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The gut-liver axis: the main role of microbiome in liver diseases

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The gut-liver axis: the main role of microbiome in liver diseases

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Editorial: The gut-liver axis: the main role of microbiome in liver diseases

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Editorial on the Research Topic
The gut-liver axis: the main role of microbiome in liver diseases

It is clear that changes in the composition of gut microbiota may contribute to the pathogenesis of liver diseases in multiple ways by influencing gut permeability, immune response, and bile salt metabolism, among other factors (Albillos et al., 2020). Therefore, this Research Topic has been initiated to offer a comprehensive overview of recent advancements regarding the role of microbiota in the multifaceted field of liver diseases, encompassing various conditions such as liver cirrhosis, autoimmune cholangitis, and non-alcoholic fatty liver disease (NAFLD).

Several articles in this Research Topic investigated the relevance of changes in microbiota to the pathogenesis of NAFLD by using completely different experimental strategies. Lin et al. used an animal model of the disease and compared the intestinal microbiome of nine mice with NAFLD induced by a high-fat diet to that of control mice. They found that specific bacteria including *Ileibacterium*, *Ruminococcaceae*, *Olsenella*, *Duncaniella*, *Paramuribaculum*, *Bifidobacterium*, and *Coriobacteriaceae_UCG_002* were specifically associated with NAFLD. Different metabolic profiles also corresponded to varying compositions of the gut microbiome. Ultimately, the authors identified a set of 8 bacterial strains, 14 genes, and 83 metabolites that strongly distinguished normal mice from those with NAFLD. In contrast, Pan et al. employed Mendelian randomization with published human data to assess the causal relationship between specific bacterial strains in the gut and the development of NAFLD. The results of this analysis showed that eight bacterial strains are strongly associated with this disease: the bacteria *Actinomycetales*, NB1n, the family *Actinomycetaceae*, the bacteria *Oxalobacteraceae*, and the genus *Ruminococcaceae UCG005* were positively correlated, whereas the family *Lactobacillaceae*, the *Christensenellaceae R7 group*, and the genus *Intestinibacter* were negatively correlated. Additionally, the analysis conducted by Pan et al. revealed that differences in the microbiome were associated with metabolic variations. The results were further validated in a mice model of NAFLD. A markedly different approach was employed by Shera et al., who transplanted fecal matter from patients who had undergone bariatric surgery into mice on a high-fat diet. This treatment significantly reduced the development of NAFLD in these mice and weakened the changes in the cytokine and T-lymphocyte patterns typically associated with this disease.

NAFLD may progress to NASH (non-alcoholic steatohepatitis), which is characterized by significant liver inflammation, and can lead to cirrhosis. The reason this progression occurs in some patients, but not in others, is largely unknown; however, the data reported by Huang et al. in this Research Topic suggest that microbiota composition is a key factor. They discovered that *Megamonas* and *Fusobacterium* were significantly more abundant in the fecal microbiome of NASH patients compared to control subjects and NAFLD patients. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed major metabolic differences in the gut microbiota between NAFLD and NASH patients.

The relevance of microbiota changes in the pathogenesis of liver cirrhosis was investigated by Li et al. by comparing microbiome and mycobiome in 45 cirrhotic patients and 30 healthy controls. Distinctive changes were identified; in cirrhotic patients, *Streptococcus*, *Akkermansia*, *Ligilactobacillus*, and *Pseudescherichia* were increased, while *Blautia*, *Anaerobutyricum*, *Gemmiger*, *Ruminococcus*, and *Dorea* were decreased. The significant changes in *mycobiota* included *an increase in Saccharomyces* alongside a concurrent decrease *in Aspergillus, Penicillium, Auricularia,* and *Cladosporium*. By combining metagenomic data with the results of metabolomic analyses, the authors developed a diagnostic testing system that was able to identify cirrhotic patients with a high accuracy (area under the curve [AUC] of 0.938 at the receiver operating characteristic [ROC] curve analysis).

In the articles by Cui et al. and Zhang et al., the association between changes in the gut microbiome and autoimmune liver diseases was assessed using Mendelian randomization (specifically for primary biliary cholangitis and primary sclerosing cholangitis).

Natural products have been claimed to exert beneficial effects on liver diseases, which can depend at least in part on changes in the composition of gut microbiota. Data supporting this hypothesis have been reported in two articles of this Research Topic. Sun S. et al. reviewed the evidence that certain traditional Chinese medicine remedies, which are claimed to be effective in treating liver cirrhosis, might be functioning not only as antioxidant, anti-inflammatory, and anti-fibrotic agents but also through modifications in gut microbiota. This has been demonstrated for purified natural products used in traditional Chinese medicine, including polyphenols such as resveratrol, alkaloids such as berberine, terpenoids such as Ginkgo Biloba, carbohydrates such as alginate, and glycosides such as FTA, a glycoside extracted from the dried fruit of *Forsythia suspensa*. Evidence of beneficial changes in microbiota composition is also available for herbal prescriptions that contain multiple compounds from various sources. Liang et al. investigated the impact of licorice on the hepatoxicity of Evodiae Fructus—a medicinal herb with analgesic, anti-tumor, and anti-inflammatory properties. They demonstrated that, in mice, Evodiae Fructus, particularly in the small-flowered forms, induces oxidative damage and inflammation in the liver. Additionally, these effects are reduced when the extract is administered along with licorice extracts. Changes in microbiota composition may contribute to licorice protective action. Licorice prevented the decrease in the abundance of *Corynebacterium* induced by Evodiae Fructus, while simultaneously causing an increase in *Candidatus Arthromitus*. Importantly, the abundance of these two bacterial genera correlated positively and negatively with biochemical markers of oxidative damage and inflammation, respectively. These results not only emphasize that natural products may protect the liver by changing gut microbiota but also highlight that toxic substances may harm the liver in a microbiota-dependent way. This concept was also addressed in the article by Tarantino et al., which explores the role of gut microbiota in the hepatotoxicity induced by drugs of abuse. The authors reviewed the literature demonstrating that opioids change the composition of intestinal microbiota by increasing Gram-positive bacterial species, including *Staphylococcus sciuri*, *Staphylococcus cohnii*, and *Staphylococcus aureus,* as well as *Enterococcus durans*, *Enterococcus casseliflavus*, *Enterococcus faecium*, and *Enterococcus faecalis*. This opioid-induced dysbiosis leads to the weakening of the intestinal barrier and, as a result, allows bacterial species to translocate to the liver. This occurrence strongly promotes inflammation and cell damage. Importantly, data are available indicating that similar mechanisms also contribute to alcohol-induced hepatotoxicity. If the hypothesis that gut dysbiosis plays a significant role in the pathogenesis of alcohol-induced liver damage is correct, then it could be suggested that interventions aimed at modifying the composition of intestinal microbiota may be beneficial for this condition. Wei et al. explored how alcohol feeding induces gut dysbiosis in mice by increasing the abundance of *Oscillibacter, Escherichia/Shigella, and Alistipes*. This increase is accompanied by a parallel rise in liver T-helper (Th) 1 and Th17 lymphocytes, along with a decrease in Regulatory T-cells (Treg) cells. These changes were completely reverted by the combined administration of *Akkermansia muciniphila* and inosine, a compound that acts as an energy source and also provides anti-inflammatory and immunomodulatory effects. Remarkably, this combined treatment promoted the growth of butyrate-producing bacteria, including not only *Akkermansia* but also *Lactobacillus* and *Clostridium IV*.

The molecular mechanisms by which intestinal microorganisms may exert beneficial or detrimental effects on the liver have been extensively investigated and crucial factors have been identified, such as bacterial translocation and endotoxin release, the release of indole and trimethylamine, bacterial metabolism of biliary acids, and changes in the level of short-chain fatty acids (Figure 1). The article by Sun R. et al. describes a new potential mechanism for liver protection involving the gut microbiome. By using quadrupole time-of-flight mass spectrometry (Q-TOF-MS)-based metabolomics, they demonstrated that, in mice, liver regeneration following partial hepatectomy is associated with a significant increase in liver steroid hormone biosynthesis, potentially linked to the observed rise in *Escherichia*, *Shigella*, *Lactobacillus*, *Akkermansia*, and *Muribaculaceae* within the gut microbiome.

In this Research Topic, we aimed to improve our understanding of the potential mechanisms that explain the connection between gut flora dysbiosis and liver damage. The 16 publications collected in this Research Topic have expanded our understanding of the microbiome's role and its involvement in liver diseases. Further exploration is needed to determine if modifying the composition of gut microbiota with probiotics, prebiotics, or symbiotics could ameliorate or prevent liver diseases.

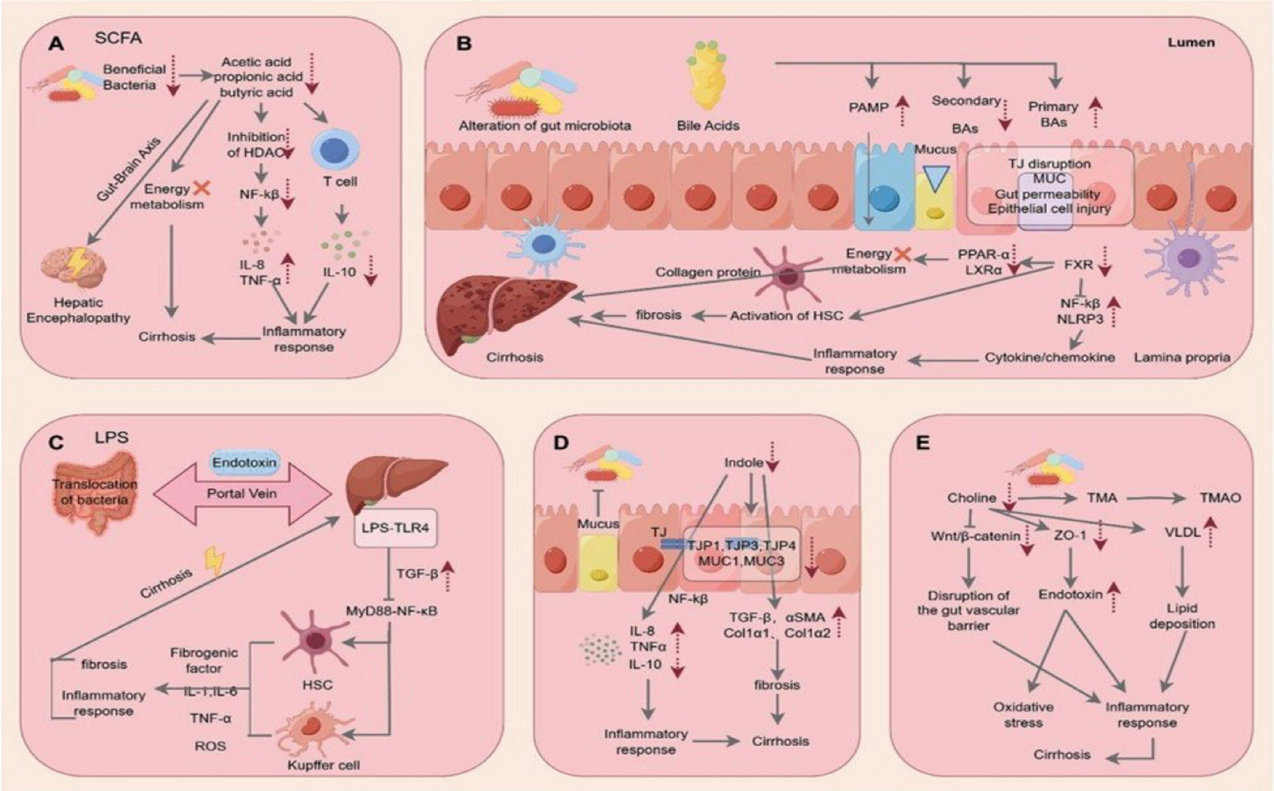

FIGURE 1
Multiple mechanisms by which the liver–gut axis may exert protective or detrimental effects on the liver: **(A)** Short-chain fatty acids; **(B)** Bile salts; **(C)** Endotoxin and bacterial translocation; **(D)** Indole; **(E)** Choline and trimethylamine (TMA) (Reproduced from the article by Sun S. et al. published in this Research Topic).

Author contributions

GT: Conceptualization, Writing – original draft, Writing – review & editing. TD: Writing – original draft, Writing – review & editing. MC: Conceptualization, Writing – original draft.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Albillos, A., de Gottardi, A., and Rescigno, M. (2020). The gut-liver axis in liver disease: Pathophysiological basis for therapy. *J Hepatol.* 72, 558–577. doi: 10.1016/j.jhep.2019.10.003

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Impact of gut microbiota on nonalcoholic fatty liver disease: insights from a leave-one-out cross-validation study

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Introduction: Enteric dysbacteriosis is strongly associated with nonalcoholic fatty liver disease (NAFLD). However, the underlying causal relationship remains unknown. Thus, the present study aimed to investigate the relationship between gut microbiota and NAFLD using Mendelian randomization (MR) and analyze the target genes potentially regulated by specific microbiota.

Methods: Bidirectional two-sample MR analysis was performed using inverse variance weighted (IVW) supplemented by MR-Egger, weighted median, simple mode, and weighted mode methods. Data were pooled from gut microbiota and NAFLD association studies. The least absolute shrinkage, selection operator regression, and the Support Vector Machine algorithm were used to identify genes regulated by these intestinal flora in NAFLD. The liver expression of these genes was verified in methionine choline-deficient (MCD) diet-fed mice.

Results: IVW results confirmed a causal relationship between eight specific gut microbes and NAFLD. Notably, the order Actinomycetales, NB1n, the family Actinomycetaceae, Oxalobacteraceae and the genus *Ruminococcaceae UCG005* were positively correlated, whereas Lactobacillaceae, the *Christensenellaceae R7 group*, and *Intestinibacter* were negatively correlated with NAFLD onset. In NAFLD, these eight bacteria regulated four genes: colony-stimulating factor 2 receptor β, fucosyltransferase 2, 17-beta-hydroxysteroid dehydrogenase 14, and microtubule affinity regulatory kinase 3 (*MAPK3*). All genes, except *MARK3*, were differentially expressed in the liver tissues of MCD diet-fed mice.

Discussion: The abundance of eight gut microbiota species and NAFLD progression displayed a causal relationship based on the expression of the four target genes. Our findings contributed to the advancement of intestinal microecology-based diagnostic technologies and targeted therapies for NAFLD.

KEYWORDS

gut microbiota, nonalcoholic fatty liver disease, Mendelian randomization, colonystimulating factor 2 receptor β, fucosyltransferase 2, 17-beta-hydroxysteroid dehydrogenase 14

1 Introduction

Nonalcoholic fatty liver disease (NAFLD) is a metabolic syndrome characterized by less than 5% lipid deposits in hepatocytes, without alcohol consumption or other factors that may induce liver damage. The progressive stages of NAFLD include fatty liver (steatosis), nonalcoholic steatohepatitis (NASH), and fibrosis/cirrhosis, which eventually progress to end-stage liver failure or hepatocellular carcinoma with a low survival rate and a poor prognosis. Unfortunately, the global prevalence of NAFLD has considerably increased from 25.24% in 2016 to 32.4% in 2022 (Younossi et al., 2016; Riazi et al., 2022).

Advances in high-throughput sequencing technology have revealed strong associations between changes in the intestinal microbiome and the development of NAFLD. Several factors, including bidirectional crosstalk via the anatomical and physiological structure of the intestine–liver axis, disruption of the intestinal mucosal barrier, gut microbiota dysbiosis, harmful bacteria, and metabolite translocation, can promote the development of NAFLD lesions (Albillos et al., 2020). Currently, there are no specific drugs are approved for the treatment of NAFLD. However, interventions targeting intestinal microecology have exhibited therapeutic effects by rectifying intestinal dysbacteriosis, repairing damaged mucosal barriers, and regulating microbial metabolite production to alleviate chronic inflammation, insulin resistance, and oxidative stress (Craven et al., 2020; Juárez-Fernández et al., 2021; Zhao et al., 2021; Pan et al., 2022). However, the established associations between intestinal microbiota and NAFLD are based on cross-sectional studies that are prone to confounding factors and reverse causality. Thus, other study designs must be used to establish the causality between the intestinal microbiota and NAFLD and identify therapeutic targets.

Randomized controlled trials are the gold standard for investigating causality in epidemiology; however, their implementation is difficult owing to ethical constraints, high costs, and time constraints (Hariton and Locascio, 2018). Mendel's law of inheritance dictates that parental alleles are randomly assigned to offspring, achieving the necessary randomization akin to randomized controlled trials without being affected by common confounding factors, including acquired or prior exposure, while excluding reverse causality. Mendelian randomization (MR) leverages genetic variation as an instrumental variable (IV), presenting an effective method for studying causal relationships between risk factors and outcomes (Hemani et al., 2018). Several genome-wide association studies (GWASs) on the intestinal microbiota and NAFLD (Bonder et al., 2016; Wang B. et al., 2016; Wang J. et al., 2016; Anstee et al., 2020; Ghodsian et al., 2021; Kurilshikov et al., 2021) have highlighted the relationships between these entities.

In the present study, we investigated the potential causality between different intestinal microbiota changes and NAFLD via a two-sample MR analysis using summary statistics from a GWAS. The least absolute shrinkage and selection operator (LASSO) regression and the Support Vector Machine (SVM) algorithm were used to screen the positively regulated genes in the intestinal flora. The expression of these genes was verified in the intestinal and liver tissues of mice fed a methionine choline-deficient (MCD) diet. This study aimed to outline a framework for developing gut microbiome-based diagnostic, preventive, and therapeutic approaches for NAFLD based on the target bacteria or genes they regulate.

2 Materials and methods

2.1 Data sources

Controls (770,180) and NAFLD cases (8,434) from a GWAS-based meta-analysis provided the summary statistics for NAFLD (Kurilshikov et al., 2021). Summary data for the 16S fecal microbiomes were obtained from a microbiome-based GWAS with 18,340 cases (Ghodsian et al., 2021). The GSE24807 Series Matrix File was downloaded from the Gene Expression Omnibus database.[1] The data file (annotated as GPL2895) included the expression profile data of 17 patients, including 5 in the control group and 12 in the NAFLD group. The ethics review committees authorized each study cited in the GWAS and informed consent was obtained from all participants.

2.2 Study design

MR analysis was performed to determine the causal relationship between the intestinal microbes and NAFLD. Quality control experiments, such as heterogeneity and gene multiplicity tests, were conducted to confirm the reliability of causality results. Three hypotheses were assumed in the MR analysis: IVs are closely related to exposure, confounding variables that affect 'exposure–outcome' are not linked to IVs, and IVs affect outcome only through exposure.

2.3 Selection of IVs

Single-nucleotide polymorphisms (SNPs) with genome-wide importance in the gut microbiota were selected for pooling. The following selection criteria were used to choose the IVs: (1) In the forward MR analysis, $p < 1.0 \times 10^{-5}$ of SNPs was selected as the potential IVs; and in the reverse MR analysis, $p < 5.0 \times 10^{-6}$ of SNPs was selected as the potential IVs (Burgess et al., 2017). (2) The linkage disequilibrium parameter (r2) was set at a threshold of 0.001, and a genetic distance of 10,000 kb was required to select SNPs (Cheng et al., 2020). (3) The strength of the selected SNPs was assessed after removing palindromic SNPs. The strength of genetic variation as IVs was assessed using the F-statistic, and an F-statistic $\geq$10 indicates a strong IV with a low likelihood of introducing significant bias in the analysis (Shang et al., 2013).

2.4 MR analysis and sensitivity analysis

Chain imbalance analysis, MR analysis, and quality control experiments were performed using the R statistical programming language and two-sample MR (Hemani et al., 2018). Five methods were used to estimate causal effects (Bowden et al., 2015; Verbanck et al., 2018; Parker et al., 2020): inverse variance weighted, MR-Egger, weighted median, simple mode, and weighted mode. IVW served as the main method. The leave-one-out approach was used for sensitivity analysis and to compute the values of the remaining cumulative effects

1 https://www.ncbi.nlm.nih.gov/geo/info/datasets.html

of the SNPs (Curtis et al., 2019). Horizontal genetic pleiotropy tests were performed using the intercept terms from the MR-Egger regression to determine whether IVs affected the outcomes through pathways other than exposure. The relationship between the gut microbiome and the risk of developing NAFLD was summarized as odds ratios (ORs) and 95% confidence intervals (95% CIs). A threshold of statistical significance was set at a Bonferroni-corrected $p < 0.05$ to address the issue of multiple comparisons. Figure 1 presents an overview of the analysis pipeline.

2.5 Feature selection process using LASSO regression and the SVM algorithm

The IVs listed in Supplementary Table S1 were converted to the corresponding SNPs using the "gwasrapidd" software package. LASSO logistic regression and the SVM algorithm were used to select the diagnostic markers for diseases using the "glmnet" and "e1071" software packages. After extracting SNPs, glmnet function of "glmnet" software package was used to fit linear model, family was set to binomial, alpha was set to 1, and then cv.glmnet was used for 10 cross-validation. When λ value reached the minimum, gene results were output.

2.6 Animals and diets

Twelve seven-week-old male wild-type C57BL/6 mice were purchased from the SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All mice were housed under specific pathogen-free laboratory animal barrier environmental conditions with a 12/12 h light/dark cycle, temperature of 24 ± 2°C, and humidity of 50 ± 10%. Mice were randomly analyzed in two groups with six mice in each group. The control group was fed normal chow (NC) and had *ad libitum* access to water. Mouse models of NASH were established by feeding the mice an MCD diet (A02082002B; Research Diets, New Brunswick, NJ, United States) for 4 weeks. $n = 6$ per group. All experiments were conducted in accordance with the established guidelines and were approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University (No. wydw2018-0252).

2.7 Quantitative real-time polymerase chain reaction

Total RNA was isolated from the liver samples and cultured cells using TRIzol™ reagent (Invitrogen). RNA was reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Takara, Shiga, Japan). qRT-PCR was performed using SYBR Green Master Mix (Thermo Fisher Scientific) in the Real-Time PCR System (Thermo Fisher Scientific) using cDNA. β-actin was used as the invariant control. The RT-qPCR results were analyzed using the comparative Ct method ($2^{-\Delta\Delta Ct}$). The primer sequences used for PCR are listed in Supplementary Table S2.

2.8 Histopathology

The liver tissues were fixed with 4% paraformaldehyde. All histological analyses were performed using paraffin-embedded tissue sections. The sections were stained with hematoxylin and eosin (Sigma, MO, United States) according to a standard procedure to visualize the lipid accumulation pattern. The Histological Activity Index (HAI) scores were quantified using ImageJ software (version 1.8.0, National Institutes of Health, Bethesda, MD, United States).

2.9 Biochemical analyses

Serum was isolated from blood via centrifugation at 1,300 rpm for 10 min and stored at −80°C until use. The levels of serum triglycerides

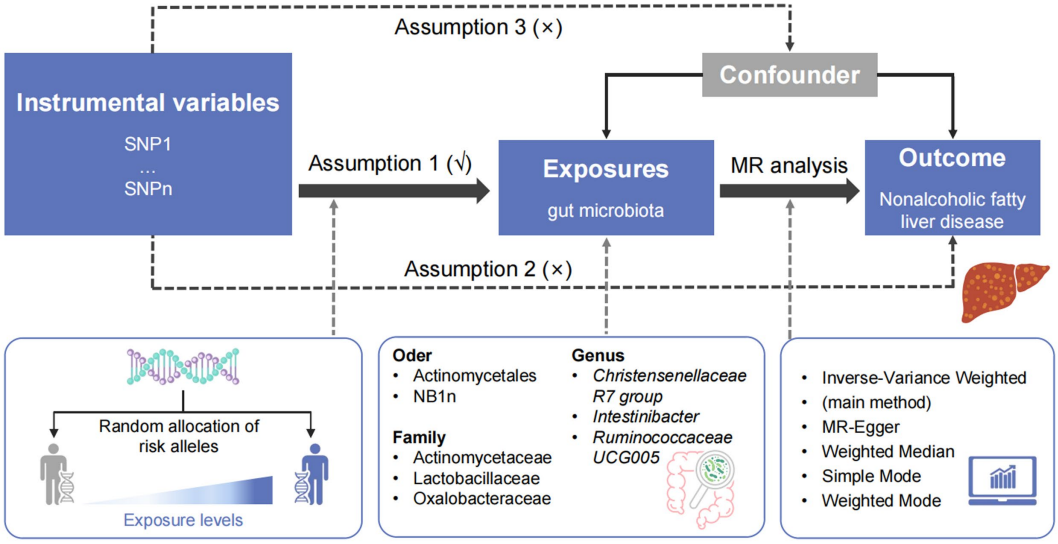

FIGURE 1
Overview of the study design. SNP, single-nucleotide polymorphism; MR, Mendelian randomization.

(TG), total cholesterol (TC), non-esterified fatty acid (NEFA), aspartate transaminase (AST), and alanine transaminase (ALT) were measured according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China).

2.10 Statistical analyses

The experimental data were analyzed using GraphPad Prism 10.1.0.316 for Windows (San Diego, CA, United States). The Shapiro–Wilk test showed that the data were normally distributed, so the data were presented as the mean ± standard deviation. Then, Student's *t*-test and one-or two-way analysis of variance were used to compare the data between groups. Statistical significance was set at $p < 0.05$.

3 Results

3.1 Selection of genetic IVs

SNPs were selected using GWAS summary statistics (Ghodsian et al., 2021). Ninety-eight SNPs were used as IVs for eight gut microbes based on the selection criteria for IVs. Supplementary Table S1 lists the details of the selected IVs.

3.2 Causal effects of gut microbiota on NAFLD

The causal relationship between the relative abundance of enterobacteria and NAFLD was analyzed at three levels (family, genus, and order) using 52-sample MR methods, primarily based on the IVW method. A total of eight intestinal microorganisms were found to be associated with NAFLD (Figure 2).

Forward MR analysis of the gut microbiota and NAFLD revealed that Actinomycetaceae, Oxalobacteraceae, *Ruminococcaceae UCG005*, Actinomycetales, and NB1n were positively associated with NAFLD. In contrast, Lactobacillaceae, *Christensenellaceae R7 group*, and *Intestinibacter* were negatively associated with NAFLD (Table 1, Figure 3, and Supplementary Table S3). Reverse MR analysis of the NAFLD-to-gut-microbiota ratio was performed to further evaluate the confounding factors and verify the relationship between enterobacteria and NAFLD. We observed that NAFLD had no effect on the eight gut microbiota mentioned above (Table 2, Figure 4, and Supplementary Table S4).

3.3 Sensitivity analysis of the reliability of the conclusion

According to the results of the MR analysis (Figure 2) and leave-one-out analysis (Figure 5) of all clusters based on different SNPs for Actinomycetaceae, Actinomycetales, *Christensenellaceae R7 group*, *Intestinibacter*, and *Ruminococcaceae UCG005*, all MR analyses were consistent with the primary analysis and the MR-Egger regression intercept term test. The Egger regression intercept test indicated that bacterial pleiotropy did not introduce bias to the results ($p > 0.05$). The results of the primary and reliability analyses using leave-one-out cross-validation supported these findings (Figure 5). Oxalobacteraceae exhibited consistent results in the multiple MR tests (Figure 2), and reliability analysis from the leave-one-out method (Figure 5) confirmed the plausibility of the outcomes. The MR-Egger regression intercept term test indicated that multipotency did not affect the abundance of Lactobacillaceae. The findings for NB1n were consistent with the main results except for those of the MR-Egger test, indicating that multipotency did not introduce bias to the results ($p > 0.05$). As expected, the results of the reliability analysis using the leave-one-out method were consistent with those of the primary analysis (Figure 5).

3.4 Identification of the key SNP-harboring genes regulated by positive gut microbiota and verification of their expression in mice with NASH

The IVs in Supplementary Table S1 were converted into 106 genes using the "gwasrapidd" pack-age, the principal component analysis (PCA) diagram shows a significant difference between our control group and the NAFLD disease group (Figure 6A). Further, the corresponding characteristic genes in NAFLD were screened using LASSO regression and the SVM algorithm. After ten cross-validations, LASSO regression identified five NAFLD characteristic genes (Figures 6B,C). However, the top five feature genes with the highest accuracy were screened using the SVM algorithm and intersected with the feature genes screened using LASSO regression (Figure 6D). Four intersection genes were accordingly identified as key genes involved in the regulation of the differential intestinal flora in NAFLD: colony-stimulating factor 2 receptor β (CSF2RB), fucosyltransferase 2 (FUT2), 17-beta-hydroxysteroid dehydrogenase 14 (HSD17B14), and microtubule affinity-regulated kinase 3 (MARK3) (Figure 6E).

To investigate the expression of the aforementioned SNP-harboring genes in the liver and colon of model mice with NASH, C57BL/6 mice were fed an NC or MCD diet for 4 weeks. The MCD diet-fed model mice with NASH exhibited a more notable infiltration of liver inflammatory cells and adipose vacuoles and increased HAI scores than those fed with NC (Figure 6F). Serum ALT, AST, TG, TC, and NEFA levels exhibited a consistent upward trend (Figures 6G,H). Furthermore, the hepatic mRNA expression of *CSF2RB* increased, whereas that of *FUT2* and *HSD17B14* decreased in mice with NASH compared to that in healthy controls (Figure 6I).

4 Discussion

NAFLD has emerged as the most common factor contributing to chronic liver disease worldwide, with an increasing global prevalence and an onset at younger ages. Intestinal inflammation, intestinal mucosal barrier destruction, bacterial flora structure disorder, and bacterial metabolite translocation are strongly associated with liver inflammation and fibrosis in NAFLD (Canfora et al., 2019; Tilg et al., 2021). The present study, using bidirectional two-sample MR analysis, revealed a causal relationship between eight intestinal flora and NAFLD and analyzed the target genes regulated by them.

Several previous studies have utilized MR analysis to analyze the causal relationship between gut bacteria and NAFLD. Consistent with these studies, we demonstrated that Lactobacillaceae,

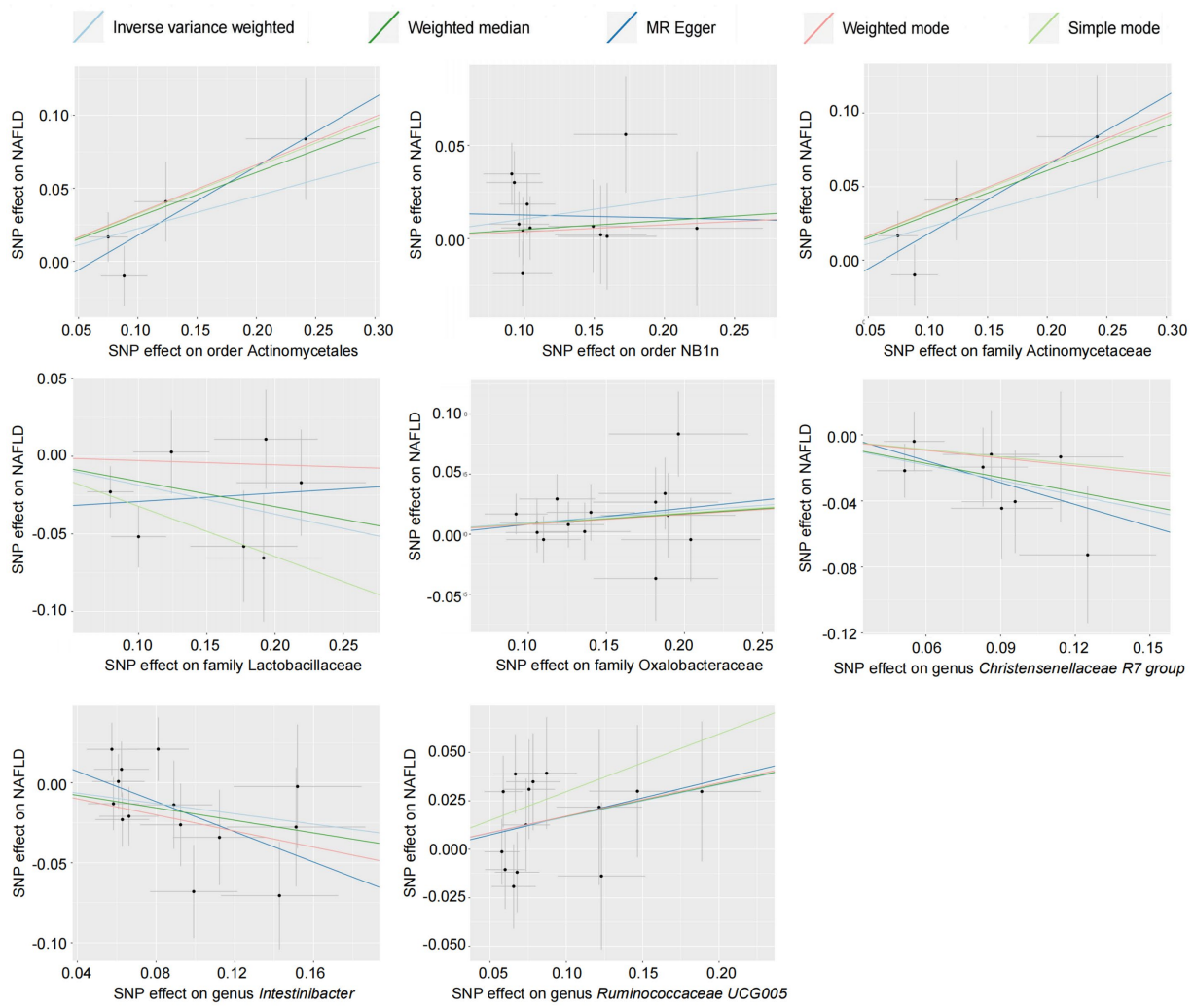

FIGURE 2
Scatter plot of the gut microbiota and NAFLD (forward results). SNP, single-nucleotide polymorphism; NAFLD, nonalcoholic fatty liver disease.

TABLE 1 Forward MR analysis of gut microbiota and NAFLD risk based on the IVW method.

Gut microbiota	SNP (*n*)	OR	95% CI	*p* value
Order				
Actinomycetales	4	1.25	1.02–1.53	0.03
NB1n	12	1.11	1.00–1.23	0.04
Family				
Actinomycetaceae	4	1.25	1.02–1.53	0.03
Lactobacillaceae	7	0.83	0.71–0.97	0.02
Oxalobacteraceae	14	1.10	1.01–1.21	0.04
Genus				
Christensenellaceae R7 group	8	0.74	0.59–0.92	0.01
Intestinibacter	14	0.85	0.73–0.99	0.03
Ruminococcaceae	14	1.18	1.01–1.38	0.03

CI: confidence interval; IVW, Inverse variance weighted; MR, Mendelian randomization; NAFLD, nonalcoholic fatty liver disease; OR: odds ratio; SNP: single-nucleotide polymorphisms.

Christensenellaceae R7 group, and *Intestinibacter* play a protective role in NAFLD, whereas Actinomycetaceae, Oxalobacteraceae, *Actinomycetales* and *Ruminococcaceae UCG005* are harmful bacteria in NAFLD (Li et al., 2023; Zhang R. et al., 2023). These results are consistent with previously reported intestinal microecological changes in NAFLD, with an increase in the abundance of Actinomycetales detected in obese mice fed a high-fat diet (Zhao et al., 2017). The abundances of probiotics, such as Lactobacillaceae (Yu et al., 2021; González-Lozano et al., 2022), *Christensenellaceae R7 group* (Tavella et al., 2021), and *Intestinibacter* (Zhang et al., 2022) were decreased and inversely correlated with the body weight ratio, NAFLD activity score, and visceral and subcutaneous adipose tissue content. In addition, Boursier et al. (2016) have reported that patients with NAFLD and fibrosis have a higher abundance of *Ruminococcaceae* members than those without fibrosis. However, some studies on the abundance and role of *Rumenococcaceae* in NAFLD present divergent findings form our conclusions. For example, the abundance of *Ruminococcaceae* members was low in patients with NAFLD (Tsai et al., 2020). Two studies compared the obesity status of the gut microecology in Latin American NASH and Asian NAFLD cohorts

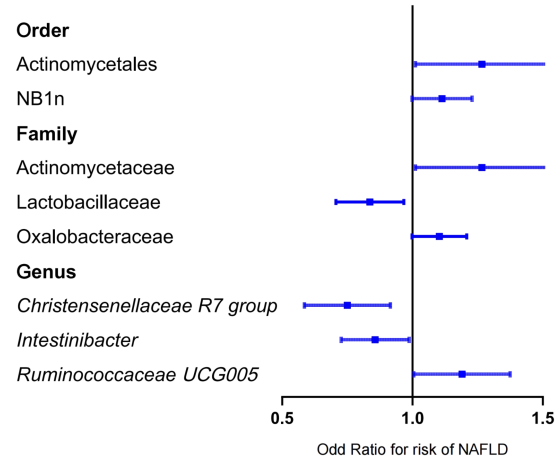

FIGURE 3
Mendelian randomization results of the positive association between gut microbiota and NAFLD based on the inverse variance weighted method. NAFLD, nonalcoholic fatty liver disease; OR, odds ratio.

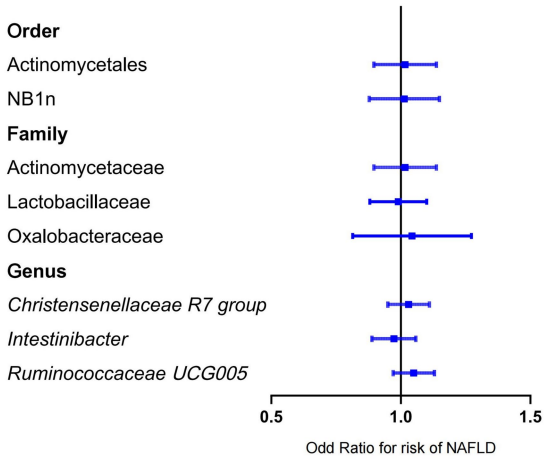

FIGURE 4
Mendelian randomization results of the negative association between gut microbiota and NAFLD based on inverse variance weighted method. NAFLD, nonalcoholic fatty liver disease; OR, odds ratio.

TABLE 2 Reverse MR results for the gut microbiota causally associated with NAFLD based on the IVW method.

Gut microbiota	SNP (*n*)	OR	95% CI	*p*-value
Order				
Actinomycetales	3	1.01	0.90–1.14	0.89
NB1n	5	1.01	0.88–1.15	0.91
Family				
Actinomycetaceae	3	1.01	0.90–1.14	0.87
Lactobacillaceae	6	0.99	0.88–1.10	0.79
Oxalobacteraceae	2	0.86	0.97–1.30	0.97
Genus				
Christensenellaceae R7 group	5	1.03	0.95–1.11	0.47
Intestinibacter	4	0.97	0.89–1.06	0.53
Ruminococcaceae	3	1.05	0.97–1.13	0.25

CI: confidence interval; IVW, Inverse variance weighted; MR, Mendelian randomization; NAFLD, nonalcoholic fatty liver disease; OR: odds ratio; SNP: single-nucleotide polymorphisms.

and revealed a significantly lower relative abundance of *Ruminococcaceae* members in non-obese patients with NASH than in non-obese controls (Wang B. et al., 2016; Duarte et al., 2018). Lee et al. (2020) reported that the severity of liver fibrosis in non-obese patients with NAFLD was negatively related to the abundance of *Ruminococcaceae*. In addition, *Faecalibacterium prausnitzii* (*Ruminococcaceae* member) was associated with decreased adipose tissue inflammation in high-fat diet-fed mice (Munukka et al., 2017), and *Ruminococcus faecalis* mitigated fibrosis in diet-induced NAFLD models (Lee et al., 2020). The discrepancies in conclusions may be attributed to the different inclusion criteria for the participants, including the presence of comorbid obesity.

The role of NB1n in NAFLD has not been previously reported, and to the best of our knowledge, this study is the first to identify NB1n as a harmful bacterium in NAFLD. While NB1n has been associated with infections (Lyu et al., 2023; Yan et al., 2023) and urolithiasis (Zhang L. et al., 2023), its role in the development of NAFLD remains unclear. On the other hand, the specific role of Oxalobacteraceae in NAFLD is also completely unclear. Oxalic acid is ingested through the diet or endogenously produced by the liver and metabolized and degraded in the gut by microorganisms, including Oxalobacteraceae members (Ermer et al., 2023). Notably, there is a significant correlation between steatosis severity and oxalate excretion in overweight children and adolescents and higher liver production and urinary oxalate excretion in ob/ob mice (Gianmoena et al., 2021). Furthermore, steatotic hepatocytes are responsible for glyoxalate detoxification (Gianmoena et al., 2021). A recent study revealed that patients with obesity have a significantly higher liver expression of Oxalobacteraceae-specific genes than lean individuals; the genus *Massilia* (Oxalobacteraceae member) was only detected in patients with obesity (Suppli et al., 2021). However, whether Oxalobacteraceae members naturally colonize the liver or translocate from the intestine remains unclear, and their role in oxalic acid synthesis in the liver requires further investigation.

We also demonstrated that the eight target gut bacteria mainly regulate the expression of three key genes in the liver during NAFLD: *CSF2RB*, *HSD17B14*, and *FUT2*. Compared with the control group, intrahepatic *CSF2RB* mRNA expression increased in the NAFLD group, whereas that of *HSD17B14* and *FUT2* decreased. CSF2RB, also called granulocyte-macrophage colony-stimulating factor/interleukin-3/interleukin-5 receptor common β-subunit, was highly activated in the liver of mice with NASH, aggravating liver tissue inflammation by promoting the production and differentiation of macrophages and granulocytes (Wessendarp et al., 2022). Moreover, the intrahepatic expression of *HSD17B14* (also known as DHRS10, a member of the 17 be-ta-hydroxysteroid dehydrogenase superfamily) was negatively correlated with NASH severity and involved in the regulation of retinol and fatty acid metabolism (Govaere et al., 2020). A large GWAS-based analysis revealed that *FUT2* (rs2519093) mutations are closely associated with low-density lipoprotein levels (Hoffmann et al., 2018). Notably, this gene is highly expressed in the digestive tract (intestine and gallbladder) of both humans and mice

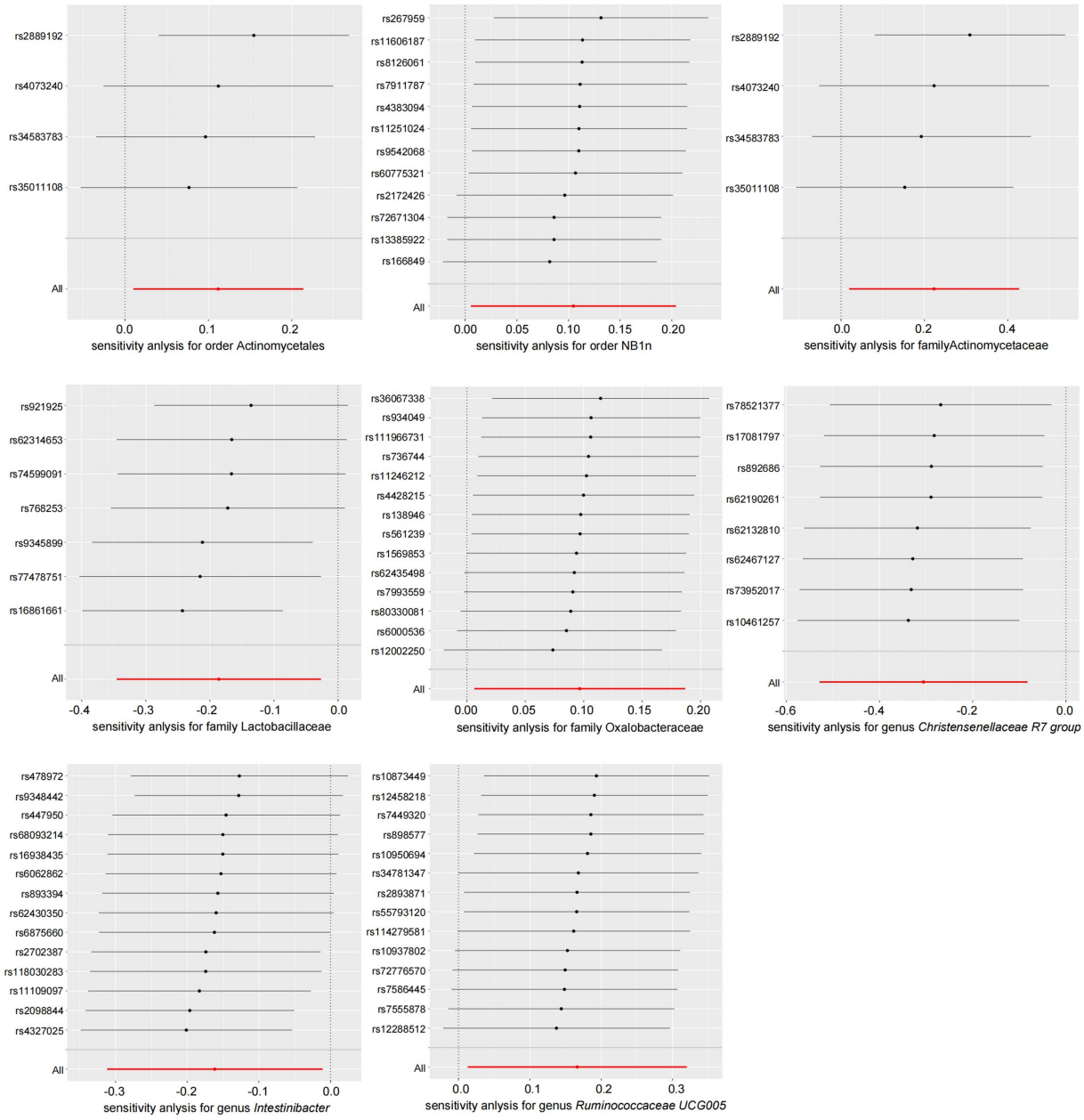

FIGURE 5
Leave-one-out plot of gut microbiota and NAFLD (forward results). NAFLD, nonalcoholic fatty liver disease.

and is primarily responsible for encoding alpha-1, 2-focusing transferase (Franks, 2011) and synthesizing H antigen (a receptor for adhesion molecules) (Xing and Guo, 2000). Approximately 20% of White individuals lack the functional *FUT* allele (i.e., the non-secretor), resulting in structural changes in the gut microbiota and disrupting mucosal immunity, which in turn increases the risk of diseases, including inflammatory bowel disease and primary sclerosing cholangitis (McGovern et al., 2010; Folseraas et al., 2012; Jostins et al., 2012). Zhou et al. (2021) have reported that Western diets decrease *FUT*2 expression in the intestinal epithelium of mice and that the loss of Fut2 activity mitigates diet-induced bile acid accumulation, insulin sensitivity, and hepatic steatosis. The specific roles of *CSF2RB*, *HSD17B14*, and *FUT2* in the enterohepatic dialog of NAFLD are unknown, warranting further studies to determine how the eight gut bacteria identified in our study regulate the involvement of these three genes in NAFLD progression.

However, this study has some limitations, necessitating exploration in future research. First, our genome-wide data is limited to European populations, and validating our findings in more diverse populations is critical. Second, several factors, including lifestyle, metabolic factors, age, and sex, that influence the gut microbiota need to be considered (Redondo-Useros et al., 2020; Yuan et al., 2022). Third, the sample size of transcriptome sequencing data needs to be further expanded. Finally, the roles of the eight gut microbes and the related key genes in NAFLD development require further evaluation in animal models.

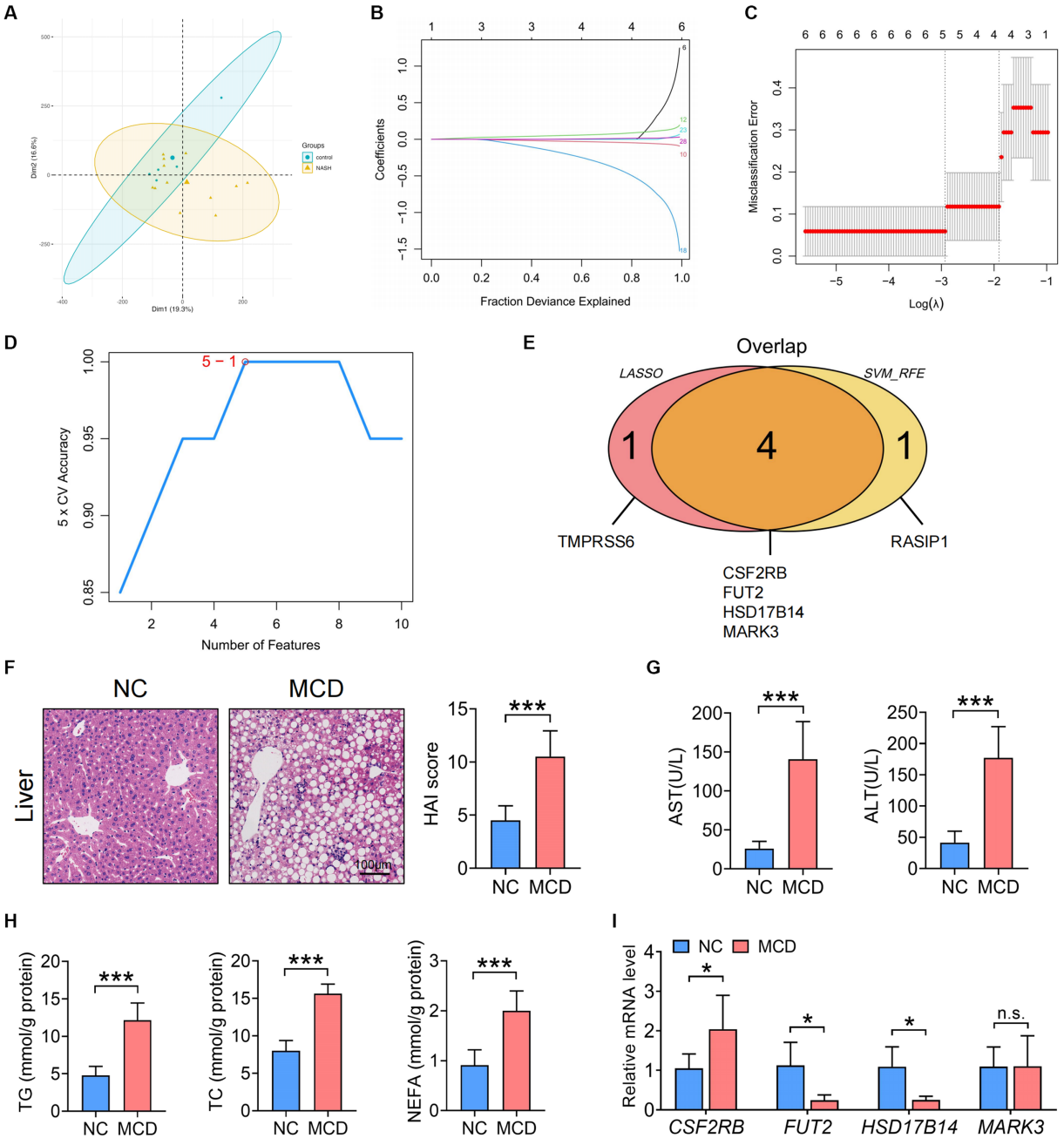

FIGURE 6
Identification of the key SNP-harboring genes corresponding to the gut microbiota and verification of their expression in the liver and colon of MCD-fed mice with NASH. **(A)** PCA results. **(B)** LASSO coefficient distribution of the differential genes. **(C)** Ten cross-validations of tuning parameter selection in the LASSO model. **(D)** The highest accuracy of the SVM model. **(E)** Wayne graphs for the LASSO and SVM algorithms. **(F)** Representative images of liver hematoxylin and eosin staining and statistical results of the HAI scores of NC or MCD diet-fed mice. Scale =100 μm. **(G)** Serum ALT and AST levels in MCD diet-fed mice. **(H)** Serum TG, TC, and NEFA levels in NC or MCD diet-fed mice. **(I)** qRT-PCR analysis of the key SNP-harbouring genes (*CSF2RB*, *FUT2*, *HSD17B14*, and *MARK3*) in the liver of NC-or MCD diet-fed mice. **(F,I)** $n = 6$ per group. Data are presented as mean ± standard deviation. **(F–H)** Two-tailed unpaired Student's *t*-test; **(I)** Two-way analysis of variance (ANOVA) followed by Sidak. MCD, methionine choline-deficient; NASH, nonalcoholic steatohepatitis; PCA, Principal component analysis; SNP, single-nucleotide polymorphism; LASSO, least absolute shrinkage and selection operator; SVM, support vector machine; alanine transaminase, ALT; aspartate transaminase, AST; HAI. Histological Activity Index; TG, triglyceride; TC, total cholesterol; NEFA, non-esterified fatty acid; NC, normal chow.

5 Conclusion

Our results suggest a causal relationship between eight gut microbes and NAFLD, highlighting three key SNP-harboring genes (*CSF2RB*, *FUT2*, and *HSD17B14*) for specific microbiome regulation. This study suggests that increasing the abundance of Lactobacillaceae, *Christensenaceae R7 group*, and *Intestinibacter* may alleviate NAFLD and that decreasing that of enterogenous Actinomycetales, NB1n, Actinomycetaceae, Oxalobacteraceae, and *Ruminococcaceae UCG005* may be potential therapeutic targets for NAFLD. In addition,

inhibiting the expression of *CSF2RB* or upregulating that of *FUT2* and *HSD17B14* may be another beneficial approach to alleviate NAFLD.

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 author.

Ethics statement

The studies involving humans were approved by the Research Ethics Committee of the University of Tartu and Danish Ethics Committee. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements. The animal study was approved by Institutional Animal Care and Use Committee of Wenzhou Medical University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

TP: Conceptualization, Writing – review & editing. LS: Data curation, Writing – original draft. YZ: Writing – original draft. FY: Data curation, Writing – original draft. YC: Conceptualization, Funding acquisition, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1320279/full#supplementary-material

References

Albillos, A., de Gottardi, A., and Rescigno, M. (2020). The gut-liver axis in liver disease: pathophysiological basis for therapy. *J. Hepatol.* 72, 558–577. doi: 10.1016/j.jhep.2019.10.003

Anstee, Q. M., Darlay, R., Cockell, S., Meroni, M., Govaere, O., Tiniakos, D., et al. (2020). Genome-wide association study of non-alcoholic fatty liver and steatohepatitis in a histologically characterised cohort. *J. Hepatol.* 73, 505–515. doi: 10.1016/j.jhep.2020.04.003

Bonder, M. J., Kurilshikov, A., Tigchelaar, E. F., Mujagic, Z., Imhann, F., Vila, A. V., et al. (2016). The effect of host genetics on the gut microbiome. *Nat. Genet.* 48, 1407–1412. doi: 10.1038/ng.3663

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

Bowden, J., Davey Smith, G. D., and Burgess, S. (2015). Mendelian randomization with invalid instruments: effect estimation and bias detection through egger regression. *Int. J. Epidemiol.* 44, 512–525. doi: 10.1093/ije/dyv080

Burgess, S., Small, D. S., and Thompson, S. G. (2017). A review of instrumental variable estimators for Mendelian randomization. *Stat. Methods Med. Res.* 26, 2333–2355. doi: 10.1002/jrsm.1346

Canfora, E. E., Meex, R. C. R., Venema, K., and Blaak, E. E. (2019). Gut microbial metabolites in obesity, NAFLD and T2DM. *Nat. Rev. Endocrinol.* 15, 261–273. doi: 10.1038/s41574-019-0156-z

Cheng, Q., Yang, Y., Shi, X., Yeung, K. F., Yang, C., Peng, H., et al. (2020). MR-LDP: a two-sample Mendelian randomization for GWAS summary statistics accounting for linkage disequilibrium andhorizontal pleiotropy. *NAR Genom. Bioinform.* 2:lqaa028. doi: 10.1093/nargab/lqaa028

Craven, L., Rahman, A., Nair Parvathy, S. N., Beaton, M., Silverman, J., Qumosani, K., et al. (2020). Allogenic fecal microbiota transplantation in patients with nonalcoholic fatty liver disease improves abnormal small intestinal permeability: a randomized control trial. *Am. J. Gastroenterol.* 115, 1055–1065. doi: 10.14309/ajg.0000000000000661

Curtis, S. W., Cobb, D. O., Kilaru, V., Terrell, M. L., Marder, M. E., Barr, D. B., et al. (2019). Environmental exposure to polybrominated biphenyl (PBB) associates with an increased rate of biological aging. *Aging* 11, 5498–5517. doi: 10.18632/aging.102134

Duarte, S. M. B., Stefano, J. T., Miele, L., Ponziani, F. R., Souza-Basqueira, M., Okada, L. S. R. R., et al. (2018). Gut microbiome composition in lean patients with NASH is associated with liver damage independent of caloric intake: a prospective pilot study. *Nutr. Metab. Cardiovasc. Dis.* 28, 369–384. doi: 10.1016/j.numecd.2017.10.014

Ermer, T., Nazzal, L., Tio, M. C., Waikar, S., Aronson, P. S., and Knauf, F. (2023). Oxalate homeostasis. *Nat. Rev. Nephrol.* 19, 123–138. doi: 10.1038/s41581-022-00643-3

Folseraas, T., Melum, E., Rausch, P., Juran, B. D., Ellinghaus, E., Shiryaev, A., et al. (2012). Extended analysis of a genome-wide association study in primary sclerosing cholangitis detects multiple novel risk loci. *J. Hepatol.* 57, 366–375. doi: 10.1016/j.jhep.2012.03.031

Franks, I. (2011). Gut microbiota: FUT2 genotype influences the gut microbiota in patients with Crohn's disease and healthy individuals. *Nat. Rev. Gastroenterol. Hepatol.* 9:2. doi: 10.1038/nrgastro.2011.237

Ghodsian, N., Abner, E., Emdin, C. A., Gobeil, É., Taba, N., Haas, M. E., et al. (2021). Electronic health record-based genome-wide meta-analysis provides insights on the genetic architecture of non-alcoholic fatty liver disease. *Cell Rep. Med.* 2:100437. doi: 10.1016/j.xcrm.2021.100437

Gianmoena, K., Gasparoni, N., Jashari, A., Gabrys, P., Grgas, K., Ghallab, A., et al. (2021). Epigenomic and transcriptional profiling identifies impaired glyoxylate detoxification in NAFLD as a risk factor for hyperoxaluria. *Cell Rep.* 36:109526. doi: 10.1016/j.celrep.2021.109526

González-Lozano, E., García-García, J., Gálvez, J., Hidalgo-García, L., Rodríguez-Nogales, A., Rodríguez-Cabezas, M. E., et al. (2022). Novel horizons in postbiotics: Lactobacillaceae extracellular vesicles and their applications in health and disease. *Nutrients* 14:5296. doi: 10.3390/nu14245296

Govaere, O., Cockell, S., Tiniakos, D., Queen, R., Younes, R., Vacca, M., et al. (2020). Transcriptomic profiling across the nonalcoholic fatty liver disease spectrum reveals

gene signatures for steatohepatitis and fibrosis. *Sci. Transl. Med.* 12:eaba4448. doi: 10.1126/scitranslmed.aba4448

Hariton, E., and Locascio, J. J. (2018). Randomised controlled trials—the gold standard for effectiveness research: study design: randomised controlled trials. *Br. J. Obstet. Gynaecol.* 125:1716. doi: 10.1111/1471-0528.15199

Hemani, G., Zheng, J., Elsworth, B., Wade, K. H., Haberland, V., Baird, D., et al. (2018). The MR-base platform supports systematic causal inference across the human phenome. *elife* 7:e34408. doi: 10.7554/eLife.34408

Hoffmann, T. J., Theusch, E., Haldar, T., Ranatunga, D. K., Jorgenson, E., Medina, M. W., et al. (2018). A large electronic-health-record-based genome-wide study of serum lipids. *Nat. Genet.* 50, 401–413. doi: 10.1038/s41588-018-0064-5

Jostins, L., Ripke, S., Weersma, R. K., Duerr, R. H., McGovern, D. P., Hui, K. Y., et al. (2012). Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* 491, 119–124. doi: 10.1038/nature11582

Juárez-Fernández, M., Porras, D., Petrov, P., Román-Sagüillo, S., García-Mediavilla, M. V., Soluyanova, P., et al. (2021). The synbiotic combination of Akkermansia muciniphila and quercetin ameliorates early obesity and NAFLD through gut microbiota reshaping and bile acid metabolism modulation. *Antioxidants* 10:2001. doi: 10.3390/antiox10122001

Kurilshikov, A., Medina-Gomez, C., Bacigalupe, R., Radjabzadeh, D., Wang, J., Demirkan, A., et al. (2021). Large-scale association analyses identify host factors influencing human gut microbiome composition. *Nat. Genet.* 53, 156–165. doi: 10.1038/s41588-020-00763-1

Lee, G., You, H. J., Bajaj, J. S., Joo, S. K., Yu, J., Park, S., et al. (2020). Distinct signatures of gut microbiome and metabolites associated with significant fibrosis in non-obese NAFLD. *Nat. Commun.* 11:4982. doi: 10.1038/s41467-020-18754-5

Li, Y., Liang, X., Lyu, Y., Wang, K., Han, L., Wang, Y., et al. (2023). Association between the gut microbiota and nonalcoholic fatty liver disease: a two-sample Mendelian randomization study. *Dig. Liver Dis.* 55, 1464–1471. doi: 10.1016/j.dld.2023.07.014

Lyu, B., Ma, J., Bai, Y., and Feng, Z. (2023). Casual effects of gut microbiota on risk of infections: a two-sample Mendelian randomization study. *Front. Microbiol.* 14:1284723. doi: 10.3389/fmicb.2023.1284723

McGovern, D. P. B., Jones, M. R., Taylor, K. D., Marciante, K., Yan, X., Dubinsky, M., et al. (2010). Fucosyltransferase 2 (FUT2) non-secretor status is associated with Crohn's disease. *Hum. Mol. Genet.* 19, 3468–3476. doi: 10.1093/hmg/ddq248

Munukka, E., Rintala, A., Toivonen, R., Nylund, M., Yang, B., Takanen, A., et al. (2017). *Faecalibacterium prausnitzii* treatment improves hepatic health and reduces adipose tissue inflammation in high-fat fed mice. *ISME J.* 11, 1667–1679. doi: 10.1038/ismej.2017.24

Pan, Z., Mao, B., Zhang, Q., Tang, X., Yang, B., Zhao, J., et al. (2022). Postbiotics prepared using *Lactobacillus paracasei* CCFM1224 prevent nonalcoholic fatty liver disease by modulating the gut microbiota and liver metabolism. *Int. J. Mol. Sci.* 23:13522. doi: 10.3390/ijms232113522

Parker, D. C., Bartlett, B. N., Cohen, H. J., Fillenbaum, G., Huebner, J. L., Kraus, V. B., et al. (2020). Association of blood chemistry quantifications of biological aging with disability and mortality in older adults. *J. Gerontol. A Biol. Sci. Med. Sci.* 75, 1671–1679. doi: 10.1093/gerona/glz219

Redondo-Useros, N., Nova, E., González-Zancada, N., Díaz, L. E., Gómez-Martínez, S., and Marcos, A. (2020). Microbiota and lifestyle: a special focus on diet. *Nutrients* 12:1776. doi: 10.3390/nu12061776

Riazi, K., Azhari, H., Charette, J. H., Underwood, F. E., King, J. A., Afshar, E. E., et al. (2022). The prevalence and incidence of NAFLD worldwide: a systematic review and meta-analysis. *Lancet Gastroenterol. Hepatol.* 7, 851–861. doi: 10.1016/S2468-1253(22)00165-0

Shang, R., Liu, Y., Xin, Z., Guo, W., Guo, Z., Hao, B., et al. (2013). Synthesis and antibacterial evaluation of novel pleuromutilin derivatives. *Eur. J. Med. Chem.* 63, 231–238. doi: 10.1016/j.ejmech.2013.01.048

Suppli, M. P., Bagger, J. I., Lelouvier, B., Broha, A., Demant, M., Kønig, M. J., et al. (2021). Hepatic microbiome in healthy lean and obese humans. *JHEP Rep.* 3:100299. doi: 10.1016/j.jhepr.2021.100299

Tavella, T., Rampelli, S., Guidarelli, G., Bazzocchi, A., Gasperini, C., Pujos-Guillot, E., et al. (2021). Elevated gut microbiome abundance of Christensenellaceae, Porphyromonadaceae and Rikenellaceae is associated with reduced visceral adipose tissue and healthier metabolic profile in Italian elderly. *Gut Microb.* 13, 1–19. doi: 10.1080/19490976.2021.1880221

Tilg, H., Adolph, T. E., Dudek, M., and Knolle, P. (2021). Non-alcoholic fatty liver disease: the interplay between metabolism, microbes and immunity. *Nat. Metab.* 3, 1596–1607. doi: 10.1038/s42255-021-00501-9

Tsai, M. C., Liu, Y. Y., Lin, C. C., Wang, C. C., Wu, Y. J., Yong, C. C., et al. (2020). Gut microbiota dysbiosis in patients with biopsy-proven nonalcoholic fatty liver disease: a cross-sectional study in Taiwan. *Nutrients* 12:820. doi: 10.3390/nu12030820

Verbanck, M., Chen, C. Y., Neale, B., and Do, R. (2018). Detection of widespread horizontal pleiotropy in causal relationships inferred from Mendelian randomization between complex traits and diseases. *Nat. Genet.* 50, 693–698. doi: 10.1038/s41588-018-0099-7

Wang, B., Jiang, X., Cao, M., Ge, J., Bao, Q., and Tang, L. (2016). Altered fecal microbiota correlates with liver biochemistry in nonobese patients with non-alcoholic fatty liver disease. *Sci. Rep.* 6:32002. doi: 10.1038/srep32002

Wang, J., Thingholm, L. B., Skiecevičienė, J., Rausch, P., Kummen, M., Hov, J. R., et al. (2016). Genome-wide association analysis identifies variation in vitamin D receptor and other host factors influencing the gut microbiota. *Nat. Genet.* 48, 1396–1406. doi: 10.1038/ng.3695

Wessendarp, M., Watanabe-Chailland, M., Liu, S., Stankiewicz, T., Ma, Y., Kasam, R. K., et al. (2022). Role of GM-CSF in regulating metabolism and mitochondrial functions critical to macrophage proliferation. *Mitochondrion* 62, 85–101. doi: 10.1016/j.mito.2021.10.009

Xing, L., and Guo, L. H. (2000). FUT2 gene involved in expression of H blood group antigen on surface of human tumor cell lines BEL-7404, SGC-7901, and SPC-A-1. *Acta Pharmacol. Sin.* 21, 997–1001.

Yan, M., Ouyang, Y. L., Xiao, L. Y., Ao, M., Gosau, M., Friedrich, R. E., et al. (2023). Correlations between gut microbiota and lichen planus: a two-sample Mendelian randomization study. *Front. Immunol.* 14:1235982. doi: 10.3389/fimmu.2023.1235982

Younossi, Z. M., Koenig, A. B., Abdelatif, D., Fazel, Y., Henry, L., and Wymer, M. (2016). Global epidemiology of nonalcoholic fatty liver disease-meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* 64, 73–84. doi: 10.1002/hep.28431

Yu, J. S., Youn, G. S., Choi, J., Kim, C. H., Kim, B. Y., Yang, S. J., et al. (2021). Lactobacillus lactis and *Pediococcus pentosaceus*-driven reprogramming of gut microbiome and metabolome ameliorates the progression of non-alcoholic fatty liver disease. *Clin. Transl. Med.* 11:e634. doi: 10.1002/ctm2.634

Yuan, S., Chen, J., Li, X., Fan, R., Arsenault, B., Gill, D., et al. (2022). Lifestyle and metabolic factors for nonalcoholic fatty liver disease: Mendelian randomization study. *Eur. J. Epidemiol.* 37, 723–733. doi: 10.1007/s10654-022-00868-3

Zhang, L., Wang, Z., Zhang, X., Zhao, L., Chu, J., Li, H., et al. (2022). Alterations of the gut microbiota in patients with diabetic nephropathy. *Microbiol. Spectr.* 10:e0032422. doi: 10.1128/spectrum.00324-22

Zhang, R., Zhao, W., Zhao, R., Zhao, Y., Zhang, Y., and Liang, X. (2023). Causal relationship in gut microbiota and upper urinary urolithiasis using Mendelian randomization. *Front. Microbiol.* 14:1170793. doi: 10.3389/fmicb.2023.1170793

Zhang, L., Zi, L., Kuang, T., Wang, K., Qiu, Z., Wu, Z., et al. (2023). Investigating causal associations among gut microbiota, metabolites, and liver diseases: a Mendelian randomization study. *Front. Endocrinol.* 14:1159148. doi: 10.3389/fendo.2023.1159148

Zhao, Z. H., Wang, Z. X., Zhou, D., Han, Y., Ma, F., Hu, Z., et al. (2021). Sodium butyrate supplementation inhibits hepatic steatosis by stimulating liver kinase B1 and insulin-induced gene. *Cell. Mol. Gastroenterol. Hepatol.* 12, 857–871. doi: 10.1016/j.jcmgh.2021.05.006

Zhao, L., Zhang, Q., Ma, W., Tian, F., Shen, H., and Zhou, M. (2017). A combination of quercetin and resveratrol reduces obesity in high-fat diet-fed rats by modulation of gut microbiota. *Food Funct.* 8, 4644–4656. doi: 10.1039/c7fo01383c

Zhou, R., Llorente, C., Cao, J., Zaramela, L. S., Zeng, S., Gao, B., et al. (2021). Intestinal α1-2-fucosylation contributes to obesity and steatohepatitis in mice. *Cell. Mol. Gastroenterol. Hepatol.* 12, 293–320. doi: 10.1016/j.jcmgh.2021.02.009

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Research reviews and prospects of gut microbiota in liver cirrhosis: a bibliometric analysis (2001–2023)

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Introduction: The gut-liver axis has emerged as a focal point in chronic liver disorders, prompting more research into the role of the gut microbiota in liver cirrhosis. In individuals with liver cirrhosis, changes in the structure and function of the gut microbiota are closely tied to clinical prognosis. However, there is a scarcity of bibliometric evaluations conducted in this particular field.

Methods: This study is aiming to conduct a complete analysis of the knowledge structure and centers pertaining to gut microbiota in liver cirrhosis using bibliometric methods. Publications on gut microbiota and liver cirrhosis from 2001 to 2023 are sourced from the Web of Science Core Collection. For the bibliometric analysis, we employ VOSviewer, CiteSpace, and the R package "bibliometrix".

Results: Our study encompasses a comprehensive collection of 3109 articles originating from 96 countries, with notable contributions from leading nations such as the United States and China. The quantity of publications concerning the gut microbiota of liver cirrhosis rises annually. The University of California San Diego, Virginia Commonwealth University, Zhejiang University are the primary research institutions. World Journal of Gastroenterology publishes the most papers in this field, while hepatology is the most frequently co-cited journal. These publications come from a total of 15,965 authors, and the most prolific authors are Bajaj Jasmohan S., Schnabl Bernd and Gillevet Patrick M., while the most co-cited authors are Bajaj Jasmohan S., Younossi Zobair M., and Reiner Wiest. In addition, "dysbiosis", "gut microbiota", "intestinal barrier", "fecal microbiota transplantation", and "complement-system" are the primary keywords of research trends in recent years.

Discussion: This study offering a comprehensive insight into the research dynamics surrounding gut microbiota in patients with liver cirrhosis. It delineates the current research frontiers and hotspots, serving as a valuable guide for scholars.

KEYWORDS

liver cirrhosis, gut microbiota, bibliometrics, VOSviewers, CiteSpace

1 Introduction

Liver cirrhosis is common worldwide and is a late manifestation of acute or chronic liver injury with multiple causes, such as excessive alcohol consumption, fatty liver, autoimmune diseases, cholestasis, and hepatitis B or C virus infections (Ginès et al., 2021). Under the long-term stimulation of pathogenic factors, hepatocytes repeatedly degenerate, become necrotic

and eventually replaced by fibrotic tissue, which induces progressive deteriorates of liver function (Hernandez-Gea and Friedman, 2011; Zhou et al., 2014; Parola and Pinzani, 2019). Common complications of liver cirrhosis encompass portal hypertension, gastrointestinal bleeding, ascites, and hepatic encephalopathy (HE), among others (Garcia-Pagan et al., 2021). Individuals suffering from decompensated liver cirrhosis often have an unfavorable prognosis and usually need liver transplantation (Fouts et al., 2012; Shen et al., 2014; Engelmann et al., 2021). The mitigation of the burden caused by liver cirrhosis and its subsequent sequelae is a critical therapeutic concern for prompt resolution.

The human digestive system houses a diverse ecosystem teeming with bacteria, viruses, fungi, and other microbial entities, together referred to as the gut microbiome which is a result of the co-evolution of the gut microbiota with its host (Brody, 2020; Weersma et al., 2020). The constitution of the gut microbiota is easily affected by numerous factors such as age (Martino et al., 2022), drugs (Weersma et al., 2020) and diet (Gentile and Weir, 2018). Its dysregulation plays a pivotal role in the pathogenesis of chronic diseases including neurodegenerative diseases (Sampson et al., 2016), inflammatory bowel diseases (Franzosa et al., 2019) and cancer (Bai et al., 2022), particularly chronic liver disease (Frost et al., 2021). As the primary organ contacting microbial products that transverse the intestinal barrier, the liver is more susceptible to the alterations in the gastrointestinal microbiota in many ways (Tranah et al., 2021). Numerous prior investigations have highlighted the intricate interplay between gut microbiota and liver cirrhosis prognosis, noting that the balance of the gut microbiota is further disrupted with disease progression or decompensation (Bajaj et al., 2014b; Trebicka et al., 2021a). Therapeutic interventions concentrating on the gut microbiota, including probiotics, fecal microbiota transplantation (FMT), and antibiotics, have been extensively studied for chronic liver disease treatment, underscoring their therapeutic potential. Subsequent research has reinforced the importance of modulating the gut-liver axis (Bajaj, 2019). The preliminary studies on FMT in the treatment of decompensated cirrhosis demonstrate its safety profile and potential to improve patient prognosis while reducing hospitalizations, thereby paving the way for larger-scale investigations (Bajaj et al., 2017, 2019).

The utilization of bibliometrics as a research evaluation approach has become increasingly prevalent, employing a blend of quantitative and qualitative scientific analysis methodologies to investigate comprehensive knowledge about developmental trends within associated fields (Wallin, 2005). Bibliometric analysis is increasingly used to study various aspects of science (Ellegaard and Wallin, 2015). It can analyze the different features of publications in a particular field over a specific time period, including information and contribution about countries, institutions, authors, journals, keywords, references, etc. (Ke et al., 2020; Wu et al., 2022). Researchers can thus distill the current research landscape, identify hotspots, and discern progression trends in specific fields or diseases, offering valuable insights and guidance for future investigations (Lu et al., 2019). As a powerful tool for disciplinal development planning, bibliometrics has been drastically utilized in research across various medical fields, encompassing gynecological and obstetrical diseases (Brandt et al., 2019), pulmonary diseases (Lin et al., 2022), autoimmune diseases (Wu et al., 2022), cardiovascular diseases (Ma et al., 2021), among others. However, systematic bibliometric studies are scarce in the field of intestinal microbiology of liver cirrhosis. Our study is aiming to provide a comprehensive bibliometric analysis of the field of gut microbiota in liver cirrhosis, thus summarizing the present research status within this domain and anticipating further trends and prospects.

2 Materials and methods

2.1 Data search and retrieval strategy

For bibliometric studies, the Web of Science Core Collection (WoSCC) is an ideal source because of its extensive coverage of scholarly literature and the number of journals it indexes. All articles utilized in this study were sourced from the WoSCC database and retrieved on August 26, 2023. The results of literature retrieval are credible and authentic. The specific search strategy is as follows. The search formula is ((TS (topic) = (gut OR intestin* OR gastrointestin*) AND TS = (microbio* OR microflora OR flora OR bacteri* OR dysbiosis OR microecology OR 16Sr* OR metagenome)) OR TS = (prebiotic* OR probiotic* OR synbiotic*)) AND TS = (Liver cirrhosis OR hepatic cirrhosis OR liver fibrosis) AND LA (language) = (English). The publication type is limited to articles or reviews, and the time period of publication is from January 1, 2001, to August 26, 2023. We collated a total of 3,109 publications, comprising 2,004 articles and 1,105 reviews, for subsequent analysis.

2.2 Data process and visualization

VOS viewer (version 1.6.19) is a commonly used software designed for bibliometric analysis, boasting a robust graphical display capability. The tool allows users to construct and examine bibliometric maps of cooperation, co-citation, and co-occurrence networks for visual analysis. Additionally, it can extract critical information from a vast number of publications (van Eck and Waltman, 2010). Analysis of countries and institutions, analysis of journals and co-cited journals, analysis of authors and co-cited authors, analysis of co-cited references, and analysis of keyword co-occurrence were all performed mostly using the software in our research. Nodes on the map produced by VOS viewer provide details about a project, such as its location, organization, journal, and author. The size of the nodes signifies the total count of these items, and the various colors represent either the categories of the items or the years of their production. The line thickness connecting the nodes denotes the degree of collaboration or co-citation between items (Wu et al., 2021, 2022).

CiteSpace (version 6.2.4) is another bibliometric tool for citation visualization analysis (Synnestvedt et al., 2005). In our research, we utilized CiteSpace to examine journal coverage through a dual map approach and to analyze citations via Citation Bursts. With CiteSpace's ability to identify and track the growth and shift of information, we can gain an intuitive understanding of cutting-edge developments, hotspots, and trends in these areas (Chen, 2004).

We employed the R package "bibliometrix" (version 4.1.3) from https://www.bibliometrix.org to analyze topic evolution and create a comprehensive publication network concerning gut microbiota in liver cirrhosis (Aria and Cuccurullo, 2017). The Journal Citation Reports 2022 was referenced to ascertain the journal's impact factor (IF) and quartile. Furthermore, Microsoft Office Excel 2021 facilitated

the analysis of the most prolific and highly cited countries/regions, journals, authors, institutions, co-cited references, and keywords throughout the articles.

3 Results

3.1 Quantitative analysis of annual publications

Based on our search criteria, 3,109 studies related to gut microbiota in liver cirrhosis were published in the past 23 years, comprising 2,004 articles and 1,105 reviews (Figure 1). Analyzing the annual publication growth rate and overall trend, we discerned two distinct phases: the initial phase (2001–2009) and the subsequent phase (2010–2023). As depicted in Figure 2, the initial phase, considered the research's nascent stage, saw fewer than 50 publications annually, averaging 30.1 papers each year. In contrast, the subsequent phase recorded an average of 195.9 publications per year. In 2020, the number of publications was 328, which was 1.3 times more than that in 2019. In 2022, the average annual number of publications was approximately 435, and 291 papers have been published to date in 2023. Since 2017, the number of papers published on gut microbiota in relation to liver cirrhosis has increased substantially, particularly over the past 5 years.

3.2 Countries/regions and institution analyses

The study of gut microbiota in liver cirrhosis has seen contributions from a total of 96 nations and regions, involving 3,437 institutions. The geographical breakdown of the top 10 nations was seen throughout Europe, Asia, and North America, with a predominant presence in Europe ($n = 5$) and Asia ($n = 3$) (Table 1). In this study, it was observed that the United States had the greatest number of published papers ($n = 821$, 19.68%) among the selected nations. China followed closely with a total of 726 papers (17.4%), while Italy and Germany had 296 (7.09%) and 206 (4.94%) articles, respectively. China and the United States together contribute roughly

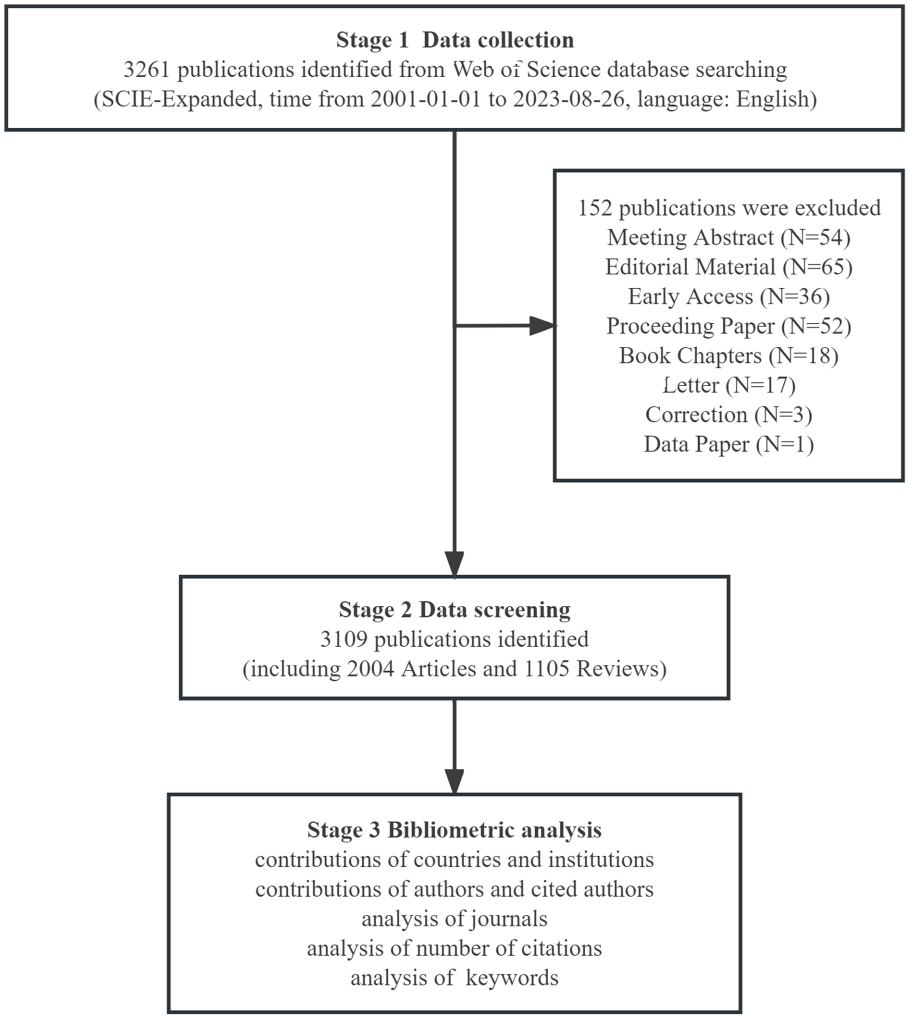
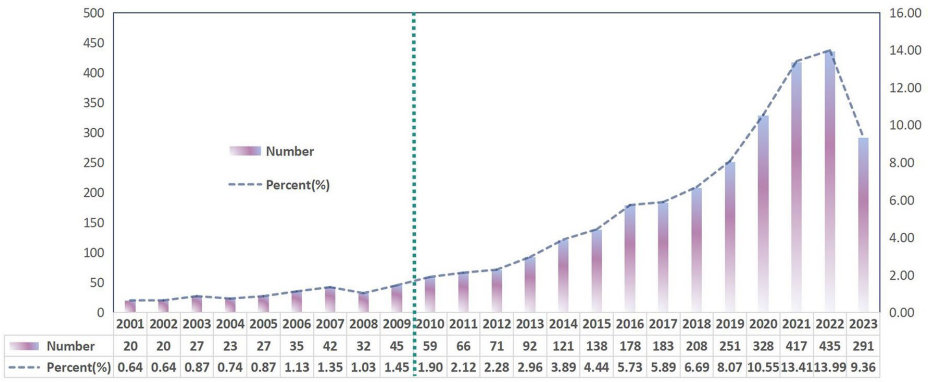


FIGURE 1
Flowchart of the publication selection.



	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023
Number	20	20	27	23	27	35	42	32	45	59	66	71	92	121	138	178	183	208	251	328	417	435	291
Percent(%)	0.64	0.64	0.87	0.74	0.87	1.13	1.35	1.03	1.45	1.90	2.12	2.28	2.96	3.89	4.44	5.73	5.89	6.69	8.07	10.55	13.41	13.99	9.36

FIGURE 2
Annual number and trends of publications on gut microbiota in liver cirrhosis.

TABLE 1 Leading 10 countries on research of gut microbiota in cirrhosis.

Rank	Country	Counts
1	The United States (North America)	821 (19.68%)
2	China (Asia)	726 (17.40%)
3	Italy (Europe)	296 (7.09%)
4	Germany (Europe)	206 (4.94%)
5	Japan (Asia)	206 (4.94%)
6	England (Europe)	201 (4.82%)
7	Spain (Europe)	178 (4.27%)
8	India (Asia)	134 (3.21%)
9	France (Europe)	116 (2.78%)
10	Canada (North America)	106 (2.54%)

37 percent of the overall amount. Following this, we conducted a screening process and using visual representation to analyze the data from 96 countries. Specifically, we focused on countries with a minimum of 5 publications, narrowing our analysis to a subset of 49 countries. This network is shown in Figures 3A,B. It is noteworthy to acknowledge the existence of several ongoing partnerships across various nations. For instance, while China has established partnerships with multiple countries, its strongest collaboration is with the United States. Similarly, the United States actively collaborates with countries including China, Germany, Spain, Canada, Japan, France, England, and Italy.

Table 2 reveals that among the top 10 institutions, three countries are represented. Notably, four of the top five institutions are in the United States, underscoring its dominant contribution to research in the realm of gut microbiota and liver cirrhosis. The four institutions that have published above 50 papers are: University of California San Diego ($n = 111$, 2.63%), Virginia Commonwealth University ($n = 105$, 2.49%), Zhejiang University ($n = 86$, 2.04%), and George Mason University ($n = 52$, 1.23%). Following this, we conducted a visual examination of 3,437 establishments, using a criterion of at least 20 publications as a threshold. This process yielded a final count of 37 institutions. Next, we constructed a collaboration network based on publication counts and inter-institutional ties. Figure 3C highlights that the University of California San Diego and Virginia Commonwealth University collaborate extensively with many other institutions, such as George Mason University, McGuire VA Medical Center and Virginia Commonwealth University, which work very closely with each other. Notably, the VA San Diego Healthcare System and the University of California San Diego also have a strong collaborative bond. In China, Shanghai University of Traditional Chinese Medicine, Fudan University and Shanghai Jiao Tong University are notably active in mutual cooperation. In addition, we also found that Zhejiang University began researching the field of gut microbiota in liver cirrhosis relatively early among several institutions with the highest publication volume in China. However, although Zhejiang University has published a lot of papers, it has seldom established close cooperation with other institutions.

3.3 Journals and co-cited journals

A survey of journals and co-cited journals reveals that research articles on gut microbiota in liver cirrhosis spanned 825 academic journals. Notably, six journals each published over 50 articles on the topic. The *World Journal of Gastroenterology* led the list with 98 articles, followed closely by *Hepatology* and *Journal of Hepatology*, both with 81 articles, then the *International Journal of Molecular Sciences* ($n = 77$), *Liver International* ($n = 59$). Among the leading 15 journals, the *Journal of Hepatology* boasted the highest impact factor (IF) at 25.7, followed by *Hepatology* at 13.5. Figure 4 depicts the journal network constructed by selecting 41 journals with a minimum of 15 pertinent publications each. The Figure 4A illustrates active collaboration among these journals. The *Journal of Hepatology*, *World Journal of Gastroenterology*, *Hepatology* and *International Journal of Molecular Science* show a strong co-citation relationship respectively, constituting a quadrangular network. Additionally, *World Journal of Gastroenterology*, *International Journal of Molecular Science* and *Nutrients* has a positive co-citation relationship with each other.

Table 3 indicates that each of the top 15 most co-cited journals amassed over 1,500 citations, with three journals garnering more than 5,000 citations. *Hepatology* stands out as the most cited journal, with a total of 13,375 co-citations, highlighting its significant influence. This is trailed by the *Journal of Hepatology* (11,300 co-citations), *Gastroenterology* (5,181 co-citations), and *Nature Reviews Gastroenterology & Hepatology* (4,787 co-citations). Impressively, all

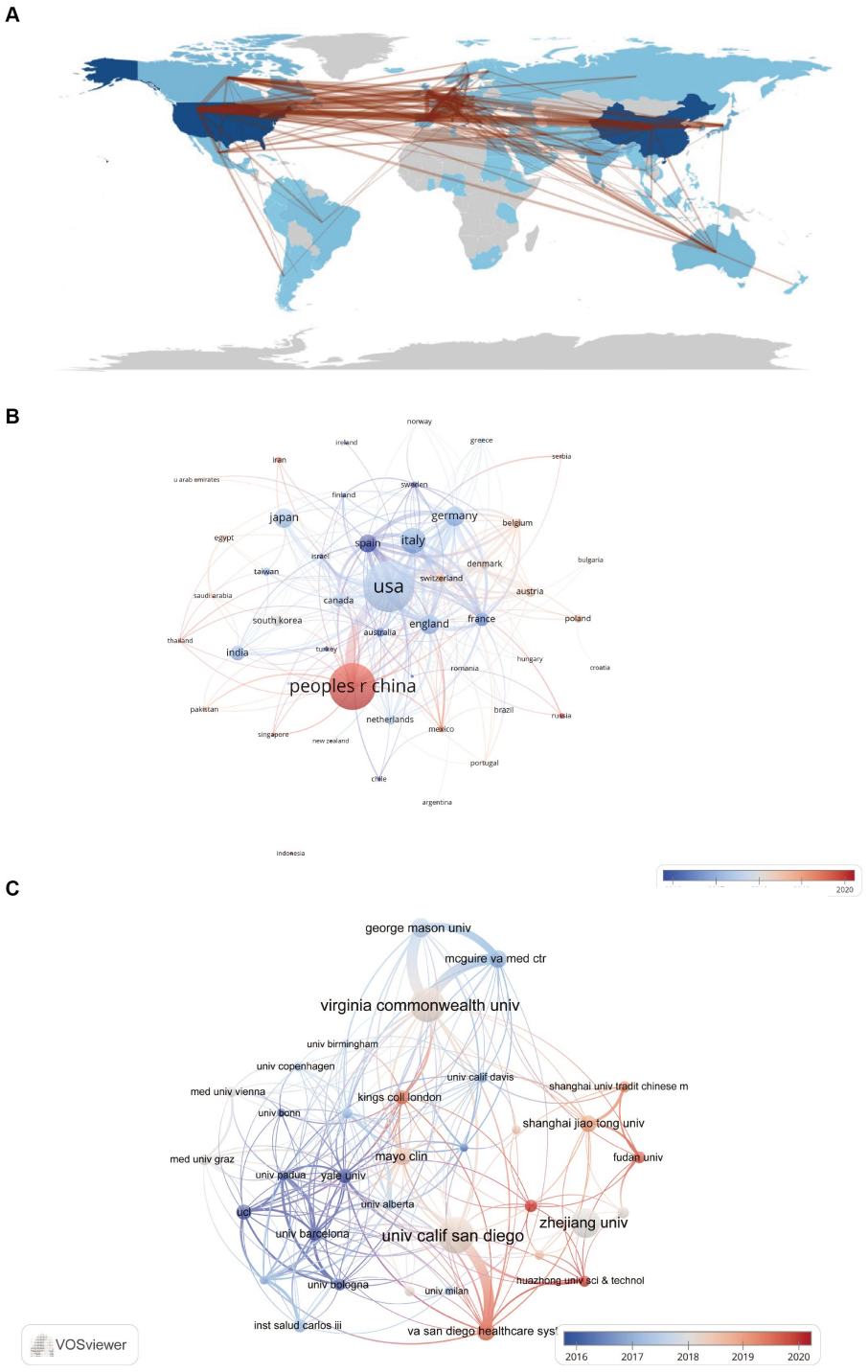


FIGURE 3
Visualization of countries/regions and institution contribution. **(A)** The geographical distribution of countries on research of gut microbiota in cirrhosis. **(B)** Visual depiction of countries involved in gut microbiota in cirrhosis studies. **(C)** Visual presentation of institutions on the research of gut microbiota in the context of cirrhosis.

the top 15 co-cited journals fall within either the first (Q1) or the second (Q2) quartiles of the journal ranking system. Of particular note, *Nature Reviews Gastroenterology & Hepatology* boasts the highest impact factor, with an IF of 65.1, succeeded by *Gastroenterology* (IF = 29.4) and *Journal of Hepatology* (IF = 25.7). The frequency with which a journal is cited serves as a significant metric for assessing its level of impact. In this research, we identified a total of 9,178 co-cited journals. After applying a screening process based on a minimum co-citation threshold of 250, a subset of 129 co-cited journals were selected to construct the co-citation network (Figure 4B). As depicted in Figure 4B, *Hepatology* shares a wide range of co-citation relationships with many journals such as *Journal of Hepatology*,

TABLE 2 Leading 10 institutions on research of gut microbiota in cirrhosis.

Rank	Institution	Counts
1	University of California San Diego (The United States)	111 (2.63%)
2	Virginia Commonwealth University (The United States)	105 (2.49%)
3	Zhejiang University (China)	86 (2.04%)
4	George Mason University (The United States)	52 (1.23%)
5	Mayo Clinic (The United States)	49 (1.16%)
6	McGuire VA Medical Center (The United States)	47 (1.11%)
7	VA San Diego Healthcare System (The United States)	46 (1.09%)
8	Shanghai Jiao Tong University (China)	44 (1.04%)
9	University College London (England)	39 (0.92%)
10	Yale University (The United States)	37 (0.88%)

Gastroenterology, *World Journal of Gastroenterology*, *Liver International*, *Clinical Gastroenterology* and *Hepatology*. In addition, it's worth noting that three important journals including *Hepatology*, *Journal of Hepatology* and *Gastroenterology* constitute a remarkable co-citation network with each other.

Figure 4C provides a dual-map overlay, showcasing the citation connections between journals and their co-cited counterparts. This overlay illustrates the subject distribution of the journals related to gut microbiota in liver cirrhosis, allowing us to rapidly comprehend the frontiers and hotspots of each discipline and the flow of knowledge between disciplines (Shen et al., 2022). The right side of the diagram displays the clusters of journals that are referenced, while the left side indicates the clusters of journals that often cite other sources. The orange and green pathways illustrate dominant citation trends, suggesting that research from the Molecular/Biology/Genetics and Health/Nursing/Medicine domains predominantly receives citations from literature in the Molecular/Biology/Immunology and Medicine/Medical/Clinical sectors.

3.4 Authors and co-cited authors

There are 15,965 authors contributing to the research. Table 4 summarizes the top 15 authors and co-citation authors according to the number of published papers and co-citation times, among which the top 15 authors all published at least 20 articles. Bajaj Jasmohan S. and Schnabl Bernd published the most papers, 82 and 66, respectively (Table 4), indicating that they have contributed to leading achievements in this field. We established a collaborative network comprised of authors who published at least 5 papers. Ultimately, 258 authors are finally shown in the picture (Figure 5A). Bajaj Jasmohan S., Schnabl Bernd, Gillevet Patrick M., Sikaroodi Masoumeh, and Lanjuan Li are represented by the largest nodes due to their most publications in this area. Furthermore, we observed intense cooperation between numerous authors. Bajaj Jasmohan S. closely collaborated with Gillevet Patrick M., Sikaroodi Masoumeh, and Fagan Andrew. Similarly, Schnabl Bernd worked closely with Lirui Wang, Fouts Derrick E., and Yi Duan. Lanjuan Li closely collaborated with Haifeng Lu and Yanfei Chen. However, it's noteworthy that despite the substantial publications of Bajaj Jasmohan S., Schnabl Bernd, and Lanjuan Li, their direct and indirect collaboration with each other remains limited.

A total of 66,339 co-cited authors were identified. After applying a screening criterion of having published 29 or more articles, a subset of 1,001 authors remained. Among these authors, it was observed that four specific authors were co-cited more than 500 occasions (Table 4). Bajaj Jasmohan S. is the most co-cited author ($n = 2,838$), demonstrating his significant impact on the research field and his essential role in promoting the field's ongoing development. The following three were Younossi Zobair M. ($n = 583$), Wiest Reiner ($n = 580$) and Fernandez Javier ($n = 534$). The entire network of co-citations was depicted (Figure 5B). As observed in Figure 5B, active collaborations exist among the various co-cited authors.

3.5 Co-cited references

In the preceding time period, an overall total of 107,643 co-cited references have been documented in the realm of gut microbiota research pertaining to cirrhosis. Each of the co-cited references enumerated in Table 5 has received a minimum of 194 citations, with three of them being co-cited in excess of 300 times. We have chosen references that have been mentioned 130 times or more in order to create a network of co-cited literature. A total of 32 co-cited references were obtained (Figure 6). As depicted in Figure 6, the article "Qin N., 2014, Nature" is an important article with the highest citation frequency and significant influence in this field. This article demonstrates a strong co-citation relationship with "Bajaj J. S., 2014, J. Hepatol.," "Chen Y. F., 2011, Hepatology," and" Kakiyama G., 2013, J. Hepatol. "among others.

3.6 Reference with citation bursts

The term "Reference with Citation Bursts" refers to scholarly papers that have been extensively cited within a specific timeframe. In the present investigation, a total of 25 references exhibiting citation bursts were found using the CiteSpace software (Figure 7). As seen in Figure 7, each bar corresponds to a single year, with the red bar indicating a period of significant citation burst (Huang et al., 2019). The earliest citation bursts for references appeared in 2010 and the latest in 2021. The article titled "Alterations of the human gut microbiome in liver cirrhosis" by Qin et al., published in Nature, had the highest citation strength (strength = 59.76) among the references.

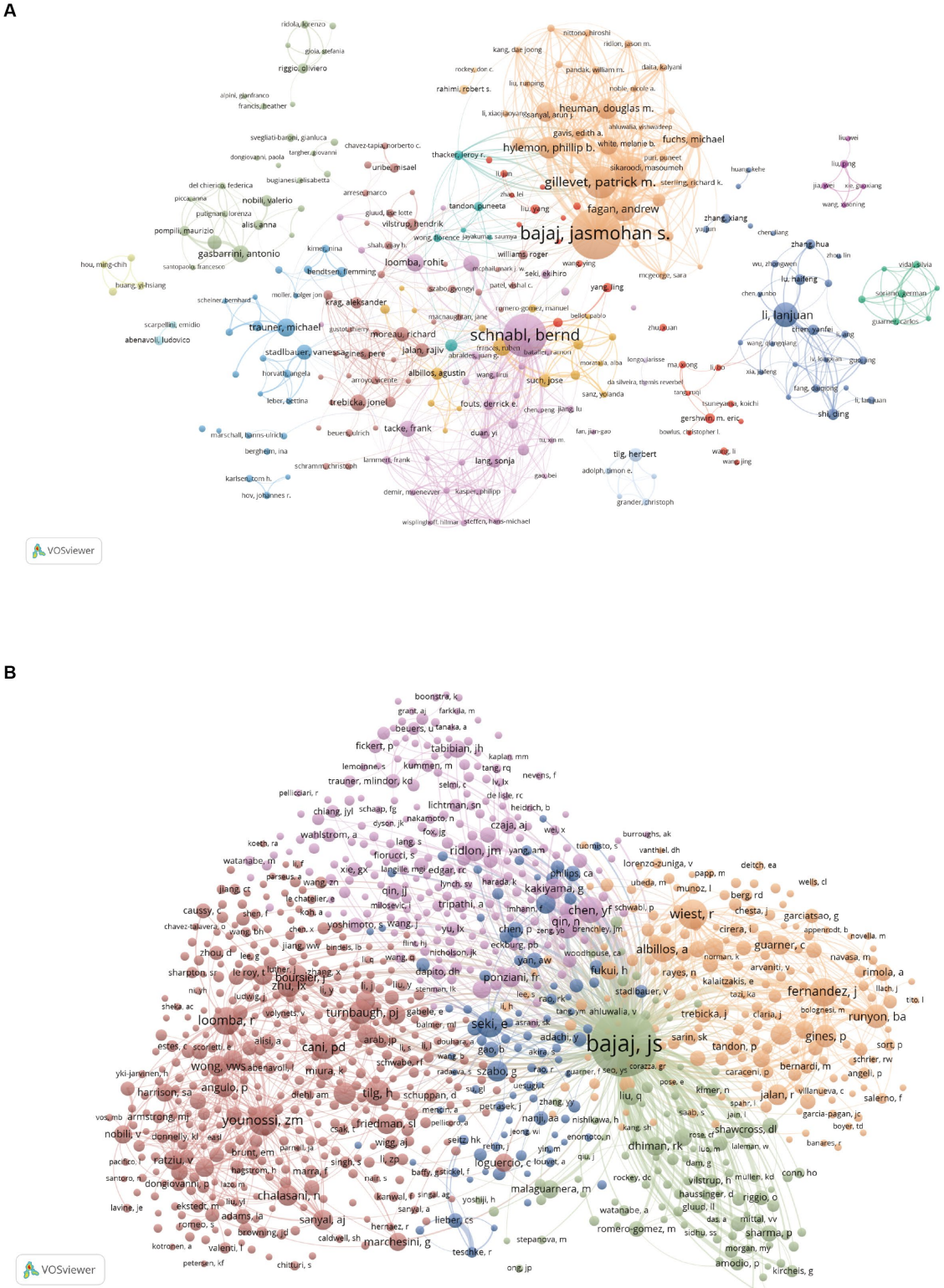


FIGURE 4
Visualization of authors and co-cited authors. **(A)** Visual representation of authors on the research of gut microbiota in the context of cirrhosis. **(B)** Visual presentation of co-cited authors on the research of gut microbiota in the context of cirrhosis.

Notably, this publication saw a significant increase in citations between 2015 and 2019. The article titled "Altered profile of human gut microbiome is associated with cirrhosis and its complications" published in the Journal of Hepatology in 2014 by Bajaj JS et al. had the second highest citation burst strength (53.38). The citation burst period for this article extended from 2015 to 2019. The three

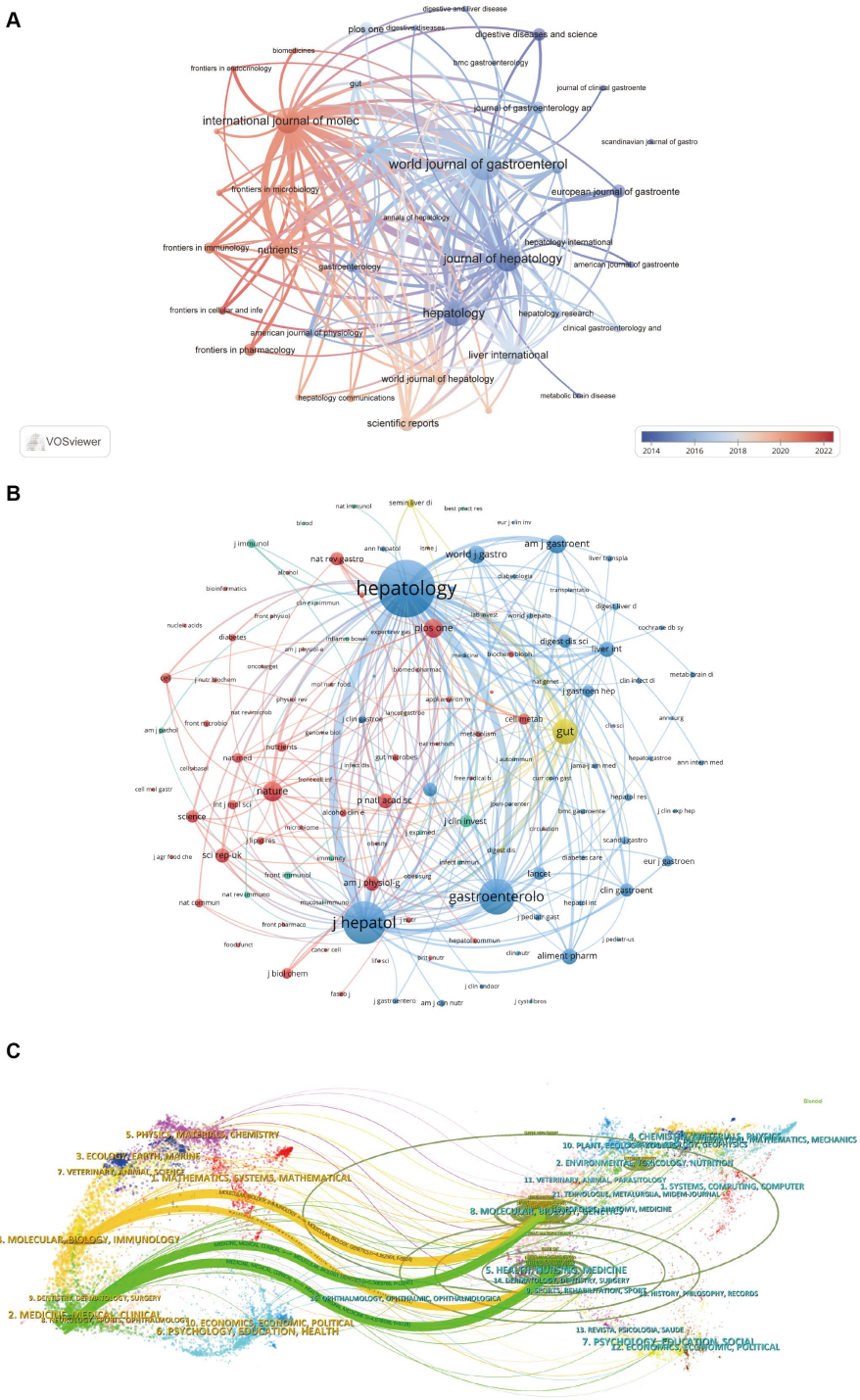


FIGURE 5
Visualization of journals and co-cited journals involvement. **(A)** Visual presentation of journals conducting research on gut microbiota in cirrhosis. **(B)** Visual presentation of co-citations among scholarly journals f on the research of gut microbiota in the context of cirrhosis. **(C)** The dual-map overlay of journals on research of gut microbiota in cirrhosis.

references, namely "The gut-liver axis in liver disease: pathophysiological basis for therapy," "Gut microbiota and human NAFLD: disentangling microbial signatures from metabolic disorders," and "Gut Microbiome-Based Metagenomic Signature for Non-invasive Detection of Advanced Fibrosis in Human Nonalcoholic Fatty Liver Disease," all continue to be a subject of significant research interest in 2023. In general, the set of 25 references exhibits a burst strength range spanning from 19.76 to 59.76, with an endurance strength varying between 2 and 5 years. Table 6 provides a comprehensive summary of the primary research topics discussed in the 25 references, arranged according to the literature order shown in Figure 7.

TABLE 3 The leading 15 journals and co-cited journals for research of gut microbiota in cirrhosis.

Rank	Journal	Count	IF	JCR	Co-cited journal	Co-citation	IF	JCR
1	World Journal of Gastroenterology	98	4.3	Q2	Hepatology	13,375	13.5	Q1
2	Hepatology	81	13.5	Q1	Journal of Hepatology	11,300	25.7	Q1
3	Journal of Hepatology	81	25.7	Q1	Gastroenterology	5,181	29.4	Q1
4	International Journal of Molecular Sciences	77	5.6	Q1	Nature reviews Gastroenterology & Hepatology	4,787	65.1	Q1
5	Liver International	59	6.7	Q2	World Journal of Gastroenterology	4,196	4.3	Q2
6	Nutrients	57	5.9	Q1	Gut	3,789	24.5	Q1
7	Scientific Reports	48	4.6	Q1	American Journal of Physiology-Gastrointestinal and Liver Physiology	2,956	4.5	Q1
8	World Journal of Hepatology	42	2.4	None	Alimentary Pharmacology & Therapeutics	1993	7.6	Q1
9	European Journal of Gastroenterology & Hepatology	40	2.1	Q4	Metabolism-Clinical and Experimental	1826	9.8	Q1
10	PLoS One	40	3.7	Q2	International Journal of Molecular Sciences	1819	5.6	Q1
11	Digestive Diseases and Sciences	39	3.1	Q3	American Journal of Gastroenterology	1808	9.8	Q1
12	Journal of Gastroenterology and Hepatology	37	4.1	Q2	Journal of Gastroenterology and Hepatology	1749	4.1	Q2
13	Frontiers in Pharmacology	36	5.6	Q1	PLoS One	1,616	3.7	Q2
14	Alimentary Pharmacology & Therapeutics	33	7.6	Q1	Liver International	1,611	6.7	Q2
15	Frontiers in Immunology	33	7.3	Q1	Clinical Gastroenterology and Hepatology	1,531	12.6	Q1

TABLE 4 The leading 15 authors and co-cited authors on research of gut microbiota in cirrhosis.

Rank	Authors	Count	Co-cited authors	Citations
1	Bajaj, Jasmohan S.	82	Bajaj, Jasmohan S.	2,838
2	Schnabl, Bernd	66	Younossi, Zobair M.	583
3	Gillevet, Patrick M.	46	Wiest, Reiner	580
4	Sikaroodi, Masoumeh	45	Fernández, Javier	534
5	Lanjuan Li	32	Loomba, Rohit	495
6	Fagan, Andrew	31	Seki, Ekihiro	461
7	Hylemon, Phillip B.	28	Yanfei Chen	454
8	Heuman, Douglas M.	24	Tilg, Herbert	448
9	Sanyal, Arun J.	23	Albillos, Agustín	438
10	White, Melanie B.	21	Cani, Patrice D.	407
11	Trauner, Michael	21	Nan Qin	384
12	Frances, Ruben	21	Turnbaugh, Peter J.	364
13	Yoshiji, Hitoshi	21	Runyon, Bruce A.	359
14	Suk, Ki Tae	21	Lixin Zhu	340
15	Loomba, Rohit	20	Boursier, Jérôme	325

TABLE 5 The leading 10 co-cited references contributing to the research of gut microbiota in cirrhosis.

Rank	Co-cited reference	Citations
1	Shen et al. (2014)	379
2	Bajaj et al. (2014b)	371
3	Chen et al. (2011)	335
4	Zhu et al. (2013)	298
5	Miele et al. (2009)	264
6	Boursier et al. (2016)	257
7	Loomba et al. (2017)	209
8	Henao-Mejia et al. (2012)	205
9	Kakiyama et al. (2013)	198
10	Bass et al. (2010)	194

3.7 Keywords co-occurrence analysis

The use of keyword co-occurrence analysis enables the expeditious identification of prominent areas of study focus within a certain academic domain. This analysis can also highlight the most essential topics and forecast potential future research directions. Table 7 displays the top 20 high-frequency keywords. Among them, cirrhosis, gut microbiota, and liver cirrhosis each occurred more than 200 times, representing the main research directions of liver cirrhosis-related gut microbiota.

As shown in Figure 8A, the keywords with the highest frequencies are non-alcoholic fatty liver disease (NAFLD) (446 times), cirrhosis (404 times), and gut microbiota (339 times). A total of five clusters were found, each reflecting a distinct study path. Additionally, the evolution of keywords over time is shown in the overlay representation

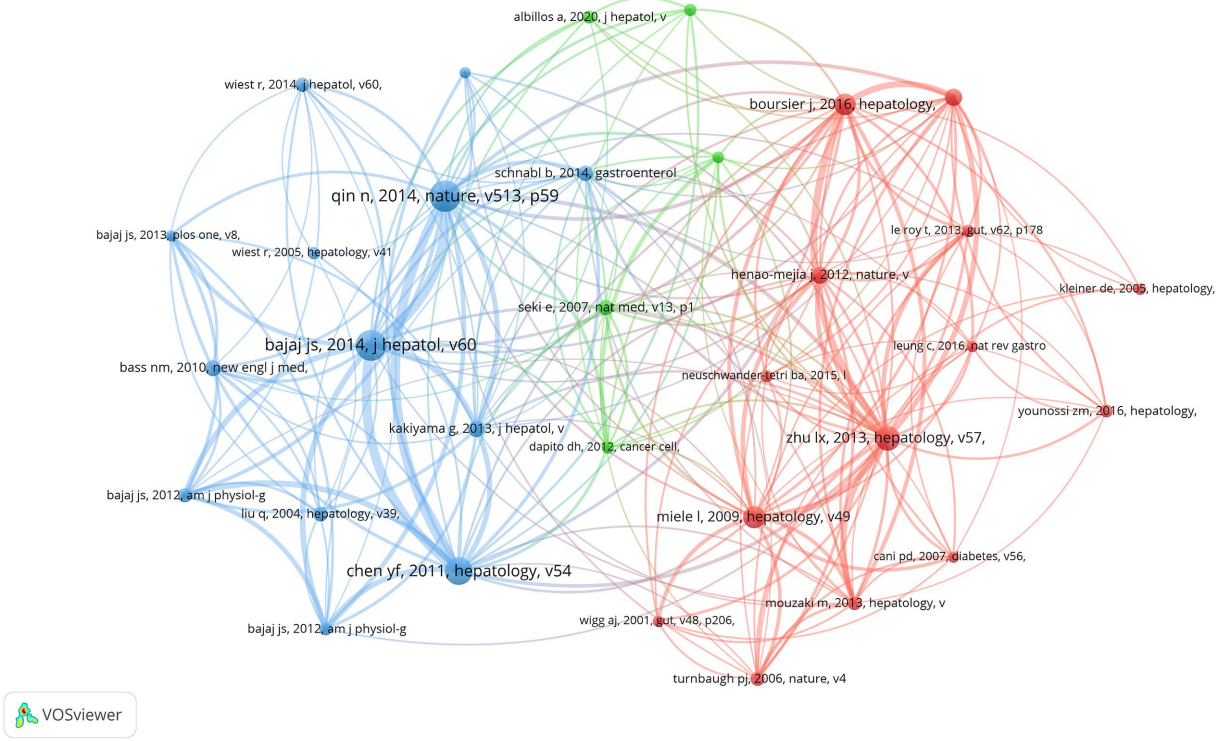
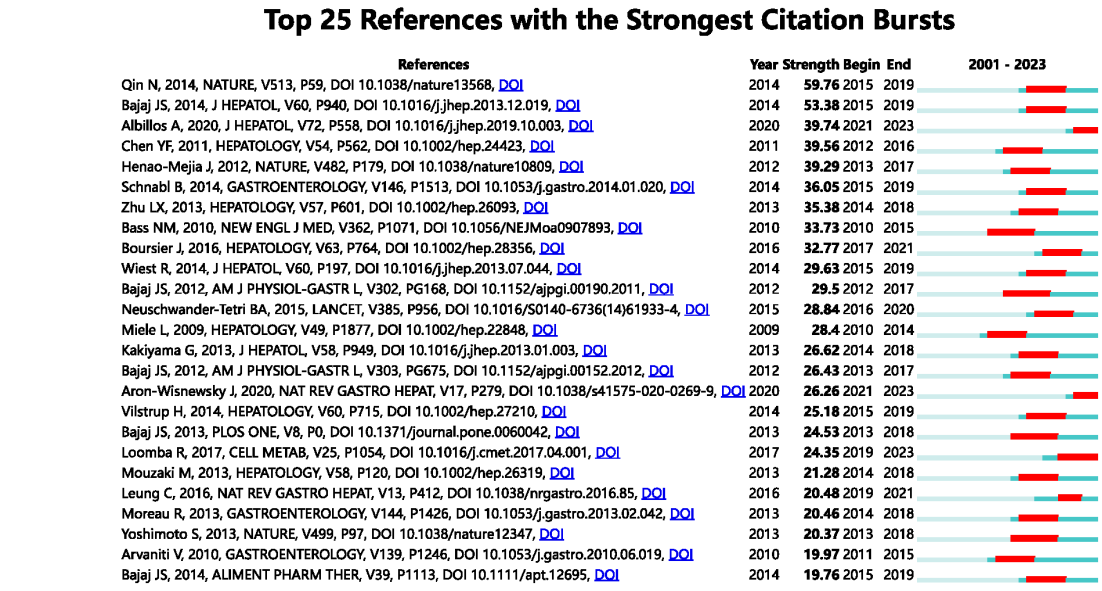


FIGURE 6
Visual presentation of references among authors on the research of gut microbiota in the context of cirrhosis.



Top 25 References with the Strongest Citation Bursts

References	Year	Strength	Begin	End	2001 - 2023
Qin N, 2014, NATURE, V513, P59, DOI 10.1038/nature13568, DOI	2014	59.76	2015	2019	
Bajaj JS, 2014, J HEPATOL, V60, P940, DOI 10.1016/j.jhep.2013.12.019, DOI	2014	53.38	2015	2019	
Albillos A, 2020, J HEPATOL, V72, P558, DOI 10.1016/j.jhep.2019.10.003, DOI	2020	39.74	2021	2023	
Chen YF, 2011, HEPATOLOGY, V54, P562, DOI 10.1002/hep.24423, DOI	2011	39.56	2012	2016	
Henao-Mejia J, 2012, NATURE, V482, P179, DOI 10.1038/nature10809, DOI	2012	39.29	2013	2017	
Schnabl B, 2014, GASTROENTEROLOGY, V146, P1513, DOI 10.1053/j.gastro.2014.01.020, DOI	2014	36.05	2015	2019	
Zhu LX, 2013, HEPATOLOGY, V57, P601, DOI 10.1002/hep.26093, DOI	2013	35.38	2014	2018	
Bass NM, 2010, NEW ENGL J MED, V362, P1071, DOI 10.1056/NEJMoa0907893, DOI	2010	33.73	2010	2015	
Boursier J, 2016, HEPATOLOGY, V63, P764, DOI 10.1002/hep.28356, DOI	2016	32.77	2017	2021	
Wiest R, 2014, J HEPATOL, V60, P197, DOI 10.1016/j.jhep.2013.07.044, DOI	2014	29.63	2015	2019	
Bajaj JS, 2012, AM J PHYSIOL-GASTR L, V302, PG168, DOI 10.1152/ajpgi.00190.2011, DOI	2012	29.5	2012	2017	
Neuschwander-Tetri BA, 2015, LANCET, V385, P956, DOI 10.1016/S0140-6736(14)61933-4, DOI	2015	28.84	2016	2020	
Miele L, 2009, HEPATOLOGY, V49, P1877, DOI 10.1002/hep.22848, DOI	2009	28.4	2010	2014	
Kakiyama G, 2013, J HEPATOL, V58, P949, DOI 10.1016/j.jhep.2013.01.003, DOI	2013	26.62	2014	2018	
Bajaj JS, 2012, AM J PHYSIOL-GASTR L, V303, PG675, DOI 10.1152/ajpgi.00152.2012, DOI	2012	26.43	2013	2017	
Aron-Wisnewsky J, 2020, NAT REV GASTRO HEPAT, V17, P279, DOI 10.1038/s41575-020-0269-9, DOI	2020	26.26	2021	2023	
Vilstrup H, 2014, HEPATOLOGY, V60, P715, DOI 10.1002/hep.27210, DOI	2014	25.18	2015	2019	
Bajaj JS, 2013, PLOS ONE, V8, P0, DOI 10.1371/journal.pone.0060042, DOI	2013	24.53	2013	2018	
Loomba R, 2017, CELL METAB, V25, P1054, DOI 10.1016/j.cmet.2017.04.001, DOI	2017	24.35	2019	2023	
Mouzaki M, 2013, HEPATOLOGY, V58, P120, DOI 10.1002/hep.26319, DOI	2013	21.28	2014	2018	
Leung C, 2016, NAT REV GASTRO HEPAT, V13, P412, DOI 10.1038/nrgastro.2016.85, DOI	2016	20.48	2019	2021	
Moreau R, 2013, GASTROENTEROLOGY, V144, P1426, DOI 10.1053/j.gastro.2013.02.042, DOI	2013	20.46	2014	2018	
Yoshimoto S, 2013, NATURE, V499, P97, DOI 10.1038/nature12347, DOI	2013	20.37	2013	2018	
Arvaniti V, 2010, GASTROENTEROLOGY, V139, P1246, DOI 10.1053/j.gastro.2010.06.019, DOI	2010	19.97	2011	2015	
Bajaj JS, 2014, ALIMENT PHARM THER, V39, P1113, DOI 10.1111/apt.12695, DOI	2014	19.76	2015	2019	

FIGURE 7
The leading 25 references with citation bursts are depicted. A red bar signifies an elevated count of citations for that specific year.

depicted in Figure 8B. Previous keywords are shown in purple, while red represents the most recent keywords. TLR, bacterial translocation, lactulose, endotoxin, intestinal permeability, and cytokines were the main themes of early studies. In contrast, we can see that red frames are popular keywords in recent years, which include gut-liver axis, metabolomics, short-chain fatty acids (SCFAs), bile acids and FMT. The results of these analyses can help researchers speculate on future research directions and progress in the field of gut microbiota in liver cirrhosis.

Trend topic Analysis of the keywords (Figure 8C) shows that as early as 2005, the main keywords are represented by selective intestinal decontamination, transit, norfloxacin. This indicates that researchers

TABLE 6 The primary scientific discoveries of the 25 references with strong citations bursts.

Rank	Strength	Main research content
1	59.76	Identified alterations of the human gastrointestinal microbiome in liver cirrhosis (Shen et al., 2014)
2	53.38	Cirrhosis is accompanied by progressive alterations in the gastrointestinal microbiome, which become more severe during decompensation (Bajaj et al., 2014b)
3	39.74	The gut-liver axis is the pathophysiological premise for gut-centered therapy in liver disease (Albillos et al., 2020)
4	39.56	Patients with cirrhosis have different fecal microbial communities compared to healthy individuals (Chen et al., 2011)
5	39.29	Dysbiosis mediated by inflammasomes controls the progression of NAFLD and adiposity (Henao-Mejia et al., 2012)
6	36.05	The combination of liver injury and intestinal dysbiosis contributes to the development of liver disease (Schnabl and Brenner, 2014)
7	35.38	Nonalcoholic steatohepatitis patients' gut microbiomes were characterized (Zhu et al., 2013)
8	33.73	Rifaximin substantially decreased the risk of hepatic encephalopathy in cirrhotic patients (Bass et al., 2010)
9	32.77	Gut dysbiosis and a change in the gut microbiota's metabolic function are associated with the severity of nonalcoholic fatty liver disease (Boursier et al., 2016)
10	29.63	Pathological bacterial translocation in liver cirrhosis (Wiest et al., 2014)
11	29.5	The gut microbiome is dramatically altered in patients with hepatic encephalopathy and is associated with cognition (Bajaj et al., 2012b)
12	28.84	This study investigates the effectiveness of obeticholic acid in treating non-alcoholic steatohepatitis in adult patients (Neuschwander-Tetri et al., 2015)
13	28.4	The disruption of intercellular tight junctions in the intestines may lead to an increase in intestinal permeability, which may have a substantial effect on the development of hepatic fat deposition (Miele et al., 2009)
14	26.62	The gut microbiota's influence on the fecal bile acid composition in individuals with cirrhosis (Kakiyama et al., 2013)
15	26.43	In cirrhosis and hepatic encephalopathy, the colonic mucosal microbiome is distinct from the stool microbiome and is associated with cognition and inflammation (Bajaj et al., 2012a)
16	26.26	A comprehensive analysis of microbiome characteristics for human NAFLD (Aron-Wisnewsky et al., 2020)
17	25.18	Practice guidelines for hepatic encephalopathy published in 2014 (Vilstrup et al., 2014)
18	24.53	Rifaximin modulates the metabiome in patients with cirrhosis and mild hepatic encephalopathy (Bajaj et al., 2013)
19	24.35	A microbiome panel based on the metagenome can accurately diagnose advanced fibrosis (Loomba et al., 2017)
20	21.28	NAFLD development may be influenced by intestinal microbiota (Mouzaki et al., 2013)
21	20.48	The central role of the microbiome in NAFLD (Leung et al., 2016)
22	20.46	Identified the diagnostic criteria of acute-on-chronic liver failure and described the development of this syndrome in European patients with acute decompensation (Moreau et al., 2013)
23	20.37	Through the senescence secretome, an obesity-induced intestinal microbial metabolite boosts liver cancer (Yoshimoto et al., 2013)
24	19.97	Infections quadruple the mortality rate in cirrhotic patients (Arvaniti et al., 2010)
25	19.76	The impact of *Lactobacillus* GG on the gut microbiota, metabolome, and endotoxemia in individuals diagnosed with cirrhosis is being investigated (Bajaj et al., 2014a)

began to study intestinal decontamination therapy and the role of intestinal bacteria in liver cirrhosis and its related complications. The main keywords between 2005 and 2015 were lactulose, bacterial translocation, endotoxin, portal-systemic encephalopathy, ascites. There is no doubt that researchers have begun to actively explore the treatment of liver cirrhosis by regulating intestinal bacteria in order to reduce the incidence of related complications. Until 2018, focuses were shifted towards dysbiosis, gut microbiota, intestinal barrier, mechanisms and so on, which indicates that the aim of researches has changed from simple sterilization towards improving the balance of gut microbiota. In 2023, complement-system is likely to exhibit research potential in the future.

Of note, although bacterial taxon is a critical topic in the study of gut microbiota, the number of keywords occurrence in this topic seems insufficient for visualization. As a result, the routine co-occurrence analysis (Figure 8A) is technically impossible to specifically demonstrate keywords related to bacterial taxa. To reveal the trend and pattern of this topic, we manually pick up related keywords from the whole keywords pool, demonstrating an independent overlay visualization of co-occurring keywords related to bacterial taxa over time (Figure 8D). Table 7 shows the leading 10 keywords related to bacterial taxa. *Lactobacillus* (49 times), *Escherichia coli* (43 times) and *Helicobacter pylori* (32 times) are the most frequent keywords. In addition, *Lactobacillus* and *Escherichia coli* show a wide correlation with other keywords. These bacterial taxa have gained major concern and exhibited critical value in the research area of liver cirrhosis.

4 Discussion

The notion of the gut-liver axis, which emerged some decades ago, has shed light on the significant involvement of gut microbiota

TABLE 7 Leading 20 keywords in the research on the gut microbiota in relation to cirrhosis and 10 keywords related to bacterial taxa.

Rank	Keyword	Counts	Total link strength
1	Non-alcoholic fatty liver disease	446	950
2	Cirrhosis	404	832
3	Gut microbiota	339	645
4	Liver cirrhosis	264	439
5	Non-alcoholic steatohepatitis	252	599
6	Probiotics	195	518
7	Inflammation	191	476
8	Microbiome	179	434
9	Hepatic encephalopathy	177	374
10	Microbiota	168	421
11	Bacterial translocation	142	336
12	Gut-liver axis	120	337
13	Fibrosis	118	284
14	Liver fibrosis	118	188
15	Dysbiosis	114	310
16	Hepatocellular carcinoma	107	246
17	Liver disease	106	190
18	Portal hypertension	95	209
19	Bile acids	91	218
20	Gut microbiome	84	188

Rank	Keyword (taxa)	Counts	Total link strength
1	*Lactobacillus*	49	22
2	*Escherichia coli*	43	17
3	*Helicobacter pylori*	32	7
4	*Akkermansia muciniphila*	31	8
5	*Clostridium difficile*	20	5
6	*Clostridium difficile* infection	20	5
7	*Helicobacter pylori* infection	15	5
8	*Bifidobacterium*	14	12
9	*Lactobacillus acidophilus*	10	3

in chronic liver illnesses. This revelation has opened up new avenues for investigating prospective treatment strategies towards different liver diseases such as alcoholic liver disease (Duan et al., 2019), NAFLD (Lei et al., 2022) and liver cancer (Ma et al., 2018). Fruitful works have indicated a wide range of novel insights into the gut microbiota in liver cirrhosis (Trebicka et al., 2021a,b). We utilized the WoSCC database to analyze 3,109 articles over the last decades in this field. From 2001 to 2009, the annual number of published papers was less than 50 and experienced a slow but steady development, manifesting that the research on the gut microbiota of liver cirrhosis was in its infancy during this period. After 2010, there was a notable surge in the quantity of published articles, with an average yearly figure of 195.9. Furthermore, the current data suggests a continuous upward trend of the ongoing study in this particular domain, indicating great scientific interest and focus.

The results pertaining to country distribution reveal that the United States, China, and Italy are the top three countries for studying gut microbiota in relation to liver cirrhosis, with the United States ranking first. We have observed that the United States cooperates actively with numerous nations, most closely with China. Regarding research institutions, the top 10 are spread across three countries. Remarkably, four-fifths of these are based in the United States, and several of these institutions maintain strong collaborative ties. For instance, George Mason University, McGuire VA Medical Center, and Virginia Commonwealth University work closely together. Three institutions in China collaborate actively, namely Shanghai Jiao Tong University, Fudan University, and Shanghai University of Traditional Chinese Medicine. Additionally, there is also active collaboration between Huazhong University of Science and Technology, the University of California San Diego, and the VA San Diego Healthcare System. Nevertheless, it's worth mentioning that despite Zhejiang

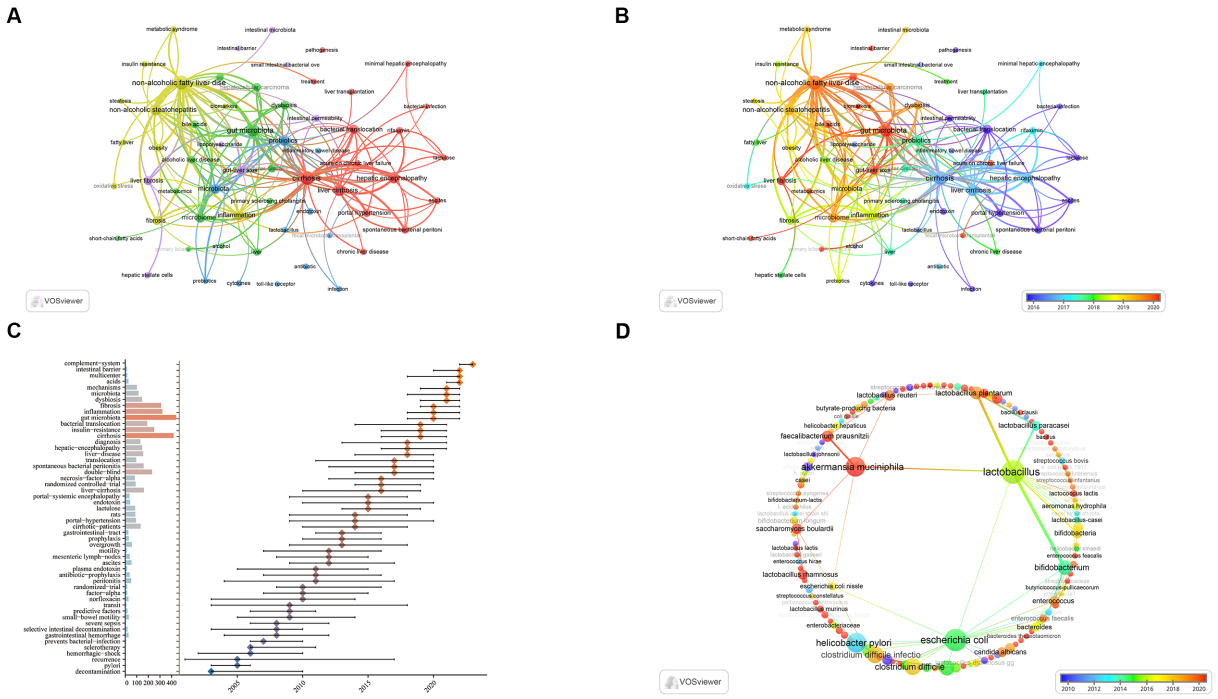


FIGURE 8
Visualization of keywords and trend topics. **(A)** Keyword cluster analysis. **(B)** Overlay visualization analysis revealed the evolution of co-occurring keywords over time. **(C)** The trend topic analysis of the keywords. **(D)** Overlay visualization analysis revealed the evolution of co-occurring keywords related to bacterial taxa over time.

University's significant publication output, its limited collaboration with other universities might potentially impede the sustained progress of academic research. Although several nations have established cooperative partnerships, there is a need for further enhancement in the scope and depth of collaboration among specialized organizations. It is very advisable to foster extensive collaboration and facilitate exchanges among research institutes across different nations in order to collectively advance the academic progress.

The *World Journal of Gastroenterology* leads in publications on gut microbiota in liver cirrhosis, with 98 articles, marking its significance in the field. Of the top 15 journals, *Journal of Hepatology* boasts the highest impact factor (IF = 25.7, Q1), followed by *Hepatology* (IF = 13.5, Q1). Most of the top co-cited journals are renowned Q1 journals, with six possessing an IF above 10, underscoring their pivotal role in the field. Current research primarily appears in Molecular/Biology/Immunology and Medicine/Medical/Clinical journals, citing work from Molecule/Biology/Genetics or Health/Nursing/Medicine realms. This reflects a comprehensive approach to research, bridging basic studies with clinical applications.

Among the top 10 authors in this field, the average number of publications per author exceeds 20 papers, with Bajaj Jasmohan S., Schnabl Bernd, and Gillevet Patrick M. publishing more than 40 papers each. Professor Bajaj Jasmohan S. has published the most papers for 82. Bajaj Jasmohan S. and his team have dedicated themselves to this field and their researches have played a vital role in the advancement of this discipline. The researchers made the observation that the gut microbiome undergoes a gradual transformation as cirrhosis advances. They proposed that the "cirrhosis dysbiosis ratio" offers a significant quantitative metric to delineate changes in the gut microbiome as cirrhosis progresses (Bajaj et al., 2014b). In 2013, they revealed a complex relationship between bile acid levels and the reciprocal regulation of intestinal microbiota in cirrhosis (Ridlon et al., 2013). In the same year, further studies investigated the relationship between the concentration of fecal bile acid and the intestinal microbiome in cirrhotic patients of varying severity. Alterations in fecal bile acid profiles in individuals with cirrhosis are influenced by pivotal microorganisms within the gut microbiota (Kakiyama et al., 2013). Bajaj Jasmohan S. and Gillevet Patrick M., upon analyzing the gut microbiome in patients with liver cirrhosis, identified a correlation between the concentrations of gut microbiota-derived metabolites in the bloodstream and the incidence of acute-on-chronic liver failure (ACLF) as well as mortality rates in these patients. The use of this biomarker has the potential to effectively and promptly identify individuals at a heightened risk of ACLF and mortality (Bajaj et al., 2020). In conclusion, their works provide a cornerstone for related studies which establish solid bridge between the pattern of microbial agents and the progression of liver cirrhosis.

In 2021, they also investigated the influence of antibiotic resistance genes (ARGs) burden in intestinal metagenomes on disease progression and prognosis. They discovered that the intestinal ARGs burden was higher in cirrhotic patients than in controls and worsened with disease progression. Nonetheless, rifaximin has a good regulatory effect on ARGs (Shamsaddini et al., 2021). Bajaj Jasmohan S. has also studied the connection between gut microbiota and inflammatory response, demonstrating that gut microbiota can promote the development of neuroinflammatory and systemic inflammatory responses in cirrhotic mice (Kang et al., 2016; Liu et al., 2020). Later, his research on gut microbiota expanded the understanding of fungi,

revealing the presence of a significant fungal dysbiosis in cirrhotic patients. The ratio of Bacteroidetes to Ascomycota can independently forecast 90 days hospitalization outcomes in patients with cirrhosis (Bajaj et al., 2018b). Furthermore, the authors offered a succinct review of ongoing research exploring the gut microbiota as a possible therapeutic focus on liver illness. This includes the investigation of probiotics, FMT, and precision medicine focused on particular microorganisms (Bajaj et al., 2022). The researchers conducted an experimental study to validate the reduction in abundance of ARGs in the gut microbiota of patients with decompensated cirrhosis after FMT. They further noted that FMT could potentially reverse the damage caused by antibiotics to the constitution and functionality of the gut microbiota in individuals with cirrhosis (Bajaj et al., 2018a, 2021). On the one hand, their recent works put forward the understanding of the relationship between gut microbiota (including fungi) and ARGS as well as inflammation in liver cirrhosis. On the other hand, they also made extensive explore on the therapeutic practice of microbial intervention towards liver cirrhosis.

Among the Chinese authors, Lanjuan Li has published most papers, reaching 32. As early as 2011, she revealed the features of the intestinal microflora in cirrhotic patients (Chen et al., 2011). By 2014, she delved deeper into the changes in gut microbiota in individuals with liver cirrhosis, constructed a gene catalog of the intestinal microbiome, and selected 15 optimal biomarkers with high specificity for distinguishing liver cirrhosis patients from healthy individuals (Shen et al., 2014). Moreover, she also disclosed the involvement of gut microbiota, bile acids, and the T helper cell 17/Interleukin 6 axis in the progression of liver fibrosis associated with hepatitis B (Chen et al., 2020). These studies also made important contribution on the microbial aspects of prognosis and pathogenesis in liver cirrhosis.

The author with the most co-citations is Bajaj Jasmohan S. ($n = 2,838$), followed by Younossi Zobair M. ($n = 583$), Wiest Reiner ($n = 580$), and Fernandez Javier ($n = 538$). The fact that Bajaj Jasmohan S. has the most published articles and is the most cited author is sufficient evidence that he facilitates the development of intestinal microbiota in liver cirrhosis. Bajaj Jasmohan S. is dedicated to studying not only the alterations in intestinal flora in liver cirrhosis, but also the effects of intestinal microbiota-derived metabolites in serum and ARGs in the gut metagenome on the progression and prognosis of liver cirrhosis. Jasmohan S. Bajaj and his team, through their clinical research, have substantiated that FMT can improve the outcomes for patients suffering from liver cirrhosis. Additionally, their investigations expanded into the realm of mycobiome, pinpointing an imbalance in fungal populations within the intestines of patients with cirrhosis.

Younossi Zobair M. has primarily centered his research on investigating advanced fibrosis and cirrhosis in patients diagnosed with NAFLD (Younossi et al., 2018, 2019). Early in 2014, Wiest Reiner summarized the issue of pathological bacterial translocation (PBT) in patients with liver cirrhosis. Liver cirrhosis can significant damage the intestinal endothelial and muco-epithelial barriers, resulting in PBT into the portal vein system. Farnesoid × receptor agonists can decrease PBT (Wiest et al., 2014; Sorribas et al., 2019). These studies have laid the foundation for the future therapies of liver cirrhosis by targeting the intestinal microbiota.

A co-citation relationship is established when multiple papers are jointly cited by one or more subsequent publications. Consequently, co-cited reference is an important method of analysis in bibliometrics research, and citation frequency can reflect an article's influence in a particular research field (Sun et al., 2022). Highly cited papers are typically of high quality, innovative, and make a substantial contribution to their field, which is the basis for promoting the continuous development of the research field. Table 4 displays the 10 most frequently co-cited papers. These studies have been co-cited a minimum of 194 times, with the first three being cited more than 300 times, which have significant impacts on this field. Lanjuan Li and Bajaj JS have each authored two publications that are ranked inside the top 10 most frequently referenced papers. The paper titled "Alterations of the human gut microbiome in liver cirrhosis" authored by Lanjuan Li in 2014, as published in *Nature*, has garnered a total of 379 citations, so establishing itself as the most often referenced work within the domain of liver cirrhosis research. The researchers have disclosed distinct modifications in the gut microbiota among persons with cirrhosis and have found gene clusters that exhibit varying levels of abundance between cirrhotic patients and those who are in good health, as determined using quantitative metagenomic analysis (Shen et al., 2014). In 2011, Lanjuan Li released a work that has been mentioned 335 times, which examined the attributes of the intestinal microbial population in individuals diagnosed with liver cirrhosis. The proliferation of Enterobacteriaceae, Streptococcaceae, and other potentially dangerous bacteria, along with the decline in helpful bacteria like Lachnospiraceae, is expected to have implications for the prognosis of individuals diagnosed with liver cirrhosis. The study titled "Altered profile of the human gut microbiome in association with cirrhosis and its complications," authored by Bajaj, Jasmohan S., was published in the esteemed *Journal of Hepatology*. This research paper garnered significant attention, being cited 371 times, making it the second most frequently referenced publication. The study findings revealed a progressive alteration in the gut microbiome imbalance as cirrhosis advanced, offering valuable insights into the relationship between cirrhosis and the human gut microbiome in the year 2014 (Bajaj et al., 2014b). Another study published by Bajaj Jasmohan S., cited 198 times, confirmed that the changes in fecal bile acid patterns in cirrhotic patients were regulated by key microorganisms in the gut microbiota (Kakiyama et al., 2013). These masterworks are in crucial role in the establishment of the connection between gut microbiota and liver cirrhosis. They collectively provided valuable and detailed evidences covering static status and dynamic trends in this connection, which constructed the academic frame and provided inspiration for further studies.

References characterized by citation bursts are those that experience frequent and vigorous discussion or citation by academics within a specific timeframe. The burst detection technique is designed to identify and record instances of significant growth in the popularity of citations within a certain timeframe. The analysis of research hotspots in an academic subject is a valuable approach for investigating the growth of scholarly interests. This method is useful in efficiently identifying prominent areas of study or specific themes within a given field. The analysis conducted revealed that the first occurrence of a surge in reference citations within the field started in 2010 and persisted until 2023. There are two studies published in 2010, one of which was researched by Bass N. M. in 2010, demonstrating that rifaximin, as a minimally absorbed antibiotic, can effectively reduce the risk of HE in patients with cirrhosis (Bass et al., 2010). Another notable study, conducted by Miele L. in 2009, underscored the notion that elevated intestinal permeability, brought about by the disruption of tight junctions amidst intestinal cells, could play a pivotal role in the onset of liver fat accumulation (Miele et al., 2009). Both of these

studies set the stage for a subsequent series of studies. As shown in Figure 7, most of the burst citations are still in progress. The most recent study started in 2021 and is still in burst citation status until 2023. Among the top 25 references exhibiting significant citation bursts, the research by Qin et al. in 2014, which delved into the changes in the functionality and composition of the gut microbiota in liver cirrhosis patients, stood out with the highest burst strength value. Using quantitative metagenomic analysis, they established a cohort reference gene set of 2.69 million genes, of which 36.1% were novel genes. In addition, they identified 66 gene clusters whose abundance varied between cirrhosis patients and healthy individuals (Shen et al., 2014). This finding has a profound influence on the gut microbiota of liver cirrhosis and lays the foundation for future research in this field.

Keyword co-occurrence analysis is a widely used method in the field of bibliometrics to find significant research themes. The analysis may serve as a valuable tool for tracking the progression of scholarly interests and gaining deeper insights into prominent areas of investigation (Pei et al., 2022). We performed overlay visualization analysis and trend topic analysis of keywords in the field. As shown in Figure 8A, the keywords that frequently appeared in the red cluster were cirrhosis, liver cirrhosis, bacterial translocation, HE, portal hypertension, ascites, lactulose, rifaximin. Numerous prior investigations have substantiated the presence of intestinal microecological dysbiosis in individuals diagnosed with liver cirrhosis. This dysbiosis has been observed to detrimentally affect the intestinal barrier's integrity, resulting in heightened bacterial translocation, infection, systemic inflammation and vasodilation, eventually culminating in acute decompensation of cirrhosis and potential organ failures (Trebicka et al., 2021b). The phenomenon of bacterial translocation originating from the gut microbiota is believed to have a significant role in the development of systemic inflammation and the deterioration of cirrhosis, ultimately impacting the prognosis of individuals with this condition (Simbrunner et al., 2023). The imbalance of gut microbiota and the subsequent translocation of bacteria are significant contributors to the development of chronic liver disorders, including cirrhosis and its associated consequences, such as spontaneous bacterial peritonitis and HE (Arab et al., 2018). As hepatic disorders become more severe, there is a concomitant rise in the frequency and intensity of pathogenic bacterial translocation. Exploring the intricate correlation between gut microbiota and bacterial translocation may offer insights into potential therapeutic targets, which could help alleviate infections and complications related to liver cirrhosis (Wiest et al., 2014). HE is a prominent complication stemming from liver cirrhosis, characterized by changes in the composition and functionality of the gut microbiota. Current therapeutic strategies predominantly focus on modulating the gut microbiota, utilizing treatments such as lactulose, rifaximin, and various other pharmaceutical agents (Hassouneh and Bajaj, 2021). One potential intervention is the administration of lactulose, which has shown beneficial outcomes for patients with minor HE resulting from bacterial-deoxyribonucleic acid (DNA) translocation. Lactulose has the ability to diminish the occurrence of bacterial-DNA translocation and mitigate the subsequent decline in neurocognitive scores (Moratalla et al., 2017). Moreover, the degradation pathways of arginine and ornithine were decreased after lactulose treatment (Wang et al., 2023). In cirrhosis, rifaximin is frequently prescribed to prevent the development of HE (Caraceni et al., 2021). Rifaximin plays a pivotal role in re-establishing the integrity of the intestinal barrier, potentially elucidating its capacity to mitigate bacterial translocation and systemic endotoxemia in patients with liver cirrhosis (Patel et al., 2022). Rifaximin can also decrease the recurrence of HE in individuals with cirrhosis and the risk of hospitalization in HE by regulating the function and activity of the gut microbiota (Bajaj, 2016; Yu et al., 2021a). The study by Shamsaddini et al. (2021) confirmed that rifaximin improves patient outcomes by regulating ARGs in the intestinal microbiome of cirrhotic patients. In conclusion, rifaximin, lactulose, and probiotics can all improve the structure of the gut microbiota to a certain extent and are reliable and efficient in the treatment of minimal HE (Wang et al., 2023). Furthermore, Sharma et al. (2013) found that treatment of HE with both lactulose and rifaximin was superior to lactulose monotherapy.

