

Mechanisms of Yangxin Tongmai Formula against Coronary Heart Disease through the Microbiota-Gut-Heart Axis

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Abstract: Coronary heart disease (CHD) is one of the most common causes of death worldwide. Disruptions in the intestinal microbiota can promote the formation of CHD through different inflammatory reactions. Yangxin Tongmai Formula (YTF) has shown beneficial effects in CHD patients. Therefore, we aimed to investigate differences in the gut microbiota between rats with CHD and healthy rats in order to identify new biomarker of gut microbiota for diagnosis and Chinese herbal medicine treatment of CHD. The study cohort included 72 CHD rats and 16 normal rats. The CHD animal models were established and divided into 9 groups, including three YTF groups, four fecal transplantation groups, the atorvastatin calcium group, and the CHD group. All rats were sacrificed after 3.5 months of feeding and experiments, and blood, fecal, heart, and small intestine were samples collected for biochemical and pathological evaluations. The abundance of the intestinal flora was analyzed by bacterial 16S rRNA sequencing. The occludin protein was detected by western blotting. The result display the CHD model was successfully created. Rats that received YTF showed improvements in the lipid indices. The myocardial tissue cells and intestinal tissues in the medium-dose YTF group were basically complete, making it difficult to observe inflammatory response. The transmission electron microscopy results showed that the tight junctions of the medium-dose YTF group was improved. The operational taxonomic unit comparison showed that the abundance of lactobacilli and bifidobacteria in CHD rats significantly were improved after treatment with YTF. The abundance of beneficial bacteria in CHD rats was significantly increased ($P < 0.01$) following fecal bacterial transplantation from normal control rats and rats that underwent the YTF treatment. In comparison with the model control group, the relative expression of occludin protein in the medium-dose YTF group was upregulated ($P < 0.05$). The abundance of the gut microbiota differed significantly between CHD rats and healthy rats. YTF and fecal transplantation successfully alleviated atherosclerosis by remodeling the gut microbiota and the mechanical barrier function of small intestinal tissue.

1. Introduction

Coronary heart disease (CHD) is one of the most common causes of death in developed and developing countries[1]. Although lipid-lowering therapies can bring LDL cholesterol to normal levels, other cardiovascular risk factors cannot be controlled in this manner. The intestinal mucosa is the largest organ with immune activity and contains tens of billions of bacteria that constitute the gut microbiota[2]. The intestinal tract is not only the main digestive organ of the human body but also the largest immune organ, and is rich in large complex microbial communities. There are more than 7000 known species of microorganisms in the human body, and microorganisms in the intestine play an important role in human health[3]. These microorganisms participate in host metabolism, provide nutrition for intestinal epithelial cells, enhance immune function, and enhance resistance to the invasion of foreign microorganisms[4]. The uniqueness of the intestinal microflora of each individual is why the intestinal microflora is regarded as the second fingerprint of humans[5]. Microbiome composition and function are influenced by the host's genes, diet, and environment, and gut microbiota and the host show a complex and precise dynamic balance[6,7]. Gut microorganisms absorb nutrients from the host by degrading compounds that cannot be decomposed during the digestive process. Once the balance between intestinal microorganisms and the host is compromised, the structure of intestinal microflora is disrupted. Intestinal microorganisms can affect host health through energy absorption, endotoxemia, short-chain fatty acids, choline, and bile acid metabolism[8,9].

Intestinal flora can promote the formation of CHD through different metabolic pathways and inflammatory reactions[10]. Advances in next-generation sequencing technologies have substantially improved the characterization of gut microbial communities. Among these approaches, 16S rRNA gene amplicon sequencing is widely used to profile bacterial community composition, whereas shotgun metagenomic sequencing provides broader information on microbial taxonomic and functional characteristics[11]. High-throughput sequencing technology based on the 16S rRNA variable region is widely used in Roche 454, Illumina HiSeq and MiSeq, and Ion Torrent high-throughput sequencing platforms[12]. These techniques have contributed to our understanding of the relationship between changes in the abundance of intestinal flora and human health. In this study, high-throughput sequencing was used to study the intestinal flora structure of CHD model rats. Changes in tight junctions in the rat small intestine were observed by transmission electron microscopy and western blotting, and the correlation between the gut microbiome and CHD was explored to determine whether gut dysbiosis represents a contributing factor to, or a consequence of, CHD remains incompletely understood[13]. While adjusting the abundance of the bacterial flora, improving the diversity of the bacterial flora, and improving the balance between the intestinal microorganisms and patient health, the prevention and treatment of CHD by the Yangxin Tongmai Formula (YTF) and fecal transplantation based on gut microbiota remodeling were also investigated[14].

2. Manuscript preparation

2.1 Laboratory animals

Ninety-two male Sprague-Dawley (SD) rats weighing 200 ± 10 g were provided by the Laboratory Animal Center of the Hunan University of Chinese Medicine (Animal Qualification Certificate No: SCXK (Xiang) 2013-0005; Animal Ethics Review No. LL2018022701).

2.2 Reagents and instruments

YTF consists of ginseng (10 g), *Salvia miltiorrhiza* (10 g), Fructus Aurantii Immaturus (10 g), Rhizoma Alismatis (10 g), and Ramulus Cinnamomi (10 g). Both YTF and atorvastatin calcium tablets were purchased from the First Affiliated Hospital of Hunan University of Chinese Medicine. YTF was prepared at three different concentrations: low dose (6 g/kg•d), medium dose (12 g/kg•d), and high dose (18 g/kg•d).

