Journal of Inflammation Research

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ORIGINAL RESEARCH

Exercise and Berberine Intervention Ameliorate High-Fat Diet-Induced MAFLD by Regulating Gut Microbiota and Hepatic Fatty Acid Beta-Oxidation

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Background: Metabolic dysfunction-associated fatty liver disease (MAFLD) is a global concern. The gut microbiota and hepatic fatty acid beta-oxidation have been shown to be important factors in the development of MAFLD. Independently, exercise and berberine can significantly ameliorate symptoms of MAFLD, although the specific mechanism is not clear; moreover, it is not known whether the combination of exercise and berberine produces a better therapeutic effect.

Methods: Over an experimental period of 17 weeks, the effects of exercise, berberine, and a combined (exercise/berberine) intervention on the composition of the gut microbiota and the expression of hepatic fatty acid beta-oxidation related proteins were examined. The mice were divided into five treatment groups: CON (control group, n = 10), HFD (high-fat diet, n = 10), HFE (high-fat diet + exercise, n = 10), HFB (high-fat diet + berberine, n = 10), and HBE (high-fat-diet + exercise + berberine, n = 10). The dose of BBR administered for oral gavage was 300 mg/kg, once per day, for 8 weeks. Mice were subjected to treadmill exercise, 5 days per week for 8 weeks, and the intensity was increased gradually.

Results: Serological and histopathological results showed that exercise, berberine and a combined (exercise/berberine) intervention effectively improved liver lipid accumulation caused by a high-fat diet. Analysis of 16S rRNA showed that the three interventions restored the species and number of gut microbiota in MAFLD mice. The functional prediction of gut microbiota revealed significant differences in beta-oxidation-related units among groups. Simultaneously, exercise and berberine intervention regulated the expression of hepatic fatty acid beta-oxidation-related proteins ACOX1, HMGCS2, and CPT-1α, with the combined intervention having a more significant effect than each intervention alone.

Conclusion: Our findings indicate that exercise and berberine ameliorate MAFLD by regulating the gut microbiota and hepatic fatty acid beta-oxidation, suggesting that their combination may be a potential therapy for MALFD.

Keywords: metabolic dysfunction-associated fatty liver disease, gut microbiota, fatty acid beta-oxidation, exercise, berberine

Introduction

Metabolic dysfunction-associated fatty liver disease (MAFLD) is the new term for non-alcoholic fatty liver disease (NAFLD). In 2020, European Association for the Study of the Liver proposed this name change, along with the use of positive diagnostic criteria. This statement regarding the name change from NAFLD to MAFLD has been widely recognized in the field of liver disease.[1] MAFLD is the leading cause of liver disease in all regions of the world. The global prevalence of MAFLD is estimated at 24% and continues to rise.[2,3] A characteristic of MAFLD is the excessive accumulation of triglycerides (TGs) and cholesterol in lipid droplets within hepatocytes.[4] Although simple steatosis is considered a benign condition, metabolic-associated fatty liver may worsen and develop into metabolic dysfunction-

Received: 1 October 2024
Accepted: 10 February 2025
Published: 26 February 2025

associated steatohepatitis (MASH), a more severe form of MAFLD involving inflammation, cell damage, and steatosis. After alcohol-related liver disease, MASH has emerged the second largest indication for end-stage liver disease, requiring liver transplantation.[5,6] Although no drugs are currently approved for the treatment of MAFLD,[7] progress has been made in the field. Resmetirom, an orally administered, liver-targeted thyroid hormone receptor-β (THR-β) agonist for the treatment of MAFLD and MASH,[8] was given accelerated approved in the US in March 2024 for use in conjunction with diet and exercise for the treatment of adults with noncirrhotic MASH with moderate to advanced liver fibrosis (consistent with stages F2 to F3 fibrosis).[9] This treatment has provided a new direction for the study of MAFLD, showing that combination therapies may produce unexpected therapeutic effects.

The pathogenesis of MAFLD is complex. It is a multifactorial complication caused by genetic predisposition, metabolic function, inflammation, gut microbiota, and environmental factors. The main manifestations are abnormal intracellular lipid storage (hepatic steatosis) and inflammatory progression.[10] Despite the in-depth study of the pathogenesis of MAFLD, which has progressed the initial "two-hit" theory[11] to the current "multiple injury model",[12] the specific mechanisms of pathogenesis remain unclear.

The liver is the central organ of fatty acid metabolism. It can take up carbohydrates and fats from adipose tissue for gluconeogenesis and ketogenesis in the liver.[13] A disruption in the liver lipid balance can cause metabolic disturbances that lead to the accumulation of fat within the liver, and various factors affecting hepatic lipid metabolism contribute to the development of fatty liver.[14] In the liver, fatty acids are broken down by beta-oxidation and esterified into TGs. In patients with MAFLD, the fatty acid beta-oxidation process is blocked, leading to excessive esterification of fatty acids to triglycerides, which causes hepatic steatosis.[15,16] Previous studies have also demonstrated that in mice fed a high-fat diet, hepatic fatty acid beta-oxidation-related protein expression decreased, and there was a significant increase in lipid droplets in the liver.[17] Anatomically, there is a close relationship between the gut and the liver: 70% to 75% of the blood supply to the liver comes from the portal vein, and bacteria and their metabolites produced in the gut can reach the liver through the portal vein, making the liver one of the most easily accessible organs for gut microbiota and their derivatives.[18] In patients with MAFLD, the disruption of gut microbiota has been observed repeatedly. The most common changes in patients with MAFLD are the decreased abundance of *Bacteroidetes* and *Ruminococcaceae* and the increased abundance of *Lactobacillaceae, Veillonellaceae*, and *Dorea*.[19] In addition, changes in the gut microbiota promote the occurrence and development of MAFLD. Two weeks after fecal microbiota from patients with hepatic steatosis were transplanted into the gut of normal mice, large quantities of triglycerides accumulated in the liver.[20] When the gut microbiota of patients with MAFLD and healthy individuals were transplanted into the gut of normal mice, the mice transplanted with gut microbiota from patients with MAFLD gained more weight and had more obvious liver steatosis.[21]

Berberine ($C_{20}H_{19}NO_5$,BBR) is an isoquinoline alkaloid isolated from berberis. BBR is widely used to treat diarrhea, insulin resistance, inflammation, and gastrointestinal diseases.[22,23] Wang et al reported that in mice with high-fat diet-induced MAFLD, BBR can ameliorate MAFLD by suppressing the accumulation of lipids in the liver, inhibiting the expression of liver inflammatory factors and oxidative stress processes, and thereby regulating the disrupted gut microbiota.[24–26] Zheng et al reported that exercise can improve MAFLD by promoting mitochondrial autophagy in the liver, increasing the range of species and number of beneficial microbiota in the gut, and upregulating the expression of hepatic fatty acid beta-oxidation-related proteins.[27–30]

These findings provide evidence that exercise and berberine can individually result in significant amelioration of MAFLD symptoms; however, it is not known if their combination could produce a better therapeutic effect or if BBR could enhance the hepatic fatty acid beta-oxidation process, and the combined effect of exercise and berberine on fatty acid beta-oxidation and the gut microbiota is still unclear. Therefore, in this study, we explored BBR and aerobic treadmill exercise as a possible treatment for MAFLD by combining traditional molecular biology research methods with high-throughput sequencing technology (16s rRNA sequencing analysis).

We investigated the effects of exercise and berberine on the gut microbiota and fatty acid beta-oxidation in mice with MAFLD. Exercise and berberine could both relieve MAFLD symptoms, increase hepatic fatty acid beta-oxidation and improve the composition of the gut microbiota; however, they were more effective as a combination treatment. Additionally, we found that the changes in the gut microbiota were closely related to the hepatic fatty acid beta-oxidation

process. These results suggest that the combination of exercise and berberine is a promising candidate for the treatment of MAFLD.

