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Original Article

Nonalcoholic Fatty Liver Disease: Changes in Gut Microbiota and Blood Lipids

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Abstract

Background and Aims: The global prevalence of nonalcoholic fatty liver disease (NAFLD) is 25%. This study aimed to explore differences in the gut microbial community and blood lipids between normal livers and those affected by NAFLD using 16S ribosomal deoxyribonucleic acid sequencing. ***Methods:*** Gut microbiome profiles of 40 NAFLD and 20 non-NAFLD controls were analyzed. Information about four blood lipids and 13 other clinical features was collected. Patients were divided into three groups by ultrasound and FibroScan, those with a normal liver, mild FL (FL1), and moderate-to-severe FL (FL2). FL1 and FL2 patients were divided into two groups, those with either hyperlipidemia or non-hyperlipidemia based on their blood lipids. Potential keystone species within the groups were identified using univariate analysis and a specificity–occupancy plot. Significant difference in biochemical parameters ion NAFLD patients and healthy individuals were identified by detrended correspondence analysis and canonical correspondence analysis. ***Results:*** Decreased gut bacterial diversity was found in patients with NAFLD. *Firmicutes/Bacteroidetes* decreased as NAFLD progressed. *Faecalibacterium* and *Ruminococcus 2* were the most representative fatty-related bacteria. Glutamate pyruvic transaminase, aspartate aminotransferase, and white blood cell count were selected as the most significant biochemical indexes. Calculation of areas under the curve identified two microbiomes combined with the three biochemical indexes that identified normal liver and FL2 very well but performed poorly in diagnosing FL1. ***Conclusions:*** *Faecalibacterium* and *Ruminococcus 2*, combined with glutamate pyruvic transaminase, aspartate aminotransferase, and white blood cell count distinguished NAFLD. We speculate that regulating the health of gut microbiota may release NAFLD, in addition to providing new targets for clinicians to treat NAFLD.

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Introduction

With a global prevalence of 25%,[1] nonalcoholic fatty liver disease (NAFLD), the liver component of a group of disorders linked to metabolic dysfunction,[2] is defined as the presence of steatosis in more than 5% of hepatocytes in the absence of heavy alcohol intake or other chronic liver illnesses.[3] NAFLD is currently slowly being recognized as a chronic disease. However, due to limited medical knowledge, most NAFLD patients ignore treatment of the disease.[4,5] NAFLD is known to have a close, bilateral association with metabolic syndrome.[6] Additionally, lipid abnormalities are linked to an increased risk of liver[7] as well as cardiovascular disease.[8] Furthermore, some studies have conclusively shown cardiovascular disease to be the leading cause of death in NAFLD patients.[9,10] Therefore, it is imperative to investigate the link between dyslipidemia and NAFLD.

Collectively, data from rodent studies support the hypothesis that gut microbiota plays a role in the development of NAFLD.[11–14] Additionally, changes in the gut microbiota can affect the gut–liver axis and are linked to the development of cirrhosis and NAFLD in human patients.[15–19] Therefore, characterizing the bacterial populations implicated in dysbiosis is critical as it may aid in the development of alternative disease management techniques. In most cases, NAFLD is diagnosed by imaging, and in routine practice, the most commonly used imaging technique is abdominal ultrasound.[2] However, whether the effects changes of the gut microbiota in FL can be diagnosed by ultrasound is up for debate. The controlled attenuation parameter (CAP) has been widely used to assess steatosis[20,21] and reportedly has outstanding performance in diagnosing more than 10% of hepatic steatosis instances.[22,23] In this study, we used abdominal ultrasound and CAP to estimate the level of FL. We also noted that how the gut microbiota changes in patients with NAFLD

Keywords: Liver; Gut microbiota; Blood lipids; Nonalcoholic fatty liver disease; Hyperlipidemia.

Abbreviations: ALT, alanine transaminase; AST, aspartate aminotransferase; AUCs, areas under the receiver operating characteristic curve; BMI, body mass index; CAP, controlled attenuation parameter; CCA, canonical correspondence analysis; DCA, detrended correspondence analysis; FL, fatty liver; FL1, mild FL; FL2, moderate-to-severe FL; HL, hyperlipidemia; NAFLD, nonalcoholic fatty liver disease; NHL, non-hyperlipidemia; NL, normal liver; OUTs, operational taxonomic units; TGs, triglyceride; WBC, white blood cell.

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and hyperlipidemia remains an open question.

The primary objective of this investigation was two-fold, firstly, to establish a classification framework for distinguishing individuals afflicted with FL through the combined use of ultrasound and CAP, and secondly, to dissect differences in the gut microbial composition in subjects with normal liver function and in those diagnosed with NAFLD, using 16S rDNA gene amplicon sequencing. Furthermore, this study delved into alterations of the gut microbiota of patients concurrently affected by NAFLD and hyperlipidemia. We used biochemical parameters such as alanine transaminase (ALT) and aspartate aminotransferase (AST) to identify significantly differences between NAFLD patients and healthy individuals.

Methods

Human patients

A total of 60 patients were enrolled in our study between January 1 and November 30, 2021. The study was approved by the institutional ethics committee of Shenzhen People's Hospital. The approval number is LL-KY-2021637. The inclusion criteria for NAFLD patients were: (1) >18 years of age; (2) newly diagnosed with NAFLD during the selection period, and confirmed by abdominal ultrasound and FibroScan; (3) not being treated with any medication and had not gained any significant weight in the preceding 6 months; (4) abdominal ultrasound and biochemical indexes were evaluated on the same day. The inclusion criteria of normal patients were (1) >18 years of age; (2). abdominal ultrasound was normal; (3) abdominal ultrasound and biochemical indexes were evaluated on the same day. The exclusion criteria for both NAFLD and normal patients were: (1) the presence of autoimmune hepatitis, primary biliary cholangitis, or primary sclerosing cholangitis; (2) hepatitis B or C viral infection; (3) antibiotics and other commonly used nonantibiotic medications, such as PPIs, laxatives, statins, antidepressants, and opioids used within the preceding month; (4) a malignancy diagnosis (<5 years); (5) human immunodeficiency virus infection; (6) chronic disorders associated with lipodystrophy or immunosuppression; (7) drug-induced steatosis or liver injury; or (8) diabetes, gout, and/or other metabolic disease.

Ultrasound and FibroScan detection

All abdominal ultrasound examinations were performed using a Mindray Resona 7A (Mindray, Shenzhen, China) convex array probe at a frequency of 1–6MHz. We also assessed liver disease severity using a FibroScan 502 Touch model (M Probe; XL Probe; Echosens, Paris, France), which included two functional examinations of liver stiffness and fat content, i.e., CAP and vibration-controlled transient elastography. Patients, were divided into three groups, of 20 each, those with a normal liver (NL), mild FL (FL1), and moderate-to-severe FL (FL2) according to the liver ultrasound performance and CAP value based on the following criteria.[24–26] (1) Patients with a normal liver echogenic structure were included in the NL group. (2). When the diaphragm and the portal vein wall could be normally observed, but there was a small and generalized increase in liver echogenicity, the patients were included in the FL1 group. (3). Patients with moderate or markedly increased liver echogenicity and mild or severe impairment in the appearance of the portal vein wall, diaphragm, and posterior right hepatic lobe were included in the FL2 group. Patients were divided into three groups according to the CAP value as follows:[27] (1) NL group, CAP<240 dB/m; (2) FL1 group, 240 dB/m<CAP<265 dB/m; and (3) FL2 group, CAP>265 dB/m.

Baseline assessment

Low-density lipoprotein, high-density lipoprotein, triglycerides (TGs), total cholesterol, gamma-glutamyl transpeptidase, ALT, AST, alkaline phosphatase, albumin, direct bilirubin, total bilirubin, and white blood cell (WBC) and platelet counts were measured after an 8 h overnight fast. Body mass index (commonly known as BMI) was defined as the weight divided by height squared (kg/m^2). Abdominal circumference was defined as the horizontal abdominal girth through the point of the iliac crest. Additionally, age and sex characteristics were also collected in our study.

Hyperlipidemia definition

In this study, hyperlipidemia (HL) was defined as (1) total cholesterol >6.5 mmol/L] and (2) TGs>2.3 mmol/L.[28] If the levels of the subject's blood lipid indicators were consistent with the above definitions, we classified the study participants as having hyperlipidemia.

Microbiome analysis by 16S rDNA sequencing

Stool samples of the patients in our study were collected and stored in a freezer at −40°C. Following the extraction of 16S rDNA and sample quality checks, variable regions V3–V4 of bacterial 16S rRNA genes were amplified with degenerate PCR primers. An Agilent 2100 Bioanalyzer was used for quality inspection and the qualified library was sequenced by selecting the corresponding Illumina sequencing HiSeq 2500 platform and PE300 (San Diego, CA, USA). Raw reads were filtered to remove adaptors and low-quality and ambiguous bases. Paired-end reads were then added to tags using the Fast Length Adjustment of Short Reads program (FLASH, v.1.2.11)[29] to derive the tags. The tags were clustered into operational taxonomic units (OTUs) with a cutoff value of 97% using UPARSE software (v7.0.1090),[30] and chimera sequences were compared with the Gold database using UCHIME (v4.2.40)[31] to detect. Representative OTU sequences were taxonomically classified using Ribosomal Database Project Classifier v.2.2 with a minimum confidence threshold of 0.6 and trained on the Greengenes database (v.201305) using QIIME (v.1.8.0).[32] USEARCH_global[33] was used to compare all of the tags back to the OTU to derive the OTU abundance statistics table for each sample. Details of the microbiome analysis are shown in the Supplementary File 1.

Statistical analysis

The statistical analysis was performed using R (https://www.r-project.org). Differences in normally distributed numerical variables were compared with *t*-tests, rank sum tests were used for non-normally distributed numerical variables, and a chi-squared tests were used for disordered classification variables. *P*-values <0.05 was used to determine whether the clinical indicators were associated with NAFLD or HL. The ace index was used to access the alpha diversity. Principal coordinate analysis was conducted to access beta diversity. Specificity–occupancy plots were used to identify potential keystone species.

