

Clinical efficacy and bacterial flora characteristics of Huazhi Rougan granules in the intervention of moderately resistant to damp-heat nonalcoholic fatty liver disease

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Author contributions

Dan Zhao, Ming-Zhong Xiao, and Chen-Xia Lu contributed to the conception of the study; Ming-Zhong Xiao contributed significantly to the analysis and manuscript preparation; Dan Zhao performed the data analyses and wrote the manuscript; Jian-Liang Xu helped perform the analysis with constructive discussions. All the authors reviewed the final manuscript.

Competing interests

The authors declare no conflicts of interest.

Acknowledgments

Project of the State Administration of Traditional Chinese Medicine: Key Laboratory of Liver and Kidney Treatment of Chronic Liver Diseases (No Z155080000004); Key project Natural Science Foundation of Hubei Province (No. 2020CFA023).

Abbreviations

NAFLD, non-alcoholic fatty liver disease; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma; CAP, controlled attenuation parameters; LSM, liver stiffness measurement; UMI, unique molecular identifier; BMI, body mass index; TCM, traditional Chinese medicine.

Peer review information

Gastroenterology & Hepatology Research thanks all anonymous reviewers for their contribution to the peer review of this paper.

Citation

Zhao D, Xiao MZ, Xu JL, Lu CX. Clinical efficacy and bacterial flora characteristics of Huazhi Rougan granules in the intervention of moderately resistant to damp-heat nonalcoholic fatty liver disease. *Gastroenterol Hepatol Res*. 2022;4(3):13. doi: 10.53388/ghr2022-09-055.

Executive editor: Chen-Xia Lu.

Received: 07 September 2022; **Accepted:** 20 September 2022;

Available online: 25 September 2022.

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Abstract

Background: To explore the clinical efficacy of Huazhi Rougan granules in treating non-alcoholic fatty liver disease (NAFLD) and its effect on the oral and intestinal flora. **Methods:** 48 patients who met the inclusion criteria were intervened with Huazhi Rougan granules, the liver and spleen CT ratio, Traditional Chinese medicine (TCM) syndrome, body mass index (BMI, WC, SMM, body fat percentage, visceral fat area), liver function, and blood lipid levels were compared before and after treatment, and the curative effect was evaluated; 6 cases of NAFLD patients whose clinical efficacy after Huazhi Rougan granule intervention was effective were randomly selected, and the patient's oral and intestinal samples were taken for further analysis. **Results:** After treatment, the total effective rate of clinical efficacy was 85.14%. The TCM symptoms of NAFLD patients were significantly alleviated compared with before treatment ($P < 0.05$). There were statistically significant differences in ALT, AST, GGT, TC, and TG before and after treatment ($P < 0.01$). After treatment, BW, BMI, WC, body fat percentage, visceral fat area, liver hardness, and fat attenuation index were significantly decreased ($P < 0.01$). Oral-gut flora analysis showed: After treatment, the Shannon index of the gut microbial community increased ($P < 0.01$). Compared with before treatment, the abundance of Firmicutes decreased, while the abundance of Bacteroidetes, Candidatus-Sa, and Tenericutes increased ($P < 0.05$). At the genus level, the abundance of Bacteroides, Lactobacillus, and Bifidobacterium increased, and Streptococcus, Oribacterium, and Peptoanaerobacter decreased ($P < 0.05$). Before and after treatment, the dominant bacterial phyla in the intestine were the same, including Bacteroidetes, Firmicutes, and Proteobacteria. The abundance of Firmicutes and Roseburia decreased after treatment ($P < 0.05$). **Conclusions:** Huazhi Rougan granules have a significant effect on the treatment of NAFLD, can improve liver function, reduce blood lipids, can effectively reduce body morphological indicators such as body mass, WC, body fat percentage, visceral fat area, liver stiffness measurement (LSM), and fat content, and has the function of protecting the liver and reducing blood pressure, promoting the role of intrahepatic fat metabolism; and significantly relieve the clinical symptoms. NAFLD patients have the gut and oral flora disturbances. Huazhi Rougan granules can restore the diversity of intestinal microbial communities in NAFLD patients and play a therapeutic role by regulating the abundance and composition of oral and Gut flora.

Keywords: non-alcoholic fatty liver disease; Huazhi Rougan granule; intestinal flora; oral flora; clinical observation

Introduction

Non-alcoholic fatty liver disease (NAFLD) is histologically defined as accumulating more than 5% to 10% fat in the liver. The prevalence of NAFLD continues to increase with the worldwide prevalence of metabolic syndrome, including obesity, type 2 diabetes, and dyslipidemia. The population prevalence of NAFLD in Asia is around 25%. While hepatocellular carcinoma and end-stage liver disease secondary to NAFLD remain uncommon, a rising trend has emerged [1]. NAFLD represents a histopathological spectrum ranging from non-alcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH). Patients with NASH risk liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) [2]. The pathogenesis of NAFLD remains unclear. Compared with the classic “two-hit” theory, the “multiple-hit theory” has been widely studied-sedentary, low-activity, high-fat diet, obesity, and other lousy living habits cause insulin resistance, which leads to the accumulation of lipids in the liver [3]. Oxidative stress and lipid peroxidation occur in liver lipid accumulation, leading to mitochondrial dysfunction, endoplasmic reticulum stress, and activation of the inflammatory cascade [4]. Gut microbiota disturbance induces intestinal endothelial barrier dysfunction, leading to translocation of bacterial components and liver inflammation, also aggravating disease progression [5].

