Research Article

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Alleviation of loperamide-induced constipation with sticky rice fermented huangjiu by the regulation of serum neurotransmitters and gut microbiota

Xinjing Feng,[a,b†] Ying Shi,[c†] Zhilei Zhou,[a,b,d] Zhongwei Ji,[a,b,d] Weibiao Zhou,[d,f] Shuguang Chen[e*] and Jian Mao[a,b,d,g*]

Abstract

BACKGROUND: Huangjiu is an important component of traditional fermented food. It is produced by cereal fermentation. Sticky rice fermented huangjiu is an abundant source of polysaccharides, oligosaccharides, proteins, amino acids, and flavor compounds (POPAF), and it has been used as a dietary supplement and pharmaceutical ingredient. The purpose of this study is to explore the alleviation of constipation using sticky rice fermented huangjiu, with the aim of providing a basis for the nutritional treatment of constipation.

RESULTS: Sticky rice fermented huangjiu was more effective in the alleviation of constipation than same concentration of ethanol treatment on serum neurotransmitters, gut microbiota, and intestinal metabolites in this 17 days constipation mouse model. Compared with ethanol treatment, the administration of sticky rice fermented huangjiu to constipated mice increased gastrointestinal motility. It alleviated the decrease in motilin (27.94%), substance P (13.85%), gastrin (63.46%), 5-hydroxytryptamine (4.55%), and short-chain fatty acid (19.80%) levels, and alleviated the increase in somatostatin levels (9.54%). Furthermore, the administration of sticky rice fermented huangjiu regulated the microbiota-mediated gut ecology through alterations in the characteristic taxa.

CONCLUSION: The results reveal that sticky rice fermented huangjiu may alleviate loperamide-induced constipation by the regulation of serum neurotransmitters and gut microbiota. These findings reveal that huangjiu is endowed with many functional components by cereal fermentation, and the bioactive substances in huangjiu can be separated and applied for medical treatment or diet therapy in the future.

Supporting information may be found in the online version of this article.

Keywords: huangjiu; constipation; serum neurotransmitters; short chain fatty acids; gut microbiota

* *Correspondence to: J Mao, National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, Wuxi, Jiangsu 214122, China. E-mail: maojian@jiangnan.edu.cn; or S Chen, Department of General Surgery, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College (CAMS & PUMC), Beijing, 100730, China. E-mail: cshuguang6@sina.com*

† *The authors contributed equally to this work.*

a *National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, Wuxi, China*

b *School of Food Science and Technology, Jiangnan University, Wuxi, China*

c *Department of Food Science and Nutrition, College of Biosystems Engineering and Food Science, Zhejiang University, Hangzhou, China*

d *Shaoxing Key Laboratory of Traditional Fermentation Food and Human Heath, Jiangnan University (Shaoxing) Industrial Technology Research Institute, Shaoxing, China*

e *Department of General Surgery, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College (CAMS & PUMC), Beijing, China*

f *Department of Food science and technology, National University of Singapore, Singapore, Singapore*

g *National Engineering Research Center of Chinese Rice Wine, Zhejiang Guyuelongshan Shaoxing Wine CO., Ltd, Shaoxing, China*

INTRODUCTION

Constipation is a chronic disease, which affects up to 15% of the global population[1] and 5%–30% of the elderly.[2] Medication is widely used to alleviate constipation,[3] including laxatives, stool softeners, and prokinetic drugs;[4] however, a large majority of such drugs may induce adverse effects such as diarrhea, vomiting, electrolyte disturbances, and stomach cramps.[5] Diet therapy has recently received increasing attention due to its promising regulatory effect on the gut microbiota.[6] Evidence indicates that functional-ingredient-rich food plays an important role in alleviating constipation without obvious side effects compared with drug treatments.[7] Traditional fermented food is produced in a unique brewing process, which possesses the advantages of strong functionality, low side effects, and high biological activity. For this reason, the use of fermented food is a promising approach to prevent or relieve constipation.

Huangjiu is an important component of traditional fermented food, which has been an important part of the daily human diet in China for thousands of years. The primary raw materials for huangjiu production are rice, wheat, and wheat bran. In the pretreatment stage, the rice starch of steamed rice is enzymatically hydrolyzed and saccharified;[8] huangjiu is subsequently endowed with plentiful polysaccharides, oligosaccharides, proteins, amino acids, and flavor substances in the low-temperature and long-term fermentation by a unique variety of species (including *Aspergillus* spp., *Rhizopus* spp., *Fusarium*, *Mucor*, *Bacillus*, *Saccharopolyspora*, lactic acid bacteria and yeasts).[9] Huangjiu is frequently ingested as an auxiliary medicine, which has potential applications in human health, although this has not been confirmed by research. Indigestible polysaccharides and oligosaccharides are considered to be potential prebiotics.[10] Numerous dietary polysaccharides, such as durian peel polysaccharide,[11] *Spirulina platensis* polysaccharide,[12] and isomaltooligosaccharide[13] have been shown to alleviate constipation. Nevertheless, these various and abundant functional components have not been isolated and applied to the treatment of constipation. In previous studies, huangjiu polysaccharides,[14] the main huangjiu oligosaccharides (isomaltooligosaccharide),[13] huangjiu peptides,[15] partial dietary proteins and amino acids,[16] and analogous volatile compounds[17] have been proven to help to restore the dysbiotic microbial community by regulating specific gut taxa. Huangjiu polyphenols have also been proven to exert anti-inflammatory effects. Polysaccharides, oligosaccharides, protein, amino acids, and flavor substances in huangjiu treatment may therefore be an effective therapy to alleviate constipation.

The gut microbiota composition of constipated patients has been shown to differ substantially from that of healthy individuals.[18] Recent findings have revealed that the pathogenesis of constipation is closely related to abnormal changes in the gut microbiota,[19] which suggests the effect of regulation of huangjiu polysaccharides and oligosaccharides on gut microbiota is a potential pathway to alleviate constipation. The possible pathways by which huangjiu alleviates constipation include (i) upregulation of beneficial gut microbiota such as *Lactobacillus*;[20] (ii) elevation of intestinal metabolite production; (iii) modification of serum neurotransmitter levels, and (iv) regulation of the 5-hydroxytryptamine (5-HT) pathway. However, the specific mechanism remains unclear.

In this study, a mouse constipation model was established by the intragastric administration of loperamide (Xian Janssen Pharmaceutical Ltd, Xian, China), and phenolphthalein tablets (Junan County Pharmaceutical Factory, Linyi, China) were used to cure constipation. The effects of sticky rice fermented huangjiu on the physiological and intestinal parameters, serum neurotransmitter levels, histological parameters, short-chain fatty acid (SCFA) content, and gut microbiota composition were investigated in comparison with ethanol treated mice. This study was thus designed to explore the anti-effect of sticky rice fermented huangjiu on constipation, which aimed to provide a basis for the nutritional treatment of constipation.

MATERIALS AND METHODS

Determination the content of functional components in huangjiu

Determination of oligosaccharides and polysaccharides content

The method used for the determination of oligosaccharides and polysaccharides followed a previous study,[21] with some modifications. A roll-type membrane filtration device (Shaoxing Haina Membrane Technology Co., Ltd, Shaoxing, China) was used to filter the huangjiu sample (Shaoxing semi-dry huangjiu, Shaoxing, China). The huangjiu (2000 mL) was ultrafiltered with a polyethersulfone 3000 Da membrane (Shaoxing Haina Membrane Technology Co., Ltd) at 1.0 MPa. Additional deionized water was added to the feed tank until the permeate volume achieved 50 L. The retentate and permeate liquid were collected separately to determine the content of total sugars and reducing sugars, using phenol-sulfuric acid method,[21] and the 3,5-dinitrosalicylic acid colorimetry method,[22] respectively. In addition, the other reagents not marked in the paper were purchased from Chinese National Pharmaceutical Group Co., Ltd. (Shaoxing, China).

The polysaccharide content in huangjiu was equivalent to the total sugar content in the retentate and the oligosaccharide content could be calculated by determining the sugar content in the permeate liquid. The total oligosaccharide content was calculated as follows:[21]

$$\text{total oligosaccharides content} = \begin{matrix}\text{total sugar content} \\ -\ \text{total reducing sugar content}\end{matrix} \quad (1)$$

Determination of protein content

The protein content in huangjiu was determined using the Kjeldahl method.[23]

Determination of free amino acids content

The method used for the determination of amino acids followed the method used in a previous study,[24] with some modifications. First, 0.8 mL 10% trichloroacetic acid was added to 0.8 mL huangjiu and shaken vigorously. Two hours later, the mixture was centrifuged at 7000×*g* for 5 min. The supernatant was filtered through a 0.22 μm syringe filter and injected into the high-performance liquid chromatography (HPLC) system. Each huangjiu was analyzed in triplicate.