Materials and Methods

Chemicals

BBR hydrochloride tablets were purchased from Northeast Pharmaceutical Group Shenyang No. 1 Pharmaceutical Co., Ltd. The primary antibodies CPT-1α (PAB33949), HMGCS2 (PAB31943), and ACOX1 (PAB35687) were all sourced from Wuhan Hualianke Biotechnology Co., Ltd. The antibody glyceraldehyde 3-phosphate dehydrogenase (GAPDH, AC001) was from AIBOTEK Biotechnology Co., Ltd. The secondary antibody HRP-conjugated Goat anti-Rabbit IgG (AS014) was from AIBOTEK Biotechnology Co., Ltd. C57BL/6J mice were purchased from SPF (Beijing) Biotechnology Co., Ltd. Mice feed was provided by Shenyang Maohua Biotechnology Co., Ltd.

Experimental Design

The experiment was conducted in accordance with the principles of the Ethics Committee of Shenyang Sport University. Fifty male SPF-grade C57BL/6J mice (8 weeks old, 20 ± 2 g) were used for the experiment. After 1 week of adaptive feeding in their cage, the mice were randomly allocated into one of the two groups: CON (n = 10) or MOD (n = 40). The CON group received a normal diet (23.07% protein, 65.08% carbohydrate, 11.85% fat) and the MOD group received a high-fat diet (20% protein, 60% fat, and 20% carbohydrate).[31] After 8 weeks, the mice in the MOD group were randomly allocated into one of four groups and given the indicated treatments: HFD (high-fat diet, n = 10), HFE (high-fat diet + exercise, n = 10), HFB (high-fat diet + BBR, n = 10), and HBE (high-fat-diet + exercise + BBR, n = 10) (Figure 1A). The control group continued to receive a normal diet. Mice in the HFE and HBE groups performed an 8-week treadmill exercise program,[32,33] as shown in Table 1. Mice in the HFB and HBE groups were intragastrically administered a berberine suspension (a mixture of 0.5% sodium carboxymethylcellulose solution and berberine powder) at a dose of 300 mg/kg,[31,34] once per day, for eight weeks. To ensure that all the mice received the same stimulation, except for the intervention, the mice in the CON, HFD, and HFE groups received an intragastric administration of 0.5% CMC-Na solution at a dose of 10 mL/kg, once per day, for 8 weeks. During the experiment, the body weight of mice was recorded every week. At the end of the experiment, the mice were anesthetized with CO_2, the abdominal cavity was opened, and blood from the abdominal aorta, the contents of the small intestine, and tissue from the liver were collected for analysis.

Biochemical Assays

Serum triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were measured using an automatic biochemical analyzer.

Histological Analysis

Hematoxylin and eosin (H&E) staining: Liver tissues were fixed in 10% formalin, dehydrated in ethanol, and embedded in paraffin. The 5-μm sections were cut and mounted on a glass slide. After staining with hematoxylin and eosin (H&E) and dehydration with alcohol, images were captured with a microscope at a resolution of 400×.

Oil Red O (ORO) staining: The frozen liver samples were sectioned at 7 μm thickness. The sections were dried, fixed in 10% buffered formalin, and then washed with running tap water. The slides were rinsed with 60% isopropanol and stained with prepared Oil Red O solution. After rinsing again with 60% isopropanol, rinsed with distilled water, and mounted with aqueous mounting media and cover slipped. Finally, images were captured with a microscope at a resolution of 400×.

16S rRNA Sequencing and Analysis

The intestinal contents of the mice were immediately placed in a −80°C freezer after being removed from the small intestine, and then sent to Shanghai Parsono Biotechnology Co., Ltd for sequencing. The steps include: total DNA extraction and quality control of the microbiome, PCR amplification, second amplification, library construction, quality inspection, and computer sequencing. The concentration and purity of total DNA in small intestinal contents were

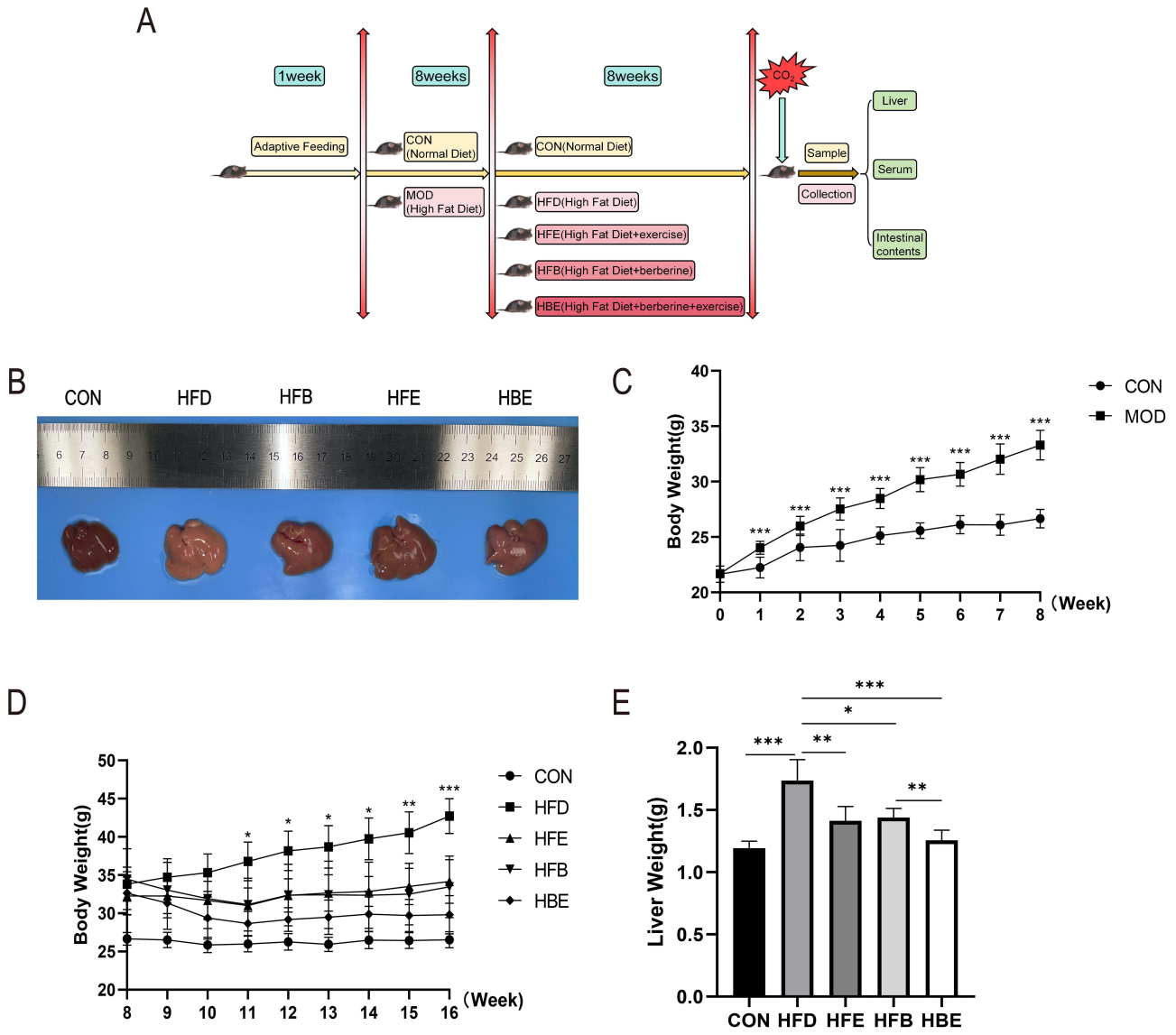

Figure 1 (A) Experimental design of a protocol for male C57BL/6J mice; **(B)** liver morphology; **(C)** changes in body weight over 0–8 weeks; **(D)** changes in body weight over 8–16 weeks; **(E)** changes in liver weight.
Notes: *: $P<0.05$, * *: $P<0.01$, * * *: $P<0.001$.
Abbreviations: CON, normal diet; MOD, high-fat diet; HFD, high-fat diet; HFE, high-fat diet + exercise; HFB, high-fat diet + BBR; HBE, high-fat diet + exercise + BBR.

analyzed using agarose gel electrophoresis. The target was the V3–V4 region of the bacterial ribosomal rRNA gene sequence; the forward primer used was ACTCCTACGGGAGGCAGCA and the reverse primer used was GGACTACHVGGGTWTCTAAT. The DNA fragments in the gut microbiota were sequenced on the Illumina platform, with de-priming, quality filtering, and denoising performed using the DADA2 method and QIIME2 (2019.4) software. The primer fragments of the sequence were removed with qiimecutadapttrim-paired, and the unmatched primer sequences were discarded. After denoising all libraries, the ASV characteristic sequences were merged and singletons ASVs were removed. A script in the R language was used to count the distribution of the sequence length, and distribution of the length of high-quality sequences contained in all samples was recorded. The results are presented in five parts: α diversity of the microbiota; β diversity of the microbiota; species composition of the gut microbiota; species differences and marker species analysis; and function prediction.

