Investigation of the effect of hawthorn after thermal processing on functional dyspepsia based on fecal metabolomics and gut microbiota

Lilin ZHANG†, Yao TIAN†, Qi LIANG†, Chunjie WU*†, Li AI*†

Abstract

Hawthorn is an important medicine food homology (MFH) species. Charred hawthorn (CH) is derived from the thermal processing of raw hawthorn (RH). Traditional Chinese medicine theory suggests that CH has a stronger effect to promote digestion than RH. In this study, based on the functional dyspepsia (FD) model in rats, the effects of RH and CH on gastrointestinal motility were investigated, and the mechanism of action was revealed from the perspectives of gut microbiota and metabolomics. FD model was established by various stimulation and chronic induction methods for 21 days. After 7 days of intervention, CH could improve the gastric emptying rate and intestinal propulsion rate; correct the abnormal levels of 20 different metabolites in the feces; regulate the metabolic pathways of vitamin A and niacin; increase the diversity and richness of intestinal microbiota and adjust the structure and composition of gut microbiota in FD rats. And the effect of CH is better than RH. The results of this study show that CH can better modulate the metabolites and gut microbiota of rats with FD, to provide a theoretical basis for the development of dietary therapy of hawthorn as a treatment plan or adjuvant treatment for FD.

Keywords: hawthorn; functional dyspepsia; gut microbiota; fecal metabolomics; thermal processing.

Practical Application: Hawthorn is an important medicine food homology (MFH) species. Charred hawthorn (CH) is derived from the thermal processing of raw hawthorn (RH). We investigated the effects of hawthorn after thermal processing on gastrointestinal motility in rats with FD from the perspectives of gut microbiota and metabolomics and revealed the related mechanism. Our study provided a theoretical basis for the development of dietary therapy of hawthorn as a treatment plan or adjuvant treatment for functional dyspepsia.

1 Introduction

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. As a widely used Chinese herbal medicine and medicinal herb, many hawthorn-related drugs are included in the pharmacopeias of many countries (Chang et al., 2002). Hawthorn, used to improve indigestion, has a long history and definite effects. Charred hawthorn (CH) was prepared from raw hawthorn (RH) by stir-frying, a thermal processing method of traditional Chinese medicine. Traditional Chinese medicine records that compared to RH, CH has a stronger effect on improving digestive function. But the effect and mechanism are unclear. Thermal processing usually changes the chemical composition of the plant and even produces new phytochemical conjugates (Wong et al., 2019). The thermal processing of hawthorn not only changes its color but also its medicinal effects. These are related to changes in their chemical composition. Traditional Chinese medicine records that compared to RH, CH has a stronger effect on improving digestive function. This effect has also been demonstrated in our previous studies (Ai et al., 2022). CH may improve digestive function in FD rats, but its mechanism remains unclear.

Functional dyspepsia (FD) is one of the most common functional gastrointestinal diseases in clinics (Wauters et al., 2020). The overall prevalence of FD is 16% in the general population (Ford et al., 2020). FD is a difficult disease to cure clinically, and its influencing factors are many and complex. Although FD is usually not life-threatening, patients’ quality of life, social behavior, and mental health would be severely affected (Hantoro et al., 2018). Its pathogenesis is still unclear, and may be related to gastrointestinal motility disorders, hypersensitivity reactions in the stomach, Helicobacter pylori infection, and psychosocial factors (Wauters et al., 2021). In response to possible pathogenesis, many common drugs have been used to treat FD. For example, anti-Helicobacter pylori drugs, proton pump inhibitors (PPI), and prokinetic drugs (Tack & Camilleri, 2018). However, these drugs are difficult to show high efficacy in FD treatment and will bring more side effects. In recent years, due to the close relationship between food and intestinal microorganisms, more and more studies have paid attention to the role of food in the treatment of gastrointestinal diseases (Pearlman & Akpotaire, 2019). A range of medicine food homology (MFH) species have been developed as functional foods, which can provide the required nutrition for the human body and play a role in the prevention and treatment of certain diseases (Hou & Jiang, 2013).
The concept of ‘medicine and food homology’ was proposed in Huang Di Nei Jing Su Wen: ‘Eating on an empty stomach as food, and administering to the patient as medication’ embodies the theory of medicine food homology (MFH) (Xia & Xiao, 2021). In recent years, the public has come to realize the inextricable relationship between food and disease. Improper diet is an important cause of some diseases. Therefore, proper diet can play an important role in the prevention and treatment of certain diseases. This is supported by many studies. For example, MFH is involved in the prevention and treatment of diabetes (a chronic metabolic disease that cannot be cured and requires long-term medication), which is considered a safe, low-cost, and stable adjuvant treatment (Gong et al., 2020). MFH has also been well studied in the treatment of hyperlipidemia (Song & Jiang, 2017) and chronic inflammation (Lu et al., 2022). In the treatment of these diseases, chemical drugs, and other traditional treatment drugs, poor taste and side effects of drugs bring some pain to patients, and even some psychological diseases, resulting in resistance to treatment. The development of MFH food can provide an effective solution to these problems.