2.3 Modeling and administration

2.3.1 YTF treatment in CHD rats

Animal processing and modeling were performed in two steps using previously described methods. After successfully establishing a CHD rat model, the animals were divided into two normal groups, namely, the normal control (group A) and the normal control + YTF high-dose group (group B), and five model groups, namely, the CHD (group C), YTF low-dose (group D), YTF medium-dose (group E), YTF high-dose (group F), and atorvastatin calcium (group G). Blood and fecal samples were collected after YTF and atorvastatin calcium treatment by gavage for 15 days.

The rats were administered intraperitoneal anesthesia with 10% chloral hydrate (0.25 mL/100 g). After 5 min of anesthesia and the absence of a struggling reaction, the rats were fixed on a rat plate and the intestinal contents were collected in a sterile tube using sterile tweezers. Sterile tubes were immediately placed in liquid nitrogen. The intestinal contents were stored at -80 °C until use.

2.3.2 Fecal transplantation

From the beginning of the treatment period, fecal samples were obtained from groups A, C, E, and G and mixed within the groups subjected to YTF treatment for fecal transplantation experiments. Fifty-five SD rats were randomly divided into four groups: rats in group Fa received fecal transplantation from group A, those in group Fb received fecal transplantation from group C, those in group Fc received fecal transplantation from group E, and those in group Fd received fecal transplantation from group G. One hundred milligrams of feces were mixed and suspended in 1 mL of sterile normal saline solution. The rats received the fecal supernatant by gavage once per day for two weeks.

2.3.3 Detection of changes in blood lipid levels

Each group was fasted for 12 h on the day before the material was collected; 2 mL and 5 mL of fresh abdominal aortic blood were sent to the First Affiliated Hospital of Hunan University of Chinese Medicine for testing on the same day; and blood lipids were detected by a Siemens Advia2400 automatic biochemical instrument.

2.3.4 Hematoxylin-eosin staining

The intact heart and aortic arch were hooked and cut with ophthalmic forceps, washed with normal saline, and fixed with 4% paraformaldehyde solution. Approximately 1 cm of the small intestine was intercepted, washed with normal saline, and fixed with 4% paraformaldehyde solution, and the pathological changes in the tissue specimens were observed by hematoxylin-eosin (HE) staining after fixation, dehydration, and transparency, wax-embedding, slicing, and deswaxed staining.

2.4 16S rRNA gene sequencing

2.4.1 Influence of YTF on the structure and function of intestinal microorganisms in CHD rats

16S rRNA gene sequencing was performed on the rat intestinal contents from each group. The intestinal microbial diversity was analyzed using Mothur (<http://www.mothur.org/>) and MEGAN and QIIME software, and the composition and differences in the intestinal microbial community were then compared [15]. Based on the KEGG and COG/EGGNOG databases (<http://eggno5.embl.de/#/app/home>), gene function classification and metabolic pathways of the samples were determined using MEGAN [16].

2.4.2 Analysis of intestinal microorganisms by 16S rRNA gene sequencing

Based on the 16S rRNA gene sequencing results, the structure and function of the intestinal microorganisms in the rats of each group were analyzed and predicted.

2.4.3 Data processing and bioinformatics analysis

Mothur software was used for operational taxonomic unit (OTU) analysis or sequence typing. High-quality sequences were artificially divided into OTUs at a sequence similarity threshold of 97% [17]. Next, the high-quality sequences were merged, and the OTUs were divided using the UCLUST software [18]. The longest sequence for each class of microorganisms was selected as the representative sequence. The BLAST package in QIIME was used to compare the OTU representative sequences with the NCBI (NCBI *et al.*, 2016) database and obtain taxonomic information for each OTU. OTUs whose abundance value was less than 0.001% of the total sequencing sample were removed, and for the remaining OTUs, a series of analyses was performed. Unique sequencing reads that clustered into OTUs were aligned using the RDP database for species annotation and abundance statistics.

The Shannon, Simpson, and Chao alpha diversity indices for each sample were calculated using a 97% similarity threshold for OTU classification. Alpha diversity indices are comprehensive indices that reflect the richness and evenness of a microbial population. The Chao index reflects the richness of the intestinal bacterial community, with a high value representing high richness of the community. Shannon's index reflects the diversity of the bacterial community, with a higher Shannon value indicating a more diverse bacterial community.

The annotated species were classified at the phylum, family, genus, and species levels, and the species abundance of each sample at different levels was analyzed. Based on the taxonomic information database of the identified microbial species, MEGAN was used to reveal the relationship between species abundance and the tree of the taxonomic system of microbial species. A species distribution map depicting the evolutionary relationships of microorganisms was generated, and the evolutionary relationships and microbial abundance in the different samples were described from a taxonomic perspective. Lastly, UniFrac was used for PCoA and UPGMA cluster tree analyses.

