Changes and roles of intestinal fungal microbiota in coronary heart disease complicated with nonalcoholic fatty liver disease

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Abstract: Background: Patients who suffered coronary heart disease (CHD) complicated with non-alcoholic fatty liver disease (NAFLD) were reported to have worse cardiac function and clinical outcomes than patients with CHD only. The mechanism was unclear. Previous study focused on the metabolism and showed it could be regulated by the microbiota. Few studies related to fungi. We aimed to investigate the characteristics of intestinal fungal microbiota in CHD patients complicated with NAFLD (CHD-NAFLD). Methods: 72 People were recruited and equally divided into three groups, including CHD patients (without NAFLD), CHD-NAFLD patients, and healthy controls (HCs). Fecal samples were collected. The Illumina sequencing of the internal transcribed spacer 3-4 rRNA was applied. Results: The BMI, uric acid and triglyceride in CHD-NAFLD patients increased compared with CHD patients. The abundance of Exophiala attenuata and Malassezia restricta in all CHD-NAFLD and CHD patients significantly reduced. The intestinal fungal microbiota in CHD-NAFLD patients showed an increase in the abundance of Preussia, Xylodon and Cladorrhinum, and a reduction in the abundance of Candida glabrata and Ganoderma. Among them, the abundance of Ganoderma was significantly lower than that in CHD patients. The ejection fraction was negatively correlated to the abundance of Xylodon. Uric acid was positively correlated with the abundance of Cladorrhinum and Preussia. Conclusions: These changes of intestinal fungal microbiota in CHD-NAFLD patients may be important factors affecting the degree of metabolic disorder. But there are few reports on these fungi. More studies are needed to confirm the effects of these fungi on human.

Keywords: Non-alcoholic fatty liver disease, coronary heart disease, intestinal microbiota

Introduction

Nonalcoholic fatty liver disease (NAFLD) was one of the most common chronic liver diseases worldwide [1, 2]. However, the pathogenesis of NAFLD was still unclear. In recent years, it was believed that NAFLD tended to be caused by various factors including genetic differences, insulin resistance, intestinal microbial dysbiosis and lipid metabolism [3]. The intestinal microbiota was found to play an important role in the occurrence and development of NAFLD [4].

It was reported that NAFLD had a closely correlation with coronary atherosclerotic heart disease (CHD). The risk of cardiovascular disease was increased in NAFLD patients [5, 6]. A observational study found that the incidence of atherosclerotic cardiovascular disease including CHD and ischemic stroke in CHD patients complicated with NALFD (CHD-NAFLD) was significantly higher than that in CHD patients [7]. The incidence and mortality of cardiovascular events in NAFLD patients significantly increased [8-13]. And the rate of coronary stenosis was higher in CHD-NAFLD patients than that in CHD patients without NAFLD [14] and the severity of CHD and cardiac function were also worse [15]. There were few studies on the mechanisms, especially from the perspective of intestinal microbiota.

A large number of recent studies also focused on the role of intestinal microbiota in CHD
There was continuous evidence that intestinal microbiota was closely related to atherosclerosis [17]. Trimethylamine N-oxide (TMAO), formed by gut microbe-dependent metabolism, is a gut microbiota-derived metabolite that enhances both platelet responsiveness and in vivo thrombosis potential in animal models and could predict incident atherothrombotic event risks in human clinical studies. The drug TMAO inhibitor for CHD targeted on the intestinal bacterial microbiota had also made some progress [18].

Gut microbiota and metabolism played pivotal roles in the progression of CHD and NAFLD. Furthermore, fungal microbiota was an important component of the intestinal microbiota and some animal experiments showed that fungi also played a role in metabolic diseases [19, 20]. However, current researches mainly focused on bacterial microbiota and there were few studies on fungal microbiota, coronary heart disease and NAFLD. Thus the characteristics of fungal microbiota in CHD patients, especially the characteristics of fungal microbiota in CHD-NAFLD patients have not been reported. This study was designed to investigate the characteristics and effects of intestinal fungal microbiota in CHD-NAFLD patients.

