Integrated 16S rRNA and metabolomics investigation of the hepatoprotective effects of Ixeris chinensis on high-fat diet-induced nonalcoholic fatty liver in mice

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Research Article

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Integrated 16S rRNA and metabolomics investigation of the hepatoprotective effects of *Ixeris chinensis* on high-fat diet-induced nonalcoholic fatty liver in mice

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Abstract

Ixeris chinensis (Thunb.) Nakai (IC) is traditionally used in Mongolian medicine prescriptions for liver diseases. This study aimed to investigate the hepatoprotective mechanism of IC on nonalcoholic fatty liver disease (NAFLD) mice by the integration of gut microbiota and metabolomics analysis. Male C57BL/6J mice were fed with chow or high-fat diet (HFD) with or without IC supplementation, and the gut microbial and metabolite profiles were studied by 16S rRNA sequencing and untargeted metabolomics, respectively. The results showed that the administration of IC resulted in significant decreases in body weight, liver index, serum biomarkers such as ALT, TG, and LDL-C, and the liver inflammatory factors IL-1β, IL-6, and TNF-α in mice fed with HFD. The 16S rRNA sequencing results showed that administration of the IC extract altered both the composition and abundance of the gut microbiota, thus leading to improvement of NAFLD. Untargeted metabolomics analysis of liver samples detected a total of 212 metabolites, of which 29 were differentially expressed. IC was found to significantly alter the levels of metabolites such as L-glutamic acid, pyridoxal, ornithine, L-aspartic acid, D-proline, and N4-acetylaminobutanal involved in the regulation of glutamine and glutamate, Vitamin B6 metabolism, and arginine and proline metabolism pathways. Correlation analysis indicated that the effects of the IC extract on metabolites were associated with alterations in the abundance of Akkermansiaceae, Lachnospiraceae, and Muribaculaceae. Our study revealed that IC has a potential hepatoprotective effect in

NAFLD and that its function is linked to improvements in the composition of the gut microbiota and its metabolites.

Keywords: *Ixeris chinensis* (Thunb.) Nakai, gut microbiota, metabolomics, nonalcoholic fatty liver disease, Mongolian medicine, hepatoprotective mechanism

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the world, affecting one-quarter of the global population (Cotter et al., 2020). It is characterized by an excessive accumulation of fat in the liver in the absence of excessive alcohol consumption (usually defined as <20 g per day for women and <30 g per day for men) (Diehl and Day, 2017). The pathological symptoms of NAFLD can be divided, in terms of increasing severity, into liver steatosis, non-alcoholic steatohepatitis, fibrotic hepatitis, advanced fibrosis, liver cirrhosis, and hepatocellular carcinoma (Tilg et al., 2021). NAFLD usually occurs in the context of metabolic syndrome and is closely associated with obesity, insulin resistance, type 2 diabetes, and dyslipidemia (Zhou et al., 2020). One of the main causes of the non-alcoholic fatty liver is diet, including both unbalanced intake and an unhealthy diet. Studies have shown that high-fat and high-carbohydrate diets are closely related to non-alcoholic fatty liver (Marchesini et al., 2016). The consumption of these high-energy diets results in low satiety, high caloric intake, and the unfavorable modulation of the gut microbiota which, in turn, accelerates obesity (Mulders et al., 2018). There is no specific effective therapy for the treatment of non-alcoholic fatty liver. The fundamental guideline for NAFLD is an improvement in diet and living habits to counteract obesity (Marjot et al., 2020).

Ixeris chinensis (Thunb.) Nakai (IC), belonging to the Asteraceae family, is a characteristically bitter-tasting Mongolian medicine (Luo, 2006). Bitterness is one of the six flavors forming the theoretical basis of Mongolian medicine. Traditionally, it promotes fat reduction. One of our studies showed that an extract of Mongolian Medicine Digta containing bitter compounds induced weight loss and reduced fat in obese rats fed on high-fat high-energy diets (Bao, 2019). IC is often used in

Mongolian medicine prescriptions, such as Digeda-8, and Chaganbong A-13, for liver protection (Bagenna, 2007). Its main chemical components are triterpenoids, steroids, glycosides, volatile oils, flavonoids, and phenolic compounds (Xi et al., 2020). In addition, IC contains luteolin, apigenin, β-sitosterol, and other chemical components with hepatoprotective activities (Liu et al., 2021). As reviewed in the literature, IC counteracts inflammation, oxidative stress, and diabetes, as well as having hepatoprotective and other pharmacological effects (Yang et al., 2013). Nevertheless, there is limited information about how IC's actions on NAFLD and its associated effects on the gut microbiota. The gut microbiota play important roles in human physiology and are considered key to the development of obesity (Diehl et al., 2017). These microorganisms interact with the host through dietary intake and physical activity, with both the microbes and their metabolites exhibiting different functions and regulating the health of the host through different organs (Kolodziejczyk et al., 2019). For example, the microbiota contribute to carbohydrate digestion (Makki et al., 2018), bile acid metabolism (Gérard et al., 2013), and vitamin synthesis (Yatsunenko et al., 2012) and also play a vital role in maintaining liver homeostasis. Imbalances in the microbiota can lead to disorders of the enterohepatic circulation that are closely associated with the development of NAFLD (Li et al., 2019). About 70% of the liver's blood supply comes from the gut through the portal vein, exposing the liver to metabolites produced by the gut microbiome (Hu, 2020). This relationship is often referred to as the liver-gut axis or liver-microbe axis (Adolph et al., 2018). Studies have shown that the microbiota are associated with non-alcoholic fatty liver and non-alcoholic steatohepatitis in humans and animals (Rabot et al., 2010; Zhu et al., 2013). Therefore, both the gut and its metabolites have become key mechanisms for the prevention and treatment of non-alcoholic fatty liver (Ni et al., 2020). The current work aimed to explore the hepatoprotective actions of IC on NAFLD through an investigation of the intestinal microbiota and its metabolites.

Materials and Methods

Materials

Whole IC plants were collected from Tongliao city, Inner Mongolia, China. The

high-fat diet was purchased from Shandong Hengrong Biotechnology Co., Ltd. (No.:SCXK (Lu) 2018 0003). Blood alanine transaminase (ALT), aspartate aminotransferase (AST), triglycerides (TG), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), and low-density lipoprotein-cholesterol (LDL-C) kits were purchased from Shenzhen Icubio Biotechnology Co., Ltd. (China) ELISA kits for measuring the cytokines IL-6, IL-1β, and TNF-α were purchased from Jiangsu Jingmei Biotechnology Co., Ltd. (China). Hematoxylin and eosin (H&E) stain was purchased from Nanjing Jiancheng Technology Co., Ltd (China).

Animals and experimental design

Six-to-eight weeks-old male C57BL/6J mice (initial body weight = 18-20 g) were obtained from Changsheng Biotechnology Co., Ltd (Liaoning, China). All mice were housed individually in a temperature-controlled room (20-25 °C) with a 14 h-10 h light-dark cycle. Mice were allowed seven days for acclimatization with *ad-libitum* access to water. The NAFLD model was induced by feeding a high-fat diet (2% cholesterol, 7% lard, 8.3% egg yolk, 16.7 % sucrose, with 66% standard normal diet) for 12 weeks, while the control group was given a standard diet. Body weights were recorded weekly. The NAFLD mice were randomly allocated to one of three experimental groups that received IC extract at 0.5 g/kg, 1.5 g/kg, and 3.0 g/kg doses, respectively, by oral gavage for 10 weeks. At the end of the experiment, blood was collected from the retro-orbital sinus and centrifuged at 3500 rpm for 10 min at 4 ℃. The supernatant was collected and stored at -80 ℃. Fresh fecal samples were collected from the cecum and were frozen in liquid nitrogen and stored at -80 ℃ for analysis. The livers were surgically removed from each mouse, the wet weights were measured, and the tissues were stored at -80 ℃ for subsequent experiments. All experiments were performed following protocols reviewed and approved by the Institutional Animal Care and Use Committee, Inner Mongolia Minzu University (approval no. NM-LL-2021-06-15-1).

Serum biochemical markers assay

An automatic biochemical analyzer (Ichem-340, Icubio, China) was used to measure the levels of ALT, AST, TG, TC, HDL-C, and LDL-C in serum. Sample

volumes of 150 μl were used for the tests.