2.4.4 Electron microscopy and occludin protein detection of small intestine tissue

A double-blade saw method was used for obtaining small intestine specimens. The small intestine tissue was cut into small pieces of approximately 1-2 m³ at low temperature and fixed in a 2.5% glutaraldehyde solution for transmission electron microscopy. The western blot protein loading was 100 µg. After denaturation in a dry bath at 100 °C for 10 min, electrophoresis was

performed at 90 V for approximately 80 min, at 200 mA for 1 h, and blocked with 5% skim milk for 1 h. The membrane containing the isolated proteins was incubated with the primary antibody (occludin 1:1000, GAPDH 1:8000) at 4 °C overnight and the secondary antibody (1:8000) at 37 °C for 1.5 h. After washing the membrane with TBST, it was developed using a gel imager. ImageJ software was used to calculate relative protein expression.

3. Results

3.1 Body state and behavioral characteristics of the rats

The rats’ dietary intake, fur status, mental state, and deaths were recorded at approximately 9:00 am. The rats were weighed on an electronic balance, and their weight was recorded. The normal control rats showed normal water consumption, normal sensitive responses, and smooth fur. From the 30th day of modeling, the weight growth of model rats gradually slowed, and their hair became haggard and colorless. On the 70th day of modeling, the model rats showed reduced food intake, weight loss, soft feces, obvious curling up behaviors, less movement, and a preference to get together. On day 90, the model rats showed a dark ear rim and purple claw armor, and their feces were no longer granular and curled up. After the drug intervention, the body weight increased in each drug intervention group, with the highest average weight increase observed in the low- and medium-dose groups of YTF. The results are presented in Figure 1.

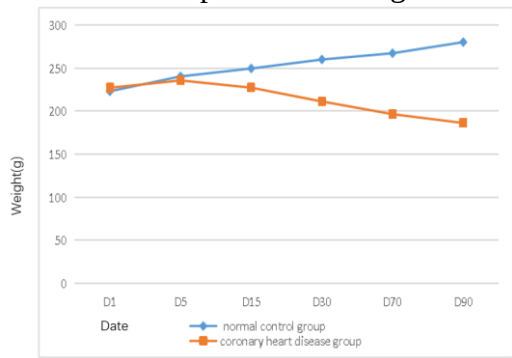


Figure 1. Weight changes in the normal control and coronary heart disease groups before and after the drug intervention.

3.1.1 Blood lipid test results

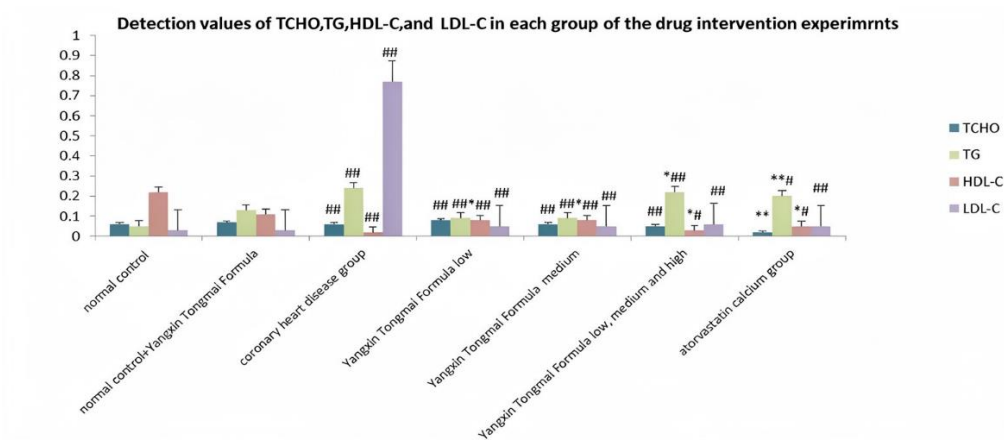


Figure 2. TCHO, TG, HDL-C, and LDL-C values in each group (n = 8; mmol/L; $\bar{x} \pm s$).

Note: In comparison with the normal control group, $*P < 0.05$, $**P < 0.01$; in comparison with the coronary heart disease group, $\#P < 0.05$, $\#\#P < 0.01$.

In the drug intervention experiment, in comparison with the normal control group, the CHD group and atorvastatin calcium group showed significantly higher total cholesterol (TCHO), triglyceride (TG), and low-density lipoprotein cholesterol (LDL-C) levels and a lower high-density lipoprotein cholesterol (HDL-C) level ($P < 0.05$ or $P < 0.01$). In contrast, the TCHO and TG values decreased; the HDL-C value increased; and the LDL-C value decreased in the atorvastatin calcium group and the YTF low-, medium-, and high-dose groups ($P < 0.05$). Thus, the YTF low-, medium-, and high-dose groups were better than the atorvastatin calcium group in reducing TCHO and TG levels ($P < 0.05$). The results are shown in Figure 2.

3.1.2 Blood lipid test results after fecal bacteria transplantation

The results of the fecal bacteria transplantation experiment showed that the TCHO, TG, and LDL-C levels in group Fb were significantly higher than those in group Fa, while the HDL-C level in group Fb was lower than that in group Fa ($P < 0.01$). However, groups Fc and Fd showed significantly higher HDL-C and significantly lower LDL-C values than group Fb ($P < 0.01$). In comparison with the CHD group, the TCHO and TG values in group Fd were lower, and the difference was statistically significant ($P < 0.01$). Moreover, the reductions in TCHO and TG values in group Fc were better than those in group Fd ($P < 0.05$). These results are consistent with the findings of drug intervention experiments using YTF. The results are shown in Figure 3.