Materials and methods

Subject enrollment

Patients who were admitted to the Department of Gastroenterology or Cardiology in Peking University People’s Hospital from January to September in 2018 were recruited. They must meet: (1) No viral hepatitis, autoimmune liver disease and alcoholic hepatitis. No chronic gastrointestinal disease and previous abdominal surgery; (2) Left ventricular ejection fraction ≥40% and no heart failure; (3) Age between 18 and 80 years. Pregnant women or after an abortion would be also excluded in this study; (4) No antibiotics for nearly 2 weeks. No drinking alcohol, spicy food, yogurt and probiotics for nearly 1 week; (5) Normal stool frequency: 3 times/Day-3 times/week without diarrhea.

This study was approved by the Conjoint Health Research Ethics Board of Peking University People’s Hospital (No. 2018PHB033-01) and informed consent forms were obtained from all the participants. The study was carefully conducted complying with the Declaration of Helsinki.

People were divided into three groups, including CHD patients (without NAFLD), CHD-NAFLD patients and healthy controls (HCs). The overall CHD patients included CHD patients and CHD-NAFLD patients. CHD diagnosis was confirmed by coronary angiography and individuals that had ≥50% stenosis in single or multiple vessels were included. NAFLD diagnosis was confirmed based on the evidence of hepatic steatosis via imaging [21]. B-ultrasound is the preferred method for imaging diagnosis of NAFLD [22]. Considering that liver biopsy was an invasive procedure, the guidelines recommended patients with undiagnosed NAFLD or suspected coexisting chronic liver disease needed the biopsy [22]. No such patients were included in this study. Therefore, this study mainly used B-ultrasound for imaging diagnosis of NAFLD. All the healthy controls enrolled were free of NAFLD, CHD and had no clinically CHD evidence such as angina and abnormal electrocardiographic.

The CHD-NAFLD patients were 1:1 matched with CHD patients and HCs according to the gender and age (±5). All the patients would receive abdominal ultrasound and biochemical tests and the overall CHD patients has performed the coronary angiography examination in the Peking University People’s Hospital. Demographic data and clinical information were carefully collected.

Sampling and sequencing

Fresh feces of each subject were collected after admission to the hospital. All samples were collected in Stool Collection Tube with Stool Satilizer and stored in -80°C freezers before further analysis in 48 hours.

DNA was extracted from stool samples using the PSP® Spin Stool DNA Plus Kit protocol (Stratec, German). The full-length primer sequences, using standard IUPAC nucleotide nomenclature, to follow the protocol targeting this region are ITS V1-V2 Amplicon PCR Forward Primer = 5'-GGAAGTAAAAGTCGTAACAAGG, PCR Reverse Primer = 5'-GCTGCGTTCTTCATCGATGC[23]. Each PCR product of the appropriate size was purified and quantified. And then, they were added to a master pool of DNA, subse-
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sequently, a 2 × 250 paired-end sequencing was performed and base called using the MiSeq Reporter software and the MiSeq system. For the alignment, the software flash was used [24]. For the quality control, high-quality sequences (QC value ≥25) were retained by the software QC tools.

**Sequencing data analysis**

The main software used for sequence analysis is Vsearch v2.8.1 [25] and Usearch v10 (bit 32). The original data was merged using a double-ended sequence by Vsearch, followed by data quality control, excision of primers and barcodes. 4786644 sequences remained and 15848 sequences were removed. Then we used vsearch to remove the redundant sequences and sequences with <30 occurrences. There are 1575125143 base pairs in the 4786644 sequences with a minimum of 250 pairs and a maximum of 490 pairs (an average of 329 pairs). A total of 1889618 redundant sequences were removed and 29525 high quality sequences remained.

The chimera was removed by ESV non-cluster denoising [26] and Usearch v10 (balanced pattern) based on the reference sequence utax reference dataset 22.08.2016.fasta and a total of 1087 chimeric sequences were removed. 2497 non-chimera sequences were obtained. The Operational Taxonomic Unit (OTU) table was generated by Vsearch and the finally obtained sequence was clustered according to a certain threshold. The sequence of which the similarity is higher than 97% was defined as an OTU. In the 70 samples, a total of 4489199 reads (3584 OTUs) were obtained. Among these OTUs, 0 OTU appeared in all samples, 28 OTUs appeared in 90% of samples and 162 OTUs appeared in 50% of samples. All samples were equally sampled to 30,000 reads with Usearch V10, resulting in a total of 978664 reads (3584 OTUs). Among them, 0 OTU appeared in all samples, 15 OTUs appeared in 90% of samples and 104 OTUs appeared in 50% of samples.