Keywords grouped within the blue cluster predominantly encompass gut microbiota microbiome dysbiosis bile acids SCFAs hepatocellular carcinoma alcoholic liver disease primary sclerosing cholangitis (PSC) and primary biliary cholangitis (PBC). It has been observed that patients afflicted with diverse liver conditions ranging from alcoholic liver disease non-alcoholic liver disease PSC to PBC consistently exhibit alterations in their gut microbiota. This dysregulation in the intestinal microbial composition can influence the severity of liver steatosis inflammation and fibrosis through various mechanisms (Tilg et al., 2016; Tang et al., 2018). Persistent liver damage can progress to conditions like liver fibrosis eventually resulting in cirrhosis and possibly hepatocellular carcinoma (Kostallari et al., 2021).

Bile acid a crucial metabolite synthesized by the gut microbiota exerts an important impact on the quantity variety and metabolic activities of the intestinal microbiota. The modulation of the bile acid reservoir's dimensions and composition reciprocally interacts with the configuration of the gut microbiota. The maintenance of this balance is of utmost importance in relation to human health and disease (Ridlon et al., 2013). The impact of bile acid on the absorption or excretion of dietary lipids signifies its potential to modify the composition of the gut microbiome or the metabolic activity of certain bacteria. Consequently regulating the metabolism of bile acid has promise for promoting human health (Collins et al., 2023). Bile acids are instrumental in the pathogenesis of NAFLD (Gottlieb and Canbay, 2019). The primary constituents of the circulating bile acid pool are the secondary bile acids deoxycholic acid and lithocholic acid together with their respective derivatives. The dimensions and constituents of this reservoir play a pivotal role in the management of primary cholangitis and NASH (Funabashi et al., 2020). For example after ursodeoxycholic acid treatment in patients with PBC the abundance of six PBC-related bacterial genera is reversed and gut microbiota emerges as a prospective therapeutic focus and diagnostic biomarker for PBC (Tang et al., 2018). Increasing evidence suggests that the dysregulation of microbial metabolites such as imbalance of secondary bile acids and reduction of SCFA-producing species is central to the pathogenic mechanisms of NAFLD (Albillos et al., 2020; Shashni et al., 2023). Liver cirrhosis patients especially with the decompensation of liver function exhibits serious intestinal microbial dysbiosis. The structure of gut microbiota in persons with this condition differs dramatically from that of healthy people. This is primarily characterized by a recent study that a notable decrease in the abundance of SCFA-producing bacteria such as Firmicutes (which is negatively correlated with liver function indicators) *Coprococcus* and *Clostridium IV* were described (Wu et al., 2023). In addition this study

also revealed that the abundance of *Oscillibacter* (also a SCFA-producing bacterium) is markedly reduced accompanied by a notable decrease in the ratio of Firmicutes and Bacteroidetes along with the progression from compensated cirrhosis to decompensated cirrhosis. Further the SCFA-producing Lachnospiraceae family can reduce liver fibrosis and the SCFA-producing families (Ruminococcaceae and Lachnospiraceae) may have a potential association with the progression of HE in cirrhotic patients (Awoniyi et al., 2023). Supplementation with probiotics targeting to enhance the population of SCFA-producing bacteria emerges as a potential therapeutic strategy for cirrhotic patients. In conclusion microbial interventions encompassing adjustments in gut microbiota composition enhancement of SCFA levels and restoration of bile acid metabolism may play a pivotal role in alleviating liver fibrosis (Fu et al., 2022).

The keywords in the green cluster are probiotics, prebiotics, microbiota, endotoxin, antibiotic, cytokines, lactobacillus, TLR, FMT, etc. Probiotics and prebiotics exert their effects via various mechanisms and are instrumental in sustaining the health of the human gut (Sanders et al., 2019; Peng et al., 2020). The therapeutic and prophylactic potential of probiotics and prebiotics in addressing diverse gut-associated ailments may hinge on their ability to alter the composition and functionality of the gut microbiota. The genera *Lactobacillus*, *Bifidobacterium*, and *Saccharomyces*, as probiotics, have been historically documented for their therapeutic efficacy. Meanwhile, *Akkermansia* spp., *Lactobacillus rhamnosus GG*, *Roseburia* spp., *Propionibacterium* spp., and *Faecalibacterium* spp. are promising candidates for future therapeutic applications (Sanders et al., 2019). The treatment of difficulties associated with severe liver illnesses, such as cirrhosis and HE, may be addressed by the administration of a diverse range of prebiotics, probiotics, and antibiotics. This therapeutic approach further underscores the significant contribution of the gut microbiota in the context of liver disorders. Enhanced comprehension of the gut microbiota and its composition in the context of liver disease has the potential to provide a more comprehensive knowledge of these complex conditions, thereby laying the groundwork for new treatment strategies (Tilg et al., 2016).

Previous research has investigated the impact of probiotic strains including several species of *Lactobacillus* and *Bifidobacterium* on the immunological function of the intestinal mucosa and the integrity of the intestinal barrier in individuals diagnosed with NAFLD. Intestinal mucosal immune function may be effectively stabilized by the administration of certain substances, hence offering protection to individuals with NAFLD against heightened permeability resulting from disturbances in the intestinal barrier (Mohamad Nor et al., 2021). Specific strains of *Lactobacillus*, including *L. acidophilus*, *L. fermentum*, and *L. plantarum*, have the potential to mitigate the progression of NASH through cholesterol reduction (Lee et al., 2021). Another randomized clinical trial confirmed that *Lactobacillus GG* can modulate the gut microbiome, metabolome, and endotoxemia in patients with cirrhosis, and the use of *Lactobacillus GG* was safe and well tolerated (Bajaj et al., 2014a). *Lactobacillus lactis* and *Pediococcus pentosaceus* have been shown to ameliorate the progression of NAFLD by regulating both the gut microbiome and metabolome functionalities in mouse models (Yu et al., 2021b). Multiple research investigations have highlighted that *Lactococcus lactis* and *Lactobacillus rhamnosus* have both preventative and therapeutic properties in relation to the advancement of liver fibrosis. Specifically, administration of *Lactobacillus rhamnosus GG* has proven efficacious in counteracting the adverse outcomes of bile acid-induced liver injury and subsequent fibrosis in mouse models. This protective mechanism is achieved via the inhibition of hepatic bile acid production and the promotion of bile acid excretion inside the liver (Liu et al., 2020; Won et al., 2023). In recent advancements, *Akkermansia* spp. is a novel promising probiotic that plays a significant role in immune-mediated liver damage, alcoholic liver disease, and NAFLD (Wu et al., 2017; Grander et al., 2018; Kim et al., 2020).

In recent years, with the development of 16S ribosomal ribonucleic acid (rRNA) sequencing, fecal metagenomic sequencing and other technologies, gut microbiota research has also risen to a new stage. With a more precise and exhaustive analysis of the structural and functional changes of intestinal flora, researchers are capable of revealing the impact of certain species on the occurrence and progression of diseases. In the future, the importance of more gut species may be discovered and become the direction of further research. Besides probiotics, prebiotics, and antibiotics, FMT has also become a potential treatment for diseases related to cirrhosis. It can be used to treat relapsed and refractory *Clostridium difficile* infections, HE, and other conditions. FMT can improve the cognitive ability of patients with perceptual encephalopathy, which is safe and effective (Bajaj et al., 2017; Cheng et al., 2021).

In addition, TLRs can recognize the gut microbiota in cirrhotic patients, triggering signaling cascades linked with the progression of cirrhosis. TLRs hold a crucial position in the occurrence, development, and treatment of cirrhosis (Fan et al., 2021). Furthermore, the gut microbiota and TLR4 are proposed as significant therapeutic targets to prevent hepatocellular carcinoma in individuals with severe liver conditions (Darnaud et al., 2013). TLR4 is also a treatment target for the prevention and management of liver failure (Engelmann et al., 2020). TLRs have multiple functions in the liver, with TLR4 notably fostering oxidative stress, instigating liver inflammation, and driving the progression of fibrosis (Seki et al., 2007; Gill et al., 2010). Numerous natural compounds have demonstrated their potential in attenuating oxidative stress, alleviating liver inflammation, and mitigating fibrosis through the inhibition of the TLR4 signaling pathway or its associated cascades (Luangmonkong et al., 2018).

The primary terms inside the yellow cluster consist of NAFLD, NASH, fatty liver, obesity, metabolic syndrome, oxidative stress, inflammation, fibrosis, insulin resistance, and other related concepts. The gut microbiota is crucial for the overall health and metabolic functions of the host. Dysbiosis, or imbalance within the gut microbiota, has been linked to the onset and progression of numerous metabolic disturbances. These include conditions such as obesity, NAFLD, and insulin resistance (Choi et al., 2021; Fan and Pedersen, 2021). The connection between gut microbiota and host metabolism is modulated by many variables, such as intestinal barrier impairments, bile acid metabolism alterations, antibiotic consumption, and microbial metabolite production. Metabolic syndrome encompasses a cluster of risk factors that, if unmanaged, frequently progress to more severe metabolic complications like type 2 diabetes mellitus and NASH (Dabke et al., 2019). Changes in the composition of gut microbiota can profoundly impact the interaction between intestinal bacteria and the host, mainly by initiating low-grade inflammatory processes leading to the progression of obesity, insulin resistance, NAFLD and other metabolic diseases (Marchesi et al., 2016; Scheithauer et al., 2020). The occurrence of dysbiosis within the gut microbiome, disturbance of the intestinal barrier, and ensuing

translocation of bacteria may initiate proinflammatory and profibrotic mechanisms, eventually culminating in the progression of cirrhosis (Pierantonelli and Svegliati-Baroni, 2019; Hyun et al., 2022). Conversely, regulating oxidative stress, fatty acid metabolism, and the balance of gut microbiota may serve as a barrier against hepatic steatosis and fibrosis, subsequently mitigating liver injury (Wang et al., 2021; Nam et al., 2023).

Within the cluster indicated by the color purple, many key components may be identified, including the gut-liver axis, liver fibrosis, intestinal barrier, intestinal microbiota, small intestine bacterial overgrowth, and lipopolysaccharide. The phenomenon of enterohepatic crosstalk has been documented in clinical investigations pertaining to liver disorders, including NAFLD, alcoholic liver disease, and PSC. Cirrhosis often occurs as a consequence of several disorders. Among microbial factors, microbial pathogen-related molecular patterns, for example, are key to driving liver inflammation and clinical complications (Tilg et al., 2022). Both dysbiosis of the intestinal microecology and disturbance of the intestinal barrier have the potential to result in bacterial translocation. Bacterial translocation and leakage of lipopolysaccharide (LPS) have been seen in animal models during the first phases of liver fibrosis. The stimulation of hepatic stellate cells is mediated by LPS via the regulation of autophagy and retinoic acid signaling pathways. Autophagy triggered by LPS can further reduce retinoic acid signaling. This leads to the downregulation of Bambi and promoting the susceptibility of hepatic stellate cells to the fibrotic response of transforming growth factor-β signaling (Chen et al., 2017; Chopyk and Grakoui, 2020). During liver fibrosis, the predominant myofibroblasts present are activated hepatic stellate cells, a distinctive marker of fibrosis (Iwaisako et al., 2014).

As cirrhotic disease progresses, liver transplantation is often eventually required. However, most patients are not eligible for transplantation, and the organ source is scarce. Therefore, further in-depth studies on the regulation of the gut-liver axis are necessary (Bajaj, 2019). In the future, it is necessary to continue to investigate the intricate link between the gut and the liver in clinical trials, which will open up a new way for future targeted therapy.

Specifically, we conduct an independent overlay visualization analysis with keywords of bacterial taxa (Figure 8D). Compared with leading keywords in Figure 8A, most taxa exhibit low occurrence and poor link strength. Studies towards many specific taxa are probably relatively isolated and lacking of extensivity. To be noteworthy, *Lactobacillus*, *Escherichia coli* (*E. coli*) and *Akkermansia muciniphila* (*A. muciniphila*) are three predominant bacterial taxa with both significant occurrence and link strength in this domain. As a contrast, we can observe that *Lactobacillus* and *A. muciniphila* are mainly linked with beneficial species while *E. coli* is mainly linked with pathogens. This is the visual evidence to understand the core species in the both sides (beneficial and harmful) of gut microbiota. In terms of time overlay, we can observe the research trend of these taxa. *Lactobacillus* is a classical probiotic in the research of liver cirrhosis. Many species have exhibited therapeutic potential (Chiva et al., 2002; Liu et al., 2020). *A. muciniphila* is a rising probiotic in recent years and has gained wide acknowledgement in different diseases such as obesity (Depommier et al., 2019), colorectal cancer (Jiang et al., 2023) and liver diseases (Rao et al., 2021). Present findings indicate its therapeutic role in early stage in including NAFLD (Depommier et al., 2019; Rao et al., 2021) and ALD (Grander et al., 2020), whilst its role in advanced liver cirrhosis has not been detected.

Most pathogens, from *Helicobacter pylori* (*H. pylori*), *E. coli* to *Clostridium difficile*, not only indicates the focus of clinical concern, but also reveals the clinical practice from disinfection to microbial regulation. The pathogenic role of *H. pylori* in liver cirrhosis has been widely discussed, which emphasize related disinfection treatment (Wang et al., 2016; Okushin et al., 2018). However, as a common but potentially harmful species, the management of *E. coli* may depend on the balance between disinfection and microbial eubiosis, which provide crucial colonization resistance against *E. coli* (Ducarmon et al., 2019; Zhang et al., 2022). Further, *Clostridium difficile* infection has raised clinical concern of excessive antibiotic use, which can unfavorably induce dysbiosis, leading the outburst of the resistant bacteria (Martin et al., 2016). The academic trend here represents the advancement of the clinical understanding and practice: in some scenarios, compared with simple eradication of harmful species, the promotion of beneficial community is as well critical.

5 Summary

This study has the following advantages: our study is the first to systematically and comprehensively analyze the gut microbiota of liver cirrhosis using bibliometric methods, which is different from previous studies. For example, Pezzino S et al. undertook a bibliometric study on the impact of the intestinal microbiome in NAFLD/NASH, and related liver cirrhosi based on the Dimensions scientific research database (Pezzino et al., 2023). Our study includes research on cirrhosis also caused by viral hepatitis, autoimmune diseases, long-term heavy alcohol consumption, obesity, cholestasis, and other causes. Our study is helpful to fully reveal the role of intestinal microbiology in this multi-etiological pathological process of liver cirrhosis because the patterns of gut microbiota in liver cirrhosis with different causes are not completely the same.

This investigation is not without its limitations. Firstly, the exclusive reliance on the WoSCC database might have inadvertently omitted relevant research from alternative databases. Furthermore, the study's focus on solely English publications could have led to the exclusion of significant research in other languages. This omission could potentially affect the comprehensiveness of our findings.

In summary, this work represents the first thorough bibliometric analysis conducted on publications pertaining to the investigation of gut microbiota in patients diagnosed with cirrhosis resulting from various etiologies. Recent years have witnessed a burgeoning interest in the gut microbiota's relationship with liver cirrhosis, mirroring the advancements in our understanding of the pathophysiological processes of liver cirrhosis. The gut microbiota is known to have a substantial effect on the development and advancement of liver cirrhosis, along with its associated consequences. The efficacy of therapeutic interventions aimed at modulating the gut microbiota has been demonstrated in patients diagnosed with liver cirrhosis, regardless of accompanying comorbidities. Hence, a comprehension of the intestinal flora's mechanism in liver cirrhosis of diverse etiologies establishes the foundation and offers substantiation for the prospective treatment of liver cirrhosis through the selective targeting of specific bacteria. This approach has the potential to enhance patient prognosis and alleviate the societal burden associated with chronic liver disease. Furthermore, it is essential for organizations and nations to actively participate in international cross-border collaboration in

order to facilitate the progress of gut microbiota in the context of liver cirrhosis.

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 author.

Author contributions

XZ: Writing – original draft. ZZ: Writing – original draft. XP: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Albillos, A., de Gottardi, A., and Rescigno, M. (2020). The gut-liver axis in liver disease: pathophysiological basis for therapy. *J. Hepatol.* 72, 558–577. doi: 10.1016/j.jhep.2019.10.003

Arab, J. P., Martin-Mateos, R. M., and Shah, V. H. (2018). Gut-liver axis, cirrhosis and portal hypertension: the chicken and the egg. *Hepatol. Int.* 12, 24–33. doi: 10.1007/s12072-017-9798-x

Aria, M., and Cuccurullo, C. (2017). Bibliometrix: an R-tool for comprehensive science mapping analysis. *J. Informetr.* 11, 959–975. doi: 10.1016/j.joi.2017.08.007

Aron-Wisnewsky, J., Vigliotti, C., Witjes, J., Le, P., Holleboom, A. G., Verheij, J., et al. (2020). Gut microbiota and human NAFLD: disentangling microbial signatures from metabolic disorders. *Nat. Rev. Gastroenterol. Hepatol.* 17, 279–297. doi: 10.1038/s41575-020-0269-9

Arvaniti, V., D'Amico, G., Fede, G., Manousou, P., Tsochatzis, E., Pleguezuelo, M., et al. (2010). Infections in patients with cirrhosis increase mortality four-fold and should be used in determining prognosis. *Gastroenterology* 139:1246. doi: 10.1053/j.gastro.2010.06.019

Awoniyi, M., Wang, J., Ngo, B., Meadows, V., Tam, J., Viswanathan, A., et al. (2023). Protective and aggressive bacterial subsets and metabolites modify hepatobiliary inflammation and fibrosis in a murine model of PSC. *Gut* 72, 671–685. doi: 10.1136/gutjnl-2021-326500

Bai, X., Wei, H., Liu, W., Coker, O. O., Gou, H., Liu, C., et al. (2022). Cigarette smoke promotes colorectal cancer through modulation of gut microbiota and related metabolites. *Gut* 71, 2439–2450. doi: 10.1136/gutjnl-2021-325021

Bajaj, J. S. (2016). Review article: potential mechanisms of action of rifaximin in the management of hepatic encephalopathy and other complications of cirrhosis. *Aliment. Pharmacol. Ther.* 43, 11–26. doi: 10.1111/apt.13435

Bajaj, J. S. (2019). Alcohol, liver disease and the gut microbiota. *Nat. Rev. Gastroenterol. Hepatol.* 16, 235–246. doi: 10.1038/s41575-018-0099-1

Bajaj, J. S., Fagan, A., Gavis, E. A., Kassam, Z., Sikaroodi, M., and Gillevet, P. M. (2019). Long-term outcomes of fecal microbiota transplantation in patients with cirrhosis. *Gastroenterology* 156:1921. doi: 10.1053/j.gastro.2019.01.033

Bajaj, J. S., Heuman, D. M., Hylemon, P. B., Sanyal, A. J., Puri, P., Sterling, R. K., et al. (2014a). Randomised clinical trial: *Lactobacillus* GG modulates gut microbiome, metabolome and endotoxemia in patients with cirrhosis. *Aliment. Pharmacol. Ther.* 39, 1113–1125. doi: 10.1111/apt.12695

Bajaj, J. S., Heuman, D. M., Hylemon, P. B., Sanyal, A. J., White, M. B., Monteith, P., et al. (2014b). Altered profile of human gut microbiome is associated with cirrhosis and its complications. *J. Hepatol.* 60, 940–947. doi: 10.1016/j.jhep.2013.12.019

Bajaj, J. S., Heuman, D. M., Sanyal, A. J., Hylemon, P. B., Sterling, R. K., Stravitz, R. T., et al. (2013). Modulation of the metabiome by rifaximin in patients with cirrhosis and minimal hepatic encephalopathy. *PLoS One* 8:e60042. doi: 10.1371/journal.pone.0060042

Bajaj, J. S., Hylemon, P. B., Ridlon, J. M., Heuman, D. M., Daita, K., White, M. B., et al. (2012a). Colonic mucosal microbiome differs from stool microbiome in cirrhosis and hepatic encephalopathy and is linked to cognition and inflammation. *Am. J. Physiol. Gastrointest. Liver Physiol.* 303, G675–G685. doi: 10.1152/ajpgi.00152.2012

Bajaj, J. S., Kakiyama, G., Savidge, T., Takei, H., Kassam, Z. A., Fagan, A., et al. (2018a). Antibiotic-associated disruption of microbiota composition and function in cirrhosis is restored by fecal transplant. *Hepatology* 68, 1549–1558. doi: 10.1002/hep.30037

Bajaj, J. S., Kassam, Z., Fagan, A., Gavis, E. A., Liu, E., Cox, I. J., et al. (2017). Fecal microbiota transplant from a rational stool donor improves hepatic encephalopathy: a randomized clinical trial. *Hepatology* 66, 1727–1738. doi: 10.1002/hep.29306

Bajaj, J. S., Liu, E. J., Kheradman, R., Fagan, A., Heuman, D. M., White, M., et al. (2018b). Fungal dysbiosis in cirrhosis. *Gut* 67, 1146–1154. doi: 10.1136/gutjnl-2016-313170

Bajaj, J. S., Ng, S. C., and Schnabl, B. (2022). Promises of microbiome-based therapies. *J. Hepatol.* 76, 1379–1391. doi: 10.1016/j.jhep.2021.12.003

Bajaj, J. S., Reddy, K. R., O'Leary, J. G., Vargas, H. E., Lai, J. C., Kamath, P. S., et al. (2020). Serum levels of metabolites produced by intestinal microbes and lipid moieties independently associated with acute-on-chronic liver failure and death in patients with cirrhosis. *Gastroenterology* 159:1715. doi: 10.1053/j.gastro.2020.07.019

Bajaj, J. S., Ridlon, J. M., Hylemon, P. B., Thacker, L. R., Heuman, D. M., Smith, S., et al. (2012b). Linkage of gut microbiome with cognition in hepatic encephalopathy. *Am. J. Physiol. Gastrointest. Liver Physiol.* 302, G168–G175. doi: 10.1152/ajpgi.00190.2011

Bajaj, J. S., Shamsaddini, A., Fagan, A., Sterling, R. K., Gavis, E., Khoruts, A., et al. (2021). Fecal microbiota transplant in cirrhosis reduces gut microbial antibiotic resistance genes: analysis of two trials. *Hepatol. Commun.* 5, 258–271. doi: 10.1002/hep4.1639

Bass, N. M., Mullen, K. D., Sanyal, A., Poordad, F., Neff, G., Leevy, C. B., et al. (2010). Rifaximin treatment in hepatic encephalopathy. *N. Engl. J. Med.* 362, 1071–1081. doi: 10.1056/NEJMoa0907893

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

Brandt, J. S., Hadaya, O., Schuster, M., Rosen, T., Sauer, M. V., and Ananth, C. V. (2019). A bibliometric analysis of top-cited journal articles in obstetrics and gynecology. *JAMA Netw. Open* 2:e1918007. doi: 10.1001/jamanetworkopen.2019.18007

Brody, H. (2020). The gut microbiome. *Nature* 577:S5. doi: 10.1038/d41586-020-00194-2

Caraceni, P., Vargas, V., Solà, E., Alessandria, C., de Wit, K., Trebicka, J., et al. (2021). The use of rifaximin in patients with cirrhosis. *Hepatology* 74, 1660–1673. doi: 10.1002/hep.31708

Chen, C. (2004). Searching for intellectual turning points: progressive knowledge domain visualization. *Proc. Natl. Acad. Sci. U.S.A.* 101, 5303–5310. doi: 10.1073/pnas.0307513100

Chen, Y.-F., Chen, J., and Li, L.-J. (2020). The role of intestinal microbiota, bile acids, and Th17/Il17 axis in hepatitis B virus-related liver fibrosis. *Chin. Med. J.* 133, 2902–2904. doi: 10.1097/CM9.0000000000001199

Chen, M., Liu, J., Yang, W., and Ling, W. (2017). Lipopolysaccharide mediates hepatic stellate cell activation by regulating autophagy and retinoic acid signaling. *Autophagy* 13, 1813–1827. doi: 10.1080/15548627.2017.1356550

Chen, Y., Yang, F., Lu, H., Wang, B., Chen, Y., Lei, D., et al. (2011). Characterization of fecal microbial communities in patients with liver cirrhosis. *Hepatology* 54, 562–572. doi: 10.1002/hep.24423

Cheng, Y.-W., Alhaffar, D., Saha, S., Khanna, S., Bohm, M., Phelps, E., et al. (2021). Fecal microbiota transplantation is safe and effective in patients with *Clostridioides difficile* infection and cirrhosis. *Clin. Gastroenterol. Hepatol.* 19, 1627–1634. doi: 10.1016/j.cgh.2020.06.051

Chiva, M., Soriano, G., Rochat, I., Peralta, C., Rochat, F., Llovet, T., et al. (2002). Effect of *Lactobacillus johnsonii* La1 and antioxidants on intestinal flora and bacterial translocation in rats with experimental cirrhosis. *J. Hepatol.* 37, 456–462. doi: 10.1016/S0168-8278(02)00142-3

Choi, H., Rao, M. C., and Chang, E. B. (2021). Gut microbiota as a transducer of dietary cues to regulate host circadian rhythms and metabolism. *Nat. Rev. Gastroenterol. Hepatol.* 18, 679–689. doi: 10.1038/s41575-021-00452-2

Chopyk, D. M., and Grakoui, A. (2020). Contribution of the intestinal microbiome and gut barrier to hepatic disorders. *Gastroenterology* 159, 849–863. doi: 10.1053/j.gastro.2020.04.077

Collins, S. L., Stine, J. G., Bisanz, J. E., Okafor, C. D., and Patterson, A. D. (2023). Bile acids and the gut microbiota: metabolic interactions and impacts on disease. *Nat. Rev. Microbiol.* 21, 236–247. doi: 10.1038/s41579-022-00805-x

Dabke, K., Hendrick, G., and Devkota, S. (2019). The gut microbiome and metabolic syndrome. *J. Clin. Invest.* 129, 4050–4057. doi: 10.1172/JCI129194

Darnaud, M., Faivre, J., and Moniaux, N. (2013). Targeting gut flora to prevent progression of hepatocellular carcinoma. *J. Hepatol.* 58, 385–387. doi: 10.1016/j.jhep.2012.08.019

Depommier, C., Everard, A., Druart, C., Plovier, H., Van Hul, M., Vieira-Silva, S., et al. (2019). Supplementation with *Akkermansia muciniphila* in overweight and obese human volunteers: a proof-of-concept exploratory study. *Nat. Med.* 25, 1096–1103. doi: 10.1038/s41591-019-0495-2

Duan, Y., Llorente, C., Lang, S., Brandl, K., Chu, H., Jiang, L., et al. (2019). Bacteriophage targeting of gut bacterium attenuates alcoholic liver disease. *Nature* 575, 505–511. doi: 10.1038/s41586-019-1742-x

Ducarmon, Q. R., Zwittink, R. D., Hornung, B. V. H., van Schaik, W., Young, V. B., and Kuijper, E. J. (2019). Gut microbiota and colonization resistance against bacterial enteric infection. *Microbiol. Mol. Biol. Rev.* 83:e00007. doi: 10.1128/MMBR.00007-19

Ellegaard, O., and Wallin, J. A. (2015). The bibliometric analysis of scholarly production: how great is the impact? *Scientometrics* 105, 1809–1831. doi: 10.1007/s11192-015-1645-z

Engelmann, C., Clària, J., Szabo, G., Bosch, J., and Bernardi, M. (2021). Pathophysiology of decompensated cirrhosis: portal hypertension, circulatory dysfunction, inflammation, metabolism and mitochondrial dysfunction. *J. Hepatol.* 75, S49–S66. doi: 10.1016/j.jhep.2021.01.002

Engelmann, C., Sheikh, M., Sharma, S., Kondo, T., Loeffler-Wirth, H., Zheng, Y. B., et al. (2020). Toll-like receptor 4 is a therapeutic target for prevention and treatment of liver failure. *J. Hepatol.* 73, 102–112. doi: 10.1016/j.jhep.2020.01.011

Fan, Y., Li, Y., Chu, Y., Liu, J., Cui, L., and Zhang, D. (2021). Toll-like receptors recognize intestinal microbes in liver cirrhosis. *Front. Immunol.* 12:608498. doi: 10.3389/fimmu.2021.608498

Fan, Y., and Pedersen, O. (2021). Gut microbiota in human metabolic health and disease. *Nat. Rev. Microbiol.* 19, 55–71. doi: 10.1038/s41579-020-0433-9

Fouts, D. E., Torralba, M., Nelson, K. E., Brenner, D. A., and Schnabl, B. (2012). Bacterial translocation and changes in the intestinal microbiome in mouse models of liver disease. *J. Hepatol.* 56, 1283–1292. doi: 10.1016/j.jhep.2012.01.019

Franzosa, E. A., Sirota-Madi, A., Avila-Pacheco, J., Fornelos, N., Haiser, H. J., Reinker, S., et al. (2019). Gut microbiome structure and metabolic activity in inflammatory bowel disease. *Nat. Microbiol.* 4, 293–305. doi: 10.1038/s41564-018-0306-4

Frost, F., Kacprowski, T., Rühlemann, M., Pietzner, M., Bang, C., Franke, A., et al. (2021). Long-term instability of the intestinal microbiome is associated with metabolic liver disease, low microbiota diversity, diabetes mellitus and impaired exocrine pancreatic function. *Gut* 70, 522–530. doi: 10.1136/gutjnl-2020-322753

Fu, K., Ma, C., Wang, C., Zhou, H., Gong, L., Zhang, Y., et al. (2022). Forsythiaside a alleviated carbon tetrachloride-induced liver fibrosis by modulating gut microbiota composition to increase short-chain fatty acids and restoring bile acids metabolism disorder. *Biomed. Pharmacother.* 151:113185. doi: 10.1016/j.biopha.2022.113185

Funabashi, M., Grove, T. L., Wang, M., Varma, Y., McFadden, M. E., Brown, L. C., et al. (2020). A metabolic pathway for bile acid dehydroxylation by the gut microbiome. *Nature* 582, 566–570. doi: 10.1038/s41586-020-2396-4

Garcia-Pagan, J. C., Francoz, C., Montagnese, S., Senzolo, M., and Mookerjee, R. P. (2021). Management of the major complications of cirrhosis: beyond guidelines. *J. Hepatol.* 75, S135–S146. doi: 10.1016/j.jhep.2021.01.027

Gentile, C. L., and Weir, T. L. (2018). The gut microbiota at the intersection of diet and human health. *Science* 362, 776–780. doi: 10.1126/science.aau5812

Gill, R., Tsung, A., and Billiar, T. (2010). Linking oxidative stress to inflammation: toll-like receptors. *Free Radic. Biol. Med.* 48, 1121–1132. doi: 10.1016/j.freeradbiomed.2010.01.006

Ginès, P., Krag, A., Abraldes, J. G., Solà, E., Fabrellas, N., and Kamath, P. S. (2021). Liver cirrhosis. *Lancet* 398, 1359–1376. doi: 10.1016/S0140-6736(21)01374-X

Gottlieb, A., and Canbay, A. (2019). Why bile acids are so important in non-alcoholic fatty liver disease (NAFLD) progression. *Cells* 8:1358. doi: 10.3390/cells8111358

Grander, C., Adolph, T. E., Wieser, V., Lowe, P., Wrzosek, L., Gyongyosi, B., et al. (2018). Recovery of ethanol-induced *Akkermansia muciniphila* depletion ameliorates alcoholic liver disease. *Gut* 67, 891–901. doi: 10.1136/gutjnl-2016-313432

Grander, C., Grabherr, F., Spadoni, I., Enrich, B., Oberhuber, G., Rescigno, M., et al. (2020). The role of gut vascular barrier in experimental alcoholic liver disease and *A. muciniphila* supplementation. *Gut Microbes* 12:1851986. doi: 10.1080/19490976.2020.1851986

Hassouneh, R., and Bajaj, J. S. (2021). Gut microbiota modulation and fecal transplantation: an overview on innovative strategies for hepatic encephalopathy treatment. *J. Clin. Med.* 10:330. doi: 10.3390/jcm10020330

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

Hernandez-Gea, V., and Friedman, S. L. (2011). Pathogenesis of liver fibrosis. *Annu. Rev. Pathol.* 6, 425–456. doi: 10.1146/annurev-pathol-011110-130246

Huang, X., Fan, X., Ying, J., and Chen, S. (2019). Emerging trends and research foci in gastrointestinal microbiome. *J. Transl. Med.* 17:67. doi: 10.1186/s12967-019-1810-x

Hyun, J. Y., Kim, S. K., Yoon, S. J., Lee, S. B., Jeong, J.-J., Gupta, H., et al. (2022). Microbiome-based metabolic therapeutic approaches in alcoholic liver disease. *Int. J. Mol. Sci.* 23:8749. doi: 10.3390/ijms23158749

Iwaisako, K., Jiang, C., Zhang, M., Cong, M., Moore-Morris, T. J., Park, T. J., et al. (2014). Origin of myofibroblasts in the fibrotic liver in mice. *Proc. Natl. Acad. Sci. U.S.A.* 111, E3297–E3305. doi: 10.1073/pnas.1400062111

Jiang, Y., Xu, Y., Zheng, C., Ye, L., Jiang, P., Malik, S., et al. (2023). Acetyltransferase from *Akkermansia muciniphila* blunts colorectal tumourigenesis by reprogramming tumour microenvironment. *Gut* 72, 1308–1318. doi: 10.1136/gutjnl-2022-327853

Kakiyama, G., Pandak, W. M., Gillevet, P. M., Hylemon, P. B., Heuman, D. M., Daita, K., et al. (2013). Modulation of the fecal bile acid profile by gut microbiota in cirrhosis. *J. Hepatol.* 58, 949–955. doi: 10.1016/j.jhep.2013.01.003

Kang, D. J., Betrapally, N. S., Ghosh, S. A., Sartor, R. B., Hylemon, P. B., Gillevet, P. M., et al. (2016). Gut microbiota drive the development of neuroinflammatory response in cirrhosis in mice. *Hepatology* 64, 1232–1248. doi: 10.1002/hep.28696

Ke, L., Lu, C., Shen, R., Lu, T., Ma, B., and Hua, Y. (2020). Knowledge mapping of drug-induced liver injury: a scientometric investigation (2010–2019). *Front. Pharmacol.* 11:842. doi: 10.3389/fphar.2020.00842

Kim, S., Lee, Y., Kim, Y., Seo, Y., Lee, H., Ha, J., et al. (2020). *Akkermansia muciniphila* prevents fatty liver disease, decreases serum triglycerides, and maintains gut homeostasis. *Appl. Environ. Microbiol.* 86:e03004. doi: 10.1128/AEM.03004-19

Kostallari, E., Valainathan, S., Biquard, L., Shah, V. H., and Rautou, P.-E. (2021). Role of extracellular vesicles in liver diseases and their therapeutic potential. *Adv. Drug Deliv. Rev.* 175:113816. doi: 10.1016/j.addr.2021.05.026

Lee, N. Y., Shin, M. J., Youn, G. S., Yoon, S. J., Choi, Y. R., Kim, H. S., et al. (2021). *Lactobacillus* attenuates progression of nonalcoholic fatty liver disease by lowering cholesterol and steatosis. *Clin. Mol. Hepatol.* 27, 110–124. doi: 10.3350/cmh.2020.0125

Lei, Y., Tang, L., Chen, Q., Wu, L., He, W., Tu, D., et al. (2022). Disulfiram ameliorates nonalcoholic steatohepatitis by modulating the gut microbiota and bile acid metabolism. *Nat. Commun.* 13:6862. doi: 10.1038/s41467-022-34671-1

Leung, C., Rivera, L., Furness, J. B., and Angus, P. W. (2016). The role of the gut microbiota in NAFLD. *Nat. Rev. Gastroenterol. Hepatol.* 13, 412–425. doi: 10.1038/nrgastro.2016.85

Lin, F., Chen, Y., Mo, W., Zhou, H., Xiao, Z., Hu, S., et al. (2022). A bibliometric analysis of autophagy in lung diseases from 2012 to 2021. *Front. Immunol.* 13:1092575. doi: 10.3389/fimmu.2022.1092575

Liu, Y., Chen, K., Li, F., Gu, Z., Liu, Q., He, L., et al. (2020). Probiotic *Lactobacillus rhamnosus* GG prevents liver fibrosis through inhibiting hepatic bile acid synthesis and enhancing bile acid excretion in mice. *Hepatology* 71, 2050–2066. doi: 10.1002/hep.30975

Liu, R., Kang, J. D., Sartor, R. B., Sikaroodi, M., Fagan, A., Gavis, E. A., et al. (2020). Neuroinflammation in murine cirrhosis is dependent on the gut microbiome and is attenuated by fecal transplant. *Hepatology* 71, 611–626. doi: 10.1002/hep.30827

Loomba, R., Seguritan, V., Li, W., Long, T., Klitgord, N., Bhatt, A., et al. (2017). Gut microbiome-based metagenomic signature for non-invasive detection of advanced fibrosis in human nonalcoholic fatty liver disease. *Cell Metab.* 25:1054. doi: 10.1016/j.cmet.2017.04.001

Lu, C., Bing, Z., Bi, Z., Liu, M., Lu, T., Xun, Y., et al. (2019). Top-100 most cited publications concerning network pharmacology: a bibliometric analysis. *Evid. Based Complement. Alternat. Med.* 2019:1704816. doi: 10.1155/2019/1704816

Luangmonkong, T., Suriguga, S., Mutsaers, H. A. M., Groothuis, G. M. M., Olinga, P., and Boersema, M. (2018). Targeting oxidative stress for the treatment of liver fibrosis. *Rev. Physiol. Biochem. Pharmacol.*:175. doi: 10.1007/112_2018_10

Ma, D., Guan, B., Song, L., Liu, Q., Fan, Y., Zhao, L., et al. (2021). A bibliometric analysis of exosomes in cardiovascular diseases from 2001 to 2021. *Front. Cardiovasc. Med.* 8:734514. doi: 10.3389/fcvm.2021.734514

Ma, C., Han, M., Heinrich, B., Fu, Q., Zhang, Q., Sandhu, M., et al. (2018). Gut microbiome-mediated bile acid metabolism regulates liver cancer via NKT cells. *Science* 360:eaan5931. doi: 10.1126/science.aan5931

Marchesi, J. R., Adams, D. H., Fava, F., Hermes, G. D. A., Hirschfield, G. M., Hold, G., et al. (2016). The gut microbiota and host health: a new clinical frontier. *Gut* 65, 330–339. doi: 10.1136/gutjnl-2015-309990

Martin, J. S. H., Monaghan, T. M., and Wilcox, M. H. (2016). *Clostridium difficile* infection: epidemiology, diagnosis and understanding transmission. *Nat. Rev. Gastroenterol. Hepatol.* 13, 206–216. doi: 10.1038/nrgastro.2016.25

Martino, C., Dilmore, A. H., Burcham, Z. M., Metcalf, J. L., Jeste, D., and Knight, R. (2022). Microbiota succession throughout life from the cradle to the grave. *Nat. Rev. Microbiol.* 20, 707–720. doi: 10.1038/s41579-022-00768-z

Miele, L., Valenza, V., La Torre, G., Montalto, M., Cammarota, G., Ricci, R., et al. (2009). Increased intestinal permeability and tight junction alterations in nonalcoholic fatty liver disease. *Hepatology* 49, 1877–1887. doi: 10.1002/hep.22848

Mohamad Nor, M. H., Ayob, N., Mokhtar, N. M., Raja Ali, R. A., Tan, G. C., Wong, Z., et al. (2021). The effect of probiotics (MCP® BCMC® strains) on hepatic steatosis, small intestinal mucosal immune function, and intestinal barrier in patients with non-alcoholic fatty liver disease. *Nutrients* 13:3192. doi: 10.3390/nu13093192

Moratalla, A., Ampuero, J., Bellot, P., Gallego-Durán, R., Zapater, P., Roger, M., et al. (2017). Lactulose reduces bacterial DNA translocation, which worsens neurocognitive shape in cirrhotic patients with minimal hepatic encephalopathy. *Liver Int.* 37, 212–223. doi: 10.1111/liv.13200

Moreau, R., Jalan, R., Gines, P., Pavesi, M., Angeli, P., Cordoba, J., et al. (2013). Acute-on-chronic liver failure is a distinct syndrome that develops in patients with acute decompensation of cirrhosis. *Gastroenterology* 144:1426. doi: 10.1053/j.gastro.2013.02.042

Mouzaki, M., Comelli, E. M., Arendt, B. M., Bonengel, J., Fung, S. K., Fischer, S. E., et al. (2013). Intestinal microbiota in patients with nonalcoholic fatty liver disease. *Hepatology* 58, 120–127. doi: 10.1002/hep.26319

Nam, Y., Kim, M., Erdenebileg, S., Cha, K. H., Ryu, D. H., Kim, H. Y., et al. (2023). *Sanguisorba officinalis* L. ameliorates hepatic steatosis and fibrosis by modulating oxidative stress, fatty acid oxidation, and gut microbiota in CDAHFD-induced mice. *Nutrients* 15:3779. doi: 10.3390/nu15173779

Neuschwander-Tetri, B. A., Loomba, R., Sanyal, A. J., Lavine, J. E., Van Natta, M. L., Abdelmalek, M. F., et al. (2015). Farnesoid × nuclear receptor ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis (FLINT): a multicentre, randomised, placebo-controlled trial. *Lancet* 385, 956–965. doi: 10.1016/S0140-6736(14)61933-4

Okushin, K., Tsutsumi, T., Ikeuchi, K., Kado, A., Enooku, K., Fujinaga, H., et al. (2018). *Helicobacter pylori* infection and liver diseases: epidemiology and insights into pathogenesis. *World J. Gastroenterol.* 24, 3617–3625. doi: 10.3748/wjg.v24.i32.3617

Parola, M., and Pinzani, M. (2019). Liver fibrosis: pathophysiology, pathogenetic targets and clinical issues. *Mol. Asp. Med.* 65, 37–55. doi: 10.1016/j.mam.2018.09.002

Patel, V. C., Lee, S., McPhail, M. J. W., Da Silva, K., Guilly, S., Zamalloa, A., et al. (2022). Rifaximin-A reduces gut-derived inflammation and mucin degradation in cirrhosis and encephalopathy: RIFSYS randomised controlled trial. *J. Hepatol.* 76, 332–342. doi: 10.1016/j.jhep.2021.09.010

Pei, Z., Chen, S., Ding, L., Liu, J., Cui, X., Li, F., et al. (2022). Current perspectives and trend of nanomedicine in cancer: a review and bibliometric analysis. *J. Control. Release* 352, 211–241. doi: 10.1016/j.jconrel.2022.10.023

Peng, M., Tabashsum, Z., Anderson, M., Truong, A., Houser, A. K., Padilla, J., et al. (2020). Effectiveness of probiotics, prebiotics, and prebiotic-like components in common functional foods. *Compr. Rev. Food Sci. Food Saf.* 19, 1908–1933. doi: 10.1111/1541-4337.12565

Pezzino, S., Sofia, M., Mazzone, C., Castorina, S., Puleo, S., Barchitta, M., et al. (2023). Gut microbiome in the progression of NAFLD, NASH and cirrhosis, and its connection with biotics: a bibliometric study using dimensions scientific research database. *Biology* 12:662. doi: 10.3390/biology12050662

Pierantonelli, I., and Svegliati-Baroni, G. (2019). Nonalcoholic fatty liver disease: basic pathogenetic mechanisms in the progression from NAFLD to Nash. *Transplantation* 103:e1. doi: 10.1097/TP.0000000000002480

Rao, Y., Kuang, Z., Li, C., Guo, S., Xu, Y., Zhao, D., et al. (2021). Gut *Akkermansia muciniphila* ameliorates metabolic dysfunction-associated fatty liver disease by regulating the metabolism of L-aspartate via gut-liver axis. *Gut Microbes* 13, 1–19. doi: 10.1080/19490976.2021.1927633

Ridlon, J. M., Alves, J. M., Hylemon, P. B., and Bajaj, J. S. (2013). Cirrhosis, bile acids and gut microbiota: unraveling a complex relationship. *Gut Microbes* 4, 382–387. doi: 10.4161/gmic.25723

Sampson, T. R., Debelius, J. W., Thron, T., Janssen, S., Shastri, G. G., Ilhan, Z. E., et al. (2016). Gut microbiota regulate motor deficits and neuroinflammation in a model of Parkinson's disease. *Cell* 167:1469. doi: 10.1016/j.cell.2016.11.018

Sanders, M. E., Merenstein, D. J., Reid, G., Gibson, G. R., and Rastall, R. A. (2019). Probiotics and prebiotics in intestinal health and disease: from biology to the clinic. *Nat. Rev. Gastroenterol. Hepatol.* 16, 605–616. doi: 10.1038/s41575-019-0173-3

Scheithauer, T. P. M., Rampanelli, E., Nieuwdorp, M., Vallance, B. A., Verchere, C. B., van Raalte, D. H., et al. (2020). Gut microbiota as a trigger for metabolic inflammation in obesity and type 2 diabetes. *Front. Immunol.* 11:571731. doi: 10.3389/fimmu.2020.571731

Schnabl, B., and Brenner, D. A. (2014). Interactions between the intestinal microbiome and liver diseases. *Gastroenterology* 146, 1513–1524. doi: 10.1053/j.gastro.2014.01.020

Seki, E., De Minicis, S., Osterreicher, C. H., Kluwe, J., Osawa, Y., Brenner, D. A., et al. (2007). TLR4 enhances TGF-beta signaling and hepatic fibrosis. *Nat. Med.* 13, 1324–1332. doi: 10.1038/nm1663

Shamsaddini, A., Gillevet, P. M., Acharya, C., Fagan, A., Gavis, E., Sikaroodi, M., et al. (2021). Impact of antibiotic resistance genes in gut microbiome of patients with cirrhosis. *Gastroenterology* 161:508. doi: 10.1053/j.gastro.2021.04.013

Sharma, B. C., Sharma, P., Lunia, M. K., Srivastava, S., Goyal, R., and Sarin, S. K. (2013). A randomized, double-blind, controlled trial comparing rifaximin plus lactulose with lactulose alone in treatment of overt hepatic encephalopathy. *Am. J. Gastroenterol.* 108, 1458–1463. doi: 10.1038/ajg.2013.219

Shashni, B., Tajika, Y., Ikeda, Y., Nishikawa, Y., and Nagasaki, Y. (2023). Self-assembling polymer-based short chain fatty acid prodrugs ameliorate non-alcoholic steatohepatitis and liver fibrosis. *Biomaterials* 295:122047. doi: 10.1016/j.biomaterials.2023.122047

Shen, Z., Hu, J., Wu, H., Chen, Z., Wu, W., Lin, J., et al. (2022). Global research trends and foci of artificial intelligence-based tumor pathology: a scientometric study. *J. Transl. Med.* 20:409. doi: 10.1186/s12967-022-03615-0

Shen, S., Park, J. W., Lu, Z.-x., Lin, L., Henry, M. D., Wu, Y. N., et al. (2014). rMATS: robust and flexible detection of differential alternative splicing from replicate RNA-Seq data. *Proc. Natl. Acad. Sci. U.S.A.* 111, E5593–E5601. doi: 10.1073/pnas.1419161111

Simbrunner, B., Caparrós, E., Neuwirth, T., Schwabl, P., Königshofer, P., Bauer, D., et al. (2023). Bacterial translocation occurs early in cirrhosis and triggers a selective inflammatory response. *Hepatol. Int.* 17, 1045–1056. doi: 10.1007/s12072-023-10496-y

Sorribas, M., Jakob, M. O., Yilmaz, B., Li, H., Stutz, D., Noser, Y., et al. (2019). FXR modulates the gut-vascular barrier by regulating the entry sites for bacterial translocation in experimental cirrhosis. *J. Hepatol.* 71, 1126–1140. doi: 10.1016/j.jhep.2019.06.017

Sun, H.-L., Bai, W., Li, X.-H., Huang, H., Cui, X.-L., Cheung, T., et al. (2022). Schizophrenia and inflammation research: a bibliometric analysis. *Front. Immunol.* 13:907851. doi: 10.3389/fimmu.2022.907851

Synnestvedt, M. B., Chen, C., and Holmes, J. H. (2005). CiteSpace II: visualization and knowledge discovery in bibliographic databases. *AMIA Annu. Symp. Proc.* 2005, 724–728.

Tang, R., Wei, Y., Li, Y., Chen, W., Chen, H., Wang, Q., et al. (2018). Gut microbial profile is altered in primary biliary cholangitis and partially restored after UDCA therapy. *Gut* 67, 534–541. doi: 10.1136/gutjnl-2016-313332

Tilg, H., Adolph, T. E., and Trauner, M. (2022). Gut-liver axis: pathophysiological concepts and clinical implications. *Cell Metab.* 34, 1700–1718. doi: 10.1016/j.cmet.2022.09.017

Tilg, H., Cani, P. D., and Mayer, E. A. (2016). Gut microbiome and liver diseases. *Gut* 65, 2035–2044. doi: 10.1136/gutjnl-2016-312729

Tranah, T. H., Edwards, L. A., Schnabl, B., and Shawcross, D. L. (2021). Targeting the gut-liver-immune axis to treat cirrhosis. *Gut* 70, 982–994. doi: 10.1136/gutjnl-2020-320786

Trebicka, J., Bork, P., Krag, A., and Arumugam, M. (2021a). Utilizing the gut microbiome in decompensated cirrhosis and acute-on-chronic liver failure. *Nat. Rev. Gastroenterol. Hepatol.* 18, 167–180. doi: 10.1038/s41575-020-00376-3

Trebicka, J., Macnaughtan, J., Schnabl, B., Shawcross, D. L., and Bajaj, J. S. (2021b). The microbiota in cirrhosis and its role in hepatic decompensation. *J. Hepatol.* 75, S67–S81. doi: 10.1016/j.jhep.2020.11.013

van Eck, N. J., and Waltman, L. (2010). Software survey: VOSviewer, a computer program for bibliometric mapping. *Scientometrics* 84, 523–538. doi: 10.1007/s11192-009-0146-3

Vilstrup, H., Amodio, P., Bajaj, J., Cordoba, J., Ferenci, P., Mullen, K. D., et al. (2014). Hepatic encephalopathy in chronic liver disease: 2014 practice guideline by the American Association for the Study of Liver Diseases and the European Association for the Study of the Liver. *Hepatology* 60, 715–735. doi: 10.1002/hep.27210

Wallin, J. A. (2005). Bibliometric methods: pitfalls and possibilities. *Basic Clin. Pharmacol. Toxicol.* 97, 261–275. doi: 10.1111/j.1742-7843.2005.pto_139.x

Wang, J., Chen, R.-C., Zheng, Y.-X., Zhao, S.-S., Li, N., Zhou, R.-R., et al. (2016). *Helicobacter pylori* infection may increase the risk of progression of chronic hepatitis B disease among the Chinese population: a meta-analysis. *Int. J. Infect. Dis.* 50, 30–37. doi: 10.1016/j.ijid.2016.07.014

Wang, X., Liu, D., Wang, Z., Cai, C., Jiang, H., and Yu, G. (2021). Porphyran-derived oligosaccharides alleviate NAFLD and related cecal microbiota dysbiosis in mice. *FASEB J.* 35:e21458. doi: 10.1096/fj.202000763RRR

Wang, M.-W., Ma, W.-J., Wang, Y., Ma, X.-H., Xue, Y.-F., Guan, J., et al. (2023). Comparison of the effects of probiotics, rifaximin, and lactulose in the treatment of minimal hepatic encephalopathy and gut microbiota. *Front. Microbiol.* 14:1091167. doi: 10.3389/fmicb.2023.1091167

Weersma, R. K., Zhernakova, A., and Fu, J. (2020). Interaction between drugs and the gut microbiome. *Gut* 69, 1510–1519. doi: 10.1136/gutjnl-2019-320204

Wiest, R., Lawson, M., and Geuking, M. (2014). Pathological bacterial translocation in liver cirrhosis. *J. Hepatol.* 60, 197–209. doi: 10.1016/j.jhep.2013.07.044

Won, S. M., Lee, N. Y., Oh, K.-K., Gupta, H., Sharma, S. P., Kim, K. H., et al. (2023). Gut *Lactobacillus* and probiotics *Lactobacillus lactis*/rhamnosis ameliorate liver fibrosis in prevention and treatment. *J. Microbiol.* 61, 245–257. doi: 10.1007/s12275-023-00014-y

Wu, H., Cheng, K., Guo, Q., Yang, W., Tong, L., Wang, Y., et al. (2021). Mapping knowledge structure and themes trends of osteoporosis in rheumatoid arthritis: a bibliometric analysis. *Front. Med.* 8:787228. doi: 10.3389/fmed.2021.787228

Wu, F., Gao, J., Kang, J., Wang, X., Niu, Q., Liu, J., et al. (2022). Knowledge mapping of exosomes in autoimmune diseases: a bibliometric analysis (2002–2021). *Front. Immunol.* 13:939433. doi: 10.3389/fimmu.2022.939433

Wu, W., Lv, L., Shi, D., Ye, J., Fang, D., Guo, F., et al. (2017). Protective effect of *Akkermansia muciniphila* against immune-mediated liver injury in a mouse model. *Front. Microbiol.* 8:1804. doi: 10.3389/fmicb.2017.01804

Wu, Z., Zhou, H., Liu, D., and Deng, F. (2023). Alterations in the gut microbiota and the efficacy of adjuvant probiotic therapy in liver cirrhosis. *Front. Cell. Infect. Microbiol.* 13:1218552. doi: 10.3389/fcimb.2023.1218552

Yoshimoto, S., Loo, T. M., Atarashi, K., Kanda, H., Sato, S., Oyadomari, S., et al. (2013). Obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome. *Nature* 499:97. doi: 10.1038/nature12347

Younossi, Z. M., Loomba, R., Anstee, Q. M., Rinella, M. E., Bugianesi, E., Marchesini, G., et al. (2018). Diagnostic modalities for nonalcoholic fatty liver disease, nonalcoholic steatohepatitis, and associated fibrosis. *Hepatology* 68, 349–360. doi: 10.1002/hep.29721

Younossi, Z. M., Ratziu, V., Loomba, R., Rinella, M., Anstee, Q. M., Goodman, Z., et al. (2019). Obeticholic acid for the treatment of non-alcoholic steatohepatitis: interim analysis from a multicentre, randomised, placebo-controlled phase 3 trial. *Lancet* 394, 2184–2196. doi: 10.1016/S0140-6736(19)33041-7

Yu, X., Jin, Y., Zhou, W., Xiao, T., Wu, Z., Su, J., et al. (2021a). Rifaximin modulates the gut microbiota to prevent hepatic encephalopathy in liver cirrhosis without impacting the resistome. *Front. Cell. Infect. Microbiol.* 11:761192. doi: 10.3389/fcimb.2021.761192

Yu, J. S., Youn, G. S., Choi, J., Kim, C.-H., Kim, B. Y., Yang, S.-J., et al. (2021b). *Lactobacillus* lactis and *Pediococcus Pentosaceus*-driven reprogramming of gut microbiome and metabolome ameliorates the progression of non-alcoholic fatty liver disease. *Clin. Transl. Med.* 11:e634. doi: 10.1002/ctm2.634

Zhang, Y., Tan, P., Zhao, Y., and Ma, X. (2022). Enterotoxigenic *Escherichia coli*: intestinal pathogenesis mechanisms and colonization resistance by gut microbiota. *Gut Microbes* 14:2055943. doi: 10.1080/19490976.2022.2055943

Zhou, W.-C., Zhang, Q.-B., and Qiao, L. (2014). Pathogenesis of liver cirrhosis. *World J. Gastroenterol.* 20, 7312–7324. doi: 10.3748/wjg.v20.i23.7312

Zhu, L., Baker, S. S., Gill, C., Liu, W., Alkhouri, R., Baker, R. D., et al. (2013). Characterization of gut microbiomes in nonalcoholic steatohepatitis (NASH) patients: a connection between endogenous alcohol and NASH. *Hepatology* 57, 601–609. doi: 10.1002/hep.26093

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Symbiotic combination of *Akkermansia muciniphila* and inosine alleviates alcohol-induced liver injury by modulating gut dysbiosis and immune responses

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Background: Alcoholic liver disease (ALD) is exacerbated by disruptions in intestinal microecology and immune imbalances within the gut–liver axis. The present study assesses the therapeutic potential of combining *Akkermansia muciniphila* (*A. muciniphila*) with inosine in alleviating alcohol-induced liver injury.

Methods: Male C57BL/6 mice, subjected to a Lieber-DeCarli diet with 5% alcohol for 4 weeks, served as the alcoholic liver injury model. Various analyzes, including quantitative reverse transcription polymerase chain reaction (qRT-PCR), ELISA, immunochemistry, 16S rRNA gene sequencing, and flow cytometry, were employed to evaluate liver injury parameters, intestinal barrier function, microbiota composition, and immune responses.

Results: Compared to the model group, the *A. muciniphila* and inosine groups exhibited significantly decreased alanine aminotransferase, aspartate aminotransferase, and lipopolysaccharide (LPS) levels, reduced hepatic fat deposition and neutrophil infiltration, alleviated oxidative stress and inflammation, and increased expression of intestinal tight junction proteins (Claudin-1, Occludin, and ZO-1). These effects were further pronounced in the *A. muciniphila* and inosine combination group compared to individual treatments. While alcohol feeding induced intestinal dysbiosis and gut barrier disruption, the combined treatment reduced the abundance of harmful bacteria (*Oscillibacter, Escherichia/Shigella, and Alistipes*) induced by alcohol consumption, promoting the growth of butyrate-producing bacteria (Akkermansia, Lactobacillus, and Clostridium IV). Flow cytometry revealed that alcohol consumption reduced T regulatory (Treg) populations while increasing those of T-helper (Th) 1 and Th17, which were restored by *A. muciniphila* combined with inosine treatment. Moreover, *A. muciniphila* and inosine combination increased the expression levels of intestinal CD39, CD73, and adenosine A2A receptor (A2AR) along with enhanced proportions of $CD4^+CD39^+$Treg and $CD4^+CD73^+$Treg cells in the liver and spleen. The A2AR antagonist KW6002, blocked the beneficial effects of the *A. muciniphila* and inosine combination on liver injury in ALD mice.

Conclusion: This study reveals that the combination of *A. muciniphila* and inosine holds promise for ameliorating ALD by enhancing the gut ecosystem, improving intestinal barrier function, upregulating A2AR, CD73, and CD39

expression, modulating Treg cells functionality, and regulating the imbalance of Treg/Th17/Th1 cells, and these beneficial effects are partly A2AR-dependent.

KEYWORDS

alcoholic liver disease, *Akkermansia muciniphila*, inosine, gut microbiota, adenosine 2A receptor, Regulatory T Cells

1 Introduction

Alcoholic liver disease (ALD) encompasses alcoholic fatty liver disease, alcoholic hepatitis, alcoholic liver fibrosis, cirrhosis, and hepatocellular carcinoma. Globally, in 2020, approximately 1.34 billion men and 312 million women engaged in harmful alcohol consumption, leading to approximately 1.78 million deaths attributed to alcohol consumption (Cabezas, 2022; Huang et al., 2023). Reports indicate that 50% of cirrhosis-related mortality is linked to alcohol consumption, which poses a significant threat to human health (Xiao et al., 2019; Avila et al., 2020).

The primary morphological characteristics of ALD include steatosis, hepatocyte ballooning degeneration, necrosis, and lobular inflammation primarily characterized by neutrophil polymorphism. With disease progression, inflammatory and fibrotic alterations may extend to the hepatic veins, eventually advancing to the stage of micronodular cirrhosis (Addolorato et al., 2020a). Dysbiosis of the intestinal microbiota is a pathogenic mechanism of ALD that results inintestinal barrier dysfunction, altered microbiota, and immune cell changes (Ge et al., 2019, 2022). An increase in intestinal permeability, resulting from a breached intestinal barrier, gut-derived bacteria and products, such as LPS, the main component of endotoxin, can bind to Toll-like receptor 4 (TLR4), increasing the recruitment of Myd88 and ultimately accelerating the nuclear translocation of NF-κB. This activation induces the expression and release of multiple inflammatory factors, which stimulate the innate immune system and damage the liver (Albillos et al., 2020; Siddiqui and Cresci, 2020). Metabolic components produced by the intestinal microbiota, such as short-chain fatty acids, amino acids, and their derivatives, act on intestinal epithelial cells and directly or indirectly affect various immune cells of the mucosal immune system. ALD leads to marked immune cell imbalances, such as the Treg/Th17 cells ratio, which is closely associated with gut microbial dysbiosis in intestinal microecology (Gao and Bataller, 2011; Szabo, 2015). Thus, modulating intestinal microecology and barrier function is crucial for maintaining immune homeostasis in the gut–liver axis.

Akkermansia muciniphila, a strictly anaerobic Gram-negative bacterium, is the only known member of the human gut-associated phylum Verrucomicrobia, comprising approximately 0.5–5% of the human intestinal microbiota (Cani et al., 2022, 2023). Its abundance is negatively correlated with various diseases, including inflammatory bowel disease, neurodegenerative diseases, obesity, and autism (Rodrigues et al., 2022). Notably, *A. muciniphila* can upregulate the RORγt$^+$ Treg cell-mediated immunosuppressive response by interacting with TLR4, which plays a key role in intestinal mucosal homeostasis in mice (Liu et al., 2022). The influence of *A. muciniphila* on host physiology depends, in part, on the small molecules produced through the joint metabolism of the host and microbial community. One such metabolite, inosine, serves as a physiological energy source and exhibits potent anti-inflammatory and immunomodulatory properties (Saveljeva et al., 2022). A previous study revealed that pretreatment with inosine reduced acute liver damage and inflammation, in part by controlling the TLR4/NF-κB signaling pathway (Guo et al., 2021). However, the additional benefits and underlying mechanisms of the combination of *A. muciniphila* and inosine in ALD remain unclear.

One of the four adenosine receptors in the G-protein-coupled receptor superfamily, the adenosine 2A receptor (A2AR), exhibits strong anti-inflammatory properties that affect immune cell regulation (Haskó et al., 2008). The ectoenzymes CD39 and CD73, which are highly expressed in Tregs, hydrolyze the extracellular ATP (eATP) produced in injured tissues to adenosine (ADO), thereby reducing inflammation. Notably, the proliferation and maintenance of Tregs are related to the expression of CD39 and CD73. A2AR agonists significantly increase Treg numbers and enhance their immunosuppressive function (Haskó et al., 2008; Xing et al., 2023). Moreover, A2AR activation alleviates intestinal inflammation in inflammatory bowel diseases, potentially by promoting interleukin (IL)-10 and transforming growth factor-beta (TGF-β) secretion (Odashima et al., 2005). In A2AR-knockout models, IL-10 and TGF-β levels are significantly decreased, accompanied by an increase in interferon-gamma (IFN-γ) levels (Nowak et al., 2010). These cytokines play important roles in the regulation of Treg, Th17, and Th1 cell differentiation. These data suggest that the adenosine A2AR pathway may be involved in the differentiation and function of immune cells to maintain immune homeostasis in the gut–liver axis; however, this hypothesis requires verification.

In the present study, we investigated the potential protective effects of *A. muciniphila* combined with inosine in ALD mice. We analyzed the intestinal microbiota components, mucosal barrier, and the balance of Treg/Th17/Th1 in the liver and spleen. To further explore the potential mechanisms, we measured the levels of CD39, CD73, and A2AR in the intestine as well as the proportions of CD39$^+$ Tregs and CD73$^+$ Tregs in the liver and spleen. Our findings demonstrate that *A. muciniphila* combined with inosine may be a promising therapy for patients with ALD.

2 Materials and methods

2.1 *Akkermansia muciniphila* culture

The *A. muciniphila* strain acquired from the American Type Culture Collection (ATCC Number: BAA-835) was cultured in an anaerobic environment at 37°C using brain-heart infusion (BHI) (CM1135B, OXOID) liquid medium supplemented with 0.5% (wt/vol)

mucin (M2378, Sigma). The cultures were incubated for 48 h, centrifuged (3,500 rpm, 10 min, 4°C), washed twice in sterile saline, and resuspended at 5×10^9 CFU/mL (measured by absorbance at 630 nm) for intragastric injection (Grander et al., 2018).

2.2 Animals and treatment

Six to eight-week-old male C57BL/6 mice were procured from Beijing Vital River Laboratory Animal Technology Co., Ltd. and housed in an SPF environment. The ALD model was developed as previously described (Zhu et al., 2022). As shown in Supplementary Figure S2, mice were first fed a liquid control diet called Lieber-DeCali (Trophic Animal Feed High-tech Co., Jiangsu, China) for 1 week, after which they were fed an adaptive alcohol diet for 1 week (the ratio of Lieber-DeCarli control diet to ethanol diet was adjusted from 2:1 1:1 to 1:2 on days 2, 4 and 6, respectively). Following the acclimation phase, the mice were given a four-week supply of the full Lieber-DeCarli ethanol diet with 5% alcohol.

To investigate the effect of *A. muciniphila* combined with inosine on ALD, mice were randomly divided into five groups: (1) normal group (N, $n = 10$), maintained on a Lieber-DeCarli control diet until euthanasia; (2) model group (M, $n = 10$); (3) *A. muciniphila* treatment group (AKK, $n = 10$); (4) inosine (I4125, Sigma-Aldrich, St. Louis, MO, United States) treatment group (I, $n = 10$); (5) and the *A. muciniphila* and inosine combination group (AKK + I, $n = 10$). To investigate whether therapeutic effects of the combined intervention are mediated via A2AR, the ALD model animals were randomized into three groups: (1) the A2AR antagonist group (KW6002, $n = 5$), (2) the *A. muciniphila* and inosine combination group (AKK + I, $n = 5$), (3) and combination therapy +A2AR antagonist group (AKK + I + KW6002, $n = 5$).

For treatment with *A. muciniphila,* mice were gavaged with 1×10^9 CFU *A. muciniphila* suspended in 0.2 mL of sterile saline every other day for 4 weeks on a complete ethanol diet. For treatment with inosine, mice were gavaged inosine (300 mg/kg) every other day for 4 weeks on a complete ethanol diet. For treatment with KW6002, mice were intraperitoneally injected with 5 mg/kg/day of KW6002 during the last 2 weeks of the complete ethanol diet. Sterile saline was used as a negative control for intragastric or intraperitoneal injections.

At the end of the experiment, all mice were euthanized, and blood, liver, spleen, fecal, and intestinal tissues were collected. The study protocols were approved by the Ethics Committee of the Health Science Center of Ningbo University (approval number 11417).

2.3 Liver and intestine histopathology

Samples of the liver and small intestine were obtained, fixed for 24 h in 10% formalin, and embedded in paraffin. Hematoxylin and eosin were used to stain liver and small intestine sections for pathological evaluation. Standard protocols were followed for the immunohistochemical studies (Zhu et al., 2022). Sections immersed in paraffin were deparaffinized and rehydrated. Liver sections were incubated with anti-MPO (PA5-16672, 1:200 dilution; Thermo Fisher Scientific, Waltham, MA, United States) and anti-F4/80 (SP115, 1:100 dilution; Abcam, Cambridge, United Kingdom) antibodies to assess macrophage and neutrophil infiltration, respectively. Small intestinal sections were incubated with anti-CD39 (ab223842, 1:1000 dilution; Abcam), anti-CD73 (ab288154, 1:500 dilution; Abcam), and anti-A2AR (ab3461, 1:200 dilution; Abcam) antibodies to evaluate the levels of intestinal CD39, CD73, and A2AR proteins. PE-coupled secondary anti-rabbit IgG was used for detection. Liver and small intestine sections were rapidly frozen, cut into 4-μm sections, and stained with Oil Red O. Images were observed with a clear field microscope (Nikon, Tokyo, Japan) and analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, United States). Each specimen was randomly selected with three independent visual fields at 20× magnification. Liver hepatic steatosis was evaluated using the Brunt scale by a pathologist who was blinded to the study.

2.4 Serum biochemical analysis and ELISA assay

An automatic biochemical analyzer (AU5800; Beckman Coulter, Brea, CA, USA) was used to measure serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. ELISA kits (MultiSciences, China) were used to measure the levels of hepatic IL-17A, IFN-γ, and IL-10 proteins, in accordance with the manufacturer's instructions. ELISA kits (Nanjing Jiengcheng Bioengineering Institute, China) were used to measure the levels of hepatic glutathione (GSH), oxidized glutathione (GSSG), and superoxide dismutase (SOD), in accordance with the manufacturer's instructions. ELISA kits (MyBioSource) were used to measure the serum levels of LPS, following the manufacturer's instructions.

2.5 Real-time reverse transcriptase polymerase chain reaction (RT-PCR) assay

The RNA Extraction Kit (74,104; QIAGEN, Hilden, Germany) was used to extract total RNA, and the PrimeScript RT Reagent Kit (RR047A, Takara Bio, Shiga, Japan) was used to reverse-transcribe the extracted RNA into cDNA. TB Green Premix Ex Taq (RR420A; Takara Bio) was used to amplify cDNA. A Thermo Fisher QuantStudio5 real-time PCR system was used to quantify the amounts of RNA. The $2^{-\Delta\Delta Ct}$ method was used to assess the final data, and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as the reference gene for mRNA expression estimates. The primer sequences are listed in Supplementary Table S1.

2.6 Western blot analysis

Small intestinal tissues were homogenized in RIPA buffer (Solarbio, China) with a mixture of protease inhibitors. The Pierce BCA Protein Assay Kit (TransGen Biotech, China) was used to quantify protein concentration. Protein samples were subjected to electrophoresis using on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to PVDF membranes (MilliporeSigma, Burlington, MA, United States). Subsequently, the membranes were blocked with skimmed milk powder (Solarbio) and incubated overnight at 4°C with the following primary antibodies: anti-ZO-1 (ab96587, 1:1000 dilution; Abcam), anti-Occludin (A2601, 1:2000 dilution; ABclonal, Woburn, MA, United States), anti-Claudin-1

(ab307692, 1:1000 dilution; Abcam:1000), and anti-GAPDH (ab181602, 1:1000 dilution; Abcam). After five washes with TBST for 5 min each, the membranes were incubated with the respective secondary antibodies (goat anti-rabbit; SA00001-2, Proteintech, Rosemount, IL, United States) for 1 h at room temperature. Protein bands were visualized using a high-sensitivity ECL chemiluminescence kit (NCM Biotech, China) and gel imager (Bio-Rad Laboratories, Hercules, CA, USA), and band intensity was quantified using ImageJ software version 2.3.0 (National Institute of Health, Bethesda, MD, USA).

2.7 16S rRNA gene sequencing analysis and bioinformatics analysis

Feces were immediately collected and stored at −80°C. Bacterial DNA was extracted using a FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. The V3-V4 hypervariable regions of the 16S rRNA gene, along with spike-ins, were amplified using primers 341F (5′-CCT ACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVG GGTATCTAATCC-3′) and subsequently sequenced on an Illumina NovaSeq 6,000 sequencer (Illumina, San Diego, CA, United States) (BioProject ID PRJNA1071471). Usearch software was used to perform similarity level clustering analysis of reads, and reads with similarity greater than or equal to 97% were clustered into one operational taxonomic units (OTUs); RDP database was used as a reference database to taxonomically annotate the obtained OTUs. Principal Coordinate Analysis (PCoA) based on Unifrac distance was used to identify the microbial structure. Linear discriminant analysis (LDA) coupled with effect size measurement (LefSe) analysis was used to identify the microbial taxa with different degrees of enrichment in different treatment groups. The Kruskal-Wallis test ($p < 0.05$) was used to identify microbial taxa that differed among multiple groups.

2.8 Short-chain fatty acids (SCFAs) identification and quantification

Gas chromatography–mass spectrometry (GC–MS) was used to quantify typical SCFA for targeted metabolomics. Fecal samples were homogenized, centrifuged, filtered, and suspended in ultrapure water. Centrifugation was used to separate the ethyl acetate layer from the filtrate, and an equal volume of ethyl acetate was combined and incubated for 30 min at 4°C. SCFA standards at various concentrations were subjected to the same procedure. The SCFAs in the samples were characterized by comparing their retention times with those of the standards. A calibration curve based on the peak areas of the standards at different concentrations facilitated the quantification of SCFA levels.

2.9 Isolation of intrahepatic lymphocytes (IHL) and flow cytometry analysis

IHLs were isolated using a previously established method (Xu et al., 2021). Briefly, following anesthesia, the mouse liver was perfused with PBS. Liver tissues were collected and digested in RPMI-1640 medium containing collagenase IV (0.05%; C4-BIOC; Sigma-Aldrich) at 37°C for 30 min. To obtain single-cell suspensions, the digested cell suspensions were filtered through 70-μm nylon cell strainers. Centrifugation (400 × *g*) at room temperature for 30 min across a 30/70% discontinuous Percoll gradient was used to isolate IHLs (17,089,102; Cytiva). At interphase, cells were harvested, carefully washed, and resuspended in full RPMI-1640 medium supplemented with 10% FBS.

Regulatory T Cells were assayed using a Mouse Regulatory T Cell Staining Kit (88–8,111-40; Thermo Fisher Scientific), according to the manufacturer's instructions. For Th1 and Th17 cell analysis, lymphocytes were first incubated with Leukocyte Activation Cocktail (550,583; BD Pharmingen, Franklin Lakes, NH, United States) for 5 h, followed by staining with Fixable Viability Stain 780 (565,388; BD Pharmingen), CD3e FITC (553,061; BD Pharmingen), CD4 APC (553,051; BD Pharmingen), and CD8a PerCP-Cy5.5 (551,162; BD Pharmingen) at 4°C for 30 min in the dark. A Fixation/Permeabilization Kit (554,714; BD Pharmingen) was used for fixation and permeabilization, followed by staining with IL-17A PE (55,952; BD Pharmingen) and IFN-γ PE (55,442; BD Pharmingen) at 4°C for 1 h in the dark. Samples were processed on a FACS Symphony A1 and analyzed using FlowJo X software version 10 (Tree Star, Ashland, OR, United States).

2.10 Statistical analysis

Statistics and graphing were performed using GraphPad Prism 8 (San Diego, CA, USA). One-way analysis of variance followed by Tukey's multiple comparison test was used for continuous variables with normal distribution for comparison among multiple groups, and Kruskal-Wallis test was used for continuous variables with skewed distribution for comparison among multiple groups. Multiple comparisons were corrected by the Benjamini-Hochberg method, and $p < 0.05$ or false discovery rate (FDR) <0.05 were considered statistically significant differences. Data are presented as the mean ± SEM.

3 Results

3.1 Combination of *Akkermansia muciniphila* and inosine alleviated liver injury in ALD mice

In this study, we established an alcoholic liver injury model using a Lieber-DeCarli ethanol diet (5% ethanol). The model group exhibited elevated serum ALT, AST and LPS levels (Figures 1A,B), hepatocyte ballooning, and fat deposition (Figures 1C–E), along with significant macrophage and neutrophil infiltration (Supplementary Figure S3), compared to the normal group. This result suggests that alcohol exposure induced significant liver injury and inflammation. Application of *A. muciniphila* or inosine alone attenuated ALT, AST and LPS levels (Figures 1A,B), decreased ethanol-induced fat accumulation (Figures 1C–E), and reduced macrophage and

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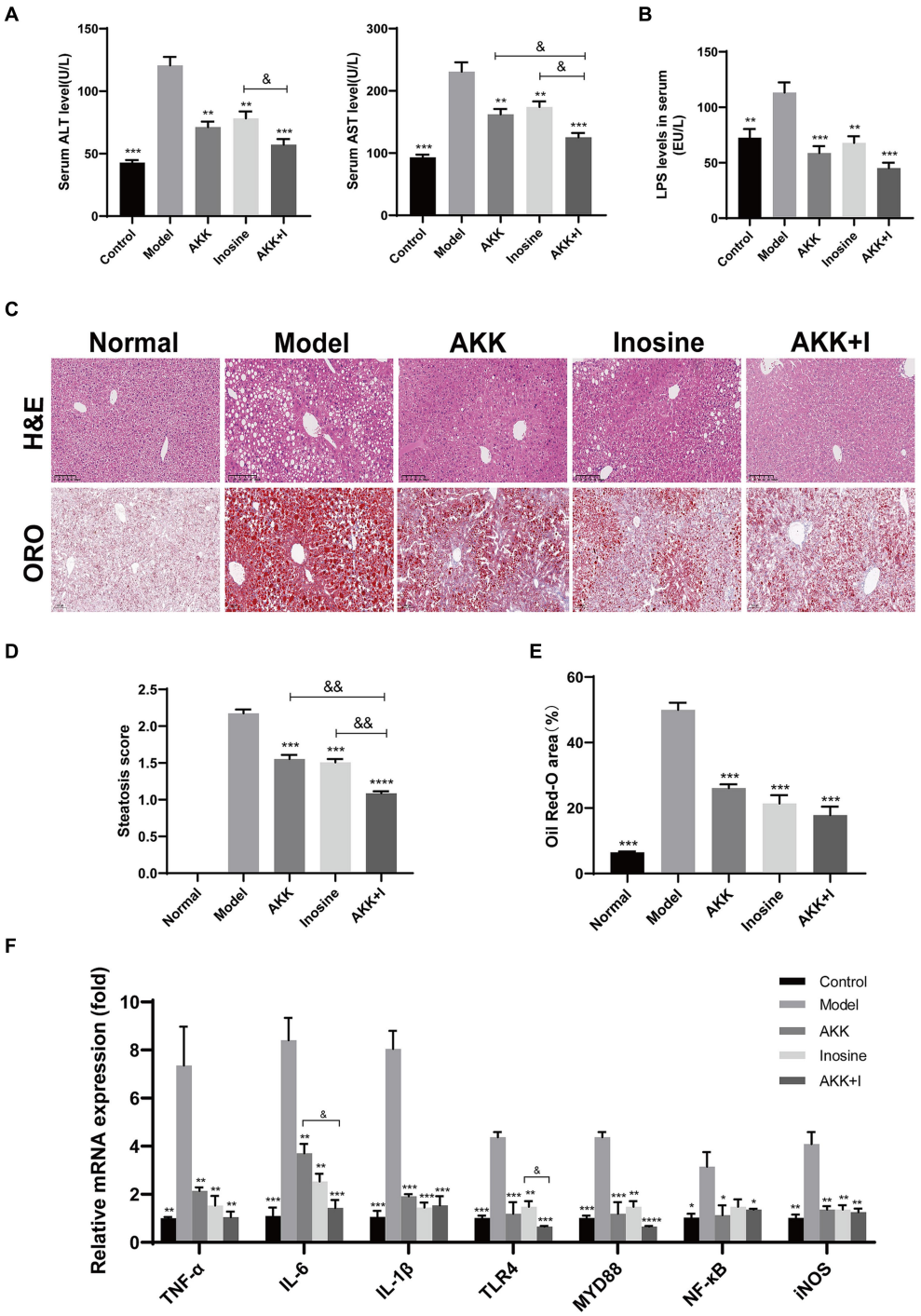

FIGURE 1
The combination of *Akkermansia muciniphila* and inosine alleviated liver injury in ALD mice. C57BL/6 mice were fed a Lieber-DeCarli diet containing 5% alcohol for 4 weeks. *A. muciniphila* (1×10^9 CFU/mouse), inosine (300 mg/kg), or a combination of *A. muciniphila* (1×10^9 CFU/mouse) and inosine (300 mg/kg) were administered orally every other day. All mice were euthanized in the 6th week. **(A)** Serum ALT and AST levels in different experimental groups. **(B)** The levels of LPS in the serum. **(C)** Representative images of hematoxylin and eosin and Oil Red O staining of liver sections (scale bar, 100 μm). **(D,E)** Statistical analysis of steatosis scores and Oil Red O staining. **(F)** Fold changes in the mRNA levels of proinflammatory cytokines determined via qRT-PCR. Data are shown as the mean ± SEM; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the model group; $\&p < 0.05$, $\&\&p < 0.01$ compared with the AKK + I group. $N = 10$ for each group.

neutrophil infiltration in the liver (Supplementary Figure S3). Notably, the combination of *A. muciniphila* and inosine further alleviated hepatic fat accumulation and attenuated ALT and AST levels, compared to treatment with *A. muciniphila* or inosine alone. These findings highlight the synergistic effect of *A. muciniphila* and inosine in ameliorating liver damage induced by chronic ethanol consumption.

3.2 Combination of *Akkermansia muciniphila* and inosine alleviates oxidative stress and inflammation in ALD mice

Given that oxidative stress is implicated in the pathogenesis of ALD, we evaluated the levels of glutathione, GSSG, and SOD in the five groups to assess susceptibility to oxidative stress. The levels of GSH, GSH/GSSG, and SOD were significantly reduced in the model group. Treatment with *A. muciniphila* significantly restored GSH levels compared to those in the model group, and the GSH/GSSG ratio was restored in both the *A. muciniphila* and inosine individual treatment groups. Notably, these effects were further pronounced in the combination treatment group (Supplementary Figures S4A,C,D). However, there were no significant differences in GSSG levels among the five groups (Supplementary Figure S4B).

To evaluate liver inflammation, we detected changes in the expression of representative inflammatory markers at the transcriptional level. The mRNA expression levels of *TNF-α*, *IL-6*, *IL-1β*, *TLR4*, *Myd88*, *NF-κB*, and *iNOS* were significantly higher in alcohol-fed mice than in normal mice, indicating that alcohol consumption induced liver inflammation (Figure 1F). Treatment with either *A. muciniphila* group or inosine decreased the transcription levels of these above indicators. Treatment with the combination further reduced the transcriptional levels of *IL-6* and *TLR4* and improved those of *IL-10* and *AMPK* (Figure 1F and Supplementary Figure S5). However, no significant differences was observed for the other indicators (*IL-2*, *COX-2*, *IκB-α*, and *Nfr2*) (Supplementary Figure S5). These results suggest that the combination of *A. muciniphila* with inosine ameliorates alcohol-induced oxidative stress and the hepatic inflammatory response.

3.3 Combination of *Akkermansia muciniphila* and inosine restored gut barrier function in ALD mice

Hematoxylin and eosin staining of mouse small intestines revealed irregular and shorter villi in the ALD model group compared to those in the normal group. Treatment with either *A. muciniphila* or inosine improved the villus lesions, and the *A. muciniphila* and inosine combination further increased the villus height-to-crypt depth ratio, indicating an improvement in villus–crypt connections during alcohol-induced barrier disruption (Figures 2A,B). Furthermore, we measured the expression of tight junction (TJ) proteins, including Claudin-1, Occludin, and ZO-1, in the small intestine. Compared with the normal group, alcohol feeding significantly reduced the mRNA expression of the genes encoding TJ proteins, whereas treatment with either *A. muciniphila* or inosine alone upregulated the expression of the genes. Notably, the combination treatment significantly restored Claudin-1 expression compared to that with *A. muciniphila* treatment alone (Figure 2C). The results of western blotting were consistent with the gene expression results; combination treatment with *A. muciniphila* and inosine further increased the expression levels of these TJ proteins compared to those in the individual treatment groups (Figures 2D–G). Overall, our findings suggest that the combined treatment with *A. muciniphila* and inosine improved intestinal barrier function in an ALD mouse model.

3.4 Combined treatment ameliorates alcohol-induced intestinal dysbiosis

We performed 16S rRNA sequencing analysis to investigate the changes in the gut microbiota in the altered intestinal environment. The results revealed alterations in bacterial abundance at the phylum and genus levels due to alcohol exposure (Figure 3A). Alcohol feeding increased the abundance of *Firmicutes* and *Proteobacteria* but decreased the proportion of *Bacteroidetes* compared to that in the normal group (Figure 3B). However, the combination of *A. muciniphila* and inosine specifically increased *Bacteroidetes, Akkermansia, Lactobacillus*, and *Clostridium IV*, while decreasing the abundance of *Firmicutes*, *Proteobacteria*, *Oscillibacter*, *Escherichia/Shigella*, and *Alistipes* (Figures 3B,C). Wilcoxon analysis and LDA scores indicated that *Bacilli, Desulfovibrionaceae*, *Mobilitalea*, and *Peptococcaceae* were significantly associated with the model group. *Verrucomicrobiaceae* and *Akkermansia* spp. were significantly enriched in the Akkermansia group. *Rikenellaceae*, *Sutterellaceae*, and *Alistipes* were the dominant taxa in the inosine group. *Barnesiell*a, *Clostridium IV*, and *Candidatus_ Saccharimonas* were enriched in the combination intervention group (Supplementary Figure S6). Overall, these results indicate that *A. muciniphila* combined with inosine effectively improved gut dysbiosis induced by ethanol feeding.

3.5 Combination of *Akkermansia muciniphila* and inosine increased SCFAs levels

SCFAs are indispensable signaling molecules in the gut–liver axis. Therefore, we conducted targeted metabolomic analysis to determine the concentrations of specific SCFAs, including acetate, propionate, isobutyrate, butyrate, 2-methylbutyrate, and valerate, in mouse fecal samples. Alcohol consumption markedly reduced the concentrations of acetic, propionic, butyric, and valeric acids (Figure 4). Conversely, *A. muciniphila* administration increased the production of propionic and butyric acids. Inosine-treated mice exhibited elevated levels of acetic, propionic, and butyric acids; however, the difference was not significant compared to the model group (Figure 4). Importantly, in the combination group, the levels of acetate, butyrate, and valerate were significantly increased compared to those in the model group. However, no significant differences were observed in isobutyrate or 2-methylbutyrate levels among the groups (Figure 4). These results suggest supplementation with both *A. muciniphila* and inosine partially reversed the alcohol-induced reduction in SCFAs.

3.6 Combined treatment regulates the imbalance of immune cells

We measured Treg, Th17, and Th1 cell numbers using flow cytometry and found that alcohol consumption decreased the proportion of Treg cells in the spleen and liver, while significantly increasing that of Th1 and Th17 cells (Figures 5A–C). Conversely, the administration of *A. muciniphila* or inosine alone increased Treg cell proportions, which were further increased by the combined *treatment with A. muciniphila* and inosine. The proportion of Th1 and Th17 cells in the *A. muciniphila* or inosine treatment groups was lower

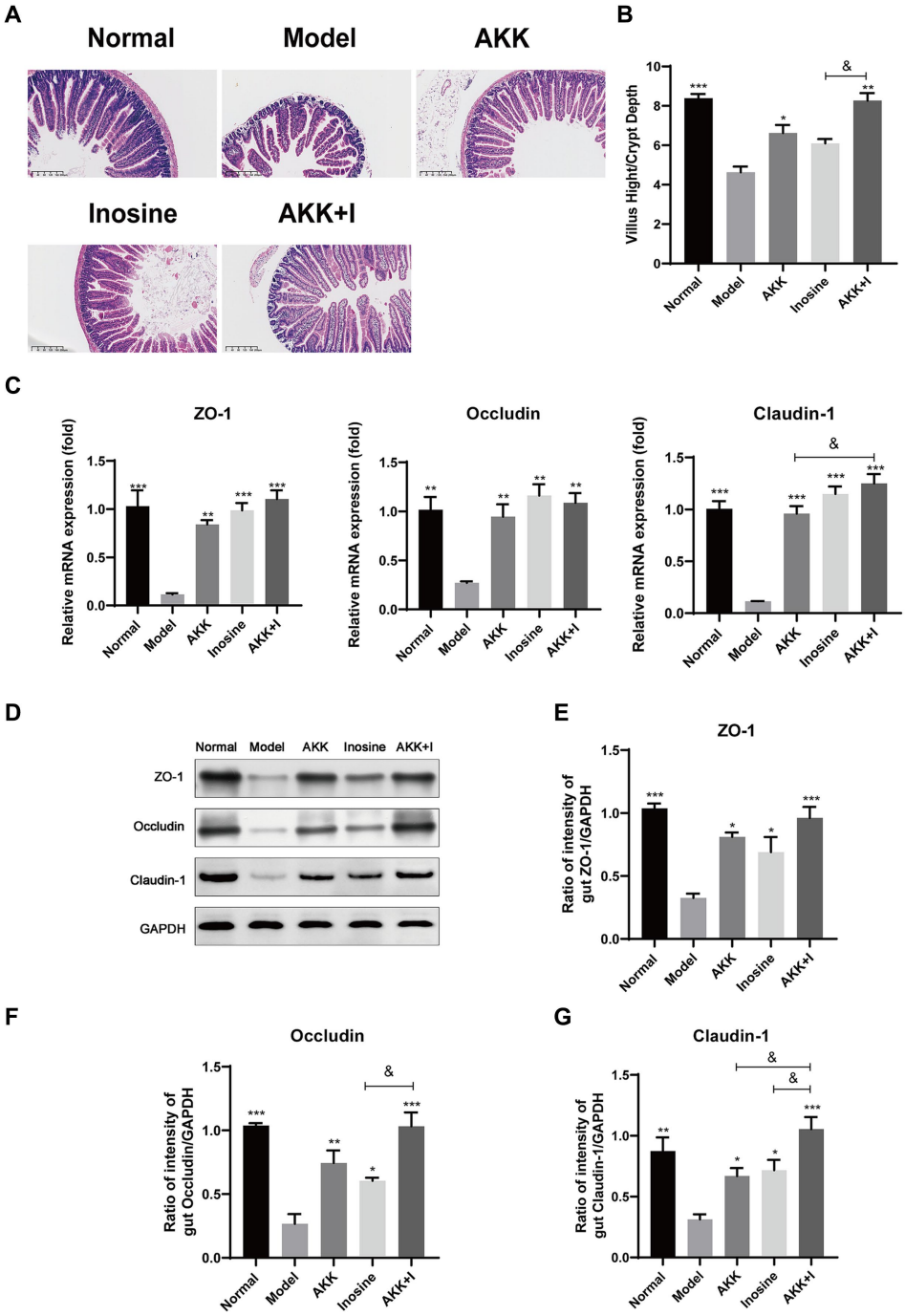

FIGURE 2
Improvement of intestinal barrier dysfunction with *Akkermansia muciniphila* and inosine treatment. ALD mice were treated as described in Figure 1. **(A)** Representative images of hematoxylin and eosin-stained histological sections of the small intestine (scale bar: 200 μm). **(B)** Measurements of the villi and crypts from three sections per group. **(C)** Fold changes in the mRNA levels of tight junction proteins determined via qRT-PCR. **(D–G)** representative Western blot images and histograms of the band densities of ZO-1, Occludin, and Claudin-1 in the small intestine of each experimental group. Data are shown as the mean ± SEM; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared to the model group; $\&p < 0.05$, $\&\&p < 0.01$ compared with the AKK + I group. $N = 10$ each group for hematoxylin and eosin-stained; $N = 6$ each group for RT-qPCR; $N = 3$ each group for Western blot.

than that in the model group, and this reduction in the proportions was more pronounced with the combined treatment (Figures 5A–C). Consequently, intrahepatic IFN-γ and IL-17A levels were significantly elevated in the model group, whereas intrahepatic IL-10 levels were remarkably decreased compared to those in the normal group (Figure 5D). Notably, the combination of *A. muciniphila* and inosine reduced IFN-γ and IL-17A levels and restored IL-10 levels (Figure 5D). Therefore, the combined treatment inhibited liver inflammation by restoring the balance of dysregulated Th17/Th1/Treg immune responses.

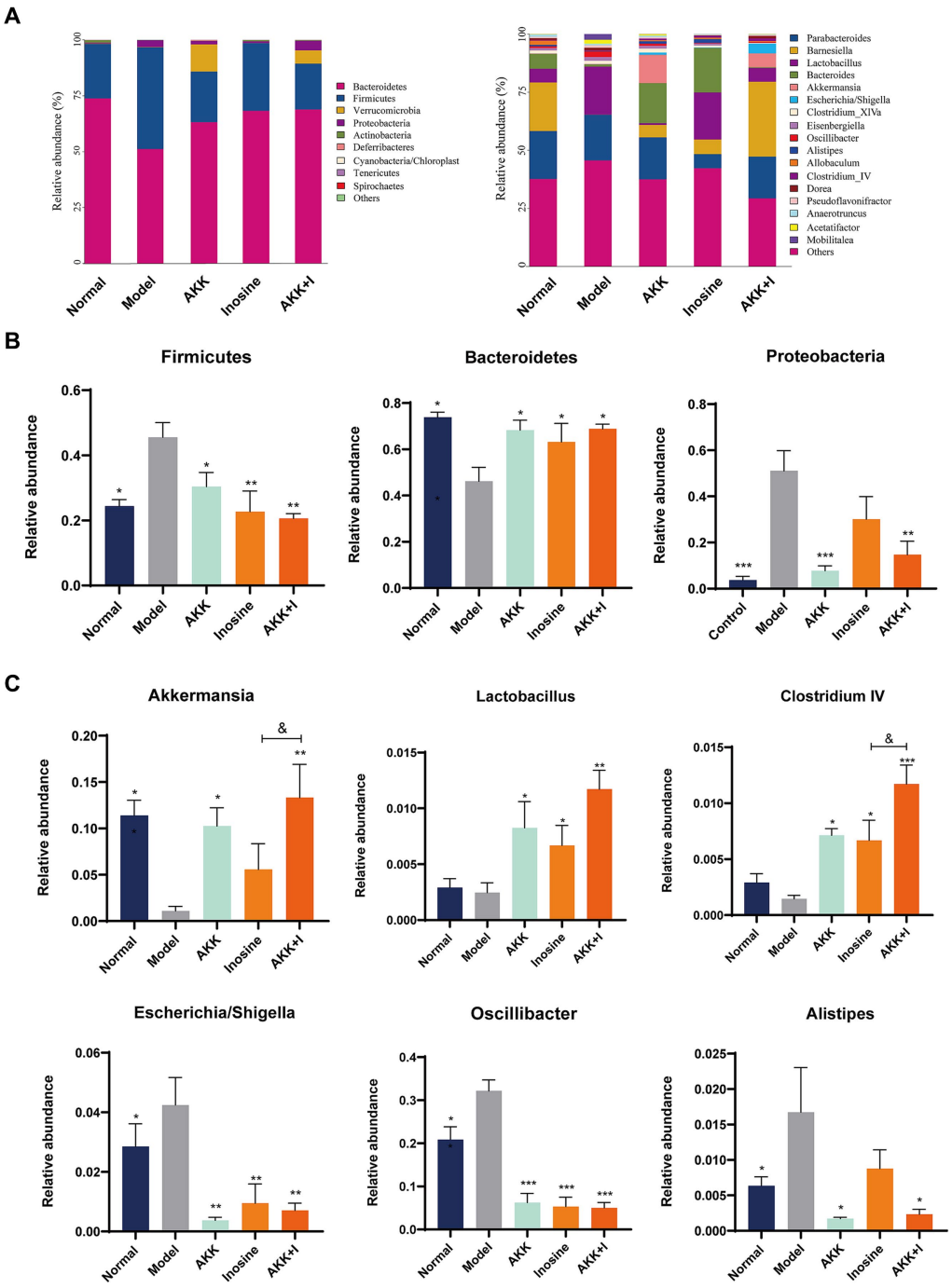

FIGURE 3
The combination of *Akkermansia muciniphila* and inosine regulates alcohol-induced intestinal dysbiosis. ALD mice were treated as described in Figure 1. **(A)** Relative abundance of predominant bacteria (>1% in each sample) at the phylum and genus levels. **(B)** Relative abundances of *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* at the phylum level in feces. **(C)** The relative abundance of *Bacteroides*, *Lactobacillus*, *Clostridium* IV, *Oscillibacter*, *Escherichia/Shigella*, and *Alistipes* at the genus level in feces. Data are shown as the mean ± SEM; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared to the model group; $\&p < 0.05$, $\&\&p < 0.01$ compared with the AKK + I group. $N = 6$ for each group.

3.7 Therapeutic effects of the combined *Akkermansia muciniphila* and inosine treatment might be mediated by CD39-CD73-A2AR pathway

The adenosine/A2AR pathway is crucial for the suppression of inflammation and immune cell differentiation. Alcohol consumption significantly reduced *CD39*, *CD73*, and *A2AR* mRNA expression levels in the small intestine compared to that in the normal group, whereas inosine administration significantly increased them (Figure 6A). In addition, *A. muciniphila*-treated mice exhibited elevated levels of these markers, but the differences were not significant compared to the model group. Treatment with the *A. muciniphila* and inosine combination significantly upregulated the mRNA expression of *CD39*, *CD73*, and

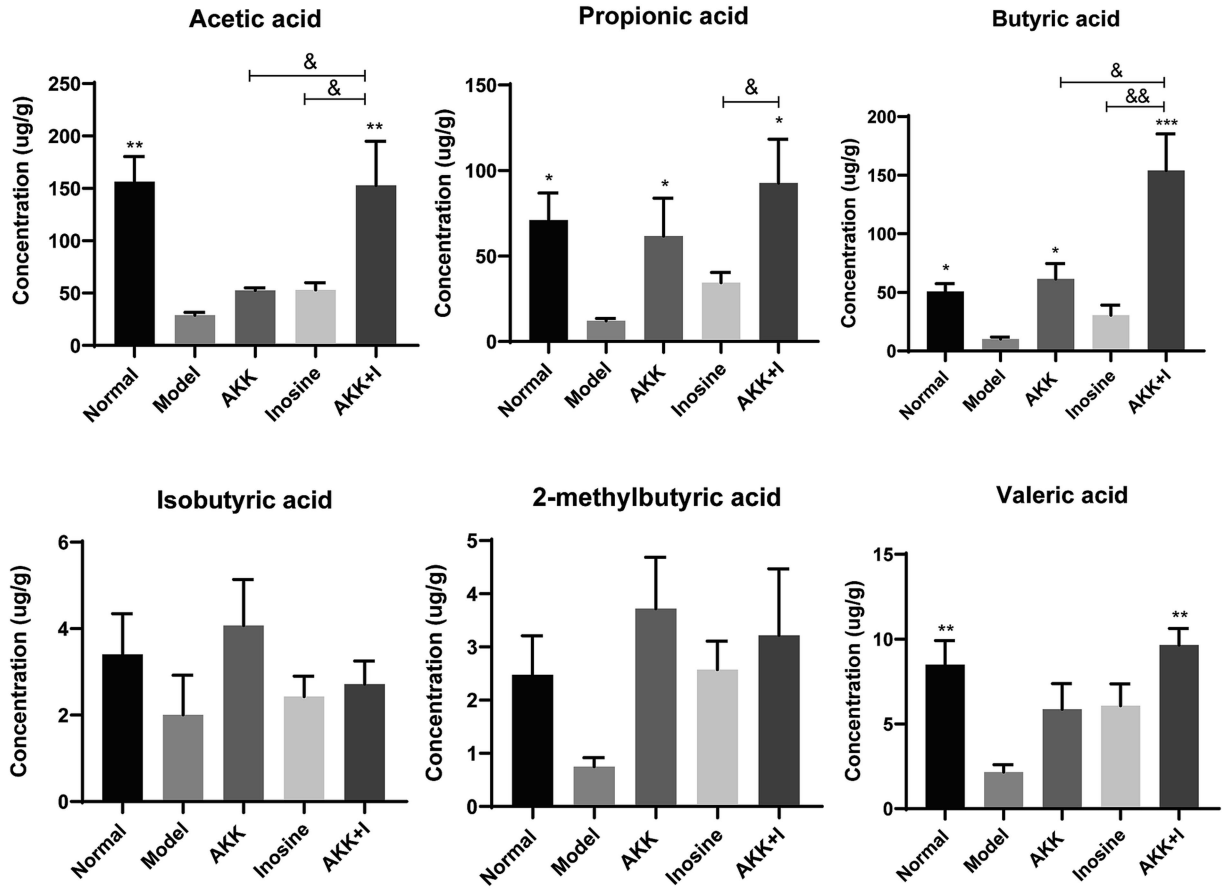

FIGURE 4
The combination of *Akkermansia muciniphila* and inosine improves SCFA production. ALD mice were treated as described in Figure 1. **(A)** Levels of SCFAs in fecal samples from different groups. Data are shown as the mean ± SEM; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared to the model group; $\&p < 0.05$, $\&\&p < 0.01$ compared with the AKK + I group. $N = 6$ for each group.

A2AR (Figure 6A). To confirm our qRT-PCR results, we performed immunohistochemistry analysis and found that both *A. muciniphila* or inosine treatment alone increased CD39 and A2AR protein expression compared to that in the ALD model group (Figures 6B,C). However, combined *A. muciniphila* and inosine treatment further upregulated CD39, CD73, and A2AR protein levels (Figures 6B,C).

As CD39 and CD73, which are crucial for immunosuppression, are highly expressed in Tregs, we examined the proportions of $CD4^+CD39^+$ Tregs and $CD4^+CD73^+$ Tregs in the spleen and liver. The proportions of $CD4^+CD39^+$ Tregs and $CD4^+CD73^+$ Tregs in the spleen were significantly decreased in the model group but restored in the *A. muciniphila* or inosine groups, and this effect was further pronounced in the combination group (Figures 6D,E). The ratio of $CD4^+CD39^+$ Tregs to $CD4^+CD73^+$ Tregs in the liver was lower than that in the normal group, with no significant differences among the treatment groups (Figures 6D,E).

3.8 Therapeutic effects of *Akkermansia muciniphila* + inosine are blocked by the A2AR antagonist KW6002

We investigated whether A2AR acts as a mediator for the beneficial effects induced by the combination of *A. muciniphila* and inosine. Compared to the *A. muciniphila* combined with inosine group, the serum LPS levels in the *A. muciniphila* + inosine plus KW6002 group were significantly increased (Figure 7A), more severe balloon-like changes and fat deposition were observed in the liver (Figures 7B,C), the villus-crypt structure of the small intestine was more severely damaged (Figure 7B), and the levels of intestinal TJ proteins were significantly decreased (Figures 7E,F). In addition, compared to the *A. muciniphila* combined with inosine group, the mRNA levels of *CD39*, *CD73*, and *A2AR* in the small intestine of mice in the *A. muciniphila* + inosine plus KW6002 group were significantly reduced (Figure 7D), the proportions of Tregs, $CD39^+$Tregs and $CD73^+$Tregs in the spleen and liver were also significantly reduced (Figure 7G; Supplementary Figure S2). KW6002 blocked the therapeutic effect of AKK combined with inosine. Compared to the *A. muciniphila* + inosine plus KW6002 group, when KW6002 was treated alone, the mice showed the more severe pathological manifestations about these indicators. Our results suggest that the protective effect of *A. muciniphila* combined with inosine for ALD is achieved, in part, through the A2AR signaling pathway.

4 Discussion

ALD is the primary cause of chronic liver disease, and its end stages are associated with higher complication rates of spontaneous

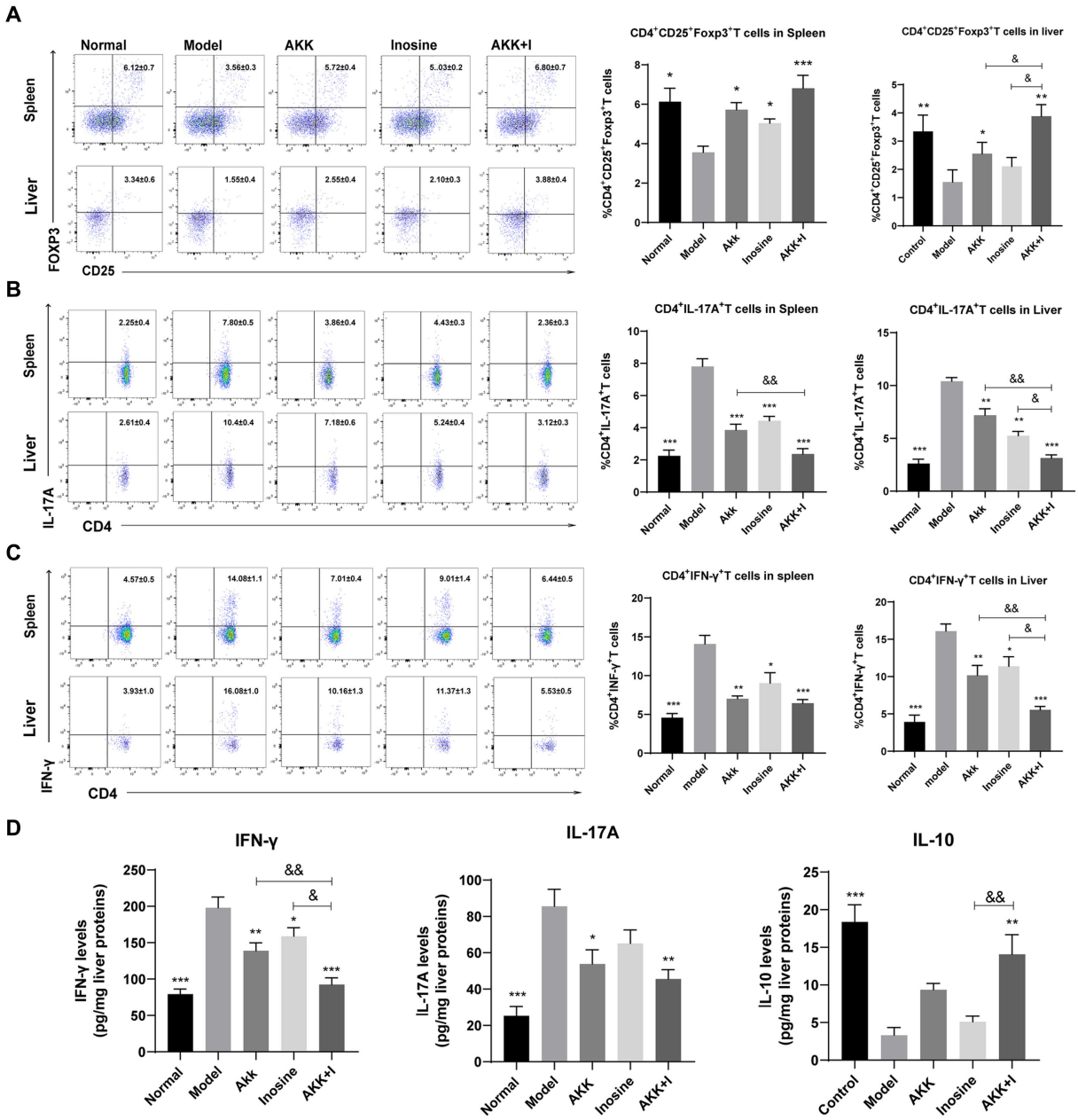

FIGURE 5
The combination of *Akkermansia muciniphila* and inosine reduces Th1 and Th17 cells but increases Treg cells in the spleen and liver. ALD mice were treated as described in Figure 1. **(A)** Representative flow cytometric plots and quantification of Tregs in the spleen and liver. **(B)** Representative flow cytometric plots and quantification of Th17 cells in the spleen and liver. **(C)** Representative flow cytometric plots and quantification of Th1 cells in the spleen and liver. **(D)** Protein levels of INF-γ, IL-17A, and IL-10 in the liver, as measured using ELISA. Data are shown as the mean ± SEM; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared to the model group; $\&p < 0.05$, $\&\&p < 0.01$ compared with the AKK + I group. $N = 7–10$ for each group.

peritonitis and hepatic encephalopathy than other liver diseases (Bhandari et al., 2020). Research indicates that a compromised mucosal barrier and severe dysbiosis of the intestinal microbiota are factors in the immunological imbalance in the gut–liver axis that causes intrahepatic inflammation in ALD (Yan et al., 2023). Similarly, in the present study, we discovered that alcohol exposure severely damaged the intestinal barrier, resulting in the depletion of potential probiotic genera, such as *Lactobacillus*, *Clostridium* IV, and *Akkermansia*, and the enrichment of opportunistic pathogens, such as *Oscillibacter*, *Escherichia/Shigella*, and *Alistipes*, in ALD. Therefore, targeting the gut microbiota is a promising therapeutic strategy for treating ALD.

The abundance of *A. muciniphila* is significantly reduced in patients with ALD (Addolorato et al., 2020b). While accumulating evidence supports the beneficial role of *A. muciniphila* in autoimmune hepatitis and non-alcoholic fatty liver disease (NAFD) (Wu et al., 2017; Qu et al., 2023), its exploration in the context of ALD is limited. In a previous study, we treated ALD with varying concentrations of inosine combined with *Lactobacillus rhamnosus* and found that 300 mg/kg inosine combined with *L. rhamnosus* demonstrated

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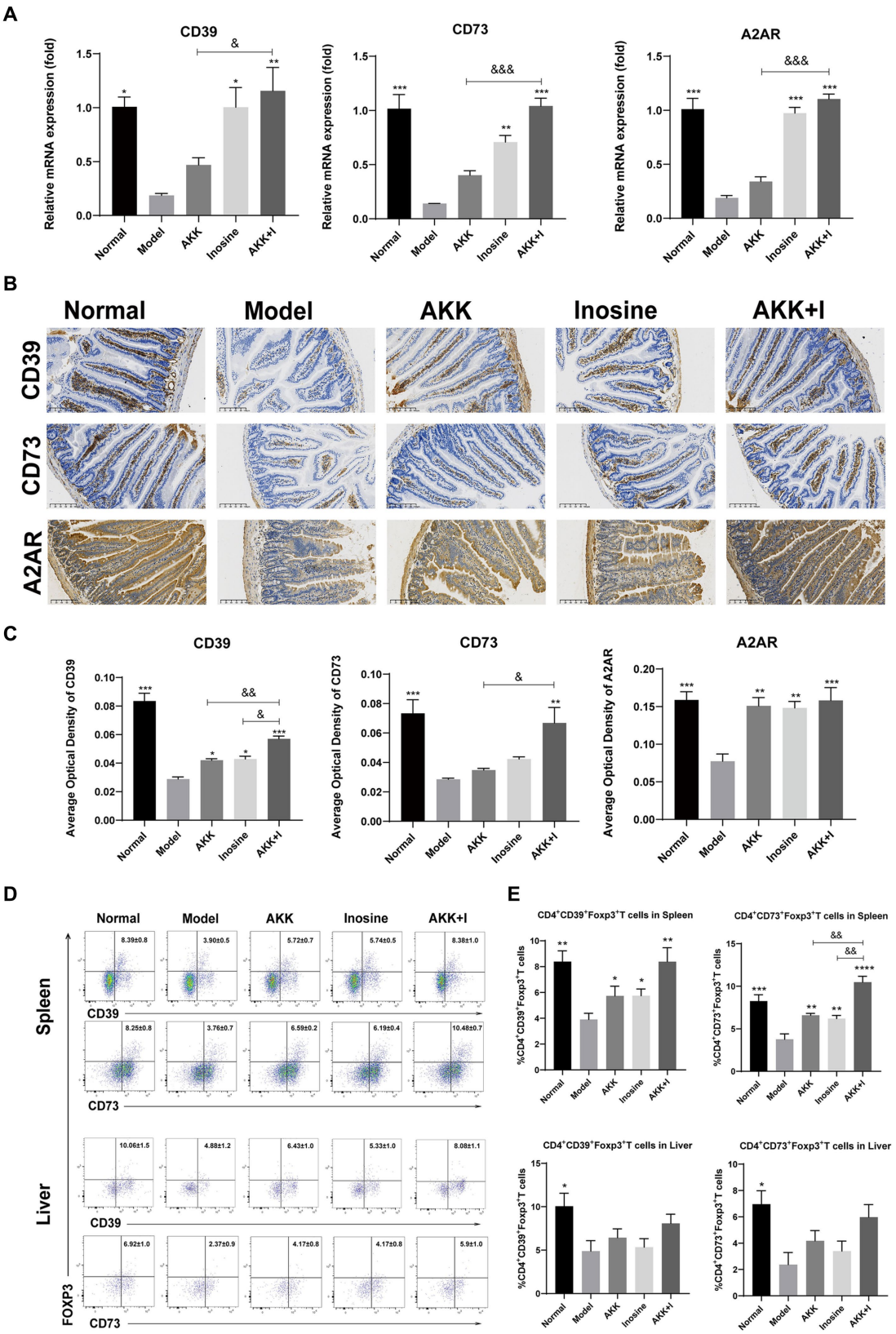

FIGURE 6
The combination of *Akkermansia muciniphila* and inosine increases the expression of CD39, CD73, and A2AR in the intestine and upregulates the ratios of CD39$^+$ and CD73$^+$ Treg cells in CD4$^+$ T cells from the spleen and liver. ALD mice were treated as described in Figure 1. **(A)** Fold change in mRNA levels of *CD39*, *CD73*, and *A2AR* determined using qRT-PCR. **(B)** Representative images of CD39, CD73, and A2AR proteins in the small intestine, detected via immunohistochemical staining (scale bar, 100 μm). **(C)** Mean optical densities of CD39, CD73, and A2AR. **(D,E)** Flow cytometric plots of

(Continued)

FIGURE 6 (Continued)

CD39$^+$ Treg cells and CD73$^+$ Treg cells, and quantitative analysis of cell percentages in the spleen and liver. Data are shown as the mean ± SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to the model group; &$p < 0.05$, &&$p < 0.01$ compared with the AKK + I group. $N = 6$ each group for RT-qPCR; $N = 10$ each group for immunohistochemical staining; $N = 7–10$ each group for Flow cytometric.

superior regulation of ALD-induced intestinal microecological disorders (Zhu et al., 2022). In the present study, the combination of *A. muciniphila* and inosine alleviated liver injury, as evidenced by the reduced expression of TNF-α, IL-6, and IL-1β; alleviated hepatocyte degeneration and liver fat accumulation; reduced infiltration of macrophages and neutrophils; and corrected the redox imbalance. In addition, the combined therapy reduced serum LPS levels, downregulated hepatic TLR4, MyD88, and NF-κB expression, and upregulated intestinal TJ protein expression levels, suggesting that its anti-inflammatory effects are closely related to the improved intestinal barrier function. To the best of our knowledge, this is the first report on the efficacy of the combination of *A. muciniphila* and inosine in preventing ALD.

In this study, the combined supplementation of *A. muciniphila* and inosine significantly decreased the relative abundances of *Oscillibacter*, *Escherichia/Shigella*, and *Alistipes*. Prior research has reported the enrichment of *Oscillibacter* in patients with chronic metabolic diseases such as NAFLD (Rodriguez-Diaz et al., 2022). *Escherichia/Shigella* increases endogenous ethanol, secondary bile acids, and endotoxin production, all of which are known to exacerbate hepatic inflammation (Baltazar-Díaz et al., 2022). Alcohol consumption increases the levels of *Alistipes*, an aggravating factor associated with intestinal inflammation (Pisani et al., 2022). In contrast, the combination treatment effectively increased the relative abundance of *Lactobacillus*, *Clostridium* IV, and *Akkermansia*, three important intestinal tract producers of SCFAs (Li et al., 2023). SCFAs, which are crucial energy sources for colon epithelial cells, play a vital role in maintaining gut barrier functions by inducing genes encoding TJs that exhibit immunomodulatory and anti-inflammatory properties (Silva et al., 2018; Gonzalez et al., 2019). Higher butyrate levels decrease intestinal chemotaxis and inflammation by promoting Treg cell differentiation by inhibiting histone deacetylase or activation of GPR signaling (Arpaia et al., 2013). In addition, butyrate reduces autoimmune responses and steatohepatitis by modulating T cell development through enterohepatic immunity (Hu et al., 2018). Based on targeted metabolomic analysis, we observed that the levels of SCFAs, including propionic, butyric, valeric, and acetic acids, markedly decreased after alcohol exposure. However, the reshaping of the microbial community by *A. muciniphila* combined with inosine was characterized by the dominance of beneficial bacteria favoring SCFA synthesis. Consequently, this combination restored acetate, propionate, and butyrate levels to appropriate concentrations. These findings suggest that an increase in the abundance of SCFA-producing bacteria may play a crucial role in the regulation of the intestinal mucosal immune response.

In addition to the gut microbiota, A2AR is also involved in maintaining intestinal homeostasis (Sun et al., 2021). Studies have indicated high expression of CD39 and CD73 in immune cells within the intestine, closely related to the intestinal lumen environment (Weinhage et al., 2015; Zhu et al., 2021). Moreover, acute *Toxoplasma gondii* infection leads to a marked decrease in CD73 expression and adenosine content in the intestinal lumen, resulting in intestinal mucosal injury and exacerbation of hepatic damage, whereas activation of adenosine A2AR significantly ameliorates intestinal mucosal injury (Francois et al., 2015). In the present study, alcohol consumption reduced the expression of A2AR, CD39, and CD73 in the small intestine. Conversely, the combination of *A. muciniphila* and inosine significantly increased intestinal CD39, CD73, and A2AR expression levels in intestinal submucosal immune cells, potentially contributing to the protective effect of this symbiotic combination on intestinal immune homeostasis and the intestinal barrier.

A2AR is highly expressed on T cell surfaces and is closely associated with their differentiation and functional changes (Vigano et al., 2019). Adenosine inhibits effector T cell function by binding to A2AR, interfering with TCR activation and the downstream signaling of co-stimulatory molecules (Alam et al., 2020). In patients with alcohol-related cirrhosis, a strong correlation was observed between the severity of their condition and the Th1 response intensity, and the secretion of IFN-γ by Th1 cells directly contributed to hepatic injury (González-Reimers et al., 2012). Moreover, patients with alcoholic hepatitis exhibit liver infiltration by Th17 cells, which correlates with hepatic damage (He et al., 2021). Our study identified a significant immune cell imbalance in the alcoholic liver model group compared to the normal group. We verified that alcohol exposure increased the number of Th1 and Th17 cells, and this increase was reversed by the combination of inosine and *A. muciniphila*. Moreover, the combination therapy significantly decreased the liver expression of IL-17A and INF-γ induced by alcohol. Tregs are a subset of immune-suppressive cells that are crucial in several liver diseases (Qu et al., 2022). In the present study, alcohol reduced the proportion of Treg cells, whereas treatment with *A. muciniphila*, alone or in combination with inosine, increased the proportion of Treg cells in ALD mice. In addition, we observed a reduced number of Tregs expressing CD39 and CD73 following alcohol intake. In the context of ALD induction using CD39-knockout mice, the absence of CD39 expression in Tregs results in the impairment of their immunosuppressive capabilities. Consequently, this leads to a heightened inflammatory response in CD39-knockout mice compared with wild-type mice (Xia et al., 2022). Similarly, Tregs derived from an autoimmune hepatitis model generated a reduced amount of anti-inflammatory substances (TGF-β and IL-10) compared to control Tregs, associated with impaired CD73 expression on the Treg surface (Huang et al., 2021). Furthermore, adenosine produced by Tregs can act on A2AR on the surface of various immune cells, such as Th1, Th2, and Th17 cells. This interaction reduces the secretion of cytokines, such as IFN-γ, IL-17, and IL-2, thereby inhibiting the differentiation and maturation of Th1 and Th2 (Zhang et al., 2023). Notably, supplementation with *A. muciniphila* combined with inosine increased the proportion of CD39$^+$ and CD73$^+$ Tregs in the liver and spleen, suggesting that *A. muciniphila* and inosine may regulate Treg cell function through the CD39-CD73-A2AR pathway. Importantly, in this study, A2AR antagonist blocked the beneficial effects of *A. muciniphila* combined with inosine in alleviating liver injury, repairing intestinal barrier function, and increasing Treg cells proportion in ALD, suggesting that A2AR may be a potential new target for the treatment of ALD.

Regarding the future directions of our study, although supplementation with *A. muciniphila* and inosine has demonstrated promising therapeutic effects in models of ALD, the specific components

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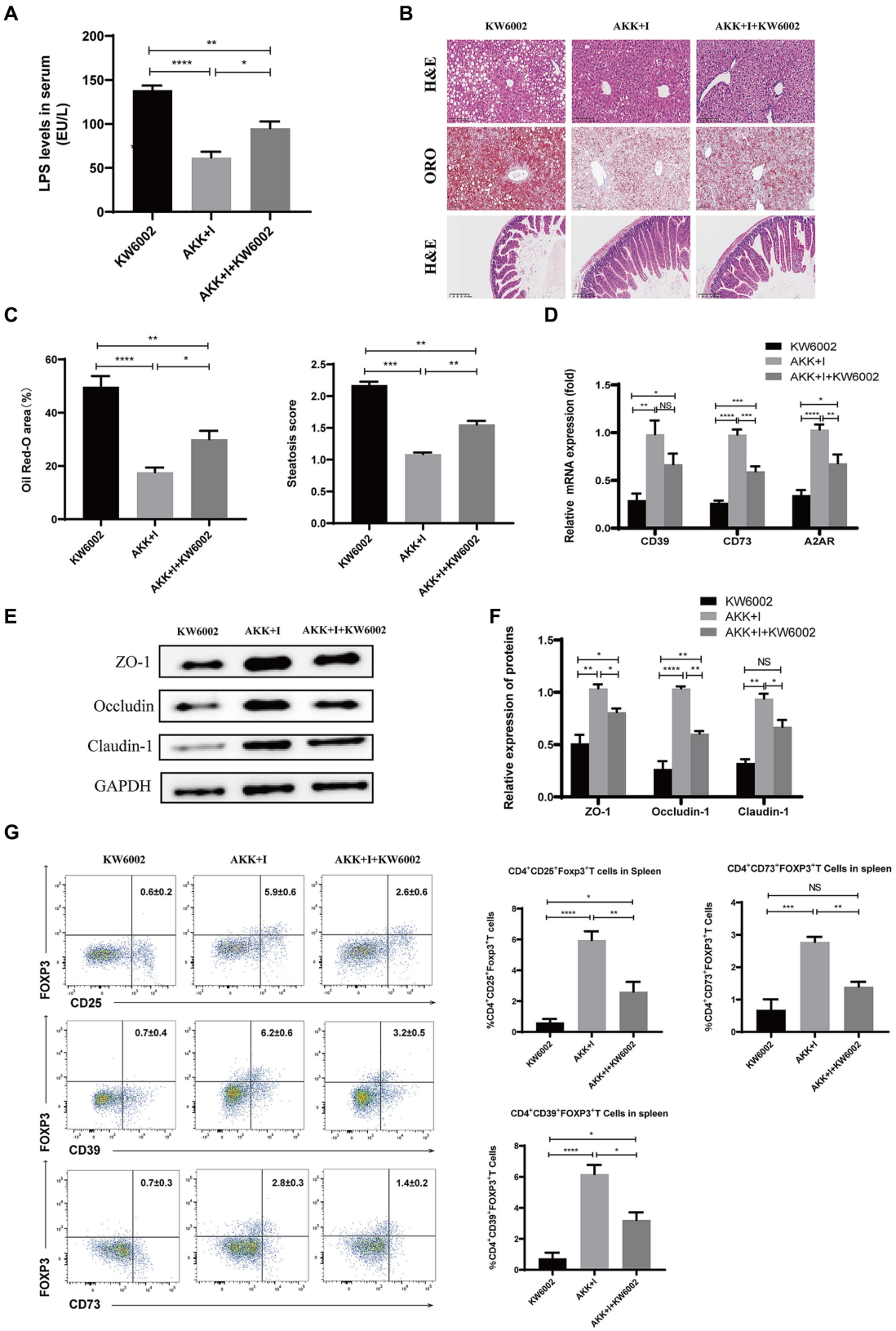

FIGURE 7
The combination of *Akkermansia muciniphila* and inosine exerts protective effects against ALD partly through A2AR. C57BL/6 mice were fed a Lieber-DeCarli diet containing 5% alcohol for 4 weeks. A combination of *A. muciniphila* (1×10^9 CFU/mouse) and inosine (300 mg/kg) was administered orally every other day. KW6002 (5 mg/kg/day) was injected intraperitoneally during the last 4 weeks of the complete ethanol diet. All mice were euthanized in the 6th week. **(A)** The levels of LPS in serum. **(B)** Representative images of H&E and Oil Red O staining of liver sections (scale bar, 100 μm), and H&E-

(Continued)

FIGURE 7 (Continued)

stained histological sections of the small intestine (scale bar: 200 μm). **(C)** Statistical analysis of steatosis scores and Oil Red O staining. **(D)** Fold change in mRNA levels of *CD39*, *CD73*, and *A2AR* determined via qRT-PCR. **(E,F)** Representative Western blot images and histograms of the band densities of ZO-1, Occludin, and Claudin-1 in the small intestine of each experimental group. **(G)** Flow cytometric plots of Tregs, $CD39^+$ Treg, and $CD73^+$ Tregs, and quantitative analysis of cell percentages in the spleen. Data are shown as the mean ± SEM; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. $N = 5$ for each group.

of *A. muciniphila* that synergizes with inosine remains unclear. Further investigation into the interactions between *A. muciniphila*'s outer membrane proteins or vesicles and inosine is warranted. Furthermore, the specific mechanism by which *A. muciniphila* combined with inosine regulates the CD39-CD73-A2AR pathway needs to be elucidated. We plan to address this gap through future experiments using knockout mouse models and *in vitro* studies. Finally, our intervention was conducted entirely in the mouse model, and the beneficial effects from those in humans warrant further investigation in clinical trials.

5 Conclusion

In conclusion, this study investigated the effects of the combination of *A. muciniphila* and inosine on the gut–liver axis in alcohol-fed mice. Our results suggest that this combined treatment can ameliorate liver injury in an ALD mouse model by restoring gut microbiota balance, regulating intestinal barrier function, reducing inflammatory cytokine levels, ameliorating oxidative stress, and increasing the proportions of Tregs while decreasing the proportions of Th1 and Th17 cells. In addition, *A. muciniphila* and inosine combination therapy increased the expression of A2AR, CD73, and CD39 in the intestinal mucosa, and increased the proportions of $CD39^+$ Treg cells and $CD73^+$ Treg cells. This modulation contributes to the regulation of the immunity of the "gut–liver axis" and inhibits the immune response in intrahepatic inflammation. Therefore, combined therapy with *A. muciniphila* and inosine holds promise as a potential therapeutic strategy for patients with ALD.

Data availability statement

16S rRNA gene sequencing data are deposited in the NCBI Sequence Read Archive (SRA) database with accession numbers PRJNA1071471.

Ethics statement

The animal study was approved by Ethics Committee of the Health Science Center of Ningbo University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

LW: Writing – original draft, Software, Methodology, Formal analysis, Conceptualization. YP: Writing – original draft, Methodology. YuG: Writing – original draft, Software, Investigation. YZ: Writing – original draft, Data curation. HJ: Writing – original draft, Formal analysis. YiG: Writing – original draft, Resources. CL: Writing – original draft, Investigation. YW: Writing – original draft, Data curation. JL: Writing – original draft, Resources. YC: Writing – review & editing, Project administration. CK: Writing – review & editing, Visualization. LX: Writing – review & editing, Visualization, Project administration, Conceptualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1355225/full#supplementary-material

References

Addolorato, G., Abenavoli, L., Dallio, M., Federico, A., Germani, G., Gitto, S., et al. (2020a). Alcohol associated liver disease 2020: a clinical practice guideline by the Italian Association for the Study of the liver (AISF). *Dig. Liver Dis.* 52, 374–391. doi: 10.1016/j.dld.2019.12.008

Addolorato, G., Ponziani, F. R., Dionisi, T., Mosoni, C., Vassallo, G. A., Sestito, L., et al. (2020b). Gut microbiota compositional and functional fingerprint in patients with alcohol use disorder and alcohol-associated liver disease. *Liver Int.* 40, 878–888. doi: 10.1111/liv.14383

Alam, M. S., Cavanaugh, C., Pereira, M., Babu, U., and Williams, K. (2020). Susceptibility of aging mice to listeriosis: role of anti-inflammatory responses with enhanced Treg-cell expression of CD39/CD73 and Th-17 cells. *Int. J. Med. Microbiol.* 310:151397. doi: 10.1016/j.ijmm.2020.151397

Albillos, A., de Gottardi, A., and Rescigno, M. (2020). The gut-liver axis in liver disease: pathophysiological basis for therapy. *J. Hepatol.* 72, 558–577. doi: 10.1016/j.jhep.2019.10.003

Arpaia, N., Campbell, C., Fan, X., Dikiy, S., van der Veeken, J., deRoos, P., et al. (2013). Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* 504, 451–455. doi: 10.1038/nature12726

Avila, M. A., Dufour, J. F., Gerbes, A. L., Zoulim, F., Bataller, R., Burra, P., et al. (2020). Recent advances in alcohol-related liver disease (ALD): summary of a gut round table meeting. *Gut* 69, 764–780. doi: 10.1136/gutjnl-2019-319720

Baltazar-Díaz, T. A., González-Hernández, L. A., Aldana-Ledesma, J. M., Peña-Rodríguez, M., Vega-Magaña, A. N., Zepeda-Morales, A. S. M., et al. (2022). Escherichia/shigella, SCFAs, and metabolic pathways-the triad that orchestrates intestinal dysbiosis in patients with decompensated alcoholic cirrhosis from Western Mexico. *Microorganisms* 10:1231. doi: 10.3390/microorganisms10061231

Bhandari, R., Khaliq, K., Ravat, V., Kaur, P., and Patel, R. S. (2020). Chronic alcoholic liver disease and mortality risk in spontaneous bacterial peritonitis: analysis of 6,530 hospitalizations. *Cureus* 12:e8189. doi: 10.7759/cureus.8189

Cabezas, J. (2022). Management of Alcohol-Related Liver Disease and its Complications. *Clin. Drug Investig.* 42, 47–53. doi: 10.1007/s40261-022-01143-9

Cani, P. D., Depommier, C., Derrien, M., Everard, A., and de Vos, W. M. (2022). *Akkermansia muciniphila*: paradigm for next-generation beneficial microorganisms. *Nat. Rev. Gastroenterol. Hepatol.* 19, 625–637. doi: 10.1038/s41575-022-00631-9

Cani, P. D., Van Hul, M., and Bachmann, R. (2023). *Akkermansia muciniphila* derived tripeptide jams the gear of sepsis, inflammation and mortality. *Gut* 73, 3–4. doi: 10.1136/gutjnl-2023-331092

Francois, V., Shehade, H., Acolty, V., Preyat, N., Delrée, P., Moser, M., et al. (2015). Intestinal immunopathology is associated with decreased CD73-generated adenosine during lethal infection. *Mucosal Immunol.* 8, 773–784. doi: 10.1038/mi.2014.108

Gao, B., and Bataller, R. (2011). Alcoholic liver disease: pathogenesis and new therapeutic targets. *Gastroenterology* 141, 1572–1585. doi: 10.1053/j.gastro.2011.09.002

Ge, L., Chen, D., Chen, W., Cai, C., Tao, Y., Ye, S., et al. (2019). Pre-activation of TLR3 enhances the therapeutic effect of BMMSCs through regulation the intestinal HIF-2α signaling pathway and balance of NKB cells in experimental alcoholic liver injury. *Int. Immunopharmacol.* 70, 477–485. doi: 10.1016/j.intimp.2019.02.021

Ge, Y., Sun, H., Xu, L., Zhang, W., Lv, J., and Chen, Y. (2022). The amelioration of alcohol-induced liver and intestinal barrier injury by *Lactobacillus rhamnosus* Gorbach-Goldin (LGG) is dependent on interleukin 22 (IL-22) expression. *Bioengineered* 13, 12650–12660. doi: 10.1080/21655979.2022.2070998

Gonzalez, A., Krieg, R., Massey, H. D., Carl, D., Ghosh, S., Gehr, T. W. B., et al. (2019). Sodium butyrate ameliorates insulin resistance and renal failure in CKD rats by modulating intestinal permeability and mucin expression. *Nephrol. Dial. Transplant.* 34, 783–794. doi: 10.1093/ndt/gfy238

González-Reimers, E., Santolaria-Fernández, F., Medina-García, J. A., González-Pérez, J. M., de la Vega-Prieto, M. J., Medina-Vega, L., et al. (2012). TH-1 and TH-2 cytokines in stable chronic alcoholics. *Alcohol Alcohol* 47, 390–396. doi: 10.1093/alcalc/ags041

Grander, C., Adolph, T. E., Wieser, V., Lowe, P., Wrzosek, L., Gyongyosi, B., et al. (2018). Recovery of ethanol-induced *Akkermansia muciniphila* depletion ameliorates alcoholic liver disease. *Gut* 67, 891–901. doi: 10.1136/gutjnl-2016-313432

Guo, W., Xiang, Q., Mao, B., Tang, X., Cui, S., Li, X., et al. (2021). Protective effects of microbiome-derived inosine on lipopolysaccharide-induced acute liver damage and inflammation in mice via mediating the TLR4/NF-κB pathway. *J. Agric. Food Chem.* 69, 7619–7628. doi: 10.1021/acs.jafc.1c01781

Haskó, G., Linden, J., Cronstein, B., and Pacher, P. (2008). Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. *Nat. Rev. Drug Discov.* 7, 759–770. doi: 10.1038/nrd2638

He, Y., Hwang, S., Ahmed, Y. A., Feng, D., Li, N., Ribeiro, M., et al. (2021). Immunopathobiology and therapeutic targets related to cytokines in liver diseases. *Cell. Mol. Immunol.* 18, 18–37. doi: 10.1038/s41423-020-00580-w

Hu, E. D., Chen, D. Z., Wu, J. L., Lu, F. B., Chen, L., Zheng, M. H., et al. (2018). High fiber dietary and sodium butyrate attenuate experimental autoimmune hepatitis through regulation of immune regulatory cells and intestinal barrier. *Cell. Immunol.* 328, 24–32. doi: 10.1016/j.cellimm.2018.03.003

Huang, D. Q., Mathurin, P., Cortez-Pinto, H., and Loomba, R. (2023). Global epidemiology of alcohol-associated cirrhosis and HCC: trends, projections and risk factors. *Nat. Rev. Gastroenterol. Hepatol.* 20, 37–49. doi: 10.1038/s41575-022-00688-6

Huang, C., Shen, Y., Shen, M., Fan, X., Men, R., Ye, T., et al. (2021). Glucose metabolism reprogramming of regulatory T cells in concanavalin A-induced hepatitis. *Front. Pharmacol.* 12:726128. doi: 10.3389/fphar.2021.726128

Li, N., Wang, H., Zhao, H., Wang, M., Cai, J., Hao, Y., et al. (2023). Cooperative interactions between Veillonella ratti and *Lactobacillus acidophilus* ameliorate DSS-induced ulcerative colitis in mice. *Food Funct.* 14, 10475–10492. doi: 10.1039/d3fo03898j

Liu, Y., Yang, M., Tang, L., Wang, F., Huang, S., Liu, S., et al. (2022). TLR4 regulates RORγt(+) regulatory T-cell responses and susceptibility to colon inflammation through interaction with *Akkermansia muciniphila*. *Microbiome* 10:98. doi: 10.1186/s40168-022-01296-x

Nowak, M., Lynch, L., Yue, S., Ohta, A., Sitkovsky, M., Balk, S. P., et al. (2010). The A2aR adenosine receptor controls cytokine production in iNKT cells. *Eur. J. Immunol.* 40, 682–687. doi: 10.1002/eji.200939897

Odashima, M., Bamias, G., Rivera-Nieves, J., Linden, J., Nast, C. C., Moskaluk, C. A., et al. (2005). Activation of A2A adenosine receptor attenuates intestinal inflammation in animal models of inflammatory bowel disease. *Gastroenterology* 129, 26–33. doi: 10.1053/j.gastro.2005.05.032

Pisani, A., Rausch, P., Bang, C., Ellul, S., Tabone, T., Marantidis Cordina, C., et al. (2022). Dysbiosis in the gut microbiota in patients with inflammatory bowel disease during remission. *Microbiol. Spect.* 10:e0061622. doi: 10.1128/spectrum.00616-22

Qu, G., Chen, J., Li, Y., Yuan, Y., Liang, R., and Li, B. (2022). Current status and perspectives of regulatory T cell-based therapy. *J. Genet. Genomics* 49, 599–611. doi: 10.1016/j.jgg.2022.05.005

Qu, D., Chen, M., Zhu, H., Liu, X., Cui, Y., Zhou, W., et al. (2023). Akkermansia muciniphila and its outer membrane protein Amuc_1100 prevent high-fat diet-induced nonalcoholic fatty liver disease in mice. *Biochem. Biophys. Res. Commun.* 684:149131. doi: 10.1016/j.bbrc.2023.149131

Rodrigues, V. F., Elias-Oliveira, J., Pereira, Í., Pereira, J. A., Barbosa, S. C., Machado, M. S. G., et al. (2022). Akkermansia muciniphila and gut immune system: a good friendship that attenuates inflammatory bowel disease, obesity, and diabetes. *Front. Immunol.* 13:934695. doi: 10.3389/fimmu.2022.934695

Rodriguez-Diaz, C., Taminiau, B., García-García, A., Cueto, A., Robles-Díaz, M., Ortega-Alonso, A., et al. (2022). Microbiota diversity in nonalcoholic fatty liver disease and in drug-induced liver injury. *Pharmacol. Res.* 182:106348. doi: 10.1016/j.phrs.2022.106348

Saveljeva, S., Sewell, G. W., Ramshorn, K., Cader, M. Z., West, J. A., Clare, S., et al. (2022). A purine metabolic checkpoint that prevents autoimmunity and autoinflammation. *Cell Metab.* 34, 106–124.e10. doi: 10.1016/j.cmet.2021.12.009

Siddiqui, M. T., and Cresci, G. A. M. (2020). Microbiota reprogramming for treatment of alcohol-related liver disease. *Transl. Res.* 226, 26–38. doi: 10.1016/j.trsl.2020.07.004

Silva, J. P. B., Navegantes-Lima, K. C., Oliveira, A. L. B., Rodrigues, D. V. S., Gaspar, S. L. F., Monteiro, V. V. S., et al. (2018). Protective mechanisms of butyrate on inflammatory bowel disease. *Curr. Pharm. Des.* 24, 4154–4166. doi: 10.2174/1381612824666181001153605

Sun, L., Li, X., Guan, H., Chen, S., Fan, X., Zhou, C., et al. (2021). A novel role of a(2A)R in the maintenance of intestinal barrier function of enteric glia from hypoxia-induced injury by combining with mGluR5. *Front. Pharmacol.* 12:633403. doi: 10.3389/fphar.2021.633403

Szabo, G. (2015). Gut-liver axis in alcoholic liver disease. *Gastroenterology* 148, 30–36. doi: 10.1053/j.gastro.2014.10.042

Vigano, S., Alatzoglou, D., Irving, M., Ménétrier-Caux, C., Caux, C., Romero, P., et al. (2019). Targeting adenosine in cancer immunotherapy to enhance T-cell function. *Front. Immunol.* 10:925. doi: 10.3389/fimmu.2019.00925

Weinhage, T., Däbritz, J., Brockhausen, A., Wirth, T., Brückner, M., Belz, M., et al. (2015). Granulocyte macrophage Colony-stimulating factor-activated CD39(+)/CD73(+) murine monocytes modulate intestinal inflammation via induction of regulatory T cells. *Cell. Mol. Gastroenterol. Hepatol.* 1, 433–449.e1. doi: 10.1016/j.jcmgh.2015.04.005

Wu, W., Lv, L., Shi, D., Ye, J., Fang, D., Guo, F., et al. (2017). Protective effect of *Akkermansia muciniphila* against immune-mediated liver injury in a mouse model. *Front. Microbiol.* 8:1804. doi: 10.3389/fmicb.2017.01804

Xia, G. Q., Cai, J. N., Wu, X., Fang, Q., Zhao, N., and Lv, X. W. (2022). The mechanism by which ATP regulates alcoholic steatohepatitis through P2X4 and CD39. *Eur. J. Pharmacol.* 916:174729. doi: 10.1016/j.ejphar.2021.174729

Xiao, J., Wang, F., Wong, N. K., He, J., Zhang, R., Sun, R., et al. (2019). Global liver disease burdens and research trends: analysis from a Chinese perspective. *J. Hepatol.* 71, 212–221. doi: 10.1016/j.jhep.2019.03.004

Xing, J., Zhang, J., and Wang, J. (2023). The immune regulatory role of adenosine in the tumor microenvironment. *Int. J. Mol. Sci.* 24:14928. doi: 10.3390/ijms241914928

Xu, L., Wang, X., Chen, Y., Soong, L., Chen, Y., Cai, J., et al. (2021). Metformin modulates T cell function and alleviates liver injury through bioenergetic regulation in viral hepatitis. *Front. Immunol.* 12:638575. doi: 10.3389/fimmu.2021.638575

Yan, C., Hu, W., Tu, J., Li, J., Liang, Q., and Han, S. (2023). Pathogenic mechanisms and regulatory factors involved in alcoholic liver disease. *J. Transl. Med.* 21:300. doi: 10.1186/s12967-023-04166-8

Zhang, C., Wang, K., and Wang, H. (2023). Adenosine in cancer immunotherapy: taking off on a new plane. *Biochim. Biophys. Acta Rev. Cancer* 1878:189005. doi: 10.1016/j.bbcan.2023.189005

Zhu, Y., Wang, X., Zhu, L., Tu, Y., Chen, W., Gong, L., et al. (2022). *Lactobacillus rhamnosus* GG combined with inosine ameliorates alcohol-induced liver injury through regulation of intestinal barrier and Treg/Th1 cells. *Toxicol. Appl. Pharmacol.* 439:115923. doi: 10.1016/j.taap.2022.115923

Zhu, Y., Zhuang, Z., Wu, Q., Lin, S., Zhao, N., Zhang, Q., et al. (2021). CD39/CD73/A2a adenosine metabolic pathway: targets for moxibustion in treating DSS-induced ulcerative colitis. *Am. J. Chin. Med.* 49, 661–676. doi: 10.1142/s0192415x21500300

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Association between gut microbiota and autoimmune cholestatic liver disease, a Mendelian randomization study

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Background: Previous studies have suggested that the gut microbiota (GM) is closely associated with the development of autoimmune cholestatic liver disease (ACLD), but limitations, such as the presence of confounding factors, have resulted in a causal relationship between the gut microbiota and autoimmune cholestatic liver disease that remains uncertain. Thus, we used two-sample Mendelian randomization as a research method to explore the causal relationship between the two.

Methods: Pooled statistics of gut microbiota from a meta-analysis of genome-wide association studies conducted by the MiBioGen consortium were used as an instrumental variable for exposure factors. The Pooled statistics for primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) were obtained from the R9 version of the FinnGen database (https://r9.finngen.fi/). Inverse-variance Weighted (IVW), cML-MA, MR-Egger regression, Weighted median (WME), Weighted mode (WM), and Simple mode (SM) were used to detect the association between intestinal flora and the causal relationship between intestinal flora and ACLD, in which IVW method was dominant, was assessed based on the effect indicator dominance ratio (odds ratio, OR) and 95% confidence interval (CI). Sensitivity analysis, heterogeneity test, gene pleiotropy test, MR pleiotropy residual sum and outlier test (MR-PRESSO) were combined to verify the stability and reliability of the results. Reverse Mendelian randomization analysis was performed on gut microbiota and found to be causally associated with ACLD.

Results: The IVW results showed that the relative abundance of the genus *Clostridium innocuum* group, genus *Butyricicoccus*, and genus *Erysipelatoclostridium* was negatively correlated with the risk of PBC, that is, increased abundance reduced the risk of PBC and was a protective, and the relative abundance of the genus *Eubacterium hallii* was positively correlated with the risk of PSC, which is a risk factor for PSC. Family *Clostridiaceae1* and family *Lachnospiraceae* were negatively correlated with the risk of PSC, which is a protective factor for PSC.

