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Altered gut microbial profile accompanied by abnormal short chain fatty acid metabolism exacerbates nonalcoholic fatty liver disease progression

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Dysregulation of the gut microbiome has associated with the occurrence and progression of non-alcoholic fatty liver disease (NAFLD). To determine the diagnostic capacity of this association, we compared fecal microbiomes across 104 participants including non-NAFLD controls and NAFLD subtypes patients that were distinguished by magnetic resonance imaging. We measured their blood biochemical parameters, 16 S rRNA-based gut microbiota and fecal short-chain fatty acids (SCFAs). Multi-omic analyses revealed that NAFLD patients exhibited specific changes in gut microbiota and fecal SCFAs as compared to non-NAFLD subjects. Four bacterial genera (*Faecalibacterium*, *Subdoligranulum*, *Haemophilus*, and *Roseburia*) and two fecal SCFAs profiles (acetic acid, and butyric acid) were closely related to NAFLD phenotypes and could accurately distinguish NAFLD patients from healthy non-NAFLD subjects. Twelve genera belonging to *Faecalibacterium*, *Subdoligranulum*, *Haemophilus*, *Intestinibacter*, *Agathobacter*, *Lachnospiraceae_UCG-004*, *Roseburia*, *Butyricicoccus*, *Actinomycetales_unclassified*, *[Eubacterium]_ventriosum_group*, *Rothia*, and *Rhodococcus* were effective to distinguish NAFLD subtypes. Of them, combination of five genera can distinguish effectively mild NAFLD from non-NAFLD with an area under curve (AUC) of 0.84. Seven genera distinguish moderate NAFLD with an AUC of 0.83. Eight genera distinguish severe NAFLD with an AUC of 0.90. In our study, butyric acid distinguished mild-NAFLD from non-NAFLD with AUC value of 0.83. And acetic acid distinguished moderate-NAFLD and severe-NAFLD from non-NAFLD with AUC value of 0.84 and 0.70. In summary, our study and further analysis showed that gut microbiota and fecal SCFAs maybe a method with convenient detection advantages and invasive manner that are not only a good prediction model for early warning of NAFLD occurrence, but also have a strong ability to distinguish NAFLD subtypes.

Keywords Non-alcoholic fatty liver disease, Gut microbiota, Short-chain fatty acids, Invasive manner

The global burden of non-alcoholic fatty liver disease (NAFLD) is substantial, both in terms of health and economic impact. With a prevalence rate of approximately 25% worldwide, There are very few drugs approved for the treatment of MASH, and it will be March 2024 before resmetirom becomes the first FDA-approved drug for the treatment of severe (F2 or F3) fibrotic MASH, and experimental studies of vitamin E in combination with Saroglitazar for the treatment of NASH have also begun. However, all of them are associated with various adverse effects[1,2]. The NAFLD compositions encompass a spectrum of conditions, ranging from hepatic lipid accumulation to non-alcoholic steatohepatitis (NASH) and liver cirrhosis, ultimately culminating in the development of hepatocellular carcinoma[3].

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There is growing evidence that the gut microbiota, microbial metabolites, and nonalcoholic fatty liver disease are closely related, which suggest a potential role of the microbiome in the etiology of NAFLD[4]. Changes of relative abundance and diversity in gut microbiota are associated with the progression of NAFLD[5], with specific gut microbiota signatures identified for each stage of NAFLD. The potential impact of the gut microbiota on extraintestinal organs is mediated through bacterial metabolism products, mainly including short-chain fatty acids (SCFAs), amino acids, and bile acids et al. These metabolites undergo alterations and play roles in the pathogenesis of NAFLD[6].

Although human cross-sectional studies have elucidated the significance of gut bacteria in the development of NAFLD[7]. The key bacterial species results are not always consistent[8–10]. There is currently a lack of a reliable and effective technique for early warning of NAFLD. To discover noninvasive, high-efficiency biomarkers of NAFLD, we recruited 104 volunteers including 21 healthy volunteers, 33 mild NAFLD patients, 20 moderate NAFLD patients, and 30 severe NAFLD patients.

In this study, we conducted a comprehensive analysis and comparison of the gut microbiome composition and functional potential between NAFLD patients and healthy controls using 16 S rRNA sequencing. In addition, by integrating metabolomics, we aim to further investigate the alterations in the gut microbiota and metabolome associated with patients with mild, moderate and severe steatosis and to determine their important role in the initiation and progression of the disease.

Methods

Ethics statement

This study was approved by Lianshui People's Hospital Affiliated to kangda college, Nanjing Medical University (Jiangsu Province, China). The study adhered to the principles outlined in the Helsinki Declaration and received ethical approval from the Medical Ethics Committee of Lianshui People's Hospital (Ethics No. 20230301-1). Written informed consent was obtained from all participants prior to sample collection.

Study design and participants

The present study was a cross-sectional investigation conducted at a single center. All patients were recruited at Lianshui People's Hospital from March to May 2023. Feces and blood samples were collected from all participants to characterize the blood biochemical parameters, gut microbiota, and fecal metabolites. The inclusion criteria for volunteers specify individuals ≥ 18 years old, with body mass index (BMI) ≥ 25. Exclusion criteria include individuals < 18 or > 65 years of age; patients with type 2 diabetes or other metabolic disorders; those adhering to special diets, such as vegetarianism, participating in weight-loss regimens, or experiencing malnutrition; individuals with a history of organ transplantation, organ failure, malignant tumors, or hereditary diseases; individuals with specific conditions leading to fatty liver, such as viral hepatitis, drug-induced liver disease, total parenteral nutrition, hepatic steatosis, autoimmune liver disease, and others; as well as individuals using immunosuppressive agents or consuming excessive alcohol (more than 10 g per day for women and more than 20 g per day for men).The specific process is shown in Fig. 1.

Grades of NAFLD

We used an MRI-PDFF value to distinguish the degree of progression of NAFLD in 104 participants. There are categorized into four groups including Healthy volunteers (also defined as non-NAFLD, None group), mild NAFLD patients (Mild group), moderate NAFLD patients (Moderate group), and severe NAFLD patients (Severe group).

The quantification of liver fat was performed using a 3 Tesla Phillips MRI machine (Philips, Netherlands). The selected sequence is the mDixon-Quant sequence, which uses low reflection angle to reduce T1 bias. Six echoes are scanned to correct for T2 effect, and fat signals are modeled to obtain the quantitative parameter assessed using MRI-estimated proton density fat fraction (MRI-PDFF)[11], which can describe the fat content and distribution in the entire liver.

When reading the PDFF value, select 1 measurement area from each of the 9 liver segments, with a size ranging from 100 to 200 (mm^2). Try to avoid blood vessels, bile ducts, lesions, and artifacts, and ultimately take the average value to represent the average fat content of the patient's entire liver. The grading criteria for liver steatosis are evaluated by recommended MRI-PDFF thresholds shown in Table 1.

Anthropometric measurements

Participants' body composition was measured using the body composition analyzer InBody-S10, including BMI, Fat percentage, Obesity degree, Waist-Hip Ratio, and Visceral fat area.