**Statistical analysis and visualization**

The basic data were statistically analyzed using SPSSv21. Except for the special annotations, the measurement data were expressed as mean ± standard error (Mean ± SD). The data analysis between groups was analyzed by one-way ANOVA. $P \leq 0.05$ was considered statistically significant. The specific different statistical methods were described in the respective sections. Unless special annotations, the data was visualized by the ggplot2.

In the diversity analysis, Usearch v10 was used for alpha and beta diversity analysis. The beta diversity was based on the bray curtis distance. Data differences were evaluated using the adnois test.

In the difference analysis, we used the following methods: 1) Using the STAMP software [27], the two groups of independent samples were compared using the t-test. $P \leq 0.05$ was considered statistically significant. 2) Lefse (Linear Discriminant Analysis Effect Size) visualizes the abundance of the fungi with a difference of more than 2 times in abundance [28]. The method we used was the Kruskal-wallis method and the wilcoxon test. $P \leq 0.05$ was considered statistically significant and the corresponding fungi was included in the lefse analysis. Data visualization was achieved at the website (http://huttenhower.sph.harvard.edu [28]).

Indicator species analysis was performed on the genus and species levels using the indic species package, permutation = 999.

Correlation analysis was performed using the psych package and the stringr package, and the $p$ value was corrected by the false discovery rate. Data visualization was performed using the pheatmap package. $P \leq 0.05$ was considered statistically significant and was labeled in the figure.

All 3584 OTU data were functionally annotated using the software FunGuild [29]. 1719 OTU data received functional annotations.

**Results**

**Clinical characteristics**

We have included three groups of 72 patients, 24 in each group. The basic information is shown. We could see that the ratio of male to female is 17/7 and the age and gender of the three groups of patients were matched. To be mentioned, though 72 patients were recruited,
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Table 1. Clinical characteristics of the patients

<table>
<thead>
<tr>
<th></th>
<th>CHD-NAFLD</th>
<th>CHD</th>
<th>HC</th>
<th>(CHD-NAFLD+CHD) VS HC</th>
<th>CHD-NAFLD VS CHD</th>
<th>CHD-NAFLD VS HC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(N = 24)</td>
<td>(N = 24)</td>
<td>(N = 22)</td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>Male/Female (N)</td>
<td>17/7</td>
<td>17/7</td>
<td>15/7</td>
<td>0.822</td>
<td>1</td>
<td>0.845</td>
</tr>
<tr>
<td>Age (Mean ± SD)</td>
<td>63.54±7.21</td>
<td>63.50±7.70</td>
<td>63.83±7.22</td>
<td>0.622</td>
<td>0.985</td>
<td>0.67</td>
</tr>
<tr>
<td>BMI</td>
<td>27.74±2.72</td>
<td>24.46±5.80</td>
<td>24.84±4.22</td>
<td>0.229</td>
<td>0.001***</td>
<td>0.014*</td>
</tr>
<tr>
<td>HBP (N)</td>
<td>18</td>
<td>17</td>
<td>11</td>
<td>0.026*</td>
<td>0.745</td>
<td>0.04*</td>
</tr>
<tr>
<td>DM (N)</td>
<td>11</td>
<td>6</td>
<td>9</td>
<td>0.659</td>
<td>0.131</td>
<td>0.736</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>25.04±11.69</td>
<td>20.45±13.28</td>
<td>17.96±10.03</td>
<td>0.128</td>
<td>0.211</td>
<td>0.038*</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>25.54±12.97</td>
<td>20.45±12.73</td>
<td>20.80±9.92</td>
<td>0.322</td>
<td>0.456</td>
<td>0.201</td>
</tr>
<tr>
<td>UA (umol/L)</td>
<td>405.21±103.08</td>
<td>371.33±112.13</td>
<td>328.04±76.40</td>
<td>0.032*</td>
<td>0.282</td>
<td>0.01**</td>
</tr>
<tr>
<td>BUN (mmol/L)</td>
<td>5.50±1.56</td>
<td>5.89±2.00</td>
<td>5.27±1.06</td>
<td>0.427</td>
<td>0.462</td>
<td>0.732</td>
</tr>
<tr>
<td>HDLC (mmol/L)</td>
<td>1.04±0.34</td>
<td>1.03±0.24</td>
<td>1.08±0.26</td>
<td>0.441</td>
<td>0.879</td>
<td>0.581</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>2.45±0.67</td>
<td>2.37±0.72</td>
<td>2.55±0.87</td>
<td>0.402</td>
<td>0.694</td>
<td>0.593</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.88±1.69</td>
<td>1.40±0.79</td>
<td>1.16±0.54</td>
<td>0.094</td>
<td>0.214</td>
<td>0.056</td>
</tr>
<tr>
<td>Cre (umol/L)</td>
<td>72.58±19.11</td>
<td>82.79±31.71</td>
<td>70.38±16.58</td>
<td>0.182</td>
<td>0.183</td>
<td>0.555</td>
</tr>
<tr>
<td>EF (%)</td>
<td>64.58±7.11</td>
<td>66.35±6.61</td>
<td>67.87±5.05</td>
<td>0.27</td>
<td>0.396</td>
<td>0.169</td>
</tr>
<tr>
<td>NCA (N)</td>
<td>1.78±0.85</td>
<td>1.63±1.10</td>
<td>1.63±1.10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMI (N)</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Statin (N)</td>
<td>24</td>
<td>24</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BMI, Body mass index; HBP, High blood pressure; DM, diabetes mellitus; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; GGT, Glutamyl Transpeptidase; ALP, Alkaline phosphatase; UA, uric acid; BUN, Blood ureanitrogen, HDL-C, High-density lipoprotein cholesterol; LDL-C, Low-density lipoprotein cholesterol; TG, Triglyceride; Cre, creatinine; EF, ejection fractions; NCA, Narrowed coronary artery; HMI, History of myocardial infarction. *P≤0.05; **P≤0.01; ***P≤0.001.