Conclusion: This study found a causal relationship between gut microbiota and ACLD. This may provide valuable insights into gut microbiota-mediated pathogenesis of ACLD. It is necessary to conduct a large-sample randomized controlled trial (RCT) at a later stage to validate the associated role of the relevant gut microbiota in the risk of ACLD development and to explore the associated mechanisms.

KEYWORDS

gut microbiota, autoimmune cholestatic liver disease, Mendelian randomization, primary biliary cholangitis, primary sclerosing cholangitis

1 Introduction

Autoimmune cholestatic liver disease (ACLD) encompasses a cadre of immune-mediated, protracted cholestatic hepatic maladies, notably primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC). The etiology of ACLD is the complicated interplay between genetic predispositions and environmental factors, precipitating the breakdown of immune tolerance toward biliary epithelial cells and subsequent immune activation (Liwinski et al., 2022; Richardson et al., 2022). Among these, PBC is characterized by progressive destruction of the small intrahepatic bile ducts as a pathological feature, whereas PSC manifests inflammation or fibrosis within both intrahepatic and extrahepatic conduits of medium and large calibers (Blesl and Stadlbauer, 2021). The pathophysiological mechanisms underlying ACLD remain unclear. In recent years, scholarly attention has pivoted toward the gut microbiota (GM) as a critical environmental determinant, with multiple investigations revealing a potential correlation between GM and ACLD. For instance, Furukawa et al. (2020) observed a decrease in the microbial diversity of patients with PBC, characterized by an elevated relative abundance of *Lactobacilli* and a diminished presence of symbiotically beneficial *Clostridium* compared to their normal counterparts. Moreover, evidence suggests that GM and its metabolites may be intricately linked to the progression of PBC toward hepatic fibrosis (Lammert et al., 2021). Analogously, investigations into PSC have revealed reduced GM diversity and altered abundance of specific flora. Fukui (2019), in an observational study, identified an upregulation of fecal *Haemophilus*, *Rothia*, *Clostridium*, *Enterococcus*, *Streptococcus*, and *Veillonella* in 43 Czech PSC patients compared to their healthy counterparts. Notwithstanding the aforementioned insights, observational studies encounter the challenge of controlling for confounding variables such as age, environment, dietary habits, and lifestyle, which may impinge upon the discernment of a causal relationship between GM and ACLD. Consequently, to clarify the potential causal nexus between GM and ACLD, we deployed a two-way Mendelian randomization approach.

Mendelian randomization (MR) is a methodological approach that leverages genetic variation to construct instrumental variables (IVs) for exposure, thereby facilitating the estimation of causal relationships between said exposure and the onset of a given malady (Greenland, 2000). It is a viable surrogate for Randomized Controlled Trials (RCTs), owing to the randomized assignment of genotypes from progenitors to progeny (Zhang et al., 2023). This randomization process ensures that the association between genetic variants and the resultant outcome remains impervious to common confounding variables, and the causal sequence maintains a plausible trajectory (Li et al., 2022). The methodological prowess of MR is now ubiquitously harnessed to dig deeper into the complicated tapestry of causal relationships between the intricacies of the GM and afflictions. In this study, we meticulously employed Genome-Wide Association Study (GWAS) amalgamated data from MiBioGen and the R9 version of the FinnGen Consortium to perform a two-sample Mendelian randomization (TSMR) analysis to assess the causal relationship between GM and ACLD.

2 Materials and methods

2.1 Study design

In the present inquiry, we applied TSMR methodology to scrutinize the complicated nexus between GM and ACLD. This investigation involved orchestration of dual, bidirectional, two-sample Mendelian randomization analyses, employing GM as the exposure, PBC and PSC as the outcomes. Our primary analytical approach predominantly involved the Inverse Variance Weighting (IVW) method, with subsequent layers of scrutiny including sensitivity analyses, heterogeneity assessments, gene multiplicity evaluations, and application of the MR-PRESSO algorithm, all orchestrated to corroborate the steadfast reliability of the derived outcomes.

MR analysis needs to satisfy the following three assumptions: (1) IVs and exposure need to be strongly associated (association assumption), (2) IVs should not be associated with the outcome through confounders (independence assumption), and (3) IVs cannot directly affect the outcome but only through exposure (exclusivity assumption). The specific process is shown in Figure 1.

2.2 Data sources

Single nucleotide polymorphisms (SNPs) intricately linked to the gut microbiota (GM) have been meticulously curated as instrumental variables for exposure. The consolidated dataset for these SNPs emanated from the MiBioGen consortium, an august assembly that scrutinized a comprehensive assemblage of 18,340 samples featuring 16S rRNA gene sequencing, drawn from a mosaic of 24 population-based cohorts, predominantly of European lineage. The microbial composition was meticulously analyzed, targeting three distinct variable regions of the 16S rRNA gene (V4, V3-V4, and V1-V2), with subsequent taxonomic classification achieved through direct taxonomic binning. To discern the complicated nuances of host genetic influence on gut bacterial taxa abundance, the endeavor embraced Microbiota Quantitative Trait Locus (mbQTL) positional analyses (Kurilshikov et al., 2021). Genetic datasets pertinent to both PBC and PSC—integral components of ACLD spectrum—were gleaned from the R9 iteration of the FinnGen Consortium, accessible at https://r9.finngen.fi/ (Kurki et al., 2023). The PBC cohort comprised 281,684 participants, with the case group numbered 557, and the healthy control cohort comprised 281,127 individuals. This collective was characterized by an exhaustive 20,167,312 SNPs. In parallel, the

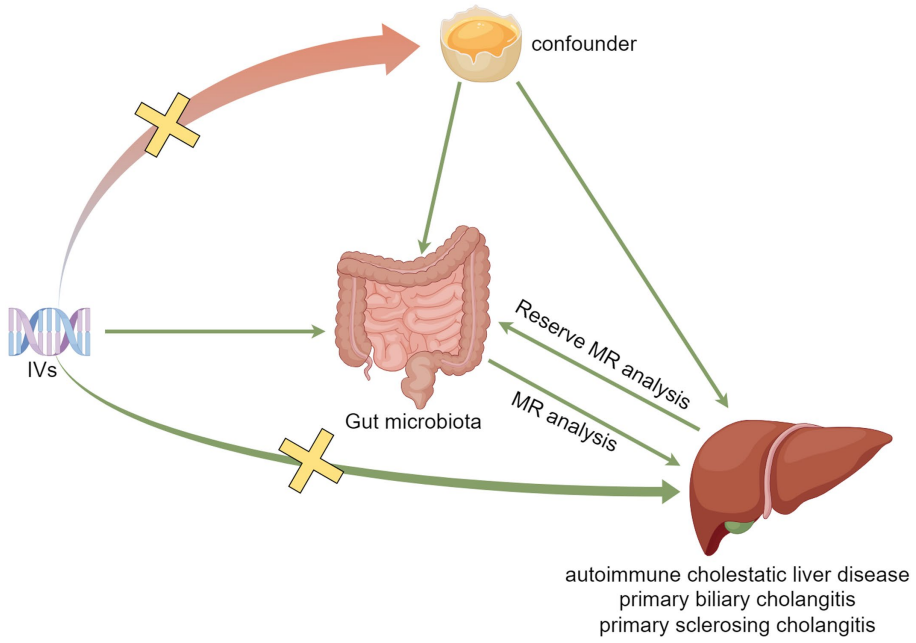


FIGURE 1
Model plot for MR analysis between GM and ACLD.

PSC dataset encompasses 332,618 participants, including 1,715 in the case group and 330,903 in the healthy control cohort, with an analogous wealth of 20,169,171 SNPs. Detailed information on ACLD data can be found in Supplementary Table 1.

2.3 Selection of instrumental variables

A meticulous screening process was undertaken to identify IVs from a cohort of 211 gut microbes, adhering to the following exclusion criteria: (1) SNPs achieving a significance threshold ($p < 1.0 \times 10^{-5}$) within the designated locus range were discerningly chosen as potential IVs (Zhang et al., 2023). (2) To guarantee the independence of the included SNPs, linkage disequilibrium (LD) was meticulously expunged using a reference panel sourced from 1,000 Genome Project European samples, with a stringent criterion of $r^2 < 0.001$ and a window size of 10,000 kb. Finally, data for the above selected SNPs were extracted from the outcome variables.

To obviate the potential sway of feeble instrumental biases in the estimation of the association effect, a rigorous evaluation of the robustness of instrumental variables was conducted. This scrutiny involved a meticulous assessment of the *F*-value, derived from the formula $F = \beta^2/se^2$ (Xie et al., 2023), where β represents the effect value and se signifies the standard error. It was posited that the absence of significant weak instrumental bias could be affirmed when the calculated *F*-value exceeded the threshold of 10 (Staiger and Stock, 1997).

2.4 Statistical methods

Within the confines of this investigation, the primary modality employed to scrutinize the potential causal linkage between gut microbial abundance and the proclivity for ACLD rested on IVW analysis. This was seamlessly complemented by an ensemble of supplementary methodologies, including cML-MA, MR-Egger, Weighted median (WME), Weighted mode (WM), and Simple mode (SM). The imprimatur of a relatively steadfast causal association was imputed when the *p*-value fell below 0.05 for the IVW methods and concordantly for cML-MA, MR-Egger, WME, WM, and SM when their vectors were aligned (Zhang et al., 2023). Furthermore, a meticulous dissection of the MR-Egger intercept term, denoted as the Egger-intercept, served as a sentinel for horizontal pleiotropy. An Egger-intercept infinitesimally proximal to 0 or statistically insignificant signaled the dearth of genetic pleiotropy (Burgess and Thompson, 2017). Additionally, the study used the MR-PRESSO methodology, an instrument adept at identifying potential outliers. Subsequent to the excision of outliers (p-value <0.05), a recalibration of the causal analysis was performed (Verbanck et al., 2018). Sensitivity analyses, executed using the leave-one-out method, were instrumental in gauging the impact of each SNP on causality. This intricate procedure involves the sequential removal of individual SNPs to ascertain the cumulative effect value of the residual SNPs (Hemani et al., 2018). To gauge the specter of heterogeneity, the study invoked the Cochran's *Q* test was used to discern potential discordance among the instrumental variables. A *p*-value less than 0.05 signified a noteworthy presence of heterogeneity. In a judicious effort to avert spurious positives due to the vicissitudes of multiple testing, a stringent false discovery rate (FDR) correction was implemented. Results attaining a *P*FDR < 0.1 were deemed statistically significant (Storey and Tibshirani, 2003; Li et al., 2022). In a bid to unravel the causal intricacies between GM and ACLD, a reciprocal MR analysis was executed for bacterial taxa pinpointed as causally entwined with

ACLD in antecedent MR exploration. The genome-wide significance threshold for the exposure dataset was rigidly set at $p < 5.0 \times 10^{-6}$, with all other criteria and parameters aligned harmoniously with those governing anterior MR scrutiny.

Statistical analyses were diligently executed employing the Two Sample MR package (version 0.5.7) (Hemani et al., 2018), the MR-PRESSO package (version 1.0) (Verbanck et al., 2018), and MR-cML (Xue et al., 2021) within the expanse of the R software (version 4.3.1).

3 Results

3.1 Instrumental variable

Eradicating 15 unclassified genera, a collective of 196 intestinal microorganisms spanning 9 phyla, 16 orders, 20 orders, 32 families, and 119 genera were judiciously included in this inquiry. Following the stringent criteria delineated in the previous section, 2,774 SNPs (Supplementary Table 2) intricately linked to the intestinal flora were identified. Notably, each of these SNPs boasted *F*-values surpassing the threshold of 10, thus effectually obviating the distortions introduced by feeble instrumental variables.

3.2 Mendelian randomization analysis

Within this investigation, a compendium of six MR methodologies was meticulously employed, with subsequent result scrutiny via the FDR correction method. In particular, Inverse-variance Weighted (IVW) outcomes unveiled six discernible gut microorganisms intricately implicated in the etiology of ACLD, each registering a noteworthy statistical significance (*P*FDR < 0.1). The relative abundance of three genera, including genus *Clostridium innocuum* group (OR = 0.54, 95% CI: 0.37–0.80, PFDR = 0.011), genus *Butyricoccus* (OR = 0.34, 95% CI: 0.15–0.74, *P*FDR = 0.034), and genus *Erysipelatoclostridium* (OR = 0.57, 95% CI: 0.36–0.90, *P*FDR = 0.063), and the relative abundance of the three types of intestinal microorganisms were negatively correlated with the risk of developing PBC, and the above three types of intestinal microorganisms were protective factors for PBC; the relative abundance of genus *Eubacterium hallii* group (OR = 1.45, 95% CI: 1.06–1.97, *P*FDR = 0.09) was positively correlated with the risk of developing PSC, i.e., it was a risk factor for PSC, family *Clostridiaceae1* (OR = 0.64, 95% CI: 0.43–0.96, *P*FDR = 0.09) and family *Lachnospiraceae* (OR = 0.63, 95% CI: 0.41–0.98, *P*FDR = 0.09) were negatively correlated with the incidence of PSC, and were protective factors for PSC thereby manifesting as protective factors. In tandem, the complementary method cML-MA-BIC lent credence to the causal associations discerned in the aforementioned six gut microbial contributions to ACLDs. Visual representations in Figures 2–5 concurred with the directional alignment of effect values across the six Two-Sample Mendelian Randomization (TSMR) methodologies, solidifying the inference that the six identified intestinal bacterial taxa were substantively and causally interwoven with the complex etiology of ACLD, thereby accentuating the robustness of the results.

3.3 Quality control

The leave-one-out method analysis meticulously demonstrated that the stepwise exclusion of SNPs engendered no substantive deviation between the amalgamated effect and cumulative effect of the residual SNPs, as elegantly delineated in Figures 6, 7. Akin to this, the discerning MR-PRESSO analysis, expounded in Figures 2, 3, corroborated the absence of outliers ($p > 0.05$), thereby amplifying the veracity of the results. Furthermore, both the Cochran Q test and the Egger-intercept test unveiled *p* values surpassing the threshold of 0.05, attesting to the dearth of significant heterogeneity within the instrumental variables of this inquiry. Notably, these findings underscore the imperviousness of the results regarding the influence of genetic pleiotropy.

3.4 Reverse MR analysis

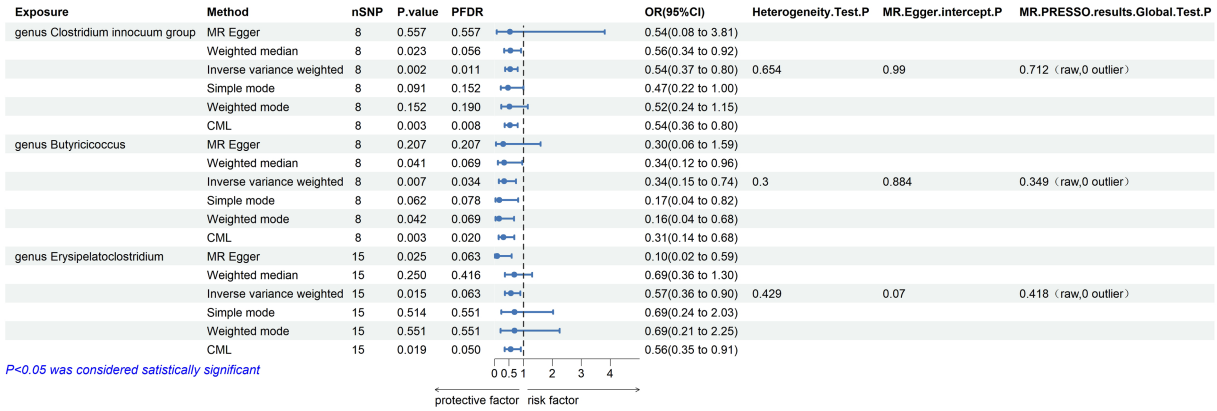
In the realm of intestinal microbiota, specifically comprising two familial entities and four generative cohorts, whose causal affiliations with ACLD were prominently underscored through the lens of forward MR analysis, a subsequent deconstruction was embarked upon via reverse MR scrutiny. The findings thereof failed to unveil any discernible causal nexus between ACLD and the intricate tapestry of intestinal flora, as substantiated by the statistical insignificance denoted in Table 1 ($p > 0.05$). The exposure data from the reverse MR analysis are detailed in Supplementary Tables 3, 4.

4 Discussion

In this study, we utilized GWAS-pooled data from MiBioGen and Finngen, subjected to analysis using TSMR. Our findings revealed a negative correlation between the risk of PBC and the relative abundance of three genera: *Clostridium innocuum* group, *Butyricicoccus*, and *Erysipelatoclostridium*. Conversely, the relative abundance of the genus *Eubacterium hallii* group exhibited a positive correlation with the risk of PSC. Furthermore, we observed a negative correlation between the development of PSC and the relative abundance of family *Clostridiaceae1* and family *Lachnospiraceae*. These results contribute to our understanding of the intricate relationships between specific microbial taxa and risks associated with PBC and PSC.

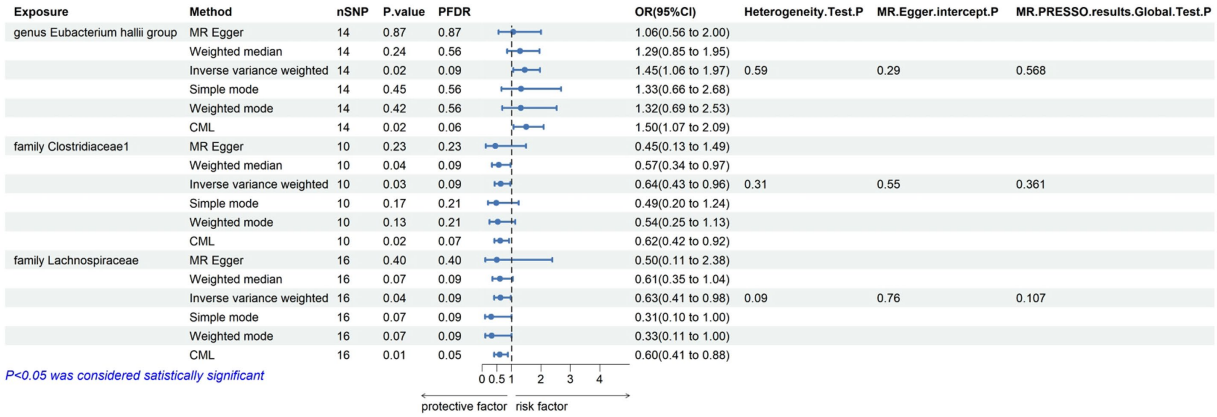
An increasing body of research underscores the role of intestinal flora in the development of ACLD through the gut-liver axis. This pathway involves microbial translocation, which is a pivotal feature of ACLD pathogenesis (Blesl and Stadlbauer, 2021). Bacteria and their metabolites, entering the circulation, are recognized by hepatic immune receptors, initiating intrahepatic inflammatory responses that contribute to the development and progression of ACLD (Tripathi et al., 2018). Moreover, structural alterations in the intestinal flora result in dysregulation of hepatic and intestinal bile acid circulation. This dysregulation precipitates cholestasis and accumulation of bile acid toxicity, ultimately inducing ACLD and progressive liver injury (Mousa et al., 2021).

A study conducted by Tedesco et al. revealed that translocation of *Lactobacillus gasseri* triggered the production of interleukin-17 (IL-17)



Exposure	Method	nSNP	P.value	PFDR	OR(95%CI)	Heterogeneity.Test.P	MR.Egger.intercept.P	MR.PRESSO.results.Global.Test.P
genus Clostridium innocuum group	MR Egger	8	0.557	0.557	0.54(0.08 to 3.81)			
	Weighted median	8	0.023	0.056	0.56(0.34 to 0.92)			
	Inverse variance weighted	8	0.002	0.011	0.54(0.37 to 0.80)	0.654	0.99	0.712（raw,0 outlier）
	Simple mode	8	0.091	0.152	0.47(0.22 to 1.00)			
	Weighted mode	8	0.152	0.190	0.52(0.24 to 1.15)			
	CML	8	0.003	0.008	0.54(0.36 to 0.80)			
genus Butyricicoccus	MR Egger	8	0.207	0.207	0.30(0.06 to 1.59)			
	Weighted median	8	0.041	0.069	0.34(0.12 to 0.96)			
	Inverse variance weighted	8	0.007	0.034	0.34(0.15 to 0.74)	0.3	0.884	0.349（raw,0 outlier）
	Simple mode	8	0.062	0.078	0.17(0.04 to 0.82)			
	Weighted mode	8	0.042	0.069	0.16(0.04 to 0.68)			
	CML	8	0.003	0.020	0.31(0.14 to 0.68)			
genus Erysipelatoclostridium	MR Egger	15	0.025	0.063	0.10(0.02 to 0.59)			
	Weighted median	15	0.250	0.416	0.69(0.36 to 1.30)			
	Inverse variance weighted	15	0.015	0.063	0.57(0.36 to 0.90)	0.429	0.07	0.418（raw,0 outlier）
	Simple mode	15	0.514	0.551	0.69(0.24 to 2.03)			
	Weighted mode	15	0.551	0.551	0.69(0.21 to 2.25)			
	CML	15	0.019	0.050	0.56(0.35 to 0.91)			

FIGURE 2
Results of MR analysis of 6 methods of GM and PBC and Egger-intercept, MR-PRESSO, and Cochran Q tests of IVW methods.



Exposure	Method	nSNP	P.value	PFDR	OR(95%CI)	Heterogeneity.Test.P	MR.Egger.intercept.P	MR.PRESSO.results.Global.Test.P
genus Eubacterium hallii group	MR Egger	14	0.87	0.87	1.06(0.56 to 2.00)			
	Weighted median	14	0.24	0.56	1.29(0.85 to 1.95)			
	Inverse variance weighted	14	0.02	0.09	1.45(1.06 to 1.97)	0.59	0.29	0.568
	Simple mode	14	0.45	0.56	1.33(0.66 to 2.68)			
	Weighted mode	14	0.42	0.56	1.32(0.69 to 2.53)			
	CML	14	0.02	0.06	1.50(1.07 to 2.09)			
family Clostridiaceae1	MR Egger	10	0.23	0.23	0.45(0.13 to 1.49)			
	Weighted median	10	0.04	0.09	0.57(0.34 to 0.97)			
	Inverse variance weighted	10	0.03	0.09	0.64(0.43 to 0.96)	0.31	0.55	0.361
	Simple mode	10	0.17	0.21	0.49(0.20 to 1.24)			
	Weighted mode	10	0.13	0.21	0.54(0.25 to 1.13)			
	CML	10	0.02	0.07	0.62(0.42 to 0.92)			
family Lachnospiraceae	MR Egger	16	0.40	0.40	0.50(0.11 to 2.38)			
	Weighted median	16	0.07	0.09	0.61(0.35 to 1.04)			
	Inverse variance weighted	16	0.04	0.09	0.63(0.41 to 0.98)	0.09	0.76	0.107
	Simple mode	16	0.07	0.09	0.31(0.10 to 1.00)			
	Weighted mode	16	0.07	0.09	0.33(0.11 to 1.00)			
	CML	16	0.01	0.05	0.60(0.41 to 0.88)			

FIGURE 3
Results of MR analysis of 6 methods of GM and PSC and Egger-intercept, MR-PRESSO, and Cochran Q tests of IVW methods.

by hepatic γδ T-cell receptor-positive (γδ TCR) cells in mice. This translocation is associated with hepatic inflammatory injury. Additionally, γδ TCR cells were isolated from the livers of patients with PSC, implying that intestinal flora may play a role in inducing cholestatic liver disease. Specifically, the mediation of γδ TCR cells to produce IL-17 has been identified as a potential mechanism contributing to the development of cholestatic liver disease (Tedesco et al., 2018). Observational studies have further supported the link between dysbiosis of gut flora and ACLD. For instance, Sabino et al. reported a higher proportion of *Enterococcus*, *Fusobacterium*, and *Lactobacillus* genera in the feces of 66 Belgian patients with PSC compared to controls (Sabino et al., 2016). Furukawa et al. (2020) also found relevant associations; they identified decreased bacterial diversity, a high abundance of *Lactobacillus*, and a low abundance of *Clostridiales* in the intestines of patients with PBC compared to the healthy population. Furthermore, in the ursodeoxycholic acid (UDCA)-treated nonresponder group, there was a significant decrease in the *Faecalibacterium* genus. This suggests that the presence of *Faecalibacterium* may influence the long-term prognosis of patients with PBC.

In alignment with prior investigations, our study identified significant associations using MR analysis. Specifically, the genus *Clostridium innocuum* exhibited a negative correlation with the risk of developing PBC. Conversely, the genus *Eubacterium hallii* and the family *Lachnospiraceae* were positively and negatively associated with the development of PSC, respectively. Earlier research has posited the absence of beneficial *Clostridiales* commensals in the intestines of PBC patients (Furukawa et al., 2020). Abe et al. (2018) reported a reduction in *Clostridium subcluster XIVa* among PBC patients compared to controls. Furthermore, patients with PBC with abnormal hepatic function exhibited decreased levels of *Clostridium cluster IV* and *Clostridium subcluster XIVa*, particularly in those with abnormal liver function. These findings suggest a potential protective role for *Clostridium* in PBC and indicate its possible influence on liver function. Scholars have affirmed that *Clostridium*, through 7-α-Dehydroxylation, converts Primary bile acids into secondary bile acids (Staley et al., 2017). Given that ACLD patients often experience bile acid metabolism dysregulation, reduced Dehydroxylation reaction, and cytotoxicity, the concentration of hydrophobic Primary bile acids increases, leading to toxicity

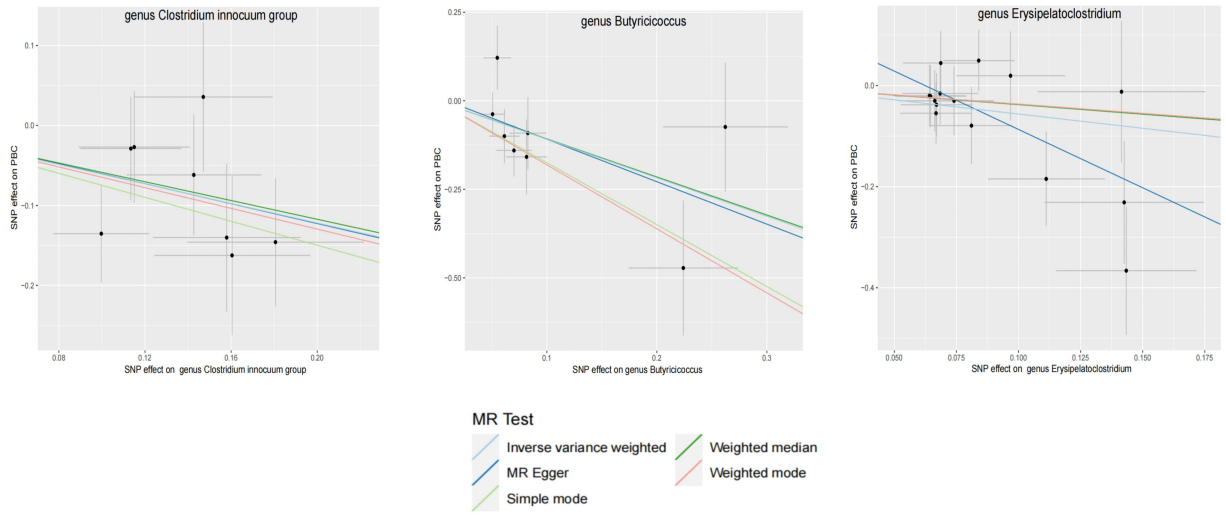


FIGURE 4
GM with causal relationship with PBC and the scatterplot of the 6 MR models.

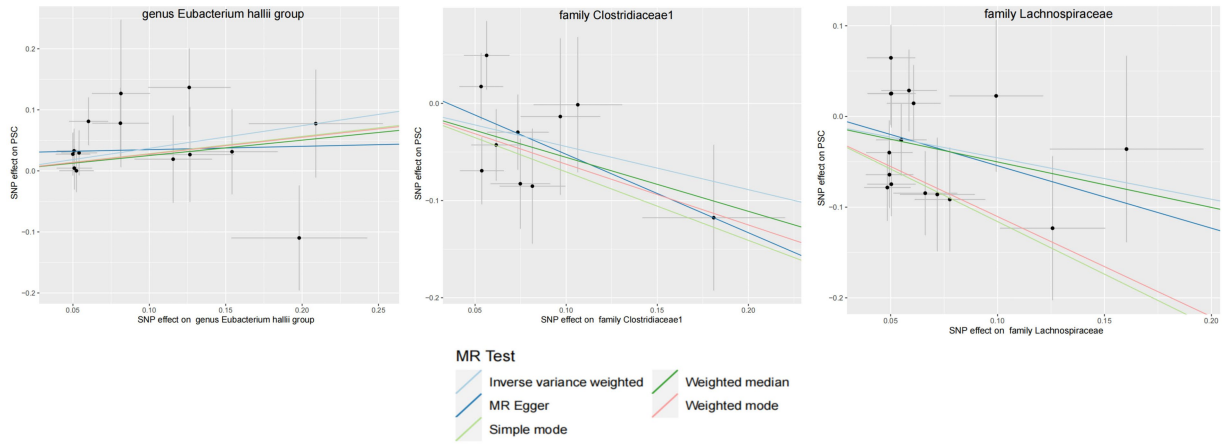


FIGURE 5
GM with causal relationship with PSC and the scatterplot of the 6 MR models.

accumulation and bile duct damage (Schmucker et al., 1990; Li et al., 2017; Torres et al., 2018; Fiorucci et al., 2021). Therefore, *Clostridium* may mitigate the risk of PBC development by improving bile acid metabolism. In a study by Kummen et al. in 2020, fecal DNA sequencing of patients with PSC revealed a significant depletion of various *Eubacterium* species (Kummen et al., 2021). Concurrently, patient treatment with UDCA was associated with elevated levels of *Eubacterium rectale*. While this study suggests a protective role for *Eubacterium* in PSC, our findings indicate that the genus *Eubacterium hallii* is a risk factor for PSC, necessitating further exploration of its role in PSC development. Quraishi et al. (2020) in the same year observed a notable reduction in the family *Lachnospiraceae* in patients with PSC, suggesting a potential protective effect against PSC. Previous research has confirmed the ability of family *Lachnospiraceae* to produce butyrate (Winston et al., 2021), a short-chain fatty acid known for its anti-inflammatory, bile acid metabolism-regulating, and intestinal barrier-improving properties (Cui et al., 2018; Ye et al., 2021; Liu et al., 2022; Wang et al., 2023). This may help mitigate inflammatory damage associated with bile acid stagnation and bacterial translocation. An earlier study in 2018 demonstrated a positive correlation between *Faecalibacterium* enrichment and *Erysipelatoclostridium* in healthy infants, which was not observed in cholestatic infants (Guo et al., 2018). This suggests a potential association between dysregulation of genus *Erysipelatoclostridium* and cholestasis. However, protective associations for PBC necessitate further investigation. While no direct study establishes the association of genus *Butyricicoccus* and family *Clostridiaceae1* with ACLD, but because genus *Butyricicoccus* is the main microbiota producing butyrate, it was found to be positively associated with the concentration of Secondary bile acids, which may contribute to the conversion of Primary bile acids to Secondary bile acids (Yang et al., 2021). Certain genera within

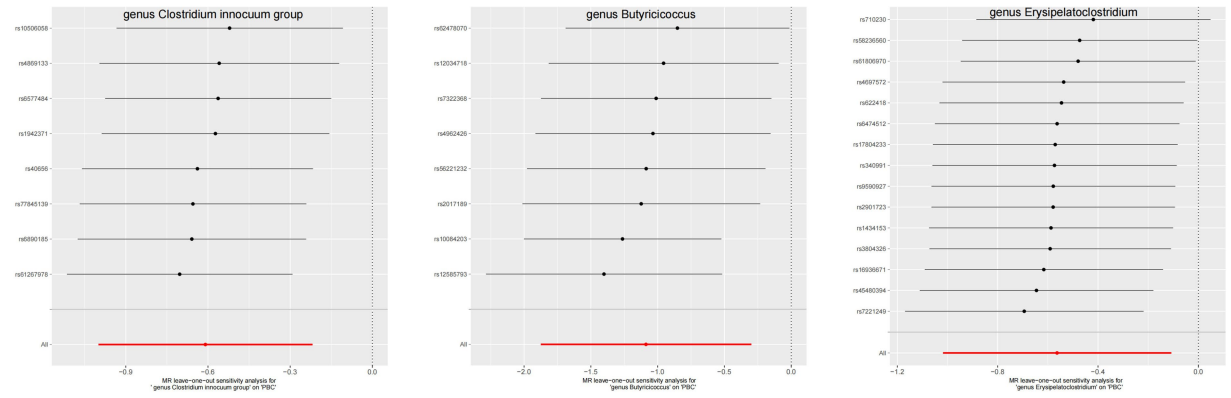


FIGURE 6
The leave-one-out plots of GM with causal relationship with PBC.

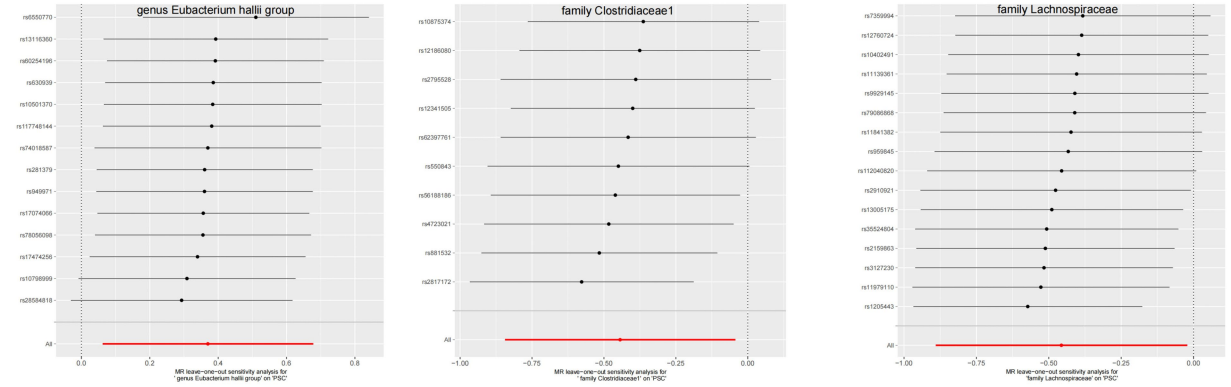


FIGURE 7
The leave-one-out plots of GM with causal relationship with PSC.

TABLE 1 **Results of reverse MR analysis.**

Exposure	Outcome	SNP	Method	*β*	*SE*	*p*
PBC	Genus *Clostridium innocuum* group	10	IVW	0.0002	0.228	0.990
PBC	Genus Butyricicoccus	12	IVW	0.017	0.012	0.164
PBC	Genus Erysipelatoclostridium	12	IVW	0.007	0.014	0.5812
PSC	Genus *Eubacterium hallii* group	5	IVW	−0.008	0.026	0.7478
PSC	Family Clostridiaceae1	5	IVW	−0.029	0.0278	0.297
PSC	Family Lachnospiraceae	5	IVW	0.012	0.025	0.625

family *Clostridiaceae*, such as *Clostridium*, have been implicated in the conversion of Primary bile acids to Secondary bile acids (Furukawa et al., 2020). Therefore, we posit that genus *Butyricicoccus* and family *Clostridiaceae1* may exert protective effects against ACLD through the production of butyrate and regulation of bile acids. Further research is warranted to validate these hypotheses.

It is also worth noting that the occurrence and development of non-alcoholic fatty liver disease (NAFLD) is also closely related to dysregulation of gut microbiota and bile acid metabolism, for example (Sorrentino et al., 2005). Qilong Zhai et al. found in an MR study that *Enterobacteriales*, *Enterobacteriaceae*, *Lachnospiraceae UCG-004*, and *Prevotella9* increased the risk of NAFLD, and *Dorea* and *Veillonella* increased the risk of NASH. *Oscillospira* and *Ruminococcaceae UCG-013* were able to reduce their risk, with *Veillonella*, *Lachnospiraceae UCG-004* being able to participate in bile acid metabolism. It has

also been suggested that dysregulation of BA homeostasis and signaling may further contribute to abnormal lipid metabolism and lipotoxicity in NAFLD (Arab et al., 2017), and functional cholestasis may be related to the pathogenesis of NASH (Segovia-Miranda et al., 2019). Thus, it can be seen that the disorder of bile acid hepatic and intestinal circulation caused by dysbiosis of gut microbiota is likely to be an important cause of the development and progression of NAFLD, which has some overlap with the mechanism of ACLD triggered by gut microbiota, and thus it has been pointed out that cholestasis and NAFLD can share some of the therapeutic targets in their treatments (Trauner and Fuchs, 2022). Most of the positive gut microbiota we found in this study exerted their promotional or inhibitory effects on ACLD by affecting bile acid metabolism, and it should be noted that two protective genera, *Clostridium* and *Butyricicoccus*, were also found to be in increased abundance in patients with NAFLD regression after bariatric surgery in this study (Pérez-Rubio et al., 2023), and thus we hypothesize that they may be able to contribute to the development of common therapeutic targets to ameliorate both cholestasis and NAFLD by modulating the gut microbiota.

In comparison with previous literature, we found some similarities in the design of the two studies, but there are obvious differences in data sources, findings and research methods, etc. The study of Yang et al. (2024) found that order *Bacillales*, family *Peptostreptococcaceae*, family *Ruminococcaceae*, genus *Anaerotruncu* was associated with a reduced risk of developing PBC, order *Selenomonadales*, *family Bifidobacteriaceae* may be a factor that increases the risk of PBC, *order Selenomonadales*, *family Rhodospirillaceae*, and *genus RuminococcaceaeUCG013* was positively associated with PSC, *order Actinomycetales*, *family Actinomycetaceae*, *genus Actinomyces*, *genus Alloprevotella*, *genus Barnesiella*, and *genus Peptococcus* were negatively correlated with PSC risk. Zhang et al. (2023) similarly found that *Order Selenomonadales*, *Order Bifidobacteriales* and *Genus Lachnospiraceae UCG_004* were associated with higher risk of PBC, and *Family Peptostreptococcaceae* and *Family Ruminococcaceae* were protective against PBC. The above two studies and the content of the present study are from different databases, so the results produce a large difference, which also indicates that there may be some differences in the gut microorganisms affecting the pathogenesis of PBC and PSC in different sample populations. This study can further add candidate gut microorganisms affecting the pathogenesis of cholestatic liver disease, providing more complete basic research content for future studies.

The strengths and limitations of this study are delineated as follows. Notably, the utilization of the MR method stands out as a strength, enabling the analysis of the causal relationship between GM and ACLD, and can be complemented by the results of previous studies. This approach offers potential candidate gut microorganisms for subsequent investigations. Additionally, the study leveraged genetic data derived from a substantial population sample, mitigating the impact of confounding factors more effectively than small observational studies. Consequently, the results were deemed more reliable. However, certain limitations merit consideration. Firstly, the GWAS data primarily originated from European populations, raising the possibility of variation in results when extrapolated to other ethnic groups. Furthermore, the reliance on 16S rRNA gene sequencing limits taxonomic resolution to the genus level, precluding an exploration of the causal relationship between GM and ACLD at the species level. In specific instances, such as the prominence of the genus *Eubacterium hallii* in this study, discrepancies in its direction of action compared to prior research on its correlation with PSC were observed. Additionally, the absence of direct evidence confirming a causal relationship between the genus *Butyricicoccus* and the family *Clostridiaceae1* with ACLD pathogenesis is acknowledged. This underscores the necessity for future studies to validate and substantiate these potential associations. Consequently, the study calls for further investigation to solidify the understanding of these relationships and their implications for ACLD development.

In summary, this study identified six intestinal microbial taxa with causal associations to PBC and PSC through Mendelian randomization analysis. This finding establishes a foundational basis for investigating the link between GM and ACLD. Nevertheless, to comprehensively illustrate the specific mechanisms underlying the protective or triggering effects of these relevant flora on ACLD, further investigations employing large sample clinical Randomized Controlled Trial (RCT), pathway analyses, and laboratory studies are imperative.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

No animal/human studies are presented in the manuscript.

Author contributions

YC: Data curation, Methodology, Writing – original draft, Investigation. YG: Methodology, Software, Writing – original draft. YK: Data curation, Software, Writing – original draft. GZ: Supervision, Writing – review & editing, Project administration.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1348027/full#supplementary-material

References

Abe, K., Takahashi, A., Fujita, M., Imaizumi, H., Hayashi, M., Okai, K., et al. (2018). Dysbiosis of oral microbiota and its association with salivary immunological biomarkers in autoimmune liver disease. *PLoS One* 13:e0198757. doi: 10.1371/journal.pone.0198757

Arab, J. P., Karpen, S. J., Dawson, P. A., Arrese, M., and Trauner, M. (2017). Bile acids and nonalcoholic fatty liver disease: molecular insights and therapeutic perspectives. *Hepatology* 65, 350–362. doi: 10.1002/hep.28709.

Blesl, A., and Stadlbauer, V. (2021). The gut-liver axis in cholestatic liver diseases. *Nutrients* 13:1018. doi: 10.3390/nu13031018

Burgess, S., and Thompson, S. G. (2017). Interpreting findings from Mendelian randomization using the MR-egger method. *Eur. J. Epidemiol.* 32, 377–389. doi: 10.1007/s10654-017-0255-x

Cui, H., Cai, Y., Wang, L., Jia, B., Li, J., Zhao, S., et al. (2018). Berberine regulates Treg/Th17 balance to treat ulcerative colitis through modulating the gut microbiota in the Colon. *Front. Pharmacol.* 9:571. doi: 10.3389/fphar.2018.00571

Fiorucci, S., Distrutti, E., Carino, A., Zampella, A., and Biagioli, M. (2021). Bile acids and their receptors in metabolic disorders. *Prog. Lipid Res.* 82:101094. doi: 10.1016/j.plipres.2021.101094

Fukui, H. (2019). Role of gut dysbiosis in liver diseases: what have we learned so far? *Diseases* 7:58. doi: 10.3390/diseases7040058

Furukawa, M., Moriya, K., Nakayama, J., Inoue, T., Momoda, R., Kawaratani, H., et al. (2020). Gut dysbiosis associated with clinical prognosis of patients with primary biliary cholangitis. *Hepatol. Res.* 50, 840–852. doi: 10.1111/hepr.13509

Greenland, S. (2000). An introduction to instrumental variables for epidemiologists. *Int. J. Epidemiol.* 29, 722–729. doi: 10.1093/ije/29.4.722

Guo, C., Li, Y., Wang, P., Li, Y., Qiu, C., Li, M., et al. (2018). Alterations of gut microbiota in cholestatic infants and their correlation with hepatic function. *Front. Microbiol.* 9:2682. doi: 10.3389/fmicb.2018.02682

Hemani, G., Zheng, J., Elsworth, B., Wade, K. H., Haberland, V., Baird, D., et al. (2018). The MR – base platformsupports systematic causal inference across the humanphenome. *eLife* 7:e34408. doi: 10.7554/eLife.34408

Kummen, M., Thingholm, L. B., Rühlemann, M. C., Holm, K., Hansen, S. H., Moitinho-Silva, L., et al. (2021). Altered gut microbial metabolism of essential nutrients in primary sclerosing cholangitis. *Gastroenterology* 160, 1784–1798.e0. doi: 10.1053/j.gastro.2020.12.058

Kurilshikov, A., Medina-Gomez, C., Bacigalupe, R., Radjabzadeh, D., Wang, J., Demirkan, A., et al. (2021). Large-scale association analyses identify host factors influencing human gut microbiome composition. *Nat. Genet.* 53, 156–165. doi: 10.1038/s41588-020-00763-1

Kurki, M. I., Karjalainen, J., Palta, P., Sipilä, T. P., Kristiansson, K., Donner, K. M., et al. (2023). FinnGen provides genetic insights from a well-phenotyped isolated population. *Nature* 613, 508–518. doi: 10.1038/s41586-022-05473-8.

Lammert, C., Shin, A., Xu, H., Hemmerich, C., O'Connell, T. M., and Chalasani, N. (2021). Short-chain fatty acid and fecal microbiota profiles are linked to fibrosis in primary biliary cholangitis. *FEMS Microbiol. Lett.* 368:fnab038. doi: 10.1093/femsle/fnab038

Li, Y., Tang, R., Leung, P. S. C., Gershwin, M. E., and Ma, X. (2017). Bile acids and intestinal microbiota in autoimmune cholestatic liver diseases. *Autoimmun. Rev.* 16, 885–896. doi: 10.1016/j.autrev.2017.07.002

Li, P., Wang, H., Guo, L., Gou, X., Chen, G., Lin, D., et al. (2022). Association between gut microbiota and preeclampsia-eclampsia: a two-sample Mendelian randomization study. *BMC Med.* 20:443. doi: 10.1186/s12916-022-02657-x

Liu, X., Wang, L., Tan, S., Chen, Z., Wu, B., and Wu, X. (2022). Therapeutic effects of Berberine on liver fibrosis are associated with lipid metabolism and intestinal flora. *Front. Pharmacol.* 13:814871. doi: 10.3389/fphar.2022.814871

Liwinski, T., Heinemann, M., and Schramm, C. (2022). The intestinal and biliary microbiome in autoimmune liver disease-current evidence and concepts. *Semin. Immunopathol.* 44, 485–507. doi: 10.1007/s00281-022-00936-6

Mousa, O. Y., Juran, B. D., McCauley, B. M., Vesterhus, M. N., Folseraas, T., Turgeon, C. T., et al. (2021). Bile acid profiles in primary sclerosing cholangitis and their ability to predict hepatic decompensation. *Hepatology* 74, 281–295. doi: 10.1002/hep.31652

Pérez-Rubio, Á., Soluyanova, P., Moro, E., Quintás, G., Rienda, I., Periañez, M. D., et al. (2023). Gut microbiota and plasma bile acids associated with non-alcoholic fatty liver disease resolution in bariatric surgery patients. *Nutrients* 15:3187. doi: 10.3390/nu15143187.

Quraishi, M. N., Acharjee, A., Beggs, A. D., Horniblow, R., Tselepis, C., Gkoutos, G., et al. (2020). A pilot integrative analysis of colonic gene expression, gut microbiota, and immune infiltration in primary sclerosing cholangitis-inflammatory bowel disease: association of disease with bile acid pathways. *J. Crohns Colitis* 14, 935–947. doi: 10.1093/ecco-jcc/jjaa021

Richardson, N., Wootton, G. E., Bozward, A. G., and Oo, Y. H. (2022). Challenges and opportunities in achieving effective regulatory T cell therapy in autoimmune liver disease. *Semin. Immunopathol.* 44, 461–474. doi: 10.1007/s00281-022-00940-w

Sabino, J., Vieira-Silva, S., Machiels, K., Joossens, M., Falony, G., Ballet, V., et al. (2016). Primary sclerosing cholangitis is characterised by intestinal dysbiosis independent from IBD. *Gut* 65, 1681–1689. doi: 10.1136/gutjnl-2015-311004

Schmucker, D. L., Ohta, M., Kanai, S., Sato, Y., and Kitani, K. (1990). Hepatic injury induced by bile salts: correlation between biochemical and morphological events. *Hepatology* 12, 1216–1221. doi: 10.1002/hep.1840120523

Segovia-Miranda, F., Morales-Navarrete, H., Kücken, M., Moser, V., Seifert, S., Repnik, U., et al. (2019). Three-dimensional spatially resolved geometrical and functional models of human liver tissue reveal new aspects of NAFLD progression. *Nat. Med.* 25, 1885–1893. doi: 10.1038/s41591-019-0660-7

Sorrentino, P., Tarantino, G., Perrella, A., Micheli, P., Perrella, O., and Conca, P. (2005). A clinical-morphological study on cholestatic presentation of nonalcoholic fatty liver disease. *Dig. Dis. Sci.* 50, 1130–1135. doi: 10.1007/s10620-005-2719-1.

Staiger, D., and Stock, J. H. (1997). Instrumental variables regression with weak instruments. *Econometrica* 65, 557–586. doi: 10.2307/2171753

Staley, C., Weingarden, A. R., Khoruts, A., and Sadowsky, M. J. (2017). Interaction of gut microbiota with bile acid metabolism and its influence on disease states. *Appl. Microbiol. Biotechnol.* 101, 47–64. doi: 10.1007/s00253-016-8006-6

Storey, J. D., and Tibshirani, R. (2003). Statistical significance for genomewide studies. *Proc. Natl. Acad. Sci. U. S. A.* 100, 9440–9445. doi: 10.1073/pnas.1530509100

Tedesco, D., Thapa, M., Chin, C. Y., Ge, Y., Gong, M., Li, J., et al. (2018). Alterations in intestinal microbiota lead to production of interleukin 17 by intrahepatic γδ T-cell receptor-positive cells and pathogenesis of cholestatic liver disease. *Gastroenterology* 154, 2178–2193. doi: 10.1053/j.gastro.2018.02.019

Torres, J., Palmela, C., Brito, H., Bao, X., Ruiqi, H., Moura-Santos, P., et al. (2018). The gut microbiota, bile acids and their correlation in primary sclerosing cholangitis associated with inflammatory bowel disease. *United European Gastroenterol J* 6, 112–122. doi: 10.1177/2050640617708953

Trauner, M., and Fuchs, C. D. (2022). Novel therapeutic targets for cholestatic and fatty liver disease. *Gut* 71, 194–209. doi: 10.1136/gutjnl-2021-324305.

Tripathi, A., Debelius, J., Brenner, D. A., Karin, M., Loomba, R., Schnabl, B., et al. (2018). The gut-liver axis and the intersection with the microbiome. *Nat. Rev. Gastroenterol. Hepatol.* 15, 397–411. doi: 10.1038/s41575-018-0011-z

Verbanck, M., Chen, C. Y., Neale, B., and do, R. (2018). Detection of widespread horizontal pleiotropy in causal relationships inferred from mendelian randomization between complex traits and diseases. *Nat. Genet.* 50, 693–698. doi: 10.1038/s41588-018-0099-7

Wang, S., Xiang, L., li, F., Deng, W., lv, P., and Chen, Y. (2023). Butyrate protects against *Clostridium difficile* infection by regulating bile acid metabolism. *Microbiol. Spectr.* 11:e0447922. doi: 10.1128/spectrum.04479-22

Winston, J. A., Rivera, A., Cai, J., Patterson, A. D., and Theriot, C. M. (2021). Secondary bile acid ursodeoxycholic acid alters weight, the gut microbiota, and the bile acid pool in conventional mice. *PLoS One* 16:e0246161. doi: 10.1371/journal.pone.0246161

Xie, J., Huang, H., Liu, Z., Li, Y., Yu, C., Xu, L., et al. (2023). The associations between modifiable risk factors and nonalcoholic fatty liver disease: a comprehensive mendelian randomization study. *Hepatology* 77, 949–964. doi: 10.1002/hep.32728

Xue, H., Shen, X., and Pan, W. (2021). Constrained maximum likelihood-based Mendelian randomization robust to both correlated and uncorrelated pleiotropic effects. *Am. J. Hum. Genet.* 108, 1251–1269. doi: 10.1016/j.ajhg.2021.05.014

Yang, Z. H., Liu, F., Zhu, X. R., Suo, F. Y., Jia, Z. J., and Yao, S. K. (2021). Altered profiles of fecal bile acids correlate with gut microbiota and inflammatory responses in patients with ulcerative colitis. *World J. Gastroenterol.* 27, 3609–3629. doi: 10.3748/wjg.v27.i24.3609

Yang, J., Ma, G., Wang, K., Yang, H., Jiang, S., Fan, Q., et al. (2024). Causal associations between gut microbiota and Cholestatic liver diseases: a Mendelian randomization study. *Front. Med.* 11:1342119. doi: 10.3389/fmed.2024.1342119.

Ye, X., Shen, S., Xu, Z., Zhuang, Q., Xu, J., Wang, J., et al. (2021). Sodium butyrate alleviates cholesterol gallstones by regulating bile acid metabolism. *Eur. J. Pharmacol.* 908:174341. doi: 10.1016/j.ejphar.2021.174341

Zhang, J., Wu, G., Tang, Y., Liu, H., Ge, X., Peng, R., et al. (2023). Causal associations between gut microbiota and primary biliary cholangitis: a bidirectional two-sample Mendelian randomization study. *Front. Microbiol.* 14:1273024. doi: 10.3389/fmicb.2023.1273024

Zhang, L., Zi, L., Kuang, T., Wang, K., Qiu, Z., Wu, Z., et al. (2023). Investigating causal associations among gut microbiota, metabolites, and liver diseases: a Mendelian randomization study. *Front. Endocrinol.* 14:1159148. doi: 10.3389/fendo.2023.1159148

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From gut to liver: unveiling the differences of intestinal microbiota in NAFL and NASH patients

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Non-alcoholic fatty liver disease (NAFLD) is increasingly recognized for its global prevalence and potential progression to more severe liver diseases such as non-alcoholic steatohepatitis (NASH). The gut microbiota plays a pivotal role in the pathogenesis of NAFLD, yet the detailed characteristics and ecological alterations of gut microbial communities during the progression from non-alcoholic fatty liver (NAFL) to NASH remain poorly understood. Methods: In this study, we conducted a comparative analysis of gut microbiota composition in individuals with NAFL and NASH to elucidate differences and characteristics. We utilized 16S rRNA sequencing to compare the intestinal gut microbiota among a healthy control group (65 cases), NAFL group (64 cases), and NASH group (53 cases). Random forest machine learning and database validation methods were employed to analyze the data. Results: Our findings indicate a significant decrease in the diversity of intestinal flora during the progression of NAFLD ($p<0.05$). At the phylum level, high abundances of Bacteroidetes and Fusobacteria were observed in both NAFL and NASH patients, whereas Firmicutes were less abundant. At the genus level, a significant decrease in Prevotella expression was seen in the NAFL group (AUC 0.738), whereas an increase in the combination of Megamonas and Fusobacterium was noted in the NASH group (AUC 0.769). Furthermore, KEGG pathway analysis highlighted significant disturbances in various types of glucose metabolism pathways in the NASH group compared to the NAFL group, as well as notably compromised flavonoid and flavonol biosynthesis functions. The study uncovers distinct microbiota characteristics and microecological changes within the gut during the transition from NAFL to NASH, providing insights that could facilitate the discovery of novel biomarkers and therapeutic targets for NAFLD.

KEYWORDS

NAFLD, gut microbiome, NASH, 16S rRNA sequencing, NAFL

1 Introduction

Non-alcoholic fatty liver disease (NAFLD) has emerged as a prominent global health challenge in the field of hepatology, characterized by abnormal accumulation of lipids in the liver, including non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH; Scavo et al., 2022; Huang et al., 2023; Ren et al., 2023). The latter often progresses to cirrhosis, liver failure, and hepatocellular carcinoma (HCC), making it the leading cause of liver transplantation (Davis et al., 2019; Huang et al., 2021). Despite advancements in understanding the pathogenesis and therapeutic approaches for NAFLD (Sun et al., 2019), significant challenges remain in the early diagnosis and treatment of the disease.

The gut microbiota, which refers to the microbial community residing within the gastrointestinal tract, plays a crucial role in host digestion, immunity, and metabolism. Dysbiosis, characterized by unhealthy alterations in the normal bacterial ecology, pervades the entire pathological continuum of NAFLD, encompassing conditions from simple steatosis to NASH, and extending through to advanced fibrosis, culminating in cirrhosis and hepatocellular carcinoma (Hoyles et al., 2018; Javdan et al., 2020; Mohr et al., 2020).

The foundational study by Demir et al. presented groundbreaking evidence supporting a causal relationship between the gut microbiota and the pathogenesis of NAFLD, as demonstrated by mouse models and fecal transplant experiments (Demir et al., 2022). The findings revealed an increase in two distinct bacterial species (Trichophylidae bacteria 609 and Barnesiella) among mice exhibiting steatosis (Jin et al., 2022). However, significant disparities were observed in terms of composition, predominant genera, as well as abundance levels for specific genera and species when comparing the gut microbiota profiles between mice and humans (Javdan et al., 2020; Mohr et al., 2020). Therefore, it is imperative to detect and differentiate the disparities between gut microbiota and NAFLD in human subjects. In recent years, numerous studies have concentrated on the characteristics of gut microbiota in human patients with steatosis, NASH, or related cirrhosis. Current research has demonstrated an increase in Proteus (Raman et al., 2013; Shen et al., 2017; Hoyles et al., 2018; Loomba et al., 2019) and Enterobacterium (Hoyles et al., 2018) within NAFLD patients. Conversely, Riederia (Zhu et al., 2013; Del et al., 2017) and Ruminococcus exhibited a reduction at the genus level (Zhu et al., 2013; Del et al., 2017). Specifically, Escherichia (Zhu et al., 2013; Hoyles et al., 2018) Doxella (Zhu et al., 2013; Del et al., 2017), and Gastrobacillus (Zhu et al., 2013; Del et al., 2017) displayed an upward trend. On the other hand, anaerobic sporococcus (Wang et al., 2016; Roeb, 2022), Bacillus faecalis (Wang et al., 2016; Hoyles et al., 2018), Bacillus (Zhu et al., 2013; Hoyles et al., 2018), Bacillus faecalis (Zhu et al., 2013; Roeb, 2022), and Prevotella (Boursier et al., 2016; Hoyles et al., 2018) all showed a downward trajectory. The microbial signatures observed in NASH patients were similar to those found in NAFLD when compared to healthy individuals serving as controls. However, these findings exhibited variations attributed to disparities in cohort demographics, including but not limited to sex, ethnicity, severity of liver disease stage, presence or absence of type 2 diabetes mellitus and BMI, as well as the patient population being either pediatric or adult. An additional factor that could complicate the interpretation of changes in microbiota composition in NAFLD is sarcopenia. Xiangya study has found that the relative abundance of six species (*Desulfovibrio piger*, *Clostridium symbiosum*, Hungatella effluvii, *Bacteroides fluxus*, Absiella innocuum, and *Clostridium citroniae*) was also positively associated with the severity of sarcopenia (Wang et al., 2022). Sarcopenia is a common complication of various chronic diseases (Tarantino et al., 2023), yet its specific molecular mechanisms remain unclear. Sarcopenia's close relationship with the gut microbiome has been documented in studies by Nikkhah et al. (2023) and Ren et al. (2021) and the prevalence of sarcopenia in patients with NAFLD has been noted by Cai et al. (2020). Additionally, discrepancies in gut microbiota composition were influenced by factors such as obesity-related metabolic diseases and the sequencing techniques employed for gut microbiota analysis. Consequently, certain contemporary studies even present contradictory trends when compared to the aforementioned results (Kim et al., 2021; Galli et al., 2022; Girdhar et al., 2023).

Therefore, in this study, 16S rRNA sequencing technology was employed to systematically analyze intestinal samples from Chinese individuals pathologically diagnosed with NAFL and NASH, and compare them with healthy controls. By investigating the variations in intestinal microflora composition at different stages of NAFLD and further exploring the correlation between alterations in the abundance of dominant strains of intestinal microflora among patients with distinct disease stages such as NAFL and NASH, functional analysis and mechanistic studies will contribute to comprehending the specific role of these microflora in NAFLD progression. The findings from this investigation are anticipated to offer novel biomarkers and therapeutic targets for diagnosing and treating NASH. Through understanding the association between gut microbiota and NASH, we can enhance our comprehension of NASH pathogenesis while developing new strategies and approaches for its prevention and treatment.

2 Materials and methods

2.1 Study design

This study aimed to investigate the role of gut microbiome in patients with NAFL or NASH through 16S rRNA gene amplicon sequencing. Patients diagnosed with NAFL or NASH, as well as a normal control population, were recruited for this study. Detailed demographics are provided in Table 1. Stool samples from the patients were collected and immediately stored in −80°C at the time of each research visit. DNA extraction of stools, amplification of bacterial 16S rRNA genes, cloning, and sequencing of polymerase chain reaction (PCR) products were performed at the BGI-Shenzhen laboratory. This study was approved by Ethics Committee of Shenzhen Hospital of Traditional Chinese Medicine (ethical approval number: K2017-024), all the participants who agreed to serve as fecal donors provided written informed consent, in accordance with national legislation and the Code of Ethical Principles for Medical Research Involving Human Subjects of the World Medical Association (Declaration of Helsinki).

2.2 Patient selection

2.2.1 Study participants

Participants were recruited from the Department of Hepatology at Shenzhen Traditional Chinese Medicine Hospital between December 2018 and December 2019. A total of 237 individuals

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TABLE 1 Characteristics of each group in detail, including clinical, metabolic, biochemical and histological profiles (mean, mean ± sd).

Variables (mean, sd)	Control	NAFL	NASH	FDR* (NASH vs. NAFL)
	N = 65	*N* = 64	*N* = 53	
Age, years	37.03 (9.19)	37.83 (9.29)	34.79 (8.32)	0.070076
Female sex, n (%)	35 (0.54)	31 (0.48)	5 (0.09)	0.000000
Smoking, n (%)				
Current smoker	26 (0.4)	25 (0.39)	9 (0.17)	0.000000
Ex-smoker	2 (0.03)	2 (0.03)	6 (0.11)	0.000000
Non-smoker	37 (0.57)	37 (0.58)	38 (0.72)	0.792146
BMI, kg/m2	22.76 (2.84)	29.26 (3.60)	29.44 (4.56)	0.000037
WC, cm	76.46 (12.57)	89.81 (10.83)	98.11 (7.70)	0.000342
CAP, dB/m	213.25 (18.84)	304.64 (35.16)	325.38 (35.84)	0.000757
E, kPa	4.31 (1.22)	5.12 (1.35)	9.25 (11.87)	0.000000
ALT, U/L	16.46 (7.00)	22.10 (7.23)	76.17 (34.50)	0.000000
AST, U/L	20.96 (8.49)	19.18 (3.89)	40.22 (15.45)	0.000000
GGT, U/L	17.94 (17.00)	27.48 (17.49)	71.45 (47.93)	0.000091
ALP, U/L	54.75 (14.00)	62.10 (16.68)	73.34 (14.43)	0.278399
TBIL, umol/L	15.58 (5.88)	15.01 (8.71)	16.39 (5.13)	0.21574
DBIL, umol/L	3.49 (1.84)	3.16 (1.82)	3.57 (1.64)	0.441257
IBIL, umol/L	12.09 (4.72)	12.03 (7.22)	12.82 (3.74)	0.003631
ALB, g/L	47.93 (2.39)	48.87 (2.30)	50.43 (3.81)	0.496058
PLT, %	258.31 (53.98)	284.52 (66.26)	273.00 (139.86)	0.000006
AFP, mg/L	2.83 (1.78)	3.23 (1.90)	1.68 (1.68)	0.000005
CR, mmol/L	68.57 (15.43)	66.59 (14.25)	78.92 (12.10)	0.000006
UA, umol/L	325.32 (84.63)	382.16 (80.30)	453.94 (83.03)	0.91268
eGFR	106.91 (13.25)	102.24 (22.07)	101.88 (16.32)	0.019299
TG, mmol/L	1.11 (0.54)	1.77 (1.73)	2.04 (0.77)	0.047391
TC, mmol/L	4.35 (0.76)	4.72 (0.99)	5.05 (0.91)	0.300345
LDL, mmol/L	6.73 (32.17)	3.09 (0.95)	3.28 (1.01)	0.818905
FBG, mmol/L	4.42 (0.87)	5.15 (0.56)	5.18 (0.63)	0.314147
HBAlc, %	5.62 (0.31)	5.70 (0.33)	5.76 (0.32)	0.004465
FBC, nmol/L	1.52 (0.63)	2.36 (0.98)	2.91 (1.41)	0.005862
FBI, pmol/L	46.85 (27.25)	86.17 (44.70)	116.34 (91.35)	0.054905
SF, umol/L	18.31 (6.98)	18.28 (6.30)	20.51 (6.10)	0.000000
FER, nmol/L	123.58 (113.66)	145.72 (117.95)	328.30 (211.70)	0.144514
CER, g/L	0.14 (0.03)	0.17 (0.04)	0.16 (0.04)	0.000000
AST/ALT	1.37 (0.43)	0.93 (0.24)	0.57 (0.21)	0.000000
SAF score	–	1.52 (0.31)	4.81 (0.74)	0.005000
HOMA-IR	1.37 (0.91)	2.75 (1.48)	3.71 (2.84)	0.005000
TyG	7.4 (0.39)	7.52 (0.64)	7.65 (0.32)	0.000000

*By Mann–Whitney test. HOMA-IR calculation formula: FPG (mmol/L) × FBI (μU/mL)/22.5. TyG calculation formula: ln [TG (mg/dl) × FBG (mg/dl)]/2.

underwent primary screening, with 32 patients excluded due to HBV infection and 10 patients excluded due to alcohol consumption. Among the remaining 205 subjects, ultrasound or Fibroscan was performed to diagnose NAFLD and categorize them into 140 NAFLD patients and 65 normal controls. 64 patients out of the 140 with NAFLD showed abnormal ALT levels (ALT≥40 U/L). All of these 140 NAFLD patients were advised to undergo a liver biopsy and provide fecal samples; however, 20 of them refused the liver biopsy and three patients had unqualified fecal specimens. Finally, a total of 53 NASH patients with confirmed biopsies, along with 64 biopsy-proven NAFLD patients and 65 healthy controls were enrolled (Figure 1).

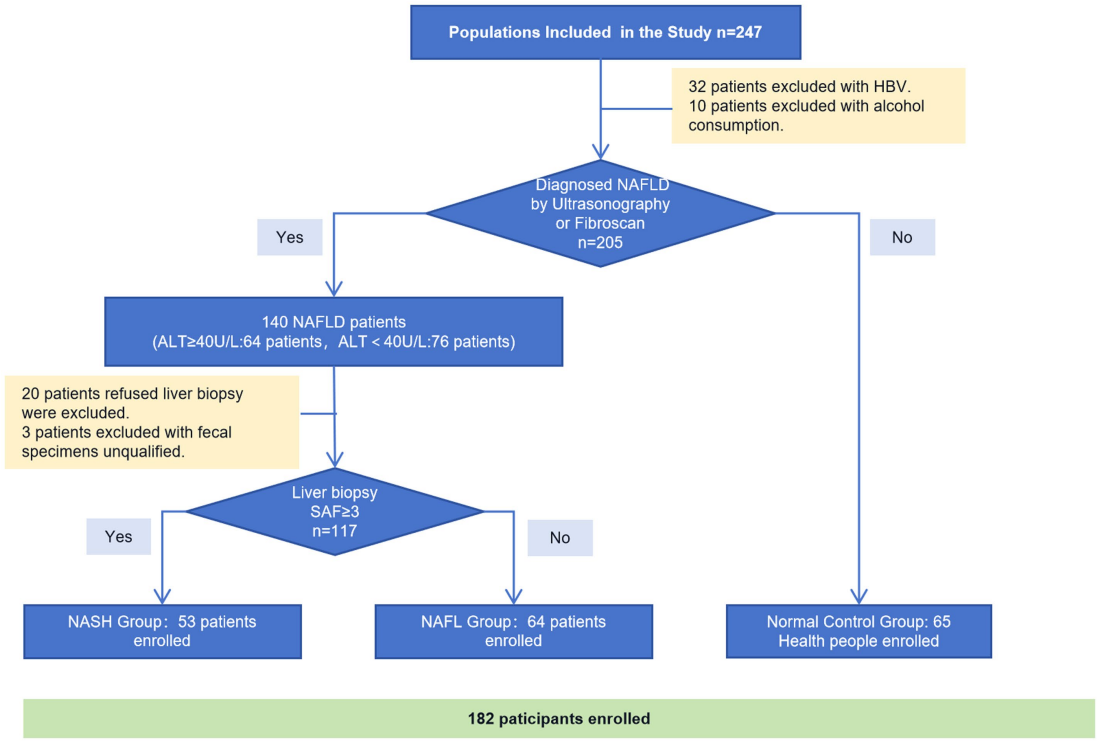

FIGURE 1
Consort flow diagram of study participants.

2.2.2 Inclusion criteria

Inclusion criteria were: (1) Presence of hepatic steatosis confirmed by liver imaging techniques such as ultrasonography or fibroscan; (2) Absence of any concurrent chronic liver diseases including viral hepatitis or autoimmune hepatitis; (3) Alcohol consumption below 20 g/day.

2.2.3 Liver biopsy and histopathology

The percutaneous liver biopsy was performed under ultrasound guidance following local anesthesia. A 16-G Tru-Cut biopsy needle (C.R. Bard Inc., Covington, United States) was inserted into the liver parenchyma to obtain an adequate amount of liver tissue containing a minimum of 10 portal tracts with an approximate length of 2.0 cm for precise diagnostic purposes. The obtained specimens were promptly fixed, embedded in paraffin, and stained with hematoxylin–eosin (HE). Subsequently, all specimens were sent to the Department of Pathology at Shenzhen Traditional Chinese Medicine Hospital for evaluation. Liver pathological images were diagnosed by two experienced pathologists who were blinded to the TE (CAP) value and color Doppler ultrasound scans. The SAF score was employed to semi-quantify each individual feature of NAFLD, encompassing steatosis, inflammatory activity, and fibrosis, in accordance with the SAF (0–7) scoring system (Bedossa et al., 2012). The classification of NAFLD comprises two subtypes: NAFL and NASH. The scoring criteria encompass hepatocyte steatosis (0–3), balloon-like degeneration (0–2), and lobular inflammation (0–2). NASH is diagnosed when both balloon-like degeneration and lobular inflammation coexist, or if the total score on each scale is ≥3; otherwise, NAFL is diagnosed.

2.2.4 Genomics DNA extraction

DNA was extracted from fecal samples using the MagPure Stool DNA kit B (Magen, China) according to the provided protocol. Subsequently, the DNA was quantified utilizing a Qubit Fluorometer with the Qubit® dsDNA BR Assay kit (Invitrogen, China), and its quality was assessed by electrophoresis on a 1% agarose gel.

2.2.5 Library construction

Variable region V4 of bacterial 16S rRNA gene was amplified with degenerated PCR primers, 515F (5'-GTGCCAGCMGCCGCG GTAA-3′) and 806R (5'-GGACTACHVGGGTWTCTAAT-3′). The forward and reverse primers were both modified with Illumina adapter, pad, and linker sequences. PCR enrichment was conducted in a 50 μL reaction system containing 30 ng of template DNA using the following cycling conditions: pre-denaturation at 95°C for 3 min, followed by 30 amplification cycles consisting of denaturation at 95°C for 45 s, annealing at 56°C for 45 s, and extension at 72°C for 45 s. A final extension step was performed in 72°C for a duration of 10 min. Subsequently, the PCR products were purified using Agencourt AMPure XP beads and eluted in Elution buffer. The quality assessment of libraries was conducted using the Agilent Technologies' bioanalyzer model number 2100. Once validated, these libraries were subjected to sequencing on Illumina HiSeq2500 platform (BGI, Shenzhen, China) following the standard protocols provided by Illumina. This resulted in the generation of paired-end reads with a length of 2 × 250 bp.

2.2.6 Sequencing and bioinformatics analysis

The raw reads were subjected to adaptor removal, as well as the elimination of low-quality and ambiguous bases. Subsequently, the Fast Length Adjustment of Short reads program (FLASH, v1.2.11) was employed to merge paired-end reads with tags in order to obtain the final tags.

The tags were denoised into operational taxonomic units (OTUs) using two methods: unoise3 with VSEARCH (v2.22.1; Rognes et al., 2016), and DADA2 with QIIME2 v2023.2 (Bolyen et al., 2019). Chimera sequences were identified by comparing them to the gold database Silva (v123; Quast et al., 2012) using VSEARCH. Subsequently, an OTU feature table was generated for further analysis using VSEARCH (v2.22.1). Taxonomic classification of the OTU representative sequences was performed with Silva v123 (Pelin et al., 2013), employing a minimum confidence threshold of 0.6, and trained on the Greengenes database v13_8 through QIIME2(v2023.2; Yokono et al., 2018). Finally, USEARCH global (Edgar, 2010) was utilized to compare all tags back to the OTUs in order to obtain an abundance statistics table for each sample.

The estimation of alpha and beta diversity was conducted using USEARCH (v11.0.667; Edgar, 2010) and QIIME2 (v2023.2) at the OTU level, respectively. The visualization of diversity analysis was generated using the R package "amplicon" (Labun et al., 2019), while the Chord diagram illustrating different classification levels was created using the R package "circlize" (Gu et al., 2014).

Differential analysis was performed using the "edgeR" (Chen et al., 2016) package and STAMP (Parks et al., 2014). Volcano plots and Manhattan plots were generated using the "ggplot2" (Ginestet, 2011) package. LDA analysis was conducted using LEfSe (Zhang et al., 2013). A false discovery rate (FDR) of 0.05 and an LDA score greater than 2 were used as indicators of statistical significance. KEGG function prediction was carried out using the PICRUSt (Wilkinson et al., 2018) and PICRUSt2 (Douglas et al., 2020) software. Organism Level Microbiome Phenotypes were predicted using BugBase (Ward et al., 2017) software. Significant species of function were determined by R (v4.2.3), based on Wilcox-test or Kruskal-test.

2.2.7 Statistical analysis

Because the data did not all satisfy the normal distribution, the Mann–Whitney test was used to test each clinical data. The statistical test of negative binomial distribution was used to analyze OTU differences by edgeR. The Wilcoxon rank sum test was used to analyze species differences between the two groups. LEfSe analysis firstly used the non-parametric Kruskal-Wallis rank sum test to detect species with significant differences between different groups in multiple groups of samples, and then used the Wilcoxon rank sum test for groups of significantly different species to analyze the differences between groups. Finally, linear discriminant analysis (LDA) was used to reduce the dimensionality of the data to evaluate the influence of species with significant differences. In the analysis of metabolic pathways, Welch's t-test was used to compare the two groups. Data sets that involved more than two groups were assessed by one-way ANOVA followed by Tukey–Kramer *post-hoc* tests. $p < 0.05$ was considered statistically significant and Story false discovery rate (FDR) of 0.05 was used as indicators of statistical significance.

3 Results

3.1 Clinical characteristics of NAFLD population

This study included 64 patients diagnosed with NAFL and 53 patients diagnosed with NASH based on pathological examination, as well as 65 individuals without NAFLD serving as controls, according to the predefined inclusion criteria. The comprehensive characteristics of each group, encompassing clinical, metabolic, biochemical, and histological profiles, are presented in Table 1. Most of the clinical indices such as BMI, CAP, E, ALT, AST, GGT, ALP, UA, etc. showed significant variance. The values of CAP, ALT, E, HOMA-IR and other clinical indices gradually increased in the control, NAFL and NASH groups, and the indices in the NAFL and NASH groups were mostly higher than the normal values of liver function tests.

3.2 Species diversity of the gut microbiome in groups

In microbiome sequencing, the UNOISE3 algorithm yielded a total of 13,686 operational taxonomic units (OTUs). A sparse profile based on observed species indicates that the sequencing data is sufficient for detecting all species present in the samples (Supplementary Figure S1). To assess differences in bacterial diversity among the three groups, sequence alignment was performed to estimate both alpha and beta diversity. Our study revealed significant differences in α diversity between the NAFL group, NASH group, and control group (Shannon index, $p < 0.05$; Figure 2B). Beta diversity was subsequently evaluated using CPCoA rankings (Figure 2C), revealing a significant variation in species composition across the three groups (variance: 1.32%; $p = 0.009$). The Venn diagram demonstrated that there was a total of 2,481 OTUs shared among all three groups, with differentially expressed OTUs numbering at 210 for the control group, 156 for NAFL, and 71 for NASH. Furthermore, the number of group-specific taxa decreased from healthy control patients to NAFL patients to NASH patients (Figure 2A).

3.3 Differences in the taxonomic composition in groups

To investigate taxonomic differences between NAFL and NASH groups at the phylum and genus levels, we employed edgeR and LEFSe for analysis. The relative abundance of bacteria was assessed across all three groups at both the phylum and genus levels, revealing statistically significant disparities. At the phylum level, each sample exhibited distinct species composition (Figure 3A), with Bacteroidetes, Firmicutes, Proteobacteria, Fusobacteria, and Actinobacteria being the predominant phyla accounting for 95% or more abundance in all three groups (Figure 3B). At the phylum level, the predominant bacteria in the NAFL group were predominantly members of the phylum Bacteroidetes (73.9%), Firmicutes (17.2%), Fusobacteria (3.4%), and Proteobacteria (4.6%). The NASH group was also dominated by members of Bacteroidetes (71%), Firmicutes (19.7%), Fusobacteria (4.1%), and Proteobacteria (4.8%). At the genus level, Bacteroides, Prevotella_9,

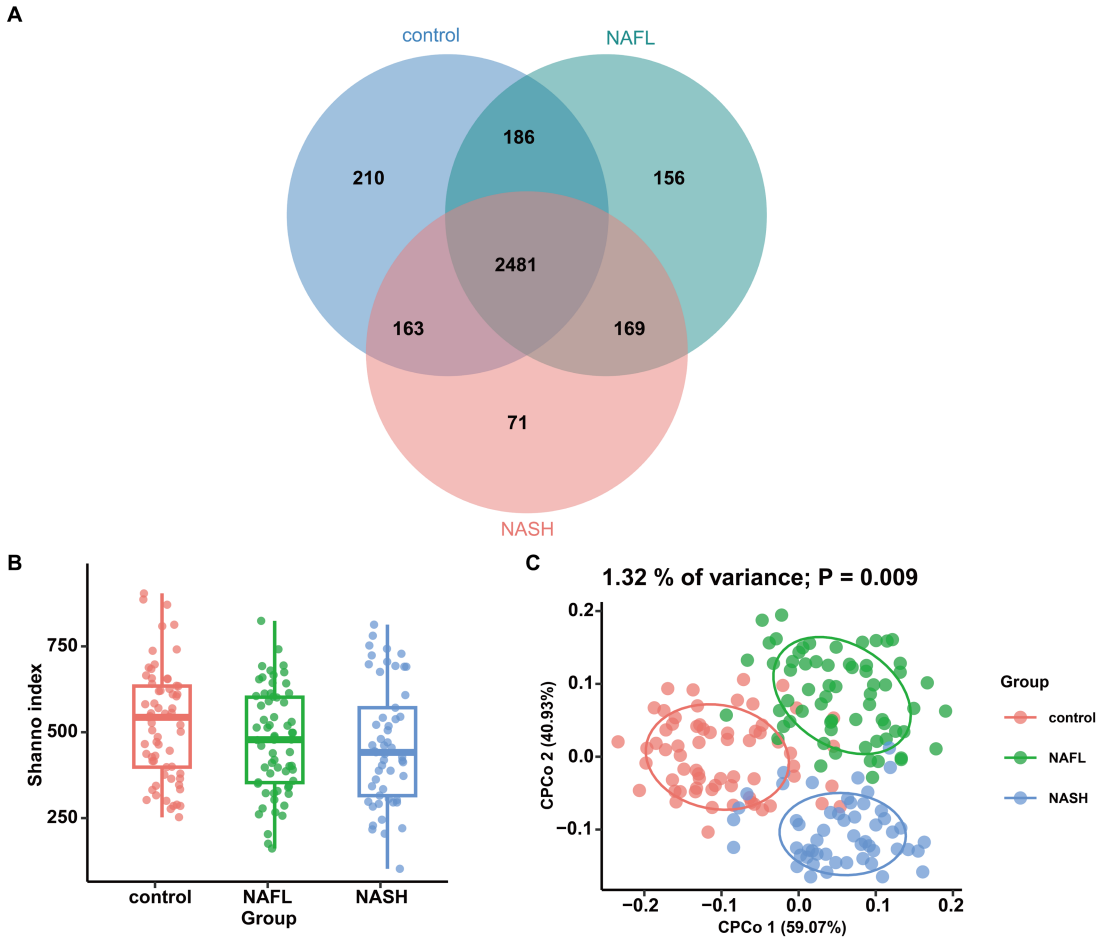

FIGURE 2
Comparisons of the alpha diversity and beta diversity about control, NAFL and NASH patients. **(A)** Venn diagram showing overlaps of the operational taxonomic units (OTUs) in the two groups (Shannon index). **(B)** Alpha diversity of the three groups, as quantified by diversity indices (Shannon, $p < 0.05$). **(C)** Principal coordinate analysis (CPCoA) plots of bacterial beta diversity of the three groups based on Euclidean. ($p = 0.009$).

Faecalibacterium, Megamonas, and Fusobacterium were the predominant genera accounting for 90% or more abundance in all three groups (Figure 3C). The results of the edgeR package analysis, illustrated by the Manhattan plot (Figure 4A) showed that there was an enrichment of Bacteroidetes and Fusobacteria, while the ratios of Firmicutes and Proteobacteria were depleted in both the NAFL group and NASH group, as depicted in Supplementary Figure S2. Compared to the NAFL group, the NASH group exhibited higher levels of Firmicutes but lower levels of Bacteroidetes at phylum level, with Bacteroides being enriched as the most abundant genus (FDR < 0.05; Supplementary Figure S2). The relative abundance of Bacteroides and Fusobacterium was significantly higher in the NAFL group, while Prevotella_9 was lower compared to the control group. In the NASH group, there was an enrichment of Megamonas and Fusobacterium. Furthermore, the NASH group exhibited higher levels of Megamonas and Fusobacterium compared to the NAFL group.

The LEfSe analysis was employed to construct a cladogram for the identification of specific bacteria associated with the three groups (Figure 4B). Megamonas and Fusobacterium were found to be significantly enriched in the fecal samples of patients belonging to the NASH group, as indicated by high LDA scores (log10 > 4). Conversely, Subdoligranulum and Ruminococcus_2 were identified as the predominant microbiota in the control group (Figure 4C).

3.4 Difference analysis of intestinal flora expression between NAFL and NASH patients

In the study cohort, we constructed a random forest classifier model for 65 healthy controls, 64 NAFL and 53 NASH to evaluate the diagnostic efficacy of intestinal microbiome markers and explore their potential as a non-invasive diagnostic tool for NAFL and NASH. Through a 10-fold cross-validation of the random forest model, the dataset was partitioned into 10 subsets, with nine of them utilized for training purposes and one reserved for testing. The accuracy rate was computed for each experiment, and the average accuracy rate from 10 iterations serves as an estimation of the algorithm's performance. This process was repeated using 10-fold cross-validation to obtain a final averaged estimation of the algorithm's accuracy. Using this approach, the first five microbial genera were selected as the best marker set for NAFL and NASH (Figure 5A), Combining the results of species abundance and LEfSe analyses, we decided to assess the potential value of Prevotella_9, Megamonas and Fusobacterium as biomarkers. The results showed that compared with the control group, Prevotella_9 distinguished NAFL with an AUC of 0.738 (95% CI = 0.580–0.896; Figure 5B). However, the

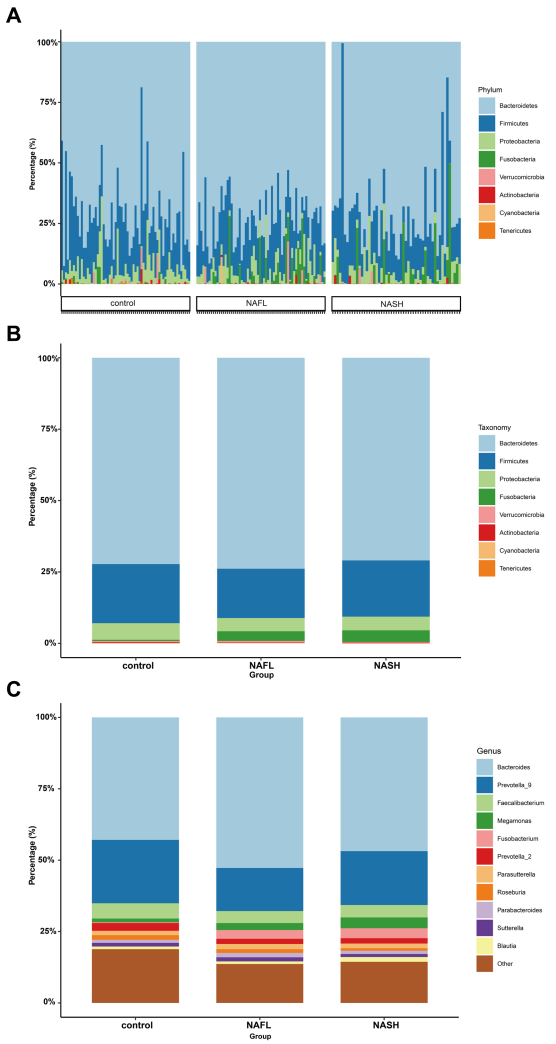

FIGURE 3
Comparisons of the taxonomic classifications of control, NAFL, NASH group. **(A)** Phyla composition for each sample. The species composition of all 182 samples is displayed, revealing significant variations in both the species distribution and relative abundance among each sample. Consequently, subsequent investigations can selectively focus on a few specific samples for comprehensive data analysis and clinical examination. $p < 0.05$. **(B)** Top 8 phyla in the three groups. $p < 0.05$. In the three groups, despite variations in the relative abundance of species, the phyla Bacteroidetes and Firmicutes consistently represented the largest fractions. This suggests that regardless of the health status—spanning from the healthy individuals, through those with NAFL, to patients with NASH—the key constituents of the gut microbiome remain fundamentally stable, without extreme shifts from dominance to depletion. **(C)** Top 11 genera in the three groups. $p < 0.05$. Upon refining the analysis down to the genus level, we discerned more defined taxonomic categories, and the distinctions between species became increasingly conspicuous. This enables the identification of potentially pathogenic bacteria associated with conditions such as NAFL or NASH. Such genus-level differential analysis is instrumental in establishing a foundation for clinical diagnosis, informing treatment strategies, and guiding preventative measures.

expression of Megamonas and Fusobacterium continues to progressively increase in the control group, NAFL group, and NASH group, with a particularly significant elevation observed in the NASH group. The combined presence of these taxa effectively distinguishes NASH with an AUC of 0.769 (95% CI = 0.610–0.927; Figure 5C). It is evident that Prevotella_9 exhibits a significant decrease in expression as the most representative bacterial taxon in NAFL, while Megamonas and Fusobacterium demonstrate a notable increase in expression as the most representative taxa in NASH. To differentiate between the microflora profiles of the NAFL and NASH groups, a simple forest analysis was conducted using single and combined data for Megamonas and Fusobacterium compared to those solely of the NAFL group (Figure 5D), this yielded an AUC value of 0.666 for single Fusobacterium alone. These findings suggest a potential correlation between elevated levels of Megamonas and Fusobacterium and disease progression.

3.5 Public data validation

To validate the robustness and generalizability of the analysis findings, we selected two sets of publicly available NAFLD-related sequence data for species diversity analysis. The respective Project ids associated with these datasets are PRJNA737039 and PRJNA540738. Upon comparing our data with the two public datasets, it is evident that the composition and variability of gut microbiota across different countries and regions are substantial. These discrepancies are likely influenced by factors such as geographical environment and dietary practices. Notably, our analysis results exhibit a higher degree of similarity with the PRJNA737039 project, with predominant species including Bacteroides, Prevotella_9, Faecalibacterium, and Roseburia. The observed differences in species composition and significant disparities may be attributed to disease classification standards, regional influences, and other variables. This suggests that the findings of our research may have greater relevance and applicability within the context of China (Supplementary Figure S3). The PRJNA737039 dataset originated from China, while the PRJNA540738 dataset was obtained from Germany. Combining species diversity analysis with LEfSe difference analysis revealed that in Project PRJNA737039, Bacteroidetes, Prevotella_9 and Faecalibacterium were significantly expressed in both NAFL and NASH groups compared to healthy controls, which is consistent with the species composition observed in our own sample. In contrast, Project PRJNA540738 exhibited a distinct pattern of species expression, including Bacteroides, Blautia, Bacillus, and Faecalibacterium. Our analysis findings exhibit a high degree of resemblance to the taxonomic composition of the Chinese dataset, while displaying significant disparities with the European data. This underscores the credibility of our analytical results and further highlights dissimilarities in gut microbiome composition between Asian and European NAFLD patients, potentially attributed to factors such as dietary habits and living environment.

3.6 Functional analysis of the gut microbiome

The Bugbase platform was utilized to predict the corresponding characteristics and content of each bacterium within the three groups. The compositional differences of aerobic bacteria, anaerobic bacteria, facultative anaerobes, mobile element carriers, biofilm formers, gram-negative species, gram-positive species, potentially pathogenic strains, and stress-tolerant organisms in these three groups are illustrated in

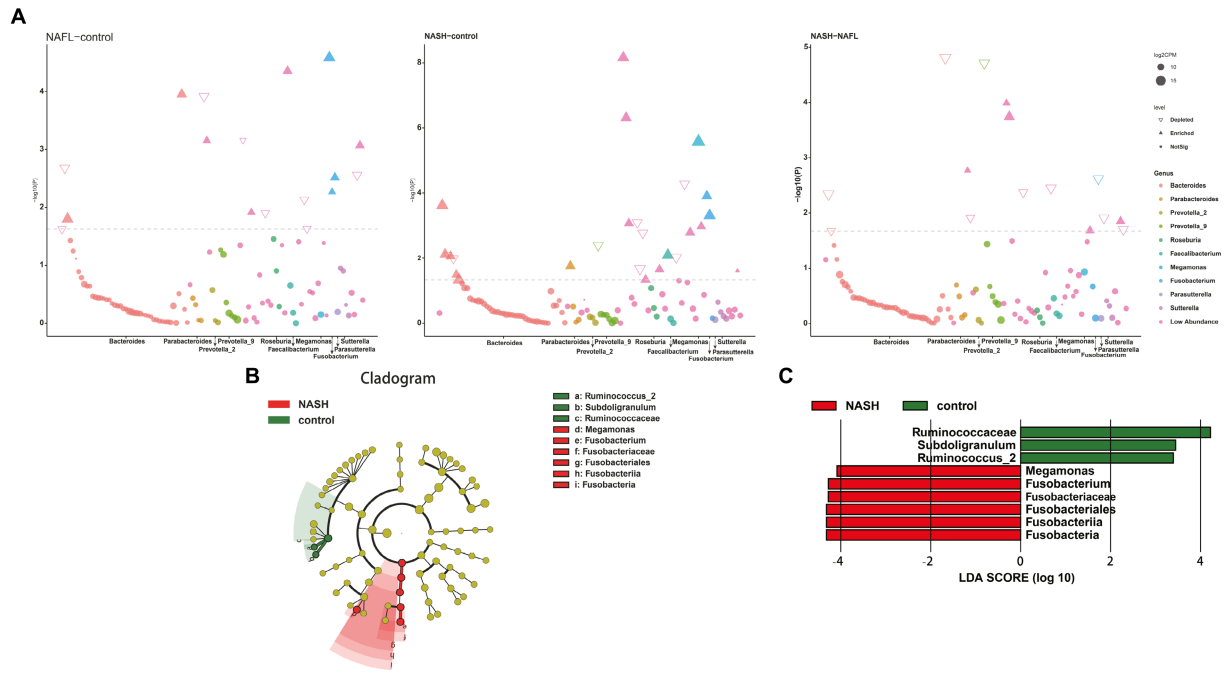

FIGURE 4
Bacterial biomarkers identified with the edgeR and linear discriminant analysis effect size (LEfSe) algorithm. **(A)** The results of the differential analysis of 10 genera including Bacteroides, Parabacteroides, Prevotella_2, Prevotella_9, Roseburia, Faecalibacterium, Megamonas, Fusobacterium, Parasutterella, Sutterella are presented. Manhattan plots for analysis of genus differences between NAFL and control groups (Figure 4A, left; $p < 0.05$). Bacteroides and Fusobacterium were significantly expressed in NAFL group compared with the control group. Manhattan plots for analysis of genus differences between NASH and control groups (Figure 4A, middle; $p < 0.05$). Faecalibacterium and Fusobacterium were significantly expressed in NASH group compared with the control group. Manhattan plots for analysis of genus differences between NASH and NAFL groups (Figure 4A,right; $p < 0.05$). Bacteroides was significantly decreased in NASH group compared with the NAFL group. **(B)** Cladogram showing the relationships between taxa at different taxonomic levels. Each circle represents a hierarchical structure, followed by phylum, class, order, family and genus. Different phyla are marked with different colors. The size of the nodes represents the abundance of the taxon. **(C)** Linear discriminant analysis (LDA) scores with the LEfSe tool for taxa, with LDA scores >4 and $p < 0.05$ shown in the histogram.

Supplementary Figure S4. Bacteroides exhibited higher enrichment in the NAFL group while Firmicutes were enriched in the NASH group with regards to their high-level phenotypes present potential pathogenicity (Supplementary Figure S4H).

PICRUSt and PICRUt2 were utilized for predictive functional analysis. KEGG pathway comparisons were conducted to investigate potential variations in the functional composition of the microbiome among NAFL, NASH, and control groups. PICRUST2 essentially encompasses the findings obtained from PICRUST (Supplementary Figure S5). In comparison to the control group, numerous pathway differences were observed in NAFL, such as increased abundance of lipid IVA biosynthesis, 6-hydroxymethyl-dihydropterin diphosphate biosynthesis I, CMP-3-deoxy-D-manno-octulosonate biosynthesis I, and 6-hydroxymethyl-dihydropterin diphosphate biosynthesis III (Chlamydia), while decreased abundance was found in superpathway of purine deoxyribonucleosides degradation, L-methionine biosynthesis III, and phosphatidylglycerol biosynthesis I (plastidic; Figure 6A). The pathways were significantly different in the NASH group compared with the control group. Specifically, 6-hydroxymethyl-dihydropterin diphosphate biosynthesis, L-lysine biosynthesis II, and superpathway of L-phenylalanine biosynthesis were more abundant in NASH while superpathway of hexuronide and hexuronate degradation, phosphatidylglycerol biosynthesis II (non-plastidic), and phosphatidylglycerol biosynthesis I (plastidic) was less abundant (Figure 6B). Compared to the control group, there were significant differences in pathways between NASH and NAFL groups. Creatinine degradation II was more abundant in NASH compared to NAFL while superpathway of UDP-N-acetylglucosamine-derived O-antigen building blocks biosynthesis, L-1,2-propanediol degradation, dTDP-L-rhamnose biosynthesis I, succinate fermentation to butanoate, and Flavone and flavonol biosynthesis were significantly less abundant than the NAFL group (Figure 6C).

4 Discussion

There is already evidence to suggest that the development of fatty liver is related to gut bacteria, including dysbiosis-induced dysfunction of the intestinal epithelial barrier. This dysfunction allows the translocation of bacterial components and leads to liver inflammation. Additionally, various metabolites produced by the gut microbiota may affect the liver, thereby regulating susceptibility to NAFLD.

In our study, 16S amplicon analysis revealed alterations in the gut microbiota composition in NAFL and NASH diseases compared to a healthy control group, as well as differences between the two diseases. The NAFL group exhibited an increased abundance of Gram-negative bacteria and a decreased abundance of Gram-positive bacteria compared to the control group. Bacterial diversity showed significant differences ($p < 0.05$) among the three groups, with species diversity

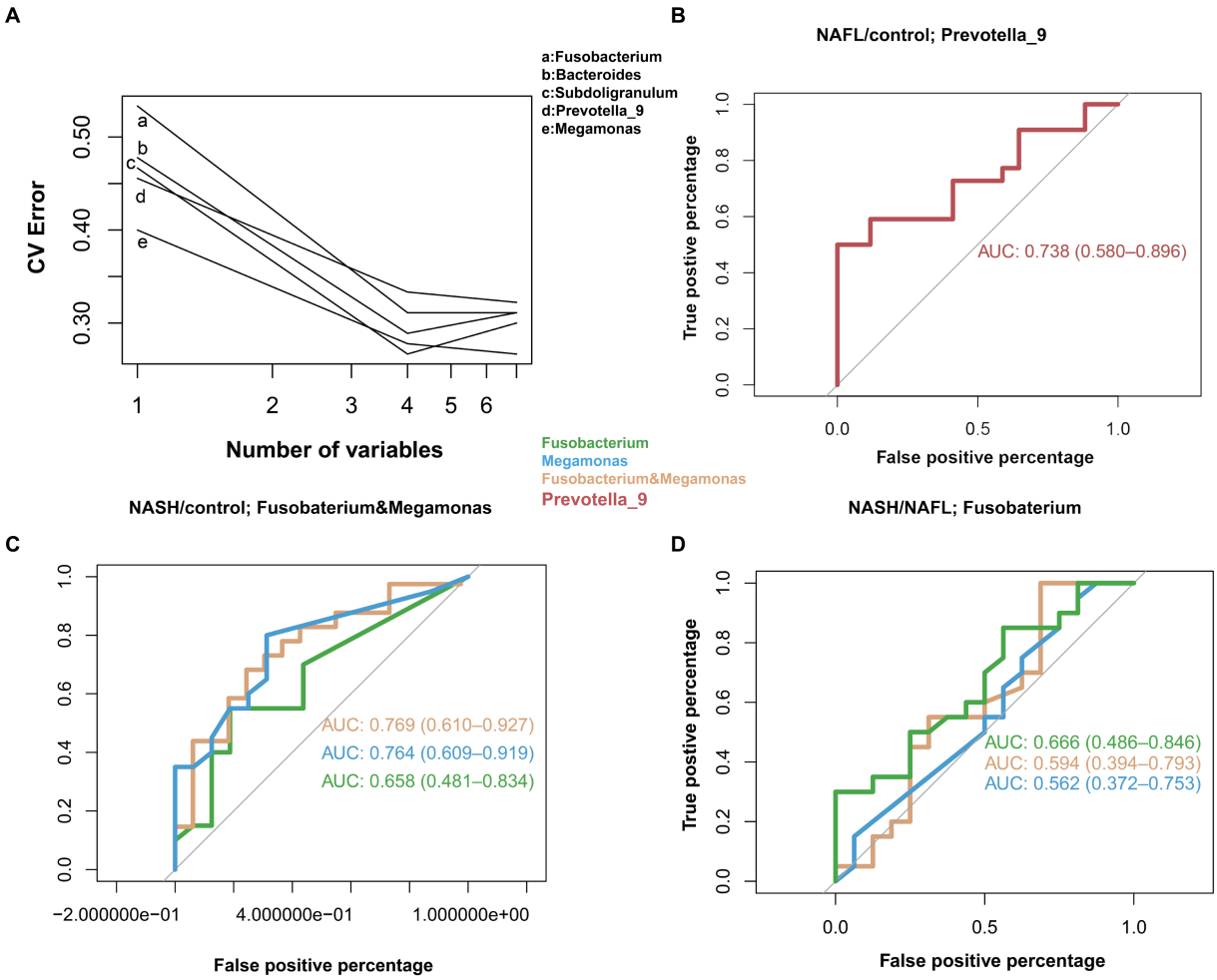

FIGURE 5
Diagnostic potential of gut microbial markers in NAFL and NASH patients. Diagnostic potential of gut microbial markers in NAFL and NASH patients. **(A)** Five microbial markers (Bacteroides, Prevotella_9, Ruminococcus_2, Subdoligranulum, Megamonas, Fusobacterium) were selected as the markers set by the random forest model. **(B)** The classification performance of the multivariable logistic regression model using relative abundance of genera (Prevotella_9) was assessed using area under the ROC. The AUC value of 0.738 with 95% CI of 0.580–0.896 between control versus NAFL group. **(C)** Classification performance of a multivariable logistic regression model using the relative abundance of genera (Megamonas, Fusobacterium, and a combination of both) was evaluated using the area under the ROC. The highest AUC value between the control group and the NASH group was 0.769, with a 95% CI of 0.610–0.927, belonging to the combination of Megamonas and Fusobacterium. **(D)** Classification performance of a multivariable logistic regression model using the relative abundance of genera (Megamonas, Fusobacteria, and a combination of both) evaluated using the area under the ROC. The highest AUC value between the NAFL and NASH groups was 0.666, 95% CI, belonging to the genus Fusobacterium.

progressively decreasing by threefold in the NASH group. This shift toward specific bacterial types promotes an increase in pathogenic abundance (Shen et al., 2017). At the phylum level, the abundances of Bacteroidetes and Fusobacteria, were higher in NAFL and NASH patients than in the control group, while the abundances of firmicutes were lower. At the genera level, a significant reduction in the abundance of Prevotella was observed in the NAFL group. In contrast, the NASH group showed a higher proportion of Megamonas and Fusobacterium ($p < 0.05$). These findings align with existing data from Asian patients but differ substantially from European case data, suggesting variations in gut microbiome composition between Eurasian populations (Quesada-Vazquez et al., 2020).

Through the analysis of three main bacterial genera, namely Prevotella_9, Megamonas, and Fusobacterium, it was observed that compared to the control group, Prevotella_9 exhibited a discriminative ability for distinguishing NAFL with an AUC of 0.738 (95% CI = 0.580–0.896; Figure 5B). Conversely, the combination of Megamonas and Fusobacterium demonstrated a discriminative potential for identifying NASH with an AUC of 0.769 (95% CI = 0.610–0.927; Figure 5C). In the NAFL group, we observed that Fusobacterium alone had a superior diagnostic performance than Megamonas alone, or Fusobacterium and Megamonas combined. More specifically, as shown in Figure 5D, the AUC of Fusobacterium is 0.666 (95% CI = 0.486–0.846), surpassing that of Megamonas alone (0.562, 95% CI = 0.372–0.753), or that of Fusobacterium and Megamonas combined (0.594, 95% CI = 0.394–0.793). These observational results suggest that an increased abundance of Prevotella_9 has diagnostic value for NAFL, while elevated levels of Megamonas and Fusobacterium may indicate a more severe disease state that has progressed to the NASH stage, especially the increase in Fusobacterium, which has more significant diagnostic meaning. In other words, changes in

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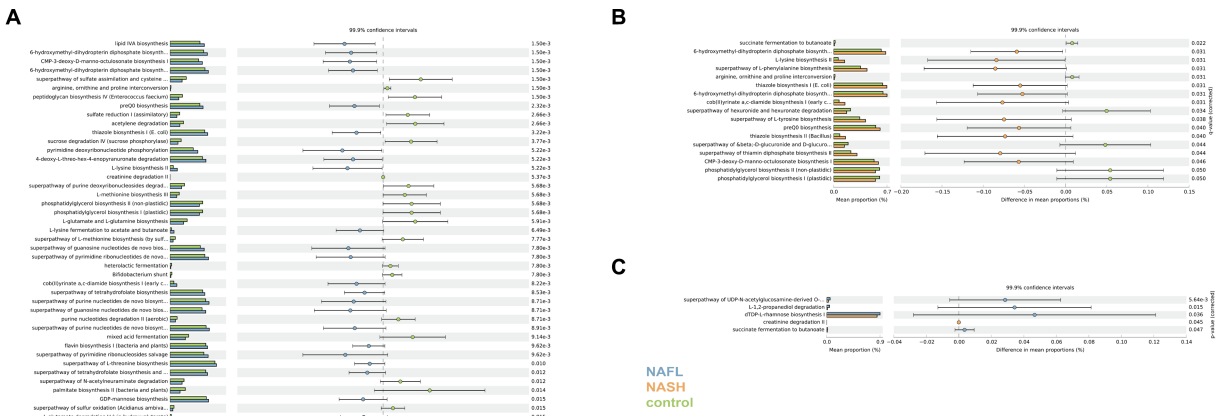

FIGURE 6
Functional analysis of the predicted metagenomes. **(A)** Difference in relative abundance of predicted microbial genes related to metabolism information between NAFLD and healthy controls. **(B)** Difference in relative abundance of predicted microbial genes related to metabolism information between NASH and healthy controls. **(C)** Difference in relative abundance of predicted microbial genes related to metabolism information between NASH and NAFLD. Data were processed through PICRUSt2's 16S rRNA sequencing data using level 3 of the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologues and using a Student's t-test. The significance of gut microbial species derived from blocked two-sided Wilcoxon tests (+indicates statistical significance FDR < 0.05, 99.9% confidence intervals).

these three main gut genera could provide a non-invasive basis for diagnosing different stages of NAFLD.

Building upon previous research, we conducted an analysis on the potential pathogenic mechanisms of Bacteroides and Fusobacterium. The gut microbiota has the ability to ferment dietary carbohydrates into ethanol, which subsequently enters the bloodstream and is eventually metabolized by the liver. Notably, Bacteroides is recognized as one of the primary producers of ethanol (Amaretti et al., 2007). Both animal studies and clinical trials have consistently demonstrated that NAFL patients exhibit significantly elevated levels of gastrointestinal ethanol compared to control groups, with a strong correlation observed between these levels and alterations in gut microbiota composition (Cope, 2000). The higher abundance of Bacteroides in the NAFL group, compared to the control group, implies that an elevated Bacteroides content may contribute to liver damage by promoting endogenous ethanol production. Fusobacterium has been reported to produce short-chain fatty acids with both anti-inflammatory and pro-inflammatory properties (Fan et al., 2019). An increase in Fusobacterium abundance can serve as a marker for tumor occurrence and intestinal inflammation (Kostic et al., 2012; Zhang et al., 2018). Fusobacterium, a pro-inflammatory bacterium known to impair the integrity of the intestinal barrier (Neubauer et al., 2019), has been observed in fatty samples and is associated with the exacerbation of liver steatosis and recurrence of NAFL (Fan et al., 2019). The Prevotella spp. and P. Capri complexes are commonly associated with non-Western dietary patterns characterized by a high intake of carbohydrates, resistant starch, and fiber content. There is a negative correlation between the relative abundance of Prevotella and Bacteroides (Carelli et al., 2023).

Prevotella has a number of classification methods, and it has been reported that a 16S rRNA sequence method was utilized to reclassify Prevotella and identify 5 genera (Adkins et al., 2017). This study unveiled a negative correlation between changes in Prevotella-9 abundance and Bacteroides, which aligns with previous research

findings. Previous studies have reported diverse roles for Prevotella in gut health, while P. Capri has demonstrated potential benefits in glucose homeostasis and host metabolism. Conversely, another study identified an association between P. Capri and insulin resistance. This study revealed a significant decrease in NAFLD when comparing levels of Prevotella 9 between healthy individuals and those at the NAFL stage, suggesting that reduced Prevotella abundance may contribute to impaired carbohydrate and starch metabolism, both of which are linked to obesity and steatosis. The impact of Prevotella on human health remains uncertain, leading to ongoing debates regarding its potential benefits or harms. However, it is undeniable that the enzymatic digestion of polysaccharides within the Prevotella library plays a crucial role in digestive dynamics and intestinal homeostasis. It should be noted that the relationship between Prevotella and inflammation, particularly NASH, still lacks clarity. In this study, although there was some recovery in Prevotella abundance from NAFL to NASH stages, it remained lower than that observed in healthy individuals. The relationship between the inflammatory response of NAFL and the progression of NASH, as well as fibrosis, remains unclear. Some studies have reported a significant increase in Prevotella abundance in NASH with fibrosis (Boursier et al., 2016). Compared to the control group, the NASH group exhibited higher abundances of Megamonas and Fusobacterium. Megamonas, a core gut bacterium, may be indicative of Asian ethnicities. It is closely associated with diseases such as inflammatory bowel disease, colorectal cancer, ankylosing spondylitis (AS), autism spectrum disorder (ASD), and obesity. Previous studies on the gut microbiota of European subjects did not report dominant presence of the genus Megamonas; it was only observed in studies conducted on Chinese individuals, suggesting its potential characteristic nature within the Asian population (Boursier et al., 2016). Studies have revealed a negative correlation between the abundance of Megamonas and the rate of weight loss, exhibiting a significant increase in the obese cohort. The average BMI of our study population exceeded 29 kg/m^2, with nearly all NASH patients displaying abnormal ALT levels. These findings suggest a substantial elevation in Megamonas abundance within the Chinese obese population, which, when combined with an augmentation in Fusobacterium abundance, further compromises intestinal barrier integrity and facilitates the progression from NAFL to NASH.

To further investigate the impact of dysbiosis on microbial metabolism, we utilized gene transcription analysis techniques. KEGG pathway analysis revealed significant alterations in metabolic pathways associated with NAFLD, including notable variations in transcription, immune system disorders, signaling molecules and their interactions, environmental adaptation, transport and catabolic metabolism. The changes of these metabolites are correlated with disease progression. In comparison to the NAFL group, the NASH group exhibited significant differences in all aspects of glucose metabolism as well as biosynthesis of flavonoids and flavonols.

In the KEGG predicted functional pathway analysis of both the NASH and NAFL groups, significant increases were observed in the biosynthesis of flavones and flavonols, glucose metabolism pathways (including the biosynthesis superpathway of O-antigen building blocks derived from UDP-N-acetylgalactosamine), degradation pathways of L-rhamnose and L-fucopyranose (including L-1,2-propanediol degradation), biosynthesis of dTDP-L-rhamnose I, and butyrate fermentation from succinate in the NAFL group. Additionally, creatinine degradation II was significantly higher in the NASH group. It has been reported that flavonoid compounds are representative substances that protect liver function by regulating CYP2E19 (Liu et al., 2021). Compared to NAFL, the expression of flavones and flavonol biosynthesis pathways is reduced in NASH. Numerous studies have demonstrated that flavonoid compounds exert physiological functions such as antioxidation, lipid-lowering, blood sugar regulation, and inflammation inhibition by modulating the gut microbiota, thereby preventing various diseases. The presence of an inflammatory response in NASH and may be associated with alterations in this pathway. Research has reported the potential role of ferritin in reflecting the inflammatory state associated with NAFLD and its progression, with elevated ferritin levels indicating inflammation and liver damage (Shah and Kowdley, 2019). Although we did not initially focus on inflammatory biomarkers such as ferritin in our study, in future research on the inflammation associated with NASH, we can consider measuring inflammatory biomarkers like ferritin in the serum of patients with NAFLD or NASH. Differences in various carbohydrate metabolisms also indicate changes in the fundamental metabolism between the two groups (Sandoval et al., 2020; Kariagina and Doseff, 2022; Tan et al., 2022). This finding provides support for the hypothesis that NAFLD is fundamentally a metabolic disorder. Our study focused on the Chinese population, with participants from various regions of China, including Shenzhen, a city known for its immigrant population. It should be noted that the relationship between gut microbiome dysbiosis and NAFLD disease cannot be extrapolated to European and American populations. Additionally, it is important to acknowledge that the use of 16S rRNA gene sequencing methods has inherent limitations in accurately determining the composition of the microbiome.

5 Conclusion

In summary, this study employed 16S amplicon analysis to uncover changes in the composition of gut microbiota during the progression of NAFLD, characterized by a gradual decrease in species diversity. At the phylum level, significant shifts were observed in the abundance of key bacterial groups including Bacteroidetes, Firmicutes, Fusobacteria. Moreover, at the genus level, a noticeable reduction was observed abundance of Prevotella within the NAFL group while an increased proportion of Megamonas and Fusobacterium was evident in the NASH group. The prominent expression of Megamonas and Fusobacterium can serve as a valuable biological marker for identifying NASH. KEGG pathway analysis revealed significant disruptions in various glucose metabolism pathways within the NASH group compared to the mild NAFL group, along with a notable deficiency in flavones and flavonols biosynthesis. Our study has elucidated significant associations between gut microbiome alterations and the progression from NAFL to NASH in patients with NAFLD. The differential microbial profiles observed in NASH patients underscore the potential of microbiota-based biomarkers for non-invasive diagnosis, which could mitigate the reliance on liver biopsies and enable earlier detection and intervention. Our findings suggest that certain bacteria could serve as targets for microbiome-modulating therapies, such as Megamonas

and Fusobacterium, opening new avenues for precision medicine in NAFLD management. Moreover, the novel takes identified in our study invite further investigation into their functional roles in NAFLD pathophysiology. The potential for microbiome-based interventions to attenuate or reverse NAFLD progression is an exciting prospect, but it is one that must be approached with rigorously designed clinical trials.

Data availability statement

The datasets analyzed in this study can be found in the China National GeneBank (CNGB) with a program ID CNP0003220.

Ethics statement

The studies involving humans were approved by Ethics Committee of Shenzhen Hospital of Traditional Chinese Medicine (ethical approval number: K2017-024). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

FH: Data curation, Writing – original draft. BL: Software, Writing – original draft. FX: Writing – original draft. YX: Data curation, Writing – review & editing. ZH: Data curation, Writing – review & editing. JL: Data curation, Writing – review & editing. JM: Software, Writing – review & editing. YZ: Software, Writing – review & editing. HZ: Software, Writing – review & editing. ZX: Software, Writing – review & editing. PG: Software, Writing – review & editing. YL: Funding acquisition, Writing – review & editing. LB: Funding acquisition, Writing – review & editing. GT: Funding acquisition, Writing – review & editing. XF: Funding acquisition, Writing – original draft. FL: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1366744/full#supplementary-material

References

Adkins, P., Middleton, J. R., Calcutt, M. J., Stewart, G. C., and Fox, L. K. (2017). Species identification and strain typing of staphylococcus agnetis and *staphylococcus hyicus* isolates from bovine milk by use of a novel multiplex pcr assay and pulsed-field gel electrophoresis. *J. Clin. Microbiol.* 55, 1778–1788. doi: 10.1128/JCM.02239-16

Amaretti, A., Bernardi, T., Tamburini, E., Zanoni, S., Lomma, M., Matteuzzi, D., et al. (2007). Kinetics and metabolism of *bifidobacterium adolescentis* mb 239 growing on glucose, galactose, lactose, and galactooligosaccharides. *Appl. Environ. Microbiol.* 73, 3637–3644. doi: 10.1128/AEM.02914-06

Bedossa, P., Poitou, C., Veyrie, N., Bouillot, J. L., Basdevant, A., Paradis, V., et al. (2012). Histopathological algorithm and scoring system for evaluation of liver lesions in morbidly obese patients. *Hepatology* 56, 1751–1759. doi: 10.1002/hep.25889

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using qiime 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9

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

Cai, C., Song, X., Chen, Y., Chen, X., and Yu, C. (2020). Relationship between relative skeletal muscle mass and nonalcoholic fatty liver disease: a systematic review and meta-analysis. *Hepatol. Int.* 14, 115–126. doi: 10.1007/s12072-019-09964-1

Carelli, L. L., D'Aquila, P., Rango, F., Incorvaia, A., Sena, G., Passarino, G., et al. (2023). Modulation of gut microbiota through low-calorie and two-phase diets in obese individuals. *Nutrients* 15:1841. doi: 10.3390/nu15081841

Chen, Y., Lun, A. T., and Smyth, G. K. (2016). From reads to genes to pathways: differential expression analysis of rna-seq experiments using rsubread and the edger quasi-likelihood pipeline. *F1000Res* 5:1438. doi: 10.12688/f1000research.8987.2

Cope, K. (2000). Increased gastrointestinal ethanol production in obese mice: implications for fatty liver disease pathogenesis. *Gastroenterology* 119, 1340–1347. doi: 10.1053/gast.2000.19267

Davis, R. P., Surewaard, B., Turk, M., Carestia, A., Lee, W. Y., Petri, B., et al. (2019). Optimization of in vivo imaging provides a first look at mouse model of non-alcoholic fatty liver disease (nafld) using intravital microscopy. *Front. Immunol.* 10:2988. doi: 10.3389/fimmu.2019.02988

Del, C. F., Nobili, V., Vernocchi, P., Russo, A., De Stefanis, C., Gnani, D., et al. (2017). Gut microbiota profiling of pediatric nonalcoholic fatty liver disease and obese patients unveiled by an integrated meta-omics-based approach. *Hepatology* 65, 451–464. doi: 10.1002/hep.28572

Demir, M., Lang, S., Hartmann, P., Duan, Y., Martin, A., Miyamoto, Y., et al. (2022). The fecal mycobiome in non-alcoholic fatty liver disease. *J. Hepatol.* 76, 788–799. doi: 10.1016/j.jhep.2021.11.029

Douglas, G. M., Maffei, V. J., Zaneveld, J. R., Yurgel, S. N., Brown, J. R., Taylor, C. M., et al. (2020). Picrust2 for prediction of metagenome functions. *Nat. Biotechnol.* 38, 685–688. doi: 10.1038/s41587-020-0548-6

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than blast. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Fan, J. G., Wei, L., and Zhuang, H. (2019). Guidelines of prevention and treatment of nonalcoholic fatty liver disease (2018, China). *J. Dig. Dis.* 20, 163–173. doi: 10.1111/1751-2980.12685

Galli, G., Amici, G., Conti, L., Lahner, E., Annibale, B., and Carabotti, M. (2022). Sex-gender differences in adult coeliac disease at diagnosis and gluten-free-diet follow-up. *Nutrients* 14:3192. doi: 10.3390/nu14153192

Ginestet, C. (2011). Ggplot2: elegant graphics for data analysis. *J. R. Stat. Soc.* 174, 245–246. doi: 10.1111/j.1467-985X.2010.00676_9.x

Girdhar, K., Dogru, Y. D., Huang, Q., Yang, Y., Tolstikov, V., Raisingani, A., et al. (2023). Dynamics of the gut microbiome, Iga response, and plasma metabolome in the development of pediatric celiac disease. *Microbiome.* 11:9. doi: 10.1186/s40168-022-01429-2

Gu, Z., Gu, L., Eils, R., Schlesner, M., and Brors, B. (2014). Circlize implements and enhances circular visualization in r. *Bioinformatics* 30, 2811–2812. doi: 10.1093/bioinformatics/btu393

Hoyles, L., Fernandez-Real, J. M., Federici, M., Serino, M., Abbott, J., Charpentier, J., et al. (2018). Molecular phenomics and metagenomics of hepatic steatosis in non-diabetic obese women. *Nat. Med.* 24, 1070–1080. doi: 10.1038/s41591-018-0061-3

Huang, S., Li, B., Liu, Z., Xu, M., Lin, D., Hu, J., et al. (2023). Three-dimensional mapping of hepatic lymphatic vessels and transcriptome profiling of lymphatic endothelial cells in healthy and diseased livers. *Theranostics* 13, 639–658. doi: 10.7150/thno.79953

Huang, Y. L., Shen, Z. Q., Huang, C. H., Teng, Y. C., and Tsai, T. F. (2021). Cisd2 protects the liver from oxidative stress and ameliorates western diet-induced nonalcoholic fatty liver disease. *Antioxidants.* 10:559. doi: 10.3390/antiox10040559

Javdan, B., Lopez, J. G., Chankhamjon, P., Lee, Y. J., Hull, R., Wu, Q., et al. (2020). Personalized mapping of drug metabolism by the human gut microbiome. *Cell* 181, 1661–1679.e22. doi: 10.1016/j.cell.2020.05.001

Jin, L., Deng, L., Bartlett, M., Ren, Y., Lu, J., Chen, Q., et al. (2022). A novel herbal extract blend product prevents particulate matters-induced inflammation by improving gut microbiota and maintaining the integrity of the intestinal barrier. *Nutrients*. 14:2010. doi: 10.3390/nu14102010

Kariagina, A., and Doseff, A. I. (2022). Anti-inflammatory mechanisms of dietary flavones: tapping into nature to control chronic inflammation in obesity and cancer. *Int. J. Mol. Sci.* 23:15753. doi: 10.3390/ijms232415753

Kim, J., Burton-Pimentel, K. J., Fleuti, C., Blaser, C., and Vergères, G. (2021). Microbiota and metabolite modifications after dietary exclusion of dairy products and reduced consumption of fermented food in young and older men. *Nutrients* 13:1905. doi: 10.3390/nu13061905

Kostic, A. D., Gevers, D., Pedamallu, C. S., Michaud, M., Duke, F., Earl, A. M., et al. (2012). Genomic analysis identifies association of fusobacterium with colorectal carcinoma. *Genome Res.* 22, 292–298. doi: 10.1101/gr.126573.111

Labun, K., Guo, X., Chavez, A., Church, G., Gagnon, J. A., and Valen, E. (2019). Accurate analysis of genuine crispr editing events with amplican. *Genome Res.* 29, 843–847. doi: 10.1101/gr.244293.118

Liu, X., Huang, L., Wang, K., Tan, W., Gao, X., Wang, X., et al. (2021). New insight and potential therapy for nafld: cyp2e1 and flavonoids. *Biomed. Pharmacother.* 137:111326. doi: 10.1016/j.biopha.2021.111326

Loomba, R., Seguritan, V., Li, W., Long, T., Klitgord, N., Bhatt, A., et al. (2019). Gut microbiome-based metagenomic signature for non-invasive detection of advanced fibrosis in human nonalcoholic fatty liver disease. *Cell Metab.* 30:607. doi: 10.1016/j.cmet.2019.08.002

Mohr, A. E., Jager, R., Carpenter, K. C., Kerksick, C. M., Purpura, M., Townsend, J. R., et al. (2020). The athletic gut microbiota. *J. Int. Soc. Sport Nutr.* 17:24. doi: 10.1186/s12970-020-00353-w

Neubauer, V., Humer, E., Mann, E., Kroger, I., Reisinger, N., Wagner, M., et al. (2019). Effects of clay mineral supplementation on particle-associated and epimural microbiota, and gene expression in the rumen of cows fed high-concentrate diet. *Anaerobe* 59, 38–48. doi: 10.1016/j.anaerobe.2019.05.003

Nikkhah, A., Ejtahed, H. S., Ettehad, M. F., Taghavi, M., Pakmehr, A., Hajipour, F., et al. (2023). The critical role of gut microbiota dysbiosis in skeletal muscle wasting: a systematic review. *J. Appl. Microbiol.* 134:lxac014. doi: 10.1093/jambio/lxac014

Parks, D. H., Tyson, G. W., Hugenholtz, , Beiko, R. G., et al. (2014). Stamp: statistical analysis of taxonomic and functional profiles. *Bioinformatics* 30, 3123–3124. doi: 10.1093/bioinformatics/btu494

Pelin, Y., Wegener, P. L., Pablo, Y., Jan, G., Elmar, P., Christian, Q., et al. (2013). The silva and "all-species living tree project (ltp)" taxonomic frameworks. *Nucleic Acids Res.* 42, D643–D648. doi: 10.1093/nar/gkt1209

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., and Glckner, F. O. (2012). The silva ribosomal rna gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Quesada-Vazquez, S., Aragones, G., Del, B. J., and Escote, X. (2020). Diet, gut microbiota and non-alcoholic fatty liver disease: three parts of the same axis. *Cells.* 9:176. doi: 10.3390/cells9010176

Raman, M., Ahmed, I., Gillevet, P. M., Probert, C. S., Ratcliffe, N. M., Smith, S., et al. (2013). Fecal microbiome and volatile organic compound metabolome in obese humans with nonalcoholic fatty liver disease. *Clin. Gastroenterol. Hepatol.* 11, 868–875.e3. doi: 10.1016/j.cgh.2013.02.015

Ren, X., Hao, S., Yang, C., Yuan, L., Zhou, X., Zhao, H., et al. (2021). Alterations of intestinal microbiota in liver cirrhosis with muscle wasting. *Nutrition* 83:111081. doi: 10.1016/j.nut.2020.111081

Ren, Z., Simons, P., Wesselius, A., Stehouwer, C., and Brouwers, M. (2023). Relationship between nafld and coronary artery disease: a mendelian randomization study. *Hepatology* 77, 230–238. doi: 10.1002/hep.32534

Roeb, E. (2022). Diagnostic and therapy of nonalcoholic fatty liver disease: a narrative review. *Visc. Med.* 38, 126–132. doi: 10.1159/000519611

Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahe, F. (2016). Vsearch: a versatile open source tool for metagenomics. *Peerj.* 4:e2584. doi: 10.7717/peerj.2584

Sandoval, V., Sanz-Lamora, H., Arias, G., Marrero, P. F., Haro, D., and Relat, J. (2020). Metabolic impact of flavonoids consumption in obesity: from central to peripheral. *Nutrients* 12:2393. doi: 10.3390/nu12082393

Scavo, M. P., Depalo, N., Rizzi, F., Carrieri, L., Serino, G., Franco, I., et al. (2022). Exosomal fzd-7 expression is modulated by different lifestyle interventions in patients with nafld. *Nutrients* 14:1133. doi: 10.3390/nu14061133

Shah, R. A., and Kowdley, K. V. (2019). Serum ferritin as a biomarker for nafld: ready for prime time? *Hepatol. Int.* 13, 110–112. doi: 10.1007/s12072-019-09934-7

Shen, F., Zheng, R. D., Sun, X. Q., Ding, W. J., Wang, X. Y., and Fan, J. G. (2017). Gut microbiota dysbiosis in patients with non-alcoholic fatty liver disease. *Hepatob. Pancreatic. Dis. Int.* 16, 375–381. doi: 10.1016/S1499-3872(17)60019-5

Sun, L., Pang, Y., Wang, X., Wu, Q., Liu, H., Liu, B., et al. (2019). Ablation of gut microbiota alleviates obesity-induced hepatic steatosis and glucose intolerance by modulating bile acid metabolism in hamsters. *Acta Pharm. Sin. B* 9, 702–710. doi: 10.1016/j.apsb.2019.02.004

Tan, P., Jin, L., Qin, X., and He, B. (2022). Natural flavonoids: potential therapeutic strategies for non-alcoholic fatty liver disease. *Front. Pharmacol.* 13:1005312. doi: 10.3389/fphar.2022.1005312

Tarantino, G., Sinatti, G., Citro, V., Santini, S. J., and Balsano, C. (2023). Sarcopenia, a condition shared by various diseases: can we alleviate or delay the progression? *Intern. Emerg. Med.* 18, 1887–1895. doi: 10.1007/s11739-023-03339-z

Wang, B., Jiang, X., Cao, M., Ge, J., Bao, Q., Tang, L., et al. (2016). Altered fecal microbiota correlates with liver biochemistry in nonobese patients with non-alcoholic fatty liver disease. *Sci. Rep.* 6:32002. doi: 10.1038/srep32002

Wang, Y., Zhang, Y., Lane, N. E., Wu, J., Yang, T., Li, J., et al. (2022). Population-based metagenomics analysis reveals altered gut microbiome in sarcopenia: data from the xiangya sarcopenia study. *J. Cachexia. Sarcopenia Muscle* 13, 2340–2351. doi: 10.1002/jcsm.13037

Ward, T., Larson, J., Meulemans, J., Hillmann, B., Lynch, J., Sidiropoulos, D., et al. (2017). *Bugbase predicts organism level microbiome phenotypes BioRxiv*, 133462. doi: 10.1101/133462

Wilkinson, T. J., Huws, S. A., Edwards, J. E., Kingston-Smith, A. H., Siu-Ting, K., Hughes, M., et al. (2018). Cowpi: a rumen microbiome focussed version of the picrust functional inference software. *Front. Microbiol.* 9:1095. doi: 10.3389/fmicb.2018.01095

Yokono, M., Satoh, S., and Tanaka, A. (2018). Comparative analyses of whole-genome protein sequences from multiple organisms. *Sci. Rep.* 8:6800. doi: 10.1038/s41598-018-25090-8

Zhang, S., Cai, S., and Ma, Y. (2018). Association between fusobacterium nucleatum and colorectal cancer: progress and future directions. *J. Cancer* 9, 1652–1659. doi: 10.7150/jca.24048

Zhang, C., Li, S., Yang, L., Huang, P., Li, W., Wang, S., et al. (2013). Structural modulation of gut microbiota in life-long calorie-restricted mice. *Nat. Commun.* 4:2163. doi: 10.1038/ncomms3163

Zhu, L., Baker, S. S., Gill, C., Liu, W., Alkhouri, R., Baker, R. D., et al. (2013). Characterization of gut microbiomes in nonalcoholic steatohepatitis (Nash) patients: a connection between endogenous alcohol and Nash. *Hepatology* 57, 601–609. doi: 10.1002/hep.26093

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Regulating bile acids signaling for NAFLD: molecular insights and novel therapeutic interventions

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Nonalcoholic fatty liver disease (NAFLD) emerges as the most predominant cause of liver disease, tightly linked to metabolic dysfunction. Bile acids (BAs), initially synthesized from cholesterol in the liver, undergo further metabolism by gut bacteria. Increasingly acknowledged as critical modulators of metabolic processes, BAs have been implicated as important signaling molecules. In this review, we will focus on the mechanism of BAs signaling involved in glucose homeostasis, lipid metabolism, energy expenditure, and immune regulation and summarize their roles in the pathogenesis of NAFLD. Furthermore, gut microbiota dysbiosis plays a key role in the development of NAFLD, and the interactions between BAs and intestinal microbiota is elucidated. In addition, we also discuss potential therapeutic strategies for NAFLD, including drugs targeting BA receptors, modulation of intestinal microbiota, and metabolic surgery.

KEYWORDS

bile acids, gut microbiota, NAFLD, TGR5, FXR, gut liver axis

1 Introduction

As the global incidence of obesity and its associated metabolic syndrome escalates, nonalcoholic fatty liver disease (NAFLD) has emerged as a primary contributor to chronic liver conditions and progressive liver fibrosis. Its global prevalence is estimated to be around 25% (Powell et al., 2021). NAFLD encompasses a continuum of pathological changes, ranging from simple steatosis (non-alcoholic fatty liver, NAFL) to nonalcoholic steatohepatitis (NASH), then progressing toward fibrosis and ultimately leading to hepatocellular carcinoma and liver failure. Approximately 5–25% of individuals with nonalcoholic steatohepatitis progress to severe liver fibrosis (Castera et al., 2019).

The development of NAFLD is inherently intricate and governed by numerous factors. Originally, the pathogenesis of NAFLD was described by the "two-hit" hypothesis: the "first hit" being lipid accumulation in the liver, which predisposes the organ to further damage, and the "second hit" comprising factors like lipotoxicity, mitochondrial damage, oxidative stress, and hepatic inflammation, which facilitate the progression from NAFL to liver fibrosis. More recently, the understanding has shifted to the "multiple-hit" hypothesis. This theory includes various detrimental factors, including insulin resistance, inflammatory mediators, dietary factors, gut microbiota imbalances, and genetic and epigenetic variations (Kumar et al., 2021). However, the exact pathogenesis of NAFLD is largely unknown, and no FDA-approved drug to treat NAFLD is currently available.

Recent studies have increasingly focused on the role of bile acids (BAs) in the pathogenesis of NAFLD due to their origin from hepatic cholesterol (Arab et al., 2017). Dysregulation in BA metabolism in NAFLD patients heightens the risk of liver damage. Research has demonstrated marked disparities in both the serum levels and composition of BAs between high-fat mouse models and NAFLD patients, and those observed in a normal control group (Jiao et al., 2018). Furthermore, elevated levels of circulating BAs have been detected in diet-induced NASH mice, correlating closely with the severity of liver fibrosis (Suga et al., 2019). In patients with NASH, enhanced BA synthesis compared to healthy individuals has been noted, and serum BA levels are predictive of liver fibrosis severity (Shlomai et al., 2013; Mouzaki et al., 2016). In addition to assisting the absorption of dietary lipids and vitamins, BAs are crucial signaling molecules that regulate lipid and glucose metabolism and modulate inflammation in various tissues. The imbalance of gut microbiota is intricately linked to NAFLD progression (Henao-Mejia et al., 2012; Aron-Wisnewsky et al., 2020). BAs, as one class of metabolites of intestinal microbiota, are frequently associated with metabolic diseases including obesity, diabetes and NAFLD. It has been demonstrated that administration of beneficial bacteria could improve NAFLD (Kolodziejczyk et al., 2019). On the other hand, metabolites derived from microbiota can also reduce NAFLD severity by mediating the beneficial effects of intestinal bacteria.

In this review, we synthesize and analyze the mechanisms through which BA signaling influences NAFLD pathogenesis, focusing particularly on the interactions between BAs and gut microbiota, as well as identifying potential therapeutic targets for the treatment of NAFLD.

2 BA synthesis and regulation

2.1 BA synthesis

BAs are synthesized predominantly from cholesterol in the liver through two primary pathways: the classical pathway and the alternative pathway, involving at least 17 enzymes (Figure 1). The classical pathway, initiated by the rate-limiting enzyme cholesterol 7a-hydroxylase (CYP7A1) and further regulated by sterol-12α-hydroxylase (CYP8B1), generates the majority of the BA pool. In contrast, the alternative pathway is chiefly controlled by sterol 27α-hydroxylase (CYP27A1) and sterol 7α-hydroxylase (CYP7B1), producing chenodeoxycholic acid (CDCA) and cholic acid (CA) in humans, and predominantly beta-muricholic acid (β-MCA) in rodents (Wahlström et al., 2016). CYP8B1 plays a crucial role in synthesizing CA, thus determining the CA to CDCA ratio (Bertaggia et al., 2017). Within hepatocytes, BAs are conjugated primarily with glycine in humans and almost exclusively with taurine in mice, forming conjugated bile acids that are expelled into the bile through the bile salt export pump (BSEP) and multidrug resistance-associated protein 2 (Falany et al., 1994). Released into the duodenum, primary bile acids are transformed into secondary bile acids like deoxycholic acid (DCA) and lithocholic acid (LCA) by intestinal bacteria via deconjugation and dehydroxylation. Subsequently, over 95% of the BAs are reabsorbed into the portal vein by the apical sodium dependent bile acid transporter (ASBT) at the terminal ileum and returned to the liver, constituting the enterohepatic circulation, while the remainder is excreted in the feces (de Aguiar Vallim et al., 2013). BAs re-enter the liver from the bloodstream via the Na^+-taurocholate cotransport polypeptide (NTCP). BA homeostasis is a critical physiological process involving the synthesis, metabolism, and recycling of bile acids, which are essential for lipid digestion and nutrient absorption. The regulation of BA levels is finely tuned by feedback mechanisms primarily mediated by BA receptors, which inhibits bile acid synthesis in the liver when intrahepatic or intestinal bile acid levels are high. Disturbances in bile acid homeostasis are associated with liver diseases such as cholestasis and NAFLD, as well as systemic disorders including obesity and diabetes (Wahlström et al., 2016). For instance, ASBT inhibitor blocks the reabsorption of BAs in the terminal ileum, thereby increasing BA excretion in feces and improving NAFLD induced by high fat diet (Rao et al., 2016).

2.2 BA receptors

The primary BA receptors are the farnesoid X receptor (FXR), or NR1H4, and the G protein-coupled bile acid receptor (TGR5). FXR is predominantly expressed the liver, intestines, white adipose tissue, adrenal glands, kidneys, and immune cells (Lefebvre et al., 2009). The binding affinity of BAs to FXR follows the order: CDCA > DCA > CA > LCA. Conversely, Tα-MCA, Tβ-MCA, and possibly UDCA act as antagonists (Sayin et al., 2013). TGR5 is expressed in brown adipose tissue (BAT), enteroendocrine L cells, white adipose tissue (WAT), gallbladder, skeletal muscle, islet α and β cells, immune cells, astrocytes and neurons, and is activated by BAs with varying efficacies (LCA > DCA > CDCA > CA) (Guo et al., 2016). Additional receptors involved in BA signaling include the vitamin D receptor (VDR), liver X receptor (LXR), pregnane X receptor (PXR), and sphingosine-1-phosphate receptor 2 (SIPR2) (Supplementary Table S1) (Schaap et al., 2014). BAs can exert a wide range of regulatory effects through these receptors, including glucose and lipid metabolism, BA homeostasis and energy expenditure, immune and inflammatory responses, and improving insulin sensitivity (Thomas et al., 2008).

2.3 BA synthesis regulation

By regulating FXR receptors in the liver and intestine, BAs can achieve self-regulation and control the transport of BAs, maintaining BA balance (Mencarelli and Fiorucci, 2010). FXR knockout mice show increased CYP7A1 expression and hepatic BA synthesis, suggesting that FXR primarily mediates the inhibitory effects of BA synthesis via CYP7A1 (Sinal et al., 2000). This suppression primarily occurs through the interaction of the small heterodimer partner (SHP) with liver receptor homolog-1 (LRH-1), curtailing CYP7A1 gene expression (Goodwin et al., 2000), a mechanism critical for averting excessive BA production and resultant liver damage. Furthermore, intestinal FXR activation by BAs at the ileum's terminal segment prompts fibroblast growth factor 15 (FGF15), analogous to human FGF19, to modulate bile acid synthesis in the liver. Upon FGF15/FGF19 is secreted, it binds to the hepatic fibroblast growth factor receptor 4 (FGFR4)/beta-klotho heterodimer complex, triggering JNK1/2 and ERK1/2 signaling cascades, leading to the inhibition of CYP7A1 expression (Potthoff et al., 2012). Experimental

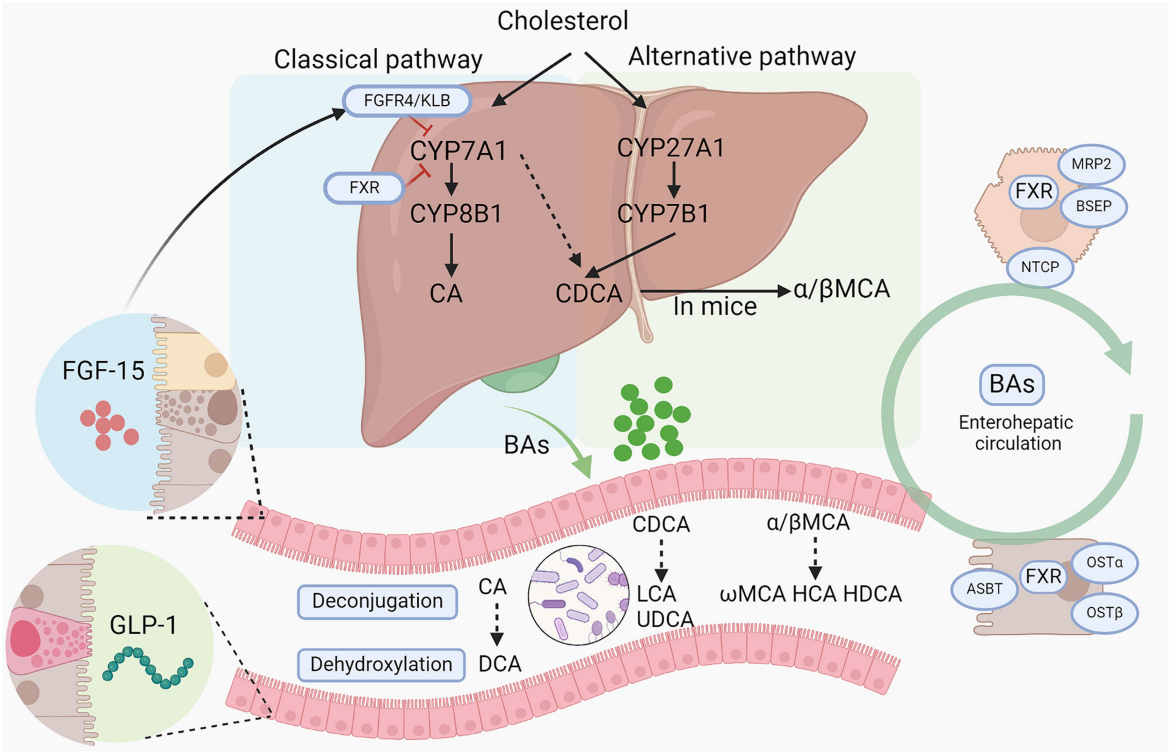

FIGURE 1
Bile acid synthesis and metabolism by gut microbiota. Bile acids are synthesized from cholesterol via classical pathway and alternative pathway. CYP7A1, CYP27A1, CYP8B1, and CYP7B1 are the main enzymes responsible for primary BA synthesis. Then conjugated BAs are released into intestine and further metabolized by gut microbiota into secondary BAs via a series of action including deconjugation, dihydroxylation, and epimerization. And primary bile acids and secondary bile acids can cooperate to stimulate the intestinal epithelium to secrete FGF15 and GLP-1 to regulate BA synthesis in turn and alter host metabolism. BAs, bile acids; CYP7A1, Cholesterol 7-alpha hydroxylase; CYP8B1, sterol 12α-hydroxylase; CYP27A1, cholesterol 7a-hydroxylase; CYP8B1, sterol-12α-hydroxylase; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; HDCA, hyodeoxycholic acid; HCA, hyocholic acid; LCA, lithocholic acid; FGF15/19, fibroblast growth factor 15/19; MCAs/ α/β-MCA, muricholic acids/α/β-muricholic acid; UDCA, ursodeoxycholic acid; GLP-1, glucagon-like peptide-1; ASBT, apical sodium-dependent BA transporter; FXR, farnesoid X receptor; OSTα/β, organic solute transporter subunit α and β; NTCP, sodium dependent taurocholate co-transporting polypeptide; BSEP, bile salt export pump; KLB: β-Klotho.

data from tissue-specific FXR knockout mice indicate intestinal FXR exerts a stronger inhibitory impact on CYP7A1 compared to hepatic FXR, which more significantly influences CYP8B1 expression and thus reduces cholic acid production (Kim et al., 2007; Kong et al., 2012). The gut-liver axis, delineating the complex interactions between gut microbiota and the liver, is closely linked to NAFLD (Song and Zhang, 2022). Microbial metabolites and other intestinal signaling molecules could reach the liver via portal and systematic circulation, regulating BAs synthesis and transport. Of note, intestinal FXR-FGF15/19 plays a critical role in the crosstalk of gut microbiota and BAs. FGF15/19 has displayed substantial protection against hepatic steatosis caused by high-fat diets (Sciarrillo et al., 2021). Continued clinical investigations are imperative to explore the therapeutic potentials of FGF19-based chimeric molecules for NAFLD therapy.

3 BA signaling in metabolism

BAs play a critical role in various metabolic processes via their receptors, mainly FXR and TGR (Figure 2). Dysregulated BAs signaling contributes to the initiation and progression of NAFLD.