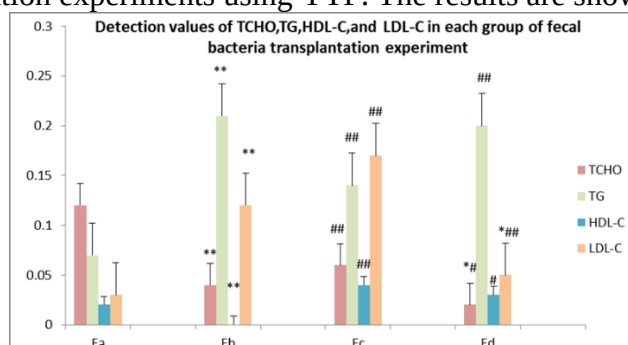


Figure 3. TCHO, TG, HDL-C, and LDL-C levels in the fecal bacteria transplantation experiment ($n = 8$; mmol/L, $\bar{x} \pm s$).

Note: In comparison with Fa, $*P < 0.05$, $**P < 0.01$; in comparison with Fb, $\#P < 0.05$, $\#\#P < 0.01$.

3.2 Histopathological examination of the coronary artery and the surrounding myocardium in rats

In the normal control group, the endothelium of the coronary artery was intact; no stenosis or deformation was found in the lumen; cardiomyocytes were arranged in an orderly manner; and there was no inflammatory necrosis. In the CHD group, the lumen became narrow due to thickening of the intima of the coronary artery, and the tissue structure was loose. Myocardial cells showed focal necrosis and disordered arrangement accompanied by inflammatory infiltration and edema. The pathological morphology in the four drug intervention groups was between that of the normal control and CHD groups. The coronary artery endothelium in the medium-dose YTF group was complete and wavy; the cardiomyocytes were arranged in order; the morphology was relatively complete; and there was slight necrosis and slight stenosis of the lumen. In the atorvastatin calcium group, the cells were arranged in a disorderly manner; there was obvious necrosis and inflammatory

infiltration; the lumen was significantly narrowed; and the intimal tissue structure was loose. The results are shown in Figure 4.

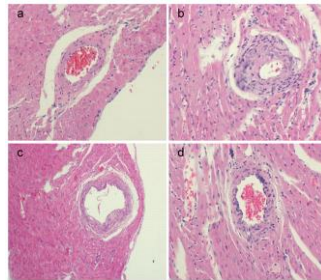


Figure 4. Histopathology of the coronary artery and surrounding myocardium in the four rats, (a)Normal control (HE $\times 400$), (b)Coronary heart disease group (HE $\times 400$), (c)Medium-dose YTF group (HE $\times 400$), (d)Atorvastatin calcium group (HE $\times 400$).

3.3 HE staining results of rat small intestinal tissue

In the normal control group, the intestinal mucosa and villi were intact, the villi were arranged in an orderly manner, the subepithelial space was not dilated, and the thickness of muscle layer was uniform. In the CHD group, the top of the intestinal villi epithelium was separated from the lamina propria, the top of intestinal villi was broken and missing, and the submucosa showed pathological manifestations such as inflammatory infiltration and necrosis. The intestinal villi of rats in the medium-dose YTF group were basically intact, and the subepithelial space was basically not dilated. The atorvastatin calcium group showed slight rupture of intestinal villi; the morphological part was obviously incomplete with typical inflammatory infiltration, and cell necrosis is not obvious. The results are shown in Figure 5.

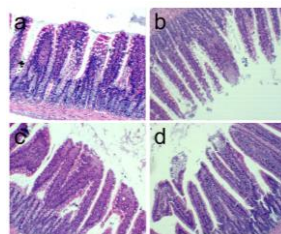


Figure 5. Histopathology of the small intestine in four rats, (a)Normal control group (HE $\times 200$), (b)Coronary heart disease group (HE $\times 200$), (c)Medium-dose YTF group (HE $\times 200$), (d)Atorvastatin calcium group (HE $\times 200$).

3.4 Diversity analysis

The Venn diagram shows the number of shared or unique OTUs between the different groups. Each ellipse represents a group. As shown in the Venn diagram, the fecal transplantation experiment indicated 428 common OTUs among the four groups (Fig. 6). A total of 1131 OTUs were identified in the Fa group, 1117 in the Fb group, 1131 in the Fc group, and 1084 for the Fd group. Of the total OTUs identified in the Fa, Fb, Fc, and Fd groups, 313, 252, 223, and 211, respectively, were unique OTUs. Thus, while YTF treatment increased the diversity of the intestinal flora of rats close to that in the normal group, the total number of microorganisms did not reach the level observed in normal rats.

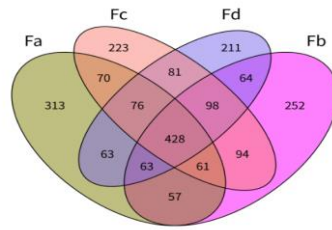


Figure 6. OTU Venn diagram.