Currently, no specific drug exists to treat NAFLD, and the targeted treatment drugs are still in the clinical trial stage. The primary interventions are lifestyle changes, dietary adjustments, increased exercise, and weight loss [6]. However, the patient is difficult to adhere to, and the curative effect is insignificant. According to the clinical symptoms, signs, tongue, and pulse of traditional Chinese medicine and other characteristic diagnosis and treatment methods, traditional Chinese medicine classifies the disease as “liver addiction”, “fei qi” and “accumulation” with TCM syndrome differentiation. Therefore, it has significant advantages in the treatment of NAFLD. Huazhi Rougan granules are recommended for treating non-alcoholic fatty liver with damp-heat middle block syndrome [7].

In this article, a self-controlled study was used to explore Huazhi Rougan granules's clinical efficacy in treating NAFLD with damp-heat and moderate resistance. Taking the “gut-liver” axis as the research entry point, 16S rDNA was used to sequence NAFLD patients' oral and intestinal microbes. To explore the changes in flora characteristics and abundance after Huazhi Rougan granules intervention and to clarify the therapeutic effect of Huazhi Rougan granules on NAFLD from the perspective of microbial mechanism.

General clinical data and methods

Participants

All cases of the CT-diagnosed NAFLD patients who visited the Department of Hepatology and Obesity, Hubei Provincial Hospital of Traditional Chinese Medicine from October 2020 to December 2021 were of damp-heat moderate resistance type.

Diagnostic criteria

Referring to the “Guidelines for the Prevention and Treatment of Non-Alcoholic Fatty Liver Disease (2018 Update)”, the diagnosis can be made if the following items are present: No history of excessive drinking (male intake of ethanol < 30 g/d < 20 g/d for women) and other specific causes of fatty liver disease (amiodarone, glucocorticoid) hormone and other drugs, hepatitis C virus type 3 infection, autoimmune hepatitis, total parenteral nutrition, and other specific diseases lead to fatty liver. The imaging findings met the diagnostic criteria for fatty liver [6].

TCM syndrome diagnosis criteria

Reference to “General Principles of Clinical Research on New Drugs of Traditional Chinese Medicine,” the sixth edition of the textbook of internal medicine of traditional Chinese medicine, and “Classification and Code of Syndrome of Traditional Chinese Medicine,” damp-heat

moderate resistance syndrome: liver area discomfort or dull pain, yellow and greasy tongue coating, loss of appetite, fatigue, mouth bitter, stringy or slippery pulse is the clinical manifestation. Two main symptoms plus one secondary symptom can be diagnosed.

Inclusion criteria and exclusion criteria

Inclusion criteria

Age ≥ 18 years old; those who meet the diagnostic criteria for non-alcoholic fatty liver disease and TCM belongs to the criteria for syndrome differentiation of damp-heat middle resistance; voluntarily sign the informed consent; liver/spleen CT ratio < 1.0; liver imaging findings meet the imaging diagnostic criteria for the diffuse fatty liver; HbA1c ≤ 6.5%; ALT, AST, TBil ≤ 2 × ULN; GFR ≥ 60 (mL/min/1.73 m²).

Exclusion criteria

1. Pregnant and lactating women, women of childbearing age who have not taken effective contraceptive measures, or male subjects who are unwilling to use contraceptive measures;
2. Those with a history of diabetes, severe primary cardiovascular disease, kidney disease, cancer, and other serious diseases that affect survival;
3. HCV antibody, HIV antibody-positive or HBsAg-positive and HBV-DNA titer-positive; those who are allergic to the components of this medicine should be excluded;
4. Participated in other clinical investigators within three months before screening;
5. Those who have used similar traditional Chinese medicines for the treatment of non-alcoholic simple fatty liver within two weeks before the screening (except for the experimental drugs);
6. The compliance is poor, and the researcher judges that it is not suitable to participate in the research.

Fall-off criteria

Adverse events occurred; violations of the trial protocol (including poor compliance and voluntary withdrawal); subjects withdrew informed consent.

Method

All subjects were given Huazhi Rougan granules orally, in one bag, three times a day. At the same time, during the study period, the subjects were given health education: avoid sitting for a long time and move less, and appropriately increase physical activity and resistance exercise according to interests and hobbies. Control calorie intake, it is recommended to reduce 2092–4184 kJ of calories per day, increase the intake of soy protein and whey protein; adjust the diet structure, make a balanced diet of moderate fat and carbohydrates, eat less red meat and deep-processed meat, and eat more fish and shrimp, avoid sugary soft drinks and refined pastries and pasta; strictly control the calorie intake of dinner and late-night snack behavior, and reduce the intake of fast food.

Observation indexes

Safety indicators

Blood analysis, urine routine, liver and kidney function, electrocardiogram, etc.

Efficacy indicators

1. Primary efficacy indicator-liver/spleen CT ratio: determination of liver/spleen CT ratio within one week before and after treatment.
2. Secondary efficacy measures
 - (1) TCM syndrome score-symptoms and signs of patients before and after treatment were scored by the same investigator and reviewed and judged by a deputy chief physician.
 - (2) Biochemical indicators: within a week before and after treatment, professional nurses collected venous blood from subjects in the morning to measure liver function (ALT, AST, GGT, ALP, TBIL), blood lipids (TC, TG, HDL-C, LDL-C).
 - (3) Body Morphology Indicators: within a week before and

after treatment, the subject's height, weight, waist circumference, liver transient elastography, and body composition indicators (including body fat rate, skeletal muscle mass, and visceral fat area) were tested, respectively. (4) Imaging indicators: the controlled attenuation parameters (CAP) and LSM were obtained by the same experienced and blinded doctor in the same machine before and after treatment with Fibrotouch controlled attenuation parameter ultrasonic measurement method.

