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Gut microbiome composition and metabolic activity in metabolic-associated fatty liver disease

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ABSTRACT

Metabolic Associated Fatty Liver Disease (MAFLD) impacts approximately 25% of the global population. Between April 2023 and July 2023, 60 patients with MAFLD, along with 60 age, ethnicity, and sex-matched healthy controls (HCs), were enrolled from the Inner Mongolia Autonomous Region, China. Analysis of gut microbiota composition and plasma metabolic profiles was conducted using metagenome sequencing and LC-MS. LEfSe analysis identified five pivotal species: Eubacterium rectale, Dialister invisus, Pseudoruminococcus massiliensis, GGB3278 SGB4328, and Ruminococcaceae bacteria. In subgroup analysis, Eubacterium rectale tended to increase by more than 2 times and more than double in the non-obese MAFLD group, and MAFLD with moderate hepatic steatosis (HS), respectively. Plasma samples identified 172 metabolites mainly composed of fatty acid metabolites such as propionic acid and butyric acid analogues. Ruminococcaceae bacteria have a strong positive correlation with β-alanine, uric acid, and L-valine. Pseudoruminococcus massiliensis has a strong positive correlation with β-alanine. Combinations of phenomics and metabolomics yielded the highest accuracy (AUC = 0.97) in the MAFLD diagnosis. Combinations of phenomics and metagenomics yielded the highest accuracy (AUC = 0.94) in the prediction of the MAFLD HS progress. Increases in Eubacterium rectale and decreases in Dialister invisus seem to be indicative of MAFLD patients. Eubacterium rectale may predict HS degree of MAFLD and play an important role in the development of non-obese MAFLD. Eubacterium rectale can generate more propionic acid and butyric acid analogues to absorb energy and increase lipid synthesis and ultimately cause MAFLD.

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Introduction

In 2020, an international panel of experts proposed Metabolic Associated Fatty Liver Disease (MAFLD) as a new term to replace Non-Alcoholic Fatty Liver Disease (NAFLD) [1]. MAFLD is now the number one liver disease, influencing over 25% of the global adult population [2]. MAFLD is characterized by an abnormal accumulation of lipids in liver cells, a condition known as hepatic steatosis (HS). HS can progress to a more severe form known as metabolic dysfunction-associated hepatic steatohepatitis (MASH), resulting in an increased risk of cirrhosis and liver cancer [3]. MAFLD is closely related to obesity and is a hepatic phenotype of the metabolic syndrome [1,4]. However, non-obese subjects, i.e. those with "non-obese MAFLD" or "lean MAFLD" is more strongly associated with a higher risk of severe liver disease than obese MAFLD [5]. MAFLD individuals with normal weight (BMI <23 kg/m^2 in Asians) have been defined as lean MAFLD [5]. The commonly used criterion defined MAFLD with BMI <25 kg/m^2 as non-obese MAFLD and MAFLD with BMI ≥30 kg/m^2 as obese MAFLD [5].

The pathogenesis of MAFLD is complex and unknown [6]. Mainstream treatment guidelines for MAFLD involve lifestyle changes aimed at weight loss [7]. Therefore, it is crucial to further investigate the mechanisms of MAFLD and innovate new therapeutic approaches. The intestinal microbiota plays a significant role in the pathogenesis of MAFLD [8]. Although a large number of studies suggest that changes in the gut microbiota may be associated with MAFLD risk, research evidence remains largely inconsistent. In addition, the mechanisms by which the gut microbiota causes and exacerbates MAFLD are unknown. In addition, the role of the gut microbiome in non-obese subjects with MAFLD or MAFLD subjects with severe HS

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is unclear due to the limited number of MAFLD subjects for subgroup analysis. The following are some shortcomings of the current study. First, 16S rRNA gene sequencing is the most common method for detecting the composition of the gut microbiota. More metagenomic studies should be conducted because 16S rRNA gene sequencing can only identify major taxa and explore microbial diversity but cannot pinpoint any specific microbial species and strains [9]. Thus, most studies focused only on the gut microbiota and did not summarise its relationship with other biomarkers such as gut microbial metabolites. Multi-omics data may reveal potential molecular mechanisms associated with MAFLD and identify new biomarkers and drug targets. In addition, gut microbial abundance may vary across populations (e.g. race, age, gender, obesity status, and MAFLD subtype), and most studies have not controlled for the effects of confounding factors on the gut microbiota.

To address these research gaps, our study aimed to comprehensively assess the composition of the microbiota and its metabolites, as well as the impact of the abundance of microbial taxa (e.g. phylum, genus, and species) on MAFLD risk. The Inner Mongolia Autonomous Region is located on the Mongolian Plateau in Central Asia, with a large temperature difference between day and night, plenty of sunshine hours, and a high variability of precipitation, with local traditional diet regarding beef, mutton, and dairy food as the main diet. The Inner Mongolia region is dominated by a high sugar, high fat, high salt, and high protein diet, and this is especially obvious for people with MAFLD. Unique geographic location, lifestyle, and dietary habits create unique population flora characteristics. This area has a slightly higher incidence of MAFLD, and interpreting region-specific features and informing disease interventions by targeting microbiota is essential. In addition, under the background of industrialization and modernization, the dietary habits and lifestyles of ethnic minorities in Inner Mongolia, such as the Mongolian people, have gradually changed. The Mongolian population has been living with Han Chinese for a long time, and their dietary habits and lifestyles have inevitably been influenced by the differences in the genotypes of the Han and Mongolian populations, which may lead to similarities and differences in the intestinal flora in their bodies. Therefore, it is necessary to study these differences with regard to the potential impact on human health. This study conducted metagenome sequencing and non-targeted liquid chromatography-mass spectrometry (LC-MS) analysis to identify potential associations between gut microbes and metabolites in MAFLD subjects.

Materials and methods

Overall study design and subject enrollment

This is a non-interventional prospective study to explore the gut microbiological profile of patients with MAFLD. Between April 2023 and July 2023, this study was conducted in the Inner Mongolia Autonomous Region, China, involving 60 MAFLD patients, including 30 individuals of Han ethnicity and 30 of Mongolian ethnicity, alongside 60 age, ethnic, and sex-matched healthy controls from the same communities as healthy controls (HCs). Adults, aged 18–65 years, were diagnosed with MAFLD according to the international expert consensus statement [1]. Two experienced ultrasound physicians blinded to the study further classified it as (1) mild HS or moderate or severe HS. Ultrasound in patients with mild HS may show normal liver size and morphology, but with enhanced echoes in the anterior field, insignificant attenuation of echoes in the posterior field, and well-defined intrahepatic ductal structures. Moderate HS may show normal liver size and morphology, or mild or moderate enlargement, with enhanced anterior field echoes, attenuated posterior field echoes, and blurred intrahepatic ductal structures, but still recognizable. Severe HS shows that the liver is obviously enlarged and full in shape, with obvious enhancement of anterior field echo, obvious attenuation of posterior field echo, and may even present an anechoic area with unclear contour and difficult to recognize tubular structure. We defined MAFLD with a BMI of $<28\ kg/m^2$ as non-obese MAFLD and MAFLD with BMI $\geq 28\ kg/m^2$ as obese MAFLD. MAFLD individuals with normal weight (BMI $<23\ kg/m^2$), has been defined as normal weight [5]. MAFLD individuals ($23\ kg/m^2 \leq$BMI $<28\ kg/m^2$) have been defined as overweight. Non-obese MAFLD included MAFLD with a normal weight or overweight body. Out of 60 MAFLD patients, 44 patients with moderate HS, and 16 patients with mild HS were included in the overall and subgroup analysis. Out of 60 MAFLD patients, 12 patients with a normal weight, 19 with overweight body, and 29 patients with obesity body were included in overall and subgroup analysis.

This study was approved by the Institutional Review Board (EC-20231214-1009). The study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all study participants. All methods were performed in accordance with relevant guidelines and regulations.

Inclusion and exclusion criteria for MAFLD

In the MAFLD study, participants must have (1) varying degrees of hepatic steatosis on abdominal ultrasound and (2) a. overweight/obesity (BMI ≥23 kg/m^2); b. presence of type 2 diabetes mellitus (fasting glucose ≥7.0 mmol/L); and c. metabolic dysfunction, with at least two of the following: (a) high waist circumference; (b) hypertension; (c) hypertriglyceridemia; (d) high-density lipoprotein cholesterol; (e) prediabetes; (f) insulin resistance; and (g) ultrasensitive C-reactive protein levels.

Exclusion criteria included (1) specific diseases that may cause steatosis, such as viral hepatitis, alcoholic liver disease, drug-induced liver disease, and autoimmune liver disease; (2) other chronic diseases, including, but not limited to, chronic infections, significant intestinal disorders, or symptoms (including, but not limited to, chronic constipation, diarrhoea, ulcerative colitis (UC), Crohn's disease (CD), intestinal obstruction, and intestinal malignancies); (3) other severe acute cardiovascular disease, renal disease, malignancies, etc., that may have interfered with data analysis in the month prior to the screening visit; (4) cirrhosis of the liver; (5) patients with dietary "abnormalities" (e.g. vegan diets) in the past 12 months or exposure to medications or interventions (e.g. drugs or interventions) that affect the composition of the intestinal microbiome in the past 3 months; (6) patients who have been exposed to medications or interventions that affect the composition of the gut microbiome (e.g. antibiotics, immunosuppressants, chemotherapy, and proton pump inhibitors) in the past 3 months; (7) excessive consumption of alcohol (>1000 ml of beer or >200 ml) or a sudden major change in diet in the 1 week after sample collection; (8) any condition unsuitable for the study, such as malaise or poor compliance.