An Agilent 1100 HPLC system coupled with a diode array detector (DAD) (Agilent Corp., Karlsruhe, Germany) using phthalaldehyde/ fluorene methoxycarbonyl derivatives was used to determine the free amino acids in huangjiu.[24] The chromatography column (ODS Hypersil; column length: 250 mm; column width 4.6 mm; inner diameter 5 μm) was obtained from Agilent Technologies (Tianjin, China). The column temperature was set to 40 °C and the flow rate was 1.0 mL·min^{-1}. The amino acids

were determined at 338 and 262 nm. The injection volume was 1 μL. Mobile phase A was a 20 mM sodium acetate buffer containing 0.02% v/v triethylamine. Mobile phase B was a 20% 20 mM sodium acetate buffer, 40% acetonitrile, and 40% methanol. Both mobile phases were adjusted to pH 7.20 with low-concentration acetic acid. The gradient was set up as follows: 8%–60% B from 0 to 27.5 min, 60% B to 100% B from 27.5 to 31.5 min, 100% B from 31.5 to 34 min, 100%–8% B from 34 to 35.5 min, and 8% B from 35.5 to 40 min. The amino acid mixture standard solution (1 mM, Wako, Japan) was used to analyze the free amino acids quantitatively.

Determination of main volatile flavor substances

The headspace solid-phase micro-extraction (HS-SPME) method was used as described in a previous study,[25] with some modifications. A Thermo Fisher Trace 1300 ISQ mass spectrometer (Thermo Fisher Scientific Inc., Massachusetts, USA) was used to perform operations. The alcohol concentration of huangjiu was diluted to 6% (v/v), and subsequently 6 mL diluted huangjiu was sucked into the headspace bottle. Then 1.5 g NaCl and 15 μL 2-octanol (internal standard, 8800 mg L^{-1}) were added to the mixture and 50/30 μm divinylbenzene/carboxen/poly (dimethylsiloxane) (DVB/CAR/PDMS) coated fibers were used to extract the volatile compounds. The huangjiu sample was equilibrated and extracted at 60 °C for 50 min. Subsequently the fiber was inserted into the injection port of the gas chromatography mass spectrometry equipment for 6 min to desorb the analytes.

Separation was carried out on a TG-Wax (30 m × 0.25 mm × 0.25 μm) with 1.0 mL min^{-1} helium as the carrier gas.[26] The injection port was set at 250 °C. The temperature program was set as follows: The detector was held at 40 °C for 3 min, then it was ramped up to 100 °C at the rate of 6 °C·min^{-1}. Subsequently the detector was heated to 230 °C at the rate of 10 °C min^{-1}, and held for 7 min. The electron energy was set to 70 eV to acquire the mass spectra (mass range of m/z33–350). The transfer and ion source temperatures were set to 240 °C and 280 °C, respectively.

Animals and experimental design

Forty-seven-week-old male pathogen-free BALB/c mice with a weight of 22 g ± 2 g were purchased from Zhejiang Vital River Laboratory Animal Technology Co., Ltd. (Jiaxing, China). The mice were housed in a pathogen-free barrier animal laboratory at the animal experiment center of Jiangnan University. The breeding environment was maintained at a temperature of 24° C ± 2 °C and relative humidity of 50% ± 20%, with a circadian cycle of 12 h light and 12 h dark (08:00 a.m. to 20:00 p.m. every day). All experiments were approved by the animal ethics committee of Jiangnan University (approval number SYXK2016-0045). The animal procedures were approved by the animal management and use committee of Jiangnan University (JN. No20201130b0960104 [334]). All animal experiments complied with Animal Research Reporting of in Vivo Experiments guidelines and the protocol was conducted in accordance with EU guidelines (directive 2010/63/EU).

During the animal trial, the weight gain of the mice was monitored on a weekly basis. After a week of adaptive feeding, the mice were randomly divided into five groups with eight mice per group, including a control group, a model group, a phenolphthalein group, a huangjiu group, and an ethanol group. The feeding details are provided in Table 1. During the feeding period, each mouse was allowed to consume standard food (190.10 g kg^{-1} crude protein, 49.60 g kg^{-1} crude fat, 592.60 g kg^{-1} carbohydrates, including 25.70 g kg^{-1} dietary fiber) and water at will. The daily consumption of huangjiu (14% alcohol content, Shaoxing, China) was set to 174 mL following Dietary Guidelines for Americans.[27] This is equivalent to the dose for a human subject weighing approximately 60 kg, from which the mouse gavage dose was calculated by the body surface area calculation method. During days 1–14, the mice in the control and model groups were intragastrically administered 0.66 mL of sterile water on a daily basis, whereas the mice in phenolphthalein group were treated with 0.66 mL of phenolphthalein solution (70 mg kg^{-1} bw). Moreover, the mice in huangjiu group were intragastrically administered 0.66 mL huangjiu, and the mice in ethanol group were treated with 0.66 mL food grade ethanol (14%, purchased from Jinan BaoHui Chemical Co., Ltd., Jinan, China) on a daily basis. The mice received intragastric gavages twice a day (0.33 mL for each intragastric administration), with an interval of 7 h between administrations. During days 15–17, the mice in the control group were given 0.2 mL of sterile water by gavage, whereas the other mice were administered 0.2 mL of loperamide solution (10 mg kg^{-1} bw) at 8:30 a.m. Subsequently the mice in the control group were respectively administered 0.33 mL of sterile water at 2: 00 PM and 8:00 PM, and the mice in model group were also gavaged as above method. In addition, the mice

Table 1. Ingredients and doses for intragastric administration

Groups	Intervention period 1–14 days	Modeling period 15–17 days
Control group	8:30 a.m.: Sterile water 0.33 mL 3:30 p.m.: Sterile water 0.33 mL	8:30 a.m.: sterile water 0.2 mL 2:00 p.m.: sterile water 0.33 mL 8:00 p.m.: sterile water 0.33 mL
Model group	8:30 a.m.: Sterile water 0.33 mL 3:30 p.m.: Sterile water 0.33 mL	8:30 a.m.: loperamide hydrochloride 0.2 mL 2:00 p.m.: sterile water 0.33 mL 8:00 p.m.: sterile water 0.33 mL
Phenolphthalein group	8:30 a.m.: Phenolphthalein solution 0.33 mL 3:30 p.m.: Phenolphthalein solution 0.33 mL	8:30 a.m.: loperamide hydrochloride 0.2 mL 2:00 p.m.: phenolphthalein solution 0.33 mL 8:00 p.m.: phenolphthalein solution 0.33 mL
Huangjiu group	8:30 a.m.: Huangjiu 0.33 mL 3:30 p.m.: Huangjiu 0.33 mL	8:30 a.m.: loperamide hydrochloride 0.2 mL 2:00 p.m.: Huangjiu 0.33 mL 8:00 p.m.: Huangjiu 0.33 mL
Ethanol group	8:30 a.m.: Food grade ethanol 0.33 mL 3:30 p.m.: Food grade ethanol 0.33 mL	8:30 a.m.: loperamide hydrochloride 0.2 mL 2:00 p.m.: food grade ethanol 0.33 mL 8:00 p.m.: food grade ethanol 0.33 mL

in the phenolphthalein, huangjiu, and ethanol groups were respectively gavaged with corresponding solutions, and the gavage time and dose was shown in Table 1. All mice were euthanized after 12 h of fasting.