Sample collection and processing

The fresh fecal samples (200–300 mg) from participants were collected and then transferred into sterile 5 mL tubes. One tube was designated for gut microbiota sequencing, while the other was allocated for metabolite detection. Each sterile tube was appropriately labeled, put in an ice box, and then instantly transported to the laboratory. Next, specimens intended for metabolite analysis were rapidly frozen in liquid nitrogen before being stored at -80 °C. The following morning, peripheral venous fasting blood samples were drawn from participants using labeled test tubes. Then centrifuged the blood samples to obtain serum, of which 0.5 mL was aliquoted into properly labeled 1.5 mL centrifuge tubes and stored at -80 °C until further use. Clinical information was obtained from patients, while basic information was obtained from volunteers.

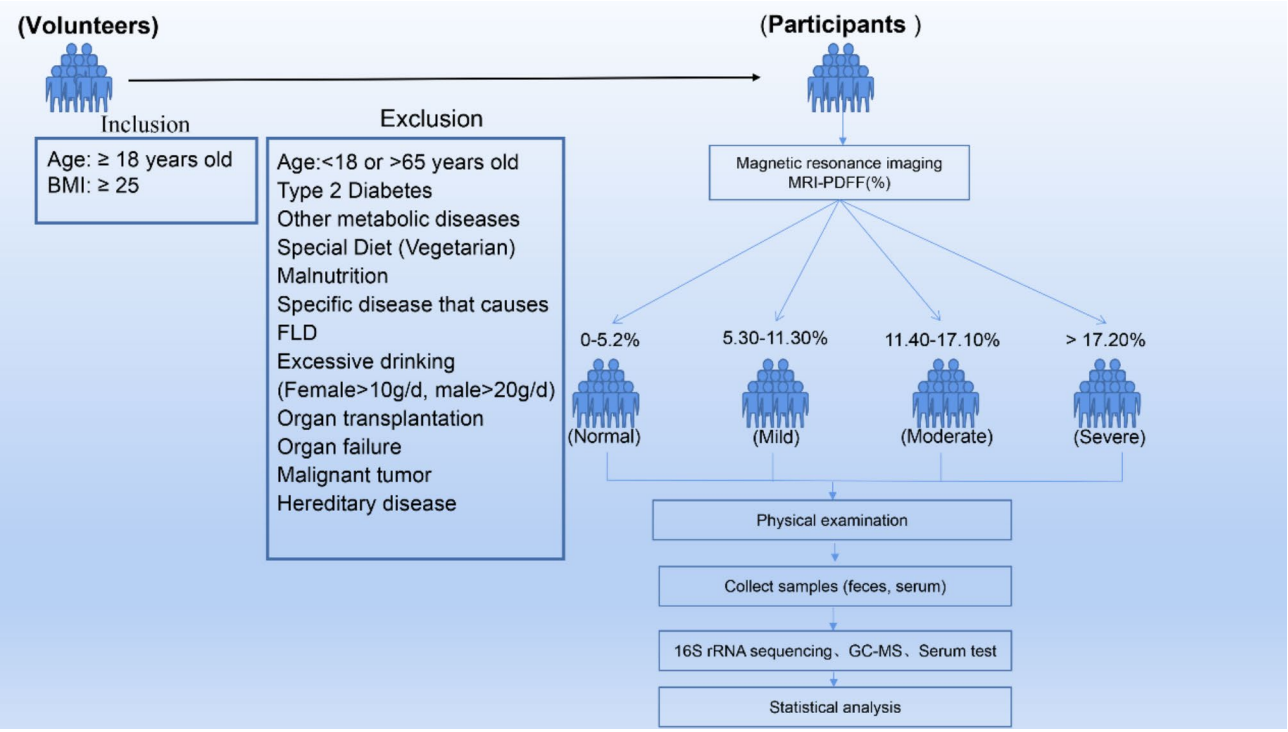

Fig. 1. Inclusion and exclusion criteria and experimental procedures of study subjects.

Grades	MRI-PDFF percentage
Normal	0-5.20%
Mild	5.30–11.30%
Moderate	11.40–17.10%
Severe	> 17.20%

Table 1. MRI-PDFF percentage and grades. MRI-PDFF, magnetic resonance imaging–estimated proton density fat fraction.

Serological indicators

Serum GLU, TC, TG, HDL-C, LDL-C, AST and ALT were measured by automatic biochemical analyzer. each index repeated measurement three times.

Gut microbiota analysis

The QIAamp DNA Stool Mini Kit was utilized to extract total bacterial DNA from frozen feces. According to the full-length 16 S primers (the V1-V9 regions of probes used 27 F, 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R, 5′-GGTTACCTTGTTACGACTT-3′) were synthesized for PCR amplification and purified using a primer pair. Sequencing was performed using the Illumina MiSeq platform at Shanghai BIOTREE Co., Ltd (Shanghai, China). The method used to assess the gut microbial profile is available in the **Supplementary Method**.

Targeted metabolomic analysis of SCFAs in fecal samples

Fecal samples were collected for quantification analysis of SCFA profile using gas chromatography-mass spectrometer (SHIMADZU GC2030-QP2020 NX), including acetate, propionate, butyrate, isobutyric acid, valeric acid, isovaleric acid, and caproic acid. SCFAs detection was performed at Shanghai BIOTREE Co., Ltd., Shanghai China. Further materials and methods details are available in the **Supplementary Method**.

Statistical analysis

Clinical parameters

Data of clinical parameters are expressed as mean ± standard deviation (SD). The significance of the differences between the groups was determined using one-way analysis of variance (ANOVA) conducted with GraphPad Prism 8 software (USA). The Wilcoxon rank-sum test was employed to compare variables exhibiting unequal variances. P value less than 0.05 was considered as statistical significance.

Sequencing data analysis

The microbiota was evaluated using QIME2 and R package 3.5.1. To compare the relative level of the rich taxa at the level of phylum, family, and genus among the groups, the one-way analysis of variance (ANOVA) and the post-hoc least significance tests were carried out. The α-diversity of intestinal flora microbial community was assessed using QIME2, which included Chao1, Shannon, and Simpson indices. Based on Jaccard, Principal coordinate analysis (PCoA) and nonmetric multidimensional scaling (NMDS) is used to evaluate β-diversity. The significant difference in β-diversity was analyzed through permutation multivariate analysis of variance (PERMDISP). In addition, LEfSe (linear discriminant analysis (LDA) effect size) was employed to identify biomarkers rich in taxa and functional pathways by calculating LDA scores (greater than 3) between groups, and R package 3.5.1 was used to draw heat maps. The data were expressed as mean ± mean standard error (SEM) and $P < 0.05$ as the significance threshold.

Targeted SCFAs metabolomics data analysis

In this study, 11 SCFAs metabolites were examined. The final dataset, which includes information on compound name, sample name, and concentration, was imported to SIMCA16.0.2 software (Umea, Sweden) for multivariate analysis. The data was scaled and logarithmically transformed in order to mitigate the impact of both noise and high variance of the variables. After these transformations, the sparse partial least squares discriminant analysis (sPLS-DA) was conducted to visually represent the distribution and the grouping patterns of the samples.

Spearman correlation analysis

The associations among the levels of gut microbiota, SCFAs, and tryptophans, as well as the relationships between these two types of metabolites and clinical parameters, were determined. The interconnections among these features were visualized using the *pheatmap* package in R software.