Specific criteria for inclusion and exclusion of HC have been thoroughly outlined in the supplementary material.

Assessment of eating habits and clinical characteristics

We collected detailed scales of personal circumstances including age, sex, BMI, alcohol consumption, smoking, physical activity, mental stress, sleep conditions, diabetes, hypertension, medical history, etc [10]. The dietary questionnaire was conducted by a full-time dietitian. A paper-based questionnaire with a combination of pictures, videos, voice conversations, and video calls was used to ensure the reliability of the dietary intake information, taking into account the volunteers' literacy level, language communication, and other interfering factors. Food types were categorized into five main groups (main food, side dishes, fruits, beverages, and other local specialties). The dietary questionnaire was designed for the Chinese population and included traditional Chinese foods such as staple foods, side dishes (a variety of meats and vegetables), fruits, and beverages (tea and coffee), as well as foods native to the Inner Mongolia region (typical European Western food, bread, fresh milk, steak, vegetable salad, rice balls, and noodles). Intake of these foods was recorded as yes or no in the past 1 month and on the last 1 day. The dietary questionnaire used in this study was designed according to the 2016 Dietary Guidelines for Chinese Residents. Given the diversity and variety of Inner Mongolian diets, which are very different from those of other ethnic groups in other parts of China, we customized and adapted the questionnaire after consulting with a local dietitian and added other foods with Inner Mongolian characteristics. The revised questionnaire also underwent two rounds of pilot testing and validation before it was officially used in this study. If some items in the questionnaire were confusing, or if respondents suggested improvements to items in the questionnaire, the questionnaire was further refined to ensure clarity, information, and variability of answers. Each volunteer collected two tubes of venous blood samples on an empty stomach in the morning in health centers, one red riser tube for blood routine, and one purple tube for metabolomics and biochemical indexes. The purple tube of blood samples needs to be centrifuged at 3000 rpm for 10 min in a high speed refrigerated centrifuge (Hengnuo 5-21 R) to obtain serum for testing. Blood routine includes blood cells, neutrophils (Neu), lymphocytes (Lym), monocytes (Mono), red blood cell distribution width (RDW), platelets (PLT), and other routine blood tests. Biochemical variables include serum K+, Na+, Cl-, Ca+, alanine aminotransferase (ALT), aspartate aminotransferase (AST), glutamyl transpeptidase (GGT), alkaline phosphatase (ALP), total bilirubin (TBIL), direct bilirubin (DBIL), indirect bilirubin (IBIL), creatinine (Cr), uric acid (URIC), Blood urea nitrogen (BUN), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG), cholesterol (CHOL), low-density lipoprotein (LDL-C), fasting plasma glucose (FPG). Blood routine was detected by the LH780 blood cell analyzer (Beckman Coulter) and Hitachi 7600 auto-analyzer. Other indexes were tested by the Cobas 6000 auto-analyzer (the COBA 6000 system E601 immunoassay analyzer) (Roche Diagnostics, IN, Germany). The testing of the above indicators is done by the Laboratory Department of our hospital.

There is , basically, no statistical difference between the groups in other important confounding factors (such as education level, personal monthly income, sleeping status, stress at work, contact with animals, frequency of eating out, physical activity levels, mental stress, smoking history, and alcohol history) that may affect the gut microbiome and MAFLD. Specific information has been thoroughly outlined in the supplementary material (Dataset S1, Supporting Information).

Sample collection

Fresh fecal samples were divided into three 100 mg portions and promptly snap-frozen in liquid nitrogen. Post snap-freezing samples were preserved at −80°C pending extraction for analysis. The plasma from the venous blood collection was centrifuged and maintained at −80°C until further testing.

Tool sample collection, DNA extraction, and metagenomic shotgun sequencing

The extraction of bacterial DNA was carried out at Novogene Bioinformatics Technology (Beijing, China) utilizing the SDS method. DNA concentration and purity were evaluated on 1% agarose gels, with subsequent dilution to 1 ng/μL in sterile water. The extent of DNA degradation and the presence of potential contamination were evaluated using 1% agarose gels. The NanoPhotometer® spectrophotometer (IMPLEN, CA, USA) was utilized to determine the DNA purity through measurements of the OD260/OD280 and OD260/OD230 ratios. Additionally, the DNA concentration was quantified using the Qubit® dsDNA Assay Kit in the Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). The samples were subjected to paired-end sequencing on an Illumina platform (insert size 350 bp, read length 151 bp, NovaSeq 6000) at Novogene Bioinformatics Technology (Beijing, China).

LC/MS nontargeted metabolomics analysis

One hundred microliters of each sample was transferred to an EP tube, followed by the addition of 400 μL of extraction solution (methanol with an isotopically labeled internal standard mixture). The samples were vortexed for 30 s, followed by sonication in an ice-water bath for 10 min, and then incubated at −40°C for 1 h to facilitate protein precipitation. The sample was centrifuged at 12,000 rpm (RCF = 13800 (×g), $R = 8.6$ cm) for 15 min at 4°C. The clear supernatant was then transferred to a new glass vial for further analysis. A quality control (QC) sample was prepared by pooling equal aliquots from each supernatant. Metabolomic analyses were conducted utilizing a UHPLC system (Vanquish, Thermo Fisher Scientific) with a UPLC HSST3 column (2.1 mm × 100 mm, 1.8 μm), coupled to the Orbitrap Exploris 120 mass spectrometer (Orbitrap MS, Thermo). The mobile phase consisted of a mixture of water (component A) containing 5 mmol/L of ammonium acetate and 5 mmol/L of acetic acid and acetonitrile (component B). The auto-sampler was maintained at 4°C, and the injection volume was set to 2 μL. The Orbitrap Exploris 120 mass spectrometer was selected for its capability to acquire MS/MS spectra using information-dependent acquisition (IDA) mode, under the control of the acquisition software (Xcalibur, Thermo). In this mode, the acquisition software of the Orbitrap Exploris 120 mass spectrometer performs a continuous evaluation of the full scan MS spectrum. The ESI source settings were as follows: a sheath gas flow rate at 50 Arb, auxiliary gas flow rate at 15 Arb, capillary temperature at 320°C, full MS resolution at 60,000, MS/MS resolution at 15,000, collision energy in the NCE mode at 10/30/60, and spray voltage at 3.8 kV for the positive mode or −3.4 kV for negative mode. The raw data was first converted to the mzXML format using ProteoWizard, followed by processing with a custom R program developed based on XCMS for tasks such as peak detection, extraction, alignment, and integration. Subsequently, an in-house MS2 database (SHANGHAI BIOTREE BIOTECH CO., LTD) was utilized for metabolite annotation, with the annotation cutoff set at 0.3 [11].

Statistical analysis

Microbes that exhibited a p-value <0.05 in the Wilcoxon rank-sum test and had a median relative abundance exceeding 0.01% of the total abundance were identified as differentially abundant between HC and MAFLD groups. To conduct a more in-depth comparison of the distinctions among various microorganisms, both LEfSe analysis and ANOSIM were employed [12]. The data preprocessing, statistical analysis, metabolite classification annotations, and functional annotations were conducted using the metabolomics R package metaX [13] and the metabolome bioinformatic analysis pipeline. Metabolite comparisons between the groups were performed utilizing principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), Student's t-test, and fold changes obtained from variability analysis. The correlation between microorganisms, metabolites, and clinical data was assessed utilizing Pearson

correlation analysis and random forest analysis. Statistical significance was defined at $p < 0.05$.

Predictive modeling of MAFLD diagnosis and severity by random forests

The continuous type data in microorganisms, metabolites, and clinical data were Z-Score normalized, and a random forest model was fitted to all the data using the R package random Forest. We use the rfcv function to do 10-fold cross-validation and repeated five times to obtain the relationship between variable selection and the average error rate of model classification, based on the Mean Decrease Accuracy value from the largest to the smallest to filter the 20 feature rows of the next modeling. Randomized stratified sampling of the samples in a ratio of 7:3 was used to obtain the training and test sets, and the performance of the random forest model fitting was performed to confirm the reliability of the predictive model by performing a calibration analysis (e.g. calibration plots).

Results

The clinical and physical variables

Weight, BMI, ALT, GGT, ALP, GLB, FPG, URIC, TG, and LDL-C were significantly lower in the HC group compared with the MAFLD group (Table 1). HDL-C was higher in the HC group compared with the MAFLD group (Table 1).