Table 1 Exercise Intervention Program

Week	Date	Speed	Time
First week	Monday	10 m/min	30 min
First week	Tuesday	10.5 m/min	30 min
First week	Wednesday	11 m/min	30 min
First week	Thursday	12 m/min	30 min
First week	Friday	12.5 m/min	30 min
Second week	Monday	12.5 m/min	45 min
Second week	Tuesday	12.5 m/min	45 min
Second week	Wednesday	12.5 m/min	45 min
Second week	Thursday	12.5 m/min	60 min
Second week	Friday	12.5 m/min	60 min
Third week	Monday—Friday	13.5 m/min	60 min
Fourth week	Monday—Friday	14.5 m/min	60 min
Fifth week	Monday—Friday	15.5 m/min	60 min
Sixth week	Monday—Friday	16.5 m/min	60 min
Seventh week	Monday—Friday	17.5 m/min	60 min
Eighth week	Monday—Friday	18.5 m/min	60 min

Western Blotting Analysis

Proteins were extracted from the liver tissue and prepared for SDS-PAGE. The separating gel was prepared at the appropriate concentration based on the molecular weight of the target protein (10% separation gel for molecular weights between 20 and 80 kDa, 8% separation gel for molecular weights between 30 and 90 kDa, 6% separation gel for molecular weights between 50 and 150 kDa; 10% separation gel is used in this experiment). In accordance with the instructions for the reagent kit, a concentrated gel was prepared, mixed, and added quickly to the separation gel without allowing bubbles to form. The electrophoresis settings were 80 V; after approximately 50 min, the voltage was adjusted to 120 V. When the blue marker line reached the bottom of the separation gel and approached the silver metal line, electrophoresis was stopped. Membrane transfer and sealing were performed, followed by incubation with the primary antibody. The primary antibody dilution was 1:1000 (1 μL of primary antibody in 1 mL of blocking solution). The PVDF membrane was cut for different molecular weights and immersed in different antibodies on a shaker overnight at 4°C in a chromatography cabinet. After the incubation with the primary antibody, the PVDF membrane was removed and placed in an incubator containing TBST solution. The membrane was washed three times on a shaker for 5–10 minutes each time. For secondary antibody incubation, the dilution was 1:10000 in blocking solution. After washing, the PVDF membrane was incubated with diluted secondary antibody on a shaker at room temperature for 1 hour. After incubation with the secondary antibody, the PVDF membrane was washed three times with TBST, and placed in the membrane in the developing area of the luminescence instrument. An appropriate amount of luminescence solution was applied, and the results were collected. The gray values of the results were obtained using ImageJ software. Finally, statistical analysis of the data was performed.

Statistical Analysis

Statistical analyses were performed using SPSS 20.0 software, and the results were presented as the mean±SD ($\overline{X} \pm S$). Statistical significance was accepted for P values of <0.05. GraphPad 8.0 was used for image rendering. The Shapiro–Wilk method was used to test whether the five groups of data were in accordance with the normal distribution; if they were not, the Kruskal–Wallis test was used. Levene's test was used to examine the homogeneity of variance for normally distributed data. If the variance was uniform, one-way ANOVA and the least significant difference (LSD) method were used to compare the five groups of data. If the variance was uneven, one-way ANOVA and Dunnett's T3 method were used to compare the five groups of data.

Results

Body Weight, Liver Weight, and Liver Morphology of Mice

Morphological observations of the mouse liver are presented in Figure 1B. For mice fed the normal diet, the surface of the liver was smooth and fine, whereas for mice fed a high-fat diet for 16 weeks, the livers were swollen, greasy, and pale. The liver color, size, and greasiness of mice were obviously improved by the different intervention methods.

During the first 8 weeks of the diet, mice in the MOD group gained weight rapidly (Figure 1C). Compared with the CON group, mice in the MOD group gained a significant amount of weight after 1 week ($P < 0.001$). In the last eight weeks of the diet, compared with the HFD group, all three interventions significantly decreased the weight of mice after 11 weeks ($P < 0.05$), and the combined intervention resulted in a more significant decrease ($P < 0.001$) (Figure 1D). At the end of the experiment, the weight of the livers of mice in the HFD group was significantly higher than that in the CON group ($P < 0.001$), and exercise and berberine significantly decreased the weight of the liver, respectively ($P < 0.05$), the effect of combined intervention was more significant ($P < 0.001$) (Figure 1E).

Exercise and Berberine Ameliorated Liver Steatosis and Altered Blood Lipid Levels in MAFLD Mice

In our study, mice in the HFD group exhibited marked hepatic steatosis, as demonstrated by hematoxylin and eosin (H&E) and oil red O staining (Figure 2B and C). H&E staining revealed a large number of fat vacuoles and a large extent of inflammatory cell infiltration, while oil red O staining showed obvious accumulation of lipid droplets. Exercise and berberine interventions reversed this change, respectively, and the combined intervention resulted in a better effect.

To explore whether exercise and berberine improved lipid metabolism in MAFLD mice, we examined the serum levels of HDL-C, TC, TG, and LDL-C (Figure 2A). The serum levels of HDL-C, TC, and LDL-C in HFD mice were significantly higher than those in the CON group ($P < 0.001$). Compared with the HFD group, TC, TG, and LDL-C were significantly lower in the HFE group ($P < 0.05$); TG was significantly decreased in the HFB group ($P<0.001$); TC, TG, and LDL-C were decreased significantly in the HBE group ($P < 0.05$, $P < 0.001$, and $P < 0.05$, respectively), and HDL-C was increased significantly ($P < 0.01$).

Effects of Exercise and Berberine on the Composition and Diversity of Gut Microbiota in MAFLD Mice

Changes in Gut Microbiota Composition

To investigate the species composition of the gut microbiota and the relative abundance at three different taxonomic levels (phylum, family, and genus), we plotted cumulative bar graphs for the 10 species with the highest relative abundance of all samples with annotation sequence information represented by cluster analysis of operational taxonomic units (OTUs). At the phylum level (Figure 3A), compared with the CON group, the abundance of *Proteobacteria* increased and that of *Firmicutes* decreased in the HFD group; all three interventions increased the abundance of *Firmicutes* and decreased the abundance of *Proteobacteria*, and the effect of exercise intervention was more obvious than that of other interventions. At the family level (Figure 3B), the dominant bacteria in each group were *Lactobacillaceae*, and the abundance of *Lactobacillaceae* in the gut of mice in the HFD group was lower than that in the CON group, although the result was reversed in the other intervention groups. At the genus level (Figure 3C), *Lactobacillus, Akkermansia*, and *Allobaculum* were the dominant bacteria in each group. The high-fat diet led to a decrease in *Lactobacillus* abundance, which was reversed by all interventions, and the most effective intervention was the drug. The abundance of *Akkermansia* was higher in the HFB and HBE groups, whereas that of *Allobaculum* was higher in the HFE and CON groups.