In this study, UHPLC-MS fecal metabolomics combined with 16S rDNA amplicon sequencing was used to analyze the gut microbiota and fecal metabolites of RH and CH in rats with FD. And we analyzed the chemical composition difference between CH and RH decoctions. Objective To investigate the mechanism of CH improving functional dyspepsia based on fecal metabolomics and gut microbiota. And to provide support for the development of functional food for hawthorn to treat FD.

2 Materials and methods

2.1 Instruments and reagents

Raw hawthorn (Tongrentang, China); Methanol, Formic acid, and Ammonium Acetate (Thermo Fisher); Water (Merck); Qiagen Gel Extraction Kit (Qiagen, Germany); TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA).

2.2 Preparation of RH and CH decoction

Preparation of CH: when the temperature reaches 250 °C, pour in 500 g of RH and stir-fry continuously for 4 min. Fry RH until the surface and sections are browned to obtain CH (Figure 1A).

50 g RH and CH were weighed and placed in the flask. They were extracted three times with 500 mL boiling water for 1.5 h each time. After filtration, the filtrate was combined and concentrated at 45 °C. Add pure water and dilute to 0.3 g/mL (Wei et al., 2019).

2.3 Animals

The Animal Care and Use Committee of Chengdu University of Traditional Chinese Medicine approved the experimental program and followed international animal studies guidelines to minimize the pain and discomfort of the animals. Fifty male SD rats (180 ± 20 g) of SPF grade were purchased from Chengdu Dasuo Experimental Animal Co., Ltd. After 3 days of adaptive feeding, all rats were randomly divided into control and model groups. The FD rat model was established by various stimulation and chronic induction methods: fasting and not prohibiting water for 24 h, water deprivation and not fasting for 24 h, tilting the cage for 24 h, fixing for 2 hours, day and night upside down, swimming for 5 min in 4 °C water, and clamping the tail for 1 min (Ai et al., 2022). Animals are exposed to different types of stimuli for a certain amount of time each day. The same stimulus is not presented two days in a row, making the animal unable to predict the stimulus it will receive.

After 21 days of continuous modeling, 5 rats were randomly selected from the control and model groups for model verification. We verified the effectiveness of the model by observing the condition of rats in the model group and control group and comparing their gastrointestinal motility. The rats were given a black semisolid paste (BSP, 5% activated carbon dissolved in 10% gum Arabic) by intragastric administration. Then the gastric emptying rate (Formula 1) and intestinal propulsion rate (Formula 2) of rats in each group were determined according to the method in the literature (Zhu et al., 2020).

The other FD rats were randomly divided into model groups, RH group, and CH group. The dosage of RH and CH decoction was 3 g/kg/d. The control and model groups were given equal amounts of normal saline. After 7 days of continuous administration, fecal samples were collected from rats in each group. The tail lift stimulated the rats to defecate, causing feces to fall on the sterile ice box wrapped in cloth. Then rat feces were placed in sterile cryopreserved tubes and stored in the -80 °C refrigerator. The gastric emptying rate and intestinal propulsion rate of rats in the control group, model group, RH group, and CH group were determined by the same method.

\[
\text{Gastric emptying rate} = \left(1 - \frac{W_1 + W_2}{W_3}\right) \times 100\% \quad (1)
\]

\[
\text{Intestine propulsion rate} = \frac{L_1}{L_2} \times 100\% \quad (2)
\]

\(W_1\): Total weight of the stomach, \(W_2\): The weight of the stomach after BSP removal, \(W_3\): The weight of BSP, \(L_1\): The movement distance of BSP, \(L_2\): The length of the small intestine.

2.4 Sequencing of 16S rDNA amplicon of microbiota

Extraction of genome DNA

Total genome DNA from fecal samples was extracted using the CTAB method. DNA concentration and purity were monitored on 1% agarose gel. According to the concentration, DNA was diluted to 1 ng/µL using sterile water.