Diversity of gut mycobiome in MAFLD patients

The Community Richness and Shannon indexes (Figure 1a) used for alpha diversity analysis of metagenome data indicated no significant difference between the HC and MAFLD groups, but we observed a tendency of decrease of the microbiome diversity in the MAFLD subjects. Similarly, beta diversity analyses using ANOSIM showed a large overlap between the two groups, with only minor differences that were almost indistinguishable (Figure 1b).

Table 1. Comparison of clinical characteristics and laboratory test indexes between MAFLD and HC groups.

Variables	HC group ($N = 60$)	MAFLD group ($N = 60$)	Test value	p
Age (year)	52.0 (47.0 – 57.0)	54.0 (47.25 – 57.75)	−0.793	0.430
Gender (male/female)	20/40	31/29	4.126	0.064
Height (cm)	163.5 (160.0 – 168.0)	165.0 (160.0 – 170.0)	−1.188	0.236
Weight (Kg)	60.50 (55.25 – 65.75)	75.5(67.625 – 82.75)	−5.374	<0.001
BMI (kg/cm^2)	23.182 ± 3.311	27.358 ± 4.199	−6.049	<0.001
Married	2/58	6/54	2.143	0.272
Personal monthly income < 3000	28/32	24/36	0.543	0.581
High School below	47/13	37/23	3.968	0.072
Never or one time per year travel	53/7	50/10	0.617	0.602
Stress at Work	37/23	39/21	0.144	0.850
Good Sleeping	38/22	39/21	0.036	1.000
Regularity of Eating	49/11	51/9	0.240	0.807
Exercise	48/12	49/11	0.054	1.000
Smoking	5/55	10/50	1.905	0.269
Alcohol	13/47	13/47	0.000	1.000
Normal delivery	58/2	59/1	0.342	1.000
Contact with Animals	25/35	26/34	0.034	1.000
Eating out	32/28	27/33	0.834	0.465
TBIL (umol/L)	9.25 (7.45 – 11.70)	9.90 (8.10 – 14.0)	−1.305	0.193
DBIL (umol/L)	4.20 (3.40 – 4.875)	4.20 (3.30 – 5.70)	−0.105	0.918
IBIL (umol/L)	5.0 (3.925 – 6.90)	6.0(4.5 – 8.3)	−2.137	0.032
ALT (IU/L)	15.50 (13.0 – 20.75)	23.00 (16.00 – 40.0)	−4.717	<0.001
AST(IU/L)	20.0 (16.25 – 24.00)	21.00 (17.00 – 27.00)	−1.404	0.161
AST/ALT	1.30 (1.07 – 1.62)	0.88 (0.69 – 1.17)	−5.745	<0.001
GGT(IU/L)	17.50 (12.0 – 25.75)	35.0 (22.0 – 55.00)	−5.840	<0.001
ALP(U/L)	23.182 ± 3.311	27.358 ± 4.199	−2.422	0.017
TP(g/L)	71.85 (69.625 – 75.675)	76.75 (72.325 – 79.875)	−3.782	<0.001
ALB(g/L)	46.196 ± 2.523	46.585 ± 4.184	−0.615	0.540
GLB(g/L)	26.355 ± 3.266	29.19 ± 4.703	−3.835	<0.001
A/G	1.778 ± 0.231	1.649 ± 0.332	2.454	0.016
Fasting blood glucose (mmol/L)	4.625 (3.965 – 5.320)	5.365 (4.7325 – 6.97)	−4.441	<0.001
UREA (mmol/L)	5.0 (4.30–6.325)	4.85 (4.095 – 5.8375)	−1.352	0.177
CREA (umol/L)	73.0 (67.0–79.75)	75.0 (65.0–79.0)	−0.433	0.667
URIC (umol/L)	289.95 ± 52.91	378.66 ± 82.26	−7.026	<0.001
TG (mmol/L)	0.975 (0.72 – 1.355)	1.91(1.46 – 2.31)	−6.157	<0.001
CHOL (mmol/L)	5.1687 ± 1.15367	5.4513 ± 1.28466	−1.268	0.207
HDL-C (mmol/L)	1.59 (1.38 – 1.80)	1.175(0.975 – 1.38)	−5.814	<0.001
LDL-C (mmol/L)	2.94 (2.51 – 3.4925)	3.43(2.85 – 3.9175)	−2.412	0.016
FIB-4*	1.0837 (0.8769 – 1.3731)	0.9926(0.7480 – 1.4647)	−1.417	0.158

Fibrosis-4 index = (Age(year)×AST(U/L)/(PLT(×10^9/L)×ALT(U/L)$^{1/2}$).

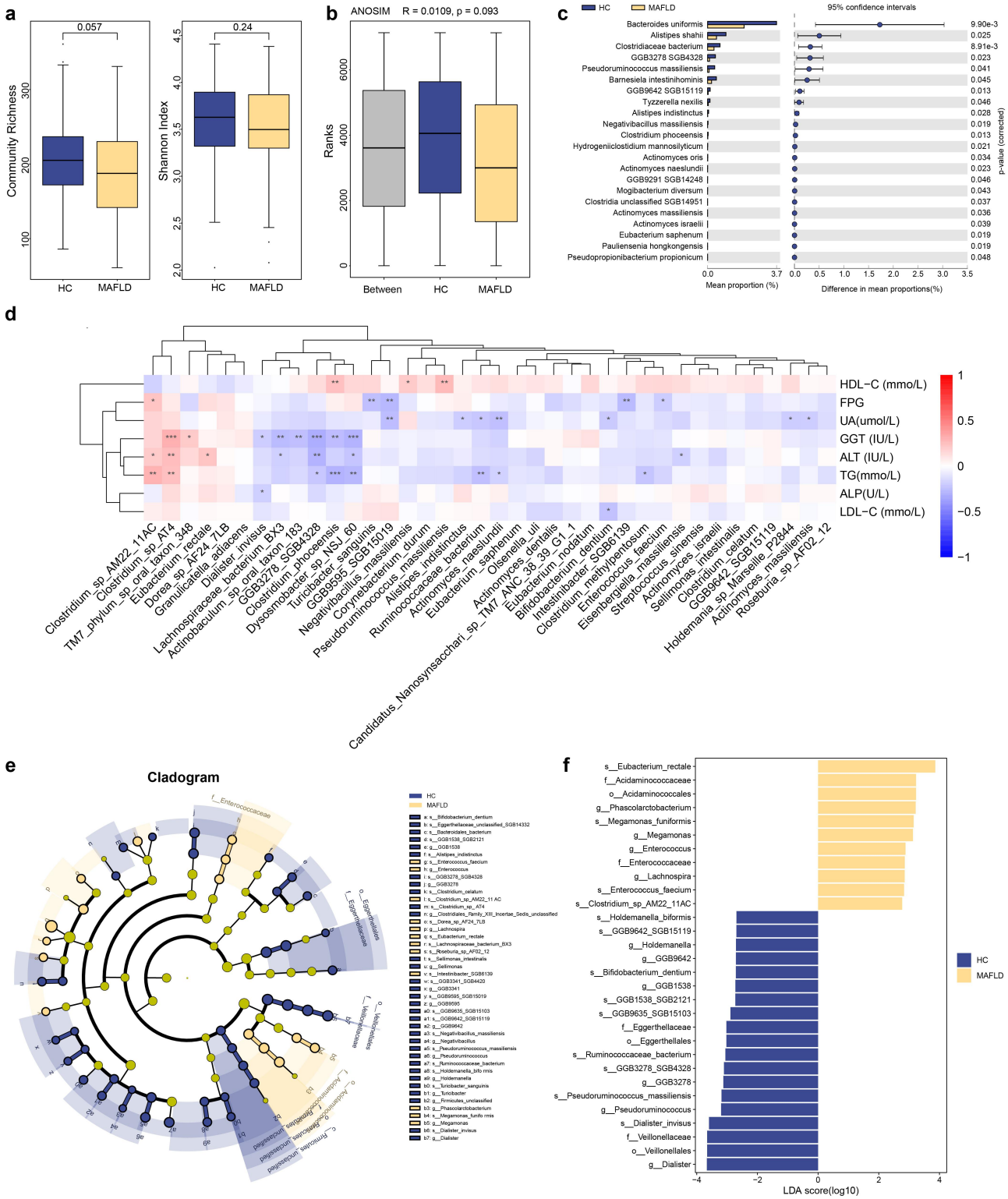

Figure 1. Diversity of the gut microbiome characteristics. (a) Alpha diversity indices (community richness and Shannon) of the intestinal bacterial communities of HC and MAFLD group. (b) Beta differences determined by ANOSIM base on metagenomic sequencing in species level. (c) Differential analysis of species by Wilcoxon rank-sum test bar plot. (d) Associations between differential species and clinical parameters. (e and f) LDA discrimination column chart.

Intestinal flora structural changes

Although their flora composition is similar, the dominant bacteria should be different. Considering the crucial impact of the gut microbiota on MAFLD, we then compared the differences in both phylum, genus, and species levels between the two groups. The Firmicutes, Bacteroidetes, and Actinobacteria collectively constituted over 90% of the total abundance and emerged as the predominant taxa in both study cohorts (online Supplementary Fig. S1A). At the genus level, Blautia and Faecalibacterium were identified as the dominant taxa in both groups (online Supplementary Fig. S1B). At the species level, *Faecalibacterium prausnitzii* and *Eubacterium rectale* were the most prevalent in both groups (online Supplementary Fig. S1C).