3.1 BA signaling in glucose metabolism

Insulin resistance is a primary characteristic of NAFLD, integral to both its development and progression. BAs are released into the digestive tract after a meal. Therefore, as postprandial messengers, BAs regulate glucose metabolism by activating various receptors. Studies using whole-body FXR knockout mice have shown a reduction in insulin sensitivity, whereas administration of the FXR agonist GW4064 significantly improves insulin resistance and glucose regulation in ob/ob mice (Cariou et al., 2006). Additionally, hepatic gluconeogenesis, vital for maintaining glucose levels, is attenuated by FXR activation, which reduces the expression of critical enzymes such as glucose 6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) (Ma et al., 2006). Moreover, BAs enhance insulin synthesis and secretion by stimulating the release of glucagon-like peptide 1 (GLP-1) via TGR5 activation. This mechanism not only protects pancreatic β-cells from apoptosis but also encourages their proliferation (Zheng et al., 2021). Furthermore, recent research indicates that TGR5, expressed in pancreatic α and β-cells, promotes insulin secretion and stabilizes glucose levels (Kumar et al., 2012, 2016). Activation of LXR increases the expression of the GLUT4 in adipose and muscular tissues,

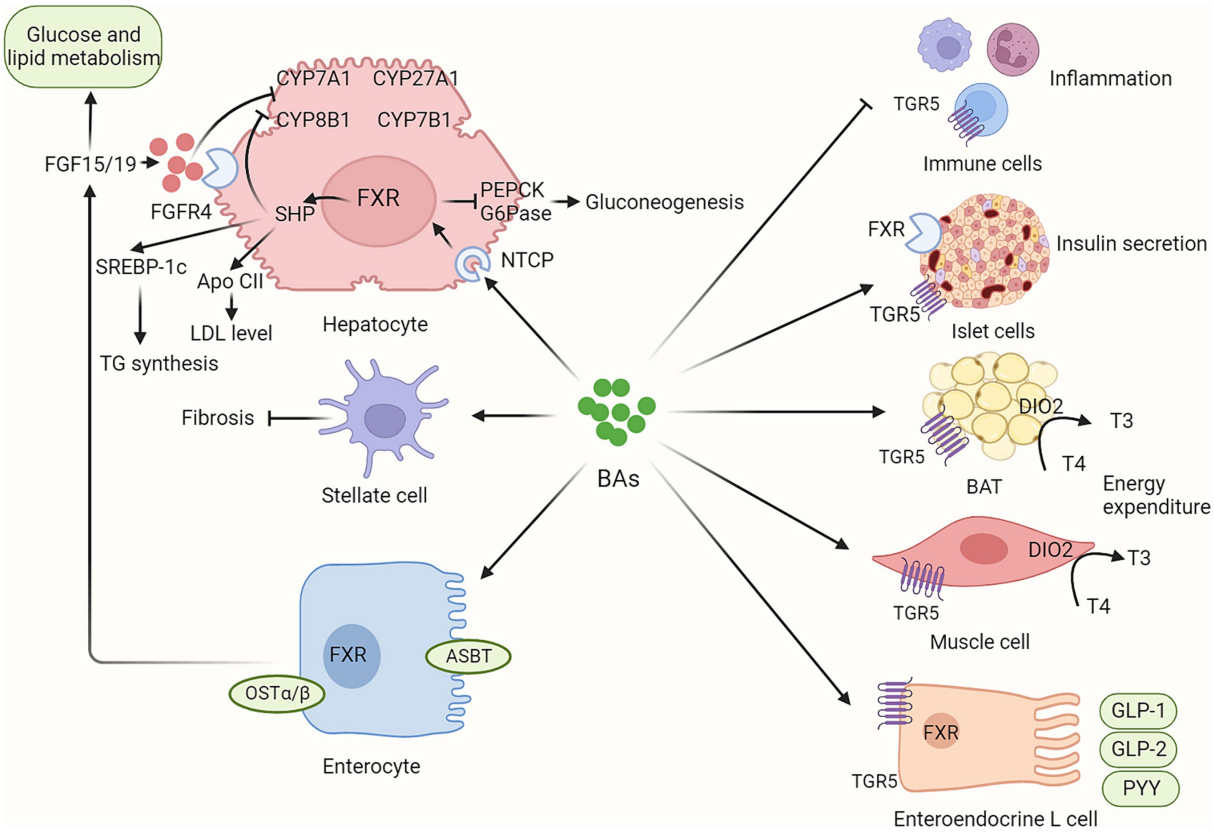

FIGURE 2
BAs contribute to host metabolism in various organs via their receptors. BAs synthesis is not only regulated by hepatic FXR/SHP signaling but also by intestinal FXR/FGF15/19 signaling. In addition, circulating FGF15/19, as metabolic hormones, could improve glucose and lipid metabolism. BAs contribute to host metabolism in multiple organs via their FXR and TGR5 receptors, including enhancing insulin secretion in pancreatic islet cells, promoting energy expenditure in brown adipose tissue and muscle tissue, inducing incretin release in enteroendocrine L cells, and regulating inflammation response in immune cells. BAs, bile acids; ASBT, apical sodium-dependent bile acid transporter; BAT, brown adipose tissue; CYP7A1, Cholesterol 7-alpha hydroxylase; CYP8B1, sterol 12α-hydroxylase; CYP27A1, cholesterol 7a-hydroxylase; CYP8B1, sterol-12α-hydroxylase; DIO2, Type II iodothyronine deionidinase; FGF15/19, fibroblast growth factor 15/19; FGFR4, fibroblast growth factor receptor 4; FXR, farnesoid X receptor; G6Pase, glucose 6-phosphatase; GLP-1, glucagon-like peptide-1; GLP-2, glucagon-like peptide-2; NTCP, sodium dependent taurocholate co-transporting polypeptide; PEPCK, phosphoenolpyruvate carboxykinase; OSTα/β, organic solute transporter subunit α and β; PYY, peptide YY; TGR5, G protein-coupled bile acid receptor.

thereby improving glucose uptake (Baranowski et al., 2014). In addition, BAs interact with FXR in ileal enterocytes to trigger FGF15/19 signaling, enhancing glucose control (Kliewer and Mangelsdorf, 2015).

3.2 BA signaling in lipid metabolism

NAFLD is marked by significant lipid accumulation in the liver and BAs exert a profound effect on lipid metabolism mainly via FXR signaling. Notably, FXR-deficient ($FXR^{-/-}$) mice exhibit marked elevations in hepatic and plasma cholesterol and triglycerides. Conversely, FXR agonists reduce these lipid parameters in db/db mice, yet show no efficacy in FXR deficient mice (Cariou et al., 2006). In NAFLD patients, FXR expression is reduced, which correlates with an increase in sterol regulatory element binding protein-1c (SREBP-1c) (Yang et al., 2010). BAs act on hepatic FXR and induce SHP expression, thereby inhibiting SREBP-1c and reducing fatty acid synthesis (Watanabe et al., 2004). FXR further inhibits the expression of the apolipoprotein (apo) B gene, decreasing very low-density lipoprotein (VLDL) secretion (Hirokane et al., 2004). Additionally, FXR boosts lipoprotein lipase activity by upregulating the expression of apolipoprotein C-II (apo-CII), a stimulator of lipoprotein lipase, while decreasing apolipoprotein C-III (apo-CIII) expression, a lipoprotein lipase inhibitor (Chavez-Talavera et al., 2017). The hydrophobicity and conjugation state of the BAs are critical for regulating intestinal cholesterol and lipid absorption. Thus, CA absence in CYP8B1 knock-out mice prevents hepatic steatosis induced by a high-fat diet (Bertaggia et al., 2017).

3.3 BA signaling in energy expenditure

Currently, obesity is believed to be the result of a disruption in energy metabolism, where energy intake exceeds energy expenditure. Thus, enhancing energy expenditure presents a good approach for NAFLD therapy. Activation of TGR5 by BAs elevates brown fat tissue energy expenditure, improve insulin resistance, and prevent obesity (Zietak and Kozak, 2016). It also results in elevated cAMP levels, which in turn activates type 2 iodothyronine deiodinase (D2). This

activation enhances the conversion of thyroxine (T4) into the more active form, triiodothyronine (T3). Subsequently, T3 boosts uncoupling protein 1 (UCP-1) expression, further enhancing energy expenditure. Therefore, TGR5 signaling mediated by BAs plays a pivotal role in maintaining energy homeostasis (Watanabe et al., 2006). In contrast, TGR5-deficient mice exhibit heightened obesity susceptibility under a high-fat diet compared to control group (Maruyama et al., 2006). A clinical trial involving 12 healthy women who received chenodeoxycholic acid (CDCA) treatment for 2 days revealed significant increases in brown fat energy expenditure. *In vitro*-cultured human brown fat cells exposed to CDCA also showed an increase in UCP1 expression levels (Broeders et al., 2015).

Therefore, regulating bile acids to enhance the TGR5 signaling pathway and increase brown fat energy expenditure could become an important target for NAFLD. However, despite its positive impact on metabolic health, therapeutic targeting of TGR5 signaling is hindered by the potential for TGR5-mediated gallstone formation and gallbladder filling. Therefore, TGR5-selective agonists, such as INT-777, RDX8940, show promising effects in improving energy metabolism, reducing inflammation and stimulating energy expenditure (Pellicciari et al., 2009; Finn et al., 2019).

3.4 BA signaling in inflammation

Recently, the association between bile acids and immune regulation is an emerging and increasingly studied field in biomedical research. Activation of the innate immune system is pivotal in initiating hepatic inflammation, while persistent low-grade inflammation is crucial to the development of NAFLD and liver fibrosis. Recent studies highlight the significance of BA signaling in regulating hepatic inflammation. TGR5 and FXR are localized in various immune cell types, such as monocytes and macrophages, as well as dendritic cells (Fiorucci et al., 2021). Research has shown that activation of TGR5 decreases cytokine production in monocytes and macrophages and exerts potent anti-inflammatory effects (Perino and Schoonjans, 2015). Moreover, TGR5 activation protects against inflammation induced by lipopolysaccharide (LPS) by suppressing the production of proinflammatory cytokines mediated by NF-κB pathway (Wang et al., 2011). On the contrary, TGR5 deficiency promotes NLRP3 inflammasome and M1 macrophage polarization (Ma et al., 2021) and TGR5($^{-/-}$) mice show increased liver inflammation (Wang et al., 2011). M1 macrophages have the capability to secrete pro-inflammatory cytokines, including IL-1β, IL-6, TNF-α. In addition, FXR agonist can also orchestrate the immunological activities of macrophages and monocytes to improve NAFLD (McMahan et al., 2013). FXR activation decreased the mRNA levels of inflammatory genes (IL-1β, IL-6 and TNF-α) induced by LPS treatment *in vitro* (Xiong et al., 2017). *In vivo*, activation of the intestinal FXR signaling inhibits inflammation and helps maintain the integrity of the intestinal barrier in inflammatory bowel disease (Gadaleta et al., 2011). BAs homeostasis also plays an important role in inflammation regulation. Overexpression of CYP7A1 has been shown to protect the liver from inflammatory infiltration and alleviate hepatic fibrosis in FXR dependent manner. Recently, 3-oxoLCA and isoallo-LCA, which are derived from LCA, have been identified as T_{H17} and Treg cell regulators to control their differentiation, suggesting BAs regulate host immune response (Hang et al., 2019). Therefore, regulating BA signaling has emerged as a promising strategy in treating NAFLD by attenuating hepatic inflammation. Recent therapeutic advances involve the use of FXR agonists, which have shown efficacy in reducing hepatic steatosis and inflammation (Zhang et al., 2009).

4 Gut microbiota and BAs

The gut microbiota is regarded as a metabolic "organ" that produces numerous metabolites to regulate host metabolism. The interaction between gut microbiota and BAs is a significant area of research because it underscores a complex, bidirectional relationship where not only does the gut microbiota influence BAs profiles, but BAs also affect the composition and function of the gut microbiota.

4.1 Microbial regulation of BAs

Microbial metabolism of BAs by gut microbiota not only increase the diversity of BAs but also promote the hydrophobicity of the BA pool. The BA pool and composition in germ-free (GF) mice exhibit significant differences compared to those in conventionally raised mice (Sayin et al., 2013), emphasizing the vital role of gut microbiota in bile acid regulation. Bile acid deconjugation is the first step of metabolism by bacteria with bile salt hydrolase (BSH) activity, which is present in strains of lactobacilli, bifidobacteria, Bacteroides, and Clostridium. Deconjugated primary BAs are further metabolized through the 7-dehydroxylation into secondary bile acid. The potent endogenous agonists of TGR5 are LCA and DCA, which are metabolically derived from CDCA and CA, respectively. This transformation is primarily governed by intestinal bacteria, including Clostridium (such as *C. scindens* and *C. sordellii*), Bacteroides (such as *B. fragilis*), and Eubacterium (such as *E. lentum*). At lower concentrations, these metabolites positively influence glucose and lipid metabolism. However, when present at higher concentrations, they exert negative effects on host health. For example, fecal DCA levels are significantly elevated in patients with NAFLD compared to those in the healthy control group (Kasai et al., 2022), thereby promoting obesity-associated hepatocellular carcinoma (HCC) by causing DNA damage (Yoshimoto et al., 2013). Therefore, maintaining the homeostasis of intestinal microflora and its metabolites is essential. For example, GF mice has increased bile acid pool and TβMCA level, which serves as an antagonist to intestinal FXR signaling, thus inducing FGF15/19 secretion to inhibit hepatic BA synthesis. Some probiotics can regulate the metabolism and synthesis of BAs, thereby influencing the body's metabolism and the progression of diseases. Recent research demonstrated that *lactobacillus rhamnosus GG* increased ileum FGF15 and subsequently reduced BA synthesis, which attenuated liver inflammation and prevents liver fibrosis (Liu et al., 2020). Levels of *Parabacteroides distasonis* are reportedly lower in individuals with NAFLD, and this bacterium can mitigate obesity and metabolic dysfunctions by modulating the production of UDCA and LCA (Wang et al., 2019).

4.2 BAs regulating gut microbiota

Gut microbiota plays a crucial role in maintaining BA homeostasis through via bioconversion of BA and enhancement of FGF15/FGF19 signaling. On the other hand, BAs, serving as the detergent molecules

in intestine, significantly shape the composition of the intestinal microflora. BAs exert antimicrobial properties, affecting the growth and survival of different strains. Zheng et al. showed that mice fed with BAs under a normal diet condition exhibited obese phenotype and similar gut microbial composition in HFD mice (Zheng et al., 2017). A recent study showed that obeticholic acid (OCA), an analog of CDCA, inhibited endogenous BA synthesis and led an increased proportion of Firmicutes in the small intestine (Friedman et al., 2018). Gut microbiota modulates NAFLD in part via regulating intestinal FXR signaling. In obese mice induced by HFD, glycine-β-muricholic acid (Gly-MCA) administration reduced the ratio of *Firmicutes* to *Bacteroidetes,* which improved insulin resistance and ameliorated obesity related metabolic dysfunction (Zhang et al., 2016). Although serum BAs concentrations are elevated in individuals with NAFLD, the proportion of the FXR antagonistic DCA has increased, whereas the levels of the FXR agonistic CDCA have decreased (Jiao et al., 2018). Therefore, FXR signaling can modulate the gut microbial composition and regulate hepatic metabolism.

5 Targeting BA metabolism for NAFLD therapy

Targeting BA metabolism has become a promising therapeutic strategy for NAFLD due to the significant impact of BA on host metabolism, energy expenditure, inflammation, and the composition of the gut microbiota. Significant efforts have been made to explore approaches that modify BAs signaling. These approaches include agonist for BA receptor, regulating gut microbiota and metabolic surgery (Figure 3).

5.1 Pharmacotherapy for NAFLD by targeting BA

Traditional pharmacotherapies for NAFLD, including PPAR agonists, insulin sensitizers, and antioxidants, have been extensively researched; however, their effectiveness varies among individuals and is generally limited. It is well-established that activation of BA receptors exhibits metabolic benefits in NAFLD. Emerging evidence strongly suggests that BA receptors are promising therapeutic targets. Currently, a wide range of FXR agonists are under investigation in clinical trials for NAFLD. OCA, a highly potent FXR agonist and a semi-synthetic derivative of CDCA, has been approved by the FDA for treating patients with PBC who are unresponsive to UDCA (Trauner et al., 2019). Meanwhile, OCA has significantly enhanced the histological features associated with NASH. However, LDL cholesterol was increased in the early stage of OCA treatment (Younossi et al., 2019). In addition to common side effects such as pruritus, long term OCA administration may increase the risk of cardiovascular disease. Several other FXR agonists, including EDP305, tropifexor and cilofexor have already undergone phase II clinical trials, demonstrating the potential to emerge as novel treatments for NAFLD (Panzitt et al., 2022). Fexaramine, a non-absorbable FXR agonist that remains in the intestine, has shown potential in reducing diet-induced obesity and improving insulin sensitivity (Fang et al., 2015). The TGR5-specific agonist INT-777, derived from CA, alleviated liver fat accumulation and improved insulin sensitivity in obese mice (Thomas et al., 2009). Recently, a novel TGR5 agonist RDX8940, has been reported to improve hepatic steatosis in western diet-fed mice and enhance the secretion of gastrointestinal hormones such as GLP-1, GLP-2, and peptide YY (Finn et al., 2019). BA receptor dual agonists target multiple receptors involved in BA signaling, which play crucial roles in metabolic regulation, inflammation control, and liver protection. Research indicates that INT-767, an agonist for both FXR and TGR5, can improve metabolic control, reduce liver fibrosis, and decrease inflammation in animal models of liver disease (Comeglio et al., 2018; Jadhav et al., 2018; Roth et al., 2018). This makes it a promising candidate for the treatment of NAFLD and NASH. Sevelamer, as a BA sequestrant, has been shown to alleviate hepatic inflammation, lipid deposition, and fibrosis by targeting LPS signaling and inducing BAs excretion (Tsuji et al., 2020). Drugs that inhibit ASBT reduce the reabsorption of ileal BAs, enhancing their synthesis from cholesterol in liver and excretion in feces through suppression of FGF15/19. ASBT inhibitors, such as IMB17-15, volixibat and elobixibat, currently being investigated for their potential to treat NAFLD and NASH (Ge et al., 2019; Jansen, 2021). Additional clinical trials and long-term follow-ups are necessary to fully understand their impact on host health.

5.2 Regulating gut microbiota for NAFLD

There are several strategies to regulate the intestinal microbiota and further improve NAFLD, including the diet and exercise, the administration of probiotics, prebiotics, and fecal microbiota transplantation (FMT). For example, high fat diet caused increased DCA levels, accompanied by alteration of gut microbiota (Lin et al., 2019). In contrast, calorie restriction significantly altered microbiota and decreased the levels of non-12α-hydroxylated BAs (Li et al., 2022). Exercise could also regulate intestinal microbiota and induce metabolic improvements by modifying circulating BAs (Aoi et al., 2023). The molecular mechanism underlying metabolic improvement by exercise may involve FXR-FGF15 signaling (Qiu et al., 2021). Increasing evidence supports that various probiotic bacteria have promising effects on NAFLD, including the genera such as *Bifidobacteria* and *Lactobacillus*, along with others like *Saccharomyces boulardii* and *Streptococcus thermophilus*. Zhao et al. showed that administration of *Lactobacillus plantarum* alleviated the severity of NAFLD in HFD induced mice (Zhao et al., 2020). *Lactobacillus gasseri* ameliorated hyperlipidemia and modulates BAs metabolism. Mechanically, probiotics reduced the inflammatory factors and increased the gut barrier. In addition, *Parabacteroides distasonis* has been reported to alleviate hepatic steatosis via increasing alternative BA synthesis pathway (Kuang et al., 2023). Prebiotics are a group of nutrients in the diet that are resistant to digestion but can be fermented by the intestinal microflora, including fructo-oligosaccharides (FOS) and inulin. The fermentation of prebiotics by gut microbiota produces short-chain fatty acids (SCFAs) and affects BAs, though this is not well-documented. SCFAs may directly regulate BA synthesis and alter the microbial composition in the gut, which in turn can modify the profile of BAs. Currently, FMT is increasingly supported by evidence as a viable therapy option for NAFLD. In contrast to probiotics, FMT offers a broad spectrum of healthy bacteria that help to reshape the gut microbial dysbiosis. It is well established that FMT from lean donors enhances insulin sensitivity in individuals with metabolic syndrome

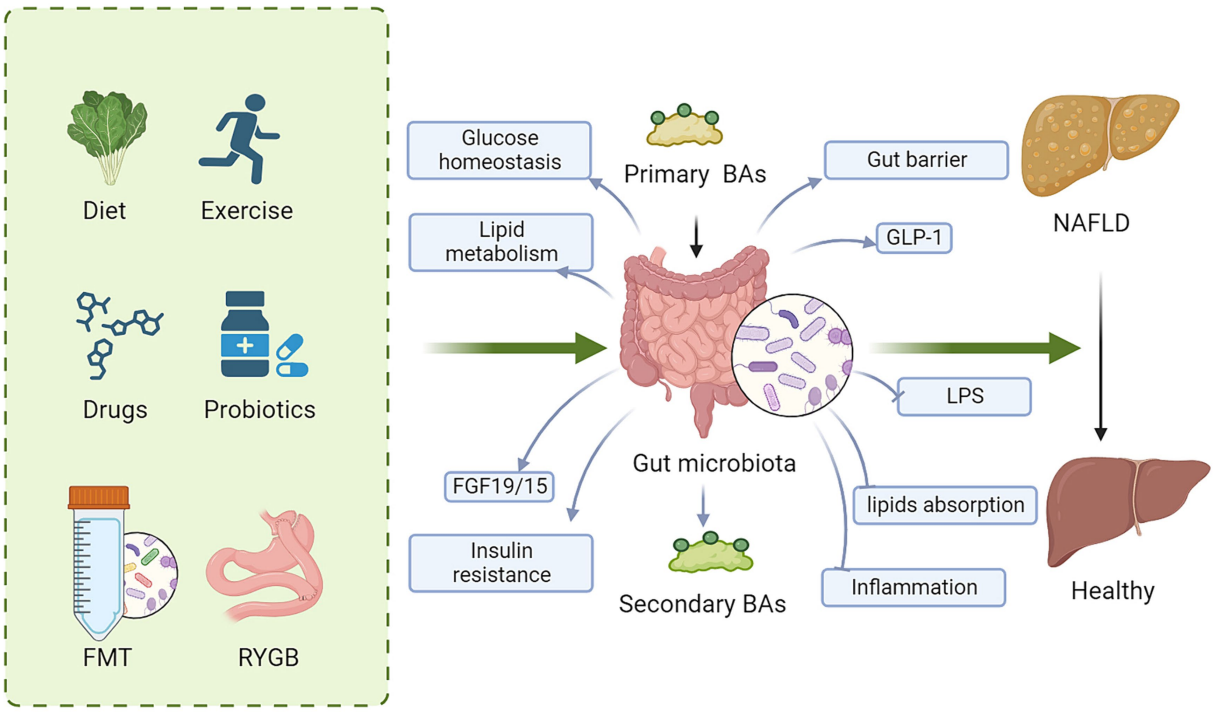

FIGURE 3
Regulating BA signaling is a promising therapeutic target for NAFLD. These approaches to regulate BA signaling for NAFLD therapy include diet, exercise, drugs, probiotics, FMT and metabolic surgery. Mechanically, targeting BAs and gut microbiota improves glucose and lipid metabolism, gut barrier, insulin resistance and GLP-1 secretion, while decreases lipid absorption, LPS, and inflammation, ameliorating hepatic steatosis. BAs, bile acids; FMT, fecal microbiota transplantation; FGF15/19, fibroblast growth factor 15/19; LPS, lipopolysaccharide; GLP-1, glucagon-like peptide-1; NAFLD, nonalcoholic fatty liver disease.

(Vrieze et al., 2012). Xue et al. performed a randomized clinical trial and discovered that FMT attenuated fatty liver disease by facilitating intestinal microflora reconstruction (Xue et al., 2022). The long-term effects and potential side effects of FMT for NAFLD require further investigation. Potential risks include changes in the recipient's gut microbial diversity that could lead to unforeseen health issues, including gastrointestinal infections, or exacerbation of other inflammatory conditions. Moreover, the durability of the treatment's effectiveness remains uncertain.

5.3 Metabolic surgery as a therapeutic option for NAFLD

Metabolic surgery commonly includes vertical sleeve gastrectomy (VSG) and Roux-en-Y gastric bypass (RYGB). Numerous clinical studies and animal experiments have provided evidence that the histopathology of NAFLD improves significantly after metabolic surgery, with this improvement being closely linked to weight loss (Aguilar-Olivos et al., 2016; Axelrod et al., 2023). However, changes in BAs and the gut microbiota also play a pivotal role in ameliorating NAFLD (Pérez-Rubio et al., 2023). Both human and animal studies have shown that following RYGB surgery, there is a significant increase in both fasting and postprandial BA levels, a marked rise in the ratio of 12α-OH/non-12α-OH BAs, and a reversal in the primary to secondary BA ratio. Additionally, levels of related factors such as FGF19 and GLP-1 are also significantly elevated (Ahmad et al., 2013; Bhutta et al., 2015; Dutia et al., 2016; Lalloyer et al., 2023). These alterations in BA levels do not manifest immediately post-surgery but exert a prolonged influence. Some studies suggest that the mechanisms underlying RYGB's alleviation of type 2 diabetes may be linked to the increase in FGF19, upregulation of CYP7A1 gene expression, and an overall rise in bile acids (Gerhard et al., 2013). Research shows that cholesterol is converted into bile acids and excreted, with the alternative BA synthesis pathway enhanced due to significant increases in CYP27A1 and CYP7B1 expression levels. This could be a mechanism through which RYGB improves NAFLD by regulating hepatic and systemic cholesterol as well as BA metabolism (Lalloyer et al., 2023). Interestingly, bile diversion surgery in diet-induced obese mice has shown that diverting bile to the ileum induces physiological changes similar to those seen with RYGB, including significant weight loss, improved glucose tolerance, and sustained improvement in liver fat deposition, with a notable increase in bile acids, especially conjugated T-β-MCA (Flynn et al., 2015). Moreover, the gut-brain axis might be involved in the metabolic surgery. BAs in serum and brain are gradually elevated after metabolic surgery, which attenuates cocaine-induced elevations in accumbal dopamine via TGR5 pathway (Reddy et al., 2018). Neuron-specific TGR5 activation exhibits anorexigenic actions via Rho-ROCK-action pathway, thus decreasing neuropeptide Y secretion (Perino et al., 2021). *In vivo*, deletion of TGR5 significantly increases food intake. Dysregulation of dopamine signaling can lead to overeating, especially of high-fat or high-sugar foods, contributing to the development of NAFLD. The specific mechanisms by which the gut-brain axis mediates the metabolic surgery effects require further investigation. While metabolic surgery can provide significant health benefits for NAFLD, it is essential to

consider the potential disadvantages, including postoperative complications, nutritional deficiencies, and irreversible alterations to the digestive system (Kheirvari et al., 2020).

6 Conclusions and perspectives

Dysregulated BA signaling has been implicated in the development and progression of NAFLD. Therefore, understanding how BAs influence host metabolism is crucial for developing strategies to manipulate BA levels effectively in treating the disease. Although the regulation of the BA signaling holds promising therapeutic potential for NAFLD, there are currently no BA analogs or drugs targeting BA signaling available for NAFLD treatment. The principal challenge lies in developing a tissue-specific drug or non-absorbed drug that regulates BA signaling, thereby improving the metabolic dysfunction in NAFLD without causing significant adverse effects. Therefore, there is an urgent need for further research to explore the relevant molecular mechanisms of BAs in the progression of NAFLD and to develop novel drugs that can be applicated early in the treatment of NALFD. In addition, reshaping the gut microbiota to gently modulate BA signaling is also an important strategy for NAFLD treatment. Notably, FMT has emerged as a safe and efficient therapeutic approach for NAFLD. Furthermore, combination therapy, integrating multiple bile acid-targeted therapies, may enhance treatment efficacy and patient outcomes. However, there are several limitations in the current approaches to targeting BAs for NAFLD treatment. First, the intricate role of BAs in liver metabolism and systemic health remains inadequately understood, potentially leading to unintended off-target effects and systemic toxicity. Second, there are significant concerns regarding the long-term safety of modifying BA pathways, particularly because high doses of BAs may act as pro-carcinogenic agents. Third, the necessity for more phase 2 and 3 clinical trials to evaluate their long-term efficacy and safety is critical. Taken together, dysregulated BA signaling has been demonstrated to be involved in the development and progression of NAFLD. Thus, understanding the mechanism by which how BAs impact the host metabolism can help us in deciding how to manipulate the levels of BAs for treat the disease.

Author contributions

MW: Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Conceptualization. WT: Writing – review & editing. GH: Writing – review & editing, Supervision.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1341938/full#supplementary-material

References

Aguilar-Olivos, N. E., Almeda-Valdes, P., Aguilar-Salinas, C. A., Uribe, M., and Méndez-Sánchez, N. (2016). The role of bariatric surgery in the management of nonalcoholic fatty liver disease and metabolic syndrome. *Metabolism* 65, 1196–1207. doi: 10.1016/j.metabol.2015.09.004

Ahmad, N. N., Pfalzer, A., and Kaplan, L. M. (2013). Roux-en-Y gastric bypass normalizes the blunted postprandial bile acid excursion associated with obesity. *Int. J. Obes.* 37, 1553–1559. doi: 10.1038/ijo.2013.38

Aoi, W., Inoue, R., Mizushima, K., Honda, A., Björnholm, M., Takagi, T., et al. (2023). Exercise-acclimated microbiota improves skeletal muscle metabolism via circulating bile acid deconjugation. *iScience* 26:106251. doi: 10.1016/j.isci.2023.106251

Arab, J. P., Karpen, S. J., Dawson, P. A., Arrese, M., and Trauner, M. (2017). Bile acids and nonalcoholic fatty liver disease: molecular insights and therapeutic perspectives. *Hepatology* 65, 350–362. doi: 10.1002/hep.28709

Aron-Wisnewsky, J., Vigliotti, C., Witjes, J., Le, P., Holleboom, A. G., Verheij, J., et al. (2020). Gut microbiota and human NAFLD: disentangling microbial signatures from metabolic disorders. *Nat. Rev. Gastroenterol. Hepatol.* 17, 279–297. doi: 10.1038/s41575-020-0269-9

Axelrod, C. L., Langohr, I., Dantas, W. S., Heintz, E. C., Vandanmagsar, B., Yang, S., et al. (2023). Weight-independent effects of Roux-en-Y gastric bypass surgery on remission of nonalcoholic fatty liver disease in mice. *Obesity (Silver Spring)* 31, 2960–2971. doi: 10.1002/oby.23876

Baranowski, M., Zabielski, P., Błachnio-Zabielska, A. U., Harasim, E., Chabowski, A., and Górski, J. (2014). Insulin-sensitizing effect of LXR agonist T0901317 in high-fat fed rats is associated with restored muscle GLUT4 expression and insulin-stimulated AS160 phosphorylation. *Cell. Physiol. Biochem.* 33, 1047–1057. doi: 10.1159/000358675

Bertaggia, E., Jensen, K. K., Castro-Perez, J., Xu, Y., Di Paolo, G., Chan, R. B., et al. (2017). Cyp8b1 ablation prevents western diet-induced weight gain and hepatic steatosis due to impaired fat absorption. *Am. J. Physiol. Endocrinol. Metab.* 313, E121–E133. doi: 10.1152/ajpendo.00409.2016

Bhutta, H. Y., Rajpal, N., White, W., Freudenberg, J. M., Liu, Y., Way, J., et al. (2015). Effect of Roux-en-Y gastric bypass surgery on bile acid metabolism in normal and obese diabetic rats. *PLoS One* 10:e0122273. doi: 10.1371/journal.pone.0122273

Broeders, E. P., Nascimento, E. B., Havekes, B., Brans, B., Roumans, K. H., Tailleux, A., et al. (2015). The bile acid Chenodeoxycholic acid increases human Brown adipose tissue activity. *Cell Metab.* 22, 418–426. doi: 10.1016/j.cmet.2015.07.002

Cariou, B., van Harmelen, K., Duran-Sandoval, D., van Dijk, T. H., Grefhorst, A., Abdelkarim, M., et al. (2006). The farnesoid X receptor modulates adiposity and peripheral insulin sensitivity in mice. *J. Biol. Chem.* 281, 11039–11049. doi: 10.1074/jbc.M510258200

Castera, L., Friedrich-Rust, M., and Loomba, R. (2019). Noninvasive assessment of liver disease in patients with nonalcoholic fatty liver disease. *Gastroenterology* 156, 1264–81.e4. doi: 10.1053/j.gastro.2018.12.036

Chavez-Talavera, O., Tailleux, A., Lefebvre, P., and Staels, B. (2017). Bile acid control of metabolism and inflammation in obesity, type 2 diabetes, dyslipidemia, and nonalcoholic fatty liver disease. *Gastroenterology* 152, 1679–1694.e3. doi: 10.1053/j.gastro.2017.01.055

Comeglio, P., Cellai, I., Mello, T., Filippi, S., Maneschi, E., Corcetto, F., et al. (2018). INT-767 prevents NASH and promotes visceral fat brown adipogenesis and mitochondrial function. *J. Endocrinol.* 238, 107–127. doi: 10.1530/JOE-17-0557

de Aguiar Vallim, T. Q., Tarling, E. J., and Edwards, P. A. (2013). Pleiotropic roles of bile acids in metabolism. *Cell Metab.* 17, 657–669. doi: 10.1016/j.cmet.2013.03.013

Dutia, R., Embrey, M., O'Brien, S., Haeusler, R. A., Agénor, K. K., Homel, P., et al. (2016). Temporal changes in bile acid levels and 12α-hydroxylation after Roux-en-Y gastric bypass surgery in type 2 diabetes. *Int. J. Obes.* 40:554. doi: 10.1038/ijo.2015.250

Falany, C. N., Johnson, M. R., Barnes, S., and Diasio, R. B. (1994). Glycine and taurine conjugation of bile acids by a single enzyme. Molecular cloning and expression of human liver bile acid CoA: amino acid N-acyltransferase. *J. Biol. Chem.* 269, 19375–19379. doi: 10.1016/S0021-9258(17)32178-6

Fang, S., Suh, J. M., Reilly, S. M., Yu, E., Osborn, O., Lackey, D., et al. (2015). Intestinal FXR agonism promotes adipose tissue browning and reduces obesity and insulin resistance. *Nat. Med.* 21, 159–165. doi: 10.1038/nm.3760

Finn, P. D., Rodriguez, D., Kohler, J., Jiang, Z., Wan, S., Blanco, E., et al. (2019). Intestinal TGR5 agonism improves hepatic steatosis and insulin sensitivity in Western diet-fed mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* 316, G412–G424. doi: 10.1152/ajpgi.00300.2018

Fiorucci, S., Distrutti, E., Carino, A., Zampella, A., and Biagioli, M. (2021). Bile acids and their receptors in metabolic disorders. *Prog. Lipid Res.* 82:101094. doi: 10.1016/j.plipres.2021.101094

Flynn, C. R., Albaugh, V. L., Cai, S., Cheung-Flynn, J., Williams, P. E., Brucker, R. M., et al. (2015). Bile diversion to the distal small intestine has comparable metabolic benefits to bariatric surgery. *Nat. Commun.* 6:7715. doi: 10.1038/ncomms8715

Friedman, E. S., Li, Y., Shen, T. D., Jiang, J., Chau, L., Adorini, L., et al. (2018). FXR-dependent modulation of the human small intestinal microbiome by the bile acid derivative Obeticholic acid. *Gastroenterology* 155, 1741–52.e5. doi: 10.1053/j.gastro.2018.08.022

Gadaleta, R. M., van Erpecum, K. J., Oldenburg, B., Willemsen, E. C., Renooij, W., Murzilli, S., et al. (2011). Farnesoid X receptor activation inhibits inflammation and preserves the intestinal barrier in inflammatory bowel disease. *Gut* 60, 463–472. doi: 10.1136/gut.2010.212159

Ge, M.-x., Niu, W.-x., Ren, J.-f., Cai, S.-y., Yu, D.-k., Liu, H.-t., et al. (2019). A novel ASBT inhibitor, IMB17-15, repressed nonalcoholic fatty liver disease development in high-fat diet-fed Syrian golden hamsters. *Acta Pharmacol. Sin.* 40, 895–907. doi: 10.1038/s41401-018-0195-3

Gerhard, G. S., Styer, A. M., Wood, G. C., Roesch, S. L., Petrick, A. T., Gabrielsen, J., et al. (2013). A role for fibroblast growth factor 19 and bile acids in diabetes remission after Roux-en-Y gastric bypass. *Diabetes Care* 36, 1859–1864. doi: 10.2337/dc12-2255

Goodwin, B., Jones, S. A., Price, R. R., Watson, M. A., McKee, D. D., Moore, L. B., et al. (2000). A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis. *Mol. Cell* 6, 517–526. doi: 10.1016/S1097-2765(00)00051-4

Guo, C., Xie, S., Chi, Z., Zhang, J., Liu, Y., Zhang, L., et al. (2016). Bile acids control inflammation and metabolic disorder through inhibition of NLRP3 inflammasome. *Immunity* 45, 802–816. doi: 10.1016/j.immuni.2016.09.008

Hang, S., Paik, D., Yao, L., Kim, E., Trinath, J., Lu, J., et al. (2019). Bile acid metabolites control T(H)17 and T(reg) cell differentiation. *Nature* 576, 143–148. doi: 10.1038/s41586-019-1785-z

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

Hirokane, H., Nakahara, M., Tachibana, S., Shimizu, M., and Sato, R. (2004). Bile acid reduces the secretion of very low density lipoprotein by repressing microsomal triglyceride transfer protein gene expression mediated by hepatocyte nuclear factor-4. *J. Biol. Chem.* 279, 45685–45692. doi: 10.1074/jbc.M404255200

Jadhav, K., Xu, Y., Xu, Y., Li, Y., Xu, J., Zhu, Y., et al. (2018). Reversal of metabolic disorders by pharmacological activation of bile acid receptors TGR5 and FXR. *Mol. Metab.* 9, 131–140. doi: 10.1016/j.molmet.2018.01.005

Jansen, P. L. M. (2021). Targeting the ileal bile salt transporter in the treatment of non-alcoholic fatty liver disease. *Hepatol. Int.* 15, 283–286. doi: 10.1007/s12072-021-10134-5

Jiao, N., Baker, S. S., Chapa-Rodriguez, A., Liu, W., Nugent, C. A., Tsompana, M., et al. (2018). Suppressed hepatic bile acid signalling despite elevated production of primary and secondary bile acids in NAFLD. *Gut* 67, 1881–1891. doi: 10.1136/gutjnl-2017-314307

Kasai, Y., Kessoku, T., Tanaka, K., Yamamoto, A., Takahashi, K., Kobayashi, T., et al. (2022). Association of Serum and Fecal Bile Acid Patterns with Liver Fibrosis in biopsy-proven nonalcoholic fatty liver disease: An observational study. *Clin. Transl. Gastroenterol.* 13:e00503. doi: 10.14309/ctg.0000000000000503

Kheirvari, M., Nikroo, N. D., Jaafarinejad, H., Farsimadan, M., Eshghjoo, S., Hosseini, S., et al. (2020). The advantages and disadvantages of sleeve gastrectomy; clinical laboratory to bedside review. *Heliyon* 6:e03496. doi: 10.1016/j.heliyon.2020.e03496

Kim, I., Ahn, S.-H., Inagaki, T., Choi, M., Ito, S., Guo, G. L., et al. (2007). Differential regulation of bile acid homeostasis by the farnesoid X receptor in liver and intestine. *J. Lipid Res.* 48, 2664–2672. doi: 10.1194/jlr.M700330-JLR200

Kliewer, S. A., and Mangelsdorf, D. J. (2015). Bile acids as hormones: the FXR-FGF15/19 pathway. *Dig. Dis.* 33, 327–331. doi: 10.1159/000371670

Kolodziejczyk, A. A., Zheng, D., Shibolet, O., and Elinav, E. (2019). The role of the microbiome in NAFLD and NASH. *EMBO Mol. Med.* 11:e9302. doi: 10.15252/emmm.201809302

Kong, B., Wang, L., Chiang, J. Y., Zhang, Y., Klaassen, C. D., and Guo, G. L. (2012). Mechanism of tissue-specific farnesoid X receptor in suppressing the expression of genes in bile-acid synthesis in mice. *Hepatology* 56, 1034–1043. doi: 10.1002/hep.25740

Kuang, J., Wang, J., Li, Y., Li, M., Zhao, M., Ge, K., et al. (2023). Hyodeoxycholic acid alleviates non-alcoholic fatty liver disease through modulating the gut-liver axis. *Cell Metab.* 35, 1752–66.e8. doi: 10.1016/j.cmet.2023.07.011

Kumar, D. P., Asgharpour, A., Mirshahi, F., Park, S. H., Liu, S., Imai, Y., et al. (2016). Activation of transmembrane bile acid receptor TGR5 modulates pancreatic islet α cells to promote glucose homeostasis. *J. Biol. Chem.* 291, 6626–6640. doi: 10.1074/jbc.M115.699504

Kumar, S., Duan, Q., Wu, R., Harris, E. N., and Su, Q. (2021). Pathophysiological communication between hepatocytes and non-parenchymal cells in liver injury from NAFLD to liver fibrosis. *Adv. Drug Deliv. Rev.* 176:113869. doi: 10.1016/j.addr.2021.113869

Kumar, D. P., Rajagopal, S., Mahavadi, S., Mirshahi, F., Grider, J. R., Murthy, K. S., et al. (2012). Activation of transmembrane bile acid receptor TGR5 stimulates insulin secretion in pancreatic β cells. *Biochem. Biophys. Res. Commun.* 427, 600–605. doi: 10.1016/j.bbrc.2012.09.104

Lalloyer, F., Mogilenko, D. A., Verrijken, A., Haas, J. T., Lamazière, A., Kouach, M., et al. (2023). Roux-en-Y gastric bypass induces hepatic transcriptomic signatures and plasma metabolite changes indicative of improved cholesterol homeostasis. *J. Hepatol.* 79, 898–909. doi: 10.1016/j.jhep.2023.05.012

Lefebvre, P., Cariou, B., Lien, F., Kuipers, F., and Staels, B. (2009). Role of bile acids and bile acid receptors in metabolic regulation. *Physiol. Rev.* 89, 147–191. doi: 10.1152/physrev.00010.2008

Li, M., Wang, S., Li, Y., Zhao, M., Kuang, J., Liang, D., et al. (2022). Gut microbiota-bile acid crosstalk contributes to the rebound weight gain after calorie restriction in mice. *Nat. Commun.* 13:2060. doi: 10.1038/s41467-022-29589-7

Lin, H., An, Y., Tang, H., and Wang, Y. (2019). Alterations of bile acids and gut microbiota in obesity induced by high fat diet in rat model. *J. Agric. Food Chem.* 67, 3624–3632. doi: 10.1021/acs.jafc.9b00249

Liu, Y., Chen, K., Li, F., Gu, Z., Liu, Q., He, L., et al. (2020). Probiotic *Lactobacillus rhamnosus* GG prevents liver fibrosis through inhibiting hepatic bile acid synthesis and enhancing bile acid excretion in mice. *Hepatology* 71, 2050–2066. doi: 10.1002/hep.30975

Ma, K., Saha, P. K., Chan, L., and Moore, D. D. (2006). Farnesoid X receptor is essential for normal glucose homeostasis. *J. Clin. Invest.* 116, 1102–1109. doi: 10.1172/JCI25604

Ma, K., Tang, D., Yu, C., and Zhao, L. (2021). Progress in research on the roles of TGR5 receptor in liver diseases. *Scand. J. Gastroenterol.* 56, 717–726. doi: 10.1080/00365521.2021.1903547

Maruyama, T., Tanaka, K., Suzuki, J., Miyoshi, H., Harada, N., Nakamura, T., et al. (2006). Targeted disruption of G protein-coupled bile acid receptor 1 (Gpbar1/M-bar) in mice. *J. Endocrinol.* 191, 197–205. doi: 10.1677/joe.1.06546

McMahan, R. H., Wang, X. X., Cheng, L. L., Krisko, T., Smith, M., El Kasmi, K., et al. (2013). Bile acid receptor activation modulates hepatic monocyte activity and improves nonalcoholic fatty liver disease. *J. Biol. Chem.* 288, 11761–11770. doi: 10.1074/jbc.M112.446575

Mencarelli, A., and Fiorucci, S. (2010). FXR an emerging therapeutic target for the treatment of atherosclerosis. *J. Cell. Mol. Med.* 14, 79–92. doi: 10.1111/j.1582-4934.2009.00997.x

Mouzaki, M., Wang, A. Y., Bandsma, R., Comelli, E. M., Arendt, B. M., Zhang, L., et al. (2016). Bile acids and dysbiosis in non-alcoholic fatty liver disease. *PLoS One* 11:e0151829. doi: 10.1371/journal.pone.0151829

Panzitt, K., Zollner, G., Marschall, H.-U., and Wagner, M. (2022). Recent advances on FXR-targeting therapeutics. *Mol. Cell. Endocrinol.* 552:111678. doi: 10.1016/j.mce.2022.111678

Pellicciari, R., Gioiello, A., Macchiarulo, A., Thomas, C., Rosatelli, E., Natalini, B., et al. (2009). Discovery of 6alpha-ethyl-23(S)-methylcholic acid (S-EMCA, INT-777) as a potent and selective agonist for the TGR5 receptor, a novel target for diabesity. *J. Med. Chem.* 52, 7958–7961. doi: 10.1021/jm901390p

Pérez-Rubio, Á., Soluyanova, P., Moro, E., Quintás, G., Rienda, I., Periañez, M. D., et al. (2023). Gut microbiota and plasma bile acids associated with non-alcoholic fatty liver disease resolution in bariatric surgery patients. *Nutrients* 15:3187. doi: 10.3390/nu15143187

Perino, A., and Schoonjans, K. (2015). TGR5 and immunometabolism: insights from physiology and pharmacology. *Trends Pharmacol. Sci.* 36, 847–857. doi: 10.1016/j.tips.2015.08.002

Perino, A., Velázquez-Villegas, L. A., Bresciani, N., Sun, Y., Huang, Q., Fénelon, V. S., et al. (2021). Central anorexigenic actions of bile acids are mediated by TGR5. *Nat. Metab.* 3, 595–603. doi: 10.1038/s42255-021-00398-4

Potthoff, M. J., Kliewer, S. A., and Mangelsdorf, D. J. (2012). Endocrine fibroblast growth factors 15/19 and 21: from feast to famine. *Genes Dev.* 26, 312–324. doi: 10.1101/gad.184788.111

Powell, E. E., Wong, V. W., and Rinella, M. (2021). Non-alcoholic fatty liver disease. *Lancet* 397, 2212–2224. doi: 10.1016/S0140-6736(20)32511-3

Qiu, Y., Yu, J., Li, Y., Yang, F., Yu, H., Xue, M., et al. (2021). Depletion of gut microbiota induces skeletal muscle atrophy by FXR-FGF15/19 signalling. *Ann. Med.* 53, 508–522. doi: 10.1080/07853890.2021.1900593

Rao, A., Kosters, A., Mells, J. E., Zhang, W., Setchell, K. D., Amanso, A. M., et al. (2016). Inhibition of ileal bile acid uptake protects against nonalcoholic fatty liver disease in high-fat diet-fed mice. *Sci. Transl. Med.* 8:357ra122. doi: 10.1126/scitranslmed.aaf4823

Reddy, I. A., Smith, N. K., Erreger, K., Ghose, D., Saunders, C., Foster, D. J., et al. (2018). Bile diversion, a bariatric surgery, and bile acid signaling reduce central cocaine reward. *PLoS Biol.* 16:e2006682. doi: 10.1371/journal.pbio.2006682

Roth, J. D., Feigh, M., Veidal, S. S., Fensholdt, L. K., Rigbolt, K. T., Hansen, H. H., et al. (2018). INT-767 improves histopathological features in a diet-induced Ob/Ob mouse model of biopsy-confirmed non-alcoholic steatohepatitis. *World J. Gastroenterol.* 24, 195–210. doi: 10.3748/wjg.v24.i2.195

Sayin, S. I., Wahlstrom, A., Felin, J., Jantti, S., Marschall, H. U., Bamberg, K., et al. (2013). Gut microbiota regulates bile acid metabolism by reducing the levels of tauro-beta-muricholic acid, a naturally occurring FXR antagonist. *Cell Metab.* 17, 225–235. doi: 10.1016/j.cmet.2013.01.003

Schaap, F. G., Trauner, M., and Jansen, P. L. (2014). Bile acid receptors as targets for drug development. *Nat. Rev. Gastroenterol. Hepatol.* 11, 55–67. doi: 10.1038/nrgastro.2013.151

Sciarrillo, C. M., Keirns, B. H., Koemel, N. A., Anderson, K. L., and Emerson, S. R. (2021). Fibroblast growth factor 19: potential modulation of hepatic metabolism for the treatment of non-alcoholic fatty liver disease. *Liver Int.* 41, 894–904. doi: 10.1111/liv.14802

Shlomai, A., Halfon, P., Goldiner, I., Zelber-Sagi, S., Halpern, Z., Oren, R., et al. (2013). Serum bile acid levels as a predictor for the severity of liver fibrosis in patients with chronic hepatitis C. *J. Viral Hepat.* 20, 95–102. doi: 10.1111/j.1365-2893.2012.01628.x

Sinal, C. J., Tohkin, M., Miyata, M., Ward, J. M., Lambert, G., and Gonzalez, F. J. (2000). Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. *Cell* 102, 731–744. doi: 10.1016/S0092-8674(00)00062-3

Song, Q., and Zhang, X. (2022). The role of gut–liver axis in gut microbiome dysbiosis associated NAFLD and NAFLD-HCC. *Biomedicines* 10:524. doi: 10.3390/biomedicines10030524

Suga, T., Yamaguchi, H., Ogura, J., Shoji, S., Maekawa, M., and Mano, N. (2019). Altered bile acid composition and disposition in a mouse model of non-alcoholic steatohepatitis. *Toxicol. Appl. Pharmacol.* 379:114664. doi: 10.1016/j.taap.2019.114664

Thomas, C., Gioiello, A., Noriega, L., Strehle, A., Oury, J., Rizzo, G., et al. (2009). TGR5-mediated bile acid sensing controls glucose homeostasis. *Cell Metab.* 10, 167–177. doi: 10.1016/j.cmet.2009.08.001

Thomas, C., Pellicciari, R., Pruzanski, M., Auwerx, J., and Schoonjans, K. (2008). Targeting bile-acid signalling for metabolic diseases. *Nat. Rev. Drug Discov.* 7, 678–693. doi: 10.1038/nrd2619

Trauner, M., Nevens, F., Shiffman, M. L., Drenth, J. P., Bowlus, C. L., Vargas, V., et al. (2019). Long-term efficacy and safety of obeticholic acid for patients with primary biliary cholangitis: 3-year results of an international open-label extension study. *Lancet Gastroenterol. Hepatol.* 4, 445–453. doi: 10.1016/S2468-1253(19)30094-9

Tsuji, Y., Kaji, K., Kitade, M., Kaya, D., Kitagawa, K., Ozutsumi, T., et al. (2020). Bile acid Sequestrant, Sevelamer ameliorates hepatic fibrosis with reduced overload of endogenous lipopolysaccharide in experimental nonalcoholic steatohepatitis. *Microorganisms* 8:925. doi: 10.3390/microorganisms8060925

Vrieze, A., Van Nood, E., Holleman, F., Salojärvi, J., Kootte, R. S., Bartelsman, J. F., et al. (2012). Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. *Gastroenterology* 143, 913–6.e7. doi: 10.1053/j.gastro.2012.06.031

Wahlström, A., Sayin, S. I., Marschall, H.-U., and Bäckhed, F. (2016). Intestinal crosstalk between bile acids and microbiota and its impact on host metabolism. *Cell Metab.* 24, 41–50. doi: 10.1016/j.cmet.2016.05.005

Wang, Y. D., Chen, W. D., Yu, D., Forman, B. M., and Huang, W. (2011). The G-protein-coupled bile acid receptor, Gpbar1 (TGR5), negatively regulates hepatic inflammatory response through antagonizing nuclear factor κ light-chain enhancer of activated B cells (NF-κB) in mice. *Hepatology* 54, 1421–1432. doi: 10.1002/hep.24525

Wang, K., Liao, M., Zhou, N., Bao, L., Ma, K., Zheng, Z., et al. (2019). *Parabacteroides distasonis* alleviates obesity and metabolic dysfunctions via production of succinate and secondary bile acids. *Cell Rep.* 26, 222–35.e5. doi: 10.1016/j.celrep.2018.12.028

Watanabe, M., Houten, S. M., Mataki, C., Christoffolete, M. A., Kim, B. W., Sato, H., et al. (2006). Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. *Nature* 439, 484–489. doi: 10.1038/nature04330

Watanabe, M., Houten, S. M., Wang, L., Moschetta, A., Mangelsdorf, D. J., Heyman, R. A., et al. (2004). Bile acids lower triglyceride levels via a pathway involving FXR, SHP, and SREBP-1c. *J. Clin. Invest.* 113, 1408–1418. doi: 10.1172/JCI21025

Xiong, X., Ren, Y., Cui, Y., Li, R., Wang, C., and Zhang, Y. (2017). Obeticholic acid protects mice against lipopolysaccharide-induced liver injury and inflammation. *Biomed. Pharmacother.* 96, 1292–1298. doi: 10.1016/j.biopha.2017.11.083

Xue, L., Deng, Z., Luo, W., He, X., and Chen, Y. (2022). Effect of fecal microbiota transplantation on non-alcoholic fatty liver disease: A randomized clinical trial. *Front. Cell. Infect. Microbiol.* 12:759306. doi: 10.3389/fcimb.2022.759306

Yang, Z.-X., Shen, W., and Sun, H. (2010). Effects of nuclear receptor FXR on the regulation of liver lipid metabolism in patients with non-alcoholic fatty liver disease. *Hepatol. Int.* 4, 741–748. doi: 10.1007/s12072-010-9202-6

Yoshimoto, S., Loo, T. M., Atarashi, K., Kanda, H., Sato, S., Oyadomari, S., et al. (2013). Obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome. *Nature* 499, 97–101. doi: 10.1038/nature12347

Younossi, Z. M., Ratziu, V., Loomba, R., Rinella, M., Anstee, Q. M., Goodman, Z., et al. (2019). Obeticholic acid for the treatment of non-alcoholic steatohepatitis: interim analysis from a multicentre, randomised, placebo-controlled phase 3 trial. *Lancet* 394, 2184–2196. doi: 10.1016/S0140-6736(19)33041-7

Zhang, S., Wang, J., Liu, Q., and Harnish, D. C. (2009). Farnesoid X receptor agonist WAY-362450 attenuates liver inflammation and fibrosis in murine model of non-alcoholic steatohepatitis. *J. Hepatol.* 51, 380–388. doi: 10.1016/j.jhep.2009.03.025

Zhang, L., Xie, C., Nichols, R. G., Chan, S. H., Jiang, C., Hao, R., et al. (2016). Farnesoid X receptor signaling shapes the gut microbiota and controls hepatic lipid metabolism. *mSystems* 1:e00070-16. doi: 10.1128/mSystems.00070-16

Zhao, Z., Chen, L., Zhao, Y., Wang, C., Duan, C., Yang, G., et al. (2020). *Lactobacillus plantarum* NA136 ameliorates nonalcoholic fatty liver disease by modulating gut microbiota, improving intestinal barrier integrity, and attenuating inflammation. *Appl. Microbiol. Biotechnol.* 104, 5273–5282. doi: 10.1007/s00253-020-10633-9

Zheng, X., Chen, T., Jiang, R., Zhao, A., Wu, Q., Kuang, J., et al. (2021). Hyocholic acid species improve glucose homeostasis through a distinct TGR5 and FXR signaling mechanism. *Cell Metab.* 33, 791–803.e7. e7. doi: 10.1016/j.cmet.2020.11.017

Zheng, X., Huang, F., Zhao, A., Lei, S., Zhang, Y., Xie, G., et al. (2017). Bile acid is a significant host factor shaping the gut microbiome of diet-induced obese mice. *BMC Biol.* 15:120. doi: 10.1186/s12915-017-0462-7

Zietak, M., and Kozak, L. P. (2016). Bile acids induce uncoupling protein 1-dependent thermogenesis and stimulate energy expenditure at thermoneutrality in mice. *Am. J. Physiol. Endocrinol. Metab.* 310, E346–E354. doi: 10.1152/ajpendo.00485.2015

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Glossary

ASBT	Apical sodium-dependent bile acid transporter
BA	Bile acid
BAs	Bile acids
BAT	Brown adipose tissue
BSH	Bile salt hydrolase
BSEP	Bile salt export pump
WAT	White adipose tissue
CA	Cholic acid
CDCA	Chenodeoxycholic acid
ChREBP	Carbohydrate response element binding proteins
CYP7A1	Cholesterol 7-alpha hydroxylase
CYP8B1	Sterol 12α-hydroxylase
CYP27A1	Sterol-27α-hydroxylase
CYP7B1	Oxysterol 7α-hydroxylase
DCA	Deoxycholic acid
DIO2	Type II iodothyronine deionidinase
ERK1/2	Extracellular regulated protein kinases
FGF15/19	Fibroblast growth factor 15/19
FGFR4	Fibroblast growth factor receptor 4
FXR	Farnesoid X receptor
FOS	Fructo-oligosaccharides
FMT	Fecal microbiota transplantation
GLP-1	Glucagon-like peptide-1
GLP-2	Glucagon-like peptide-2
Gly-MCA	Glycine-β-muricholic acid
GLUT4	Glucose transporter 4
G6Pase	Glucose 6-phosphatase
HFD	High-fat diet
HCC	Hepatocellular carcinoma
HDCA	Hyodeoxycholic acid
HCA	Hyocholic acid
LCA	Lithocholic acid
LXR	Liver X receptor
LRH-1	Liver receptor homolog-1
LPS	Lipopolysaccharide
MCAs/α/β-MCA	Muricholic acids/α/β-muricholic acid
MRP2	Multidrug resistance-associated protein 2
NAFLD	Nonalcoholic fatty liver disease
NAFL	Non-alcoholic fatty liver
NASH	Nonalcoholic steatohepatitis
NTCP	Sodium dependent taurocholate co-transporting polypeptide
OSTα/β	Organic solute transporter subunit α and β
OCA	Obeticholic acid
PYY	Peptide YY
PXR	Pregnane X receptor

(Continued)

Glossary (Continued)

PEPCK	Phosphoenolpyruvate carboxykinase
RYGB	Roux-en-Y gastric bypass
SHP	Small heterodimer partner
SREBP-1c	Steroid response element binding protein-1c
SCFAs	Short-chain fatty acids
SIPR2	Sphingosine-1-phosphate receptor 2
TGR5	G protein-coupled bile acid receptor
UCP1	Uncoupling protein1
UDCA	Ursodeoxycholic acid
VDR	Vitamin D receptor

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Multi-omics analysis of the biological mechanism of the pathogenesis of non-alcoholic fatty liver disease

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Background: Non-alcoholic fatty liver disease (NAFLD) is a type of liver metabolic syndrome. Employing multi-omics analyses encompassing the microbiome, metabolome and transcriptome is crucial for comprehensively elucidating the biological processes underlying NAFLD.

Methods: Hepatic tissue, blood and fecal samples were obtained from 9 NAFLD model mice and 8 normal control mice. Total fecal microbiota DNA was extracted, and 16S rRNA was amplified, to analyze alterations in the gut microbiota (GM) induced by NAFLD. Subsequently, diagnostic strains for NAFLD were screened, and their functional aspects were examined. Differential metabolites and differentially expressed genes were also screened, followed by enrichment analysis. Correlations between the differential microbiota and metabolites, as well as between the DEGs and differential metabolites were studied. A collinear network involving key genes-, microbiota-and metabolites was constructed.

Results: *Ileibacterium* and *Ruminococcaceae*, both belonging to Firmicutes; *Olsenella*, *Duncaniella* and *Paramuribaculum* from Bacteroidota; and *Bifidobacterium*, *Coriobacteriaceae_UCG_002* and *Olsenella* from Actinobacteriota were identified as characteristic strains associated with NAFLD. Additionally, differentially expressed metabolites were predominantly enriched in tryptophan, linoleic acid and methylhistidine metabolism pathways. The functions of 2,510 differentially expressed genes were found to be associated with disease occurrence. Furthermore, a network comprising 8 key strains, 14 key genes and 83 key metabolites was constructed.

Conclusion: Through this study, we conducted a comprehensive analysis of NAFLD alterations, exploring the gut microbiota, genes and metabolites of the results offer insights into the speculated biological mechanisms underlying NAFLD.

KEYWORDS

non-alcoholic fatty liver disease, gut microbiota, differential metabolites, differentially expressed genes, multi-omics

1 Introduction

The gut microbiota (GM) constitutes a complex, dynamic and spatially heterogeneous ecosystem hosting numerous interacting microorganisms (Fan and Pedersen, 2021). Renowned as not only the largest "micro-ecosystem," but also the "second largest gene pool" in the human body (Ren et al., 2020; Li C. et al., 2021). GM plays a pivotal role in human health (Álvarez-Mercado et al., 2019). Alterations in the GM composition can significantly impact metabolic health, with abnormal changes in its abundance contributing to various common metabolic disorders, such as obesity, type 2 diabetes, non-alcoholic fatty liver disease, and cardiometabolic disease (de la Cuesta-Zuluaga et al., 2018; Safari and Gerard, 2019; Sanchez-Rodriguez et al., 2020; Wu et al., 2020). Marshall proposed the concept of the entero-hepatic axis (Marshall, 1998), elucidating the reciprocal regulation and influence of substances, cells and cytokines between the liver and intestine through the portal vein system. Moreover, studies have validated the metabolic interaction and immune correlation between the liver and intestine.

Studies have shown that in healthy individuals, there is usually a stable proportion of bacteria within the GM. Disruption of the GM can lead to structural, functional and diversity changes in intestinal tissues. Moreover, an increase in pathogenic bacteria could cause inflammation, energy metabolism disorders and immune disorders in host tissues, potentially leading to various diseases (Singh et al., 2017), including non-alcoholic fatty liver disease (NAFLD). According to the latest nomenclature standards, Metabolic Associated Steatotic Liver Disease (MASLD) has replaced the previous term Non-Alcoholic Fatty Liver Disease (NAFLD). However, for the sake of clarity and reference in this study, we will continue to use the term NAFLD after introducing MASLD. MASLD is a newer term. It is defined by hepatic steatosis and the presence of at least one cardio-metabolic risk factor. It emphasizes the central role of metabolic factors in fatty liver disease. This name more clearly points out the key role of metabolic disorders (such as obesity, diabetes, hyperlipidemia, etc.) in the occurrence of disease (Abdelhameed et al., 2024). NAFLD encompasses liver fat accumulation exceeding 5%, excluding alcohol and known liver damage factors, and includes non-alcoholic hepatic steatosis, non-alcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma (Powell et al., 2021). Research by Li F. et al. (2021) confirmed increased abundances of *Escherichia*, *Prevotella* and *Streptococcus*, alongside decreased abundances of *Coprococcus*, *Faecalibacterium* and *Ruminococcus* in patients with NAFLD. Similarly, a study by Loomba et al. (2017) also identified GM imbalances in NAFLD, specifically increased abundances of *Proteobacteria* and *Enterobacteria* and decreased abundances of *Ruminococcus* and *Firmicutes*. Further investigations revealed the crucial role of GM metabolites in the onset and progression of NAFLD. However, comprehensive analyses integrating transcriptome-metabolome-and microbiome data remain an area needing further exploration.

The advantage of using mice as a multi-omics model for NAFLD research lies in the following reasons. First, biological similarity. The biology of mice is similar to that of humans, especially in terms of metabolic and liver diseases. Therefore, by studying NAFLD in mouse models, the pathogenesis and pathophysiological processes of the disease can be better understood. Second, controllability. Mouse models can be more easily controlled and manipulated, and experimental conditions such as diet, environment, and gene expression can be precisely controlled to better understand the development and progression of NAFLD. Third, experimental resources and costs. Mouse models are relatively inexpensive and resources are more readily available, and mouse experiments can be conducted more cost-effectively than human studies. Fourth, evaluation of potential treatment strategies. Studies conducted in mouse models can provide important information for the development and evaluation of potential NAFLD treatment strategies, such as drug therapy, dietary interventions, and gene editing.

In this study, we extensively elucidated the intricate biological mechanisms involved in the pathogenesis of NAFLD, examining genes, species, metabolites and metabolic pathways through multi-omics analyses encompassing the microbiome, metabolome and transcriptome. Specifically, we initially employed 16S rRNA sequencing to analyze differences in the species diversity and composition within the GM between patients with NAFLD and healthy controls (HCs). Subsequently, we identified differential microbiota, and metabolites as well as differentially expressed genes (DEGs), exploring their respective functions. Additionally, we delved into the driving mechanisms associated with key strains of NAFLD by conducting correlation analyses among the genes, microbiota and metabolites.

2 Materials and methods

2.1 Construction of a nonalcoholic fatty liver model

Male C57 mice aged 8 weeks and weighing between 18 and 22 g were randomly allocated into two groups: 9 mice in the high-fat group and 8 in the control group. The high-fat group was fed through 85% basal feed +15% lard +1.5% cholesterol; the low-fat group was fed through normal feed. The feed composition includes 97 g/kg moisture, 194.8 g/kg crude protein, 46 g/kg crude fat, 20 g/kg crude fiber, 56 g/kg crude ash, 13.4 g/kg calcium, 8.2 g/kg total phosphorus, 0.146 mg/kg aflatoxin B1, and < 10 CFU/g total colony count. All mice had *ad libitum* access to food and water throughout the study. The high-fat group received a diet high in -fat content, whereas the control group was provided with a standard normal diet. After a feeding period of 13 weeks, blood samples were collected from the eyeball, fecal samples were collected and liver tissue was obtained through laparotomy. This study received approval from the ethics committee of Kunming Medical University.

2.2 Lipid panel tests

The blood collected from the eyeballs in each group at a range of 2–8°C by centrifugation at 3000 rpm for 15 min. Subsequently, the resulting and the supernatant underwent analysis using a biochemical analyzer (SMT-120VP, Smart).

2.3 Fat expression detection using oil red O staining

The liver tissues obtained from the mice in each group were initially fixed in paraformaldehyde, and subsequently treated with the

OCT embedding agent, and rapidly frozen and embedded on the frozen table of microtome. To visualize lipid content, frozen sections were immersed in an oil red O working solution in darkness for 8–10 min. Afterwards, differentiation was achieved by briefly submerging the sections in 60% isopropyl alcohol for 3–5 s. The staining of nuclei was accomplished using a haematoxylinstaining solution (G1004, Servicebio), followed by a series of washing steps with water to remove excess stain. Once washed, dried slightly, the sections were sealed using a glycerine gelatine sealing medium to preserve the samples.

2.4 Data extraction

The total fecal microbiota DNA was extracted and the V3-V4 regions of the 16S rRNA gene were amplified. DNA extraction kits were available for different types of samples in order to ensure DNA extraction efficiency and quality. (OMEGASoilDNAKit; OMEGAWaterDNAKi; OMEGAStool DNAKit). The PCR primer (Supplementary Table 1) was designed against the conserved region to target the variable region of the 16S /ITS2 rDNA gene. After 35 cycles of PCR, sequencing adapters and barcodes were added for amplification. PCR amplification products were detected by 1.5% agarose gel electrophoresis. The target fragments were recovered using the AxyPrep PCR Cleanup Kit. The PCR product was further purified using the Quant-iT PicoGreen dsDNA Assay Kit. The library was quantified on the Promega QuantiFluor fluorescence quantification system. The pooled library was loaded on Illumina platform using a paired-end sequencing protocol (2 × 250 bp). The composition of the fecal microbiota was evaluated using the quantitative insights into microbial ecology (QIIME) software (Release 138) for microbiota analysis.

Additionally, the collected tissue samples were thawed on ice, and metabolites were extracted using a 50% methanol buffer. Briefly, 20 μL of each sample was mixed with 120 μL of precooled 50% methanol, vortexed for 1 min, and then incubated at room temperature for 10 min. The extraction mixture was stored overnight at −20°C. The next day, the mixture was centrifuged at 4,000 *g* for 20 min, and the supernatants were carefully transferred into new 96-well plates. The samples were then stored at −80°C until further analysis by LC–MS. In addition, quality control (QC) samples were prepared by combining 10 μL of each extraction mixture. QC samples with a relative standard deviation (RSD) greater than 30% were removed from the analysis to ensure data quality. Subsequently, the extracted metabolites were annotated and quantified using the metaX software in combination with the KEGG and HMDB databases to identify and characterize the metabolites of interest.

2.5 Species diversity analysis

The sequencing depth was assessed using the Good's coverage index. Various a Alpha-diversity indexes, including Chao1, observed species, Shannon and Simpson, were compared to analyze the species richness and uniformity (Mohakud et al., 2019). Beta-diversity analysis was used to evaluate the species' complexity among different groups. In this study, principal coordinate analysis was used to illustrate the differences between the different groups. Subsequently, the analysis of similarities (Anosim) was used to analyze whether differences between the two groups were significantly greater than those within the groups (Draiko et al., 2019).

2.6 Species composition diversity analysis

The GM composition was further analyzed, comparing the distribution of the GM at both the phylum and genus levels. Characteristic strains were identified using linear discriminant analysis effect size (LEfSE), and operational taxonomic units with a relative abundance exceeding 0.5% were selected for analysis based on the linear discriminant analysis (LDA) scores (Segata et al., 2011).

2.7 Functional prediction for the diagnostic strains

Receiver operating characteristic (ROC) curves were calculated using the "pROC" R package (version 1.18.0), and the area under the ROC curve (AUC) values were used to access the distinguish ability of characteristic strains (Robin et al., 2011). Functional prediction of the diagnostic strains (AUC > 0.8) was assessed, and differences between NAFLD mice and HCs were studied using the "PICRUSt2" R package (version 0.2.3) (Douglas et al., 2020).

2.8 Differential analysis of the metabolites

The metabolic differences between the NAFLD mice and HCs were compared through the construction of an orthogonal partial least squares discriminant analysis (OPLS-DA) model. This model's validity was assessed using a permutation test. Subsequently, three methods, were employed to select metabolites: the multiple change method (FC value), the t test method (*p* value, and FDR value), and the partial least squares discriminant analysis method (VIP value). The differentially expressed metabolites between the two groups were identified using the following criteria: VIP > 1, FC ≥ 1, and $p < 0.05$. In addition, enrichment analysis of these differentially expressed metabolites was performed using the online platform "Metaboanalyst" (Chong et al., 2019).

2.9 Function analysis of the DEGs in NAFLD

The mRNA expression levels between the NAFLD mice and HCs were compared using the "limma" R package (version 3.52.4) ($|\log FC| > 0.5$, $p < 0.05$), and visualized using the 'ggplot2' R package and Kolde R (2019). _pheatmap: Pretty Heatmaps_. R package version 1.0.12.[1] Then, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of DEGs were conducted using the 'clusterprofiler' R package (version 4.4.4) (Wu et al., 2021).

1 https://CRAN.R-project.org/package=pheatmap

2.10 Construction of a gene-microbiota-metabolite interaction network

The correlations between the characteristic strains and differential metabolites were assessed using the 'Spearman' package ($|r| \geq 0.3$, $p < 0.05$). Metabolites in the pair were regarded as the key metabolites 1, and diagnostic strains were regarded as key microbiota. Similarly, the correlations between the DEGs and differential metabolites were studied, and the DEGs in the pair were regarded as key genes, whereas metabolites were regarded as key metabolites 2. Collinear networks of the key microbiota-metabolites 1 and key genes-metabolites 2 were constructed using 'Cytoscape' (version 3.7.1) (Shannon et al., 2003). Finally, key metabolites 1 and 2 were combined, and a collinear network of key genes-microbiota-metabolites was constructed using 'Cytoscape.'

2.11 Verification of the expression of differential genes using RT-PCR

Total RNA was extracted from the control and model groups, and the reverse transcription reaction was subsequently performed, followed by qPCR reaction with 2 × Universal Blue SYBR Green qPCR Master Mix kit. The PCR cycling conditions were as follows: pre-denaturation at 95°C for 1 min; denaturation for 20 s at 95°C, annealing for 20 s at 55°C,and final extension for 30 s at 72°C; total, 40 cycles.

2.12 Statistical analysis

Statistical analysis was performed using R software (version 4.2.3) and the maps were plotted using Prism GraphPad, and the experimental data are presented as mean ± SEM. The Welch's *t*-test was used to compare the measurement data between groups. $p < 0.05$ for statistical significance; $p < 0.01$ for high statistical significance; $p < 0.001$ for extremely high statistical significance.

3 Results

3.1 Construction of non-alcoholic fatty liver model

Upon examination, liver tissues from the model group of C57 mice exhibited a distinctive yellow hue and enlarged sizes (Figure 1A). Moreover, the levels of TG, TC and LDL were significantly increased, whereas HDL levels showed a significant decrease (Figure 1B). These findings collectively suggest the successful establishment of the NAFLD model (Figure 1).

3.2 Fat formation observed upon oil red O staining

Excessive fat deposition represents a crucial pathological characteristic of NAFLD. To validate the successful establishment of the model, we conducted observations of adipogenesis using oil red O staining. Results demonstrated a substantial increase in fat content within the model group compared to the control group, further substantiating the successful construction of the model (Figure 2).

3.3 Species diversity analysis

The goods coverage curve approaching 1, indicated that sufficient sequencing data were acquired, affirming a reasonable sequencing depth (Figure 3A). No significant differences in alpha-diversity indexes were observed between the NAFLD and HC groups,

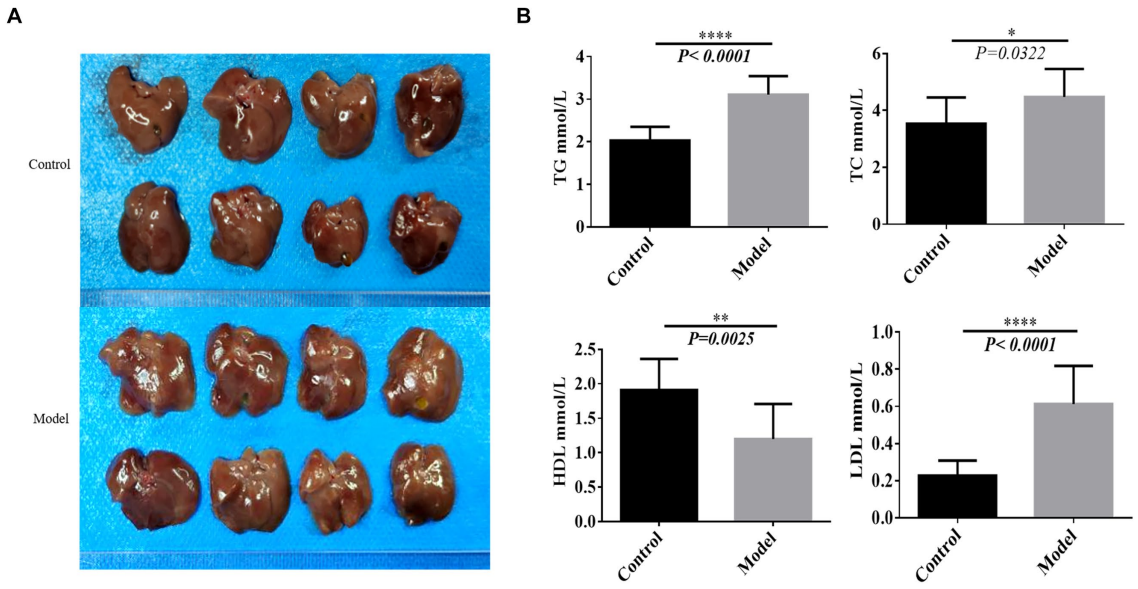

FIGURE 1
Construction of the nonalcoholic fatty liver model. **(A)** The liver sample of each tissue. **(B)** Lipid panel. *p<0.05, **p<0.01, ****p<0.0001.

suggesting that the richness and uniformity of bacterial communities remained largely unchanged owing to NAFLD (Figure 3B). However, the PCoA results showed a distinct clustering of patients with NAFLD and HCs (Figure 3C). Furthermore, the ANOSIM analysis demonstrated greater differences between groups than within groups (ANOSIM statistics = 0.6233, $p = 0.001$), signifying the meaningfulness of grouping and substantial alterations in the structure and composition of the GM induced by NAFLD.

3.4 Analysis of the species composition diversity

At the phylum level, the gut microbiota primarily comprised Firmicutes, Bacteroidetes, Actinobacteria, Desulfobacterota, Patescibacteria, Proteobacteria, Verrucomicrobiota, Campylobacterota, Deferribacterota, and Synergistota. Comparing the NAFLD and HC groups, revealed significant decreases in the abundances of Firmicutes

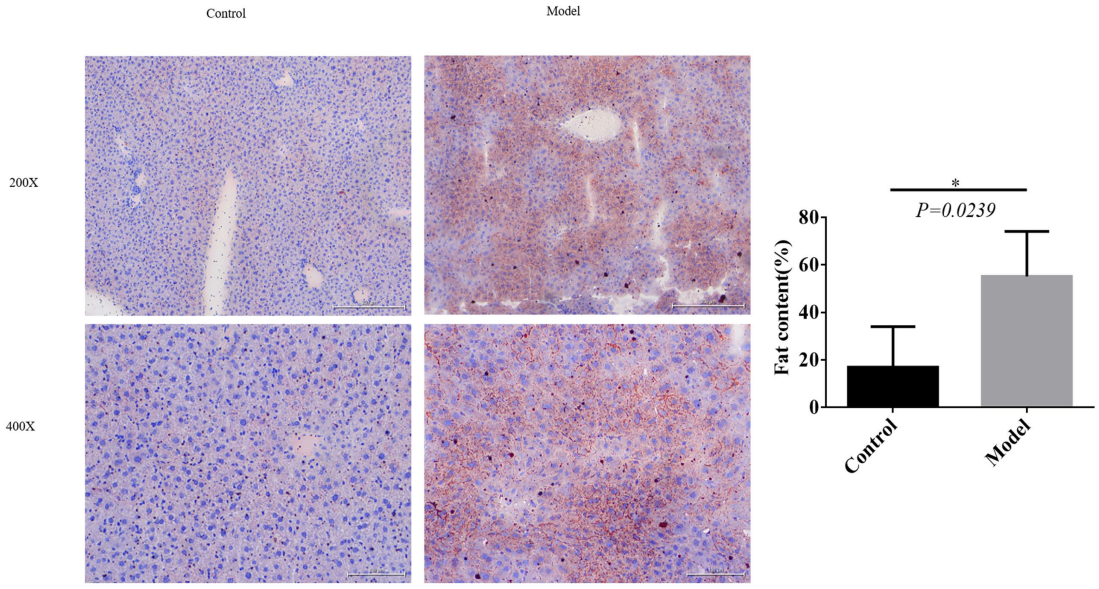

FIGURE 2
Fat formation observed upon oil red O staining. *p<0.05.

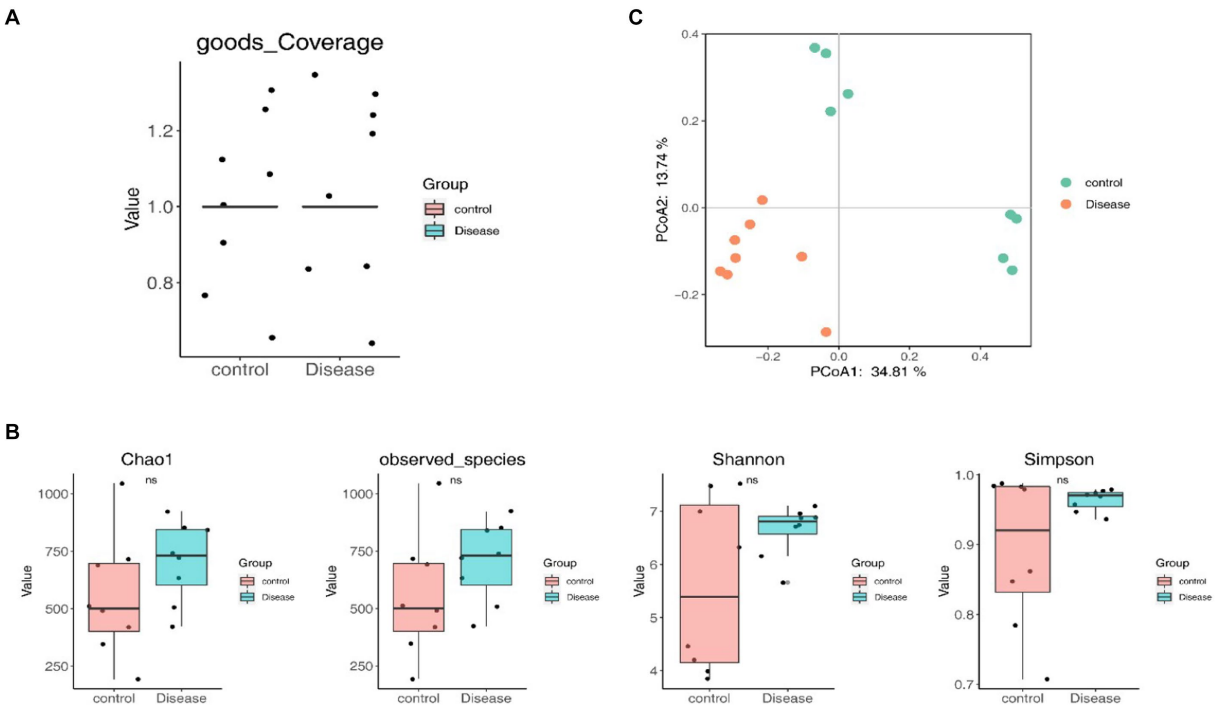

FIGURE 3
Species diversity analysis. **(A)** The goods coverage curve. **(B)** Alpha-diversity indexes between the NAFLD and HC groups. **(C)** The PCoA result.

and Synergistota in the NAFLD groups ($p < 0.05$), whereas Patescibacteria and Deferribacterota showed decreased abundance, Others demonstrated significantly decreased abundances in the NAFLD group ($p < 0.05$) (Figure 4A and Supplementary Table 2). At the genus level, the NAFLD group exhibited prominent representation of *Muribaculaceae, Allobaculum, Dubosiella, Ligilactobacillus, Ruminococcaceae, Paramuribaculum, Bifidobacterium*, and *Desulfovibrio*, among others. The HC group showed a predominant presence of *Ligilactobacillus, Muribaculaceae, Lactobacillus, HT002, Dubosiella, Allobaculum, Candidatus*, and *Clostridia*, among others. Notably, the NAFLD group demonstrated significantly increased abundances of *Muribaculaceae, Allobaculum, Paramuribaculum*, and *Ruminococcaceae*, and decreased abundances of *Ligilactobacillus, Lactobacillus*, and *HT002* (Figure 4B and Supplementary Table 3). Further investigation using LEfSE analysis identified 46 differentially abundant key gut microbiota, including 35 with notable roles in the NAFLD groups and 11 in the HC groups. Among these, *Ileibacterium, Ruminococcaceae* (Firmicutes), *Olsenella, Duncaniella* and *Paramuribaculum* (Bacteroidota), and *Bifidobacterium, Coriobacteriaceae_UCG_002* and *Olsenella* (Actinobacteriota) were characteristic strains of NAFLD. On the other hand, *HT002, Lactobacillus* (Firmicutes), *Bacteroides* (Bacteroidota) were characteristic strains of HC (Figures 4C,D and Supplementary Table 4).

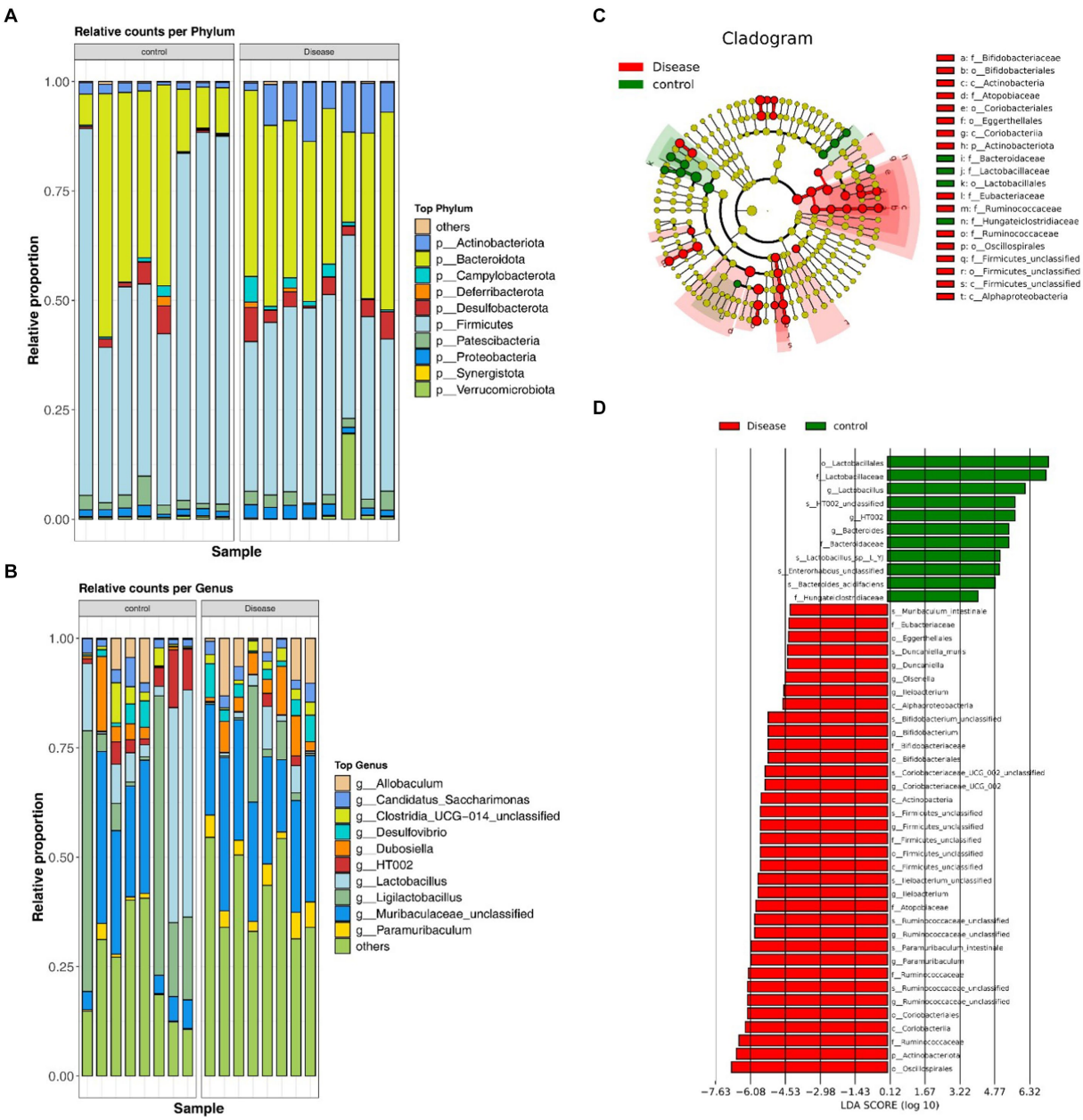

FIGURE 4
Analysis of the species composition diversity. **(A)** The distribution of the GM at the phylum level. **(B)** The distribution of the GM at the genus level. **(C)** LEfSE analysis of the clustering tree. **(D)** Different microflora in the NAFLD and HC groups.

3.5 Screening of the 13 diagnostic strains, associated with liver protection

In this study, the ROC curves of the characteristic strains were calculated, and 13 diagnostic strains were obtained with an AUC value >0.8 ($AUC_{Bifidobacterium} = 0.969$, $AUC_{Olsenella} = 1.000$, $AUC_{Enterorhabdus} = 0.812$, $AUC_{Bacteroides} = 0.812$, $AUC_{Duncaniella} = 1.000$, $AUC_{Muribaculum} = 0.922$, $AUC_{Paramuribaculum} = 0.953$, $AUC_{Ileibacterium} = 0.891$, $AUC_{Turicibacter} = 0.922$, $AUC_{HT002} = 0.883$, $AUC_{Lactobacillus} = 0.979$, $AUC_{Ruminococcaceae} = 0.984$, $AUC_{Ileibacterium} = 0.891$) (Figure 5A). In addition, the functional prediction of the diagnostic strains was assessed, and the results showed that 121 pathways were significantly different between the two groups. Among these groups, methylerythritol phosphate pathway, glycogen degradation, l-ornithine, l-valine, l-tryptophan, and l-isoleucine biosynthesis pathways, among others, were significantly highly expressed in the NAFLD groups ($p < 0.05$) (Figure 5B).

3.6 The function of 2,497 differential metabolites mainly enriched in tryptophan, linoleic acid and methylhistidine metabolism pathways

The constructed OPLS-DA model revealed distinct differences in metabolite composition between the two groups ($Q2 = 0.837$, $R2Y = 0.999$) (Figure 6A). A total of 2,497 differentially regulated metabolites, including 947 down-regulated and 1,551 up-regulated metabolites, were identified in the NAFLD group, meeting the criteria of VIP > 1, FC ≥ 1, and $p < 0.05$ (Figures 6B,C). In addition, these differentially regulated metabolites were predominantly enriched in pathways related to tryptophan, alpha-linolenic acid, linoleic acid and methylhistidine metabolism (Figure 6D).

3.7 The functions of 2,510 DEGs are associated with the occurrence of diseases

A total of 2,510 DEGs (1,531 down-regulated and 979 up-regulated) were screened in the NAFLD groups compared with the HC groups (Figures 7A,B). In addition, these DEGs exhibited significant enrichment in 92 GO, categories, notably involving processes such as small molecule catabolic, processes and the regulation of small GTPase mediated signal transduction (Figure 7C). Furthermore, the KEGG results revealed associations of these DEGs with pathways related to cholesterol metabolism, amino acid biosynthesis, and primary bile acid biosynthesis, among others (Figure 7D).

3.8 Correlation analysis among the genes, microbiota and metabolites

As shown in Figure 8A, characteristic strains of the HC groups—*Bacteroides, HT002* and *Lactobacillus* demonstrate strong negative correlations with docosahexaenoic acid, n-isobutyrylglycine and taurine. Conversely, the key microbiota of the NAFLD groups exhibit strong positive correlations with cholanoic acid, 5-methyltetrahydrofolic acid etc. The correlation between the key metabolites 2 and genes is shown in Figure 8B. Shisa8 and Lrrc8e display strong positive correlations with cis-4-hydroxyequol, whereas Gm38220 and Gm42977 exhibit strong negative correlations with Glu-Ile. Moreover, the constructed network involving 8 key strains, 14 key genes and 83 key metabolites elucidates potential regulatory interactions. Notably, Mup-ps16 has been indicated to potentially regulate the metabolism of 3,7-dihydroxy-12-oxocholanoic acid through interactions with *Duncaniella* and *Olsenella*. Additionally, Proser2 shows potential regulatory capabilities across the metabolism influenced by most diagnostic microbiota (Figure 8C and Supplementary Table 5).

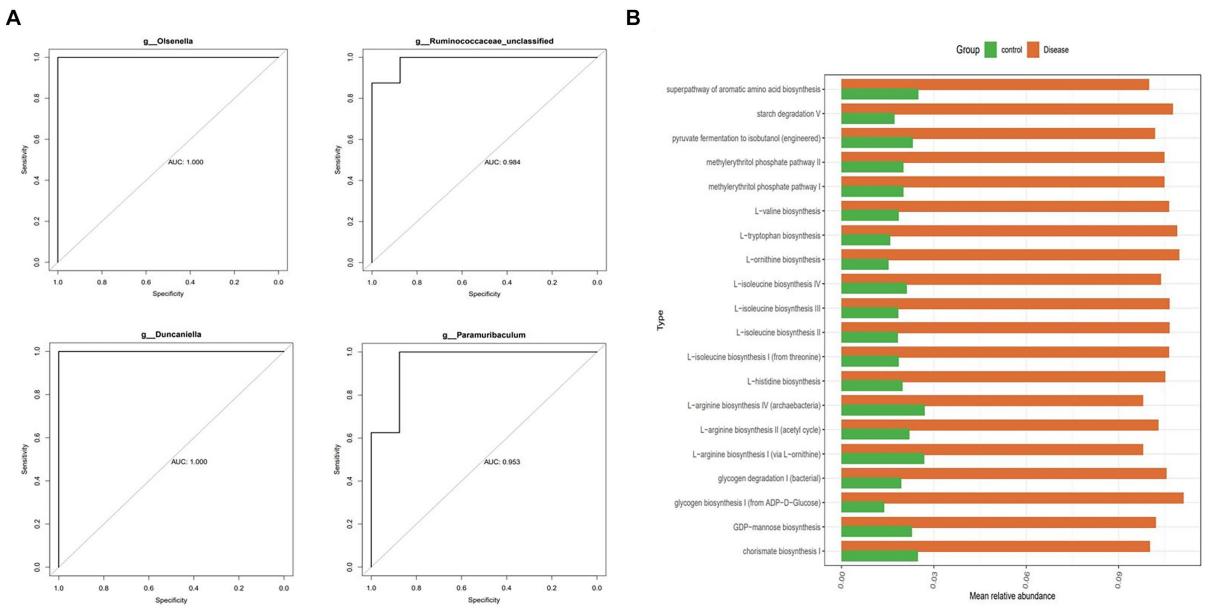

FIGURE 5
The 13 diagnostic strains related to liver protection and their function prediction analysis. **(A)** The ROC curve characteristic pressure calculation and 4/13 diagnostic strains produced AUC values >0.8. **(B)** Functional prediction analysis of diagnostic strains.

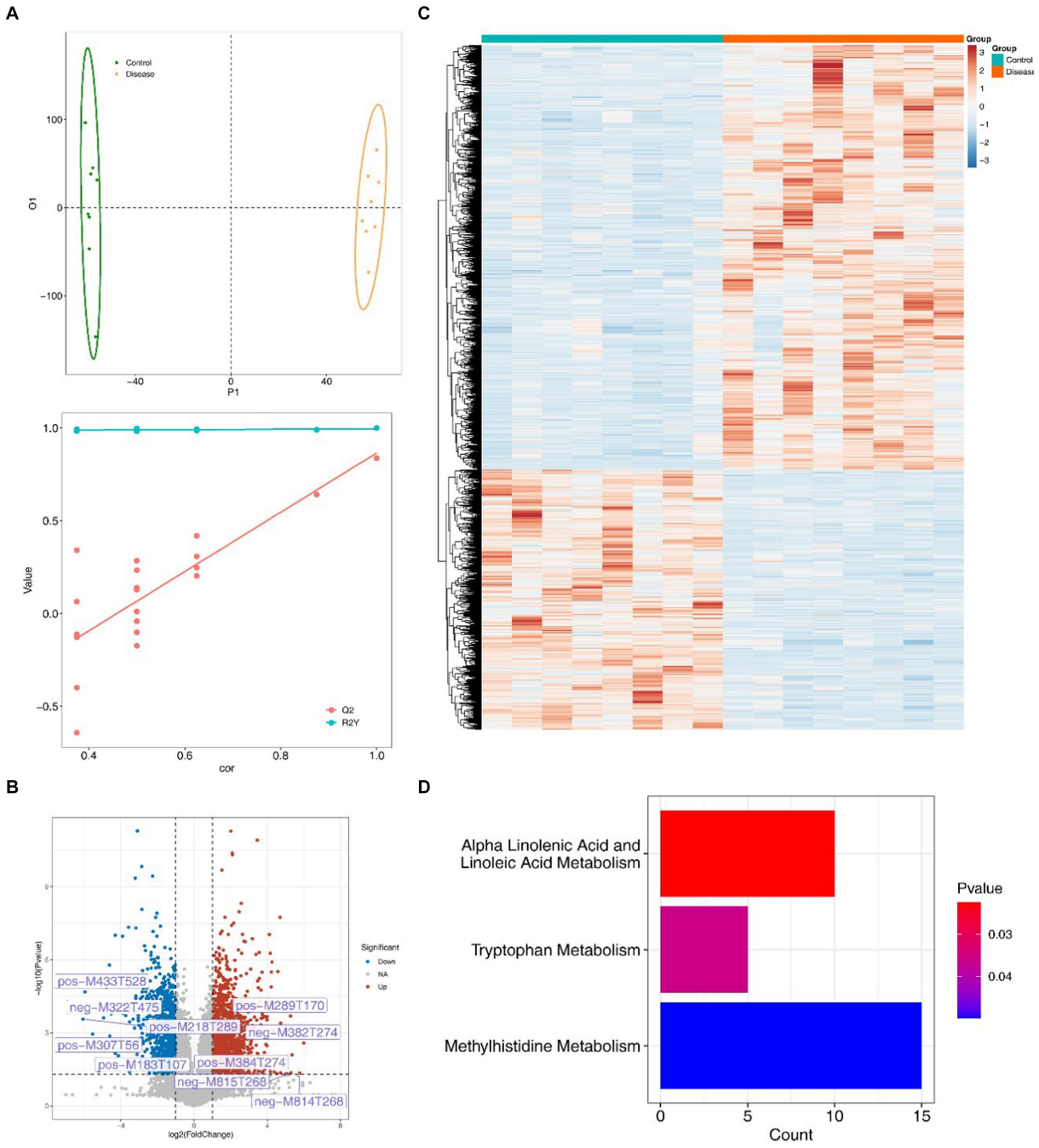

FIGURE 6
2,497 different metabolites and their functional enrichment metabolic pathways. **(A)** The OPLS-DA model shows differences in the metabolite composition between the two groups. **(B)** The volcanic map of different metabolites in the NAFLD group with VIP > 1, FC ≥ 1, $p < 0.05$. **(C)** Heat map of differential metabolite expression in the NAFLD group. **(D)** Differential metabolite enrichment pathway.

3.9 Verification of the expression of differential genes using RT-PCR

Based on the results of multi-omics joint analyses, we further verified the expression of differential genes using RT-PCR. The results showed that compared with that in the control group, the expression of Slc22a7 in the model group was significantly increased, whereas the expressions of Hsd3b5, Zfp334, Ace2, Dbp and Proser2 were significantly decreased. Cyp2b9 and Ccr7 showed no significant differences. In addition to Dbp, Hsd3b5, Cyp2b9 and Ccr7, Slc22a7, Zfp334, Ace2 and Proser2 expressions were consistent with the results of multi-omics analysis (Figure 9).

4 Discussion

With the advancement in people's living standards, NAFLD has emerged as the most prevalent liver disease globally. An increasing

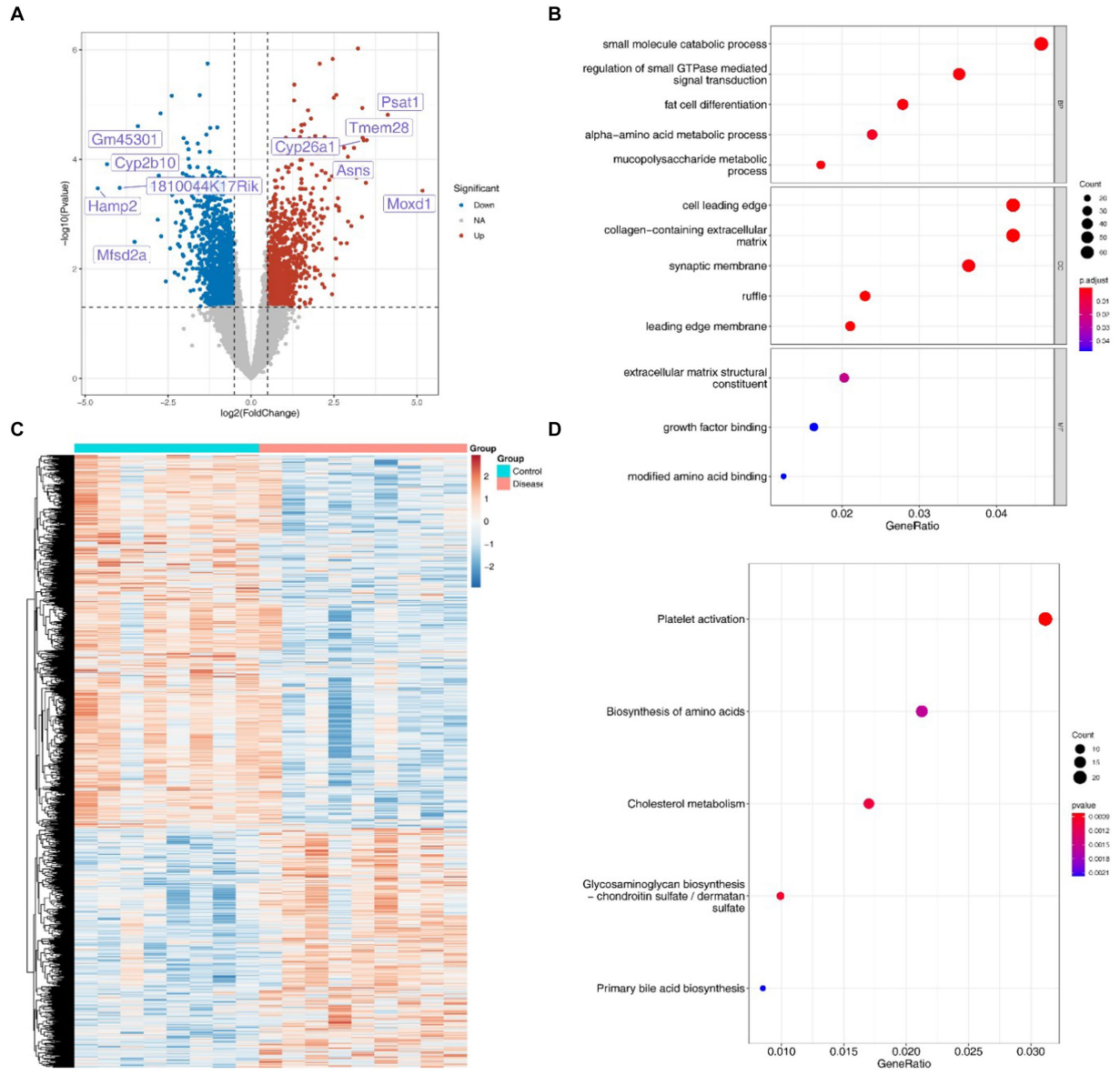

FIGURE 7
Differentially expressed genes and their enrichment analysis. **(A)** Volcanic map of 2,510 DEGs associated with disease occurrence. **(B)** Heat maps of 2,510 DEGs associated with disease occurrence. **(C)** GO enrichment analysis of DEGs. **(D)** KEGG enrichment analysis of DEGs.

body of research has established connections between imbalances in gut flora and a spectrum of host diseases, such as fatty liver disease, metabolic syndrome, inflammatory bowel disease, and colon cancer. The direct linkage between the intestine and liver through the portal vein, enables the intestinal microflora to remotely influence liver health (gut–intestinal microbiota – liver axis). Consequently, disruptions in intestinal microflora can alter the biological metabolism beyond the liver, potentially contributing to the onset and development of NAFLD. However, comprehensive joint analyses integrating transcriptomic, microbiomic and metabolomic data in NAFLD remain unreported.

In this study, we observed significant increases in the abundances of *Bacteroides*, *Muribaculaceae, Allobaculum, Paramuribaculum* and *Ruminococcaceae*, alongside notable decreases in the abundances of *Ligilactobacillus, Lactobacillus* and *Enterococcus faecalis HT002* within the NAFLD group. Specifically, *Ileibacterium valens, Ruminococcaceae, Duncaniella frettoniella, Paramuribaculum, Coriobacteriaceae, Marmorella UCG_002* and *Olsenella* were identified as characteristic strains associated with NAFLD. Previous studies have highlighted the negative association of the *Ruminococcaceae* family with hepatic markers, including liver weight, serum transaminase levels and the degree of hepatic steatosis and inflammation (Milton-Laskibar et al., 2022). Moreover, our findings align with previous reports indicating increased abundances of *Blautia, Romboutsia, Faecalibaculum* and *Ileibacterium* alongside decreased levels of *Allobaculum* and *Enterorhabdus* in NAFLD mice (Gu et al., 2022). Our results are consistent with those of previous literature reports. The relationship between the other five strains and NAFLD has not been reported in detail. However, Fang et al. (2022) found that *Olsenella* is negatively correlated with lower levels of free fatty acids, a pivotal factor in alcoholic liver disease- development. Additionally, Zhou et al. (2022) investigated the intestinal microecology in hepatocellular carcinoma

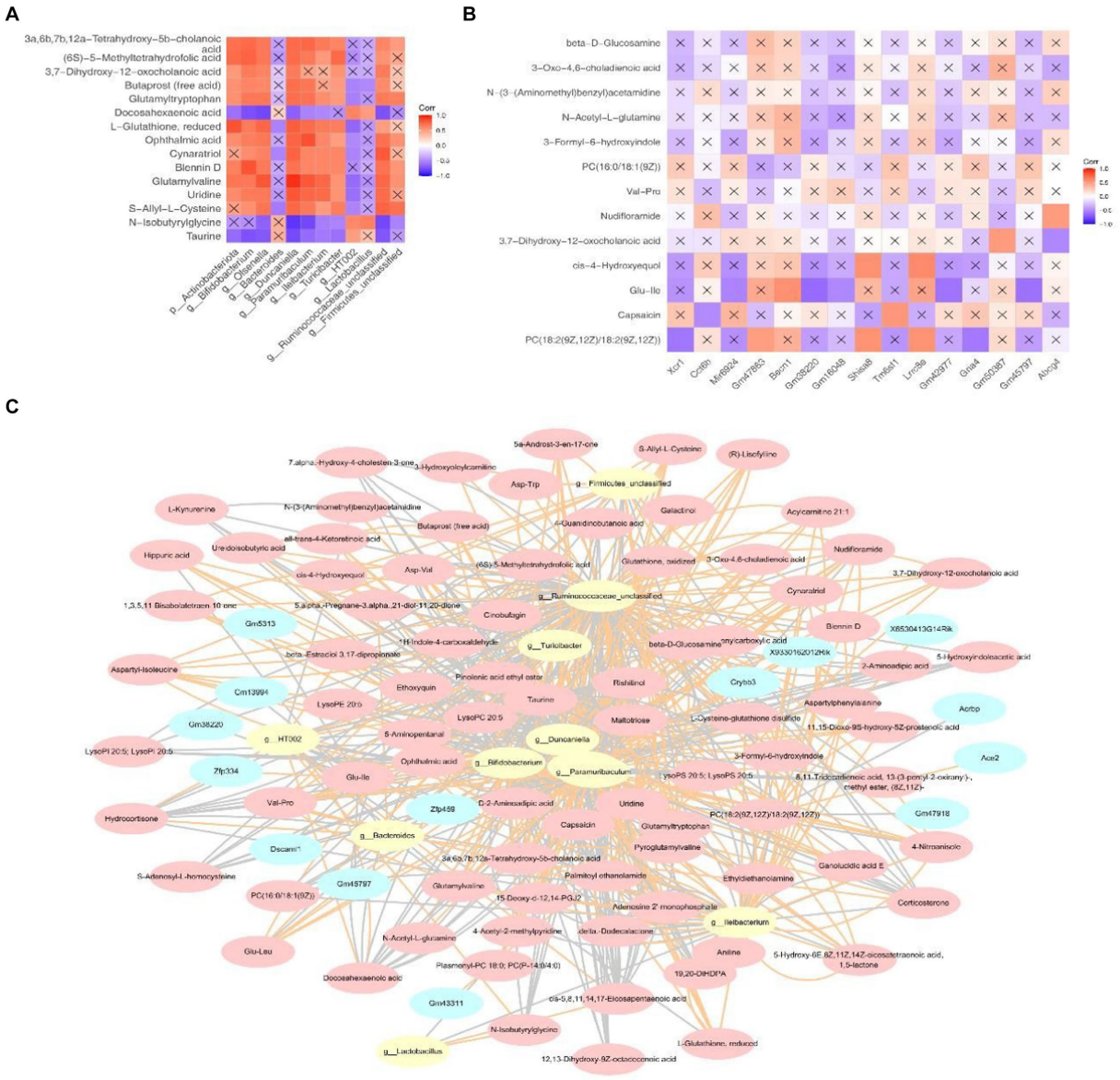

FIGURE 8
The correlation between the key metabolites, microbiota and DEGs and the collinear network diagram. **(A)** Correlation between the key metabolites and microbiota. **(B)** Correlations between some differential metabolites and DEGs. In Figures, the "X" indicates non-significant ($p > 0.05$). **(C)** Collinear network diagram of the genes –microbiome –metabolites.

model mice induced by diethylnitrosamine, emphasizing *Coriobacteriaceae* as a dominant bacterial group at the genus level. This further suggests the potential importance of *Olsenella* and *Coriobacteriaceae* in liver diseases.

Jiang et al. (2023) discovered that free fatty acid receptor4 (FFAR4) deficiency hindered the protective effects of high endogenous n-3 polyunsaturated fatty acids on intestinal barrier dysfunction and hepatic steatosis. Moreover, FFAR4 deficiency decreased gut microbiota diversity and increased the prevalence of *Rikenella*, *Anaerotruncus*, and *Enterococcus*, while reducing that of *Dubosiella*, *Ruminococcaceae UCG-010*, *Ruminococcaceae UCG-014*, *Coriobacteriaceae UCG-002*, *Faecalibaculum*, *Ruminococcaceae UCG-009*, and *Akkermansia*. Alterations in the abundances of these specific bacterial genera led to changes in the overall production of free fatty acids and their interaction manner with FFAR4, ultimately diminishing the protective mechanisms against hepatic steatosis. These findings underscore the potential significance of *Ruminococcaceae* and *Coriobacteriaceae* in liver diseases.

We observed significantly higher expression levels of the methylerythritol phosphate, glycogen degradation, l-ornithine, l-valine, l-tryptophan, and l-isoleucine biosynthesis pathways in the NAFLD group. Isoprenoids were associated with the methylerythritol phosphate pathway. Glycogen degradation was found to be correlated with liver metabolism, processes, contributing to the onset and development of NAFLD. L-ornithine and L-aspartic acid (LOLA) act as effective amino-decreasing agents in hepatic encephalopathy (Teunis et al., 2022). LOLA facilitates ammonia removal through urea synthesis and glutamine production via the action of glutamine synthetase. Plausible mechanisms of LOLA in NAFLD involve enhanced ammonia removal, increased antioxidative capacity, and

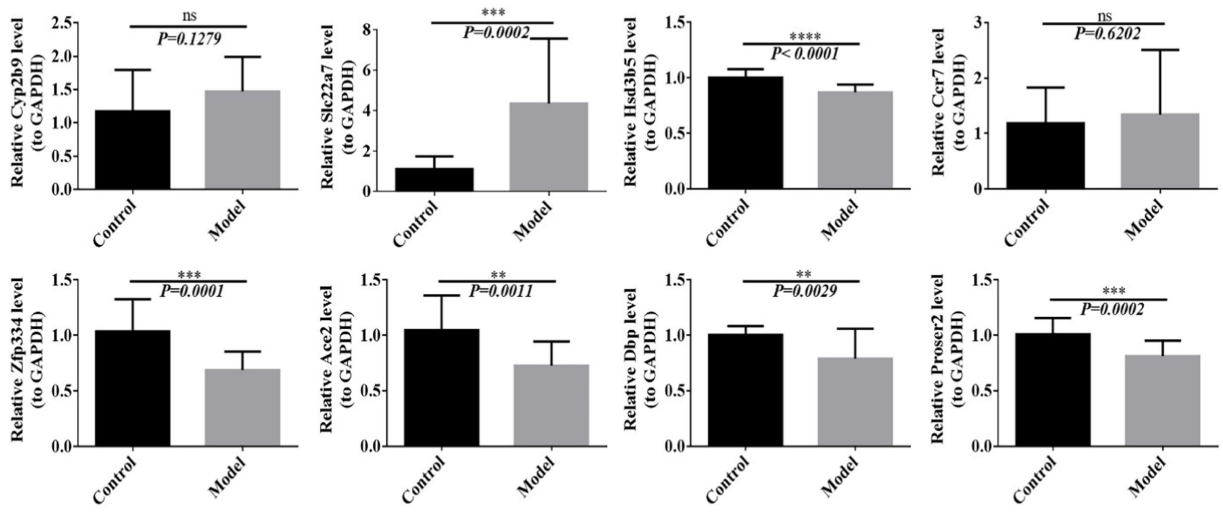

FIGURE 9
The expression of differential genes was detected by RT-PCR. **p<0.01, ***p<0.001, ****p<0.0001.

reduced lipid peroxidation via the action of glutamine and glutathione. Additionally, LOLA improves hepatic microcirculation by producing l-arginine-derived NO (Canbay and Sowa, 2019).

Furthermore, we identified key metabolites significantly enriched in tryptophan metabolism, alpha-linolenic acid linoleic acid metabolism and methylhistidine pathways. Notably, NAFLD, a known risk factor for cirrhosis, is linked with metabolic conditions such as obesity, type 2 diabetes, dyslipidaemia, and atherosclerosis, conditions potentially mitigated by tryptophan metabolism. The canine pathway, a significant route in tryptophan metabolism is modulated by indoleamine 2, 3-dioxygenase, whose increased activity is positively associated with inflammation and fibrosis in NAFLD (Teunis et al., 2022). In addition, substituting linoleic acid or long-chain n-3 polyunsaturated fatty acids has shown potential in preventing the onset of NAFLD associated with a western diet (Musso et al., 2010; Yuan et al., 2016).

The key microflora associated with NAFLD, such as *Muribaculaceae, Allobaculum, Dubosiella, Ligilactobacillus, Ruminococcaceae, Paramuribaculum*, and *Bifidobacterium*, exhibited strong positive correlations with cholinic acid, 5-methyltetrahydrofolic acid and other substances. A study has indicated that primary bile acids and deoxycholic acids tend to accumulate in mice on a choline-deficient diet. Moreover, the introduction of *Aerococcus, Oscillospiraceae, Ruminococcaceae, Bilophila, Muribaculaceae, Helicobacter* and *Alistipes* has been linked to increased liver steatosis, lobular inflammation and fibrogenesis in mice, consequently promoting the progression of NAFLD (Jian et al., 2022). These findings align with our analyses.

In this study, our analysis revealed associations of DEGs with processes such as small molecule catabolism and the regulation of small GTPase-mediated signal transduction. Notably, NAFLD can progress into NASH, where the activation of the inflammasome protein scaffold (NLRP3) plays a pivotal role in NASH-related inflammation. Moreover, our findings indicate that Mup-ps16 could regulate the metabolism of 3,7-dihydroxy-12-oxocholanoic acid through the action of *Duncaniella* and *Olsenella*. Similarly, Proser2 appears to regulate metabolism across most diagnostic microbiota. Mouse major urinary proteins, primarily expressed in the liver, and circulating major urinary proteins regulate metabolism by suppressing hepatic gluconeogenesis and lipid metabolism (Charkoftaki et al., 2019). In line with our observations, Xiang et al. (2021) suggested that *Olsenella* and *Slackia* genera potentially contribute positively to fat and energy metabolism, correlating with increased chicken abdominal fat deposition. Based on these findings, we hypothesize that Mup-ps16 and *Duncaniella* may regulate the production of 3, 7-dihydroxy-12-oxocholanoic acid, impacting energy metabolism by inhibiting liver gluconeogenesis and lipid metabolism, potentially affecting the progression of non-alcoholic fatty liver.

In summary, *Ileibacterium valens, Ruminococcaceae, Olsenella, Duncaniella Frettoniella, Paramuribaculum, Coriobacteriaceae*, and *Marmorella UCG_002* were identified as the characteristic strains associated with NAFLD. Additionally, significant upregulation of pathways such as the methylerythritol phosphate, glycogen degradation, l-ornithine, l-valine, l-tryptophan, and l-isoleucine biosynthesis pathways was observed in the NAFLD group.

Furthermore, our findings suggest that Mup-ps16 might influence the metabolism of 3,7-dihydroxy-12-oxocholanoic acid through the action of *Duncaniella* and *Olsenella*. Similarly, Proser2 seems to regulate metabolism across most diagnostic microbiota. Based on these observations, we hypothesize that Mup-ps16 and *Duncaniella* could potentially regulate the production of 3, 7-dihydroxy-12-oxocholanoic acid, influencing energy metabolism by inhibiting liver gluconeogenesis and lipid metabolism, thereby affecting the progression of non-alcoholic fatty liver.

In the future, we will focus on conducting an in-depth study of the combined mechanisms of gene–strain metabolic processes in NAFLD.

Data availability statement

The datasets generated/analyzed for this study can be found in the SRA database https://www.ncbi.nlm.nih.gov/sra/PRJNA1068522 after publication/on 2026-03-01.

Ethics statement

The studies involving humans were approved by the Ethics Committee of Project Application of the First Affiliated Hospital of

Kunming Medical University (July 4th, 2023). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements. The animal study was approved by the Ethics Committee of Kunming Medical University (kmmu20231209). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JL: Conceptualization, Data curation, Formal analysis, Writing – original draft, Project administration, Software, Writing – review & editing. RZ: Conceptualization, Methodology, Validation, Writing – original draft. HL: Investigation, Supervision, Writing – review & editing. YZ: Formal analysis, Funding acquisition, Methodology, Writing – original draft. ND: Writing – original draft, Investigation, Resources. QQ: Writing – review & editing, Formal analysis, Resources. HB: Writing – review & editing, Project administration, Supervision, Visualization. LZ: Writing – review & editing, Project administration, Supervision, Validation. OL: Writing – original draft, Methodology, Software, Visualization. LS: Writing – original draft, Formal analysis, Software. MM: Writing – original draft, Investigation, Visualization. JY: Funding acquisition, Project administration, Writing – review & editing, Supervision.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1379064/full#supplementary-material

References

Abdelhameed, F., Kite, C., Lagojda, L., Dallaway, A., Chatha, K. K., Chaggar, S. S., et al. (2024). Non-invasive scores and serum biomarkers for fatty liver in the era of metabolic dysfunction-associated Steatotic liver disease (Masld): a comprehensive review from Nafld to Mafld and Masld. *Curr. Obes. Rep.* doi: 10.1007/s13679-024-00574-z

Álvarez-Mercado, A. I., Navarro-Oliveros, M., Robles-Sánchez, C., Plaza-Díaz, J., Sáez-Lara, M. J., Muñoz-Quezada, S., et al. (2019). Microbial population changes and their relationship with human health and disease. *Microorganisms* 7:68. doi: 10.3390/microorganisms7030068

Canbay, A., and Sowa, J. P. (2019). L-ornithine L-aspartate (Lola) as a novel approach for therapy of non-alcoholic fatty liver disease. *Drugs* 79, 39–44. doi: 10.1007/s40265-018-1020-5

Charkoftaki, G., Wang, Y., McAndrews, M., Bruford, E. A., Thompson, D. C., Vasiliou, V., et al. (2019). Update on the human and mouse lipocalin (Lcn) gene family, including evidence the mouse Mup cluster is result of an "evolutionary bloom". *Hum. Genomics* 13:11. doi: 10.1186/s40246-019-0191-9

Chong, J., Wishart, D. S., and Xia, J. (2019). Using MetaboAnalyst 4.0 for comprehensive and integrative metabolomics data analysis. *Curr. Protoc. Bioinformatics* 68:e86. doi: 10.1002/cpbi.86

de la Cuesta-Zuluaga, J., Corrales-Agudelo, V., Velásquez-Mejía, E. P., Carmona, J. A., Abad, J. M., and Escobar, J. S. (2018). Gut microbiota is associated with obesity and cardiometabolic disease in a population in the midst of westernization. *Sci. Rep.* 8:11356. doi: 10.1038/s41598-018-29687-x

Douglas, G. M., Maffei, V. J., Zaneveld, J. R., Yurgel, S. N., Brown, J. R., Taylor, C. M., et al. (2020). Picrust2 for prediction of metagenome functions. *Nat. Biotechnol.* 38, 685–688. doi: 10.1038/s41587-020-0548-6

Draiko, C. V., Yamarat, K., Panza, A., and Draleru, J. (2019). Knowledge, skills and competency retention among health workers one year after completing helping babies breathe training in South Sudan. *Pan Afr. Med. J.* 33:175. doi: 10.11604/pamj.2019.33.175.17560

Fan, Y., and Pedersen, O. (2021). Gut microbiota in human metabolic health and disease. *Nat. Rev. Microbiol.* 19, 55–71. doi: 10.1038/s41579-020-0433-9

Fang, C., Zhou, Q., Liu, Q., Jia, W., and Xu, Y. (2022). Crosstalk between gut microbiota and host lipid metabolism in a mouse model of alcoholic liver injury by chronic baijiu or ethanol feeding. *R. Soc. Chem. Food Funct.* 13, 596–608. doi: 10.1039/D1FO02892H

Gu, C., Zhou, Z., Yu, Z., He, M., He, L., Luo, Z., et al. (2022). The microbiota and it's correlation with metabolites in the gut of mice with nonalcoholic fatty liver disease. *Front. Cell. Infect. Microbiol.* 12:870785. doi: 10.3389/fcimb.2022.870785

Jian, J., Nie, M. T., Xiang, B., Qian, H., Yin, C., Zhang, X., et al. (2022). Rifaximin ameliorates non-alcoholic steatohepatitis in mice through regulating gut microbiome-related bile acids. *Front. Pharmacol.* 13:841132. doi: 10.3389/fphar.2022.841132

Jiang, X., Yang, Q., Qu, H., Chen, Y., and Zhu, S. (2023). Endogenous n-3 Pufas improve non-alcoholic fatty liver disease through Ffar4-mediated gut–liver crosstalk. *Nutrients* 15:586. doi: 10.3390/nu15030586

Li, C., Pi, G., and Li, F. (2021). The role of intestinal Flora in the regulation of bone homeostasis. *Front. Cell. Infect. Microbiol.* 11:579323. doi: 10.3389/fcimb.2021.579323

Li, F., Ye, J., Shao, C., and Zhong, B. (2021). Compositional alterations of gut microbiota in nonalcoholic fatty liver disease patients: a systematic review and meta-analysis. *Lipids Health Dis.* 20:22. doi: 10.1186/s12944-021-01440-w

Loomba, R., Seguritan, V., Li, W., Long, T., Klitgord, N., Bhatt, A., et al. (2017). Gut microbiome-based metagenomic signature for non-invasive detection of advanced fibrosis in human nonalcoholic fatty liver disease. *Cell Metab.* 25, 1054–62.e5. doi: 10.1016/j.cmet.2017.04.001

Marshall, J. C. (1998). The gut as a potential trigger of exercise -induced inflammatory responses. *Can. J. Physiol. Pharmacol.* 76, 479–484. doi: 10.1139/y98-049

Milton-Laskibar, I., Cuevas-Sierra, A., Portillo, M. P., and Martínez, J. A. (2022). Effects of resveratrol Administration in Liver Injury Prevention as induced by an obesogenic diet: role of ruminococcaceae. *Biomedicines* 10:1797. doi: 10.3390/biomedicines10081797

Mohakud, S., Ray, B., Naik, S., and Deep, N. (2019). Isolated sacral vertebral hydatid cyst presenting with pathological fracture. *J. Neurosci. Rural Pract.* 10, 565–566. doi: 10.1055/s-0039-1695700

Musso, G., Gambino, R., Cassader, M., and Pagano, G. (2010). A meta-analysis of randomized trials for the treatment of nonalcoholic fatty liver disease. *Hepatology* 52, 79–104. doi: 10.1002/hep.23623

Powell, E. E., Wong, V. W., and Rinella, M. (2021). Non-alcoholic fatty liver disease. *Lancet* 397, 2212–2224. doi: 10.1016/S0140-6736(20)32511-3

Ren, Z., Fan, Y., Li, A., Shen, Q., Wu, J., Ren, L., et al. (2020). Alterations of the human gut microbiome in chronic kidney disease. *Adv. Sci.* 7:2001936. doi: 10.1002/advs.202001936

Robin, X., Turck, N., Hainard, A., Tiberti, N., Lisacek, F., Sanchez, J. C., et al. (2011). Proc: an open-source package for R and S+ to analyze and compare roc curves. *BMC Bioinformatics* 12:77. doi: 10.1186/1471-2105-12-77

Safari, Z., and Gerard, P. (2019). The links between the gut microbiome and non-alcoholic fatty liver disease (Nafld). *Cell. Mol. Life Sci.* 76, 1541–1558. doi: 10.1007/s00018-019-03011-w

Sanchez-Rodriguez, E., Egea-Zorrilla, A., Plaza-Díaz, J., Aragón-Vela, J., Muñoz-Quezada, S., Tercedor-Sánchez, L., et al. (2020). The gut microbiota and its implication in the development of atherosclerosis and related cardiovascular diseases. *Nutrients* 12:605. doi: 10.3390/nu12030605

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., et al. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* 13, 2498–2504. doi: 10.1101/gr.1239303

Singh, R. K., Chang, H. W., Yan, D., Lee, K. M., Ucmak, D., Wong, K., et al. (2017). Influence of diet on the gut microbiome and implications for human health. *J. Transl. Med.* 15:73. doi: 10.1186/s12967-017-1175-y

Teunis, C., Nieuwdorp, M., and Hanssen, N. (2022). Interactions between tryptophan metabolism, the gut microbiome and the immune system as potential drivers of non-alcoholic fatty liver disease (Nafld) and metabolic diseases. *Meta* 12:514. doi: 10.3390/metabo12060514

Wu, T., Hu, E., Xu, S., Chen, M., Guo, P., Dai, Z., et al. (2021). clusterProfiler 4.0: a universal enrichment tool for interpreting omics data. *Innovation* 2:100141. doi: 10.1016/j.xinn.2021.100141

Wu, H., Tremaroli, V., Schmidt, C., Lundqvist, A., Olsson, L. M., Krämer, M., et al. (2020). The gut microbiota in prediabetes and diabetes: a population-based cross-sectional study. *Cell Metab.* 32, 379–390.e3. doi: 10.1016/j.cmet.2020.06.011

Xiang, H., Gan, J., Zeng, D., Li, J., Yu, H., Zhao, H., et al. (2021). Specific microbial taxa and functional capacity contribute to chicken abdominal fat deposition. *Front. Microbiol.* 12:643025. doi: 10.3389/fmicb.2021.643025

Yuan, F., Wang, H., Tian, Y., Li, Q., He, L., Li, N., et al. (2016). Fish oil alleviated high-fat diet-induced non-alcoholic fatty liver disease via regulating hepatic lipids metabolism and metaflammation: a transcriptomic study. *Lipids Health Dis.* 15:20. doi: 10.1186/s12944-016-0190-y

Zhou, W., Zheng, Y., Shang, J., Wang, H., Wang, Y., Lu, H., et al. (2022). Intestinal microecology in mice bearing diethylnitrosamine-induced primary hepatocellular carcinoma. *J. Zhejiang Univ.* 51, 438–453. doi: 10.3724/zdxbyxb-2022-0283

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Licorice processing involving functions of Evodiae Fructus on liver inflammation and oxidative stress are associated with intestinal mucosal microbiota

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Background: This study aimed to investigate the effects of licorice processing of different Evodiae Fructus (EF) specifications on liver inflammation and oxidative stress associated with the intestinal mucosal microbiota.

Materials and methods: The 25 Kunming mice were divided into control (MCN), raw small-flowered Evodiae Fructus (MRSEF), raw medium-flowered EF (MRMEF), licorice-processed small-flowered EF (MLSEF), and licorice-processed medium-flowered EF (MLSEF) groups. The EF intervention groups were given different specifications of EF extract solutions by gavage. After 21 days, indices of liver inflammation and oxidative stress and intestinal mucosal microbiota were measured in mice.

Results: Compared with the MCN, malondialdehyde (MDA), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) levels were significantly increased in the MRMEF. Although the trends of oxidative stress and inflammatory indexes in the MLSEF and MLMEF were consistent with those in the raw EF groups, the changes were smaller than those in the raw EF groups. Compared to the raw EF groups, the MLSEF and MLMEF showed closer approximations of metabolic function to the MCN. The abundance of *Corynebacterium* in MRMEF was significantly lower than that in the MCN, and it was not significantly different from the MCN after licorice processing. The probiotic *Candidatus Arthromitus* was enriched in the MLSEF. The probiotic *Lactobacillus* was enriched in the MLMEF. Correlation analysis revealed significant negative correlations between IL-1β, some metabolic functions and Corynebacterium.

Conclusion: The effects of medium-flowered EF on oxidative stress and inflammatory factors in the liver of mice were stronger than those of small-flowered EF. The licorice processing can reduce this difference by modulating the abundance of *Corynebacterium* and intestinal mucosal metabolic function.

KEYWORDS

small-flowered Evodiae Fructus, medium-flowered Evodiae Fructus, specification, licorice processing, liver inflammation, oxidative stress, intestinal mucosal microbiota

Background

Euodiae Fructus (EF) is a medicinal herb with significant therapeutic value. It is derived from the dried nearly ripe fruits of the plants *Euodia rutaecarpa* (Juss.) Benth., *Euodia rutaecarpa* (Juss.) Benth. var. *officinalis* (Dode) Huang, or *Euodia rutaecarpa* (Juss.) Benth. var. *bodinieri* (Dode) Huang (National Pharmacopoeia Committee, 2020). Natural herbal medicines exhibit characteristics such as multiple targets, multiple actions, and multiple levels of effects. In traditional Chinese medicine (TCM), EF is often used for treating gastrointestinal disorders, headaches, oral ulcers, and vomiting (Xiao et al., 2023). Modern pharmacological studies have demonstrated that EF is rich in alkaloids, lignans, and flavonoids, which possess pharmacological properties such as analgesic, anti-tumor, and anti-inflammatory effects (Xia et al., 2023).

Ever since the book "Supplementary Records of Famous Physicians,"classic Chinese medical books such as "Bencaojing Jizhu" have documented that EF has trace amounts of toxicity. Fortunately, throughout its history of medicinal use, it has been discovered that the addition of licorice juice during the processing of EF can inhibit the generation of toxic metabolites, thereby reducing its hepatotoxicity (Ren et al., 2023). For example, Zhang et al. (2018) intervened in mice with 1, 5, and 10 times clinical doses of EF and found that licorice processing significantly reduced the degree of liver pathological damage and the elevated levels of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase, and alkaline phosphatase. This is supported by the findings of Shan et al. (2021) that an overdose of EF altered the morphology of mouse liver, while licorice processing effectively reduced the acute toxicity of aqueous extracts, volatile oils and ethanolic extracts of EF. The liver toxicity of EF might be related to cytochrome P450 (CYP450) (Zhang et al., 2015; Liu et al., 2020; Zhang W. et al., 2021), oxidative stress (Cai et al., 2014), inflammatory response (Liao et al., 2014) and other biochemical reactions. The research on these mechanisms of the preparation of licorice processing EF is insufficient. It is necessary to explore the different functions of EF on liver inflammation and oxidative stress before and after licorice processing, that may help to explain the mechanism of processing attenuated toxin.

In recent years, it has become increasingly clear that the vast microbial community residing in the gastrointestinal tract plays a critical and intricate role in drug absorption and overall liver health (Gratz et al., 2010; Pabst et al., 2023). The intestine is an important site for the metabolism of orally administered drugs in the body, and upon entering the intestine, the drug components are first exposed to the microbiota. Therefore, the intestinal microbiota influences the transformation and absorption of certain drug components even prior to the liver first-pass effect (Džidić-Krivić et al., 2023). The intestinal microbiota play important roles in the dual effects of effectiveness and hepatotoxicity of Chinese medicine. For example, the reactive metabolite genipin dialdehyde intermediate, generated through the gut microbiota-mediated transformation of geniposide, plays a critical role in geniposide-induced liver damage (Li et al., 2019). Another example is the anticancer drug irinotecan, which, after metabolism by bacterial β-glucuronidase, can cause severe diarrhea (Kodawara et al., 2016). In fact, an animal study has shown that the prevention and treatment of colorectal cancer by EF are associated with the modulation of gut microbial metabolites and colonic epithelial signaling pathways (Wang M. X. et al., 2021). This includes promoting the enrichment of bacteria producing short-chain fatty acids and reducing the abundance of pro-inflammatory bacteria (Wang M. X. et al., 2021; Wang et al., 2023). We discuss the effects of processing EF on its efficacy and hepatotoxicity associated with the intestinal mucosal microbiota (Long et al., 2018a,b). This will lead to a better understanding of the traditional process of EF.

In traditional Chinese medicine clinics, Evodiae Fructus is divided into different specifications and grades according to the size of the fruit:small, medium, and large flowers, and the ancients hold that small-flowered EF is of superior quality. It is worth noting that the large- flowered EF is usually fully matured, and the seeds separate from the husk, which is inconsistent with the description of 'nearly mature' in the Chinese Pharmacopoeia (National Pharmacopoeia Committee, 2020; Zhang W. et al., 2021). Therefore, we took raw or licorice-processed small-flowered and medium-flowered specifications of EF to make extract solutions, intervened in mice at a 5-fold clinically equivalent dose, and examined liver inflammation and oxidative stress indicators and intestinal mucosal microbiota. Our results will contribute to the elucidation of the toxicological properties and the detoxification mechanisms of licorice processing on different specifications of EF.

Materials and methods

Drugs and reagents

Processing of licorice: Mix licorice juice with raw EF in a ratio of 3:50, stir well, and stir-fry over low heat until dry (Zhang et al., 2018). EF dry extract: the raw small-flowered (fruit diameter less than 3 mm) and medium-flowered EF (fruit diameter 3 to 5mm), and their licorice-processed were crushed to powder and sieved, weighed equal amounts of powder, respectively, with 12 times the amount of water, refluxed and extracted three times, each time 40 min, combined two filtrates, 50°C water bath concentrated to powder, set aside. Table 1 shows the yield and the amount of raw drug of the four dried extracts of EF, along with the content of limonin and evodiamine in each. The general feed was provided by the laboratory animal center of the Hunan University of Chinese Medicine and produced by Jiangsu Medison Biomedical Co. Ltd. Malondialdehyde (MDA), superoxide dismutase (SOD), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) kits were purchased from Konodi Biotechnology Co., Ltd.

EF extract solution configuration

Referring to the literature (Zhang et al., 2018), the daily dose for mice was calculated using 5 times the pharmacopoeial dosage (25 g/d) of dried EF extract converted into raw drug quantity and body surface area of mice. Different specifications of EF extract powder were weighed and mixed with a certain amount

TABLE 1 Extraction yield of EF dry extract and limonin and evodiamine contents in dry extracts.

Sample	The amount of raw herbs per g of dry extract(g)	Limonin content in dry extract (mg/g)	Evodiamine content in dry extract (mg/g)
Raw small-flowered EF	3.5721	15.76	0.67
Raw medium-flowered EF	2.4040	18.97	0.78
Licorice-processed small-flowered EF	2.0613	8.84	0.43
Licorice-processed medium-flowered EF	2.7686	15.46	0.91

of distilled water to obtain solutions of EF extract with varying specifications, which were prepared and used immediately. The raw small-flowered EF extract solution (0.91 g/kg·d), raw medium-flowered EF extract solution (1.35 g/kg·d), licorice-processed small-flowered EF (1.58 g/kg·d), licorice-processed small-flowered EF extract solution (1.17 g/kg·d).

Animals

Twenty-five SPF-grade male (Zhou et al., 2024) Kunming mice weighing 20 ± 2 g were provided by Hunan Slaccas Jingda Laboratory Animal Co., Ltd., with license number SCXK2019-0004. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Hunan University of Chinese Medicine and approved by the Animal Experimental Ethics Committee of Hunan University of Chinese Medicine (Ethics No: LL2022112302).

Animal grouping and administration

After 3 days of adaptive feeding, 25 mice were randomly divided into the following groups: control (MCN), raw small-flowered EF (MRSEF), raw medium-flowered EF (MRMEF), licorice-processed small-flowered EF (MLSEF), and licorice-processed medium-flowered EF (MLMEF) group. The experimental procedure is shown in Figure 1. The MRSEF, MRMEF, MLSEF, and MLMEF groups were gavaged with extract solutions of raw small-flowered EF, raw medium-flowered EF, licorice-processed small-flowered EF, and licorice-processed medium-flowered EF, respectively. The administration was performed twice a day for 21 consecutive days, 0.35 ml/ time. The MCN group was gavaged with distilled water at the same frequency and amount.

Biochemical index detection

After 21 days of intervention with EF extract solution, euthanasia was performed on all mice through rapid cervical dislocation. The liver tissues weighing 0.1 g were collected and placed in 1.5 ml centrifuge tubes, followed by the addition of 0.9 ml of physiological saline. The mixture was homogenized at 4°C. After centrifugation at 1000 g for 20 min, the supernatant was collected. According to the instructions provided with the assay kit, the levels of MDA, SOD, TNF-α, IL-1β, and IL-6 in the liver tissues were measured (Wu Y. et al., 2022).

Intestinal mucosa collection

The mice were dissected under aseptic conditions, the jejunum to ileum section of the intestine was removed and cut with surgical scissors, rinsed in saline to remove the attached contents, placed on sterile filter paper to remove water, and the intestinal mucosa was scraped on the weighing paper with a slide. The scraped intestinal mucosa was put into a sterile tube, weighed, and stored at −80°C (Li et al., 2023).

DNA extraction and PCR amplification

Total microbial genomic DNA was extracted from each sample tube according to the OMEGA Soil DNA Kit instructions. The quality and concentration of DNA were assessed using 1.0% agarose gel electrophoresis and a NanoDrop® ND-2000 spectrophotometer (Thermo Scientific Inc., USA), respectively (Zhou et al., 2022).

The PCR amplification region is the V3-V4 region of the 16S rRNA gene, with the forward primer 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and the reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The samples were purified using the OMEGA DNA purification column, and checked by 1.8% agarose gel electrophoresis. The PCR products were recovered using the Monarch DNA Recovery Kit. The amplification reaction system and conditions refer to our previously published literature (Liu et al., 2023). Finally, the PCR products were sequenced using the Illumina Novaseq 6000 sequencing platform. Shanghai Personal Biotechnology Co. performed the sequencing work.

Bioinformatics analysis

The raw sequencing data was processed through modifications, trimming, filtering, denoising, merging, and removal of chimeras by the DADA2 method to obtain high-quality sequences (Callahan et al., 2016). The obtained data was analyzed and visualized using QIIME2 (v2019.4) (Caporaso et al., 2010) and R language (v4.2.0). Alpha diversity of the microbial community was assessed using Chao1, Observed species, Shannon, and Simpson indices. Beta diversity of the microbial community was evaluated using non-metric multidimensional scaling (NMDS) analysis. Random forest analysis was employed to rank the importance of the microbial community and identify the key core species in each group. Subsequently, the correlation analysis based on the Spearman correlation coefficient was performed to investigate the relationship between the biochemical indicators in the liver and the core genus.

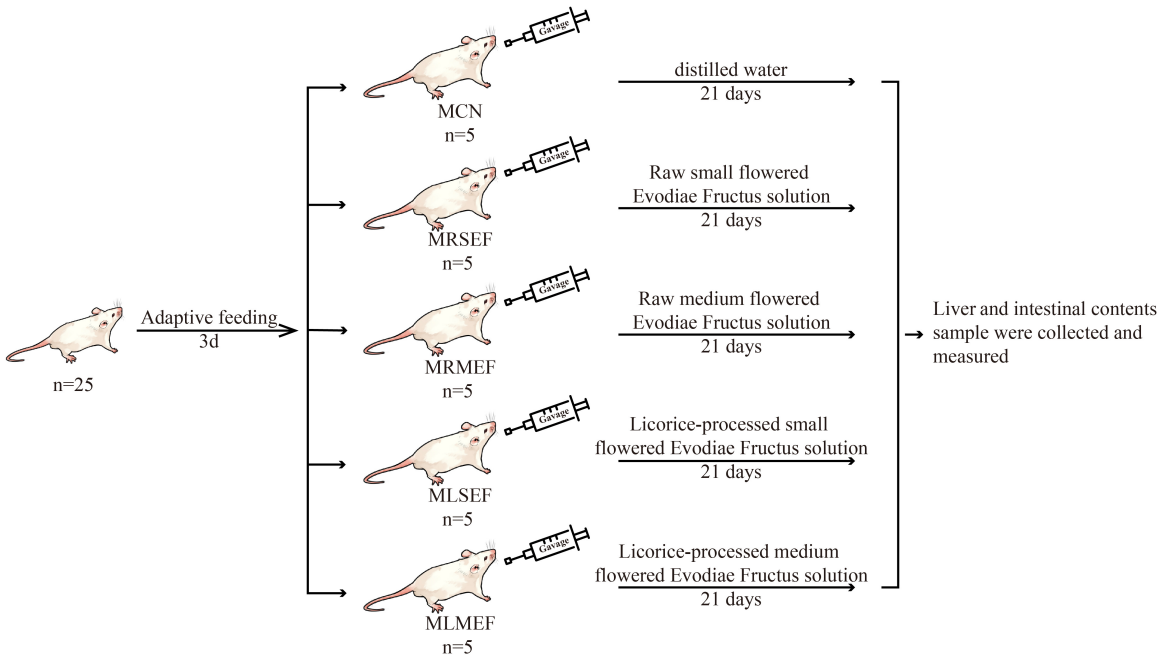

FIGURE 1
Animal experiment process. MCN: Control group; MRSEF: Raw small-flowered EF group; MRMEF: Raw medium-flowered EF group; MLSEF: Licorice-processed small-flowered EF group; MLMEF: Licorice-processed medium-flowered EF group.

Finally, functional analysis of the gut microbiota in mice intestinal mucosa was conducted using the PICRUSt2 software (v2.2.0-b) (Langille et al., 2013), which provided predicted metabolic function information of different bacterial samples.

Statistical analysis

The data were statistically analyzed using SPSS 22.0 software. When the measurement data conformed to normal distribution and met variance homogeneity, one-way ANOVA was used to compare the differences between multiple groups, and the Least Significant Difference (LSD) method was selected for two-way comparison between groups. When the data did not conform to normal distribution or the variance was not uniform, the Kruskal Wallis test with Bonferroni correction was used in the nonparametric test, and $P < 0.05$ indicated that the differences were statistically significant (Yi et al., 2023).

Results

Effect of licorice-processed EF extract solution on liver oxidative stress and inflammatory factors

As shown in Figures 2A, B, the MDA content in the liver of mice increased after the intervention of EF extract solution, with the highest content in the MRMEF group, which was significantly higher than the MCN group ($P < 0.01$), indicating that the oxidation products in the MRMEF group were increased. SOD content was opposite to MDA content, and SOD content in the liver of mice decreased after treatment with EF extract solutions, with greater decreases in the MRSEF and MRMEF groups, with no significant difference. Compared with MCN, the inflammatory factors in the MRSEF, MRMEF, MLSEF and MLMEF groups all showed increasing trends (Figures 2C–E). TNF-α and IL-6 contents were significantly higher in the MRMEF group than that in the MCN group ($P < 0.01$), and IL-1β contents were significantly higher in the MRSEF group than in the MCN and MLMEF groups ($P < 0.01$). In contrast, there was no significant difference between the two groups MLSEF, MLMEF and the MCN group ($P > 0.05$). This indicates that the intervention of raw EF increased the level of oxidative stress and promoted the level of inflammatory factors in the liver of mice. Among them, medium-flower EF caused the most damage to the liver.

Analysis of sequencing quality and number of amplicon sequence variants (ASVs)

Based on the dilution curve (Figure 3A), it is evident that with increasing sequencing depth, the number of detected species in each sample has reached a plateau, indicating that the current samples are adequate for subsequent analysis. Moreover, at various taxonomic levels (Figure 3B), the overall classification count in the control group is consistently lower than that in the four EF extract intervention groups. Using Qiime software, sequences were clustered into ASVs based on a 100% sequence similarity threshold. The Up-Set plot illustrates the shared or unique ASV counts among the groups. As depicted in Figure 3C, the groups'

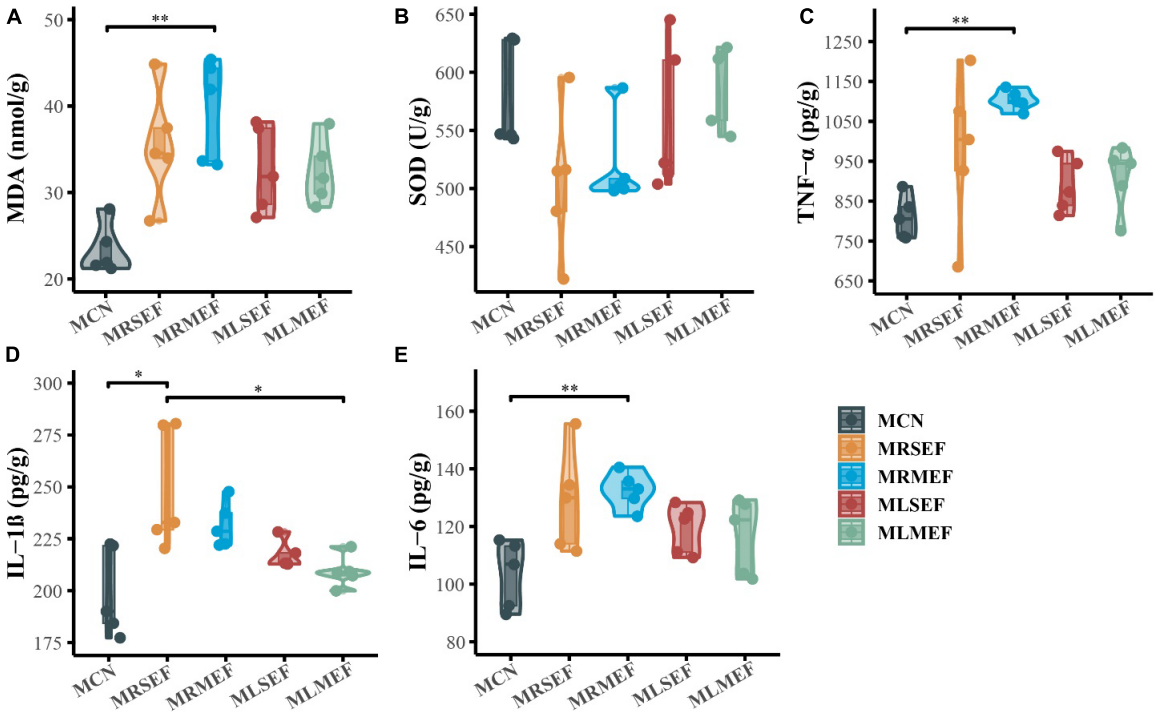

FIGURE 2
Liver oxidative stress and inflammatory factor levels. **(A)** MDA content. **(B)** SOD content. **(C)** TNF-α content. **(D)** IL-1β content. **(E)** IL-6 content. MCN: Control group; MRSEF: Raw small-flowered EF group; MRMEF: Raw medium-flowered EF group; MLSEF: Licorice-processed small-flowered EF group; MLMEF: Licorice-processed medium-flowered EF group. *$P < 0.05$, **$P < 0.01$.

