

Original Research Article

Comparative studies on the regulatory effects of raw and charred hawthorn on functional dyspepsia and intestinal flora

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Abstract

Purpose: To compare the effects of raw hawthorn (RH) and charred hawthorn (CH) on functional dyspepsia (FD) and intestinal flora (IF).

Methods: A rat model of FD was established through use of a chronic stimulator. Rat models were evaluated by the rat's physical state, body weight, diet, and histopathological examination. After RH or CH administration, the digestive function of the rats was evaluated by determining gastric emptying and intestinal propulsion rate, diversity of intestinal flora.

Results: RH and CH both improved gastric emptying and intestinal propulsion rate in FD group when compared to control group ($p < 0.05$). CH yielded higher treatment effectiveness than RH. Sixteen phyla of microbiomes were recognized from all samples. After FD model establishment, the relative abundance of *Lactobacillus*, *Lachnospiraceae* and *Bacteroidales* decreased compared to normal control rats. On the other hand, the relative abundance of *Helicobacter* and *Bacteroides* in the model control group increased compared to normal control. After RH and CH treatment, the relative abundance of all dysregulated phyla was restored to varying degrees, but the levels after CH treatment were similar to those of the normal control group.

Conclusion: The relative abundance of intestinal flora of FD model rats is significantly different from that of rats in normal control group. Thus, RH and CH intervention improves digestive function, and the mechanisms may be related to adjustment of gut dysbacteriosis.

Keywords: Raw Hawthorn, Charred Hawthorn, Functional dyspepsia, Intestinal flora,

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INTRODUCTION

Functional dyspepsia (FD) is one of the most prevalent functional diseases of the digestive tract, and the incidence of FD is increasing with each passing year. The global prevalence of

uninvestigated dyspepsia in adults is 20.8 % [1]. FD is characterized by an extended course, tending towards recrudescence of symptoms that include epigastric pain and burning, feeling bloated after a meal, early satiation, distension in the epigastric region, and nausea and vomiting in

the absence of obvious organic pathologic changes [2]. All of these symptoms impact the quality of patient life. The precise mechanism of FD is not fully understood, and many current treatments are therapeutically unsatisfactory; hence, there is a need to develop a more effective treatment program.

Hawthorn refers to the ripe fruit of *Crataegus pinnatifida* Bge. var. *major* N.E.Br. or *Crataegus pinnatifida* Bge, belonging to the Rosaceae family of plants. In addition to being consumed as a food, hawthorn is a traditional Chinese herbal medicine that has been used for over a thousand years to treat dyspepsia, cardiodynia, hernia, and postpartum blood stasis. A series of investigations have demonstrated that hawthorn has numerous pharmacological effects on the digestive system [3]. Processing (pao zhi) is an integral step before clinical application of Traditional Chinese Medicine (TCM). In clinical practice, hawthorn is always charred for the treatment of dyspepsia. Some studies have also shown that CH is more effective in promoting gastric emptying and small intestine propelling in normal mice and atropine-loaded mice [4]. However, the mechanism behind the efficacy of hawthorn it cannot, as yet, be fully explained by any theory.

This paper studied the differences between the effects of RH and CH on the digestive system and intestinal flora of FD rats. In addition, this paper explores for the first time the possible mechanism by which of CH improves the digestive function.

EXPERIMENTAL

Materials and reagents

RH was collected from the Chengdu *Hehuachi* Chinese Herbal Medicine Market, which was identified by ChunJie Wu (College of Pharmacy, Chengdu University of Traditional Medicine) as being the dried fruit of *Crataegus pinnatifida* Bunge.

Drug preparation

To prepare the CH, the empty heating pot was preheated to 250 °C, and the RH was then added into the pot. The RH was stirred continuously even cooking until the surface of was brown.

Traditionally, RH and CH are boiled to treat dyspepsia. In this study, 50 g of CH or RH was weighed and extracted with boiling water twice for 1.5 h. The resultant filtrate was concentrated to 150 ml at 60 °C.