Differential analysis of intestinal microbes

The results revealed 9 phyla, 20 genera, and 30 species. At the phylum level, Candidatus Saccharibacteria exhibited higher abundance in the HC group compared to the MAFLD group (online Supplementary Fig. S1D). At the genus level, 18 genera, such as *Dialister*, and Clostridiales Family XIII Incertae Sedis unclassified, exhibited higher abundance in HC than MAFLD, whereas 2 genera, such as *Phascolarctobacterium* and *Lachnospira*, were significantly lower in HC (online Supplementary Fig. S1E). Thirty-eight species, including *Clostridium* sp. AM22 11AC and Lachnospiraceae bacterium B×3, indicated differences between the HC and MAFLD groups ($p < 0.05$) (Figure 1c). Additionally, 20 species demonstrated significant associations with various clinical parameters (Figure 1d). LEfSe analysis identified key gut microbiota differences, with 11 species in HC and 3 in MAFLD (Figure 1e,f). A decrease in the levels of *Dialister invisus*, *Pseudoruminococcusmassiliensis*, GGB3278 SGB4328, and Ruminococcaceae bacteria, and an increase in the levels of *Eubacterium rectale* (*E. rectale*) was identified as indicative of potential biomarkers for detecting MAFLD at the species level.

KEGG orthology metabolic pathway and MetaCyc metabolic pathway analysis

K18120 (4-hydroxybutyrate dehydrogenase [EC:1.1.1.61]) exhibited the most significant statistical variances in abundance within the MAFLD group (online Supplementary Fig. S1F). K10542 (methyl-galactoside transport system ATP-binding protein [EC:7.5.2.11]) exhibited the largest statistical enrichment in the MAFLD group (online Supplementary Fig. S1F). Gluconeogenesis III (PWY66–399) showed the most substantial statistical difference in the HC group compared to the MAFLD patients (online Supplementary Fig. S1G).

Plasma metabolite changes in patients with MAFLD

In the comparison between HC and MAFLD, 172 differential metabolites were identified for their biological relevance. The validation of the PLS-DA model confirmed that it was not subject to "overfitting" (Figure 2a,b). The metabolic differences between the two groups were visually represented through volcano plots (Figure 2c). In the comparison between HC and MAFLD groups, significant enrichment was observed in several metabolic pathways, including central carbon metabolism in cancer, protein digestion and absorption, D-amino acid metabolism, aminoacyl–tRNA biosynthesis, tyrosine metabolism, and biosynthesis of amino acids in KEGG IDs analysis (Figure 2d). Additionally, 26 of 27 lipid metabolites were correlated with clinical traits (Figure 2e). These key metabolites with the smallest *p*-values primarily consisted of amino acids, peptides, and analogues: L-valine, alpha-ketoisovaleric acid, and β-alanine; benzoic acids and derivatives: eudesmic acid; stilbene glycosides:trans-piceid; organoheterocyclic compounds: pyrrolidine; zearalenones:(S,E)-zearalenone; indolyl carboxylic acids and derivatives: indolepyruvate; and other metabolites: 4-methyl-2-oxovaleric acid and uric acid, which have strong correlations with clinical indicators in the context of MAFLD (Figure 2f).

Correlation between differential bacteria and differential metabolites

Five key species with relative abundances above 0.01% were identified by LEfSe and the top 10 metabolites were selected for correlation analysis (Figure 2g). Ruminococcaceae bacteria have a strong positive correlation with β-alanine, uric acid, and L-valine. *Pseudoruminococcus massiliensis* has a strong positive correlation with β-alanine.

MAFLD diagnosis and prediction of MAFLD HS progress based on multiomics data

Metabolomics data (AUC = 0.94) (Figure 3a) and phenomics data (AUC = 0.91) (Figure 3c) were the top-performing datasets in MAFLD diagnosis. Gut metagenomics data were the worst-performing data (AUC = 0.78) (Figure 3b). Combinations of phenomics and metabolomics yielded the highest accuracy (AUC = 0.97) in the MAFLD diagnosis (Figure 3d). In addition, cross-

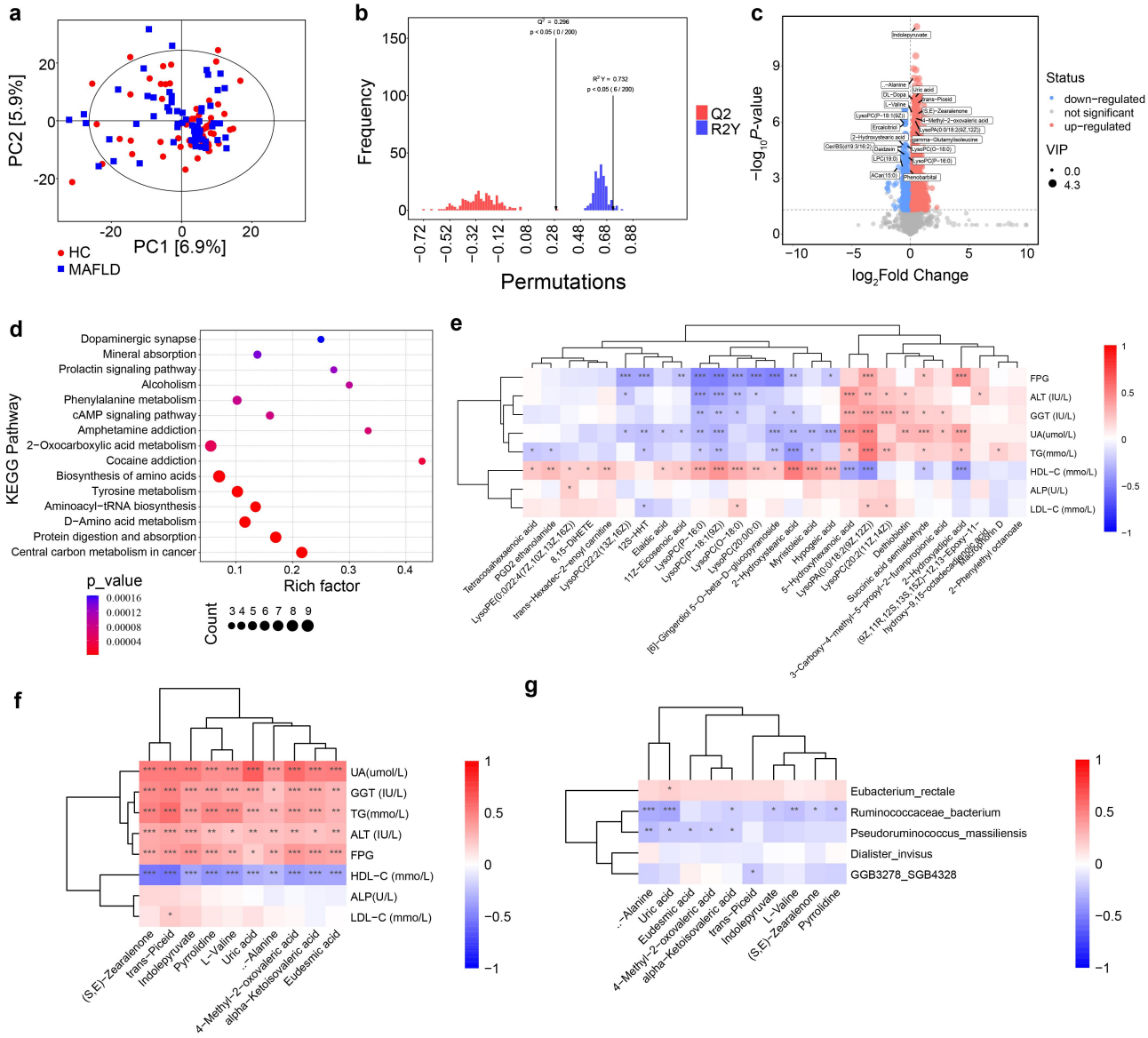

Figure 2. Overview of altered serum metabolism. (a) PCA score plot. (b) OPLS-DA permutation histogram. (c) A volcano plot is a graphical representation of differential metabolism between HC and MAFLD. (d) KEGG IDs of the differential metabolites. (e) Correlation between lipid metabolites and clinical index. (f) Correlation between top 10 metabolites and clinical index. (g) Correlation between key bacteria and top differential metabolites.

validation, random forest ntree, and calibration analysis (e.g. calibration plots) confirm that the predictive models are more reliable (online Supplementary Fig. S2–3).

Metabolomics data (AUC = 0.85) (Figure 3e) and phenomics data (AUC = 0.92) (Figure 3g) were the top-performing dataset in the prediction of the MAFLD HS progress degree. Gut metagenomics data were the worst-performing data (AUC = 0.82) (Figure 3f). Combinations between phenomics and gut metagenomics yielded the highest accuracy (AUC = 0.94) in the prediction of the MAFLD HS progress degree (Figure 3h).

