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Research paper

Structural and functional characteristics of the fecal-associated microbiome in dampness-heat constitution

Jianhua Zhen[a,1], Pengfei Zhao[a,1], Yini Li[a], Lu Zhao[a], Guangrui Huang[a,*], Anlong Xu[a,b,*]

[a] School of Life Sciences, Beijing University of Chinese Medicine, 100029 Beijing, China
[b] State Key Laboratory of Bio-control, Department of Biochemistry, School of Life Sciences, Sun Yat-Sen University, 510006 Guangzhou, China

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ABSTRACT

Introduction: Susceptibility to lipid metabolic dysfunction are more frequent in individuals with dampness-heat (DH) constitution, which might be caused by the changes in the gut microbiome. This study is to explore the structural and functional characteristics of the fecal-associated microbiome (FAM) in DH constitution and screen FAM-related biomarkers of DH constitution.

Methods: Fecal samples were collected. The FAM structure was described with alpha/beta diversity indexes and relative abundances of dominant taxa; the distribution/functional differences of the FAM between DH and balanced constitutions were analyzed by Wilcoxon rank-sum test, MetagenomeSeq and LEfSe analysis, and the specific OTUs were screened to construct ROC curve.

Results: There was a significant difference between the groups in the sweet preference, ACE and Chao1 indexes. In PCoA and PLS-DA, the bacterial communities in the balanced samples and DH samples clustered separately. Notably, there were 115 differentially distributed OTUs between groups identified in the MetagenomeSeq analysis and 213 OTUs identified with the Wilcoxon rank-sum test. Predicted functions showed that 12 metabolic pathways were differentially distributed between groups, including the pathway related to glycerolipid metabolism. The AUC of ROC curve based on the 4 screened specific OTUs was 0.91, and the relative abundance of these 4 OTUs could result in changes in lipid metabolism.

Conclusion: Unique FAM structural characteristics were identified in DH constitution, which might involve in changes in lipid metabolism and susceptibility to lipid metabolic dysfunction. The 4 screened specific OTUs could be used as potential biomarkers of DH constitution to assist clinical diagnosis.

1. Background

Molecular assessment of individual differences between healthy subjects and associated disease risks are of substantial interest in clinical medicine, as they are the basis of personalized medicine and play important roles in guiding the prevention and treatment of related diseases. An increasing number of researchers have been trying to explore the mechanism of individual susceptibility differences from the perspective of genes, proteins, metabolites and so on, but the resulting observations are not applicable in the clinic because of the complexity and expense. For example, the susceptibility to coronary atherosclerotic heart disease has been confirmed to be associated with the polymorphisms of multiple genes, such as rs266729, rs822395, rs1501299, rs2241766 and rs6458155 [1–3]. The detection of these genotypes

Abbreviations: FAM, fecal-associated microbiome; DH, dampness-heat; TCM, Traditional Chinese Medicine; BA, balanced; PBC, primary biliary cirrhosis; HLA, human leukocyte antigen; TC, total cholesterol; TG, triglyceride; *ChREBP*, carbohydrate-responsive element-binding protein; *SREBP-1*, sterol regulatory element-binding protein-1; *Fiaf*, fasting-induced adipocyte factor; OTU, operational taxonomic unit; FLASH, Fast Length Adjustment of Short Reads; QIIME, Quantitative Insights Into Microbial Ecology; PCoA, Principal coordinate analysis; PLS-DA, partial least squares discriminant analysis; VIF, variance inflation factor; db-RDA, distance-based redundancy analysis; PICRUSt, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; KEGG, Kyoto Encyclopedia of Genes and Genomes; ROC, receiver operating characteristic; LDL, low-density lipoprotein; BMI, body mass index; Sobs, the observed richness; ApoA1, apolipoprotein A1; HBDH, hydroxybutyrate dehydrogenase; CK, creatine kinase; AST, aspartate aminotransferase; P, phosphorus; GLU, glucose; ALP, alkaline phosphatase; BUN, blood urea nitrogen; B2 MG, β2-microglobulin; SCFA, short-chain fatty acid; GLP-1, glucagon-like peptide-1; PYY, peptide YY; GPR, G-protein coupled receptor

* Corresponding author.
E-mail addresses: jianhuazhen@bucm.edu.cn (J. Zhen), zhaopengfei@bucm.edu.cn (P. Zhao), LYN@bucm.edu.cn (Y. Li), zhaol@bucm.edu.cn (L. Zhao), hgr@bucm.edu.cn (G. Huang), xuanlong@bucm.edu.cn (A. Xu).
[1] These authors contributed equally for this study.

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makes the medical expenses so high that many patients cannot afford them, which limits their clinical applications.

Based on traditional Chinese medicine (TCM) theory and long-term clinical observations, healthy individuals have been grouped into distinctive types under the concept of "constitution". Each constitution has its own clinical presentations and biological features as well as certain susceptibilities to some diseases, so the TCM constitution theory is used widely in the clinic, with simplicity and high operability/accuracy. A previous study showed that phlegm-dampness (PD) constitution was a risk factor for hypertension and obesity (*OR = 2.56,* 95 % *CI = 2.42–2.70; OR = 2.107,* 95 % *CI = 1.485-2.991*) [4,5], while primary biliary cirrhosis (PBC) was related to qi-deficient constitution, qi-stagnation constitution and yin-deficient constitution [6]. As a result, it is feasible to judge individual susceptibility differences based on the TCM constitution.

As mentioned above, individual susceptibility differences are based on genetic and metabolic changes, so the related judging criteria, TCM constitutions, should also have unique genetic and metabolic backgrounds. Chen et al. investigated the relationship between TCM constitutions and human leukocyte antigen (HLA) and observed associations of *DR*04* with blood-stasis constitution, *DQ*09* with qi-deficient constitution and PD constitution [7]. Yu et al. discovered that the expression of lipid synthesis-related genes, such as *DGAT2*, *ACSL1* and *ABCA1*, was downregulated in individuals of yang-deficient constitution, and this result was consistent with the reduction in very low-density lipoprotein/low-density lipoprotein (LDL), fatty acids and unsaturated fatty acids in serum samples in individuals of yang-deficient constitution [8].