Amplicon generation

16S rRNA genes of distinct regions were amplified using a specific primer (515F-806R) with the barcode. All PCR reactions were carried out with 15 µL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs); 2 µM of forward and reverse primers, and about 10 ng template DNA. Thermal cycling consisted of initial denaturation at 98 °C for 1 min, followed by...
30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 30 s. Finally, 72 °C for 5 min.

**PCR products quantification and qualification**

Mix the same volume of 1X loading buffer (containing SYBR green) with PCR products and operate electrophoresis on 2% agarose gel for detection. PCR products were mixed in equidensity ratios. Then, the mixture of PCR products was purified with Qiagen Gel Extraction Kit (Qiagen, Germany).

**Library preparation and sequencing**

Sequencing libraries were generated using TruSeq* DNA PCR-Free Sample Preparation Kit (Illumina, USA) following the manufacturer’s recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an Illumina platform and 250 bp paired-end reads were generated.

**2.5 Fecal untargeted metabolomics analysis**

**Metabolites extraction**

Samples (100 mg) were individually grounded with liquid nitrogen and the homogenate was resuspended with prechilled 80% methanol by the good vortex. Samples were incubated on ice for 5 min and then were centrifuged at 15,000 g, 4 °C for 20 min. Some of the supernatants were diluted to a final concentration containing 53% methanol by LC-MS grade water. Samples were subsequently transferred to a fresh Eppendorf tube and then were centrifuged at 15000 g, 4 °C for 20 min. Finally, the supernatant was injected into the LC-MS/MS system analysis (Want et al., 2013).

**UHPLC-MS/MS analysis**

UHPLC-MS/MS analyses were performed using a Vanquish UHPLC system (Thermo Fisher, Germany) coupled with an Orbitrap Q ExactiveTM HF-X mass spectrometer (Thermo Fisher, Germany). Samples were injected onto a HypeSiGold column (100 mm × 2.1 mm, 1.9μm) using a 17-min linear gradient at a flow rate of 0.2mL/min. The eluents for the positive polarity mode were eluent A (0.1% formic acid (FA) in Water) and eluent B (Methanol). The eluents for the negative polarity mode were eluent A (5 mM ammonium acetate, pH 9.0) and eluent B (Methanol). The solvent gradient was set as follows: 2% B, 1.5 min; 2-100% B, 3 min; 100% B, 10 min;100-2% B, 10.1 min; 2% B, 12 min. Q ExactiveTM HF-X mass spectrometer was operated in positive/negative polarity mode with a spray voltage of 3.5 kV, capillary temperature of 320 °C, sheath gas flow rate of 35 psi, and aux gas flow rate of 10 L/min, S-lens RF level of 60, Aux gas heater temperature of 350 °C.
Data processing and metabolite identification

The raw data files generated by UHPLC-MS/MS were processed using Compound Discoverer 3.1 (CD 3.1, Thermo Fisher) to perform peak alignment, peak picking, and quantitation for each metabolite. The main parameters were set as follows: retention time tolerance, 0.2 minutes; actual mass tolerance, 5 ppm; signal intensity tolerance, 30%; signal/noise ratio, 3; and minimum intensity, et al. After that, peak intensities were normalized to the total spectral intensity. The normalized data were used to predict the molecular formula based on additive ions, molecular ion peaks, and fragment ions. And then peaks were matched with the McCloud, Mzvault, and MassList database to obtain accurate qualitative and relative quantitative results. Statistical analyses were performed using the statistical software R (R version R-3.4.3), Python (Python 2.7.6 version), and CentOS (CentOS release 6.6). When data were not normally distributed, normal transformations were attempted using area normalization method.

2.6 Component analysis for the decoctions of RH and CH

The decoctions of RH and CH were extracted with methanol and diluted to 10 mg/mL. The LC-MS (Thermo Fisher, Germany) was used to identify their chemical composition. Samples were injected onto an Eclipse Plus C18 column (50 mm × 2.1 mm, 1.8 μm) using a 30-min linear gradient at a flow rate of 0.2 mL/min. The eluents: A (0.1% FA in Water) and eluent B (Methanol). The solvent gradient was set as follows: 0 ~ 3 min, 20% ~ 30% B, 3 ~ 5 min, 30% ~ 30% B; 5 ~ 17 min, 30% ~ 37% B; 17 ~ 19 min, 37% ~ 40% B; 19 ~ 21 min, 40% ~ 40% B; 21 ~ 24 min, 40% ~ 70% B; 24 ~ 27 min, 70% ~ 100% B; 27 ~ 30 min, 100% ~ 20% B; 30 ~ 32 min, 20% ~ 20% B. The mass spectrometer was operated in positive/negative polarity mode with a spray voltage of 3.5 kV, a capillary temperature of 320 °C, a sheath gas flow rate of 35 psi, and an aux gas flow rate of 10 L/min, an S-lens RF level of 60, Aux gas heater temperature of 350 °C.