Subgroup analyses of different ethnic groups with MAFLD

Subgroup analysis is shown in the Figure 4a. Alpha-beta did not show significant differences between the groups (Figure 4b–c), but we observed a tendency of decrease of the microbiome diversity in the Mongolian MAFLD subjects. It pinpointed *Bifidobacterium pseudocatenulatum* and GGB4596 SGB6358 as key species in the Han and Mongolian populations, respectively, utilizing the LEfSe method (Figure 4d–g).

In correlation analysis, *Bifidobacterium pseudocatenulatum* has a strong negative correlation with HDL-C (Figure 4h–i).

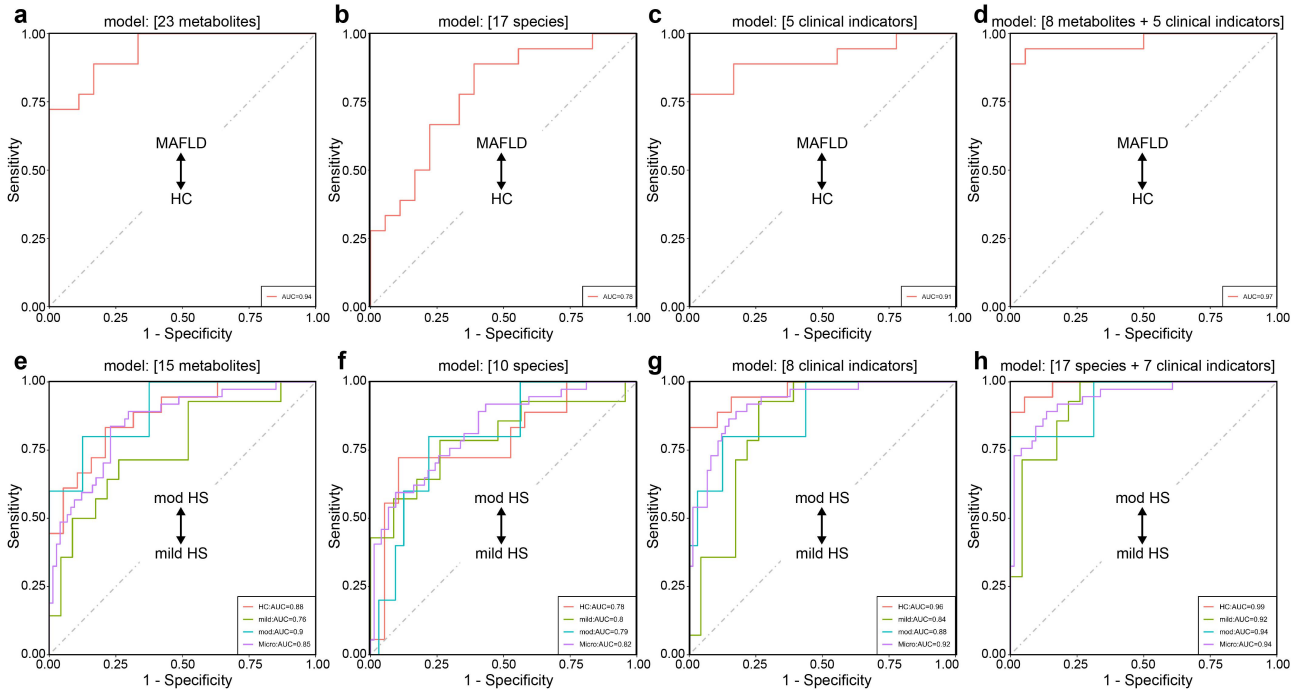

Figure 3. MAFLD diagnosis and prediction of MAFLD HS progress based on multiomics data. Metabolomics data (AUC = 0.94) (a) and phenomics data (AUC = 0.91) (c) were the top-performing dataset in the MAFLD diagnosis. Gut metagenomics data (b) were the worst-performing data (AUC = 0.78). Combinations of phenomics and metabolomics (d) yielded the highest accuracy (AUC = 0.97) in the MAFLD diagnosis. Metabolomics data (AUC = 0.85) (e) and phenomics data (AUC = 0.92) (g) were the top-performing dataset in the prediction of the MAFLD HS progress degree. Gut metagenomics data were the worst-performing data (AUC = 0.82) (f). Combinations between phenomics and gut metagenomics yielded the highest accuracy (AUC = 0.94) in the prediction of the MAFLD HS progress degree (h).

Subgroup analyses in different levels of MAFLD severity

Subgroup analysis is shown in the Figure 5a. Alpha diversity of moderate HS was lower than that of mild HS, but beta diversity has no significant differences (Figure 5b–c). The species *E. rectale* and *Dialister invisus* were identified as the most characteristic species of moderate HS and mild HS, respectively (Figure 5d–g).

Subgroup analyses in different body types with MAFLD

Subgroup analysis is shown in the Figure 6a. Alpha-beta diversity analyses showed no significant variations, but we observed a tendency of decrease of the microbiome diversity in the non-obese MAFLD subjects (Figure 6b–c). *E. rectale* was identified as the most characteristic bacteria in the non-obese MAFLD group (Figure 6d–i).

Discussion

Few studies have assessed the gut microbiome in MAFLD patients using metagenome sequencing. This analysis identified potential key gut microbe-metabolite associations, providing a basis for further investigation into the pathogenesis of MAFLD.

The diversity of the gut microbiota reflects the species richness in a given ecosystem and its variation or spatial distance across ecological niches. Many studies have reported that gut microecological dysbiosis is associated with altered microbial diversity, although these results have shown inconsistency. An updated meta-analysis to explore the profiles of intestinal dysbiosis in MAFLD patients in regions around the world found significant differences in β-diversity were reported by over 75% (37/47) of the included studies, and the remaining 10 studies reported nonsignificant results [14]. In our present study, we found that no disparities existed in microbial diversity between MAFLD and control groups, similar to the results of a recently published article [15], which could be explained by the similar dietary habits brought up from the same geographical location.

Table 2 shows various gut microbial species in patients with MAFLD based on relevant literature, highlighting their associations with clinical variables. LEfSe identified 28 differential species as potential biomarkers and most of them were enriched in species

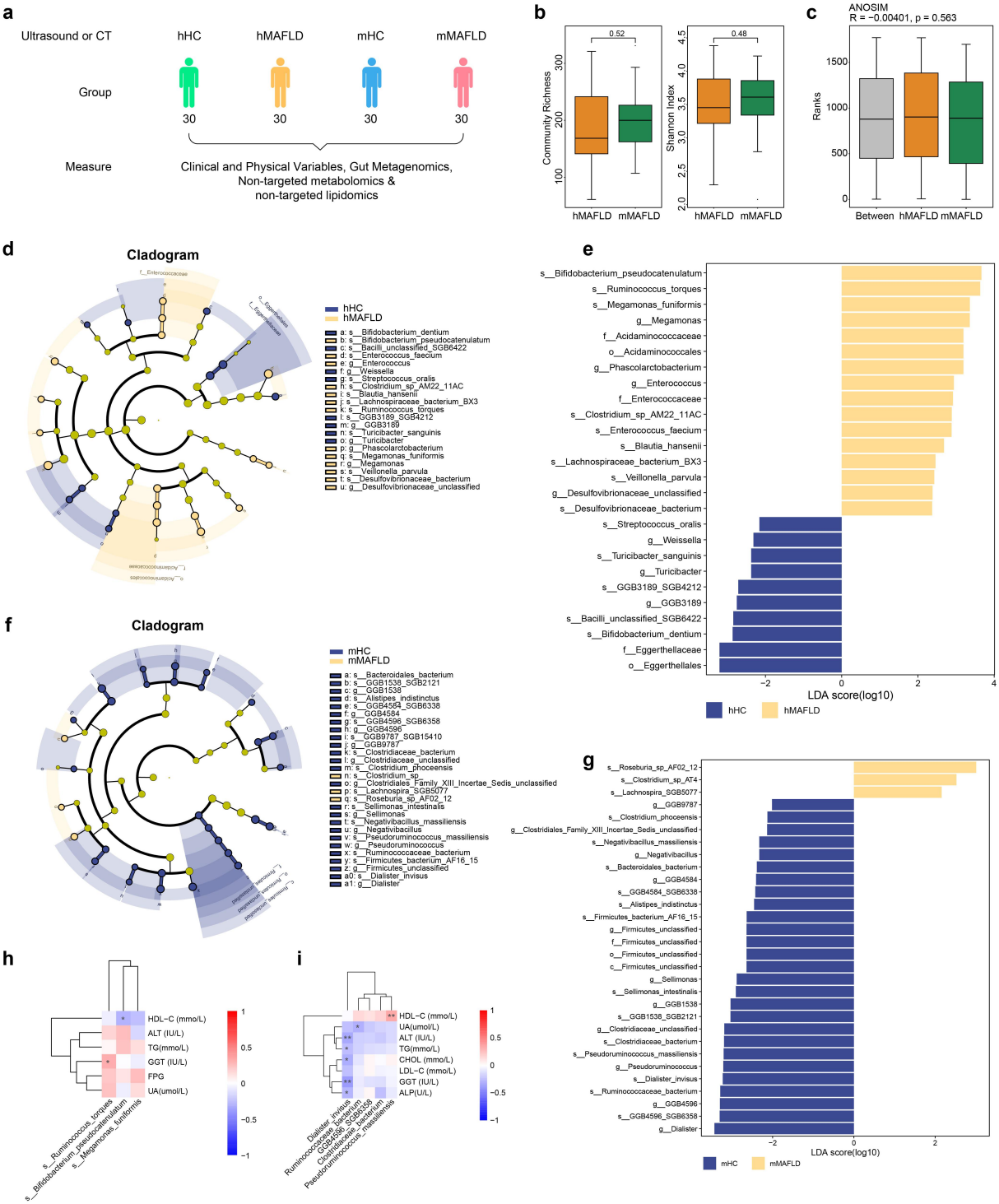

Figure 4. Subgroup analyses of different ethnic groups with MAFLD in microbes. (a) Han and Mongolian Chinese queue. (b and c) Alpha diversity indices and beta differences in species level. (d and e) LDA discrimination column chart in han Chinese queue. (f and g) LDA discrimination column chart in Mongolian Chinese queue. (h) Associations between species and various clinical parameters in han Chinese queue. (i) Associations between species and various clinical parameters in Mongolian Chinese queue.