Dampness-heat (DH) constitution, which is characterized by greasy hair and face, acne, drippy scrotum or yellowish leucorrhea, and yellowish and thick tongue fur in the clinic, is caused by nutritional diet or excessive drinking or humid environments. Among the women of childbearing age in Shanghai, aversion to vegetables and job stress were the high-risk factors for DH constitution [9]; and in another multicentered clinical investigation, tobacco and alcohol use were significantly correlated with DH constitution [10]. Individuals with DH constitution were more likely to suffer from frequent and painful urination, as well as psoriasis and arteriosclerosis obliterans [11–13]. Low sperm quality (sperm concentration and normalized sperm rate) and abnormal sex hormone (higher follicle-stimulating hormone and estradiol) have also been observed in infertile males with DH constitution [14,15]. Notably, compared with that in balanced (BA) constitution individuals, plasma cholesterol and unsaturated lipid contents are significantly higher, while genes related to lipid metabolism (such as *ABCA13, LCN* and *MPO*) are upregulated and the frequency of the $\varepsilon 4/\varepsilon 4$ genotype in *ApoE* increases remarkably in individuals with DH constitution [16,17]. Therefore, susceptibility to lipid metabolism disorders is more frequent in DH constitution, which has been supported by the results of some clinical research. For instance, Zhang et al. found that PD and DH constitutions are the third highest-risk constitutions for hyperlipidemia, and the total cholesterol (TC), triglyceride (TG) and LDL in the serum of individuals with these two constitutions are distinctly higher than those of individuals with other unbalanced constitutions [18].

As an important player in host material metabolism and nutrition transformation, the gut microbiome participates in the hydrolysis or synthesis of nutrients by a variety of enzymes in bacteria (such as the hydrolysis process of resistant starch and L-tryptophan), and it can also regulate the expression of host metabolism-related genes to influence metabolism indirectly, such as regulating the expression of carbohydrate-responsive element-binding protein (*ChREBP*), sterol regulatory element-binding protein-1 (*SREBP-1*) and fasting-induced adipocyte factor (*Fiaf*) to influence fat synthesis and storage [19]. Based on the above-mentioned information, it is hypothesized that DH constitution subjects have certain structural characteristics of the gut microbiome, which could result in changes in lipid metabolism and susceptibility to lipid metabolic dysfunction. To validate this hypothesis, we collected fecal samples in this study from BA constitution and DH constitution individuals to analyze the structures and functions of the fecal-associated microbiome (FAM) and screened specific operational taxonomic units (OTUs) as potential biomarkers of DH constitution to assist clinical diagnosis.

2. Methods

2.1. Study design, subject recruitment and sample collection

This was a cross-sectional study. All subjects were recruited from Beijing University of Chinese Medicine in Beijing, China. The subjects were between 18 and 40 years old and did not use any specific medicine (especially antibiotics) within the 3 months before enrollment. The constitutions were identified according to the "Classification and determination of constitution in TCM" [20] published by the China Association of Chinese Medicine on April 9, 2009 and further confirmed by a traditional Chinese medicine clinician. Additionally, subjects infected by HBV, HCV, HIV or *Treponema pallidum* were excluded, as well as those with chronic disease and abnormal indexes in medical reports. Female subjects who were pregnant, breastfeeding or planning to become pregnant within 6 months were also excluded, as well as subjects participating in other clinical studies. This study was approved by the Ethics Committee of the Beijing University of Chinese Medicine Third Affiliated Hospital (protocol number: KTPJ-BZYSY-2017-01). Written informed consent was obtained from all subjects. All experiments were performed in accordance with the approved guidelines.

According to a well-defined clinical protocol, fecal samples were collected and transported to the laboratory on dry ice within two hours and stored at −80 °C before subsequent processing.

2.2. DNA extraction, Illumina sequencing and bioinformatics analysis of 16S rDNA amplicons

Total genomic DNA was individually extracted from fecal samples using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quantity and quality of the isolated DNA were measured by agarose gel electrophoresis. DNA samples were frozen at −20 °C for further analysis. The diluted DNA was used as a template for PCR amplification of bacterial 16S rDNA with barcoded primers and Takara Ex Taq (Takara). For bacterial diversity analysis, the V3-V4 variable regions of the 16S rDNA were amplified with the universal primers 338 F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTC TAAT-3′). Amplicon quality was visualized using gel electrophoresis, and the DNA was purified with AMPure XP beads (Agencourt) and amplified for another round of PCR. After another round of purification with AMPure XP beads, the final amplicons were quantified using a Qubit dsDNA assay kit. Equal amounts of purified amplicons were pooled for subsequent sequencing. Pairs of reads from the original DNA fragments were merged using Fast Length Adjustment of Short Reads (FLASH) software (FLASH v1.2.11) [21], and sequences were analyzed using Quantitative Insights Into Microbial Ecology (QIIME) software (v1.70) [22]. Sequences were assigned to OTUs at 97 % similarity; a representative sequence was selected for each OTU, and the RDP classifier was employed to assign taxonomic data to each representative sequence [23].

Representative sequences were assigned at different taxonomic levels (from phylum to species) with the SILVA 128/16S rDNA database for bacteria following the Bayesian approach with a 97 % cutoff value. Bacterial diversity was determined by performing a sampling-based OTU analysis and was displayed as a rarefaction curve [23]. Bacterial richness and diversity across samples were assessed using the following alpha indexes: the observed richness (Sobs), ACE, Chao1, Shannon and Shannoneven. The Wilcoxon rank-sum test was used to compare bacterial diversity. Principal coordinate analysis (PCoA) and analysis of