The microbiota information of two people in the 24 HCs was missed. So in the analysis of microbiota, 70 samples were used. The basic information is shown in (Table 1).

The levels of uric acid and triglyceride in CHD patients were higher than those in HCs. These clinical indexes in CHD-NAFLD patients were further increased and the uric acid in CHD-NAFLD patients was significantly higher than that in HCs (P<0.05). The BMI of CHD patients was not significantly different from that of the HCs, but the BMI of CHD-NAFLD patients was significantly higher than that of the HCs (P<0.05). These results indicated that the changes of BMI, uric acid and triglyceride in CHD-NAFLD patients are higher than those in CHD patients.

In terms of cardiac function, the echocardiographic ejection fraction of CHD-NAFLD patients was lower than that of CHD patients. The number of narrowed coronary artery was higher than that of CHD patients. The narrowed coronary artery was defined as the coronary artery with more than 70% stenosis including left main coronary artery, left anterior descending artery, left circumflex artery and right coronary artery.

Diversity of the fecal fungal microbiota

We used Shannon index and chao1 index to assess the α-diversity of the fungal microbiota. Principal coordinate analysis (PCoA) was used for the β-diversity of the fungal microbiota.

The difference of Shannon index and chao1 index between the overall CHD patients and the HCs was analyzed and was not statistically different (Figure 1A and 1B). The PCoA analysis showed that there was no statistically significant difference in the composition pattern of the fungal microbiota between the overall CHD patients and the HCs (Figure 1C).

The difference of Shannon index and chao1 index between CHD patients, CHD-NAFLD patients and the HCs was also not statistically different (Figure 1D and 1E). For the CHD-NAFLD patients, the PCoA analysis showed no significant difference with either CHD patients or HCs (Figure 1F). These results didn’t show a distinctive fungal composition in different groups.
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The diversity of the fecal fungal microbiota. A. The Shannon index in the overall CHD patients. B. The Chao1 index in the overall CHD patients. C. The PCoA analysis of the overall CHD patients. D. The Shannon index in CHD-NAFLD patients. E. The Chao1 index in CHD-NAFLD patients. F. The PCoA analysis of CHD-NAFLD patients. The “CHD+CHD-NAFLD” stood for the overall CHD patients. ns, not significant. 70 samples were used in each analysis. Kruskal-Wallis H test was used in the comparsion of Shannon index and Chao1 index. In the comparsion of PCoA analysis, adonis test was used.

Figure 1. The diversity of the fecal fungal microbiota. A. The Shannon index in the overall CHD patients. B. The Chao1 index in the overall CHD patients. C. The PCoA analysis of the overall CHD patients. D. The Shannon index in CHD-NAFLD patients. E. The Chao1 index in CHD-NAFLD patients. F. The PCoA analysis of CHD-NAFLD patients. The “CHD+CHD-NAFLD” stood for the overall CHD patients. ns, not significant. 70 samples were used in each analysis. Kruskal-Wallis H test was used in the comparsion of Shannon index and Chao1 index. In the comparsion of PCoA analysis, adonis test was used.