2.7 Statistic analysis of data

Data are presented as mean ± Standard Error of Mean (SEM). Statistical significance was analyzed using SPSS software (version 18.0, USA). The comparisons between different groups were made using one-way analysis of variance (ANOVA) or non-parametric tests. Statistical significance was defined by p < 0.05.

3 Results and analysis

3.1 Model verification and effect of RH and CH on gastrointestinal motility in FD rats

Before molding, the rats had normal behaviors such as eating and sleeping. After modeling, there was no significant change in the control group, but the rats in the model group showed a gradual loss of appetite, and the fur color of the rats was messy and dim. The gastric emptying rate and intestinal propulsion rate were significantly different between the control group and the model group (P<0.001) (Figure 1B), suggesting that the FD model was successfully constructed.

After 7 days of continuous administration, the gastric emptying rate (Figure 1C) and intestinal propulsion rate (Figure 1D) of rats in each group were determined. Gastric emptying and intestinal propulsion capacity were significantly reduced in the model group (P<0.001). After the intervention of RH and CH, the gastrointestinal motility of FD rats could be significantly improved. The effect of CH is better than RH. This is consistent with previous research (Ai et al., 2022).

3.2 Effects of RH and CH on the overall structure and composition of gut microbiota in FD rats

The overall structure and composition of gut microbiota in each group were analyzed by selecting some flora with greater relative abundance at phylum and genus levels. At the phylum level, 6 floras with the highest abundance were selected (Figure 2A), including Firmicutes, Bacteroidota (Bacteroidetes), Proteobacteria, unidentifed Bacteria, Actinobacteriota, Desulfbacterota. Firmicute and Bacteroidota were dominant floras at the phylum level. The relative abundance of Firmicute was more than 50%, and its relative abundance in the model group was significantly higher than that in the control group (P<0.05). The relative abundance of Bacteroidota was between 10% and 30%, and the relative abundance of Bacteroidota in the model group was significantly lower than that in the control group (P<0.05). RH and CH tended to improve the relative abundance of Bacteroidota in FD rats, but this was not statistically significant. It is worth noting that compared with the control group, the ratio of Firmicutes to Bacteroidota (F/B) in the model group was significantly increased (P<0.01), and CH intervention significantly reduced the F/B ratio (P<0.05) (Figure 2B). The relative abundance of unidentifed Bacteria and Actinobacteriota in the model group were both less than 10%, which was significantly lower than that in the control group (P<0.001). RH had no significant effect, while CH significantly increased the relative abundance of unidentifed Bacteria and Actinobacteriota in FD rats (P<0.01).

As shown in Figure 2C, the top 10 floras with the highest abundance were selected at the genus level. After statistical analysis of the abundances of each flora in four groups, CH was found to significantly improve the relative abundances of Ligilactobacillus, Bacteroides, Dubosiella, and Limosilactobacillus in FD rats. Ligilactobacillus and Bacteroides were dominant floras at genus level, with relative abundances between 30%-60% and 5%-15%, respectively. Compared with the control group, the relative abundance of Ligilactobacillus and Limosilactobacillus in the model group was significantly increased (P<0.001). CH significantly decreased the relative abundances of Ligilactobacillus (P<0.05) and Limosilactobacillus in FD rats. RH decreased the relative abundance of Limosilactobacillus, but not Ligilactobacillus. Compared with the control group, the relative abundance of Bacteroides (P<0.01) and Dubosiella (P<0.001) in the model group was significantly reduced. CH significantly decreased the relative abundances of Bacteroides (P<0.05) and Dubosiella (P<0.001) in FD rats, but RH had no effect.

3.3 Effects of RH and CH on metabolic profile in FD rats

Effects of RH and CH on differential metabolites in FD rats

Differential metabolites were screened according to VIP, FC, and P-value. VIP is the variable importance in the projection of the first principal component of the PLS-DA model. It represents
the metabolite's contribution to the grouping (Heischmann et al., 2016). FC is 'fold change', which is the ratio of each metabolite to the mean quantitative values of all biological replicates in the comparison group. P-value is calculated by t-test and indicates the level of difference in significance. The metabolites with VIP > 1 and P-value < 0.05 and fold change ≥2 or FC≤0.5 were considered differential metabolites. A total of 77 metabolites significantly different between the control group and the model group were identified, which may be potential biomarkers of FD. A total of 99 metabolites were significantly different between RH group and model group, and 157 metabolites were significantly different between CH group and model group.