Detrended correspondence analysis (DCA) was employed to identify broad structural changes in microbial communities. In correspondence analysis, the arch effect, where the data points are arranged in a horseshoe-like pattern, is removed using detrending. The ordination method is known as DCA. Canonical correspondence analysis (CCA) was performed to determine the most important biochemical index

shaping microbial community composition and organization. Thus, DCA and CCA were employed to select the most ideal biochemical indexes. Finally, areas under the receiver operating characteristic curve (AUCs) were compared to determine the efficacy of the gut microbiota and biochemical index for identifying significant FL.

Results

Baseline patient characteristics

This study included 20 patients with ultrasound-proven NL, 20 patients with ultrasound-proven FL1, and 20 patients with ultrasound-proven FL2. The FL1 and FL2 patients were divided into two groups, an HL and a non-HL (NHL) group. Finally, there were a total of 40 NHL patients and 20 HL patients in our study. Table 1 and Supplementary Tables 1 and 2 summarize the characteristics of each group. Among all the HL and NHL patients, only ALT was statistically significant among the NL, FL1, and FL2 patients. Meanwhile, whether in all patients or the NHL patients, TGs, gamma-glutamyl transpeptidase, direct bilirubin, platelet, and abdominal circumference were statistically significant among the NL, FL1, and FL2 patients. Furthermore, only BMI was statistically significant among the NL, FL1, and FL2 patients, both in the HL and NHL groups. Age and weight were only statistically significant among the NL, FL1, and FL2 patients in the HL group.

Decreased gut bacterial diversity is present in patients with NAFLD

We compared the microbial diversity using data from 16S rDNA gene amplicon sequencing, based on patient liver ultrasound results and blood lipid levels. For comparison, the beta diversity, based on the Bray–Curtis distance, and the alpha diversity based on the ace metric were both plotted (Fig. 1). In all patients, 6% lower ace alpha diversity was found in cases of NAFLD; in NHL patients, 5% lower ace alpha diversity was found in NAFLD cases. In terms of beta diversity, no significant differences were found in all patients (p=0.26), hyperlipidemia patients (p=0.356), or nonhyperlipidemic patients (p=0.337).

Top 10 bacteria in terms of OTU abundance differed slightly among the three groups

We evaluated the relative abundance of NL, FL1, and FL2 based on various groupings and selected the 10 most abundant levels (Fig. 2 and Supplementary Table 3). In the three groups, the top 10 phyla were *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, *Actinobacteria*, *Verrucomicrobia*, unclassified, *Synergistetes*, *Candidatus_Saccharibacteria*, and *Cyanobacteria*. However, the relative order of abundance of *Fusobacteria*, *Actinobacteria*, and *Verrucomicrobia* were different. Among the three groups, from NL, FL1, and FL2, the proportion of *Firmicutes* gradually decreased while the proportion of *Bacteroidetes* gradually increased. That is, *Firmicutes/Bacteroidetes* decreased as NAFLD progressed.

Changes in FL-related microbiota are more prevalent in all patients and NHL patients than in HL patients

Univariate analysis was used to assess differences in various microbial taxa by fatty severity in all, NHL, and HL patients (Table 2 and Supplementary Tables 4–6). Comparison of NL, FL1, and FL2 patients, found *Faecalibacterium*, *Lachnospira,* and *Lachnospiracea_incertae_sedis* to differ in all patients and the NHL patient group. *Fusicatenibacter*, *Lachnoanaerobaculum*, and *Victivallis* were only found different in the all-patients group. Whether in the all-patients group or the NHL patients group, the relative abundance of *Faecalibacterium*, *Lachnospira*, and *Lachnospiracea_incertae_sedis* in NL patients was the highest, indicating that the abundance of these three gut bacteria was reduced in patients with NAFLD, as well as in patients with NAFLD but without HL. Interestingly, in both the all-patients group and the NHL patients group, the abundance of *Faecalibacterium* for FL2 patients was higher than that of FL1. This indicated that although the abundance of these bacteria was lower in NAFLD, the degree of reduction was inversely proportional to the severity of the disease. Conversely, the abundance of *Lachnospiracea_incertae_sedis* gradually decreased between the NL, FL1, and FL2 patients, signaling that the reduction in its abundance positively correlated with NAFLD progression.

Comparing FL1 and FL2 patients, *Mogibacterium* abundance was different in the all-patients group and the NHL patients group, *Ruminococcus 2* differed in the all-patients group and the HL patients group, *Fusicatenibacter* and *Lachnoanaerobaculum* abundance only differed among the all-patients group, and *Cyanobacteria* and *Solobacterium* only differed in abundance among the NHL patients group. In the three groups, the abundance of *Cyanobacteria*, *Mogibacterium*, *Lachnoanaerobaculum*, and *Solobacterium* was close to 0. As such, the differences in abundance of the three bacteria may be unreliable. The abundance of *Ruminococcus 2* in the FL2 group was significantly higher than in the FL1 group both in the all-patient group and in the HL patients group, indicating that *Ruminococcus 2* was associated with severe FL and HL. The abundance of *Fusicatenibacter* gradually decreased in NL, FL2, and FL1 patients. Moreover, *Fusicatenibacter* differed significantly in abundance between FL1 and FL2 in all patients as well as between the NL and FL1 in the all-patient group and the NHL patient group. But there was no difference between FL1 and FL2 in the NHL patient group and the HL patient group. This phenomenon may indicate that the change in abundance of *Fusicatenibacter* may be more strongly related to patients with mild rather than severe NAFLD.

Potential keystone species within the group, identified using a specificity–occupancy plot

The OTU of species and phyla with total relative abundance above 0.01% were retained from the OTU table; then, the specificity and occupancy of each group were calculated separately according to the retained OTU table. Specificity was defined as the mean abundance of an OTU in the samples of a group. Occupancy was defined as the relative frequency of occurrence of the OTU in the samples of a group. These values were calculated as follows:[34,35]

$$\text{Specificity} = \frac{\text{Average relative abundance of an OTU across samples in a subgroup}}{\text{Sum of the average relative abundance of the OTU across all study subgroups}}$$

$$\text{Occupancy} = \frac{\text{Number of samples detected by an OTU in a subgroup}}{\text{Number of samples detected across all study samples}}$$

To locate potential keystone species attributed to each group, we selected the specie showing an OTU with specificity and occupancy equal to or greater than 0.7 for each specific group. *Firmicutes* were found in the three groups, while *Proteobacteria* was found only in the FL1 group (Fig. 3). At the genus level, there were a total of six specific genus species in the NL group, none of which were observed in the other two groups (Fig. 4). The six genus species were *Faecalibacterium*, *Clostridium_XIVa*, *Streptococcus*, *Ruminococcus*, *Oscillibacter*, and *Flavonifractor*. *Klebsiella,* and *Veillonella* found in the FL1 group. Only *Lachnospiracea_incertae_sedis* was found in the FL2 group.

Table 1. Baseline information of all the study patients

Variables	Total (*n*=60)	NL (*n*=20)	FL1 (*n*=20)	FL2 (*n*=20)	*p*[a]	*p*[b]	*p*[c]	*p*[d]	*p*[e]
Age[g], year	47.07±11.43	45.9±11.25	50.65±10.36	44.65±12.27	0.22	0.58	0.1	0.17	0.74
Sex[h]					0.82	1	0.75	1	1
Female	27 (45)	9 (45)	8 (40)	10 (50)					
Male	33 (55)	11 (55)	12 (60)	10 (50)					
HL[h]					<0.01	<0.01	1	<0.01	<0.01
HL	40 (67)	20 (100)	10 (50)	10 (50)					
NHL	20 (33)	0 (0)	10 (50)	10 (50)					
LDL[g], mmol/L	2.95±0.97	3.18±0.55	2.53±1.14	3.14±1	0.05	0.11	0.08	0.03	0.88
TG[f], mmol/L	1.41 (1, 2.71)	0.9 (0.76, 1.19)	1.83 (1.33, 3.41)	2.24 (1.78, 4.46)	<0.01	<0.01	0.27	<0.01	<0.01
HDL[f], mmol/L	1.1 (0.9, 1.33)	1.33 (1.06, 1.54)	1.06 (0.88, 1.18)	1 (0.88, 1.19)	0.06	0.02	0.88	0.1	0.05
TC[g], mmol/L	5.03±1.28	4.75±0.88	4.77±1.43	5.57±1.34	0.07	0.17	0.08	0.96	0.03
GGT[f], U/L	27 (16, 44.25)	16 (13.75, 18.5)	32.5 (25.08, 46)	38 (28.75, 51.5)	<0.01	<0.01	0.4	<0.01	<0.01
ALT[f], U/L	20 (14.9, 28)	15.5 (13.5, 18)	20 (14.82, 28)	29 (20.75, 51.25)	<0.01	<0.01	0.02	0.04	<0.01
AST[f], U/L	22 (17, 42.7)	18 (14, 24.5)	22 (18.75, 41.4)	36.65 (22.75, 46.6)	<0.01	0.01	0.08	0.14	<0.01
ALP[f], U/L	67 (60.75, 69.93)	64.6 (58, 68.12)	67.35 (57.75, 68.62)	68.6 (62.9, 76.5)	0.08	0.1	0.07	0.42	0.06
ALB[f], g/L	42.63 (14.88, 45.65)	40.88 (16.2, 46.15)	41.85 (12.4, 44.7)	43.2 (16.35, 44.75)	0.82	0.72	0.6	0.59	0.96
TP[f], g/L	68.25 (62.25, 71.83)	65.8 (62.08, 71.3)	70 (62.32, 71.48)	68.55 (66.15, 74.12)	0.54	0.29	0.84	0.51	0.25
DB[f], μmol/L	3.4 (2.2, 4.7)	2.25 (1.9, 2.8)	4.07 (2.52, 4.74)	3.95 (3.25, 4.78)	<0.01	<0.01	0.88	0.03	<0.01
TBIL[f], μmol/L	11.15 (8.05, 15.82)	11.1 (8.67, 15.45)	11.1 (7.92, 16.52)	11.45 (7.75, 15.5)	0.99	0.96	0.94	0.91	1
WBC[f], $\times 10^9$/L	6.59 (5.9, 8.02)	6.53 (6.15, 7.62)	7.11 (5.84, 8.58)	6.56 (5.7, 7.58)	0.8	0.86	0.54	0.5	0.86
PLT[f], $\times 10^9$/L	239.5 (218, 267.5)	221 (201.75, 239.5)	250 (231, 273)	248 (235.25, 270.25)	0.03	<0.01	0.94	0.02	0.03
Abdominal circumference[g], cm	93.13±11.58	84.95±10.63	93.85±9.24	100.6±9.39	<0.01	<0.01	0.03	<0.01	<0.01
Height[g], cm	165.72±8.85	167.3±7.69	164.5±9.16	165.35±9.78	0.6	0.3	0.78	0.45	0.49
Weight[f], kg	71 (64.5, 75.5)	72 (61.5, 77.25)	67.5 (59.5, 75)	74 (69.5, 79.75)	0.1	0.68	0.04	0.97	0.05
BMI[f]	25.39 (24.1, 27.34)	24.49 (22.72, 25.99)	25.08 (23.6, 25.83)	27.37 (25.69, 28.66)	<0.01	0.02	0.02	0.59	<0.01