Alpha Diversity and Beta Diversity Analysis

The alpha diversity analysis (Figure 3E) showed that the abundance, diversity, evenness, and sequencing coverage of the gut microbiota were significantly different between the groups ($P < 0.01$). Compared with the CON group, the Chao1 index, observed species, Shannon and Simpson indexes, and Pielou's evenness were higher in the HFD group, and the

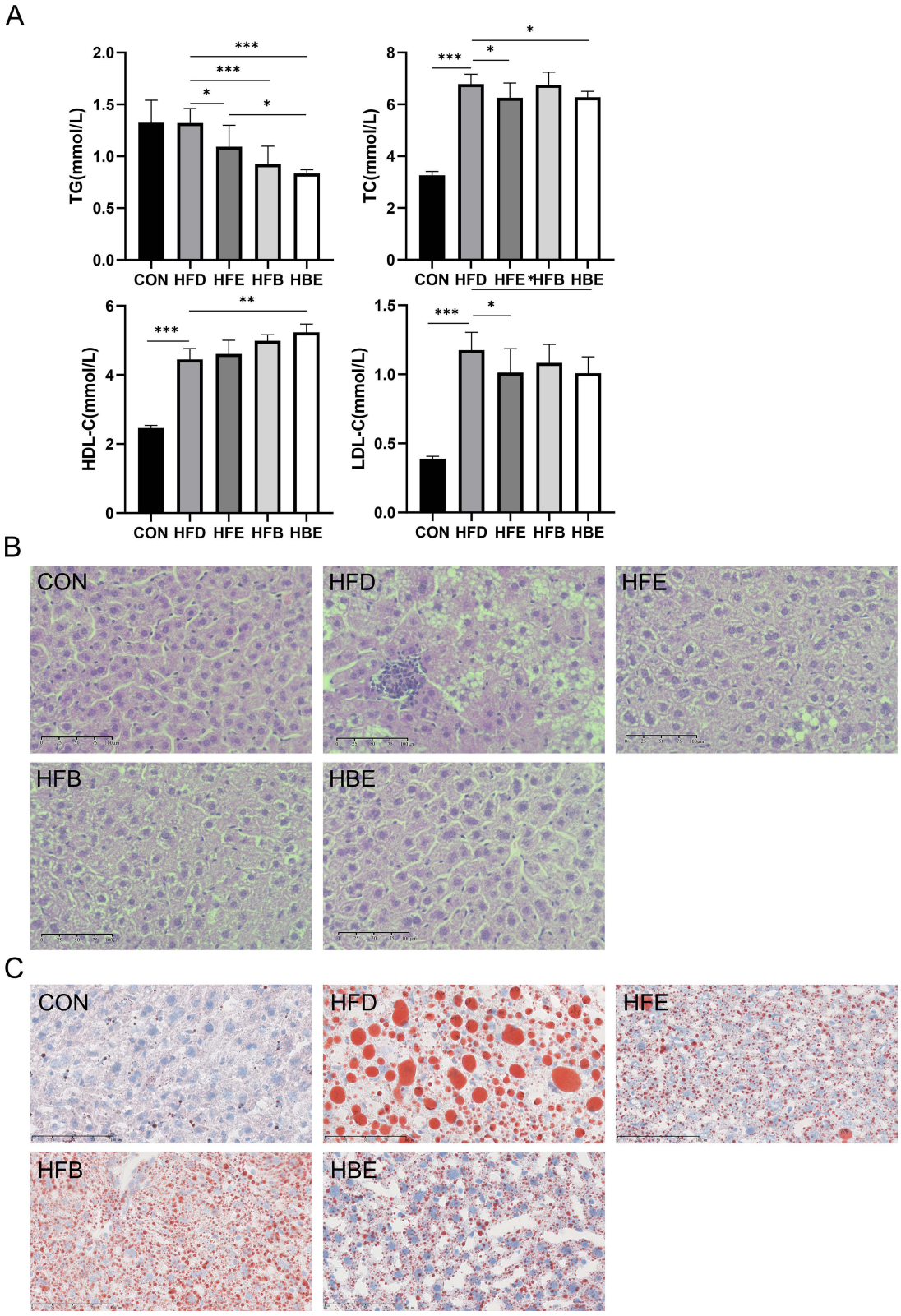

Figure 2 Results of liver tissue staining and changes in serum lipid levels. (**A**) Serum TG, TC, HDL-C, LDL-C levels; (**B**) liver tissue HE staining (×400); (**C**) liver tissue oil red O staining.

Notes: (×400) *: $P<0.05$, * *: $P<0.01$, * * *: $P<0.001$.

Abbreviations: CON, normal diet; HFD, high-fat diet; HFE, high-fat diet + exercise; HFB, high-fat diet + BBR; HBE, high-fat diet + exercise + BBR.

https://doi.org/10.2147/JIR.S498782

different interventions resulted in a decrease in the indexes. The variation in the abundance curve (Figure 3D) and the alpha diversity performance results were consistent, namely a high-fat diet increases the evenness of the gut microbiota. Our results indicate that a high-fat diet increases the abundance and evenness of the gut microbiota, and that the exercise and BBR interventions reversed this change caused by the high-fat diet.

We used hierarchical clustering analysis and the intergroup difference analysis to describe beta diversity. Through the hierarchical clustering analysis (Figure 3F), we found significant differences in the microbial community composition between the HFD group and the other four groups. The microbial community composition of the HFE, HFB, and HBE groups was similar, and that of the HBE and HFB groups was the most similar. At the genus level, *Lactobacillus, Akkermansia*, and *Allobaculum* were the most abundant, with significant differences between groups. Subsequently, we used the anosim test method (Figure 3G) to conduct statistical tests on the microbial community and determine whether there were significant differences between multiple groups of samples. The intra-group distance between the samples in the HFD group was significantly lower than that of the other groups after pairwise comparison; moreover, the test statistic R was greater than 0, indicating that the intra-group difference was smaller than the intergroup difference. There was also a significant difference in the inter-group distance between the other groups and the HFD group ($P < 0.01$).

Species Differences and Marker Species Analysis

We further analyzed the differences between species using the LEfSe analysis method, with a comparison strategy of one against-all, an LDA threshold of 4, and the Wilcoxon test method for validation. Taxonomic units with significant differences between groups were displayed in a bar chart (Figure 4A), and taxonomic branching diagrams were used to display the classification hierarchy relationships of the main taxonomic units in the sample community (Figure 4B). The results indicate that at the phylum level, *Proteobacteria* and *Actinobacteria* were more abundant in the HFD group, *Firmicutes* was more abundant in the HFE group, and *Verrucomicrobia* was more abundant in the HFB group. At the family level, *promicromonosporaceae, Neisseriaceae, Prevotellaceae, Pasteurellaceae*, and *Streptococcaceae* were more abundant in the HFD group; *Lactobacillaceae* and *Verrucomicrobiaceae* were more abundant in the HFB group; *Erysipelotrichaceae* was more abundant in the HFE group; *Enterococcaceae, Peptostreptococcaceae*, and