The microbiota at phylum and genus level

Among all the identified OTUs, the Ascomycota and Basidiomycota were the two most abundant phylum (Figure 2A). For the CHD patients and CHD-NAFLD patients, the Ascomycota and Basidiomycota were also the dominant phylum (Figure 2C).

At the genus level, the composition of fungal microbiota of the overall CHD patients and HCs was analyzed (Figure 2B). Phyllactinia, Alternaria and Candida were the main genus of the fungal microbiota in the overall CHD patients and HCs.

For the CHD patients and CHD-NAFLD patients, Phyllactinia, Alternaria and Candida were also the main genus of the fungal microbiota (Figure 2D).

The characteristic of fungal microbiota of the overall CHD patients

At the genus level, indicating species was used to find the characteristic of the fungal microbiota. We found that the abundance of Thermosascus in the overall CHD patients was lower than in HCs, which was the characteristic fungi of the overall CHD patients (Figure 3A).

At the species level, indicating species found that the abundance of Exophiala attenuata and Malassezia restricta in the overall CHD patients was lower than that in HCs, which was the characteristic of the fungal microbiota of the overall CHD patients (Figure 3B).

The characteristic of fungal microbiota of the CHD patients

Compared with the HCs, the abundance of Microascus (P = 0.028), Microascus brevicaulis (P = 0.028) was significantly increased in the CHD patients (Figure 4).

The characteristic microbiota was analyzed using indicating species. We found that the indicating species in CHD patients was Chaetomium (P = 0.004) and Cryptococcus arboriformis (P = 0.038) (Figure 3B). Among the three
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Figure 2. The composition of the fungi at the phylum and genus level. A. The fungi at the phylum level in the overall CHD patients. B. The fungi at the genus level in the overall CHD patients. The top 13 genus in abundance was listed. C. The fungi at the phylum level in the CHD-NAFLD and CHD patients. D. The fungi at the genus level in the CHD-NAFLD and CHD patients. The top 13 genus in abundance was listed. 70 samples were used in each analysis.

The characteristic of fungal microbiota of the CHD-NAFLD patients

Compared with HCs, CHD-NAFLD patients had significantly higher abundance of *Preussia* (*P* = 0.031) (Figure 5). Compared with CHD patients, the abundance of *Coprinopsis* and *Phyllactinia* was significantly higher and the abundance of *Ganoderma* was relatively lower in CHD-NAFLD patients using the Lefse [28].

The indicating species analysis found that the indicating species of CHD-NAFLD patients was *Candida glabrata* (*P* = 0.025) (Figure 3B). Compared with the other two groups, the abundance of *Candida glabrata* was the lowest in CHD-NAFLD patients.

At present, there are few reports on fungi such as *Preussia* and *Candida glabrata*. Previous studies reported that *Ganoderma* had protective effects on atherosclerosis and NAFLD [19, 20]. It was suggested that the reduction of abundance of *Ganoderma* in CHD-NAFLD patients might be an important factor affecting the degree of metabolic disorder.
Figure 3. The specific microbiota at the genus and species level. A. The specific fungal microbiota at the genus level. B. The specific fungal microbiota at the species level. The R3.5.1 with indicpecies package was used. Permutation test was performed. The shape of the graph represents the comparison in enrichment (circle) or depletion (triangle) between three groups. The size of the graph indicates the relative abundance. *P≤0.05; **P≤0.01; ***P≤0.001. 70 samples were used in analysis.
Figure 4. The comparison of fungal microbiota in CHD patients. A. The comparison of fungal microbiota between CHD patients and HCs at genus level. B. The comparison of fungal microbiota between CHD patients and HCs at species level. The t test and STAMP was used. 70 samples were used in each analysis.
Figure 5. The comparison of fungal microbiota in CHD-NAFLD patients. A. The comparison of fungal microbiota between CHD-NAFLD patients and HCs at genus level. B. The comparison of fungal microbiota between CHD-NAFLD patients and HCs at species level. C. The comparison of fungal microbiota between CHD-NAFLD patients and CHD patients using the Lefse. The t-test and STAMP was used. 70 samples were used in analysis.
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A