[a]Comparison of groups NL, FL1, and FL2; [b]Comparison of groups NL and NNL; [c]Comparison of groups FL1 and FL2; [d]Comparison of groups NL and FL1; [e]Comparison of groups NL and FL2; [f]Non-normally distributed numerical variables, medians (first quartile, third quantile); [g]Normally distributed numerical variables, mean±standard deviation; [h]Disordered classification variables, percentages. ALB, albumin; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate aminotransferase; DB, direct bilirubin; GGT, γ-glutamyl transpeptidase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PLT, platelet; TB, total bilirubin; TC, total cholesterol; TG, triglycerides; WBC, white blood cell.

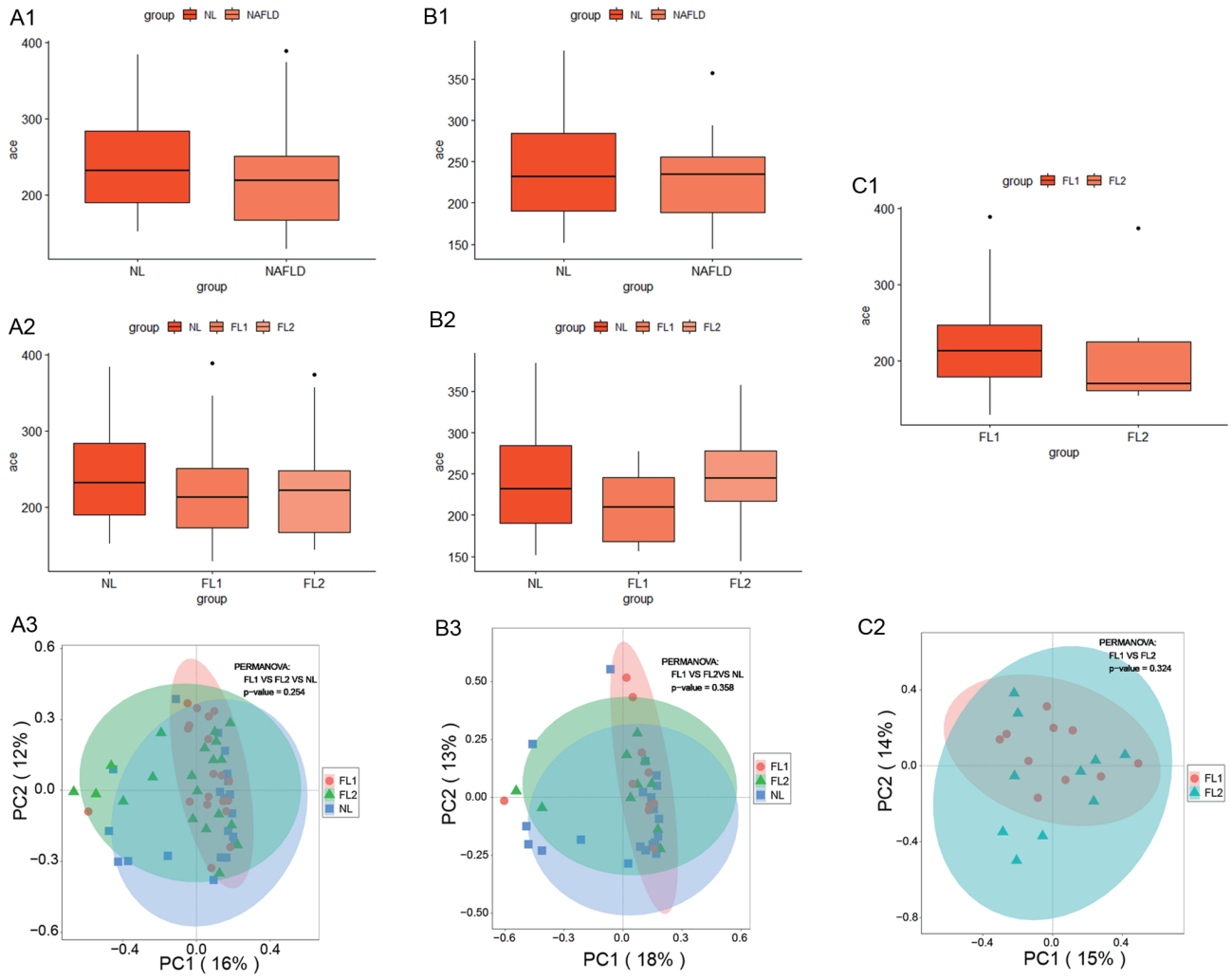

Fig. 1. Comparison of the diversity of gut microbial communities in all, hyperlipidemia, and nonhyperlipidemia patient groups. (A–B) The alpha and beta diversity of A, all (n=60), B, nonhyperlipidemia (n=40), and C, hyperlipidemia (n=20) patients were divided according to their liver ultrasound performance and blood lipid levels. Alpha diversity was based on the ace metric. The box plots show the median, the boxes represent the 25–75th percentile, the black dots above the box plot represent discrete values, and the whiskers show the 10–90th percentile. The principal coordinates analysis maps were created using relative operational taxonomic unit abundance data and the Bray–Curtis distance, with the ellipse representing the 95% confidence interval. FL1, mild fatty liver; FL2, moderately severe fatty liver; NAFLD, nonalcoholic fatty liver disease; NL, normal liver.

ALT and AST levels and WBC count were significant biochemical indexes for gut microbial community changes in all-patients group

To explore which clinical factors influenced changes in the gut microbial community, we first used DCA to calculate the values of four gradient lengths (Supplementary Table 7). We found that the largest value was greater than 4 and, accordingly, decided to use CCA (Fig. 5). The significance of clinical factors associated with gut microbial community composition was then assessed using Monte Carlo permutation tests (Supplementary Table 8). Finally, we found that ALT (p=0.001), AST (p=0.001), and WBC count (p=0.019) were significant biochemical indexes for microbial community changes in the all-patient group.

Microbiome combined with biochemical index reflects FL severity in the all-patient group

Based on the above results, *Faecalibacterium* and *Ruminococcus 2* were selected as the most ideal and symbolic fatty-related bacteria taxa. Furthermore, ALT, AST, and WBC count were chosen as the most significant biochemical indexes. To ascertain the discriminatory capability between NAFLD patients and healthy individuals, we opted for AUCs (Fig. 6), confirming the distinct classification potential of the two gut microbiota taxa and the three biochemical indices. While diagnosing NL patients, the AUCs of the biochemical index, bacteria taxa, and (bacterial + biochemical) were found to be 0.78, 0.77, and 0.86, respectively. When distinguishing FL1, the AUCs of the biochemical index, bacteria taxa, and (bacterial + biochemical) were 0.50, 0.61, and 0.51, respectively. For diagnosing FL2, the AUCs of the biochemical index, bacteria taxa, and (bacteria + biochemical) were 0.78, 0.66, and 0.85, respectively.