H&E staining

The left liver lobe tissue was fixed with 4 % formalin for at least 48 h, after which the liver tissue was dehydrated with an alcohol gradient, cleared with xylene, embedded in paraffin, and sliced into 5μm-thick sections. The sections were then deparaffinated, stained with H&E, and examined under a microscope (AXIO Vert.A1, Zeiss).

Liver IL-6 , IL-1β, and TNF-α analysis

Liver homogenates (10%) were prepared by the addition of 100 mg liver tissue to 0.9% sodium chloride solution at a ratio of 1:9 and homogenized using an electric tissue grinder in an ice bath. The homogenate was then centrifuged at 3500 rpm for 10 min and the levels of IL-6, IL-1β, and TNF-α were measured in the supernatant using enzyme-linked immunosorbent assay kits, according to the manufacturer's instructions. A total of 10 μl sample was used for the test, and the absorbance was measured at 450 nm.

Gut microbiota analysis

DNA was extracted from fresh mouse feces and was amplified and purified by PCR. The library was prepared using a TruSeq DNA PCR-Free Library Preparation Kit (Illumina, San Diego, CA, USA). Library quantification was performed by Qubit, and sequencing of the 16S rRNA genes was performed on the Illumina Miseq PE250 platform. Sequences were evaluated on the KEGG and Tax4Fun databases for functional group prediction.

Metabolomic analysis of liver samples

Twenty-five milligrams of the samples were weighed into an EP tube and 50 μL extract solution (methanol:acetonitrile: water = 2:2:1, with isotopically-labelled internal standard mixture) were added. The samples were then homogenized at 35 Hz for 4 min and sonicated for 5 min in an ice-water bath. The homogenization and sonication cycle was repeated three times, after which the samples were incubated for 1 h at -40 ℃ and centrifuged at 12 000 rpm for 15 min at 4 ℃. The supernatant was transferred to a fresh glass vial for analysis. Quality control (QC) samples were

prepared by mixing an equal aliquot of the supernatants from all of the samples. The liver samples were analyzed for untargeted metabolite profiles using the LC-MS/MS (Q Exactive Orbitrap, Thermo Fisher Scientific, Waltham, MA, USA). LC-MS/MS analyses were performed using a UHPLC system (Vanquish, Thermo Fisher Scientific) with a UPLC BEH Amide column (2.1 mm × 100 mm, 1.7μm) coupled to Q Exactive HFX mass spectrometer (Orbitrap MS, Thermo Fisher). The mobile phase consisted of mmol/L ammonium acetate and 25 ammonium hydroxide in water（pH = 9.75） (A) and acetonitrile (B). The auto-sampler temperature was 4 ℃, and the injection volume was 2 μL.

The QE HFX mass spectrometer was used for its ability to acquire MS/MS spectra in the information-dependent acquisition (IDA) mode under the control of the acquisition software (Xcalibur, Thermo Fisher). In this mode, the acquisition software continuously evaluates the full-scan MS spectrum. The ESI source conditions were set as follows: sheath gas flow rate, 30 Arb; Aux gas flow rate, 25 Arb; capillary temperature, 350 ℃; full MS resolution, 120 000; MS/MS resolution, 7500; collision energy, 10/30/60 in the NCE mode; spray voltage, 3.6 kV (positive) or -3.2 kV (negative), respectively.

Statistical analysis

GraphPad Prism 8.0.2 software was used for the statistical analysis and processing of data. The physiological characteristics of mice are expressed as mean ± standard error (X ± SEM). In the experiments, one-way ANOVA was used for the comparison of multiple groups of data, and the LSD test was used for multiple comparisons. $P<0.05$ indicates that the difference is statistically significant. In the analysis of intestinal flora, Usearch software was used to perform de-chimerism and cluster analysis of the data to obtain the OTUs (Operational Taxonomic Units) for α-diversity and β-diversity analysis. Linear discriminant analysis (LDA) was used to estimate the communities or species that differed significantly between the samples. XploreMET (Metabo-Profile Biotechnology, Shanghai, China) was used for the processing of the raw metabolomics data, aggregation of the QC samples to establish a reference database, comparison of metabolic signals, correction and normalization

of missing values, and identification of metabolites. This was followed by partial least squares discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA). Spearman correlation coefficients were used to determine the correlations between differential flora and differential metabolites.

Results

IC extract reduced body weight, liver index, and fat tissue in HFD diet-induced NAFLD mice

To investigate whether IC could prevent HFD diet-induced NAFLD, HFD mice received 0.5, 1.5, or 3 g/kg IC extract by oral gavage for 10 weeks. We found that the body weight, liver index, perirenal fat, and epididymal fat were significantly increased in the HFD groups compared with the control mice ($p < 0.05$). Administration of IC extract resulted in significant decreases in body weight, liver index, perirenal fat, and epididymal fat in comparison with the HFD group ($p < 0.05$) (Fig. 1).

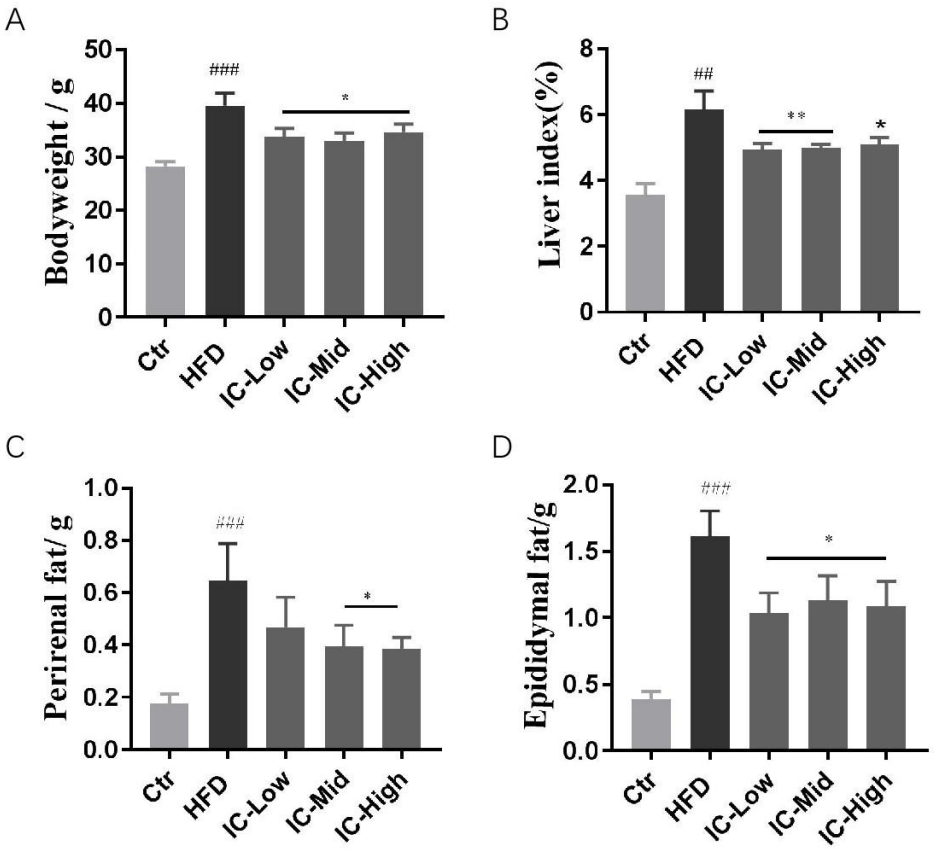

FIGURE 1. Effects of IC extract on body weight (A), liver index (B), perirenal fat (C), and epididymal fat (D). The data represent means ± SEM (n=6). $^{\#}P<0.05$ versus

the control group; *P<0.05 and **P<0.01 versus the HFD group, respectively.

Effect of IC extract on serum biochemical markers

As shown in Table 1, the serum levels of ALT, TG, and LDL-C were significantly elevated in the HFD group compared with the control group ($p < 0.05$). Notably, IC administration significantly reduced the serum levels of ALT, TG, and LDL-C after all three doses in the HFD mice ($p < 0.05$). Additionally, although the serum AST level was not significantly increased in HFD mice, there was a significant reduction in the AST level at the high IC extract dose of IC (P<0.05). Although the HFD induced higher TC levels, these were not significantly reduced by treatment with the IC extract.