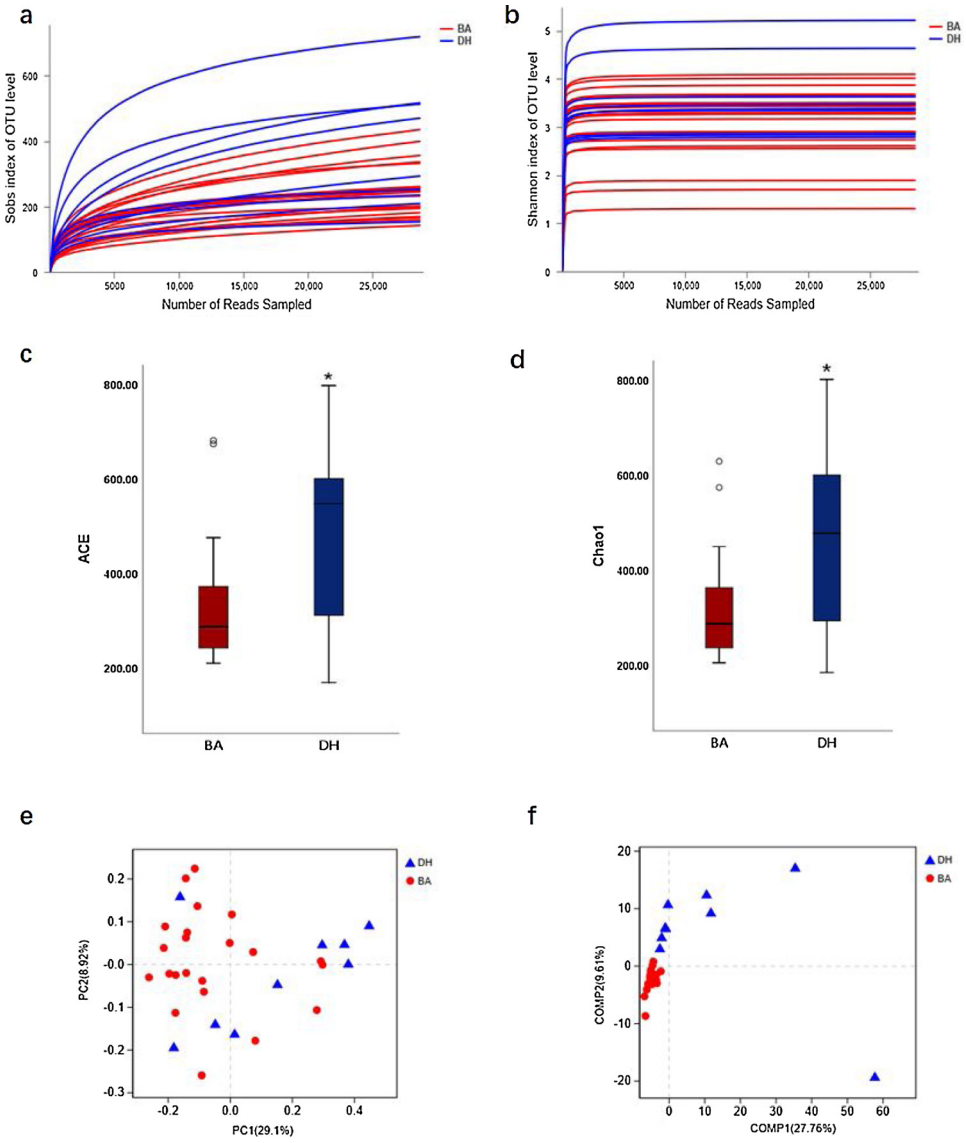

Fig. 1. Comparison of FAM structures in the BA and DH samples. (a) Rarefaction analysis of bacterial 16S rDNA sequences was performed to evaluate whether further sequencing would likely detect additional taxa, and plateaus of each curve indicated that no further sequencing was needed. Different colors represent different groups. (b) Shannon index curves were constructed to evaluate the diversity of samples, and the plateau indicated that the majority of alpha diversity had been covered. Different colors represent different groups. Box plots depict differences in bacterial diversity between BA and DH constitution according to (c) the ACE index and (d) the Chao1 index. *, $P < 0.05$. (e) PCoA at the OTU level with $P = 0.015$ in Adonis analysis. (f) Partial least square discriminant score plot of the FAM between the BA and DH samples at the OTU level. BA, balanced constitution; DH, dampness-heat constitution.

similarities (ANOSIM, Adonis) using unweighted UniFrac distance metrics were carried out, and an R package was used to visualize the interactions among bacterial communities in different samples. In addition, partial least squares discriminant analysis (PLS-DA) was also performed to compare bacterial composition between samples [24]. MetagenomeSeq, Wilcoxon rank-sum test and LEfSe were employed to identify distinguishing taxa between the two groups at multiple levels, and the LEfSe results were visualized using taxonomic bar charts and cladograms [25]. Relationships between the top 20 genera and the clinical characteristics selected in variance inflation factor (VIF) analysis were explored by calculating distance-based redundancy analysis (db-RDA) and Spearman's correlation coefficients based on Bray-Curtis distance. The results were clustered and visualized using an R package. Functional compositions of the bacterial communities were predicted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) dataset [26]. A receiver operating characteristic (ROC) curve was constructed to determine the diagnostic values of OTUs as potential biomarkers of DH constitution. In addition to Spearman's correlation between the biomarkers and serum lipids, analysis of multivariate association with linear models based on these OTUs and LDL was performed using the free online platform of Majorbio Cloud Platform (www.majorbio.com).

2.3. Statistical analysis

The statistical calculations were carried out with a test using the SPSS software package (SPSS v25.0, SPSS Inc., Chicago, IL, USA). The normality of the distribution of variables was tested by the Shapiro-Wilk test. Spearman's correlation was used to evaluate the associations between the bacterial community based on Bray-Curtis distance and the clinical characteristics. The independent samples T-test procedure was used to analyze the variables found to have a normal distribution. The variables found to have a nonnormal distribution were analyzed using the Mann-Whitney *U* test [27]. The chi-square test was used to analyze the count data. Significance was declared at $P < 0.05$.

3. Results

3.1. Characteristics of the subjects included in the study

Our study population was composed of 22 subjects with BA constitution (herein referred to as BA) as the control and 9 subjects with DH constitution (herein referred to as DH) (see Supplementary Table S1). There was no significant difference between the BA and DH groups in the distribution of gender, age, height, weight, body mass index (BMI), occupation, type of delivery and feeding, taste and diet

preference, sleep and exercise habits, health stage, and all blood tests and urine tests, except for sweet preference ($P = 0.038$, Supplementary Table S1). The results showed that the subjects with DH constitution liked sweets more than those with BA constitution.