Sample collection, DNA extraction, and 16S rRNA sequencing

We performed 16S rRNA gene sequencing of oral and stool samples from 48 NAFLD patients before and after treatments. Patients were instructed to collect buccal swab specimens and fecal samples into DNA storage tubes (CW2654, CwBiotech, Beijing, China), then store the pieces at room temperature and send them to the laboratory. Bacterial DNA was extracted using an intestinal DNA extraction kit (Qiagen Fecal DNA Extraction Kit, Qiagen, Hilden, Germany); buccal swab bacterial DNA was extracted using an oral DNA extraction kit. After subsequent quantitative and qualitative analysis and quality control of the extracted DNA, splicing and linking libraries were constructed, and electrophoresis and qubit concentration measurements were performed on the spliced and ligated libraries. Using the Illumina Nova-seq6000 sequencing platform and PE150 sequencing strategy, the library that passed the quality control was sent for sequencing.

Bioinformatics analysis

Extract unique molecular identifier (UMI) pairing relationships by linking libraries. All sequences corresponding to each paired UMI were extracted from the assembled library. Primers and UMI tags were excised using Cutadapt V1.2.1, and each UMI was built into a full-length 16S sequence using the software SPAdes V3.13.1 with default parameters. Mothur V1.42.0 and SILVA_132_SSURef_Nr99 databases were used for all the above lines to perform species annotation with default parameters. Alpha bacterial diversity of gut microbiomes was estimated by qiime1 V1.8.0. Differences between groups were calculated by the Mann-Whitney U test using the scipy 1.3.1 package in python3.6. Beta diversity was analyzed by R3.6.1 package vegan2.5–3.

Criteria for judging the efficacy of fatty liver

Clinical recovery: liver/spleen CT ratio ≥ 1 ; significantly effective: liver/spleen CT ratio decreased by 2 grades (severe $\rightarrow$ mild); valid: liver/spleen CT ratio decreased by 1 grade (severe $\rightarrow$ moderate, or moderate $\rightarrow$ mild); invalid: the liver/spleen CT ratio did not change or even worsened.

Total effective rate = (clinical recovery + markedly effective + effective) number of cases/total number of cases $\times$ 100%.

Statistical analysis

Statistical product and service solutions (SPSS) 23.0 statistical software was used for data analysis. Measurement data conforming to normal distribution were statistically described as mean $\pm$ standard deviation, and the comparison of intra-group changes before and after treatment was performed by paired t-test. Otherwise, the paired Wilcoxon signed-rank test was used. Counting data were statistically described by frequency (%) and constituent ratio. Kendall's tau-b correlation analysis was used to detect the relationship between the differential bacteria and the changes in TCM syndrome scores before and after treatment. Spearman correlation analysis was used to detect the relationship between the differential bacteria and the changes in clinical indicators before and after treatment. Hypothesis tests were all two-sided tests; the test level was $\alpha = 0.05$, and $P < 0.05$ was considered statistically significant.

Results and discussion

Enrollment and baseline characteristics

Forty-eight patients with NAFLD were in our hospital from October 2020 to December 2021. Among them were 23 females and 25 males, with an average age of (48.3 ± 9.4) y (range, 29–68 y). Stratification of overweight and obesity in patients according to BMI, six cases were not overweight (NOW) (body mass index (BMI) 18.5–23.9 kg/m²), 19 were overweight (BMI 24–27.9 kg/m²), and 23 cases were obese (BMI ≥ 28 kg/m²). The liver and spleen CT ratio were used to determine the severity of fatty liver, and the results showed that 6 cases were mild fatty liver, 13 moderate fatty liver, and 29 severe fatty liver (Figure 1).

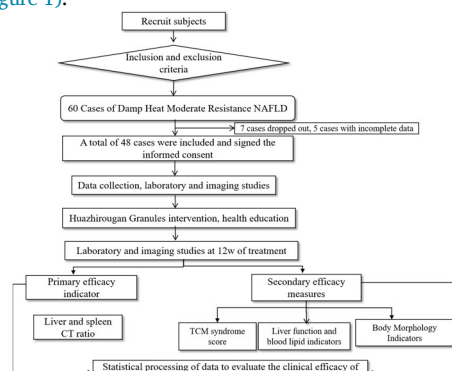


Figure 1 Flow chart of clinical efficacy observation

Security assessment

Before and after treatment, there were no abnormalities in the safety indicators such as routine (blood, urine) routine, renal function, and electrocardiogram. Among them, one patient had diarrhea, which was determined by the expert group to be related to the use of traditional Chinese medicine rhubarb; 1 patient developed herpes zoster during the test, which was determined not to be associated with this drug. All patients had no adverse events, and the safety was good.

Clinical efficacy evaluation

As shown in Table 1, after 12 weeks of treatment, the clinical efficacy was determined by the liver/spleen CT ratio, and the results showed that the total effective rate was 85.41%.

After treatment, 11 cases of TCM syndromes were clinically cured. Four cases were markedly effective, 15 cases were practical, and 18 cases were ineffective. The total effective rate was 62.5%, and the inefficient rate was 37.5%. Compared with before treatment, according to the paired t-test within the group, the TCM syndromes of the patients were significantly relieved ($P < 0.01$), as shown in Table 2.

