome in CA-low group; F: Heatmap for the correlation between SCFAs and differential microbiome in CA-high group. PRU: Prucalopride; CA-low: Cinnamic acid with low dose; CA-high: Cinnamic acid with high dose.

absorbed into the bloodstream mainly in the duodenum and jejunum and indirectly affected on the intestinal tract and the intestinal flora. However, no relevant experiments were designed to prove this in our study. Therefore, the study of absorption and metabolism of CA in STC mice will help to systematically elucidate the mechanism of action of CA in the treatment of STC.

SCFAs have been verified to affect the gut motility and contractility, colonic transit time, mucus production and the gut-brain axis. The alterations of the intestinal microbiome could regulate the production of SCFAs by changing the intestinal environment. Butyrate stimulates the Na and Cl absorption in the intestine and accelerates colon transit[12]. Butyrate also significantly increased the

Table 1 Concentration of 5-hydroxytryptamine and vasoactive intestinal peptide in each group of mice

Groups (n = 8)	5-HT (ng/mL)	VIP (ng/mL)
Control	66.83 ± 4.50	14.64 ± 2.08
STC Model	53.48 ± 4.23[b]	30.73 ± 3.70[b]
PRU	67.69 ± 5.00[d]	18.18 ± 1.92[d]
CA-low	65.31 ± 4.44[d]	19.28 ± 1.30[d]
CA-high	66.00 ± 5.93[d]	19.31 ± 1.75[d]

[b]P < 0.01 versus the control group.
[d]P < 0.01 versus the slow transit constipation model group.
5-HT: 5-hydroxytryptamine; CA: Cinnamic acid; PRU: Prucalopride; VIP: Vasoactive intestinal peptide; STC: Slow transit constipation.

colonic muscle contractions and promoted colonic transit by increasing the proportion of choline acetyltransferase in rats' enteric nervous system[30]. In addition, another study *in vitro* has shown that butyrate, propionate, and valerate induced the phasic contractions in the middle and distal colon *via* connecting the mucosal receptors to enteric and/or vagal nerves[31]. More and more studies have indicated that the intestinal microbiome regulates the level of SCFAs. A study predicted that the genus of *Coprococcus*, Roseburia, and *Faecalibacterium* increased the level of butyrate in constipation patients [6]. de Meij *et al*[32] found an increase in *Bacteroides fragilis*, *Bacteroides ovatus*, *Bifidobacterium longum*, *Parabacteroides spp*., and a decrease in *Alistipes finegoldii* in children with STC compared to healthy children. Parthasarathy and his colleagues found by 16S ribosomal RNA gene sequencing that the colonic mucosal microbiota of STC patients differed from that of healthy patients——increased abundant of *Bacteroidetes spp.* and decreased abundant of *Firmicutes spp*. (*Faecalibacterium, Lactococcus, and Roseburia*). And they revealed that *Firmicutes spp*. were associated with faster colonic transport, and methane (slowing intestinal motility) production was related to the composition of the fecal microbiota but not to constipation or colonic transport[5]. Moreover, the abundance of *Prevotella* is positively correlated with the fiber content of the diet[33]. *Clostridium spp*., and *Ruminococcus spp*. were responsible for the significant fraction of AA, BA and PA production[34]. Our study found that the main types of SCFAs (including AA, BA, CA-1, IBA, IVA, PA and VA) were decreased in the loperamide-induced mice with STC. After being treated by CA, most of SCFAs level were increased with the specific microbiome regulated by CA. The level of BA and VA was significantly increased with *g_UCG.005*, PA was increased with the abundance of *g_Paenalcaligenes* and *g_Psychrobacter* significantly. But the level of CA-1 was significantly decreased with *g__norank_f__Rs-E47_termite_group*.

CONCLUSION

Conclusively, this study provided experimental evidence that CA was an effective agent in treating STC. This conclusion was followed by the results that CA ameliorated the infiltration of neutrophils and lymphocytes, increasing the number of goblet cells and the colon mucosa secretory function. CA significantly improved the diversity and abundance of the beneficial microbiome. Furthermore, the changed abundance of *Firmicutes, Akkermansia, Lachnoclostridium, Monoglobus, UCG.005, Paenalcaligenes, Psychrobacter* and *Acinetobacter* were involved in the production of AA, BA, PA and VA. Our results identified that CA could ameliorate the composition and abundance of the intestinal microbiome to regulate the production of SCFAs in STC.

ARTICLE HIGHLIGHTS

Research background

Slow transit constipation (STC) is a disorder with delayed colonic transit. Cinnamic acid (CA) is an organic acid in natural plants with low toxicity and biological activities to modulate the intestinal microbiome.

Research motivation

We found CA to be very effective in treating STC.

Research objectives

We intend to explore the potential effects of CA on the intestinal microbiome and the primary endogenous metabolites.

Research methods

Loperamide was applied to induce STC in mice. The treatment effects of CA on STC mice were assessed from the 24 *h* defecations, fecal moisture and intestinal transit rate. We used the enzyme-linked immunosorbent assay, Hematoxylin-eosin and Alcian blue and Periodic acid Schiff staining, 16S rDNA and gas chromatography-mass spectrometry to explore the potential effects of CA on the intestinal microbiome and the primary endogenous metabolites-short-chain fatty acids (SCFAs) and evaluate the therapeutic effects of CA in STC.

Research results

CA ameliorated the symptoms and the pathology of STC and treated STC effectively. CA significantly increased the concentration of 5-HT and reduced VIP. CA significantly improved the diversity and abundance of the beneficial microbiome. The production of SCFAs (including acetic acid, butyric acid, propionic acid and valeric acid) was significantly promoted by CA.

Research conclusions

CA could treat STC effectively by ameliorating the composition and abundance of the intestinal microbiome to regulate the production of SCFAs.

Research perspectives

CA is effective in treating STC mice, and further studies are needed to better advance its clinical application.

FOOTNOTES

Author contributions: Jiang JG and Luo Q conceived the project and wrote the manuscript; Luo Q performed the central part of the experiments and analyzed data, with contributions from Li SS, Tan TY, and Xiong K; Yang T and Xiao TB participated in the experimental design and manuscript draft preparation and revision; All authors read and approved the final manuscript.

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ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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ORCID number: Jin-Guang Jiang 0000-0001-7665-8755; Qian Luo 0000-0003-0096-1736; Shuang-Shuang Li 0000-0001-7408-5797; Tian-Ying Tan 0000-0003-2133-3886; Kai Xiong 0000-0002-4063-0627; Tao Yang 0000-0002-5787-2542; Tian-Bao Xiao 0000-0003-2622-948X.

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