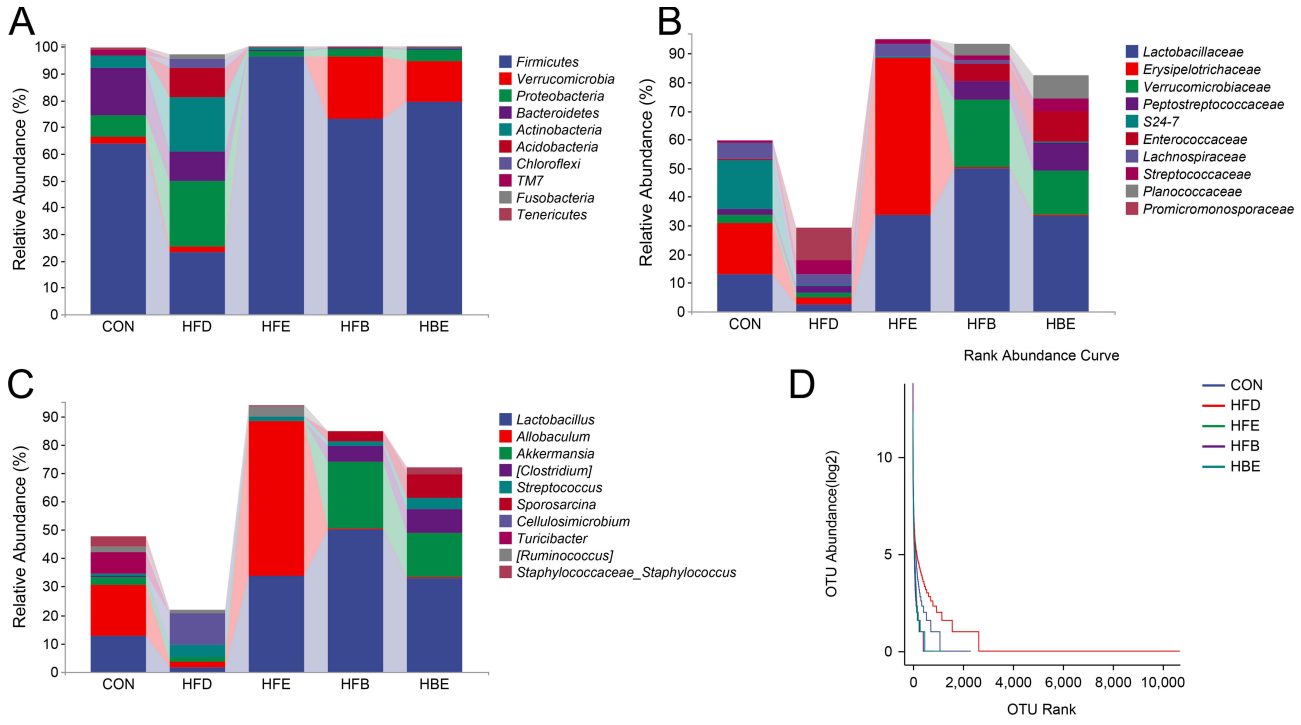

Figure 3 Continued.

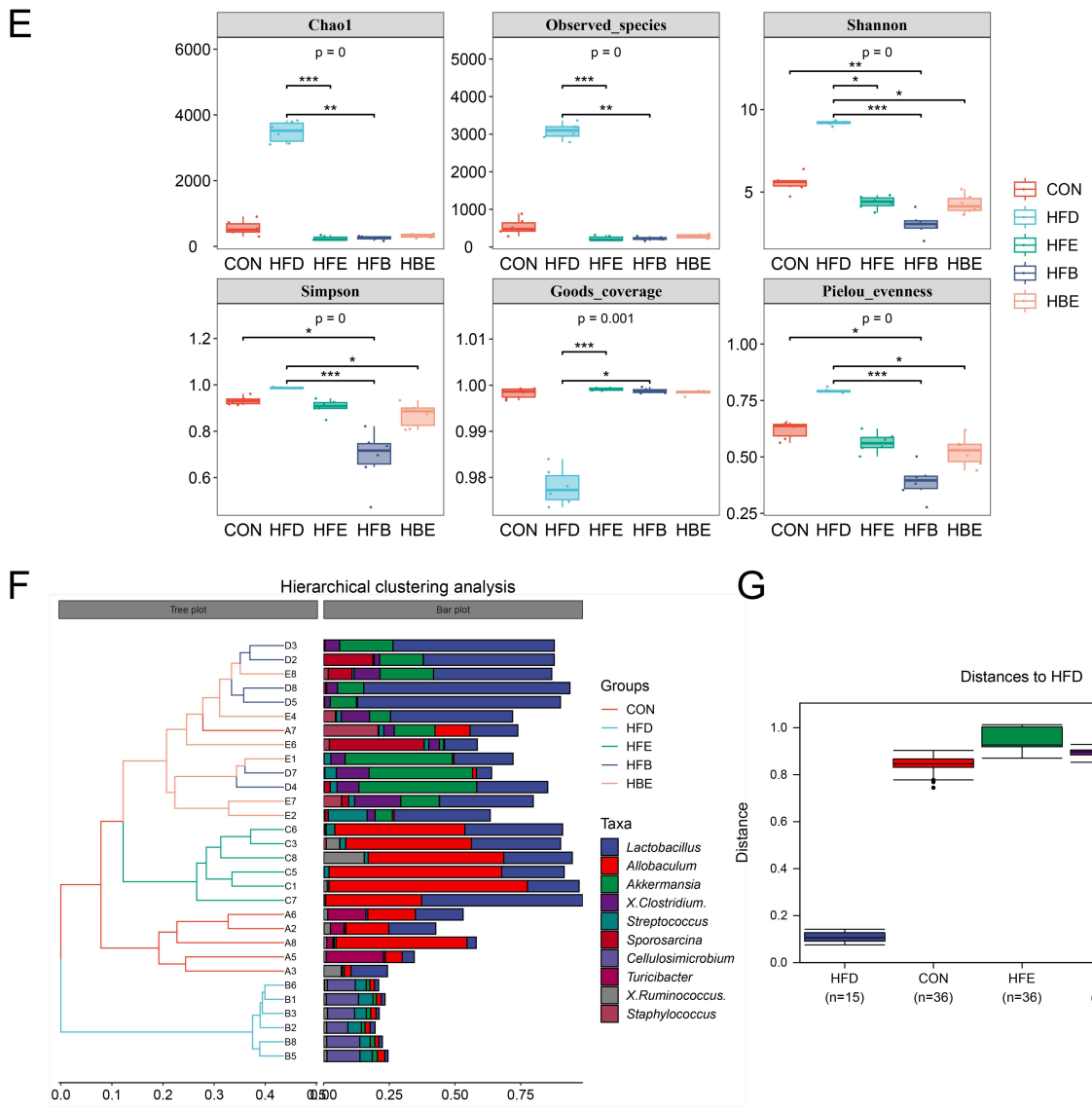

Figure 3 Changes in the composition and diversity of the gut microbiota. **(A)** Composition of the gut microbiota at the phylum level; **(B)** composition of the gut microbiota at the family level; **(C)** composition of the gut microbiota at the genus level; **(D)** rank abundance curve; **(E)** α diversity index; **(F)** hierarchical clustering analysis. On the left is a hierarchical clustering tree, in which samples are clustered according to their similarity to each other: the shorter the branch length between the samples, the more similar the two samples. On the right is a stacked histogram of the top 10 genera in abundance; **(G)** intergroup difference analysis. Comparison with the HFD group.

Notes: **P*<0.05, * **P*<0.01, * * **P*<0.001.

Abbreviations: CON, normal diet; HFD, high-fat diet; HFE, high-fat diet + exercise; HFB, high-fat diet + BBR; HBE, high-fat diet + exercise + BBR.

Planococcaceae were more abundant in the HBE group. At the genus level, *Cellulosimicrobium, Prevotella, Neisseria*, and *Streptococcus* were more abundant in the HFD group; *Lactobacillus* and *Akkermansia* were more abundant in the HFB group; *Allobaculum* was more abundant in the HFE group; and *Enterococcus* was more abundant in the HBE group.

Subsequently, we used the Kruskal–Wallis test to statistically analyze significant differences at the genus level in bacterial communities between groups (Figure 4C). The results showed that compared with the CON group, the abundance of *Neisseria, Streptococcus, Cellulosimicrobium*, and *Prevotella* in the HFD group was significantly increased ($P < 0.05$); the different interventions significantly reduced the abundance of *Neisseria, Cellulosimicrobium*, and *Prevotella* ($P < 0.05$), berberine intervention significantly reduced the abundance of *Streptococcus* ($P < 0.01$). A high-fat diet reduced the abundance of *Allobaculum* and *Lactobacillus*, and all interventions significantly increased the abundance of *Lactobacillus* ($P < 0.05$), berberine intervention caused the most significant effect ($P < 0.01$). Exercise