3.2. Overall structure of FAM communities

In the present study, 31 fecal samples were sequenced using an Illumina MiSeq platform, and a total of 1,668,568 raw sequences were generated. After quality trimming and chimera checking, 886,135 high-quality sequences with an average length of 435 bp and an average of 28,585 reads per sample were recovered for downstream analysis. After alignment in the SILVA 128/16S rDNA database for bacteria, unique representative sequences were classified into 1,010 OTUs at a 97 % similarity level, from which 24 phyla, 40 classes, 76 orders, 129 families, 328 genera and 634 species were detected. Different indexes (Sobs, ACE, Chao1, Shannon and Shannoneven) were employed to analyze the alpha diversity of the bacterial community (Supplementary Table S2). The rarefaction curves of the Sobs and Shannon diversity indexes for each sample reached plateaus, indicating that the majority of the diversity was detected (Fig. 1a and b). As revealed by the ACE and Chao1 indexes (Fig. 1c and d and Supplementary Table S2), the diversity of the bacterial community in DH samples was significantly increased compared with that of the bacterial community in the BA samples ($P = 0.043$ and 0.047).

To evaluate the extent of the similarity of the bacterial communities, PCoA based on unweighted UniFrac distance at the OTU level was employed. As shown in Fig. 1e, the samples of the different groups were clearly separated (Fig. 1e, ANOSIM R = 0.2229, $P = 0.01$; Adonis $R^2 = 0.074$, $P = 0.017$). Then, PLS-DA, a supervised analysis suitable for high-dimensional data, was also performed (Fig. 1f), and the bacterial communities in the BA samples and DH samples obviously clustered separately. These two results suggested that the overall structures of the bacterial communities in the two groups were significantly different.

3.3. Common and distinct bacterial taxa in the analyzed groups

The bacterial communities in the BA samples and the DH samples were analyzed at different taxonomic levels (Fig. 2). *Bacteroidaceae, Lachnospiraceae, Ruminococcaceae, Prevotellaceae* and *Veillonellaceae*, the top 5 most abundant families, together comprised 81.82 % of all the sequences (Fig. 2a). *Bacteroidaceae* was the most abundant family, accounting for 26.42 % of the sequences. In contrast, the abundances of the other detected families, including *Streptococcaceae* and *Peptostreptococcaceae*, were less than 1 %. At the genus level, *Bacteroides, Prevotella 9, Faecalibacterium, [Eubacterium] rectale group* and *Dialister* were the 5 most abundant genera, comprising 26.42 %, 15.39 %, 7.23 %, 4.78 % and 3.36 % of the sequences, respectively (Fig. 2b). At the genus level, an average of approximately 126 genera were detected per sample. Consistent with the significant interindividual variation, only 26 genera were found across all samples, while 28 genera were found only in the BA samples and 50 genera were found only in the DH samples (Fig. 2c and d). At the OTU level, only 22 OTUs were found across all samples (Fig. 2e), which was similar to the results at the genus level.

The bacterial composition of the DH samples was significantly different from that of the BA samples. Notably, there were 115 differentially distributed OTUs between the DH and BA groups in the MetagenomeSeq analysis and 213 OTUs via the Wilcoxon rank-sum test (Supplementary Tables S3 and S4). To identify the distinguishing taxa within the groups, the LEfSe method was implemented (Fig. 3a). At the family level, *Acidaminococcaceae* and *Spirochaetaceae* were significantly enriched in the DH samples, while *Alcaligenaceae* was significantly enriched in the BA samples; at the genus level, *Phascolarctobacterium*, *Megamonas* and *Treponema 2* exhibited significantly higher abundances in the DH samples than in the BA samples (Fig. 3b).

3.4. FAM species associated with the clinical indexes in the analyzed groups

Additionally, the associations between the bacterial community based on Bray-Curtis distance and the clinical characteristics (except for the indexes in the blood and urine tests) in the BA and DH samples were analyzed with db-RDA (Fig. 4a). The results showed that sex, type of feeding, greasy diet and sweets preference were the most influential factors on the bacterial community (Supplementary Table S5). Although height and weight showed significant impacts on the bacterial community, BMI had no relevant influence. Then, the association between the top 20 genera and the clinical characteristics in the BA and DH subjects was analyzed with Spearman's correlation (Fig. 4b and c). In Fig. 4b, there was no significant relationship between the top 20 genera and sex or type of feeding, while greasy diet was negatively related to *Bacteroidales S24-7 group* and positively related to *Lachnoclostridium* ($R = -0.403$, $P < 0.05$ and $R = 0.378$, $P < 0.05$), which was the most strongly positive, and sweets preference was negatively related to *Alistipes* and *Dialister* ($R = -0.467$, $P < 0.01$ and $R = -0.402$, $P < 0.05$), the former of which was the most strongly negative. Clinical indexes in blood/urine in Fig. 4c were selected by VIF analysis with low internal correlation (Supplementary Table S6), and *Bacteroidales S24-7 group* was positively related to LDL and phosphorus (P) ($R = 0.414$, $P < 0.05$ and $R = 0.451$, $P < 0.05$); *Dialister* was positively related to apolipoprotein A1 (ApoA1) ($R = 0.392$, $P < 0.05$); and *Alistipes* was positively related to hydroxybutyrate dehydrogenase (HBDH), creatine kinase (CK) and aspartate aminotransferase (AST) ($R = 0.402$, $P < 0.05$, $R = 0.361$, $P < 0.05$ and $R = 0.459$, $P < 0.01$), with the last correlation the strongest.

As shown in Fig. 4b, there were also negative relationships found between BMI and *Bifidobacterium* ($R = -0.441$, $P < 0.05$), type of delivery and *Lactobacillus/ Bacteroidales S24-7 group* ($R = -0.424$, $P < 0.05$ and $R = -0.388$, $P < 0.05$), light diet and *Ruminococcaceae UCG-002* ($R = -0.449$, $P < 0.05$), and tea consumption and *Subdoligranulum* ($R = -0.426$, $P < 0.05$). Only *Ruminococcaceae UCG-002* was found to be significantly correlated with ApoA1, P, AST and glucose (GLU) in blood ($R = 0.462$, $P < 0.01$; $R = 0.376$, $P < 0.05$; $R = 0.398$, $P < 0.05$; and $R = -0.360$, $P < 0.05$). The strongest negative correlation shown in Fig. 4c was found between *Escherichia-Shigella* and alkaline phosphatase (ALP) ($R = -0.532$, $P < 0.05$), and *Bacteroides* was positively related to blood urea nitrogen (BUN) and β2-microglobulin (B2 MG) ($R = 0.364$, $P < 0.05$ and $R = 0.364$, $P < 0.05$), which represents renal function.