B

Candida glabrata
Debaryomyces renali
Wallenia sebi
Sporobolomyces symmetricus
Malassezia sympodialis
Stereum hirsutum
Cladosporium halotolerans
Wallenia mellicola
Rhodotorula mucilaginosa
Hypsizygus marmoreus
Paathyrella candideeana
Erythrobasidium hasegawianum
Tilletia holci
Candida takata
Cyberlindnera fabiani
Alternaria eichhorniae
Xylophoma rimosissimus
Holtermanniella takashimae
Schizophyllum commune
Arthrinium phaeospermum
Mortierella polygonia
Zygogomycetes paraballii
Agaricus subrufescens
Lentinula edodes
Kernia pachyploea
Resiciniuc bicolor
Preussia pilosella
Colletotrichum biltii
Microstrotia phylloplanum
Microstrotia phylloplanum
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**Correlation analysis between clinical indexes and fungal microbiota at genus and species levels**

The fungal microbiota of all samples was included and Spearman’s correlation analysis was performed between fungal abundance and clinical indexes (**Figure 6**).

The ejection fraction of the overall CHD patients was lower than that of HCs. Correlation analysis found that the ejection fraction also negatively correlated with the abundance of Xylocladon \((P<0.05)\). The abundance of Xylocladon in CHD-NAFLD patients was higher than that of CHD patients and HCs (the abundance of Xylocladon in CHD-NAFLD, CHD, HCs: 0.0904, 0.0438, 0.0312).

Among the participants, UA increased in CHD-NAFLD patients. Correlation analysis showed that UA positively correlated with the abundance of Preussia, Cladorrhinum, Zygosaccharomyces parabailii and Alternaria eichhorniae. Compared with CHD patients and HCs, the abundance of Preussia and Cladorrhinum was the highest in CHD-NAFLD patients (the abundance of Preussia in CHD-NAFLD, CHD, HCs: 0.5233, 0.3372, 0.2468; the abundance of Cladorrhinum in CHD-NAFLD, CHD, HCs: 0.1598, 0.0796, 0.0807).

**Function annotation of fungal microbiota**

A total of 3,584 OTU data were annotated with the software FunGuild [29] and 1719 were functionally annotated. According to the nutrition method, the fungus can be divided into pathotroph, symbiotroph and saprotroph. And it was further subdivided into 12 categories including animal pathogens, arbuscular mycorrhizal fungi, ectomycorrhizal fungi, ericoid mycorrhizal fungi, foliar endophytes, lichenicolous fungi, lichenized fungi, mycoparasites, plant pathogens, undefined root endophytes, undefined saprotrophs and wood saprotrophs [29].

We compared the OTU levels under each function annotation. The animal pathogens increased in CHD patients compared with HCs and it was further increased in CHD-NAFLD patients, which was the characteristic of the disorder in fungal microbiota (**Figure 7**).

**Discussion**

The change of intestinal microbiota was an important factor in the occurrence and progression of CHD and NAFLD. At present, most research focused on bacterial microbiota [17]. There was no research on the characteristics of fungal microbiota in CHD patients and CHD-NAFLD patients. Therefore, this study analyzed the characteristics of the microbiota of CHD-NAFLD patients from the perspective of fungal microbiota.

In this study, there was no significant difference in the \(\alpha\) and \(\beta\) diversity of the microbiota between the overall CHD patients and HCs. At present, the diversity of the bacterial microbiota in the overall CHD patients and HCs was still controversial in previous studies and the differences in diversity reported by different studies were not consistent [30, 31]. About the diversity of fungal microbiota, there was no previous reports.

There was no significant difference in the \(\alpha\) and \(\beta\) diversity of the CHD-NAFLD patients compared with CHD patients and HCs, which suggested that the richness and diversity of the fungal microbiota had no significant difference between CHD patients, CHD-NAFLD patients and HCs. The diversity of gut microbiota represents the homeostasis of gut microbes and have been reported to be correlated with human health [32, 33], but it was not positively correlated with the human health in all cases [34-37].