Discussion

In this study, we found substantial variations in gut micro-

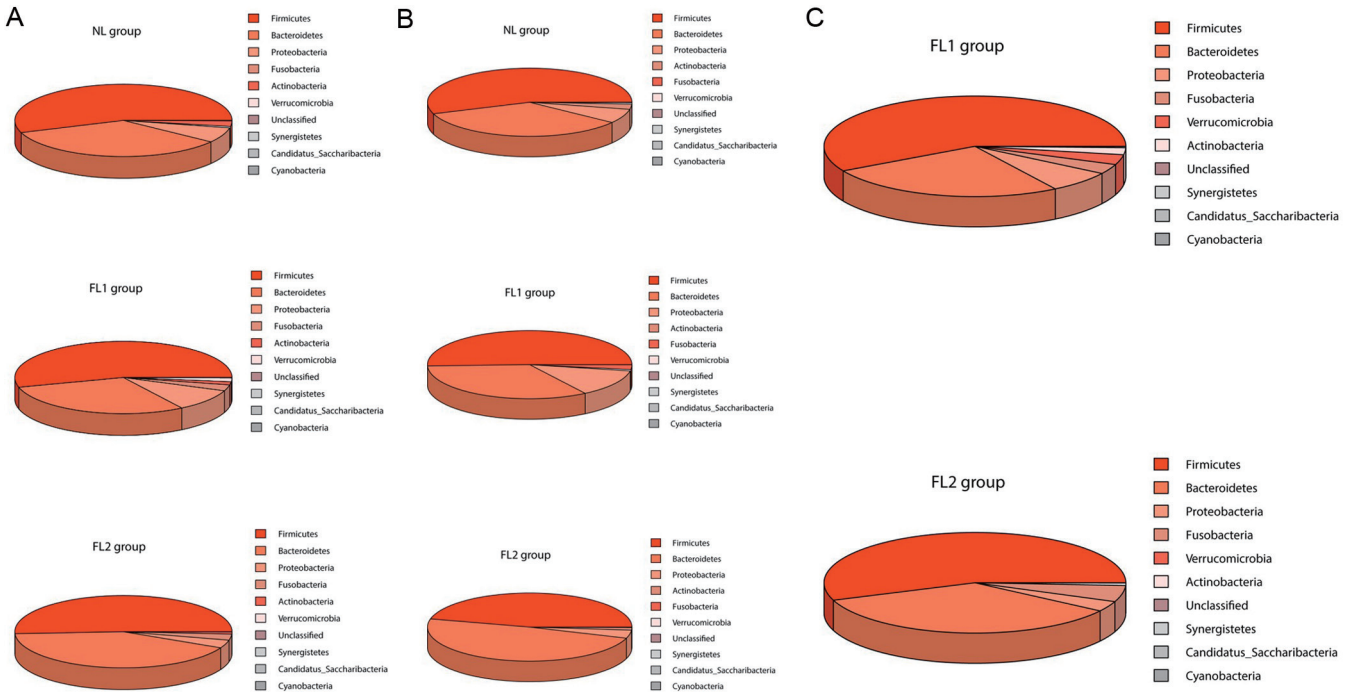

Fig. 2. Top 10 bacteria in all patients, hyperlipidemic, and nonhyperlipidemic patient groups. (A–C) The top 10 bacteria of A, all (n=60), B, nonhyperlipidemia (n=40), and C, hyperlipidemia (n=20) patients were stratified by liver ultrasound performance and blood lipid levels. FL1, mild fatty liver; FL2, moderately severe fatty liver; NL, normal liver.

biota modifications based on NAFLD severity in all, NHL, and HL patients. We focused on two microorganisms, *Faecalibacterium* and *Ruminococcus 2*, as potential targets for distinguishing between FL and healthy individuals using univariate analysis and a specificity–occupancy plot. We also found that ALT, AST, and WBC count were the best biochemical indexes related to FL, based on the result of the CCA. Using the two microbiomes and the three biochemical indexes, NL and FL2 patients could be easily identified. However, this approach performed poorly in diagnosing FL1.

Previous studies showed that the alpha diversity of gut microbiota was lower in FL cases,[36,37] which was also found in this study. Whether with or without HL, the alpha diversity of NAFLD decreased and the ace index in the NHL patient group was 5% higher than in the all-patient group. That is, HL would further decrease the abundance of gut microbial communities in NAFLD patients. The results of this study are in accord with existing results indicating the influence of environmental variables over genetic traits in determining human intestinal microbiota.[38]

It was reported that an increased proportion of *Firmicutes* was dominantly linked with NAFLD in mice as well as humans.[39–41] In this study, among the three groups, the specificity and occupancy of *Firmicutes* were ≥ 0.7. Remarkably, prominent associations with NAFLD in our study all derived from the *Firmicutes* phylum and, diversely, included *Faecalibacterium, Lachnospiracea_incertae_sedis*, *Ruminococcus 2*, *Fusicatenibacter*, *Gemmiger*, and *Roseburia*. These results were also found to be the case in a large population sample.[36]

Much evidence suggests that the presence of *Faecalibacterium* is significantly lower in NAFLD than in non-NAFLD patients.[15,42–45] *Faecalibacterium prausnitzii*, a species of the *Faecalibacterium* genus,[46] as well as an oxygen-sensitive, butyrate-producing bacterium, plays a significant part in maintaining a healthy gut.[47] *Faecalibacterium prausnitzii* levels have been reported to be lower in patients with intestinal and metabolic disorders, such as inflammatory bowel disease, irritable bowel syndrome, and celiac disease.[48,49] It is therefore not surprising that Faecalibacterium decreased not only in NAFLD but also in HL patients in our study. Furthermore, *Faecalibacterium* produce short-chain fatty acids[50] that have an anti-inflammatory function by regulating immune cell chemotaxis, reactive oxygen species release, and cytokine release.[51] Additionally, a clinical study demonstrated the direct anti-inflammatory activity of butyrate at the site of inflammation.[52] Accordingly, a decrease in the amount of *Faecalibacterium* may lessen short-chain fatty acid levels in the gut, intensifying gut inflammation involved in the pathogenesis of NAFLD.

Ruminococcus 2 has the genetic potential to worsen the onset of FL disease because it was more prevalent in the FL2 patients in our study. More importantly, the abundance of *Ruminococcus 2* in FL2 patients was higher than in both all FL1 and HL patients, but not in NHL patients. A Chinese population study found that *Ruminococcus 2* was positively correlated with serum lipids,[53] and a survey showed that dietary fiber intervention for 4 days inhibited the growth of *Ruminococcus 2*.[54] That is, *Ruminococcus 2* may through influence human lipid metabolism rather than another way to aggravate FL. Furthermore, NAFLD patients may be able to adjust their diet to include more dietary fiber to combat the disease; however, further research is needed to establish whether this is possible.

In this study, oddly, *Fusicatenibacter* decreased in NAFLD cases but its abundance in FL1 patients was lower than that in FL2 patients. A study showed that the abundance of *Fusicatenibacter* decreased in the presence of NAFLD.[55] Furthermore, *Fusicatenibacter saccharivorans*, the lone species in the *Fusicatenibacter* genus,[56] decreased in cases of NAFLD

Table 2. Differences in gut microbiome in different groups

	All patients (*n*=60)					NHL patients (*n*=40)					HL patients (*n*=20)
	NL vs. FL1 vs. FL2	NL vs. FL	NL vs. FL1	NL vs. FL2	FL1 vs. FL2	NL vs. FL1 vs.FL2	NL vs. FL	NL vs. FL1	NL vs. FL2	FL1 vs. FL2	FL1 vs. FL2
Cyanobacteria	0.08	0.36	0.81	0.09	0.05	0.02	0.8	0.1	0.09	0.01	0.55
Anaerostipes	0.05	0.04	0.27	0.01	0.24	0.05	0.04	0.37	0.02	0.25	0.48
Bilophila	0.51	0.25	0.32	0.32	1	0.1	0.04	0.06	0.15	0.53	0.57
Butyricicoccus	0.1	0.38	0.06	0.7	0.08	0.09	0.19	0.03	0.98	0.13	0.39
Christensenella	0.51	0.31	0.5	0.27	0.58	0.09	0.03	0.08	0.06	0.85	0.48
Cloacibacillus	0.16	0.06	0.07	0.11	0.78	0.08	0.03	0.09	0.04	0.57	0.8
Enterococcus	0.07	0.02	0.07	0.02	0.76	0.05	0.01	0.05	0.01	1	0.73
Faecalibacterium	<0.01	<0.01	<0.01	<0.01	0.19	<0.01	<0.01	<0.01	0.04	0.06	0.97
Faecalicoccus	0.15	0.1	0.39	0.05	0.33	0.1	0.07	0.41	0.03	0.34	0.73
Fusicatenibacter	0.03	0.11	0.02	0.74	0.03	0.09	0.14	0.03	0.78	0.11	0.17
Lachnoanaerobaculum	0.02	0.09	0.63	<0.01	<0.01	0.15	0.17	0.68	0.06	0.08	0.08
Lachnospira	<0.01	<0.01	<0.01	<0.01	0.94	<0.01	<0.01	0.01	0.02	0.84	0.85
Lachnospiracea_incertae_sedis	0.04	0.01	0.06	0.02	0.46	<0.01	<0.01	0.02	<0.01	0.53	0.8
Mogibacterium	0.08	0.91	0.21	0.36	0.03	0.09	0.75	0.31	0.15	0.03	0.4
Roseburia	0.08	0.33	0.05	0.8	0.06	0.06	0.15	0.02	0.98	0.09	0.48
Ruminococcus 2	0.06	0.64	0.49	0.13	0.02	0.52	0.97	0.5	0.56	0.28	0.03
Solobacterium	0.56	0.66	0.93	0.36	0.33	0.09	0.56	0.51	0.06	0.03	0.58
Victivallis	0.04	0.22	1	0.08	0.08	0.05	0.16	1	0.05	0.17	1

FL1, mild fatty liver; FL2, moderately severe fatty liver; NL, normal liver; HL, hyperlipidemia; NHL, non-hyperlipidemia.