level between MAFLD patients and healthy controls, of which *E. rectale* and *Dialister invisus* were the most significant species in MAFLD and control groups, respectively. There is still controversy about the role of *E. rectale*. *E. rectale* is a beneficial bacterium that produces short-chain fatty acids, which are beneficial

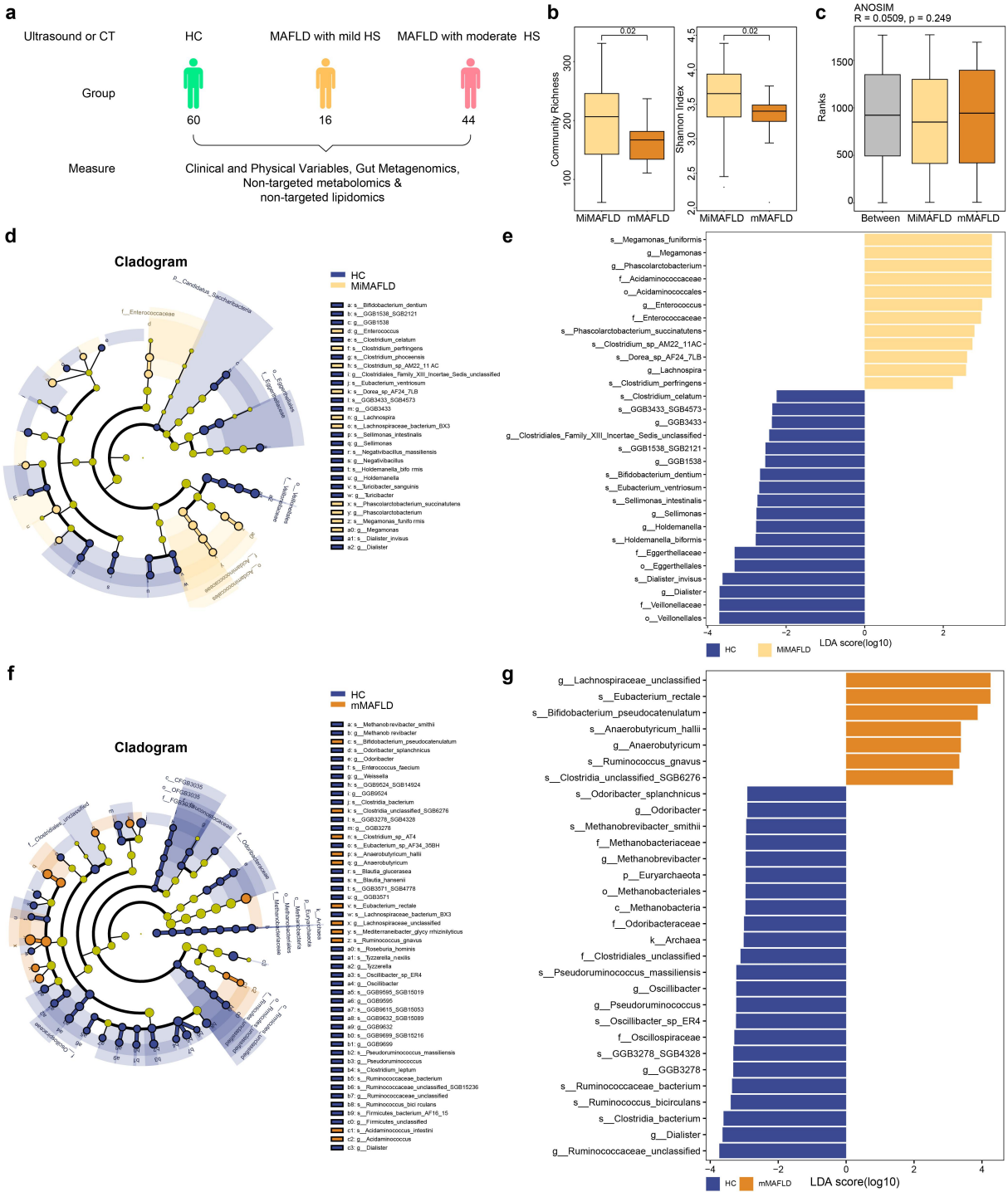

Figure 5. Subgroup analyses in different levels of MAFLD severity in microbes. (a) MAFLD with mild HS and MAFLD with moderate HS queue. (b and c) Alpha diversity indices and beta differences in species level. (d and e) LDA discrimination column chart in MAFLD with mild HS queue. (f and g) LDA discrimination column chart in MAFLD with moderate HS queue.

for COVID-19 patients [16]. Previous studies found that *E. rectale* was depletion in metabolic disease such as heart failure patients [17], coronary heart disease (CHD) [18], and obesity with NAFLD [5,19], but a study also revealed that *E. rectale* was increased in CHD [20]. A study confirms that *E. rectale* is harmful bacteria for Prader–Willi syndrome [21]. We observed an increased relative abundance of *E. rectale* in MAFLD

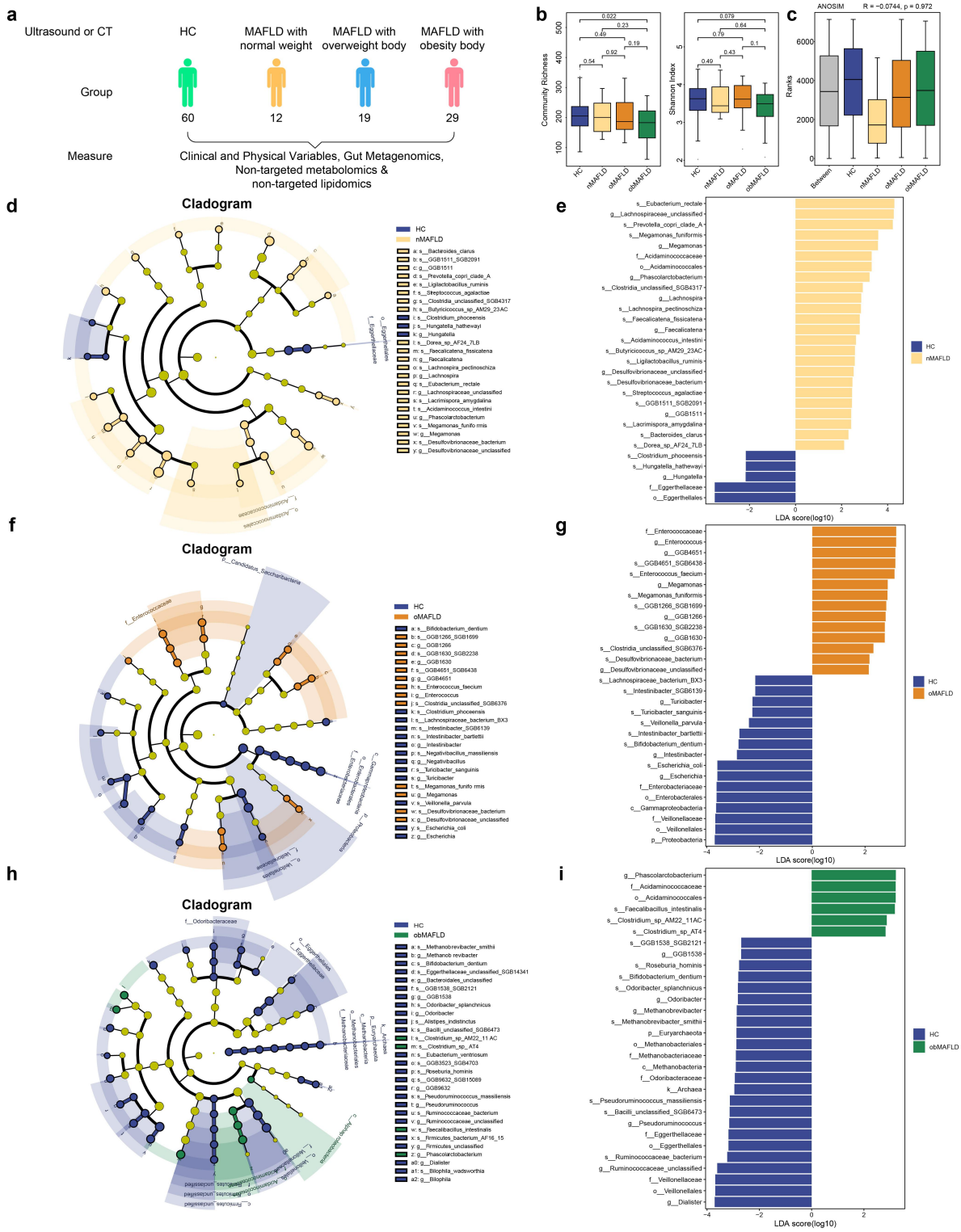

Figure 6. Subgroup analyses in different body types with MAFLD in microbes. MAFLD with normal weight, MAFLD with overweight body, and MAFLD with obesity body queue (a). (b and c) Alpha diversity indices and beta differences in species level. (d and e) LDA discrimination column chart in MAFLD with normal weight. (f and g) LDA discrimination column chart in MAFLD with overweight body. (h and i) LDA discrimination column chart in MAFLD with obesity body.