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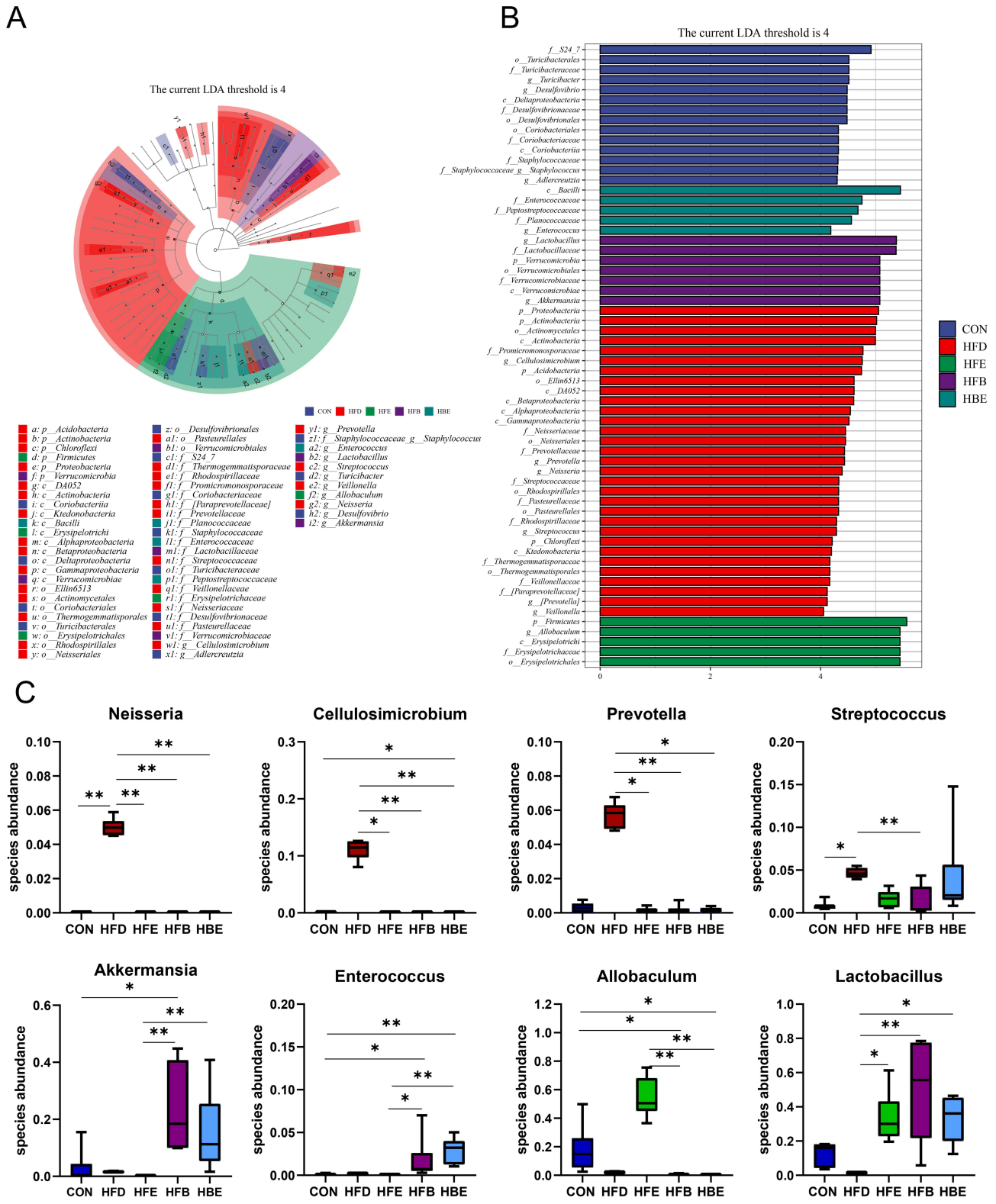

Figure 4 Species differences and marker species analysis. (**A**) Evolutionary branching diagrams for divergent species. Circles radiating from inside to outside represent taxonomic levels from phylum to genus (or species); (**B**) marker bacteria (LDA score (log 10) > 2) in the five groups; (**C**) differential species statistics.
Notes: **P*<0.05, ***P*<0.01.
Abbreviations: CON, normal diet; HFD, high-fat diet; HFE, high-fat diet + exercise; HFB, high-fat diet + BBR; HBE, high-fat diet + exercise + BBR.

significantly increased the abundance of *Allobaculum* compared with the HFB and HBE groups ($P < 0.01$); compared with the HFE group, the abundance of *Akkermansia* in the HFB group was significantly increased ($P < 0.01$), and the abundance of *Enterococcus* in the HBE group was significantly increased ($P < 0.01$).

Functional Prediction

Sample functional differences were expanded at low dimensions using a sample difference distance matrix combined with principal coordinate analysis (Figure 5A). The HFD group had a greater horizontal projection distance from the other four groups, indicating that the HFD group was significantly different from the other four groups in their functional composition in the corresponding dimensions. The smallest distance on the horizontal projection, which was found between the CON group and the HBE group, indicates that the functional composition of the two groups was most similar in the corresponding dimensions. Analysis using the KEGG database revealed that the KEGG class I metabolic pathways with the highest relative abundance in the intestinal content of included metabolism, genetic information processing, and environmental information processing (Figure 5C).

After the metabolic pathway abundance was determined, we analyzed lipid and fatty acid metabolism pathways (Figure 5B) and found significant differences between groups ($P < 0.05$). Further study of the fatty acid metabolism of acyl-coenzyme A oxidase 1 (ACOX1) and hydroxymethylglutaryl-CoA synthase (HMGCS) indicated significant differences between groups ($P < 0.01$).

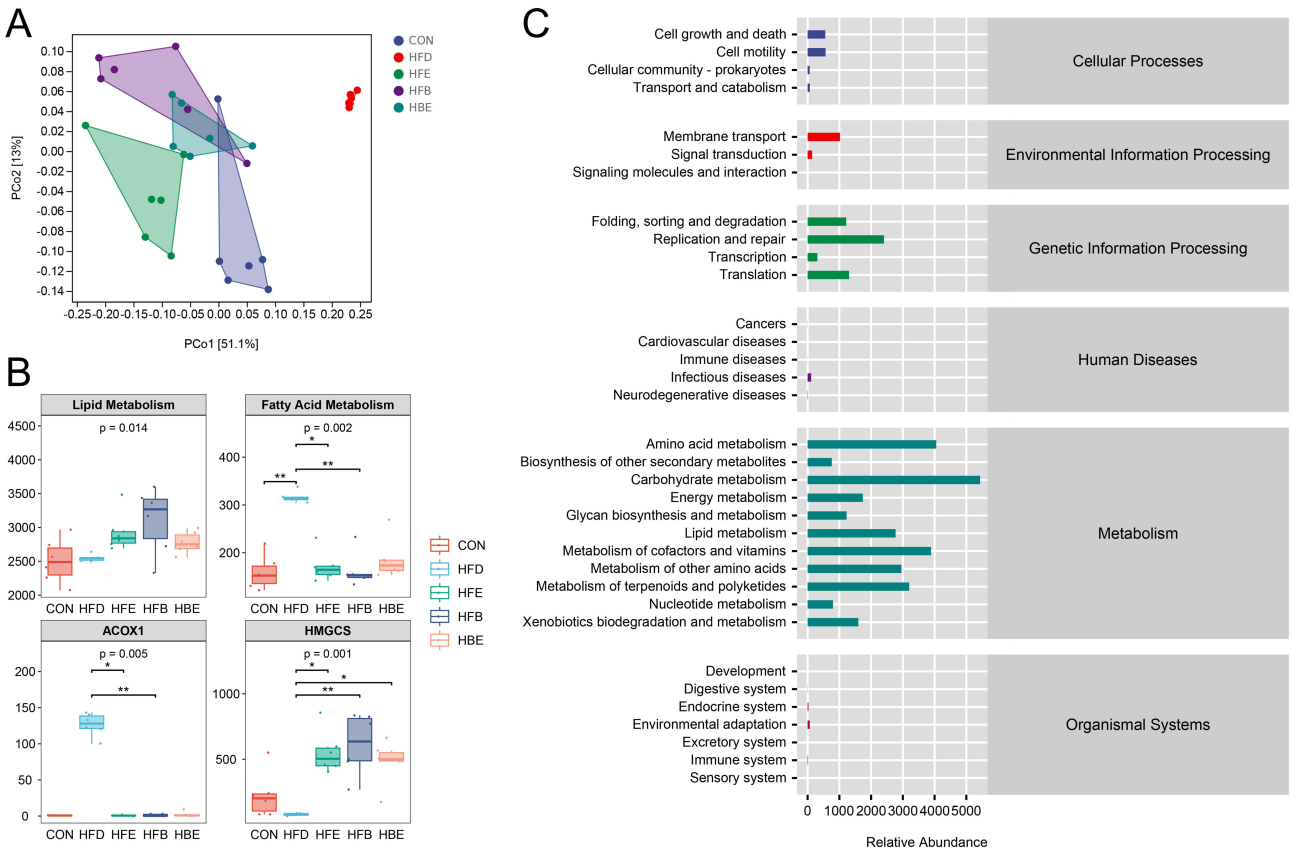

Figure 5 Functional prediction. (**A**) Functional unit PCoA analysis. The closer the projection distance between two points on the coordinate axis, the more similar the functional composition of these two samples in the corresponding dimension; (**B**) metabolic pathway statistics; (**C**) differential testing of lipid metabolism pathways.
Notes: * $P<0.05$, ** $P<0.01$.
Abbreviations: CON, normal diet; HFD, high-fat diet; HFE, high-fat diet + exercise; HFB, high-fat diet + BBR; HBE, high-fat diet + exercise + BBR.