Determination of water content in feces

The mice feces were collected each day. Each mouse was placed in a clean cage with dry filter paper (Wuxi Hengkang Medical Technology Co., Ltd, Wuxi, China) at the bottom. The subsequent feces were gathered within 5 h, and the numbers of fecal pellets were recorded. After weighing the wet fecal weight, the samples were dried in an oven at 60 °C for 24 h.[28] The fecal water content was calculated as follows:

$$\text{Fecal water content}\,(\%) = \frac{\text{wet weight}\,(\text{g}) - \text{dry weight}\,(\text{g})}{\text{dry weight}\,(\text{g})} \times 100 \quad (2)$$

Determination of defecation time of the first black stool

On day 16, the mice in the control group were administered sterile water, while the other mice were treated with loperamide solution. One hour later, all mice were intragastrically gavaged with 0.2 mL activated carbon (Beijing InnoChem Science & Technology Co., Ltd, Beijing, China) mixed with sterile water, phenolphthalein solution, huangjiu, or food-grade ethanol. Each mouse was placed in a clean cage with absorbent paper at the bottom. The time for each mouse to expel the first black stool was recorded. The longest defecation time of the first black stool in the model group was regarded as the termination time. Any mouse whose first black stool defecation time exceeded the termination time was excluded from the calculation.

Determination of gastrointestinal (GI) transit rates

All mice were fasted for 12 h prior to euthanization but were allowed to drink water at will. On day 18, the mice in the control group were administered sterile water, while the other mice were treated with loperamide solution. Thirty minutes later, the mice were intragastrically gavaged with 0.2 mL activated carbon mixed with sterile water, phenolphthalein solution, huangjiu or food-grade ethanol, and after a further 30 min, they were euthanized by carbon dioxide inhalation. The total length of the small intestine and the ink-advancing length were measured. The GI transit rates[29] were calculated as follows:

$$\text{GI transit rates}\,(\%) = \frac{\text{ink-advancing length}\,(\text{cm})}{\text{small intestine length}\,(\text{cm})} \times 100 \quad (3)$$

Colon histological analysis

Colon segments were obtained from the euthanized mice and fixed in paraformaldehyde solution. The fixed tissues were embedded in paraffin, cut into 4 μm thick sections, and stained with hematoxylin and eosin. The histological sections were then observed and photographed using an optical microscope.

Determination of serum neurotransmitter levels

Blood samples were obtained by eyeball extirpation and 1.5 mL blood was subsequently collected from orbital venous plexus to the anticoagulant Eppendorf tube. After allowing the blood samples to stand at room temperature for 2 h, sera were obtained by centrifugation at 3000×*g* for 15 min. The serum concentrations of neurotransmitters substance P (SP), somatostatin (SS), motilin (MTL), gastrin (Gas), and 5-HT were determined using an SP enzyme-linked immunosorbent assay (ELISA) kit, an SS ELISA kit, an MTL ELISA kit, a Gas ELISA kit, and a 5-HT ELISA kit (Nanjing SenBeiJia Biological Technology Co., Ltd, Nanjing, China) in accordance with the operation instructions.

Determination of SCFAs in feces

The method described by Wang[29] was adopted as a reference for the fecal sample pretreatment. Briefly, 20 mg of fecal sample was suspended in 500 μL of saturated sodium chloride solution, and 20 μL of 10% H_2SO_4 was added to the mixture. Next, 800 μL of anhydrous ether was added to extract the SCFAs in the feces. The mixed liquor was subsequently centrifuged at 18 000×*g* for 15 min. The water in diethyl ether was absorbed by 0.25 g of anhydrous Na_2SO_4. The mixture was then centrifuged at 18 000×*g* for 5 min to collect the upper layer of diethyl ether. The SCFAs in the diethyl ether layer were determined by gas chromatography–mass spectrometry using a Rtx-Wax column (column length: 30 m, inner diameter: 25 μm, purchased from Guangzhou Ai Xin Biology Science and Technology Co., Ltd, Guangzhou, China). The detection was performed using a Thermo Fisher Trace 1300 ISQ mass spectrometer (Thermo Fisher Scientific Inc.). The helium was used as the carrier gas, with a flow rate of 2 mL min^{-1}, and an injection volume of 1 μL. The temperature was increased to 140 °C at the rate of 7.5 °C min^{-1}, followed by an increase to 200 °C at the rate of 200 °C min^{-1}. The temperature was held for 3 min, and the ionization temperature was set at 20 °C.

16S rDNA sequencing and analysis

The method described by Zhang[30] was adopted as a reference for the 16S rDNA sequencing and analysis. The feces were subjected to gut microbiota analysis. Briefly, the bacterial DNA was extracted using the FastDNA Spin Kit (MP Biomedicals, Santa Ana, CA, USA) for feces. The V3-V4 variable regions were amplified using barcoded primers (341F: 5′- CCT AYG GGR BGC ASC AG-3′ and 806R 5′-GACTACHVGGGT WTCTAAT-3′). Polymerase chain reaction (PCR) amplification was performed as follows: initiation at 95 °C for 5 min, 20 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a final extension of 72 °C for 10 min. The purified amplicons were pooled in equimolar sections and paired-end sequenced. A library was constructed and sequenced using a MiSeq sequencer. The data were subsequently analyzed using the QIIME toolkit (QIIME 1). High-quality data with >97% similarity were defined as an operational taxonomic unit (OTU) by UCLUST (Version 1.2.22), while sequences shorter than 110 nucleotides or sequences with overlap less than 10 bp were removed. Usearch (Version 8.1.1861) was used to identify and remove the chimeric sequences. The taxonomy of each 16S rDNA gene sequence was analyzed by UCLUST against the Silva 138 16S rRNA database using a 90% confidence threshold. An OTU table was then generated for further analysis.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8, and all data were expressed as means ± SEMs. The differences among multiple comparisons were analyzed using a one-way ANOVA followed by Fisher's least significant difference (LSD) test. Differences where $P < 0.05$ were considered significant.

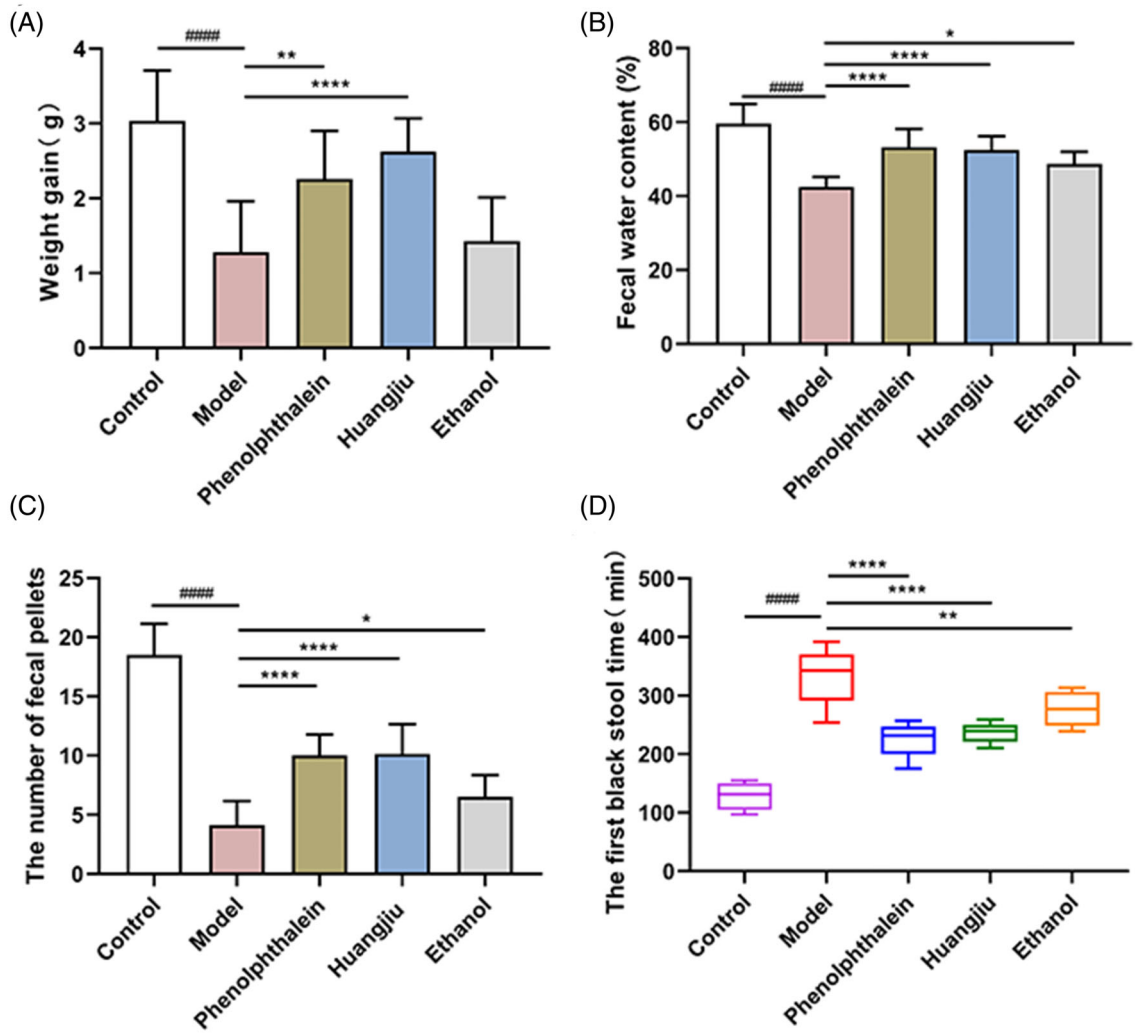

Figure 1. Effect of huangjiu on physiological parameters in constipated mice. (A) Weight gain. (B) Fecal water content. (C) The number of fecal pellets. (D) The first black stool time. Data were represented as means ± SEMs ($n = 5$). $^{\#\#\#\#}P < 0.0001$ versus the control group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ versus the model group.