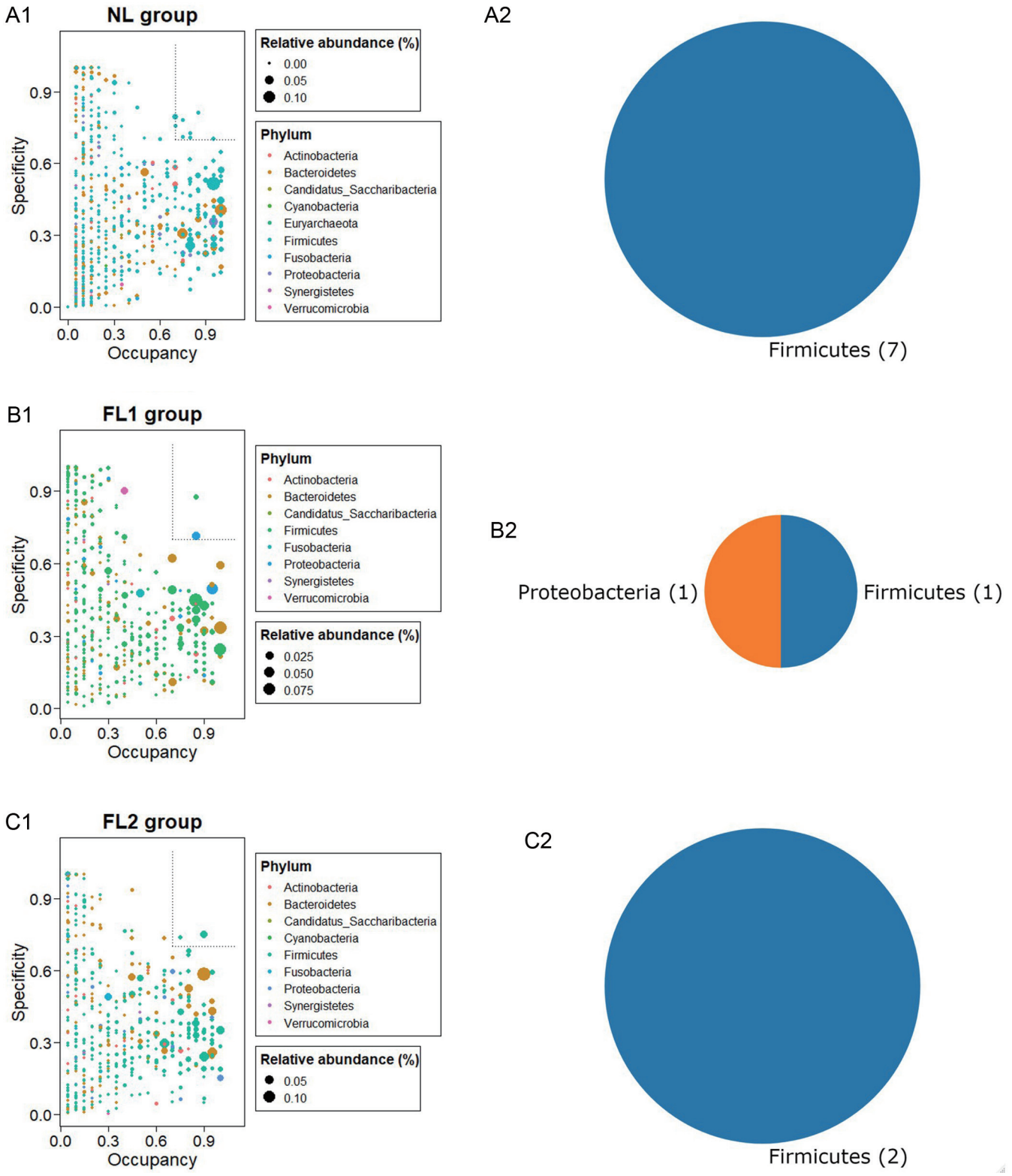

Fig. 3. Specificity–occupancy plot of the phyla identified in the all-patient group. Specificity–occupancy plots show the relative abundance above 0.01% for the operational taxonomic units (OUTs) in each group; the x-axis represents occupancy, i.e. how well an OTU is distributed across all three groups [normal liver and mild fatty liver [FL)], and moderately severe FL; the y-axis represents specificity, i.e. whether they were also found in other groups (A1, B1, C1). Pie charts show the number of OTUs representing keystone phylum species in each group (A2, B2, C2). FL1, mild fatty liver; FL2, moderately severe fatty liver; NL, normal liver.

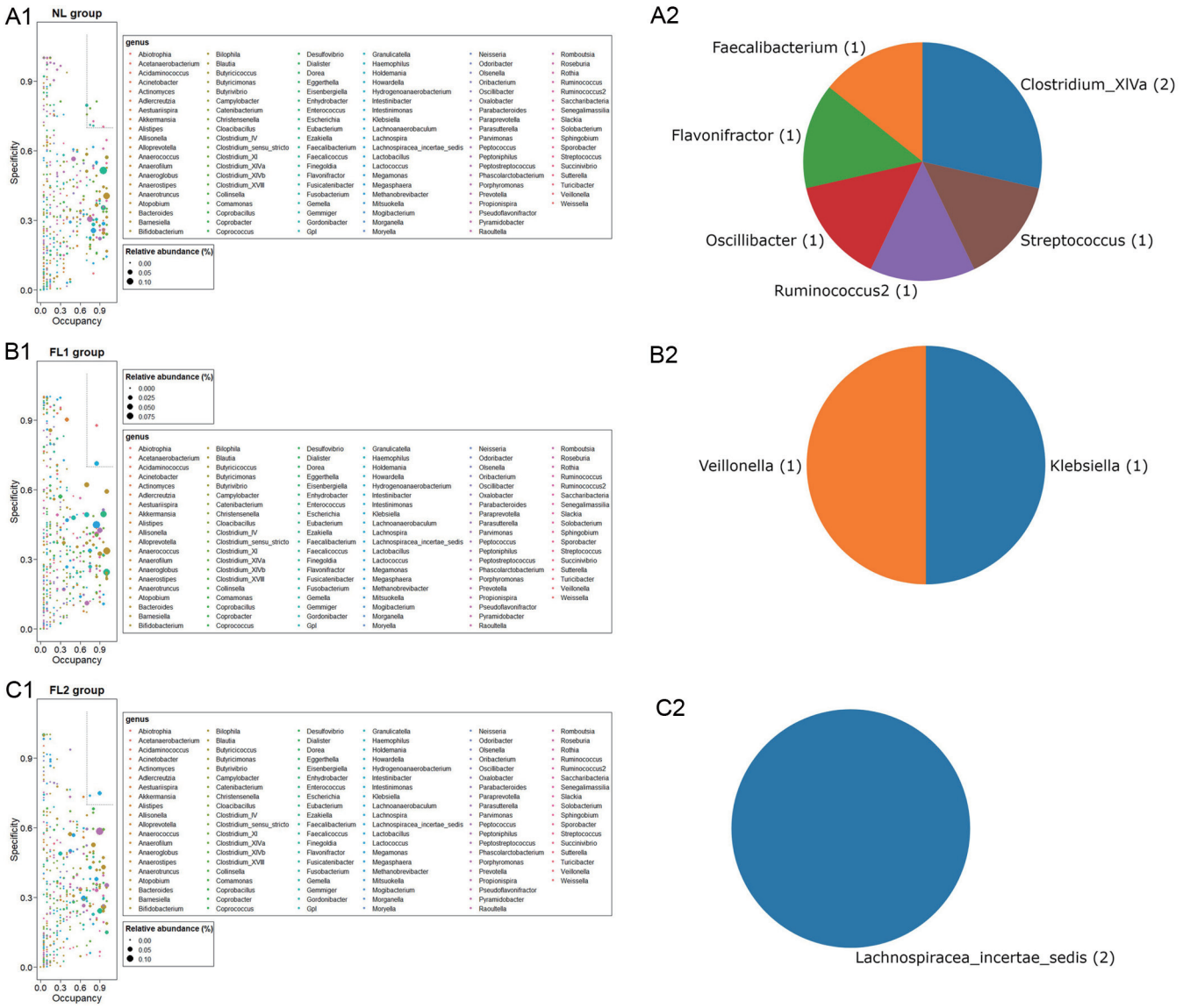

Fig. 4. Specificity–occupancy plot of genera identified in the all-patient group. The specificity–occupancy plots show the relative abundance above 0.01% for operational taxonomic units (OTUs) in each group; the x-axis represents occupancy, i.e. how well an OTU is distributed across all three groups [(normal liver, mild fatty liver (FL)], and moderately severe FL; the Y-axis represents specificity, i.e. whether they are also found in other groups (A1, B1, C1). Pie charts show the number of OTUs representing keystone genus species in each group (A2, B2, C2). FL1, mild fatty liver; FL2, moderately severe fatty liver; NL, normal liver.

with coronary artery disease[57] but increased in cases of NAFLD in obese youth.[58] An early-life nutrition study found this species to be associated with a diet high in processed foods.[59] Although the difference in the ages of FL1 and FL2 patients was not statistically significant (p=0.1), the mean age of FL1 patients was 50.65 and that of FL2 patients was 44.65 years. Younger people tend to eat more processed foods. Therefore, we hold the hypothesis that although *Fusicatenibacter* decreased in NAFLD patients, those with severe NAFLD tend to be more obese or eat more processed foods, thereby increasing its abundance to a higher level than in FL1 patients. Further study is needed to support this.

ALT and AST levels are common biomarkers of liver injury.[60] Although both were increased in the patients with NAFLD among the three groups in our study, the changes between them can be considered mild increases in aminotransferase levels (increases of <5 times the upper reference limit).[61–63] In the Western world, NAFLD is the most common cause of minor changes in liver enzyme levels.[60] Furthermore, the liver enzyme levels in HL patients were higher than in NHL patients. It was reported that metabolic syndromes, such as HL, increased the suspicion of the presence of NAFLD.[64,65] Thus, in our study, the levels of ALT and AST in NAFLD cases with HL participants were pronouncedly increased.