TABLE 1. Serum biochemical markers in each group (X±S, n=6)

Groups	ALT(U/L)	AST（U/L）	TG（mg/dL）	TC（mg/dL）	HDL-C（mmoL/L）	LDL-C（mmoL/L）
Control	31.26±8.65	76.00±13.71	0.32±0.01	2.43±0.13	2.26±0.07	1.18±0.18
HFD	57.13±28.60$^{\#}$	87.00±16.35	0.38±0.02$^{\#}$	4.93±1.15$^{\#\#}$	3.79±0.12	3.64±0.68$^{\#\#}$
IC 0.5g/kg	35.67±7.45*	88.67±3.67	0.27±0.01**	3.50±0.50	3.98±0.12	2.56±0.15*
IC 1.5g/kg	20.50±10.10**	82.50±11.29	0.31±0.01*	3.43±0.29	3.75±0.10	2.46±0.08*
IC 3.0g /kg	30.00±19.49**	75.71±9.69*	0.30±0.02*	3.65±0.42	3.80±0.11	2.55±0.10*

$^{\#}$($p < 0.05$),versus the control group;**P<0.01，*P<0.05, versus the HFD group.

Effect of IC extract on liver histopathology

To assess morphological changes in liver tissues, the livers of the experimental animals were collected and compared using H&E staining. In the control group, the structure of the liver lobule was complete and the liver cells were arranged radially around the central vein. In the HFD group, the cells showed prominent macro-vesicular steatosis compared with the control mice. The liver sections from the IC group showed mild cellular swelling without steatosis compared with cells from the HFD group; the structure of the hepatic lobule in the IC group was more complete and the degree of steatosis was significantly lower than those in the HFD group (Figure 2).

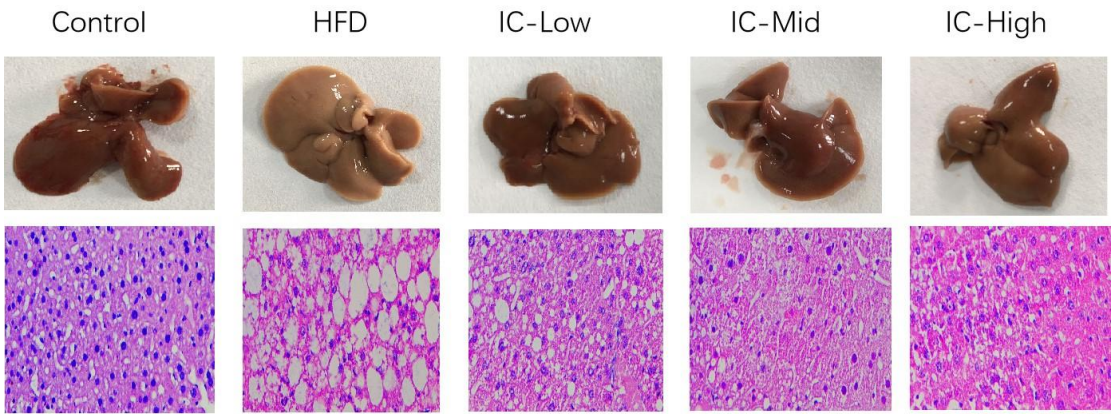

FIGURE 2. Histopathological changes in liver tissues from the five experimental mouse groups

Effect of IC extract on liver IL-6, IL-1β, and TNF-α

As shown in Table 2, the levels of IL-6, IL-1β, and TNF-α were increased in the HFD group compared with the control group. After treatment with IC extract (0.5, 1.5, and 3 g/kg), the IL-1β levels declined in comparison with the HFD group ($p < 0.05$). IC extract at the 0.5- and 1.5-g/kg doses reduced the concentrations of IL-6 ($P<0.05$), while IC extract at the 3 g/kg dose reduced the TNF-α level ($P<0.05$).

TABLE 2. Liver IL-6 , IL-1β, and TNF-α levels in each group (X±S, n=6)

Groups	IL-6（pg/ml）	IL-1β（pg/ml）	TNF-α（pg/ml）
Control	18.95±0.26	17.91±0.28	106.99±2.91
HFD	19.38±0.34	18.74±0.50	115.60±2.59
IC 0.5g/kg	17.32±0.97**	15.80±0.62**	107.27±2.32
IC 1.5g/kg	18.33±0.25*	16.79±0.40**	111.68±2.59
IC 3.0g /kg	18.57±0.20	17.42±0.28*	99.70±6.22**

**P<0.01，*P<0.05, versus the HFD group.

Gut microbiota analysis

The Venn diagram (Figure 3) shows the OTUs shared between groups and those that are unique to each group. As seen in the Figure, 585 OTUs (31.25%) of the total OTUs were common to all three groups. A further 135 OTUs were shared between the control group and the HFD group, while 181 OTUs were shared between the HFD

and the IC groups. Treatment with the IC extract increased the number of OTUs.

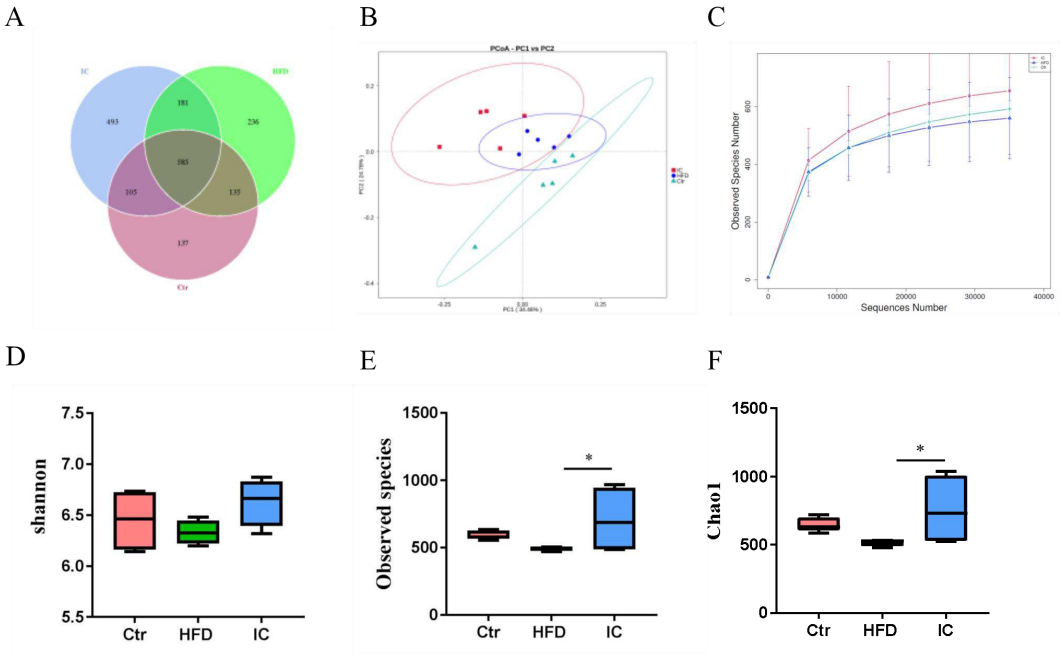

FIGURE 3. α and β diversity in the fecal samples from the three experimental groups. A. Venn diagram of OTUs. B. Principal coordinate analysis (PCoA) ordination plot for the three experimental groups. C. Rarefaction curves showing the average observed species number for three groups. D. Shannon index. E. Observed species. F. Chao1.

The α-diversity analysis reflects the abundance and diversity of a single sample and includes the Chao1, Observed species, and Shannon indices. As shown in Figure 3 D-F, the Shannon, Chao1, and observed species indices of the IC group are higher than those in the HFD group, indicating that the species diversity is high, and the distribution is even. β-diversity reflects the presence of significant differences in the microbial communities between the samples. In this study, principal coordinate analysis (PCoA) was used to identify differences in species diversity. The distance of each coordinate point represents the degree of aggregation and dispersion between samples. The closer the distance, the closer the species composition of the sample; the larger the distance, the greater the difference between the samples. As shown in

Figure 3 (B and C), the samples of the control group, HFD group, and the IC group are distinct, indicating that the species structure is quite different, while the distance between the samples within the groups are close, indicating that the community structure is similar.