RESULTS

The content of functional components (POPAF) in huangjiu

Polysaccharides, oligosaccharides, proteins, amino acids, and flavor substances (POPAF) are the five substances with highest huangjiu content, and their content in huangjiu is much more than other trace substances; POPAF was therefore used to represent the main functional components of huangjiu in the experiment. The POPAF content was determined and is presented in Table A1 in the supporting information.

Sticky rice fermented huangjiu improved physiological parameters in constipated mice

In this study, dry and hard feces and difficult defecation were observed in the model group. In contrast, more defecation and large and soft feces were observed in the other groups. The constipation model was therefore considered to have been successfully established. The effects of huangjiu on the physiological parameters of the constipated mice are shown in Fig. 1. The results reveal that huangjiu treatment significantly alleviated the weight loss decline in constipated mice ($P < 0.0001$; Fig. 1(A)) whereas ethanol treatment did not have this effect. As shown in Fig. 1(B), C), the water content and number of feces in the model group were significantly lower than those in the control group ($P < 0.05$) on day 17. In contrast, the fecal parameters were markedly ameliorated upon huangjiu administration ($P < 0.0001$). Notably, ethanol intervention similarly improved the fecal parameters in constipated mice ($P < 0.05$) but its effect was not as distinct as that of huangjiu treatment.

The defecation time of the first black stool was measured on day 16 to characterize the GI motility in the constipated mice. As shown in Fig. 1(D), the defecation time of the first black stool was significantly longer for mice in the model group than in the control group ($P < 0.05$). The mice that received an intragastric administration of phenolphthalein, huangjiu, or ethanol showed a significantly shorter defecation time than those in the model group ($P < 0.05$). The huangjiu treatment significantly improved the GI motility of the mice, and the effect was equal to that achieved by phenolphthalein administration. The relieving effect of huangjiu on constipation was significantly better than that of the ethanol intervention.

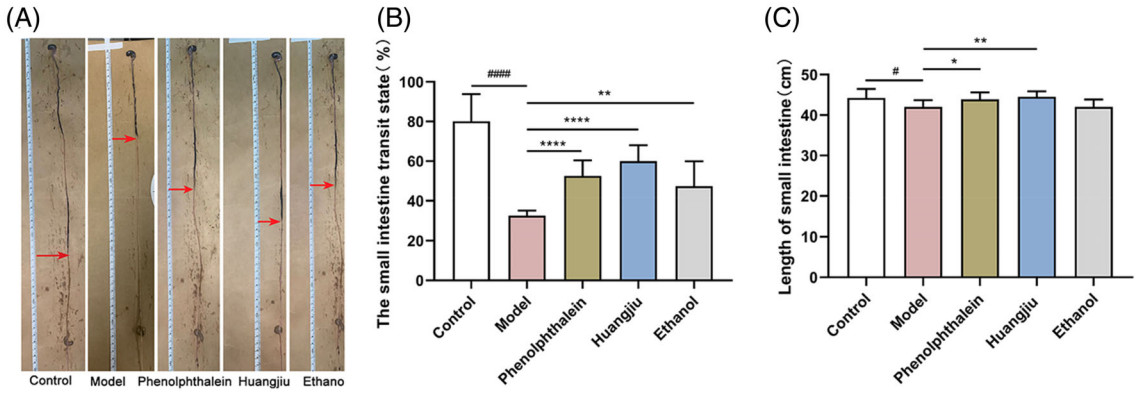

Figure 2. Effect of huangjiu on intestinal parameters in constipated mice. (A) Propulsion distance of active charcoal meal in small intestine. (B) GI transit rates. (C) Small intestine length. Data were represented as means ± SEMs ($n = 5$). $^{\#}P < 0.05$, $^{\#\#\#\#}P < 0.0001$ versus the control group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ versus the model group.

Sticky rice fermented huangjiu alleviated gastrointestinal motility in constipated mice

The GI transit rates were measured on day 18 to determine the GI motility in the constipated mice. Figure 2(A) shows the total small intestine length and the distance travelled by the activated carbon. The mice in the model group showed significantly shorter GI transit rates than those in the control group (Fig. 2(B)), which reflects the successful establishment of the constipation model. In contrast, the huangjiu, phenolphthalein, and ethanol treatments dramatically suppressed the decline in the GI transit rate caused by loperamide ($P < 0.01$). In general, a better mitigatory effect was observed in the huangjiu group compared with the ethanol group. Figure 2(C) indicates that the average small intestine length in the model group was significantly shorter than that in the control group ($P < 0.05$), which was reversed by the huangjiu treatment ($P < 0.01$). No statistical differences were observed between the model and ethanol groups. These results indicate that the loperamide treatment significantly reduced GI motility, a change that was strikingly inhibited by the huangjiu treatment.

Sticky rice fermented huangjiu alleviated serum neurotransmitter disturbances and intestinal tissue injury in constipated mice

The serum neurotransmitter (SP, SS, MTL, Gas, and 5-HT) levels were quantified to further examine the therapeutic mechanism of huangjiu in constipation. As shown in Fig. 3(A)–(D), the Gas, SP, and MTL levels in the control group were significantly higher than those in the model group ($P < 0.05$), which indicates that constipation disturbed the serum neurotransmitter levels. The MTL and SP levels were significantly upregulated, whereas the SS levels were evidently downregulated ($P < 0.05$), which suggests that huangjiu treatment was able to restore the neurotransmitter concentrations in the serum. In contrast, ethanol administration did not alleviate the neurotransmitter imbalance. Figure 3(E) shows that constipation significantly reduced the serum 5-HT levels. Notably, huangjiu treatment restored the 5-HT levels in the constipated mice to the normal level found in the control group. In contrast, no difference in 5-HT levels was noted between the model and ethanol groups.

Histological staining was used to evaluate changes in colon features to further explore the mitigatory effect of huangjiu in constipated mice. Figure 3(F) shows a histopathological image of colon tissue. The colon sections in the control group show intact colonic epithelium, which appear as a single layer of columnar epithelial cells with undamaged morphology. The lamina propria contained abundant intestinal glands, in which a large number of goblet cells were observed. No visible inflammatory infiltration was found in the tissue section. In contrast, a small amount of lymphocyte infiltration was observed in the model and ethanol groups (black arrow in Fig. 3(F)), which indicates slight colon inflammation.

Sticky rice fermented huangjiu upregulated SCFA content in constipated mice

The fecal SCFAs were quantitated to characterize and monitor the alterations that occurred in constipated and treated mice. As shown in Fig. 4, the loperamide treatment significantly reduced the SCFA content ($P < 0.05$). The change was notably reversed by the administration of huangjiu and phenolphthalein ($P < 0.05$), whereas the ethanol treatment failed to elevate the SCFA content. The fecal content of acetic acid, propionic acid, and butyric acid in the constipated mice were significantly lower than those in the control group ($P < 0.01$; Fig. 4(B)–(D)). A remarkable improvement in the acetic acid and propionic acid content was observed in the huangjiu treatment group ($P < 0.05$), but no difference was noted between the model and ethanol groups.