After 12 weeks of treatment, the blood lipid indexes TC, TG, and LDL-C of the patients were decreased compared with those before treatment, and the t-test showed that the difference between TC and TG before and after treatment was statistically significant ($P < 0.05$); there was no significant difference in HDL-C and LDL-C, as shown in Table 3.

As shown in Table 4, after treatment, the patient's body weight, BMI, waist circumference, lipid accumulation index, body fat percentage, and visceral fat area were significantly decreased compared with those before treatment ($P < 0.01$).

Compared with before treatment, the liver stiffness and fat attenuation index of patients after treatment were significantly decreased ($P < 0.01$), as shown in Table 5.

Table 1 Statistics of comprehensive efficacy after treatment

Efficacy	Number of cases (cases)	Percentage (%)
Clinical recovery	27	56.25
Significantly effective	4	8.33
Valid	10	20.83
Invalid	7	14.58

Table 2 Comparison of TCM syndrome scores before and after treatment ($\bar{x} \pm s$)

	Before treatment	After treatment	T value	P
liver area discomfort or dull pain	1.958 ± 0.503	1.083 ± 1.007	5.226	< 0.001**
Yellow and greasy tongue coating	2.708 ± 1.203	1.583 ± 1.301	6.002	< 0.001**
Fatigue	0.895 ± 0.660	0.479 ± 0.504	4.708	< 0.001**
Loss of appetite	0.1667 ± 0.376	0	3.066	0.004**
Bitter taste	0.583 ± 0.577	0.291 ± 0.459	4.013	< 0.001**
Total scores	6.270 ± 1.659	3.437 ± 2.041	9.101	< 0.001**

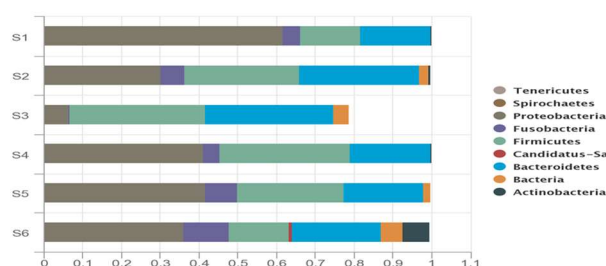
**, at the 0.01 level (two-tailed), a significant difference; *at the 0.05 level (two-tailed), a significant difference (the same below).

Table 3 Comparison of blood lipid indexes ($\bar{x} \pm s$)

	Before treatment	After treatment	T value	P
TC (mmol/L)	6.952 ± 1.604	5.122 ± 1.436	5.695	< 0.001**
TG (mmol/L)	6.012 ± 1.937	2.285 ± 1.527	12.62	< 0.001**
HDL-C (mmol/L)	1.147 ± 0.212	1.130 ± 0.204	0.712	0.48
LDL-C (mmol/L)	3.446 ± 0.965	3.353 ± 1.028	-0.999	0.323

Table 4 Comparison of body morphological indexes ($\bar{x} \pm s$)

	Before treatment	After treatment	T value	P
Weight (kg)	79.225 ± 11.823	77.231 ± 11.706	2.742	0.009**
BMI (kg/m ²)	28.276 ± 3.441	27.571 ± 3.489	2.772	0.008**
waist circumference (cm)	96.375 ± 8.451	94.343 ± 9.455	3.589	0.001**
Lipid Accumulation index	200.557 ± 74.779	69.793 ± 49.960	11.85	< 0.001**
SMM (kg)	28.397 ± 6.174	31.621 ± 5.903	-5.42	< 0.001**
F (%)	35.509 ± 6.702	32.472 ± 6.431	4.777	< 0.001**
Visceral fat area	132.990 ± 37.350	124.075 ± 39.110	3.395	0.002**



Before treatment

	Before treatment	After treatment	T value	P
LSM (KPA)	7.844 ± 3.253	6.544 ± 2.317	6.052	< 0.001**
CAP (db/m)	292.704 ± 36.082	278.276 ± 33.627	4.035	< 0.001**

Table 5 Comparison of liver stiffness ($\bar{x} \pm s$)**Oral-gut flora abundance changes**

Randomly selected 6 NAFLD patients with practical clinical efficacy after Huazhi Rougan granules intervention (the main efficacy index was clinically cured, markedly valuable, and functional) and obtained OTU absolute abundance and annotation information based on 16S rDNA sequencing. Descriptive analysis of sequence numbers of the oral-gut microbiota at the phylum and genus levels.

Species composition and abundance changes of oral flora

According to the relative abundance table of oral bacteria, at the phylum level, the dominant bacteria of oral bacteria were Proteobacteria, Bacteroidetes, and Firmicutes, which were consistent with the results of the predominant bacteria after treatment. As shown in Figure 2.

After 12 weeks of treatment, the abundance of oral Bacteroidetes, Candidatus Saccharibacteria, and Tenericutes increased, while the quantity of Firmicutes decreased ($P < 0.05$), as shown in Figure 3.

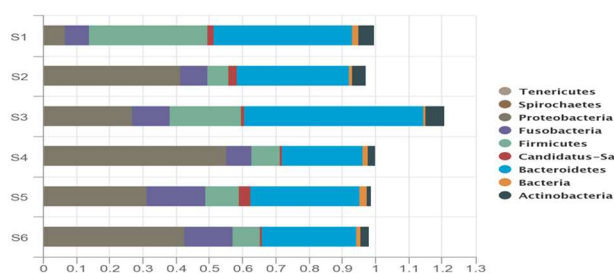
At the genus level, the oral bacteria genera were sorted according to their relative abundance before and after treatment, which showed that the dominant genera were Neisseria, Prevotella, Veillonella, Streptococcus, and Fusobacterium (Figure 4). The quantity of Bacteroides, Lactobacillus, and Bifidobacterium increased compared with before treatment (Figure 5).