Results

General characteristics of the study participants

We enrolled 104 patients, a mean age of 36.9y (range 21 to 61), and marginal female preponderance (57.7%). The participants were categorized into four subgroups including normal, mild, moderate, and severe groups using their histological characteristics. A total of 21 healthy control (Normal) participants and 83 NAFLD patients took part in the study (NAFLD patients included those in the mild [Mild, $n = 33$] group, those in the moderate [Moderate, $n = 20$] group, and those in the severe [Severe, $n = 30$] group).

First, we compared the anthropometric and biochemical characteristics of control participants, and those with NAFLD (mild-, moderate-, and severe) (Table 2). The detailed clinical phenotype data were showed in Table 2. The participants with different grades of NAFLD had significantly higher BMI, fat percentage (%), obesity degree, visceral fat area, and TG than the normal participants ($p < 0.05$). However, the participants with severe NAFLD only had significantly higher ALT, and AST concentration than the normal participants ($p < 0.05$). As moreover, there was no difference in age, TC, LDL-C, HDL-C, and waist-hip ratio compared the patients with mild-, moderate-, and severe-NAFLD to normal controls ($p > 0.05$, Table 2).

Parameter	Normal	Mild	Moderate	Severe	P_{-vale}
Samples	21	33	20	30	
Sex(F/M)	13/8	17/16	7/13	7/23	
Age (mean ± SD)	38.38 ± 10.88	38.55 ± 9.75	38.75 ± 11.17	35.2 ± 8.80	0.50
Anthropometric measurements (mean ± SD)					
BMI (kg/m^2)	28.06 ± 2.84^b	31.42 ± 4.15^a	32.79 ± 4.62^a	31.81 ± 3.88^a	0.001
MRI-PDFF (%)	3.09 ± 0.70^d	7.32 ± 2.17^c	12.85 ± 2.19^b	23.27 ± 6.71^a	0.001
Fat percentage (%)	31.06 ± 5.37^b	35.27 ± 5.88^a	35.41 ± 5.83^a	33.56 ± 5.3^{a,b}	0.03
Obesity degree	131.32 ± 14.34^b	146.15 ± 18.35^a	151.41 ± 20.99^a	145.99 ± 17.01^a	0.002
Waist-Hip Ratio	0.92 ± 0.04	0.95 ± 0.10	0.96 ± 0.13	0.97 ± 0.096	0.32
Visceral fat area	112.64 ± 28.76^b	155.27 ± 58.15^a	183.97 ± 62.35^a	161.30 ± 47.467^a	0.001
Serological indicators (mean ± SD)					
AST (U/L)	19.38 ± 5.68^b	18.85 ± 4.07^b	23.95 ± 8.02^b	39.27 ± 22.24^a	< 0.001
ALT (U/L)	23.10 ± 12.08^b	23.64 ± 8.07^b	35.45 ± 15.95^b	75.77 ± 51.76^a	< 0.001
TC (mmol/L)	4.46 ± 0.72	4.90 ± 0.87	4.61 ± 0.92	4.67 ± 0.70	0.25
LDL-C (mmol/L)	2.70 ± 0.58	3.09 ± 0.80	2.88 ± 0.77	2.94 ± 0.61	0.25
HDL-C (mmol/L)	1.06 ± 0.14	1.10 ± 0.16	1.03 ± 0.12	1.07 ± 0.18	0.48
TG (mmol/L)	1.36 ± 0.79^b	2.07 ± 1.20^a	2.26 ± 1.68^a	2.35 ± 1.52^a	0.008
GLU (mmol/L)	5.05 ± 0.84	5.05 ± 0.64	5.20 ± 0.62	5.17 ± 0.58	0.80

Table 2. Baseline characteristics of patients with MRI-PDFF-confirmed NAFLD. BMI, Body mass index; SD, standard deviation; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TC, total cholesterol; TG, triglycerides.

Alterations of gut microbiota composition in normal and NAFLD patients based on 16s rRNA sequencing

The gut microbiota is closely associated with the etiology of NAFLD. In order to identify effective biomarkers capable of distinguishing between different grades of NAFLD and normal participants, we conducted a 16 S rRNA-based analysis on fecal samples to determine their relative abundance (Figure S1).

The alpha diversity results from Simpson index showed a significant difference among Normal, Mild, Moderate, and Severe groups, suggesting that the overall structure of the gut microbiota was obviously changed with the NAFLD status ($p = 0.039$, Fig. 2b). Similar result from Shannon index among four groups, but with no statistical difference ($p = 0.075$, Fig. 2a).

Moreover, both bacteria diversity (Shannon and Simpson) was significantly reduced in subjects with mild-, moderate-, and severe-NAFLD ($p < 0.05$, paired samples t-test, Figure S2a–f) when compared with the healthy control respectively. These results suggest subjects with NAFLD had a significant decrease in bacteria richness compared to normal subjects.

Based on principal coordinates analysis (PCoA) and multidimensional scaling (NMDS) of Jaccard dissimilarities, patients with different subgroups including Normal, Mild, Moderate, and Severe showed distinct clustering of their gut microbiota composition ($p < 0.05$, PERMDISP test, Fig. 2c,d). Gut microbiota composition of these three NAFLD subtypes was distinct from that of controls ($p < 0.05$. PERMDISP test, Figure S3a–f).

The taxonomic analysis conducted at the family and genus levels revealed significant alterations in the relative abundance of specific taxa across different groups (Figs. S4–8). Subsequently, we performed the linear discriminative analysis (LDA) effect size (LEfSe) analysis for Mild, Moderate, and Severe independently with the control group. Eight bacterial family were identified to be associated with different NAFLD subtypes (mild-, moderate-, and severe-NAFLD) ($p < 0.05$, LDA > 3, LEfSe, Figure S6–8a). At the family level, subjects with NAFLD subtypes shared similar compositional alterations driven by depletion of *Ruminococcaceae*, and *Pasteureuaceae* compared with controls. Moreover, fiver bacterial family showed inconsistent trends. For example, *Actinomycetales* family decreased in Mild group, *Oxalobacteraceae* family in Moderate group, and *Anaerovoracaceae*, *Micrococcaceae*, *Nocardiaceae* family in Severe group (Fig. S6–8a).

A total of 19 bacteria genera were identified to be associated with different NAFLD subtypes. Four bacterial genera including *Faecalibacterium*, *Subdoligranulum*, *Haemophilus*, *and Roseburia* were found to be significantly decreased in all three NAFLD subtypes (**$p < 0.05$**, **LDA > 3**, Fig. 2e–g; Figure S6–8b). As moreover, the bacteria *Weissella*, and *Butyricicoccus*, in Mild group, *Eubacterium_hallii_group*, *Lachnospiraceae_UCG_004*, and *Intestinibacter*, in Moderate group,, *Rothia*, *Erysipelotrichaceae_UCG_003*, and *Eubacterium_ventriosum_group* in Severe group were found to be significantly decreased ($p < 0.05$, **LDA > 3**, Fig. 2e-g; Figure S6–8b). the bacteria Actinomycetales_unclassified in Mild group, Lactobacillus, and Herminiimonas in Moderate group, Tyzzerella, Rhodococcus, and Eubacterium_nodatum_group in Severe group were found to be significantly increased ($p < 0.05$, **LDA > 3**, Fig. 2e-g; Figure S6–8b).The subsequent ROC analysis demonstrated that these gut bacterial sets exhibited discriminative potential in distinguishing NAFLD subtypes (Mild, Moderate, and Severe) from the control group, with corresponding AUC values of 0.84, 0.83, and 0.90 respectively (Fig. 3b–d).