Sticky rice fermented huangjiu reshaped the gut microbial structure in constipated mice

The fecal microbiota was characterized by 16S rDNA sequencing to investigate the regulation of the gut microbiota. Significant differences in the Chao1 index ($P < 0.001$) and no notable differences in the Shannon index and Simpson index were observed between the control and model groups (Fig. 5(A)–(C)). The huangjiu treatment restored the Chao1 level ($P < 0.05$) and significantly reduced the Simpson index ($P < 0.01$). In contrast, the ethanol treatment showed no effect on the Simpson index and Chao1 index in the constipated mice. The Shannon index in the ethanol group was also lower than that in the model group ($P < 0.05$). These results indicate that, compared with ethanol treatment, huangjiu treatment more effectively restored the gut microbial diversity.

The Bray–Curtis algorithm was used to obtain a principal coordinate analysis (PCoA) map, and R software was used to identify and remove the chimeric sequences. Figure 5(D) shows notable differences in PCoA distributions between the control and model groups ($P_{control} = 0.005$). The huangjiu group samples were mainly

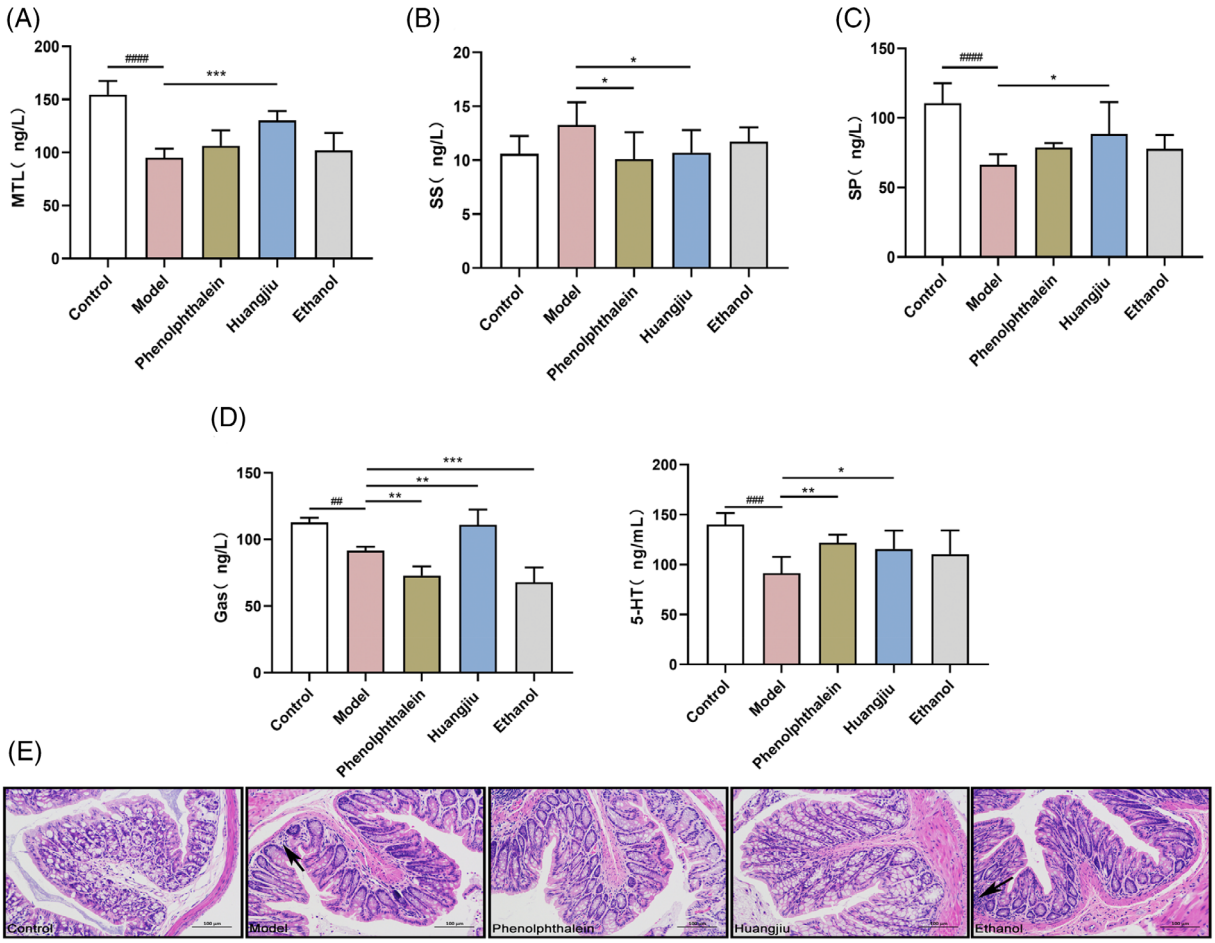

Figure 3. Effect of huangjiu on the serum neurotransmitter levels and histological changes in constipated mice. (A) MTL concentrations. (B) SS concentrations. (C) SP concentrations. (D) Gas concentrations. (E) 5-HT concentrations. (F) Colon histopathological features. Data were represented as means ± SEMs ($n = 5$). $^{\#\#}P < 0.05$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$ versus the control group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the model group.

distributed directly under the control group, whereas the phenolphthalein and ethanol group samples were scattered between the model and control groups. Compared with the model group, phenolphthalein, huangjiu and ethanol could significantly change the β-diversity of gut microbiota in constipated mice ($P_{phenolphthalein} = 0.033$, $P_{huangjiu} = 0.014$, $P_{ethanol} = 0.006$).

The PCoA map indicated that loperamide administration reshaped the fecal microbiota structure. Huangjiu treatment notably led to a partial recovery of the β-diversity, whereas ethanol treatment did not achieve a similar effect. Sticky rice fermented huangjiu played a role in re-establishing the homeostasis of the disordered gut microbiota, which could restore normal intestinal peristalsis in the constipated mice, thereby alleviating constipation.

The phylum changes in the gut microbiota are shown in Fig. 6. No significant differences in the abundance of Firmicutes and Bacteroidetes were found between the control and model groups (Fig. 6(C)), whereas huangjiu treatment dramatically upregulated the abundance of Bacteroidetes and the Firmicutes/ Bacteroidetes ratio. A distinct increase in Verrucomicrobia abundance was also observed in the model group ($P < 0.001$), whereas huangjiu intervention led to a significant reduction in Verrucomicrobia abundance (Fig. 6(C)). In contrast, ethanol treatment had no beneficial regulatory effect on the gut microbiota structure.

The differences in the gut microbiota were further analyzed at the genus level. As shown in Fig. 6(B), *Lactobacillus, Bacteroides*, *Muribaculum*, *Acetivibrio, Alloprevotella*, *Alitipes*, *Akkermansia*, and others accounted for a relatively high proportion of the gut microbiota in all groups. At the genus level, loperamide treatment decreased the abundances of *Lactobacillus* and *Bacteroides* and increased those of *Akkermansia* and *Candidatus Bacilloplasma* compared with the control group (Fig. 6(D)). Huangjiu treatment markedly upregulated the abundance of *Lactobacillus* and downregulated the abundance of *Akkemansia* and *Candidatus Bacilloplasma* in the constipated mice, whereas ethanol treatment failed to restore the changes to the normal level. In conclusion, exposure to huangjiu markedly restored the gut microbiota composition of the constipated mice.

A linear discriminant analysis effect size (LEfSe) of the gut microbiota (LDA score = 3.0) was used to explore the ability of huangjiu to reshape the microbial composition. Particular taxa including *Lachnospirales*, *Oscillospirales*, *Lachnospiraceae*, *Akkermansiaceae*, *Akkermansia-muciniphila*, *Akkermansia*, *Prevotellaceae NK3B31 group*, and *Ruminococcus* were over-represented in feces relative to the control group with loperamide treatment (Fig. 7(A), B)). Importantly, administration of huangjiu particularly enhanced the abundances of *Lactobacillales*, *Burkholderiales*, *Lactobacillaceae*, *Lactobacillus-murinus*, and *Lactobacillus* to the levels similar