Establishment of FD rat model

The experimental program was approved by the Animal Care and Use Committee of Chengdu University of Traditional Chinese Medicine (No.20180209) and followed the international guidelines for animal studies [5] to minimize the pain and discomforts of the animals. Before experiments, rats were kept for 7 days at a temperature of 23 ± 2 °C and a relative humidity of 55 ± 2 % on a 12 h light-dark cycle with a standard aseptic diet and distilled water ad libitum. Eighty male Sprague-Dawley (SD) rats (7 weeks of age) of specific pathogen free (SPF) clean grade weighing 180 – 220 g were supplied by the Chengdu *Dashuo* Experimental Animal Co., Ltd (Chengdu, China). Eighty rats were divided into a normal control group (NC, $n = 20$) and a stress group (SG, $n = 60$) randomly. Rats within the SG group were clamped 2 cm from the base of their tail for 30 min per day to make them angry, nervous and depressed [6]. The food intake was recorded of every day and the body weight every 3 days. In addition, the behavioral characteristics of the rats were observed for 21 days.

Histopathological examination

After 21 days of chronic stimulation, 6 rats in NC and 18 rats in the SG were randomly selected and sacrificed by vertebral dislocation. Stomach and duodenum tissues were collected immediately. The samples were opened to observe whether the tissues were whether normal and smooth, congested, edematous or eroded. The stomach and duodenum were subjected to histological examination after fixing with 4% paraformaldehyde and embedding in paraffin.

Pathological slices of rat stomach and duodenum were examined under an Olympus microscope (Olympus, Tokyo, Japan) after H&E staining to determine morphological features [7].

Gastric emptying and intestinal propulsion

The rest of the rats in the SG were divided equally into three groups: the model control group (MG), the RH group (RG), and the CH group (CG). The RG and CG were given RH and CH, respectively, for 7days, at a dose of 3 g/kg per day. Gastric emptying and intestinal propulsion rates were measured to evaluate the digestive function according to a published method, with some modifications [8]. Briefly, on the seventh day, 6 rats were randomly selected from every group and fasted for 24 hours. One hour after the last RH or CH administration, all

rats were given 2g (Q_0) of a semisolid paste (3.2 g starch, 3.2 g glucose, 4 g CMC-Na, 100 mL distilled water, 0.14 mL pure black ink). Twenty minutes later, the rats were sacrificed by cervical dislocation. The stomach (from cardia to pylorus) was exposed and removed by laparotomy to measure the stomach weight (Q_1) and stomach net weight (Q_2) and determine the gastric residual rate (GR) according to the formula 1. The small intestine (from the pylorus to the ileocecal) was then removed to separate the mesentery and placed on filter paper and straightened gently to measure the distance of ink advancement (L_1) and intestinal length (L_2). The intestinal propulsion rate (IR) for each rat was calculated according to the formula 2.

$$GR (\%) = \frac{Q_1 - Q_2}{Q_0} \times 100 \dots\dots\dots (1)$$

$$IR (\%) = \frac{L_1}{L_2} \times 100 \dots\dots\dots (2)$$

Polymerase chain reaction (PCR) amplification and sequencing

Stool samples were obtained from the colon of the remaining rats under sterile conditions. To amplify the V3–V4 region of the 16S rRNA gene for Illumina deep sequencing, universal primers, 338 F: 5'-ACTCCTACGGGAGGCAGCA-3' and 806 R: 5'-GGACTACHVGGGTWTCTAAT-3', were used. The PCR was performed in a total reaction volume of 20 μ L: 13.25 μ L H₂O, 2.0 μ L 10 \times PCR ExTaq Buffer, 0.5 μ L DNA template (100 ng/mL), 1.0 μ L prime 1 (10 mmol/L), 1.0 μ L prime 2 (10 mmol/L), 0.25 μ L dNTP 2.0 μ L, ExTaq (5U/mL). After an initial denaturation at 95 °C for 5 min, amplification was performed by 30 cycles of incubations for 30 sec at 95 °C, 20 sec at 58 °C, and 6 sec at 72 °C, followed by a final extension at 72 °C for 7 min. Then the amplified products were purified and recovered using a 1.0% agarose gel electrophoresis method. The library construction and sequencing steps were performed by Beijing Biomarker Technologies Co. Ltd (Beijing, China).