We finally selected 4 genera including *Faecalibacterium*, *Subdoligranulum*, *Haemophilus*, and *Roseburia* as a gut bacterial biomarker set for NAFLD occur. We conducted a correlation analysis between the relative abundance of bacterial genera and seven key blood biochemical indices to identify pivotal bacteria that exhibit a strong association with the occurrence of NAFLD. *Faecalibacterium* and *Subdoligranulum* were negative to NAFLD risk indicators (MRI-PDFF, AST, ALT) (Spearman's correlation, FDR < 0.1; Fig. 3a). In addition, *Faecalibacterium* and *Subdoligranulum* were also negative to obesity indicators (BMI, and obesity degree), and *Haemophilus* were negatively associated with fat percentage ($p < 0.05$).

Short-chain fatty acids signatures in different subtypes of NAFLD

The fecal microbiota in NAFLD patients presented a significant reduction or disappearance in the relative abundance of several beneficial genera belonging to *Ruminococcaceae*, *Lachnospiraceae*, and *Pasteureuaceae*, which may generate SCFAs[7]. To support the findings related to the diminished SCFA-generating microbiomes, we analyzed the expression differences of SCFAs in the early and late stages of NAFLD (mild-, moderate-, and severe-NAFLD).

A distinct separation was observed between the Mild, Moderate, and Severe group with the healthy non-NAFLD group using the sparse partial least squares discriminant analysis (sPLS-DA) model established based on the detected fecal SCFAs (Fig. 4).

As illustrated in (Fig. 5a–f, Figure S9), acetic acid and butyric acid levels were significantly reduced in three stages of NAFLD ccompared with non-NAFLD. Subsequently, we determined the fecal relative abundance of eleven SCFAs. Between healthy subjects and different subtypes of NAFLD, the relative abundance of acetic acid and butyric acid (%) tended to be lower in NAFLD (Fig. 5g–l). Specially, the relative abundance of acetic acid (%) and butyric acid (%) decreased significantly in severe NAFLD (Fig. 5i,l). Nevertheless, other short-chain fatty acids, including propionic acid, isobutyric acid, isovaleric acid, valeric acid, hexanoic acid, heptanoic acid, octanoic acid, and nonanoic acid, were not found to be significantly different in early and late NAFLD (Fig. 6a–f; Figure S9).The aforementioned findings collectively suggest that acetic acid and butyric acid may exert pivotal roles in the pathogenesis and progression of NAFLD.

The area under the curves (AUCs) were utilized to evaluate the value of SCFAs in the early identification of healthy subjects and NAFLD (Fig. 7). The results showed that the AUCs of acetic acid were 0.70, 0.84, and 0.70 for mild-, moderate- and severe-NAFLD against healthy (non-NAFLD) subjects, respectively (Fig. 7b–d). The AUCs of butyric acid were 0.83, 0.68, and 0.62 for mild-, moderate- and severe-NAFLD against healthy subjects, respectively (Fig. 7b–d).

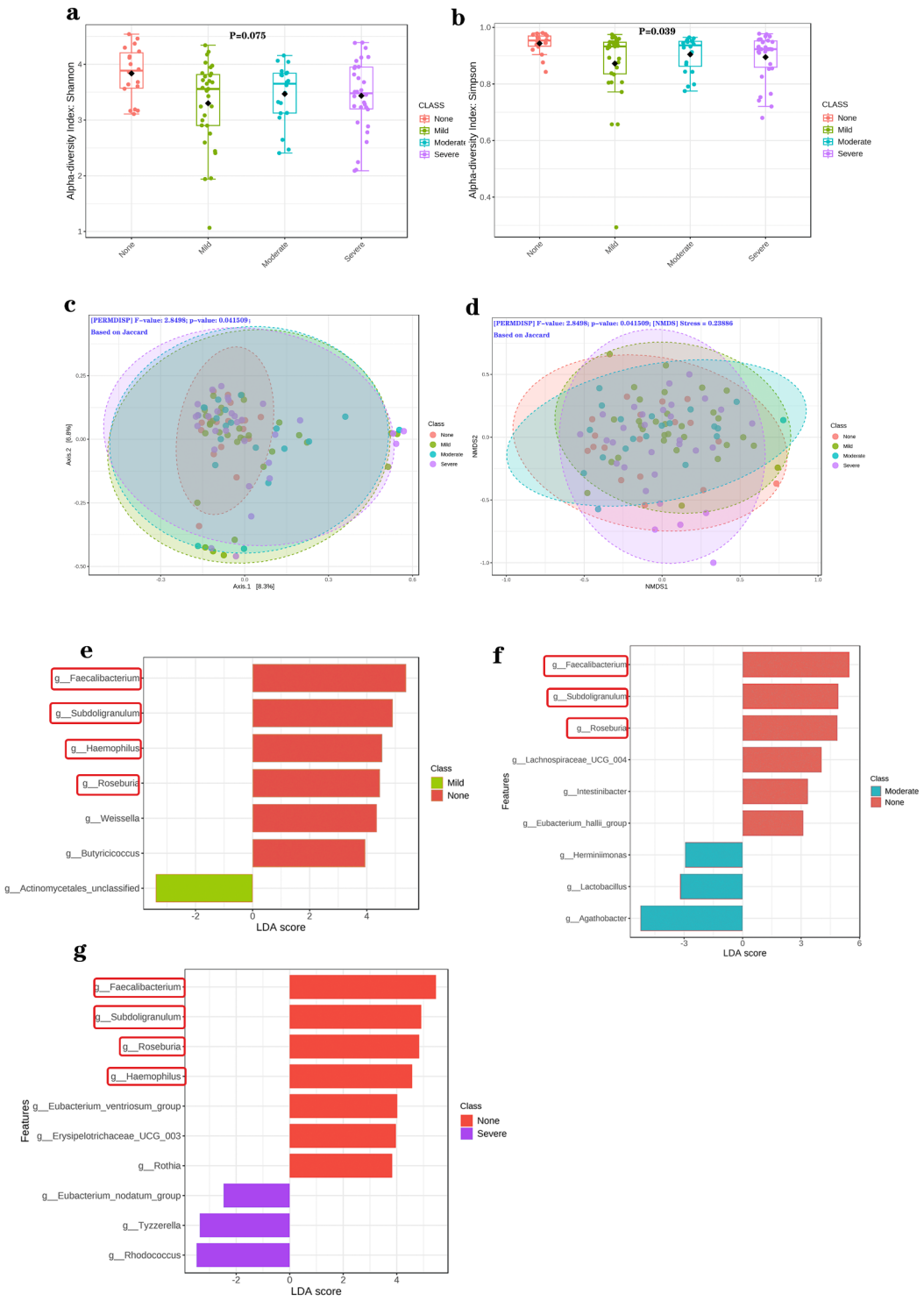

Fig. 2. Major alterations in the gut microbiome profiles of the NAFLD subtypes group compared with the non-NAFLD control group (**a**) and (**b**), Shannon and Simpson indices on OTU levels. (**c**) and (**d**), principal coordinates analysis (PCoA) and nonmetric multidimensional scaling (NMDS) of Jaccard dissimilarities. (**e**–**g**), the linear discriminative analysis (LDA) effect size (LEfSe) analysis.