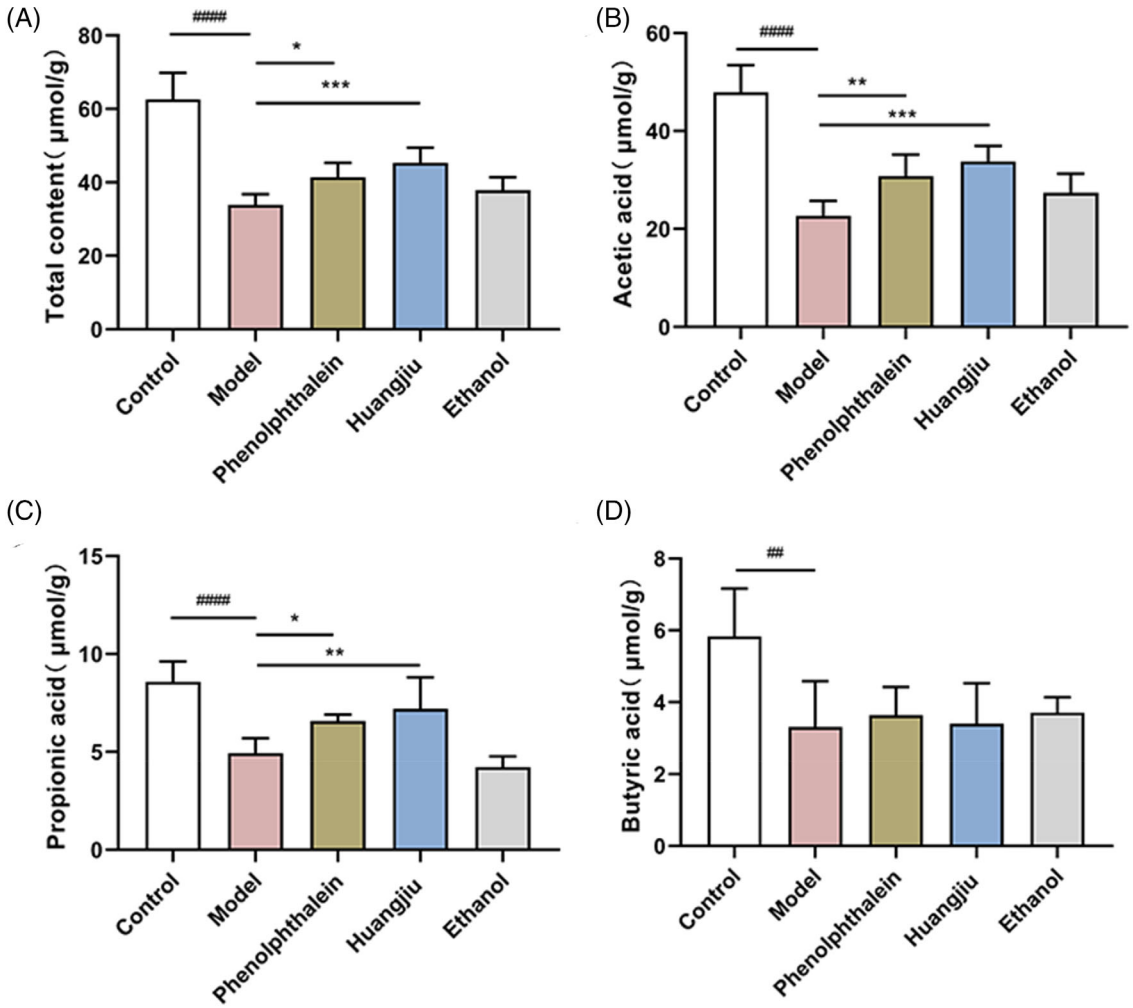

Figure 4. Effect of huangjiu on fecal SCFAs content in constipated mice. (A) Total content. (B) Acetic acid content. (C) Propionic acid content. (D) Butyric acid content. Data were represented as means ± SEMs ($n = 5$). $^{\#\#}P < 0.01$, $^{\#\#\#\#}P < 0.0001$ versus the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the model group.

to those in the control group (Fig. 7(C), (D)). Furthermore, huangjiu treatment increased the relative abundance of *Burkholderiales*, *Coriobacteriales*, *Eggerthellaceae*, *Desulfovibrionaceae*, *Lactobacillaceae*, *Lactobacillus-murinus*, *Enterorhabdus*, and *Lactobacillus* (Fig. 7(C), (D)) in constipated mice. These findings indicated that huangjiu consumption could partially restore the alterations of intestinal community structures and attenuate gut microbiota dysbiosis induced by constipation.

DISCUSSION

In this study, reduced defecation, dry and hard feces, and longer GI transit time were observed in constipated mice in the 17-day constipation mouse model. Constipation generally leads to a lower gastric emptying rate, thereby resulting in the accumulation of harmful substances in the GI tract.[31,32] Beverages with low alcohol concentrations appear to reverse the changes by stimulating gastric motility.[33] Interestingly, huangjiu treatment was found to have a better therapeutic effect on gastrointestinal transport, serum neurotransmitters, gut microbiota, and intestinal metabolites than ethanol intervention. These results suggest that huangjiu intake can be used as an effective diet therapy for constipation treatment.

During the production process of huangjiu, sticky rice and wheat are hydrolyzed and saccharified by environmental microorganisms. Functional components with diverse structures and biological effects are produced during the low temperatures and from long-term fermentation by a unique variety of species. The potentially bioactive food substance could be used for the prevention and treatment of constipation. For instance, chitosan oligosaccharides have been proven to regulate AQP3/4 and ENaC-β/γ at protein levels to recover water-electrolyte metabolism that has deteriorated, thereby alleviating constipation.[30] Konjac glucomannan can enhance gastrointestinal motility by restoring serum neurotransmitters levels, including MTL, AchE, ET-1, 5-HT, and NO[4]. Dietary supplementation of dendrobium huoshanense polysaccharides revealed a positive impact on intestinal barrier function. Huangjiu peptides also contributed to the restoration of the dysbiotic microbial community by regulating specific gut microbiota. It was suggested that the metabolism of dietary components should be an important way to attenuate constipation. The distal regions of the colon are generally deficient in carbohydrates, while indigestible huangjiu polysaccharides and

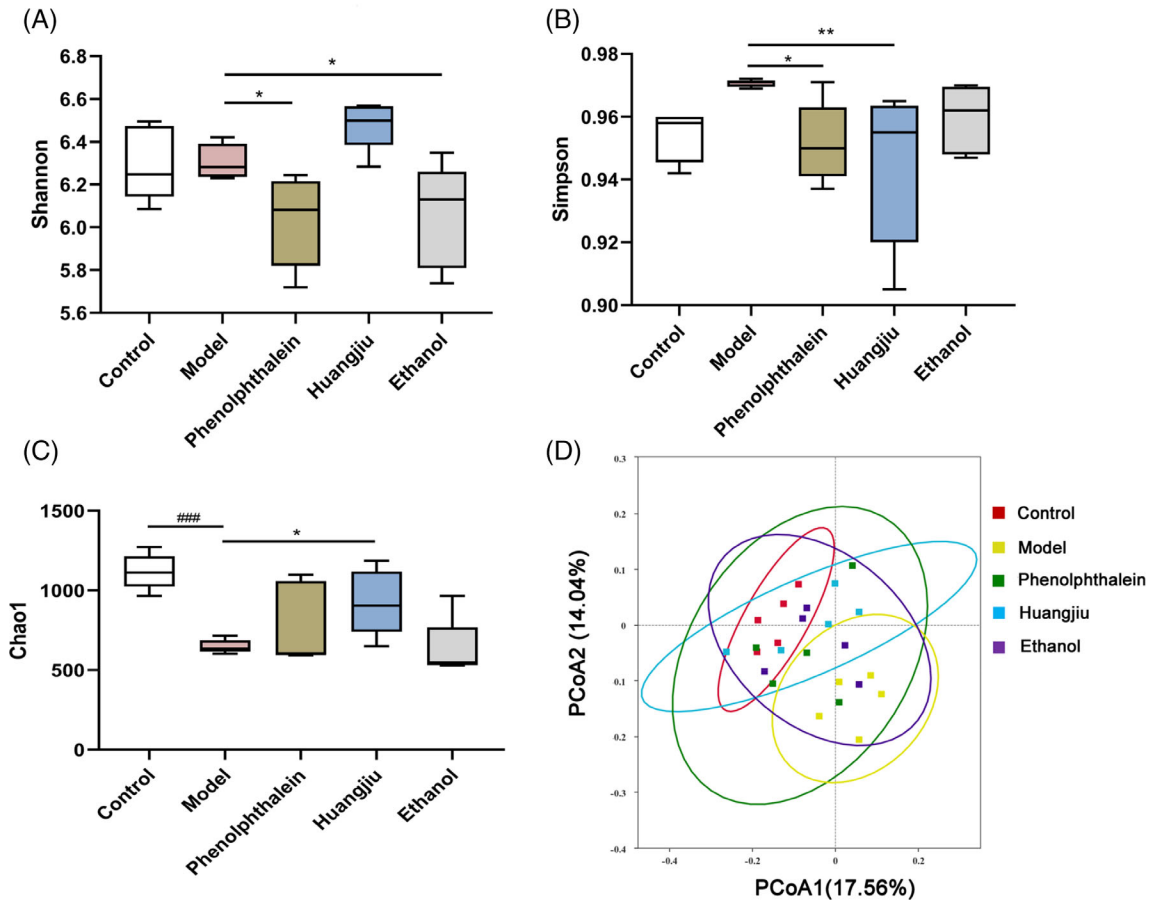

Figure 5. Effect of huangjiu on the α-diversity index and β-diversity in constipated mice. (A) α-diversity index: Shannon. (B) α-diversity index: Simpson. (C) α-diversity index: Chao1. (D) Principal coordinate analysis (PCoA) map. Data were represented as means ± SEMs ($n = 5$). $^{###}P < 0.001$ versus the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ versus the model group.