3.5. Functional predictions

The PICRUSt algorithm was employed to predict bacterial function in the two groups. The Wilcoxon rank-sum test outputs showed a series of metabolic pathways presenting significantly different distributions in each group (Fig. 5 and Supplementary Table S7). Pathways related to benzoate degradation, dioxin degradation, C5-branched dibasic acid metabolism, xylene degradation, glycerolipid metabolism, valine, leucine and isoleucine biosynthesis, and chloroalkane and chloroalkene degradation were remarkably enriched in DH constitution samples, while pathways related to polyketide sugar unit biosynthesis, biosynthesis of vancomycin group antibiotics, pore ion channels, membrane and intracellular structural molecules, and chaperones and folding catalysts were enriched in BA constitution samples.

3.6. Potential biomarkers of DH constitution in the FAM

As mentioned above, LEfSe analysis clearly displayed the differences between the BA and DH samples. This analysis identified a genus-based signature that correctly distinguished the DH constitution from the BA constitution. In another analysis, the Wilcoxon rank-sum test, 4 OTUs

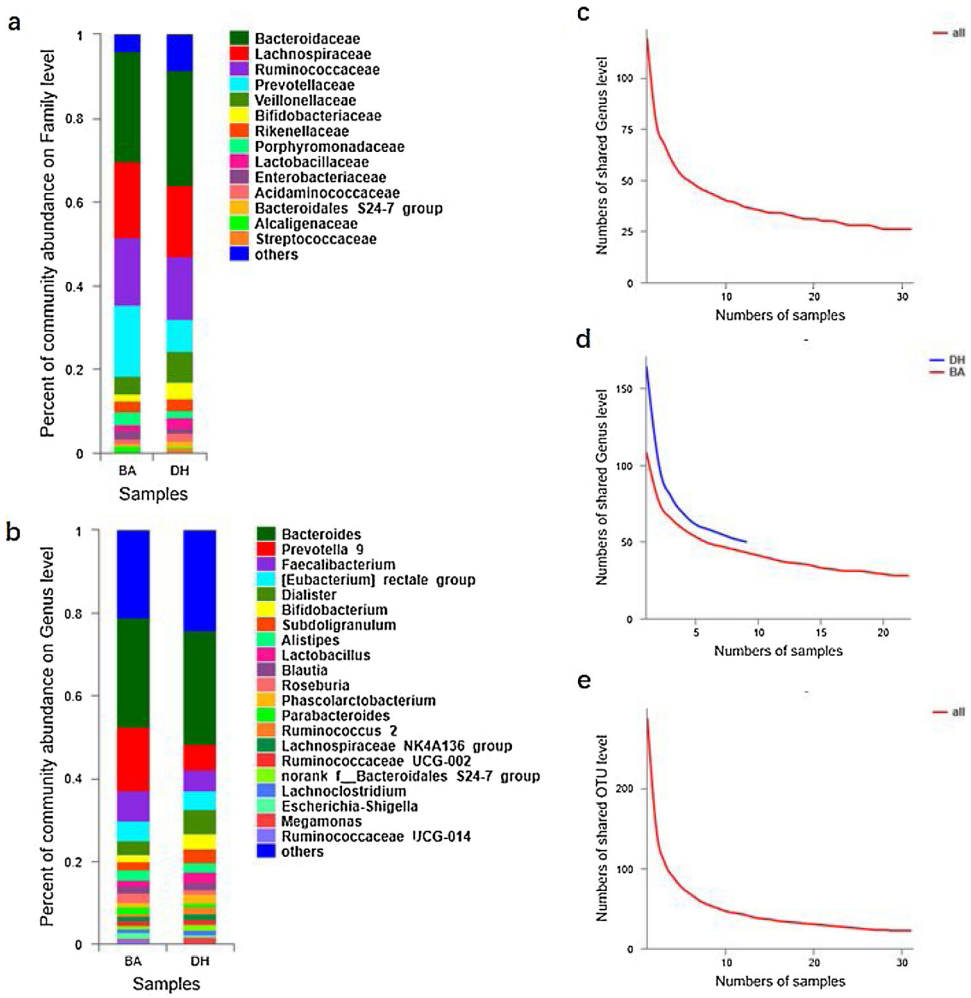

Fig. 2. Composition of bacterial communities across samples at the family and genus levels, and core taxa analysis at the genus and OTU levels. (a) Relative abundance of bacterial families among the BA and DH groups. (b) Relative abundance of bacterial genera among the BA and DH groups. (c) Core genera analysis based on all samples. (d) Core genera analysis based on the separate BA and DH samples. (e) Core OTU analysis based on all samples. BA, balanced constitution; DH, dampness-heat constitution.

were enriched in DH samples with $P < 0.001$, which were also observed in the MetagenomeSeq analysis (Fig. 6a). Then, a ROC curve was constructed based on these 4 OTUs between the DH and BA samples, and the area under the curve (AUC) was 0.91 (Fig. 6b). Therefore, we hypothesized that these 4 OTUs could be potential biomarkers of DH constitution in the FAM. According to the taxonomy, these biomarkers included a member of the genus *Ruminiclostridium 9*, a member of the family *Lachnospiraceae*, a member of the family *Peptococcaceae* and a member of the class *Cyanobacteria*, three of which were in *Firmicutes* and one in *Cyanobacteria* (Supplementary Table S8).

In addition, the association between potential indicative OTUs and serum lipids in the DH samples was analyzed with Spearman's correlation (Fig. 6c). There were positive relationships between OTU647 and TC as well as OTU655 and TC/ApoB, and the linear association between OTU655 and ApoB was significant (Fig. 6d).