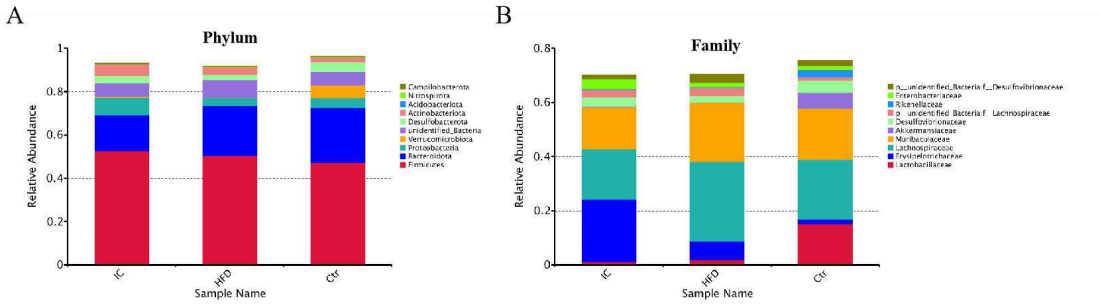

FIGURE 4. Gut microbiota compositions at the phylum and family levels for the three experimental groups.

Analysis of species abundance

A species profiling histogram can visually reveal the more abundant species and their relative abundance in the different samples in terms of different taxonomic levels. As shown in Figure 4A, at the phylum level, each group of samples included mostly Firmicutes, Bacteroidota, Proteobacteria, Verrucomicrobiota, Desulfobacterota, Actinobacteriota, Acidobacteriota, Nitrospirota, and Campilobacterota.

As shown in Figure 4B, at the family level, each group of samples included mostly Lactobacillaceae, Erysipelotrichaceae, Lachnospiraceae, Muribaculaceae, Akkermansiaceae, Desulfovibrionacea, Unidentified_Bacteria_Lachnospiraceae, Rikenellaceae, Enterobacteriaceae, unidentified_Bacteria_and Desulfovibrionaceae. More precisely, the abundance of Lachnospiraceae and Muribculaceae in the IC group was significantly reduced in contrast to the HFD group ($P<0.05$), while the abundance of Erysipelotrichaceae was significantly decreased in the IC group in comparison with the HFD group. There was a significant decrease in the numbers of Akkermaniaceae in the HFD group compared with the control group ($P<0.05$), which was reversed by IC treatment (Figure 5). Tax4Fun function prediction found that the most enriched bacterial metabolic pathways related to NAFLD were “amino acid

metabolism", "carbohydrate metabolism", "energy metabolism", "nucleotide metabolism", "metabolism of cofactors and vitamins", "glycan biosynthesis and metabolism", "membrane transport", "replication and repair", "translation", and "signal transduction" (**Figure 6**).

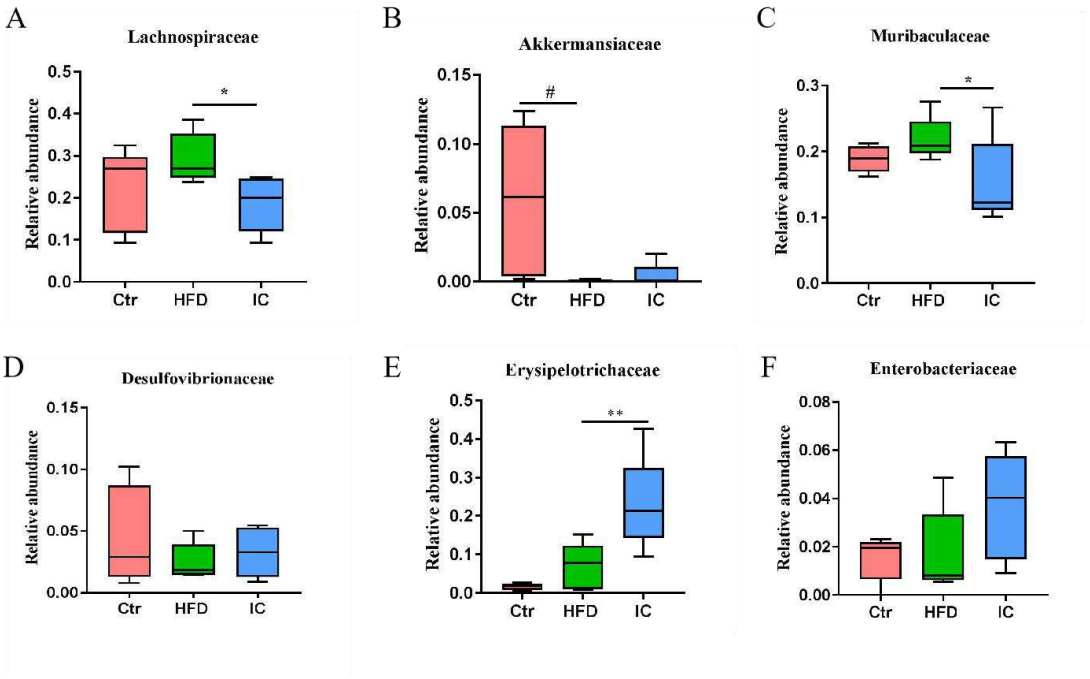

FIGURE 5. Relative abundance of differentially expressed individual gut microbiota at the phylum and family levels (n=5).