ASV counts are ranked as follows: MRSEF (1436) > MCN (1294) > MRMEF (1128) > MLMEF (828) > MLSEF (761). These findings preliminarily suggest that licorice-processed EF further diminishes species richness in the mouse intestinal mucosa.

Effect of licorice-processed EF extract solution on the diversity of intestinal mucosal microbiota

We evaluated the species richness of different groups using the Chao1 and Observed species indices, and assessed species diversity using the Shannon and Simpson indices. As shown in Figures 3D–G, except for the MRSEF group, the MRMEF, MLSEF, and MLMEF groups exhibited varying degrees of decline in the Chao1, Observed species, Shannon, and Simpson indices compared to the MCN group. Among them, the MLSEF and MLMEF groups showed the largest decrease in intestinal mucosal richness indices, and the MLSEF group had the lowest values for all four diversity indices among the five groups. These results indicate that EF extract reduced species richness and diversity in the mouse intestinal mucosa. Furthermore, licorice processing exacerbated the decrease in intestinal mucosal microbial richness, with a greater impact observed in small-flower EF.

Subsequently, we used NMDS analysis to evaluate the distribution characteristics of different microbial communities. In NMDS analysis, a smaller Stress value is better, and values below 0.2 are generally considered acceptable (Wu Z. et al., 2022). As shown in Figure 3H, the Stress value for this analysis was 0.00879. Compared to the other groups, the distribution areas of MCN overlapped with the MRSEF and MRMEF groups, while the distribution areas of MCN were relatively separated from the MLMEF and MLSEF groups. This indicates that the EF extract solution intervention did not alter the microbial community structure in the intestinal mucosa. However, licorice-processed EF exhibited a certain degree of regulatory effect on the mouse intestinal mucosal microbiota.

Effects of licorice-processed EF extract solution on the taxonomic composition and core genera of the intestinal mucosal microbiota

By ASVs analysis and species annotation, a total of 23 distinct phyla and 326 genera were identified. We selected the top 10 abundant phyla and genera for graphical representations (Figures 4A–D). As shown in Figures 4 A, B, Firmicutes (70.24%) and Bacteroidetes (17.83%) dominated the majority of the samples. Compared to MCN (78.58%), the abundance of Firmicutes and Bacteroidetes increased in the MRSEF (84.39%), MRMEF (92.65%), MLSEF (90.96%), and MLMEF (93.87%) groups. Following intervention with EF extract solution, the dominance of the dominant phyla in the intestinal mucosal microbiota was further expanded. At the genus level (Figures 4C, D), *Candidatus Arthromitus* was the most abundant genus in the intestinal mucosa. Compared with MCN (27.59%), the relative abundance of *Candidatus Arthromitus* increased to different degrees in

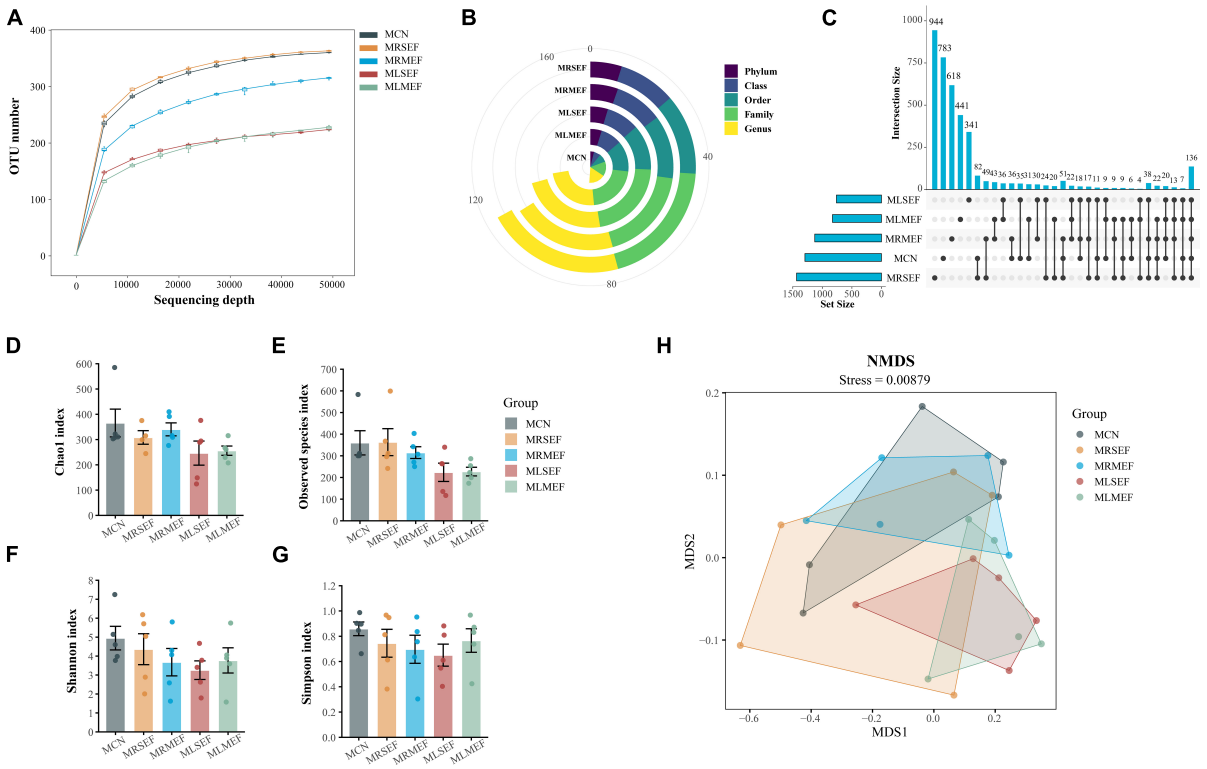

FIGURE 3
Analysis of ASV number and diversity of intestinal mucosal microbiota. **(A)** Dilution curve of Observed species. **(B)** Number of classifications for each level. **(C)** Up-set plot. **(D)** Chao1 index. **(E)** Observed species index. **(F)** Shannon index. **(G)** Simpson index. **(H)** NMDS analysis. MCN: Control group; MRSEF: Raw small-flowered EF group; MRMEF: Raw medium-flowered EF group; MLSEF: Licorice-processed small-flowered EF group; MLMEF: Licorice-processed medium-flowered EF group.

the MRSEF (48.97%), MRMEF (49.17%), MLSEF (67.83%), and MLMEF (31.38%) groups.

In addition, the relative abundance of *Corynebacterium* and *Staphylococcus* decreased in all four groups (Figures 5A, B). Specifically, the relative abundance of *Corynebacterium* in the MRMEF was significantly lower than that in the MCN group ($P < 0.05$), and the relative abundance of *Staphylococcus* in the MRSEF group was significantly lower than that in the MCN group ($P < 0.05$). Although both *Staphylococcus* and *Corynebacterium* also showed a decreasing trend in the MLSEF and MLMEF groups, there was no significant difference in their relative abundance compared to the MCN group ($P > 0.05$). Licorice processing can alleviate the changes of these two genera of bacteria in the intestinal mucosa induced by the administration of EF to a certain extent.

We further screened the top-ranking core bacterial genera in each group by constructing a random forest model. As illustrated in Figures 5C, the core genera in the MCN group included *Corynebacterium*, *Jeotgalicoccus*, and *Psychrobacter*. A genus under the Erysipelotrichaceae, cc_115, contributed more to the MRSEF group, and *Akkermansia* contributed more to the MRMEF group. The core genera of the MLSEF group include *Bifidobacterium*, *Candidatus Arthromitus*, *Shigella*, *Adlercreutzia*, *Lactococcus*, *Clostridium*, *Oscillospira*, and *Blautia*. The core genera of the MLMEF group include *Streptococcus*, *Acinetobacter*, *Lactobacillus*, *Atopostipes*, *Sporosarcina*, *Facklamia*, and *Aerococcus*.

Effect of licorice-processed EF extract solution on intestinal microbiota functionality

Based on the intestinal microbiota data, we conducted a functional analysis of the metabolic pathways involved in the intestinal mucosal microbiota using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. As shown in Figure 6A, metabolism was identified as the predominant primary functional category within the mouse intestinal mucosal microbiota. The primary functional category was further divided into 31 secondary functional categories. Among them, carbohydrate metabolism, amino acid metabolism, metabolism of cofactors and vitamins, metabolism of terpenoids and polyketides, metabolism of other amino acids, and lipid metabolism exhibited the highest abundances. The secondary functional categories were further divided into 182 sub-functions, of which we selected and presented the top 20 abundant functions. These functions may represent the main pathways affected by the administration of EF extract solution within the mouse intestinal mucosal microbiota, including biosynthesis of ansamycins, D-glutamine and D-glutamate metabolism, D-alanine metabolism, secondary bile acid biosynthesis, and biosynthesis of vancomycin group antibiotics.

Based on Figure 6B, compared to the MCN, the MRSEF and MRMEF groups showed increased abundances in 17 out

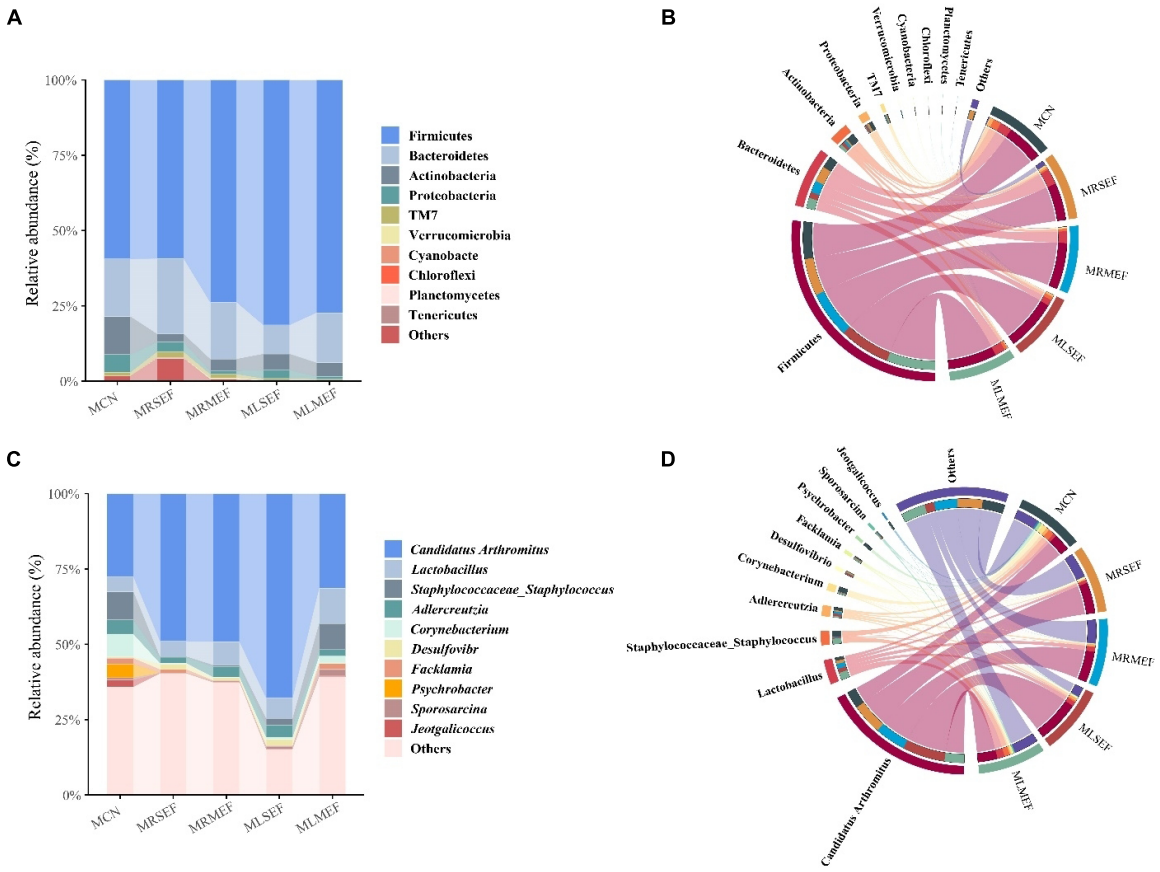

FIGURE 4
Taxonomic composition of intestinal mucosal microbiota. **(A)** Relative abundance at the phylum level. **(B)** Chord diagram of the phylum level. **(C)** Relative abundance at the genus level. **(D)** Chord diagram of the genus level.

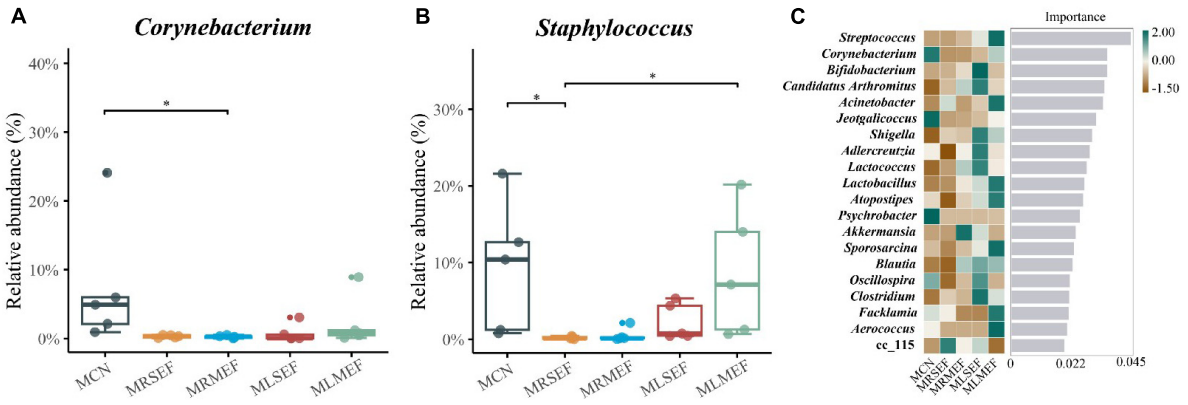

FIGURE 5
The effect of licorice-processed EF extract solution on the core genera of the intestinal mucosal microbiota. **(A)** Differences in the relative abundance of *Corynebacterium*. **(B)** Differences in the relative abundance of *Staphylococcus*. **(C)** Random forest analysis. The heat map of the relative abundance distribution of the genera in each group is shown on the left. MCN: Control group; MRSEF: Raw small-flowered EF group; MRMEF: Raw medium-flowered EF group; MLSEF: Licorice-processed small-flowered EF group; MLMEF: Licorice-processed medium-flowered EF group. *$P < 0.05$.

of 20 level 3 functions, with the exceptions of valine, leucine and isoleucine biosynthesis, pantothenate and CoA biosynthesis, and terpenoid backbone biosynthesis. Notably, the MRMEF group exhibited significantly higher abundance in functions such as peptidoglycan biosynthesis, aminoacyl-tRNA biosynthesis, and ribosome compared to the MCN group ($P < 0.05$). After intervention with licorice-processed EF extract solution, the MLSEF and MLMEF showed similar abundance to the

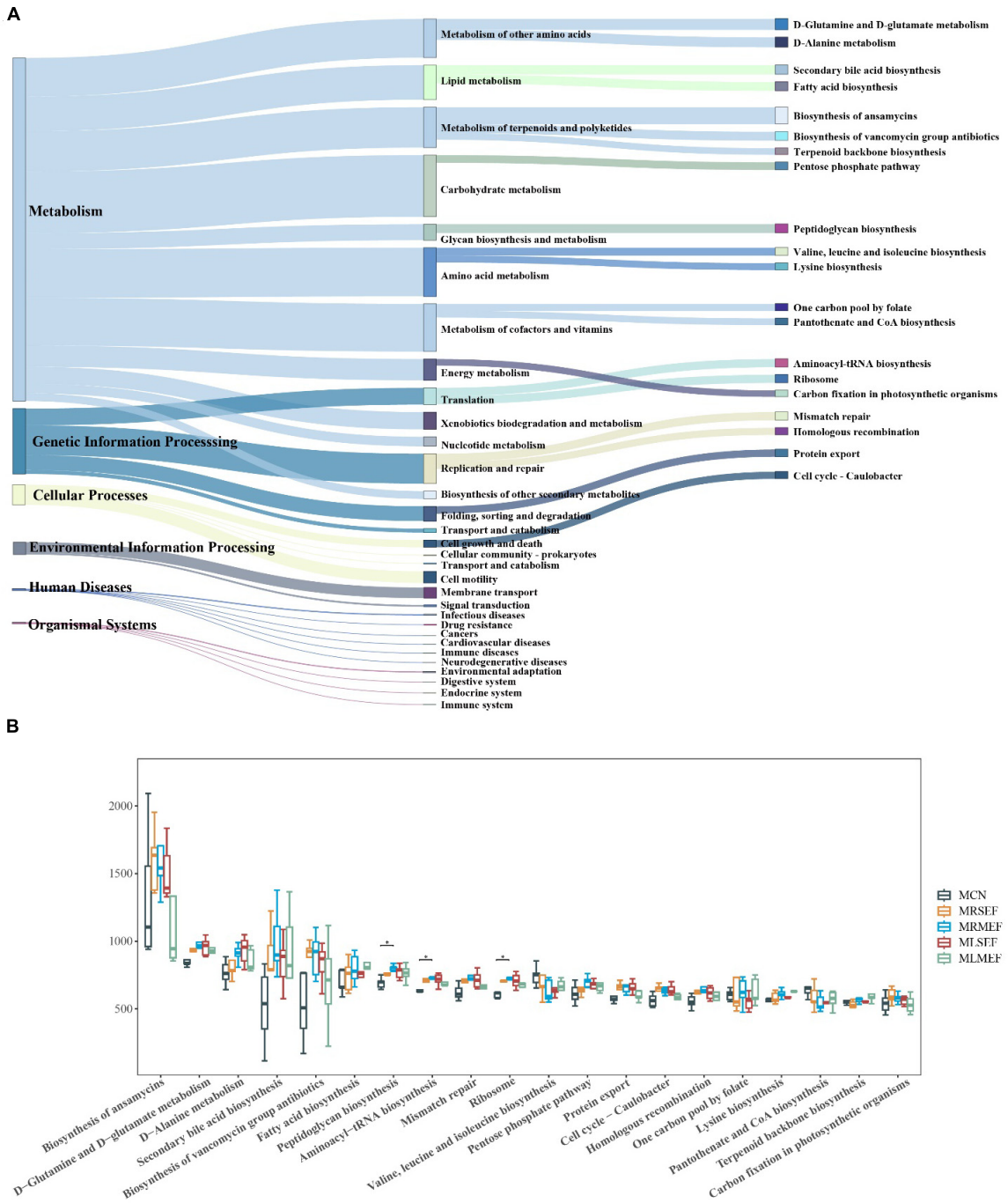

FIGURE 6
The effect of EF extract solution on intestinal microbiota functionality. **(A)**: Sankey diagram of KEGG function prediction. The first column shows the six classes of primary metabolic pathways, the second column shows the secondary metabolic functions, and the third column shows the tertiary metabolic functions in the top twenty in abundance in mouse intestinal mucosa. **(B)** Difference diagram of metabolic functions in the top 20 in abundance. MCN: Control group; MRSEF: Raw small-flowered EF group; MRMEF: Raw medium-flowered EF group; MLSEF: Licorice-processed small-flowered EF group; MLMEF: Licorice-processed medium-flowered EF group. *$P < 0.05$.

MCN group in functions such as biosynthesis of ansamycins, biosynthesis of vancomycin group antibiotics, mismatch repair, ribosome, homologous recombination, and carbon fixation in photosynthetic organisms. Among them, the MLMEF group demonstrated more consistent effects on intestinal mucosal metabolic functions after processing. Compared to the MRMEF, the MLMEF group showed a closer resemblance to the MCN group in the abundance of 14 functions, including biosynthesis of ansamycins, D-glutamine and D-glutamate metabolism, and D-alanine metabolism. This suggests that excessive raw EF extract solution disrupts the metabolic functions of the mouse intestinal mucosa, while processing with licorice can alleviate the impact of raw EF on mouse intestinal mucosal metabolic functions.

Correlation analysis of intestinal mucosal microbiota with liver biochemical indicators and metabolic functions

In order to explore the correlation between intestinal mucosal microbiota and liver biochemical indexes and their functions, we did the correlation analysis between the top 10 genera of random forest contribution and liver biochemical indexes and the top 20 abundant intestinal mucosal flora functions in Figure 6. In Figure 7A, MDA and SOD were positively correlated with *Lactobacillus*, *Streptococcus* and *Acinetobacter*, and TNF-α, IL-1β and IL-6 were positively correlated with *Bifidobacterium*, *Shigella* and *Lactococcus*, and with *Corynebacterium* and *Jeotgalicoccus* were negatively correlated. Among them, IL-1β was significantly negatively correlated with *Corynebacterium* and *Jeotgalicoccus* ($P < 0.05$).

In Figure 7B, *Candidatus Arthromitus* showed significant positive correlations with d-alanine metabolism, fatty acid biosynthesis, mismatch repair, protein export, and cell cycle-caulobacter ($P < 0.05$). It showed significant negative correlations with valine, leucine and isoleucine biosynthesis, one carbon pool by folate, lysine biosynthesis, and pantothenate and CoA biosynthesis ($P < 0.05$). *Acinetobacter* exhibited a significant positve correlation with D-alanine metabolism and fatty acid biosynthesis. *Lactobacillus* demonstrated significant negative correlations with the biosynthesis of ansamycins and fatty acid biosynthesis, but a significant positive correlation with lysine biosynthesis ($P < 0.05$). *Adlercreutzia* showed a significant negative correlation with fatty acid biosynthesis, but significant positive correlations with valine, leucine and isoleucine biosynthesis, one carbon pool by folate, and pantothenate and CoA biosynthesis ($P < 0.05$). It is worth noting that *Corynebacterium* displayed significant negative correlations with several important metabolic functions, including D-glutamine and D-glutamate metabolism, secondary bile acid biosynthesis, and biosynthesis of vancomycin group antibiotics ($P < 0.01$).

Discussion

Oxidative stress is typically caused by an imbalance between reactive oxygen species and the body's antioxidant defense system, and it is one of the most common mechanisms of cell death in organ injuries, including liver damage (Pizzino et al., 2017). Among them, MDA is the final product of lipid oxidation, while SOD is an antioxidant enzyme and one of the body's cellular defense mechanisms against oxidative stress. Therefore, the balance between them can reflect the level of oxidative stress response, which affects liver health. After intervention with raw EF extract solution, SOD levels had no difference before and after administration of EF. MDA level significantly increased in MRMEF, meanwhile, there was no difference in MDA level between MC and MLMEF. It means that the intervention of raw medium-flowered EF caused liver damage in mice, and licorice processing mitigated the damage. Licorice-processed EF relieves the damage degree of the liver by reducing lipid peroxidation. It remains to be further studied that whether licorice-processed EF can cause other changes in the molecular mechanisms of oxidative stress.

TNF-α, IL-1β and IL-6 are all pro-inflammatory cytokines, and they play complex roles in the inflammatory response, that can both help the host defend against infection and promote inflammation. Their increased levels contribute to the development of inflammation, thus mediating liver injury. In a recent report, (Zhang M. et al., 2021) used EF of different doses to intervene in rats and found that licorice processing significantly reduced the levels of TNF-α, IL-1β, and IL-6 in serum by EF. We found a similar pattern that the magnitude of changes in inflammation markers decreased after intervention with licorice-processed EF solution, indicating that licorice processing has a certain alleviating effect on the liver inflammatory damage caused by EF intervention. Interestingly, we also found the differences in the inflammatory response mediated by different sizes of EF. In Figures 2C–E, IL-1β significantly increased in MRSEF, while TNF-α, and IL-6 levels significantly increased in MRMEF. This may be related to the difference in chemical composition in different sizes of EF. And licorice processing narrows down the differences in liver damage caused by different specifications.

Despite the intervention of the raw EF and licorice-processed EF, there was no obvious change in the structure of the microbiota (Figures 3D–G). However different sizes of EF before and after processing caused differences in the core bacterial genera, which may penetrate their inflammatory reactions. Following the intervention of EF extract solution, except for the MLMEF group, the abundance of *Candidatus Arthromitus* in the intestinal mucosa increased. Among them, the MLSEF group exhibited the highest abundance. *Candidatus Arthromitus* is one of the most abundant genera in the intestinal mucosal microbiota. Previous studies have indicated that *Candidatus Arthromitus* potentially plays a role in promoting the growth of probiotics and shaping adult intestinal metabolism (Hedblom et al., 2018; Vallianou et al., 2021). Wu et al. (2020) reported that the processing of licorice can reduce the hepatotoxicity of *Tripterygium wilfordii*, and this mechanism may be associated with the modulation of fatty acid metabolism. In the correlation analysis (Figure 6B), *Candidatus Arthromitus* showed significant positive correlations with metabolic pathways including fatty acid biosynthesis, D-alanine metabolism, and mismatch repair. In addition, probiotics such as *Bifidobacterium* (He et al., 2019), *Adlercreutzia* (Ziętak et al., 2016), *Lactococcus* (Casalta and Montel, 2008), *Oscillospira* (Yang et al., 2021), and *Blautia* (Liu et al., 2021) were enriched in the MLSEF group. This indicated that licorice processing small-flower EF could improve the intestinal microbiota by promoting probiotics such as *Candidatus Arthromitus*, *Bifidobacterium*, and thus regulating fatty acid metabolism.

However, this effect may change with the gradual ripening of EF. In terms of medium-flower EF, the maximum enrichment of *Lactobacillus* genus was observed in the MLMEF group. *Lactobacillus* may be the key genus of licorice for regulating intestinal microbiota according to the previous findings of Wang et al. (2020). The majority of *Lactobacillus* is recognized as probiotics, exhibiting functions such as maintaining intestinal mucosal integrity, modulating immunity, and alleviating gastrointestinal inflammation (Heeney et al., 2018; Ji et al., 2020). Research has suggested that the therapeutic effects of Evodiamine on ulcerative colitis associated with an increase in *Lactobacillus acidophilus* abundance and acetate production (Wang M. X. et al., 2021). Therefore, the alleviation of inflammation in processed

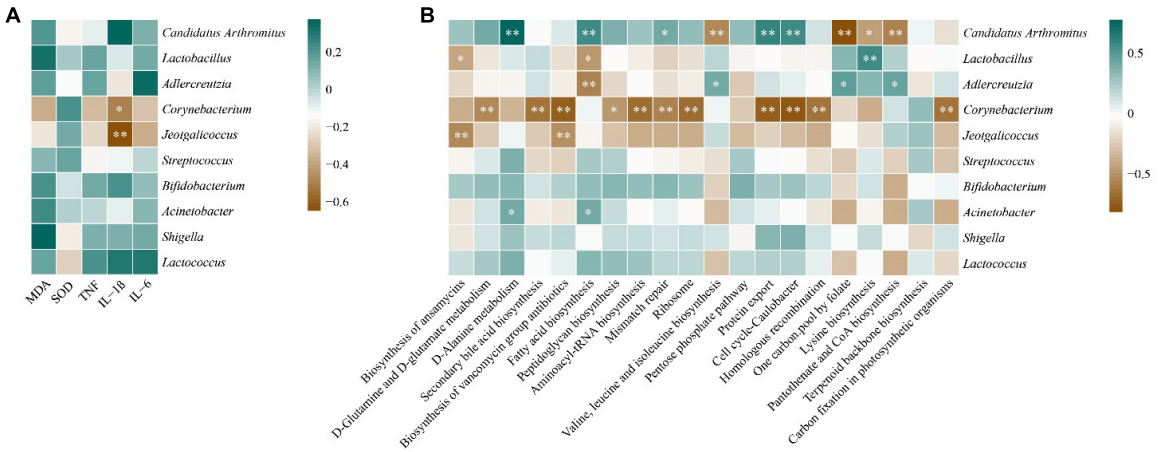

FIGURE 7
Correlation analysis. **(A)**: Heat map of correlation between core genera and liver biochemical indices. **(B)** Heat map of correlation between core genera and metabolic functions. *$P < 0.05$, **$P < 0.01$.

medium-flowered EF may be attributed to the elevation of *Lactobacillus* abundance. Despite the positive correlations between *Candidatus Arthromitus*, *Bifidobacterium*, and *Lactobacillus* with MDA, IL-1β, TNF-α, and IL-6 levels, opportunistic pathogens such as *Shigella*, *Staphylococcus*, and *Acinetobacter* were also enriched in the MLSEF or MLMEF groups (Qi, 2010; Amorim and Nascimento, 2017). Licorice processing only mitigated the liver inflammation of EF, and the presence of these opportunistic pathogens may be associated with the induction of inflammation under excessive intervention of EF. Moreover, compared with the MCN group, *Corynebacterium* and *Jeotgalicoccus* exhibited lower abundance in all four EF solution intervention groups. *Jeotgalicoccus* is a natural producer of terminal olefins, with limited clinical reports (Nusantara Putra et al., 2019). *Corynebacterium* is an opportunistic pathogen commonly found in immunocompromised patients (Sengupta et al., 2015; Kalt et al., 2018). It is significantly and negatively associated with several dominant metabolic functions, including peptidoglycan biosynthesis, aminoacyl-tRNA biosynthesis, and ribosome, which are significantly elevated in the MRMEF group. Furthermore, *Corynebacterium* is negatively correlated with MDA, TNF-α, and IL-6, and significantly negatively correlated with IL-1β. Compared to the intervention group of raw EF, the abundance of *Corynebacterium* is higher in licorice-processed EF. This aligns with the previous mention that licorice may cause moderate increases in inflammatory factors to exert its anti-infective effects (Wang et al., 2020). This suggests that the addition of licorice juice is the key to influencing the intestinal bacteria and pharmaceutical effect of EF. Licorice-processed EF not only reduces the pathogenic bacteria in the intestinal mucosal microbiota and adjusts the balance of the intestinal mucosal microbiota, but also enhances the mice's resistance to infection by regulating the abundance of *Corynebacterium*.

As one of the most commonly used herbal medicines in Asia, the reliability of EF therapeutic efficacy has been tested for centuries (Li and Wang, 2020). Licorice-processed EF is commonly used in clinical preparation, widely approved as less toxic than raw products. Its toxicity is suspected to be size related, which is still far from certain. Therefore, investigating the scientific connotation of processing different specifications of EF with licorice to reduce its toxicity is of significant importance for promoting the use of EF and ensuring patient safety.

Conclusion

Based on the above, excessive intervention with raw EF extract solution resulted in liver oxidative damage and increased levels of inflammatory factors in mice. The effect of medium-flowered EF on oxidative stress and inflammatory factors in the liver of mice was stronger than that of small-flowered EF. Licorice processing can alleviate the liver inflammation caused by different sizes of EF extracts, restore intestinal mucosal function closer to the normal group, and potentially enhance the mice's resistance to infection. This is closely related to the regulation of *Corynebacterium* abundance. Moreover, the licorice processing influenced the modulatory effect of EF on the intestinal microbiota. The licorice-processed small-flowered EF can promote the growth of beneficial bacteria such as *Candidatus Arthromitus* and *Bifidobacterium*, thereby regulating fatty acid metabolism. While licorice-processed medium-flowered EF promotes the growth of the probiotic *Lactobacillus*.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found here: NCBI-PRJNA989095.

Ethics statement

The animal study was approved by the Animal Experimental Ethics Committee of Hunan University of Chinese Medicine. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

XL: Project administration, Supervision, Writing—original draft, Writing—review and editing. QT: Formal analysis, Resources, Writing—review and editing. LC: Data curation, Methodology, Writing—review and editing. YZ: Investigation, Resources, Writing—review and editing. YP: Funding acquisition, Project administration, Validation, Writing—review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Amorim, A. M., and Nascimento, J. D. (2017). Acinetobacter: An underrated foodborne pathogen? *J. Infect. Dev. Ctries.* 11, 111–114. doi: 10.3855/jidc.8418

Cai, Q. Y., Wei, J. J., Zhao, W., Shi, S., Zhang, Y., Wei, R. R., et al. (2014). Toxicity *of Evodiae fructus* on rat liver mitochondria: The role of oxidative stress and mitochondrial permeability transition. *Molecules* 19, 21168–21182. doi: 10.3390/molecules191221168

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J., and Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Casalta, E., and Montel, M. C. (2008). Safety assessment of dairy microorganisms: The *Lactococcus* genus. *Int. J. Food Microbiol.* 126, 271–273. doi: 10.1016/j.ijfoodmicro.2007.08.013

Džidić-Krivić, A., Kusturica, J., Sher, E., Selak, N., Osmančević, N., Karahmet Farhat, E., et al. (2023). Effects of intestinal flora on pharmacokinetics and pharmacodynamics of drugs. *Drug Metab. Rev.* 55, 126–139. doi: 10.1080/03602532.2023.2186313

Gratz, S. W., Mykkanen, H., and El-Nezami, H. S. (2010). Probiotics and gut health: A special focus on liver diseases. *World J. Gastroenterol.* 16, 403–410. doi: 10.3748/wjg.v16.i4.403

He, L., Liu, Y. W., Guo, Y. F., Nenqun Xiao, N. Q., and Tan, Z. J. (2019). Influences of Aflatoxin B1 on main intestinal bacteria communities and enzyme activities in mice. *Toxin Rev.* 38, 121–126. doi: 10.1080/15569543.2018.1426611

Hedblom, G. A., Reiland, H. A., Sylte, M. J., Johnson, T. J., and Baumler, D. J. (2018). Segmented filamentous bacteria - metabolism meets immunity. *Front. Microbiol.* 9:1991. doi: 10.3389/fmicb.2018.01991

Heeney, D. D., Gareau, M. G., and Marco, M. L. (2018). Intestinal *Lactobacillus* in health and disease, a driver or just along for the ride? *Curr. Opin. Biotechnol.* 49, 140–147. doi: 10.1016/j.copbio.2017.08.004

Ji, X. L., Hou, C. Y., Gao, Y. Y., Xue, Y. Q., Yan, Y., and Guo, X. D. (2020). Metagenomic analysis of gut microbiota modulatory effects of jujube (*Ziziphus jujuba* Mill.) polysaccharides in a colorectal cancer mouse model. *Food Funct.* 11, 163–173. doi: 10.1039/c9fo02171j

Kalt, F., Schulthess, B., Sidler, F., Herren, S., Fucentese, S. F., Zingg, P. O., et al. (2018). *Corynebacterium* Species Rarely Cause Orthopedic Infections. *J. Clin. Microbiol.* 56, e1200–e1218. doi: 10.1128/JCM.01200-18

Kodawara, T., Higashi, T., Negoro, Y., Kamitani, Y., Igarashi, T., Watanabe, K., et al. (2016). The inhibitory effect of ciprofloxacin on the β-glucuronidase-mediated deconjugation of the irinotecan metabolite SN-38-G. *Basic Clin. Pharmacol. Toxicol.* 118, 333–337. doi: 10.1111/bcpt.12511

Langille, M. G., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., et al. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* 2013, 814–821. doi: 10.1038/nbt.2676

Li, M. L., and Wang, C. H. (2020). Traditional uses, phytochemistry, pharmacology, pharmacokinetics and toxicology of the fruit of *Tetradium ruticarpum*: A review. *J. Ethnopharmacol.* 263:113231. doi: 10.1016/j.jep.2020.113231

Li, X. Y., Zhu, J. Y., Wu, Y., and Tan, Z. J. (2023). Correlation between kidney function and intestinal biological characteristics of adenine and folium sennaeinduced diarrhea model in mice. *Turk. J. Gastroenterol.* 34, 4–14. doi: 10.5152/tjg.2022.211010

Li, Y., Pan, H., Li, X. B., Jiang, N., Huang, L. Y., Lu, Y. F., et al. (2019). Role of intestinal microbiota-mediated genipin dialdehyde intermediate formation in geniposide-induced hepatotoxicity in rats. *Toxicol. Appl. Pharmacol.* 377:114624. doi: 10.1016/j.taap.2019.114624

Liao, W. Q., Li, B., Li, L., and Zhao, J. N. (2014). Study on molecular mechanism of *Euodiae fructus* on liver toxicity in MICE. *Chin. J. Tradit. Chin. Med.* 24, 4865–4868. doi: 10.4268/cjcmm20142432

Liu, J., Qiao, B., Deng, N., Wu, Y., Li, D. D., and Tan, Z. J. (2023). The diarrheal mechanism of mice with a high-fat diet in a fatigued state is associated with intestinal mucosa microbiota. *3 Biotech* 13:77. doi: 10.1007/s13205-023-03491-5

Liu, X. M., Mao, B. Y., Gu, J. Y., Wu, J. Y., Cui, S. M., Wang, G., et al. (2021). Blautia-a new functional genus with potential probiotic properties? *Gut Microbes* 13, 1–21. doi: 10.1080/19490976.2021.1875796

Liu, Y. T., Liu, C., Liu, Y. M., Ge, Q. L., and Sun, C. (2020). Cytochrome P450 mediated bioactivation of rutaevin, a bioactive and potentially hepatotoxic component of *Evodia rutaecarpa*. *Chem. Res. Toxicol.* 33, 3054–3064. doi: 10.1021/acs.chemrestox.0c00475

Long, C. X., Liu, Y. W., He, L., Yu, R., Li, D. D., Tan, Z. J., et al. (2018b). Bacterial lactase genes diversity in intestinal mucosa of dysbacterial diarrhea mice treated with Qiweibaizhu powder. *3 Biotech* 8:423. doi: 10.1007/s13205-018-1460-3

Long, C. X., Liu, Y. W., He, L., Tan, Q. Q., Yu, Z. Z., Xiao, N. Q., et al. (2018a). Bacterial lactase genes diversity in intestinal mucosa of mice with dysbacterial diarrhea induced by antibiotics. *3 Biotech* 8:176. doi: 10.1007/s13205-018-1191-5

National Pharmacopoeia Committee (2020). *Pharmacopoeia of the people's Republic of China*. Beijing: Medical Science Press.

Nusantara Putra, F. J., Putri, S. P., and Fukusaki, E. (2019). Metabolomics-based profiling of three terminal alkene-producing Jeotgalicoccus spp. during different growth phase. *J. Biosci. Bioeng.* 127, 52–58. doi: 10.1016/j.jbiosc.2018.06.014

Pabst, O., Hornef, M. W., Schaap, F. G., Cerovic, V., Clavel, T., and Bruns, T. (2023). Gut-liver axis: Barriers and functional circuits. *Nat. Rev. Gastroenterol. Hepatol.* 20, 447–461. doi: 10.1038/s41575-023-00771-6

Pizzino, G., Irrera, N., Cucinotta, M., Pallio, G., Mannino, F., Arcoraci, V., et al. (2017). Oxidative stress: Harms and benefits for human health. *Oxid. Med. Cell Longev.* 2017:8416763. doi: 10.1155/2017/8416763

Qi, Z. T. (2010). *Medical microbiology*, 2nd Edn. Beijing: Science publisher.

Ren, K., Wang, R. J., Fang, S. N., Ren, S. M., Hua, H. M., Wang, D. M., et al. (2023). Effect of CYP3A inducer/inhibitor and licorice on hepatotoxicity and in vivo metabolism of main alkaloids of *Euodiae fructus* based on UPLC-Q-Exactive-MS. *J. Ethnopharmacol.* 303:116005. doi: 10.1016/j.jep.2022.116005

Sengupta, M., Naina, P., Balaji, V., and Anandan, S. (2015). *Corynebacterium amycolatum*: An unexpected pathogen in the ear. *J. Clin. Diagn. Res.* 9, Dd01–Dd3. doi: 10.7860/JCDR/2015/15134.7002

Shan, Q. Y., Tian, G., Wang, J. L., Hui, H., Shou, Q. Y., Fu, H. Y., et al. (2021). Change in the active component of processed *Tetradium ruticarpum* extracts leads to improvement in efficacy and toxicity attenuation. *J. Ethnopharmacol.* 264:113292. doi: 10.1016/j.jep.2020.113292

Vallianou, N., Dalamaga, M., Stratigou, T., Karampela, I., and Tsigalou, C. (2021). Do antibiotics cause obesity through long-term alterations in the gut microbiome? A review of current evidence. *Curr. Obes. Rep.* 10, 244–262. doi: 10.1007/s13679-021-00438-w

Wang, J., Yuan, T. J., Chen, L. T., Zhao, X. L., Li, J. S., and Di, L. Q. (2020). Total saponins of *Glycyrrhiza inflata* and its decoction on intestinal flora in rats with liver injury. *Chin. Tradit. Herb. Drug* 51, 101–108. doi: 10.7501/j.issn.0253-2670.2020.01.015

Wang, M. X., Zhou, B. Q., Cong, W. H., Zhang, M., Li, Z. W., Li, Y., et al. (2021). Amelioration of AOM/DSS-induced murine colitis-associated cancer by evodiamine intervention is primarily associated with gut microbiota-metabolism-inflammatory signaling axis. *Front. Pharmacol* 12:797605. doi: 10.3389/fphar.2021.797605

Wang, P. Y., Cai, M., Yang, K., Sun, P. L., Xu, J., Li, Z. H., et al. (2023). Phenolics from *Dendrobium officinale* leaf ameliorate dextran sulfate sodium-induced chronic colitis by regulating gut microbiota and intestinal barrier. *J. Agric. Food Chem.* 44, 16630–16646. doi: 10.1021/acs.jafc.3c05339

Wu, H., Yu, X. H., Ma, G. C., Ma, Z. J., and Zhang, C. E. (2020). Metabolomics study on reduction of hepatotoxic of *Tripterygium wilfordii* processed by liquorice based on LC-MS. *Tradit. Herb. Drug* 1, 5501–5508. doi: 10.7501/j.issn.0253-2670.2020.21.014

Wu, Y., Peng, X. X., Li, X. Y., Li, D. D., Tan, Z. J., and Yu, R. (2022). Sex hormones influence the intestinal microbiota composition in mice. *Front Microbiol.* 13:964847. doi: 10.3389/fmicb.2022.964847

Wu, Z., Zeng, W. Z., Zhang, X., and Yang, J. F. (2022). Characterization of acidic tea polysaccharides from yellow leaves of wuyi rock tea and their hypoglycemic activity via intestinal flora regulation in rats. *Foods* 11:617. doi: 10.3390/foods11040617

Xia, H. M., Dai, Y. P., Zhao, C. X., Zhang, H. M., Shi, Y. S., and Lou, H. X. (2023). Chromatographic and mass spectrometric technologies for chemical analysis of *Euodiae fructus*: A review. *Phytochem. Anal.* 34, 5–29. doi: 10.1002/pca.3187

Xiao, S. J., Xu, X. K., Chen, W., Xin, J. Y., Yuan, W. L., Zu, X. P., et al. (2023). Traditional Chinese medicine *Euodiae fructus*: Botany, traditional use, phytochemistry, pharmacology, toxicity and quality control. *Nat. Prod. Bioprospect.* 13:6. doi: 10.1007/s13659-023-00369-0

Yang, J. P., Li, Y. N., Wen, Z. Q., Liu, W. Z., Meng, L. T., and Huang, H. (2021). Oscillospira - a candidate for the next-generation probiotics. *Gut Microbes* 13:1987783. doi: 10.1080/19490976.2021.1987783

Yi, X., Zhou, K., Jiang, P., Deng, N., Peng, X. X., and Tan, Z. J. (2023). Brain-bacteria-gut axis and oxidative stress mediated by intestinal mucosal microbiota might be an important mechanism for constipation in mice. *3 Biotech* 13:192. doi: 10.1007/s13205-023-03580-5

Zhang, F. L., He, X., Zhai, Y. R., He, L. N., Zhang, S. C., Wang, L. L., et al. (2015). Mechanism-based inhibition of CYPs and RMs-induced hepatoxicity by rutaecarpine. *Xenobiotica* 45, 978–989. doi: 10.3109/00498254.2015.1038742

Zhang, W., Wang, M. J., Song, H. J., Gao, C. F., Wang, D. M., Hua, H. M., et al. (2021). CYP3A4 inducer aggravates big flower *Evodiae fructus*-induced hepatotoxicity whereas limonin attenuates its hepatotoxicity. *J. Ethnopharmacol.* 264:113277. doi: 10.1016/j.jep.2020.113277

Zhang, M., Gao, M., Wu, S. S., Zhou, L. F., Cao, L., Qiao, R. F., et al. (2021). Hepatotoxicity comparison of crude and licorice-processed *Euodiae fructus* in rats with stomach excess-cold syndrome. *Front. Pharmacol.* 12:756276. doi: 10.3389/fphar.2021.756276

Zhang, S. R., Liu, S. L., Zhong, Z. G., Chen, B., and Wei, H. (2018). The relationship of dose-time-toxicity of different processed products of *Evodia rutaecarpa* (Juss.) Benth on hepatotoxicity in mice. *Lishizhen. Med.* 29, 881–884. doi: 10.3969/j.issn.1008-0805.2018.04.037

Zhou, K., Peng, M. J., Deng, N., Tan, Z. J., and Xiao, N. Q. (2022). Lactase bacteria in intestinal mucosa are associated with diarrhea caused by high-fat and high-protein diet. *BMC Microbiol.* 22:226. doi: 10.1186/s12866-022-02647-2

Zhou, M. S., Li, X. Y., Liu, J., Wu, Y., Tan, Z. J., and Na Deng, N. (2024). Adenine's impact on mice's gut and kidney varies with the dosage administered and relates to intestinal microorganisms and enzyme activities. *3 Biotech* 14:88. doi: 10.1007/s13205-024-03959-y2024-2-22

Ziętak, M., Kovatcheva-Datchary, P., Markiewicz, L. H., Ståhlman, M., Kozak, L. P., and Bäckhed, F. (2016). Altered microbiota contributes to reduced diet-induced obesity upon cold exposure. *Cell Metab.* 23, 1216–1223. doi: 10.1016/j.cmet.2016.05.001

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Bariatric-induced microbiome changes alter MASLD development in association with changes in the innate immune system

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Introduction: Metabolic dysfunction-associated steatotic liver disease (MASLD) affects nearly 25% of the population and is the leading cause for liver-related mortality. Bariatric surgery is a well-known treatment for MASLD and obesity. Understanding the fundamental mechanisms by which bariatric surgery can alter MASLD can lead to new avenues of therapy and research. Previous studies have identified the microbiome's role in bariatric surgery and in inflammatory immune cell populations. The host innate immune system modulates hepatic inflammation and fibrosis, and thus the progression of MASLD. The precise role of immune cell types in the pathogenesis of MASLD remains an active area of investigation. The aim of this study was to understand the interplay between microbiota composition post-bariatric surgery and the immune system in MASLD.

Methods: Eighteen morbidly obese females undergoing sleeve gastrectomy were followed pre-and post-surgery. Stool from four patients, showing resolved MASLD post-surgery with sustained weight loss, was transplanted into antibiotic treated mice. Mice received pre-or post-surgery stool and were fed a standard or high-fat diet. Bodyweight, food intake, and physiological parameters were tracked weekly. Metabolic parameters were measured post-study termination.

Results: The human study revealed that bariatric surgery led to significant weight loss ($p > 0.05$), decreased inflammatory markers, and improved glucose levels six months post-surgery. Patients with weight loss of 20% or more showed distinct changes in blood metabolites and gut microbiome composition, notably an increase in *Bacteroides*. The mouse model confirmed surgery-induced microbiome changes to be a major factor in the reduction of markers and attenuation of MASLD progression. Mice receiving post-surgery fecal transplants

had significantly less weight gain and liver steatosis compared to pre-surgery recipients. There was also a significant decrease in inflammatory cytokines interferon gamma, interleukin 2, interleukin 15, and mig. This was accompanied by alterations in liver immunophenotype, including an increase in natural killer T cells and reduction of Kupfer cells in the post-surgery transplant group.

Discussion: Our findings suggest surgery induced microbial changes significantly reduce inflammatory markers and fatty liver progression. The results indicate a potential causal link between the microbiome and the host immune system, possibly mediated through modulation of liver NKT and Kupffer cells.

KEYWORDS

metastatic dysfunction-associated steatotic liver disease, obesity, host innate immune system, microbiome, fecal transplant

1 Introduction

The prevalence of obesity in adults has doubled in the last decade alone, confirming that the obesity epidemic of the late 1900s is only worsening (Park et al., 2005; Hruby and Hu, 2015). Obesity is understood as the underlying mechanism for several comorbidities, one being metabolic associated steatotic liver disease (MASLD). MASLD is the leading cause of chronic liver disease and for liver-related mortalities globally (Younossi et al., 2016; Ding et al., 2023). The rates of MASLD in the population have increased in parallel with the rise in obesity, affecting over 25% of the global population (The GBD 2015 Obesity Collaborators, 2017; Pouwels et al., 2022). While the disease itself can be relatively asymptomatic and in some cases, reversible, nearly 25% MASLD cases progress into stages in which the liver becomes overly fibrotic and eventually scarred (Ismail and Pinzani, 2009; Fallowfield and Hayes, 2011; Bashiardes et al., 2016). Given its rise and the detrimental outcomes of MASLD, understanding the underlying mechanisms of MASLD progression, and identifying potential therapeutic strategies for MASLD remains a leading area of research.

Bariatric surgery has been a very well established treatment for eligible patients with both obesity and MASLD. Many studies have shown the beneficial effects of bariatric surgery in MASLD (Gulinac et al., 2023). It is thought the main effect of bariatric surgery on MASLD is through weight loss. However, MASLD improvement can often occur before significant weight loss is achieved (Seeberg et al., 2022). This suggests that the effects of bariatric surgery on MASLD are potentially multifactorial.

The gut microbiome has recently emerged as a critical factor contributing to the progression of MASLD, indicating it to be a promising avenue for investigation (Durack and Lynch, 2019; Afzaal et al., 2022). Dysregulation of the gut microbiota has been associated with compromised gut barrier function, which can contribute to translocation of bacteria and endorse hepatic inflammation, and with the modulation of certain metabolites (Bashiardes et al., 2016; Chopyk and Grakoui, 2020; Afzaal et al., 2022). The gut microbiome also plays an important role in the host innate immune system (Zheng et al., 2020). This is important because several immune cell populations and inflammatory mediators have been implicated in the development and progression of MASLD (Carter and Friedman, 2022; Huby and Gautier, 2022). The liver, as a central organ in the immune response, is constantly exposed to gut-derived antigens and microbial products through the portal circulation, making it susceptible to immune-mediated damage. Studies have shown that various immune cells, including macrophages, T cells, and dendritic cells, infiltrate the liver and contribute to the inflammatory milieu that characterizes MASLD (Ma et al., 2018; Bai et al., 2023).

One specific subset of immune cells, known as natural killer T (NKT) cells, has garnered increasing attention due to their involvement in metabolic conditions like MASLD (Kumar and Delovitch, 2014; Krijgsman et al., 2018). NKT cells are a subset of T cells that express both a T cell receptor and a natural killer cell receptor (Kumar and Delovitch, 2014; Krijgsman et al., 2018; Nilsson et al., 2020). With two receptors, the cells can recognize lipid antigens presented by CD1d molecules, making them critical in lipid metabolism and immune regulation (Dowds et al., 2014). Several studies have shown that NKT cells modulate hepatic lipid metabolism and introduce a pro-inflammatory environment (Nilsson et al., 2020; Zheng et al., 2022). More specifically, the activation of NKT cells has been associated with a prevalence of pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF-α) or interferon-gamma (IFN-γ). Some studies, however, have shown the opposite. Certain subsets of NKT cells have been found to play a protective role, producing anti-inflammatory cytokines like interleukin-4 (IL-4) and interleukin-10 (IL-10) instead (Nilsson et al., 2020).

Given the contradictory results across studies, the precise role of NKT cells in the pathogenesis of MASLD remains an active area of investigation. Understanding the interplay between gut microbiota changes in bariatric surgery and the protective or pathogenic proliferation of NKT cells in the context of liver inflammation could provide novel insight in the underlying mechanisms of MASLD. Therefore, the aim of this study is to investigate the role of the microbiome post-bariatric surgery in MASLD and its effects on NKT cells in the liver disease setting.

2 Methods

2.1 Human cohort recruitment

A human study using a small cohort design was employed to investigate a causal relationship between the gut microbiome and MASLD after bariatric surgery. Adult patients were recruited from the Bariatric Surgery Program at the University of California, Los Angeles.

Patients eligible for the study had to meet the following requirements: biologically female, an intention to undergo sleeve gastrectomy, diagnosis of MASLD, and in line with the criteria listed in the Guidelines for Clinical Application of Laparoscopic Bariatric Surgery of American Gastrointestinal and Endoscopic Surgeons (SAGES Guidelines Committee, 2008). Recruiting patients of the same gender, surgical procedure intent, and from the same program minimized confounding factors that could impact the gut microbiome composition. Diagnosis of MASLD was based on a patient history of non-excessive alcohol use, absence of viral hepatitis, and either pathology and imaging consistent with steatosis or elevated transaminases. Exclusion criteria included cirrhosis, use of medications interfering with or affecting intestinal motility, excessive substance abuse, a history of major gastrointestinal surgeries or procedures, irritable bowel syndrome, inflammatory bowel disease, pregnancy, or recent antibiotic or probiotic use. The demographic information of each participant was collected as well as stool and blood samples before surgery and six months after surgery.

Ethical approval of the study was obtained from the University of California, Los Angeles Institutional Review Board (IRB #13-001552). All patient research procedures followed the principles of the Declaration of Helsinki.

2.2 Human cytokine profiling

Fasting blood samples were obtained from patients both before and 6 months after undergoing bariatric surgery. Subsequently, the levels of C-reactive protein (CRP) (Abcam, ab260058) and lipopolysaccharide-binding protein (LBP) (Abcam, ab279407) were quantified using an enzyme-linked immunosorbent assay (ELISA) kit, following the manufacturer's recommended protocols. The following cytokines were measured: HGF, IL-1beta, IL-6, IL-8, MCP-1, NGF, and TNF-α. Fast blood serum was also analyzed for metabolites using 16S sequencing.

2.3 Human serum metabolite profiling

Fasting serum was collected from patient and sent to Metabolon Inc. for processing.

2.4 Human stool collection and 16S sequencing

Stool samples were collected from the participants within one week prior to their scheduled surgery, as well as at 6 and 12 months post-surgery. Fresh stool samples were promptly collected and immediately frozen, ensuring preservation at a temperature of-80°C. Subsequently, the frozen stool samples were divided into aliquots for subsequent analysis. DNA extraction was performed using the ZymoBIOMICS DNA Microprep Kit (Zymo Research, United States), following the manufacturer's instructions.

To target the V4 region of the 16S ribosomal RNA gene, PCR amplification was conducted utilizing the 515\u00B0F-806 R primer set (Tong et al., 2014). Following the amplification step, the samples underwent 250 × 2 paired-end sequencing using an Illumina HiSeq platform (Illumina, San Diego, CA, United States) (Zheng et al., 2022). The resulting raw fastq files were subjected to processing using the DADA2 pipeline in R, employing the SILVA 132 database and default parameters for taxonomy assignment (Callahan et al., 2016).

After initial pre-processing in R using DADA2, the data were subsequently integrated into QIIME 2 version 2019.10 (Bolyen et al., 2019). To ensure data quality, amplicon sequence variants (ASVs) that were sparsely represented were filtered out if they were not present in at least 15% of all samples, consistent with our previous publications (Dong et al., 2020; Hussain et al., 2020). The sequence depths ranged from 60,710 to 269,258 per sample.

2.5 Statistical analysis of human data

Physiological metrics were analyzed after calculating the mean values and standard deviations of quantitative demographic information, including age, body weight, and body mass index (BMI). The Shapiro–Wilk test was done to test for normality of continuous data from the human trial. Data deemed to be not normally distributed if the Shapiro–Wilk test had a p-value <0.05. Parametric test were done on normally distributed data and non-parametric tests were done on non-normally distributed data. A paired t-test was used to statistically compare respective mean values of normally distributed data. A paired Wilcoxon signed-rank test was used to statistically compare non-normally distributed data: body weight, BMI, fasting glucose, and inflammatory cytokines in patients before and after surgery. A Fisher's exact test was used to assess categorical demographic information, like ethnicity and race.

Changes to microbial composition were analyzed using several metrics. Alpha diversity was computed using the Shannon Index within the QIIME2 framework (Bolyen et al., 2019). The Shannon index incorporates both species and evenness. The microbiome dataset was rarefied to a standardized sequence depth of 60,710 reads. The statistical significance of the Shannon Index was determined using analysis of variance (ANOVA) after confirming normality using the Shapiro–Wilk test, adjusting for subject ID as a covariate. Beta diversity was analyzed using the robust Aitchison Distance Metric within the DEICODE package of QIIME2 (Martino et al., 2019). The Aitchison Distance Metric has demonstrated superior discriminatory capacity compared to other metrics, such as UniFrac or Bray-Curtis (Lozupone and Knight, 2005; Martino et al., 2019). Beta diversity was evaluated using permutational multivariate analysis of variance, specifically employing the 'adonis' package in R (Version 4.1.2), adjusting for subject ID as a covariate (Dong et al., 2021). Differential abundance analysis of genera was computed using MaAsLin2, in which subject ID was a random effect (Mallick et al., 2021).

Metabolite data was transformed using a median-scale normalization approach–one in which each metabolite value was divided by its median value. Metabolite levels over different time points were statistically compared using ANOVA, adjusting for subject ID as a covariate. p-values were adjusted for multiple hypothesis testing using the Benjamin-Hochberg correction method (Benjamini and Hochberg, 1995; Glueck et al., 2008). A heatmap was generated using the 'ggplots' and heatmap.2 function in R to visually depict significant metabolites. p-values deemed significant were those less than 0.05, adjusted for multiple hypothesis testing if appropriate.

2.6 Mouse model

A mouse model was designed to engraft the microbiome of human patients into antibiotic treated mice. 64 C57/B16 male mice between 6–8 weeks old at the start of the study were separated into 16 cages with four mice in each cage. Since we were evaluating the role of the immune system in our model, ferm-free mice were not used because of their altered immune systems (Kennedy et al., 2018). Eight cages received donor stool collected prior to surgery and eight cages received donor stool collected six months post-surgery. Within those time point groups, half was assigned a standard double-irradiated diet (10% fat by kcal, 0% fructose, and 0% cholesterol) and the other was assigned a Western high-fat double-irradiated diet (40% fat, 20% fructose, 2% cholesterol) for the duration of the study. Each cage was provided with individually packaged 1-kg bags of food, with a new bag introduced during each cage change.

A combination of systemically absorbed antibiotics (ampicillin, cefoperazone, and clindamycin) and non-systemically absorbed antibiotics (ertapenem, neomycin, and vancomycin) were given on alternating weeks in their drinking water for 21 days prior to the beginning of the study. This process has been shown to lead to stable engraftment of human microbiota in non-germ-free animals. The concentration of antibiotics in the mice's water was maintained at 1 g/L *ad libitum*.

Stool samples for fecal transplant were prepared using donor stool from four patients who met the following criteria: diagnoses of MASLD prior to surgery that resolved after surgery and achieved significant and sustained weight loss of at least 20% six months following surgery. Resolution of MASLD was determined either by biopsy or by imaging. Approximately 1 g of frozen stool per time point and donor was resuspended in 15 mL of pre-reduced PBS and filtered through a 100 µm filter. Mice were then orally administered 200 µL of this solution. The microbial concentration of the preparation was determined using a Petroff-Hausser counting chamber, and approximately 10^10 cells were administered during each gavage procedure.

This research was conducted in compliance with the guidelines and approval of the UCLA Animal Research Committee and the Institutional Animal Care and Use Committee.

2.7 Mouse body composition and glucose tolerance testing

The body weight of each mouse and average food intake per mouse were recorded once a week. At the end of the 90-day period, a body composition analysis, including lean body mass and fat mass, was conducted using an EchoMRI machine. To assess glucose tolerance, mice were fasted for 9 h and then injected intraperitoneally 2 g/kg of glucose. Serum glucose levels were measured at 0, 30 min, 60 min, and 90 min using a glucometer.

2.8 Mouse sample processing

Mice were fasted 9 h before being euthanized. Isoflurane was used as the primary method for euthanasia. Animals were exposed to a 10% concentration of isoflurane. Heart puncture and phlebotomy were carried out as a secondary method. Serum samples were obtained via heart puncture, and the liver and gastrointestinal tract were collected.

2.9 Liver steatosis staining and quantification

Frozen OCT-embedded liver tissue was stained with Oil red O. The quantification of staining was performed using ImageJ. Hematoxylin and eosin staining was conducted by the histology core at the University of California, Los Angeles. A blinded pathologist calculated MASLD activity scores based on the hematoxylin and eosin staining. Liver triglyceride content was quantified using a calorimetric triglyceride assay kit (Abcam ab65336), per the manufacturer protocol.

2.10 Serum inflammatory markers and hepatic immune flow cytometry

IL-6, TNF-α, CCL2, IL-4, and TGF-β were quantified using the Millipore Milliplex Cytokine Kit (Melamud et al., 2022). This kit also includes other cytokines such as IL-1β, IL-2, IL-15, IL-10, IL-12p70, IL-13, IL-17A, IFN-γ, MCP-1, MIP-1a, MIP-1b, GM-CSF, CCL3, CCL4, MIG, and CCL5, which play critical roles in various inflammatory and immune responses, providing valuable insights into MASLD and HCC development (Kennedy et al., 2018).

The activation of hepatic immune cells was assessed using flow cytometry and cell surface marker staining (Hildreth et al., 2023). The liver of each mouse was perfused with PBS and EDTA, followed by incubation with HBSS containing collagenase IV and DNAase I. Each liver was homogenized, filtered through a cell strainer, and centrifuged. The resulting pellet was resuspended and separated using a Percoll gradient. Flow cytometry antibodies targeting specific markers were used, including Alexa Fluor® 488 anti-mouse Ly-6C, Alexa Fluor® 647 anti-mouse F4/80, APC/Cyanine7 anti-mouse CD45.2, Brilliant Violet 650™ anti-mouse CD3, Brilliant Violet 750™ anti-mouse/human CD11b, PE anti-mouse α-GalCer:CD1d complex, PE/Dazzle™ 594 anti-mouse CD335 (NKp46), PerCP/Cyanine5.5 anti-mouse CD4, Brilliant Violet 421™ anti-mouse/human CD45R/B220, and Brilliant Violet 510™ anti-mouse CD8a. The antibodies were obtained from BioLegend.

The flow cytometry panel allowed for the characterization of various immune cell populations, including T cells (CD3, CD4, and CD8a), B cells (CD45R/B220), macrophages (F4/80), monocytes (Ly-6C), and NKT cells.

2.11 Statistical analysis for mouse data

A paired *t*-test was used to statistically compare groups. Shapiro–Wilk test was done to test for normality.

3 Results

3.1 Bariatric surgery leads to decreased obesity markers

Eighteen morbidly obese females were recruited for the human cohort study. Their demographic and physical characteristics were recorded (Table 1). Non-Hispanic White individuals made up 44.4% of the cohort and Hispanic individuals made up 38.9%. Asians made up 11.1% of the cohort and African Americans made up 5.6%. The

TABLE 1 Cohort characteristics.

Race and ethnicity (%)	
Non-Hispanic White	44.4
Hispanic	38.9
Asian	11.1
African American	5.6

Average (SD) ($n = 18$)	Six months pre-surgery	Six months post-surgery	p-value
Age (yr)	37.1 (9.4)	37.1 (9.4)	–
Body Mass Index	44.7 (4.9)	33.9 (4.8)	<0.001
Weight (kg)	118.5 (18.8)	89.7 (16.9)	<0.001

The demographic and physical characteristics of 18 females recruited for the human cohort study. SD: Standard Deviation.

mean pre-surgery BMI was 44.7 ± 4.9 and the mean pre-surgery body weight 118.5 ± 18.8 kg. The cohort exhibited a statistically significant reduction in BMI and body weight six months after bariatric with a mean post-surgery bodyweight of 89.7 ± 16.9 kg (Figure 1A), and a post-surgery BMI of 33.9 ± 4.8 ($p < 0.001$) (Figure 1B). Blood fasting glucose decreased by 7.9% and the inflammatory markers C-reactive protein (CRP) and lipopolysaccharide binding protein (LBP) decreased by approximately 87.5 and 42.3%, respectively, six months post-surgery (Figures 1C–E).

3.2 Bariatric surgery reduces blood metabolites and introduces changes in gut microbiome composition

Six months post-bariatric surgery, patients demonstrated a noteworthy decline in several blood metabolites. Specifically, hepatocyte growth factor (HGF), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α) levels significantly decreased (Figures 2A–C). No significant differences were observed in interleukin-1 beta (IL-1B), interleukin-8 (IL-8), monocyte chemoattractant protein 1 (MCP1), or nerve growth factor (NGF) concentrations.

Upon analysis of beta diversity, patients who maintained a sustained weight loss of 20% or greater from their baseline had distinct differences in their gut microbiomes both at baseline and six months post-surgery compared to those who did not sustain weight loss (Figure 3A). Patients that sustained weight loss also exhibited significantly lower alpha diversity both at baseline and post-surgery (Figure 3B). There was no significant difference between alpha or beta diversity between individual patients' samples pre-surgery of six months afterwards. Patients who sustained weight loss had higher levels of *Bacteroides* at baseline, with an upward trend in relative abundance over the duration of the study while patients who did not sustain weight loss over time had lower levels of *Bacteroides* at baseline and a relatively unchanged level overtime.

46 blood metabolites were identified as significantly different at the six-month post-surgery time point when compared to their baseline values. Of these, 31 metabolites were found to be linked to lipid metabolism, while 9 were associated with amino acid metabolism (Figure 3C).

Gut Differential Analysis reveals 12 patients who sustained weight loss to have altered genera six months post-surgery. *Bacteroides*, which had the largest relative abundance amongst both groups, increased noticeably after the sleeve gastrectomy procedure; 22.5% at baseline to 25.5% for patients without sustained weight loss and 36.6 to 42.8% six months after surgery for patients who did (Figures 3D,E). Other genera that were altered pre-and post-surgery include *Acetanaerobacterium, Phascolarctobacterium, Ruminococcaceae UCG-005, Ruminococcus 2, Klebsiella, Blautia, Lachnospiraceae NK4A136 group, Lachnospiraceae NK4A136 group, (f) Lachnospiraceae, Capriciproducens, Agathobacter, and Veillonella*.

3.3 Bariatric surgery-induced gut microbiome changes in a preventative mouse model result in body weight reduction and attenuation of MASLD progression

Stool samples were collected from four donors who met the criteria for fecal transplantation. (Table 2). Mice with baseline donor stools (donor stool collected pre-surgery) gained significantly more weight on either diet compared to those who received the donor stool six months post-surgery (Figures 4A,B). No significant difference was seen in the average food intake in either of the two groups (Figures 4C,D). A measurement of body fat percentage using EchoMRI showed that mice on either diet who received the post-surgery stool had significantly lower body fat percentage and higher average lean body mass compared to their respective pre-surgery groups (Figures 4D,E). Histological analysis of the liver revealed that pre-surgery groups on either diet had significantly elevated hepatic steatosis compared to their respective post-surgery groups (Figure 4G). This was verified both by quantification of oil red o lipid staining using ImageJ and quantification of liver triglycerides (Figures 4H–J).

Mice who received the post-surgery stool had several significantly lower inflammatory cytokines in their serums as compared to mice who received the pre-surgery stool, regardless of diet. Specifically, interferon gamma (IFN-y), interleukin-2 (IL-2), interleukin 15 (IL-15), and mig levels were significantly lower (Figures 5A–D). No other cytokines tested were significantly different.

3.4 Bariatric surgery-induced gut microbiome alters immunophenotype and reduces inflammation

The immunophenotype of the livers reflected an increase in natural killer T cells (NKT cells) in both the post-surgery groups compared to their respective pre-surgery groups, but the groups receiving a high fat diet demonstrated a significant decrease overall in the percentage of NKT cells (Figures 6A,C). Cytotoxic T-cells (CTLs) were lower in the post-surgery group on both diets with no significant differences in the quantity of CTLs between diet groups (Figures 6B,D). A decrease in Kupffer cells was evident in the post-surgery groups on either diet with a notable elevation of Kupffer cell quantity overall in the groups receiving the high fat diet (Figures 7A,C). There was a

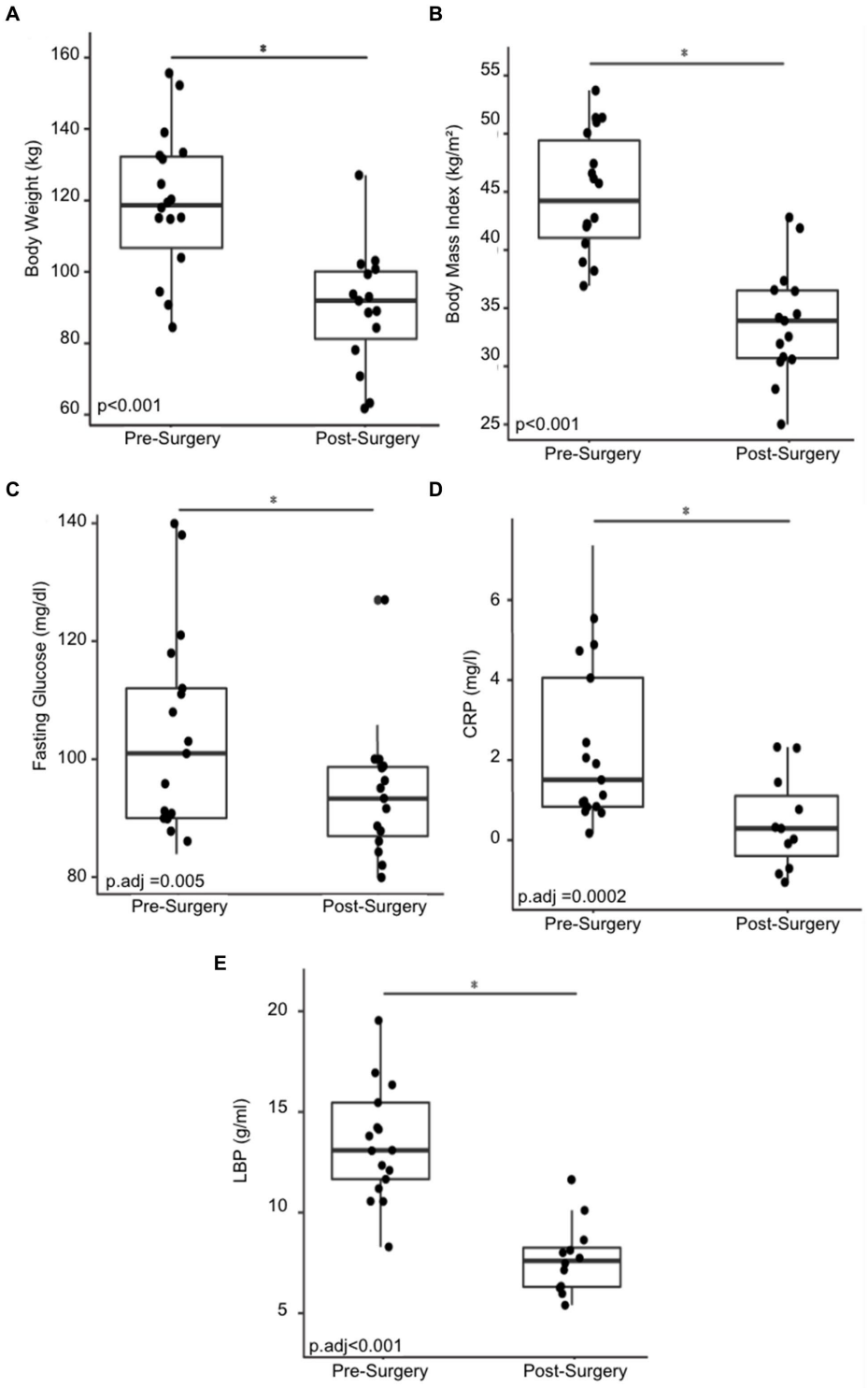

FIGURE 1
Changes in physical and physiological metrics post-bariatric surgery. **(A)** Body weight of 18 cohort members before and six months post-surgery. **(B)** BMI before and six months post-surgery. **(C)** Fasting glucose before and six months post-surgery. **(D)** C-reactive protein (CRP) before and six months post-surgery. **(E)** Change in lipopolysaccharide binding protein (LBP) before and six months post-surgery. "*" indicates *p*-value<0.05.

significant decrease in inflammatory macrophages of the post-surgery group on either diet, and a notable increase in inflammatory macrophages overall in the groups receiving the high fat diet (Figures 7B,D).

4 Discussion

Bariatric surgery is one of the leading treatment options for a long-term positive outcome for obese individuals with MASLD. The

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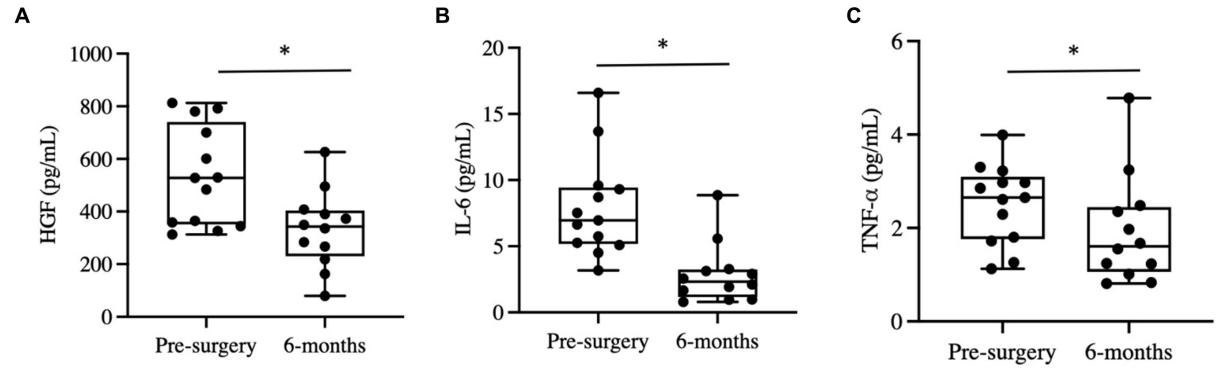

FIGURE 2
Changes in blood cytokines post-bariatric surgery. **(A)** Hepatocyte growth factor (HGF) before and six months post-surgery. **(B)** Interleukin-6 before and six months post-surgery. **(C)** Tumor necrosis factor alpha (TNF-α). "*" indicates *p*-value<0.05.

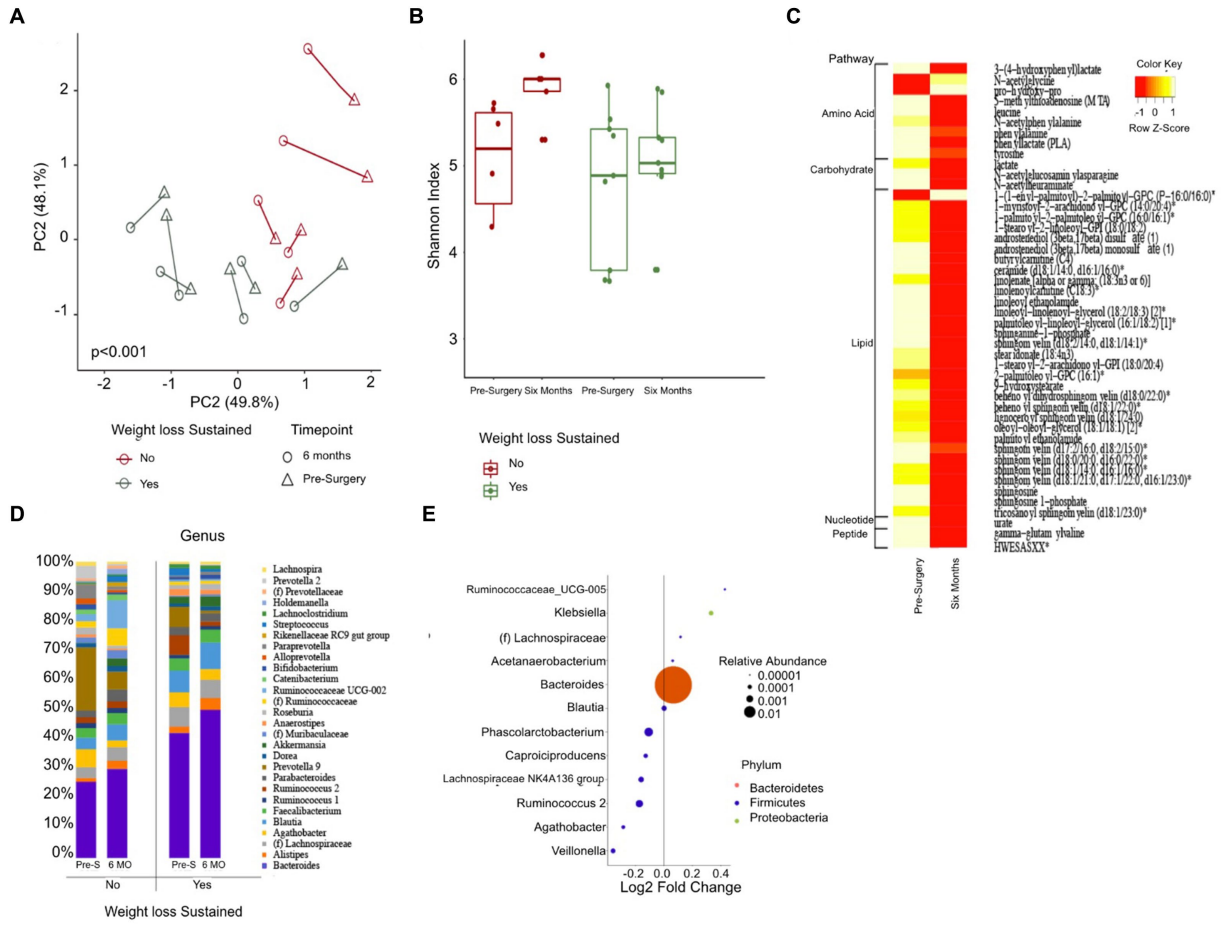

FIGURE 3
Changes in gut microbiome composition and serum metabolites following bariatric surgery. **(A)** Principal coordinate analysis plot (PCoA) measuring beta diversity. Each patient sample is indicated by either a triangle for pre-surgery or circle for six-months post-surgery, color-coded in either green for sustained weight loss or red for otherwise. Shapes are connected by a line to indicate an individual patient's sample over time. **(B)** Shannon Index quantifying alpha diversity. **(C)** Heatmap of serum metabolites before and six months post-surgery, categorized by major biochemical pathway. Only metabolites that were significantly different are shown. **(D)** Stacked bar plot showing the average relative abundance of each genera. Only genera with a relative abundance of at least 1% are included. **(E)** Results of differential abundance testing by MaAsLin, showing genera that are different from baseline. The test is adjusted for sustained weight loss (SWL) with patient ID as a random effect.

primary purpose of this study was to demonstrate that the microbiome is partially responsible for MASLD improvement following bariatric surgery. The long-term positive trends observed in the human cohort reflected those seen in previously published data on more extensive

TABLE 2 Microbiome donor characteristics.

Donor	Race/ethnicity	Age	Body mass index		% Body weight loss at six months	Fatty liver resolution on imaging
			Pre-surgery	Post-surgery		
A	Non-Hispanic White	25	51.0	37.1	20.1	Yes
B	Hispanic	21	42.2	30.9	23.3	Yes
C	Asian	45	36.9	25.1	35.3	Yes
D	Hispanic	43	38.2	31.0	20.2	Yes

The demographic and physical characteristics of four cohort members who had fatty liver disease prior to surgery that resolved after six months after treatment.

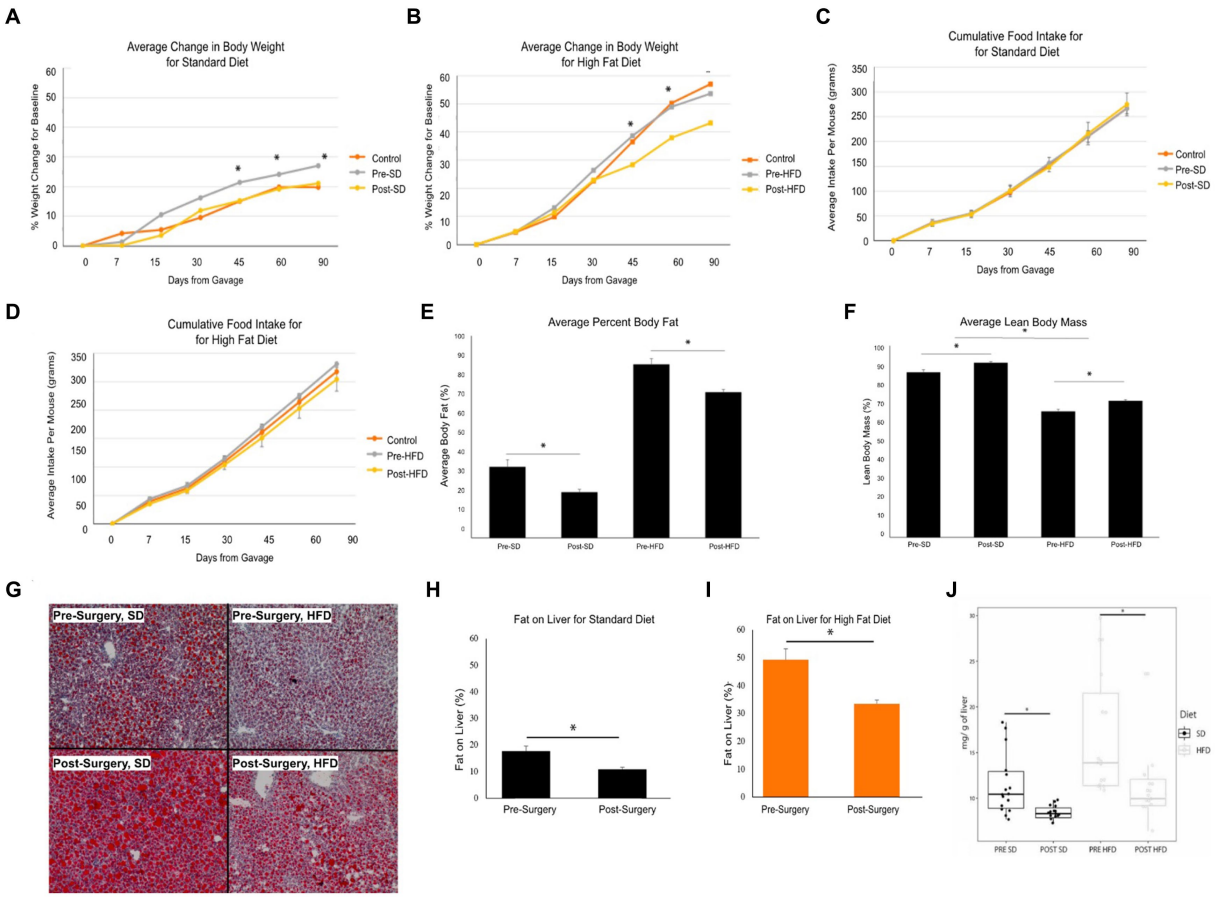

FIGURE 4
Bariatric surgery induced microbiome changes protect against MASLD progression in mice. **(A)** Average change in body weight for mice on a standard diet (SD) over 90 days. Orange indicates the control group with a vehicle gavage; gray indicates the group which received the pre-surgery stool transplant; yellow indicates the group that received the post-surgery stool transplant. **(B)** Average change in body weight for mice on high fat diet (HFD) over 90 days. **(C)** Cumulative food intake for mice on a standard diet over 90 days. **(D)** Cumulative food intake for mice on a high fat diet over 90 days. Comparison of average percent body fat between mice receiving pre-surgery versus post-surgery stool transplant on either diet following 90 days of gavage. **(E)** Comparison of average percent body fat of mice receiving pre-surgery versus post-surgery stool transplant on either diet following 90 days of gavage. **(F)** Comparison of average lean body mass of mice receiving pre-surgery versus post-surgery stool transplant on either diet following 90 days of gavage. **(G)** Images of Oil red o-stained liver samples from pre-surgery or post-surgery transplants on either diet. **(H)** Quantification of fat on liver for mice on standard diet using ImageJ. **(I)** Quantification of fat on liver from mice on high fat diet using ImageJ. **(J)** Quantification of mouse liver triglycerides. "*" indicates p-value<0.05.

cohorts and confirm that the treatment is effective (Courcoulas et al., 2014). Notably, individuals who underwent sleeve gastrectomy achieved a total body weight loss of 22.7% and a decrease in BMI of 21.6% six months after surgery. This decrease was associated with significant downwards trends in concentrations of C-reactive protein and lipopolysaccharide-binding protein, both notable inflammatory markers associated with obesity. Previous studies examining visceral adipocyte tissue in obese patients have revealed that expressions of hepatocyte growth factor (HGF), interleukin-6 (IL-6), and tumor necrosis α (TNF-α) are significantly elevated in obese patients compared to lean ones (Park et al., 2005; Gletsu-Miller and Wright, 2013; Muratsu et al., 2017). Likewise, studies incorporating bariatric

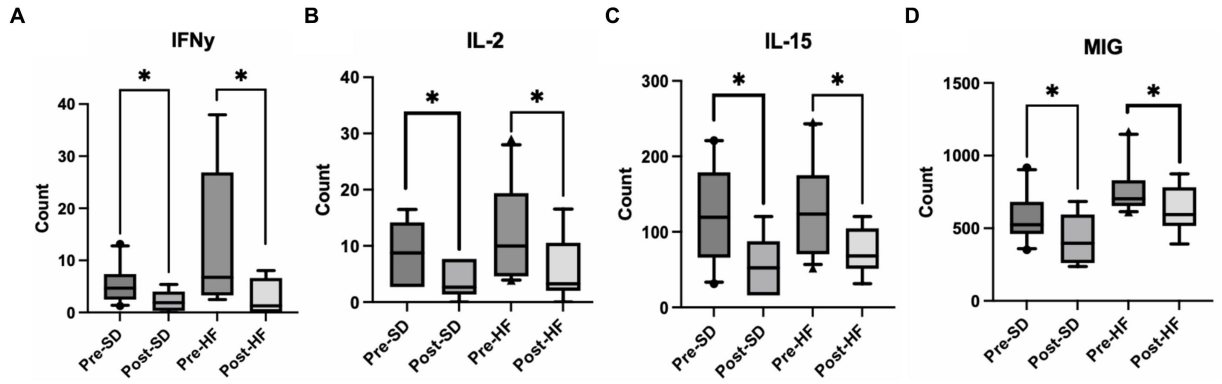

FIGURE 5
Post bariatric microbiome changes alters inflammatory cytokines in the serum. These four groups are consistent across graphs: Mice who received pre-surgery stool and were on a standard diet (Pre-SD); mice who received post-surgery stool and were on a high fat diet (Pre-HFD); mice who received post-surgery stool and were on a standard diet (Post-SD); mice who received post-surgery stool and were on a high fat diet (Post-SD). Comparison of **(A)** interferon gamma (IFN-y), **(B)** interleukin-2 (IL-2), **(C)** interleukin-15 (IL-15), **(D)** Mig between the pre-and post-surgery groups within their respective diets. "*" indicates p-value<0.05.

surgery and its role in obesity show a decrease in adiposity and in serum levels of IL-6, and TNF-α (Casimiro et al., 2020). Elevated levels of HGF have also been found to be associated with increased insulin resistance, a marker of severe obesity (Muratsu et al., 2017). As anticipated with the differences in physiological markers for patients in our cohort, measurements of inflammatory cytokine markers HGF, IL-6, and TNF-α were significantly lower six months after surgery compared to before. These results match the trends of more extensive bariatric surgery trials referenced.

A comparison of the microbiome composition of individuals before and after surgery reveals significant long-term differences. *Bacteroides* have previously been shown to have a negative correlation with physiological markers of obesity–body weight, BMI, and body fat (Aoun et al., 2020; Zsálig et al., 2023). The findings in this study concur with these trends; individuals in our study had increased levels of *Bacteroides* after surgery. Those who sustained weight loss after surgery had a more pronounced increase compared to those who did not. The exact mechanisms by which *Bacteroides* are correlated with weight loss remain unclear. However, multiple studies propose that these species may contribute to weight management through their potential involvement in gut-barrier integrity and metabolism (Ghosh et al., 2021; Zafar and Saier, 2021). *Bacteroides* have previously been shown to promote tight junction expression between epithelial cells in the small intestine, reducing permeability and potentially hindering the translocation of pro-inflammatory pathogens from the gut lumen into the bloodstream (Zhou et al., 2022). Consequently, an increase in the population could protect against systemic inflammation, a hallmark of obesity-related dysfunction. *Bacteroides* are also known for their ability to synthesize SCFAs as byproducts of complex dietary fibers. SCFAs are positively associated with glucose homeostasis and satiety (Ghosh et al., 2021; Zafar and Saier, 2021). Ghosh et al. (2021) particularly emphasized the potential impact of *Bacteroides*-synthesized SCFAs on insulin sensitivity. Several mouse studies and human studies have shown that SCFA are altered after bariatric surgery, with butyrate and propionate being positively associated with weight loss.

In order to demonstrate a causal relationship between changes in the gut microbiome induced by bariatric surgery and the progression of MASLD, fecal samples pre-surgery and post-surgery of cohort members who had resolution of MASLD post-surgery were transplanted into antibiotic treated mice. Mice who had received the pre-surgery microbiome showed increased body weight and a more rapid increase in body fat complemented with more severe progressed MASLD and fat on the liver, demonstrated in the histological staining and quantification. These trends were observed regardless of diet with no significant differences in food intake amongst those receiving the pre-surgery or post-surgery transplant, matching those of the findings in *Tremaroli et al.*; mice receiving the pre-surgery transplant had gained greater weight than those receiving the post-surgery transplant with no difference in respiratory levels, activity, or average food intake (Tremaroli et al., 2015). Mice receiving the post-surgery transplant also had a significant decrease in interferon gamma (IFN-y), interleukin-2 (IL-2), interleukin 15 (IL-15), and mig levels in their serum as compared to the pre-surgery transplant group. The active role of these cytokines is linked to growth of hepatocytes and a more rapid progression of MASLD (Chang et al., 2015; Duan et al., 2022). Moreover, in *Tarantino et al.*, IL-15 is identified as a predictor for intima-media thickness independent of age, suggesting the role of the cytokine in the onset and progression of atherosclerosis in obese individuals (Tarantino et al., 2021). While the exact mechanism by which the gut microbiome can modulate the development of obesity and MASLD remains unknown, we propose that the microbiome is affecting the progression of MASLD via the host innate immune system, specifically via natural killer T cells (NKT).

Hepatic lipid accumulation triggers a complex interplay of immune responses centered around Kupffer cells, which subsequently contributes to the progression of fibrosis, cirrhosis, and ultimately hepatocyte death (Chiang et al., 2011; Krijgsman et al., 2018). At the forefront of the immune defense, the innate immune system orchestrates rapid reactions through lymphocyte infiltration and localized inflammation. NKT cells, recognized by their expression of both CD3 and a unique natural killer cell marker, play a distinct role in this context. These cells exhibit an unconventional antigen recognition mechanism, responding to lipid antigens presented by

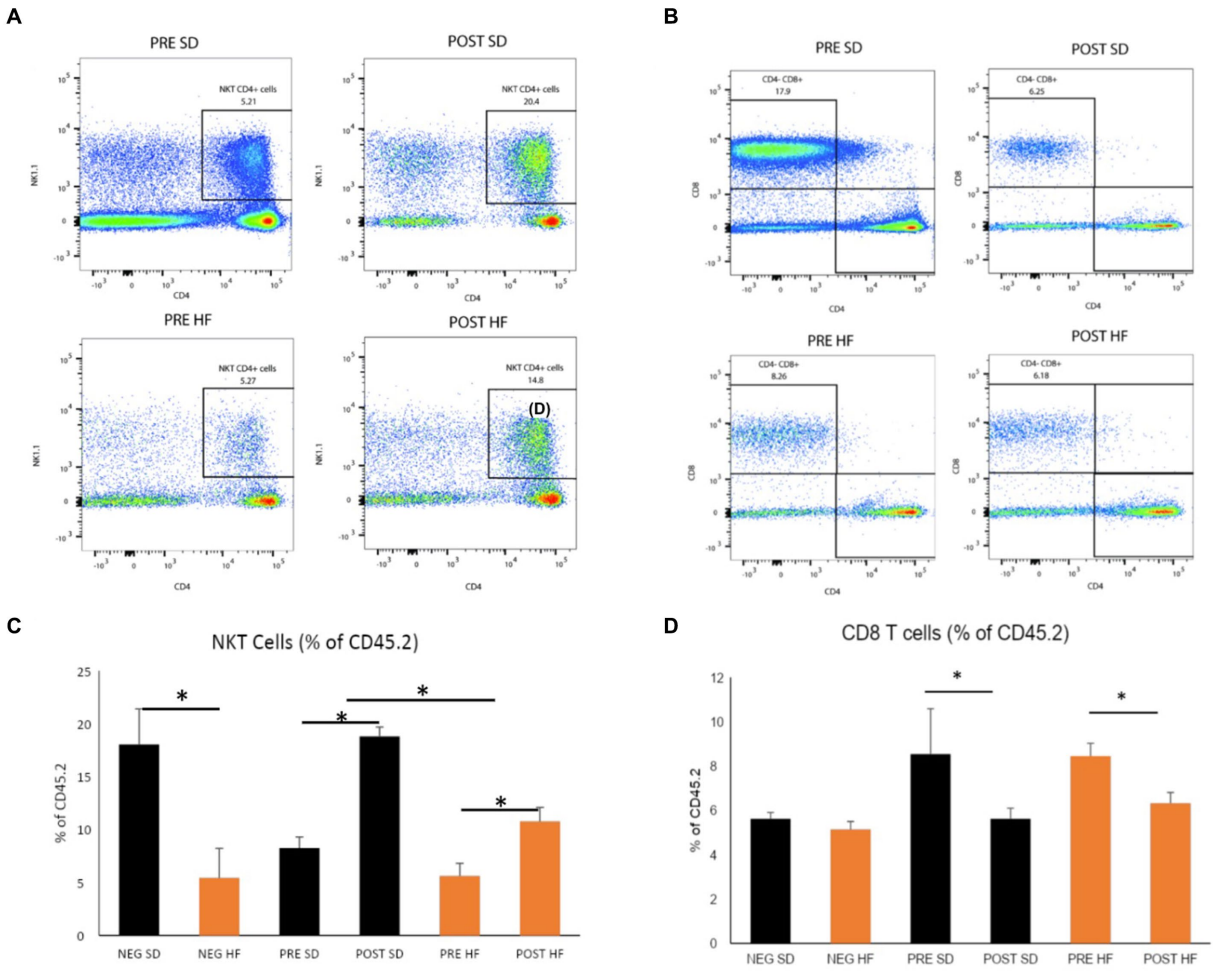

FIGURE 6
Natural killer T and CD8 T cells are altered by microbial changes post-bariatric surgery. Flow cytometry data displayed in density plot for four groups: Mice who received pre-surgery stool and were on a standard diet (Pre SD); mice who received post-surgery stool and were on a high fat diet (Pre HFD); mice who received post-surgery stool and were on a standard diet (Post SD); mice who received post-surgery stool and were on a high fat diet (Post SD). **(A)** Comparison of NKT cell count in black square box labeled "NKT CD4+ Cells." Mice who received post-surgery stool had a higher count of NKT cells compared to the pre-surgery group on either diet. **(B)** Comparison of CD8 T cells cell count in black square box labeled "CD4+ CD8 + ." Mice who received post-surgery stool had a lower count of CD8 T cells compared to the pre-surgery group on either diet. **(C)** Quantification by percentage of density plot **(A)**. **(D)** Quantification by percentage of 606 density plot **(B)**. "*" indicates *p*-value<0.05.

CD1d molecules. This specialized characteristic suggests their potential involvement in the pathogenesis of fatty liver diseases, where lipid accumulation serves as a hallmark feature.

In the studies presented in this paper, mice with the pre-surgery microbiome had higher weight gain and lower levels of NKT cells, worsened fatty liver pathogenesis, and increased insulin resistance as compared to their counterparts. This trend is consistent with those demonstrated in earlier findings. Previous murine studies consistently reveal a decrease in NKT cells in obese phenotypes (Lynch et al., 2012; Subramanian et al., 2018). It should be noted, however, that there exists unclear information about the role of NKT cells in obesity; while murine models consistently show a depletion in NKT cells, several human studies have conflicted results (Ohmura et al., 2010). This is possibly due to the stage at which human MASLD is clinically evaluated compared to murine studies. Existing murine models focus on the inhibitory role of NKT cells in early stages of fibrosis while later human studies suggest the promotion of progression in later stages of fibrosis (Teige et al., 2010; Lee et al., 2022). A recently published paper using knockdown and overexpression of CD1d, showed that overexpression of CD1d was protective in human and mouse MASLD models (Lei et al., 2024).

Kupffer cells, the resident macrophages of the liver, are regulated by the recruitment of inflammatory cytokines by cytotoxic T cells (CTL) (Kolios, 2006; Zhang and Bansal, 2020). Kupffer cells were significantly decreased in post-surgery mice in either group, indicating a relationship between the transplanted microbiome and macrophage activation. Active macrophages further enhance the pro-inflammatory environment by amplifying the cytokine response (Kolios, 2006). It is likely that dysbiosis in obesity phenotypes may contribute to the recruitment of cytokines by CTL and subsequent activation of Kupffer cells (Krijgsman et al., 2018; Zhang and Bansal, 2020). This could have a dual impact on the increase in cytokine secretion and depletion in NKT cells.

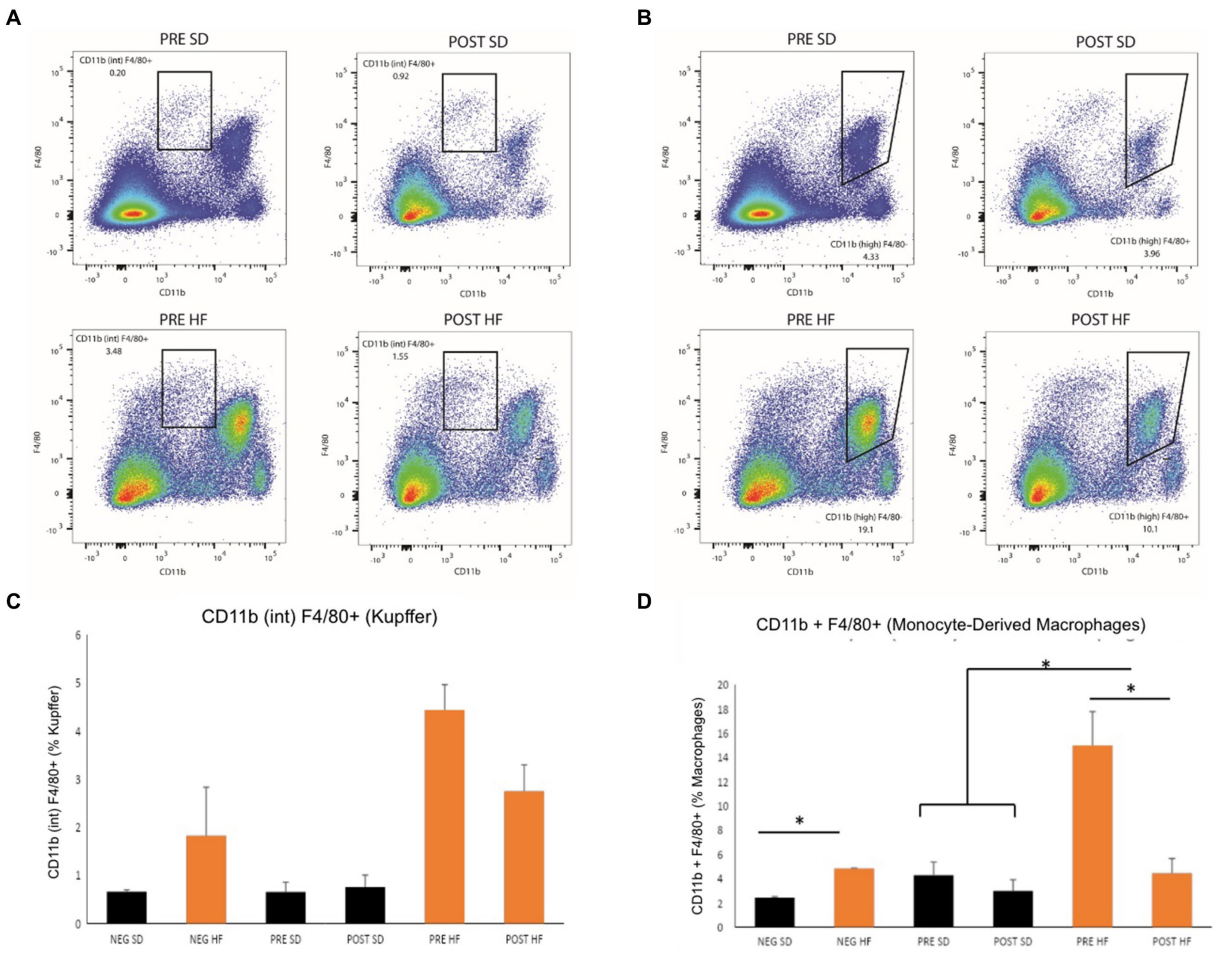

FIGURE 7
Mice in pre-surgery group have higher level Kupffer cells and macrophages. Flow cytometry data displayed in density plot for four groups: Mice who received pre-surgery stool and were on a standard diet (Pre SD); mice who received post-surgery stool and were on a high fat diet (Pre HFD); mice who received post-surgery stool and were on a standard diet (Post SD); mice who received post-surgery stool and were on a high fat diet (Post SD). **(A)** Comparison of Kupffer cell count in black square box labeled "CD11b (int) F4/80." Mice who received post-surgery stool had a visually lower quantity of Kupffer cells than mice in the pre-surgery group on either diet. **(B)** Comparison of Monocyte-Derived macrophage count in black square box labeled "CD11b (high)." Mice who received post-surgery stool had a lower count of macrophages compared to the pre-surgery group on either diet. **(C)** Quantification by percentage of density plot **(A)**. **(D)** Quantification by percentage of 606 density plot **(B)**. "*" indicates p-value<0.05.

In conclusion, the findings in this study suggest that bariatric surgery induces microbiome changes that interacts with the host innate immune system, influencing the progression of MASLD. The human cohort study confirms that the microbiome of an obese individual undergoes distinct changes after bariatric surgery both physically and with biological markers of obesity. These findings were replicated in the mouse model using fecal transplants from qualifying cohort members that recovered from MASLD. Mice who received the pre-surgery stool reflected an obese phenotype with greater inflammatory markers than those who received the post-surgery stool, regardless of diet. Quantification of innate immune cells using flow cytometry suggests a depletion of NKT cells in mice receiving the pre-surgery stool, paired with an increase in pro-inflammatory cytokines, findings that were opposite for the post-surgery group. These findings suggest that NKT cells may have a protective role early on against the development MASLD.

4.1 Limitations

While our study offers valuable insights into the interplay between microbiome and host innate system on obesity and MASLD, several limitations should be considered. First, despite the observed association between the study phenotypes and their respective immune profile, the exact molecular mechanisms underlying the influence of microbiome changes on NKT cell signaling remain a subject of ongoing investigation. While we tried to explore as many immune cells as we could, not all immune cells were studied. For example, regulatory T cells play a significant role in inflammation and may play a role in MASLD. Therefore, it is possible that other immune cells not measured may play a critical role between the microbiome and MASLD. It should also be noted that microbial transfer has pleiotropic effects that extend beyond the scope of this study. These effects could include, but are not limited to, alterations to bile acids,

SCFAs, and hormone signaling pathways. Future studies should involve manipulating NKT cell activity through knockdown and overexpression techniques within a similar microbial transfer model. This will help determine whether NKT cell expression is a crucial mediator between the gut microbiome and MASLD.

Data availability statement

Raw sequencing data from the human cohort are available on the NIH NCBI BioProject (PRJNA885868) https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA885868.

Ethics statement

The studies involving humans were approved by University of California, Los Angeles Institutional Review Board (IRB #13-001552). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by UCLA Animal Research Committee and the Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SS: Visualization, Writing – original draft, Writing – review & editing. WK: Investigation, Visualization, Writing – original draft, Writing – review & editing. JY: Methodology, Investigation, Writing – original draft, Writing – review & editing. CC: Methodology, Investigation, Writing – original draft, Writing – review & editing. NA-J: Investigation, Writing – original draft, Writing – review & editing. VL: Investigation, Writing – original draft, Writing – review & editing. AB: Investigation, Writing – original draft, Writing – review & editing. YC: Investigation, Writing – original draft, Writing – review & editing. ED: Investigation, Writing – original draft, Writing – review & editing. ZL: Methodology, Writing – original draft, Writing – review & editing. EM: Methodology, Funding Acquisition, Writing – original draft, Writing – review & editing. JP: Conceptualization, Writing – original draft, Writing – review & editing. CS: Methodology, Investigation, Supervision, Funding Acquisition, Writing – original draft, Writing – review & editing. SP: Visualization, Writing – original draft, Writing – review & editing. DZ: Visualization, Writing – original draft, Writing – review & editing. ML: Writing – original draft, Writing – review & editing. LH: Visualization, Writing – original draft, Writing – review & editing. JJ: Conceptualization, Visualization, Project Administration, Supervision, Writing – original draft, Writing – review & editing. TD: Conceptualization, Methodology, Investigation, Visualization, Funding Acquisition, Project Administration, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Afzaal, M., Saeed, F., Shah, Y. A., Hussain, M., Rabail, R., Socol, C. T., et al. (2022). Human gut microbiota in health and disease: unveiling the relationship. *Front. Microbiol.* 13:999001. doi: 10.3389/fmicb.2022.999001

Aoun, A., Darwish, F., and Hamod, N. (2020). The influence of the gut microbiome on obesity in adults and the role of probiotics, prebiotics, and synbiotics for weight loss. *Prev. Nutr. Food Sci.* 25, 113–123. doi: 10.3746/pnf.2020.25.2.113

Bai, Y. M., Liang, S., and Zhou, B. (2023). Revealing immune infiltrate characteristics and potential immune-related genes in hepatic fibrosis: based on bioinformatics, transcriptomics and q-PCR experiments. *Front. Immunol.* 14:1133543. doi: 10.3389/fimmu.2023.1133543

Bashiardes, S., Shapiro, H., Rozin, S., Shibolet, O., and Elinav, E. (2016). Non-alcoholic fatty liver and the gut microbiota. *Mol. Metab.* 5, 782–794. doi: 10.1016/j.molmet.2016.06.003

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B Methodol.* 57, 289–300. doi: 10.1111/j.2517-6161.1995.tb02031.x

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Carter, J. K., and Friedman, S. L. (2022). Hepatic stellate cell-immune interactions in NASH. *Front. Endocrinol.* 13:867940. doi: 10.3389/fendo.2022.867940

Casimiro, I., Hanlon, E. C., White, J., De Leon, A., Ross, R., Moise, K., et al. (2020). Reduction of IL-6 gene expression in human adipose tissue after sleeve gastrectomy surgery. *Obes. Sci. Pract.* 6, 215–224. doi: 10.1002/osp4.396

Chang, C. C., Wu, C. L., Su, W. W., Shih, K. L., Tarng, D. C., Chou, C. T., et al. (2015). Interferon gamma-induced protein 10 is associated with insulin resistance and incident diabetes in patients with nonalcoholic fatty liver disease. *Sci. Rep.* 5:10096. doi: 10.1038/srep10096

Chiang, D. J., Pritchard, M. T., and Nagy, L. E. (2011). Obesity, diabetes mellitus, and liver fibrosis. *Am. J. Physiol-Gastrointest. Liver Physiol.* 300, G697–G702. doi: 10.1152/ajpgi.00426.2010

Chopyk, D. M., and Grakoui, A. (2020). Contribution of the intestinal microbiome and gut barrier to hepatic disorders. *Gastroenterology* 159, 849–863. doi: 10.1053/j.gastro.2020.04.077

Courcoulas, A. P., Yanovski, S. Z., Bonds, D., Eggerman, T. L., Horlick, M., Staten, M. A., et al. (2014). Long-term outcomes of bariatric surgery: a National Institutes of Health Symposium. *JAMA Surg.* 149, 1323–1329. doi: 10.1001/jamasurg.2014.2440

Ding, Y., Deng, Q., Yang, M., Niu, H., Wang, Z., and Xia, S. (2023). Clinical classification of obesity and implications for metabolic dysfunction-associated fatty liver disease and treatment. *Diabetes Metab. Syndr. Obes.* 16, 3303–3329. doi: 10.2147/DMSO.S431251

Dong, T. S., Katzka, W., Lagishetty, V., Luu, K., Hauer, M., Pisegna, J., et al. (2020). A microbial signature identifies advanced fibrosis in patients with chronic liver disease mainly due to NAFLD. *Sci. Rep.* 10:2771. doi: 10.1038/s41598-020-59535-w

Dong, T. S., Luu, K., Lagishetty, V., Sedighian, F., Woo, S. L., Dreskin, B. W., et al. (2021). The intestinal microbiome predicts weight loss on a calorie-restricted diet and is associated with improved hepatic steatosis. *Front. Nutr.* 8:718661. doi: 10.3389/fnut.2021.718661

Dowds, C. M., Kornell, S. C., Blumberg, R. S., and Zeissig, S. (2014). Lipid antigens in immunity. *Biol. Chem.* 395, 61–81. doi: 10.1515/hsz-2013-0220

Duan, Y., Pan, X., Luo, J., Xiao, X., Li, J., Bestman, P. L., et al. (2022). Association of inflammatory cytokines with non-alcoholic fatty liver disease. *Front. Immunol.* 13:880298. doi: 10.3389/fimmu.2022.880298

Durack, J., and Lynch, S. V. (2019). The gut microbiome: relationships with disease and opportunities for therapy. *J. Exp. Med.* 216, 20–40. doi: 10.1084/jem.20180448

Fallowfield, J., and Hayes, P. (2011). Pathogenesis and treatment of hepatic fibrosis: is cirrhosis reversible? *Clin. Med.* 11, 179–183. doi: 10.7861/clinmedicine.11-2-179

Ghosh, S., Whitley, C. S., Haribabu, B., and Jala, V. R. (2021). Regulation of intestinal barrier function by microbial metabolites. *Cell. Mol. Gastroenterol. Hepatol.* 11, 1463–1482. doi: 10.1016/j.jcmgh.2021.02.007

Gletsu-Miller, N., and Wright, B. N. (2013). Mineral malnutrition following bariatric surgery. *Adv. Nutr.* 4, 506–517. doi: 10.3945/an.113.004341

Glueck, D. H., Mandel, J., Karimpour-Fard, A., Hunter, L., and Muller, K. E. (2008). Exact calculations of average power for the Benjamini-Hochberg procedure. *Int. J. Biostat.* 4:3. doi: 10.2202/1557-4679.1103

Gulinac, M., Miteva, D. G., Peshevska-Sekulovska, M., Novakov, I. P., Antovic, S., Peruhova, M., et al. (2023). Long-term effectiveness, outcomes and complications of bariatric surgery. *World J. Clin. Cases* 11, 4504–4512. doi: 10.12998/wjcc.v11.i19.4504

Hildreth, A. D., Padilla, E. T., Tafti, R. Y., Legala, A. R., and O'Sullivan, T. E. (2023). Sterile liver injury induces a protective tissue-resident cDC1-ILC1 circuit through cDC1-intrinsic cGAS-STING-dependent IL-12 production. *Cell Rep.* 42:112141. doi: 10.1016/j.celrep.2023.112141

Hruby, A., and Hu, F. B. (2015). The epidemiology of obesity: a big picture. *PharmacoEconomics* 33, 673–689. doi: 10.1007/s40273-014-0243-x

Huby, T., and Gautier, E. L. (2022). Immune cell-mediated features of non-alcoholic steatohepatitis. *Nat. Rev. Immunol.* 22, 429–443. doi: 10.1038/s41577-021-00639-3

Hussain, S. K., Dong, T. S., Agopian, V., Pisegna, J. R., Durazo, F. A., Enayati, P., et al. (2020). Dietary protein, fiber and coffee are associated with small intestine microbiome composition and diversity in patients with liver cirrhosis. *Nutrients* 12:1395. doi: 10.3390/nu12051395

Ismail, M., and Pinzani, M. (2009). Reversal of liver fibrosis. *Saudi J. Gastroenterol.* 15, 72–79. doi: 10.4103/1319-3767.45072

Kennedy, E. A., King, K. Y., and Baldridge, M. T. (2018). Mouse microbiota models: comparing germ-free mice and antibiotics treatment as tools for modifying gut bacteria. *Front. Physiol.* 9:1534. doi: 10.3389/fphys.2018.01534

Kolios, G. (2006). Role of Kupffer cells in the pathogenesis of liver disease. *World J. Gastroenterol.* 12, 7413–7420. doi: 10.3748/wjg.v12.i46.7413

Krijgsman, D., Hokland, M., and Kuppen, P. J. K. (2018). The role of natural killer T cells in Cancer—a phenotypical and functional approach. *Front. Immunol.* 9:367. doi: 10.3389/fimmu.2018.00367

Kumar, V., and Delovitch, T. L. (2014). Different subsets of natural killer T cells may vary in their roles in health and disease. *Immunology* 142, 321–336. doi: 10.1111/imm.12247

Lee, S. W., Park, H. J., Van Kaer, L., and Hong, S. (2022). Roles and therapeutic potential of CD1d-restricted NKT cells in inflammatory skin diseases. *Front. Immunol.* 13:979370. doi: 10.3389/fimmu.2022.979370

Lei, Z., Yu, J., Wu, Y., Shen, J., Lin, S., Xue, W., et al. (2024). CD1d protects against hepatocyte apoptosis in non-alcoholic steatohepatitis. *J. Hepatol.* 80, 194–208. doi: 10.1016/j.jhep.2023.10.025

Lozupone, C., and Knight, R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* 71, 8228–8235. doi: 10.1128/AEM.71.12.8228-8235.2005

Lynch, L., Nowak, M., Varghese, B., Clark, J., Hogan, A. E., Toxavidis, V., et al. (2012). Adipose tissue invariant NKT cells protect against diet-induced obesity and metabolic disorder through regulatory cytokine production. *Immunity* 37, 574–587. doi: 10.1016/j.immuni.2012.06.016

Ma, C., Han, M., Heinrich, B., Fu, Q., Zhang, Q., Sandhu, M., et al. (2018). Gut microbiome–mediated bile acid metabolism regulates liver cancer via NKT cells. *Science* 360:2. doi: 10.1126/science.aan5931

Mallick, H., Rahnavard, A., McIver, L. J., Ma, S., Zhang, Y., Nguyen, L. H., et al. (2021). Multivariable association discovery in population-scale meta-omics studies. *PLoS Comput. Biol.* 17:e1009442. doi: 10.1371/journal.pcbi.1009442

Martino, C., Morton, J. T., Marotz, C. A., Thompson, L. R., Tripathi, A., Knight, R., et al. (2019). A novel sparse compositional technique reveals microbial perturbations. *mSystems* 4:e00016-19. doi: 10.1128/mSystems.00016-19

Melamud, M. M., Ermakov, E. A., Boiko, A. S., Kamaeva, D. A., Sizikov, A. E., Ivanova, S. A., et al. (2022). Multiplex analysis of serum cytokine profiles in systemic lupus erythematosus and multiple sclerosis. *Int. J. Mol. Sci.* 23:13829. doi: 10.3390/ijms232213829

Muratsu, J., Iwabayashi, M., Sanada, F., Taniyama, Y., Otsu, R., Rakugi, H., et al. (2017). Hepatocyte growth factor prevented high-fat diet-induced obesity and improved insulin resistance in mice. *Sci. Rep.* 7:130. doi: 10.1038/s41598-017-00199-4

Nilsson, J., Hörnberg, M., Schmidt-Christensen, A., Linde, K., Nilsson, M., Carlus, M., et al. (2020). NKT cells promote both type 1 and type 2 inflammatory responses in a mouse model of liver fibrosis. *Sci. Rep.* 10:21778. doi: 10.1038/s41598-020-78688-2

Ohmura, K., Ishimori, N., Ohmura, Y., Tokuhara, S., Nozawa, A., Horii, S., et al. (2010). Natural killer T cells are involved in adipose tissues inflammation and glucose intolerance in diet-induced obese mice. *Arterioscler. Thromb. Vasc. Biol.* 30, 193–199. doi: 10.1161/ATVBAHA.109.198614

Park, H. S., Park, J. Y., and Yu, R. (2005). Relationship of obesity and visceral adiposity with serum concentrations of CRP, TNF-alpha and IL-6. *Diabetes Res. Clin. Pract.* 69, 29–35. doi: 10.1016/j.diabres.2004.11.007

Pouwels, S., Sakran, N., Graham, Y., Leal, A., Pintar, T., Yang, W., et al. (2022). Non-alcoholic fatty liver disease (NAFLD): a review of pathophysiology, clinical management and effects of weight loss. *BMC Endocr. Disord.* 22:63. doi: 10.1186/s12902-022-00980-1

SAGES Guidelines Committee (2008). Guidelines for clinical application of laparoscopic bariatric surgery [internet]. Available at: https://www.sages.org/publications/guidelines/guidelines-for-clinical-application-of-laparoscopic-bariatric-surgery/

Seeberg, K. A., Borgeraas, H., Hofsø, D., Småstuen, M. C., Kvan, N. P., Grimnes, J. O., et al. (2022). Gastric bypass versus sleeve gastrectomy in type 2 diabetes: effects on hepatic steatosis and fibrosis: a randomized controlled trial. *Ann. Intern. Med.* 175, 74–83. doi: 10.7326/M21-1962

Subramanian, S., Goodspeed, L., Wang, S., Ding, Y., O'Brien, K., Getz, G., et al. (2018). Deficiency of invariant natural killer T cells does not protect against obesity but exacerbates atherosclerosis in Ldlr−/− mice. *Int. J. Mol. Sci.* 19:510. doi: 10.3390/ijms19020510

Tarantino, G., Citro, V., Balsano, C., and Capone, D. (2021). Age and Interleukin-15 levels are independently associated with intima-media thickness in obesity-related NAFLD patients. *Front. Med.* 8:634962. doi: 10.3389/fmed.2021.634962

Teige, A., Bockermann, R., Hasan, M., Olofsson, K. E., Liu, Y., and Issazadeh-Navikas, S. (2010). CD1d-dependent NKT cells play a protective role in acute and chronic arthritis models by ameliorating antigen-specific Th1 responses. *J. Immunol.* 185, 345–356. doi: 10.4049/jimmunol.0901693

The GBD 2015 Obesity Collaborators (2017). Health effects of overweight and obesity in 195 countries over 25 years. *N. Engl. J. Med.* 377, 13–27. doi: 10.1056/NEJMoa1614362

Tong, M., Jacobs, J. P., McHardy, I. H., and Braun, J. (2014). Sampling of intestinal microbiota and targeted amplification of bacterial 16S rRNA genes for microbial ecologic analysis. *Curr. Protoc. Immunol.* 107, 7.41.1–7.41.11. doi: 10.1002/0471142735.im0741s107

Tremaroli, V., Karlsson, F., Werling, M., Ståhlman, M., Kovatcheva-Datchary, P., Olbers, T., et al. (2015). Roux-en-Y gastric bypass and vertical banded gastroplasty induce long-term changes on the human gut microbiome contributing to fat mass regulation. *Cell Metab.* 22, 228–238. doi: 10.1016/j.cmet.2015.07.009

Younossi, Z. M., Koenig, A. B., Abdelatif, D., Fazel, Y., Henry, L., and Wymer, M. (2016). Global epidemiology of nonalcoholic fatty liver disease—meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* 64, 73–84. doi: 10.1002/hep.28431

Zafar, H., and Saier, M. H. (2021). Gut *Bacteroides* species in health and disease. *Gut Microbes* 13, 1–20. doi: 10.1080/19490976.2020.1848158

Zhang, L., and Bansal, M. B. (2020). Role of Kupffer cells in driving hepatic inflammation and fibrosis in HIV infection. *Front. Immunol.* 11:1086. doi: 10.3389/fimmu.2020.01086

Zheng, D., Liwinski, T., and Elinav, E. (2020). Interaction between microbiota and immunity in health and disease. *Cell Res.* 30, 492–506. doi: 10.1038/s41422-020-0332-7

Zheng, S., Yang, W., Yao, D., Tang, S., Hou, J., and Chang, X. (2022). A comparative study on roles of natural killer T cells in two diet-induced non-alcoholic steatohepatitis-related fibrosis in mice. *Ann. Med.* 54, 2232–2244. doi: 10.1080/07853890.2022.2108894

Zhou, B., Dong, C., Zhao, B., Lin, K., Tian, Y., Zhang, R., et al. (2022). *Bacteroides fragilis* participates in the therapeutic effect of methotrexate on arthritis through metabolite regulation. *Front. Microbiol.* 13:1015130. doi: 10.3389/fmicb.2022.1015130

Zsálig, D., Berta, A., Tóth, V., Szabó, Z., Simon, K., Figler, M., et al. (2023). A review of the relationship between gut microbiome and obesity. *Appl. Sci.* 13:610. doi: 10.3390/app13010610

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The integrated analysis of gut microbiota and metabolome revealed steroid hormone biosynthesis is a critical pathway in liver regeneration after 2/3 partial hepatectomy

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Introduction: The liver is the only organ capable of full regeneration in mammals. However, the exact mechanism of gut microbiota and metabolites derived from them relating to liver regeneration has not been fully elucidated.

Methods: To demonstrate how the gut-liver axis contributes to liver regeneration, using an LC-QTOF/MS-based metabolomics technique, we examine the gut microbiota-derived metabolites in the gut content of C57BL/6J mice at various points after 2/3 partial hepatectomy (PHx). Compound identification, multivariate/univariate data analysis and pathway analysis were performed subsequently. The diversity of the bacterial communities in the gastrointestinal content was measured using 16S rRNA gene sequencing. Then, the integration analysis of gut microbiota and metabolome was performed.

Results: After 2/3 PHx, the residual liver proliferated quickly in the first 3 days and had about 90% of its initial weight by the seventh day. The results of PLS-DA showed that a significant metabolic shift occurred at 6 h and 36 h after 2/3 PHx that was reversed at the late phase of liver regeneration. The α and β-diversity of the gut microbiota significantly changed at the early stage of liver regeneration. Specifically, *Escherichia Shigella*, *Lactobacillus*, *Akkermansia*, and *Muribaculaceae* were the bacteria that changed the most considerably during liver regeneration. Further pathway analysis found the most influenced co-metabolized pathways between the host and gut bacteria including glycolysis, the TCA cycle, arginine metabolism, glutathione metabolism, tryptophan metabolism, and purine and pyrimidine metabolism. Specifically, steroid hormone biosynthesis is the most significant pathway of the host during liver regeneration.

Discussion: These findings revealed that during liver regeneration, there was a broad modification of gut microbiota and systemic metabolism and they were strongly correlated. Targeting specific gut bacterial strains, especially increasing the abundance of *Akkermansia* and decreasing the abundance of *Enterobacteriaceae*, may be a promising beneficial strategy to modulate systemic metabolism such as amino acid and nucleotide metabolism and promote liver regeneration.

KEYWORDS

steroid hormone biosynthesis, liver regeneration, 2/3 partial hepatectomy, gut microbiota, metabolomics

1 Introduction

The liver is the largest organ of the mammal's internal organs and is mainly responsible for maintaining internal balance, involving the elimination of waste, the storage and balancing of glucose and glycogen, the regulation of amino acid metabolism, detoxification, drug metabolism, and more. The liver is the only internal organ in mammals with the amazing capacity to regenerate to 100% of its initial weight, guaranteeing that the liver-to-weight ratio is consistently maintained at the level necessary for maintaining bodily homeostasis (Tremaroli and Bäckhed, 2012). Liver regeneration is a highly coordinated biological process. Many changes in gene and protein expression, the production of growth factor, and tissue remodeling occurred during this process. Multiple types of cells and extrahepatic factors cooperated during liver regeneration (Mao et al., 2014). Based on this characteristic of the liver, hepatic resection has been established as a safe primary treatment for a variety of diseases of the liver, such as hepatic trauma, intrahepatic gallstones, hepatic cysts, hepatic tumors, and liver transplantation (Rahbari et al., 2021); however, successful restoration of the patient's hepatic function in the postoperative period is critical for their good prognosis and quality of life.

After hepatic resection, hepatocytes that have not undergone final differentiation show remarkable proliferative capacity (Liu et al., 2015), in addition to cytokines, growth factors and mitogens also participate in the process of hepatic regeneration. Energy production and the provision of precursors for the manufacture of chemicals essential for hepatic cell proliferation and tissue remodeling are dependent on active metabolism (Fausto et al., 2012; Michalopoulos, 2014; Monga, 2014). Liver regeneration is accompanied by changes in system metabolism. Metabolic change is a sign of various liver diseases, and liver regeneration after PHx is also characterized by a changed status of metabolism, in which the body undergoes significant changes in metabolites reflected in the serum, liver and urine (Kajiura et al., 2019). Metabolomics is a method of comprehensive and systematic analysis and quantitate of metabolites in biological matrix using high-throughput technologies. A number of studies have been conducted on rodent partial hepatectomy models with metabolomics techniques to explore the alteration of metabolism related to liver regeneration (Kajiura et al., 2019). These studies have pointed out that metabolic pathways including bile acid metabolism have an essential role in the whole complex process of liver regeneration (de Haan et al., 2018).

Gut microecology is essential in regulating metabolic activities (Tremaroli and Bäckhed, 2012; Cani, 2014), modulating immunity (Viaud et al., 2013) and protecting the host from pathogenic microorganisms, etc (Fukuda et al., 2011). Many diseases are at risk due to dysbiosis of the intestinal flora, and in this regard, the connection between the liver and the gut is becoming more well-recognized (Tripathi et al., 2018). The gut-liver axis, which is the integrated functional and physiological relationship between the liver and the gut, is the focus of current research. The intestinal flora is validated can influence the pathophysiology of the liver through the gut-liver axis. Numerous liver illnesses, such as alcoholic liver disease and nonalcoholic fatty liver disease, primary sclerosing cholangitis, and hepatocellular carcinoma, etc. are linked to changes in the composition of the gut microbiota. In addition, gut flora has been shown to influence the regenerative capacity of the liver. Studies have shown that the gut microbiota acts through the gut-liver axis to link a wide range of metabolites to liver regeneration and that these metabolites are important signaling and energy substrates for hepatocytes (Yin et al., 2023). A shift in the gut microbiota ratio results in modifications to the metabolites, which in turn affect hepatocyte proliferation and metabolism, thus reducing liver injury and enhancing liver regeneration (Liu et al., 2016). In general, the etiology of liver disease and the process of liver regeneration are significantly influenced by gut bacteria.

With the development of metabolomics and advanced bioinformatics analysis, the relationship between the functional activity of the gut microbiota and changes in endogenous metabolites [bile acids (Huang et al., 2006), short-chain fatty acids (Yin et al., 2023), tryptophan (Xu et al., 2022), etc.] and liver regeneration has been reported; yet, the connection between the gut microbiota and the metabolites derived directly from them and liver regeneration has not been fully elucidated. In this study, gut microbiota and metabolites in the gut content were analyzed in an integrated manner, and this comprehensive and integrated approach may provide a clearer understanding of the function of gut microbiota and metabolites derived from them and the underlying mechanisms of liver regeneration.

2 Methods

2.1 Animals and treatment

Thirty male C57BL/6J mice, 6 weeks old, were kept in a 12 h light/12 h dark environment (lights on at 6:00 a.m. and lights off at 6:00 p.m.). The mice were acquired from Changzhou Cavens Laboratory Animal Co. (Changzhou, China). The Animal Ethics Committee of Nanjing University Medical School Affiliated Drum Tower Hospital accepted all protocols pertaining to animal care and experimental procedures. Five groups (n = 6) of mice were used: the Sham group (PHx-0h), the PHx-6h group, the PHx-36h group, the PHx-72h group, and the PHx-168h group. The PHx surgery was performed as previously reported (Kong et al., 2018). Briefly, the

mice were anesthetized with isoflurane, the mouse abdomen was skinned and disinfected, then fully exposes the liver and ligate the roots of the left and middle lobes of the liver with 5-0 silk thread respectively and then both lobes are removed. The control group (sham operation group) only underwent laparotomy and suturing. The mice were sacrificed at different time points after PHx. Serum and tissues were collected and used for further analysis. The detailed information can be found in Supplementary Material.

2.2 Untargeted metabolomics of the gut content by LC-QTOF/MS

The same extraction and analysis procedures were applied as previously reported with a few adjustments (Sun et al., 2023a; Sun et al., 2023b). To summarize, 20 mg of gut material was added to 100 μL of normal saline, and 50 μL of the gut content combination was then mixed with 200 μL of methanol that contained 1 μg/mL of 4-chlorophenylalanine as an internal standard (IS). Following a 3-min vortex and 10 min of centrifugation at 12,000 rpm, 200 μL of the supernatant was moved to a fresh Eppendorf tube and dried using an SPD 2010-230 SpeedVac Concentrator (Thermo Savant, Holbrook, United States). The residue was re-dissolved with 100 μL 50% methanol-water and centrifuged at 18,000 rpm for 10 min. Finally, 80 μL supernatant was transferred to an LC vial and 5 μL was injected and analysed. The chromatographic and mass spectrometric parameters were the same as reported before (Sun et al., 2023b). Briefly, the LC-Q/TOF-MS (AB SCIEX TripleTOF® 5600 LC-Q/TOF-MS, Foster City, CA) was used for the untargeted metabolomics analysis both in positive and negative mode. TOF MS ranged from m/z 50–1,200. Then compound identification, multivariate and univariate data analysis were performed. Pathway analysis was carried out using the online metabolomics data analysis tool MetaboAnalyst 6.0 (www.metaboanalyst.ca) with the KEGG library (*Mus musculus*). Enrichment analysis was also performed using chemical structures. The detailed information can be found in Supplementary Material.

2.3 16S rDNA gene sequencing

The 16S rDNA gene amplicon sequencing of gut microbiota was performed by Suzhou PANOMIX Biomedical Tech Co. Ltd. (Suzhou, China) using an Illumina MiSeq platform (Illumina, San Diego, CA, United States) (Sun et al., 2023b). The detailed information can be found in Supplementary Material.

2.4 Integration analysis of microbiome and metabolome

The integration analysis of the microbiome and metabolome was conducted using metorigin (https://metorigin.met-bioinformatics.cn) (Yu et al., 2022). *Mus musculus* (house mouse) was selected as the host.

2.5 Statistical analysis

Statistical analysis was performed using the R project (version 4.2.1) and GraphPad Prism 7.2 software (La Jolla, CA, United States). The data was displayed as mean ± standard deviation. A one-way ANOVA was used to examine group differences, and the Bonferroni *post hoc* test was used afterward. A significance level of $P < 0.05$ was applied to all statistical tests.

3 Results

3.1 The liver regenerated to normal after 2/3 PHx in mice

Serum and liver samples were collected at five time points (0 h, 6 h, 36 h, 72 h, and 168 h) after surgery to inquire about the changes in the gut microbiota and the metabolites during liver regeneration progresses following 2/3 PHx. According to the liver index, the remaining liver grew more quickly in the first 3 days and then reached over 90% of its initial weight again on the seventh day (Figures 1A, B). Following 2/3 PHx, the serum's total bile acids (TBA) considerably rose in the early stages (Figure 1C). ALT (Figure 1D), AST (Figure 1E), and ALP (Figure 1F) were significantly increased during the early stages of liver regeneration and recovered to normal after 72 h.

3.2 A significant metabolic change in the gut content occurred after 2/3 PHx

The typical total ion current (TIC) chromatograms of mouse gut content in positive and negative modes are shown in Supplementary Figure S1. 23,225 and 26,524 chromatographic features were deconvoluted and extracted respectively. The compounds identified include organic acids, amino acids, carbohydrates, purines, pyrimidines and fatty acids. An overview of the metabolomics data was obtained using unsupervised principal component analysis (PCA). In the PCA analysis, no outlier was discovered based on the scatter plots (Figures 2A, B). The partial least squares discriminant analysis (PLSDA) showed that the 6 h group, the 36 h group, and the Sham group were clearly separated from one another, while the 72 h group and the 168 h group were closer to the Sham group (Figures 2C, D). This suggests that during the liver regeneration process, PHx induced a significant metabolic change in the gut content at an early stage and returned to normal at the late stage.

3.3 Metabolites in the gut content changed significantly during liver regeneration after 2/3 PHx

To select the differential metabolites in the gut content during liver regeneration after 2/3 PHx, the average of each of the two groups was divided to determine the fold change (FC), and the Benjamini–Hochberg method was used to adjust the Student's t-test to determine the *P*-value. Metabolites in the gut contents with an FC of greater than 2 or less than 0.5 and a *P*-value of less than 0.05 are considered to be differential metabolites and displayed in the volcano plot (Supplementary Figures S2A–D, positive mode; -Supplementary Figures S3A–D, negative mode). The heat maps of differential metabolites in the gut contents of the positive and negative modes are shown in Supplementary Figures S4, S5,

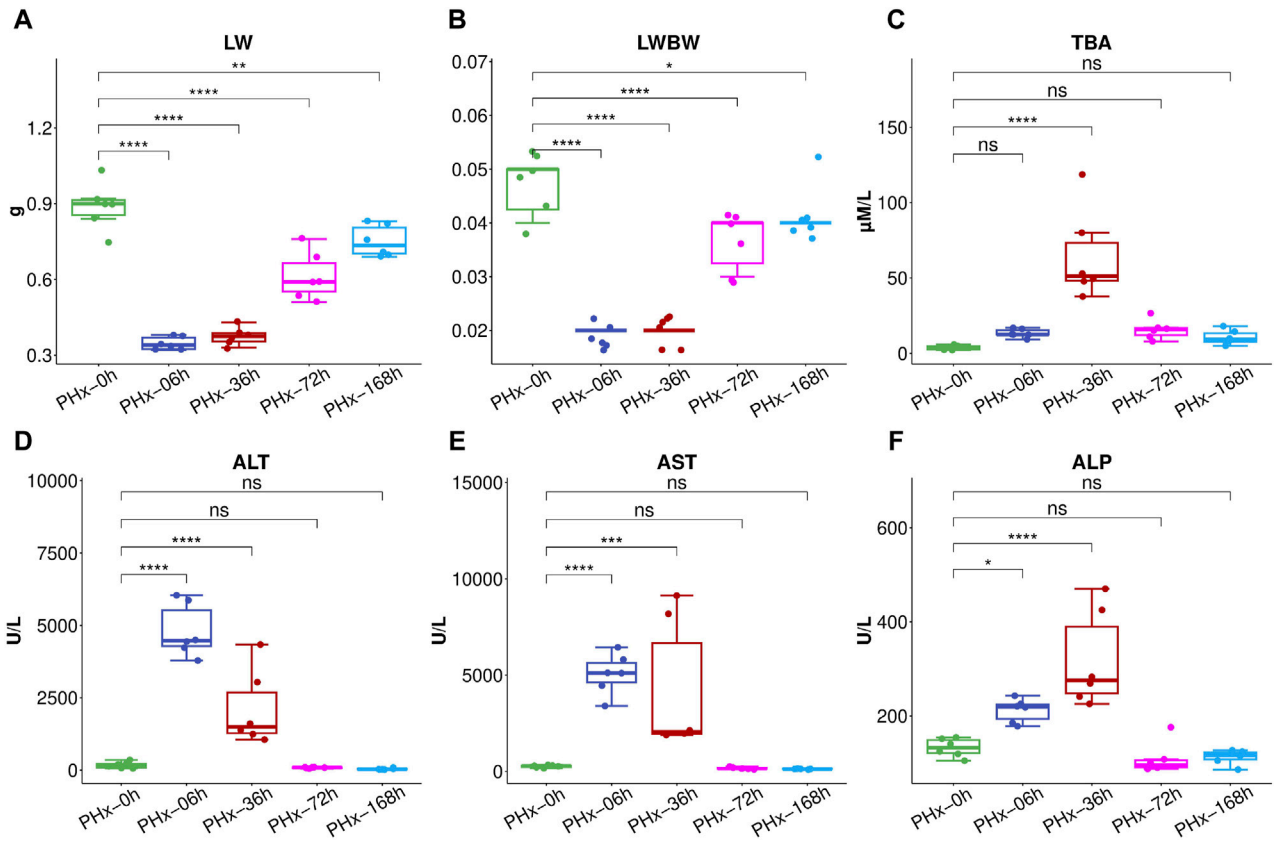

FIGURE 1
After 2/3 PHx, liver weight **(A)** and liver index **(B)** at various phases. The following biomarkers were assessed for the Sham group and 36, 72, and 168 h after PHx: total bile acids (TBA) **(C)**, Alanine aminotransferase (ALT) **(D)**, aspartate aminotransferase (AST) **(E)**, alkaline phosphatase (ALP) **(F)**. The data (n = 6) are displayed as mean ± SD. *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$.

respectively. Specifically, metabolites in TCA cycle (succinic acid), nucleotides (cAMP, AMP, Uridine), amino acids (L-cystine), the steroid hormones (cortisone, corticosterone, deoxycorticosterone and 17-hydroxyprogesterone) all peaked at the early phase (6 h or 36 h) during liver regeneration after 2/3 PHx, and returned to normal at the late phase (Supplementary Figure S6).

3.4 Energy metabolism, amino acid metabolism and nucleotide metabolism are the significantly changed metabolic pathways during liver regeneration after 2/3 PHx

The significantly changed metabolites in the gut content were selected and further analyzed by MetaboAnalyst (www.metaboanalyst.ca). In order to analyze the metabolites of gut contents for overrepresentation and pathway analysis, the differential metabolites were mapped to KEGG metabolic pathways.

The pathway analysis showed that glyoxylate and dicarboxylate metabolism, the citrate cycle (TCA cycle), alanine, aspartate and glutamate metabolism, cysteine and methionine metabolism, purine metabolism and pyrimidine metabolism were the most changed pathways (Figure 3A). Accordingly, the enrichment analysis showed that the most enriched metabolites set including carboxylic acids and derivatives, purine nucleotides/nucleotides, pyrimidine/nucleotides, benzene and substituted derivatives, steroids and steroid derivatives, indoles and derivatives and fatty acyls etc (Figure 3B).

3.5 Alterations of gut microbial diversity and abundance during liver regeneration after 2/3 PHx

16S rRNA sequencing was used to investigate the bacterial communities. The α diversity of gut microbiota is shown in Figure 4A. The Chao1 index and the Shannon index showed that the α diversity of the gut bacteria increased significantly. The PCoA results representing the β diversity (Figure 4B) showed that the composition of microbial communities at various times after 2/3 PHx was significantly separated, indicating a dramatic alteration of the microbial composition. The considerable distance between the PHx-6 h group and the PHx-36 h group with the sham group suggested that the abundance of the microbial community could be obviously modulated during liver regeneration, and the changed abundance of the microbial will return at the late phase of liver regeneration.

The top 20 most prevalent bacteria at the phylum level and family level in the gut contents are presented in Figures 4C, D, respectively. Specifically, compared with the Sham group, At the

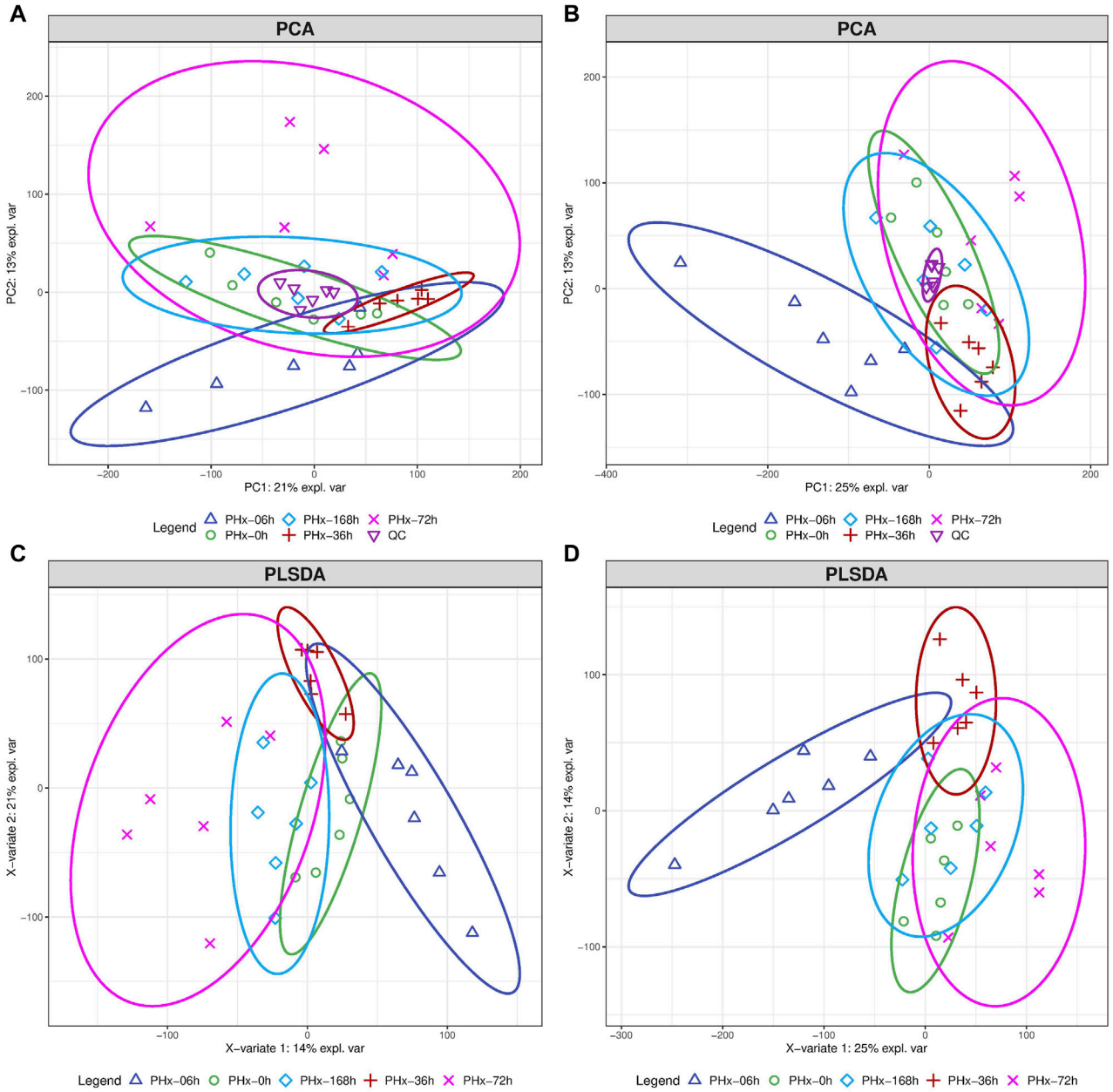

FIGURE 2
The total alteration of metabolites at different phases following a 2/3 PHx. The score plots for PCA model of mouse gut content at positive mode **(A)** and negative mode **(B)**. Score plots for the PLS-DA model's positive mode **(C)** and negative mode **(D)**.

early phase, PHx markedly decreased the relative abundance of *Firmicutes* and *Verrucomicrobia* and increased the relative abundances of *Bacteroidetes* and *Proteobacteria* at the genus level. At the family level, PHx markedly decreased the relative abundance of *Akkermansiaceae* and *Lactobacillales* and increased the relative abundances of *Muribaculaceae* and *Enterobacteriaceae* at the early phase. These changes all returned to normal at the late phase of PHx. Figure 4E shows the biological evolution tree of each group.