Species composition and abundance changes of gut microbiota

The Shannon index describes the diversity of microbial communities. After 12 weeks of treatment, the results showed that the Shannon index of NAFLD patients increased, and the variety of intestinal microbial communities increased ($P < 0.01$). As shown in Figure 6.

At the phylum level, according to the relative abundance of its flora, the dominant bacteria in the intestinal tract were relatively consistent before and after treatment. The common bacteria were Bacteroidetes, Firmicutes, and Proteobacteria (Figure 7). The abundance of Firmicutes decreased compared to before treatment ($P < 0.05$), and proteobacteria were also lower than before, but the difference was not statistically significant. As shown in Figure 8.

The intestinal bacteria genera before and after treatment were sorted according to their relative abundance from large to trim at the genus level. The results showed that the dominant bacteria genera were Bacteroides, Lactobacillus, and Sutterella. After treatment, the main species of intestinal bacteria were as follows: Bacteroides and Ruminococcus. As shown in Figure 9. Compared with before treatment, the abundance of Roseburia, Lactobacillus, Anaerostignum, Blautia, Sutterella, Faecalibacterium, Oxalobacter, Klebsiella, and Marseillibacter decreased ($P > 0.05$) (Figure 10).



After treatment

Figure 2 Example of the composition map of the oral flora at the level of oral flora

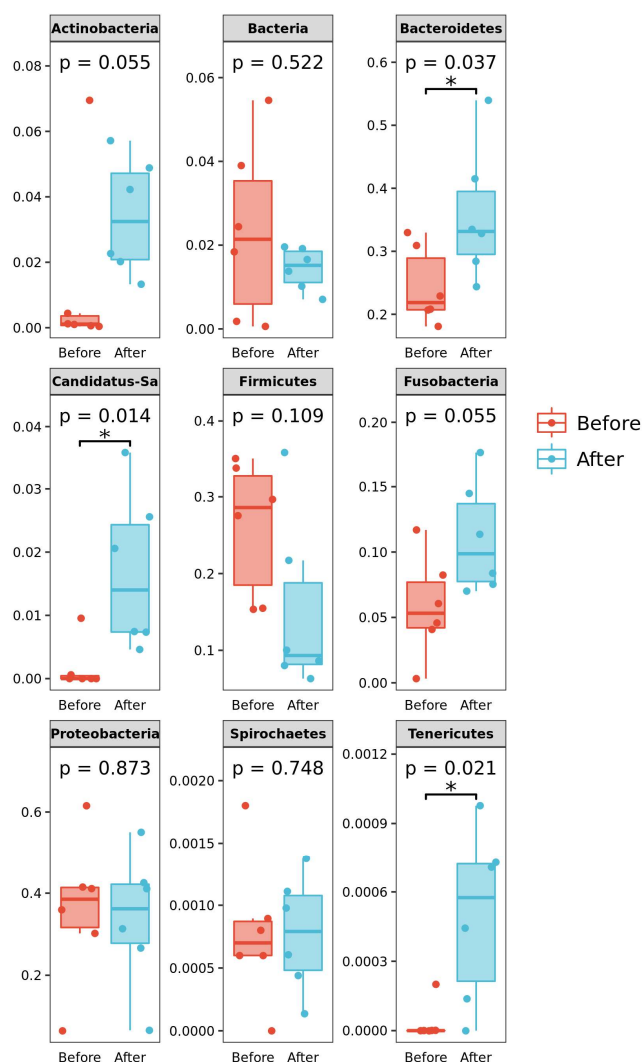


Figure 3 Changes in the abundance of oral flora at the phylum level before and after treatment