Bioinformatic analysis

The bioinformatic analysis in this study was completed on the Biomarker biocloud platform

(www.biocloud.org). To obtain the raw tags, paired-end reads were merged by FLASH (v1.2.7, <http://ccb.jhu.edu/software/FLASH/>) (Magoč, 2011). Raw tags were then filtered and clustered in the next steps. The merged tags were compared to the primers, and the tags with more than six mismatches were discarded by the FASTX-Toolkit. Tags with an average quality score <20 in a 50 bp sliding window were truncated using Trimmomatic (available from <http://www.usadellab.org/cms/?page=trimmomatic>) (Bolger, 2014) and tags shorter than 350 bp were removed. We identified possible chimeras by employing UCHIME, a tool included in mothur (<http://drive5.com/uchime>).

The denoised sequences were clustered using USEARCH (version 10.0) and tags with similarity $\geq 97\%$ were regarded as operational taxonomic units (OTUs). Taxonomy was assigned to all OTUs by searching against the Silva databases (Release128, <http://www.arb-silva.de>) using the uclust within QIIME.

Statistical analysis

Data are presented as mean \pm SD. Statistical comparisons were made by one-way analysis of variance (ANOVA) using SPSS software (version 18.0, USA), followed by Dunnett *t* multiple comparison test. A P value of less than 0.05 was considered as the significance level.

RESULTS

Validation data for FD model

An FD model in rats was developed through of a chronic stimulator as described in as described in the Experimental section After 21 days rats in the SG presented with losses in body weight, dull and messy hair, slow reaction times, sluggish spirits, pasty and loose stools, poor appetites, and lassitude. These signs are similar to the symptoms of FD that are observed in humans. Compared with the NC, the body weight and food intake of SG rats were obviously decreased (Table 1), indicating that the SG rats show the symptoms of dyspepsia.

Table 1: Body weight and food intake of rats

Group	0	3	6	9	12	15	18
Body weight (mg)							
NC	207.5	219.5	239.6	257.4	271.3	284.2	299.5
SG	209.3	216.0	228.7	237.6	243.1	247.5	251.8
Food intake (mg)							
NC	22.1	22.5	22.8	23.2	23.4	23.9	24.3
SG	22.3	21.7	21.2	20.7	20.2	19.8	19.6

Histological features

The tissue structure of the stomach and duodenum of SG was morphologically normal with distinct structural layers and regular structural arrangements. Duodenal villi were neatly arranged and structurally intact. Mucosal epithelial cells arranged densely, and the morphology of the cells was regular. There was no fibrous hyperplasia in the submucosal connective tissue, vasodilation or inflammatory cell infiltration (Figure 1). The results show that the process of model establishment did not cause organic diseases and that the FD rat model was developed successfully.

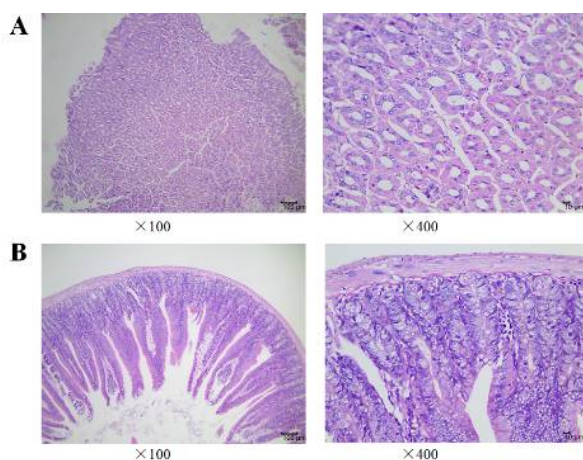


Figure 1: Stomach (A) and duodenum (B) sections of FD rats

Effect of RH and CH on gastric emptying and intestinal propulsion

Compared to NC, rats in MG showed a significant increase of semisolid paste residue in the stomach ($p < 0.05$), and a significant decrease of intestinal propelling ($p < 0.01$). RH and CH both significantly enhanced gastric emptying and intestinal propulsion ($p < 0.05$), although CH was more effective than RH (Figure 2).