LefSe was also used to evaluate the species and relative abundance of gut microbiota in each group. Following PHx, the evolutionary branch diagram displayed the most distinct bacteria at each time point (Figure 5A). The results (Figure 5B) showed that at the phylum level, *Verrucomicrobiota*, *Bacteroidota*, *Proteobacteria*, and *Firmicutes* are the most differentially enriched bacteria in the 0 h, 6 h, 36 h, and 168 h groups, respectively. At the class level, *Verrucomicrobiae*, *Bacteroidia*, *Gammaproteobacteria* and *Bacilli* are the most differentially enriched bacteria in the 0 h, 6 h, 36 h, and 168 h groups, respectively. At the order level, *Verrucomicrobiales*, *Bacteroidales*, *Enterobacterales*, *Bifidobacteriales* and *Lactobacillales* are the most differentially enriched bacteria in the 0 h, 6 h, 36 h, 72 h, and 168 h groups, respectively. At the family level, *Akkermansiaceae*, *Muribaculaceae*, *Enterobacteriaceae*, *Bifidobacteriaceae* and *Lactobacillaceae* are the most differentially enriched bacteria in the 0 h, 6 h, 36 h, 72 h, and 168 h groups, respectively. At the genus level, *Akkermansia*,

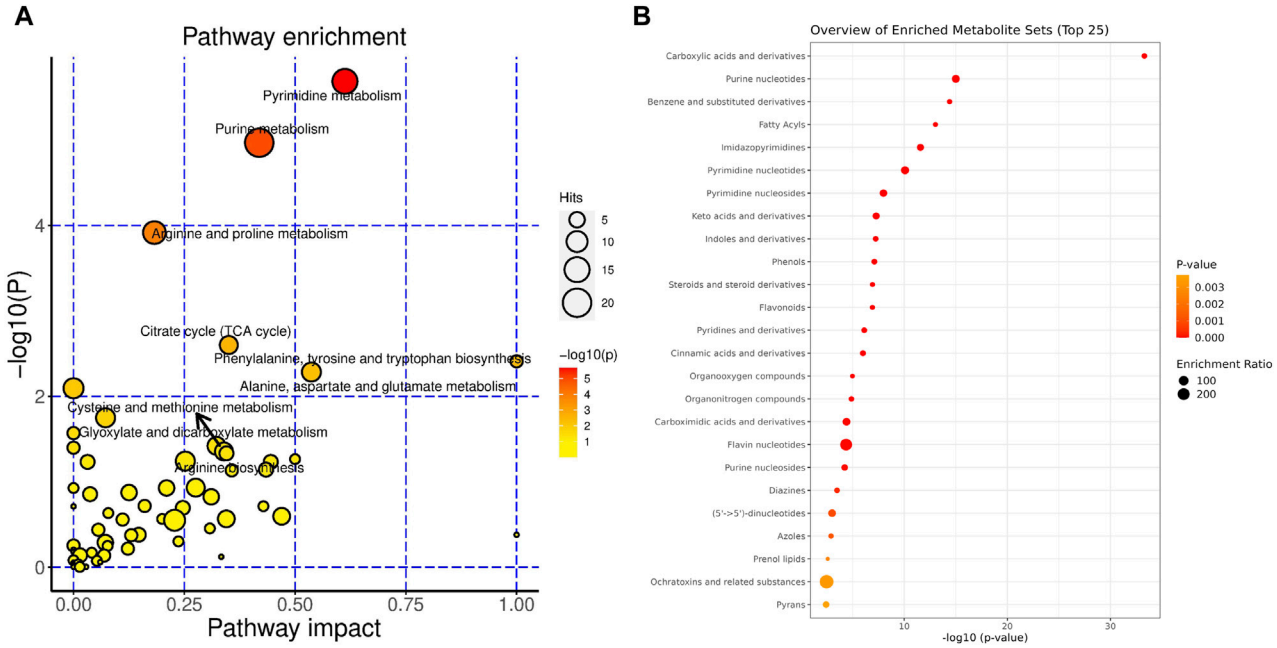

FIGURE 3
Metabolites in the gut content significantly changed during liver regeneration after 2/3 partial hepatectomy. Metabolic pathways that are modulated considerably during liver regeneration: **(A)**. The classification of metabolites that are significantly modulated during liver regeneration **(B)**.

Muribaculaceae, *Escherichia Shigella*, *Bifidobacterium* and *Lactobacillus* were the most differentially enriched bacteria in the 0 h, 6 h, 36 h, 72 h, and 168 h groups, respectively.

3.6 Gut microbiota and metabolites derived from them were closely related

The correlation between the gut microbiota and metabolites derived by them was analyzed, and the metabolites and pathways in the host and microbiota respectively and co-metabolized by the host and microbiota were shown in the venn plot (Figures 6A, B). A total of 130 metabolites and two pathways were co-metabolized by the host and microbiota. Spearman's correlation analysis between the metabolites in the gut contents and the differential bacteria at the genus level was performed in order to better evaluate the possible association between the gut microbiota and metabolites derived by them (Supplementary Figure S7). The correlations of gut bacteria and metabolites were shown in Figures 6C, D for 6 h and 36 h after PHx, respectively. Specifically, at 6 h, morin, sphinganine, oxycodone, L-theanine and prednisone, etc. were negatively correlated with *Mucispirillum*, *Anaerofustis*, *Rikenellaceae*, *Oscillibacter* and *Muribaculum*, whereas positively correlated with *Pelomonas*, *Porphyromonas*, *Corynebacterium*, *Actinomyces* and *Allobaculum*. L-alanine, sebacic acid, N-trans-feruloyltyramine, oxoadipic acid and suberic acid, etc., have the opposite trend with these bacteria (Figure 6C). At 36 h, oleanolic acid, progesterone, thymidine, carvedilol and creatine were negatively correlated with *Klebsiella*, *Erysipelatoclostridiaceae*, *Prevotella 9*, *Enterococcus* and *Oscillibacter*, and positively correlated with *RF39*, *ASF356*, *Enterobacter*, *Dialister* and *Gordonibacter*. Deoxycorticosterone, phenylacetylglycine, DOPA, guanidoacetic acid, trans-muconic acid and 5,7-Dihydroxyflavone etc. have the opposite trend with these bacteria (Figure 6D).

3.7 Network analysis of gut microbiota and metabolites derived by them revealed that steroid hormone biosynthesis is the most important altered metabolic pathway in the host

A co-occurrence network analysis among the differential microbiota and metabolites derived by them was carried out by contrasting the 6 and 36-h groups with the Sham group. Steroid hormone biosynthesis is the most important altered metabolic pathway in the host. Aminobenzoate degradation, degradation of flavonoids and caprolactam degradation are the most important changed metabolic pathways in the bacteria at 6 h after 2/3 PHx. Aminobenzoate degradation and ubiquinone and other terpenoid-quinone biosynthesis are the most important changed metabolic pathways in the bacteria at 36 h after 2/3 PHx. The TCA cycle, glycolysis, tryptophan metabolism, glutathione metabolism, taurine and hypotaurine metabolism, biosynthesis of unsaturated fatty acids, purine and pyrimidine metabolism were the important metabolic pathways co-metabolized by the host and bacteria at 6 h after 2/3 PHx (Figure 7). Glyoxylate and dicarboxylate metabolism, the TCA cycle, vitamin B6 metabolism, purine and pyrimidine metabolism were the important metabolic pathways co-metabolized by the host and bacteria at 36 h after 2/3 PHx (Figure 8). These results suggested that gut microbiota and metabolites derived by them dynamically changed during liver regeneration.

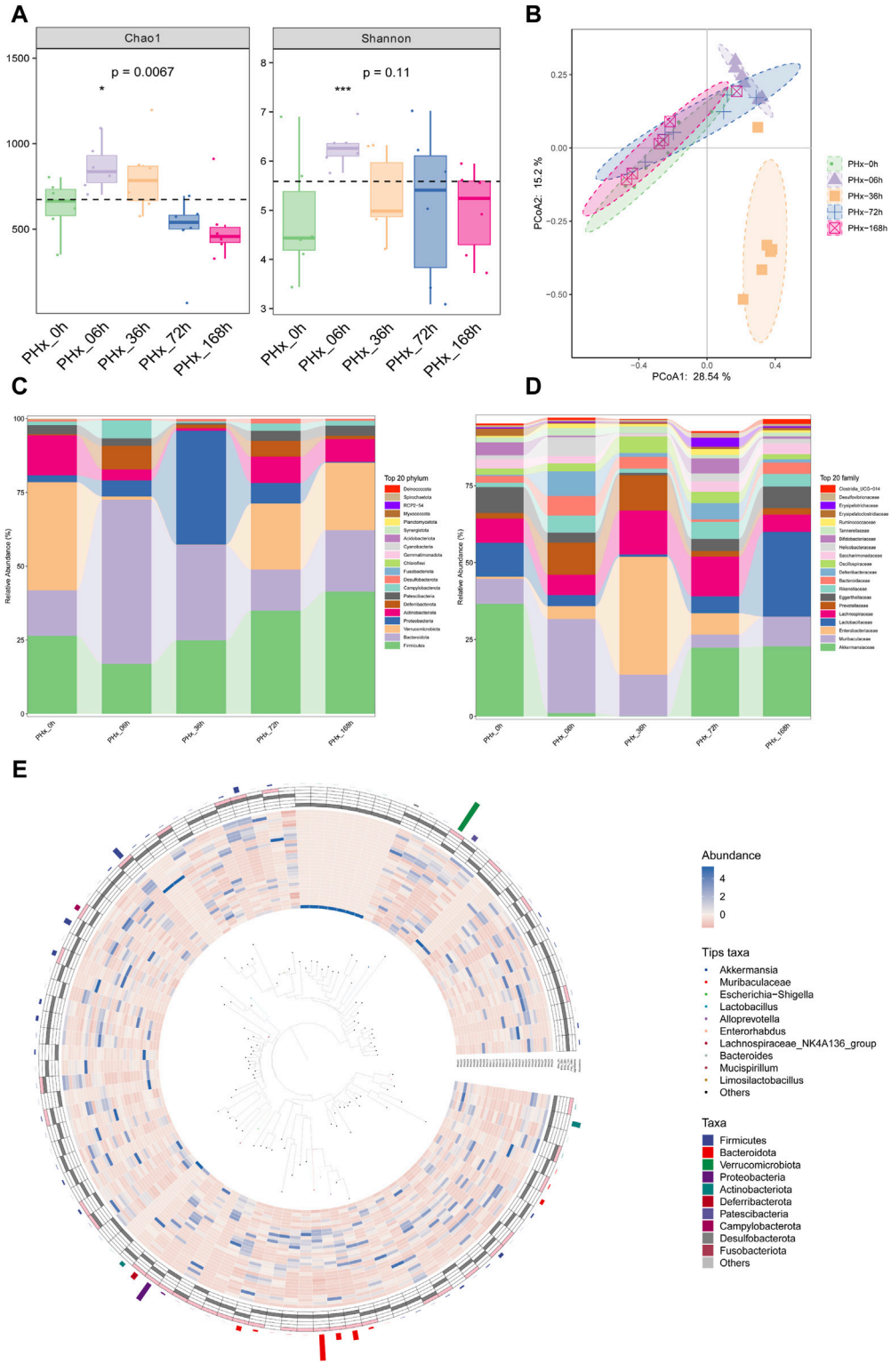

FIGURE 4
Alterations in composition of gut microbiota during liver regeneration after 2/3 partial hepatectomy. **(A)** α diversity of gut microbiota (Chao1 and Shannon index). **(B)** PCoA of β diversity. **(C)** Alteration of gut microbiota composition at phylum levels. **(D)** Alteration of gut microbiota composition at family levels. **(E)** Graphical Phylogenetic Analysis (GraPhlAn) Phylogenetic tree.

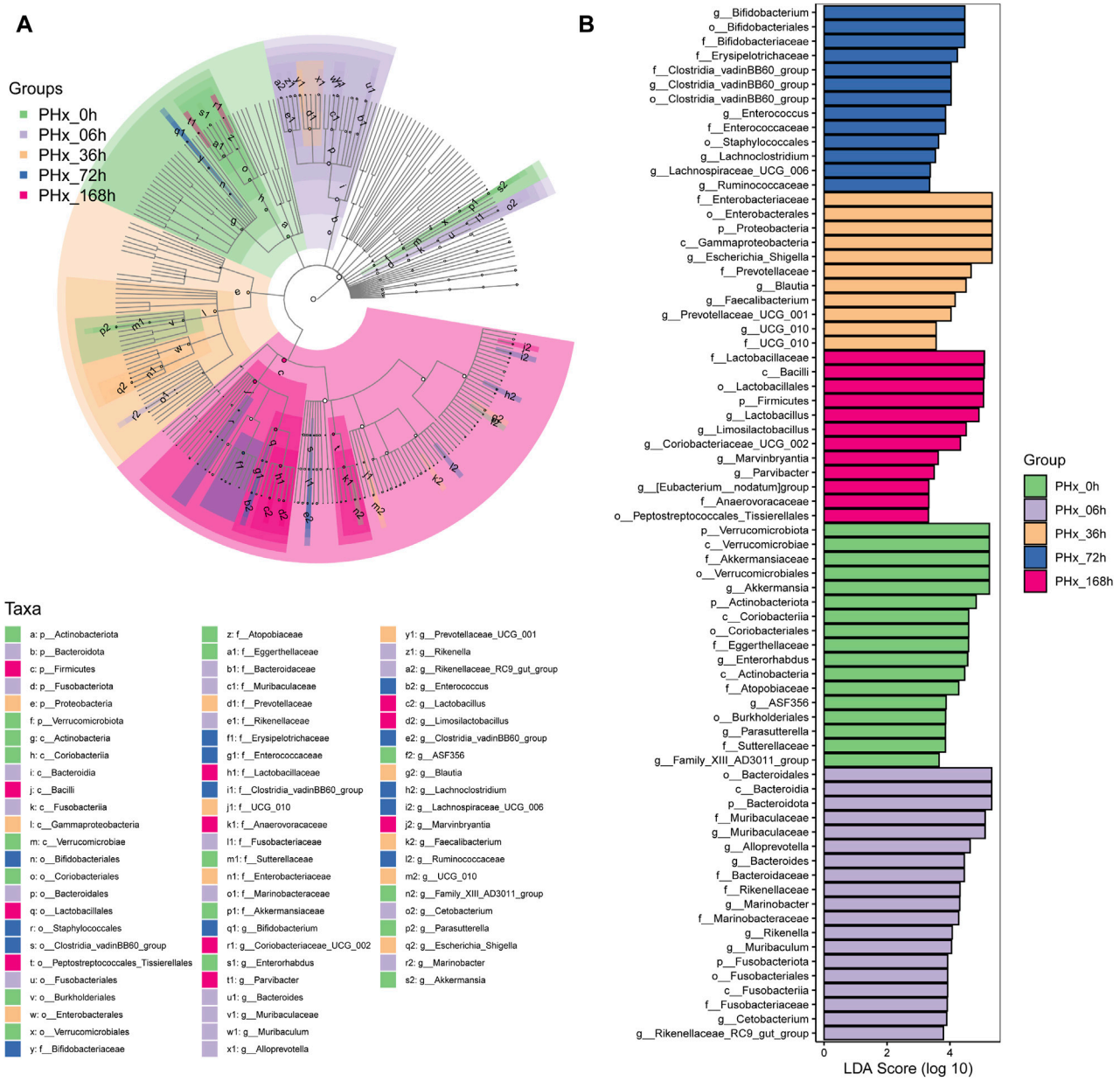

FIGURE 5
Identifying the distinctive taxa that differ the most during liver regeneration following a 2/3 PHx. **(A)** Characteristic taxa are analyzed using linear discriminant analysis (LDA) effect size (LEfSe) across five experimental groups. **(B)** Use LDA to characterize taxa with a threshold score greater than 3.0. The influence of distinctive taxa within specific groups is shown by the bar length of the LDA.

4 Discussion

The complicated biological process of liver regeneration involves successive alterations in cytokines, metabolites, and gene expression, among others. After 2/3 PHx, gut-liver axis interaction is crucial to liver regeneration. Gut microbiota plays a key role in the gut-liver axis and influences many areas of human health and diseases. It is also found to be essential in liver regeneration. Liver regeneration is delayed in Germ-free mice and after the use of antibiotics in normal mice (Cornell et al., 1990; Wu et al., 2015). Reconstitution of normal gut microbiota in GF mice could ameliorate liver regeneration (Amin et al., 2020). Several studies also demonstrate the beneficial effects of probiotics and specific gut microbiota on the promotion of liver regeneration (Xie et al., 2021; He et al., 2023). By stimulating the phospholipid biosynthesis of the hepatic membrane, gut bacteria may contribute to liver regeneration (Yin et al., 2023). These findings demonstrate how crucial gut microbiota is to liver regeneration. However, the underlying mechanisms of gut-liver axis interaction involving gut microbiota in liver regeneration are not fully understood, and the modulation of gut microbiota and the metabolites derived from them after PHx and additional investigation are still required to determine the underlying mechanisms. In this study, we discovered that there was a notable shift in the gut microbiota's composition at different

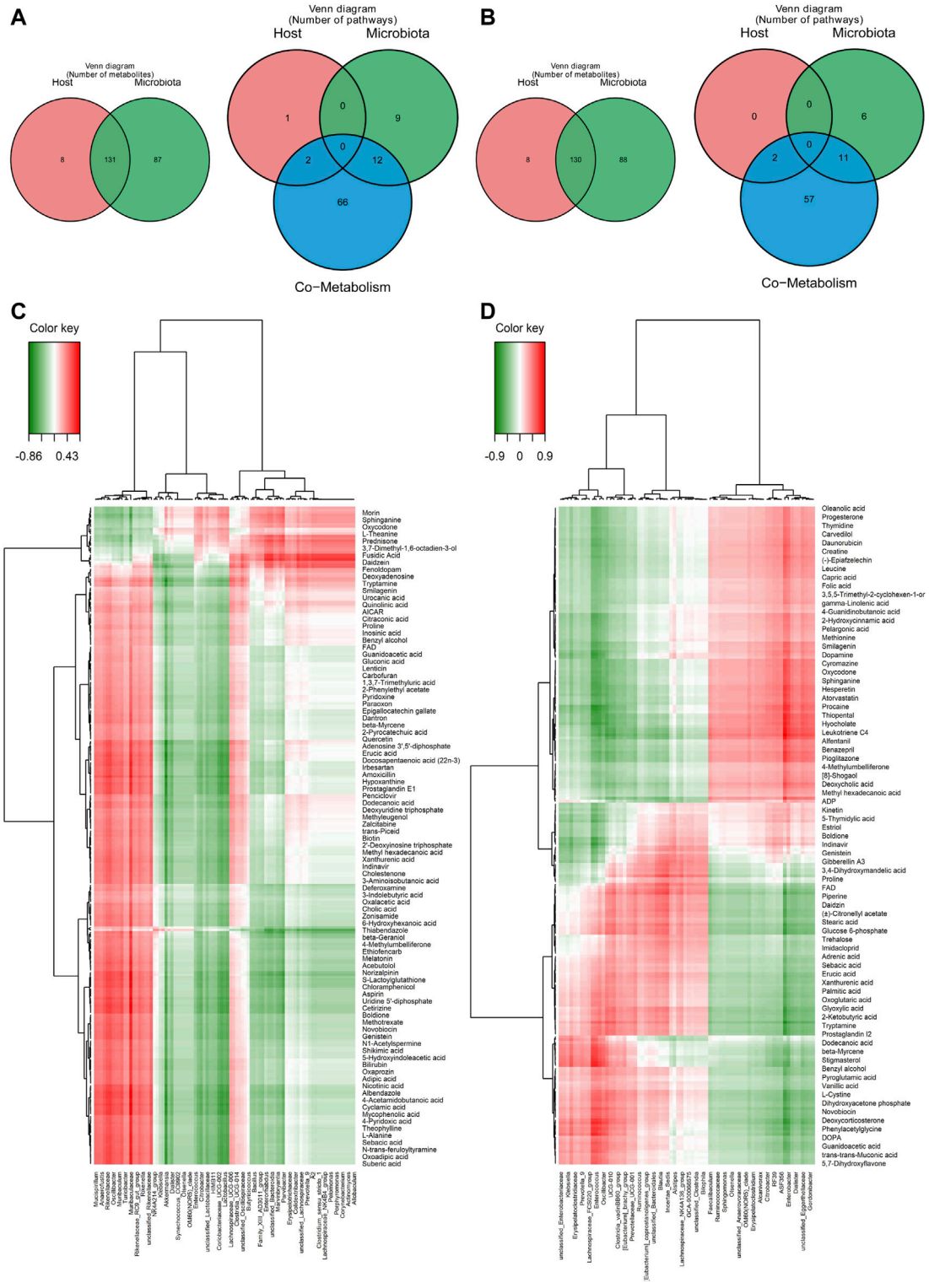

FIGURE 6
Correlation study between the gut microbiota and the considerably altered metabolites in the gut content. **(A)** Metabolites and pathways in the microbiota and host significantly altered 6 hours after 2/3 PHx. **(B)** Metabolites and pathways in the microbiota and host significantly altered 36 h after 2/3 PHx. **(C)** A correlation heatmap showing the major metabolite changes in the gut microbiota and gut content 6 h and after 2/3 PHx. **(D)** A correlation heatmap showing the metabolites that were significantly altered in the gut microbiota and gut content 36 h after 2/3 PHx.

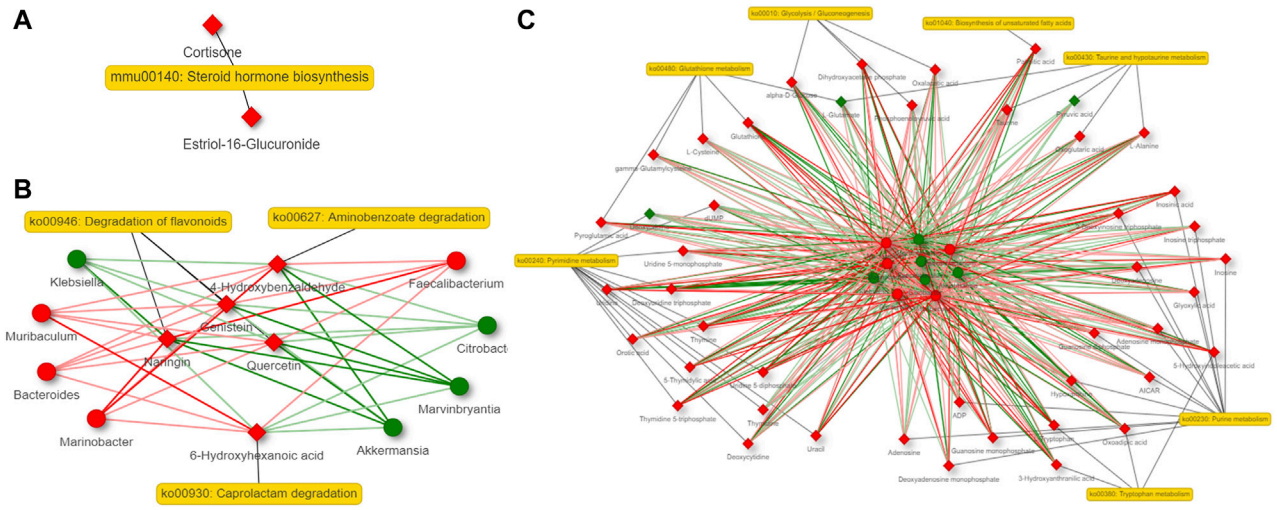

FIGURE 7
Network analysis of host metabolites **(A)**, bacteria metabolites **(B)** and co-metabolism **(C)** at 6 h after 2/3 PHx. Microbes are shown by round nodes, and metabolites are shown by cubic nodes.

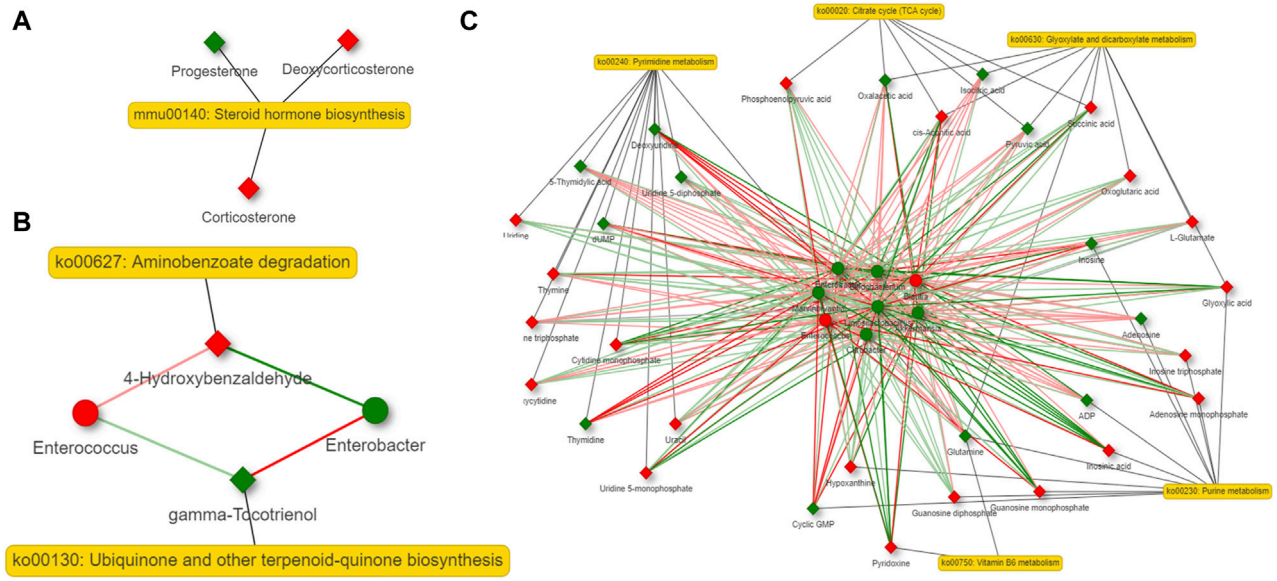

FIGURE 8
Network analysis of host metabolites **(A)**, bacteria metabolites **(B)** and co-metabolism **(C)** at 36 h after 2/3 PHx. Microbes are shown by round nodes, and metabolites are shown by cubic nodes.

stages during liver regeneration, especially at 6 h and 36 h after PHx. The metabolites in the gut content also showed an obvious alteration at various time points after 2/3 PHx. The correlation between gut microbiota and metabolites derived by them showed that several metabolic pathways are changed during liver regeneration, including pathways in host-specific metabolism, microbiota-specific metabolism and host and microbiota co-metabolized.

Our study showed the alteration of gut microbial composition after PHx by 16S rRNA sequencing. The process of liver regeneration causes a dynamic shift in the composition of the gut flora. The α and β diversity of the gut bacteria both changed. This is in line with other research (Liu et al., 2016). Specifically, at the genus level, PHx markedly decreased *Firmicutes* and *Verrucomicrobia* and increased *Bacteroidetes* and *Proteobacteria*. The ratio between *Firmicutes*/*Bacteroidetes* is reported to be associated with obesity, diabetes, NAFLD/NASH, inflammatory bowel disease and even cancer. The elevated F/B ratio that happened within the first 6 h after PHx might be more efficient in producing energy for the regenerative liver. *Muribaculaceae*, *Prevotellaceae*, *Rikenellaceae* and *Bacteroidaceae* were the most predominant family in the *Bacteroidetes* phylum, whereas *Lactobacillaceae*, *Lachnospiraceae*, *Oscillospiraceae* and *Ruminococcaceae* were the most prevalent members of the phylum

Firmicutes. These bacteria can provide energy, and have the ability to produce several metabolites and cytokines that shape the gut environment, maintain intestinal barrier function and be beneficial for maintaining gut health. Thus, we hypothesized that these bacteria were involved in modulating the metabolites in the body and promoting liver regeneration (Grigor'eva, 2020; Kusnadi et al., 2023; Stojanov et al., 2020; An et al., 2023).

An important factor in liver regeneration is metabolism. Glucose, triglycerides, cholesterol, and amino acids were significantly changed during liver regeneration (Bollard et al., 2010; Huang and Rudnick, 2014; Caldez et al., 2018). Consistently, metabolic genes are altered during liver regeneration. Through the portal vein, the body absorbs metabolites from the gut microbiota, which serve as energy substrates and vital signaling molecules. Lipopolysaccharide (LPS), bile acids, short-chain fatty acids (SCFAs) and tryptophan metabolites, etc. were the main metabolites produced by gut microbiota. Several studies have revealed the multifaceted roles of these metabolites in liver regeneration. The appropriate amount of LPS, the major component of the outer wall of the Gram-negative bacteria, is beneficial for liver regeneration, mainly by promoting hepatocyte DNA replication (Zheng and Wang, 2021). SCFAs (acetic acid, propionic acid and butyric acid) are produced by gut microbiota during the degradation of dietary fiber. SCFAs are considered energy sources for host colonocytes, they can also improve the metabolism homeostasis, shape the gut environment and maintain intestinal barrier function, thus promoting liver regeneration (Ríos-Covián et al., 2016; Murugesan et al., 2018). In our study, these metabolites were not detected. This is because LPS has a molecular weight of 10–20 kDa and can not be detected by LC-TOF/MS. Similarly, SCFAs also can not be detected because they are small polar volatile compounds and GC/MS is more suitable for detecting them. In addition, the functions of these metabolites have been well elucidated. Thus we mainly focus on the metabolites including amino acids, organic acids, purine and pyrimidine nucleotides and lipids, etc.

Our results show obvious alteration of metabolites in these pathways, including glycolysis, the TCA cycle, amino acid metabolism, purine and pyrimidine metabolism, etc. Consequently, we deduced that these pathways contribute significantly to the metabolic process involved in liver regeneration. These metabolic pathways were basic life activities and performed both by the host and bacteria. However, it's difficult to determine whether these metabolites were produced by host or bacterial. Enzymes produced by the gut microbiota known as microbial-host isozymes operate similarly to host enzymes and may be involved in gut metabolism and preserving host metabolic homeostasis (Wang et al., 2023). We speculated that the microbial-host-isozymes also changed during liver regeneration and contributed to the alteration of metabolites.

Glycolysis and the TCA cycle are metabolic pathways that generate energy. Additionally, they provide the intermediary molecules needed for additional pathways. Pyruvate, lactate and succinate, etc. can be converted into acetate, propionate and butyrate by the gut microbiota. They appear to be the natural ligands of G-protein-coupled receptor (GPR), activate the downstream signaling pathways, improve the metabolism homeostasis and promote liver regeneration (Portincasa et al., 2022). According to these findings, these metabolic intermediates modulate energy metabolism and liver regeneration in a significant way. This effect may be due to the control of intestinal cells as well as the gut microbiota.

Amino acids are the building blocks of protein. Protein synthesis is necessary for cell proliferation, which allows cells to grow and divide rapidly during liver regeneration. The liver has a pivotal role in amino acid metabolism. Besides, amino acids also function as signaling molecules and participate in many essential critical cellular functions. We found that arginine metabolism, taurine and hypotaurine metabolism, and glutathione metabolism were identified to be attributable to liver regeneration. Glutathione is a critical intracellular antioxidant, its concentration was doubled after PHx, which can maintain immune system homeostasis and activate hepatic NF-κB to promote liver regeneration (Irino et al., 2016). Taurine is a sulfur-containing amino acid, it can conjugate with bile acids to increase their aqueous solubility and decrease their cytotoxicity (Murakami et al., 2016). Taurine is also involved in many physiological functions including antioxidation, calcium modulation, and membrane stabilization. Taurine and hypotaurine metabolism is a pathway that is enriched during liver regeneration, consistent with the change of bile acids. The taurine-conjugated bile acid, taurocholic acid, can reduce the expression of connective tissue growth factor in hepatocytes through ERK-YAP signaling (Yu et al., 2018), and YAP is reported to be a core transcription coregulator in controlling organ growth including liver size (Jiang et al., 2019). The oxidative metabolite of L-arginine, nitric oxide (NO), plays a crucial role as a modulator of hepatocyte proliferation during liver regeneration. L-glutamine, a precursor for molecules including glutathione, nucleotides, and nucleic acids, can also function as a signaling molecule. L-arginine, L-glutamine, and BCAA supplements assist in speeding the recovery process of the liver following a hepatectomy (Kurokawa et al., 2012; Ceyhan et al., 2019).

Nuclear bases of nucleic acids, purine and pyrimidine nucleotides, are also important energy carriers. They also serve as building blocks for the synthesis of SAM, NAD, and other nucleotide cofactors. After PHx, the liver immediately starts to restore itself, which is a process that needs massive nucleotides, amino acids and energy. Nucleotide metabolism is a key metabolic process after PHx in the synthesis of DNA and RNA. Pyrimidine nucleoside uridine is found to be a potent regeneration-promoting factor (Liu et al., 2022). Nucleotides are also co-metabolized by the host and gut microbiota. The catabolism of purine nucleotides in the regenerating liver is accelerated and the pyrimidine pool is enlarged during liver regeneration (Usami et al., 1996). In our study, the purine and pyrimidine nucleotides were also significantly changed, these results suggested that PHx modifies the metabolism of pyrimidines and purines. A preoperative supply of nucleoside is effective for increasing hepatocyte DNA synthesis and hepatic regeneration after PHx (Usami et al., 1996).

According to earlier research, the body's endogenous metabolites and gut bacteria are crucial for liver regeneration. Different from these studies, our study mainly focuses on the metabolites in the gut content, which we think can demonstrate the direct relationship that exists between the host's gut flora and itself, and further reveal the function of gut microbiota-derived metabolites in liver regeneration. We further analyzed the results by correlation analysis to reveal the metabolites produced by the host and the gut microbiota, or co-metabolized. Our results revealed that steroid hormone biosynthesis is the most significantly changed pathway during liver regeneration, which is a host-specific metabolic pathway. Besides, bile acid metabolism is also enriched as a changed pathway. Thus we speculate that cholesterol metabolism, including primary bile acid biosynthesis and steroid hormone biosynthesis are important metabolic pathways during

liver regeneration. Steroid hormones and bile acids (BAs) are both steroid molecules produced by the degradation of cholesterol. Cholesterol is an essential substance for liver recovery after PHx. It was metabolized to primary bile acids in hepatocytes and converted into secondary bile acids by the gut microbiota. In many liver illnesses, the metabolism of primary bile acid is a crucial pathway. BAs break down fats and help absorb them into the body. What's more, they also have variate functions as signaling molecules. BAs are reported to be involved in the proliferation phase of liver regeneration. The right dosage of BAs may promote the growth of hepatocytes and cause the regeneration of the liver. However, the liver is harmed by too many BAs. Steroid hormones are found to be differential metabolites and the pathway was enriched in our study, which has not been reported by the previous studies. The role of steroid hormones in liver regeneration is complex. The steroid hormones progesterone, dehydroepiandrosterone (DHEA) (Kitamura et al., 1999), methandrastenolone, norethanedrolon, testosterone, and deoxycorticosterone were reported to accelerate this process (Gershbein, 1967). A low dose of dexamethasone ameliorates the transient impairment of liver regeneration, although it can not accelerate liver regeneration (Shibata et al., 2012). Controversially, cortisone, hydrocortisone and prednisolone depressed liver regeneration (Gershbein, 1973). Aldosterone, fludrocortisone, methylprednisolone have no apparent effects on hepatic regeneration (Glanemann et al., 2004). From a meta-analysis, pre-operative steroids may have a clinically significant benefit in liver regeneration (Richardson et al., 2014). The reason for the alteration in steroids and their function and mechanism in liver regeneration need to be further investigated.

The aminobenzoate degradation pathway was the most significant gut microbiota-specific metabolic pathway in the gut content of mice during liver regeneration. It was the third-largest xenobiotic biodegradation pathway by gut bacteria. This pathway can be cross-regulated between aerobic and anaerobic pathways of catabolized aromatic compounds, thus increasing the cell fitness in organisms, especially the gut that survive in environments subject to changing oxygen concentrations (Valderrama et al., 2012; Sridharan et al., 2014). 4-hydroxybenzaldehyde is an intermediate derived from tryptophan and other indole ring-containing compounds (Rudney and Parson, 1963). It was reported that 4-hydroxybenzaldehyde could regulate gut microbiota and inhibit lipid accumulation (Li et al., 2021). It can also sensitize *Acinetobacter baumannii* to antibiotics (Shin et al., 2018). The gut microbiota can metabolize tryptophan to produce indoles and their derivatives. They have the ability to upregulate the expression of genes critical for preserving the gut mucosal epithelial barrier and tight junctions in epithelial cells. Several bacteria participate in the degradation of these compounds, i.e., *Bacteroides,* etc. increased and *Akkermansia,* etc., decreased. These bacteria and metabolites and the process of their metabolism may contribute to maintaining homeostasis after PHx and promoting liver regeneration.

Some limitations remain in this study. Firstly, this study only involved samples from mice, whereas human samples would provide more meaningful information. We believe the animal study could provide references for further clinical studies. Secondly, the earlier time points between 6 and 36 h will be included in the future study. Thirdly, the results will be more reliable and solid if targeted metabolomics is used further to correctly quantify the differential metabolites. Finally, the impact of particular gut bacterial species and the metabolites they produce on liver regeneration, as well as the underlying mechanisms need to be further elucidated. Further study is ongoing to try to answer these questions.

5 Conclusion

In conclusion, our study demonstrated that the gut microbiota composition and gut microbiota-derived metabolites in the gut content changed markedly during liver regeneration. Untargeted metabolomics showed that PHx significantly modulates the metabolites involved in glycolysis, the TCA cycle, arginine metabolism, glutathione metabolism, tryptophan metabolism, purine and pyrimidine metabolism, which were co-metabolized by the host and gut microbiota. In the host, steroid hormone biosynthesis is the most significantly changed pathway. For the gut microbiota, *Escherichia Shigella*, *Lactobacillus*, *Akkermansia*, and *Muribaculaceae* were changed considerably during liver regeneration. Aminobenzoate degradation pathway was the most significant gut microbiota-specific metabolic pathway during liver regeneration. To the best of our knowledge, this is the first thorough explanation of the metabolites derived from the gut microbiota and how they relate to changes in the gut microbiota during liver regeneration after 2/3 PHx. This discovery advances our knowledge of the metabolic mechanism underlying liver regeneration and offers a novel, potentially useful therapeutic target for it. Additional research will be done on the underlying role and mechanism of metabolites originating from gut bacteria in liver regeneration.

Data availability statement

The data presented in the study are deposited in the MetaboLights repository, accession number MTBLS10775.

Ethics statement

The animal study was approved by the Animal Ethics Committee of Nanjing University Medical School Affiliated Drum Tower Hospital. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

RS: Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Supervision, Writing–original draft, Writing–review and editing. FF: Methodology, Writing–original draft. DJ: Methodology, Writing–original draft. HY: Methodology, Writing–original draft. ZX: Methodology, Writing–original draft. BC: Data curation, Writing–original draft, Writing–review and editing. JL: Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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SUPPLEMENTARY FIGURE S1
Typical LC-QTOF/MS chromatograms of gut content in positive **(A)** and negative modes.

SUPPLEMENTARY FIGURE S2
Metabolites (positive mode) in the gastrointestinal content significantly altered during liver regeneration following a 2/3 PHx. The volcano plots showed the Sham group compared with those of the 6 h group **(A)**, 36 h group **(B)**, 72 h group **(C)**, and 168 h group **(D)**.

SUPPLEMENTARY FIGURE S3
Metabolites (negative mode) in the gastrointestinal content significantly altered during liver regeneration following a 2/3 PHx. The volcano plots showed the Sham group compared with those of the 6 h group **(A)**, 36 h group **(B)**, 72 h group **(C)**, and 168 h group **(D)**.

SUPPLEMENTARY FIGURE S4
The heat map shows the significantly changed metabolites in the gut content in positive mode.

SUPPLEMENTARY FIGURE S5
The heat map shows the significantly changed metabolites in the gut content in negative mode.

SUPPLEMENTARY FIGURE S6
The box plot shows the typical significantly changed metabolites in the gut content.

SUPPLEMENTARY FIGURE S7
The parameters of regularized Canonical Correlation Analysis (rCCA) between gut microbiota and its metabolites.

References

Amin, A., Ngatiman, H. M., Wheatley, A. M., Aqilah, R. N., Fariha, M. N., Shanahan, F., et al. (2020). *Reintroduction of gut microbiota improves liver regeneration in germ-free mice*.

An, J., Kwon, H., Kim, Y. J., He, R., Gao, S., Yao, H., et al. (2023). The firmicutes/bacteroidetes ratio as a risk factor of breast cancer mechanism of metabolic response to hepatectomy by integrated analysis of gut microbiota, metabolomics, and proteomics. *J. Clin. Med.* 12 (6), e0206722. doi:10.3390/jcm12062216

Bollard, M. E., Contel, N. R., Ebbels, T. M., Smith, L., Beckonert, O., Cantor, G. H., et al. (2010). NMR-based metabolic profiling identifies biomarkers of liver regeneration following partial hepatectomy in the rat. *J. proteome Res.* 9 (1), 59–69. doi:10.1021/pr900200v

Caldez, M. J., Van Hul, N., Koh, H. W. L., Teo, X. Q., Fan, J. J., Tan, P. Y., et al. (2018). Metabolic remodeling during liver regeneration. *Dev. Cell* 47 (4), 425–438. doi:10.1016/j.devcel.2018.09.020

Cani, P. D. (2014). Metabolism in 2013: the gut microbiota manages host metabolism. *Nat. Rev. Endocrinol.* 10 (2), 74–76. doi:10.1038/nrendo.2013.240

Ceyhan, E., Ozen, B., Ozen, A., Berber, B., Rasulzade, L., and Uyanoglu, M. (2019). Branched-chain amino acids (BCAAs) promotes liver regeneration by decreasing SOCS3 expression to enhance IL-6/STAT3 proliferative signals. *Planta Medica* 85 (18), P–279. doi:10.1055/s-0039-3399973

Cornell, R. P., Liljequist, B. L., and Bartizal, K. F. (1990). Depressed liver regeneration after partial hepatectomy of germ-free, athymic and lipopolysaccharide-resistant mice. *Hepatol. Baltim. Md* 11 (6), 916–922. doi:10.1002/hep.1840110603

de Haan, L., van der Lely, S. J., Warps, A. K., Hofsink, Q., Olthof, P. B., de Keijzer, M. J., et al. (2018). Post-hepatectomy liver regeneration in the context of bile acid homeostasis and the gut-liver signaling axis. *J. Clin. Transl. Res.* 4 (1), 1–46. doi:10.18053/jctres.04.201801.001

Fausto, N., Campbell, J. S., and Riehle, K. J. (2012). Liver regeneration. *J. hepatology* 57 (3), 692–694. doi:10.1016/j.jhep.2012.04.016

Fukuda, S., Toh, H., Hase, K., Oshima, K., Nakanishi, Y., Yoshimura, K., et al. (2011). Bifidobacteria can protect from enteropathogenic infection through production of acetate. *Nature* 469 (7331), 543–547. doi:10.1038/nature09646

Gershbein, L. L. (1967). Liver regeneration in the presence of high levels of steroid hormones. *Proc. Soc. Exp. Biol. Med. Soc. Exp. Biol. Med. (New York, NY)* 125 (4), 1063–1067. doi:10.3181/00379727-125-32277

Gershbein, L. L. (1973). Effect of adrenal cortical steroids on liver regeneration. *J. Surg. Oncol.* 5 (5), 491–508. doi:10.1002/jso.2930050510

Glanemann, M., Münchow, S., Schirmeier, A., Al-Abadi, H., Lippek, F., Langrehr, J. M., et al. (2004). Steroid administration before partial hepatectomy with temporary inflow occlusion does not influence cyclin D1 and Ki-67 related liver regeneration. *Langenbeck's archives Surg.* 389 (5), 380–386. doi:10.1007/s00423-004-0507-6

Grigor'eva, I. N. (2020). Gallstone disease, obesity and the firmicutes/bacteroidetes ratio as a possible biomarker of gut dysbiosis. *JAMA Surg.* 11 (1), 13. doi:10.3390/jpm11010013

He, R., Gao, S., Yao, H., Zhao, Z., Tong, J., and Zhang, H. (2023). Mechanism of metabolic response to hepatectomy by integrated analysis of gut microbiota, metabolomics, and proteomics. *Microbiol. Spectr.* 11 (3), 02067222–e302022. doi:10.1128/spectrum.02067-22

Huang, J., and Rudnick, D. A. (2014). Elucidating the metabolic regulation of liver regeneration. *Am. J. pathology* 184 (2), 309–321. doi:10.1016/j.ajpath.2013.04.034

Huang, W., Ma, K., Zhang, J., Qatanani, M., Cuvillier, J., Liu, J., et al. (2006). Nuclear receptor-dependent bile acid signaling is required for normal liver regeneration. *Sci. (New York, NY)* 312 (5771), 233–236. doi:10.1126/science.1121435

Irino, Y., Toh, R., Nagao, M., Mori, T., Honjo, T., Shinohara, M., et al. (2016). 2-Aminobutyric acid modulates glutathione homeostasis in the myocardium. *Sci. Rep.* 6 (1), 36749–36810. doi:10.1038/srep36749

Jiang, Y., Feng, D., Ma, X., Fan, S., Gao, Y., Fu, K., et al. (2019). Pregnane X receptor regulates liver size and liver cell fate by yes-associated protein activation in mice. *Hepatol. Baltim. Md* 69 (1), 343–358. doi:10.1002/hep.30131

Kajiura, D., Yamanaka-Okumura, H., Hirayama, A., Tatano, H., Endo, K., Honma, M., et al. (2019). Perioperative serum and urine metabolome analyses in patients with hepatocellular carcinoma undergoing partial hepatectomy. *Nutr. Burbank, Los Angel. Cty. Calif.* 58, 110–119. doi:10.1016/j.nut.2018.06.002

Kitamura, T., Tanaka, K., Morita, K., Saito, S., Kiba, T., Numata, K., et al. (1999). Dehydroepiandrosterone (DHEA) facilitates liver regeneration after partial hepatectomy in rats. *Life Sci.* 65 (17), 1747–1756. doi:10.1016/s0024-3205(99)00427-0

Kong, B., Sun, R., Huang, M., Chow, M. D., Zhong, X. B., Xie, W., et al. (2018). Fibroblast growth factor 15–dependent and bile acid–independent promotion of liver regeneration in mice. *Hepatol. Baltim. Md* 68 (5), 1961–1976. doi:10.1002/hep.30041

Kurokawa, T., An, J., Tsunekawa, K., Shimomura, Y., Kazama, S., Ishikawa, N., et al. (2012). Effect of L-arginine supplement on liver regeneration after partial hepatectomy in rats. *World J. Surg. Oncol.* 10, 99. doi:10.1186/1477-7819-10-99

Kusnadi, Y., Saleh, M. I., Ali, Z., Hermansyah, H., Murti, K., Hafy, Z., et al. (2023). Firmicutes/Bacteroidetes ratio of gut microbiota and its relationships with clinical parameters of type 2 diabetes mellitus. a Syst. Rev. *Open Access Maced J Med Sci* 11, 67–72. doi:10.3889/oamjms.2023.10478

Li, J., Deng, Q., Zhang, Y., Wu, D., Li, G., Liu, J., et al. (2021). Three novel dietary phenolic compounds from pickled raphanus sativus L. Inhibit lipid accumulation in obese mice by modulating the gut microbiota composition. *Mol. Nutr. food Res.* 65 (6), e2000780. doi:10.1002/mnfr.202000780

Liu, H. X., Keane, R., Sheng, L., and Wan, Y. J. (2015). Implications of microbiota and bile acid in liver injury and regeneration. *J. hepatology* 63 (6), 1502–1510. doi:10.1016/j.jhep.2015.08.001

Liu, H. X., Rocha, C. S., Dandekar, S., and Wan, Y. J. (2016). Functional analysis of the relationship between intestinal microbiota and the expression of hepatic genes and pathways during the course of liver regeneration. *J. hepatology* 64 (3), 641–650. doi:10.1016/j.jhep.2015.09.022

Liu, Z., Li, W., Geng, L., Sun, L., Wang, Q., Yu, Y., et al. (2022). Cross-species metabolomic analysis identifies uridine as a potent regeneration promoting factor. *Cell Discov.* 8 (1), 6. doi:10.1038/s41421-021-00361-3

Mao, S. A., Glorioso, J. M., and Nyberg, S. L. (2014). Liver regeneration. *Transl. Res. J. laboratory Clin. Med.* 163 (4), 352–362. doi:10.1016/j.trsl.2014.01.005

Michalopoulos, G. K. (2014). Advances in liver regeneration. *Expert Rev. gastroenterology hepatology* 8 (8), 897–907. doi:10.1586/17474124.2014.934358

Monga, S. P. (2014). Role and regulation of β-catenin signaling during physiological liver growth. *Gene Expr.* 16 (2), 51–62. doi:10.3727/105221614X13919976902138

Murakami, S., Fujita, M., Nakamura, M., Sakono, M., Nishizono, S., Sato, M., et al. (2016). Taurine ameliorates cholesterol metabolism by stimulating bile acid production in high-cholesterol-fed rats. *Clin. Exp. Pharmacol. physiology* 43 (3), 372–378. doi:10.1111/1440-1681.12534

Murugesan, S., Nirmalkar, K., Hoyo-Vadillo, C., García-Espitia, M., Ramírez-Sánchez, D., and García-Mena, J. (2018). Gut microbiome production of short-chain fatty acids and obesity in children. *Eur. J. Clin. Microbiol. Infect. Dis. official Publ. Eur. Soc. Clin. Microbiol.* 37 (4), 621–625. doi:10.1007/s10096-017-3143-0

Portincasa, P., Bonfrate, L., Vacca, M., De Angelis, M., Farella, I., Lanza, E., et al. (2022). Gut microbiota and short chain fatty acids: implications in glucose homeostasis. *Int. J. Mol. Sci.* 23 (3), 1105. doi:10.3390/ijms23031105

Rahbari, N. N., Birgin, E., Bork, U., Mehrabi, A., Reißfelder, C., and Weitz, J. (2021). Anterior approach vs conventional hepatectomy for resection of colorectal liver metastasis: a randomized clinical trial. *JAMA Surg.* 156 (1), 31–40. doi:10.1001/jamasurg.2020.5050

Richardson, A. J., Laurence, J. M., and Lam, V. W. (2014). Use of pre-operative steroids in liver resection: a systematic review and meta-analysis. *HPB official J. Int. Hepato Pancreato Biliary Assoc.* 16 (1), 12–19. doi:10.1111/hpb.12066

Ríos-Covián, D., Ruas-Madiedo, P., Margolles, A., Gueimonde, M., de Los Reyes-Gavilán, C. G., and Salazar, N. (2016). Intestinal short chain fatty acids and their link with diet and human health. *Front. Microbiol.* 7, 185. doi:10.3389/fmicb.2016.00185

Rudney, H., and Parson, W. W. (1963). THE CONVERSION OF P-HYDROXYBENZALDEHYDE TO THE BENZOQUINONE RING OF UBIQUINONE IN RHODOSPIRILLUM RUBRUM. *J. Biol. Chem.* 238, 3137–3138. doi:10.1016/s0021-9258(18)51882-2

Shibata, T., Mizuguchi, T., Nakamura, Y., Kawamoto, M., Meguro, M., Ota, S., et al. (2012). Low-dose steroid pretreatment ameliorates the transient impairment of liver regeneration. *World J. gastroenterology* 18 (9), 905–914. doi:10.3748/wjg.v18.i9.905

Shin, B., Park, C., Imlay, J. A., and Park, W. (2018). 4-Hydroxybenzaldehyde sensitizes Acinetobacter baumannii to amphenicols. *Appl. Microbiol. Biotechnol.* 102 (5), 2323–2335. doi:10.1007/s00253-018-8791-1

Sridharan, G. V., Choi, K., Klemashevich, C., Wu, C., Prabakaran, D., Pan, L. B., et al. (2014). Prediction and quantification of bioactive microbiota metabolites in the mouse gut. *Nat. Commun.* 5, 5492. doi:10.1038/ncomms6492

Stojanov, S., Berlec, A., Štrukelj, B., Rahbari, N. N., Birgin, E., Bork, U., et al. (2020). The influence of probiotics on the firmicutes/bacteroidetes ratio in the treatment of obesity and inflammatory bowel disease anterior approach vs conventional hepatectomy for resection of colorectal liver metastasis: a randomized clinical trial. *Microorganisms* 8 (11), 31–40. doi:10.3390/microorganisms8111715

Sun, R., Fei, F., Wang, M., Jiang, J., Yang, G., Yang, N., et al. (2023a). Integration of metabolomics and machine learning revealed tryptophan metabolites are sensitive biomarkers of pemetrexed efficacy in non-small cell lung cancer. *Cancer Med.* 12 (18), 19245–19259. doi:10.1002/cam4.6446

Sun, R., Jin, D., Fei, F., Xu, Z., Cao, B., and Li, J. (2023b). Mushroom polysaccharides from Grifola frondosa (Dicks.) Gray and Inonotus obliquus (Fr.) Pilat ameliorated dextran sulfate sodium-induced colitis in mice by global modulation of systemic metabolism and the gut microbiota. *Front. Pharmacol.* 14, 1172963. doi:10.3389/fphar.2023.1172963

Tremaroli, V., and Bäckhed, F. (2012). Functional interactions between the gut microbiota and host metabolism. *Nature* 489 (7415), 242–249. doi:10.1038/nature11552

Tripathi, A., Debelius, J., Brenner, D. A., Karin, M., Loomba, R., Schnabl, B., et al. (2018). The gut-liver axis and the intersection with the microbiome. *Nat. Rev. Gastroenterology hepatology* 15 (7), 397–411. doi:10.1038/s41575-018-0011-z

Usami, M., Furuchi, K., Ogino, M., Kasahara, H., Kanamaru, T., Saitoh, Y., et al. (1996). The effect of a nucleotide-nucleoside solution on hepatic regeneration after partial hepatectomy in rats. *Nutr. Burbank, Los Angel. Cty. Calif.* 12 (11-12), 797–803. doi:10.1016/s0899-9007(96)00292-4

Valderrama, J. A., Durante-Rodríguez, G., Blázquez, B., García, J. L., Carmona, M., and Díaz, E. (2012). Bacterial degradation of benzoate: cross-regulation between aerobic and anaerobic pathways. *J. Biol. Chem.* 287 (13), 10494–10508. doi:10.1074/jbc.M111.309005

Viaud, S., Saccheri, F., Mignot, G., Yamazaki, T., Daillère, R., Hannani, D., et al. (2013). The intestinal microbiota modulates the anticancer immune effects of cyclophosphamide. *Sci. (New York, NY)* 342 (6161), 971–976. doi:10.1126/science.1240537

Wang, K., Zhang, Z., Hang, J., Liu, J., Guo, F., Ding, Y., et al. (2023). Microbial-host-isozyme analyses reveal microbial DPP4 as a potential antidiabetic target. *Sci. (New York, NY)* 381 (6657), eadd5787. doi:10.1126/science.add5787

Wu, X., Sun, R., Chen, Y., Zheng, X., Bai, L., Lian, Z., et al. (2015). Oral ampicillin inhibits liver regeneration by breaking hepatic innate immune tolerance normally maintained by gut commensal bacteria. *Hepatol. Baltim. Md* 62 (1), 253–264. doi:10.1002/hep.27791

Xie, C., Zhang, Z., Yang, M., Cao, C., Zhou, Y., Zhu, Z., et al. (2021). Lactiplantibacillus plantarum AR113 exhibit accelerated liver regeneration by regulating gut microbiota and plasma glycerophospholipid gut microbiota and short chain fatty acids: implications in glucose homeostasis. *Front. Microbiol.* 12 (3), 800470. doi:10.3389/fmicb.2021.800470

Xu, Z., Jiang, N., Xiao, Y., Yuan, K., and Wang, Z. (2022). The role of gut microbiota in liver regeneration. *Front. Immunol.* 13, 1003376. doi:10.3389/fimmu.2022.1003376

Yin, Y., Sichler, A., Ecker, J., Laschinger, M., Liebisch, G., Höring, M., et al. (2023). Gut microbiota promote liver regeneration through hepatic membrane phospholipid biosynthesis. *J. hepatology* 78 (4), 820–835. doi:10.1016/j.jhep.2022.12.028

Yu, B., Jin, G. N., Ma, M., Liang, H. F., Zhang, B. X., Chen, X. P., et al. (2018). Taurocholate induces connective tissue growth factor expression in hepatocytes through ERK-YAP signaling. *Cell. physiology Biochem. Int. J. Exp. Cell. physiology, Biochem. Pharmacol.* 50 (5), 1711–1725. doi:10.1159/000494790

Yu, G., Xu, C., Zhang, D., Ju, F., and Ni, Y. (2022). MetOrigin: discriminating the origins of microbial metabolites for integrative analysis of the gut microbiome and metabolome. *IMeta* 1 (1), e10. doi:10.1002/imt2.10

Zheng, Z., and Wang, B. (2021). The gut-liver Axis in health and disease: the role of gut microbiota-derived signals in liver injury and regeneration. *Front. Immunol.* 12, 775526. doi:10.3389/fimmu.2021.775526

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Candida tropicalis ZD-3 prevents excessive fat deposition by regulating ileal microbiota and bile acids enterohepatic circulation in broilers

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Introduction: Evidence suggests that the dietary intake of *Candida tropicalis*ZD-3 (ZD-3) has various health benefits, but the treatment mechanisms and effects remain unclear. The aim of this study investigates the effect of ZD-3 on reducing fat deposition in broilers and the underlying mechanism.

Methods: 180 one-day-old, yellow-feathered broilers were randomly divided into three groups: control (CON) group fed a basal diet, an active *Candida tropicalis* ZD-3 (ZD) group supplemented with ZD, and a heat-inactivated Candida tropicalis ZD-3 (HZD) group supplemented with HZD. The experiment lasted for 28 d.

Results: The ZD and HZD treatments significantly reduced the abdominal fat index ($p < 0.05$), decreased TG levels in serum and liver ($p < 0.05$), altered the ileal microbial composition by reducing the Firmicutes to Bacteroidetes (F/B) ratio. Additionally, the ZD and HZD treatments reduced liver cholesterol by decreasing ileal FXR-FGF19 signaling and increasing liver FXR-SHP signaling ($p < 0.05$). The ZD and HZD treatments also changed liver PC and TG classes lipid composition, regulating liver lipid metabolism by promoting TG degradation and modulating the signal transduction of the cell membrane.

Discussion: Overall, ZD-3 was effective in improving lipid metabolism in broilers by regulating the ileal microbial composition and BAs enterohepatic circulation. This study provides a theoretical basis for the development and application of ZD-3 for the regulation of lipid metabolism in broilers.

KEYWORDS

***Candida tropicalis* ZD-3, gut microbiota, bile acid metabolism, lipid metabolism, yellow-feathered broiler**

1 Introduction

In the poultry industry, high energy diets and genetic breeding methods are used to artificially select chickens to achieve unexampled feed conversion ratios and growth rates (Tallentire et al., 2016). Fast-growing broilers are frequently linked to excessive abdominal fat deposition because one unit of fat consumed for deposition provides three times as much energy as one unit of lean meat consumed for deposition, a trait detrimental to the interests of producers and consumers (Nie et al., 2015). In broilers, more than 85% of abdominal fat is considered unnecessary because it is viewed as a waste of dietary energy (Fouad and El-Senousey, 2014). Excessive fat deposition can lead to a fatty liver, which is associated with high mortality and morbidity in broilers. Global broiler

production yields approximately 3 million tons of abdominal fat per year, leading to many economic losses in the poultry industry and posing a significant challenge to sustainable agricultural profitability (Wen et al., 2019). Researchers have discovered that abdominal fat cells exhibit greater metabolic activity and possess higher heritability (0.82) than breast and leg muscles, resulting in fat accumulation (Abdalla et al., 2018). Accumulation of excess fat has become a perplexing and increasingly prevalent problem. Consequently, understanding the underlying mechanisms that contribute to excessive fat deposition has garnered significant attention.

When consumed in adequate amounts, probiotics offer health benefits to the host and have preclinical potential for reducing obesity (Larsen et al., 2023). Therefore, probiotics are considered a non-invasive but potentially effective treatment for obesity and related metabolic diseases (Delzenne et al., 2011). Recently, the cholesterol reducing effects of probiotics have garnered significant research interest, and consumers prefer their safety, high efficacy, and cost-effectiveness to pharmaceutical interventions (Tang et al., 2021). Many probiotic formulations have been developed to control cholesterol levels. However, most studies have used live bacteria. Additionally, studies have suggested that postbiotics also play a role (Salminen et al., 2021). The term postbiotics is a newly recognized concept encompassing various bioactive substances, such as inactive or heat-inactivated microbial cells, cellular components, and metabolic byproducts, that have the potential to promote beneficial biological effects when ingested by consumers (Cuevas-González et al., 2020). Research on postbiotics is still in its early stages, but they have been found to have a significant impact on regulating fat metabolism (Depommier et al., 2019).

In our previous study, we found that diets fermented with ZD-3 were effective in reducing total cholesterol (TC) levels and had the potential to modulate abdominal fat morphology and lipid metabolism in early white feathered broilers but the effect was not evident in later stages (Niu et al., 2020). However, the mechanism of action and the effect of heat-inactivated ZD-3 on fat metabolism remain unclear. Probiotics modulate lipid metabolism by regulating intestinal microbiota and their associated metabolites. The alteration in the gut microbiota, often referred to as the "second brain," can affect the energy regulation, glucose (GLU) equilibrium, and lipid metabolism of the host (Li et al., 2020). It has been shown that interactions between host ileal microbiota and bile acid (BA) metabolism ameliorate hyperlipidemia and related metabolic diseases (Wang Y. et al., 2023). The primary factor contributing to hyperlipidemia is the accumulation of lipids in the bloodstream and liver. Therefore, the principal approach for treating hyperlipidemia involves restoring the metabolism of lipids, particularly cholesterol and BAs (Zhang Y. et al., 2022). BAs regulate host metabolism and immunity through the farnesoid X receptor (FXR) activated by BAs. Probiotics reduce BA absorption and promote liver bile acid synthesis through the ileum liver FXR-fibroblast growth factor 19 (FGF19) axis (Pathak et al., 2018). The liver is a crucial organ for energy metabolism and plays a significant role in maintaining the lipid balance by regulating lipid synthesis and breakdown (Feng et al., 2020). Lipidomics allows for the identification and evaluation of how lipids, either independently or in conjunction with proteins, regulate cellular and subcellular functions such as signaling and gene expression. The utilization of lipidomic analysis of the liver has the potential to provide new perspectives for investigating lipid metabolism. Hence, the utilization of probiotics to regulate the gut microbiota and improve liver lipid metabolism is a promising research field.

In this study, we evaluated the regulatory effects of active and heat-inactivated ZD-3 on lipid metabolism and its effects on ileum microbiota and BA metabolism and then studied lipid changes in the liver by using lipidomics, providing a theoretical basis for the application and development of ZD-3 and the reduction of fat deposition in broilers.

2 Materials and methods

2.1 Treatment of *Candida tropicalis* ZD-3

The strains of ZD-3 were obtained from the College of Animal Science & Technology Shihezi University (Shihezi, China). The activated strains were inoculated with YPD at 37°C for 24 h. The ZD-3 were harvested using centrifugation at 4,000 g for 10 min and then washed twice with phosphate buffered saline (pH = 7.4). Next, *Candida tropicalis* ZD-3 (ZD) was obtained by lyophilization (1×10^9 CFU/kg). Heat-inactivated *Candida tropicalis* ZD-3 (HZD) were prepared from the bacterial pellets by subjecting them to a 90°C water bath for 30 min and then lyophilization. Plate culture analysis confirmed the absence of active bacterial growth.

2.2 Animals and experimental design

Animal experiments were conducted in accordance with China's national guidelines and in compliance with the regulations of the Bioethics Committee of Shihezi University (Xinjiang, China) (Approval no: A2023-202). In total, 180 one-day-old male, yellow-feathered broilers were obtained from a business incubator (Xinjiang Taikun Group Co., Ltd.) and randomly divided into three groups, each consisting of six replicates, with 10 birds in each replicate. Broilers in the CON group were fed a basal diet. Broilers in the ZD group were fed the basal diet with added active *Candida tropicalis* ZD-3 (1×10^9 CFU/kg). Broilers in the HZD group were fed a basal diet containing heat-inactivated *Candida tropicalis* ZD-3 (1×10^9 CFU/kg). The compositions and nutrient levels of the diets are listed in Table 1. The basal diet complied with the nutritional requirements of the yellow-feathered broiler chicks (NY/T 3645–2020). Broilers were raised in multilevel coops. Each coop contained three levels, five cages per level, and five birds in each cage. Each cage was equipped with two nipple drinkers and one feeder. The broilers had *ad libitum* access to water to fulfill their nutritional requirements. The cages were under 24 h lighting, and the temperature was initially set at 35°C, gradually decreasing to 25°C by the fourth week. The experiment lasted for 28 d.

2.3 Sample collection

At 28 days, after a 12 h fast, six broilers were randomly selected from each treatment group (one bird from each replicate) and sacrificed using cervical dislocation. The broilers were weighed, and blood samples (obtained from the jugular vein), liver tissue, abdominal adipose tissue, and ileal contents were collected. The blood was centrifuged at 3,000 g for 10 min at 4°C to separate the serum, which was stored at −20°C for future use. The liver and abdominal fat were collected and weighed; a portion of each was fixed in 4% paraformaldehyde, and the remainder was frozen in liquid nitrogen and stored at −80°C. Ileal contents were also collected, frozen in liquid nitrogen, and stored at −80°C for

TABLE 1 Diet compositions and nutrient contents of the experimental diets.

Item	1–28 d
Ingredients, %DM	
Yellow corn	54.80
Soybean meal	37.70
Vegetable oil	2.50
Premix[a]	5.00
Nutrient content, %DM	
Metabolizable energy, MJ/kg	12.37
Crude protein	20.98
Ether extract	5.07
Crude ash	7.12
Crude fiber	3.41
Calcium	1.01
Total phosphorus	0.71
Available phosphate	0.45
Methionine	0.53
Methionine + cysteine	0.95
Threonine	0.86
Lysine	1.31

[a]Premix provided the following per kg of diets: Ca 8.1 g/kg; P 2.97 g/kg; Nacl 2.90 g/kg; Lys 0.256 g/kg; Met 1.813 g/kg; VA 8800 IU/kg; VD 3000 IU/kg; VE 30 mg/kg; VK_2 1.65 mg/kg; VB_1 2.5 mg/kg; VB_2 6.6 mg/kg; VB_3 11 mg/kg; VB_4 500 mg/kg; VB_5 60 mg/kg; VB_6 4.0 mg/kg; VH 0.2 mg/kg; VB_{11} 1.0 mg/kg; VB_{12} 0.02 mg/kg; VC 50 mg/kg; Fe^{2+} 80.0 mg/kg; Cu^{2+} 8.0 mg/kg; Zn^{2+} 60.0 mg/kg; Mn^{2+} 70.0 mg/kg; I 0.5 mg/kg; Se^{4+} 0.3 mg/kg.

subsequent analysis. Feces were collected and mixed in repeat units 3 days before slaughter.

2.4 Growth performance

Body weight and feed intake of broilers were monitored weekly in a replicated fashion. These measurements were used to calculate the average daily feed intake (ADFI), average daily gain (ADG), and feed: gain ratio (F/G).

2.5 Carcass trait

Carcass, liver, and abdominal fat were measured and reported as a proportion of the live weight. The thickness of the subcutaneous fat (encompassing the skin) in front of the caudal vertebra was determined using Vernier calipers.

2.6 Serum and liver lipid indices and fecal total BAs concentration

TC, triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and GLU in serum; TC, TG, HDL-C, and LDL-C in liver; and fecal total BAs concentration were detected according to the instructions of commercial kits [Glu kit (A154-1-1), TG assay kit (A110-1-1), TC assay kit (A111-1-1), HDL-C assay kit (A112-1-1), LDL-C assay kit (A113-1-1), and BAs assay kit (E003-2-1)] (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.7 Histomorphology analysis

HE staining: Liver and abdominal adipose tissue samples were fixed, stained with hematoxylin for 5 min, washed with running water, dehydrated with gradient alcohol, and stained with eosin for 5 min. Oil Red O staining: Fresh frozen tissue sections were fixed and stained with Oil Red O. They were then immersed in 60% isopropyl alcohol for 5 s, followed by a hematoxylin counterstain for 5 min. The slides were fixed and observed under a light microscope.

2.8 RT-qPCR analysis

RNA was isolated from liver and ileum tissues by using TRIzol reagent (Invitrogen, CA, United States), as described in our previous study (Nie et al., 2015). RT-qPCR analysis was conducted using a Light Cycler 96 System (Roche Applied Science, Penzberg, Germany) with primers listed in Table 2, and *β-actin* was used as an internal control for the target genes. The $2^{-\Delta\Delta Ct}$ method was used to standardize the fold change in mRNA levels.

2.9 16S RNA sequencing

DNA was isolated from the samples using a DNA extraction kit (M5635-02) (Omega Bio-Tek, Norcross, GA, United States). The following steps were performed according to Niu's method with slight modifications; details are provided in the Supplementary material (Niu et al., 2020).

2.10 LC/MS analysis of BAs

The liver tissue was homogenized, and BAs were extracted using a methanol extraction method, along with the addition of internal standards. Subsequently, the analysis was conducted on the BAs by using a combination of liquid chromatography and electrospray ionization tandem mass spectrometry; details are provided in the Supplementary material (Bhargava et al., 2020).

2.11 Lipidomics analysis

Liver tissue was homogenized, and lipids were extracted using a chloroform/methanol extraction method with the addition of internal standards. Subsequently, lipid analysis was conducted using a combination of liquid chromatography and electrospray ionization tandem mass spectrometry; details are provided in the Supplementary material (Werner et al., 2018).

TABLE 2 A list of primer sequences used in this study.

Gene	Forward sequence (5′-3′)	Reverse sequence (5′-3′)	Gene Bank No.
β-actin	ATTGTCCACCGCAAATGCTTC	AAATAAAGCCATGCCAATCTCGTC	NM_205518.2
FAS	TCAGGGTGTTCTGGAATGCAA	AATCCTGGTGGGCAATCGTAG	NM_205155.4
ACC	AATCCTGGTGGGCAATCGTAG	GGAACATTCAGGATACGC	NM_205505.2
CPT	GCCCTGATGCCTTCATTCAA	ATTTTCCCATGTCTCGGTAGTGA	NM_001012898.1
SREBP	TCACCGCTTCTTCGTGGAC	CTGAAGGTACTCCAACGCATC	NM_204126.3
LPL	AGTCAGAGTGAAGTCAGGCGAAAC	CTGCTCCAGGCACTTCACAAATA	NM_205282.2
PPARα	TGCACTGGAACTGGATGATAGTGA	TCCTACATTTACAAGACCAGGACGA	NM_001001464.1
PPARγ	TGTGAAGTTCAACGCACTGGAATTA	GGAGCTCCAAAGCT TGCAACA	NM_001001460.2
CYP7A1	AGGTAACGCCCTAGATGC	GCACTGTGGGCACTCTT	NM_001001753.2
CYP27A1	CTGATGTCCCGACGCA	TCCGCATCGGGTATTT	XM_422056.8
CYP8B1	GGAGACGAAGACCCAATG	CTTACTTAGAAACCCTGAACTG	NM_001005571.1
FXR	ATGGGATCTGAAATGAATTTAAT	ATGCCAAATAAATGAGGAC	NM_001396910.1
SHP	CCCCAAAGAATATGCCTACC	TGTCTGCGTTGCCGATG	NM_001030893.3
FGF19	ATTCGTCCAGACGGCTACAA	GGGATCCATGCTGTCCGTTT	NM_204674.3
IBABP	ATGGCATTCACAGGCAAATATGA	TGCTGAGTCCAGGTGAA	NM_001277700.2
ASBT	ATGCAGTCTTACTTACTTTCTCG	AACTGACAGAGGAAACCC	NM_001319027.2

2.12 Statistical analysis

One-way ANOVA was conducted using SPSS 22.0, significant differences were assessed using Duncan's method, and correlation analysis was conducted using Spearman analysis. $p < 0.05$ indicates statistical significance, and $p < 0.01$ indicates high significance. All data are presented as mean ± SD. Data were entered into SPSS version 22.0, and correlation coefficients were calculated based on Spearman correlation distances. Heat maps were constructed using ORIGIN 2021 to assess the bivariate relationships between variables.

3 Results

3.1 Effect of ZD-3 on growth performance, fat deposition, and serum lipid indices in broilers

No significant differences were observed in Body weight, ADFI, ADG, and F/G among the three groups (Table 3). However, ZD and HZD treatments significantly reduced the abdominal fat index and subcutaneous fat thickness compared with the CON ($p < 0.05$) (Table 3). Treatment with ZD significantly reduced the serum GLU, TG, TC, and LDL-C levels relative to that of the CON ($p < 0.05$). Treatment with HZD resulted in a decreasing trend in TG and LDL-C levels relative to those of the CON. No significant differences were observed in the HDL-C levels among the three groups (Table 3).

3.2 Effect of ZD-3 on tissue morphology, liver lipid indices, and lipid gene expression in broilers

HE staining revealed that of the CON, the hepatic cell arrangement was lax and chaotic, with numerous fat vacuoles of varying sizes and quantities. When broilers were treated with ZD or HZD, the fat droplets were reduced (Figure 1A). No significant differences were observed in the liver weight among the three groups (Figure 1B). The ZD and HZD treatments significantly decreased the average size of abdominal adipocytes relative to that in the CON ($p < 0.05$) (Figure 1C) and significantly reduced the liver TG levels relative to those in the CON ($p < 0.05$). No significant differences were observed in the TC and HDL-C levels among the three groups (Figure 1D). The ZD and HZD treatments significantly increased the levels of lipoprotein lipase (LPL) and carnitine palmitoyl transferase (CPT) and decreased the levels of fatty acid synthase (FAS), acetyl CoA carboxylase (ACC), and sterol-regulatory element-binding protein 1c (SREBP) in the liver compared with those of the CON ($p < 0.05$). Treatment with ZD and HZD significantly decreased the levels of ACC and SREBP in abdominal fat compared with those of the CON ($p < 0.05$) (Figure 1E).

3.3 Effect of ZD-3 on ileal microbiota structure in broilers

A comprehensive analysis of the broiler ileum microbiota using 16S rRNA sequencing technology revealed the presence of 2,431 operational taxonomic units (OTUs), comprising 21 phyla, 51 classes, 87 orders, 165 families, 346 genera, and 467 species. The α diversity of the ileum microbiota in broilers exposed to different treatments was assessed using OTU levels. The Chao1 index of the ileal microbiota of the ZD and HZD was higher than that of the CON, and the Shannon and Simpson indices of the ileal microbiota of the ZD group were higher than those of the CON and HZD groups (Figure 2A). The ZD and HZD treatments resulted in a higher abundance of ileum microbiota in broilers, with an increase in the number of OTUs by 19.94 and 16.53%, respectively, than that of the CON (Figure 2B). The principal coordinate analysis (PCoA) of β diversity revealed that the ileum microbiota of the ZD and HZD was similar but different from that of the CON (Figure 2C). Additional analysis of the composition

TABLE 3 Effects of active and heat-inactivated ZD-3 on growth performance, fat deposition, and serum lipid indices in yellow-feathered broilers.

Items	Treatment			SEM	*p*-value
	CON	ZD	HZD		
Growth performance					
Body weight, g	701.98	692.86	694.30	7.418	0.878
ADG, g/d	23.46	23.38	23.45	0.262	0.991
ADFI, g/d	54.38	52.91	53.62	0.428	0.397
F/G	2.32	2.26	2.29	0.026	0.747
Fat deposition					
Abdominal fat index, %	1.43a	0.79b	0.92b	0.088	0.002
Subcutaneous fat thickness, cm	0.17a	0.11b	0.09b	0.010	0.003
Serum lipid indices					
GLU, mmol/L	12.48a	9.80b	10.88b	0.374	0.009
TC, mmol/L	3.78a	2.83ab	3.61b	0.167	0.019
TG, mmol/L	0.52a	0.35ab	0.44b	0.026	0.034
LDL-C, mmol/L	0.80a	0.53ab	0.63b	0.047	0.050
HDL-C, mmol/L	1.76	1.90	1.96	0.231	0.944

[ab] indicates significant differences compared to CON at $p < 0.05$, respectively.

of ileum microbiota at the phylum level revealed that the ZD and HZD treatments decreased the relative abundance of *Firmicutes* by 10.23 and 1.60%, respectively, and significantly increased the relative abundance of *Bacteroidetes* by 231.85 and 325.99%, respectively ($p < 0.05$), decreasing in the F/B ratio compared with that of the CON (Figure 2D). Additional analysis of the composition of the ileum microbiota at the genus level revealed that the ZD treatment increased the abundance of *Enterococcus*, *Gallibacterium*, *Weissella*, and *Rothia* compared with that of the CON (Figure 2D). LEfSe analysis revealed significant differences in the abundances of 20 species among the three groups, with 12 species being most abundant in the ZD group and eight species in the HZD group (Figure 2E).

3.4 Effect of ZD-3 on liver BA profile and enterohepatic circulation of BAs in broilers

OPLS-DA was used to identify clustering trends in the data. The results of the ZD and HZD differed slightly from those of the CON (Figure 3A). A permutation test further validated the reliability of the model (Figure 3B). Hierarchical clustering heatmaps of the BA content among the three groups showed that when broilers were treated with ZD and HZD, the liver BA profile differed from that of the CON (Figure 3C). The liver BA change revealed that the ZD and HZD treatments significantly increased the concentration of total BAs and primary BAs relative to that of the CON ($p < 0.05$) (Figure 3D). The ZD and HZD treatments significantly increased the levels of Norcholic acid (NorCA), Nordeoxycholic Acid (NorDCA), and Taurolithocholic acid sodium salt (TLCA) compared with those of the CON but decreased the 3β-Cholic acid (β-CA) levels ($p < 0.05$). The ZD treatment significantly increased the levels of Tauro deoxycholic acid sodium salt (TDCA), and the HZD treatment significantly increased the levels of Cholic acid (CA), Taurocholic acid Sodium Salt (TCA), and Tauro-β-muricholic acid Sodium Salt (T-β-MCA) compared with those of the CON ($p < 0.05$) (Figure 3E). In this study, BAs from the three groups of samples were combined for KEGG pathway enrichment analysis for the biological interpretation of higher-level system functions. The metabolites identified using non-targeted metabolomics were primarily associated with the biosynthesis of primary BAs and taurine and hypo taurine metabolism (Figure 3F). The ZD and HZD treatments significantly increased the concentration of total BAs in the fecal matter compared with that of the CON ($p < 0.05$) (Figure 3G). ZD and HZD significantly increased the levels of cholesterol 7-α-hydroxylase (CYP7A1), sterol 12-α-hydroxylase (CYP8B1), and FXR in the liver and decreased the levels of FXR, FGF19, and apical sodium-dependent BA transport (ASBT) in the ileum compared with those of the CON ($p < 0.05$) (Figure 3H).

3.5 Effect of ZD-3 on liver lipid composition in broilers

A total of 3,229 lipid metabolites were detected in liver tissues across the three groups, namely 612 TG, 436 Phosphatidylcholine (PC), 278 Phosphatidylethanolamine (PE), 187 Ceramides (Cer), 178 Cardiolipin (CL), 161 methylated phosphatidylcholine (MePC), 152 Diglyceride (DG), and 147 Hexosylceramides (Hex1Cer) (Figure 4A). The control and treatment groups were distinguished using the OPLS-DA results (Figure 4B). However, the ZD and HZD groups could not be distinguished (Figure 4C). Heat map visualization revealed a significant decrease in the relative abundances of TG, PC, Lyso-phosphatidylethanolamine (LPE), and Zymosteryl (ZyE) and a significant increase in the relative abundances of Phosphatidylglycerol (PG), Lyso-phosphatidylcholine (LPC), and Phosphatidylserine (PS) in the ZD and HZD groups compared with the CON ($p < 0.05$) (Figure 4D). A total of 141 differential abundance (DA) lipids were identified (abundance increased more than 2.5 times and decreased more than 1.5 times compared with CON). Heat map visualization

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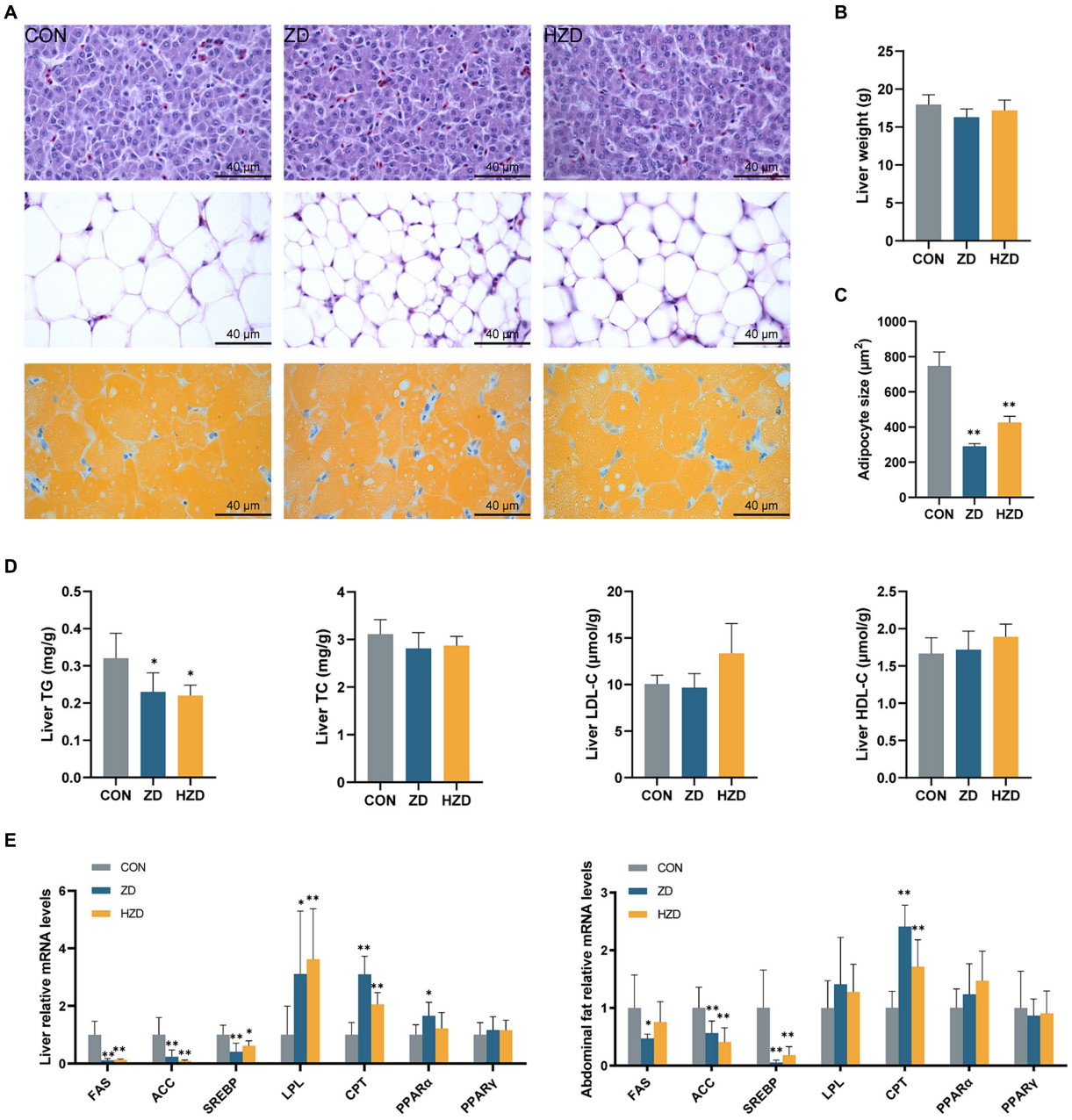

FIGURE 1
Effects of active and heat-inactivated ZD-3 on liver and abdominal fat metabolism in yellow-feathered broilers. **(A)** Representative images of H/E staining and Oil Red O staining of liver and abdominal fat sections (100 ×, scale bar = 40 μm). **(B)** Liver weight. **(C)** Distribution of adipocyte size in abdominal fat. **(D)** Liver lipid parameters indices: GLU, TG, TC, HDL-C, and LDL-C. **(E)** Lipid-related gene expression in the liver and abdominal fat (FAS, fatty acid synthase; ACC, acetyl CoA carboxylase; SREBP, sterol-regulatory element-binding protein 1c; LPL, lipoprotein lipase; CPT, carnitine palmitoyl transferase; PPAR-α, peroxisome proliferator-activated receptor-α; PPAR-γ, peroxisome proliferator-activated receptor-γ). Values are represented as means ± SD ($n = 6$). *$p < 0.05$, **$p < 0.01$ and indicate the significant differences between the CON.

(Figure 4E) confirmed that the individual lipids in the liver could be clearly classified into three groups, which was consistent with the analyses shown in Figure 4D. In this study, DA lipids from the three groups of samples were combined for lipid functional enrichment analysis, lipid classification, chemical and physical properties, and function and subcellular component interpretation of the higher-level system functions. We found that the metabolites identified through non-targeted metabolomics were primarily associated with membrane components, headgroups with positive charges/zwitterions, mitochondria, and glycerophospholipids (GP) (Figure 4F).

3.6 Correlation of ileal microbiota structure, liver BA profile, liver lipid composition, and fat deposition in broilers

Further analysis of Pearson correlations showed correlations among ileal microbial community, liver BA profile, liver lipid composition, and fat deposition. TCA had significant positive correlations with *Jeotgalicoccus* and *Enterococcus*. glycocholicacid (GCA) had significant positive correlations with *Enterococcus* ($p < 0.05$). T-β-MCA had significant positive correlations with

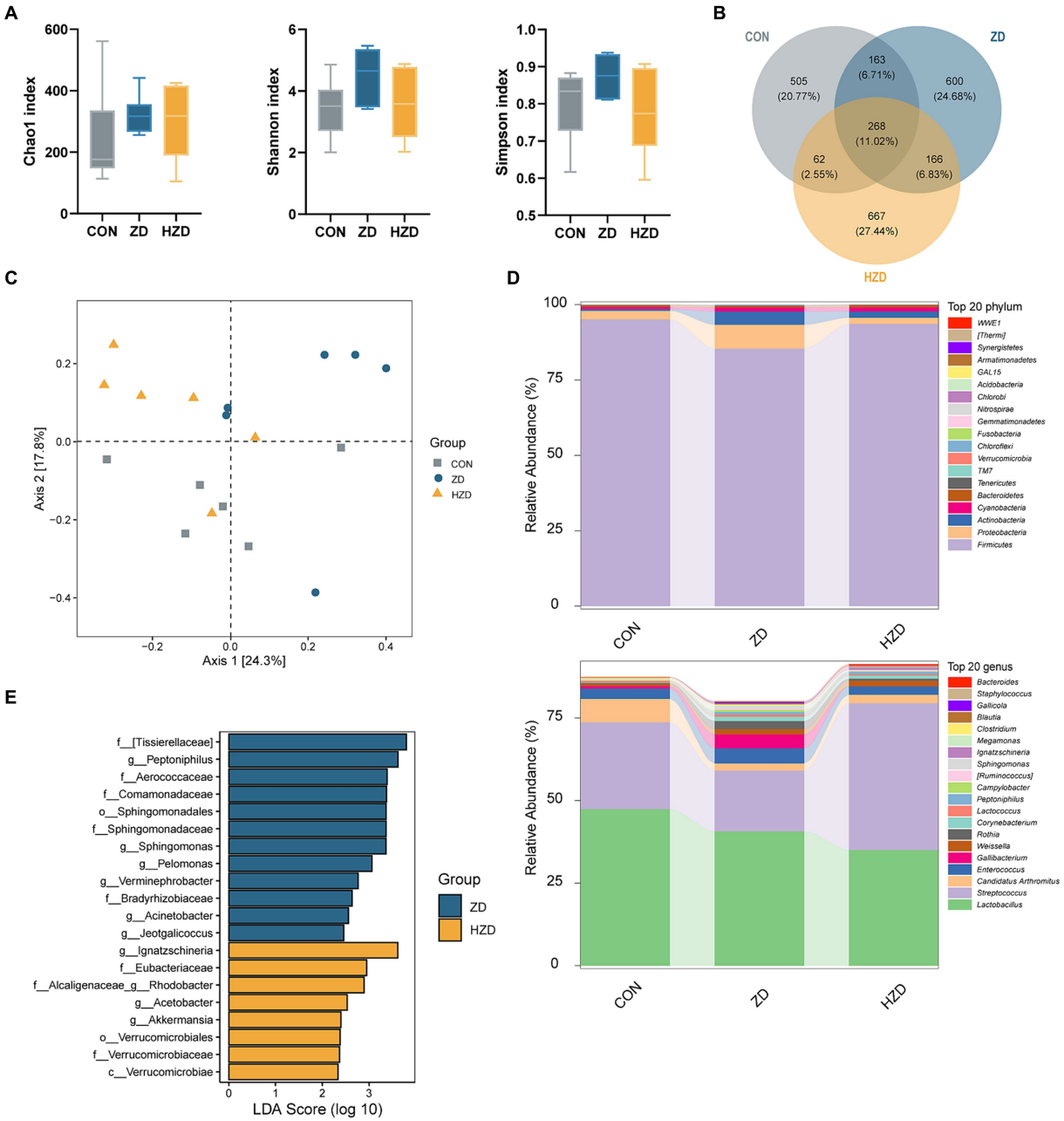

FIGURE 2
Effects of active and heat-inactivated ZD-3 on ileal microbiota structure in yellow-feathered broilers. **(A)** Alpha-diversity assessed using richness (Chao1 index) and diversity (Shannon index and Simpson index). **(B)** Venn diagram of OTUs among the groups. **(C)** Beta diversity calculated using Bray-Curtis based PCoA. **(D)** Relative abundance of microbiota at the phylum and genus level. **(E)** LDA score (LDA > 2) among the groups. Values are represented as means ± SD ($n = 6$).

Ignatzschineria ($p < 0.05$). TLCA had significant positive correlations with *Peptoniphilus* and *Jeotgalicoccus* ($p < 0.05$) (Figure 5A). MePC (34.5) had significant positive correlations with TCA, Lithocholic acid (LCA), and TLCA ($p < 0.05$). PC (33:6) and PC (12:0/18:2) had significant positive correlations with CA, TCA, GCA, TLCA, and T-β-MCA ($p < 0.05$). PE (18:3/18:2) had significant positive correlations with CA, TCA, TLCA, and T-β-MCA ($p < 0.05$). PE (38:7) had significant positive correlations with LCA, TLCA, and T-β-MCA ($p < 0.05$). DG (18:1/22:0) had significant negative correlations with TCA and T-β-MCA ($p < 0.05$). MePC (36:5e) and PE (20:4e) had significant negative correlations with TCA, GCA, LCA, and TLCA ($p < 0.05$). PC (32:1), PC (21:5e), and TG (16:1/18:2/18:3) had significant negative correlations with LCA and TLCA ($p < 0.05$). WE (21:1) had significant negative correlations with CA, TCA, and T-β-MCA ($p < 0.05$) (Figure 5B). The abdominal fat index had significant negative correlations with *Peptoniphilus*, TLCA, MePC34.5, PC (33:6), PC (12:0/18:2), and PE (18:3/18:2) and positive correlations with DG (18:1/22:0), PC (21:5e), TG (27:0/18:2/18:2), TG (6:0/6:0/13:0), and ZyE (35:1) ($p < 0.05$). Subcutaneous fat thickness had significant negative correlations

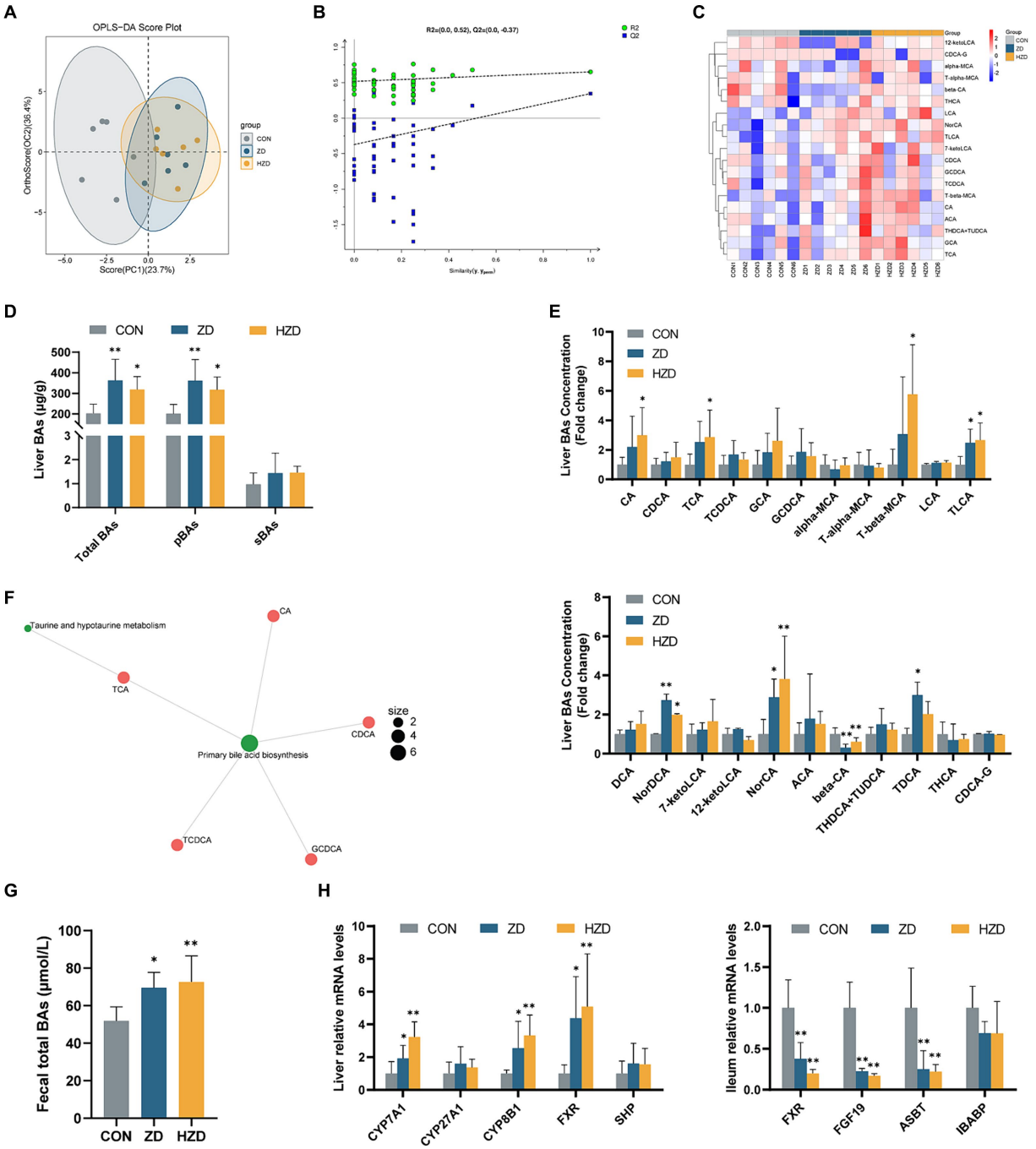

FIGURE 3
Effects of active and heat-inactivated ZD-3 on the enterohepatic circulation of BAs in yellow-feathered broilers. **(A)** Orthogonal partial least squares-discriminant analysis (OPLS-DA) based on the liver BAs metabolites. **(B)** Corresponding validation plots based on the liver BAs metabolites. **(C)** Hierarchical clustering heatmaps of the BAs contents in liver among the groups. **(D)** Liver BAs concentration: total BAs; pBAs, primary BAs; sBAs, secondary BAs. **(E)** Levels of BAs in the liver. **(F)** KEGG metabolite molecular network diagram based on the liver BAs metabolites. **(G)** Fecal total BAs concentration: total BAs. **(H)** BAs-related gene expression in the liver and ileum (CYP7A1, cytochrome P450 family 7 subfamily A member 1; CYP27A1, cytochrome P450 family 27 subfamily A member 1; CYP8B1, steps sterol 12-α-hydroxylase; FXR, farnesoid x receptor; SHP, small heterodimer partner; FGF19, fibroblast growth factor 19; ASBT, apical sodium-dependent BA transporter; IBABP, ileum BA binding protein). Values are represented as means ± SD ($n = 6$). *$p < 0.05$, **$p < 0.01$ and indicate the significant differences between the CON.

with *Jeotgalicoccus*, *Ignatzschineria*, TLCA, MePC34.5, PC (33:6), PC (12:0/18:2), PE (18:3/18:2), and PE (38:7) and positive correlations with PC (21:5e), PC (32:1), PE (20:4e), TG (16:1/18:2/18:3), TG (27:0/18:2/18:2), and TG (6:0/6:0/13:0) ($p < 0.05$) (Figure 5C).

4 Discussion

The results of the present study showed a decrease in broiler body weight and F/G under the influence of *Saccharomyces cerevisiae* tropicalis, which is consistent with the previous study (Niu et al.,

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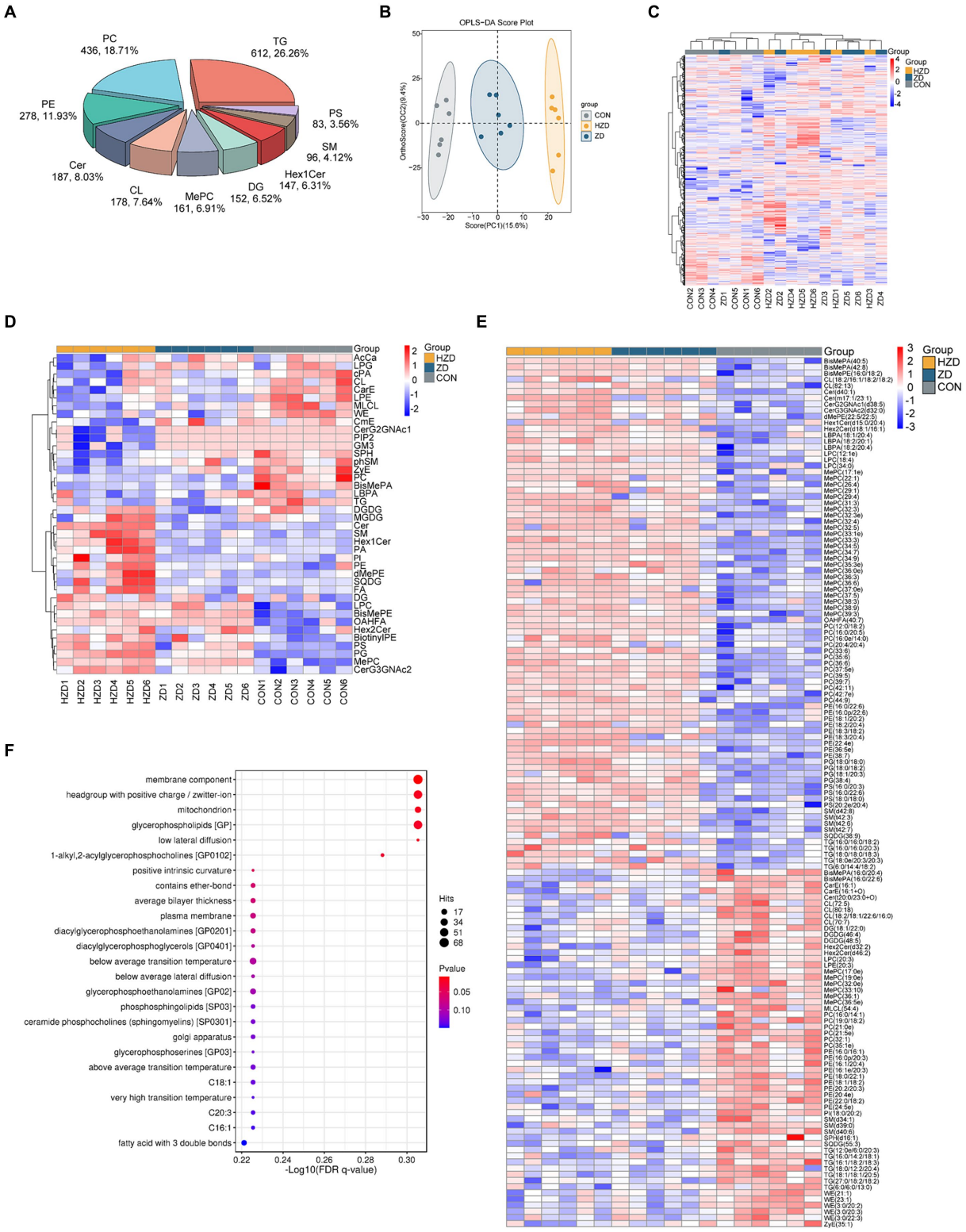

FIGURE 4
Effects of active and heat-inactivated ZD-3 on liver lipid composition in yellow-feathered broilers. **(A)** Percentages of the top 10 different lipid classes. **(B)** OPLS-DA based on the liver lipid metabolites. **(C)** Heatmaps based on total liver lipid content. **(D)** Hierarchical clustering heatmaps based on liver lipid classes. **(E)** Heatmaps of liver differential abundance (DA) of individual lipids (abundance increased more than 2.5 times and decreased more than 1.5 times compared with CON). **(F)** Lipid metabolic pathway analysis based on the liver lipid metabolites. The color indicates the significance of the enrichment *p* value, and the redder the color, the more significant the enrichment result; the size of the bubble point indicates the number of differential lipids enriched to the item, and the larger the bubble point, the more the enriched lipids are ($n = 6$).

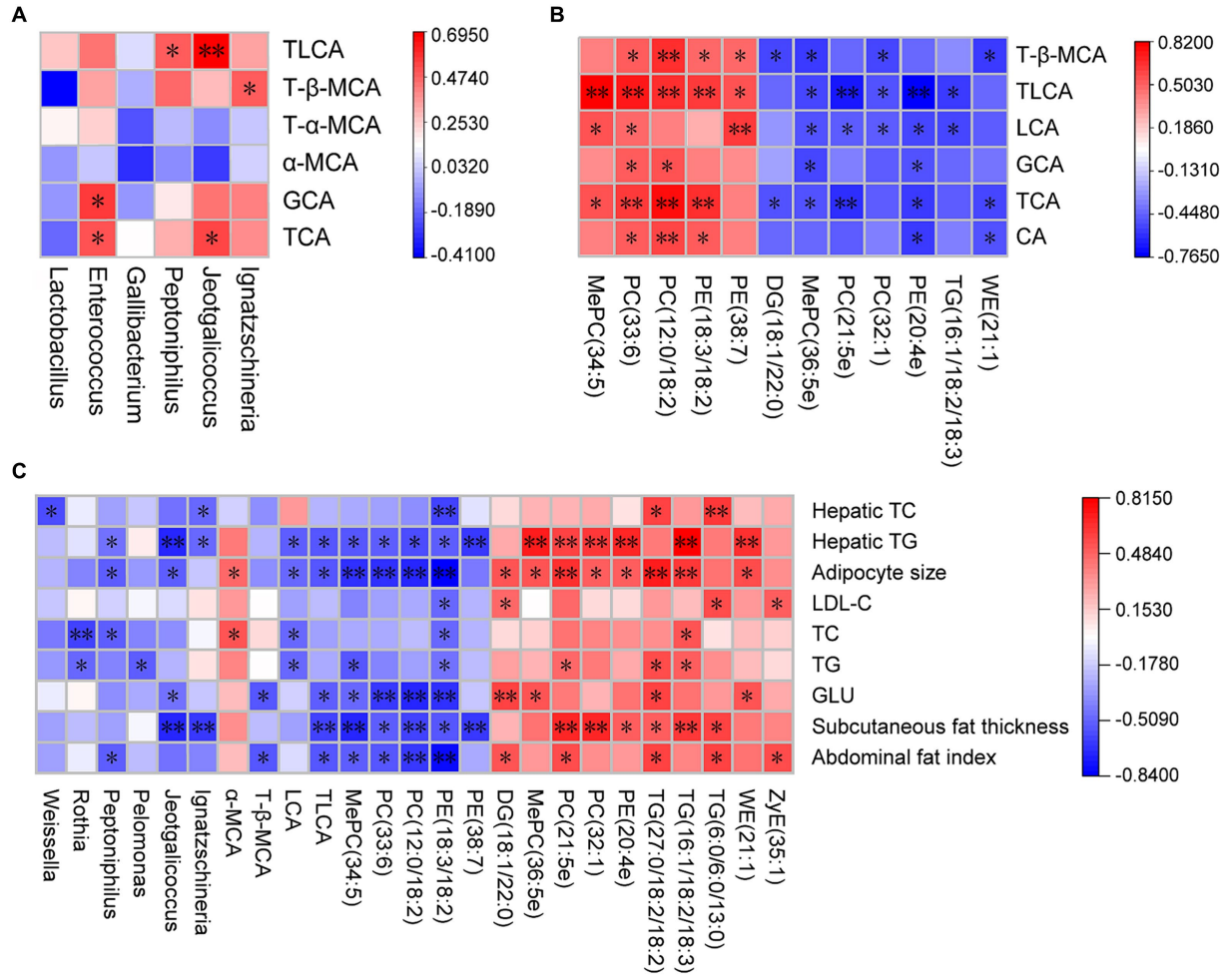

FIGURE 5
Correlation of ileal microbiota structure, liver BA profile, liver lipid composition and fat deposition in active and heat-inactivated ZD-3-treated broilers. **(A)** Correlation between ileal microbiota structure and liver BA profile. **(B)** Correlation between liver BA profile and liver lipid composition. **(C)** Correlation between ileal microbiota structure, liver BA profile, liver lipid composition, and fat deposition. Cells are colored based on Spearman's correlation coefficient: red represents a negative correlation, and blue represents a positive correlation, $*p < 0.05$, $**p < 0.01$.

2019). Probiotics prevent excessive abdominal fat deposition by inhibiting lipid synthesis and promoting fatty acid decomposition (Wang et al., 2022). In addition, ZD and HZD treatments reduced subcutaneous fat thickness and abdominal fat percentage, which suggests that ZD-3 can regulate fat metabolism in broilers, and heat-inactivated ZD-3 can play the same role and explain the loss of body weight in broilers. ZD-3 could reduce abdominal fat deposition and F/G without affecting other growth performance in broilers, thereby enhancing economic efficiency. Therefore, ZD-3 has the potential to be a novel probiotic preparation with lipid-lowering effects. This is also evidenced by the serum lipid indices. In this study, under the influence of ZD-3, GLU enters tissue cells for oxidative catabolism to provide energy and reduce fat deposition in broilers through energy metabolism. TG in broilers is mainly stockpiled in the abdominal fat, and serum TG levels and abdominal fat usually decrease simultaneously (Kim and Voy, 2021). The results showed that the addition of ZD-3 effectively reduced serum TG levels. To some extent, probiotics play a role in lowering serum TC levels (Qu et al., 2020). Meanwhile, ZD-3 has a beneficial effect on lowering serum TC and LDL-C, and the underlying mechanism may be that its cell walls adsorb cholesterol, or the bacterial cells transport cholesterol out of the body, promoting lipid metabolism (Ooi and Liong, 2010).

Hepatocytes are the major parenchymal cells of the liver that play a crucial role in the maintenance of liver and systemic lipid metabolism (Zhou et al., 2022). In this study, ZD-3 reduced the liver weight and intracellular lipid vacuoles. In this study, the surface area of adipocytes in abdominal fat was reduced in broilers fed ZD-3. Thus, ZD-3 can inhibit the enlargement of fat cells, leading to a decrease in fat accumulation. Consistent with the serum lipid results, the TG level in the liver of broilers fed ZD-3 was also significantly reduced. The ZD-3 strain demonstrated significant lipid-lowering effect, confirmed by serum and liver lipid indices and histomorphology tests. In general, cell viability is the main factor affecting the function of probiotics (Brandão et al., 2021). Our results indicate that heat-inactivated ZD-3 retains some physiological activity to provide lipid-lowering benefits to the host.

Additionally, ZD-3 regulated the expression of genes involved in lipid metabolism in broilers. Peroxisome proliferator-activated receptor-α (PPAR-α) is highly expressed in broiler liver and is primarily involved in the regulation of fatty acid oxidation and glucose

metabolism (Gu et al., 2022). CPT is a major regulator in the control of fatty acid β-oxidation in mitochondria (Dai et al., 2018). The mRNA levels of PPARα and CPT were increased in broilers fed with ZD-3, which may lead to a reduction in fat deposition by promoting fatty acid oxidation in the liver. LPL is a key enzyme that hydrolyzes TG to release fatty acids and glycerol (Huang et al., 2023). The increased expression of the LPL gene in the livers of broilers fed ZD-3 indicates that it may promote lipolysis, which is corroborated by the decrease in TG levels in the liver. SREBP and its response genes ACC and FAS activate fatty acid (FA) biosynthesis (Currie et al., 2013). In addition, ZD-3 reduced the mRNA expression of SREBP, ACC, and FAS in abdominal fat and liver tissue, implying that it reduces FA synthesis and fat deposition. Our study suggests that ZD-3 reduces the accumulation of fat in the liver and abdominal fat tissue by decreasing the mRNA expression related to *de novo* FA synthesis and increasing the mRNA expression related to FA oxidation.

It has been shown that probiotic supplements can effectively change the composition of the intestinal microbiota and influence its regulation of lipid metabolism, preventing the accumulation of abdominal fat (Helmink et al., 2019). Our study unequivocally demonstrated that ZD-3 led to changes in the composition of ileal microbial communities and an increase in microbial diversity in broilers. The principal microbial communities in the ileum of the broilers were *Firmicutes* and Proteobacteria (He et al., 2019). An increased abundance of *Firmicutes* usually disturbs host energy metabolism and results in fatty liver disease. The addition of ZD-3 led to a reduction in the abundance of *Firmicutes* in the ileum, which may lead to a decrease in liver lipids. In addition, ZD-3 increased the abundance of *Bacteroidetes* and decreased the F/B ratio, which has traditionally been considered an indicator of obesity. In this study, the addition of ZD-3 to the diet did not alter the dominance of *Firmicutes* in the ileum but increased the relative abundance of *Bacteroidetes* to regulate fat deposition. At the genus level, the abundance of *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Gallibacterium*, *Weissella*, and *Rothia* underwent significant changes in this study, which have been documented as potentially related to lipid metabolism (Turnbaugh et al., 2009). There are reports that *Enterococcus* has good cholesterol-lowering activity, which may improve lipid metabolism and prevent liver damage (Yang et al., 2021). In this study, ZD-3 increased the abundance of *Enterococcus*, which helped regulate cholesterol content to reduce fat deposition. It has been shown that mice fed a high-fat diet have a significantly reduced gut microbiota of *Weissella* (Liu et al., 2021), whereas its abundance was significantly increased by feeding ZD-3. In addition, *Rothia* has been enriched in the ZD group, which is beneficial to the host and contributes to regulating GLU metabolism, reducing lipid levels, and improving inflammation (Wang M. et al., 2023). These bacteria can produce bile salt hydrolase (BSH), a crucial component of the interaction between microbes and the host, which regulates the metabolism of host cholesterol and BAs (Liu et al., 2023). Notably, the alteration of the BA profile induced by BSH-producing bacteria may have a significant impact on promoting lipolysis. In addition, the microbial composition related to lipid metabolism was identified through LEfSe analysis. *Sphingomonas* synthesizes sphingosine, which subsequently combines with FA derivatives to form sphingomyelin, decreasing lipid accumulation in the liver (Zhang X. et al., 2022). *Jeotgalicoccus* produces cytochrome P450 enzymes for the decarboxylation and hydroxylation of fatty acids, which affects their levels in the host organism (Rude et al., 2011). *Acetobacter* synthesizes acetyl coenzyme A synthetase, which regulates the tricarboxylic acid cycle (Guertin and Wellen, 2023). These results suggest that active and heat-inactivated ZD-3 may improve host lipid metabolism by altering the composition and function of the gut microbiota and possibly by affecting the enterohepatic circulation of BA.

In the liver, cholesterol is primarily excreted from the body by being converted into bile acids. Cholesterol is converted to BAs and secreted into the ileum to facilitate the absorption of dietary lipids. Enterohepatic circulation of BAs is a highly efficient physiological mechanism (Chambers et al., 2019). Our research suggests that ZD-3 altered the liver BA profile and increased the relative concentration of BAs and the levels of CA, TCA, and GCA in liver tissue. These changes may have been influenced by alterations in the ileal microbiota (Collins et al., 2023). Spearman analysis revealed statistically significant positive correlations between TCA and the bacterial species *Enterococcus* and *Jeotgalicoccus*, and GCA had significant positive correlations with *Enterococcus*. ZD-3 has been found to contribute to the conversion of cholesterol into primary BA, and changes in BA profile were mainly reflected in the enrichment of taurine-conjugated BAs. Taurine metabolism prevents obesity by modulating mitochondrial function and promoting browning of adipose tissue (Guo et al., 2019). In this study, primary BA were primarily released as taurine-conjugated BAs in the ileum to activate the enterohepatic axis and reduce liver cholesterol levels. To gain insight into the process of BAs enterohepatic circulation, we analyzed the gene expression in broilers. In this study, the liver mRNA levels of CYP7A1 and CYP8B1 were significantly increased in broilers fed ZD-3. This result suggests that ZD-3 mainly enhances the classical pathway, accelerating the conversion of cholesterol. As CA is an activator of FXR, FXR expression was increased in the liver of broilers fed with ZD-3. When FXR is activated, it induces downstream FGF19 release, which regulates BAs metabolism (Jia et al., 2023). FXR activation down-regulates the expression of the target gene FAS and inhibits the expression of SREBP via the small heterodimer partner (SHP) and stimulates the expression of PPARα and peroxisome proliferator-activated receptor-γ (PPARγ), indirectly promoting the entry of fatty acids into mitochondria for β-oxidation (Shen et al., 2011; Xu et al., 2022). These results suggest that FXR has a significant impact on the regulation of lipid metabolism in the liver. The substantial buildup of taurine-conjugated BAs in the intestine can alleviate the occurrence of fatty liver by suppressing intestinal FXR (Huang et al., 2019). In addition, the production of BSH by the gut microbiota leads to an increase in the excretion of unconjugated BAs in the faces, facilitating the conversion of cholesterol into BAs (He and You, 2020). In our study, ZD-3 suppressed the FXR-FGF19 signaling pathway in the intestine by increasing the abundance of BSH-producing microbiota and promoting the formation of taurine-conjugated BAs. In the ileum, 95% of BAs are absorbed into the liver in the presence of ASBT and ileal BA binding protein (IBABP), completing enterohepatic circulation and regulating the size of the BA pool (Al-Dury and Marschall, 2018). ASBT inhibitors have been shown to improve hepatic steatosis in mice by modulating BA metabolism (Rao et al., 2016). In this study, ZD-3 treatment resulted in the downregulation of ileum ASBT expression, leading to increased BAs elimination, and decreased liver cholesterol levels. As a result, ZD-3 may reduce cholesterol accumulation in the liver and regulate lipid metabolism through a combination of decreased ileal FXR-FGF19 signaling via the gut microbiota-BA-FXR pathway and increased liver FXR-SHP signaling. Heat-inactivated ZD-3 could play the same role, possibly because the cell

wall, outer membrane proteins, and metabolites of ZD-3 retain some biological activity.

In this study, we found that ZD-3 induced changes in liver lipid biomarkers, which may explain the reduction in blood lipid levels and abdominal fat deposition. Significant but variable changes were observed in glycolipids (TG and DG), phospholipids (PC, PE, PG, and PS), and sphingolipids (Cer and Hex1Cer). The lipid classes may play a crucial role in regulating lipid metabolism in the liver of ZD-3. Of the 141 lipid molecules screened, downregulated lipids mainly belonged to the TG, DG, and Carboxylesterase (CarE) classes. The reduction in liver TGs was due to changes in the levels of the LPL, SREBP, ACC, and FAS genes. In hepatocytes, the reduction of CarE results in a decreased rate of lipid movement toward preexisting cytoplasmic lipid droplets, leading to a reduction in their size. This effect reduced weight gain, lowered blood lipid levels, and improved fatty liver disease (Lian et al., 2018). GP are the most abundant phospholipids found in living organisms. They form biofilms, are components of bile and membrane surfactants, and are involved in the recognition and signaling of proteins by cell membranes (Wang et al., 2020). We found multiple altered GP components in broilers fed ZD-3, such as PC, PE and MePC. PC, a major component of cell membranes, is involved in the secretion of very-low-density lipoproteins in the liver. Abnormally low or high levels can lead to steatosis (Kim et al., 2018). Lipolysis of the PE class was the main cause of the increase in free fatty acids. MePC is a methylated phospholipid and an important precursor of PC formation. Heatmap visualization showed that PC (33:6), PC (12:0/18:2), PC (32:1), PC (21:5e), PE (18:3/18:2), PE (38:7), PE (16:0/16:1), PE (20:4e), MePC (34:5), and MePC (36:5e) may be key lipids that mediate cell membrane signal transduction to regulate liver lipid metabolism. Spearman's analysis suggested that elevated levels of liver CA, TCA, GCA, LCA, and TLCA caused the GP changes in liver tissue.

Sphingolipids (SP) are components of cell membranes and lipoproteins involved in intracellular and extracellular signal transduction and the induction or activation of genes that regulate lipid metabolism (Summers et al., 2019). Cer serves as the central component of the SP pathway and can be converted into Sphingomyelin (SM) and HexCer. SM is a primary constituent of cell membranes. Changes in the levels of SM molecules in the cell membrane cause corresponding changes in cholesterol levels (St Clair et al., 2020). Thus, these altered SP might also play a role in regulating liver lipid metabolism by ZD-3. We also found higher levels of WE (21:1), WE (23:1), and ZyE (35:1) in the CON, and these DA lipids might be markers of lipid accumulation. The DA lipids examined in this study had various physiological functions, either beneficial or detrimental to lipid metabolism, and mainly belonged to the classes of PC and TG. Therefore, ZD-3 may regulate liver fat deposition by promoting TG degradation and modulating signal transduction in the cell membrane.

The liver is the center of fat transport. Changes in liver lipids in broilers can impact alterations in abdominal fat lipids and potentially influence systemic lipid changes. In this study, ZD-3 reduced abdominal fat deposition by altering the structure of ileal microbiota, liver BA profile, and liver lipid composition, and *Jeotgalicoccus*, *Ignatzschineria*, TCA, TLCA, MePC (34:5), PC (12:0/18:2), PE (18:3/18:2), and TG (16:1/18:2/18:3) may be markers for avoiding excessive fat deposition.

5 Conclusion

The addition of ZD-3 to feed helps prevent excessive fat deposition in yellow-feathered broilers by influencing the composition of *Firmicutes* and *Bacteroidetes* in the ileum microbiota, inhibiting ileal FXR-FGF19 and activating the liver FXR-SHP signaling pathway, which promotes the conversion of cholesterol to BAs and its excretion in the feces, and altering the liver lipid composition of TG and PC classes.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA1072686.

Ethics statement

The animal study was approved by regulations of the Bioethics Committee of Shihezi University (Xinjiang, China). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JF: Conceptualization, Data curation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. FW: Data curation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. SN: Methodology, Writing – review & editing. LD: Investigation, Software, Validation, Writing – review & editing. XP: Visualization, Writing – review & editing. JN: Investigation, Writing – review & editing. WZ: Funding acquisition, Writing – review & editing. CN: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1419424/full#supplementary-material

References

Abdalla, B. A., Chen, J., Nie, Q., and Zhang, X. (2018). Genomic insights into the multiple factors controlling abdominal fat deposition in a chicken model. *Front. Genet.* 9:262. doi: 10.3389/fgene.2018.00262

Al-Dury, S., and Marschall, H. U. (2018). Ileal bile acid transporter inhibition for the treatment of chronic constipation, Cholestatic pruritus, and NASH. *Front. Pharmacol.* 9:931. doi: 10.3389/fphar.2018.00931

Bhargava, P., Smith, M. D., Mische, L., Harrington, E., Fitzgerald, K. C., Martin, K., et al. (2020). Bile acid metabolism is altered in multiple sclerosis and supplementation ameliorates neuroinflammation. *J. Clin. Invest.* 130, 3467–3482. doi: 10.1172/jci129401

Brandão, L. R., de Brito Alves, J. L., da Costa, W. K. A., Ferreira, G. A. H., de Oliveira, M. P., Gomes da Cruz, A., et al. (2021). Live and ultrasound-inactivated *Lacticaseibacillus casei* modulate the intestinal microbiota and improve biochemical and cardiovascular parameters in male rats fed a high-fat diet. *Food Funct.* 12, 5287–5300. doi: 10.1039/d1fo01064f

Chambers, K. F., Day, P. E., Aboufarrag, H. T., and Kroon, P. A. (2019). Polyphenol effects on cholesterol metabolism via bile acid biosynthesis, CYP7A1: a review. *Nutrients* 11:2588. doi: 10.3390/nu11112588

Collins, S. L., Stine, J. G., Bisanz, J. E., Okafor, C. D., and Patterson, A. D. (2023). Bile acids and the gut microbiota: metabolic interactions and impacts on disease. *Nat. Rev. Microbiol.* 21, 236–247. doi: 10.1038/s41579-022-00805-x

Cuevas-González, P. F., Liceaga, A. M., and Aguilar-Toalá, J. E. (2020). Postbiotics and paraprobiotics: from concepts to applications. *Food Res. Int.* 136:109502. doi: 10.1016/j.foodres.2020.109502

Currie, E., Schulze, A., Zechner, R., Walther, T. C., and Farese, R. V. Jr. (2013). Cellular fatty acid metabolism and cancer. *Cell Metab.* 18, 153–161. doi: 10.1016/j.cmet.2013.05.017

Dai, J., Liang, K., Zhao, S., Jia, W., Liu, Y., Wu, H., et al. (2018). Chemoproteomics reveals baicalin activates hepatic CPT1 to ameliorate diet-induced obesity and hepatic steatosis. *Proc. Natl. Acad. Sci. USA* 115, E5896–e5905. doi: 10.1073/pnas.1801745115

Delzenne, N. M., Neyrinck, A. M., Bäckhed, F., and Cani, P. D. (2011). Targeting gut microbiota in obesity: effects of prebiotics and probiotics. *Nat. Rev. Endocrinol.* 7, 639–646. doi: 10.1038/nrendo.2011.126

Depommier, C., Everard, A., Druart, C., Plovier, H., Van Hul, M., Vieira-Silva, S., et al. (2019). Supplementation with *Akkermansia muciniphila* in overweight and obese human volunteers: a proof-of-concept exploratory study. *Nat. Med.* 25, 1096–1103. doi: 10.1038/s41591-019-0495-2

Feng, K., Lan, Y., Zhu, X., Li, J., Chen, T., Huang, Q., et al. (2020). Hepatic lipidomics analysis reveals the antiobesity and cholesterol-lowering effects of tangeretin in high-fat diet-fed rats. *J. Agric. Food Chem.* 68, 6142–6153. doi: 10.1021/acs.jafc.0c01778

Fouad, A. M., and El-Senousey, H. K. (2014). Nutritional factors affecting abdominal fat deposition in poultry: a review. *Asian Australas. J. Anim. Sci.* 27, 1057–1068. doi: 10.5713/ajas.2013.13702

Gu, Y., Duan, S., Ding, M., Zheng, Q., Fan, G., Li, X., et al. (2022). Saikosaponin D attenuates metabolic associated fatty liver disease by coordinately tuning PPARα and INSIG/SREBP1c pathway. *Phytomedicine* 103:154219. doi: 10.1016/j.phymed.2022.154219

Guertin, D. A., and Wellen, K. E. (2023). Acetyl-CoA metabolism in cancer. *Nat. Rev. Cancer* 23, 156–172. doi: 10.1038/s41568-022-00543-5

Guo, Y. Y., Li, B. Y., Peng, W. Q., Guo, L., and Tang, Q. Q. (2019). Taurine-mediated browning of white adipose tissue is involved in its anti-obesity effect in mice. *J. Biol. Chem.* 294, 15014–15024. doi: 10.1074/jbc.RA119.009936

He, Y., Yang, Y., Dong, Y., Yan, C., and Zhang, B. (2019). The effects of Flavomycin and Colistin sulfate pre-treatment on Ileal bacterial community composition, the response to salmonella typhimurium and host gene expression in broiler chickens. *Microorganisms* 7:574. doi: 10.3390/microorganisms7110574

He, Y. J., and You, C. G. (2020). The potential role of gut microbiota in the prevention and treatment of lipid metabolism disorders. *Int. J. Endocrinol.* 2020, 8601796–8601799. doi: 10.1155/2020/8601796

Helmink, B. A., Khan, M. A. W., Hermann, A., Gopalakrishnan, V., and Wargo, J. A. (2019). The microbiome, cancer, and cancer therapy. *Nat. Med.* 25, 377–388. doi: 10.1038/s41591-019-0377-7

Huang, Y., Li, L., Chen, L., Chen, X., Gao, P., Qi, Y., et al. (2023). 6-OR: PAR2 downregulates adipose LPL through MIF—A novel adipose mechanism contributing to the development of hypertriglyceridemia. *Diabetes* 72:6-OR. doi: 10.2337/db23-6-OR

Huang, F., Zheng, X., Ma, X., Jiang, R., Zhou, W., Zhou, S., et al. (2019). Theabrownin from Pu-erh tea attenuates hypercholesterolemia via modulation of gut microbiota and bile acid metabolism. *Nat. Commun.* 10:4971. doi: 10.1038/s41467-019-12896-x

Jia, W., Li, Y., Cheung, K. C. P., and Zheng, X. (2023). Bile acid signaling in the regulation of whole body metabolic and immunological homeostasis. *Sci. China Life Sci.* 67, 865–878. doi: 10.1007/s11427-023-2353-0

Kim, Y. C., Seok, S., Byun, S., Kong, B., Zhang, Y., Guo, G., et al. (2018). AhR and SHP regulate phosphatidylcholine and S-adenosylmethionine levels in the one-carbon cycle. *Nat. Commun.* 9:540. doi: 10.1038/s41467-018-03060-y

Kim, M., and Voy, B. H. (2021). Fighting fat with fat: n-3 polyunsaturated fatty acids and adipose deposition in broiler chickens. *Front. Physiol.* 12:755317. doi: 10.3389/fphys.2021.755317

Larsen, I. S., Choi, B. S., Föh, B., Kristensen, N. N., Ouellette, A., Haller, R. F., et al. (2023). Experimental diets dictate the metabolic benefits of probiotics in obesity. *Gut Microbes* 15:2192547. doi: 10.1080/19490976.2023.2192547

Li, M., Xu, J., Zhang, Y., Chu, S., Sun, S., Huo, Y., et al. (2020). Comparative analysis of fecal metabolite profiles in HFD-induced obese mice after oral administration of huangjinya green tea extract. *Food Chem. Toxicol.* 145:111744. doi: 10.1016/j.fct.2020.111744

Lian, J., Nelson, R., and Lehner, R. (2018). Carboxylesterases in lipid metabolism: from mouse to human. *Protein Cell* 9, 178–195. doi: 10.1007/s13238-017-0437-z

Liu, J., Sun, J., Yu, J., Chen, H., Zhang, D., Zhang, T., et al. (2023). Gut microbiome determines therapeutic effects of OCA on NAFLD by modulating bile acid metabolism. *NPJ Biofilms Microbiomes* 9:29. doi: 10.1038/s41522-023-00399-z

Liu, J., Wu, S., Cheng, Y., Liu, Q., Su, L., Yang, Y., et al. (2021). Sargassum fusiforme alginate relieves hyperglycemia and modulates intestinal microbiota and metabolites in type 2 diabetic mice. *Nutrients* 13:2887. doi: 10.3390/nu13082887

Nie, C., Zhang, W., Ge, W.-X., Yanfeng, L., Wang, Y.-Q., and Liu, J. (2015). Effect of cottonseed meal fermented with yeast on the lipid-related gene expression in broiler chickens. *Braz. J. Poult. Sci.* 17, 57–64. doi: 10.1590/1516-635XSPECIALISSUENutrition-PoultryFeedingAdditives057-064

Niu, J., Zhang, J., Wei, L., Ma, X., Zhang, W., and Nie, C. (2020). Cottonseed meal fermented by Candida tropical reduces the fat deposition in white-feather broilers through cecum bacteria-host metabolic cross-talk. *Appl. Microbiol. Biotechnol.* 104, 4345–4357. doi: 10.1007/s00253-020-10538-7

Niu, J. L., Zhang, J., Wei, L. Q., Zhang, W. J., and Nie, C. X. (2019). Effect of fermented cottonseed meal on the lipid-related indices and serum metabolic profiles in broiler chickens. *Animals (Basel)* 9:930. doi: 10.3390/ani9110930

Ooi, L. G., and Liong, M. T. (2010). Cholesterol-lowering effects of probiotics and prebiotics: a review of *in vivo* and *in vitro* findings. *Int. J. Mol. Sci.* 11, 2499–2522. doi: 10.3390/ijms11062499

Pathak, P., Xie, C., Nichols, R. G., Ferrell, J. M., Boehme, S., Krausz, K. W., et al. (2018). Intestine farnesoid X receptor agonist and the gut microbiota activate G-protein bile acid receptor-1 signaling to improve metabolism. *Hepatology* 68, 1574–1588. doi: 10.1002/hep.29857

Qu, T., Yang, L., Wang, Y., Jiang, B., Shen, M., and Ren, D. (2020). Reduction of serum cholesterol and its mechanism by *Lactobacillus plantarum* H6 screened from local fermented food products. *Food Funct.* 11, 1397–1409. doi: 10.1039/c9fo02478f

Rao, A., Kosters, A., Mells, J. E., Zhang, W., Setchell, K. D., Amanso, A. M., et al. (2016). Inhibition of ileal bile acid uptake protects against nonalcoholic fatty liver

disease in high-fat diet-fed mice. *Sci. Transl. Med.* 8:357ra122. doi: 10.1126/scitranslmed.aaf4823

Rude, M. A., Baron, T. S., Brubaker, S., Alibhai, M., Del Cardayre, S. B., and Schirmer, A. (2011). Terminal olefin (1-alkene) biosynthesis by a novel p450 fatty acid decarboxylase from Jeotgalicoccus species. *Appl. Environ. Microbiol.* 77, 1718–1727. doi: 10.1128/aem.02580-10

Salminen, S., Collado, M. C., Endo, A., Hill, C., Lebeer, S., Quigley, E. M. M., et al. (2021). The international scientific Association of Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of postbiotics. *Nat. Rev. Gastroenterol. Hepatol.* 18, 649–667. doi: 10.1038/s41575-021-00440-6

Shen, L. L., Liu, H., Peng, J., Gan, L., Lu, L., Zhang, Q., et al. (2011). Effects of farnesoid X receptor on the expression of the fatty acid synthetase and hepatic lipase. *Mol. Biol. Rep.* 38, 553–559. doi: 10.1007/s11033-010-0140-0

St Clair, J. W., Kakuda, S., and London, E. (2020). Induction of ordered lipid raft domain formation by loss of lipid asymmetry. *Biophys. J.* 119, 483–492. doi: 10.1016/j.bpj.2020.06.030

Summers, S. A., Chaurasia, B., and Holland, W. L. (2019). Metabolic messengers: ceramides. *Nat. Metab.* 1, 1051–1058. doi: 10.1038/s42255-019-0134-8

Tallentire, C. W., Leinonen, I., and Kyriazakis, I. (2016). Breeding for efficiency in the broiler chicken: a review. *Agron. Sustain. Dev.* 36:66. doi: 10.1007/s13593-016-0398-2

Tang, C., Kong, L., Shan, M., Lu, Z., and Lu, Y. (2021). Protective and ameliorating effects of probiotics against diet-induced obesity: a review. *Food Res. Int.* 147:110490. doi: 10.1016/j.foodres.2021.110490

Turnbaugh, P. J., Hamady, M., Yatsunenko, T., Cantarel, B. L., Duncan, A., Ley, R. E., et al. (2009). A core gut microbiome in obese and lean twins. *Nature* 457, 480–484. doi: 10.1038/nature07540

Wang, Y., Ai, Z., Xing, X., Fan, Y., Zhang, Y., Nan, B., et al. (2022). The ameliorative effect of probiotics on diet-induced lipid metabolism disorders: a review. *Crit. Rev. Food Sci. Nutr.* 64, 3556–3572. doi: 10.1080/10408398.2022.2132377

Wang, R., Li, B., Lam, S. M., and Shui, G. (2020). Integration of lipidomics and metabolomics for in-depth understanding of cellular mechanism and disease progression. *J. Genet. Genomics* 47, 69–83. doi: 10.1016/j.jgg.2019.11.009

Wang, Y., Xing, X., Ma, Y., Fan, Y., Zhang, Y., Nan, B., et al. (2023). Prevention of high-fat-diet-induced dyslipidemia by *Lactobacillus plantarum* LP104 through mediating bile acid enterohepatic Axis circulation and intestinal Flora. *J. Agric. Food Chem.* 71, 7334–7347. doi: 10.1021/acs.jafc.2c09151

Wang, M., Yan, L. Y., Qiao, C. Y., Zheng, C. C., Niu, C. G., Huang, Z. W., et al. (2023). Ecological shifts of salivary microbiota associated with metabolic-associated fatty liver disease. *Front. Cell. Infect. Microbiol.* 13:1131255. doi: 10.3389/fcimb.2023.1131255

Wen, C., Yan, W., Sun, C., Ji, C., Zhou, Q., Zhang, D., et al. (2019). The gut microbiota is largely independent of host genetics in regulating fat deposition in chickens. *ISME J.* 13, 1422–1436. doi: 10.1038/s41396-019-0367-2

Werner, E. R., Keller, M. A., Sailer, S., Seppi, D., Golderer, G., Werner-Felmayer, G., et al. (2018). A novel assay for the introduction of the vinyl ether double bond into plasmalogens using pyrene-labeled substrates. *J. Lipid Res.* 59, 901–909. doi: 10.1194/jlr.D080283

Xu, S., Jia, P., Fang, Y., Jin, J., Sun, Z., Zhou, W., et al. (2022). Nuclear farnesoid X receptor attenuates acute kidney injury through fatty acid oxidation. *Kidney Int.* 101, 987–1002. doi: 10.1016/j.kint.2022.01.029

Yang, L., Xie, X., Li, Y., Wu, L., Fan, C., Liang, T., et al. (2021). Evaluation of the cholesterol-lowering mechanism of *Enterococcus faecium* strain 132 and *Lactobacillus paracasei* strain 201 in hypercholesterolemia rats. *Nutrients* 13:1982. doi: 10.3390/nu13061982

Zhang, Y., Gu, Y., Jiang, J., Cui, X., Cheng, S., Liu, L., et al. (2022). Stigmasterol attenuates hepatic steatosis in rats by strengthening the intestinal barrier and improving bile acid metabolism. *NPJ Sci. Food* 6:38. doi: 10.1038/s41538-022-00156-0

Zhang, X., Hu, Y., Ansari, A. R., Akhtar, M., Chen, Y., Cheng, R., et al. (2022). Caecal microbiota could effectively increase chicken growth performance by regulating fat metabolism. *Microb. Biotechnol.* 15, 844–861. doi: 10.1111/1751-7915.13841

Zhou, B., Luo, Y., Ji, N., Hu, C., and Lu, Y. (2022). Orosomucoid 2 maintains hepatic lipid homeostasis through suppression of de novo lipogenesis. *Nat. Metab.* 4, 1185–1201. doi: 10.1038/s42255-022-00627-4

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The signatures and crosstalk of gut microbiome, mycobiome, and metabolites in decompensated cirrhotic patients

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Background: Numerous studies have confirmed that gut microbiota plays a crucial role in the progression of cirrhosis. However, the contribution of gut fungi in cirrhosis is often overlooked due to the relatively low abundance.

Methods: We employed 16S ribosomal RNA sequencing, internal transcribed spacer sequencing, and untargeted metabolomics techniques to investigate the composition and interaction of gut bacteria, fungi, and metabolites in cirrhotic patients.

Results: Cirrhotic patients exhibited significant differences in the diversity and composition of gut microbiota and their metabolites in cirrhotic patients compared to healthy individuals. Increase in pathogenic microbial genera and a decrease in beneficial microbial genera including bacteria and fungi were observed. Various clinical indexes were closely connected with these increased metabolites, bacteria, fungi. Additionally, endoscopic treatment was found to impact the gut microbiota and metabolites in cirrhotic patients, although it did not significantly alter the gut ecology. Finally, we constructed a cirrhosis diagnostic model based on different features (bacteria, fungi, metabolites, clinical indexes) with an AUC of 0.938.

Conclusion: Our findings revealed the characteristics of gut microbial composition and their intricate internal crosstalk in cirrhotic patients, providing cutting-edge explorations of potential roles of gut microbes in cirrhosis.

KEYWORDS

cirrhosis, gut dysbiosis, bacteria, fungi, metabolome

Introduction

Human gastrointestinal tracts harbor trillions of microbes (about 10^{12}-10^{14}), including bacteria, fungi, archaea, and viruses, which are involved in many physiological functions and disease pathogenesis (Gomaa, 2020). In recent years, the importance of intestines to human health and dysregulation of the gut microbes in different diseases has been implicated based on high-throughput sequencing technology (Lynch et al., 2016). Due to its unique anatomical location, the liver is regarded as the first organ to encounter gut

microbes and their products through the gut epithelial barrier. The blood supply to the liver comes from the hepatic artery and portal vein. Portal blood contributes to 75% of the total blood flow to the liver and transfers nutrients, enteric-borne microbes, and pathogen-associated molecular patterns to liver originating from the intestine (Llorente and Schnabl, 2015; Hsu and Schnabl, 2023). Thus, the liver and gastrointestinal tract are interconnected, known as the gut-liver axis (Tripathi et al., 2018). Appreciable evidence suggests that gut microbes play a central role in the gut-liver axis and are closely related to liver diseases (Schnabl and Brenner, 2014; Pabst et al., 2023). However, the contributions of gut microbes to the occurrence and progression of liver diseases and the feasibility of clinical applications targeting gut microbes have yet to be fully elucidated.

Liver cirrhosis (LC) is a common outcome of chronic liver diseases, characterized by tissue fibrosis and the transformation of normal liver architecture into abnormal nodules (Kisseleva and Brenner, 2020). LC progression eventually leads to the development of portal hypertension and its associated complications with high mortality, accounting for 1.2 million deaths per year and nearly 3.5% of global mortality (Moon et al., 2020). The gut microbes have been proven to be related to the induction and progression of liver injury (Yan et al., 2011). Gut bacterial dysbiosis may adversely disorder liver homeostasis, leading to the progression and complications of LC (Qin et al., 2014; Lachar and Bajaj, 2016). Accumulating studies have revealed significant microbial dysbiosis with substantially decreased richness, diversity, and altered composition of the gut bacteria translocation in LC patients compared with healthy individuals (Bajaj et al., 2014; Shao et al., 2018).

In addition, bacterial and fungal communities do have significant impacts on the homeostasis of the intestinal microecosystem, although colonizing fungi account for a minor component of the gut microbiota (Dworecka-Kaszak et al., 2016). Traditional culture-dependent methods can only detect small proportions of fungi. With the development of high-throughput sequencing technology, fungal sequencing methods, including 18S ribosomal RNA sequencing and fungal-specific internal transcribed spacer DNA sequencing, have gradually replaced traditional methods for studying fungi. With the growing knowledge of the characteristics and species of fungi, complex relationships between gut fungi and human health were discovered, and the alterations of fungal communities may contribute to the occurrence of liver diseases (Zeng and Schnabl, 2022). Clinical studies suggested that fungal colonization and translocation could increase the mortality rate of LC patients in intensive care unit (ICU; Theocharidou et al., 2016; Verma et al., 2022). Consistently, a cross-sectional study has revealed the presence of dysregulated intestinal fungi in cases of cirrhosis and hepatocellular carcinoma by ITS (internal transcribed spacer) DNA sequencing (Bajaj et al., 2018; Zhang et al., 2023). Although bacteria and fungi are dysregulated in cirrhosis, previous efforts mainly focused on bacteria, the impact of the differences in fungi and the crosstalk between fungi and bacteria in patients with cirrhosis is barely known, especially in those with endoscopic treatment.

In the present study, we performed 16S ribosomal RNA and ITS sequencing, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) technology to reveal gut microbial characteristics and explore the connections among intestinal bacteria, fungi, and metabolites in patients with liver cirrhosis. Our study reveals the complex interactions within the intestinal microbiota of cirrhotic patients, providing new insights for the diagnosis and treatment of liver cirrhosis.

Materials and methods

Patients and sample collection

A total of 45 patients with decompensated liver cirrhosis who were admitted to the Department of Gastroenterology and Hepatology of Beijing You'an Hospital affiliated with Capital Medical University from January to May 2023 were enrolled, while another 30 healthy people without any chronic diseases were selected as control group. Patients were diagnosed with liver cirrhosis in terms of clinical symptoms, medical history, laboratory tests, imaging tests, or histological examinations, and complications included esophagogastric varices (EGV), ascites, hepatic encephalopathy (HE), portal vein thrombosis (PVT). The demographic and clinical data of the subjects involved were collected through the electronic medical record system. Exclusion criteria included the following conditions: aged$<$ 18 or $>$75; ursodeoxycholic acid, probiotics, or antibiotics treatment before sample collection; hepatic encephalopathy $>$ grade 2 and/or other cognitive disorders not allowing for informed consent; suffered from malignant cancers including hepatocellular carcinoma, severe cardiopulmonary failure, and other diseases (gastrointestinal intestinal polyps, inflammatory bowel disease, necrotizing enteritis, diabetes, etc.). Feces samples were collected in the morning after an overnight fast, delivered to the laboratory within 2 h on dry ice, and stored at -80°C until further analyses. In addition, to further explore the short-term effects of endoscopic treatment of esophagogastric variceal on the gastrointestinal flora, we also collected the feces of patients who underwent endoscopic treatment 10–14 days. The Ethics Committee of Beijing You'an Hospital authorized the project (LL-2022-065-K), and Informed consent was obtained from all individual participants included in the study.

Clinical data analysis

Descriptive and comparative statistics were used to assess and compare clinical data between two groups. Quantitative data were represented as Mean $\pm$ SE (standard error) and categorical variables were represented by the number of cases and percentage. Continuous data was performed by using *t*-test, the chi-square test and the Fisher's precision probability test for categorical variables. *P*-value $<$ 0.05 was considered statistically significant.

DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted using the DNeasy Power Water DNA Isolation Kit (Qiagen, Germany, Cat: 14900-100-NF) according to the manufacturer's instructions with minor

modifications for separate extraction of bacterial and fungal genomic DNA.