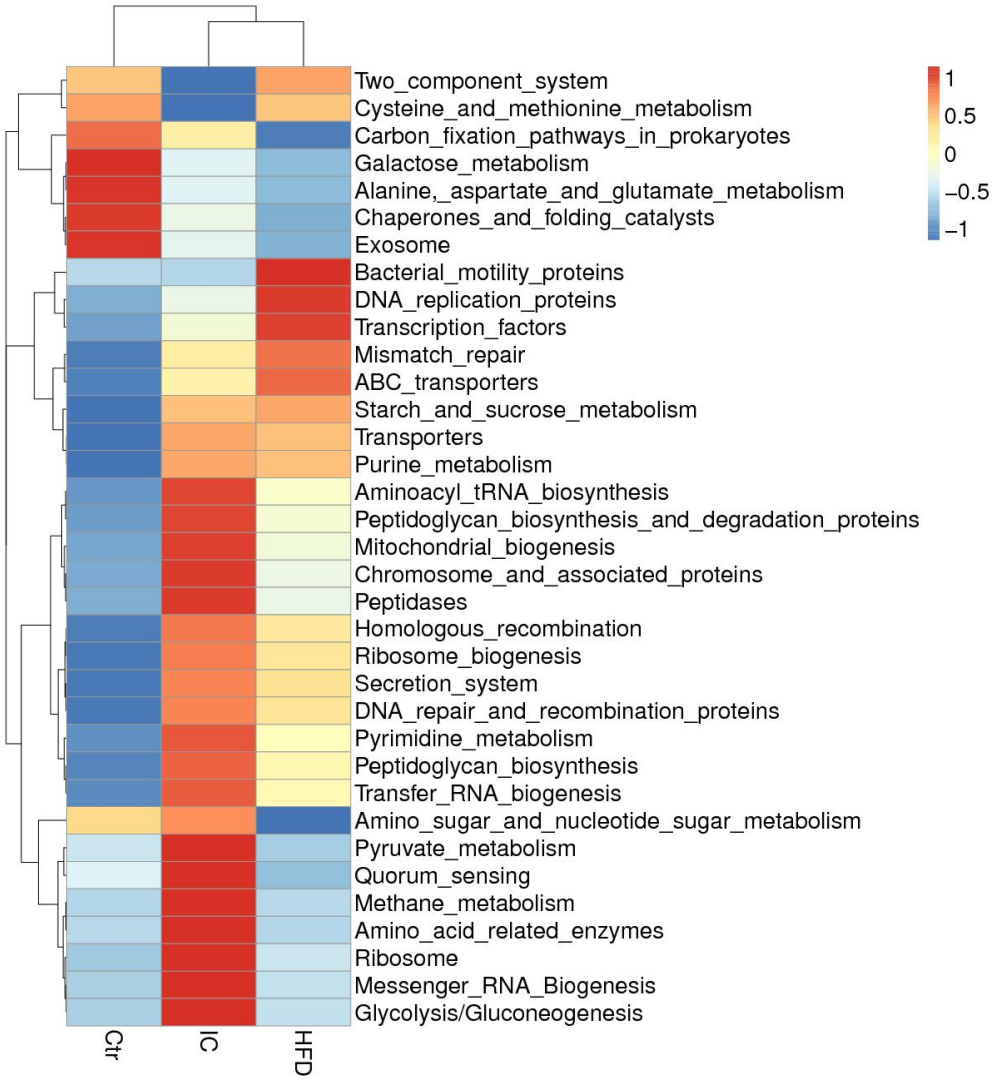

FIGURE 6. Tax4Fun functional prediction

Untargeted metabolomics study

Data quality and identification of metabolites

As shown in Figure 7 (A-C), a total of 212 metabolites were detected from 15 liver tissue samples. Of these, 35% were associated with organic acids and derivatives, 27% with lipid and lipid-like molecules, 25% with organoheterocyclic compounds, 8% with organic oxygen compounds, and 5% with nucleosides, nucleotides, and analogs. The results of the principal component analysis (PCA) showed that there were significant differences between the three groups of samples, indicating significant differences in the metabolic status of the three groups of mice. Moreover, the distances of the QC samples were observed to be extremely close, indicating a

high reliability of the sample data.

As shown in Figure 7D, among 29 total metabolites, 23 were down-regulated and 4 were up-regulated in comparison with the control and HFD groups. In addition, IC extract treatment up-regulated 11 metabolites and down-regulated 4 metabolites compared with the HFD group.

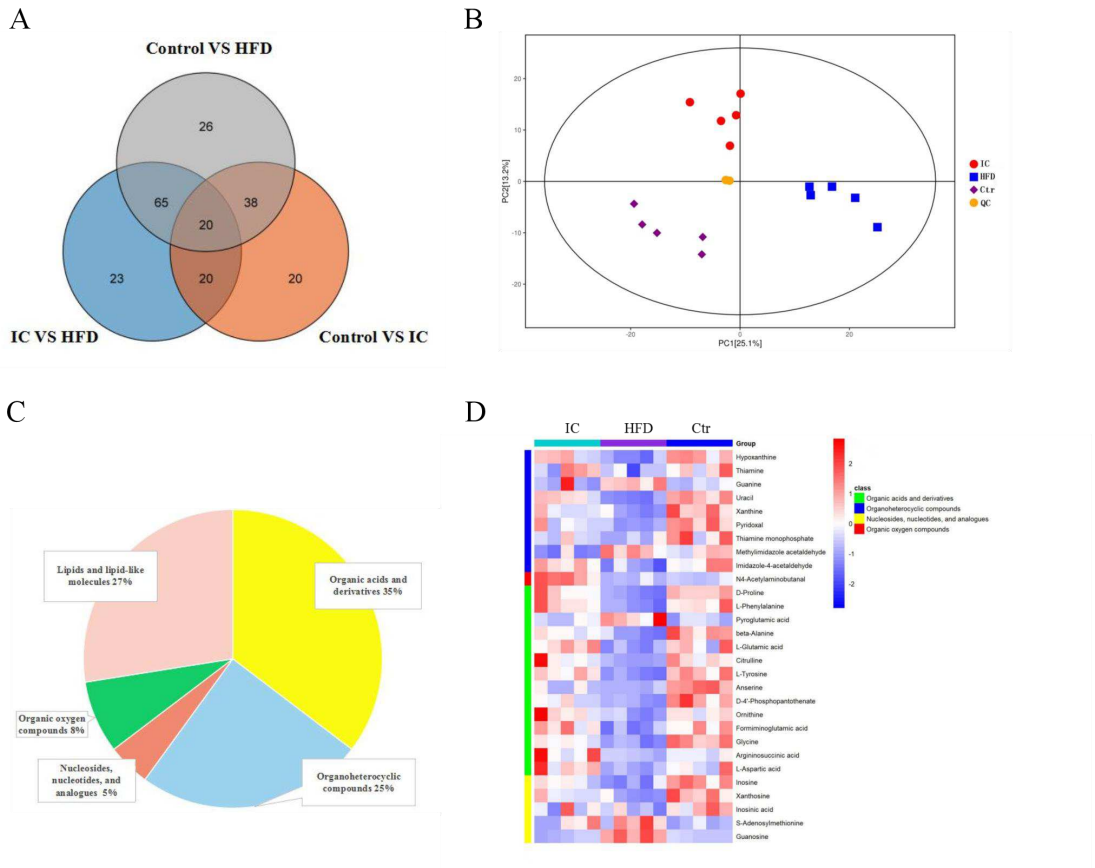

FIGURE 7. Metabolite classes and compositions in the samples. A. The overall differential metabolites. B. Serum metabolic profiles of the three groups using a principal component analysis (PCA) score plot. C. Pie chart indicating the abundance ratio of different chemical classes of annotated metabolites identified by untargeted metabolic profiling in liver samples. D. Heatmap showing the differences in expression of the differential metabolites in the three groups. Red and blue colors indicate higher or lower expression, respectively, in each sample.

OPLS-DA analysis

The OPLS-DA scatter plot (Figure 8 A and B) shows that there was a significant separation between the samples of the control vs HFD group and IC vs HFD group. As shown in Figure 8 (C and D), the corresponding OPLS-DA model was established multiple times (the number of times n = 200) to obtain the R2 and Q2 values of the random model. In control vs HFD and IC vs HFD, the R2Y values (respectively R2Y=0.92; R2Y=0.97) were close to 1, indicating that the established model conforms to the real situation of the sample data, and the Q2 values (respectively Q2Y=0.73; Q2Y=0.50) were close to 1, indicating that if a new sample is added to the model, an approximate distribution will be obtained. Thus, the original model has good stability and shows no over-fitting.

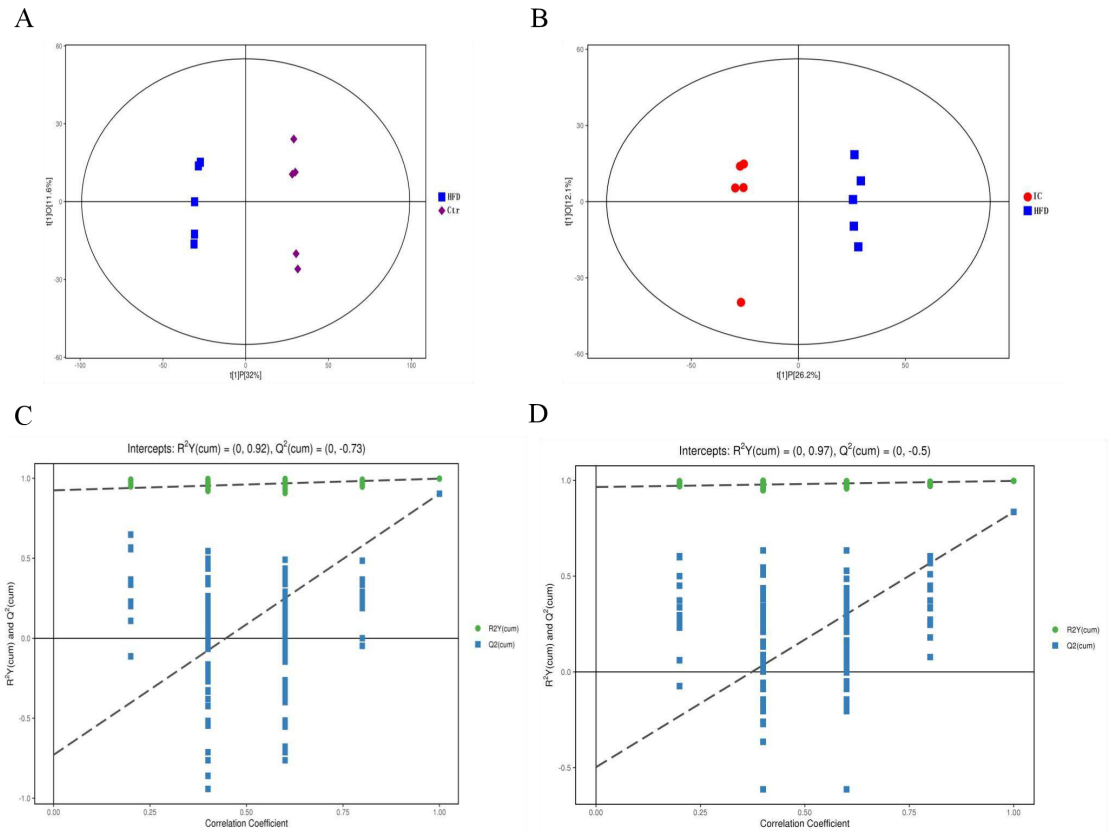

FIGURE 8. Overall metabolite profile differences between two groups: A. OPLS-DA score scatter plot. B. OPLS-DA permutation plot.