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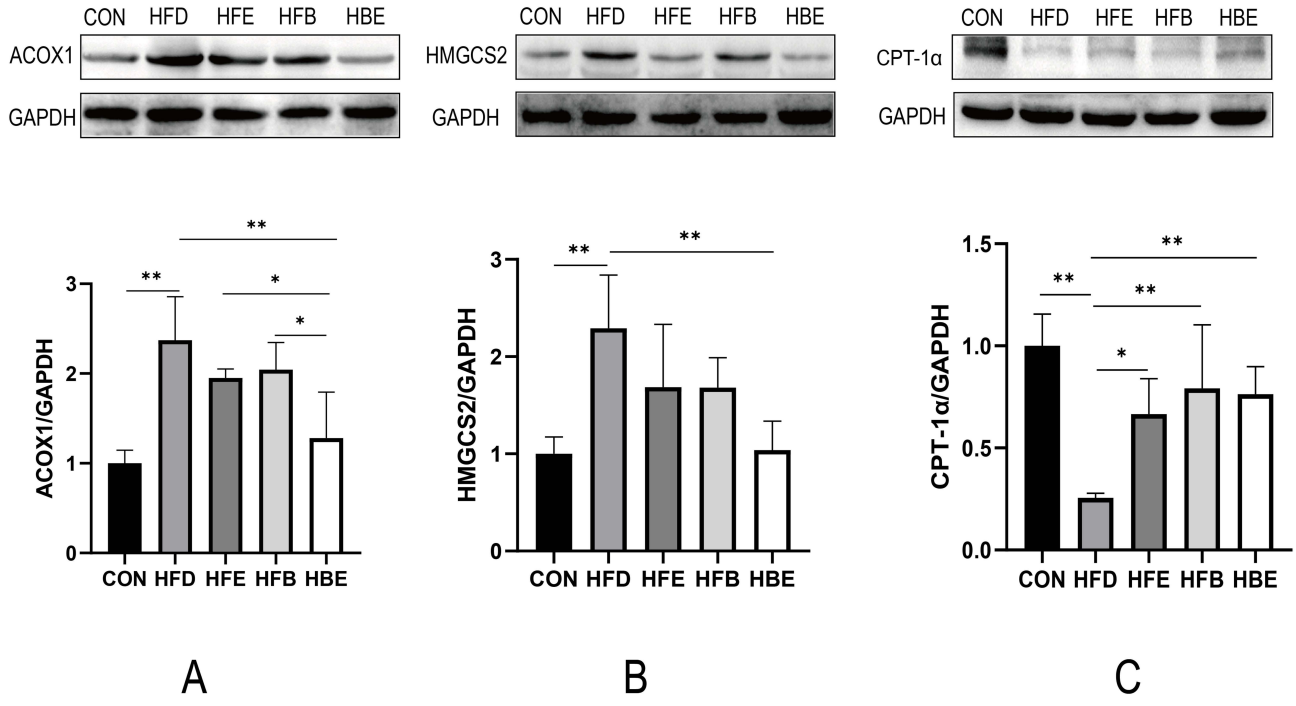

Figure 6 Western blotting assay of mouse liver tissue. (**A**) ACOX1 protein expression; (**B**) HMGCS2 protein expression; (**C**) CPT-1α protein expression.
Notes: *: *P*<0.05, * *: *P*<0.01.
Abbreviations: CON, normal diet; HFD, high-fat diet; HFE, high-fat diet + exercise; HFB, high-fat diet + BBR; HBE, high-fat diet + exercise + BBR.

Exercise and Berberine Regulate the Expression of Hepatic Fatty Acid β-Oxidation-Related Proteins

The expression of ACOX1, HMGCS2, and CPT-1 α in each group was detected using Western blotting. The results are shown in Figure 6. Compared with the CON group, the expression of the ACOX1 and HMGCS2 proteins in the HFD group was increased significantly ($P < 0.01$), and the expression of CPT-1α protein was decreased significantly ($P < 0.01$). Compared with the HFD group, the expression of ACOX1 and HMGCS2 was decreased ($P > 0.05$) in the HFE and HFB groups and decreased significantly in the HBE group ($P < 0.01$), expression of CPT-1α protein was increased in the HFE group ($P < 0.05$) and significantly increased in the HFB and HBE group ($P < 0.01$).

Discussion

With the prevalence of obesity and metabolic diseases, MAFLD has become an increasingly serious global phenomenon.[7] Many therapeutic approaches, such as exercise and berberine intervention, have been shown to significantly improve MAFLD,[25,26,28] but the underlying mechanisms remain unclear. Previous studies have shown that the development of MAFLD is associated with unhealthy eating patterns, high-calorie diets (eg, foods containing fatty acids and cholesterol or fructose-containing beverages), which increases visceral obesity and stimulates abnormal liver lipid accumulation leading to MASH.[35,36] Therefore, in the present experiment, mice were fed a high-fat diet (60% fat, 20% protein, and 20% carbohydrate) for 16 weeks to establish a model of MAFLD.[31] Gut microbiota and fatty acid beta-oxidation have been reported to play critical roles in both the development and treatment of MAFLD,[15,37,38] but a link between them has not been confirmed. This study explored whether the combination of berberine and exercise could play a therapeutic role in MAFLD by regulating the gut microbiota and fatty acid beta-oxidation process, and whether the change of gut microbiota is related to the hepatic fatty acid beta-oxidation.

After 16 weeks of a high-fat diet, mouse body weight, serum TC, and LDL-C levels were significantly increased, and the appearance of the liver was swollen and greasy. Histological observations indicated a large amount of fat accumulation in hepatocytes, which indicates that the model of MAFLD was successfully established.[1] Exercise and berberine

intervention was started from the 9th week, similar to results in previous studies,[29,39,40] the body weight, serum indexes, and appearance of mice were obviously improved, and the combined intervention of exercise and berberine showed more significant effect than single intervention. In addition, compared with other groups, mice fed a normal diet exhibited generally higher levels of serum TG, suggesting that sedentary behavior may cause elevated serum triglyceride levels in mice; however, our histological observations did not show significant liver lipid accumulation in mice fed a normal diet, indicating that the increased serum triglyceride levels of sedentary mice did not increase the liver burden.

In recent years, the gut–liver axis has been widely studied.[41] There is a close relationship between the gut and the liver, and the basis of this interaction is the gut mucosal barrier.[42] However, a high-fat diet destroys the gut mucosal barrier function of mice and changes the composition of the gut microbiota.[43] After high-throughput sequencing of the contents of the gut, we found that the α diversity index of the gut microbiota was significantly increased in MAFLD mice and that the exercise and berberine intervention restored this diversity. This indicates that a high-fat diet increases the abundance, diversity, and evenness of the gut microbiota in mice, contrasting previous studies showing that a high-fat diet leads to decreases in bacterial α diversity in the feces, small intestine, and colon of mice.[44–47] This may be the result of the overabundance of harmful bacteria. In addition, we found that the Good's coverage index decreased in MAFLD mice, whereas the Good's coverage index in the three intervention groups was close to that of the control group, suggesting that MAFLD mice have a larger proportion of undetected species in their gut, further illustrating that a high-fat diet enriched species diversity in our study. In the analysis of microbiota β diversity, we found that the microbiota composition of mice in the three intervention groups was similar to that of mice in the control group; moreover, compared with the exercise intervention group, the composition of the drug intervention group and the combined intervention group were more similar. This may indicate that berberine has a greater effect on the gut microbiota of MAFLD mice than exercise.