4. Discussion

4.1. Structural and functional characteristics of the FAM in the DH constitution and the related susceptibility difference

In this study, the structure of the FAM in the DH constitution was diverse and complex, as well as that in the BA constitution, and there were some structural commonalities in the FAM among the subjects of the same constitution, which resulted in the distinction between groups. Analysis of alpha diversity indexes showed that there was no significant difference in community diversity and evenness between the two constitutions (Shannon index and Shannoneven index), but there was higher community richness in DH constitution than BA constitution individuals (ACE, $P = 0.043$; Chao1, $P = 0.048$; Fig. 1a–d and Supplementary Table S2). In PCoA and PLS-DA, which revealed the beta diversity indexes of FAM, samples from different constitutions separated distinctively, and the ANOSIM and Adonis analyses showed significant differences between the groups (Fig. 1e and f; ANOSIM $R = 0.2229$, $P = 0.01$; Adonis $R^2 = 0.074$, $P = 0.017$). In the LEfSe analysis, the different relevant bacteria were enriched in the two constitutions across all taxonomic levels (Fig. 3); furthermore, 115 OTUs showed dramatically different distributions between DH and BA constitutions in the MetagenomeSeq analysis, as well as 213 OTUs in the Wilcoxon rank-sum test (Supplementary Tables S3 and S4), some of which were dominant bacterial taxa with relative abundances greater than 1 % and contributed to distinguishing the DH and BA constitutions well in the LEfSe analysis. Therefore, the structure of FAM in DH constitution individuals was unique, and the functional changes caused by the alteration in abundances of some dominant bacterial taxa might be responsible for the susceptibility to lipid metabolic dysfunction.

Then, we predicted the changes in metabolic pathways in DH constitution individuals based on the 16S rDNA gene (Fig. 5). Notably, the

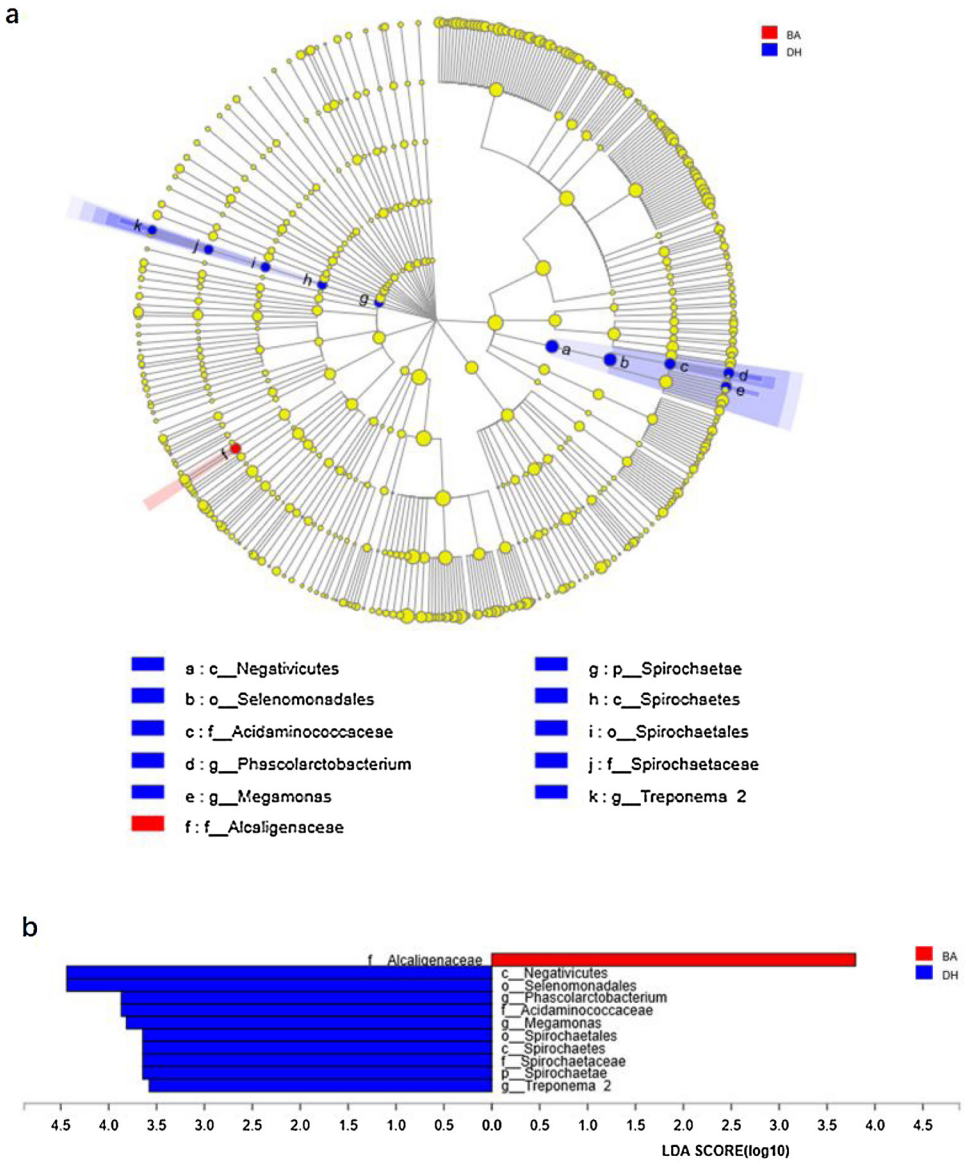

Fig. 3. Distinct taxa identified in the BA and DH groups using LEfSe analysis. (a) Cladogram constructed using the LEfSe method to display the phylogenetic distribution of bacteria that were remarkably enriched in the BA and DH groups. (b) The LDA score showed significant bacterial differences within the groups at different levels.

pathway related to glycerolipid metabolism was enriched in DH constitution, which was consistent with the results of a previous study on plasma metabolomics that suggested changes in lipid metabolism in DH constitution [28]. In this study, a greasy diet, as one of the three main causes of DH constitution and the most important environmental factors modulating the FAM structure, was negatively related to *Bacteroidales S24-7 group* and positively related to *Lachnoclostridium* abundances (Fig. 4a and b), which was consistent with previous studies [29,30], and the abundance of *Bacteroidales S24-7 group* was significantly related to the concentration of serum LDL (Fig. 4c). As short-chain fatty acid (SCFA) producers, *Bacteroidales S24-7 group* produces mainly butyrate, while *Lachnoclostridium* produces acetic acid, and these two SCFAs both could induce the secretion of glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) in enteroendocrine cells through G-protein coupled receptors (GPRs, such as GPR41 and GPR43) to participate in insulin secretion and gluconeogenesis, which are part of carbohydrate and lipid metabolism [31]. In addition, acetic acid could activate the parasympathetic nervous system directly to regulate carbohydrate and lipid metabolism by stimulating the secretion of insulin and ghrelin [32]. In summary, changes in lipid metabolism caused by greasy diet via affecting FAM in DH constitution were the basis of susceptibility to lipid metabolic dysfunction.