We firstly compared the overall CHD patients with HCs. In the overall CHD patients, we found that the abundance of Thermoascus and Malassezia restricta decreased compared with HCs, which was the characteristic for the overall CHD patients. There are few reports on Thermoascus. Some studies mentioned it was the pathogens of dialysis-related peritonitis [38] and its relationship with the human remained to be further studied.
Figure 7. Function annotation of fungal microbiota. The comparison of OTUs of function annotation in fungal microbiota between CHD patients, CHD-NAFLD patients and HCs. The software FunGuild was used. 70 samples were used in analysis.
The natural habitat of Malassezia was the skin of humans and other warm-blooded animals. It was classified into at least 14 species, 8 of which are isolated from human skin, including *Malassezia restricta*. *Malassezia* produced a variety of enzymes including lipases and phospholipases, which triggered an inflammatory response by releasing unsaturated free fatty acids in sebum and caused seborrheic dermatitis [39, 40]. Whether *Malassezia* was clustered in seborrheic dermatitis remained controversial and the influence of *Malassezia* on lipid metabolism in human remained to be further studied [41]. Therefore, it was speculated that *Malassezia restricta* appeared in the intestinal microbiota and its abundance in the overall CHD patients reduced, which might be related to lipid metabolism disorder of the overall CHD patients.

The intestinal fungal microbiota in CHD-NAFLD patients showed an increase in the abundance of *Preussia*, *Xylodon* and *Cladorrhinum* and a reduction in the abundance of *Candida glabra* and *Ganoderma*. Previous studies reported that α-glucosidase inhibitors can be extracted from the products of *Preussia minimoides* [42], suggesting that the species in *Preussia* might involve in sugar metabolism and the specific mechanism remained to be confirmed. It was reported that *Candida glabrata* was present in plaques in patients with atherosclerosis [43]. However, it was not clear whether the presence of the fungi in the coronary atherosclerotic plaque was related to the severity of coronary artery disease. Some studies concluded that there was no correlation in it [44].

*Ganoderma* was reported to have a protective effect on atherosclerosis in a mouse model [45]. Some species of *Ganoderma* could improve the area of atherosclerotic plaque and was possibly through the regulation of macrophages and release of nitric oxide to protect atherosclerosis [20, 46, 47]. Some products of the *Ganoderma* also had a certain therapeutic effect on NAFLD mice [19] and hypoglycemic and hypolipidemic effects in diabetic mice [48]. The abundance of *Ganoderma* significantly reduced in CHD-NAFLD patients compared with CHD patients, which might aggravate the degree of metabolic disorder and the progression of atherosclerosis.

Correlation analysis showed that uric acid positively correlated with the abundance of *Cladorrhinum* and *Preussia*. The CHD-NAFLD showed an increase in both the UA and the abundance of *Cladorrhinum* and *Preussia*. It suggested that the abundance of *Preussia* and *Cladorrhinum* in CHD-NAFLD patients might be related to the disorder of purine metabolism and uric acid. Serum uric acid levels are closely related to cardiovascular risk factors such as hypertension and metabolic syndrome [49]. However, there are few reports on *Preussia*. *Cladorrhinum* was reported to be one of the pathogens of fungal keratitis [50]. The relationship between these two fungi and purine metabolism still needed further research.

In terms of cardiac function, the ejection fraction in CHD-NAFLD patients was the lowest compared with the CHD patients and HCs. Correlation analysis showed a negative correlation between ejection fraction and the abundance of *Xylodon*. The abundance of *Xylodon* increased in CHD-NAFLD patients. It suggested that the changes in abundance of *Xylodon* might be related to cardiac function. But the function of *Xylodon* in human was not reported yet and further research was needed.

Notably, this study has some limitations. Firstly, it is a correlation study and had no animal research on the function of the key differential fungi. And there are few previous reports about the metabolism function of the differential fungi. Thus, more studies are needed to confirm the function of the fungi. Secondly, considering that our study was a single center study, more multi-center study was needed to confirm the results. Thirdly, the study has a small sample size.

The changes of intestinal fungal microbiota in CHD-NAFLD patients may be important factors affecting the degree of metabolic disorder. But there are few reports on these fungi. More studies are needed to confirm the effects of these fungi on human.

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Disclosure of conflict of interest
None.

Abbreviations
ALP, Alkaline phosphatase; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; BMI, Body mass index; BUN, Blood urea nitrogen; CHD, coronary atherosclerotic heart disease; CHD-NAFLD, CHD patients complicated with NAFLD; GGT, Glutamyl transpeptidase; HCs, healthy controls; HDL-C, High-density lipoprotein cholesterol; LDL-C, Low-density lipoprotein cholesterol; NAFLD, nonalcoholic fatty liver disease; OTUs, Operational Taxonomic Units; PCoA, Principal coordinate analysis; PCR, Polymerase Chain Reaction; TG, Triglyceride; UA, Uric acid.

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