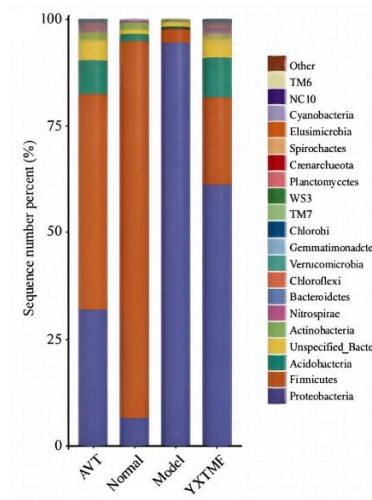


Figure 7. Histogram of the relative distribution of each group at the phylum level (top 20 species with their relative abundance).

Note: Vertical axis: sample name information; Horizontal axis: taxonomic abundance. Column maps of different colors represent the abundance levels of different bacterial genera.

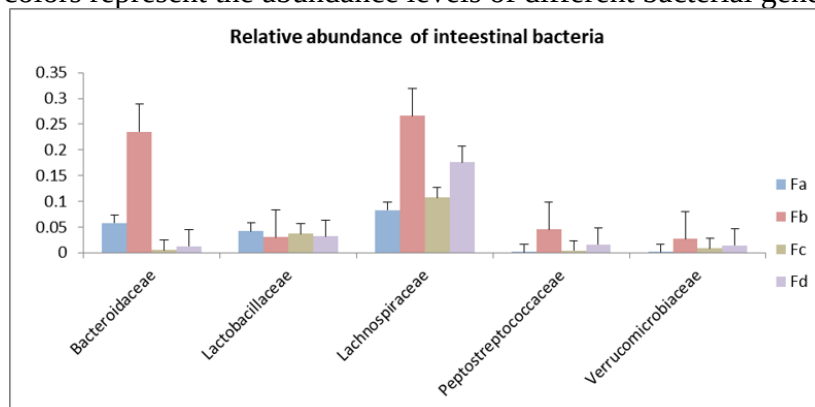


Figure 8. Relative abundance of intestinal bacteria.

While Firmicutes were low in number in groups C and G and high in groups A and D, Bacteroidetes showed the highest abundance in group C and the lowest abundance in group D (Fig. 7). The abundance of Firmicutes and Bacteroidetes in the treatment group was similar to that in the normal group. In the fecal transplantation experiment, Firmicutes showed the highest abundance in group Fd and the lowest abundance in group Fb, whereas Bacteroides showed the highest abundance in group Fb and the lowest abundance in group Fa (Fig. 8).

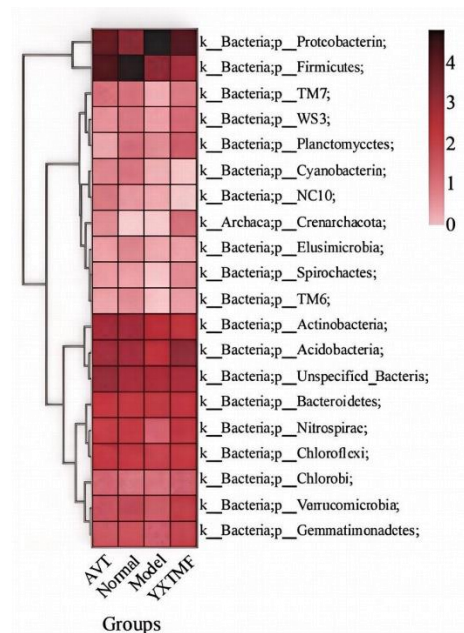


Figure 9. Thermal image of the phylum classification level.

The cluster tree above the graph represents the degree of similarity in abundance distribution of species clusters in each sample. The middle panel represents the $\log_{10}$ thermogram (absolute abundance).

While Firmicutes and Bacteroidetes constituted the largest populations of bacteria, most of the samples were not significantly different in their abundance distribution (Fig. 9). Nevertheless, the abundance of TM7 in groups A and B was significantly higher than that in other groups, while the abundance of Verrucomicrobia and Cyanobacteria was significantly lower than that in the other groups. The abundance of Spirochaetes in groups C and A was the lowest, while the Verrucomicrobia abundance in group C was significantly higher than that in the other groups (Fig. 9). Therefore, we can speculate that CHD development might be positively correlated with Verrucomicrobia abundance.

3.5 Difference analysis

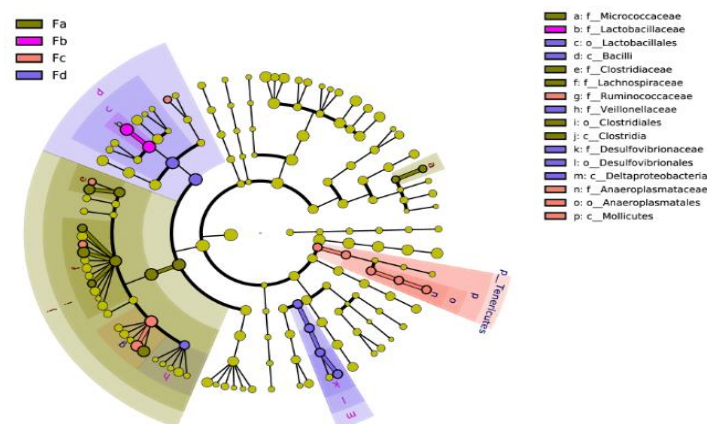


Figure 10. Cladogram of the results of LefSe analysis after fecal bacteria transplantation.