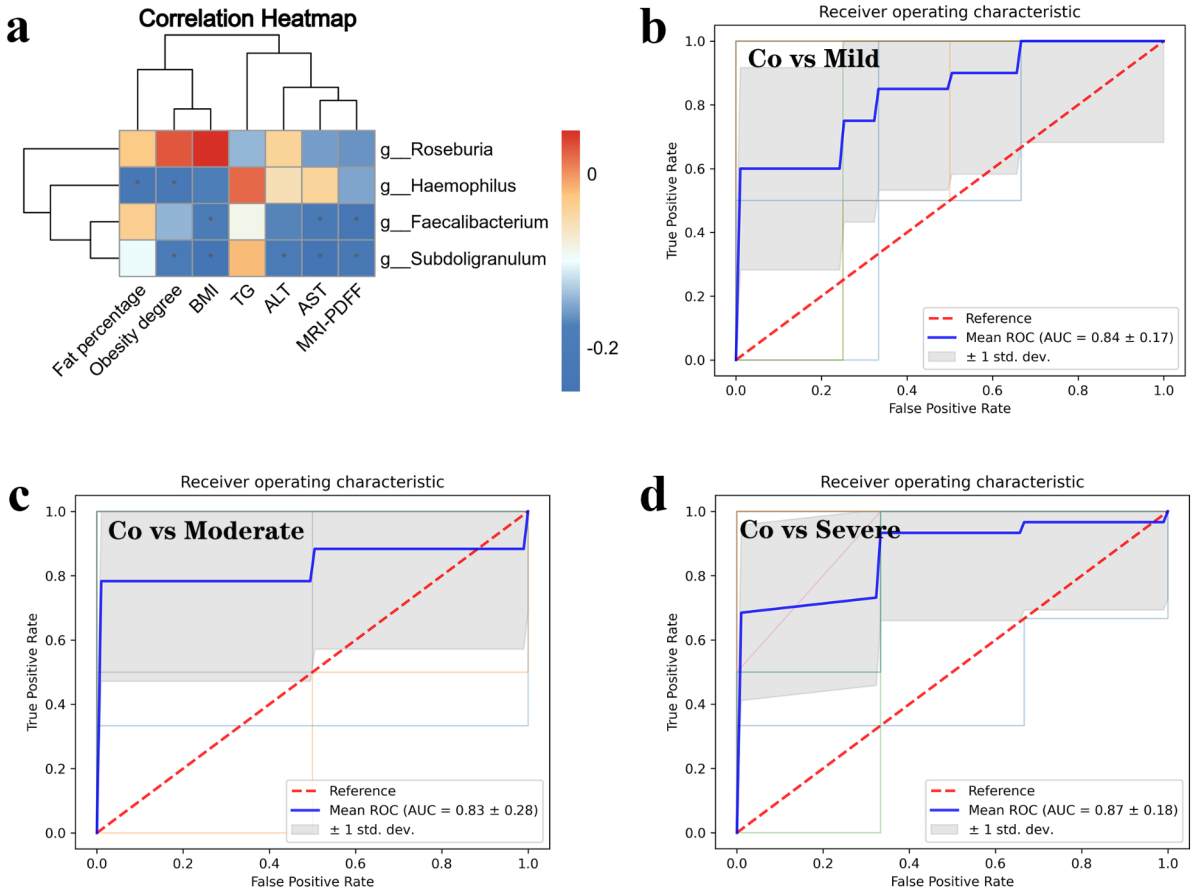

Fig. 3. Heatmap of Spearman correlation coefficients between four specific species and key blood biochemical indices (**a**) and four specific species to build the prediction model yielded an AUC based on ROC analysis (**b**–**d**).

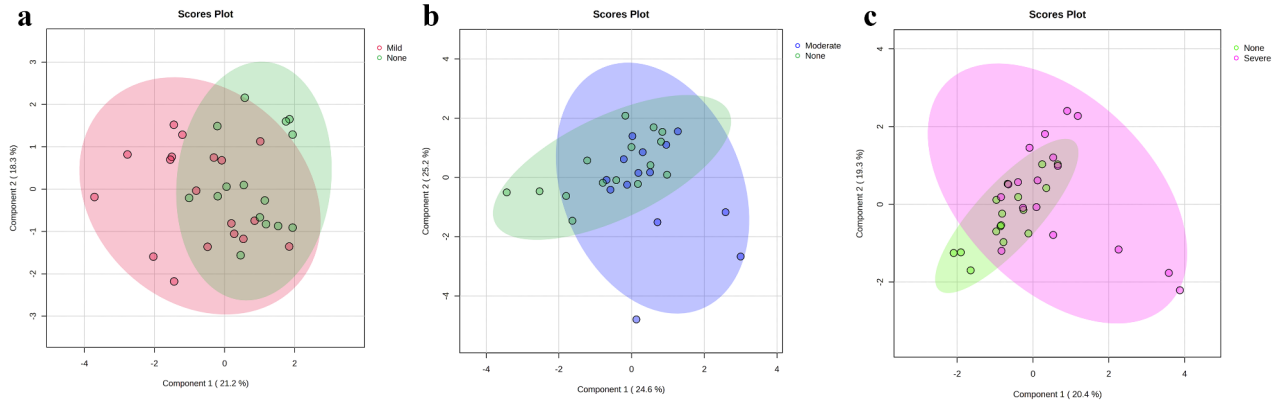

Fig. 4. Sparse partial least squares discriminant analysis (sPLS-DA) score plot of fecal SCFAs profiles compared health non-NAFLD to mild-NAFLD (**a**), moderate-NAFLD (**b**), and severe-NAFLD (**c**).