4.2. Potential biomarkers of DH constitution in FAM

Based on the unique FAM structure in DH constitution, it is possible to construct an objective diagnostic model for DH constitution in TCM as the assistance in the clinic. As a result, 4 OTUs were selected as potential biomarkers, which showed significant distributions between the two constitutions with $P < 0.001$ in the Wilcoxon rank-sum test. These 4 biomarkers were also found in the result of MetagenomeSeq analysis, while 3 of them were found in the result of LEfSe analysis with LDA > 2. ROC curve was constructed based on these 4 OTUs, in which the AUC was 0.91, with a significant diagnostic value. These potential biomarkers were all members from nondominant taxa, including *Lachnospiraceae*, *Cyanobacteria*, *Ruminiclostridium 9* and *Peptococcaceae* (Supplementary Table S8), and were all enriched in subjects with DH constitution. As mentioned above, *Lachnospiraceae* could activate GPRs

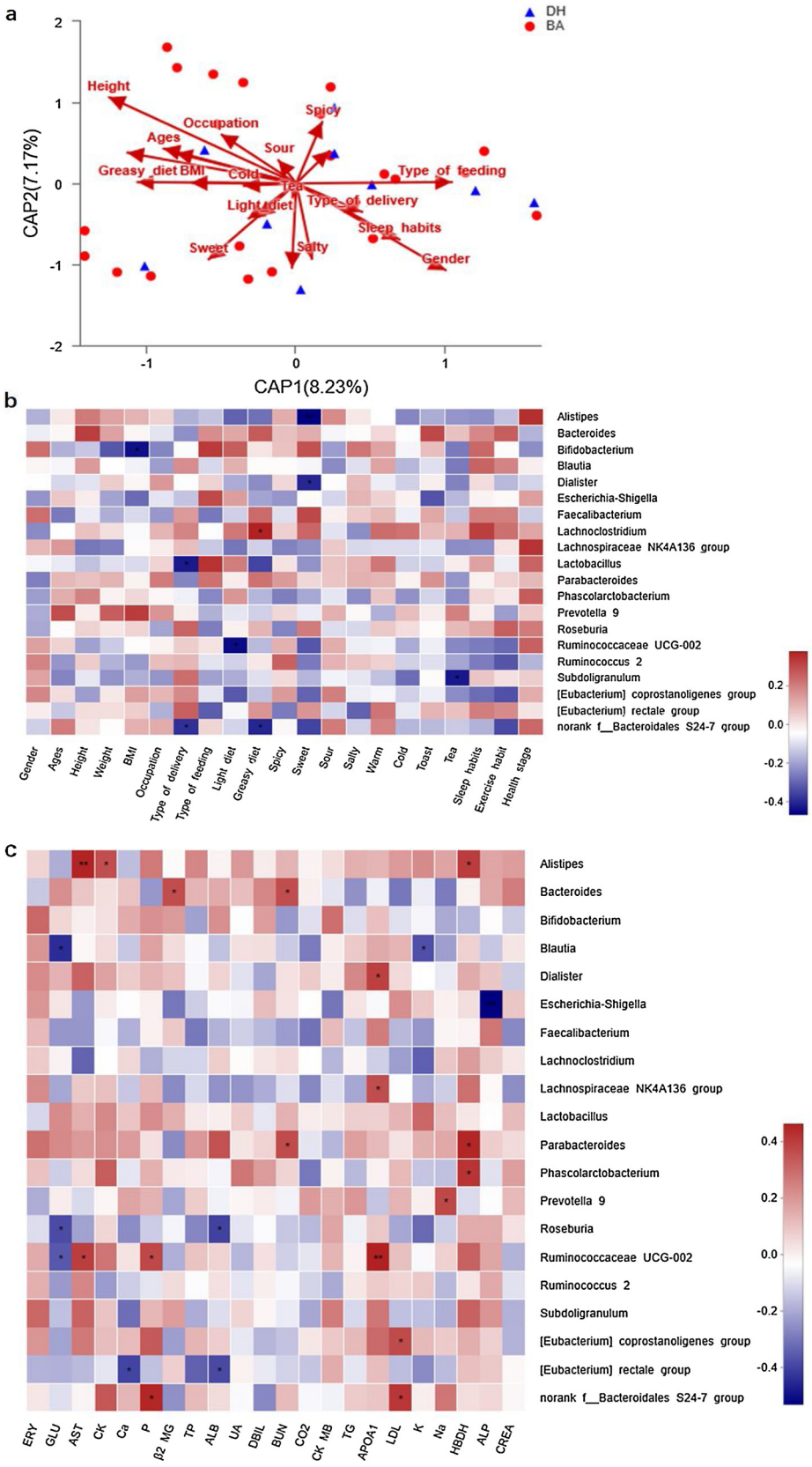

Fig. 4. Associations of the bacterial community with clinical characteristics in BA and DH samples. (a) db-RDA analysis showed the association between the bacterial community based on Bray-Curtis distance and the clinical characteristics (except for the indexes in blood and urine test). (b) and (c) show the Spearman's correlations between the top 20 genera and the clinical characteristics. (b) Spearman's correlation values ranged from -0.467 (blue) to 0.378 (red). (c) Spearman's correlation values ranged from -0.532 (blue) to 0.459 (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

and the parasympathetic nervous system by producing acetic acid to regulate carbohydrate and lipid metabolism; the exact function of *Ruminiclostridium 9* is still unknown, but as a butyrate producer, it might also regulate lipid metabolism via GPRs. In some published articles, *Cyanobacteria* was frequently found in the gut microbiome in herbivores and thought to be related to chlorophyll in the diet [33–35]; however, in recent research, it has been validated that *Cyanobacteria* is positively correlated with plasma LDL and lysophosphatidylcholine