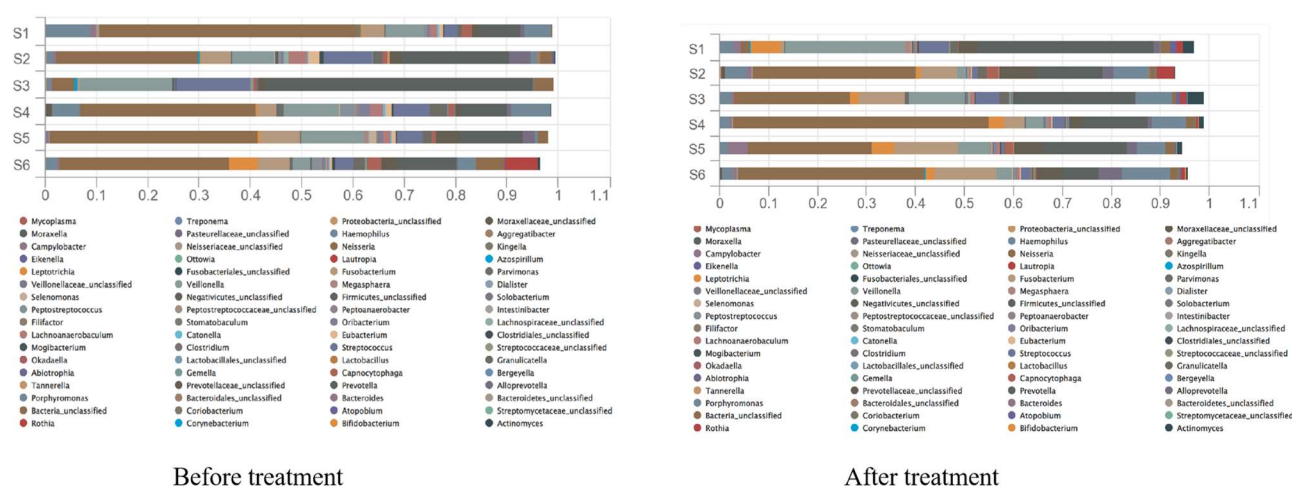


Figure 4 Example of the compositional map of oral flora at the genus level

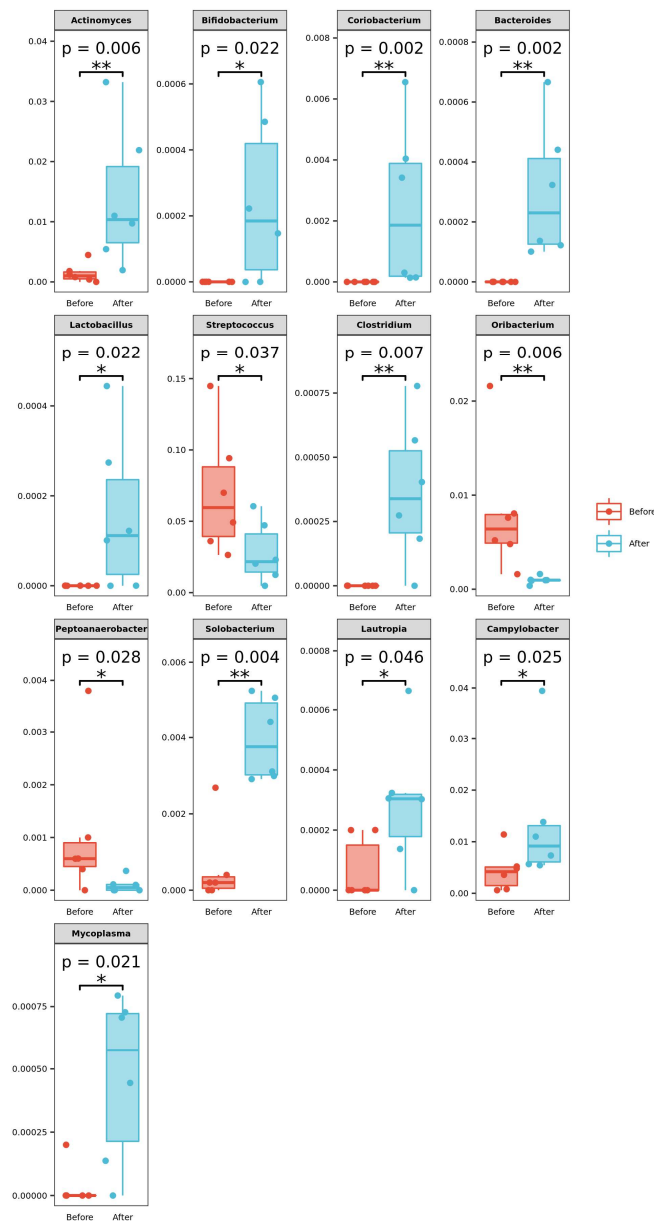


Figure 5 Changes in the abundance of oral flora at the genus level before and after treatment

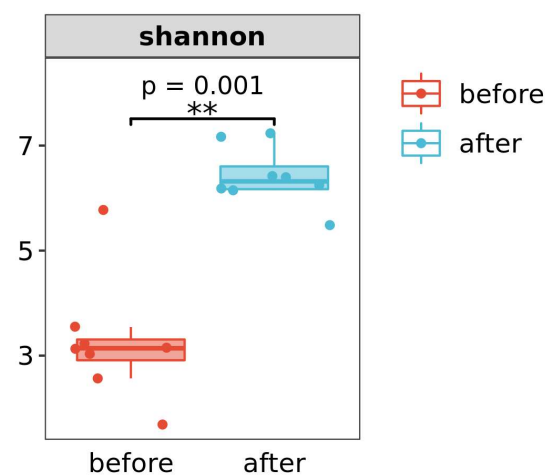
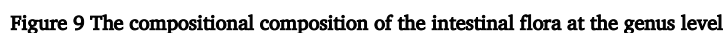


Figure 6 Comparison of species diversity of gut microbiota before and after treatment