Changes of metabolite and biological metabolic pathways

The analysis of the differential metabolites was carried out by univariate

statistical analysis (Student's t-test). Six representative differential metabolites, namely, D-proline, L-aspartic acid, pyridoxal, ornithine, L-glutamic acid, and N4-acetylaminobutanal, that were identified from the key altered pathways are illustrated in Figure 9 A-F. The results showed that IC treatment altered the metabolites in the HFD group.

The KEGG database was used to analyze the pathways involved in the regulation of differential metabolites, and to display the metabolic pathways in the form of bubble charts. As shown in Figure 11, in the IC extract vs HFD group, nine significantly enriched metabolic pathways were identified, namely, in order of impact, "biosynthesis of phenylalanine, tyrosine and tryptophan", "metabolism of glutamine and glutamate", "Vitamin B6 metabolism", "β-alanine metabolism", "alanine, aspartic acid and glutamate metabolism", "arginine and proline metabolism", "histidine metabolism", "glutathione metabolism", and "pantothenate and CoA biosynthesis". The three most significant metabolic pathways associated with the improvement of non-alcoholic fatty liver by treatment with IC extract were "glutamine and glutamate metabolism", "vitamin B6 metabolism", and "arginine and proline metabolism" pathways.

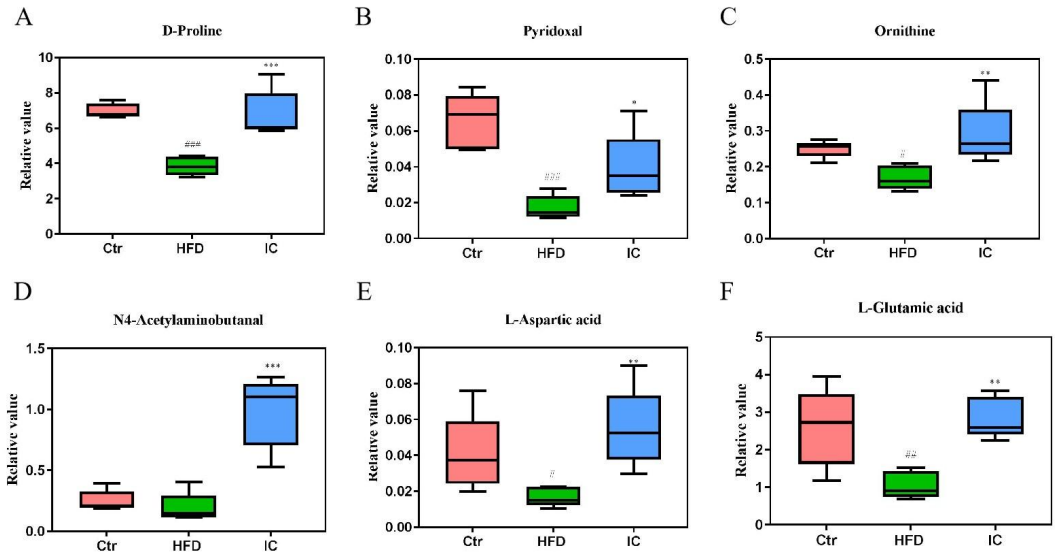

FIGURE 9. Significant differential expression of individual metabolites. Six identified metabolites that differed significantly among control, HFD, and IC groups ($p < 0.05$).

Correlation analysis between differential bacteria and liver metabolism

Spearman correlation coefficients were used to analyze the relationships between differential gut microbiota and liver metabolites during the IC intervention in HFD mice (Figure 11). Akkermansiaceae were up-regulated by IC intervention and were positively correlated with D-proline, pyridoxal, L-phenylalanine, and L-tyrosine amino acids and vitamins, while Lachnospiraceae, down-regulated by IC, were negatively correlated with N4-acetylaminobutanal and ornithine. Muribaculaceae, significantly down-regulated by IC intervention, were positively correlated with L-glutamic acid and L-aspartic acid.

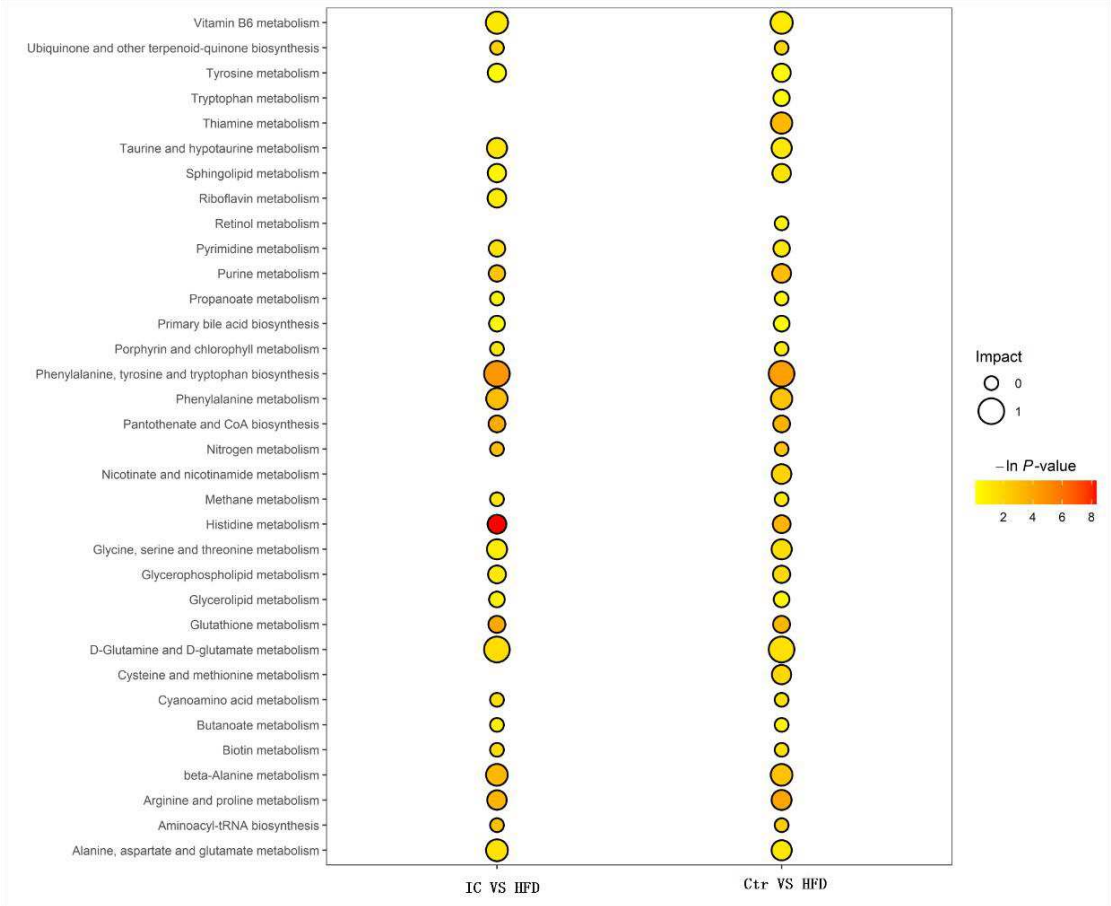

FIGURE 10. Bubble plot of KEGG pathway analysis.