oligosaccharides provide the critical energy source for colonic microbiota, and ultimately create a more advantageous luminal environment and form a more balanced bacterial composition.[14,34] Minority plant proteins, residual-exogenous peptides, and amino acids escape digestion in the stomach and small intestine, and are utilized by proteolytic bacteria in the distal colon,[16] subsequently remodeling the structure of the gut microbiota.

Importantly, sticky rice fermented huangjiu could positively regulate the levels of certain serum neurotransmitters that related to intestinal peristalsis. Motilin plays a role in promoting gastrointestinal peristalsis by promoting the contraction of the duodenum and release of pepsin.[2] Somatostatin exerts an effect on inhibiting intestinal peristalsis by reducing the release of acetylcholine and gastrointestinal hormones.[35] Substance P is known to stimulate the release of acetylcholine, thereby accelerating colonic peristalsis.[36] Gas can promote gastrointestinal motility and contraction of the pyloric sphincter.[37] Similar to the previous research, serum neurotransmitter levels were disturbed in constipated mice[2] and ethanol treated mice, which could be partially restored via huangjiu. Eatables with abundant bioactive substances (e.g., konjac glucomannan,[4] *Durio zibethinus* rind polysaccharides,[11] isomalto-oligosaccharides,[13] and yellow tea extract[38]) have been confirmed to enhance gastrointestinal motility by regulating serum neurotransmitters levels, thereby alleviating constipation. These results illustrated that the POPAF-enriched sticky rice fermented huangjiu could contribute to the regulation of serum neurotransmitters in constipated mice, and effectively relieve constipation.

Loperamide administration led to remarkable disruption of the intestinal microbial structure, and an obvious restoration of specific bacterial genera and intestinal metabolites was observed with sticky rice fermented huangjiu treatment compared to ethanol intake. Changes in habitual dietary intake were responsible for differences in gut microbiota.[39] An increased Firmicutes/Bacteroidetes ratio frequently leads to a metabolic imbalance, and this has been observed in constipated patients.[40] As in a previous study,[41] the relative abundance of Verrucomicrobia was increased by loperamide administration, restored by sticky rice fermented huangjiu, relative to the ethanol group. Sticky rice fermented huangjiu treatment also restored the abundance of Bacteroidetes and Lactobacillus, which might contribute to the secretion of SCFAs.

The main difference between sticky rice fermented huangjiu and edible ethanol lies in the content of functional components. Polysaccharides, oligosaccharides, proteins, amino acids, and flavor compounds are the five substances with highest content, and their content is much higher than other trace substances in huangjiu. Short-chain fatty acids are the main final metabolites of POPAF fermented by gut microbiota,[42,43] which are closely related to constipation.[39] Similar research reports proposed chitosan oligosaccharides,[30] konjac mannan oligosaccharides,[44] konjac glucomannan,[4] and black tea

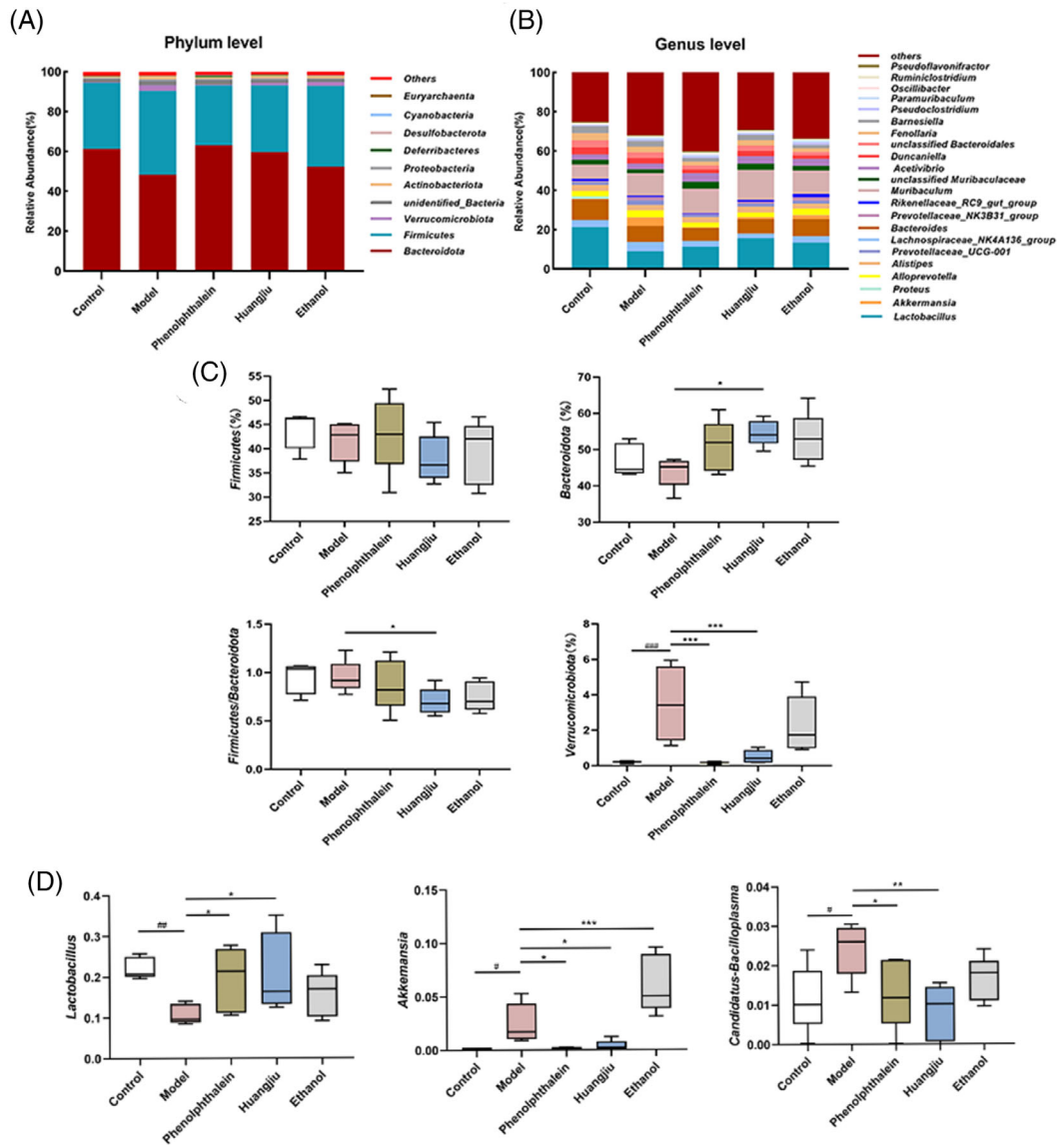

Figure 6. Effect of huangjiu on the phylum level and genus level structures of the gut microbiota. (A) Changes of gut microbiota at phylum level. (B) Changes of gut microbiota at genus level. (C) Relative abundances of Firmicutes and Bacteroidetes, the Firmicutes/ Bacteroidetes ratio, relative abundance of Verrucomicrobia. (D) Relative abundances of *Lactobacillus*, *Akkemansia* and *Candidatus Bacilloplasma*. Data were represented as means ± SEMs ($n = 5$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the model group.