White blood cell count is a reliable, easily accessible, and low-cost inflammatory marker,[66] as well as an important predictor of NAFLD in Chinese people.[67,68] There are two possible avenues for WBC involvement at the start of NAFLD.[69] NAFLD is viewed as a liver-based manifestation of metabolic syndrome.[70,71] As a relationship between WBC count and metabolic syndrome components has been documented in previous studies,[71,72] metabolic syndrome may link WBC

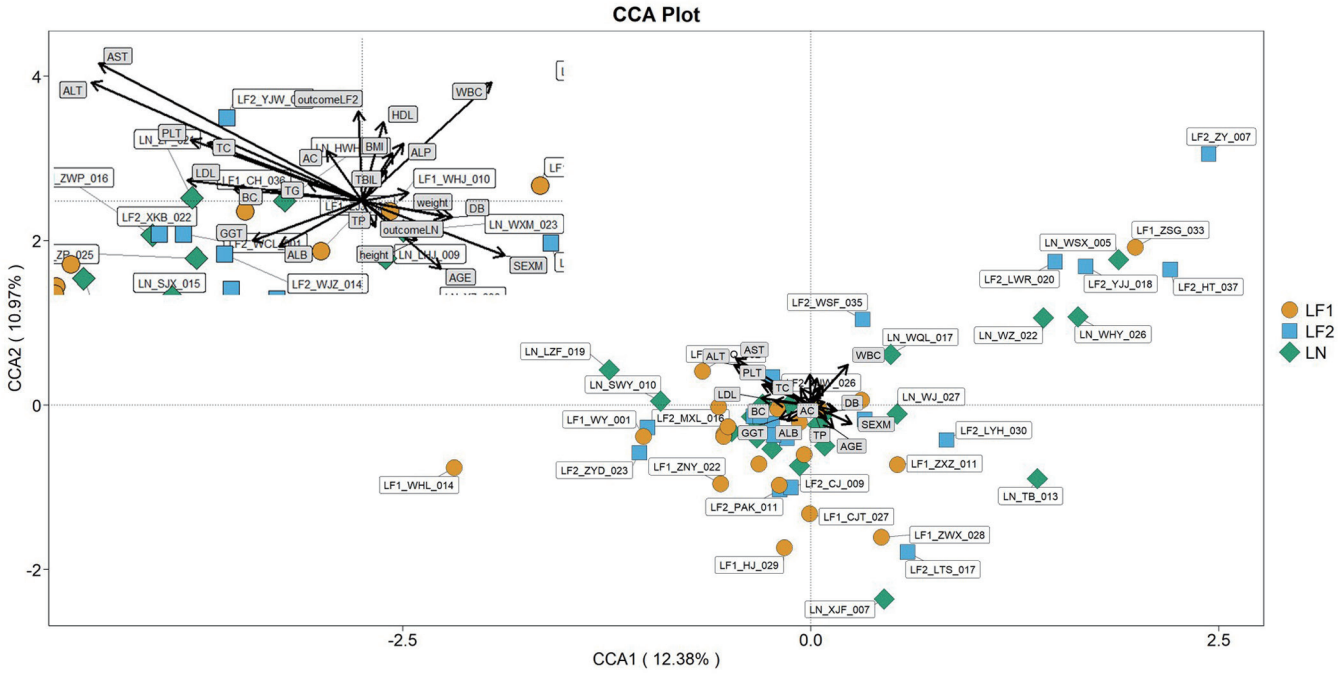

Fig. 5. Canonical correspondence analysis plot. The subplot in the top left corner is an enlarged version of the middle of the plot. Dots are used to represent different samples and arrows point from the origin to represent different clinical factors. The codes near the single dots are the sample identifications. The lengths of the arrows represent the intensity of the impact of the clinical factor on the community change, and the longer the length of the arrow, the greater the impact of the clinical factor. The angle between the arrow and the coordinate axis represents the correlation between this clinical factor and the coordinate axis; the smaller the angle, the higher the correlation. The vertical distance from the sample point to the extremely extended line of the clinical factor arrow indicates the strength of the effect of the environmental factor on the sample; the closer the sample point is to the arrow, the stronger the effect of the clinical factor on the sample. Samples located in the same direction as an arrow indicate that environmental factors are positively correlated with changes in the sample species community, and samples located in the opposite direction of an arrow indicate that environmental factors are negatively correlated with changes in the sample species community. The values in the labels of the axes in the images represent the proportion of species community variation, explained by the clinical factor combinations represented by the axes. LDL, low-density lipoprotein; HDL, high-density lipoprotein; TGs, triglyceride; TC, total cholesterol; GGT, γ-glutamyl transpeptidase; ALT, alanine transaminase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; ALB, albumin; DB, direct bilirubin; TB, total bilirubin; WBC, white blood cell; PLT, platelet; BMI, body mass index.

count and NAFLD. In our study, WBC count was selected as one of the most significant biochemical indexes associated with NAFLD. That may help reduce the medical burden on society because of its low cost, but further research on this is necessary. Furthermore, WBC count is commonly used to determine inflammatory state[73] and, accordingly, may be related to NAFLD. There are several limitations to our study. First, this was a small case study that included only one Chinese population, which may have led to bias. Secondly, the levels of NAFLD were classified by ultrasound but not pathology.

Conclusions

This study identified two bacteria, *Faecalibacterium* and *Ruminococcus 2* as being associated with NAFLD. The two bacteria, combined with three biochemical indexes, ALT, AST, and WBC count, helped to better distinguish moderate-to-severe NAFLD and healthy individuals. We speculate that regulating the health of gut microbiota may help to overcome NAFLD and provide new targets for clinicians to treat NAFLD.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conception of the study (KY, JZ, JX, FD, HW, HL), design of the study (KY, JZ, JX, FD, HW, HL), acquisition of the data (KY, JZ, JX, FD, ZD, WL, LW), interpretation of the data (K, JZ, JX, FD, ZD, WL, LW, ZL, WH), and drafting or substantive revision of the manuscript (KY, JZ, JX, F, HL, ZD).

Ethical statement

The study conformed to the ethical guidelines of the Helsinki Declaration (as revised in 2013) and is approved by the institutional ethics committee of Shenzhen People's Hospital (LL-KY-2021637). All subjects gave written informed consent in accordance with the Declaration of Helsinki. The written informed consent was obtained from patients.

Data sharing statement

Data that support the findings of this study are available in National Library of Medicine, reference number [PRJNA935081], reviewer link: https://dataview.ncbi.nlm.nih.gov/object/PRJNA935081?reviewer=ag0vq4auv2scd217s1s12r5eem.

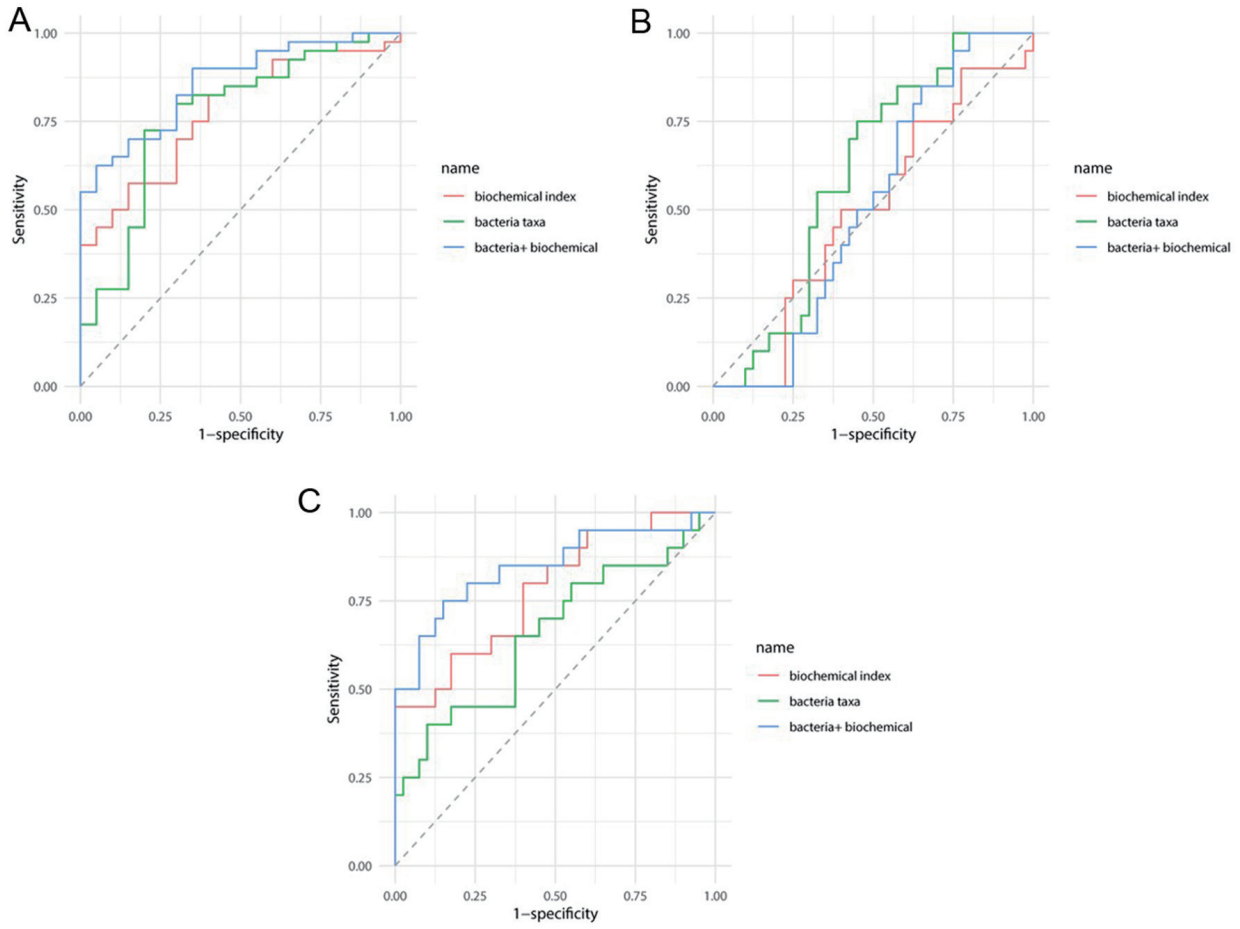

Fig. 6. Receiver operating characteristic (ROC) analysis. (A) ROC of the normal liver (NL) group. (B) ROC of the mild fatty liver (FL) group (FL1). (C) ROC of the moderate-to-severe FL group (FL2). The red line represents the biochemical index, the green line represents bacteria taxa, and the blue line represents (bacteria + biochemical).

References

[1] Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. Hepatology 2016;64(1):73–84. doi:10.1002/hep.28431, PMID:26707365.

[2] Powell EE, Wong VW, Rinella M. Non-alcoholic fatty liver disease. Lancet 2021;397(10290):2212–2224. doi:10.1016/S0140-6736(20)32511-3, PMID:33894145.

[3] European Association for the Study of the Liver (EASL)., European Association for the Study of Diabetes (EASD)., European Association for the Study of Obesity (EASO). EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. J Hepatol 2016;64(6):1388–1402. doi:10.1016/j.jhep.2015.11.004, PMID:27062661.

[4] Alexander M, Loomis AK, Fairburn-Beech J, van der Lei J, Duarte-Salles T, Prieto-Alhambra D, *et al*. Real-world data reveal a diagnostic gap in non-alcoholic fatty liver disease. BMC Med 2018;16(1):130. doi:10.1186/s12916-018-1103-x, PMID:30099968.