subjects. The elevated relative abundance of *E. rectale* may also be a regional microbial signature of the Inner Mongolia Autonomous Region of China. *Dialister* belongs to the phylum Thick-walled Bacteria, family Veronococcaceae, and mainly metabolises carbohydrates, producing succinic and acetic acids, propionic

Table 2. The highlighted different gut microbiome species between HC vs. MAFLD, which show correlation with the clinical variables.

Species	Diseases or phenotypes	Reference (PMID)	Reference (N)
Eubacterium rectale	Decrease in COVID-19	37743871	16
	Decrease in heart failure	28328981	17
	Decrease in coronary artery disease	30192713	18
	Decrease in obese patients with NAFLD	36756620	19
	Increase in rural residents with coronary heart disease	36519129	20
	Increase in Prader–Willi syndrome	37728722	21
Dialister invisus	Decrease in MAFLD	36539432	23
	Decrease in MAFLD	35047580	24
	Decrease in obesity	37536958	25
	Decrease in acute pancreatitis	37389346	26
	Decrease in cerebral palsy and epilepsy	36923492	27
Pseudoruminococcus massiliensis	Producing butyrate	34965174	28
Bifidobacterium dentium	Decrease in type 1 diabetes	37062177	31
	Increase in ability live long	35040752	32
Sellimonas intestinalis	Increase in Homeostasis gut recovery	33206037	33
	Increase in Schizophrenia	37124355	34
Alistipes indistinctus	Increase in CRC	37291572	36
	Increase in Inflammatory bowel disease (IBD)	36558391	37
	Decrease in osteoporosis	35456425	38
Turicibacter sanguinis	Reducing host serum triglyceride levels	31477894	42

acid, and butyric acid [22]. *Dialister invisus* is known to confer benefits to MAFLD [23,24]. Additionally, *Dialister invisus* decreased in individuals with obesity, acute pancreatitis, cerebral palsy and epilepsyt [25–27]. *Pseudoruminococcus massiliensis* seems to produce butyrate and have commensal behavior with the host epithelium, and its role in intestinal ecology should be studied further [28]. *Ruminococcus gnavus* is considered an important group of microbiota altered in IBD and allergic diseases [29,30]. The mechanism of action of Ruminococcaceae bacteria in MAFLD needs to be further investigated. Short-chain fatty acid (SCFA)-producing bacteria, such as *Bifidobacterium dentium*, are good for type 1 diabetes and the ability to live long [31,32]. *Sellimonas intestinalis* is a potential biomarker of homeostasis gut recovery [33]. Gut microbiota in Schizophrenia were notably enriched in *Sellimonas intestinalis* [34]. *Alistipes* is a relatively new genus of bacteria, which are highly relevant in dysbiosis and disease [35]. *Alistipes indistinctus* may promote deterioration of CRC and Inflammatory bowel disease (IBD) [36,37]. *Alistipes indistinctus* may alleviate osteoporosis [38]. Bacteria from the genus *Turicibacter* are important members of the mammalian gut microbiota and have been associated with changes in dietary fat and body weight [39]. Colonisation of *Turicibacter* strains is capable of modifying genes involved in bile acid and lipid metabolism in mice, and has been referred to as a regulator of the host's lipid biology [40,41]. *Turicibacter sanguinis*, the most well-studied species in the genus *Turicibacter*, reduced host serum triglyceride levels in addition to altering intestinal expression of genes associated with lipid metabolism in mice [42]. Although SCFA is associated with metabolism, the role of SCFA in energy homeostasis is currently unclear. Several animal and human studies have shown that obesity is associated with high levels of SCFA [43]. Gut microbiota ferment undigested carbohydrates (e.g. resistant starch and dietary fiber) and proteins in the small intestine to produce SCFA, which increase energy absorption and then synthesize lipids and glucose from scratch throughout the body, providing about 10% of an individual's energy needs and potentially contributing to obesity [43]. The Inner Mongolia region is dominated by a high sugar, high fat, high salt and high protein diet, and this is especially obvious for people with MAFLD. The continuous supply of carbohydrates in the gut is accompanied by an increase in the proportion of *E. rectale* and other butyrate-producing bacteria in MAFLD individuals, which promotes energy extraction in the gut. Our study also revealed enhanced butyrate metabolism (K18120 (4-hydroxybutyrate dehydrogenase) and K18122 (4-hydroxypropionic acid coenzyme A transferase)) in MAFLD subjects. Interaction analyses of the bacteria found *E. rectale* is an important butyric acid-producing bacterium that can be cross-fed with *Pseudoruminococcus massiliensis* and *Bifidobacterium*

dentium to maintain butyric acid concentrations and to promote the growth of pro-inflammatory bacteria such as *Alistipes indistinctus* in the colon.

The human gut bacterial microbiome is unique to each individual and its composition is largely influenced by factors such as disease, geography, lifestyle, diet, drugs, environment and genetics [44]. We analyzed by subgroups further to find relevant factors affecting the characteristic flora of MAFLD. We found that no disparities existed in microbial beta diversity in MAFLD from different Region, gender, age, BMI, HS, urbanization, ethnic groups (online Supplementary Fig. S4), Which hints at the low significance of subgroup analyses. In addition, due to the small sample sizes of the subgroup analyses, therefore we performed subgroup analysis and did not strictly ensured that other important factors related to the pathogenesis of MAFLD and changes in the gut microbiome such as body type, degree of HS, metabolic disorders like diabetes and hypertension were not statistically different between the two groups. We found no significant differences in microbial alpha-beta diversity among MAFLD populations from different ethnic groups, which may be related to dietary habits from the same geographic location. Therefore we did not bother to discuss the role of genetic predisposition, smoking, alcohol consumption, lifestyle, etc. in shaping these microbial patterns. Dietary factors, often mixed with other factors such as geography and ethnicity, are considered to be one of the most important factors influencing the gut microbial structure of populations. The diversity of dietary habits contributes to some extent to the heterogeneity of gut microbes among individuals in the population. The Mongolian people in the Inner Mongolia region are an ethnic minority with a dietary culture that distinguishes them from the Han Chinese, and therefore have their own unique flora characteristics. With the increase of urbanization level, in recent years, the government has been part of the dispersed Mongolian herders to live together, or Han-Mongolian living together, living routine, habits, environment, and then there is a more homogeneous diet, the population as a whole intestinal flora is more similar to the population, resulting in different factors under the population of intestinal flora differences are small. We found in a sampling dietary survey that local herders almost eat meat for every meal, and high sugar, high-fat, and high protein are more common than Han people, and also confirmed that the Oscillospiraceae for the ethnic minorities intestinal core genera may be related to the diet of the local people. Mongolians are dominated by Oscillospiraceae, Oscillibacter, Oscillibacter_sp_ER4. From the macrogenomic and metabolic characterization findings the organism has a butyrate kinase-mediated pathway, and thus it was inferred that Oscillospira is a butyrate producer and can utilize gluconate, a common animal-derived sugar that is both produced by the human host and ingested by the host through a diet rich in animal products [45,46]. Besides, *Bifidobacterium pseudocatenulatum* and GGB4596 SGB6358 are key species in the Han and Mongolian populations, respectively. Producing SCFAs *Bifidobacterium pseudocatenulatum* that can biotransform polyphenols, were positively associated with better cognitive performance [47]. The Mongolian and Han MAFLD cohorts exhibit distinct intestinal signature bacteria (Lefse), likely influenced by additional, currently unknown covariates as well as intrinsic microbial ecological factors, which need to look into further [8]. With respect to heterogeneity of gut flora in populations of different geographic and cultural origins, to some extent these factors are confounding. To date not enough populations have been studied to address which of these differences are due to diet, which are due to host genetics, and which are due to environmental exposures. Understanding the effect of each factor on the gut flora of a population is difficult, and new research tools will need to be continually developed in the future to achieve research objectives.