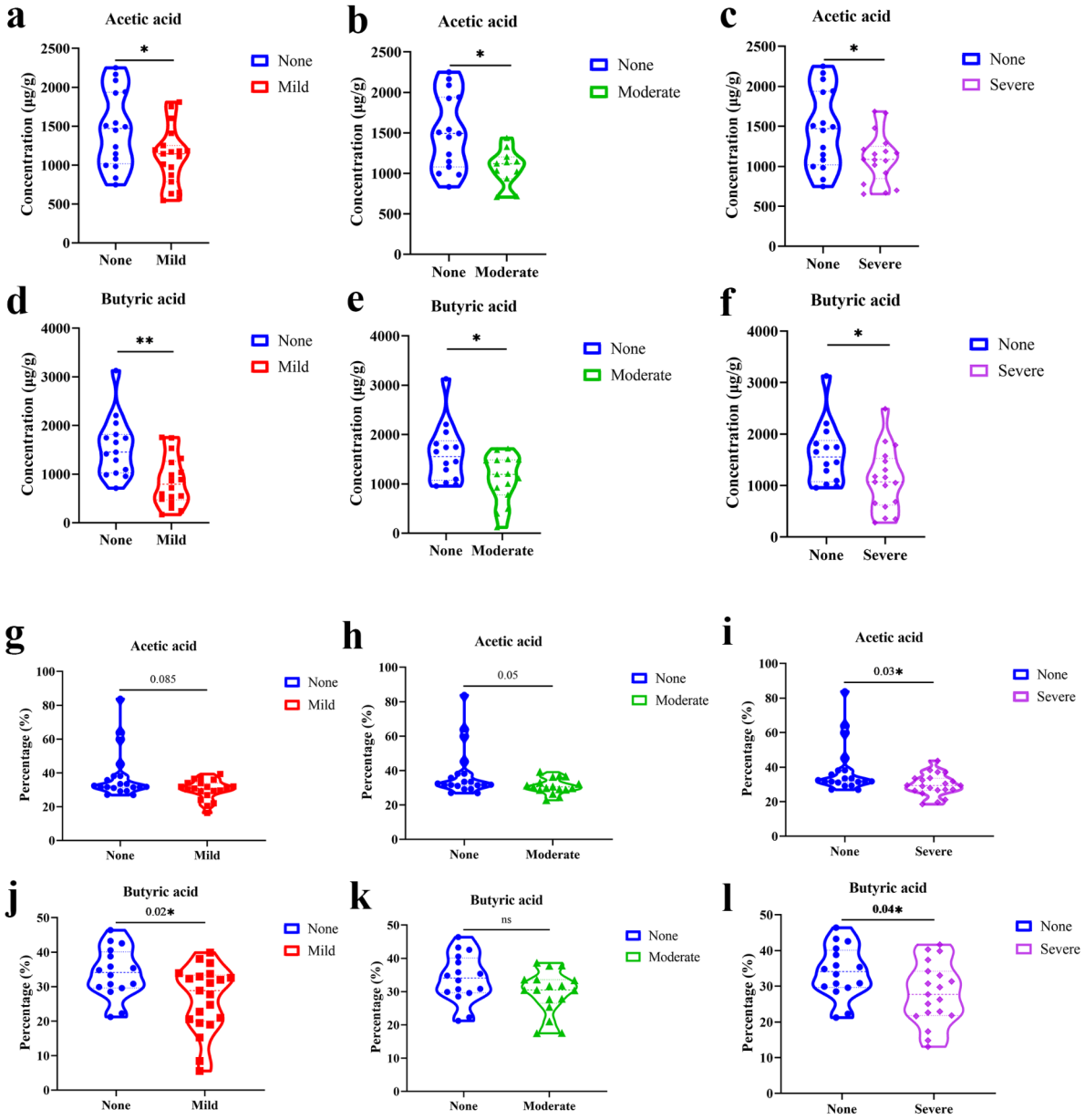

Fig. 5. The difference of short-chain fatty acids (SCFAs) concentrations (**a**–**f**) and relative abundance (**g**–**l**) among subjects with health non-NAFLD to mild-NAFLD, moderate-NAFLD, and severe-NAFLD.

Next, we correlated the two key SCFAs with the twelve key blood biochemical indices by Spearman's correlation (Fig. 7a). We observed that acetic acid was negative to the indicator of MRI-PDFF and fat percentage.

Discussion

Current diagnosis of NAFLD rests on both clinical and biopsy-based information, with liver biopsy being the sole diagnostic procedure capable of reliably assessing its severity[12]. However, this invasive method is associated with potentially lethal side effects. Consequently, accurately predicting and timely intervening to prevent the occurrence of NAFLD remains a challenge. This analysis by synthesis based on blood biochemical indexes, gut microbiota, and fecal SCFAs reveals an intimate relationship between gut microbiota and the development of NAFLD, thereby facilitating its clinical diagnosis.

We conducted a comprehensive analysis of their blood biochemical parameters, gut microbiota based on 16 S rRNA sequencing, and fecal metabolites. Our study examined and compared the profiles of blood parameters, gut microbiota and fecal SCFAs across various groups. In the end, we successfully derived a noninvasive NAFLD biomarker set including four gut genera (*Faecalibacterium*, *Subdoligranulum*, *Haemophilus*, and *Roseburia*) and

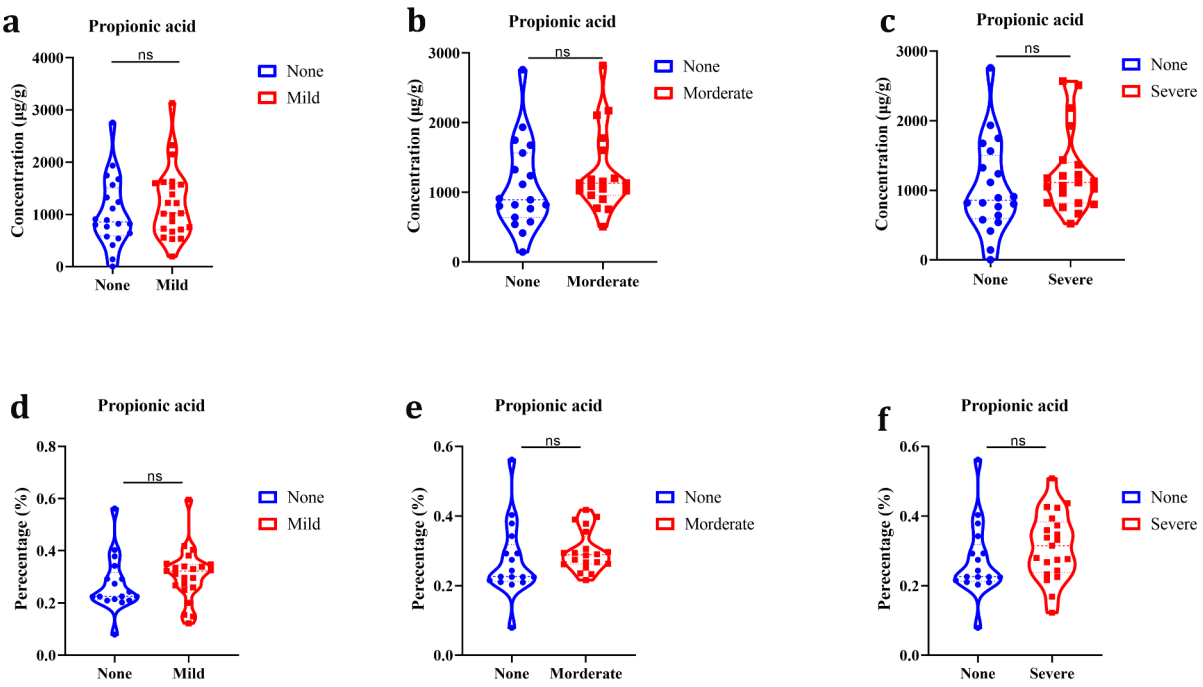

Fig. 6. The difference of Propionic acid concentrations and relative abundance (**a**–**f**) among subjects with health non-NAFLD to mild-NAFLD, moderate-NAFLD, and severe-NAFLD.

two fecal SCFAs profiles (acetic acid, and butyric acid), which can accurately distinguish NAFLD subtypes from non-NAFLD subjects.