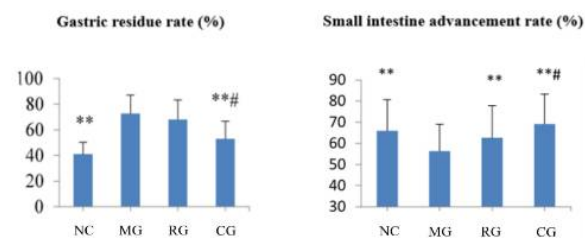


Figure 2: Gastric residue rate and intestinal propulsion rate. Data are presented as mean \pm SD; ** $p < 0.01$ vs. the FD model rats; # $p < 0.05$ vs. RH-treated rats

Effect of RH and CH on intestinal flora of FD rats

A total of 16 phylum and 159 genera of microbiomes were found in all rat stool samples (Figure 4). The microbiome composition for all groups was similar at the phyla level. The abundance of probiotics, *Lactobacillus*, *Lachnospiraceae*, and *Bacteroidales* was higher in the NC rats relative to the MG rats. On the contrary, the abundance of *Helicobacter* and *Bacteroides* was higher in the FD MG relative to the NC. After both RH and CH treatment, the relative abundance of *Lactobacillus* and *Bacteroidales* was significantly increased, although the degree of relative abundance growth was greater for CH. At the same time, the relative abundance of *Helicobacter* and *Bacteroides* in both CG and RG was significantly decreased, although the decrease was greater for CG and normal levels were reached (Figure 3).

DISCUSSION

FD is defined by Rome III Committees [9] as a chronic and recurring disease without any organic lesions, which mainly manifests as a sensation of pain or burning in the epigastrium, early satiety, fullness during or after a meal, vomiting, or a combination of the latter symptoms. Increasing evidence suggests that psychosocial factors and psychiatric disorders, especially anxiety and depression, are associated with FD [10]. Therefore, a rat stress stimulation model was chosen for use in this study. Psychological and physiological stresses, such as clamping the tails, have been shown to inhibit antral motility and gastric emptying in animals. This principal has been widely used in establishing a model for FD [11]. Stress stimulation, without any medical intervention, was the most relevant model of FD pathogenesis with simulation of a pathological state that includes tension and, to some extent, patient anxiety.

In this experiment, the rat FD model was validated by recording food and water intake, measuring changes in body weight, observing the rats' ethologic characteristics, testing gastric emptying and intestinal propulsion, and conducting histological examinations. Compared to the rats in the NC, the model rats showed several FD symptoms, such as dull and messy hair, slow reaction times, sluggish spirits, pasty and loose stools, bad appetites, lassitude, decreased food intake, and slower increases in body weight. Histological examination showed no organic lesions. All of these results showed that