extract[45] could relieve constipation by upregulating the fecal levels of SCFAs in constipated mice. Lactobacillus mainly comprises acetic acid producers[46] and propionic acid is mostly produced through the succinate pathway by Bacteroidetes.[39] The increased SCFAs may directly[47] or indirectly[48] contribute to greater secretion of 5-HT, which promoted intestinal motility in the constipated mice. 5-HT is mainly secreted by enterochromaffin (EC) cells. It is reported that 5-HT is regarded as the activator of GI motility and is considered to stimulate smooth muscle contraction in the colon and accelerate colonic transit by promoting cholinergic pathways.[49]Enterochromaffin cells are found to secrete 5-HT with external stimuli (such as acid, food, and high osmotic pressure). The possible pathways by which 5-HT alleviates constipation are as follows:[2]5-HT is transported to bind to receptors located in intestinal smooth muscle, ultimately promoting intestinal smooth muscle contraction. In addition, 5-HT promotes the secretion of serum neurotransmitters and contraction of muscle by acting on enteric neurons, which stimulates peristalsis in the intestines. 5-Hydroxytryptophan is generated from tryptophan with the action of hydroxylase, which is subsequently converted to 5-HT by 5-HTP decarboxylase. Tryptophan was generally ingested through the diet, and the content of synthesis precursor (tryptophan) increased with the intake of huangjiu protein and amino acids. Similarly, the intestinal osmotic pressure of constipated mice increased further with the action of huangjiu oligosaccharides, polysaccharides, and other substances, which promoted the secretion of 5-HT. Notably, the composition of ethanol was single compared to huangjiu, for the reason, the intake of ethanol could not stimulate the secretion of 5-HT in mice. The effect of huangjiu on 5-HT was significantly better than ethanol at the same dose, which was attributed to the POPAF in huangjiu. In the study, POPAF-enriched huangjiu effectively increased the abundance of SCFA-producing

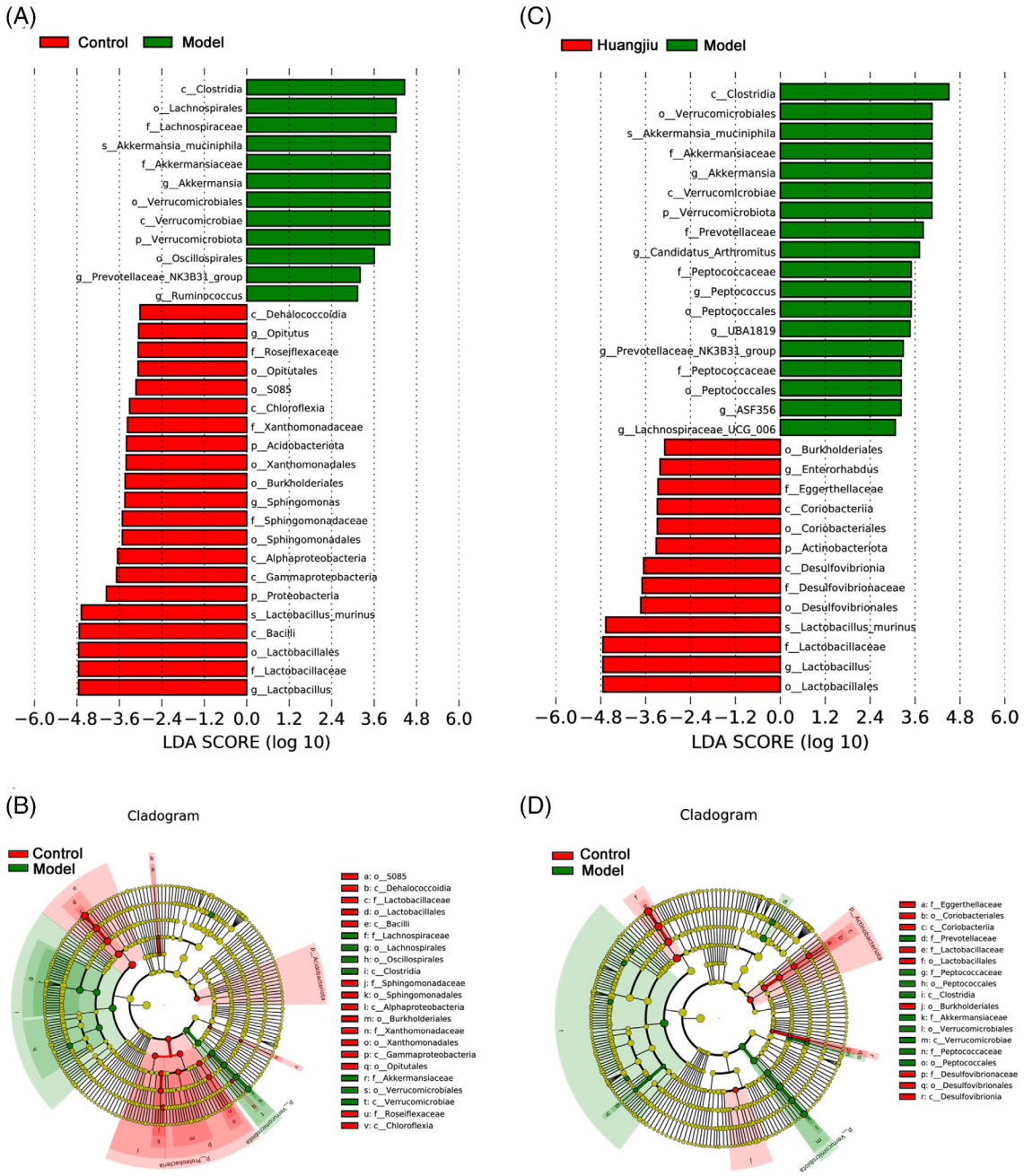

Figure 7. Identification of most characteristic taxa by LEfSe analysis between groups at the different taxonomic levels. (A, B) The differences between control and model groups. (C, D) The differences between model and huangjiu groups.

bacteria, which contributed to the release of SCFAs. Subsequently the increased SCFAs directly stimulated gastrointestinal peristalsis or indirectly stimulated the secretion of 5-HT by EC cells, subsequently directly promoting gastrointestinal peristalsis. *Lactobacillus*has also been confirmed to upregulate the expression of SERT on intestinal epithelial cells, thereby contributing to the transfer of 5-HT.[50]Moreover, the secretion of excitatory serum neurotransmitters (MTL, SP and Gas) was evidently raised, and the inhibitory serum neurotransmitters (SS) was obviously reduced with the stimulation of 5-HT, which finally enhanced gastrointestinal motility and alleviated constipation. Moreover, the specific restoration of *Akkermansia*,[51]*Enterorhabdus*, *Eggerthellaceae*[51]and *Candidatus Bacilloplasma*[52]by POPAF-enriched sticky rice fermented huangjiu might represent a fundamental step in the mitigation of constipation.

CONCLUSIONS

In summary, the present study explored alleviation of constipation by sticky rice fermented huangjiu. Huangjiu was found to alleviate loperamide-induced constipation in mice, including improving physiological parameters; accelerating gastric emptying rates; restoring Gas, MTL, SS, SP, and 5-HT levels; increasing fecal SCFAs content; and regulating the gut microbiota composition. Our findings suggest that rice fermented huangjiu may

enhance intestinal peristalsis by regulating the metabolic pathway between SCFAs and 5-HT, further demonstrating that the bioactive substances play a positive role in constipated mice. The potential mechanism might also be related to the regulation of specific gut microbial taxa such as *Lactobacillus*, *Akkermansia*, *Candidatus Bacilloplasma*, and *Enterorhabdus*. Consuming huangjiu in the daily diet can effectively maintain gut microbial community homeostasis and regulate intestinal and serum metabolites levels, which contribute to enhance gastrointestinal motility in constipated mice. These findings suggest that huangjiu is endowed with many functional components by cereal fermentation, and the bioactive substances in sticky rice fermented huangjiu can be separated and applied for medical treatment or diet therapy in the future.

ETHICS STATEMENT

This animal experiment was examined and approved by the Animal Experiment Center of Jiangnan University (license number SYXK2016-0045). It complied with the ARRIVE guidelines and the protocol was conducted in accordance with EU guidelines (directive 2010/63/EU).

AUTHOR CONTRIBUTIONS

X.F.: Formal analysis, investigation, writing – original draft, visualization. Y.S.: Methodology, formal analysis, investigation, writing – review and editing. Z.Z.: Investigation, supervision. Z.J.: Methodology. S.C.: Conceptualization, project administration. J.M.: supervision, funding acquisition.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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