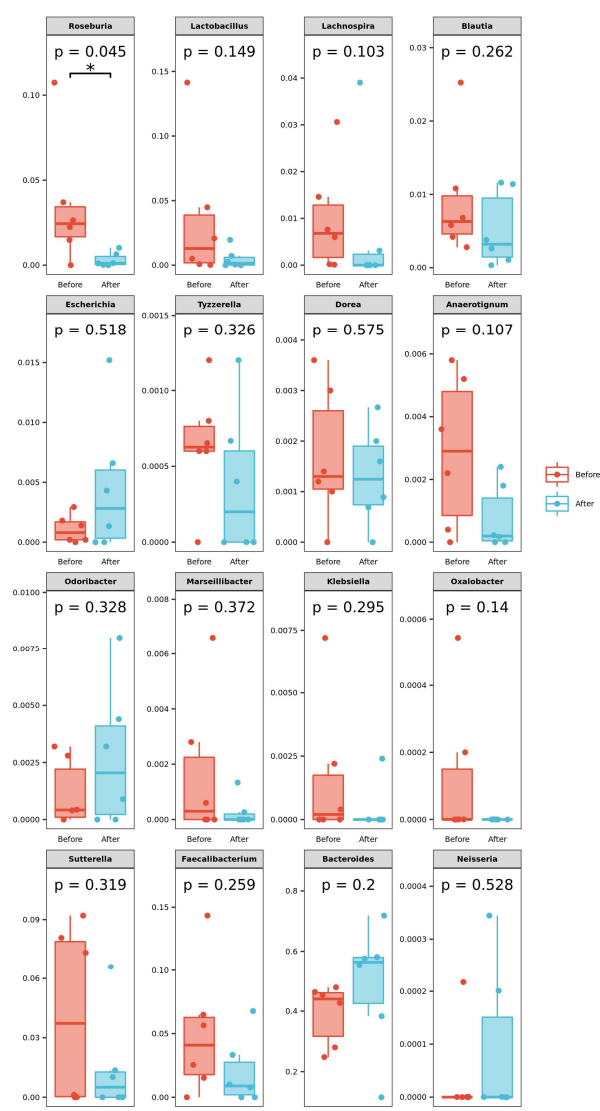


Figure 10 Changes in the abundance of gut microbiota at the genus level before and after treatment

Study on the correlation between different bacterial genera and clinical indicators before and after treatment

Kendall's tau-b correlation analysis was used to detect the relationship between oral differential bacteria and the changes in TCM syndrome scores before and after treatment. Spearman correlation analysis was used to detect the relationship between oral differential bacteria and the changes in various clinical indicators before and after treatment.

The difference in oral Actinomyces before and after treatment was positively correlated with the change of TCM syndrome score with yellow and greasy tongue coating ($r = 0.732$, $P = 0.025$) and negatively correlated with the change in body fat percentage ($r = -0.746$, $P = 0.034$); the change of Bacteroides abundance was positively correlated with the change of TCM syndrome score of fatigue ($r = 0.650$, $P = 0.039$). Changes in Lactobacillus correlated with changes in lipid accumulation index, visceral fat area and LDL-c ($r = 0.804$, $p = 0.016$; $r = 0.874$, $P = 0.005$; $r = -0.770$, $P = 0.025$); The change value of Oribacterium was positively correlated with the change value of visceral fat area ($r = 0.895$, $P = 0.034$); the change value of Coriobacterium was negatively correlated with the change value of GGT ($r = -0.821$, $P = 0.012$); the change value of Bifidobacterium was positively correlated with the change value of SMM ($r = 0.750$, $P = 0.032$). As is shown in Figure 11.

The difference in Blautia before and after treatment was positively

correlated with the change of TCM syndrome score with yellow and greasy tongue coating ($r = 0.661$, $P = 0.043$); it was positively correlated with the shift in GGT ($r = 0.889$, $P = 0.003$). The change value of Anaerostignum was positively correlated with the change value of visceral fat area ($r = 0.792$, $P = 0.019$) and positively correlated with the change value of TG ($r = 0.816$, $P = 0.014$) (Figure 12).

NAFLD is a type of liver disease associated with metabolic dysfunction characterized by diffuse bullous steatosis of hepatocytes, of which lipid metabolism disorder is the most common [8]. In the past 20 years, the prevalence and incidence of metabolic-related fatty liver disease related to overweight/abdominal obesity, type 2 diabetes, and metabolic syndrome have increased rapidly, with a global majority of up to 25%, which has now become the largest Chronic liver disease in China [9].

Intestinal microbiota dysbiosis causes intestinal endothelial barrier dysfunction, leading to bacterial translocation that causes liver inflammation, and various metabolites produced by gut microbiota may affect the liver, thereby increasing susceptibility to NAFLD [10]. The complex interaction between microbial antigens and the cytosolic inflammasomes affects the activation of the inflammatory cascade and the development of hepatic fibrosis [11].

Ren Shimeng performed 16S-rDNA sequencing of intestinal flora in serum and stool samples of 88 NAFLD patients and 30 healthy subjects and found that NAFLD patients had reduced Alpha diversity and reduced abundance of Firmicutes phylum. At the genus level, the genera Roseburia and Subdoligranulum decreased in abundance [12]. Another 16S rRNA high-throughput sequencing technology study has shown that the intestinal flora of NAFLD damp-heat syndrome is severely dysregulated, such as the increased abundance of Prevotellaceae, Blautia, and Lachnospiraceae [13].

The main components of Huazhi Rougan granules include *Artemisia scopariae herba*, *Cassia*, *Rhubarb*, *Alisma*, *Polyporus*, *Hawthorn*, *Atractylodes*, *Atractylodes*, *tangerine peel*, *Trichosanthis fructus*, *Ligustrum lucidum*, *Eclipta*, *Lycium barbarum*, *Cirsii herba*, *Bupleurum*, *Licorice*, etc. Many previous studies have shown that Huazhi Rougan granules can significantly reduce the expression levels of IL-18 and IL-1 β in serum of NAFLD patients, improve liver function, reduce blood lipid levels, improve intestinal barrier, reduce endotoxemia, alleviate liver damage [14–23].

This study showed that after 12 weeks of treatment with Huazhi Rougan granules, 27 cases were clinically cured. Four were markedly effective, ten were effective, and seven were ineffective. The total effective rate was 85.14%. Resistant NAFLD has an excellent curative effect. After treatment, the patient's TCM syndrome liver area discomfort or dull pain, fatigue, loss of appetite, bitter mouth, and yellow and greasy tongue coating were significantly decreased compared with before treatment, and the levels of ALT, AST, GGT, ALP, TC, and TG were greatly improved compared with those before treatment. And the difference was statistically significant ($P < 0.01$). After treatment, BW, BMI, WC, body fat percentage, CAP, and LSM decreased significantly ($P < 0.05$), and skeletal muscle mass increased compared with before treatment.