The volcano plots can visually show the overall distribution of differential metabolites (Figure 3). Compared to the control group, the content of most fecal metabolites in FD rats was significantly increased. The intervention of RH and CH could change this trend. Compared to the FD model group, the content of most fecal metabolites in RH and CH groups were significantly reduced. By comparing and analyzing the different metabolites in the groups, the differential metabolites that tended to return to normal levels after the intervention of RH and CH were retained. A total of 23 metabolites were screened, of which 14 metabolites were associated with abnormal metabolic status in FD model rats regulated by RH, and 20 metabolites were associated with.

Figure 2. (A) The 6 floras with the highest abundance at the phylum level; (B) The ratio of Firmicutes to Bacteroidota; (C) The top 10 floras with the highest abundance at the genus level (*represents comparison with the control group: P<0.05; **represents comparison with the control group: P<0.01; ***represents comparison with the control group: P<0.001; #represents comparison with the model group: P<0.05; ##represents comparison with the model group: P<0.01; ###represents comparison with the model group: P<0.001).
Hawthorn and functional dyspepsia

CH (Table 1). Some metabolites were annotated using the KEGG database, HMDB database, and LIPID Maps database.

**Effect of RH and CH on the potential biomarkers of FD**

KEGG is a powerful tool for metabolic analysis (Jia et al., 2016). The enrichment analysis of differential metabolites based on the KEGG Pathway can identify the main biochemical metabolic pathways and signal transduction pathways involved in differential metabolites. The metabolic pathways enrichment of differential metabolites was performed, when ratios were satisfied by \( x/n > y/N \), metabolic pathways were considered as enrichment, and when the \( P \)-value of metabolic pathway < 0.05, metabolic pathways were considered as statistically significant enrichment. After analyzing 77 differential metabolites between the control group and the model group, 21 KEGG pathways were obtained, and 4 of which were significant (Figure 4). They may play an important role in the pathogenesis of FD. We listed five differential metabolites that are closely related to these four pathways (\( \gamma \)-Tocopherol, 4-Hydroxybenzoic acid, Vitamin A, Nicotinic acid, and Corticosterone) as potential biomarkers of FD. Based on these five biomarkers, we investigated the effect of RH and CH on FD rats. As shown in Figure 5A, there were significant differences in the contents of 5 biomarkers between the model group and the control group. RH significantly regulated the content of Vitamin A and Corticosterone but did not affect \( \gamma \)-tocopherol, 4-hydroxybenzoic acid, and Nicotinic acid. CH tended to promote the recovery of 5 biomarkers to normal levels. And it significantly regulates the content of Vitamin A and Nicotinic acid (\( P<0.01 \)).

**Correlation of differential metabolites and gut microbiota after CH intervention**

To explore the potential functional relationship between gut microbiota and differential metabolites, Spearman's correlation analysis was performed on the 20 differential metabolites and the top 10 microbiota in relative abundance at genus level associated with CH intervention on FD. The results of the correlation analysis between the differential metabolites and the gut microbiota were shown in Figure 5B. Most metabolites were positively correlated with *Ligilactobacillus* and *Limosilactobacillus* and negatively correlated with *Dubosiella*, *Romboutsia*, and *Lactobacillus*. A few metabolites showed a significant correlation with *Bacteroides* and *Faecalibaculum*. *Clostridium_sensu_stricto_1*, *Collinsella*, and *Escherichia-Shigella* were the three microbiotas with the lowest relative abundance and no metabolites were significantly associated with them. Only the top 7 microbiota in relative abundance showed significant correlations with metabolites, indicating that the