The cladogram displays taxonomic hierarchies from phylum to lower taxa from inner to outer rings, with connecting lines indicating taxonomic affiliation. Each circular node stands for one

taxon: yellow nodes denote non-significant intergroup differences, while colored nodes represent signature microorganisms whose abundance is significantly elevated in the corresponding colored group. Colored sectors mark taxonomic ranges of these signature microbes.

In the YTF intervention experiment (Figure 10), nine bacterial taxa exhibited inter-group abundance discrepancies between healthy and CHD model rats. Group A had reduced *Moraxellaceae* and *Pasteurellaceae* abundance relative to other groups. Group C showed lower *Streptococcaceae* and *Turicibacteraceae* compared with group A; *Methanobacteriaceae* and *Verrucomicrobiaceae* abundance were markedly decreased versus drug-treated groups. We hypothesize that YTF suppresses endotoxin production to boost probiotic activity. Meanwhile, *Verrucomicrobiaceae* may modulate obesity-related gut flora, thereby exerting protective effects against CHD.

3.6 Correlation statistical analysis

Correlation analysis revealed microbial interactions among gut taxa (Figure 11). Lactic acid bacteria were positively correlated with fecal anaerobic bacteria. These microbes degrade dietary fiber into short-chain fatty acids (SCFAs). SCFAs enter the tricarboxylic acid cycle and react with acetyl-CoA to generate substantial energy, promoting obesity and accelerating atherosclerosis progression. *Prevotella* showed a negative correlation with fecal cocci. Although *Prevotella* degrades carbohydrates and polysaccharides, it may trigger gastrointestinal inflammation. It also produces nicotinamide (NAM) metabolites, mitigating diet-provoked oxidative stress and inflammation and accelerating glucose metabolism. Elevated intestinal *Akkermansia* reduces the risk of diabetes and cardiovascular diseases.

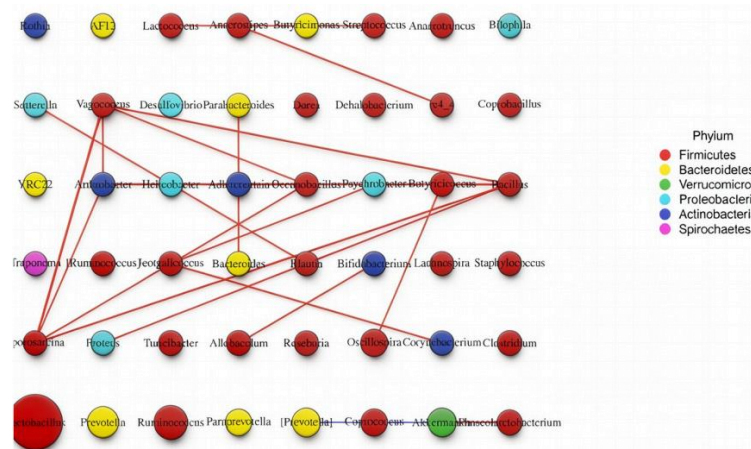


Figure 11. Species interaction network at the genus classification level.

Note: The circle represents a species; the size of the circle represents its relative abundance; the red line represents a positive correlation; and the blue represents a negative correlation. The thicker the lines, the greater the absolute value of the correlation coefficient.

As shown in Figure 12, groups A, B, D and E shared similar gut microbiota structures; groups C and G exhibited comparable profiles, whereas group F displayed an intermediate microbiota pattern. These results suggest that YTF effectively remodels gut microbial composition, and the regulatory effect on intestinal microecology may be dosage-dependent.

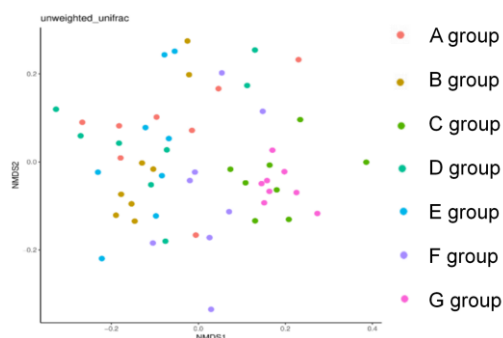


Figure 12. NMDS analysis.

Note: The distance between points indicates the degree of sample flora difference; thus, the closer the two points, the higher the similarity of the microbial community structure between the two samples and the smaller the difference.

3.7 Transmission electron microscopy (TEM) of the small intestine in rats

TEM was used to assess the ultrastructure of intercellular junctions in small-intestinal mucosa (Figure 13). The normal control group exhibited intact, well-defined tight junctions and desmosomes, together with densely-packed microvilli. By contrast, the CHD model group displayed decreased and blurred tight junctions, fewer desmosomes, and sparse, reduced microvilli. Compared with the model group, YTF-treated and atorvastatin-treated groups showed restored, better-defined tight junctions and increased desmosome counts, indicating ameliorated mechanical barrier function of the small-intestinal mucosa.

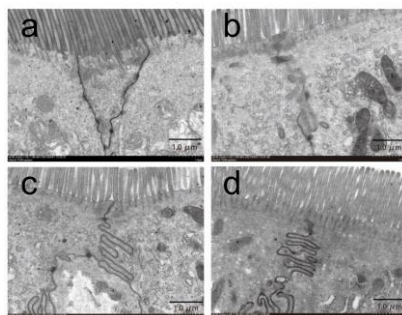


Figure 13. Small intestine tissue of rats in each group (transmission electron microscopy, $\times 6000$), (a)Normal control group, (b)Coronary heart disease group, (c)YTF medium-dose group, (d)Atorvastatin calcium group.