The study's results showed that the abundance of Lactobacillus, Bifidobacterium, and Bacteroides in the oral cavity of NAFLD patients increased after 12 weeks of treatment with Huazhi Rougan granules. Studies have shown that Lactobacillus and Bifidobacterium can reduce inflammatory factors such as IL-1 β , TNF- α , IL-6, and IL-8, regulate liver inflammatory pathways, and effectively improve the outcome of NAFLD. After treatment, intestinal Firmicutes, Roseburia, Blautia, and Faecalibacterium abundance decreased. A study finds Firmicutes associated with changes in the relative abundance of obesity [24]. Roseburia exerts protective effects by regulating immune cells and factors such as butyrate and flagellin, tryptophan metabolism, the brain-gut axis, etc. [25]. The acetate production by Blautia inhibits insulin signaling and fat accumulation in adipocytes by activating G protein-coupled receptors GPR41 and GPR43, which in turn promotes the metabolism of unbound lipids and glucose in other tissues and alleviates obesity-related diseases [26, 27].

This study found that the abundance of *Sutterella* decreased after 12 weeks of treatment compared with before treatment. Studies have shown that the quantity of *Sutterella* is negatively correlated with the levels of inflammatory cytokines (IL-12, IL-13, interferon- γ). It can adhere to intestinal epithelial cells, promote the secretion of IL-8, and has a slight proinflammatory effect but does not destroy the integrity of the monolayer epithelial cells. This study may indicate that Huazhi Rougan granules can alleviate the inflammatory state in patients with NAFLD.

Huazhi Rougan granules has a remarkable curative effect in the

treatment of NAFLD, can protect the liver and reduce enzymes, promote the metabolism of fat in the liver, and significantly relieve the clinical symptoms of NAFLD patients. Huazhi Rougan granule can increase the diversity of intestinal microbial communities in patients with NAFLD, improve the structure of oral-intestinal flora in patients with NAFLD, reduce the abundance of pathogenic bacteria, and increase the number of beneficial bacteria, thereby reducing liver inflammation and improving steatosis. In summary, Huazhi Rougan granules are effective, safe, and worthy of clinical promotion and application.

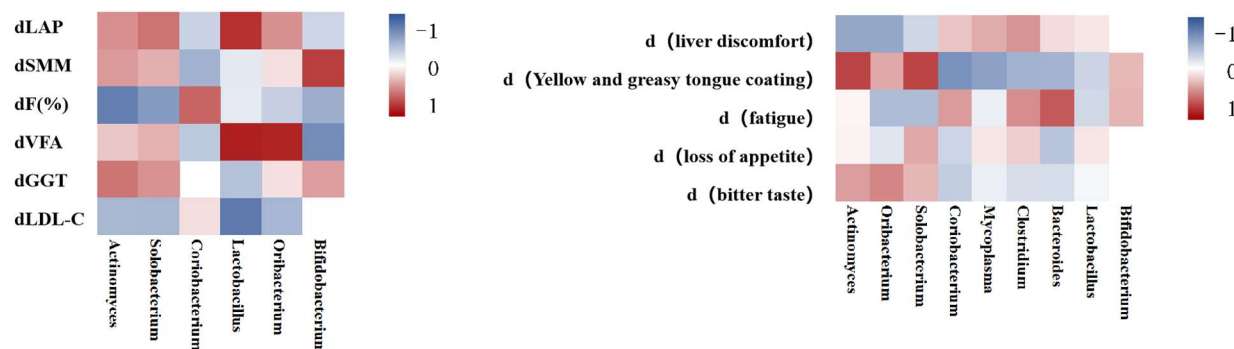


Figure 11 Heat map of the correlation between oral differential bacteria and clinical index changes

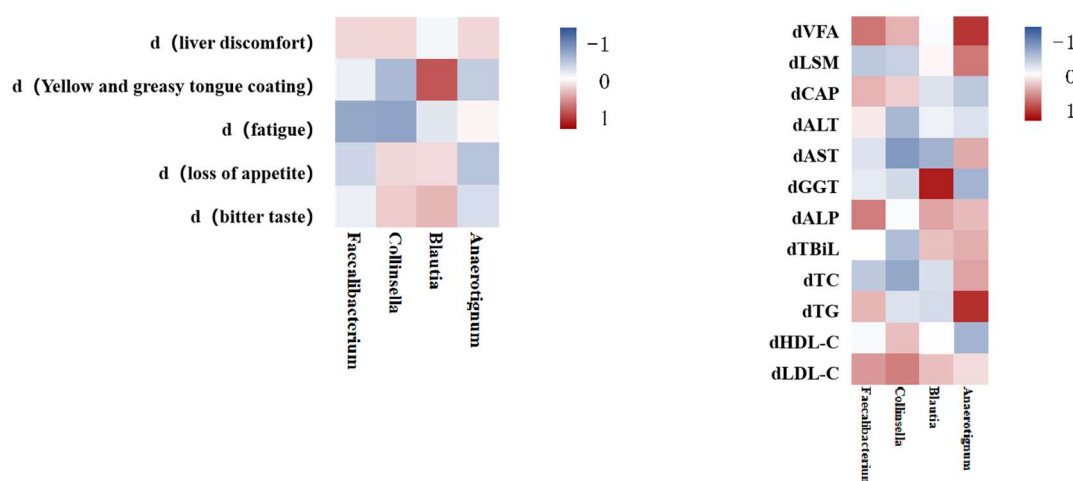


Figure 12 Heatmap of the correlation between the intestinal differential bacteria and the changes in clinical indicators

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