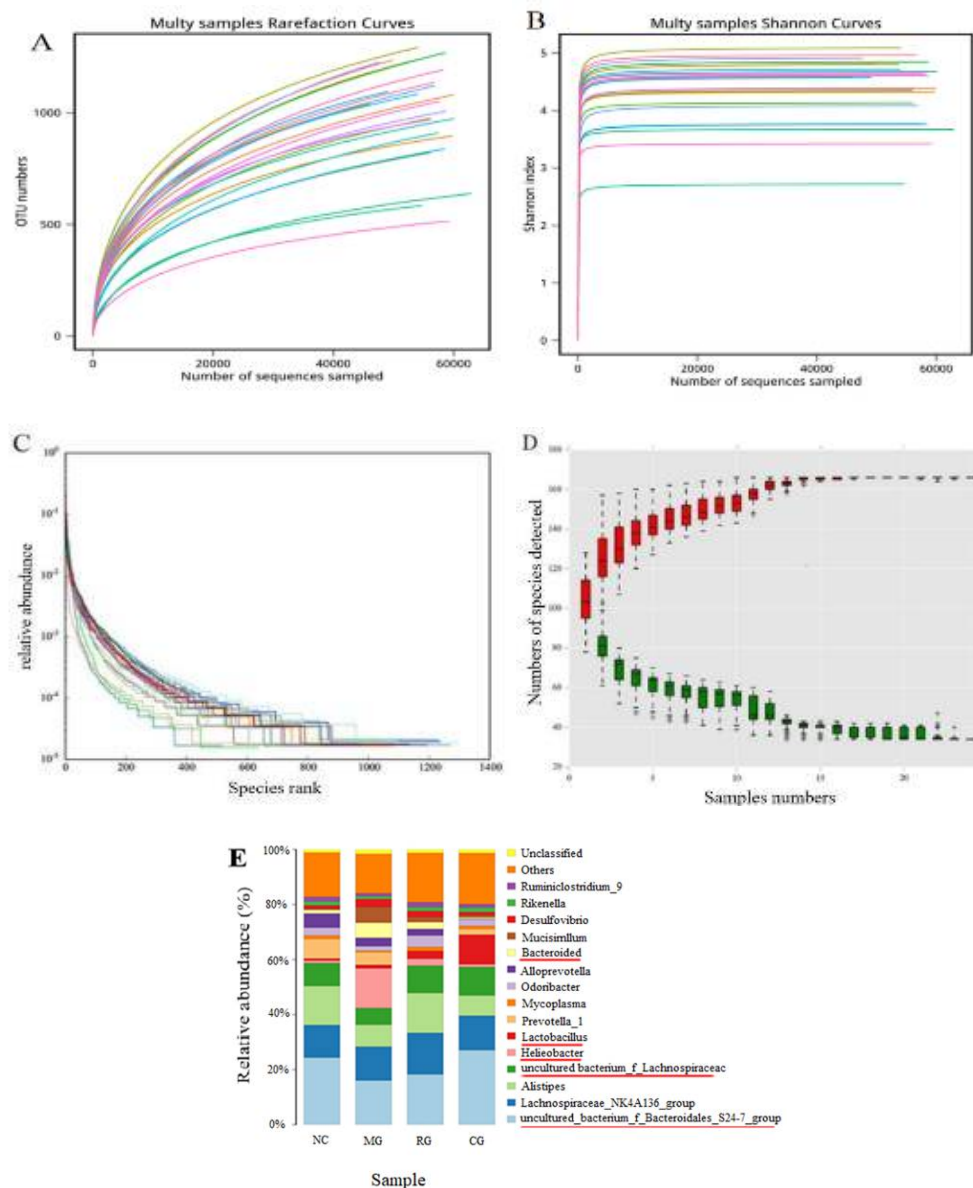


Figure 3: Effects of RH and CH on the intestinal flora of FD rats. (A) Rarefaction Curve; (B) Shannon Index; (C) Rank Abundance Curve; (D) Species Detected Accumulation Curve; (E) intestinal flora species distribution

we successfully established an FD model. CH and RH were shown to promote digestion, as indicated by the gastric residue rate and intestinal propulsion rate tests.

In recent years, many researchers have shown that the intestinal flora is related to our health. When the balance of intestinal flora is broken, the host may develop an illness, such as type 2 diabetes, irritable bowel syndrome (IBS), and FD [12,13]. Research has shown that some traditional Chinese herbal medicine may act by adjusting the composition or equilibrium of the intestinal flora [14]. Therefore, we tried to reveal the mechanism of RH and CH in treating FD from the perspective of intestinal flora. *Bacteroidetes* is one of the predominant microflora in the

human gut. Many *Bacteroidetes* are known to participate in the metabolic processing of polysaccharides, steroids, and bile acids. In addition, a decrease in the relative abundance of *Bacteroidetes* is suggested to be related to obesity, IBS, diabetes, and other conditions [15,16]. Many researchers have reported that *Lactobacillus* has a number of health benefits, such as anti-pathogenic, immunostimulatory, and catabolic activities [17,18]. Most importantly, some research showed that *Lactobacillus* can regulate and control emotion and anxiety of mice across the brain-gut axis [19]. Members of the *Lachnospiraceae* family have been shown to be associated with the production of butyric acid, a substance that is important for both microbial and host epithelial cell growth [20]. In addition, the

increases of relative abundance of harmful bacteria, such as *Helicobacter* and *Bacteroides* are likely associated with celiac disease [21], FD, gastritis, peptic ulcer disease, and even gastric cancer [22,23].

CONCLUSION

The findings of this study demonstrate that the relative abundance of the intestinal flora in FD rats established by stress stimulation is significantly different from that found in rats within NC. The results indicate that RH and CH interventions are effective in the treatment of FD, and that CH is a more effective than RH. This may be due to the fact that CH is more effective in adjusting gut dysbacteriosis. This study therefore provides a new train of thought on the strategy to treat FD. However, further *in vitro* and *in vivo* studies are required in this regard.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. C.-J. Wu conceived the research. Z, -Q. Wei and X. Chen drafted the manuscript. All authors have read and approved the manuscript for publication.

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