Clinical studies strongly support the role of gut microbial dysbiosis in NAFLD[13]. The present study showcases the ability of a fundamental gut microbiome signature to accurately discern NAFLD. In our study, four gut genera, *Faecalibacterium*, *Subdoligranulum*, *Haemophilus*, and *Roseburia*, decreased in three NAFLD subtypes than non-NAFLD subjects. In line with our results, previous studies have associated NAFLD/NASH with a decreased abundance of *Faecalibacterium*[14,15]. *Ren et al.*[16] revealed the decreased abundance of *Roseburia* and *Subdoligranulum* in the NAFLD group. Our findings concluded a core set of microbial genera that have wide-ranging implications for NAFLD, revealing previously unnoticed similarities in the condition.

The gut microbiota is also closely related to the progression of NAFLD. Previous studies have showed significant differences in the composition of gut microbiota in patients with NAFLD at different stages[17,18]. In our study, combination of five genera including *Faecalibacterium*, *Subdoligranulum*, *Haemophilus*, *Roseburia* and *Butyricicoccus* can distinguish mild NAFLD from non-NAFLD with an AUC of 0.84 (±0.17). Several genera including *Faecalibacterium*, *Subdoligranulum*, *Intestinibacter*, *Agathobacter*, *Lachnospiraceae_UCG-004*, *[Eubacterium]_ventriosum_group*, and *Actinomycetales_unclassified* can distinguish moderate NAFLD from non-NAFLD with an AUC of 0.83 (±0.28). Eight genera including *Faecalibacterium*, *Subdoligranulum*, *Haemophilus*, *Intestinibacter*, *Lachnospiraceae_UCG-004*, *Agathobacter*, *Rothia*, and *Rhodococcus* can distinguish moderate NAFLD from non-NAFLD with an AUC of 0.90 (±0.16). It is close to their original AUC value calculated by two hepatic enzymes (ALT, AST) (shown in Supplemental Table 1). Therefore, the specific genera we have identified still demonstrate a robust capacity to differentiate NAFLD subtypes across diverse populations, while also exhibiting significant predictive value for the progression of NAFLD.

The gut microbiota is capable of generating various bioactive metabolites, which can be absorbed into the enterohepatic circulation and subsequently enter the bloodstream of the host. In our present study, we found the changes of relative abundance of genera *Faecalibacterium*[19], *Roseburia*[20], *Agathobacter*[21], *Lachnospiraceae_UCG-004*[22] et al. which contribute to the production of SCFAs. Among the extensive range of microbial metabolites three SCFAs namely butyrate, propionate, and acetate have been demonstrated to exert a significant impact on various of NAFLD/NASH pathogenesis[23,24]. The gut microbiota may regulate the expression of liver lipid metabolism-related genes by regulating global histone acetylation and methylation in multiple host tissues, including the liver, mainly through the synthesis of SCFAs[25,26]. The dominant bacterial species detected in faecal samples from human subjects exhibited high butyrate production capacity, including *Faecalibacterium prausnitzii* and *Roseburia inulinivorans*. Similarly, the genera with high propionate production capacity included *Bacteroides uniformis* and *Prevotella copri*[27,28].The feature importance in the random forest model based on mean square error calculation indicates that *Romboutsia* is the most important feature for predicting acetate content[29]. Previous studies have showed that *Agathobacter* was highly correlated with acetic acid[30,31].

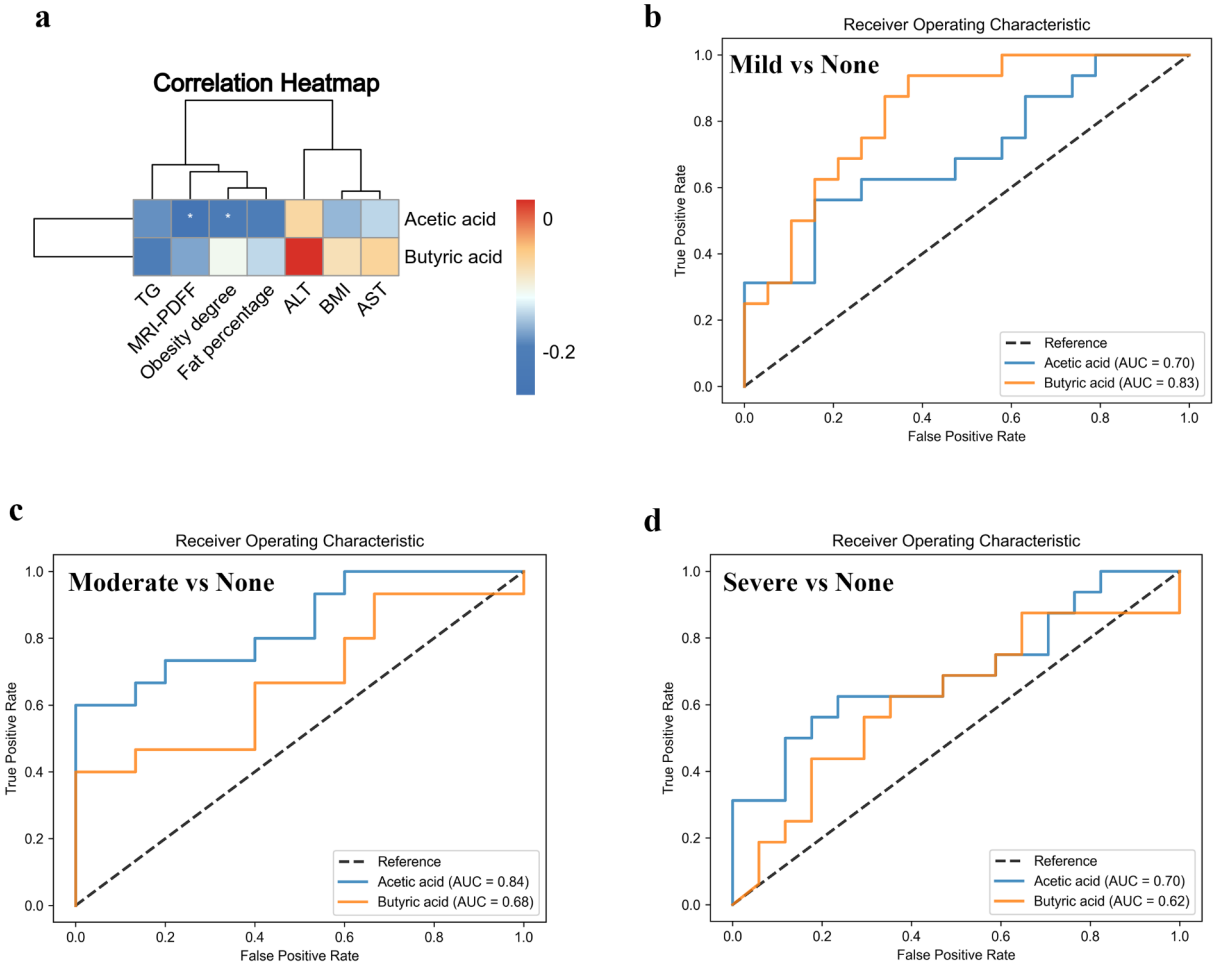

Fig. 7. Heatmap of Spearman correlation coefficients between two key SCFAs and key blood biochemical indices (**a**) and two key SCFAs to build the prediction model yielded an AUC based on ROC analysis (**b**–**d**). Receiver operating characteristic (ROC) curve analysis of biomarker panel included by acetic acid and butyric acid for discriminating the healthy non-NAFLD subjects and NAFLD subtypes (mild-, moderate-, and severe-NAFLD).