Equal concentrations of bacterial and fungal DNA (~10 ng) were used for PCR amplification of the bacterial 16S rRNA V4 hypervariable region and fungal ITS2 region. The V4 primers are forward 5′ -GTGCCAGCMGCCGCGGTAA-3′ and reverse 5′-GGACTACHVGGGTWTCTAAT-3′ (Sun et al., 2013). For fungi, the ITS2 intergenic region were amplified using the forward primer (5′-GCATCGATGAAGAACGCAGC-3′) and reverse (5′-TCCTCCGCTTATTGATATGC-3′; Zhang et al., 2023). 16S rRNA V4 hypervariable region and ITS2 region were amplified used the specific primer with the barcode. Sequencing libraries were generated using Illumina TruSeq DNA PCR-Free Library Preparation Kit (Illumina, USA) following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an Illumina NovaSeq platform and 250 bp paired-end reads were generated.

Bioinformatics and statistical analysis

Raw bacterial and fungal amplicon sequences were processed and analyzed with the EasyAmplicon (Liu et al., 2023) pipeline (version 1.19, https://github.com/YongxinLiu/EasyAmplicon). First, FastQC (version 0.12.1) was used to control the raw data quality and confirm the absence of primers. Subsequently, the software VSEARCH (Rognes et al., 2016; version 2.22.1) was used to merge pair-end reads (-fastq_mergepairs), filter (-fastq_maxee_rate 0.01), and dereplicate (-derep_fulllength). The obtained high-quality, unique sequences were denoised into amplicon sequence variants (ASVs) with the software USEARCH (Edgar, 2010; version 11.0.667). Chimeras were identified and removed from the bacterial and fungal data using VSEARCH(-uchime_ref) against the SILVA (Quast et al., 2012) v123 database and UNITE (Abarenkov et al., 2023) v9.0 database, respectively. Finally, based on the chimera-free ASVs, we constructed feature tables using VSEARCH (-usearch_global). Species classification of bacterial and fungal ASVs was done based on the RDP (Cole et al., 2014) v18 database and UNITE v9.0 database, with USEARCH (-sintax), respectively. Bacterial and fungal sequences of all samples were rarefied to 62,889 and 5,766 for downstream analysis, respectively.

Alpha diversity of bacterial and fungal communities was calculated using USEARCH (-alpha_div) based on the richness index. The differences between groups were assessed using Tukey's HSD test. Beta diversity was calculated using USEARCH (-cluster_agg, -beta_div) through principal coordinate analysis (PCoA) based on the Bray-Curtis distance. Permutational multivariate analysis of variance (PERMANOVA) with the ADONIS test was used to assess the differences between groups. Linear discriminant analysis (LDA) effect size (LEfSe) was used to determine biomarkers at the genus level between groups (LDA score>3). The functional composition of bacterial and fungal communities was predicted based on the MetaCyc (Caspi et al., 2018) database (https://metacyc.org/) using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2; Douglas et al., 2020) software. The *P*-values of metabolic pathways differing between groups were calculated by Welch's *t*-test and corrected using the Benjamini-Hochberg false discovery rate (FDR). Orthogonal partial least squares discriminant analysis (OPLS-DA) was used to determine the differences in metabolite composition among samples. The screening criteria for differential metabolites were: *P*-value of Student's *t*-test < 0.05, Variable Importance in the Projection (VIP) values obtained from the OPLS -DA model was >1, and the absolute value of $\log_2$(fold change) was >0. The VIP values were used to assess the contribution of the different metabolites to the differences between groups. Pathway enrichment analysis of differential metabolites was done based on the Kyoto Encyclopedia of Genes and Genomes (Kanehisa, 2000) pathway database. Metabolic pathways that were significantly enriched (*P*-value $<$ 0.05) were identified by the hypergeometric test.

Associations among differential bacteria-clinical indexes, differential fungi-clinical indexes, and differential metabolite-clinical indexes, as well as bacteria-fungi-differential metabolites in liver cirrhotic patients, were determined based on the Spearman's correlation analysis (correlation coefficient > 0.4, *P*-value $<$ 0.05). The random forest model used to distinguish liver cirrhosis patients from healthy individuals was constructed using the R package "randomForest." The specific construction process of the model is as follows. The first step is to determine the training and test sets. The training set consisted of 34 liver cirrhosis patients and 23 healthy individuals. The test set consisted of 11 liver cirrhosis patients and seven healthy individuals. Random forest models were then constructed using the training set (ntree = 1,000) and the importance of different features (bacteria, fungi, metabolites or clinical indexes) was evaluated (importance = TRUE). Subsequently, the optimal number of features to use was determined based on the 10-fold cross-validation results and models were further optimized to select the best mtry value. Finally, the prediction accuracy was tested in the test set and area under curve (AUC) values were calculated. All above Bioinformatic analysis results were visualized using the Wekemo Bioincloud (Gao et al., 2024; https://www.bioincloud.tech), ImageGP (Chen T. et al., 2022), STAMP (Parks et al., 2014; version 2.1.3), R packages "ggplot2" (https://ggplot2.tidyverse.org), "circlize" (http://cran.r-project.org/web/packages/circlize/), and "pheatmap" (https://cran.r-project.org/web/packages/pheatmap/).

Untargeted metabolomic data preparation and analysis

100 μL of sample was taken, mixed with 400 μL of extraction solution [MeOH: ACN, 1:1 (v/v)], the extraction solution contains deuterated internal standards, the mixed solution was vortexed for 30 s, sonicated for 10 min in 4°C water bath, and incubated for 1 h at −40°C to precipitate proteins. Then the samples were centrifuged at 12,000 rpm (RCF = 13,800 (×g), *R*= 8.6 cm) for 15 min at 4°C. The supernatant was transferred to a fresh glass vial for analysis. The quality control (QC) sample was prepared by mixing an equal aliquot of the supernatant of samples.

LC-MS/MS analyses were performed using an UHPLC system (Vanquish, Thermo Fisher Scientific) with a Waters ACQUITY UPLC BEH Amide (2.1 × 50 mm, 1.7 μm) coupled to Orbitrap Exploris 120 mass spectrometer (Orbitrap MS, Thermo). The mobile phase consisted of 25 mmol/L ammonium acetate and 25 mmol/L ammonia hydroxide in water (pH = 9.75) (A) and acetonitrile (B). The auto-sampler temperature was 4°C, and the injection volume was 2 μL. The Orbitrap Exploris 120 mass spectrometer was used for its ability to acquire MS/MS spectra on information-dependent acquisition (IDA) mode in the control of the acquisition software (Xcalibur, Thermo). In this mode, the acquisition software continuously evaluates the full scan MS spectrum. The ESI source conditions were set as following: sheath gas flow rate as 50 Arb, Aux gas flow rate as 15 Arb, capillary temperature 320°C, full MS resolution as 60,000, MS/MS resolution as 15,000, collision energy: SNCE 20/30/40, spray voltage as 3.8 kV (positive) or −3.4 kV (negative), respectively.

The raw data were converted to the mzXML format using ProteoWizard and processed with an in-house program, which was developed using R and based on XCMS, for peak detection, extraction, alignment, and integration. Then a MS2 database was applied in metabolite annotation. The cutoff for annotation was set at 0.3.

Results

Clinical characteristics of participants

A total of 75 participants were included in this study: 45 liver cirrhotic patients and 30 healthy controls. The baseline clinical and demographic data for all groups were shown in Table 1. Age ($P = 0.067$) and gender ($P = 0.745$) were matched without significant differences between LC and C groups. Serum levels of ALT, TBIL, PT, and INR were elevated, whereas WBC, HB, PLT, ALB, and PTA were decreased in the LC group compared with healthy controls ($P < 0.05$). In cirrhotic patients, the median CTP scores and MELD scores were five and nine, respectively. The distribution of etiologies of LC was as follows: HBV/HCV, 25 patients; ALD, ten patients; NAFLD, five patients; AIH, five patients. Additionally, 15 patients were accompanied by EGV complications and underwent endoscopic treatment.

Characterization of the intestinal microbiome and mycobiome in decompensated liver cirrhotic patients

A total of 10,274,244 high-quality 16S rRNA reads were obtained from fecal samples of 45 patients with liver cirrhosis (LC group) and 30 healthy individuals (C group). After sequence processing, the final number of 6,861 ASVs was obtained. The rarefaction curve of richness indicates that the current sample was sequenced at a reasonable depth (Supplementary Figure 1A). One hundred and twenty-five and 102 ASVs were specific to cirrhotic patients and healthy individuals, respectively (Supplementary Figure 1B). In terms of alpha diversity, the Shannon index was significantly lower in cirrhotic patients than in healthy individuals ($P < 0.01$, Tukey's HSD test; Figure 1A). In terms of beta diversity, the results of principal coordinate analysis (PCoA) based on Bray-Curtis distance showed that the gut bacterial community structure of cirrhotic patients was significantly different from that of healthy individuals ($P = 0.001$, PERMANOVA with ADONIS test; Figure 1B). After alignment with the RDP database, 6,861 ASVs were annotated to a total of 6 phyla, 12 classes, 19 orders, 33 families, and 83 genera. On average, more than half of the bacteria in different subgroups were from Firmicutes (Figure 1C). *Faecalibacterium*, *Blautia*, and *Bifidobacterium* were the three genera with the highest average relative abundance (Figure 1D). Subsequently, LEfSe was used to further identify bacterial genera with significant differences in abundance across subgroups (Figure 1E). There were 17 and 25 bacterial genera enriched (LDA score > 3) in cirrhotic patients and healthy individuals, respectively. Among them, *Streptococcus*, *Akkermansia*, *Ligilactobacillus*, *Pseudescherichia* were signifantly enriched in liver cirrhotic patients, while *Blautia*, *Anaerobutyricum*, *Gemmiger*, *Ruminococcus*, and *Dorea* were markedly decresed (LDA score >4). In addition, the functional composition of the bacterial communities was enriched in bacterial-associated metabolic pathways (Supplementary Figure 1C). Several metabolic pathways differed significantly from LC and healthy individuals (Supplementary Figure 1D).

In terms of gut mycobiome, a total of 9,594,331 high-quality ITS rRNA reads were obtained from fecal samples of 45 cirrhotic patients and 30 healthy individuals. After sequence processing, the final number of ASVs was 901. The rarefaction curve of richness indicates that the current sample was sequenced at a reasonable depth (Supplementary Figure 2A). There were 18 and 60 ASVs specific to cirrhotic patients and healthy individuals, respectively (Supplementary Figure 2B). The Shannon index was significantly lower in cirrhotic patients than in healthy individuals ($P < 0.01$, Tukey's HSD test; Figure 2A). The results of PCoA showed that the gut fungal community structure of cirrhotic patients was significantly different from that of healthy individuals ($P = 0.004$, PERMANOVA with ADONIS test; Figure 2B). After alignment with the UNITE database, 901 ASVs were annotated to a total of 8 phyla, 20 classes, 51 orders, 101 families, 116 genera, and 125 species. On average, more than 80% of the fungi in the different subgroups were from Ascomycota (Figure 2C). *Saccharomyces*, *Candida*, and *Aspergillus* were the three genera with the highest average relative abundance (Figure 2D). Subsequently, LEfSe analysis indicated that the *Saccharomyces* was significantly increased and *Aspergillus*, *Penicillium*, *Auricularia*, as well as *Cladosporium* were reduced in cirrhotic patients (LDA score >4). In addition, we analyzed the functional composition of the fungal communities (Supplementary Figure 2C) and predicted 69 fungal-related metabolic pathways. There were 15 metabolic pathways with mean relative abundance differences >0.3% between groups, of which six were enriched in liver cirrhotic patients, including aerobic respiration II (cytochrome c; yeast), aerobic respiration I (cytochrome c), pentose phosphate pathway, glycolysis III (from glucose), CDP-diacylglycerol biosynthesis I, and adenosine ribonucleotides *de novo* biosynthesis (Supplementary Figure 2D).

TABLE 1 Demographics and clinical characteristics of subjects.

Clinical variables	LC ($n = 45$)	C ($n = 30$)	t/χ^2-value	P-value
Age	56.2 ± 1.6	51.6 ± 2.0	−1.86	0.067
Gender, male, *n* (%)	33 (73.3)	23 (76.7)	0.11	0.745
WBC (10^9/L)	3.3 ± 0.3	6 ± 0.3	6.53	<0.001
Hb (g/L)	110 ± 4.2	153 ± 2.6	8.61	<0.001
PLT (10^9/L)	95.9 ± 13.3	240 ± 11.3	7.67	<0.001
ALT (U/L)	22.5 ± 1.7	27.6 ± 2.7	1.68	0.097
AST (U/L)	31.8 ± 2	23.9 ± 1.3	−3.31	0.001
TBIL (μmol/L)	22 ± 1.9	15.2 ± 0.8	−3.21	0.002
ALB (g/L)	36.8 ± 0.7	43.4 ± 1.5	4.54	<0.001
PT (s)	11.3 ± 0.3	10.2 ± 0.1	3.40	0.001
PTA (%)	66.27 ± 1.8	103.3 ± 1.4	14.96	<0.001
INR	1.2 ± 0.2	1 ± 0.2	−5.07	<0.001
CTP scores	5 (5, 7)	-	-	-
MELD scores	9 (8,11)	-	-	-
Etiology		-	-	-
HBV/HCV	25 (55.6)	-	-	-
ALD	10 (22.2)	-	-	-
NAFLD	5 (11.1)	-	-	-
AIH	5 (11.1)	-	-	-
Complications				
EGV, *n* (%)	15 (33.3)	-	-	-
PVT, *n* (%)	11 (24.4)	-	-	-
HE, *n* (%)	2(4.4%)	-	-	-
Ascites, *n* (%)	9 (20)	-	-	-

LC, liver cirrhosis; C, healthy controls; WBC, white blood cell; Hb, hemoglobin; PLT, blood platelet; ALT, alanine transaminase; AST, aspartate aminotransferase; TBIL, total bilirubin; PT, prothrombin time; PTA, prothrombin activity; INR, international normalized ratio; CTP scores, Child-Turcotte-Pugh scores; MELD scores, Model for End-Stage Liver Disease scores; HBV/HCV, Hepatitis B/C Virus; ALD, alcoholic liver disease; NAFLD, non-alcoholic fatty liver disease; AIH, autoimmune liver disease; EGV, esophageal and gastric variceal; PVT, portal vein emboli; HE, hepatic encephalopathy.

Characterization of the intestinal metabolome in liver cirrhotic patients

Based on the untargeted metabolomic technology, we detected 11,382 variables in fecal samples from 45 cirrhotic patients and 30 healthy individuals. Of these, 727 variables were enriched in cirrhotic patients [VIP score>1, $P < 0.05$, $|\log_2(\text{fold change})| > 0$], and 814 variables were enriched in healthy individuals (Supplementary Figure 3A). OPLS-DA analysis similarly demonstrated significant differences in the metabolite composition of the samples from different subgroups (Figure 3A). After metabolite annotation, we finally identified 130 differential metabolites. Sixty-eight of these metabolites were enriched in cirrhotic patients, and another 62 were enriched in healthy individuals (Supplementary Figure 3B). Ranking all differential metabolites by VIP score, among the top 20 differential metabolites: 5,6-dihydroyangonin, glycyrrhizin, diammonium glycyrrhizinate, bilirubin, (S)-2-acetamido-4-amino-4-oxobutanoic acid, and protoporphyrin IX were enriched in cirrhotic patients (Figure 3B). To further explore the biological processes in which the differential metabolites might be involved, we performed metabolic pathway annotation and enrichment analysis based on the KEGG PATHWAY Database. The results revealed that 49 differential metabolites were annotated to 75 metabolic pathways. Among them, the "porphyrin metabolism" pathway showed the most significant enrichment effect ($P = 0.053$, hypergeometric test; Supplementary Figure 3C). Bilirubin, biliverdin, protoporphyrin IX, and coproporphyrin I were jointly involved in this metabolic pathway.

The effects of endoscopic treatment on gut bacteria, fungi, and metabolites

In this study, 15 cirrhotic patients had fecal samples collected before and after undergoing the endoscopic treatment. Based on this, we further explored the effects of endoscopic treatment on the intestinal bacteria, fungi, and metabolome of cirrhotic patients.

A total of 8,506,082 high-quality 16S rRNA reads were obtained from fecal samples of 15 treatment-before cirrhotic patients (TB group), 15 treatment-after cirrhotic patients (TA group), and 30 healthy individuals (C group). A total of 8,009,700 high-quality ITS rRNA reads were obtained. The average number of reads per sample was 133,495 (5,771–280,279). After sequence processing, 7,069 ASVs (bacteria) and 712 ASVs (fungi) were obtained. The rarefication curve of richness indicated that the current sample was sequenced at a reasonable depth (Supplementary Figures 4A, E). Compared with the TB group, the alpha diversity and beta diversity of the TA group did not change significantly for either bacterial or fungal communities (Figures 4A–D). Species classification results of bacteria and fungi showed that at the phylum and genus levels, the TA group had a similar species composition as the TB group (Supplementary Figures 4B, C, F, G). LEfSe results showed that the relative abundance of several bacterial and fungal genera changed significantly after endoscopic treatment (Supplementary Figures 4D, H). Among them, the bacterial genus *Acinetobacter* and the fungal genus *Cutaneotrichosporon* (LDA score >4) were enriched in the TB group, while the bacterial genus *Megamonas* (LDA score > 4) and the fungal genera *Coniochaeta* and *Ascotricha* (LDA score > 3) were decreased. In contrast, no differential metabolic pathways related to bacteria and fungi were found in predicting community functional composition based on the MetaCyc database using PICRUSt2 software.

A total of 11,156 variables were detected in the metabolomic analysis of 15 before- and 15 after-treatment cirrhotic patients. Of these, 43 variables were enriched in the TA group [VIP score > 1, $P < 0.05$, $|\log_2(\text{fold change})| > 0$], and 192 variables were enriched in the TB group (Supplementary Figure 4I). The OPLS-DA analysis demonstrated that before and after endoscopic treatment, samples showed significant differences in metabolite compositions (Figure 4E). After metabolite annotation, we finally identified 16 differential metabolites. Only nandrolone was enriched in the TA group, and the other 15 metabolites were enriched in the TB group (Supplementary Figure 4J, Figure 4F). Metabolic pathway annotation and enrichment analysis based on the KEGG PATHWAY Database showed that four differential metabolites were annotated to seven metabolic pathways. Among them, the "butanoate metabolism" pathway showed the most significant enrichment effect ($P = 0.0058$, hypergeometric test; Supplementary Figure 4K). Maleic acid and D-malic acid (enriched in the TB group) were involved in this metabolic pathway.

Multi-omics integration analysis

To explore the association between clinical indexes and differential bacteria, fungi, and metabolites, we performed the Spearman's correlation analysis based on 45 cirrhotic patients and 30 healthy individuals. Depending on the number of significant factors and the size of the *p*-values, we finally demonstrated the ten most highly correlated features (differential bacteria, fungi, and metabolites) in heatmaps (Figure 5). Overall, there were significant associations between the clinical indexes ALB, HGB, MELD, PLT, PTA, and WBC with various features. Among bacterial genera, *Acinetobacter* (enriched in cirrhotic patients) significantly negatively correlated with HGB and PTA indexes (correlation coefficient<-0.6, $P < 0.001$). *Dorea* (enriched in healthy individuals) significantly positively correlated with ALB, HGB, and PTA indexes (correlation coefficient>0.6, $P < 0.001$). Among the fungal genera, *Saccharomyces* (enriched in cirrhotic patients) significantly negatively correlated with the PLT index (correlation coefficient<-0.4, $P < 0.001$). *Auricularia* (enriched in healthy individuals) significantly correlated not only with ALB, HGB, and PTA indexes (correlation coefficient>0.5, $P < 0.001$) but also with the MELD index (correlation coefficient<-0.5, $P < 0.001$). Among the metabolites, 4-Acetyl-2-methylpyrimidine and diammonium glycyrrhizinate (enriched in cirrhotic patients) significantly negatively correlated with the PTA index (correlation coefficient<-0.5, $P < 0.001$). 5,6-DHET (enriched in healthy individuals) significantly positively correlated with the PTA, ALB, and HGB indexes (correlation coefficient>0.5, $P < 0.001$).

Then we performed the Spearman's correlation network analysis for the bacterial genera ($n = 83$), fungal genera ($n = 116$), and enriched metabolites ($n = 68$) in 45 cirrhotic patients (LC group). The results showed significant associations (|correlation coefficient|>0.4, $P < 0.05$, FDR corrected) between 74 bacterial genera, 59 fungal genera, and 53 enriched metabolites (Supplementary Figure 5A). Overall, there were 519 positive and 149 negative correlations between different features. Compared to fungi, there are more links between bacteria and enriched metabolites. We also found 3 LC-enriched metabolites were associated with bacterial and fungal genera, but the associations differed (Supplementary Table 1). Within bacteria, 51 genera were associated with each other, of which 10 were enriched in cirrhotic patients (LDA score>3), including *Streptococcus*, *Ligilactobacillus*, and *Enterococcus*. The top three bacterial genera ranked by degree were *Oscillibacter*, *Anaerobutyricum* and *Coprococcus*. Within fungi, 46 genera were associated with each other, with *Pichia* enriched in cirrhotic patients (LDA score>3). The top three fungal genera ranked by degree were *Papiliotrema*, *Absidia*, and *Aureobasidium*. Within metabolites, associations existed among 36 enriched metabolites. The top three metabolites ranked by degree were 7-Methylxanthine, L-Ribulose, and Theophylline. For contrast, we also performed the Spearman's correlation network analysis for the bacterial genera ($n = 83$), fungal genera (n = 116), and enriched metabolites ($n = 62$) in 30 healthy individuals (C group). The results showed significant associations (|correlation coefficient|>0.4, $P < 0.05$, FDR corrected) between 48 bacterial genera, 77 fungal genera, and 46 enriched metabolites (Supplementary Figure 5B). Overall, there were 301 positive and 32 negative correlations between different features.

To explore the potential of different biomarkers for diagnosing liver cirrhosis disease, 45 liver cirrhotic patients and 30 healthy individuals were divided into a training set (34LC and 23C) and a test set (11LC and 7C) to construct random forest models based on the 16S data, ITS data, metabolites data, clinical indexes, and a combination of them, respectively. Figures 6A, C, E, G, I showed the top 20 features of different data types that contributed most to the accuracy of predicted sample grouping. The random forest model constructed based on 16S data had the lowest AUC value of 0.75 (Figure 6B). The genera *Acinetobacter* (enriched in LC patients), *Kandleria*, and *Parasutterella* (enriched in healthy individuals) were three of the most important features to this

model. Models constructed based on ITS data or clinical indexes had close AUC values of 0.839 and 0.844, respectively (Figures 6D, F). The fungal genera *Auricularia*, *Hypsizygus*, and *Cladosporium* (enriched in healthy individuals), as well as the clinical indexes PTA, MELD, and HGB were key features. The model constructed on the basis of metabolites data had a high AUC value of 0.909 (Figure 6H). Metabolites MeAIB, 5-Aminopentanamide, and N-Acetylglutamine enriched in LC patients were the top three important features. Compared to other models, the random forest model constructed by combining all four types of features had the highest AUC value of 0.938 (Figure 6J).

Discussion

It has been widely known that gut microbial dysbiosis is a bidirectional process with the development of cirrhosis because of pathological interactions of the gut-liver axis (Albillos et al., 2020). When the equilibrium between the intestinal microbiome and the microenvironment is disturbed, increased microbial translocation and dysbiosis result in liver injury and are closely linked to the progression of cirrhosis (Acharya et al., 2017). Meanwhile, abnormal liver function and portal hypertension easily occur in the progression of cirrhosis, which will result in intestinal mucosal barrier damage and weakening of the inhibitory effect of the liver on intestinal harmful microbes (Wiest et al., 2014; Acharya and Bajaj, 2017). The intestinal fungi and bacteria share micro-habitats and complexly interact with microbial communities, which have been shown to affect various biological processes maintaining the stability of the intestinal environment (Huang X. et al., 2023). Bacterial and fungal dysbiosis threaten the delicate equilibrium that distinguishes cirrhosis and is associated with a high mortality risk in patients with advanced liver disease through the gut-liver axis (Maraolo et al., 2020; Chen L. et al., 2022). For these reasons, our study focused on the interconnections among cirrhosis-associated gut bacteria, gut fungi, microbial metabolites, and disease conditions.

Previous studies have reported that cirrhotic patients exhibited frequent gastrointestinal bacteria disturbances, which are characterized by a decreased diversity, overgrowth of *Enterococcus*, Enterobacteriaceae, and Bacteroidaceae, and a reduction of the abundance of probiotic microorganisms, such as Lachnospiraceae and Ruminococcaceae (Gómez-Hurtado et al., 2016; Wang et al., 2020). Our results in the present study showed that intestinal bacterial alpha diversity was lower than healthy controls (Figure 1A) and identified several compositional bacterial differences in cirrhotic patients. At the genus level, the LC group's relative abundance of pathogenic bacteria such as *Acinetobacter*, *Enterococcus* and *Streptococcus* significantly increased (Figure 1E), which may cause an inflammatory reaction to aggravate liver injury. It has been reported that *Enterococcus* and *Streptococcus* are significantly abundant in patients with liver diseases, such as autoimmune hepatitis (Ponziani et al., 2018; Zhong et al., 2021; Huang X. Y. et al., 2023). Meanwhile, we observed decreased beneficial bacterium abundance of *Anaerobutyricum*, *Blautia*, *Coprococcus*, *Dorea*, *Gemmiger*, and *Ruminococcus* in LC group (Figure 1E). KEGG analysis suggested that the variations in gut bacteria led to significant differences in metabolic pathways (Supplementary Figure 1D). In the LC group, more bacteria were enriched in "fatty acid elongation—saturated," "(5Z)-dodec-5-enoate biosynthesis," and "inosine-5′-phosphate biosynthesis III" pathways. Notably, *Blautia*, *Coprococcus*, and *Ruminococcus* were mainly involved in producing short-chain fatty acids, which are widely thought to be beneficial microbial substances correlated with a reduced risk of liver fibrosis and cirrhosis (Zhou et al., 2019). Moreover, our study also exhibited strong linkages between gut bacteria and cirrhosis clinical features (Figure 5A), especially the severity of LC, including the MELD score, CTP score, and PTA positively associated with potentially pathogenic taxa and inverse correlations with the probiotic ones.

Analogous to intestinal bacteria disturbances, cirrhotic patients exhibited intestinal fungi disorders with decreased fungal diversity (Figure 2A) and a relative increase in abundance of opportunistic fungal pathogens. In our study, Ascomycota and Basidiomycota were the most common intestinal fungi at the phyla level in LC patients and healthy controls (Figure 2C). Compared with healthy controls, LC patients had an increased abundance of Ascomycota and a decreased abundance of Basidiomycota (Figures 2C, E). As reported, the Basidiomycota to Ascomycota ratio was used to define fungal dysbiosis, and the gut fungal/bacterial metric (Ascomycota/Bacteroidetes) was independently associated with the risk of hospitalization (Bajaj et al., 2018). At the genus level, our results found an increment of *Saccharomyces* and a reduction of *Aspergillus*, and *Penicillium* associated with the clinical variables (Figures 2E, 5B). *Aspergillus*, *Cryptococcus*, *Debaryomyces*, *Malassezia*, *Penicillium*, and *Saccharomyces* have been more vigorously studied in recent years to highlight the relationship between gut fungi and human diseases, including liver diseases (Suhr and Hallen-Adams, 2015; Doron et al., 2023). Of them, some species of *Saccharomyces* were significantly associated with the severity of the disease in cirrhotic patients (Costa et al., 2014). The increased fecal abundance of *Saccharomyces* was also validated in a cohort of patients with chronic hepatitis B virus (HBV) infection and HBV-associated cirrhosis (Mou et al., 2018). Zhang et al. (2023) have confirmed the decreased alpha diversity and increased *Saccharomycetes* in LC patients with various etiologies. Intriguingly, some edible mushroom genera from dietary sources such as Auricularia and Hypsizygus were found in negatively related to the severity of the liver cirrhosis in our data. Edible mushrooms are healthy food with great therapeutic value as they contain abundant bioactive metabolites with antioxidant, anti-aging, antibacterial, anti-inflammatory properties. When exploring the role of gut fungi in diseases, in addition to colonizing fungi in the intestine itself, fungi from dietary sources may also have a certain effect. In summary, our study demonstrated that intestinal microbial disorders of patients with LC exhibited the lack of potentially beneficial microbes, including an overgrowth of potentially pathogenic microbes (bacteria and fungi), which may affect and reflect the clinical features and severity of liver cirrhosis.

An essential function of the gut microbiota is metabolism, which supplies nutrients to both microbes and host. Recently, increasing evidence has shown that microbial metabolites play pivotal roles in communicating among gut bacteria, fungi, and hosts in various ways, leading to co-occurrence patterns of fungi and bacteria (Gao et al., 2021). Microbial dysbiosis can result in aberrant translocation of microbial components or

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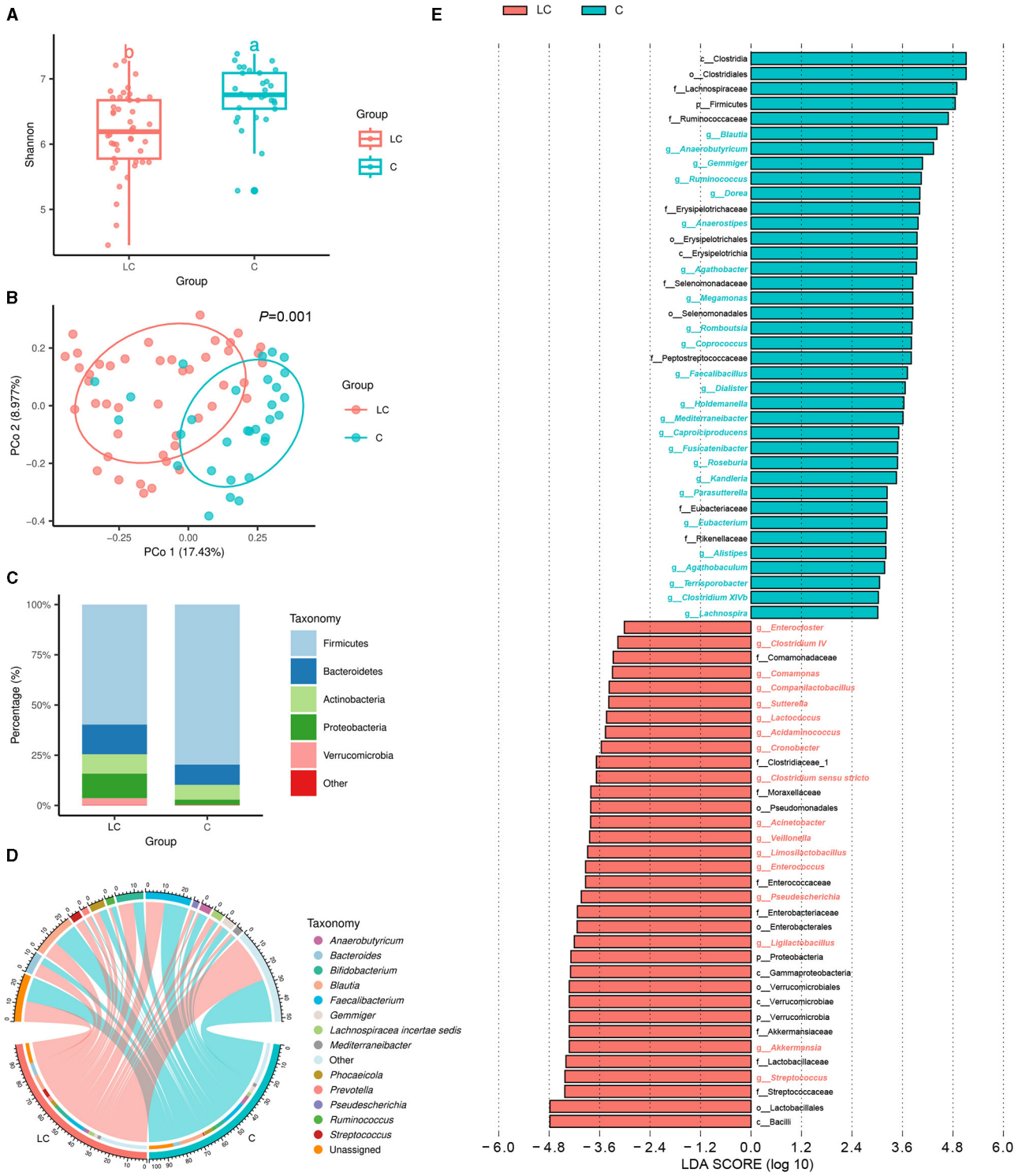

FIGURE 1
Characterization of intestinal bacterial communities in liver cirrhosis patients. **(A)** Alpha diversity of bacterial communities between groups based on Shannon index. **(B)** Principal coordinate analysis (PCoA) based on Bray-Curtis distance. **(C)** Bacterial community composition (phylum level). **(D)** Bacterial community composition (genus level). **(E)** LEfSe analysis reveals differential bacteria between groups (LDA score > 3).

microbial-derived metabolites (such as bile acids, fatty acids, and tryptophan metabolites) from the gut to the liver via the portal circulation, which can affect the onset and progression of LC (Trebicka et al., 2020). Consistent with the results of intestinal microbial sequencing, we observed the raised prevalence of the "fatty acid elongation—saturated" pathway and the reduced relative abundance of unsaturated and short-chain fatty acid-producing bacteria in patients with LC, suggesting a loss of beneficial fatty acid in the intestinal environment resulting in more pervasive gastrointestinal phenotypes. These metabolites may be involved in

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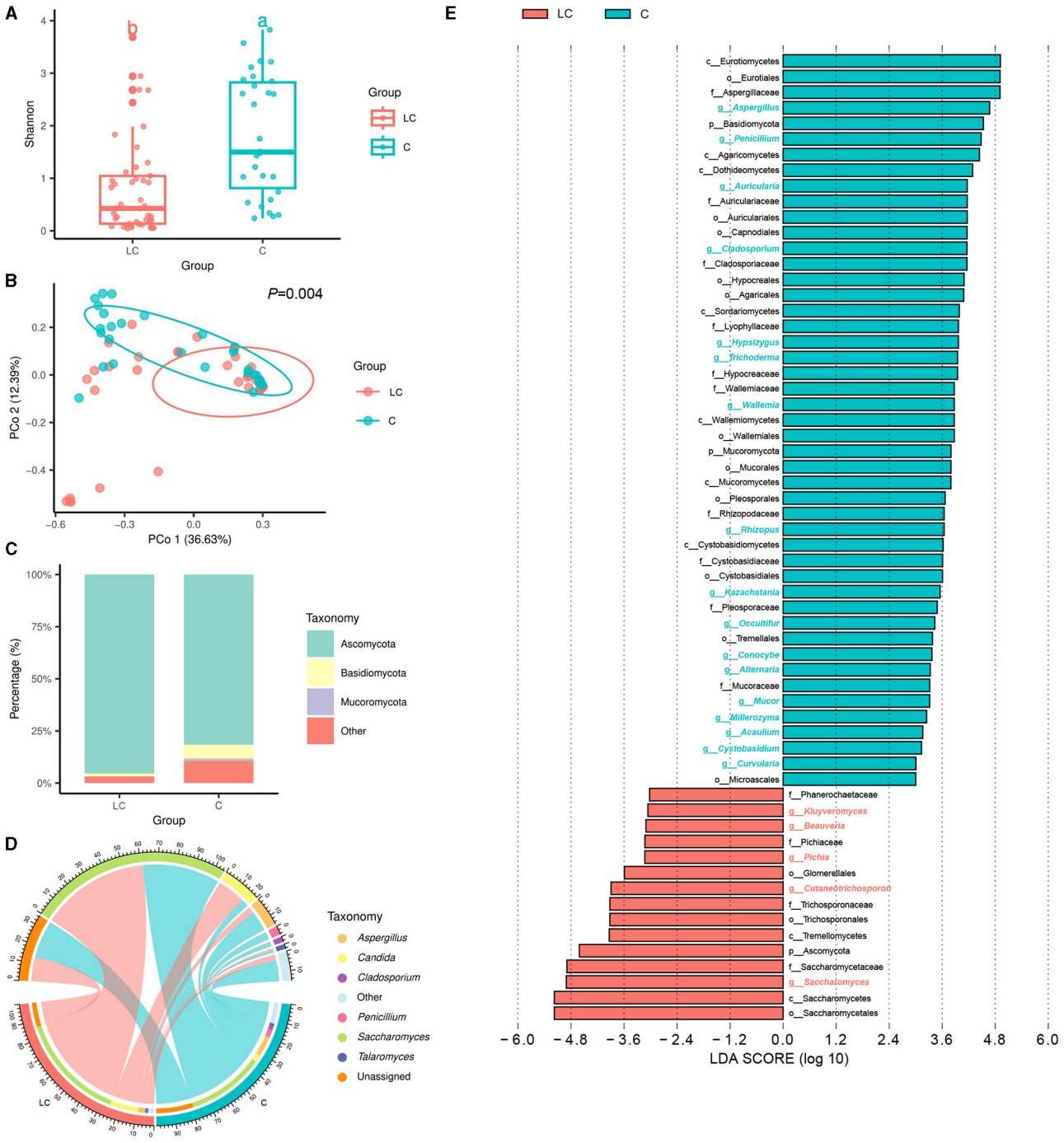

FIGURE 2
Characterization of intestinal fungal communities in liver cirrhosis patients. **(A)** Alpha diversity of fungal communities based on Shannon index. **(B)** Principal coordinate analysis (PCoA) based on Bray-Curtis distance. **(C)** Fungal community composition (phylum level). **(D)** Fungal community composition (genus level). **(E)** LEfSe analysis reveals differential fungi between groups (LDA score > 3).

regulating bacterial and fungal homeostasis and play an essential role in the development and progression of cirrhosis. This also suggests that metabolites in the gut are probably not just from the same species but may be regulated by multiple ones.

Esophageal and gastric varices is one of the potentially life-threatening complications of LC, resulting in high mortality (Lesmana et al., 2020). Esophageal and gastric variceal bleeding (EGVB) can represent a risk factor for the development of bacterial infections in up to 45% of patients (Tandon and Garcia-Tsao, 2008). In our study, we exhibited the gut microbial ecology in LC patients with EGV. Endoscopic procedures (endoscopic variceal ligation, endoscopic injection sclerotherapy, and endoscopic tissue adhesive) remain the mainstay recommended by domestic and international guidelines for treating EGV and controlling

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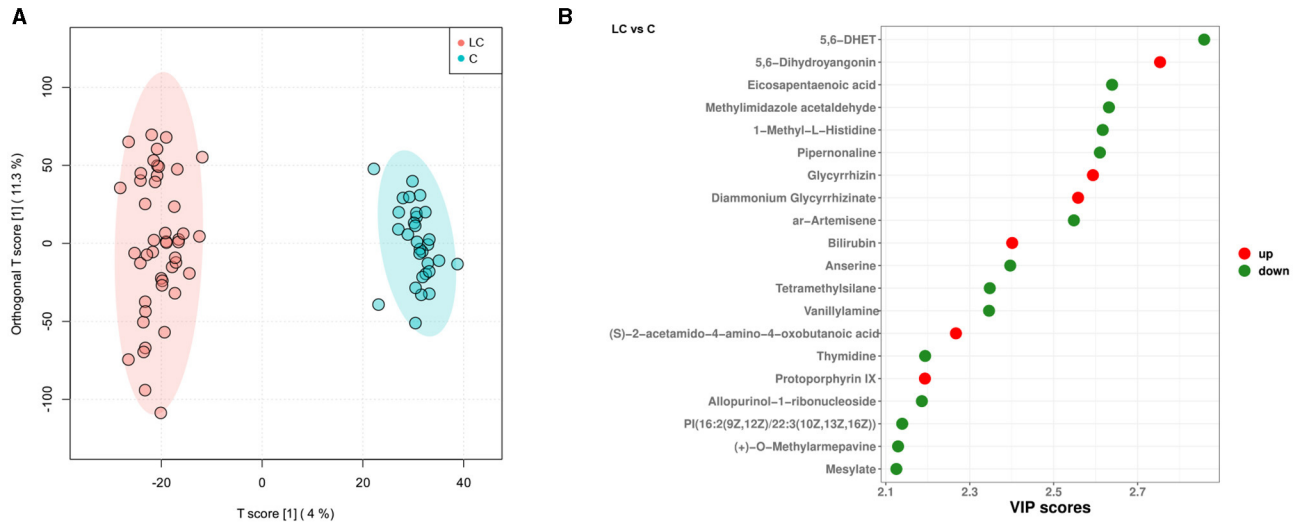

FIGURE 3
Characterization of intestinal metabolites in liver cirrhosis patients. **(A)** OPLS-DA analysis reveals differences in metabolite composition of samples between groups. Red color represents 45 liver cirrhosis patients (LC group) and blue color represents 30 healthy individuals (C group). **(B)** Top20 intergroup differential metabolites (sorted by VIP score).

acute bleeding. Changes caused by the endoscopic treatment in portal vein pressure, blood flow, and gastric function may alter gut microbiota composition and abundance. A total of 15 LC patients with EGV who underwent endoscopic treatment were enrolled to investigate the potential roles of endoscopic treatment on gut microbial changes. Finally, no significant differences were found in diversity of gut bacteria and fungi (Figures 4A–D), composition at the phylum level (Supplementary Figures 4B, F), and microbial metabolic pathways before and after endoscopic procedures. However, several bacterial and fungal genera were differentiated after endoscopic treatment (Supplementary Figures 4D, H), which may affect subsequent EGV bleeding. However, it will need long-term follow-up and monitoring.

The gut bacteria and fungi contribute to the intestinal ecosystem through their key roles in host interactions and homeostasis. Fungal–bacterial interactions underlie both the pathogenesis and the progression of disease (Krüger et al., 2019). Previous study found that gut fungi can produce antimicrobial peptides to influence bacterial colonization (Kombrink et al., 2019), while bacteria can modulate fungal commensalism, pathogenesis and growth by affecting fatty acids generation (García et al., 2017; McCrory et al., 2024). The most widely studied pathogenic fungi being antagonized by commensal bacteria (such as *Streptococcus* and *Clostridium difficile*) is *C. albicans* (Santus et al., 2021). Except that, data on the interactions between other fungi and bacteria are limited. Our study preliminarily explored a cirrhosis-specific interkingdom network among bacterial genera, fungal genera, and enriched metabolites (Supplementary Figure 5, Supplementary Table 1), and the type of interactions between these bacteria and fungi might have clinical relevance. Three cirrhosis-enriched metabolites were associated with bacterial and fungal genera, but the associations differed (Supplementary Table 1). Although the roles of these interaction are still unclear, these findings can broaden our knowledge of the function and crosstalk of gut microbiota.

Gut microbes have been used as novel molecular biomarkers, mainly for disease risk prediction modeling, and have proved their value in several diseases (e.g., diabetes, enteritis, and bowel cancer). Previous studied have interrogated shotgun metagenomic, untargeted metabolomic profiles and random forest machine learning algorithm to evaluate the diagnostic accuracy based on a set of liver fibrosis/cirrhosis-related bacterial and metabolomic signatures, and the result were effective (AUC: 0.72–0.91; Loomba et al., 2017; Lee et al., 2020; Oh et al., 2020). The construction of disease risk models for liver cirrhosis was also attempted in our study with multiple data combinations. Our modeling was based on gut microbiome data and clinical indexes from 45 patients with cirrhosis and 30 healthy individuals, with AUCs ranging from 0.750 to 0.938. Moreover, we found that the efficacy of diagnostic models based on fungal signatures is not inferior to those based on bacterial or metabolic signatures. Combining the metagenomic signature with key clinical indicators (PTA, MELD, and HGB) accurately distinguished cirrhosis in etiologically can improve the diagnostic efficacy of liver cirrhosis.

Of course, several issues should also be addressed. Firstly, we used an insufficient number of samples (case: 45 vs. control:30), thus we did not classify cirrhosis patients in detail, and differences in disease subtypes, which has an impact on our screening of differential bacteria, fungi, and metabolites. Secondly, the overall performance of our models was not promising enough, there is still much room for improvement in the AUC of the risk prediction model as well as its stability. Thirdly, this study mainly used amplicon sequencing to study bacteria and fungi in the gut microbiota. Therefore, more large-scale research must be done on the genes and functions of bacteria and fungi by mNGS sequencing. We plan to remedy these deficiencies in subsequent studies.

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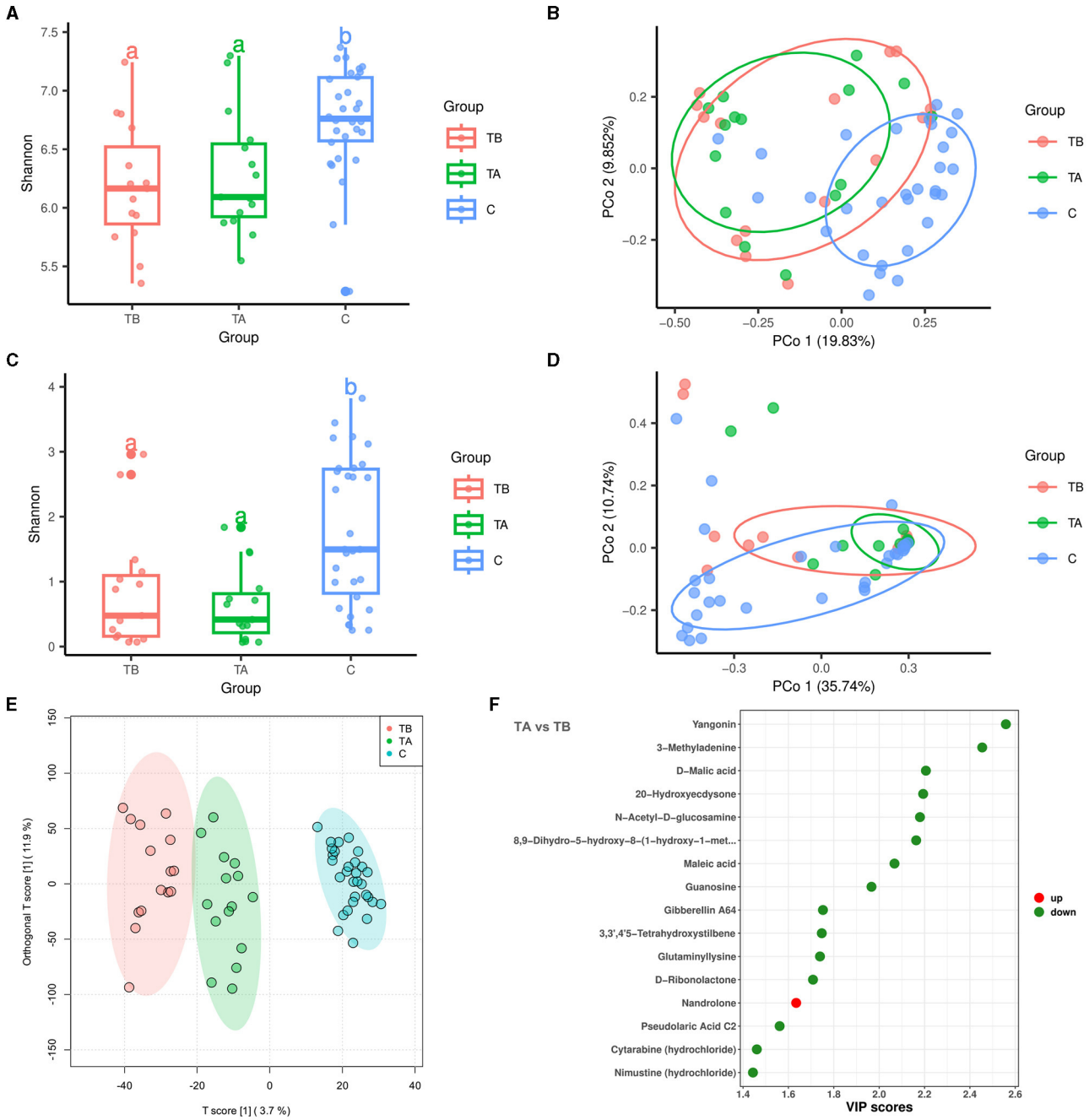

FIGURE 4
Effect of endoscopic treatment on intestinal bacteria, fungi, and metabolites in liver cirrhosis patients. Red, green, and blue colors represent 15 treatment-before cirrhotic patients (TB group), 15 treatment-after cirrhotic patients (TA group), and 30 healthy individuals (C group), respectively. **(A)** Alpha diversity of bacterial communities based on Shannon index. **(B)** Principal coordinate analysis (PCoA; bacteria) based on Bray-Curtis distance ($P = 0.001$, PERMANOVA with ADONIS test). **(C)** Alpha diversity of fungal communities based on Shannon index. **(D)** Principal coordinate analysis (PCoA; fungi) based on Bray-Curtis distance. **(E)** OPLS-DA analysis reveals differences in metabolite composition of samples between groups. **(F)** Differential metabolites in patients with cirrhosis before and after treatment (sorted by VIP score).

Conclusion

In conclusion, our study presented the cirrhosis-associated intestinal microbial characteristics and highlighted the gut bacteria-fungi-metabolite interactions in patients with cirrhosis. Meanwhile, we constructed a predicted diagnosis of cirrhosis using a random forest model based on identified intestinal microbial and clinical features. This interaction provides a new direction and theoretical basis for exploring gut microbiota-based diagnostic and therapeutic strategies for cirrhotic patients. Further mechanistic studies of fungal, bacterial, and metabolic ecological interactions in liver cirrhosis are required.

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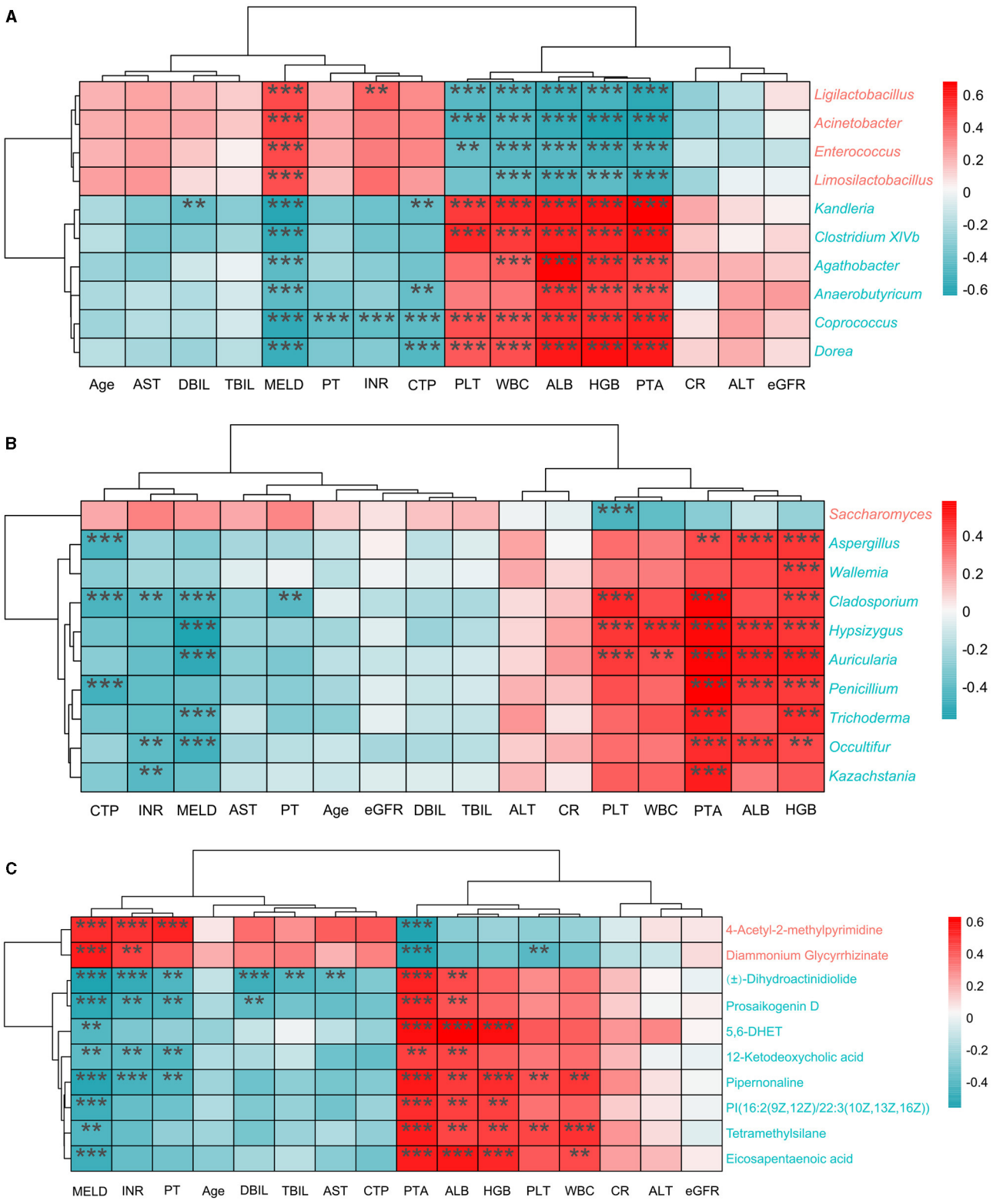

FIGURE 5
Correlation analysis of clinical indexes with different features. **(A)** Top10 differential bacterial genera most correlated with clinical indexes. **(B)** Top10 differential fungal genera most correlated with clinical indexes. **(C)** Top10 differential metabolites most correlated with clinical indexes. The red color in the heatmap represents positive correlation and the blue color represents negative correlation. $P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (FDR corrected).

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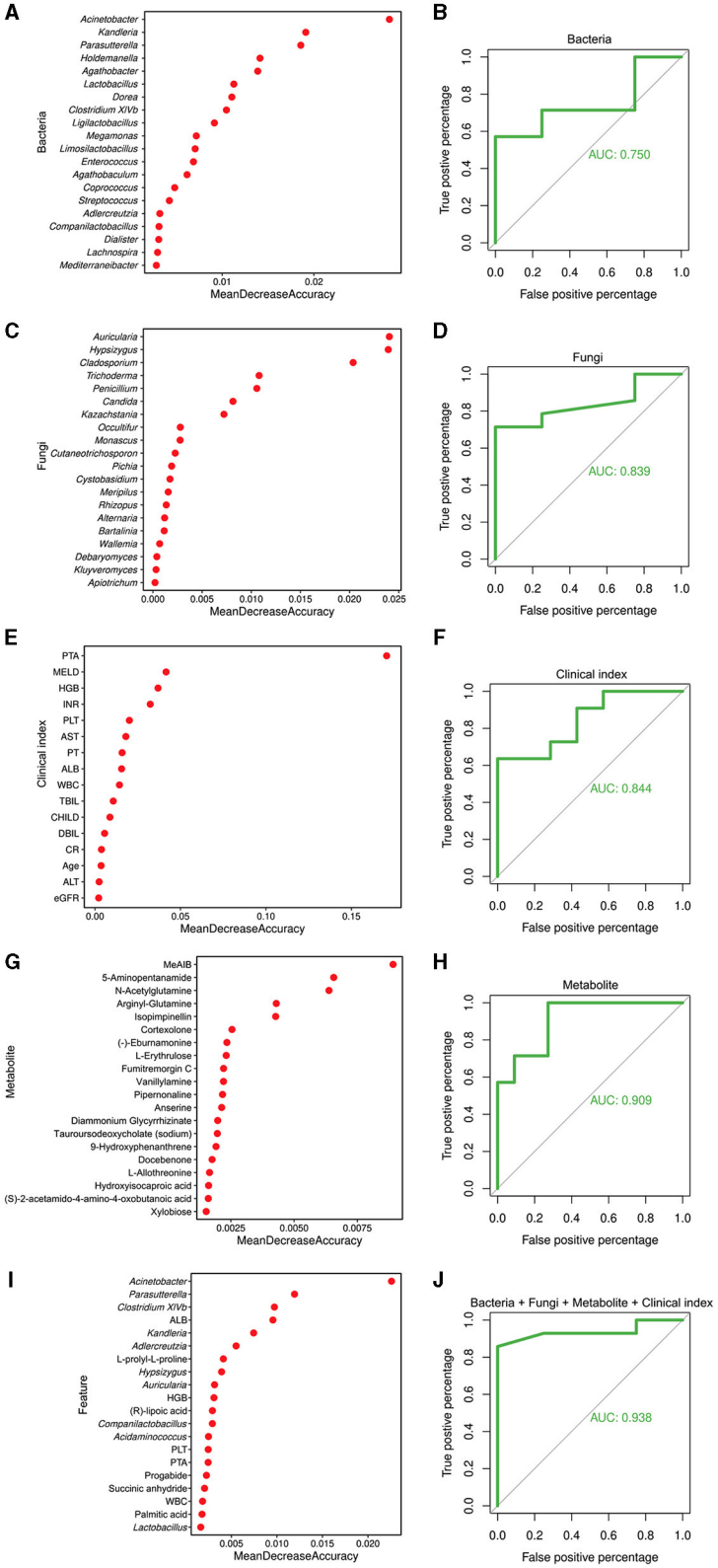

FIGURE 6
Random forest model for the diagnosis of cirrhosis. **(A)** Top 20 bacterial genera contributing most to the accuracy of predicting sample grouping. **(B)** Bacterial genera used to discriminate between cirrhotic patients and healthy individuals with an AUC of 0.750. **(C)** Top 20 fungal genera contributing most to the accuracy of predicting sample grouping. **(D)** Fungal genera used to discriminate between cirrhotic patients and healthy individuals with an AUC of 0.839. **(E)** Top 20 clinical indexes contributing most to the accuracy of predicting sample grouping. **(F)** Clinical indexes used to discriminate between cirrhotic patients and healthy individuals with an AUC of 0.844. **(G)** Top 20 metabolites contributing most to the accuracy of predicting sample grouping. **(H)** Metabolites used to discriminate between cirrhotic patients and healthy individuals with an AUC of 0.909. **(I)** Top 20 features contributing most to the accuracy of predicting sample grouping. **(J)** Bacterial and fungal genera, metabolites, and clinical indexes used to discriminate between cirrhotic patients and healthy individuals with an AUC of 0.938.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA1028775.

Ethics statement

The studies involving humans were approved by the Ethics Committee of Beijing You'an Hospital authorized the project. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

YL: Formal analysis, Writing – original draft. DL: Formal analysis, Methodology, Writing – original draft. YH: Data curation, Writing – original draft. ZZ: Investigation, Writing – original draft. AZ: Data curation, Writing – original draft. CF: Resources, Writing – original draft. LL: Conceptualization, Funding acquisition, Writing – review & editing. ZH: Funding acquisition, Methodology, Software, Writing – review & editing. HD: Conceptualization, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1443182/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Analysis of intestinal bacterial communities in cirrhotic patients. **(A)** Rarefaction curves. **(B)** Presence of ASVs (relative abundance > 0.1%) within different subgroups. **(C)** PCA analysis of the functional composition of communities in different subgroups. **(D)** Differential pathways in different subgroups (difference between proportions > 0.17%, q-value < 0.01). Pathway names not shown in full are adenosylcobalamin biosynthesis from cobyrinate a,c-diamide I, superpathway of guanosine nucleotides *de novo* biosynthesis I, superpathway of guanosine nucleotides *de novo* biosynthesis II.

SUPPLEMENTARY FIGURE 2
Analysis of intestinal fungal communities in cirrhotic patients. **(A)** Rarefaction curves. **(B)** Presence of ASVs (relative abundance > 0.1%) within different subgroups. **(C)** PCA analysis of the functional composition of communities in different subgroups. **(D)** Differential pathways (difference between proportions > 0.3%, q-value < 0.01) in different subgroups. Pathway names not shown in full are NAD/NADP-NADH/NADPH mitochondrial interconversion (yeast), phospholipid remodeling (phosphatidylethanolamine, yeast).

SUPPLEMENTARY FIGURE 3
Analysis of intestinal metabolites in cirrhotic patients. **(A)** Distribution of metabolites differing between groups. Metabolites enriched in group C are shown in blue, metabolites enriched in group LC are shown in red, and metabolites without significant differences are shown in gray. **(B)** Distribution of the 130 identified differential metabolites among samples. Darker colors indicate higher relative abundance of metabolites. Red color represents 45 patients with liver cirrhosis (LC group) and blue color represents 30 healthy control individuals (C group). **(C)** Results of KEGG pathway enrichment analysis of differential metabolites (Top 30). Vertical coordinates are KEGG pathways enriched for differential metabolites. The horizontal coordinate, Rich factor, is the ratio of the number of differential metabolites in the corresponding pathway to the total number of metabolites detected and annotated in that pathway. A larger value indicates a greater degree of enrichment. The size of the dot represents the number of differential metabolites enriched. The color of the dots is the hypergeometric test p-value, with redder values indicating more significant enrichment.

SUPPLEMENTARY FIGURE 4
Analysis of intestinal bacterial and fungal communities and metabolites in patients with liver cirrhosis after the endoscopic treatment. **(A)** Rarefaction curves (bacteria). **(B)** Bacterial community composition (phylum level). **(C)** Bacterial community composition (genus level). **(D)** LEfSe analysis reveals differential bacteria between groups (LDA score > 3). **(E)** Rarefaction curves (fungi). **(F)** Fungal community composition (phylum level). **(G)** Fungal community composition (genus level). **(H)** LEfSe analysis reveals differential fungi between groups (LDA score > 3). **(I)** Distribution of differential metabolites in cirrhotic patients before and after treatment. Red color represents metabolites enriched in TA group and blue color represents metabolites enriched in TB group. **(J)** Distribution of 16 identified differential metabolites in samples between groups. **(K)** Results of KEGG pathway enrichment analysis of 16 identified differential metabolites.

SUPPLEMENTARY FIGURE 5
Correlation network of enriched metabolites with bacteria and fungi. **(A)** LC group network. **(B)** C group network. Blue triangles, red squares and cyan circles represent bacterial genera, fungal genera, and LC-enriched metabolites, respectively. Larger nodes have more associations with other nodes. Red lines represent positive correlations and blue lines represent negative correlations. Spearman's correlation coefficient|>0.4, P < 0.05 (FDR corrected).

References

Abarenkov, K., Nilsson, R. H., Larsson, K.-H., Taylor, A. F. S., May, T. W., Frøslev, T. G., et al. (2023). The UNITE database for molecular identification and taxonomic communication of fungi and other eukaryotes: sequences, taxa and classifications reconsidered. *Nucl. Acids Res*. 2023:gkad1039. doi: 10.1093/nar/gkad1039

Acharya, C., and Bajaj, J. S. (2017). Gut microbiota and complications of liver disease. *Gastroenterol. Clin. N. Am*. 46, 155–169. doi: 10.1016/j.gtc.2016.09.013

Acharya, C., Sahingur, S. E., and Bajaj, J. S. (2017). Microbiota, cirrhosis, and the emerging oral-gut-liver axis. *JCI Insight* 2:94416. doi: 10.1172/jci.insight.94416

Albillos, A., de Gottardi, A., and Rescigno, M. (2020). The gut-liver axis in liver disease: pathophysiological basis for therapy. *J. Hepatol*. 72, 558–577. doi: 10.1016/j.jhep.2019.10.003

Bajaj, J. S., Heuman, D. M., Hylemon, P. B., Sanyal, A. J., White, M. B., Monteith, P., et al. (2014). Altered profile of human gut microbiome is associated with cirrhosis and its complications. *J. Hepatol*. 60, 940–947. doi: 10.1016/j.jhep.2013.12.019

Bajaj, J. S., Liu, E. J., Kheradman, R., Fagan, A., Heuman, D. M., White, M., et al. (2018). Fungal dysbiosis in cirrhosis. *Gut* 67, 1146–1154. doi: 10.1136/gutjnl-2016-313170

Caspi, R., Billington, R., Fulcher, C. A., Keseler, I. M., Kothari, A., Krummenacker, M., et al. (2018). The MetaCyc database of metabolic pathways and enzymes. *Nucl. Acids Res*. 46, D633–D639. doi: 10.1093/nar/gkx935

Chen, L., Zhu, Y., Hou, X., Yang, L., and Chu, H. (2022). The role of gut bacteria and fungi in alcohol-associated liver disease. *Front. Med*. 9:840752. doi: 10.3389/fmed.2022.840752

Chen, T., Liu, Y. X., and Huang, L. (2022). ImageGP: an easy-to-use data visualization web server for scientific researchers. *iMeta* 1:5. doi: 10.1002/imt2.5

Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., Sun, Y., et al. (2014). Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucl. Acids Res*. 42, D633–D642. doi: 10.1093/nar/gkt1244

Costa, L. J., Abreu, F. M. d. L., Garcia, V. E., Soares, L. A., and Davisson, C. M. I. T. (2014). The effect of *Saccharomyces boulardii* in patients eligible for liver transplantation. *Nutricion Hospitalaria* 31, 778–784. doi: 10.3305/nh.2015.31.2.7949

Doron, I., Kusakabe, T., and Iliev, I. D. (2023). Immunoglobulins at the interface of the gut mycobiota and anti-fungal immunity. *Semin. Immunol*. 67:101757. doi: 10.1016/j.smim.2023.101757

Douglas, G. M., Maffei, V. J., Zaneveld, J. R., Yurgel, S. N., Brown, J. R., Taylor, C. M., et al. (2020). PICRUSt2 for prediction of metagenome functions. *Nat. Biotechnol*. 38, 685–688. doi: 10.1038/s41587-020-0548-6

Dworecka-Kaszak, B., Dabrowska, I., and Kaszak, I. (2016). The mycobiome—a friendly cross-talk between fungal colonizers and their host. *Ann. Parasitol*. 62, 175–184.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Gao, B., Zhang, X., and Schnabl, B. (2021). Fungi-bacteria correlation in alcoholic hepatitis patients. *Toxins* 13:20143. doi: 10.3390/toxins13020143

Gao, Y., Zhang, G., Jiang, S., and Liu, Y. X. (2024). Wekemo bioincloud: a user-friendly platform for meta-omics data analyses. *Imeta* 3:e175. doi: 10.1002/imt2.175

García, C., Tebbji, F., Daigneault, M., Liu, N.-N., Köhler, J. R., Allen-Vercoe, E., et al. (2017). The human gut microbial metabolome modulates fungal growth via the TOR signaling pathway. *mSphere* 2:17. doi: 10.1128/mSphere.00555-17

Gomaa, E. Z. (2020). Human gut microbiota/microbiome in health and diseases: a review. *Antonie Van Leeuwenhoek* 113, 2019–2040. doi: 10.1007/s10482-020-01474-7

Gómez-Hurtado, I., Such, J., and Francés, R. (2016). Microbiome and bacterial translocation in cirrhosis. *Gastroenterología y Hepatología* 39, 687–696. doi: 10.1016/j.gastrohep.2015.10.013

Hsu, C. L., and Schnabl, B. (2023). The gut-liver axis and gut microbiota in health and liver disease. *Nat. Rev. Microbiol*. 21, 719–733. doi: 10.1038/s41579-023-00904-3

Huang, X., Hu, M., Sun, T., Li, J., Zhou, Y., Yan, Y., et al. (2023). Multi-kingdom gut microbiota analyses define bacterial-fungal interplay and microbial markers of pan-cancer immunotherapy across cohorts. *Cell Host Microbe* 31, 1930–1943.e1934. doi: 10.1016/j.chom.2023.10.005

Huang, X. Y., Zhang, Y.-H., Yi, S.-Y., Lei, L., Ma, T., Huang, R., et al. (2023). Potential contribution of the gut microbiota to the development of portal vein thrombosis in liver cirrhosis. *Front. Microbiol*. 14:1217338. doi: 10.3389/fmicb.2023.1217338

Kanehisa, M. (2000). KEGG: kyoto encyclopedia of genes and genomes. *Nucl. Acids Res*. 28, 27–30. doi: 10.1093/nar/28.1.27

Kisseleva, T., and Brenner, D. (2020). Molecular and cellular mechanisms of liver fibrosis and its regression. *Nat. Rev. Gastroenterol. Hepatol*. 18, 151–166. doi: 10.1038/s41575-020-00372-7

Kombrink, A., Tayyrov, A., Essig, A., Stöckli, M., Micheller, S., Hintze, J., et al. (2019). Induction of antibacterial proteins and peptides in the coprophilous mushroom *Coprinopsis cinerea* in response to bacteria. *ISME J*. 13, 588–602. doi: 10.1038/s41396-018-0293-8

Krüger, W., Vielreicher, S., Kapitan, M., Jacobsen, I. D., and Niemiec, M. J. (2019). Fungal-bacterial interactions in health and disease. *Pathogens* 8:20070. doi: 10.3390/pathogens8020070

Lachar, J., and Bajaj, J. (2016). Changes in the microbiome in cirrhosis and relationship to complications: hepatic encephalopathy, spontaneous bacterial peritonitis, and sepsis. *Semin. Liv. Dis*. 36, 327–330. doi: 10.1055/s-0036-1593881

Lee, G., You, H. J., Bajaj, J. S., Joo, S. K., Yu, J., Park, S., et al. (2020). Distinct signatures of gut microbiome and metabolites associated with significant fibrosis in non-obese NAFLD. *Nat. Commun*. 11:4982. doi: 10.1038/s41467-020-18754-5

Lesmana, C. R. A., Raharjo, M., and Gani, R. A. (2020). Managing liver cirrhotic complications: overview of esophageal and gastric varices. *Clin. Mol. Hepatol*. 26, 444–460. doi: 10.3350/cmh.2020.0022

Liu, Y.-X., Chen, L., Ma, T., Li, X., Zheng, M., Zhou, X., et al. (2023). EasyAmplicon: an easy-to-use, open-source, reproducible, and community-based pipeline for amplicon data analysis in microbiome research. *iMeta* 2:83. doi: 10.1002/imt2.83

Llorente, C., and Schnabl, B. (2015). The gut microbiota and liver disease. *Cell. Mol. Gastroenterol. Hepatol*. 1, 275–284. doi: 10.1016/j.jcmgh.2015.04.003

Loomba, R., Seguritan, V., Li, W., Long, T., Klitgord, N., Bhatt, A., et al. (2017). Gut microbiome-based metagenomic signature for non-invasive detection of advanced fibrosis in human nonalcoholic fatty liver disease. *Cell Metab*. 25, 1054–1062.e1055. doi: 10.1016/j.cmet.2017.04.001

Lynch, S. V., Phimister, E. G., and Pedersen, O. (2016). The human intestinal microbiome in health and disease. *N. Engl. J. Med*. 375, 2369–2379. doi: 10.1056/NEJMra1600266

Maraolo, A. E., Scotto, R., Zappulo, E., Pinchera, B., Moriello, N. S., Nappa, S., et al. (2020). Novel strategies for the management of bacterial and fungal infections in patients with liver cirrhosis: focus on new antimicrobials. *Expert Rev. Anti-Infect. Ther*. 18, 191–202. doi: 10.1080/14787210.2020.1725473

McCrory, C., Lenardon, M., and Traven, A. (2024). Bacteria-derived short-chain fatty acids as potential regulators of fungal commensalism and pathogenesis. *Trends Microbiol*. 4:4. doi: 10.1016/j.tim.2024.04.004

Moon, A. M., Singal, A. G., and Tapper, E. B. (2020). Contemporary epidemiology of chronic liver disease and cirrhosis. *Clin. Gastroenterol. Hepatol*. 18, 2650–2666. doi: 10.1016/j.cgh.2019.07.060

Mou, H., Yang, F., Zhou, J., and Bao, C. (2018). Correlation of liver function with intestinal flora, vitamin deficiency and IL-17A in patients with liver cirrhosis. *Exp. Therapeut. Med*. 2018:6663. doi: 10.3892/etm.2018.6663

Oh, T. G., Kim, S. M., Caussy, C., Fu, T., Guo, J., Bassirian, S., et al. (2020). A universal gut-microbiome-derived signature predicts cirrhosis. *Cell Metab* 32, 878–888.e876. doi: 10.1016/j.cmet.2020.06.005

Pabst, O., Hornef, M. W., Schaap, F. G., Cerovic, V., Clavel, T., and Bruns, T. (2023). Gut-liver axis: barriers and functional circuits. *Nat. Rev. Gastroenterol. Hepatol*. 20, 447–461. doi: 10.1038/s41575-023-00771-6

Parks, D. H., Tyson, G. W., Hugenholtz, P., and Beiko, R. G. (2014). STAMP: statistical analysis of taxonomic and functional profiles. *Bioinformatics* 30, 3123–3124. doi: 10.1093/bioinformatics/btu494

Ponziani, F. R., Bhoori, S., Castelli, C., Putignani, L., Rivoltini, L., Del Chierico, F., et al. (2018). Hepatocellular carcinoma is associated with gut microbiota profile and inflammation in nonalcoholic fatty liver disease. *Hepatology* 69, 107–120. doi: 10.1002/hep.30036

Qin, N., Yang, F., Li, A., Prifti, E., Chen, Y., Shao, L., et al. (2014). Alterations of the human gut microbiome in liver cirrhosis. *Nature* 513, 59–64. doi: 10.1038/nature13568

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucl. Acids Res*. 41, D590–D596. doi: 10.1093/nar/gks1219

Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ* 4:2584. doi: 10.7717/peerj.2584

Santus, W., Devlin, J. R., and Behnsen, J. (2021). Crossing kingdoms: how the mycobiota and fungal-bacterial interactions impact host health and disease. *Infect Immun*. 89:20. doi: 10.1128/IAI.00648-20

Schnabl, B., and Brenner, D. A. (2014). Interactions between the intestinal microbiome and liver diseases. *Gastroenterology* 146, 1513–1524. doi: 10.1053/j.gastro.2014.01.020

Shao, L., Ling, Z., Chen, D., Liu, Y., Yang, F., and Li, L. (2018). Disorganized gut microbiome contributed to liver cirrhosis progression: a meta-omics-based study. *Front. Microbiol*. 9:3166. doi: 10.3389/fmicb.2018.03166

Suhr, M. J., and Hallen-Adams, H. E. (2015). The human gut mycobiome: pitfalls and potentials–a mycologists perspective. *Mycologia* 107, 1057–1073. doi: 10.3852/15-147

Sun, D. L., Jiang, X., Wu, Q. L., and Zhou, N. Y. (2013). Intragenomic heterogeneity of 16S rRNA genes causes overestimation of prokaryotic diversity. *Appl. Environ. Microbiol*. 79, 5962–5969. doi: 10.1128/AEM.01282-13

Tandon, P., and Garcia-Tsao, G. (2008). Bacterial infections, sepsis, and multiorgan failure in cirrhosis. *Semin. Liv. Dis*. 28, 026–042. doi: 10.1055/s-2008-1040319

Theocharidou, E., Agarwal, B., Jeffrey, G., Jalan, R., Harrison, D., Burroughs, A. K., et al. (2016). Early invasive fungal infections and colonization in patients with cirrhosis admitted to the intensive care unit. *Clin. Microbiol. Infect*. 22, 189.e181–189.e187. doi: 10.1016/j.cmi.2015.10.020

Trebicka, J., Bork, P., Krag, A., and Arumugam, M. (2020). Utilizing the gut microbiome in decompensated cirrhosis and acute-on-chronic liver failure. *Nat. Rev. Gastroenterol. Hepatol*. 18, 167–180. doi: 10.1038/s41575-020-00376-3

Tripathi, A., Debelius, J., Brenner, D. A., Karin, M., Loomba, R., Schnabl, B., et al. (2018). The gut-liver axis and the intersection with the microbiome. *Nat. Rev. Gastroenterol. Hepatol*. 15, 397–411. doi: 10.1038/s41575-018-0011-z

Verma, N., Roy, A., Singh, S., Pradhan, P., Garg, P., and Singh, M. (2022). Factors determining the mortality in cirrhosis patients with invasive candidiasis: a systematic review and meta-analysis. *Med. Mycol*. 60:myab069. doi: 10.1093/mmy/myab069

Wang, R., Tang, R., Li, B., Ma, X., Schnabl, B., and Tilg, H. (2020). Gut microbiome, liver immunology, and liver diseases. *Cell. Mol. Immunol*. 18, 4–17. doi: 10.1038/s41423-020-00592-6

Wiest, R., Lawson, M., and Geuking, M. (2014). Pathological bacterial translocation in liver cirrhosis. *J. Hepatol*. 60, 197–209. doi: 10.1016/j.jhep.2013.07.044

Yan, A. W., Fouts, D. E., Brandl, J., Stärkel, P., Torralba, M., Schott, E., et al. (2011). Enteric dysbiosis associated with a mouse model of alcoholic liver disease. *Hepatology* 53, 96–105. doi: 10.1002/hep.24018

Zeng, S., and Schnabl, B. (2022). Roles for the mycobiome in liver disease. *Liver Int*. 42, 729–741. doi: 10.1111/liv.15160

Zhang, L., Chen, C., Chai, D., Li, C., Qiu, Z., Kuang, T., et al. (2023). Characterization of the intestinal fungal microbiome in patients with hepatocellular carcinoma. *J. Transl. Med*. 21:3940. doi: 10.1186/s12967-023-03940-y

Zhong, X., Cui, P., Jiang, J., Ning, C., Liang, B., Zhou, J., et al. (2021). Streptococcus, the Predominant Bacterium to Predict the Severity of Liver Injury in Alcoholic Liver Disease. *Frontiers in Cellular and Infection Microbiology*. 11. doi: 10.3389/fcimb.2021.649060

Zhou, D., and Fan, J.-G. (2019). Microbial metabolites in non-alcoholic fatty liver disease. *World Journal of Gastroenterology*. 25, 2019–2028. doi: 10.3748/wjg.v25.i17.2019

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Potential mechanisms of traditional Chinese medicine in the treatment of liver cirrhosis: a focus on gut microbiota

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Cirrhosis, a pathological stage that develops from various chronic liver diseases, is characterized by liver fibrosis, pseudolobular formation, and chronic inflammation. When it progresses to the decompensated phase, the mortality rate of cirrhosis can reach 80%. The role of gut microbiota in the progression of liver diseases has received significant attention. Numerous studies have shown that regulating gut microbiota has significant therapeutic effects on preventing and reversing liver cirrhosis. This article reviewed the mechanisms by which gut microbiota influence liver cirrhosis, explaining the effective therapeutic effects of traditional Chinese medicine. Through multi-directional regulation involving signaling pathways, gut microbiota diversity, and restoration of intestinal barrier function, traditional Chinese medicine has been promising in ameliorating liver cirrhosis, providing treatment options and pharmacological guidance for the occurrence and development of liver cirrhosis.

KEYWORDS

liver cirrhosis, gut microbiota, traditional Chinese medicine, natural products, microbial metabolites

1 Introduction

More than 2 million people worldwide die from liver disease annually, accounting for 4% of all deaths, mainly due to liver cirrhosis and liver cancer (Devarbhavi et al., 2023). As the disease progresses, various complications of liver cirrhosis are key prognostic factors. Data show that about 4–12% of patients with cirrhosis experience more than one decompensated event per year [ascites, variceal bleeding, hepatic encephalopathy (HE)], and progression of cirrhosis to HE occurs in more than 40% of patients over a 5-year period. Secondly, during hospitalization, 25–50% of patients with cirrhosis develop bacterial infections, and 20% of patients suffer from acute kidney injury (Moon et al., 2020). The above data are more conservative for European countries (Peng et al., 2016; Pimpin et al., 2018). Given that liver cirrhosis and its complications are still a major burden in the global health problem, timely reverse this is crucial.

The etiology of liver cirrhosis is complex and multifaceted, with common causes involving viral hepatitis, alcohol-related liver disease (ALD), and non-alcoholic fatty liver disease (NAFLD). Epidemiological data reveal that over 40% of global liver cirrhosis cases are infected with hepatitis B virus, while more than 20% are infected with hepatitis C virus (Huang et al.,

2023). In addition to alcohol consumption, factors such as obesity, type 2 diabetes, metabolic syndrome, and hypertension also increase the likelihood of chronic liver disease progressing to cirrhosis (Younossi, 2019). These factors gradually lead to liver cell damage, degeneration and necrosis, and then liver cell regeneration and fibrous connective tissue proliferation, liver fibrosis formation, and finally progress to cirrhosis. Its pathological evolution is mainly composed of the following four aspects: (1) The role of pathogenic factors leads to extensive degeneration and necrosis of hepatocytes and collapse of the reticular fiber scaffolds of hepatic lobules (Romanelli and Stasi, 2016); (2) The remaining hepatocytes are not regenerated along the original scaffold, but form irregular nodular clusters of hepatocytes (regenerated nodules) (Iredale, 2007). (3) Various cytokines (such as TGF-β1) promote the production of fibrosis, extending and expanding in the central venous area and the portal area of the lobule, forming fibrous septa (Nishimura et al., 2021); (4) The proliferative fiber tissue makes these fiber intervals interconnect, wrap around the regenerated nodules or re-segment the residual hepatic lobules and transform them into false lobules, forming the typical morphological changes of cirrhosis. The above pathological changes caused the narrowing, occlusion and distortion of the vascular bed, The vessels were squeezed by regenerating nodules, and the branches of intrahepatic portal vein, hepatic vein and hepatic artery lost their normal relationship, and anastomosed branches appeared (Pu et al., 2023). Hepatic blood circulation disorder is the pathological basis of portal hypertension, aggravates hepatic cell ischemia and hypoxia, and promotes the further development of cirrhosis (Kaji and Yoshiji, 2020; Mahmoudi et al., 2022; Violi et al., 2023). Clinically, liver cirrhosis is roughly divided into compensated and decompensated liver function. Most patients in the compensatory stage are asymptomatic or have mild symptoms, and there are no abnormalities or mild abnormalities in liver function tests; when the stage progresses to decompensation, patients will have significant symptoms, mainly manifested by liver function decline and portal hypertension. The transition from compensated to decompensated cirrhosis represents a significant reduction in life expectancy and an increase in mortality (Ginès et al., 2021). Therefore, it is of great significance to accurately grasp the "critical point" of progression from compensated to decompensated and delay or reverse the progression of early liver cirrhosis to improve the quality of life and survival rate of patients.

In recent years, numerous studies have highlighted the close relationship between gut health and the normal functioning of various physiological systems, particularly the dynamic balance between the gut and the liver via the portal vein system. With the progression to the decompensated stage, the gut microbiota changes progressively in the direction of more serious disorders, and conversely, the gut microbiota dysbiosis will also break the balance state of enterohepatic circulation, affect the occurrence and development of liver diseases, and become an important driving factor for complications such as hepatic encephalopathy (HE) and spontaneous bacterial peritonitis (SBP) (Trebicka et al., 2021). In other words, there is a causal relationship between the gut microbiota and cirrhosis. Therefore, early regulation of gut microbiota is expected to become a new approach for liver cirrhosis treatment (Tsiaoussis et al., 2015). Compared to Western medicine, traditional Chinese medicine (TCM) is more widely accepted by patients owing to its advantages, including fewer adverse effects, enhanced specificity, reduced dependency, and diminished drug resistance. TCM has been widely used in the treatment of various clinical diseases. For example, flavonoids directly regulate insulin secretion, glucose metabolism-related enzymes, and related signaling pathways, thereby playing a significant role in treating diabetes and its complications (Bai et al., 2019). Similarly, natural products also have significant implications in regulating intestinal microecology and treating liver cirrhosis. There is a two-way promoting effect between citrus fruits rich in flavanone and intestinal flora. Intestinal flora plays a significant metabolic potential for polyphenols. Polyphenols can also inversely increase the abundance of beneficial bacteria, such as *Bifidobacterium* and *Lactobacillus* spp., and positively regulate the production of SCFA and intestinal PH value. Thus, the bacteria and the host can achieve a win-win situation (Tang et al., 2022). Baicalin mitigates diethylnitrosamine-induced cirrhosis by inhibiting oxidative stress and inflammation (Wang et al., 2024). Forsythiside A (FTA) demonstrates its good hepatoprotective effects by improving liver fibrosis by reversing the decreased bacterial richness, displaying anti-inflammatory and antioxidant properties, and improving the metabolism of bile acids (BAs) (Zhao et al., 2021). Emodin, belonging to the anthraquinone class of compounds, has hepatoprotective and anticancer effects against liver damage induced by various factors such as alcohol, high-fat diet (HFD), and lipopolysaccharides (LPS) (Hu et al., 2020).

Based on the close relationship between the intestinal flora and the liver, the aim of this study was to explore the role and potential mechanisms of gut ecological imbalance in liver cirrhosis. Furthermore, the study also aimed to assess the feasibility of TCM interventions targeting gut microbiota to improve liver cirrhosis, providing insights for future research directions and clinical management strategies. The interplay among liver cirrhosis, gut microbiota, and TCM is illustrated in Figure 1.

2 Gut microbiota and cirrhosis

The human body is consisted by a complex ecosystem, including bacteria, fungi, viruses, and archaea, which typically coexist with the host to maintain homeostasis. The microbial community is extensive and diverse. The number of bacterial cells in humans is tenfold higher than that of animals, with over 70% of bacteria concentrated within the intestine (Neish, 2009). The gut microbiota primarily consists of anaerobic bacteria (Lynch and Pedersen, 2016), with Firmicutes and Bacteroidetes as dominant species. Studies have indicated that changes in the ratio of Firmicutes to Bacteroidetes might affect lipid metabolism and the central nervous system, potentially leading to obesity (Ley et al., 2005; Turnbaugh et al., 2006; Koliada et al., 2017). In recent years, an increasing number of researchers have analyzed the close relationship between gut microbiota and human physiology and pathology from various perspectives, such as the brain-gut axis, liver-gut axis, and lung-gut axis. Shao et al. demonstrated a significant alleviating effect on depression by targeting the brain-gut axis to regulate the peripheral microenvironment (Shao et al., 2023). Li et al. (2023) found that the lung-gut axis plays an important role in various pulmonary diseases such as COVID-19 and asthma, by reshaping the gut microbiota in rat models.

Therefore, the balance of gut microbiota is crucial for human health and diseases, often referred to as the "new virtual metabolic organ" within the body (Lankelma et al., 2017). At birth, a baby's gut microbiota begins to establish itself. Disruptions in the composition

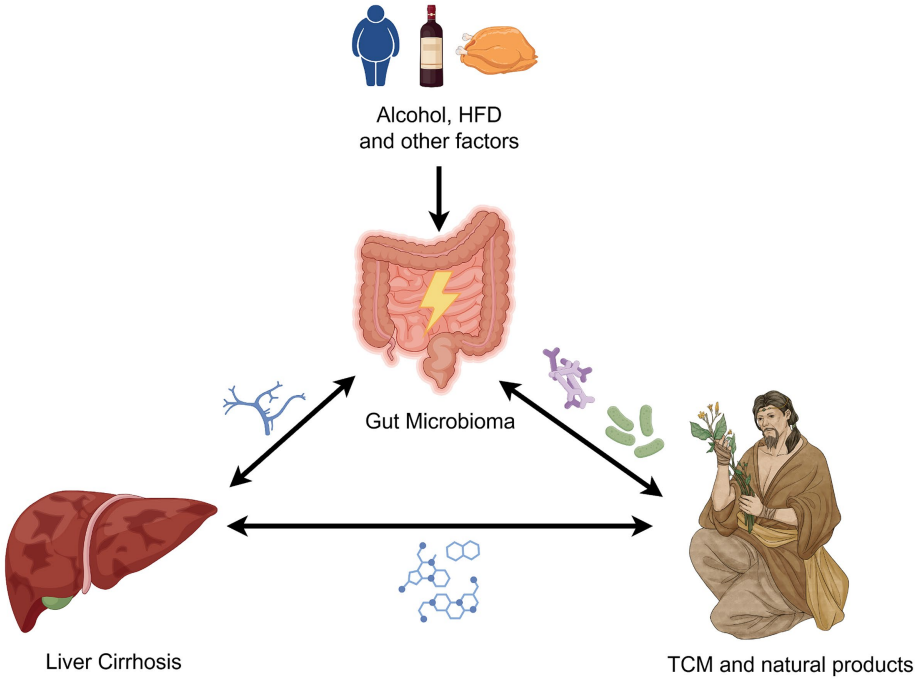

FIGURE 1
Links among liver cirrhosis, gut microbiota, and TCM. The figure is drawn with Figdraw.com.

and diversity of intestinal bacteria during early childhood, termed ecological imbalance, significantly impacts metabolism and immunity in adulthood (Milani et al., 2017). The gut and liver interact through various pathways, including the biliary tract and systemic circulation and the enterohepatic circulation facilitated by the portal vein (Giuffrè et al., 2020). Nutrients and microbial metabolites absorbed in the intestine enter the liver via the portal vein, where they are absorbed or stored in hepatic cells. Microorganisms and their metabolites are closely related to the immune and metabolic functions of the liver (Hsu and Schnabl, 2023). Conversely, the liver uses various immune cells, such as Kupffer cells, NKT cells, and Th17 cells, to regulate the intestinal barrier, prevent bacterial translocation (BT), and inhibit inflammatory responses (Gola et al., 2021). Besides, the maturation of gut microbiota is closely related to the synthesis and transport of BAs. Oral administration of BAs to postnatal mice can increase the richness and maturity of their gut microbiota (Van Best et al., 2020). Similarly, Lv et al. (2016) using metagenomic sequencing, observed a decrease in beneficial gut bacteria and an increase in Firmicutes and Proteobacteria phyla, considered opportunistic pathogens, in cholestatic patients. Additionally, upon conversion into secondary BAs, signals are transmitted through the nuclear farnesoid X receptor (FXR) and G-protein-coupled membrane receptor 5 (TGR5) in the intestinal epithelium to regulate host metabolism (Wahlström et al., 2016). In summary, the connection between gut microbiota and the liver is bidirectional, sustaining host health through synergistic effects.

The gut microbiota is widely involved in host metabolism, and its dysbiosis can alter the host's metabolic phenotype. Intestinal ecological disorders are closely related to cirrhosis and end-stage liver disease. Li et al. conducted a quantitative metagenomic association analysis on 98 patients with liver cirrhosis and 83 healthy controls, revealing a significant difference in the abundance of 75,245 genes believed to be related to liver cirrhosis (Qin et al., 2014). Similarly, an analysis of the fecal microbiome of patients with cirrhosis resulting from alcohol consumption and hepatitis B virus infection demonstrated a decrease in functional genes related to amino acids, lipids, nucleotides, and other metabolic pathways. The abundance of these functional genes negatively correlated with the Child-Pugh score (Chen et al., 2014), indicating that the structure and function of intestinal flora are significant in cirrhosis and its diverse complications. Therefore, exploring the changes in gut microbiota among patients with liver cirrhosis is expected to aid in intervening in the development of liver cirrhosis and its associated complications by targeting gut microbiota as a therapeutic approach.

3 Mechanisms of gut microbiota involved in the occurrence and development of live cirrhosis

The occurrence and development of liver cirrhosis are significantly influenced by the gut microbiota. Research indicates that changes in the gut microbiome can damage the liver by inducing inflammatory responses, exacerbating liver fibrosis, and affecting host metabolism (Martínez-Montoro et al., 2022; Wang et al., 2022; Zhang et al., 2023). Moreover, greater changes in the gut microbiota are associated with an increased risk of infection and reduced survival in liver cirrhosis (Fang et al., 2022). The gut microbiota known to be involved in the occurrence and development of chronic liver diseases such as cirrhosis. However, the specific mechanism by which changes in the gut microbiota leads to cirrhosis remains unclear. Current hypotheses propose mechanisms involving ecological dysbiosis, intestinal barrier

disruption, abnormal enterohepatic circulation and BA metabolism, and the translocation of microbial-derived metabolites (Figure 2).

3.1 Dysbiosis of gut microbiota

Dysbiosis of the gut microbiota mainly involves quantitative and qualitative changes in intestinal flora, where qualitative changes mainly lie in the composition and quantity of bacteria (Wegierska et al., 2022). Compared to the healthy population, the gut microbiota of patients with liver cirrhosis increases in Proteobacteria and Clostridium but decreases in Bacteroidetes at the phylum level (Qin et al., 2014; Bajaj et al., 2015; Trebicka et al., 2021). There is a reduction in the Bacteroides genus, with significant enrichment observed in Veillonella, Streptococcus, Clostridium, and the β-Proteus genus (Qin et al., 2014). Marco et al. demonstrated lower levels of Lachnospiraceae in fecal samples from patients with liver cirrhosis compared to healthy individuals, highlighting its important role in liver immune and metabolic processes through the production of short-chain fatty acids (SCFAs) (Zamparelli et al., 2017). In addition, beneficial bacteria like Ruminococcaceae and Clostridium Cluster XIV showed a significant decrease in the proportion of gut microbiota in patients with liver cirrhosis (Bajaj, 2019). Bajaj et al. introduced the cirrhosis dysbiosis ratio (CDR) to reflect the ratio of native to non-native microbiota in their fecal microbiota, and demonstrated correlation between CDR and the severity of liver cirrhosis, which further confirmed the significance of gut microbiota diversity in disease progression (Bajaj et al., 2014). The significant reduction of Clostridium XIV, Ruminococcae, and Trichrospiridae, which belong to the native flora, and the enrichment of potential pathogens Enterobacteriaceae and Bacteroidetes, synergistically promoted the accumulation of endotoxins in metabolites, and endotoxin levels could be used as an important indicator of the severity of HE. In addition, the abundance of Actinomycetes, Bacteroidetes and Firmicutes decreased to varying degrees in patients with cirrhosis infection, suggesting a causal relationship with the increased risk of systemic inflammation in patients with cirrhosis (Kulkarni et al., 2024). Families such as Lachnospiraceae and Ruminococcus, similar to acetate producers, are abundant in healthy individuals but significantly insufficient in patients with liver cirrhosis. Pathogenic bacterial overgrowth includes Enterobacteriaceae, Staphylococcus, and Enterococcus (Chen et al., 2011). *Veillonella atypica*, *Veillonella parvula*, and the Streptococcus

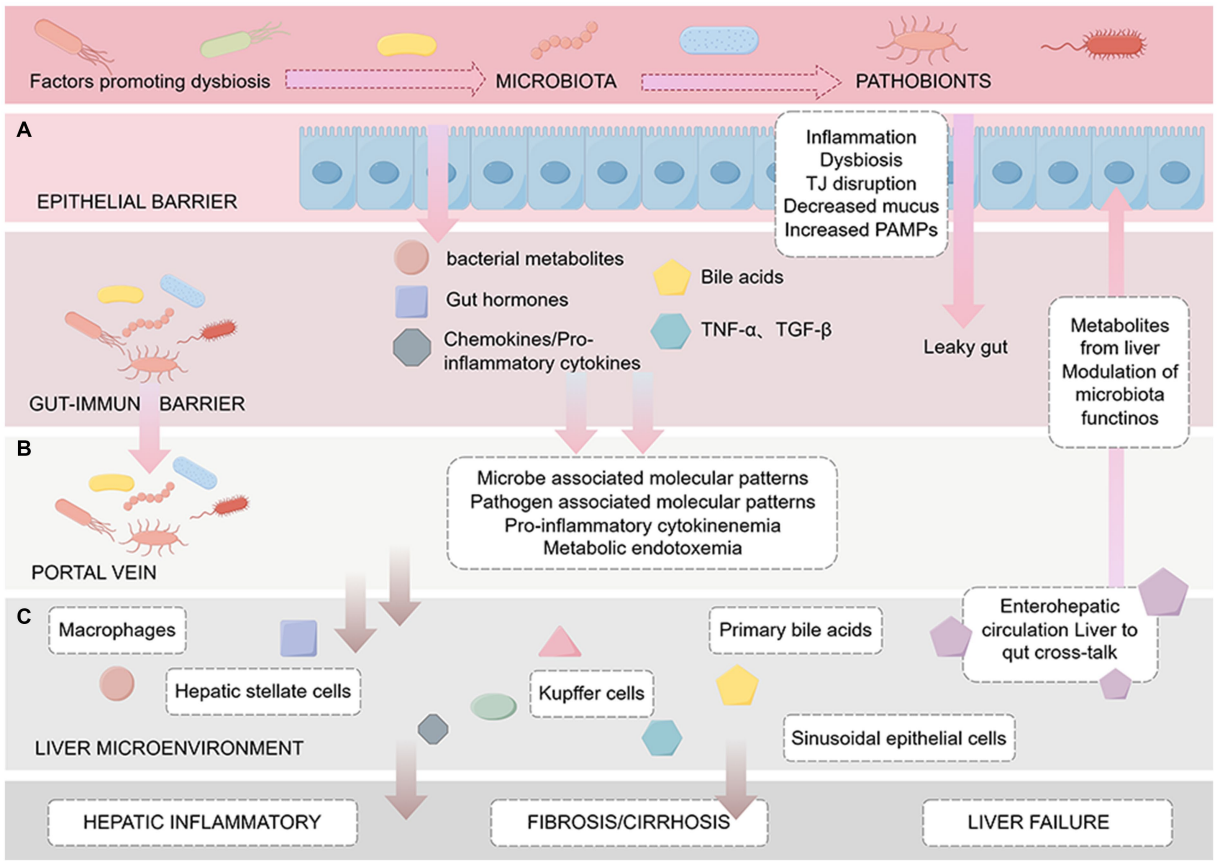

FIGURE 2
Pathogenesis of cirrhosis based on the enterohepatic circulation. The figure is drawn with Figdraw.com. The figure is divided into three sections from top to bottom: **(A)** Intestinal barrier: The effect of intestinal microbes, metabolites such as BAs and endotoxin on intestinal mucosal barrier function, including increased permeability and bacterial translocation. **(B)** Portal vein: Factors delivered to the liver via the portal vein, such as cytokines and microbiota-associated molecular patterns (MAMPs), which induce the activation of immune cells such as Kupffer cells, leading to inflammation and endotoxemia. **(C)** Liver microenvironment: After microbial-derived metabolites reach the liver through the portal vein, they trigger a series of reactions in the liver, including the activation of macrophages and HSCs, and may lead to liver fibrosis/cirrhosis, portal hypertension, and eventually liver failure.

genus were identified as pivotal components in disrupted gut microbiota of patients with liver cirrhosis. These bacteria produce rich sialidase enzymes that degrade human mucin O-glycans, leading to intestinal barrier disruption (Patel et al., 2022). Additionally, these bacteria trigger the secretion of chemokines such as CXCL8 and tumor necrosis factor-α (TNF-α) (Horvath et al., 2019), inducing inflammatory responses and increasing intestinal permeability (Chen et al., 2015). The phenomenon of oral colonization bacteria expanding in the intestine may be attributed to reduced gastric acid secretion, diminishing bacterial clearance in the stomach (Tsuda et al., 2015). Notably, Bifidobacteria and Lactobacillus, typically regarded as beneficial gut bacteria with anti-inflammatory properties (Yu et al., 2021; Song et al., 2023), are enriched in the intestinal tract of patients with alcoholic cirrhosis, suggesting potential adverse effects in liver cirrhosis patients. Therefore, attention should be paid to the administration of medications to such patients (Dubinkina et al., 2017).

Quantitative changes in gut microbiota are mainly characterized by small intestinal bacterial overgrowth (SIBO), which is related to factors such as decreased intestinal peristalsis, reduced gastric acid secretion, and impaired BA secretion, leading to prolonged content retention in the small intestine (Nishimura et al., 2021; Efremova et al., 2023). SIBO has been shown to be a risk factor for clinical decompensation of liver cirrhosis, which is manifested by increased abundance of *Firmicutes* and *Fusobacterium* at the phylum level; at the genus level, the abundance of *Blautia*, *Fusicatenibacter*, *Acinetobacter*, *Oribacterium*, and *Haemophilus* increased, with *Blautia* as a major contributor to the induction of SIBO in all overproliferating bacteria. Cirrhotic patients with SIBO had more severe intestinal flora dysbiosis, characterized by a higher Firmicutes/Bacteroidetes ratio, compared to those without SIBO (Maslennikov et al., 2022), suggesting this ratio as a crucial indicator reflecting cirrhosis progression. SIBO facilitates the translocation of harmful microorganisms and their metabolites (especially LPS) due to increased intestinal permeability, finally leading to endotoxemia (Augustyn et al., 2019). Activation of NF-κβ by endotoxemia triggers the production of pro-inflammatory cytokines, inducing liver inflammation and insulin resistance (IR), a significant factor in the pathogenesis of cirrhosis and its complications (Clarembeau et al., 2020). Moreover, SIBO generates excess acetate and disrupts the homeostasis of BA metabolism, promoting the deconjugation of BA (Bala et al., 2006). Acetate harms the colon mucosa, while unbound BAs adversely affect lipid metabolism and intestinal mucosa, accelerating the progression of liver cirrhosis (Nafday et al., 2005; Hartmann et al., 2018). The diversity of fecal microbiota in patients in the decompensated stage was lower than that in the compensated stage, and the *Clostridium* XIV, Ruminococcaceae and Trichromyceae belonging to the native flora were significantly reduced, while the potential pathogenic bacteria Enterobacteriaceae and Bacteroides were enriched, and these two changes synergistically promoted the accumulation of metabolite endotoxins, and the increase of gut permeability led to SIBO and pathological BT. The overall translocation of viable bacteria to mesenteric lymph nodes is an important feature of decompensated cirrhosis, which in turn can damage the host's systemic and local immune defenses, leading to further exacerbation and a vicious cycle (Giannelli et al., 2014). A meta-analysis involving 306 samples revealed a significant correlation between SIBO and various complications of liver cirrhosis, such as HE, spontaneous bacterial peritonitis, malnutrition, and ascites (Maslennikov et al., 2018). However, due to the lack of an optimal diagnostic standard for SIBO (Massey and Wald, 2021), it cannot be definitively concluded that SIBO directly leads to these complications, necessitating further research for confirmation. The abundance changes of gut microbiota in patients with liver cirrhosis are shown in Table 1.

3.2 Intestinal barrier dysfunction

Potential harmful bacteria and their effector molecules, known as pathogen-associated molecular patterns (PAMPs), originating from the intestine, directly cross the damaged intestinal barrier and enter the liver, facilitated by various pattern recognition receptors. Activated liver cells release damage-associated molecular patterns, which, in conjunction with hepatic stellate cells (HSCs) and liver immune cells, particularly Kupffer cells, exacerbate liver inflammation and fibrosis (Guan et al., 2022). Therefore, the dysfunction of the intestinal barrier is an important prerequisite for PAMPs translocation to the liver and is a key factor influencing the pathogenesis of liver diseases. The intestinal barrier, composed of a mucous layer and an epithelial layer, together with the gut microbiota and immune system, maintains the normal function of the intestinal function (Tilg et al., 2022). These physical, immune, and biological barriers interact with each other (Figure 3), and any disruption at any crosslink will increase susceptibility to liver disease.

3.2.1 Physical barriers

The mucus layer is mainly composed of secretion products from goblet cells, with its outer layer colonized by microbial communities and its inner layer containing minimal bacteria (Tsiaoussis et al., 2015). Mucus, predominantly composed of mucin (MUC), are divided into two types: transmembrane mucin (MUC1, MUC4, MUC15, MUC16, etc.) and secretory mucin (primarily MUC2) (Chopyk and Grakoui, 2020). Microorganisms competed for attachment sites and energy for colonization by degrading the O-and N-linked glycans and glycosaminoglycans of MUC2 (Martens et al., 2009). The protective effect of mucus in MUC2-deficient mice was significantly weakened, allowing direct entry of bacteria into epithelium and crypts, thereby inducing colitis-related cytokine expression (Johansson et al., 2008). Transmembrane mucin not only protected intestinal epithelial cells and acted as a lubricant but also provided immune protection and inhibits cell apoptosis (Constantinou et al., 2011; Paone and Cani, 2020). For example, MUC1 overexpression generated anti-MUC1 antibodies that could damage colon cells through cell-dependent cytotoxicity, leading to chronic intestinal inflammation such as ulcerative colitis (UC) (Apostolopoulos et al., 2015). Preventive treatment with anti-inflammatory milk fat globule membrane (MFGM) upregulated colonic MUC2, MUC4, Reg3g, and other gene expressions in mice, enhancing mucosal barrier integrity and reducing the risk of microbial-induced intestinal inflammation and secondary liver injury (Wu et al., 2022). Fecal samples from liver cirrhosis patients showed potential pathogenic bacteria rich in sialidase (e.g., *Veillonella atypica*, *Veillonella parvula*, and Streptococcus spp.) degrading MUC2 O-glycans, leading to barrier damage (Patel et al., 2022). Additionally, isoproterenol can reduce the number of goblet cells and mucus thickness in mice, impairing the intestinal mucosal

TABLE 1 Changes in gut microbiota abundance in patients with cirrhosis.

Classification	Common bacteria species	Change trend	Mechanism	Reference
Phylum	*Bacteroidetes*	Down	The synthesis of SCFAs (acetate, butylhydrochloric acid and palmitate) decreased; Secondary BAs production was insufficient and BA uncoupling was impaired.	Qin et al. (2014), Zamparelli et al. (2017), and Bajaj (2019)
	Proteobacteria	Up	Promoting the translocation of cells and bacteria, making them the most harmful bacteria in the gut microbiota, has the potential to stimulate liver fibrosis.	Zamparelli et al. (2017) and Maslennikov et al. (2018)
	Fusobacteria	Up	The transformation from primary BA to secondary BA was disordered. It produces LPS that travels with the impaired intestinal barrier into the liver, increases oxidative stress and activates TLR4, producing proinflammatory and profibrotic effects.	Zamparelli et al. (2017)
Family	*Enterobacteriaceae*	Up	It releases potent endotoxins and produces endogenous ethanol, which disrupts intestinal barrier function, increases intestinal permeability, stimulates the innate immune system, and leads to chronic inflammation of the intestinal wall and liver.	Bajaj et al. (2014), Bajaj (2019), and Massey and Wald (2021)
	Veillonelaceae	Up	It is the most representative fibrosis-associated bacterium and positively interacts with ursodeoxycholic acid and propionate.	Wahlström et al. (2016) and Kulkarni et al. (2024)
	Streptococcaceae	Up	It was negatively correlated with acetic acid level and positively correlated with Child-Pugh score.	Wahlström et al. (2016)
	Lachnospiraceae	Down	Reduction of 7α-dehydroxylation; Inhibition of this bacterium leads to a decrease in SCFA production, which in turn raises colonic PH and increases ammonia content.	Maslennikov et al. (2018), Bajaj (2019), and Kulkarni et al. (2024)
	Ruminococcaceae	Down	It affects the 7α-dehydroxylation process. The production of SCFA decreased, and the levels of inflammatory cytokines in the blood are increased.	Wahlström et al. (2016), Maslennikov et al. (2018), Bajaj (2019), and Kulkarni et al. (2024)
	Enterococcaceae	Up	Promote endotoxin release, cause intestinal barrier damage and BT; It activates proinflammatory cytokines, exacerbates hepatocyte injury, and plays a key role in the complications of infectious cirrhosis, especially SBP.	Kulkarni et al. (2024), Zamparelli et al. (2017), and Maslennikov et al. (2023)
	Porphyromonadacea	Up	Promoting inflammation and disrupting the normal microbiome; It is associated with cognitive impairment in patients with liver cirrhosis.	Maslennikov et al. (2023)
	Alcaligeneceae	Up	It is associated with bacterial infection in patients with liver cirrhosis.	Maslennikov et al. (2023)
	Staphylococcaceae	Up	It can change gastric hemodynamics and increase the incidence and severity of portal hypertensive gastropathy. It is also closely related to liver failure and endotoxemia.	Zamparelli et al. (2017) and Kulkarni et al. (2024)
Genus	*Bifidobacterium*	Up	It was negatively correlated with the expression level of aspartate aminotransferase and prothrombin time.	Yu et al. (2021)
	Lactobacillus	Up	Promoting the production and accumulation of endogenous ethanol.	Zamparelli et al. (2017) and Yu et al. (2021)
	Faecalibacterium prausnitzii	Down	The production of butyrate is reduced, weakening its anti-inflammatory protective and oxidative stress-ameliorating properties in the gut.	Wahlström et al. (2016)
	Prevotella	Up	Promotes ethanol metabolism in the intestine.	Wahlström et al. (2016) and Manzoor et al. (2022)
	Clostridium	Down	Involved in 7α-dehydroxylation, the reduction of its genus leads to a lack of substrates for the conversion of primary BA to secondary BA.	Wahlström et al. (2016) and Zamparelli et al. (2017), and Maslennikov et al. (2018)
	Blautia	Down	The production of SCFA decreased; BA transformation regulation is limited; It also attenuated its anti-inflammatory properties.	Bajaj et al. (2012) and Meijnikman et al. (2022)

(Continued)

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TABLE 1 (Continued)

Classification	Common bacteria species	Change trend	Mechanism	Reference
	Oscillospira	Down	Reduced production of SCFA with nutritional, metabolic, and immunomodulatory effects.	Baltazar-Díaz et al. (2022)
	Pseudobutyrivibrio	Down		Zamparelli et al. (2017)
	Coprococcus	Down		Wahlström et al. (2016) and Bajaj (2019)
	Roseburia	Down		Qin et al. (2014) and Zamparelli et al. (2017)
	Atopobium	Up	Promotes the production of high levels of CH_3SH, which directly contributes to the occurrence of hepatic encephalopathy.	Manzoor et al. (2022)
	Veillonella	Up	It produces propionate, which has proinflammatory features.	Chen et al. (2011), Chen et al. (2016), and Wahlström et al. (2016)
	Dialister	Up		Chen et al. (2016), Manzoor et al. (2022), and Wu et al., 2023
	Megasphaera	Up		Chen et al., 2016 and Manzoor et al. (2022)
	Akkermansia muciniphila	Down	It reduces the production of intestinal mucus and TJ expression, weakens the protective function of the intestinal barrier, and aggravates liver injury and disease susceptibility.	Lee et al. (2020) and Liu et al. (2019)
	Faecalibacterium prausnitzii	Down	As a key beneficial commensal, its reduction in abundance attenuated the anti-inflammatory effect on the liver.	Ponziani et al. (2018) and Oh et al (2020)

epithelial and vascular barriers, facilitating BT to the liver and exacerbating liver damage (Sorribas et al., 2020). This multifaceted dysfunction of the intestinal barrier significantly contributes to the severity and complications of liver cirrhosis.

Intestinal epithelial cells include five specialized types: stem cells, goblet cells, neuroendocrine cells, Paneth cells, and intestinal absorptive cells (Pott and Hornef, 2012). They form a protein network that mediates selective permeability through transcellular and paracellular pathways (Tsukita et al., 2001). The protein network is composed of various adhesive complexes such as desmosomes, adhesive junctions (AJs), tight junctions (TJs), and gap junctions (Groschwitz and Hogan, 2009), among which TJ is recognized as the most important. Besides, Claudins, Occludin, and cytoskeleton-connected adhesive molecules (mainly microfilaments) considered as particularly important TJs (Tsiaoussis et al., 2015). Compared to healthy individuals, the expression levels of Occludin and Claudin-1 in intestinal epithelial cells were reduced in patients with liver cirrhosis, negatively correlated with the Child-Pugh score (Assimakopoulos et al., 2012). As a dynamic permeability tight barrier, intestinal epithelial TJs could prevent potential pathogenic bacteria, toxins, and other harmful substances from invading and permit the passage of nutrients, water, and ions (Hossain and Hirata, 2008). Mutation or defects in TJs cause TJPs contraction, enlarging intercellular spaces and enabling harmful macromolecules such as bacteria and toxins to infiltrate the portal vein, promoting the occurrence and development of liver cirrhosis (Twardowska et al., 2022). Additionally, oxidative stress is a key mechanism in intestinal barrier damage (Mohamed et al., 2021; Mo et al., 2022), inducing intestinal epithelial cell apoptosis, reducing the expression of TJPs, and increasing intestinal permeability (Li et al., 2022).

3.2.2 Immune barriers

Following the disruption of the physical barrier structure, activation of the intestinal mucosal immune system occurs, with humoral immunity dominated by secreted immunoglobulin A (SIgA). SIgA not only prevents the recognition and uptake of bacteria, toxins, and other harmful molecules by intestinal mucosal epithelial cells (Mantis et al., 2011) but also contributes to the elimination of pathogens and toxins through intestinal mechanical peristalsis. Furthermore, it attenuates the systemic immune response and immune tolerance triggered by external antigenic stimulation and prevents the intestinal mucosal immune barrier system from eliciting an immune response to beneficial gut microbiota (Perez-Lopez et al., 2016). These intricate mechanisms involve T cells, interleukins (IL)-4, IL-6, transforming growth factor (TGF)-β, and tumor necrosis factor (TNF)-α within gut-associated lymphoid tissue (GALT) (Hooper and Macpherson, 2010). Prolonged pathogen stimulation of the immune system, coupled with inhibition of regulatory cells such as macrophages, dendritic cells, and M cells, disrupts immune homeostasis, thereby instigating intestinal inflammation. Multiple immune cells through complex interactions accelerates pro-inflammatory and fibrotic processes in the liver (Winer et al., 2016). Porowski et al. calculated the hepatic elimination ratio (LER) of IL-6, hepatocyte growth factor (HGF), TNF-α, and TGF-β introduced from the GALT and portal vein. In healthy individuals, LER values were 0.4, 0.2, 0.4, and 0.3 respectively, while in liver transplant patients, they were-0.1, −0.5, 0.1, and-0.2, respectively. Negative elimination rates indicate cytokine synthesis surpassing degradation in patients with liver cirrhosis (liver

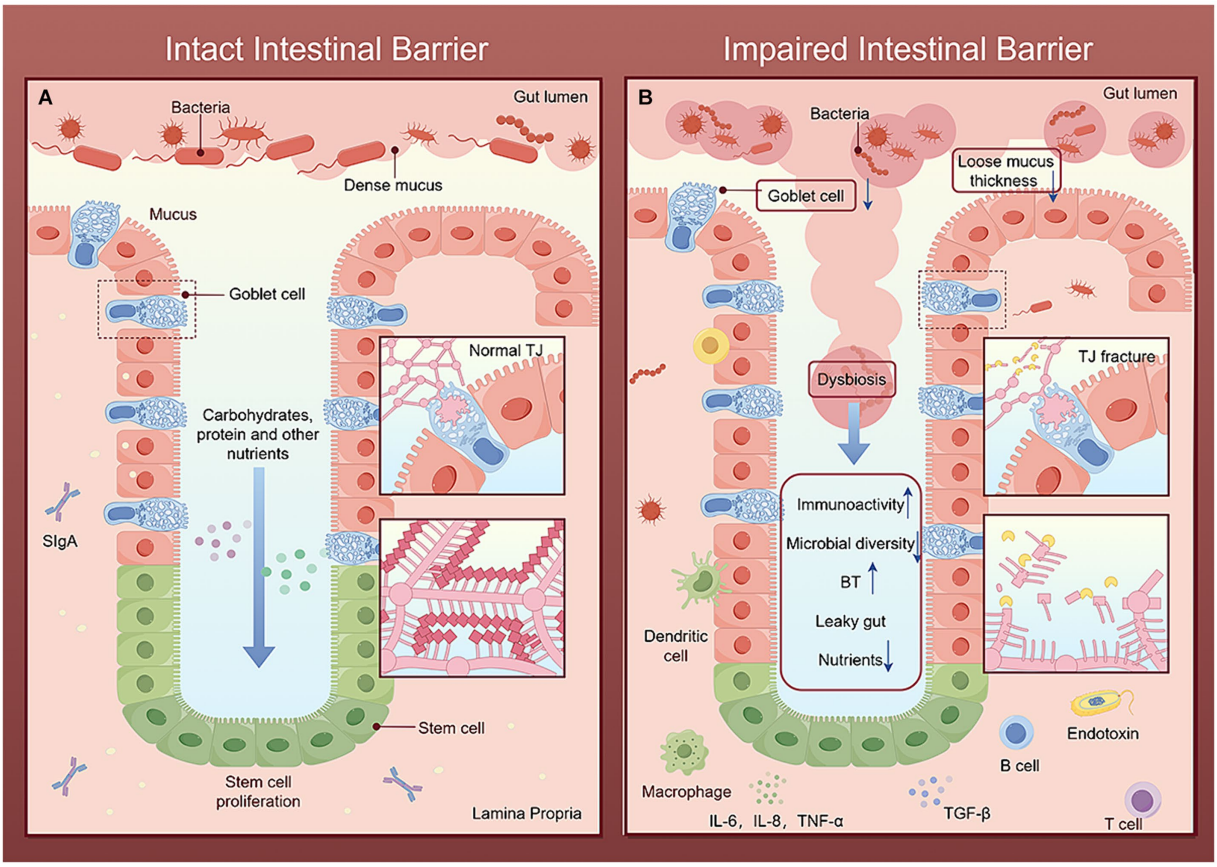

FIGURE 3
Comparison of intact and impaired intestinal barriers. The figure is drawn with Figdraw.com. **(A)** In the intact intestinal barrier, the intestinal microbial diversity is normal, the mucus layer limits the colonization and transfer of bacteria, the beneficial bacteria repel the invasion of pathogenic bacteria, and the complex structure of TJ together to maintain the selective permeability of intestinal epithelial cells, allowing nutrients and water ingested by the host to pass through and preventing harmful microorganisms and their metabolites from entering the portal vein through the intestinal barrier. Immune cells secrete various cytokines to timely remove invading pathogens and toxins and strengthen the intestinal barrier. **(B)** After the intestinal barrier is damaged, the mucus layer becomes loose and thick. Harmful microorganisms and their metabolites break through the mucus layer, destroy the intestinal tight-junction apparatus, and compete with beneficial bacteria and intestinal epithelial cells for nutrients, leading to increased intestinal permeability. Bacteria, toxins and other harmful molecules stimulate the intestinal immune barrier system, leading to enhanced immune activity and reduced microbial diversity. Finally, it further increases BT and intestinal leakage.

failure), with levels of these growth factors and cytokines closely related to the severity of liver cirrhosis (Porowski et al., 2015). Moreover, the severe immune deficiency induced by systemic inflammation and immune cell dysfunction in patients with liver cirrhosis plays a pivotal role in the pathogenesis of chronic acute liver failure (ACLF) (Martin-Mateos et al., 2019). In addition, endotoxins can severely damage the intestinal mucosal barrier and immune system of the body through oxidative stress (Wang and Wang, 2022). This particular subject was further elucidated in subsequent sections.

3.2.3 Biological barriers

We explored the composition and changes in gut microbiota in the above discussion. Here, we delve into the interplay and mechanisms between the biological barrier, comprised of gut microbiota, and other barriers. Firstly, mucoglycans serve as nutrients for beneficial bacteria, enabling them to compete for mucosal colonization sites and inhibit pathogen colonization (Paone and Cani, 2020), thereby influencing mucus properties by interfering with glycosyltransferase expression, MUC2 glycosylation, and transmembrane mucin glycosylation (Schroeder, 2019; Pelaseyed and Hansson, 2020). Furthermore, microbial metabolites such as acetate and butyrate contribute to epithelial homeostasis by modulating goblet cells (Wrzosek et al., 2013). *Lactobacillus reuteri* mediates the PI3K/Akt Nrf-2/HO-1-NF-κβ and PKC-Nrf-2/HO-1-NF-κβ signaling pathways, enhancing the expression of TJPs, inhibiting TNF-induced apoptosis of intestinal epithelial cells (Zhou et al., 2022), and activating the Wnt/β-catenin pathway to stimulate the proliferation of intestinal epithelial cells, thereby regulating the physical barrier function of the intestine (Wu et al., 2020).

Microorganisms and the intestinal mucosal immune system interact with each other, jointly maintaining a symbiotic relationship between the host and microorganisms (Hooper et al., 2012). SIgA on the surface of the intestinal mucosa selectively binds to symbiotic bacteria, preventing their penetration of the epithelial barrier (Macpherson et al., 2000). Microbial colonization also facilitates the development and function of the intestinal immune system (O'Hara and Shanahan, 2006). The Toll-like receptor (TLR) family and nucleotide-binding oligomerization domain proteins Nod1 and Nod2

serve as primary receptor systems for microbial regulation of the innate immune system in the intestinal mucosa (Kelly and Conway, 2005). For example, LPS not only aids in the repair of damaged intestinal epithelium through MyD88-dependent processes (Rakoff-Nahoum and Medzhitov, 2007), but also activates the expression of the antimicrobial lectin Reg III γ, serving as an additional barrier to inhibit bacterial infiltration into the intestinal mucosa (Vaishnava et al., 2011). Therefore, excessive microbial stimulation may lead to the abnormal activation of intestinal immune cells, leading to intestinal inflammation. In summary, damages to the intestinal biological barrier inevitably affect the physical and immune barriers of the host, ultimately increasing the risk of various chronic liver diseases such as cirrhosis.

3.3 Abnormal metabolism of BAs

3.3.1 Synthesis and pathways of BAs

BAs originate from cholesterol oxidation in the liver through two primary pathways. Under normal conditions, cholesterol 7α-hydroxylase (CYP7A1) initiates the classical pathway to produce the majority of primary BAs. CYP7A1 regulates the overall rate of BA synthesis, while sterol 12α-hydroxylase regulates the cholic acid (CA) to chenodeoxycholic acid ratio in the BA pool. In an adaptive response, alternative pathways, initiated by sterol-27 hydroxylase, play crucial roles in intestinal metabolism, cellular signaling, and microbial composition (Li and Chiang, 2014). Intestinal microbiota modify human BAs via four mechanisms: deconjugation of glycine or taurine, dehydroxylation, dehydrogenation, and epimerization (Guzior and Quinn, 2021), further activating BAs receptors. There is a close relationship between metabolic disorders of BAs and liver cirrhosis, along with its complications and intestinal dysbiosis.

3.3.2 Bidirectional regulation of BAs and gut microbiota

Changes in gut microbiota composition and activity due to factors such as antibiotics, diet, and exercise significantly impact BA synthesis and signal transduction (Collins et al., 2023). Bacterial bile salt hydrolase (BSH), found in various gut bacteria like Clostridium, Enterococcus, Bifidobacterium, Lactobacillus, and Bacteroidetes, catalyzes the decoupling, oxidation, and 7α-dehydroxylation of conjugated BA, regulating the production level of secondary BAs (Ridlon et al., 2016). In addition, the composition and number of Firmicutes also affect secondary BA levels by altering 7α-dehydroxylation (Lee et al., 2022). Conversely, BAs influence microbiota composition and function, playing a decisive role in both quantitative and qualitative changes, as well as metabolic activities of gut microbiota. For example, studies suggest that glycine-and taurine-preferred BSH can inhibit the growth of *Clostridium difficile*, possibly by increasing the concentration of microbial conjugated BA, which inhibits the germination, growth, and toxin expression of *Clostridium difficile* (Foley et al., 2023). Moreover, BA administration in neonatal mice could significantly drive the maturation of the intestinal microbiota, improving its diversity and richness (Van Best et al., 2020). However, BAs may also harm intestinal bacteria due to their toxic effects, mediating oxidative stress and DNA damage (Collins et al., 2023). Overall, BA metabolism intricately links gut microbiota to physiology and pathology of the liver, offering a broader perspective for the treatment of liver diseases through the modulation of gut microbiota.

3.3.3 Nuclear receptor FXR

FXR is a core regulatory factor in the biosynthesis of BAs and enterohepatic circulation. Dysfunction of the microbiota leads to decreased BA activity, reducing unconjugated BA and secondary BA production, weakening FXR activity, and exacerbating cirrhosis through various pathways: (1) In hepatocytes, FXR interacts with nuclear receptors such as peroxisome proliferator-activated receptors (PPAR)-α and liver X receptor (LXR)α to form heterodimers, regulating downstream target gene expression. Weakened FXR activity decreases the expression of PPAR-α and LXRα, negatively impacting liver energy metabolism (Preidis et al., 2017); (2) The inhibitory and regulatory effects on TLR4 transcription levels and NLRP3 inflammasome are weakened, promoting the production of inflammatory factors such as IL-1β and NOS2, further exacerbating the inflammatory response (Vavassori et al., 2009; Han et al., 2018); (3) Fat synthesis, high-density lipoprotein (HDL) formation, liver HDL uptake, and fatty acid β-oxidation, are affected, disrupting lipid metabolism and worsening liver steatosis and fibrosis (Ding et al., 2015); (4) Enhanced HSC activation increases collagen synthesis and deposition, accelerating liver fibrosis progression (Zhou et al., 2020). (5) Dysbiosis and the release of inflammatory markers (LPS) (Ridlon et al., 2015) further lead to BT, endotoxemia, and intestinal barrier damage, significantly correlating with liver cirrhosis progression and cognitive impairment (Zhang et al., 2022).

3.4 Microbial metabolites

The changes in gut microbiota observed during the occurrence and progression of liver cirrhosis are mainly attributed to the diversity, richness, and the presence of potential pathogenic byproducts such as endotoxins. Intestinal bacteria are the key source of LPS, and the liver is a vital site for LPS clearance. Therefore, abnormal production, kinetics and metabolism of LPS can reverse interfere with liver function and intestinal flora (Fuke et al., 2019). SCFA is the most abundant among all microbial metabolites, and about 500-600mmol of SCFAs are produced in the human intestine every day (Marrocco et al., 2022). SCFAs entering the liver supply more than 30% of the liver's total energy source (Mann et al., 2024). As a tryptophan catabolite produced by the intestinal microbiota, indole is an indispensable signaling molecule regulating intestinal homeostasis (Roager and Licht, 2018). Although less than 1% of bacteria in the healthy gut contain genes for trimethylamine (TMA) synthesis, this is sufficient to regulate the balance of choline metabolism, indicating that these trace intestinal microorganisms and choline are essential for host health (Rath et al., 2017). Under anaerobic conditions, 1g of *Escherichia coli* produces 0.8 g of ethanol per hour, and excessive ethanol will significantly change the abundance of some intestinal flora, especially Bacteroidetes and Proteobacteria (Yan et al., 2023). In brief, metabolites produced by gut microbes are key mediators of gut microbiota-cirrhosis crosstalk.

The previously discussed physical, immune, and biological barriers within the intestine are critical in preventing harmful microorganisms and their metabolites from infiltrating the liver. However, dysbiosis or compromised intestinal barriers can lead to the

abnormal accumulation of harmful metabolites in portal circulation, triggering a cascade of pro-inflammatory reactions associated with liver diseases (Ceccarelli et al., 2014). Yet, the specific mechanisms through which certain metabolites contribute to the occurrence and development of liver cirrhosis are not fully understood. Here, we delved into the pathological effects and mechanisms of SCFAs, endotoxins, indole, choline, and endogenous ethanol on the liver (Figure 4).

3.4.1 Short chain fatty acids

The main products of anaerobic fermentation in the intestine are SCFAs, with acetic acid, propionic acid, and butyric acid being the most abundant (Vallianou et al., 2021). These SCFAs help maintain energy homeostasis through multiple pathways. Firstly, SCFAs, especially butyric acid, act as preferred energy sources for intestinal epithelial cells. Upon absorption, butyric acid is converted into cellular energy, inhibiting NLRP3 inflammasome activation and autophagy, thus protecting the intestinal barrier against LPS damage and maintaining the normal cell function (Feng et al., 2018). Moreover, acetate, propionate, and butyrate contribute to glucose, cholesterol, and lipid metabolism, directly impacting hepatic energy levels (Den Besten et al., 2013). Additionally, they indirectly influence the energy metabolism of peripheral organs by affecting the activity of hormones and the nervous system (Chambers et al., 2015; Perry et al., 2016). Therefore, SCFAs play an important role in human physiology and pathology (Koh et al., 2016).

SCFAs are closely related to the occurrence and severity of liver cirrhosis (Cao et al., 2023). There is a correlation between a decrease in the number of SCFA-producing bacteria compared with compensated patients and the occurrence of decompensated events (Bajaj et al., 2023). Reduced SCFA levels, particularly butyric acid, have been observed in fecal samples from cirrhotic patients, positively correlating with Child-Pugh classification (Jin et al., 2019). Furthermore, abnormal SCFA production correlates with liver cirrhosis severity. Diminished levels of beneficial bacteria such as Lachnospiraceae, Roseburia, Blautia, and Ruminococcaceae lead to decreased butyrate levels, impacting TJ and mucin (MUC) expression and altering intestinal barrier function and permeability. Consequently, pro-inflammatory cytokines were released into the liver (Woodhouse et al., 2018), affecting NF-κB signaling pathways and histone deacetylase inhibition (Neis et al., 2016). SCFAs have demonstrated an important role in regulating endothelial function, immune response, and vascular tone. The imbalance in SCFA production due to dysbiosis of the flora, particularly decreased Bacteroidetes abundance, disrupt these regulatory mechanisms, contributing to portal hypertension (Toshida et al., 2023).

3.4.2 Endotoxin

Endotoxins, a component of LPS, constitute the outermost layer of Gram-negative bacteria cell walls, exerting diverse biological effects on ALD, viral hepatitis, cirrhosis, and its complications (Porras et al., 2017; Sehgal et al., 2020). Alcohol consumption and HFD-induced

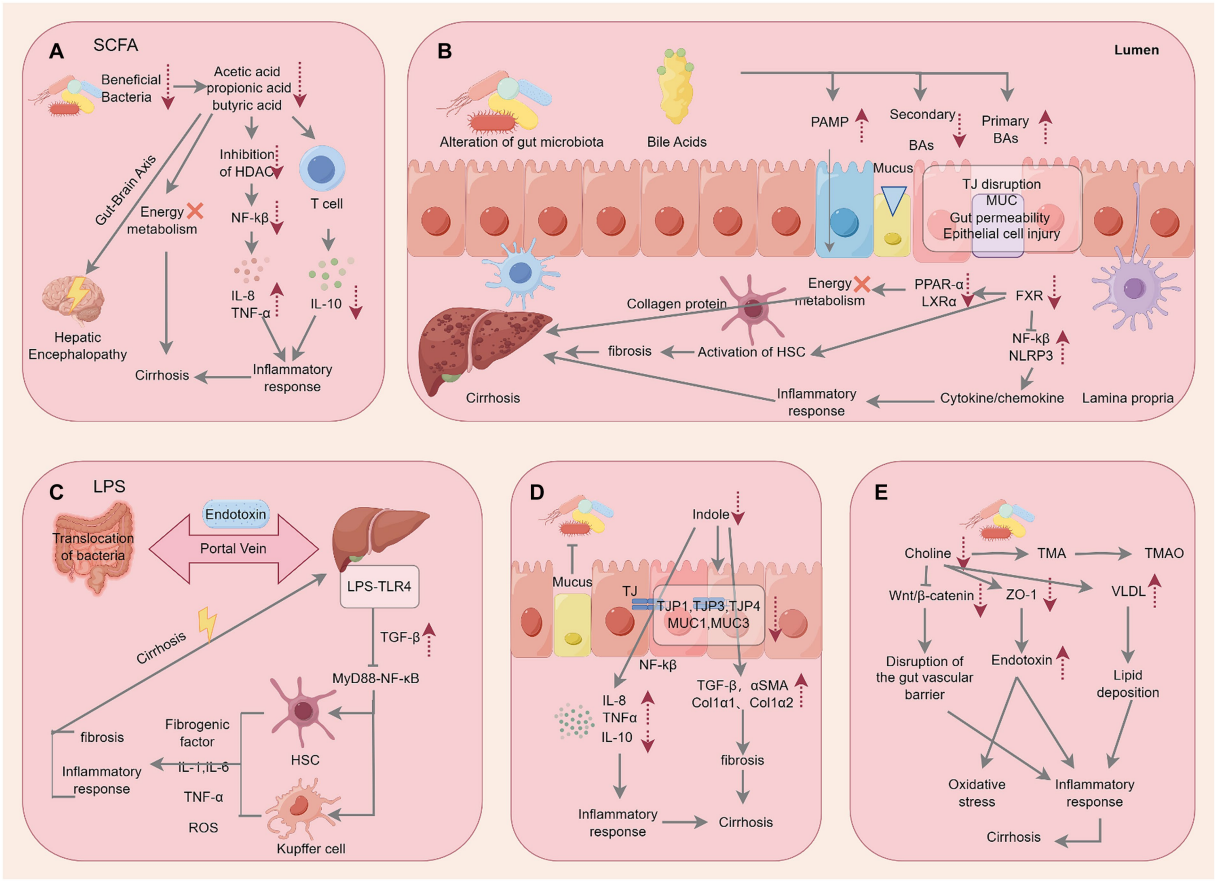

FIGURE 4
Specific mechanisms by which microbial metabolites act on cirrhosis. The figure is drawn with Figdraw.com.

dysbiosis trigger potential pathogen translocation through the disrupted intestinal barriers. LPS binds to TLR4, stimulating Kupffer cells in the liver and the NF-κB pathway and promoting the expression of inflammatory factors, such as TNF-α, IL-1, and IL-6. On the other hand, LPS-TLR4 can increase the permeability of intestinal TJs and activate HSC, leading to inflammatory reactions in the intestine and liver. Besides, it also promotes the excessive secretion and deposition of collagen and other extracellular matrix proteins, ultimately leading to the development of liver cirrhosis (Roderburg and Luedde, 2014). Interestingly, LPS in activating immune signal transduction through TLR4-related pathways and affecting liver physiological functions is structurally dependent, indicating that a few LPS may act as antagonists to inhibit the occurrence of inflammatory responses (Di Lorenzo et al., 2019). In the gut microbiota of patients diagnosed with liver cirrhosis, increased abundance of the Enterobacteriaceae family, encoding heptyltransferase I (HpeI), a key enzyme involved in LPS synthesis, were observed and categorized into seven clusters. Specifically, the first cluster of HpeI serves as a biomarker for liver cirrhosis, offering diagnostic and treatment guidance from the perspective of gut microbiota (Lin et al., 2021).

Oxidative stress plays a crucial role in the progression of chronic liver disease to cirrhosis and its complications (Assimakopoulos et al., 2011). Liver damage weakens antioxidant effects, elevating oxidative stress and the production of reactive oxygen species (ROS), which subsequently harm the intestinal mucosa (Moustafa et al., 2009). It is specifically characterized by high levels of lipid peroxidation, protein oxidation, and an imbalance in glutathione redox status (Spahr et al., 2007). Damaged intestinal mucosa enhances permeability, promotes BT, coupled with collateral circulation and liver damage, finally leading to endotoxemia. The ensuing cycle exacerbates oxidative stress, intestinal barrier dysfunction, and organ damage (Liu et al., 2023), underscoring a significant correlation between liver cirrhosis, endotoxemia, and oxidative stress. Cheon et al. found that elevated endotoxin levels and subsequent increased ammonia levels activate the NLRP3 inflammasome through mitochondrial oxidative stress, resulting in neuroinflammation, brain edema, and the development of HE (Bajaj, 2014; Cheon et al., 2023). In addition, entero-derived endotoxins and oxidative stress are important factors in triggering portal hypertension in decompensated cirrhosis (Tripathi et al., 2018; Liu et al., 2023). Alterations in specific gut microbiota (Corynebacterium, Lautropia) and their excessive accumulation of metabolites, particularly LPS, are strongly associated with sepsis and related events in patients with decompensated cirrhosis (Philips et al., 2023). In addition, Sandler et al. proposed that LPS-induced inflammatory response and oxidative stress are predictive of progression to early compensated cirrhosis and end-stage liver failure in patients with chronic HBV or HCV infection (Sandler et al., 2011). Therefore, the exploration of drugs that target the regulation of LPS and its producing bacteria is expected to provide a therapeutic strategy for liver cirrhosis at different stages of progression.

3.4.3 Indole

Indole can be produced by various gut microbiota such as *Escherichia coli*, Clostridium, and Bacteroidetes (Lee et al., 2015). Studies have found that indole can alter the composition of gut microbiota and increase gene expression of TJPs and mucins between epithelial cells, indicating enhanced intestinal epithelial barrier (Shimada et al., 2013). Moreover, indole can reduce TNF-α-mediated activation of NF-κβ, inhibit the expression of pro-inflammatory chemokine IL-8, and increase the expression of anti-inflammatory cytokine IL-10. Furthermore, it confers resistance to pathogen colonization, particularly against *Escherichia coli*, thereby attenuating intestinal inflammation (Bansal et al., 2010). Zhao et al. observed that indole intervention in HFD-treated rats resulted in significant downregulation of genes related to liver fibrosis and collagen synthesis (Zhao et al., 2019). However, subsequent studies have shown that indole or its metabolites do not consistently exhibit a beneficial role in liver disease. Liu et al. found that indole-3-propionic acid (IPA) could reduce the gap in gut microbiota diversity between CCl4-induced mice and healthy counterparts, activated HSCs and the TGF-β1/Smads signaling pathway, leading to hepatocyte apoptosis, increased expression of extracellular matrix components such as collagen I and α-SMA, ultimately promoting liver fibrosis (Liu et al., 2021). These contrasting findings underscore the necessity for thorough validation and consideration when evaluating the therapeutic potential of IPA in managing liver cirrhosis. Nevertheless, Li et al. analyzed 560 metabolites in serum metabolomics of patients with cirrhosis at different stages and found that IPA was the most significant indicator of decompensated metabolic biomarker reduction, suggesting that IPA can help accurately predict the stage of cirrhosis (Li et al., 2024).

3.4.4 Trimethylamine

Gut microbiota metabolism involves the conversion of choline into TMA, which is absorbed by the host and subsequently converted into trimethylamine N-oxide (TMAO) in the liver (Arias et al., 2020). Imbalances in gut microbiota reduce the bioavailability of choline and increase portal venous influx of TMA, both implicated in metabolic abnormalities associated with liver cirrhosis (Albillos et al., 2020). Mourier et al. established a fatty liver model using a methionine/choline-deficient diet, revealing downregulation of the Wnt/β-catenin pathway in mice, leading to disruption of the intestinal vascular barrier (Mouries et al., 2019). Sawada et al. observed reduced expression of TJPs like ZO-1 in choline-deficient rats compared to controls, resulting in intestinal endotoxin accumulation in the portal vein, triggering liver inflammation and oxidative stress (Sawada et al., 2019). In addition, deficiencies in methionine and choline promote the accumulation of cholesterol and fat in the liver by inhibiting the assembly/export of very low-density lipoprotein (Simon et al., 2020). Excessive fat deposition in liver cells is the main cause of liver inflammation, implicating its role in diverse liver diseases (Li et al., 2023).

3.4.5 Endogenous ethanol

In fact, Enterobacteriaceae and *Klebsiella pneumoniae* in the gut produce endogenous ethanol through the fermentation of carbohydrates, and ethanol metabolism produces toxic acetaldehyde (Salaspuro, 1997). Numerous studies have proved that when alcohol accumulates in the intestine due to long-term drinking, obesity and other factors, it will increase the expression of inflammatory cytokines to disrupt intestinal homeostasis, resulting in portal endotoxemia (Sheth et al., 2007; Kwon et al., 2014). In addition, ROS and ammonia produced by ethanol metabolism may cause oxidative stress and hepatocyte necrosis (Lieber, 1999). What's more, its metabolite acetaldehyde may damage TJs of intestinal epithelial cells, destroy the intestinal barrier, cause pathological BT and intestinal leakage, and promote the progression of cirrhosis (Yan et al., 2023). In conclusion,

endogenous ethanol can worsen liver inflammation through multiple pathways and has a significant effect on fibrosis.

4 Protective effects of TCM on liver cirrhosis induced by intestinal dysbiosis

Given the advantages of TCM, including its multi-component, multi-target, multi-path approaches, and mild effects, as well as its pharmacological activities like anti-inflammatory, antioxidant, anti-fibrotic, and hepatoprotective effects, it holds significant promise in the treatment of liver cirrhosis. Recent studies have highlighted the substantial potential of TCM in modulating ALD, NAFLD, liver cirrhosis, and liver cancer, primarily through targeted regulation of gut microbiota (Li et al., 2019; Liu et al., 2022). Currently, extensive research has been conducted on natural products and compounds for managing liver cirrhosis by targeting gut microbiota, with notable contributions from high-throughput screening and multi-omics research. Despite numerous studies, including clinical trials, demonstrating the significant therapeutic efficacy of TCM in treating liver diseases, the precise mechanism of action remains incompletely understood. This article was aimed to introduce the natural products (Table 2) and herbal compound formulations (Table 3) identified in recent research that focus on regulating gut microbiota to intervene in cases of liver cirrhosis.

4.1 Natural products of TCM

4.1.1 Polyphenols

Resveratrol (RSV) is primarily derived from natural plants such as *Polygonum cuspidatum*, Cassia seed, and grapes (Zhang et al., 2021). RSV has various pharmacological activities, including anti-inflammatory, antioxidant, anti-lipogenic, and anti-tumor effects, which could be closely related to its capacity for modulating gut microbiota, thereby ameliorating liver cirrhosis (Kim et al., 2011; Gambini et al., 2015). Recently, Li et al. found that in a CCl4-induced liver fibrosis rat model, RSV reversed the reduced expression of ZO-1, Occludin, and other proteins and mRNA induced by CCl4, accelerating the repair of damaged intestinal mucosal barriers. Moreover, while *Staphylococcus xylosus* and *Staphylococcus lentus* accelerate BT and enhance intestinal permeability, RSV inhibits the excessive proliferation of *Staphylococcus lentus* by inhibiting its biofilm formation, leading to the regulation of intestinal flora imbalance, adjustment of gut microbiota balance, and substantial amelioration of liver fibrosis (Li et al., 2022).

4.1.2 Alkaloids

Berberine (BBR) a quaternary ammonium alkaloid derived from Rhizoma coptidis, has become a novel therapeutic approach in recent years (Song et al., 2020). It relies on its antibacterial, anti-inflammatory, anti-fibrotic, and anti-hyperlipidemic activities to target the regulation of gut microbiota in the treatment of liver diseases. BBR can enhance intestinal anaerobic bacteria to stimulate butyrate production, elevate the levels of beneficial bacteria (especially Clostridium) in the intestines, thereby reducing host liver inflammation, regulating lipid metabolism, and improving liver fibrosis. Additionally, BBR serves as an FXR and TGR5 agonist, pivotal in modulating the metabolic homeostasis of BAs, thereby intervening in liver cirrhosis by mediating the crosstalk between BAs metabolism and gut microbiota (Liu et al., 2022). However, studies have indicated that BBR itself can induce dysbiosis of the microbiota, leading to mild diarrhea in the host (Yue et al., 2019). Therefore, in future, special attention should be paid to the adverse reactions or intolerance of BBR in the drug development.

Corydalis saxicola Bunting (CSB) has significant therapeutic benefits in various liver diseases (Qin et al., 2023). Recently, Qin et al. demonstrated for the first time that palmitine (PAL) is the main active component of CSB and plays a pivotal role, particularly in conferring anti-fibrotic attributes. Further analysis using metagenomic sequencing methods revealed that the anti-fibrotic properties of PAL depended on the gut microbiota and could significantly reverse the decrease in the abundance of s_*Lactobacillus_reuteri*, s_*Lactobacillus_murinus* and s_*Lactobacillus_johnsonii* induced by CCL4. These three bacteria were directly negatively correlated with the degree of liver fibrosis. In addition, the levels of LPS, TNF-α, IL-6 and IL-1β in the liver of mice were reduced to varying degrees after PAL treatment, repair the intestinal barrier, and increase the immune response of intestinal epithelial cells against inflammatory stimuli, thereby elucidating the specific mechanism underlying PAL's hepatoprotective effects (Qin et al., 2023).

4.1.3 Terpenoids

Curcumol (CU), a sesquiterpenoid compound extracted from turmeric, has garnered attention for its crucial anti-inflammatory, anti-fibrotic, and immunomodulatory roles in liver and intestinal diseases (Liu et al., 2019; Yang et al., 2020). Recent CU can effectively alleviate liver cirrhosis (Zhang et al., 2024), alter the fecal microbiota composition in rats afflicted with liver fibrosis, reduce the abundance of Bacteroidetes, increase the abundance of Firmicutes and Proteobacteria, and inhibit the activity of TLR4/NF-κβ signaling pathway, leading to reduced expression of inflammatory factors (IL-6, TNF-α, IL-8) (Zheng et al., 2022). These findings suggest that CU has a potential as an alternative therapeutic drug targeting the liver and intestine.