Alpha diversity of mMAFLD group was lower than that of miMAFLD, but beta diversity has no significant differences. We identified signature microbial taxa in MAFLD patients, as well as taxa that change between different HS subgroups. In addition, we found a trend towards increased levels of HS in key gut microbial taxa, suggesting that gut microecological dysregulation contributes to disease progression. In addition, the abundance of *E. rectale* was found to be significantly overrepresented in patients with MAFLD, and the degree of enrichment increased with increasing HS. These findings suggest that *E. rectale* may be potent in inducing MAFLD and imply the potential of gut microbiota as non-invasive biomarkers for predicting MAFLD severity. Clostridium difficile cluster species including *E. rectale* should have a higher relative abundance in MAFLD patients, while still little is known about *E. rectale*. These findings are partially different from previous studies [17–19], in which we found that butyric acid-producing bacteria showed higher relative abundance than healthy populations, rather than lower abundance or depletion. We believe that this can be explained by several points [20]: first, as we mentioned previously, the severity of MAFLD will be represented by different gut ecologies under different pathological

conditions and the depletion of butyric acid-producing bacteria is a gradual process; second, these key features identified in our study also include both pathogenic and beneficial Clostridia, and competition between them may lead to an increase in the MAFLD phenotypic salvage; and third, the increase in the relative abundance of butyrate-producing bacteria may be related to intestinal immunomodulation, in which intestinal microecological dysregulation inversely promotes an increase in butyrate-producing bacteria and mediates the release of anti-inflammatory cytokines. *E. rectale*, a butyrate-producing species with beneficial effects, exhibits anti-inflammatory properties by mediating immunity to regulatory T cells [48]. Finally, as mentioned earlier, patients with severe HS consume more high sugar and high fat in their daily lives, which promotes the growth of *E. rectale*, enhances intestinal energy absorption, and accelerates steatosis.

The relationship between gut microbiota composition and MAFLD in non-obese patients is unknown. Although no significant differences were observed between the non-obese and other groups in terms of alpha diversity and beta diversity, we observed a trend towards decreased microbiome diversity in non-obese MAFLD subjects. We found that the reduced abundance of butyric acid-producing *E. rectale* may play an important role in the development of MAFLD in non-obese individuals. Research has shown that butyric acid is the main energy source for intestinal epithelial cells and can increase lipid synthesis. In addition, both propionic acid and butyric acid can stimulate fat breakdown in adipocytes, which may influence the development of hepatic inflammation. Individuals with non-obese MAFLD have a poor prognosis and are largely asymptomatic during the course of the disease. The disease is primarily detected by the incidental finding of MAFLD on ultrasound or imaginal modalities or routine laboratory tests, and the diagnosis is reliably made by liver biopsy or imaging, but is difficult to screen and monitor on a large scale. Therefore, there is an urgent need to identify individuals who are at high risk for developing MAFLD or who are in the early stages of the disease, as lifestyle interventions can reverse the disease while it is in its first stages. Overall, the results of this study raise the possibility of using characteristic gut microbiota such as *E. rectale* for early clinical warning of MAFLD development.

Metabolites associated with gut microbiota, such as choline and tryptophan metabolites, SCFAs, bile acids, endogenous ethanol, and lipopolysaccharides, have been implicated in the development of MAFLD [3]. This study confirmed the disruption of lipid homeostasis in MAFLD patients. This can be influenced by a number of factors other than diet and environment.

We found significantly elevated plasma levels of valine, L-phenylalanine, eudesmic acid, gallic acid, and 1,2'-di-O-galloylhamamelofuranose in subjects with MAFLD. The elevated levels of BCAAs and AAAs observed in the progression of MAFLD are believed to be a reaction to increased inflammation, oxidative stress, and liver damage [49,50]. Additionally, we observed significantly decreased plasma levels of serine and oleoyl glycine metabolites in MAFLD subjects. Previous studies have linked MAFLD with serine deficiency, emphasizing the importance of serine and glycine as essential metabolites in the synthesis of glutathione, which is crucial for inhibiting the accumulation of intermediate products resulting from fatty acid oxidation [51,52].

The pathogenesis of MAFLD may be exacerbated by elevated levels of free fatty acids, which can trigger inflammation and contribute to fat accumulation [53]. Elevated circulating triglyceride levels have been observed not only in patients with MAFLD but also in individuals with obesity, diabetes, or insulin resistance, all of which are risk factors for MAFLD [54]. Flavonoids have been shown to alleviate hepatic steatosis, oxidative stress, inflammatory cell infiltration, and fibrosis in HFD-induced rat models of MAFLD [55]. Our findings revealed that daidzein may alleviate MAFLD by directly regulating hepatic de novo lipogenesis and insulin signaling and indirectly controlling adiposity and adipocytokines through alterations in adipocyte metabolism [56]. Polyphenols, which are plentiful secondary metabolites found in edible plants, exhibit a wide range of advantageous pharmacological effects in humans [57]. The main microbial metabolite of polyphenols, 4-hydroxyphenylacetic acid (4-HPA), is involved in antioxidant action, mitigating liver, kidney, and lung damage [58–60].

Bile acids (BAs) are essential metabolites derived from the host and modified by microbes, playing a critical role in the regulation of lipid balance. Glycocholic acid is a bile acid with anticancer activity [61]. Higher levels of specific gut microbiota-derived metabolites of bile acids (taurocholic acid) might be positively associated with both a higher MRS and MAFLD risk [62]. Glycoursodeoxycholic acid can predict the severity of ALD [63].

This study has several limitations. First, the inherent global diversity in the gut microbiota composition (interindividual and inter-population) raises questions about the generalizability of our findings to other regions. However, the strength of our large sample multi-omics study is well articulated by the common

flora characteristics of MAFLD such as a decrease in *Dialister invisus* and *Eubacterium rectale*, which provides more evidence, and the characteristic bacteria associated with the pathogenesis such as *E. rectale*, which is well advised for our region. In the future, we will continue to explore the common and individual bacteria of different ethnic groups in different regions through large-sample studies to provide more insights into treatment of diseases. In addition, previous research has highlighted the significant impact of diet on gut microbial composition and function [64]. Although our study excluded individuals with "atypical" dietary habits (e.g. vegetarian diets) within the previous 12 months, we did not enforce strict dietary adjustments for all participants, allowing them to maintain their daily dietary habits. Future studies focusing on the dietary patterns of patients may provide valuable insights and aid in the development of individualized treatment approaches. Detection of intestinal flora in human feces requires consideration of several factors, such as experimental design, sample handling, and analytical methods. Although we strictly control other factors affecting the flora results, the sample sizes of some subgroups were relatively small (for example, There were only 12 cases of MAFLD patients with normal body weight), which might lead to a reduction in the stability and reliability of the results of subgroup analysis and increase the risk of false positive or false negative results. In the future, we will expand the sample size to provide more evidence and insights on the pre-results. Ultimately, we hope that our research will contribute to the advancement of new diagnostic and therapeutic strategies for MAFLD.

Conclusion

Increases in *Eubacterium rectale* and decreases in *Dialister invisus* seem to be indicative of MAFLD patients. *Eubacterium rectale* may predict HS degree of MAFLD and play an important role in the development of non-obese MAFLD. *Eubacterium rectale* can generate more propionic acid and butyric acid analogues to absorb energy, increase lipid synthesis, and ultimately cause MAFLD. The observed association between the Ruminococcaceae bacteria and β-alanine or uric acid in these patients could offer fresh insights into the disease mechanism.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contributions

Conceptualization, D.-Y.Z., and F.-H.B.; methodology, D.-Y.Z., and F.-H.B.; software, D.-Y.Z., D.-L., S.-J.C. and F.-H.B.; validation, D.-Y.Z., S.-J.C., Q.W. and F.-H.B.; formal analysis, D.-Y.Z., Q.W., D.-L., and F.-H.B.; investigation, D.-Y.Z., Q.W., J.-R.C., and X.-L.Z.; resources, J.-R.C., and X.-L.Z; data curation, Q.W.; writing – original draft preparation, D.-Y.Z., and F.-H.B.; writing – review and editing, D.-Y.Z., and F.-H.B.; visualization, D.-Y.Z., S.-J.C. and F.-H.B.; supervision, F.-H.B.; project administration, D.-Y.Z., and F.-H.B.; funding acquisition, D.-Y.Z., and F.-H.B. All authors have read and agreed to the published version of the manuscript.

Data availability statement

This paper does not report the original code. Original data for creating all graphs in the paper are provided in Data S1. The data have been deposited, and you can visit http://www.ncbi.nlm.nih.gov/bioproject/PRJNA1181792. Metabolomics data have been deposited on the EMBL-EBI MetaboLights database (DOI: 10.1093/nar/gkad1045, PMID:37971328) with the identifier MTBLS11532.

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

Institutional review board statement

This study was approved by the Institutional Review Board (EC-20231214-1009). The study was conducted in accordance with the Declaration of Helsinki. All methods were performed in accordance with relevant guidelines and regulations.

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