[5] Lazarus JV, Ekstedt M, Marchesini G, Mullen J, Novak K, Pericàs JM, *et al*. A cross-sectional study of the public health response to non-alcoholic fatty liver disease in Europe. J Hepatol 2020;72(1):14–24. doi:10.1016/j.jhep.2019.08.027, PMID:31518646.

[6] Younossi Z, Anstee QM, Marietti M, Hardy T, Henry L, Eslam M, *et al*. Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. Nat Rev Gastroenterol Hepatol 2018;15(1):11–20. doi:10.1038/nrgastro.2017.109, PMID:28930295.

[7] Jarvis H, Craig D, Barker R, Spiers G, Stow D, Anstee QM, *et al*. Metabolic risk factors and incident advanced liver disease in non-alcoholic fatty liver disease (NAFLD): A systematic review and meta-analysis of population-based observational studies. PLoS Med 2020;17(4):e1003100. doi:10.1371/journal.pmed.1003100, PMID:32353039.

[8] Yusuf S, Hawken S, Ounpuu S, Dans T, Avezum A, Lanas F, *et al*. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. Lancet 2004;364(9438):937–952. doi:10.1016/S0140-6736(04)17018-9, PMID: 15364185.

[9] Targher G, Day CP, Bonora E. Risk of cardiovascular disease in patients with nonalcoholic fatty liver disease. N Engl J Med 2010;363(14):1341–1350. doi:10.1056/NEJMra0912063, PMID:20879883.

[10] Adams LA, Lymp JF, St Sauver J, Sanderson SO, Lindor KD, Feldstein A, *et al*. The natural history of nonalcoholic fatty liver disease: a population-based cohort study. Gastroenterology 2005;129(1):113–121. doi:10.1053/j.gastro.2005.04.014, PMID:16012941.

[11] Farrell G, Schattenberg JM, Leclercq I, Yeh MM, Goldin R, Teoh N, *et al*. Mouse Models of Nonalcoholic Steatohepatitis: Toward Optimization of Their Relevance to Human Nonalcoholic Steatohepatitis. Hepatology 2019;69(5):2241–2257. doi:10.1002/hep.30333, PMID:30372785.

[12] Chiu CC, Ching YH, Li YP, Liu JY, Huang YT, Huang YW, *et al*. Nonalcoholic Fatty Liver Disease Is Exacerbated in High-Fat Diet-Fed Gnotobiotic Mice by Colonization with the Gut Microbiota from Patients with Nonalcoholic Steatohepatitis. Nutrients 2017;9(11):1220. doi:10.3390/nu9111220, PMID:29113135.

[13] Henao-Mejia J, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T, *et al*. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature 2012;482(7384):179–185. doi:10.1038/nature10809, PMID:22297845.

[14] Nguyen TL, Vieira-Silva S, Liston A, Raes J. How informative is the mouse for human gut microbiota research? Dis Model Mech 2015;8(1):1–16. doi:10.1242/dmm.017400, PMID:25561744.

[15] Loomba R, Seguritan V, Li W, Long T, Klitgord N, Bhatt A, *et al*. Gut Microbiome-Based Metagenomic Signature for Non-invasive Detection of Advanced Fibrosis in Human Nonalcoholic Fatty Liver Disease. Cell Metab 2017;25(5):1054–1062.e5. doi:10.1016/j.cmet.2017.04.001, PMID:28467925.

[16] Boursier J, Mueller O, Barret M, Machado M, Fizanne L, Araujo-Perez F, *et al*. The severity of nonalcoholic fatty liver disease is associated with gut dysbiosis and shift in the metabolic function of the gut microbiota. Hepatology 2016;63(3):764–775. doi:10.1002/hep.28356, PMID:26600078.

[17] Bajaj JS, Heuman DM, Hylemon PB, Sanyal AJ, White MB, Monteith P, *et al*. Altered profile of human gut microbiome is associated with cirrhosis