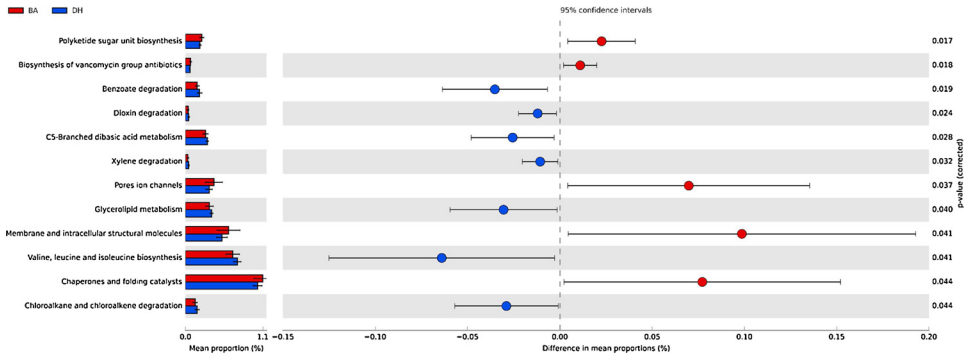

Fig. 5. Wilcoxon rank-sum test outputs of predicted gene function enriched in different groups using PICRUSt. BA, balanced constitution; DH, dampness-heat constitution.

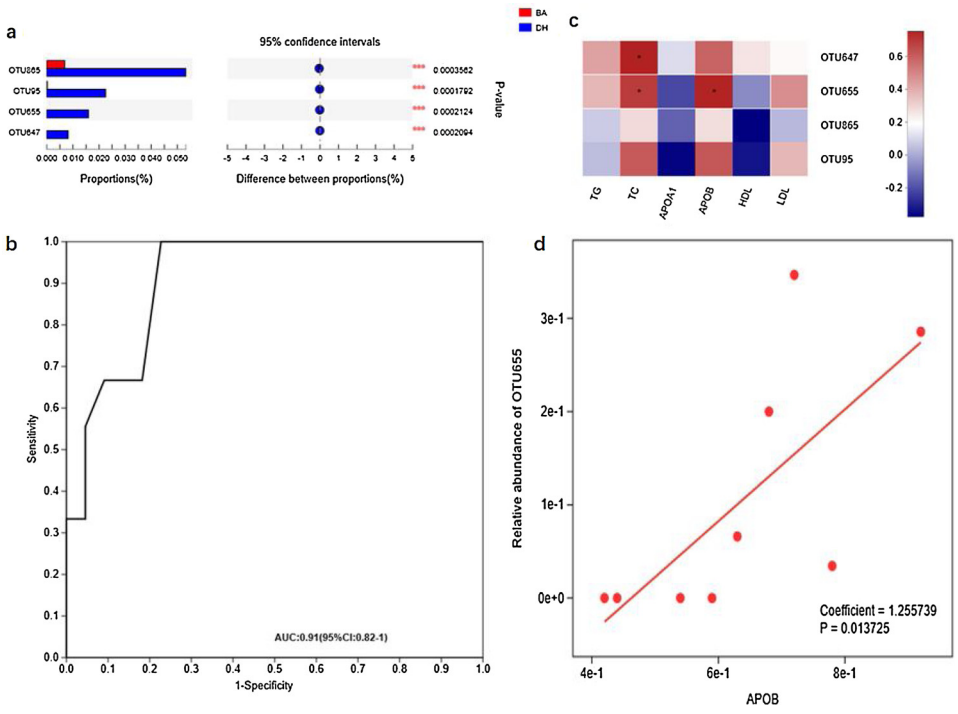

Fig. 6. Indicative OTUs as biomarkers of DH constitution in the Wilcoxon rank-sum test and their associations with serum lipids. (a) Indicative OTUs as biomarkers of DH constitution in the Wilcoxon rank-sum test. (b) Indicative OTUs were used to construct ROC curve to predict the diagnostic power. (c) Spearman's correlations between indicative OTUs and serum lipids. Spearman's correlation values ranged from -0.377 (blue) to 0.752 (red). (d) Linear model based on OTU655 and serum ApoB. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

[36,37], which are participants in lipid metabolism. *Peptococcaceae* is correlated with high fatty diet and is positively related to weight, but further mechanistic research is still needed [38,39]. In this study, in all positive relationships between the 4 potential biomarkers and the serum lipid metabolic indexes, the linear correlation between the relative abundance of OTU655 and the concentration of serum ApoB was the strongest (Fig. 6c and d), which was consistent with the results in previous studies [17]. Therefore, the changes in function caused by the alteration of these 4 potential biomarkers could explain the susceptibility to lipid metabolic dysfunction in DH constitution well.

However, as there were only 9 subjects with DH constitution in this study, our data might not be powerful enough to represent the FAM structure and function of DH constitution, and further studies need to be performed to explore the correlation between FAM dysbiosis and the lipid metabolic disorder in DH constitution via metagenomic, transcriptomic, proteomic and metabonomic analyses of a large population.

5. Conclusion

Unique FAM structural characteristics were identified in DH constitution, which were correlated with changes in lipid metabolism and susceptibility to lipid metabolic dysfunction. The 4 selected specific OTUs could be used as potential biomarkers of DH constitution to assist clinical diagnosis.

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Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the Ethics Committee of the Beijing University of Chinese Medicine Third Affiliated Hospital, Beijing, China (protocol number: KTPJ-BZYSY-2017-01). All experiments were performed in accordance with the approved guidelines.

CRediT authorship contribution statement

Jianhua Zhen: Data curation, Formal analysis, Writing - original draft, Writing - review & editing, Visualization. **Pengfei Zhao:** Methodology, Investigation. **Yini Li:** Data curation, Formal analysis. **Lu Zhao:** Data curation, Formal analysis. **Guangrui Huang:** Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Anlong Xu:** Conceptualization, Resources, Project administration, Funding acquisition.

Declaration of Competing Interest

All authors declare they have no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.eujim.2020.101166.

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