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**Figure 3.** The volcano plots of differential metabolites between groups (The horizontal coordinate represents the fold change of metabolites in different groups (log2FoldChange). The ordinate indicates the significance level of difference (-log10p-value). Each dot in the volcano plots represents a metabolite, with significantly upregulated metabolites represented by red dots and significantly downregulated metabolites represented by green dots, and the size of the dots represents the VIP value. ESI+: Positive ion model; ESI-: Negative ion model).
Table 1. 20 differential metabolites were related to CH.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Model (VS Control)</th>
<th>RH (VS Model)</th>
<th>CH (VS Model)</th>
<th>Functional annotation</th>
</tr>
</thead>
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<tr>
<td>Cefradine</td>
<td>↑***</td>
<td>↓#</td>
<td>/</td>
<td>cpd:C06897, HMDB0015428,</td>
</tr>
<tr>
<td>3-Acetoxyurs-12-en-23-oic acid</td>
<td>↑**</td>
<td>↓#</td>
<td>↓##</td>
<td>/</td>
</tr>
<tr>
<td>Vitamin A</td>
<td>↑***</td>
<td>↓#</td>
<td>↓##</td>
<td>cpd:C00473, HMDB0006216, LMPR01090001</td>
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<tr>
<td>O1-[4-(tert-butyl) benzoyl]-2-(tert-butyl sulfonyl) ethanenhydroximamide</td>
<td>↑*</td>
<td>/</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td>Oleamide</td>
<td>↑*</td>
<td>↓#</td>
<td>↓##</td>
<td>cpd:C19670, HMDB0002117, LMFA08010004</td>
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<tr>
<td>LPE 17:1</td>
<td>↑*</td>
<td>/</td>
<td>↓##</td>
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<td>↑*</td>
<td>/</td>
<td>↓##</td>
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<td>/</td>
<td>↓##</td>
<td>/</td>
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<tr>
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<td>↑*</td>
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<td>/</td>
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<tr>
<td>Nicotinic acid</td>
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<td>/</td>
<td>/</td>
<td></td>
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<td>23-Norcholic acid</td>
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<td>/</td>
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<td>/</td>
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<td>cpd:C02140, HMDB0001547, LMST0203186</td>
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</table>

***represents comparison with the control group: P<0.001; **represents comparison with the control group: P<0.01; *represents comparison with the control group: P<0.05; ##represents a comparison with the model group: P<0.001. ###represents comparison with the model group: P<0.01; #represents the comparison with the model group: P<0.05; ↑represents an up-regulation of the content; ↓represents a reduction of the content.

**Figure 4.** The KEGG pathway of enrichment (Each circle represents a metabolic pathway and the larger the circle, the more differential metabolites in that pathway, and the darker the color, the more prominent the pathway. ESI+: Positive ion model, ESI-: Negative ion model).
Figure 5. (A) The content variation of five biomarkers in the control, FD model, RH, and CH group. (** represents comparison with the control group: P<0.001; * represents comparison with the control group: P<0.01; * represents comparison with the control group: P<0.05; ## represents comparison with the model group: P<0.01; # represents the comparison with the model group: P<0.05); (B) The Spearman's correlation analysis between gut microbiota and differential metabolites (Red is a positive correlation, green is a negative correlation. * indicates a significant correlation; *P < 0.05; **P<0.01).
m microbiota with greater relative abundance played an important role while the microbiota with smaller abundance did not play an obvious role when CH was applied to FD rats.

### 3.4 Component analysis for the decoctions of RH and CH

The additive ions, molecular ion peaks, and fragment ions were used to predict the molecular formulas. And then peaks were matched with the McCloud (https://www.mzcloud.org/). Finally, 33 major compounds were identified (Table 2). We compared the peak areas of three flavonoids, five organic acids, and 5-HMF in CH and RH. The peak areas of Quercetin (Figure 6A), hyperoside (Figure 6B), Citric acid (Figure 6C), Malic acid (Figure 6D), and Chlorogenic acid (Figure 6E) in CH are lower than those in RH. However, the peak areas of Ursolic acid (Figure 6F) and 5-HMF (Figure 6G) in CH were higher than those in RH. Rutin could not be detected in CH (Figure 6H).

### 4 Discussion

#### 4.1 Thermal processing is responsible for the difference in composition between RH and CH

Stir-fried hawthorn is the traditional processing method of Chinese medicine. Thermal processing reduces the content of most organic acids and flavonoids in hawthorn, while also producing some new compounds. This may be the result of the reaction between various components during the thermal processing of hawthorn. It is worth noting that the Maillard reaction often reacts in the thermal processing of most foods (Nooshkam et al., 2019). Carbonyl compounds react with amino compounds to produce a series of complex products (Kanzler & Haase, 2020). And 5-hydroxymethylfurfural (5-HMF) is an important product of the Maillard reaction, which marks the addition products of the Maillard reaction. 5-HMF is produced by the Maillard reaction during thermal processing of foods containing reduced sugars and amino acids (Zhao et al., 2013). We found that the content of 5-HMF in CH is higher than that in RH. This indicates that

<table>
<thead>
<tr>
<th>Table 2. Chemical composition of RH and CH decoctions.</th>
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Food Sci. Technol, Campinas, 43, e002823, 2023
Hawthorn and functional dyspepsia

Maillard reaction may have occurred in the thermal processing of hawthorn. Compared to other components, the higher content of 5-HMF in RH and CH may be due to the Maillard reaction in both RH and CH during the preparation of the decoctions.