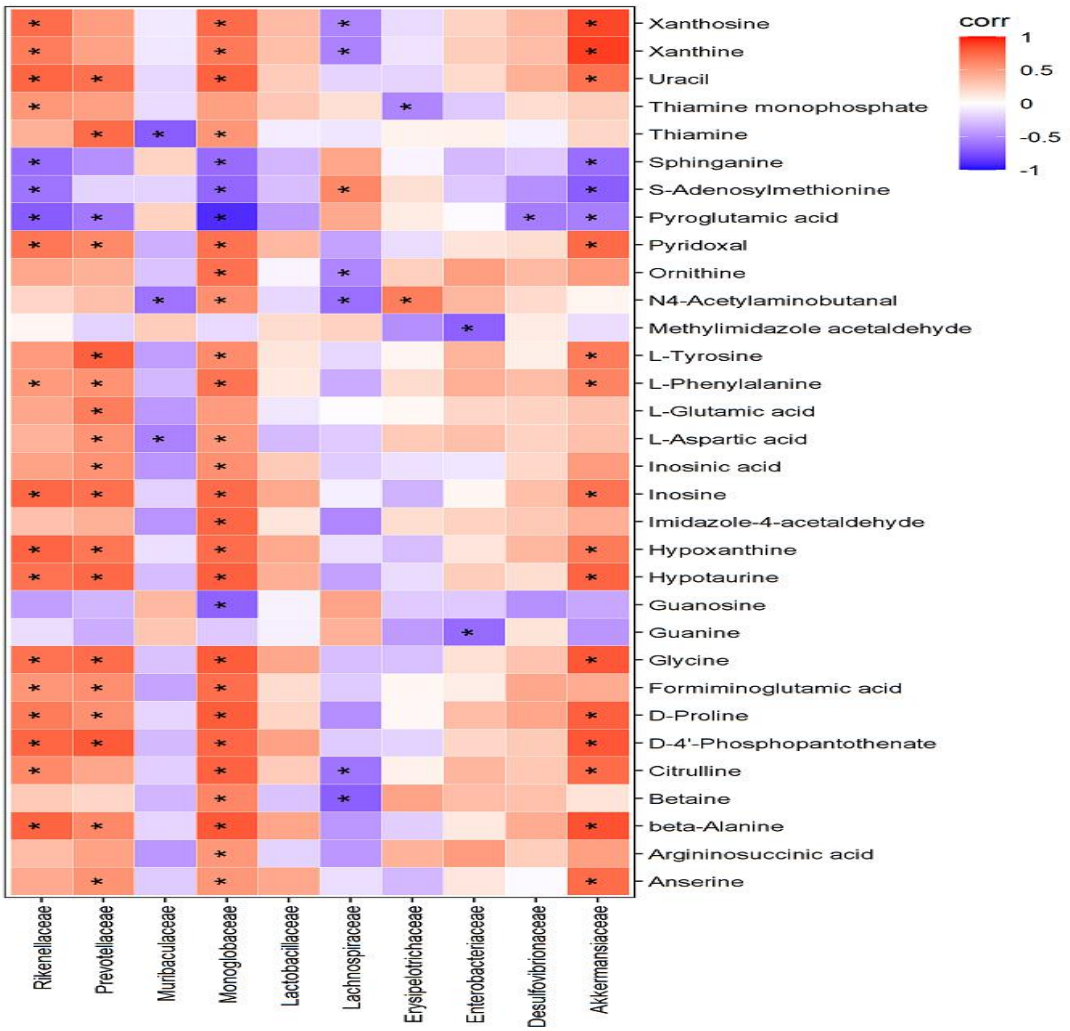

FIGURE 11. Spearman correlation heatmap of differentially expressed microflora and serum metabolites（n=5）,*P<0.05.

Discussion

This study investigated the hepatoprotective effects of IC extract on mice with non-alcoholic fatty liver induced by feeding a high-fat diet, analyzing body weight and the liver index, fat tissues, serum transaminase, liver inflammatory factors, liver pathology, correlation with gut microbiota, and liver metabolites. The HFD contained 2% cholesterol, 7% lard, 8.3% egg yolk, 16.7% sucrose, and 66% basic feed (Zeng et al, 2015), and was fed for 12 weeks to successfully induce a NAFLD mouse model. By 12 weeks, the body weights of the mice in the two groups differed significantly.

After treatment with the IC extract, there were significant reductions in the body weight, liver index, and perirenal and epididymal adipose tissues in comparison with the HFD group, indicating an improvement in the liver fat accumulation. Weight gain can significantly promote liver fat accumulation, and weight loss is the most effective way to remove fat (Marchesini et al, 2015). According to MRI, a 5% reduction in body mass index can reduce liver fat by 25% (Patel et al，2015).

NAFLD covers a spectrum of liver conditions whose dominant feature is abundant hepatic triglyceride (TG) accumulation (Haas et al, 2016). NAFLD is strongly related to obesity, diabetes, and dyslipidemia, and is thus considered to be the hepatic manifestation of metabolic syndrome (Cheung et al, 2010). On the other hand, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are considered to be markers of liver damage in many liver diseases including fatty liver (Pratt and Kaplan, 2000). Further research has suggested that these two enzymes can be considered as indicators of liver metabolic function (Sookoian et al, 2012). Hence, the levels of serum ALT, AST, TG, TC, HDL-C, and LDL-C were measured in this study. The results showed that there were significant increases in serum ALT, TG, and LDL-C in the HFD group compared with the control group, indicating abnormal liver metabolic function in the HFD mice. In contrast, the serum ALT, TG, and LDL-C levels were significantly reduced after treatment with the IC extract compared with the HFD group, indicating improved lipid absorption and metabolism. Although the serum AST levels in the HFD group tended to increase, they did not differ significantly from those in the control group, and treatment with IC extract significantly reduced the serum AST level compared with the HFD group. The liver plays an important role in orchestrating glycolipid metabolism. We speculated that IC treatment improved lipid metabolism, reducing lipid accumulation in the liver and thus lowering the lipid levels in the serum. Taken together, these results indicate that IC treatment improves the metabolic function of the liver.

This study also compared pathological changes in the liver appearance and morphology between the groups. The livers in the HFD mice were the color of faded blood and showed marked steatosis compared with those of the control group,

confirming the establishment of a non-alcoholic fatty liver model after being fed with a high-fat diet for 12 weeks; Compared with the HFD group, the livers in the IC extract group were similar to those of the control group. This was observed for all concentrations of IC extract. Furthermore, the livers did not appear fatty, indicating that the IC extract protected against fat accumulation resulting from the high-fat diet-induced non-alcoholic fatty liver. The histological findings of the H&E staining showed that the liver cells in the control group were even in size and neatly arranged. In contrast, the liver cells in the HFD group were swollen with many fat vacuoles of different sizes. Compared with the HFD group, the IC extract groups also showed a certain degree of hepatocellular swelling; however, there were no visible fat vacuoles. Non-alcoholic fatty liver can be classified into non-alcoholic fatty liver (steatosis), non-alcoholic steatohepatitis, liver cirrhosis, and hepatocellular carcinoma according to pathological symptoms. The high-fat diet-induced non-alcoholic fatty liver model usually causes only steatosis and mild inflammation (Haas et al, 2016). Therefore, we speculated that the results indicated steatosis of the liver and minor inflammation.

NAFLD is associated with insulin resistance in both the adipose tissue and liver (Anstee et al, 2013). Liver insulin resistance and liver fat accumulation can promote the release of a variety of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), leading to liver inflammation and cellular damage (Bonsembiant et al, 2021). We observed a tendency towards raised levels of TNF-α, IL-6, and IL-1β in the HFD group, although the differences were not significant when compared with the control group, which was consistent with the histopathological results that a high-fat diet induced a non-alcoholic fatty liver model and slight inflammation but not inflammatory non-alcoholic steatohepatitis. Compared with the HFD group, the IC extract groups showed significantly reduced levels of liver IL-6 and IL- 1β, while treatment with 3g/kg IC extract significantly lowered the IL-1β and TNF-α levels. Previous studies have also reported increases in serum and liver TNF-α levels in patients with non-alcoholic steatohepatitis, with the levels associated with the severity of the histopathology (Haukeland et al, 2006). Similarly, in animal models, serum IL-6 levels have also

been found to increase in correspondence with the degree of liver inflammation and fibrosis (van der et al, 2008) suggesting that the three IC extract doses used have potential inhibitory effects on liver inflammatory factors.