and its complications. J Hepatol 2014;60(5):940–947. doi:10.1016/j.jhep.2013.12.019, PMID:24374295.
[18] Caussy C, Tripathi A, Humphrey G, Bassirian S, Singh S, Faulkner C, *et al*. A gut microbiome signature for cirrhosis due to nonalcoholic fatty liver disease. Nat Commun 2019;10(1):1406. doi:10.1038/s41467-019-09455-9, PMID:30926798.
[19] Qin N, Yang F, Li A, Prifti E, Chen Y, Shao L, *et al*. Alterations of the human gut microbiome in liver cirrhosis. Nature 2014;513(7516):59–64. doi:10.1038/nature13568, PMID:25079328.
[20] Sasso M, Tengher-Barna I, Ziol M, Miette V, Fournier C, Sandrin L, *et al*. Novel controlled attenuation parameter for noninvasive assessment of steatosis using Fibroscan(®): validation in chronic hepatitis C. J Viral Hepat 2012;19(4):244–253. doi:10.1111/j.1365-2893.2011.01534.x, PMID:22404722.
[21] Sasso M, Miette V, Sandrin L, Beaugrand M. The controlled attenuation parameter (CAP): a novel tool for the non-invasive evaluation of steatosis using Fibroscan. Clin Res Hepatol Gastroenterol 2012;36(1):13–20. doi:10.1016/j.clinre.2011.08.001, PMID:21920839.
[22] Sasso M, Beaugrand M, de Ledinghen V, Douvin C, Marcellin P, Poupon R, *et al*. Controlled attenuation parameter (CAP): a novel VCTE™ guided ultrasonic attenuation measurement for the evaluation of hepatic steatosis: preliminary study and validation in a cohort of patients with chronic liver disease from various causes. Ultrasound Med Biol 2010;36(11):1825–1835. doi:10.1016/j.ultrasmedbio.2010.07.005, PMID:20870345.
[23] de Lédinghen V, Vergniol J, Capdepont M, Chermak F, Hiriart JB, Cassinotto C, *et al*. Controlled attenuation parameter (CAP) for the diagnosis of steatosis: a prospective study of 5323 examinations. J Hepatol 2014;60(5):1026–1031. doi:10.1016/j.jhep.2013.12.018, PMID:24378529.
[24] Kim SH, Lee JM, Kim JH, Kim KG, Han JK, Lee KH, *et al*. Appropriateness of a donor liver with respect to macrosteatosis: application of artificial neural networks to US images—initial experience. Radiology 2005;234(3):793–803. doi:10.1148/radiol.2343040142, PMID:15665225.
[25] Hernaez R, Lazo M, Bonekamp S, Kamel I, Brancati FL, Guallar E, *et al*. Diagnostic accuracy and reliability of ultrasonography for the detection of fatty liver: a meta-analysis. Hepatology 2011;54(3):1082–1090. doi:10.1002/hep.24452, PMID:21618575.
[26] Dasarathy S, Dasarathy J, Khiyami A, Joseph R, Lopez R, McCullough AJ. Validity of real time ultrasound in the diagnosis of hepatic steatosis: a prospective study. J Hepatol 2009;51(6):1061–1067. doi:10.1016/j.jhep.2009.09.001, PMID:19846234.
[27] Cao YT, Xiang LL, Qi F, Zhang YJ, Chen Y, Zhou XQ. Accuracy of controlled attenuation parameter (CAP) and liver stiffness measurement (LSM) for assessing steatosis and fibrosis in non-alcoholic fatty liver disease: A systematic review and meta-analysis. EClinicalMedicine 2022;51:101547. doi:10.1016/j.eclinm.2022.101547, PMID:35844772.
[28] Joint committee issued Chinese guideline for the management of dyslipidemia in adults. [2016 Chinese guideline for the management of dyslipidemia in adults]. Zhonghua Xin Xue Guan Bing Za Zhi 2016;44(10):833–853. doi:10.3760/cma.j.issn.0253-3758.2016.10.005, PMID:27903370.
[29] Magoč T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 2011;27(21):2957–2963. doi:10.1093/bioinformatics/btr507, PMID:21903629.
[30] Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods 2013;10(10):996–998. doi:10.1038/nmeth.2604, PMID:23955772.
[31] Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 2011;27(16):2194–2200. doi:10.1093/bioinformatics/btr381, PMID:21700674.
[32] Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, *et al*. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 2010;7(5):335–336. doi:10.1038/nmeth.f.303, PMID:20383131.
[33] Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 2010;26(19):2460–2461. doi:10.1093/bioinformatics/btq461, PMID:20709691.
[34] Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods 2013;10(10):996–998. doi:10.1038/nmeth.2604, PMID:23955772.
[35] Xiao L, Hu L, Chu H, Chen L, Yan J, Wang W, *et al*. Retrorsine Cooperates with Gut Microbiota to Promote Hepatic Sinusoidal Obstruction Syndrome by Disrupting the Gut Barrier. J Clin Transl Hepatol 2022;10(6):1086–1098. doi:10.14218/JCTH.2021.00398, PMID:36381109.
[36] Ruuskanen MO, Åberg F, Männistö V, Havulinna AS, Méric G, Liu Y, *et al*. Links between gut microbiome composition and fatty liver disease in a large population sample. Gut Microbes 2021;13(1):1–22. doi:10.1080/19490976.2021.1888673, PMID:33651661.
[37] Astbury S, Atallah E, Vijay A, Aithal GP, Grove JI, Valdes AM. Lower gut microbiome diversity and higher abundance of proinflammatory genus Collinsella are associated with biopsy-proven nonalcoholic steatohepatitis. Gut Microbes 2020;11(3):569–580. doi:10.1080/19490976.2019.1681861, PMID:31696774.
[38] Lim MY, You HJ, Yoon HS, Kwon B, Lee JY, Lee S, *et al*. The effect of heritability and host genetics on the gut microbiota and metabolic syndrome. Gut 2017;66(6):1031–1038. doi:10.1136/gutjnl-2015-311326, PMID:27053630.
[39] Bäckhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, *et al*. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci U S A 2004;101(44):15718–15723. doi:10.1073/pnas.0407076101, PMID:15505215.
[40] Ley RE, Bäckhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. Proc Natl Acad Sci U S A 2005;102(31):11070–11075. doi:10.1073/pnas.0504978102, PMID:16033867.
[41] Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. Nature 2006;444(7122):1022–1023. doi:10.1038/4441022a, PMID:17183309.
[42] Iino C, Endo T, Mikami K, Hasegawa T, Kimura M, Sawada N, *et al*. Significant decrease in Faecalibacterium among gut microbiota in nonalcoholic fatty liver disease: a large BMI- and sex-matched population study. Hepatol Int 2019;13(6):748–756. doi:10.1007/s12072-019-09987-8, PMID:31515740.
[43] Shen F, Zheng RD, Sun XQ, Ding WJ, Wang XY, Fan JG. Gut microbiota dysbiosis in patients with non-alcoholic fatty liver disease. Hepatobiliary Pancreat Dis Int 2017;16(4):375–381. doi:10.1016/S1499-3872(17)60019-5, PMID:28823367.
[44] Wong VW, Tse CH, Lam TT, Wong GL, Chim AM, Chu WC, *et al*. Molecular characterization of the fecal microbiota in patients with nonalcoholic steatohepatitis—a longitudinal study. PLoS One 2013;8(4):e62885. doi:10.1371/journal.pone.0062885, PMID:23638162.
[45] Da Silva HE, Teterina A, Comelli EM, Taibi A, Arendt BM, Fischer SE, *et al*. Nonalcoholic fatty liver disease is associated with dysbiosis independent of body mass index and insulin resistance. Sci Rep 2018;8(1):1466. doi:10.1038/s41598-018-19753-9, PMID:29362454.
[46] Duncan SH, Hold GL, Harmsen HJM, Stewart CS, Flint HJ. Growth requirements and fermentation products of Fusobacterium prausnitzii, and a proposal to reclassify it as Faecalibacterium prausnitzii gen. nov., comb. nov. Int J Syst Evol Microbiol 2002;52(Pt 6):2141–2146. doi:10.1099/00207713-52-6-2141, PMID:12508881.
[47] Miquel S, Martín R, Rossi O, Bermúdez-Humarán LG, Chatel JM, Sokol H, *et al*. Faecalibacterium prausnitzii and human intestinal health. Curr Opin Microbiol 2013;16(3):255–261. doi:10.1016/j.mib.2013.06.003, PMID:23831042.
[48] Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermúdez-Humarán LG, Gratadoux JJ, *et al*. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc Natl Acad Sci U S A 2008;105(43):16731–16736. doi:10.1073/pnas.0804812105, PMID:18936492.
[49] De Palma G, Nadal I, Medina M, Donat E, Ribes-Koninckx C, Calabuig M, *et al*. Intestinal dysbiosis and reduced immunoglobulin-coated bacteria associated with coeliac disease in children. BMC Microbiol 2010;10:63. doi:10.1186/1471-2180-10-63, PMID:20181275.
[50] Zhang J, Guo Z, Xue Z, Sun Z, Zhang M, Wang L, *et al*. A phylo-functional core of gut microbiota in healthy young Chinese cohorts across lifestyles, geography and ethnicities. ISME J 2015;9(9):1979–1990. doi:10.1038/ismej.2015.11, PMID:25647347.
[51] Sun M, Wu W, Liu Z, Cong Y. Microbiota metabolite short chain fatty acids, GPCR, and inflammatory bowel diseases. J Gastroenterol 2017;52(1):1–8. doi:10.1007/s00535-016-1242-9, PMID:27448578.
[52] Morrison DJ, Preston T. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. Gut Microbes 2016;7(3):189–200. doi:10.1080/19490976.2015.1134082, PMID:26963409.
[53] Zeng Q, Li D, He Y, Li Y, Yang Z, Zhao X, *et al*. Discrepant gut microbiota markers for the classification of obesity-related metabolic abnormalities. Sci Rep 2019;9(1):13424. doi:10.1038/s41598-019-49462-w, PMID:31530820.
[54] Tian T, Zhang X, Luo T, Wang D, Sun Y, Dai J. Effects of Short-Term Dietary Fiber Intervention on Gut Microbiota in Young Healthy People. Diabetes Metab Syndr Obes 2021;14:3507–3516. doi:10.2147/DMSO.S313385, PMID:34385825.
[55] Rodriguez-Diaz C, Taminiau B, García-García A, Cueto A, Robles-Díaz M, Ortega-Alonso A, *et al*. Microbiota diversity in nonalcoholic fatty liver disease and in drug-induced liver injury. Pharmacol Res 2022;182:106348. doi:10.1016/j.phrs.2022.106348, PMID:35817360.
[56] Takada T, Kurakawa T, Tsuji H, Nomoto K. Fusicatenibacter saccharivorans gen. nov., sp. nov., isolated from human faeces. Int J Syst Evol Microbiol 2013;63(Pt 10):3691–3696. doi:10.1099/ijs.0.045823-0, PMID:23625266.
[57] Hu X, Zhou R, Li H, Zhao X, Sun Y, Fan Y, *et al*. Alterations of Gut Microbiome and Serum Metabolome in Coronary Artery Disease Patients Complicated With Non-alcoholic Fatty Liver Disease Are Associated With Adverse Cardiovascular Outcomes. Front Cardiovasc Med 2022;8:805812. doi:10.3389/fcvm.2021.805812, PMID:35047580.
[58] Testerman T, Li Z, Galuppo B, Graf J, Santoro N. Insights from shotgun metagenomics into bacterial species and metabolic pathways associated with NAFLD in obese youth. Hepatol Commun 2022;6(8):1962–1974. doi:10.1002/hep4.1944, PMID:35344283.
[59] Matsuyama M, Morrison M, Cao KL, Pruilh S, Davies PSW, Wall C, *et al*. Dietary intake influences gut microbiota development of healthy Australian children from the age of one to two years. Sci Rep 2019;9(1):12476. doi:10.1038/s41598-019-48658-4, PMID:31462648.
[60] Giannini EG, Testa R, Savarino V. Liver enzyme alteration: a guide for clinicians. CMAJ 2005;172(3):367–379. doi:10.1503/cmaj.1040752, PMID:15684121.
[61] Green RM, Flamm S. AGA technical review on the evaluation of liver chemistry tests. Gastroenterology 2002;123(4):1367–1384. doi:10.1053/gast.2002.36061, PMID:12360498.
[62] Pratt DS, Kaplan MM. Evaluation of abnormal liver-enzyme results in asymptomatic patients. N Engl J Med 2000;342(17):1266–1271. doi:10.1056/NEJM200004273421707, PMID:10781624.
[63] Gopal DV, Rosen HR. Abnormal findings on liver function tests. Interpreting results to narrow the diagnosis and establish a prognosis. Postgrad Med 2000;107(2):100–114. doi:10.3810/pgm.2000.02.869, PMID:10689411.

[64] Harrison SA, Kadakia S, Lang KA, Schenker S. Nonalcoholic steatohepatitis: what we know in the new millennium. Am J Gastroenterol 2002;97(11):2714–2724. doi:10.1111/j.1572-0241.2002.07069.x, PMID:12425538.
[65] Brunt EM. Nonalcoholic steatohepatitis. Semin Liver Dis 2004;24(1):3–20. doi:10.1055/s-2004-823098, PMID:15085483.
[66] Schmidt MI, Duncan BB, Sharrett AR, Lindberg G, Savage PJ, Offenbacher S, *et al*. Markers of inflammation and prediction of diabetes mellitus in adults (Atherosclerosis Risk in Communities study): a cohort study. Lancet 1999;353(9165):1649–1652. doi:10.1016/s0140-6736(99)01046-6, PMID:10335783.
[67] Chung GE, Yim JY, Kim D, Kwak MS, Yang JI, Chung SJ, *et al*. Associations between White Blood Cell Count and the Development of Incidental Nonalcoholic Fatty Liver Disease. Gastroenterol Res Pract 2016;2016:7653689. doi:10.1155/2016/7653689, PMID:28070183.
[68] Wang S, Zhang C, Zhang G, Yuan Z, Liu Y, Ding L, *et al*. Association between white blood cell count and non-alcoholic fatty liver disease in urban Han Chinese: a prospective cohort study. BMJ Open 2016;6(6):e010342. doi:10.1136/bmjopen-2015-010342, PMID:27251683.
[69] Day CP, James OF. Steatohepatitis: a tale of two "hits"? Gastroenterology 1998;114(4):842–845. doi:10.1016/s0016-5085(98)70599-2, PMID:9547102.
[70] Tarantino G, Finelli C. What about non-alcoholic fatty liver disease as a new criterion to define metabolic syndrome? World J Gastroenterol 2013;19(22):3375–3384. doi:10.3748/wjg.v19.i22.3375, PMID:23801829.
[71] Jimba S, Nakagami T, Takahashi M, Wakamatsu T, Hirota Y, Iwamoto Y, *et al*. Prevalence of non-alcoholic fatty liver disease and its association with impaired glucose metabolism in Japanese adults. Diabet Med 2005;22(9):1141–1145. doi:10.1111/j.1464-5491.2005.01582.x, PMID:16108839.
[72] Gharipour M, Ramezani MA, Sadeghi M, Khosravi A, Masjedi M, Khosravi-Boroujeni H, *et al*. Sex based levels of C-reactive protein and white blood cell count in subjects with metabolic syndrome: Isfahan Healthy Heart Program. J Res Med Sci 2013;18(6):467–472. PMID:24250693.
[73] Pearson TA, Mensah GA, Alexander RW, Anderson JL, Cannon RO 3rd, Criqui M, *et al*. Markers of inflammation and cardiovascular disease: application to clinical and public health practice: A statement for healthcare professionals from the Centers for Disease Control and Prevention and the American Heart Association. Circulation 2003;107(3):499–511. doi:10.1161/01.cir.0000052939.59093.45, PMID:12551878.