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Structural changes of gut microbiota in a rat non-alcoholic fatty liver disease model treated with a Chinese herbal formula

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ABSTRACT

Accumulating evidence indicates that disruption of the gut microbiota by a high-fat diet (HFD) may play a pivotal role in the progression of metabolic disorders such as non-alcoholic fatty liver disease (NAFLD). In this study, the structural changes of gut microbiota were analyzed in an HFD-induced NAFLD rat model during treatment with an ancient Chinese herbal formula (CHF) used in clinical practice – *Qushi Huayu Fang*. CHF treatment significantly reduced body weight, alleviated hepatic steatosis, and decreased the content of triglycerides and free fatty acids in the livers of the rats. Gut microbiota of treated and control rats were profiled with polymerase chain reaction-denaturing gradient gel electrophoresis and bar-coded pyrosequencing of the V3 region of 16S rRNA genes. Both analyses indicated that the CHF-treated group harbored significantly different gut microbiota from that of model rats. Partial least squares discriminant analysis and taxonomy-based analysis were further employed to identify key phylotypes responding to HFD and CHF treatment. Most notably, the genera *Escherichia*/*Shigella*, containing opportunistic pathogens, were significantly enriched in HFD-fed rats compared to controls fed normal chow ($P < 0.05$) but they decreased to control levels after CHF treatment. *Collinsella*, a genus with short chain fatty acid producers, was significantly elevated in CHF-treated rats compared to HFD-fed rats ($P < 0.05$). The results revealed that the bacterial profiles of HFD-induced rats could be modulated by the CHF. Elucidation of these differences in microbiota composition provided a basis for further understanding the pharmacological mechanism of the CHF.

Introduction

Non-alcoholic fatty liver disease (NAFLD), a manifestation of the metabolic syndrome in the liver, is closely associated with insulin resistance, obesity, and diabetes mellitus [27]. Although it is a major threat to public health, the pathogenesis of NAFLD is not yet well understood, nor are there effective therapies [28].

Emerging evidence in the last two decades indicates a possible role of gut microbiota in the etiology of NAFLD [1]. Small intestinal bacterial overgrowth is commonly found in NAFLD patients and results in increased intestinal permeability, endogenous ethanol production, and choline deficiency [3,11,44]. In addition, the gut microbiota promotes absorption of monosaccharides and short chain fatty acids by fermentation, and thus increases de novo hepatic lipogenesis and enhances fat storage by regulating lipoprotein lipase activity [12]. NAFLD is typically associated with low-grade, chronic inflammation [13], and a major cell wall component of Gram-negative bacteria, lipopolysaccharide (LPS), is known to be potent in promoting inflammation [5]. When binding to CD14 and toll-like receptor 4 on the surface of immune cells, a low concentration of LPS from gut microbiota can trigger a series of inflammatory processes [31,45], which in turn contributes to the pathogenesis of NAFLD. Consequently, the gut microbiota has become a novel target for potential therapies [14], with probiotics, prebiotics, and antibiotics being tested for treatment or prevention of NAFLD [4,19,22].

Traditional medicine has long been used in China, and most of the formulas are orally administered. Recent research has revealed that many ingredients in these herbs could only be absorbed and they exert their biological effects with the help of the host's gut microbiota [8]. Furthermore, intriguing but limited data also

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Table 1
Herbs used in the Chinese herbal formula.

Chinese name	Pharmaceutical name	Family name	Production place	Processing method
Yin chen	*Artemisia capillaries* Thunb. (above-ground parts, dried)	*Compositae*	Anhui Province, China	Ethanol extraction
Hu zhang	*Polygonum cuspidatum* Sieb. et Zucc (rhizome, root, dried)	*Polygonaceae*	Jiangsu Province, China	Ethanol extraction
Jiang huang	*Curuma longa* L. (rhizome, dried)	*Zingiberaceae*	Sichuang Province, China	Ethanol extraction
Tian ji huang	*Hypericum japonicum* (whole plant, dried)	*Clusiaceae*	Jiangxi Province, China	Mixed at an equal mass ratio in distilled water, then concentrated to a relative density of 1.08–1.12 (80 °C), followed by ethanol precipitation
Zhi zi	*Gardenia jasminoides Ellis* (fruit, dried)	*Rubiaceae*	Fujian Province, China	

show that the efficacy of some traditional herbs may be related to modulation of gut microbiota [48]. One formula, *Qushi Huayu Fang*, has a long history of use in clinical practice to alleviate NAFLD. However, to date, no systematic and credible explanations have been proposed for its mechanism. Whether *Qushi Huayu Fang* can modulate the host's gut ecosystem while alleviating NAFLD symptoms and whether gut bacteria are a potential target for the herbal formula are still unanswered questions.