Although there is no evidence that the Maillard reaction is related to changes in the pharmacological effects of hawthorn before and after thermal processing, the Maillard reaction is a point that cannot be ignored in the hot processing of hawthorn.

Figure 6. (A) The peak areas of Quercetin; (B) The peak areas of Hyperoside; (C) The peak areas of Citric acid; (D) The peak areas of Malic acid; (E) The peak areas of Chlorogenic acid; (F) The peak areas of Ursolic acid; (G) The peak areas of 5-HMF; (H) The peak areas of Rutin.
4.2 CH could regulate the structure and composition of gut microbiota in FD rats

The relationship between the gut microbiome and human health is receiving increasing attention. It is now well established that the gut microbiome influences the progression of chronic diseases such as metabolic diseases, gastrointestinal diseases, and colorectal cancer (Hills et al., 2019). To explain the relationship between FD and gut microbiota, we investigated gut microbiota differences between FD rats and normal rats, and the effects of RH and CH intervention on them.

Firmicutes and Bacteroidota are two microbiotas with relatively high abundance at the phylum level, which play an important role in regulating host energy metabolism (Luo et al., 2020). The relative abundance of Firmicutes in the gut microbiota of FD rats was greater than that of normal rats and the opposite was true for Bacteroidota. Changes in the ratio of Firmicutes to Bacteroidota (F/B) may be an important reason for FD. Changes in the ratio of gut microbiota may change the metabolic characteristics of the microbiota, which will affect host health (Magne et al., 2020). The increase in the F/B ratio is closely related to many diseases, such as obesity, irritable bowel syndrome, and Sepsis (Lankelma et al., 2017). Food can have a great influence on the F/B ratio in the gut microbiota (de Wit et al., 2012). Although investigations have shown that the relative abundance of Firmicutes in the gut microbiota of FD people is significantly higher than that of normal people (Tziatziou et al., 2020), the relationship between the F/B ratio and FD deserves further study.

The richness and diversity of gut microbiota in FD rats were lower than in normal rats. The intervention of RH and CH could reverse the condition and significantly improve the richness and diversity of gut microbiota in FD rats. There is no significant difference between RH and CH. However, CH could better regulate the relative abundance of Firmicutes and Bacteroidota than RH. At the genus level, CH could also improve abnormal levels of Ligilactobacillus, Bacteroides, Dubosiella, and Limosilactobacillus in the gut microbiota of FD rats. In conclusion, CH may regulate gut microbiota structure and composition in FD rats.

4.3 CH could alleviate metabolic disorders in FD rats

Compared to the normal group, 77 intestinal metabolites were significantly altered in the FD model group. CH regulates 20 of these metabolites back to normal levels. These intestinal metabolites were annotated by KEGG, HMDB, and the LIPID Maps database. They are mainly lipids and lipid-like molecules, organic acids and derivatives, amino acids and polypeptide analogs. Some small molecules cannot be recognized, they may be metabolites of some bacteria or metabolites of the host, and may also be some useless exogenous components. Whether they have a biological role is unclear. We identified five potential biomarkers of FD based on KEGG. CH can regulate its content in rat feces so that it tends to return to a normal level. In particular, it is important in the regulation of Vitamin A and Nicotinic acid. Vitamin A and Nicotinic acid metabolism pathways may play an important role in CH intervention in FD.

Preformed vitamin A (all-trans-retinol and its esters) and provitamin A (beta-carotene) are essential dietary nutrients that provide a source of retinol. Retinol oxidation provides retinal, which is essential for vision, and retinoic acid, a transcription factor ligand that plays an important role in regulating cell genes (Dawson, 2000). Vitamin A regulates various processes including reproduction, embryogenesis, vision, growth, cellular differentiation and proliferation, maintenance of epithelial cellular integrity, and immune function (Bar-El Dadon & Reifen, 2017). It plays an important role in the whole life activities of the human body, including the regulation of gastrointestinal function. Vitamin A mediated the regulation of intestinal epithelium and mucosal immune cells and regulated the expression of tight junction proteins to ensure intestinal homeostasis (Cantorna et al., 2019). We detected significantly higher levels of vitamin A in the feces of FD rats than in normal rats, indicating that FD rats have problems with vitamin A uptake, which may be responsible for decreased gastrointestinal motility in FD rats. However, CH can reduce the intestinal fecal content of FD rats, promote intestinal uptake of vitamin A, and improve the gastrointestinal motility of FD rats.