The benefits of medicinal herbs on metabolic disorders have been shown to be associated with changes in the composition of the gut microbiota and their metabolites, which may alter metabolic pathways. The composition of the gut microbiota composition appears to be linked with the health of the host. The microbiome contributes to carbohydrate digestion (Makki et al, 2018), bile acid metabolism (Gérard, 2013), and vitamin (Yatsunenko et al, 2012) and amino acid synthesis (Laparra and Sanz, 2010). Under normal circumstances, the relationship between the human host and the gut microbiome is mutually beneficial (Lynch et al, 2016). Accordingly, gut microbiota dysbiosis, defined as an imbalance of the intestinal microbiome, is closely associated with the pathogenesis of NAFLD (Boursier et al, 2016). In this study, 16S rRNA sequences were used for analyzing differences in the composition and abundance of the gut microbiota among the groups. Consistent with previous studies, the α-diversity analysis showed that the Chao1, observed_species, and Shannon indices of the control and IC groups were higher than those of the HFD group, indicating that the number and diversity of species in the microbiota were higher in the control and IC groups than in the HFD group. NAFLD patients have been found to have reduced microbiota diversity compared with healthy subjects (Shen et al, 2017). The β-diversity analysis showed that the gut microbiota composition differed among groups but was similar within the groups. The OTU analysis showed that IC treatment increased the abundance of the Akkermansiaceae family, while significantly decreasing that of the Lachnospiraceae and Muribaculaceae families. The functional prediction results showed that both carbohydrate metabolism and amino acid metabolism were involved in the function of the microbiota. The fecal microbiota revealed that the bacterial composition in mice fed with a standard diet was dominated by the Bacteroidaceae and Akkermansiaceae families but was devoid of Lachnospiraceae and Muribaculaceae. In contrast, HFD increased the numbers of bacteria belonging to the Lachnospiraceae and

Muribaculaceae families, while reducing both Bacteroidaceae and Akkermansiaceae (Liu et al, 2021). Akkermansiaceae have been found to be related to improvements in NAFLD symptoms (Hu et al, 2020), while Lachnospiraceae are related to the induction of NAFLD (Le et al, 2013).

Additionally, the alteration in the microbiome composition and its' metabolites directly affect the tight junctions between epithelial cells (Lee et al., 2018), influencing the reactions to microorganisms and antigens. As described above, the administration of IC modified the hepatic immune response. The gut microbiota influence the immune response and control the stability of the intestine (Hooper and Machpherson, 2010). Thus, it is possible that treatment with IC extract alters the composition of the microbiome which, in turn, reduces the immune response and inflammation caused by NAFLD.

To investigate the mechanisms underlying the effects of IC on NAFLD, we carried out liver metabolomics analysis. It is well-known that the gut-liver axis is a critical link whereby the gut microbiota impact liver metabolism. Glutamine and glutamate are important energy substrates. They are oxidized by the intestine and immune cells to produce energy, allowing the cells to function optimally and to grow (Kong et al, 2018). In addition to their role as energy substrates, free glutamine and glutamate are specific precursors of glutathione. Dietary supplementation of glutamine increases the glutathione content and improves oxidative stress in mice fed on a high-fat diet (Lin et al, 2014). Studies have found that glutamine supplementation is able to protect mice with non-alcoholic steatohepatitis induced by a Western diet (Sellmann et al, 2015). Another study showed that oral glutathione administration for four months in patients with non-alcoholic fatty liver disease reduces both the serum ALT and TG levels and, as glutathione can improve liver metabolism, it follows that it has a protective effect on non-alcoholic fatty liver disease (Honda et al, 2017). In this study, it was found that the glutamine and glutamate metabolism pathway had one of the highest impact factors, and L-glutamate is a differential metabolite that regulates this pathway. L-glutamic acid was found to be significantly lowered in the HFD group and significantly increased in the IC group,

suggesting that IC regulation of this pathway may account for its protective role against non-alcoholic fatty liver.

Vitamin B6 plays an important role in cell metabolism (Hellmann et al, 2010). Vitamin B6 is composed of six vitamins, namely, pyridoxine, pyridoxal, and pyridoxamine, and their related 5'-phosphate derivatives (Mascolo et al, 2020). Vitamin B6 participates in a variety of biological reactions including the synthesis and degradation of amino acids and the metabolism of sugars and fatty acids (Percudani et al, 2009). Studies have shown that vitamin B6 intake is negatively correlated with non-alcoholic fatty liver. Compared with healthy individuals, NAFLD patients have lower levels of vitamin B6 in their diets (Ferro et al, 2017). In addition, vitamin B6 supplementation in mice on a high-fat diet has been shown to improve insulin resistance and liver fat accumulation (Liu et al, 2016). Moreover, vitamin B6 also has antioxidant and anti-inflammatory effects (Dalto et al, 2017). In this study, the vitamin B6 metabolic pathway was found to be significantly enriched in the IC extract group, indicating that it is activated by IC extract. Furthermore, pyridoxal, a differential metabolite involved in the regulation of vitamin B6 metabolic pathways, was significantly reduced in the HFD group, and significantly increased in the IC extract group, suggesting that IC extract can supplement the content of vitamin B6 in the body, leading to reduced liver fat accumulation and inflammation.

Arginine is a functional amino acid that has attracted much attention due to its ability to improve glucose tolerance, lipid metabolism, and insulin sensitivity, leading to a reduction in obesity (Wang et al, 2016). Studies have shown that supplementing arginine through diet can reduce obesity in rats and improve human metabolic characteristics (McKnight et al, 2010). Proline has a unique structure compared with other amino acids, which allows it to affect the 3D structures of proteins and mediate important protein interactions (Kay et al, 2000). In addition, increased proline in plant cells has been related to increased oxidative stress, suggesting that proline may protect against the damage caused by oxidative stress (Verbruggen et al, 2008). The pathological mechanism underlying NAFLD is complex and influenced by many factors, among which oxidative stress is considered to be the main factor leading to

liver damage and disease progression (Friedman et al, 2018). In the current study, many of the differential metabolites are known to be involved in the regulation of this pathway, namely, ornithine, aspartic acid, L-glutamate, dextroproline, and N4-acetaminobutanal. These metabolites were significantly reduced in the HFD model group, while the opposite effect was seen in the IC extract group, demonstrating that the concentrations of these metabolites can be increased and that the arginine and proline metabolic pathways are regulated by the IC extract. Finally, up-regulation of this pathway can reduce obesity and improve oxidative stress, thus leading to improved lipid metabolism and protection against the development of NAFLD.

In summary, pharmacological methods integrated with gut microbiota and metabolomics were used to evaluate the hepatoprotective mechanism of IC extract on non-alcoholic fatty liver disease induced by a high-fat diet in mice. The results of the pharmacological study showed that IC has the potential to reduce body weight, reduce the levels of serum alanine aminotransferase and liver inflammatory factors, and improve liver pathological symptoms. The mechanism of action could involve regulating the intestinal bacteria Akkermansiaceae, Lachnospiraceae and Muribaculaceae, as well as their associated metabolites D-proline, pyridoxal, N4-acetylaminobutanal and ornithine, which are involved in the regulation of “carbohydrate metabolism”, “glutamine and glutamate metabolism”, “vitamin B6 metabolism”, and “arginine and proline metabolism” pathways. The present study provided a framework for the exploration of natural medicine on metabolic diseases in holistic and systematic manners. Further investigations are required to determine the relevance of identified bacteria and metabolites in hepatoprotective mechanisms.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee, Inner Mongolia Minzu University (approval no. NM-LL-2021-06-15-1).

AUTHOR CONTRIBUTIONS

GB and MF designed and funded the project. WJ, TB, HY and LD performed the animal experiment and analyzed the data. WJ, SC, NL and MF analyzed and interpreted the gut microbiota and metabolomics data. WJ and SC wrote the original draft. JZ, GB and MF revised the manuscript. All authors approved the final version.

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