3.8 Detection of occludin protein in the small intestine of rats

As shown in figures 14 and 15, in comparison with the normal control group, the model control group showed significantly downregulated relative expression of occludin ($P < 0.05$). In comparison with the model control group, the relative expression of occludin protein in the YTF group was upregulated ($P < 0.05$), suggesting that the YTF can rebuild the barrier function of the small intestine. The relative expression of occludin protein was also upregulated in the atorvastatin group ($P < 0.01$).

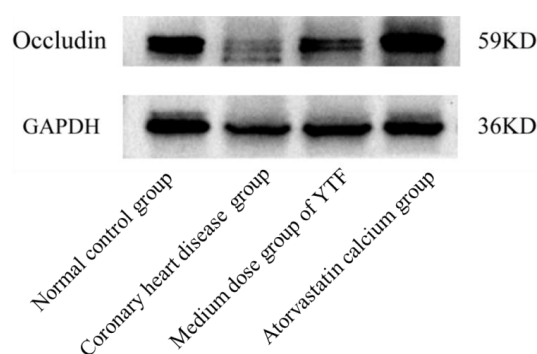


Figure 14. Electrophoresis of occludin protein in small intestine specimens.

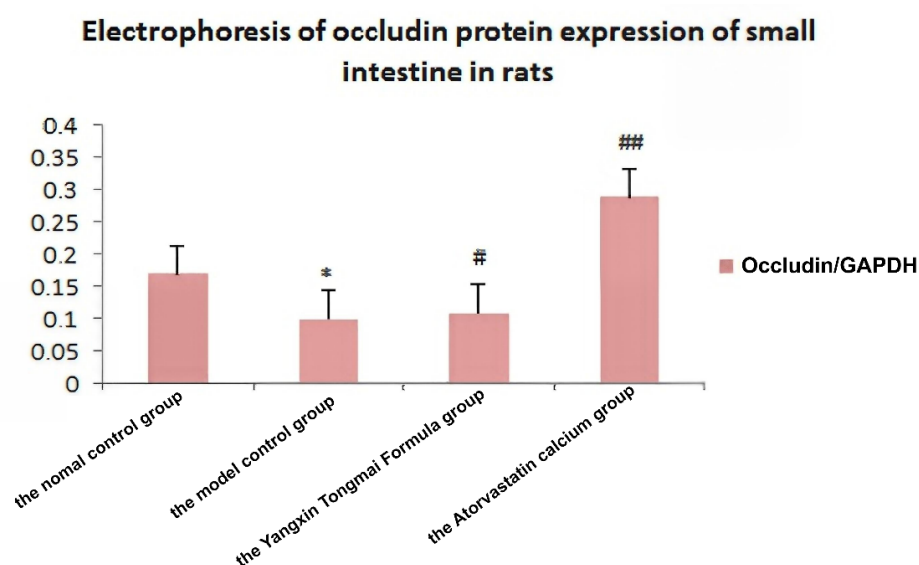


Figure 15. Comparison of the relative expression of occludin protein in rats of each group ($\bar{x} \pm s$, $n = 5$).

Note: In comparison with the normal group, * $P < 0.05$; in comparison with the model group, # $P < 0.05$, ## $P < 0.01$.

4. Discussion

The gut represents the human body's largest microecosystem. Cumulative evidence indicates that intestinal flora dysbiosis is tightly linked to the onset and progression of CHD, and serves as one critical pathogenic mechanism of CHD. With advances in molecular biology, the gut microecosystem has been recognized as a promising therapeutic target for CHD treatment[19-21].

Traditional Chinese medicine exhibits distinct advantages for CHD management. YTF, formulated by Qin Bowei for thoracic obstruction-cardiodynia via tonifying heart-qi and unblocking blood vessels, exerts therapeutic efficacy against CHD. YTF comprises *Ginseng*, *Salvia miltiorrhiza*, *Fructus Aurantii Immaturus*, *Alisma orientalis*, and *Ramulus Cinnamomi*. Its herbal components perform respective pharmacological actions: ginseng tonifies heart-qi; *Salvia miltiorrhiza* activates blood circulation to resolve stasis; *Fructus Aurantii Immaturus* regulates qi and eliminates phlegm; *Alisma orientalis* promotes diuresis and nourishes yin; and *Ramulus Cinnamomi* regulates qi and dispels phlegm.

Ginseng mainly contains saponins, polysaccharides, and other components, which show anti-inflammatory and anti-atherosclerotic effects, reduce blood pressure, and inhibit platelet aggregation[22]. Phenolic acids, flavonoids and triterpenoids in *Salvia miltiorrhiza* exert

anticoagulant, anti-platelet, anti-atherosclerotic, lipid-modulating, hypotensive, cardioprotective, anti-inflammatory and antioxidant properties[23,24]. Alkaloids and flavonoid glycosides of *Fructus Aurantii Immaturus* enhance myocardial contractility, boost coronary perfusion, modulate heart rate and cardiac output, and confer antithrombotic and anti-platelet activities[25,26]. Triterpenes and sesquiterpenes from *Alisma orientalis* lower serum lipids and glucose, alleviate inflammation and oxidative injury, and induce vasodilation[27]. Volatile oils and other compounds in *Ramulus Cinnamomi* contribute to anti-inflammation, vasodilation, blood-pressure reduction, anti-platelet aggregation and anticoagulation [28,29]. Collectively, YTF yields vascular-warming protective effects in CHD patients.