In the gut of MAFLD mice, the abundance of *Proteobacteria* increased and that of *Bacteroidetes* and *Firmicutes* decreased, confirming the results of the study by Yang et al[48,49] Berberine intervention increased the abundance of *Firmicutes* and decreased the abundance of *Bacteroidetes*, which was similar to the results of Wang et al[50] We also found that the interventions of exercise and drugs resulted in similar effects. At the genus level, a high-fat diet increased the abundance of *Cellulosimicrobium, Prevotella, Neisseria*, and *Streptococcus* in the gut. *Prevotella* and *Streptococcus* have been shown to increase in the gut microbiota of patients with cirrhosis,[51,52] and *Neisseria* was significantly more abundant in the salivary microbiota of patients with MAFLD. Our study showed that both exercise and berberine restored the abundance of these four genera of gut microbiota to different degrees. In addition, we found that berberine caused a significant increase in the abundance of *Akkermansia*. Previous studies have shown that berberine can stimulate the secretion of gut mucins and indirectly promote the growth of *Akkermansia*.[53] *Akkermansia muciniphila* was shown to prevent fatty liver disease in obese mice by regulating triglyceride synthesis in the liver and maintaining gut homeostasis.[54] These results suggest that *Akkermansia* plays an important role in the amelioration of MAFLD by berberine. *Allobaculum* is an active glucose assimilate,[55] and its overabundance is associated with low levels of circulating leptin.[56] Our results showed that a high-fat diet reduced the abundance of *Allobaculum* in mice and that exercise significantly increased its abundance. Previous studies have also shown that a high-fat diet can decrease the abundance of *Allobaculum*, and interventions such as orange peel extract, nobiletin, and berberine can reverse the reduction in the abundance of *Allobaculum* caused by a high-fat diet, increasing the abundance of *Allobaculum*.[57–59] However, in this study, we did not find evidence of this effect of berberine; this may be due to difference in the concentration of berberine used. *Lactobacillus*, as a beneficial bacterium, plays an important role in the treatment of fatty liver-related diseases. *Lactobacillus* acidophilus can suppress non-alcoholic fatty liver disease-associated hepatocellular carcinoma through the production of valeric acid[60] and *Lactobacillus oris* ameliorates non-alcoholic fatty liver in mice and inhibits endogenous cholesterol biosynthesis.[61] In this study, a high-fat diet reduced the abundance of *Lactobacillus*, consistent with previous studies,[62] and different interventions significantly reversed the decrease in *Lactobacillus* abundance.

Functional prediction of the gut microbiota revealed that the abundance of microbiota relating to lipid metabolism and energy metabolism was higher, and previous studies have shown that lipid metabolism is closely related to MAFLD.[63,64] Therefore, we examined the lipid metabolic pathway and its downstream fatty acid metabolism pathways. There were significant differences in the functional abundance of fatty acid beta-oxidation-related genes ACOX1 and HMGCS

between groups; therefore, we detected the expression of fatty acid beta-oxidation-related proteins ACOX1, HMGCS2, and CPT-1α in the liver using Western blotting. The process of fatty acid beta-oxidation is mainly performed in two kinds of organelles: peroxisomes and the mitochondria. In the initial stage of peroxisome beta-oxidation, acyl-coenzyme A oxidase 1 (ACOX1) catalyzes the desaturation of acyl-CoA to generate 2-trans-enoyl-CoA; simultaneously, ACOX1 is a rate-limiting enzyme, and its activity affects the rate of peroxisome beta-oxidation.[16,65,66] It has been found that after suppressing expression of the liver-specific ACOX1 gene in mice, the liver steatosis caused by hunger or high-fat diet in mice is obviously improved.[67] Shang et al also found that Shenge formula reduced obesity and fatty liver induced by a high-fat diet through the inhibition of ACOX1 expression.[68] In this study, we found that the expression of the ACOX1 protein in the liver of high-fat diet-fed mice increased significantly, and was decreased significantly by exercise and berberine intervention, indicating that such an intervention could ameliorate MAFLD by reducing the expression of ACOX1. Hydroxymethylglutaryl CoA synthase2 (HMGCS2), which is widely expressed in the liver, is a mitochondrial enzyme that catalyzes the second reaction of acetyl coenzyme A to synthesize ketones. The rate of conversion from acetyl coenzyme A to these ketone bodies is limited by HMGCS2.[69,70] The disorder of ketone production in patients with MAFLD is related to the degree of liver fat accumulation.[71,72] Asif et al reported that after suppressing the expression of HMGCS2 in the liver of mice, the liver fat content and TG levels were significantly increased; HMGCS2 overexpression in MAFLD mice improved hepatosteatosis and glucose homeostasis; ketone production and HMGCS2 expression in mice were impaired by a high-fat diet.[73] Therefore, a high-fat diet can decrease HMGCS2 expression, as shown by Luo et al[74,75] However, some studies have shown that mice fed a high-fat diet for 16 weeks exhibited PPARα-mediated fatty acid beta-oxidation and hepatic ketone body production, with a significant increase in HMGCS2 mRNA expression,[76] and exercise can ameliorate MAFLD induced by high-fat diet through the inhibition of HMGCS2 expression.[77] In mice fed a high-fat diet for 16 weeks, we found that the expression of the HMGCS2 protein increased significantly and that exercise and berberine decreased the expression of the HMGCS2 protein; moreover, and the expression of the HMGCS2 protein was the closest to normal following the combined intervention. These results suggest that a high-fat diet increases the expression of the HMGCS2 protein in the liver and that exercise and berberine ameliorate MAFLD by down-regulating the expression of the HMGCS2 protein, with a stronger effect observed from their combined intervention. Carnitine palmitoyl transterase-1 (CPT-1α) is a key enzyme of fatty acid beta-oxidation, which can transport fatty acids into the mitochondria for oxidation.[78,79] Under the conditions of high-fat diet or hypoxia, the expression of CPT-1α in the liver of mice is decreased, meaning that fatty acids in the cells cannot enter the mitochondria for oxidative decomposition, resulting in the accumulation of liver lipids.[80–82] However, betaine can increase the protein expression level of CPT-1α, promote the beta-oxidation of liver mitochondria, reduce the TG content in the liver, and clearly ameliorate symptoms related of MAFLD.[83] Our study also found that high-fat diet reduced the protein expression of CPT-1α in the liver and exacerbated the abnormal accumulation of lipids in the liver of mice. Exercise and berberine increased the protein expression of CPT-1α in the liver and significantly ameliorated the associated symptoms of MAFLD, indicating that exercise and berberine could affect MAFLD by promoting some stages of mitochondrial beta-oxidation.

Conclusion

MAFLD is considered a multifactorial illness since it is associated with genetic predisposition, inflammation, gut microbiota, and environmental factors, which are thought to be causal factors in its development and progression. As a result, the combination of multiple intervention methods may be an effective treatment approach. In the present experiment, our results show that both exercise and berberine can alleviate the symptoms of MAFLD by regulating the process of hepatic fatty acid beta-oxidation and gut microbiota, with their joint intervention showing a more significant effect. We also found a close relationship between the changes in gut microbiota and hepatic fatty acid beta-oxidation. We believe this could provide a basis for further research and development into microbiota-targeted drugs. Additionally, based on the Western blotting results, we believe that exercise and berberine intervention do not simply promote hepatic fatty acid beta-oxidation but instead inhibit or promote certain stages of fatty acid beta-oxidation. However, in any stage, the joint intervention has shown more significant effects. Therefore, exercise combined with berberine may be a promising therapeutic agent for MAFLD.

Data Sharing Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical Approval and Consent to Participate

All experiments in the present study were conformed to the Guide for the Care and Use of Laboratory Regulations and were approved by the Institutional Experiment Committee of Shenyang Sport University.

Funding

This work was supported by 2023 Shengjing Hospital 345 Talent Project. Traditional Chinese Medicine Abnormal Allergic Specialty Construction Project (Z211).

Disclosure

Xiaojian Zhang and Yanbin Cheng are co-first authors for this study. The authors report no conflicts of interest in this work.

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