The production of SCFAs is attributed to the metabolic activity of intestinal bacteria in response to the digestion of dietary fiber. The most prevalent SCFAs in the colon are acetic acid, propionic acid, and butyric acid, accounting for 90–95% of their total content[32]. However, the impact of SCFAs on the severity of NAFLD has not been well explored. In our study, since the core gut microbiome are more closely related to their metabolites-SCFAs. The relationship between fecal SCFAs and the occurrence of NAFLD was further investigated, followed by an analysis of the correlation between fecal SCFAs and NAFLD subtypes. We found fecal acetic and butyric acids significantly decreased in NAFLD patients, which was in accordance with existing study shown that the decreases of fecal SCFAs linked to NAFLD occurs[33] and may cause the development of NAFLD. In our study, butyric acid distinguished mild-NAFLD from non-NAFLD with AUC value of 0.83. And acetic acid distinguished moderate-NAFLD and severe-NAFLD from non-NAFLD with AUC value of 0.84 and 0.70. In detail, it was found that the acetate enhances liver fat oxidation by improving mitochondrial modifications and activating AMP-activated protein kinase[34] while propionate could stimulate leptin secretion, thereby inhibiting the generation of new lipogenesis[35]. Butyrate is an SCFA derived from the gut microbiota, mainly metabolized by colon cells as the main energy source, and exhibits anti-inflammatory effects[36,37]. A study demonstrated that a synbiotic comprising butyrate-producing commensal bacteria (*Faecalibacterium prausnitzii*) coupled with potato starch exhibited protective properties against chronic excessive ethanol-induced hepatic steatosis, liver injury and intestinal damage[38]. The dietary supplementation of butyrate not only ameliorated liver injury, inflammation, and fibrosis induced by methionine-choline deficiency but also improved gut dysbiosis[39]. The use of butyrate has recently emerged as a potent therapeutic agent for mitigating NASH[23]. As previously stated, propionic acids, in addition to butyrate and acetate, play a pivotal role in the mitigation of fatty liver disease. *Scheiman et al.*[40] showed that propionic acid, produced by the gut flora through the breakdown of lactic acid, is significantly higher in athletes than in the general population and can increase heart rate and maximal oxygen consumption, as well as improve resting energy expenditure and lipid oxidation in fasting humans. In addition,

the increased levels of propionic acid in inhibiting body weight and hepatic fat accumulation[41], and reducing steatosis and inflammation in diet-induced obese mice also been found[42]. All of these studies highlight the importance of propionic acid in fatty liver disease. It is noteworthy that the levels of propionic acid in this study were not significantly different between the non-NAFLD and NAFLD populations. The study by *Louis et al.*[43] demonstrates that the primary pathway for propionate production is via glycolysis in the gut flora, in addition to peptide and amino acid fermentation. However, amino acid metabolizing bacteria represent less than 1% of the gut flora, suggesting that changes in the gut flora in the glycolytic metabolic pathway may be the primary factor influencing propionate levels. Some studies identified *Bacteroides*, *Prevotella* and other dominant species that undergo glycolysis to produce propionic acid in the human gut. It is noteworthy that the study by *Silva* et al. and colleagues demonstrated a significantly elevated concentration of propionic acid in the faeces of patients with NAFLD[44] in comparison to the control group. This outcome could be attributed to the limitations of the aforementioned study sample size. In a study comparing the gut microbiota of African and European children on different diets, it was found that African children on diets high in dietary fibre and plant protein had almost four times the gut levels of propionic acid and butyric acid than European children on diets high in sugar and animal protein ($p < 0.05$)[45]. The differences in ethnicity and diet between this study and the study by *H.E. et al.*. may also explain the inconsistency in the propionic acid results. In the present study, the higher abundance of *Bacteroides* and *Prevotella* did not change significantly between the two groups[27,28]. This may be the reason why the two groups exhibited an insignificant difference in propionic acid. This suggests that propionic acid is not a useful biomarker for differentiating between NAFLD and non-NAFLD. It is worth noting that since NAFLD often develops from overweight and obesity, there is a significant proportion of overweight or obese patients in the NAFLD population, and relevant studies have shown that among patients with non-alcoholic fatty liver disease, obese individuals account for approximately 80%, and the prevalence of NAFLD is only 16% in individuals with normal BMI and no metabolic risk factors[46,47]. Furthermore, in a meta-analysis of 356,367 subjects and 48 studies by Li et al., the prevalence of NAFLD in Chinese men was found to be 24.81%, almost twice that of women (13.16%)[48], They may explain the large differences in BMI and sex ratio between controls and the severe steatosis group in the present study.

At present, the primary methods for assessing hepatic steatosis include ultrasound (US), controlled attenuation parameter (CAP), and magnetic resonance imaging-proton density fat fraction (MRI-PDFF), each with its unique strengths and limitations. While US is the most commonly used method for diagnosing NAFLD, its accuracy in diagnosing mild hepatic steatosis is limited, failing to reliably detect steatosis below 20%. It provides only a qualitative rather than a quantitative diagnosis of fatty liver. CAP exhibits accurate detection of low-grade hepatic steatosis but struggles to effectively distinguish between high-grade and adjacent-grade steatosis. Furthermore, CAP's discriminative ability diminishes with increasing BMI and is influenced by the use of M and XL probes[49]. In contrast, MRI-PDFF precisely and quantitatively detects hepatic steatosis across all levels, making it potentially the most accurate method for evaluating NAFLD-related hepatic steatosis[50,51]. However, fecal microbiota and short-chain fatty acid analysis show promising distinctions across all levels of hepatic steatosis, suggesting their potential superiority over the sole use of US and CAP.

Limitations of study

The primary limitation of our study lies in its cross-sectional design, which restricts the ability to make causal inferences. Additionally, we were unable to explain the production of endogenous SCFA, visceral and liver extraction, or tissue utilization in this study. Although further clinical trials are necessary to confirm its efficacy, a novel concept is proposed for predicting NAFLD recurrence and distinguishing between different grades using non-invasive testing methods such as gut microbiota and fecal SCFAs. This approach aims to facilitate the daily monitoring of NAFLD. In addition, with regard to difference by BMI and sex ratio in the control and severe NAFLD groups, we currently have no effective method of propensity matching to control for these factors, although such differences are common in both normal populations and NAFLD.

Conclusion

In summary, our study and subsequent analysis have demonstrated that gut microbiota and fecal SCFAs are not only a good prediction model for early warning of NAFLD occurrence, but also offer the convenience of non-invasive detection methods. Moreover, we have identified specific combinations of gut microbiota genera that exhibit strong discriminatory ability in distinguishing between different subtypes of NAFLD.

Data availability

All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

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Declarations

Competing interests
The authors declare no competing interests.

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