Gut microbiota disturbance and lipopolysaccharide (LPS) release disrupt tight-junction proteins (e.g., occludin, ZO-1), increase intestinal permeability, and drive microbial metabolite translocation into systemic circulation. Circulating microbial products further trigger inflammatory cascades and aggravate cardiovascular damage[30]. In CHD rat models, gut dysbiosis (decreased beneficial microbes and enriched pathogenic taxa) correlates with impaired intestinal barrier and excessive inflammation. These observations support the interconnection among gut microbial dysbiosis, intestinal barrier dysfunction, and CHD.

Following drug administration, serum lipid profiling and 16S rRNA high-throughput sequencing were performed in rats from each group. YTF markedly decreased TCHO, TG and LDL-C, and elevated HDL-C to ameliorate dyslipidemia. Comparison between CHD and control rats revealed significant differences in gut microbiota abundance, whereas microbial diversity was comparable. Structural discrepancies were prominent at the phylum level: CHD rats exhibited higher *Bacteroidetes* but lower *Firmicutes* abundance; across all animal groups, *Firmicutes* dominated, followed by *Bacteroidetes*.

Veillonella and *Veillonella dispar* were detected in both CHD and control groups, with significantly higher abundance in CHD rats ($P=0.03$), despite notable inter-individual variation within groups. As opportunistic pathogens colonizing oral, genitourinary, respiratory and intestinal tracts, *Veillonella* may trigger endocarditis and other infectious diseases[31-33]. Our findings support a close correlation between elevated *Veillonella* and CHD.

Fecal microbiota transplantation (FMT) revealed enriched probiotics, especially higher *Coprococcus* abundance, in the YTF-treated donor microbiota. Recipient rats receiving fecal material from normal control or YTF-treated donors displayed reduced TCHO, TG, LDL-C and increased HDL-C. These results indicate that YTF improves lipid profiles via intestinal probiotics and validate the gut-microbiota-heart axis as a therapeutic mechanism for CHD intervention.

Our previous work demonstrated that YTF mitigates gut dysbiosis and partially recovers microbial diversity in CHD rats with blood-stasis syndrome. Further evidence indicated that YTF promotes beneficial taxa such as *Lactobacillus* and *Bifidobacterium*, while inhibiting pathobionts including *Escherichia coli*. Hence, restoration of intestinal microbial homeostasis may partially mediate the cardioprotective effects of YTF. YTF treatment significantly elevated *Lactobacillus* abundance and reduced *Streptococcus* abundance. It is proposed that YTF modulates gut microbiota and mitigates inflammation by suppressing *Streptococcus* and limiting lipopolysaccharide-endotoxin release, conferring protective effects against CHD. Moreover, YTF significantly lowered the levels of *Parabacteroides*, *Bacteroides uniformis* and *Bacteroides caccae* in CHD rats ($P<0.05$). *Bacteroides* participate in carbohydrate fermentation, and the degradation of polysaccharides, bile acids and steroids[34]; they facilitate intestinal mucosal angiogenesis and sustain gut homeostasis[35]. *Bacteroides caccae* colonizes the intestinal mucosal surface to block pathogen adhesion and invasion.

Gut dysbiosis contributes to multiple metabolic-related disorders and represents a potential driver for CHD[36,37]. Trimethylamine N-oxide (TMAO) serves as a promising cardiovascular

biomarker. The metabolism of choline, TMAO and betaine within the phosphatidylcholine pathway is gut-microbiota-dependent[38]. Intestinal microbes modulate host cholesterol metabolism, oxidative stress and inflammatory responses, thereby facilitating cardiovascular disease progression[39,40]. Intestinal-derived peptide hormones also exert cardiovascular bioactivities. A 2015 Cleveland Medical Center study pioneered the concept of targeting intestinal metabolism and gut microbiota for heart-disease therapy[41]. Clinical evidence reveals distinct gut microbial profiles in CHD patients, characterized by expanded pathobionts and depleted beneficial taxa including *Bacteroides*. For instance, a ganoderma meroterpene derivative alleviates obesity-related atherosclerosis via enriching *Parabacteroides merdae* and boosting branched-chain amino acid (BCAA) catabolism.

Our data demonstrate that CHD model rats exhibit compromised gut microbiota homeostasis, metabolic capacity and anti-pathogen competence, implying impaired intestinal function. Hence, intestinal function monitoring and gut microbiota intervention to enrich beneficial microbes may benefit CHD patients, yet clinical translation requires further validation in human subjects. In conclusion, YTF and fecal microbiota transplantation attenuated atherosclerosis by remodeling gut microbiota and restoring small-intestinal mechanical barrier function. CHD rats displayed marked alterations in gut microbial abundance rather than microbial α -diversity.

5. Conclusions

Our results demonstrate that YTF and fecal transplantation successfully alleviated atherosclerosis by remodeling the gut microbiota. The abundance of gut microbiota differed significantly between CHD rats and healthy rats; however, the two groups showed no significant differences in the diversity of intestinal flora.

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