Nowadays, advanced technologies have facilitated the exploration of gut microbiota, especially with the development of high-throughput sequencing technology. Researchers are able to look in detail at the structure of gut microbiota, and study how external factors, such as age and drug use among others, influence gut microbiota. In this study, gut microbial composition during *Qushi Huayu Fang* treatment in a high-fat diet (HFD)-induced NAFLD rat model was monitored by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and bar-coded pyrosequencing. Using a microbiome-wide association strategy [34,41,47], the modulation of this Chinese herbal formula (CHF) in the gut microbiome was validated, and key phylotypes closely related to NAFLD development and CHF treatment were expected to be identified.

Materials and methods

Preparation of the CHF

The compound was prepared from five herbs, as listed in Table 1. Herbs were obtained from qualified suppliers and on the basis of standards specified in the Chinese Pharmacopoeia (1995 edition) and Chinese Materia Medica in Shanghai. Extracts of the herbs (Table 1) were mixed at a mass ratio of 13:7:7:7:7 (13 parts *Artemisia capillaries* Thunb., and 7 parts of each of the other four herbs). High-performance liquid chromatography was used for quality control by monitoring three active components in the formula: polydatin (retention time: 10 min; concentration: 1.779 mg g^{-1}), emodin (19 min; 5.394 mg g^{-1}), and geniposide (35 min; 2.850 mg g^{-1}). The final solution was stored at 4 °C at two concentrations, 0.93 and 0.47 g mL^{-1}, as high and low dosages, respectively. The formula has been issued a patent by the State Intellectual Property Office of PR China under the ID: ZL200610009140.0.

Animals and experimental protocol

Ethics statement

All procedures of the experiments in this study were approved by the Animal Ethics Committee of the Shanghai University of Traditional Chinese Medicine (Approval No. SCXK 2003-0003). The care and use of animals were carried out under the Guidelines for Animal Experiment of the Shanghai University of Traditional Chinese Medicine (Shanghai, China), and all efforts were made to minimize the number of animals and their suffering [49].

Twenty-six male Sprague–Dawley rats were purchased from the Shanghai Laboratory Animal Co. Ltd., China, and raised in a specific pathogen-free barrier system in the Laboratory Animal Center of the Shanghai Traditional Chinese Medicine University. After acclimatization, rats were divided into two groups: one group ($n = 21$) was fed an HFD (w/w, 87.5% normal chow, 10% lard, 2% cholesterol, and 0.5% sodium cholate), and a control group ($n = 5$) was fed normal chow (NC). After 6 weeks on the diet, HFD-fed rats were randomly divided into three subgroups (each $n = 7$). Two of the subgroups maintained the HFD diet in conjunction with high (H) and low (L) dosage (0.93 g/100 g body weight and 0.47 g/100 g body weight, respectively) CHF (*Qushi Huayu Fang*) through intragastric administration. The third subgroup remained on the HFD without additional treatment (HFD). Food intake in the groups was controlled to the same level. Control volumes (equivalent to those of the CHF compound given to experimental groups) containing water were administrated to NC- and HFD-fed rats. Body weight was measured every 14 days. At the end of week 10 (i.e. the CHF treatment lasted for 4 weeks), all animals from the four groups were sacrificed and their livers were removed and stored at −70 °C for histological and lipid content analysis, including oil Red O and hematoxylin–eosin (H&E) staining, TG and FFA analysis (see Supplementary Information). Liver index was calculated as the ratio of liver-to-body weight. Before sacrifice, fecal samples were collected from 23 animals, including 6 from HFD, 5 NC, 5 CHF (H), and 7 CHF (L). Unfortunately, the feces collected from the other three animals were insufficient, so they were omitted from the gut microbiota analysis. All stool samples were stored at −70 °C.

PCR-DGGE and multivariate analysis of the 16S rRNA gene V3 region

DNA was extracted from each fecal sample by the bead-beating method [50]. Bacterial universal primers P2 (5′-ATTACCGCGGCTGCTGG-3′) and P3 (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3′) were used to amplify the 16S rRNA gene V3 region from each DNA sample by hot-start touchdown PCR [30]. PCR amplification reactions, DGGE analysis, and phylogenetic identification of major bands were performed as described previously [46] and the DGGE denaturing gradient was 30–54% (7 M urea and 40% deionized formamide were considered to be the 100% denaturant). The sequences retrieved from major DGGE bands have been deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) with accession numbers JF729214–JF729235.

The gel images were converted into digital data using Quantity One 4.4.0 (Bio-Rad, Hercules, CA, USA). Principal component analysis (PCA) was employed to compare the gut microbiota composition between treatment groups in the MATLAB 7.11.0 (R2010b) environment (The MathWorks, Inc., Natick, MA, USA).