Nicotinic acid and its derivative nicotinamide belong to the vitamin B series compounds, which are essential nutrients in the human body and play an important role in promoting normal growth and development of the human body (Kirkland & Meyer-Ficca, 2018). Nicotinic acid is produced by the gut microbiota and can act on the GPR109A receptor to exert the same effect as butyrate and inhibit intestinal inflammation (Liu et al., 2022). Studies have shown that nicotinic acid can improve intestinal permeability in patients with Parkinson’s disease (Karunaratne et al., 2020). Although both intestinal permeability and intestinal inflammation may be underlying causes of FD, the relationship between nicotinic acid and FD has not been clarified. Normal nicotinic acid levels are generally considered a healthy condition, and nicotinic acid deficiency is the cause of some diseases. In this study, we found that the nicotinic acid content in the feces of FD rats was significantly higher than that of normal rats, which may be caused by changes in gut microbiota structure or weakened intestinal nicotinic acid intake. Interestingly, CH could significantly correct the high content of nicotinic acid in the feces of FD rats.

4.4 There is a close relationship between gut microbiota and metabolites

From the results of correlation analysis, we found that the microbiota with higher relative abundance was more closely related to intestinal metabolites. This is only a correlation of mathematical relationship change trends, which cannot be used as strong evidence to explain the relationship between microbiota and metabolites, but it can also suggest that there may be a relationship between them. Interestingly, with the exception of a few metabolites, most metabolites were positively correlated with Ligilactobacillus and Limosilactobacillus and negatively correlated with Dubosiella, Romboutsia and Lactobacillus. Ligi lactobacillus and Limosilactobacillus are both beneficial gut microbiota (Liang et al., 2021; Saviano et al., 2021). Clinically, they can be used to adjust the balance of gut microbiota and inhibit the proliferation of undesirable gut microorganisms. Studies have shown that
**Limosilactobacillus** can promote colonic motility while reducing small intestinal motility (West et al., 2020). This may account for the change in its relative abundance in our studies. **Dubosiella** is a microbiota closely related to lipid metabolism (Li et al., 2020), with 16 metabolites significantly correlated with it. Of the 200 pairs of correlation analysis, 64 pairs showed significant correlation, including 27 pairs of positive correlation and 37 pairs of negative correlation. This suggests that metabolic and microbiota co-changes are important features of CH intervention in FD.

From what has been discussed above, CH may improve gastrointestinal motility in FD rats. In addition to the apparent modification of the rat's own metabolic disorders, CH regulates a number of as-yet unidentified metabolites that may be metabolites of FD-associated microorganisms. We therefore believe that the gut microbiome plays an important role in CH therapy and is closely related to changes in the composition of hawthorn after stir-frying.

**5 Conclusion**

In this study, UHPLC-MS fecal metabolomics combined with 16S rDNA amplicon sequencing was used to evaluate the effects of RH and CH on FD in rats. The results showed that CH was more effective than RH in improving the gut microbiota and metabolic status of FD rats. CH can correct abnormal levels of 20 different metabolites in the feces of FD rats; regulate the metabolic pathways of vitamin A and niacin; increase the diversity and richness of the gut microbiota and adjust the structure and composition of the gut microbiota, to improve the gastrointestinal motility of FD rats. However, more studies are needed to explore the specific functions of the gut microbiota related to FD and the true relationship between them and metabolites, as well as to find the exact biomarkers and signature microbiota for CH intervention in FD. In the analysis of the components of RH and CH decoctions, in addition to the changes of flavonoids and organic acids, the high content of 5-HMF in CH decoction suggests that Maillard reaction may react during the thermal processing of hawthorn.

In the treatment of many diseases, MFH species have great potential and broad application prospects. Based on our study, CH will be developed as a functional food in the future and applied to FD treatment as a dietary therapy. For the treatment of chronic diseases that are difficult to cure and prone to relapse, the development of safe, low-cost, stable diet therapy as a treatment regimen or adjuvant therapy will be an emerging field, and CH development will be a good example.

Conflict of interest

No conflict of interest is associated with this work.

Availability of data and material

The data used to support the findings of this study are available from the first author upon request.

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**Author contributions**

Lilin Zhang and Li Ai conceived the project. Chunjie Wu provided supervision. Lilin Zhang, Qi Liang, and Yao Tian performed the research. Lilin Zhang, Yao Tian, and Chunjie Wu analyzed the data. Lilin Zhang and Li Ai wrote the article. All authors have read and approved the manuscript for publication.

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**References**