Ginkgolide A (GA), a diterpenoid compound extracted from the roots and leaves of *Ginkgo biloba* trees, has been widely used in clinical practice to treat cardiovascular and neurological disorders (Wang et al., 2023). However, recent studies have found that GA has great potential in managing liver diseases (Ye et al., 2016; Jeong et al., 2017). Enterococcus and Bacteroides, but not Lactobacillus, were involved in GA regulation of intestinal flora imbalance in cirrhotic mice. It can also activate the progesterone X receptor (PXR), a potential therapeutic target for liver cirrhosis, thereby upregulating the expression of PXR in the liver and intestines. By improving the expression of TJPs such as ZO-1 and Occludin, inhibiting pathological translocation of intestinal bacteria, and regulating the inflammatory cytokine environment within the liver, GA plays an important role in maintaining the balance of the gut-liver axis during the treatment of liver cirrhosis (Mohandas and Vairappan, 2020a,b).

Glaucocalyxin A (GLA), a natural n-pentane diterpene isolated from the aerial parts of Rabdosia japonica (Chen et al., 2021), has recently been implicated in improve gut microbiota dysbiosis in a mouse model of liver fibrosis. GLA treatment could decrease the Bacteroidetes to Firmicutes ratio. At the class level, it decreased abundance of Clostridia and increased availability of Bacteroidia. At

TABLE 2 Summary of natural products targeting gut microbiota for the treatment of cirrhosis.

Type	Natural products	Research design	Regulatory strains	Possible mechanism	Reference
Polyphenolics	Resveratrol	CCl4 (0.5μL/g)-induced male mouse models were given RSV 30mg/kg daily for 4 weeks	Staphylococcus_lentus↓, Staphylococcus_xylosus↓	Enhance the expression of TJPs and intestinal barrier, reduce intestinal permeability and inhibit BT.	Li et al. (2022)
Alkaloids	Berbine		Firmicutes↑, especially *Clostridium scindens*↑	Maintain BA metabolic homeostasis, increase butyrate production to regulate lipid metabolism, relieve inflammatory response and immune disorders.	Liu et al. (2022)
	Palmatine	Based on its clinical dose design for humans (1.2g/60kg/d), male rat models induced by CCl4 (1ml/kg) were given PAL, high dose (108mg/kg/ d) and low dose (54mg/kg/ d) respectively.	s_lactobacillus_reuteri↑, s_lactobacillus_murinus↑, s_lactobacillus_johnsonii↑	Enhance the expression of ZO-1, Claudin-1 and Occludin, decrease the expression of LPS, TNF-α, IL-6 and IL-1β, enhance intestinal barrier, and reduce inflammatory response, hepatocyte steatosis and collagen fiber deposition.	Qin et al. (2023)
Terpenoids	Curcumol	CCl4(5 mL/kg)-induced male mouse models were given daily intragastric administration of CU30 mL/kg (1 mg/mL CU solution and anhydrous ethanol) for 6 weeks	Bacteroidetes↓, Firmicutes↑, Proteobacteria↑	Inhibition of TLR4/NF-κβ signaling pathway and decrease the expression of downstream inflammatory factors.	Zheng et al. (2022)
	Ginkgolide A	CCl4(0.5ml/kg)-induced male Swiss albino mouse models were given GA100 mg/kg daily for 2 weeks	enterococcus↓, Bacteroidaceae↓	Inhibition of BT, restore the expression of PXR and related genes in the gut-liver axis, reduce the expression of TLR4/MyD88/NF-κβ axis and TNF-α, and improve the expression of ZO-1 、Occuldin	Mohandas and Vairappan (2020a, 2020b)
	Glaucocalyxin A	CCl4(0.01mL/g)-induced male C57BL/6J mice were intraperitoneally injected with GLA daily at a high dose (5mg/kg/ day) and a low dose (10mg/kg/ day) for 6 weeks	Bacteroidetes/Firmicutes ↑, Clostridia↓, Erysipelotrichales↓, Lachnospiraceae↓, Bacteroidia↑, Muribaculaceae↑	Reduce the expression of inflammatory factors and alleviate liver fibrosis.	Qu et al. (2023)
	EFT+SMP	CCl4(20%, 0.1ml/10g)-induced male mouse models were given EFT+SMP mixture 0.2ml/20g daily for 6 weeks	Firmicutes↓, Acfinobacteria↑, Candida phylum↑, Tenericutes↑	Increase intestinal microbial diversity in mice, reduce the production of bacterial metabolites LPS, LysoPE and LysoPC, inhibit kupffer cell activation and liver inflammatory response,then exerts potential anti-fibrotic effects.	Tan et al. (2020)
	Artesunate	CCl4、intragastric administration of 10% ethanol and HFD induced male rats were gavaged with AS (25 mg/kg) daily.	*Lactobacillus*↑, *Eubacterium* ↑, *Clostridium*↑, *Desulfotomaculum*↓, *Desulfosporosinus*↓	Increase intestinal microbial diversity, regulate intestinal permeability, reduce BT, reduce intestinal mucosal barrier damage and inflammatory response.	Chen et al. (2016)
	Cichorium pumilum Jacq Extract	5%2,4,6-trinitrobenzenesulfonic acid (60mg/kg) and 50% ethanol induced male rats were gavaged with CGEA at a high dose (150 mg/kg/ d) and a low dose (100mg/kg/d) for 2 weeks	Ochrobactrum ↓, Bacteroidetes↓, Ruminococcus ↑, *Acinetobacter* ↑, Bifidobacterium↑, Firmicutes↑, Proteobacteria↑, Acidobacteria↑	Inhibit the activation of MAPK-Akt signaling pathway, inhibit the phosphorylation of Akt and MAPKs proteins and the expression of inflammatory factors such as NO and IL-6 induced by LPS, reduce inflammation and anti-fibrosis.	Han et al. (2021)

(Continued)

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TABLE 2 (Continued)

Type	Natural products	Research design	Regulatory strains	Possible mechanism	Reference
Glycosides	Forsythiaside A	CCl4 (2mL/kg)-induced male C57BL/6 mouse model was administered with FTA (10 mL/kg) by gavage for 4 weeks	Firmicutes/Bacteroidetes↓, Mucispirillum↓, Lactobacillus↓, prevotellaceae_ UCG-001↑, Ruminococcus_1↑, Mucispirillum↑, Lactobacillus↑	Increase TJP expression and prevent intestinal injury, reduce endotoxin and inflammatory factor levels, maintain BA metabolic homeostasis, and increase SCFAs content (especially butyrate).	Fu et al. (2022)
Carbohydrates	Sodium alginate	CCl4(10%, 5mL/kg)-induced SPF male C57BL/6 mice were intraperitoneally injected with SA at high dose (100 mg/kg) and low dose (50 mg/kg) for 4 weeks	Lactobacillus↑, Lachnospiraceae↑, Faecalibaculum↑, Marvinbryantia↑, Phascolarctobacterium↑, Oscillospiraceae↑, Monoglobus↓, Erysipelotrichaceae↓, Ileibacterium↓	Regulate intestinal flora, protect intestinal barrier, reduce inflammation level, reduce liver fibrosis and liver injury.	He et al. (2023)
	Lycium barbarum L. oligosaccharides	CCl4(10mL/kg)-induced male C6BL/2019J mouse were gavaged with LBO dissolved in normal saline (200mg/kg bw)	bacillus ↑, Tyzzerella↑, Fournierella↑, corobacteriaceae UCG-002↑, but could not fully restore the intestinal flora of mice to a healthy state	Enhance intestinal barrier function, reduce intestinal permeability and inflammatory response, and significantly alleviate liver fibrosis and mitochondrial metabolism disorders	Zhang et al. (2023)
	Dendrobium officinale polysaccharide	CCl4 (30%, 2mL/kg)-induced male rats were treated by gavage of DOP solution at high (800 mg/kg), middle (400 mg/kg) and low (200 mg/kg) dose twice a week for 8 weeks		The expression of Claudin-1, ZO-1, Bcl-2 and other TJP proteins was up-regulated, and the expression of Bax and caspase-3 proteins was down-regulated, which may benign regulate the intestinal mucosal barrier. At the same time, inhibition of LPS-TLR4-NF-κB signaling pathway, decreased the expression of TGF-β and TNF-α, increased the expression of IL-10, and decreased the expression of α-SMA and collagen I.	Wang et al. (2020)
	Garlic polysaccharide	ALF mouse model was established by gavage of alcohol (56%, 6 mL/kg) for 30 days and then treated with GP, high dose group (250 mg/kg), low dose group (150 mg/kg).	Lachnospiraceae↑, Lactobacillus↑, Facklamia↓, Firmicutes↓	Remodeled intestinal microecology, down-regulated the expression of TGF-β1 and TNF-α proteins, increased the content of decorin, inhibited the activation of HSCs, and reduced the production of ECM.	Wang et al. (2018)

CCl4, carbon tetrachloride; TJ, tight junction; BT, bacterial translocation; TLR, toll-like receptor; NF-κβ, nuclear factor kappa-betta; PAL, palmatine; ZO, zonula occludens; LPS, lipopolysaccharide; LysoPE, lysophosphatidylethanolamine; LysoPC, lysophosphatidylcholine;TNF, tumor necrosis factor; IL, interleukin; GA, ginkgolide A; PXR, Pregnane X receptor; MyD88, myeloid differentiation factor-88; GLA, glaucocalyxin A; EFT, *E. fukienensis* Hsu.; SMP, *S. miltiorrhiza* Bge; AS, artesunate; DOP, *Dendrobium officinale* polysaccharide; GP, garlic polysaccharide; CGEA, *Cichorium pumilum* jacq; MAPK, mitogen-activated protein kinases; FTA, forsythiaside A; SCFA, short-chain fatty acid; BA, bile acid; LBO, *Ycium barbarum L. oligosaccharides*.

10.3389/fmicb.2024.1407991

TABLE 3 Summary of Herbal compound prescriptions targeting gut microbiota for the treatment of cirrhosis.

Herbal compound prescriptions	Composition	Regulatory strains	Possible mechanism	Reference
BJJP	turtle shell, donkey-hide gelatin, nidus vespae, pillbug, ground beetle, dung beetle, saltpeter, bupleurum, scutellaria, pinellia, codonopsis, rhizoma zingiberis, magnolia officinalis, cassia twig, radix paeoniae alba, rhizoma belamcandae, peach kernel, cortex moutan, *rheum officinale*, trumpet creeper, semen lepidii, pyrrosia leaf, fringed pink	Bifidobacteria↑, Lactobacillus↑, Faecalibacterium↑, Blautia↑, *Escherichia coli*↓, Bacteroides↓, Ruminococcus↓, Parabacteroides↓, Prevotella↓	Increase carbohydrate and vitamin metabolite levels in mice, reduce intestinal permeability and inflammatory response, improve mitochondrial dysfunction and liver fibrosis	Chi et al. (2023)
DHZCP	rhubarb, soil locust, leech, locust, grub, peach kernel, true lacquertree dried lacquer, scutellaria baicalensis, radix paeoniae alba, amygdala amara, rehmannia, licorice	Firmicutes/Bacteroidetes↓,Prevotella↑,alloprevotella↑,phascolarctobacterium↑,muribaculaceae unclassified↑,lachnospiraceae unclassified↑,clostridiales unclassified↑,desulfovibrio↓, colidextribacter↓	Regulate microbial metabolites, enhance the damaged intestinal barrier, reduce BT, and reduce inflammatory response and liver fibrosis by inhibiting the TLR4/MyD88/NF-κβ pathway	He et al. (2024)
YCWLP	artemisia Capillaris Herba, Polyporus Umbellatus, Alismatis Rhizoma, Atractylodes Macrocephalae Rhizoma stir-fried with wheat bran, Poria, Cinnamomi Ramulus	Christensenella↑, Ruminococcus↑, Barnesiella↑, Bifidobacterium↑, Coprococcus↑, Anaerostipes↑,	Increase the production of butyrate, improve the metabolism of amino acid, sphingolipid and glucose in rats by regulating related microbial metabolites, and then reduce the content of ammonia in the body, regulate inflammation and immune response	Zhang et al. (2021a, 2021b)
GSG	Codonopsis radix, Radix buuri, Radix sinensis, Radix *salvia miltiorrhiza*, Atractylodes atractylodes, Polygonum cuspetatum, peach kernel, Fructus aurantii, Poria, dandelion, Prunella sinensis, Biejia, Radix paeoniae alba	Bacteroidetes/Firmicutes↑, Lactobacillus↑, Akkermansia↑, Bacteroides↑, Oscillospira↑, Acinetobacter↑, Allobaculum↓	Reduce intestinal permeability, reduce BT, and enhance the intestinal barrier	Zhao et al. (2021)
ZGHYD	Astragalus membranaceus, Turtle shell, Three Lengs, Zedoary turmeric, Chuanxiong, *Salvia miltiorrhiza*, Hedyotis diffusa, Scutellaria barbata	Eubacteriaceae↑, Lactococcus↑, Micrococcaceae↑, Rothia↑, Eubacteriaceae↑, Rothia↑, Acidaminococcus↑, Fusobacteriaceae↓, erysipelotricaceae↓	Promote SCFAs production, enhance intestinal barrier, inhibit inflammatory response, and alleviate liver injury and fibrosis	Wenlin et al. (2023)
QJ	Astragali radix, Angelicae sinensis radix, Trionycis carapax, Eupolyphaga steleophaga, Salviae miltiorrhizae radix et rhizoma, Carthami flos, Persicae semen, Sparganii rhizome, Curcumae rhizome, Glycyrrhizae radix et rhizome	Lactobacillaceae↑, rumen bacterium↑, Rikenellaceae↑, Limosilactobacillus Reuteri↑,*Escherichia coli*↓, Muribaculaceae↓, Prevotellaceae↓	Restore the structure of intestinal tissue, increase the expression levels of Claudin-1, Occludin and ZO-1, restore the TJ apparatus damaged by CCl4, and inhibit liver inflammation and liver fibrosis by repairing the intestinal epithelial barrier	Li et al. (2024)

BJJP, Biejiajian Pill; DHZCP, Dahuang Zhechong Pill; YCWLP, YinChenwuling Powder; GSG, Ganshuang granules; ZGHYD, Zhenggan Huayu Decoction; QJ, Qijia Rougan decoction; BT,bacterial translocation; TLR, toll-like receptor; MyD88, myeloid differentiation factor-88; NF-κβ, nuclear factor kappa-betta; SCFA, short-chain fatty acid.

10.3389/fmicb.2024.1407991

the order level, the abundance of Erysipelotrichales decreased, while that of Bacteroidia increased. At the family level, Lachnospiraceae became less abundant in the GLA group, whereas the presence of Muribaculaceae and Bacteroidaceae increased. GLA can also reduce the levels of inflammatory factors that are closely related to the gut microbiota, such as TNF-α, TGF-β, and ROS, thereby reducing the extent of liver fibrosis and addressing the imbalance of intestinal flora caused by liver diseases (Qu et al., 2023).

In addition, Yang et al. explored the synergistic effect of a blend of triterpenoids extracted from E. fukienensis Hsu. (EFT) and phenolic acids derived from the roots of *S. miltiorrhiza* Bge (SMP) on liver fibrosis. Their findings underscored a significant reduction in the Firmicutes/Bacteroidetes ratio in the EFT+SMP group compared to the model group, concomitant with a decrease in the levels of gut microbiota metabolites lysophosphatidylethanolamine (LysoPE) and lysophosphatidylcholine (LysoPC), both of which have pro-fibrotic effects, thereby inhibiting the activation of Kupffer cells. These findings suggest that the potential association between EFT+SMP and gut microbiota may serve as a mechanism for the management of liver fibrosis (Tan et al., 2020).

Artesunate (AS) is synthesized based on dihydroartemisinin, a derivative of artemisinin, and is mainly used for malaria treatment (Rueangweerayut et al., 2012). AS has beneficial therapeutic effects on liver diseases (Kong et al., 2019). Chen et al. found that AS intervention group exhibited a significant increase in intestinal microbial diversity (primarily *Lactobacillus*), compared to the cirrhosis model group, and resulted in a deceased incidence and quantity of BT, contributing to the protection of the intestinal barrier, reduction of inflammation, and mitigation of liver injury. These findings demonstrated that AS effectively reduced the prevalence of liver cirrhosis induced by variouspathogenic factors, such as CCl4, alcohol, and HFDs (Chen et al., 2016).

Previous studies have underscored the significant anti-liver fibrosis activity and "probiotic-like effect" of the ethyl acetate extract of *Cichorium pumilum* Jacq (CGEA) (Qin et al., 2014; Lee et al., 2015). Therefore, building upon the gut-liver axis concept, Chang et al. further explored the effects of CGEA on the gut microbiota and intestinal barrier and discovered that CGEA promoted the growth of *Bifidobacteria*, increased the abundance of *Ruminococcus*, and consequently improved the homeostasis of the gut-liver axis. It is essential to highlight that lactate, the main compound in CGEA, is significant for the anti-inflammatory process. By inhibiting the phosphorylation of mitogen-activated protein kinase (MAPK) and Akt signaling pathways triggered by LPS, CGEA can effectively suppress the expression of inflammatory factors such as IL-6 and NO, thereby alleviating the liver's inflammatory response (Han et al., 2021).

4.1.4 Carbohydrates

Sodium alginate (SA), a natural polysaccharide extracted from seaweed or Sargassum of brown algae post-iodine and mannitol extraction, has emerged as a potential drug for liver protection and regeneration promotion (Li et al., 2022; Xia et al., 2023). Chen et al. underscored that liver fibrosis resulted in reduced gut microbiota diversity in rats, elevated colonization of harmful bacteria (such as Erysipelotrichaceae, Helicobacter, and Monolobus), and compromised the intestinal barrier. SA intervention remedied these pathological changes and increase the abundance of beneficial bacteria (such as Lactobacillus, Lachnospiraceae, and Eubacteria), thus improving liver fibrosis and inflammatory responses by restoring the stability of gut microbiota (He et al., 2023).

Lycium barbarum L. oligosaccharides (LBO) are oligosaccharides derived from *Lycium barbarum*. CCl4-induced liver fibrosis in mice results in increased ROS expression and mitochondrial dysfunction (Bi et al., 2024). Zhang et al. conducted further research and discovered a potential association between the pathological changes observed in liver fibrosis and dysbiosis of the gut microbiota. LBO could effectively treat liver fibrosis, mainly due to the restoration of gut microbiota, significantly increasing the enrichment of commensal bacteria Bacillus, Tyzzerella, Fournierella and Coriobacteriaceae UCG-002, further improves metabolic disorders caused by bacterial dysbiosis, repairs intestinal mucosal damage, regulates colonic epithelial permeability, and enhances mitochondrial function (Zhang et al., 2023).

Dendrobium officinale polysaccharide (DOP) have been previously shown to have anti-inflammatory, anti-oxidative, immune, hypoglycemic and other pharmacological properties (Liu et al., 2021; Wang et al., 2022; Chen et al., 2023), and play a significant therapeutic effect on acetaminophen induced liver injury by targeting the intestinal mucosal barrier (Lin et al., 2018). Subsequently, Wang et al. further explored and found that the mechanism of DOP in maintaining intestinal balance involved regulating the expression levels of TJPs such as occludin, claudin-1, ZO-1 and caspase-3 proteins, inhibiting the activation of LPS-TLR4-NF-κB signaling pathway, and reducing the content of inflammatory factors TGF-β and TNF-α, increasing the expression of anti-inflammatory factor IL-10, which significantly reduces the expression of α-SMA and collagen I, thereby reducing hepatocyte apoptosis and liver fibrosis (Wang et al., 2020).

Garlic, which is commonly used as a food spice, is also one of the traditional Chinese medicines and can be widely used in the treatment of cancer, nervous system and various metabolic diseases (Zhang et al., 2020; Tedeschi et al., 2022). In an experimental study on alcoholic liver fibrosis (ALF), garlic polysaccharide (GP) reversed the negative effects of changes in intestinal dominant flora on ALF, enriched Lachnospiraceae and Lactobacillus again, and reduced the diversity of potential pathogenic bacteria (Firmicute and Facklamia). It also regulates the protein expression of TGF-β1 (a key pro-fibrotic factor), TNF-α, and decorin (an anti-fibrotic agent), inhibits HSCs activation and reduces extracellular matrix (ECM) accumulation, ultimately demonstrating its hepatoprotective activity (Wang et al., 2018).

4.1.5 Glycosides

FTA is a glycoside compound extracted from the dried fruit of *Forsythia suspensa* and serves as a natural hepatoprotective agent (Gong et al., 2023). Fu et al. found that FTA not only restored the disrupted structure of intestinal microbiota, specifically, Ace index increased significantly, Shannon index decreased significantly, Lachnospiraceae_NK4A136_group, Ruminococcaceae_UCG-014, Lactobacillus、Bacteroides, Prevotellaceae_UCG-001, Helicobacter and Alloprevotellabut were adjusted to benefit the health of the host, also promoted the production of SCFA (particularly butyric acid). Furthermore, FTA reduced the levels of inflammatory factors such as LPS and TNF-α in serum, upregulated the mRNA expression of ZO-1, Claudin-1, and Occludin in a dose-dependent manner, thereby

mitigating intestinal permeability. Meanwhile, FTA regulates the expression of genes related to BAs metabolism (such as FXR, CYP7A1.), thereby facilitating the communication between gut microbiota and the liver, underscoring its hepatoprotective and anti-fibrotic properties (Fu et al., 2022).

4.2 Herbal compound prescriptions

Limited research on the regulation of intestinal microbiota by TCM compounds for liver cirrhosis has been conducted. Biejiajian pill (BJJP), composed of 23 Chinese herbs (including turtle shell, donkey-hide gelatin, nidus vespae, pillbug, ground beetle, dung beetle, saltpeter, bupleurum, scutellaria, pinellia, codonopsis, rhizoma zingiberis, magnolia officinalis, cassia twig, radix paeoniae alba, rhizoma belamcandae, peach kernel, cortex moutan, *rheum officinale*, trumpet creeper, semen lepidii, pyrrosia leaf, fringed pink)., showed promising results in a prospective randomized controlled trial. After 48 weeks of BJJP treatment, patients with hepatitis B cirrhosis/liver fibrosis showed a significantly higher improvement rate of fibrosis compared to the model group. This improvement was associated with an increase in the abundance of beneficial bacteria (such as Bifidobacterium, Lactobacillus, and Faecalibacterium) and a decrease in the abundance of potential pathogenic bacteria (such as *Escherichia coli*, Bacteroidetes, Ruminococcus, and Parabacteroides), demonstrating the close relationship between microbial community composition, reduced inflammatory response, and improved liver fibrosis and injury (Chi et al., 2023).

Moreover, He et al. investigated Dahuang Zhechong pill (DHZCP, consisting of rhubarb, soil locust, leech, locust, grub, peach kernel, true lacquertree dried lacquer, scutellaria baicalensis, radix paeoniae alba, amygdala amara, rehmannia, and licorice), and found that it could maintain the normal Firmicutes and Bacteroidetes ratio in liver fibrosis rats. DHZCP increased the abundance of beneficial bacteria, especially those producing SCFA (such as Prevotella, Ruminococcus, and Clostridium), while reducing the abundance of harmful bacteria (Desulfovibrio, Colidextribacter) and the production of LPS. DHZCP also regulated the protein and mRNA expression levels of ZO-1, TLR4, and MyD88, significantly improved the intestinal barrier, and reduced BT and endotoxemia, underscoring the anti-liver fibrotic effects of DHZCP on gut microbiota (He et al., 2024).

YinChenWuling Powder (YCWLP) is a combination of artemisia Capillaris Herba, Polyporus Umbellatus, Alismatis Rhizoma, Atractylodes Macrocephalae Rhizoma stir-fried with wheat bran, Poria, and Cinnamomi Ramulus. It is worth noting that hippuric acid can serve as a biomarker of host metabolic health, and its production level is strongly correlated with gut microbiota activity (Wang et al., 2020). Zhang et al. suggested a decrease in hippuric acid levels in rats with CCl4-induced liver fibrosis and a significant increase after the treatment with YCWLP (Tedeschi et al., 2022). YCWLP could alleviate the severity of CCl4-induced liver fibrosis by reshaping the gut microbiotaby regulating bacterial genera (such as *Christensenella*, *Ruminococcus*, and *Barnesiella*) associated with liver fibrosis. Moreover, by increasing the production level of butyrate, YCWLP could regulate the microbial metabolites in rat feces. This, in turn, helped correct metabolic disorders of amino acids, sphingolipids, and glucose, thereby reducing the generation and accumulation of ammonia. These effects highlight the potential of YCWLP in maintaining its hepatoprotective, anti-inflammatory, and immunomodulatory effects (Zhang et al., 2021a).

Ganshuang granules (GSG), composed of Codonopsis radix, Radix buuri, Radix sinensis, Radix *salvia miltiorrhiza*, Atractylodes adactylies, Polygonum cuspetatum, peach kernel, Fructus aurantii, Poria, dandelion, Prunella sinensis, Biejia, and Radix paeoniae alba, have good therapeutic effects on liver fibrosis and cirrhosis (Brial et al., 2021; Zhang et al., 2021b). Zhao et al. further investigated its underlying mechanism by targeting intestinal flora and found that GSG could mitigate xidative stress, inflammatory response, and liver fibrosis in a CCl4-induced rat model. The potential mechanisms in treating liver fibrosis may be closely related to reshaping the homeostasis of gut microbiota and enhancing the intestinal barrier (Zhao et al., 2021).

Zhenggan Huayu decoction (ZGHYD) is a herbal formulation composed of *Astragalus membranaceus*, Turtle shell, Three Lengs, Zedoary turmeric, Chuanxiong, *Salvia miltiorrhiza*, *Hedyotis diffusa*, and *Scutellaria barbata*. Research has shown that ZGHYD has the potential to increase the abundance of beneficial bacteria, while it may also enrich certain pathogenic bacteria, specifically *Enterococcus*, *Rothia*, and *Leuconostoc*. *Rothia*, interestingly, was positively correlated with the levels of type III procollagen peptide (PIIINP) and spleen thickness. PIIINP levels and spleen thickness, which are indicators of liver damage, showed a positive correlation with the presence of these pathogens. Enterococcus is a gram-negative bacterium whose abundance is positively correlated with LPS, serum IL-6, IL-1, and other inflammatory cytokines. These findings suggest that ZGHYD may not completely correct the dysregulated intestinal flora, but adjust the composition of certain pathogenic and beneficial bacteria. Nevertheless, ZGHYD demonstrated a notable positive effect in reversing liver fibrosis, surpassing the efficacy of Entecavir as a standalone treatment (Wenlin et al., 2023).

Recent studies have confirmed that Qijia Rougan decoction, composed of Astragali radix, Angelicae sinensis radix, Trionycis carapax, Eupolyphaga steleophaga, Salviae miltiorrhizae radix et rhizoma, Carthami flos, Persicae semen, Sparganii rhizome, Curcumae rhizome, and Glycyrrhizae radix et rhizome, has the effect of alleviating liver inflammation and anti-fibrosis. It was further found that the mechanism involved in targeting 10 species of bacteria and 37 metabolites in the intestinal tract (the most critical bacteria were Turicibacter, Faecalibaculum, Prevotellaceae UCG 001 and Peptococcaceae). After QJ, the intestinal tissue structure of mice returned to normal, the expression levels of Claudin-1, Occludin and ZO-1 increased, the TJ apparatus damaged by CCl4 was restored, and liver inflammation and liver fibrosis were inhibited by repairing the intestinal epithelial barrier (Li et al., 2024). Targeting enterohepatic circulation has become a potential drug for the treatment of liver fibrosis/cirrhosis.

4.3 Strengths and limitations

A growing body of research underscores the significant therapeutic impact of TCM on gut microbiota and liver cirrhosis, driving ongoing exploration into new Chinese herbal remedies. However, numerous herbal medicines face challenges in clinical application due to their limited bioavailability. It has been observed that a favorable gut microenvironment can significantly influence the

metabolism of active ingredients in TCM, thereby enhancing their bioavailability (Gong et al., 2020).

Despite the rapid expansion of research on TCM interventions targeting gut microbiota in liver cirrhosis, we still face many problems that need to be solved in the future. Firstly, there is a scarcity of clinical studies investigating the effects of TCM on gut microbiota, with most studies conducted in animal models or *in vitro* settings. Moreover, it is possible that other herbal extracts may possess similar bioactivity in the treatment of liver fibrosis, and these potential effects may still be undiscovered. Consequently, the next step should involve conducting extensive clinical research to explore the therapeutic potential of alternative medicines through clinical research. Secondly, the quality of existing literature varies, and not all TCM treatments have demonstrated beneficial effects on gut microbiota and liver health, as evidenced by the example of ZGHYD discussed earlier. Therefore, it is necessary to further conduct high-quality research to explore the efficacy, safe dosage, and adverse effects of TCM on the human body.

5 Conclusion

In conclusion, regardless of physiological or pathological conditions, there exists a close relationship between gut microbiota and liver cirrhosis. It is noteworthy that patients with liver cirrhosis experience significant pathological changes in the abundance of gut microbiota, intestinal barrier function, BAs metabolism, and microbial metabolites. In addition, there are varying degrees of liver and systemic inflammatory responses, liver fibrosis, and metabolic dysregulation. However, regulating the imbalanced gut microbiota has shown promising effects in reversing these pathological changes observed in both the gut and liver associated with cirrhosis. Thus, the gut microbiota emerges as a pivotal factor in liver cirrhosis.

Recent studies have shed light on the efficacy of TCM in targeting gut microbiota to treat liver cirrhosis. This review summarized the specific mechanisms involved in modulating gut microbiota for the treatment of liver cirrhosis using TCM. Various compounds, including polyphenols, alkaloids, terpenoids, and glycosides, have been investigated. These TCM components impact liver cirrhosis through four primary mechanisms: (1) regulating gut microbiota composition and abundance; (2) Enhancing the intestinal barrier, reducing intestinal permeability, and safeguarding against the entry of PAMPs into the portal circulation; (3) modulating genes involved in BA metabolism and activating the FXR receptor to maintain the metabolic homeostasis of BAs; (4) influencing the production and expression of key metabolites (such as LPS and SCFA) by modulating microbe-derived metabolites, thereby mitigating damage through the portal system.

While research in this field is still in its early stages, the therapeutic potential of TCM targeting gut microbiota modulation in liver cirrhosis is undeniable. Given its multifaceted composition and ability to address multiple factors, TCM has the capacity to slow down the progression of liver cirrhosis and inspire novel ideas in drug development. Thus, it represents a significant milestone in gut microbiota-based liver cirrhosis research.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Albillos, A., De Gottardi, A., and Rescigno, M. (2020). The gut-liver axis in liver disease: pathophysiological basis for therapy. *J. Hepatol.* 72, 558–577. doi: 10.1016/j.jhep.2019.10.003

Apostolopoulos, V., Stojanovska, L., and Gargosky, S. E. (2015). Muc1 (Cd227): a multi-tasked molecule. *Cell. Mol. Life Sci.* 72, 4475–4500. doi: 10.1007/s00018-015-2014-z

Arias, N., Arboleya, S., Allison, J., Kaliszewska, A., Higarza, S. G., Gueimonde, M., et al. (2020). The relationship between choline bioavailability from diet, intestinal microbiota composition, and its modulation of human diseases. *Nutrients* 12:2340. doi: 10.3390/nu12082340

Assimakopoulos, S. F., Gogos, C., and Labropoulou-Karatza, C. (2011). Could antioxidants be the "magic pill" for cirrhosis-related complications? A pathophysiological appraisal. *Med. Hypotheses* 77, 419–423. doi: 10.1016/j.mehy.2011.05.034

Assimakopoulos, S. F., Tsamandas, A. C., Tsiaoussis, G. I., Karatza, E., Triantos, C., Vagianos, C. E., et al. (2012). Altered intestinal tight junctions' expression in patients with liver cirrhosis: a pathogenetic mechanism of intestinal hyperpermeability. *Eur. J. Clin. Investig.* 42, 439–446. doi: 10.1111/j.1365-2362.2011.02609.x

Augustyn, M., Grys, I., and Kukla, M. (2019). Small intestinal bacterial overgrowth and nonalcoholic fatty liver disease. *Clin. Exp. Hepatol.* 5, 1–10. doi: 10.5114/ceh.2019.83151

Bai, L., Li, X., He, L., Zheng, Y., Lu, H., Li, J., et al. (2019). Antidiabetic potential of flavonoids from traditional Chinese medicine: A review. *Am. J. Chin. Med.* 47, 933–957. doi: 10.1142/S0192415X19500496

Bajaj, J. S. (2014). The role of microbiota in hepatic encephalopathy. *Gut Microbes* 5, 397–403. doi: 10.4161/gmic.28684

Bajaj, J. S. (2019). Altered microbiota in cirrhosis and its relationship to the development of infection. *Clin. Liver Dis.* 14, 107–111. doi: 10.1002/cld.827

Bajaj, J. S., Betrapally, N. S., and Gillevet, P. M. (2015). Decompensated cirrhosis and microbiome interpretation. *Nature* 525, E1–E2. doi: 10.1038/nature14851

Bajaj, J. S., Heuman, D. M., Hylemon, P. B., Sanyal, A. J., White, M. B., Monteith, P., et al. (2014). The cirrhosis Dysbiosis ratio defines changes in the gut microbiome associated with cirrhosis and its complications. *J. Hepatol.* 60, 940–947. doi: 10.1016/j.jhep.2013.12.019

Bajaj, J. S., Peña-Rodriguez, M., Sikaroodi, M., et al. (2023). A longitudinal trans-kingdom gut microbial approach towards decompensation in outpatients with cirrhosis. *Gut* 72, 759–771. doi: 10.1136/gutjnl-2022-328403

Bajaj, J. S., Ridlon, J. M., Hylemon, P. B., Thacker, L. R., Heuman, D. M., Smith, S., et al. (2012). Linkage of gut microbiome with cognition in hepatic encephalopathy. *Am. J. Physiol. Gastrointest. Liver Physiol.* 302, G168–G175. doi: 10.1152/ajpgi.00190.2011

Bala, L., Ghoshal, U. C., Ghoshal, U., Tripathi, P., Misra, A., Gowda, G. A. N., et al. (2006). Malabsorption syndrome with and without small intestinal bacterial overgrowth: A study on upper-gut aspirate using 1H Nmr spectroscopy. *Magn. Reson. Med.* 56, 738–744. doi: 10.1002/mrm.21041

Baltazar-Díaz, T. A., González-Hernández, L. A., Aldana-Ledesma, J. M., Peña-Rodríguez, M., Vega-Magaña, A. N., Zepeda-Morales, A. S. M., et al. (2022). Escherichia/Shigella, Scfas, and metabolic pathways-the triad that orchestrates intestinal Dysbiosis in patients with decompensated alcoholic cirrhosis from Western Mexico. *Microorganisms* 10:1231. doi: 10.3390/microorganisms10061231

Bansal, T., Alaniz, R. C., Wood, T. K., and Jayaraman, A. (2010). The bacterial signal indole increases epithelial-cell tight-junction resistance and attenuates indicators of inflammation. *Proc. Natl. Acad. Sci. USA* 107, 228–233. doi: 10.1073/pnas.0906112107

Bi, Y., Liu, S., Qin, X., Abudureyimu, M., Wang, L., Zou, R., et al. (2024). Fundc1 interacts with Gpx4 to govern hepatic ferroptosis and fibrotic injury through a mitophagy-dependent manner. *J. Adv. Res.* 55, 45–60. doi: 10.1016/j.jare.2023.02.012

Brial, F., Chilloux, J., Nielsen, T., Vieira-Silva, S., Falony, G., Andrikopoulos, P., et al. (2021). Human and preclinical studies of the host-gut microbiome co-metabolite hippurate as a marker and mediator of metabolic health. *Gut* 70, 2105–2114. doi: 10.1136/gutjnl-2020-323314

Cao, X., Zolnikova, O., Maslennikov, R., Reshetova, M., Poluektova, E., Bogacheva, A., et al. (2023). Differences in fecal short-chain fatty acids between alcoholic fatty liver-induced cirrhosis and non-alcoholic (metabolic-associated) fatty liver-induced cirrhosis. *Meta* 13:859. doi: 10.3390/metabo13070859

Ceccarelli, S., Nobili, V., and Alisi, A. (2014). Toll-like receptor-mediated signaling cascade as a regulator of the inflammation network during alcoholic liver disease. *World J. Gastroenterol.* 20, 16443–16451. doi: 10.3748/wjg.v20.i44.16443

Chambers, E. S., Viardot, A., Psichas, A., Morrison, D. J., Murphy, K. G., Zac-Varghese, S. E., et al. (2015). Effects of targeted delivery of propionate to the human colon on appetite regulation, body weight maintenance and adiposity in overweight adults. *Gut* 64, 1744–1754. doi: 10.1136/gutjnl-2014-307913

Chen, L., He, X., Wang, H., Fang, J., Zhang, Z., Zhu, X., et al. (2023). *Dendrobium officinale* polysaccharide prevents neuronal apoptosis via Tet2-dependent Dna demethylation in high-fat diet-induced diabetic mice. *Int. J. Biol. Macromol.* 233:123288. doi: 10.1016/j.ijbiomac.2023.123288

Chen, Y., Ji, F., Guo, J., Shi, D., Fang, D., and Li, L. (2016). Dysbiosis of small intestinal microbiota in liver cirrhosis and its association with etiology. *Sci. Rep.* 6:34055. doi: 10.1038/srep34055

Chen, Y. X., Lai, L. N., Zhang, H. Y., Bi, Y. H., Meng, L., Li, X. J., et al. (2016). Effect of artesunate supplementation on bacterial translocation and dysbiosis of gut microbiota in rats with liver cirrhosis. *World J. Gastroenterol.* 22, 2949–2959. doi: 10.3748/wjg.v22.i10.2949

Chen, Y., Qin, N., Guo, J., Qian, G., Fang, D., Shi, D., et al. (2014). Functional gene arrays-based analysis of fecal microbiomes in patients with liver cirrhosis. *BMC Genomics* 15:753. doi: 10.1186/1471-2164-15-753

Chen, P., Stärkel, P., Turner, J. R., et al. (2015). Dysbiosis-induced intestinal inflammation activates Tnfri and mediates alcoholic liver disease in mice. *Hepatology (Baltimore, Md.)* 61, 883–894. doi: 10.1002/hep.27489

Chen, Y., Yang, F., Lu, H., Wang, B., Chen, Y., Lei, D., et al. (2011). Characterization of fecal microbial communities in patients with liver cirrhosis. *Hepatology (Baltimore, Md.)* 54, 562–572. doi: 10.1002/hep.24423

Chen, J., Zhang, W., Pan, C., Fan, J., Zhong, X., and Tang, S. (2021). Glaucocalyxin A induces cell cycle arrest and apoptosis via inhibiting Nf-κB/p65 signaling pathway in melanoma cells. *Life Sci.* 271:119185. doi: 10.1016/j.lfs.2021.119185

Cheon, S. Y., Kim, M. Y., Kim, J., Kim, E. J., Kam, E. H., Cho, I., et al. (2023). Hyperammonemia induces microglial Nlrp3 inflammasome activation via mitochondrial oxidative stress in hepatic encephalopathy. *Biom. J.* 46:100593. doi: 10.1016/j.bj.2023.04.001

Chi, X., Cheng, D. Y., Sun, X., Liu, S. A., Wang, R. B., Chen, Q., et al. (2023). Efficacy of Biejiajian pill on intestinal microbiota in patients with hepatitis B cirrhosis/liver fibrosis: A randomized double-blind controlled trial. *Chin. J. Integr. Med.* 29, 771–781. doi: 10.1007/s11655-023-3542-2

Chopyk, D. M., and Grakoui, A. (2020). Contribution of the intestinal microbiome and gut barrier to hepatic disorders. *Gastroenterology* 159, 849–863. doi: 10.1053/j.gastro.2020.04.077

Clarembeau, F., Bale, G., and Lanthier, N. (2020). Cirrhosis and insulin resistance: current knowledge, pathophysiological mechanisms, complications and potential treatments. *Clin. Sci.* 134, 2117–2135. doi: 10.1042/Cs20200022

Collins, S. L., Stine, J. G., Bisanz, J. E., Okafor, C. D., and Patterson, A. D. (2023). Bile acids and the gut microbiota: metabolic interactions and impacts on disease. *Nat. Rev. Microbiol.* 21, 236–247. doi: 10.1038/s41579-022-00805-x

Constantinou, P. E., Danysh, B. P., Dharmaraj, N., and Carson, D. D. (2011). Transmembrane mucins as novel therapeutic targets. *Expert Rev. Endocrinol. Metab.* 6, 835–848. doi: 10.1586/eem.11.70

Den Besten, G., Lange, K., Havinga, R., et al. (2013). Gut-derived short-chain fatty acids are vividly assimilated into host carbohydrates and lipids. *Am. J. Physiol. Gastrointest. Liver Physiol.* 305, G900–G910. doi: 10.1152/ajpgi.00265.2013

Devarbhavi, H., Asrani, S. K., Arab, J. P., Nartey, Y. A., Pose, E., and Kamath, P. S. (2023). Global burden of liver disease: 2023 update. *J. Hepatol.* 79, 516–537. doi: 10.1016/j.jhep.2023.03.017

Di Lorenzo, F., De Castro, C., Silipo, A., et al. (2019). Lipopolysaccharide structures of gram-negative populations in the gut microbiota and effects on host interactions. *FEMS Microbiol. Rev.* 43, 257–272. doi: 10.1093/femsre/fuz002

Ding, L., Yang, L., Wang, Z., and Huang, W. (2015). Bile acid nuclear receptor Fxr and digestive system diseases. *Acta Pharm. Sin. B* 5, 135–144. doi: 10.1016/j.apsb.2015.01.004

Dubinkina, V. B., Tyakht, A. V., Odintsova, V. Y., Yarygin, K. S., Kovarsky, B. A., Pavlenko, A. V., et al. (2017). Links of gut microbiota composition with alcohol dependence syndrome and alcoholic liver disease. *Microbiome* 5:141. doi: 10.1186/s40168-017-0359-2

Efremova, I., Maslennikov, R., Poluektova, E., Vasilieva, E., Zharikov, Y., Suslov, A., et al. (2023). Epidemiology of small intestinal bacterial overgrowth. *World J. Gastroenterol.* 29, 3400–3421. doi: 10.3748/wjg.v29.i22.3400

Fang, J., Yu, C. H., Li, X. J., Yao, J. M., Fang, Z. Y., Yoon, S. H., et al. (2022). Gut dysbiosis in nonalcoholic fatty liver disease: pathogenesis, diagnosis, and therapeutic implications. *Front. Cell. Infect. Microbiol.* 12:997018. doi: 10.3389/fcimb.2022.997018

Feng, Y., Wang, Y., Wang, P., Huang, Y., and Wang, F. (2018). Short-chain fatty acids manifest Stimulative and protective effects on intestinal barrier function through the inhibition of Nlrp3 Inflammasome and autophagy. *Cell. Physiol. Biochem.* 49, 190–205. doi: 10.1159/000492853

Foley, M. H., Walker, M. E., Stewart, A. K., O'Flaherty, S., Gentry, E. C., Patel, S., et al. (2023). Bile salt hydrolases shape the bile acid landscape and restrict Clostridioides difficile growth in the murine gut. *Nat. Microbiol.* 8, 611–628. doi: 10.1038/s41564-023-01337-7

Fu, K., Ma, C., Wang, C., Zhou, H., Gong, L., Zhang, Y., et al. (2022). Forsythiaside A alleviated carbon tetrachloride-induced liver fibrosis by modulating gut microbiota composition to increase short-chain fatty acids and restoring bile acids metabolism disorder. *Biomed. Pharmacother.* 151:113185. doi: 10.1016/j.biopha.2022.113185

Fuke, N., Nagata, N., Suganuma, H., and Ota, T. (2019). Regulation of gut microbiota and metabolic Endotoxemia with dietary factors. *Nutrients* 11:2277. doi: 10.3390/nu11102277

Gambini, J., Inglés, M., Olaso, G., Lopez-Grueso, R., Bonet-Costa, V., Gimeno-Mallench, L., et al. (2015). Properties of resveratrol: in vitro and in vivo studies about metabolism, bioavailability, and biological effects in animal models and humans. *Oxidative Med. Cell. Longev.* 2015:837042. doi: 10.1155/2015/837042

Giannelli, V., Di Gregorio, V., Iebba, V., et al. (2014). Microbiota and the gut-liver axis: bacterial translocation, inflammation and infection in cirrhosis. *World J. Gastroenterol.* 20, 16795–16810. doi: 10.3748/wjg.v20.i45.16795

Ginès, P., Krag, A., Abraldes, J. G., Solà, E., Fabrellas, N., and Kamath, P. S. (2021). Liver cirrhosis. *Lancet (London, England)* 398, 1359–1376. doi: 10.1016/S0140-6736(21)01374-X

Giuffrè, M., Campigotto, M., Campisciano, G., Comar, M., and Crocè, L. S. (2020). A story of liver and gut microbes: how does the intestinal flora affect liver disease? A review of the literature. American journal of physiology-gastrointestinal and liver. *Physiology* 318, G889–G906. doi: 10.1152/ajpgi.00161.2019

Gola, A., Dorrington, M. G., Speranza, E., Sala, C., Shih, R. M., Radtke, A. J., et al. (2021). Commensal-driven immune zonation of the liver promotes host defence. *Nature* 589, 131–136. doi: 10.1038/s41586-020-2977-2

Gong, X., Li, X., Bo, A., Shi, R. Y., Li, Q. Y., Lei, L. J., et al. (2020). The interactions between gut microbiota and bioactive ingredients of traditional Chinese medicines: A review. *Pharmacol. Res.* 157:104824. doi: 10.1016/j.phrs.2020.104824

Gong, L., Zhou, H., Zhang, S., Wang, C., Fu, K., Ma, C., et al. (2023). Cd44-targeting drug delivery system of exosomes loading Forsythiaside A combats liver fibrosis via regulating Nlrp3-mediated Pyroptosis. *Adv. Healthc. Mater.* 12:e2202228. doi: 10.1002/adhm.202202228

Groschwitz, K. R., and Hogan, S. P. (2009). Intestinal barrier function: molecular regulation and disease pathogenesis. *J. Allergy Clin. Immunol.* 124, 3–20. doi: 10.1016/j.jaci.2009.05.038

Guan, H., Zhang, X., Kuang, M., and Yu, J. (2022). The gut–liver axis in immune remodeling of hepatic cirrhosis. *Front. Immunol.* 13:946628. doi: 10.3389/fimmu.2022.946628

Guzior, D. V., and Quinn, R. A. (2021). Review: microbial transformations of human bile acids. *Microbiome* 9:140. doi: 10.1186/s40168-021-01101-1

Han, C. Y., Rho, H. S., Kim, A., Kim, T. H., Jang, K., Jun, D. W., et al. (2018). Fxr inhibits endoplasmic reticulum stress-induced Nlrp3 Inflammasome in hepatocytes and ameliorates liver injury. *Cell Rep.* 24, 2985–2999. doi: 10.1016/j.celrep.2018.07.068

Han, C., Wu, X., Zou, N., Zhang, Y., Yuan, J., Gao, Y., et al. (2021). Cichorium pumilum Jacq extract inhibits Lps-induced inflammation via Mapk signaling pathway

and protects rats from hepatic fibrosis caused by abnormalities in the gut-liver Axis. *Front. Pharmacol.* 12:683613. doi: 10.3389/fphar.2021.683613

Hartmann, P., Hochrath, K., Horvath, A., et al. (2018). Modulation of the intestinal bile acid–Fxr–Fgf15 axis improves alcoholic liver disease in mice. *Hepatology (Baltimore, Md.)* 67, 2150–2166. doi: 10.1002/hep.29676

He, X., Liang, J., Li, X., Wang, Y., Zhang, X., Chen, D., et al. (2024). Dahuang zhechong pill ameliorates hepatic fibrosis by regulating gut microbiota and metabolites. *J. Ethnopharmacol.* 321:117402. doi: 10.1016/j.jep.2023.117402

He, C., Wang, W., Wei, G., Wang, Y., Wei, Y., Wang, J., et al. (2023). Sodium alginate combined with oxymatrine ameliorates Ccl4-induced chemical hepatic fibrosis in mice. *Int. Immunopharmacol.* 125:111144. doi: 10.1016/j.intimp.2023.111144

Hooper, L. V., Littman, D. R., and Macpherson, A. J. (2012). Interactions between the microbiota and the immune system. *Science* 336, 1268–1273. doi: 10.1126/science.1223490

Hooper, L. V., and Macpherson, A. J. (2010). Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nat. Rev. Immunol.* 10, 159–169. doi: 10.1038/nri2710

Horvath, A., Rainer, F., Bashir, M., Leber, B., Schmerboeck, B., Klymiuk, I., et al. (2019). Biomarkers for oralization during long-term proton pump inhibitor therapy predict survival in cirrhosis. *Sci. Rep.* 9, 1–9. doi: 10.1038/s41598-019-48352-5

Hossain, Z., and Hirata, T. (2008). Molecular mechanism of intestinal permeability: interaction at tight junctions. *Mol. BioSyst.* 4, 1181–1185. doi: 10.1039/b800402a

Hsu, C. L., and Schnabl, B. (2023). The gut–liver axis and gut microbiota in health and liver disease. *Nat. Rev. Microbiol.* 21, 719–733. doi: 10.1038/s41579-023-00904-3

Hu, N., Liu, J., Xue, X., et al. (2020). The effect of emodin on liver disease -- comprehensive advances in molecular mechanisms. *Eur. J. Pharmacol.* 882:173269. doi: 10.1016/j.ejphar.2020.173269

Huang, D. Q., Terrault, N. A., Tacke, F., Gluud, L. L., Arrese, M., Bugianesi, E., et al. (2023). Global epidemiology of cirrhosis — aetiology, trends and predictions. *Nat. Rev. Gastroenterol. Hepatol.* 20, 388–398. doi: 10.1038/s41575-023-00759-2

Iredale, J. P. (2007). Models of liver fibrosis: exploring the dynamic nature of inflammation and repair in a solid organ. *J. Clin. Invest.* 117, 539–548. doi: 10.1172/Jci30542

Jeong, H. S., Kim, K. H., Lee, I. S., Park, J. Y., Kim, Y., Kim, K. S., et al. (2017). Ginkgolide A ameliorates non-alcoholic fatty liver diseases on high fat diet mice. *Biomed. Pharmacother.* 88, 625–634. doi: 10.1016/j.biopha.2017.01.114

Jin, M., Kalainy, S., Baskota, N., Chiang, D., Deehan, E. C., McDougall, C., et al. (2019). Faecal microbiota from patients with cirrhosis has a low capacity to ferment non-digestible carbohydrates into short-chain fatty acids. *Liver Int.* 39, 1437–1447. doi: 10.1111/liv.14106

Johansson, M. E. V., Phillipson, M., Petersson, J., Velcich, A., Holm, L., and Hansson, G. C. (2008). The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc. Natl. Acad. Sci. U. S. A.* 105, 15064–15069. doi: 10.1073/pnas.0803124105

Kaji, K., and Yoshiji, H. (2020). Can portal hypertension and hepatic decompensation be predicted? *J. Gastroenterol.* 55, 662–663. doi: 10.1007/s00535-020-01669-9

Kelly, D., and Conway, S. (2005). Bacterial modulation of mucosal innate immunity. *Mol. Immunol.* 42, 895–901. doi: 10.1016/j.molimm.2004.12.003

Kim, S., Jin, Y., Choi, Y., and Park, T. (2011). Resveratrol exerts anti-obesity effects via mechanisms involving down-regulation of adipogenic and inflammatory processes in mice. *Biochem. Pharmacol.* 81, 1343–1351. doi: 10.1016/j.bcp.2011.03.012

Koh, A., De Vadder, F., Kovatcheva-Datchary, P., et al. (2016). From dietary Fiber to host physiology: short-chain fatty acids as key bacterial metabolites. *Cell* 165, 1332–1345. doi: 10.1016/j.cell.2016.05.041

Koliada, A., Syzenko, G., Moseiko, V., Budovska, L., Puchkov, K., Perederiy, V., et al. (2017). Association between body mass index and Firmicutes/Bacteroidetes ratio in an adult Ukrainian population. *BMC Microbiol.* 17:120. doi: 10.1186/s12866-017-1027-1

Kong, Z., Liu, R., and Cheng, Y. (2019). Artesunate alleviates liver fibrosis by regulating ferroptosis signaling pathway. *Biomed. Pharmacother.* 109, 2043–2053. doi: 10.1016/j.biopha.2018.11.030

Kulkarni, A. V., Avadhanam, M., Karandikar, P., Rakam, K., Gupta, A., Simhadri, V., et al. (2024). Antibiotics with or without Rifaximin for acute hepatic encephalopathy in critically ill patients with cirrhosis: A double-blind, randomized controlled (AriE) trial. *Off. J. Am. Coll. Gastroenterol* 119, 864–874. doi: 10.14309/ajg.0000000000002575

Kwon, H. J., Won, Y. S., Park, O., et al. (2014). Aldehyde dehydrogenase 2 deficiency ameliorates alcoholic fatty liver but worsens liver inflammation and fibrosis in mice. *Hepatology (Baltimore, Md.)* 60, 146–157. doi: 10.1002/hep.27036

Lankelma, J. M., van Vught, L. A., Belzer, C., Schultz, M. J., van der Poll, T., de Vos, W. M., et al. (2017). Critically ill patients demonstrate large interpersonal variation in intestinal microbiota dysregulation: a pilot study. *Intensive Care Med.* 43, 59–68. doi: 10.1007/s00134-016-4613-z

Lee, J. W., Cowley, E. S., Wolf, P. G., Doden, H. L., Murai, T., Caicedo, K. Y. O., et al. (2022). Formation of secondary Allo-bile acids by novel enzymes from gut Firmicutes. *Gut Microbes* 14:2132903. doi: 10.1080/19490976.2022.2132903

Lee, S. M., Han, H. W., and Yim, S. Y. (2015). Beneficial effects of soy milk and fiber on high cholesterol diet-induced alteration of gut microbiota and inflammatory gene expression in rats. *Food Funct.* 6, 492–500. doi: 10.1039/c4fo00731j

Lee, J. H., Wood, T. K., and Lee, J. (2015). Roles of indole as an interspecies and interkingdom signaling molecule. *Trends Microbiol.* 23, 707–718. doi: 10.1016/j.tim.2015.08.001

Lee, G., You, H. J., Bajaj, J. S., Joo, S. K., Yu, J., Park, S., et al. (2020). Distinct signatures of gut microbiome and metabolites associated with significant fibrosis in non-obese Nafld. *Nat. Commun.* 11:4982. doi: 10.1038/s41467-020-18754-5

Ley, R. E., Bäckhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D., and Gordon, J. I. (2005). Obesity alters gut microbial ecology. *Proc. Natl. Acad. Sci.* 102, 11070–11075. doi: 10.1073/pnas.0504978102

Li, T., and Chiang, J. Y. L. (2014). Bile acid signaling in metabolic disease and drug therapy. *Pharmacol. Rev.* 66, 948–983. doi: 10.1124/pr.113.008201

Li, Z., Dong, J., Wang, M., et al. (2022). Resveratrol ameliorates liver fibrosis induced by nonpathogenic Staphylococcus in Balb/c mice through inhibiting its growth. *Mol. Med. (Cambridge, Mass.)* 28:52. doi: 10.1186/s10020-022-00463-y

Li, Y., Huang, J., Zhang, S., Yang, F., Zhou, H., Song, Y., et al. (2022). Sodium alginate and galactooligosaccharides ameliorate metabolic disorders and alter the composition of the gut microbiota in mice with high-fat diet-induced obesity. *Int. J. Biol. Macromol.* 215, 113–122. doi: 10.1016/j.ijbiomac.2022.06.073

Li, T., Wan, M., Qing, C., Guan, X., Pi, J., Lv, H., et al. (2023). Lung protection of Chimonanthus nitens Oliv. Essential oil driven by the control of intestinal disorders and dysbiosis through gut-lung crosstalk. *Life Sci.* 333:122156. doi: 10.1016/j.lfs.2023.122156

Li, N., Wang, B., Wu, Y., Luo, X., Chen, Z., Sang, C., et al. (2019). Modification effects of SanWei GanJiang powder on liver and intestinal damage through reversing bile acid homeostasis. *Biomed. Pharmacother.* 116:109044. doi: 10.1016/j.biopha.2019.109044

Li, X., Wang, C., Zhu, J., Lin, Q., Yu, M., Wen, J., et al. (2022). Sodium butyrate ameliorates oxidative stress-induced intestinal epithelium barrier injury and mitochondrial damage through Ampk-Mitophagy pathway. *Oxidative Med. Cell. Longev.* 2022:3745135. doi: 10.1155/2022/3745135

Li, X., Xu, X., Li, Y., Chen, W., Fang, Q., and Chen, Y. (2024). Serum metabolomic profiling of patients with liver cirrhosis at different stages. *Cell. Mol. Biol.* 70, 68–76. doi: 10.14715/cmb/2024.70.4.11

Li, X., Xu, X., Tao, S., Su, Y., Wen, L., Wang, D., et al. (2024). Gut microbes combined with metabolomics reveal the protective effects of Qijia Rougan decoction against Ccl4-induced hepatic fibrosis. *Front. Pharmacol.* 15:1347120. doi: 10.3389/fphar.2024.1347120

Li, H., Zheng, J., Xu, Q., Yang, Y., Zhou, J., Guo, X., et al. (2023). Hepatocyte adenosine kinase promotes excessive fat deposition and liver inflammation. *Gastroenterology* 164, 134–146. doi: 10.1053/j.gastro.2022.09.027

Lieber, C. S. (1999). Role of S-adenosyl-L-methionine in the treatment of liver diseases. *J. Hepatol.* 30, 1155–1159. doi: 10.1016/s0168-8278(99)80274-8

Lin, G., Luo, D., Liu, J., Wu, X., Chen, J., Huang, Q., et al. (2018). Hepatoprotective effect of polysaccharides isolated from *Dendrobium officinale* against acetaminophen-induced liver injury in mice via regulation of the Nrf2-Keap1 signaling pathway. *Oxidative Med. Cell. Longev.* 2018:6962439. doi: 10.1155/2018/6962439

Lin, S., Zhang, H., Wang, X., Lin, T., Chen, Z., Liu, J., et al. (2021). Abundance of lipopolysaccharide Heptosyltransferase I in human gut microbiome and its association with cardiovascular disease and liver cirrhosis. *Front. Microbiol.* 12:756976. doi: 10.3389/fmicb.2021.756976

Liu, H., Alhassan, N., Yoon, K. T., Almutlaq, L., and Lee, S. S. (2023). Oxidative stress triggers hyperdynamic circulation via central neural activation in portal hypertensive rats. *Hepatol. Int.* 17, 689–697. doi: 10.1007/s12072-023-10481-5

Liu, J., Kong, L., Shao, M., Sun, C., Li, C., Wang, Y., et al. (2022). Seabuckthorn polysaccharide combined with astragalus polysaccharide ameliorate alcoholic fatty liver by regulating intestinal flora. *Front. Endocrinol.* 13:1018557. doi: 10.3389/fendo.2022.1018557

Liu, H., Liang, J., Zhong, Y., Xiao, G., Efferth, T., Georgiev, M. I., et al. (2021). *Dendrobium officinale* polysaccharide alleviates intestinal inflammation by promoting small extracellular vesicle packaging of miR-433-3p. *J. Agric. Food Chem.* 69, 13510–13523. doi: 10.1021/acs.jafc.1c05134

Liu, H., Nguyen, H. H., Hwang, S. Y., and Lee, S. S. (2023). Oxidative mechanisms and cardiovascular abnormalities of cirrhosis and portal hypertension. *Int. J. Mol. Sci.* 24:16805. doi: 10.3390/ijms242316805

Liu, F., Sun, C., Chen, Y., du, F., Yang, Y., and Wu, G. (2021). Indole-3-propionic acid-aggravated Ccl4-induced liver fibrosis via the Tgf-β1/Smads signaling pathway. *J. Clin. Transl. Hepatol.* 9, 917–930. doi: 10.14218/Jcth.2021.00032

Liu, X., Wang, L., Tan, S., Chen, Z., Wu, B., and Wu, X. (2022). Therapeutic effects of Berberine on liver fibrosis are associated with lipid metabolism and intestinal Flora. *Front. Pharmacol.* 13:814871. doi: 10.3389/fphar.2022.814871

Liu, H., Wang, J., Tao, Y., Li, X., Qin, J., Bai, Z., et al. (2019). Curcumol inhibits colorectal cancer proliferation by targeting miR-21 and modulated Pten/Pi3K/Akt pathways. *Life Sci.* 221, 354–361. doi: 10.1016/j.lfs.2019.02.049

Liu, G., Zhao, Q., and Wei, H. (2019). Characteristics of intestinal bacteria with fatty liver diseases and cirrhosis. *Ann. Hepatol.* 18, 796–803. doi: 10.1016/j.aohep.2019.06.020

Lu, F., Geng, J. B., Zhang, J. W., and Dong, Y. (2021). Effect of entecavir plus Ganshuang granule on fibrosis and cirrhosis in patients with chronic hepatitis B. *J. Tradit. Chin. Med.* 41, 624–629. doi: 10.19852/j.cnki.jtcm.2021.03.015

Lv, L. X., Fang, D. Q., Shi, D., Chen, D. Y., Yan, R., Zhu, Y. X., et al. (2016). Alterations and correlations of the gut microbiome, metabolism and immunity in patients with primary biliary cirrhosis. *Environ. Microbiol.* 18, 2272–2286. doi: 10.1111/1462-2920.13401

Lynch, S. V., and Pedersen, O. (2016). The human intestinal microbiome in health and disease. *N. Engl. J. Med.* 375, 2369–2379. doi: 10.1056/Nejmra1600266

Macpherson, A. J., Gatto, D., Sainsbury, E., Harriman, G. R., Hengartner, H., and Zinkernagel, R. M. (2000). A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science* 288, 2222–2226. doi: 10.1126/science.288.5474.2222

Mahmoudi, A., Atkin, S. L., Jamialahmadi, T., Banach, M., and Sahebkar, A. (2022). Ffect of curcumin on attenuation of liver cirrhosis via genes/proteins and pathways: A system pharmacology study. *Nutrients* 14:4344. doi: 10.3390/nu14204344

Mann, E. R., Lam, Y. K., and Uhlig, H. H. (2024). Short-chain fatty acids: linking diet, the microbiome and immunity. *Nat. Rev. Immunol.*, 1–19. doi: 10.1038/s41577-024-01014-8

Mantis, N. J., Rol, N., and Corthésy, B. (2011). Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. *Mucosal Immunol.* 4, 603–611. doi: 10.1038/mi.2011.41

Manzoor, R., Ahmed, W., Afify, N., Memon, M., Yasin, M., Memon, H., et al. (2022). Trust your gut: the association of gut microbiota and liver disease. *Microorganisms* 10:1045. doi: 10.3390/microorganisms10051045

Marrocco, F., Delli Carpini, M., Garofalo, S., Giampaoli, O., de Felice, E., di Castro, M. A., et al. (2022). Short-chain fatty acids promote the effect of environmental signals on the gut microbiome and metabolome in mice. *Commun. Biol.* 5, 1–13. doi: 10.1038/s42003-022-03468-9

Martens, E. C., Roth, R., Heuser, J. E., and Gordon, J. I. (2009). Coordinate regulation of glycan degradation and polysaccharide capsule biosynthesis by a prominent human gut symbiont. *J. Biol. Chem.* 284, 18445–18457. doi: 10.1074/jbc.M109.008094

Martínez-Montoro, J. I., Kuchay, M. S., Balaguer-Román, A., Martínez-Sánchez, M. A., Frutos, M. D., Fernández-García, J. C., et al. (2022). Gut microbiota and related metabolites in the pathogenesis of nonalcoholic steatohepatitis and its resolution after bariatric surgery. *Obes. Rev.* 23:e13367. doi: 10.1111/obr.13367

Martin-Mateos, R., Alvarez-Mon, M., and Albillos, A. (2019). Dysfunctional immune response in acute-on-chronic liver failure: it takes two to tango. *Front. Immunol.* 10:973. doi: 10.3389/fimmu.2019.00973

Maslennikov, R., Ivashkin, V., Efremova, I., Poluektova, E., Kudryavtseva, A., and Krasnov, G. (2022). Gut dysbiosis and small intestinal bacterial overgrowth as independent forms of gut microbiota disorders in cirrhosis. *World J. Gastroenterol.* 28, 1067–1077. doi: 10.3748/wjg.v28.i10.1067

Maslennikov, R., Pavlov, C., and Ivashkin, V. (2018). Small intestinal bacterial overgrowth in cirrhosis: systematic review and meta-analysis. *Hepatol. Int.* 12, 567–576. doi: 10.1007/s12072-018-9898-2

Maslennikov, R., Poluektova, E., Zolnikova, O., Sedova, A., Kurbatova, A., Shulpekova, Y., et al. (2023). Gut microbiota and bacterial translocation in the pathogenesis of liver fibrosis. *Int. J. Mol. Sci.* 24:16502. doi: 10.3390/ijms242216502

Massey, B. T., and Wald, A. (2021). Small intestinal bacterial overgrowth syndrome: A guide for the appropriate use of breath testing. *Dig. Dis. Sci.* 66, 338–347. doi: 10.1007/s10620-020-06623-6

Meijnikman, A. S., Davids, M., Herrema, H., Aydin, O., Tremaroli, V., Rios-Morales, M., et al. (2022). Microbiome-derived ethanol in nonalcoholic fatty liver disease. *Nat. Med.* 28, 2100–2106. doi: 10.1038/s41591-022-02016-6

Milani, C., Duranti, S., Bottacini, F., Casey, E., Turroni, F., Mahony, J., et al. (2017). The first microbial colonizers of the human gut: composition, activities, and health implications of the infant gut microbiota. *Microbiol. Mol. Biol. Rev.* 81, e00036–e00017. doi: 10.1128/Mmbr.00036-17

Mo, J., Ni, J., Zhang, M., Xu, Y., Li, Y., Karim, N., et al. (2022). Mulberry Anthocyanins ameliorate Dss-induced ulcerative colitis by improving intestinal barrier function and modulating gut microbiota. *Antioxidants (Basel, Switzerland)* 11:1674. doi: 10.3390/antiox11091674

Mohamed, N. I., Suddek, G. M., and El-Kashef, D. H. (2021). Molsidomine alleviates acetic acid-induced colitis in rats by reducing oxidative stress, inflammation and apoptosis. *Int. Immunopharmacol.* 99:108005. doi: 10.1016/j.intimp.2021.108005

Mohandas, S., and Vairappan, B. (2020a). Pregnane X receptor activation by its natural ligand Ginkgolide-A improves tight junction proteins expression and attenuates bacterial translocation in cirrhosis. *Chem. Biol. Interact.* 315:108891. doi: 10.1016/j.cbi.2019.108891

Mohandas, S., and Vairappan, B. (2020b). Ginkgolide-A attenuates bacterial translocation through activating Pxr and improving antimicrobial peptide Reg 3A in experimental cirrhosis. *Life Sci.* 257:118111. doi: 10.1016/j.lfs.2020.118111

Moon, A. M., Singal, A. G., and Tapper, E. B. (2020). Contemporary epidemiology of chronic liver disease and cirrhosis. *Clin. Gastroenterol. Hepatol.* 18, 2650–2666. doi: 10.1016/j.cgh.2019.07.060

Mouries, J., Brescia, P., Silvestri, A., Spadoni, I., Sorribas, M., Wiest, R., et al. (2019). Microbiota-driven gut vascular barrier disruption is a prerequisite for non-alcoholic steatohepatitis development. *J. Hepatol.* 71, 1216–1228. doi: 10.1016/j.jhep.2019.08.005

Moustafa, A. H. A., Ali, E. M. M., Mohamed, T. M., and Abdou, H. I. (2009). Oxidative stress and thyroid hormones in patients with liver diseases. *Eur. J. Intern. Med.* 20, 703–708. doi: 10.1016/j.ejim.2009.08.003

Nafday, S. M., Chen, W., Peng, L., Babyatsky, M. W., Holzman, I. R., and Lin, J. (2005). Short-chain fatty acids induce colonic mucosal injury in rats with various postnatal ages. *Pediatr. Res.* 57, 201–204. doi: 10.1203/01.Pdr.0000150721.83224.89

Neis, E. P. J. G., Bloemen, J. G., Rensen, S. S., van der Vorst, J. R., van den Broek, M. A., Venema, K., et al. (2016). Effects of liver resection on hepatic short-chain fatty acid metabolism in humans. *PLoS One* 11:e0166161. doi: 10.1371/journal.pone.0166161

Neish, A. S. (2009). Microbes in gastrointestinal health and disease. *Gastroenterology* 136, 65–80. doi: 10.1053/j.gastro.2008.10.080

Nishimura, N., Kaji, K., Kitagawa, K., Sawada, Y., Furukawa, M., Ozutsumi, T., et al. (2021). Intestinal permeability is a mechanical rheostat in the pathogenesis of liver cirrhosis. *Int. J. Mol. Sci.* 22:6921. doi: 10.3390/ijms22136921

O'Hara, A. M., and Shanahan, F. (2006). The gut flora as a forgotten organ. *EMBO Rep.* 7, 688–693. doi: 10.1038/sj.embor.7400731

Oh, T. G., Kim, S. M., Caussy, C., Fu, T., Guo, J., Bassirian, S., et al. (2020). A universal gut microbiome-derived signature predicts cirrhosis. *Cell Metab.* 32, 878–888.e6. doi: 10.1016/j.cmet.2020.06.005

Paone, P., and Cani, P. D. (2020). Mucus barrier, mucins and gut microbiota: the expected slimy partners? *Gut* 69, 2232–2243. doi: 10.1136/gutjnl-2020-322260

Patel, V. C., Lee, S., Mcphail, M. J. W., et al. (2022). Rifaximin-α reduces gut-derived inflammation and mucin degradation in cirrhosis and encephalopathy: Rifsys randomised controlled trial. *J. Hepatol.* 76, 332–342. doi: 10.1016/j.jhep.2021.09.010

Pelaseyed, T., and Hansson, G. C. (2020). Membrane mucins of the intestine at a glance. *J. Cell Sci.* 133:jcs240929. doi: 10.1242/jcs.240929

Peng, Y., Qi, X., and Guo, X. (2016). Child-Pugh versus meld score for the assessment of prognosis in liver cirrhosis: A systematic review and Meta-analysis of observational studies. *Medicine* 95:e2877. doi: 10.1097/Md.0000000000002877

Perez-Lopez, A., Behnsen, J., Nuccio, S. P., and Raffatellu, M. (2016). Mucosal immunity to pathogenic intestinal bacteria. *Nat. Rev. Immunol.* 16, 135–148. doi: 10.1038/nri.2015.17

Perry, R. J., Peng, L., Barry, N. A., Cline, G. W., Zhang, D., Cardone, R. L., et al. (2016). Acetate mediates a microbiome-brain-β cell axis promoting metabolic syndrome. *Nature* 534, 213–217. doi: 10.1038/nature18309

Philips, C. A., Ahamed, R., Abduljaleel, J. K. P., Rajesh, S., and Augustine, P. (2023). Identification and analysis of gut microbiota and functional metabolism in decompensated cirrhosis with infection. *J. Clin. Transl. Hepatol.* 11, 15–25. doi: 10.14218/Jcth.2021.00428

Pimpin, L., Cortez-Pinto, H., Negro, F., Corbould, E., Lazarus, J. V., Webber, L., et al. (2018). Burden of liver disease in Europe: epidemiology and analysis of risk factors to identify prevention policies. *J. Hepatol.* 69, 718–735. doi: 10.1016/j.jhep.2018.05.011

Ponziani, F. R., Putignani, L., Paroni Sterbini, F., Petito, V., Picca, A., del Chierico, F., et al. (2018). Influence of hepatitis C virus eradication with direct-acting antivirals on the gut microbiota in patients with cirrhosis. *Aliment. Pharmacol. Ther.* 48, 1301–1311. doi: 10.1111/apt.15004

Porowski, D., Wirkowska, A., Hryniewiecka, E., Wyzgał, J., Pacholczyk, M., and Pączek, L. (2015). Liver failure impairs the intrahepatic elimination of Interleukin-6, tumor necrosis factor-alpha, hepatocyte growth factor, and transforming growth factor-Beta. *Biomed. Res. Int.* 2015:934065. doi: 10.1155/2015/934065

Porras, D., Nistal, E., Martínez-Flórez, S., Pisonero-Vaquero, S., Olcoz, J. L., Jover, R., et al. (2017). Protective effect of quercetin on high-fat diet-induced non-alcoholic fatty liver disease in mice is mediated by modulating intestinal microbiota imbalance and related gut-liver axis activation. *Free Radic. Biol. Med.* 102, 188–202. doi: 10.1016/j.freeradbiomed.2016.11.037

Pott, J., and Hornef, M. (2012). Innate immune signalling at the intestinal epithelium in homeostasis and disease. *EMBO Rep.* 13, 684–698. doi: 10.1038/embor.2012.96

Preidis, G. A., Kim, K. H., and Moore, D. D. (2017). Nutrient-sensing nuclear receptors Pparα and Fxr control liver energy balance. *J. Clin. Invest.* 127, 1193–1201. doi: 10.1172/Jci88893

Pu, W., Wang, X., Zhong, X., Zhao, D., Zeng, Z., Cai, W., et al. (2023). Dysregulation of lipid metabolism in the pseudolobule promotes region-specific autophagy in hepatitis B liver cirrhosis. *Hepatol. Commun.* 7:e0187. doi: 10.1097/Hc9.0000000000000187

Qin, F., Chen, Y., Wang, F. F., Tang, S. Q., and Fang, Y. L. (2023). Corydalis saxicola bunting: A review of its traditional uses, Phytochemistry, pharmacology, and clinical applications. *Int. J. Mol. Sci.* 24:1626. doi: 10.3390/ijms24021626

Qin, H. Y., Cheng, C. W., Tang, X. D., et al. (2014). Impact of psychological stress on irritable bowel syndrome. *World J. Gastroenterol* 20:14126. doi: 10.3748/wjg.v20.i39.14126

Qin, J., Luo, Z., Wang, Q., Tang, C., Meng, M., Huang, Z., et al. (2023). Integrating metabonomics and metagenomics sequencing to study the anti-liver fibrosis effects of palmatine in Corydalis saxicola bunting. *J. Ethnopharmacol.* 315:116666. doi: 10.1016/j.jep.2023.116666

Qin, D., Nie, Y., and Wen, Z. (2014). Protection of rats from thioacetamide-induced hepatic fibrosis by the extracts of a traditional Uighur medicine Cichorium glandulosum. *Iran. J. Basic Med. Sci.* 17, 879–885

Qin, N., Yang, F., Li, A., Prifti, E., Chen, Y., Shao, L., et al. (2014). Alterations of the human gut microbiome in liver cirrhosis. *Nature* 513, 59–64. doi: 10.1038/nature13568

Qu, R., Zhang, W., Ma, Z., Ma, Q., Chen, M., Lan, T., et al. (2023). Glaucocalyxin A attenuates carbon tetrachloride-induced liver fibrosis and improves the associated gut microbiota imbalance. *Chem. Biol. Drug Des.* 102, 51–64. doi: 10.1111/cbdd.14241

Rakoff-Nahoum, S., and Medzhitov, R. (2007). Regulation of spontaneous intestinal tumorigenesis through the adaptor protein MyD88. *Science* 317, 124–127. doi: 10.1126/science.1140488

Rath, S., Heidrich, B., Pieper, D. H., and Vital, M. (2017). Uncovering the trimethylamine-producing bacteria of the human gut microbiota. *Microbiome* 5:54. doi: 10.1186/s40168-017-0271-9

Ridlon, J. M., Harris, S. C., Bhowmik, S., Kang, D. J., and Hylemon, P. B. (2016). Consequences of bile salt biotransformations by intestinal bacteria. *Gut Microbes* 7, 22–39. doi: 10.1080/19490976.2015.1127483

Ridlon, J. M., Kang, D. J., Hylemon, P. B., and Bajaj, J. S. (2015). Gut microbiota, cirrhosis and alcohol regulate bile acid metabolism in the gut. *Dig. Dis.* 33, 338–345. doi: 10.1159/000371678

Roager, H. M., and Licht, T. R. (2018). Microbial tryptophan catabolites in health and disease. *Nat. Commun.* 9:3294. doi: 10.1038/s41467-018-05470-4

Roderburg, C., and Luedde, T. (2014). The role of the gut microbiome in the development and progression of liver cirrhosis and hepatocellular carcinoma. *Gut Microbes* 5, 441–445. doi: 10.4161/gmic.29599

Romanelli, R. G., and Stasi, C. (2016). Recent advancements in diagnosis and therapy of liver cirrhosis. *Current Drug Targets* 17, 1804–1817. doi: 10.2174/1389450117666160613101413

Rueangweerayut, R., Phyo, A. P., Uthaisin, C., Poravuth, Y., Binh, T. Q., Tinto, H., et al. (2012). Pyronaridine-artesunate versus mefloquine plus artesunate for malaria. *N. Engl. J. Med.* 366, 1298–1309. doi: 10.1056/Nejmoa1007125

Salaspuro, M. (1997). Microbial metabolism of ethanol and acetaldehyde and clinical consequences. *Addict. Biol.* 2, 35–46. doi: 10.1080/13556219772840

Sandler, N. G., Koh, C., Roque, A., Eccleston, J. L., Siegel, R. B., Demino, M., et al. (2011). Host response to translocated microbial products predicts outcomes of patients with Hbv or Hcv infection. *Gastroenterology* 141, 1220–1230.e3. doi: 10.1053/j.gastro.2011.06.063

Sawada, Y., Kawaratani, H., Kubo, T., Fujinaga, Y., Furukawa, M., Saikawa, S., et al. (2019). Combining probiotics and an angiotensin-ii type 1 receptor blocker has beneficial effects on hepatic fibrogenesis in a rat model of non-alcoholic steatohepatitis. *Hepatol. Res.* 49, 284–295. doi: 10.1111/hepr.13281

Schroeder, B. O. (2019). Fight them or feed them: how the intestinal mucus layer manages the gut microbiota. *Gastroenterol. Rep.* 7, 3–12. doi: 10.1093/gastro/goy052

Sehgal, R., Bedi, O., and Trehanpati, N. (2020). Role of microbiota in pathogenesis and Management of Viral Hepatitis. *Front. Cell. Infect. Microbiol.* 10:341. doi: 10.3389/fcimb.2020.00341

Shao, J., Ma, X., Qu, L., Ma, P., Huang, R., and Fan, D. (2023). Ginsenoside Rh4 remodels the periphery microenvironment by targeting the brain-gut axis to alleviate depression-like behaviors. *Food Chem.* 404:134639. doi: 10.1016/j.foodchem.2022.134639

Sheth, P., Seth, A., Atkinson, K. J., Gheyi, T., Kale, G., Giorgianni, F., et al. (2007). Acetaldehyde dissociates the Ptp1B-E-cadherin-beta-catenin complex in Caco-2 cell monolayers by a phosphorylation-dependent mechanism. *Biochem. J.* 402, 291–300. doi: 10.1042/Bj20060665

Shi, H., Shi, H., Ren, F., Chen, D., Chen, Y., and Duan, Z. (2017). Naringin in Ganshuang granule suppresses activation of hepatic stellate cells for anti-fibrosis effect by inhibition of mammalian target of rapamycin. *J. Cell. Mol. Med.* 21, 500–509. doi: 10.1111/jcmm.12994

Shimada, Y., Kinoshita, M., Harada, K., Mizutani, M., Masahata, K., Kayama, H., et al. (2013). Commensal bacteria-dependent indole production enhances epithelial barrier function in the colon. *PLoS One* 8:e80604. doi: 10.1371/journal.pone.0080604

Simon, J., Nuñez-García, M., Fernández-Tussy, P., Barbier-Torres, L., Fernández-Ramos, D., Gómez-Santos, B., et al. (2020). Targeting hepatic Glutaminase 1 ameliorates non-alcoholic Steatohepatitis by restoring very-low density lipoproteins triglyceride assembly. *Cell Metab.* 31, 605–622.e10. doi: 10.1016/j.cmet.2020.01.013

Song, D., Hao, J., and Fan, D. (2020). Biological properties and clinical applications of berberine. *Front. Med.* 14, 564–582. doi: 10.1007/s11684-019-0724-6

Song, Q., Zhang, X., Liu, W., Wei, H., Liang, W., Zhou, Y., et al. (2023). *Bifidobacterium pseudolongum*-generated acetate suppresses non-alcoholic fatty liver disease-associated hepatocellular carcinoma. *J. Hepatol.* 79, 1352–1365. doi: 10.1016/j.jhep.2023.07.005

Sorribas, M., De Gottardi, A., Moghadamrad, S., et al. (2020). Isoproterenol disrupts intestinal barriers activating gut-liver-Axis: effects on intestinal mucus and vascular barrier as entry sites. *Digestion* 101, 717–729. doi: 10.1159/000502112

Spahr, L., Bresson-Hadni, S., Amann, P., Kern, I., Golaz, O., Frossard, J. L., et al. (2007). Allopurinol, oxidative stress and intestinal permeability in patients with cirrhosis: an open-label pilot study. *Liver Int.* 27, 54–60. doi: 10.1111/j.1478-3231.2006.01382.x

Tan, Y., Li, Y., Zhou, F., Guo, J., Wang, T., Shi, Y., et al. (2020). Administration of a mixture of triterpenoids from yeyachun and phenolic acids from danshen ameliorates carbon tetrachloride-induced liver fibrosis in mice by the regulation of intestinal flora. *J. Pharmacol. Sci.* 143, 165–175. doi: 10.1016/j.jphs.2020.04.007

Tang, R., Yu, H., Ruan, Z., Zhang, L., Xue, Y., Yuan, X., et al. (2022). Effects of food matrix elements (dietary fibres) on grapefruit peel flavanone profile and on faecal microbiota during in vitro fermentation. *Food Chem.* 371:131065. doi: 10.1016/j.foodchem.2021.131065

Tedeschi, P., Nigro, M., Travagli, A., Catani, M., Cavazzini, A., Merighi, S., et al. (2022). Therapeutic potential of Allicin and aged garlic extract in Alzheimer's disease. *Int. J. Mol. Sci.* 23:6950. doi: 10.3390/ijms23136950

Tilg, H., Adolph, T. E., and Trauner, M. (2022). Gut-liver axis: pathophysiological concepts and clinical implications. *Cell Metab.* 34, 1700–1718. doi: 10.1016/j.cmet.2022.09.017

Toshida, K., Itoh, S., Kosai-Fujimoto, Y., Ishikawa, T., Nakayama, Y., Tsutsui, Y., et al. (2023). Association of gut microbiota with portal vein pressure in patients with liver cirrhosis undergoing living donor liver transplantation. *JGH Open* 7, 982–989. doi: 10.1002/jgh3.13018

Trebicka, J., Macnaughtan, J., Schnabl, B., Shawcross, D. L., and Bajaj, J. S. (2021). The microbiota in cirrhosis and its role in hepatic decompensation. *J. Hepatol.* 75, S67–S81. doi: 10.1016/j.jhep.2020.11.013

Tripathi, D. M., Vilaseca, M., Lafoz, E., Garcia-Calderó, H., Viegas Haute, G., Fernández-Iglesias, A., et al. (2018). Simvastatin prevents progression of acute on chronic liver failure in rats with cirrhosis and portal hypertension. *Gastroenterology* 155, 1564–1577. doi: 10.1053/j.gastro.2018.07.022

Tsiaoussis, G. I., Assimakopoulos, S. F., Tsamandas, A. C., Triantos, C. K., and Thomopoulos, K. C. (2015). Intestinal barrier dysfunction in cirrhosis: current concepts in pathophysiology and clinical implications. *World J. Hepatol.* 7, 2058–2068. doi: 10.4254/wjh.v7.i17.2058

Tsuda, A., Suda, W., Morita, H., Takanashi, K., Takagi, A., Koga, Y., et al. (2015). Influence of proton-pump inhibitors on the luminal microbiota in the gastrointestinal tract. *Clin. Transl. Gastroenterol.* 6:e89. doi: 10.1038/ctg.2015.20

Tsukita, S., Furuse, M., and Itoh, M. (2001). Multifunctional strands in tight junctions. *Nat. Rev. Mol. Cell Biol.* 2, 285–293. doi: 10.1038/35067088

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., and Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444, 1027–1031. doi: 10.1038/nature05414

Twardowska, A., Makaro, A., Binienda, A., Fichna, J., and Salaga, M. (2022). Preventing bacterial translocation in patients with leaky gut syndrome: nutrition and pharmacological treatment options. *Int. J. Mol. Sci.* 23:3204. doi: 10.3390/ijms23063204

Vaishnava, S., Yamamoto, M., Severson, K. M., Ruhn, K. A., Yu, X., Koren, O., et al. (2011). The antibacterial lectin Regiiiγ promotes the spatial segregation of microbiota and host in the intestine. *Science* 334, 255–258. doi: 10.1126/science.1209791

Vallianou, N., Christodoulatos, G. S., Karampela, I., Tsilingiris, D., Magkos, F., Stratigou, T., et al. (2021). Understanding the role of the gut microbiome and microbial metabolites in non-alcoholic fatty liver disease: current evidence and perspectives. *Biomol. Ther.* 12:56. doi: 10.3390/biom12010056

Van Best, N., Rolle-Kampczyk, U., Schaap, F. G., et al. (2020). Bile acids drive the newborn's gut microbiota maturation. *Nat. Commun.* 11:3692. doi: 10.1038/s41467-020-17183-8

Vavassori, P., Mencarelli, A., Renga, B., Distrutti, E., and Fiorucci, S. (2009). The bile acid receptor Fxr is a modulator of intestinal innate immunity. *J. Immunol.* 183, 6251–6261. doi: 10.4049/jimmunol.0803978

Violi, F., Pignatelli, P., Castellani, V., Carnevale, R., and Cammisotto, V. (2023). Gut dysbiosis, endotoxemia and clotting activation: A dangerous trio for portal vein thrombosis in cirrhosis. *Blood Rev.* 57:100998. doi: 10.1016/j.blre.2022.100998

Wahlström, A., Sayin, S. I., Marschall, H. U., and Bäckhed, F. (2016). Intestinal crosstalk between bile acids and microbiota and its impact on host metabolism. *Cell Metab.* 24, 41–50. doi: 10.1016/j.cmet.2016.05.005

Wang, H., Chang, Y., Liu, X., Liu, L., Hua, M., and Li, A. (2024). Protective effects of baicalin on diethyl nitrosamine-induced liver cirrhosis by suppressing oxidative stress and inflammation. *Chem. Biol. Drug Des.* 103:e14386. doi: 10.1111/cbdd.14386

Wang, H. Y., Ge, J. C., Zhang, F. Y., Zha, X. Q., Liu, J., Li, Q. M., et al. (2022). *Dendrobium officinale* polysaccharide promotes M1 polarization of tams to inhibit tumor growth by targeting Tlr2. *Carbohydr. Polym.* 292:119683. doi: 10.1016/j.carbpol.2022.119683

Wang, Y., Guan, M., Zhao, X., and Li, X. (2018). Effects of garlic polysaccharide on alcoholic liver fibrosis and intestinal microflora in mice. *Pharm. Biol.* 56, 325–332. doi: 10.1080/13880209.2018.1479868

Wang, L., and Wang, J. (2022). Honokiol ameliorates Dss-induced mouse colitis by inhibiting inflammation and oxidative stress and improving the intestinal barrier. *Oxidative Med. Cell. Longev.* 2022:1755608. doi: 10.1155/2022/1755608

Wang, X. X., Xie, C., Libby, A. E., Ranjit, S., Levi, J., Myakala, K., et al. (2022). The role of Fxr and Tgr5 in reversing and preventing progression of Western diet–induced

hepatic steatosis, inflammation, and fibrosis in mice. *J. Biol. Chem.* 298:102530. doi: 10.1016/j.jbc.2022.102530

Wang, K., Yang, X., Wu, Z., Wang, H., Li, Q., Mei, H., et al. (2020). *Dendrobium officinale* polysaccharide protected Ccl4-induced liver fibrosis through intestinal homeostasis and the Lps-Tlr4-Nf-κB signaling pathway. *Front. Pharmacol.* 11:240. doi: 10.3389/fphar.2020.00240

Wang, L., Zhao, Y., Su, Z., Zhao, K., Li, P., and Xu, T. (2023). Ginkgolide A targets forkhead box O1 to protect against lipopolysaccharide-induced septic cardiomyopathy. *Phytother. Res.* 37, 3309–3322. doi: 10.1002/ptr.7802

Wegierska, A. E., Charitos, I. A., Topi, S., Potenza, M. A., Montagnani, M., and Santacroce, L. (2022). The connection between physical exercise and gut microbiota: Implications for competitive sports athletes. *Sports Med.* 52, 2355–2369. doi: 10.1007/s40279-022-01696-x

Wenlin, C., Fang, L., Yuncheng, Z., Jinzhen, L. V., and Daguo, Y. (2023). Effects of Zhenggan Huayu decoction combined with entecavir on gut microbiota in patients with chronic hepatitis B fibrosis. *J. Tradit. Chin. Med.* 43, 559–567. doi: 10.19852/j.cnki.jtcm.20230208.002

Winer, D. A., Luck, H., Tsai, S., and Winer, S. (2016). The intestinal immune system in obesity and insulin resistance. *Cell Metab.* 23, 413–426. doi: 10.1016/j.cmet.2016.01.003

Woodhouse, C. A., Patel, V. C., Singanayagam, A., and Shawcross, D. L. (2018). Review article: the gut microbiome as a therapeutic target in the pathogenesis and treatment of chronic liver disease. *Aliment. Pharmacol. Ther.* 47, 192–202. doi: 10.1111/apt.14397

Wrzosek, L., Miquel, S., Noordine, M. L., Bouet, S., Chevalier-Curt, M. J., Robert, V., et al. (2013). Bacteroides thetaiotaomicron and *Faecalibacterium prausnitzii* influence the production of mucus glycans and the development of goblet cells in the colonic epithelium of a gnotobiotic model rodent. *BMC Biol.* 11:61. doi: 10.1186/1741-7007-11-61

Wu, Z., Liu, X., Huang, S., Li, T., Zhang, X., Pang, J., et al. (2022). Milk fat globule membrane attenuates acute colitis and secondary liver injury by improving the mucus barrier and regulating the gut microbiota. *Front. Immunol.* 13:865273. doi: 10.3389/fimmu.2022.865273

Wu, H., Xie, S., Miao, J., Li, Y., Wang, Z., Wang, M., et al. (2020). *Lactobacillus reuteri* maintains intestinal epithelial regeneration and repairs damaged intestinal mucosa. *Gut Microbes* 11, 997–1014. doi: 10.1080/19490976.2020.1734423

Wu, Z., Zhou, H., Liu, D., and Deng, F. (2023). Alterations in the gut microbiota and the efficacy of adjuvant probiotic therapy in liver cirrhosis. *Front. Cell. Infect. Microbiol.* 13:1218552. doi: 10.3389/fcimb.2023.1218552

Xia, W., Lai, G., Li, Y., Zeng, C., Sun, C., Zhang, P., et al. (2023). Photo-crosslinked adhesive hydrogel loaded with extracellular vesicles promoting hemostasis and liver regeneration. *Front. Bioeng. Biotechnol.* 11:1170212. doi: 10.3389/fbioe.2023.1170212

Yan, M., Man, S., Sun, B., Ma, L., Guo, L., Huang, L., et al. (2023). Gut liver brain axis in diseases: the implications for therapeutic interventions. *Signal Transduct. Target. Ther.* 8:443. doi: 10.1038/s41392-023-01673-4

Yang, X., Wang, Z., Kai, J., Wang, F., Jia, Y., Wang, S., et al. (2020). Curcumol attenuates liver sinusoidal endothelial cell angiogenesis via regulating Glis-Prox1-Hif-1α in liver fibrosis. *Cell Prolif.* 53:e12762. doi: 10.1111/cpr.12762

Ye, N., Wang, H., Hong, J., Zhang, T., Lin, C., and Meng, C. (2016). Pxr mediated protection against liver inflammation by Ginkgolide A in Tetrachloromethane treated mice. *Biomol. Ther.* 24, 40–48. doi: 10.4062/biomolther.2015.077

Younossi, Z. M. (2019). Non-alcoholic fatty liver disease – A global public health perspective. *J. Hepatol.* 70, 531–544. doi: 10.1016/j.jhep.2018.10.033

Yu, J. S., Youn, G. S., Choi, J., Kim, C. H., Kim, B. Y., Yang, S. J., et al. (2021). Lactobacillus lactis and *Pediococcus pentosaceus*-driven reprogramming of gut microbiome and metabolome ameliorates the progression of non-alcoholic fatty liver disease. *Clin. Transl. Med.* 11:e634. doi: 10.1002/ctm2.634

Yue, S. J., Liu, J., Wang, W. X., Wang, A. T., Yang, X. Y., Guan, H. S., et al. (2019). Berberine treatment-emergent mild diarrhea associated with gut microbiota dysbiosis. *Biomed. Pharmacother.* 116:109002. doi: 10.1016/j.biopha.2019.109002

Zamparelli, M. S., Rocco, A., Compare, D., and Nardone, G. (2017). The gut microbiota: A new potential driving force in liver cirrhosis and hepatocellular carcinoma. *United European Gastroenterol J* 5, 944–953. doi: 10.1177/2050640617705576

Zhang, Y. L., Li, Z. J., Gou, H. Z., Song, X. J., and Zhang, L. (2022). The gut microbiota-bile acid axis: A potential therapeutic target for liver fibrosis. *Front. Cell. Infect. Microbiol.* 12:945368. doi: 10.3389/fcimb.2022.945368

Zhang, L. X., Li, C. X., Kakar, M. U., Khan, M. S., Wu, P. F., Amir, R. M., et al. (2021). Resveratrol (Rv): A pharmacological review and call for further research. *Biomed. Pharmacother.* 143:112164. doi: 10.1016/j.biopha.2021.112164

Zhang, D., Liu, Z., and Bai, F. (2023). Roles of gut microbiota in alcoholic liver disease. *Int. J. Gen. Med.* 16, 3735–3746. doi: 10.2147/Ijgm.S420195

Zhang, S. N., Liu, Q., Li, X. Z., Yang, W. D., and Zhou, Y. (2024). Liver protein and metabolite biolabels reveal hepatoprotective effects and active compounds of Eucommiae folium: exploration of new application of herb based on multi-omics and bioinformatics. *J. Pharm. Biomed. Anal.* 239:115870. doi: 10.1016/j.jpba.2023.115870

Zhang, Y., Liu, X., Ruan, J., Zhuang, X., Zhang, X., and Li, Z. (2020). Phytochemicals of garlic: promising candidates for cancer therapy. *Biomed. Pharmacother.* 123:109730. doi: 10.1016/j.biopha.2019.109730

Zhang, Z., Lu, W., Liu, P., Li, M., Ge, X., Yu, B., et al. (2023). Microbial modifications with *Lycium barbarum* L. oligosaccharides decrease hepatic fibrosis and mitochondrial abnormalities in mice. *Phytomedicine* 120:155068. doi: 10.1016/j.phymed.2023.155068

Zhang, Y., Zhao, M., Jiang, X., Qiao, Q., Liu, T., Zhao, C., et al. (2021b). Comprehensive analysis of fecal microbiome and metabolomics in hepatic fibrosis rats reveal Hepatoprotective effects of Yinchen Wuling powder from the host-microbial metabolic Axis. *Front. Pharmacol.* 12:713197. doi: 10.3389/fphar.2021.713197

Zhang, Y., Zhao, M., Liu, Y., Liu, T., Zhao, C., and Wang, M. (2021a). Investigation of the therapeutic effect of Yinchen Wuling powder on Ccl4-induced hepatic fibrosis in rats by 1H Nmr and Ms-based metabolomics analysis. *J. Pharm. Biomed. Anal.* 200:114073. doi: 10.1016/j.jpba.2021.114073

Zhao, J., Miao, J., Wei, X., Guo, L., Li, P., Lei, J., et al. (2021). Traditional Chinese medicine Ganshuang granules attenuate Ccl4 -induced hepatic fibrosis by modulating gut microbiota. *Chem. Biodivers.* 18:e2100520. doi: 10.1002/cbdv.202100520

Zhao, Z. H., Xin, F. Z., Xue, Y., Hu, Z., Han, Y., Ma, F., et al. (2019). Indole-3-propionic acid inhibits gut dysbiosis and endotoxin leakage to attenuate steatohepatitis in rats. *Exp. Mol. Med.* 51, 1–14. doi: 10.1038/s12276-019-0304-5

Zheng, Y., Wang, J., Wang, J., Jiang, R., and Zhao, T. (2022). Gut microbiota combined with metabolomics reveal the mechanism of curcumol on liver fibrosis in mice. *Biomed. Pharmacother.* 152:113204. doi: 10.1016/j.biopha.2022.113204

Zhou, J., Cui, S., He, Q., Guo, Y., Pan, X., Zhang, P., et al. (2020). Sumoylation inhibitors synergize with Fxr agonists in combating liver fibrosis. *Nat. Commun.* 11:240. doi: 10.1038/s41467-019-14138-6

Zhou, Q., Wu, F., Chen, S., et al. (2022). *Lactobacillus reuteri* improves function of the intestinal barrier in rats with acute liver failure through Nrf-2/Ho-1 pathway. *Nutrition (Burbank, Los Angeles County, Calif.)* 99-100:111673. doi: 10.1016/j.nut.2022.111673

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An urgent need for longitudinal microbiome profiling coupled with machine learning interventions

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1 Introduction: lack of universal gut microbial signatures associated with metabolic liver disease

Our understanding of the role of gut microbes in human health and disease has come a long way since John M. Whipps et al. first defined the term "microbiome." Since the early 2000s, with the gradual lowering of the cost of commercial DNA sequencing, health science has been flooded with 16S rRNA data. Unfortunately, despite a plethora of pre-clinical and clinical publications on the gut–liver axis, the majority of our understanding of the microbiota–liver reciprocal interaction remains limited to correlation analysis. The most exciting part of such metagenomic studies is the sheer amount of "big data" generated, which is rather easy to correlate with physiological variables; the bigger the metagenomic data and number of independent variables, the more the chances to find "significant" associations. Notably, our limited understanding of the gut–liver axis is derived from the microbiome, rather than the microbiota. Therefore, the conclusions obtained through microbiome-related correlation studies often do not reflect causation and are not representative of a universal phenomenon, and there have been almost no true microbial markers of dysbiosis linked to chronic liver disease.

2 Discussion

2.1 The boundary between the known and the unknown

Translational potentials of pre-clinical microbiota–liver associations in clinical disease prediction and treatment have not been very successful despite enormous numbers of interventional and observational studies. In fact, the relevance of utilizing rodents' gut microbial signatures in understanding human diseases has been criticized due to the massive difference in the gut microbes between both species, attributed to the gastrointestinal biogeography and genetic makeup (Nguyen et al., 2015). Especially in the high-fat diet model of metabolic disease, there is a lack of consistency between good and bad microbes. In fact, diseases have also been associated with the strains belonging to probiotics (e.g., *Lactobacillus*) and commensals (e.g., *Akkermansia* and *Faecalibacterium*) (Dey and Ray Chaudhuri, 2023). Furthermore, confounding results are obtained from chemical-induced metabolic disease models that are physiologically mostly irrelevant (Dey, 2020) and data from germ-free mice that possess innate defects

in various physiological processes (Jans and Vereecke, 2024). The clinical progression of metabolic liver disease is distinct from pre-clinical models of chronic liver disease in terms of timeline, cellular phenotype, pathological complexity, the difference in immune responses, and metabolic machinery (Liu et al., 2013). Furthermore, the innate and general differences between human and animal disease models, such as biological differences (e.g., genetic makeup, organ anatomy, and liver functional capacity), extent of disease complexity (e.g., model specificity and co-influence of comorbidities), ability to perform controlled experiments, and predictive validity, make it challenging to conclude on clinical gut microbial phenotypes based on pre-clinical data. Although there have been emerging reports of *in vitro* models of the human distal intestine (Qi et al., 2023), these models are physiologically irrelevant given their host-independent nature.

Due to the advent of culturomics techniques and controlled clinical studies, pre-clinical gut microbial patterns that were initially considered associated with disease conditions, such as Firmicutes-to-Bacteroides ratio, enrichment of energy-harvesting species, and specific metabolic functions, seem to be falling apart. For instance, γ-proteobacteria, due to the presence of lipopolysaccharide (LPS), were previously thought to simply cause hepatic inflammation. However, studies have identified that the LPS–TLR4–inflammation axis cannot be generalized due to huge differences in LPS structure dictating the extent of immune response (Picarello, 2022) and that the majority of the luminal LPS-supplying Bacteroides rather display immunosuppressive characteristics (d'Hennezel et al., 2017). Today, it is strongly recognized that the good, bad, and ugly nature of the gut microbiota is condition-specific. Factors such as the availability of preferred nutrients, pathoadaptive mutations, and potential to evade the mucosal immune response have been recognized as critical factors that define a specific microbial species as commensal or pathobiont (Dey and Ray Chaudhuri, 2023; Dey, 2024). The bottom line is that there is no proper clinical definition of dysbiosis and eubiosis in terms of specific microbial features. However, the only aspect almost universally accepted is that loss of gut microbial diversity is associated with chronic liver disease, and when we talk about the diversity, it is the community effects not disease causation by a single species.

2.2 A critical lack of liver-specific longitudinal studies

A recent systematic review and meta-analysis of 54 clinical studies have indicated substantial inter-study heterogeneity in gut microbial taxonomic identification, in which the enrichment of inflammation-inducing genera was more closely associated with non-alcoholic fatty liver disease, but no genera were identified to provide long-term disease risk-predictive value (Su et al., 2024). To date, there have been more than 100 cross-sectional studies to identify the predominant gut microbes associated with metabolic liver disease, yet no absolute core microbiome related to progressive liver disease has been identified. Although longitudinal studies are considered superior to cross-sectional studies and that informed understanding of disease pathogenesis can only be obtained through the former, there have been only a few longitudinal studies undertaken to link the gut microbiome with liver health. One study from Kyoto (Japan) evaluated the gut microbial alterations from pre-transplantation to 2 months post-surgery in 38 liver transplant patients (Kato et al., 2017). Data show an initial decline and later increase of microbial diversity, along with the overall increase of Bacteroides, Enterobacteriaceae, Streptococcaceae, and Bifidobacteriaceae, while a depletion in the abundance of Lactobacillaceae, Enterococcaceae, Clostridiaceae, Peptostreptococcaceae, and Ruminococcaceae was noted. The authors acknowledged that variations in antibiotic regimes, food, synbiotics, and patient heterogeneity (e.g., various donors and underlying diseases) likely influence the study's findings. Confounding variables and data on relative microbial abundance were among the limitations. In line, a relatively recent study from the National Institutes of Health, USA, investigated the gut–liver axis in hepatitis C patients, taking into account varied degrees of fibrosis severity (Ali et al., 2023). The investigation was performed 6 months after HCV was undetectable ($n = 23$) and before ($n = 29$) attaining a durable virologic response. Data suggest that increased hepatic fibrosis was correlated with *Anaerostipes hadrus*, while *Bacteroides vulgatus* with portal inflammation in HCV. A prolonged virologic response suggests that *Methanobrevibacter smithii* may have a beneficial effect on indicators of the severity of liver disease. Although this longitudinal investigation made it possible to compare HCV-infected individuals with a supposedly improved viral clearance state, they were unable to report gut microbial patterns in healthy controls due to the unavailability of samples. Another recent study from Stanford examines the gut microbial composition at various body sites (including gut) and correlated with host multi-omics, immunological, and clinical indicators (including hepatic) ($n = 86$) over 6 years to comprehend the dynamic interaction between the human microbiomes and host throughout health and illness (Zhou et al., 2024). Despite identifying microbial compositional and diversity patterns associated with host physiological parameters and metabolites, no temporal associations were derived between the gut microbiota and the measured liver-specific parameters (e.g., transaminases). Emerging studies claim the causative effects of oral microbiota in the pathogenesis of chronic metabolic disease, including liver disease (Gupta and Dey, 2023), and there has been no longitudinal study undertaken in this line to date. Thus, understanding the true nature of gut microbial dynamics under the course of hepatic disease pathogenesis and remission remains largely unknown.

2.3 Machine learning in pattern recognition along the gut–liver axis

Beyond the availability of longitudinal gut microbial data, a fundamental difficulty in analyzing large-scale microbiome big-data lies in their high dimensionality (Advani and Ganguli, 2016). In classically designed experiments, a small number of carefully selected variables (V) are measured to test a specific hypothesis, with a large number of measurements (M) for each variable. Thus, the measurement density is very large (M/V

→ ∞). Such datasets are referred to as low-dimensional, and much of classical statistics operates within this framework. In contrast to this classical scenario, the recent technological capacity for high-throughput sequencing has led to a different statistical regime. It is commonplace to simultaneously measure many variables (V), such as the abundance of hundreds of taxa at the individual level. However, due to constraints on time or resources, often it is possible only to make a limited number of simultaneous measurements. Thus, while both M and V are large, the measurement density (M/V) is much smaller than in conventional experiments. Such datasets are referred to as high dimensional, that is, they consist of a small number of points in a high-dimensional space. Microbiome datasets consist of the composition of thousands of microbial taxa in an individual gut. Hence, understanding the role of gut microbiome in liver health requires us to use machine learning (ML) approaches to dissect such a high-dimensional dataset. Utilizing these approaches enhances our understanding of the complex host–microbe reciprocal, helping in tracking disease progression over time or monitoring treatment responses, which is valuable for personalized medicine.

In recent years, ML approaches have been widely used to shed light on how gut microbiome impacts various liver diseases. These studies have identified microbiome biomarkers associated with metabolic liver diseases (Ruuskanen et al., 2021; Zhang et al., 2021; Liu et al., 2022; Park et al., 2024). However, not only that majority of these studies are mostly cross-sectional in nature but also these ML approaches suffer from a number of limitations. First, it is extremely difficult to detect patterns in microbiome datasets that can provide useful biological insight with translational value. High-dimensional data analysis techniques such as PCA, ICA, or t-SNE are useful for reducing dimensionality and detecting patterns. However, since the resulting axes represent linear combinations of a large number of features (e.g., taxa abundance), interpreting the analysis or making experimental predictions is often difficult (Donoho and Tanner, 2009; Furchtgott et al., 2017). Identifying patterns becomes even more challenging as the proportion of relevant features decreases at higher taxonomic levels (Donoho and Tanner, 2009). In fact, decades of research related to the gut microbiome have shown that specific combinations of a few microbes can be associated with a disease. This indicates that the composition of a small subset of microbes may be most relevant for making accurate computational inferences. Therefore, there is a need to develop novel methods to detect sparse patterns in high-dimensional datasets.

The second challenge is to extract meaningful models from high-dimensional datasets that can predict interventional outcomes and guide translational implications of research findings. Building complex models describing microbial networks involves hundreds of parameters. Unfortunately, in most cases, the parameters remain completely unknown. As Von Neumann once said: "*with four parameters I can fit an elephant, and with five I can make him wiggle his trunk.*" The challenges involving microbial networks are clearly exacerbated since even the simplest of models would require hundreds of parameters. Hence, the available data are always limited and can never constrain the space of models completely. Given the underconstrained nature of the problem, there exist an infinite number of combinations of the parameters that can successfully replicate the data. Hence, making predictions based on such underconstrained gut microbial networks has proven to be challenging. Moreover, most of the studies trained their models on specific datasets, such as Western or Chinese datasets, that may not generalize to different populations, limiting their overall applicability. Often these studies used a small patient population size. Some of these studies suffered from the absence of population characteristics such as the lack of lifestyle information (e.g., diet, socioeconomic status, tobacco, and alcoholism). Furthermore, in some cases, the lack of patient clinical, metagenomic, and metabolomic profiles hampered garnering a comprehensive understanding of the various causal aspects of the disease.

3 Conclusion

The complexity of the gut microbial dynamics and its function in metabolic liver disease remains largely unknown. The progressive character and course of liver disorders are not well captured by pre-clinical and cross-sectional investigations. We suggest using cutting-edge ML methods in conjunction with longitudinal research to address these issues. Hence, it is imperative to first develop methods to detect sparse relevant features that can then be used to find patterns in the data and train predictive models. By addressing these limitations and building new computational approaches, we can fully harness the potential of ML approaches to deepen our understanding of the gut microbiome's role in liver disease and other areas. By doing this, we can pinpoint certain gut microbial patterns associated with the advancement of liver disease, resulting in the development of more potent preventative and therapeutic approaches. For gut–liver axis research to reach its full potential, machine learning must be included.

Author contributions

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References

Advani, M., and Ganguli, S. (2016). Statistical mechanics of optimal convex inference in high dimensions. *Phys. Rev. X* 6:031034. doi: 10.1103/PhysRevX.6.031034

Ali, R. O., Quinn, G. M., Umarova, R., Haddad, J. A., Zhang, G. Y., Townsend, E. C., et al. (2023). Longitudinal multi-omics analyses of the gut-liver axis reveals metabolic dysregulation in hepatitis C infection and cirrhosis. *Nat. Microbiol.* 8, 12–27. doi: 10.1038/s41564-022-01273-y

Dey, P. (2020). The role of gut microbiome in chemical-induced metabolic and toxicological murine disease models. *Life Sci.* 258:118172. doi: 10.1016/j.lfs.2020.118172

Dey, P. (2024). Good girl goes bad: understanding how gut commensals cause disease. *Microb. Pathog.* 190:106617. doi: 10.1016/j.micpath.2024.106617

Dey, P., and Ray Chaudhuri, S. (2023). The opportunistic nature of gut commensal microbiota. *Crit. Rev. Microbiol.* 49, 739–763. doi: 10.1080/1040841X.2022.2133987

d'Hennezel, E., Abubucker, S., Murphy, L. O., and Cullen, T. W. (2017). Total lipopolysaccharide from the human gut microbiome silences toll-like receptor Signaling. *mSystems* 2, 10-1128. doi: 10.1128/mSystems.00046-17

Donoho, D., and Tanner, J. (2009). Observed universality of phase transitions in high-dimensional geometry, with implications for modern data analysis and signal processing. *Philos. Trans. A Math. Phys. Eng. Sci.* 367, 4273–4293. doi: 10.1098/rsta.2009.0152

Furchtgott, L. A., Melton, S., Menon, V., and Ramanathan, S. (2017). Discovering sparse transcription factor codes for cell states and state transitions during development. *Elife* 6:e20488. doi: 10.7554/eLife.20488

Gupta, U., and Dey, P. (2023). The oral microbial odyssey influencing chronic metabolic disease. *Arch. Physiol. Biochem.* 2023, 1–17. doi: 10.1080/13813455.2023.2296346

Jans, M., and Vereecke, L. (2024). A guide to germ-free and gnotobiotic mouse technology to study health and disease. *FEBS J.* 2024:17124. doi: 10.1111/febs.17124

Kato, K., Nagao, M., Miyamoto, K., Oka, K., Takahashi, M., Yamamoto, M., et al. (2017). Longitudinal analysis of the intestinal microbiota in liver transplantation. *Transpl. Dir.* 3:e144. doi: 10.1097/TXD.0000000000000661

Liu, Y., Méric, G., Havulinna, A. S., Teo, S. M., Åberg, F., Ruuskanen, M., et al. (2022). Early prediction of incident liver disease using conventional risk factors and gut-microbiome-augmented gradient boosting. *Cell Metab.* 34, 719–730.e714. doi: 10.1016/j.cmet.2022.03.002

Liu, Y., Meyer, C., Xu, C., Weng, H., Hellerbrand, C., Ten Dijke, P., et al. (2013). Animal models of chronic liver diseases. *Am. J. Physiol.-Gastroint. Liver Physiol.* 304, G449–G468. doi: 10.1152/ajpgi.00199.2012

Nguyen, T. L., Vieira-Silva, S., Liston, A., and Raes, J. (2015). How informative is the mouse for human gut microbiota research? *Dis. Model. Mech.* 8, 1–16. doi: 10.1242/dmm.017400

Park, I.-G., Yoon, S. J., Won, S.-M., Oh, K.-K., Hyun, J. Y., Suk, K. T., et al. (2024). Gut microbiota-based machine-learning signature for the diagnosis of alcohol-associated and metabolic dysfunction-associated steatotic liver disease. *Sci. Rep.* 14:16122. doi: 10.1038/s41598-024-60768-2

Picarello, D. M. (2022). Investigating how the LPS structure affects the immune response of gram-negative bacterial outer membranes. *Biophys. J.* 121:487a. doi: 10.1016/j.bpj.2021.11.344

Qi, Y., Yu, L., Tian, F., Zhao, J., and Zhai, Q. (2023). In vitro models to study human gut-microbiota interactions: Applications, advances, and limitations. *Microbiol. Res.* 270:127336. doi: 10.1016/j.micres.2023.127336

Ruuskanen, M. O., Åberg, F., Männistö, V., Havulinna, A. S., Méric, G., Liu, Y., et al. (2021). Links between gut microbiome composition and fatty liver disease in a large population sample. *Gut Microbes* 13:1888673. doi: 10.1080/19490976.2021.1888673

Su, X., Chen, S., Liu, J., Feng, Y., Han, E., Hao, X., et al. (2024). Composition of gut microbiota and non-alcoholic fatty liver disease: a systematic review and meta-analysis. *Obes. Rev.* 25:e13646. doi: 10.1111/obr.13646

Zhang, T., Zhang, S., Jin, C., Lin, Z., Deng, T., Xie, X., et al. (2021). A predictive model based on the gut microbiota improves the diagnostic effect in patients with cholangiocarcinoma. *Front. Cell. Infect. Microbiol.* 11:751795. doi: 10.3389/fcimb.2021.751795

Zhou, X., Shen, X., Johnson, J. S., Spakowicz, D. J., Agnello, M., Zhou, W., et al. (2024). Longitudinal profiling of the microbiome at four body sites reveals core stability and individualized dynamics during health and disease. *Cell Host Microbe.* 32, 506–526.e509. doi: 10.1016/j.chom.2024.02.012

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Could chronic opioid use be an additional risk of hepatic damage in patients with previous liver diseases, and what is the role of microbiome?

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Summary: Among illicit drugs, addiction from opioids and synthetic opioids is soaring in an unparalleled manner with its unacceptable amount of deaths. Apart from these extreme consequences, the liver toxicity is another important aspect that should be highlighted. Accordingly, the chronic use of these substances, of which fentanyl is the most frequently consumed, represents an additional risk of liver damage in patients with underlying chronic liver disease. These observations are drawn from various preclinical and clinical studies present in literature. Several downstream molecular events have been proposed, but recent pieces of research strengthen the hypothesis that dysbiosis of the gut microbiota is a solid mechanism inducing and worsening liver damage by both alcohol and illicit drugs. In this scenario, the gut flora modification ascribed to non-alcoholic fatty liver disease performs an additive role. Interestingly enough, HBV and HCV infections impact gut–liver axis. In the end, the authors tried to solicit the attention of operators on this major healthcare problem.

KEYWORDS

opioids, fentanyl, liver toxicity, gut flora dysbiosis, CYP 3A4, interleukins

Introduction

According to *Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5)*, drug abuse refers to the excessive use of sedatives/hypnotics/anxiolytics/stimulants, alcohol, caffeine, cannabis, hallucinogens, inhalants, and opioids. Both substance abuse and dependence were merged into the category of substance use disorders (SUDs) (American Psychiatric Association, 2013). Drug addiction involves the mesolimbic system. When rewarding stimuli are experienced, the dopaminergic mesolimbic system is activated leading to the release of dopamine from the targeted nuclei (Swanson, 2000; Baik, 2013). Contextually, the concentration of dopamine grows in the reward areas of the brain, evoking the so-called "stereotypical exhilaration and relaxation effects." As a consequence, this dopamine flood in the reward pathway is distinctively associated with the addiction to the drug (Comer and Cahill, 2019). A

recent survey on the adult population from European regions revealed that substance-attributable mortality rates were highest for tobacco smoking, followed by alcohol and illicit drugs (Peacock et al., 2018). Among illicit drugs, opioid abuse is skyrocketing in an unprecedented way with its unbearable burden of deaths (Wilson et al., 2020). Fentanyl is one of major contributors to both fatal and non-fatal overdoses in the United States. In this country, it is reported that in 2017, more than 70,237 fatalities were the result of drug overdoses. Of these, a high number such as approximately 50,000 were due to opioids, but coincidently over 20,000 of those deaths were caused by fentanyl alone (National Institute on Drug Abuse, 2023). Specifically, in the time interval 2017–2019, death rates involving synthetic opioids increased from 9.0 per 100,000 population to 9.9 in 2018 and accounted for an astonishingly 67.0% of opioid-involved deaths in 2018. The rates, quite similar among men and women, both aged ≥25 years, need to force healthcare system to set up comprehensive surveillance and prevention measures to try to reduce this high toll (Wilson et al., 2020). Fentanyl users exhibit a false sense of wellbeing, feelings of euphoria and relaxation, extreme happiness, quickly dissolving into difficulty thinking, speaking or walking, confusion, dizziness and coma. Without immediate treatment by trained professionals, death comes swiftly in minutes. The signs recognizing opioid overdose are small, constricted "pinpoint pupils," falling asleep or losing consciousness, slow, weak or no breathing, choking or gurgling sounds, limp body, cold and/or clammy skin, and discolored lips and nails (Centre for Disease Control and prevention, 2023). Impaired liver function, evaluated by the means of Child-Pugh score, was identified as one of the most significant factors in determining variation in serum fentanyl concentrations (Kokubun et al., 2012). The last finding is primary to understanding that reduced metabolic processes could induce potential side effects of fentanyl, mainly when highly prevalent non-alcoholic fatty liver disease (NAFLD) and alcoholic liver disease (ALD) co-exist (Alkhouri et al., 2022), but not only.

Aim of the review

This narrative review was organized with summarizing the history of research in this field and clarifying trends. It has been presented as a 'conceptual frame', providing a "rationale" for predictions about the relationships among variables, where the contents are separated according to various physio-pathological aspects. In this model, the central body is partitioned in sections, each composed of mechanistic events, which are discussed and evaluated. As core investigation, we reviewed evidence on the interference of NAFLD and/or ALD, without overlooking HBV and HCV infections, with opioid use/abuse. We intended to show that not only NAFLD and ALD may occur in the context of these drugs hepatotoxicity but also that these and other previous hepatic pathologies could influence the susceptibility to opioid hepatotoxicity. In this context, it may be useful to draw the attention of physicians and healthcare authorities on a possible, sometime severe, risk of liver damage subsequent to the chronic use of these substances, with fentanyl as a cornerstone, in patients with hidden or full-fledged ALD, NAFLD, or viral infections.

Methods

To prepare this narrative review, the authors first interrogated PubMed,[1] Scopus,[2] and Embase[3] to track recent evidence using the following keywords: Opioids addiction, opioids analogs, opiod overdose, opioid antagonist, opium contamination, substance use disorders, fentanyl pharmacokinetics, adverse drug events, nonalcoholic fatty liver disease, hepatic steatosis, gut-liver axis, gut microbiome, obesity, metabolic syndrome, alcoholic liver disease, hepatotoxicity, anesthetics toxicity, microsomal enzymes, liver enzymes, drug induced liver injury, microRNAs, animal models, prognostic factors, viral hepatitis, coronavirus-19 (revised on September 2024). As acronyms were used SUD, NAFLD, ALD, CYP450, COVID-19, ALT/AST, HCV, HBV, COVID-19, DILI, PKs, ADEs, and their combination, thesaurus system such as the Medical Subject Headings (MeSH) terms of the National Library of Medicine was referred to for selecting the appropriate keywords directly related to the topic of interest. Full tests and abstracts, when the previous ones were not available, were screened from January 2000 to April 2023, and successively on September 2024, after duplicates removed. Among the available published articles in English, both preclinical and clinical studies (case reports, epidemiological surveys, RCTs) were critically evaluated with particular attention on key results, limitations, suitability of the methods used to test the initial hypothesis, quality of the results obtained, interpretation of the results, and impact of the conclusions in the area. Additional references were identified in the list of references of previously retrieved articles. The exclusion criteria comprehended no methods described, scarce interpretation of the results, and content redundancy or gray literature. This search ended up in summing up a number of findings, mostly concerning inner mechanisms, linked to liver damage. The final step was citing and listing the researched references.

Liver toxicity: from the beginning

The pioneering studies dealing with liver toxicity of fentanyl are based on its side effects as an anesthetic. Other potential hepatotoxicity can be inferred by the whole class of opioids with same therapeutical use. First of all, the terminology commonly adopted in this field of research should be clarified. Minimum alveolar concentration (MAC) is the concentration of a vapor in the alveoli of the lungs that is needed to prevent motor response to surgical stimulus (Minimum Alveolar Concentration, 2023). The 0.3 MAC indicates a comparable level of anesthetic potency, using the response of male Sprague–Dawley rats to tail clamping, and determining the 50% effective dose [ED50] value related to thiopental (Shingu et al., 1983).

A historical research on a single sub-anesthetic dose of fentanyl showed that it had no effect on liver enzymes. While rats receiving the same dose, i.e., 15.6 /mcg/kg bodyweight of fentanyl (0.3MAC), for 6 consecutive days showed increase of transaminases, mainly GPT, also known as ALT, after repeated injections of fentanyl, with no evidence

1 https://pubmed.ncbi.nlm.nih.gov/, accessed on April 2023

2 https://www.scopus.com/search/form.uri?display=basic&zone=header&origin=#basic, accessed on April 2023

3 https://www.embase.com/, accessed on April 2023

of liver necrosis (Fassoulaki et al., 1986). The previous findings were not consistent with the results of another study using the hypoxic rat model. In fact, fentanyl caused the most severe hepatic injury with centrilobular localization when compared with several anesthetics, with exception of halothane (Fassoulaki et al., 1984).

Still, to make matters more complicated, all the anesthetics have long ago been hypothesized that could affect the liver through perturbing the permeability of liposome membranes and consequently impairing cell functions (Fassoulaki et al., 1984). Accordingly, it has been evidenced that enflurane, isoflurane, halothane, and fentanyl in an animal-based study were associated with the same degree of post-anesthetic hepatic "dysfunction," which was judged minimal both in cirrhotic rats (if they were exposed by inhalation to carbon tetrachloride in air at weekly intervals for 12 weeks to induce cirrhosis) and in non-cirrhotic ones (Baden et al., 1985).

In eliciting hepatotoxicity, the role of the liver detoxification system is expected to be highlighted. Starting with the finding that isolated rat hepatocytes metabolize morphine to various compounds (morphinone-glutathione conjugate, normorphine, and morphinone), the addition of morphine to the isolated hepatocytes induced a marked decrease in the level of intracellular glutathione (GSH) and resulted in cell death. The formation of glutathione conjugate was correlated with the loss of GSH. The cytotoxicity of morphinone was higher than that of morphine (Nagamatsu et al., 1986). Following this line of research, a study showed that fatty livers (likely of alcoholic and non-alcoholic origin) have a weak compensation of hepatic GSH regulation, which fails under stress conditions, thus increasing the fatty liver's susceptibility to oxidative damage (Grattagliano et al., 2003).

To confuse the issue whether opioids could induce liver necrosis, a very recent case report of a middle-aged man with a chronic history of drug abuse who presented, after an overdose of cocaine and heroin, with acute liver failure, renews interest in opioid hepatotoxicity (Dolkar et al., 2022).

It is necessary to stress that the kind of hepatic lesions evidenced in old articles focusing on the liver toxicity, extrapolating this datum on the basis of anesthetics, is different in severity, thus leading to uncertainties. Although there are several lines of evidence about a direct hepatic injury from opioids, ascertaining the presence/absence of hepatotoxicity of these drugs is rarely so simplistically cut and dried, and certainly research in this area is still in its infancy, while the topic is a matter of intense socio-political debate yet. The pieces of research dealing with hepatotoxicity by opioids are shown in Table 1.

TABLE 1 Findings from preclinical and clinical studies concerning the liver toxicity induced by opioids.

Authors	Results from the study	Reference number (year)
Kokubun et al.	Child-Pugh score determines variation in serum fentanyl concentrations, inducing potential side effects.	Kokubun et al. (2012)
Fassoulaki et al.	Findings of ↑ serum ALT after 6 days of 15.6 mcg/kg of fentanyl in male Sprague–Dawley rats with no liver necrosis	Fassoulaki et al. (1986)
Fassoulaki et al.	Fentanyl caused most severe hepatic injury with centrilobular localization in the hypoxic rat model	Fassoulaki et al. (1984)
Baden et al.	Fentanyl gave minimal hepatic dysfunction in both cirrhotic and non-cirrhotic rats like enflurane, isoflurane, and halothane.	Baden et al. (1985)
Nagamatsu et al.	The cytotoxicity of morphinone was higher than that of morphine due to marked ↓ levels of intracellular glutathione resulting in cell death.	Nagamatsu et al. (1986)
Dolkar et al.	A middle-aged man with a chronic history of drug abuse presented with acute liver failure after an overdose of cocaine.	Dolkar et al. (2022)

Similar features of alcohol-induced liver damage and opioid cytotoxicity

Before pointing out the additional effects of contextual habits, i.e., heavy alcohol drinking and opioid use/abuse, the liver cytotoxicity by excessive ethanol consumption should be opportunely addressed. Interaction between CYP2E1, ethanol metabolites, and enhanced lipid peroxidation is linked to the pathogenesis of alcoholic liver disease. That said, even CYP2A6, CYP3A415 and.

CYP3A4 induction also leads to consequent generation of acetaldehyde and lipid peroxidation-derived protein-aldehyde adducts in the liver (Niemelä et al., 2000), which are hybrid compounds resulting by interaction of alcohol metabolites with other complex molecules, confirming previous findings in the liver of alcohol-treated rats (Niemelä et al., 1998). Moreover, when looking at the early phase of histological liver damage in patients abusing of alcohol beverages, the presence of protein adducts in the centrilobular region of the liver (the same one interested in opioid cytotoxicity) shows that "adducts" formation is one of the leading events in the molecular processes displayed during the ALD (Niemelä, 1999).

Bidirectional relationship between alcohol/opioids and gut flora

Alcoholic and/or opioid addiction, separately and contextually, is modified by gut microbiome, in the sense that these substances, impacting on gut flora, alter drug biotransformation pathways, but it is also true the opposite. Morphine-induced microbial dysbiosis associated with the gut barrier disruption was completely reversed by transplanting placebo-treated microbiota into morphine-treated animals (Banerjee et al., 2016). Morphine metabolism and elimination, both of them, are dominant in assessing their efficacy and its adverse

effects (Smith, 2009). The same is valid for alcohol toxicity, in that among the four main parameters, absorption, distribution, metabolism, and excretion only metabolism plays a central role. In fact, the elimination of ethanol from the body occurs primarily through metabolism (92–98% of dose) (Jones, 2019).

Indeed, that gut microbiota exerts an important role during enterohepatic circulation either before drug absorption or through various microbial enzymatic reactions in the gut, which is a cogent finding (Zhang et al., 2018). The importance of gut microbiota in providing information on drug metabolism is evidenced by *in vivo*, *in vitro*, *ex vivo*, *in silico,* and multi-omics approaches (Dhurjad et al., 2022).

Gut–liver axis: the intersection between microbiome, liver metabolism, and inflammation

It is well-established that disruption of intestinal epithelial integrity bears as consequence the bacterial translocation from the gut (Schulzke et al., 2009). Indeed, both of these processes are mediated by toll-like receptor (TLR2 and TLR4) signaling in morphine-treated mice (Meng et al., 2013). Following the previous line of research, demonstrating that morphine treatment gives place to a primary Gram-positive bacterial dissemination, authors using a murine model of poly-microbial sepsis showed the induced, sustained upregulation of interleukin (IL)-17A and IL-6. The over-expression of IL-17A compromised intestinal epithelial barrier function, increased gut permeability, maintained bacterial dissemination, and elevated systemic inflammation. In keeping with previous findings, analysis of the gut microbiome showed that morphine treatment induced enrichment of the Firmicutes phylum mostly, and specifically the Gram-positive bacterial species *Staphylococcus sciuri*, *Staphylococcus cohnii*, and *Staphylococcus aureus,* as well as *Enterococcus durans*, *Enterococcus casseliflavus*, *Enterococcus faecium*, and *Enterococcus faecalis* in the gut microbiome. IL-17A neutralization protected barrier integrity and improved survival in morphine-treated animals (Meng et al., 2015). At this point, it is mandatory to report another gut dysbiosis induced by chronic alcoholic beverages. Alcohol is one of the main factors that alters the proper functioning of the gut, leading to a disruption of the intestinal barrier integrity that increases the permeability of the mucosa with a trend for a depletion of bacteria with anti-inflammatory activity, such as *Bacteroidetes* and *Firmicutes* phyla, and an increase in bacteria with pro-inflammation activity, such as *Proteobacteria* (Mutlu et al., 2012). Gut flora function, especially related to bile acid metabolism, can modulate alcohol-associated injury from the less to the more severe form, i.e., alcoholic cirrhosis (Bajaj, 2019). Microbiota changes might also alter brain function, and the gut–brain axis might be a potential target to reduce alcoholic relapse risk. Particularly, the expansion of *Bifidobacterium* and *Lactobacillus* suggests that probiotic interventions for patients with alcohol-related disorders could be useful (Dubinkina et al., 2017). It is salient to analyze another emerging aspect involving gut flora modifications and systemic inflammation, process not only linked to life styles (addiction and obesity) but also to the frailty of old people, who for chronic inflammatory diseases, such as rheumatoid arthritis or osteoarthritis, are on opioid therapy for persistent ache. Pain in the elderly population, burdened by physiological, pharmacological, and psychological aspects, is a major problem of caring in the geriatric setting. In a random chart review of 300 US veterans, 44% of those receiving an analgesic also received opioids (Chau et al., 2008). Opioids are one of the major causes of adverse drug events (ADEs) during hospitalization or shortly after discharge. Out of 10,917 patient records, 357 ADEs were identified, of which 28 (8%) involved opioids (Schutijser et al., 2020). Chronic low-grade inflammation has been speculated to accelerate the aging process, as well as frailty. Intestinal homeostasis employs a crucial role in healthy aging (Xu et al., 2021). In this sense, the prebiotic consumption modifies the intestinal microbiota but unfortunately has little action on markers of inflammation (Mysonhimer et al., 2023).

Returning to the intestinal microbiota, several lines of evidence suggest that obesity-related NAFLD could impact on patients who habitually use/abuse opioids. In a recent study, 72 cirrhotics chronically on opioids were age and severity—by model for end-stage liver disease and prior hepatic encephalopathy (HE)—balanced with 72 cirrhotics on no opioids. Stool microbiota composition (multi-tagged sequencing); predicted functionality, as estimated using so-called PiCRUST; endotoxemia; and inflammatory markers were comparatively evaluated. Significant gut flora modification was observed in the opioid cohort, especially in encephalopathic patients on opioids with lower abundance of the autochthonous families compared to others (Clostridiales XIV and *Lachnospiraceae*) along with a decrease in *Bacteroidaceae* relative abundance. PiCRUST showed the highest aromatic amino acid and endotoxin production in opioid users, who also had higher levels of IL-6. In contrast with previous findings addressing the intestinal permeability, in the aforementioned study, addicts were not characterized by increased levels of IL-17. Interestingly, the hypothesis of the authors was that opioids, being associated with obesity in that population, may predispose to the development or worsening of NAFLD. Surprisingly, alcoholic etiology did not impact on the gut flora (Acharya et al., 2017). The interference of opioids on cytokine profile is testified by the fundamental role of Il-6 in the development of morphine tolerance (Liu et al., 2019). The inflammatory cytokines, taken previously into account, engage a salient role also in patients with NAFLD, as possible liver co-morbidity of opioids dependents, mainly if the attention is focused on the microRNA-26a (miR-26a) that exhibits anti-inflammatory immune effects on immune cells (Yu et al., 2022). The mir-26a-IL-6-IL-17 axis attenuated NAFLD through inhibition of IL-6 in a murine model of high-fat diet-induced obesity. Moreover, IL-17 neutralization markedly decreased total liver weight, triglyceride deposition in liver (thus, hepatic steatosis), and serum transaminase (ALT) concentration when compared with the control group (He et al., 2017). Consequently, mir-26a, regulating insulin sensitivity and metabolism of glucose and lipids (Fu et al., 2015), was reduced in the livers of patients with NAFLD (Xu et al., 2021). Still, miR-26a-5p is hypothesized to carry out a protective role against DILI via targeting Bid, a pro-apoptotic member of the Bcl-2 family (Zhang et al., 2022). The reported findings have a certain significance in the light that neurovascular and immune system alterations are mediated by miRNA networks that function as regulators of cellular response to opioids (Grimm et al., 2023). Summing up, many molecular processes displayed, in the aforementioned studies, zero in both immunological and microbiological aspects. They are visible in Table 2 and Figure 1.

Continuing the focus on the impact of gut flora as foremost, but not unique, mechanism of liver damage, in addition to the role of

TABLE 2 Gut flora alterations associated with liver damage in course of opioid addiction and in presence of alcoholic and non-alcoholic fatty liver disease.

Authors	Findings from the study	Reference number (year)
Zhang et al.	Intestinal flora can influence pharmacokinetics, modulating therapeutic effects and side effects of drugs.	Zhang et al. (2018)
Meng et al.	Morphine induced in mice gut epithelial barrier dysfunction and subsequent bacteria translocation mediated by TLR2 and 4 signaling.	Meng et al. (2013)
Meng et al.	Morphine treatment induced enrichment of the Firmicutes phylum and specifically the Gram-positive bacterial species *Staphylococcus sciuri*, *Staphylococcus cohnii*, *Staphylococcus aureus, Enterococcus durans*, *Enterococcus casseliflavus*, *Enterococcus faecium*, and *Enterococcus faecalis* in the gut microbiome, using a murine model of poly-microbial sepsis due to activation of TLR2 induced sustained upregulation of IL-17A and IL-6.	Meng et al. (2015)
Mutlu et al.	Alcohol led to a disruption of the intestinal barrier integrity with ↑ permeability of the mucosa with depletion of bacteria with anti-inflammatory activity, such as *Bacteroidetes* and *Firmicutes* phyla, and ↑ in bacteria with pro-inflammation activity, such as *Proteobacteria* in 48 alcoholics.	Mutlu et al. (2012)
Bajaj et al.	Bile acid metabolism, related to gut flora, modulated alcohol-associated injury until alcoholic cirrhosis.	Bajaj et al. (2019)
Acharya et al.	Patients with encephalopathy+opioid use showed lower abundance of the autochthonous families Clostridiales XIV and *Lachnospiraceae* compared to others with ↓ in *Bacteroidaceae* relative abundance. PiCRUST evidenced highest aromatic amino acid and endotoxin production in opioid users, who had higher levels of IL-6.	Acharya et al. (2017)
Liu et al.	The interference of opioids on inflammatory cytokines is testified by the fundamental role of Il-6 in the development of morphine tolerance.	Liu et al. (2019)
Yu et al.	Interleukins exert a central role also in patients with NAFLD, mediated by microRNA-26a. exhibiting anti-inflammatory/immune effects.	Yu et al. (2022)
He et al.	The mir-26a-IL-6-IL-17 axis attenuated NAFLD through inhibition of IL-6 in a murine model of high-fat diet-induced obesity. IL-17 neutralization markedly decreased total liver weight, hepatic triglyceride deposition, and serum ALT concentration when compared with the control group.	He et al. (2017)
Xu et al.	Mir-26a, regulating insulin sensitivity and metabolism of glucose and lipids, was reduced in the livers of patients with NAFLD.	Xu et al. (2021)
Zhang et al.	MiR-26a-5p plays a protective role against DILI via targeting Bid, a pro-apoptotic member of the Bcl-2 family.	Zhang et al. (2022)
Grimm et al.	Neurovascular and immune system alterations are mediated by miRNA networks functioning as regulators of cellular response to opioid abuse.	Grimm et al. (2023)
Meijnikman et al.	There is an endogenous production of alcohol in NAFLD patients. *Lactobacillaceae* correlated with postprandial peripheral ethanol concentrations.	Meijnikman et al. (2022)

glucose dysmetabolism (Bedogni et al., 2012), as well as the involvement of lipids (Pei et al., 2020) in the onset and progression of NAFLD, recently the endogenous production of alcohol is emerging. In fact, to test the hypothesis that the gut microbiota of patients with NAFLD produce enough ethanol to be a driving force in the development and evolution of this highly prevalent liver disease, the authors performed both a prospective and intervention clinical study, whose results suggested that microbial ethanol could be considered fundamental to the pathogenesis of NAFLD. Furthermore, *lactobacillaceae* correlated with postprandial peripheral ethanol concentrations (Meijnikman et al., 2022). This last finding apparently closes the circle. In conclusion, based on the above studies, there is likely an association between the intestinal microbiota and liver disease, but a causal relationship has yet to be confirmed (Schwenger et al., 2019).

Coming back to metabolic dysfunctions in opioid addicts, not always overlapping, as evident in Table 3, a systematic review comprehending 46 articles and 37,407 participants suggested that opioids increase serum triglycerides (thus, inducing or worsening hepatic steatosis) and may reduce blood glucose and low-density lipoproteins (LDLs). However, these effects are temporary, even though long-term drug dependence exacerbates glucose and lipid-associated diseases such as diabetes mellitus and atherosclerosis (Rezaei and Rezaei, 2021). In keeping with precedent data on LDL, the authors of a recent meta-analysis showed that opium abuse significantly decreased total cholesterol compared to that of controls but only in diabetic patients. On the contrary, concerning body mass index (BMI), the differences were not statistically significant. Those researchers concluded that nutritional deficiencies, weight loss, and lipid dysregulation due to liver dysfunction (as sequela of previous diseases) may explain the findings (Ojo et al., 2019). On the other side, compelling results show that chronic heroin administration produces a state of fasting hyperinsulinemia (again, another driver of NAFLD) even in the absence of obesity (Passariello et al., 1983). Further data show that alteration of glucose metabolism evidences similarities between opiate addicts and non-insulin dependent diabetics (Ceriello et al., 1987). Indeed, the lack of involvement of BMI in opioid abusers is challenged by other pieces of research.

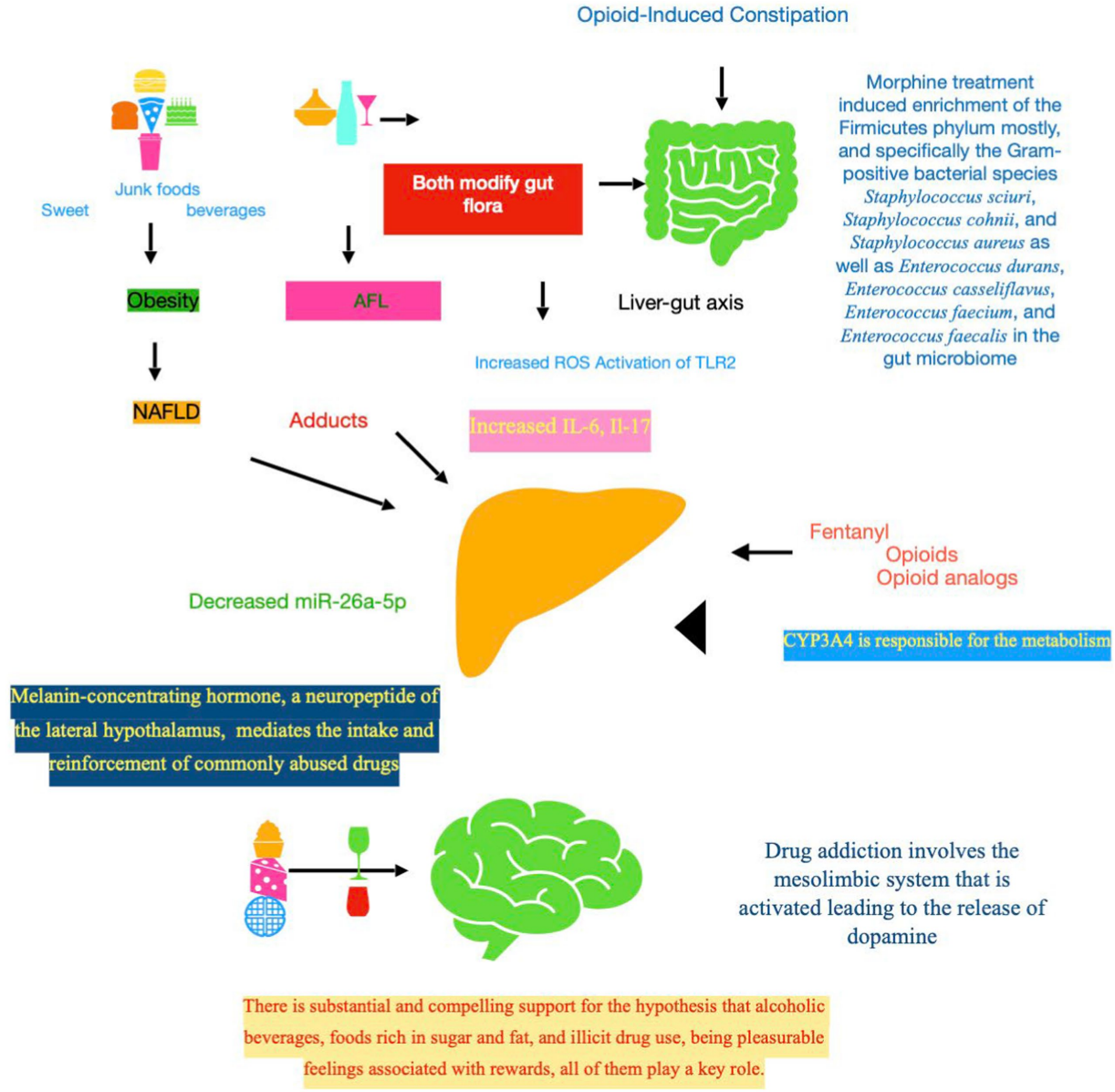

FIGURE 1
Downstream signaling events in opioid-related liver toxicity. First of all, it should be stressed that fentanyl contextually impacts on three levels, which are intertwined and bidirectional, i.e., CNS, gut flora, and liver. The complex ecosystem known as the gut microbiota, encompassing bacteria, viruses, and fungi living in the gastrointestinal system, plays a fundamental role in the control of the host energy metabolism. The liver–gut axis, mediated by intestinal microbiota, could be the link between co-existing liver disease, such as NAFLD and ALD, and the drug addiction. Acetaldehyde, a major toxic metabolite, is one of the principal culprits mediating fibrogenic of alcohol in the liver. Mechanistically, acetaldehyde promotes adduct formation, leading to functional impairments of key proteins, including enzymes. Indeed, gut microbiota of individuals with NAFLD produce enough ethanol to be a driving force in the development and progression of this disease. Gut microbial dysbioses are linked to aberrant immune responses, which are often accompanied by abnormal production of inflammatory cytokines (Il-6 and Il-17). The toxicity of opioids could be mediated also by *CYP3A4,* key enzyme involved in the fentanyl metabolism. MCH, a neuropeptide primarily transcribed in the lateral hypothalamus, displays multiple functions, mostly controlling feeding behavior and energy homeostasis and regulating the stress axis and emotion. MCH signaling pathway is a potential target to treat obesity, and also anxiety and depression that are at the basis of addiction. NAFLD, non-alcoholic fatty liver disease; ALD, alcoholic liver disease; ROS, reactive oxygen species; TRL, toll-like receptor; IL, interleukin; CYP, cytochrome P450 enzyme; SNC, central nervous system; MCH, melanin-concentrating hormone.

Starting with the growing consensus that the overconsumption of readily available and highly palatable foods likely contributes to the growing rates of obesity worldwide (Johnson and Wardle, 2014), the high intake of ultra-processed foods was associated with an increased risk of NAFLD, as evidenced in 6,545 participants who were recruited in National Health and Nutrition Examination Surveys 2011–2018 (Liu et al., 2022), but the point is that both preference and consumption of sweet substances often parallel the self-administration of several drugs of abuse, and under certain conditions, the termination of intermittent access to sweet substances produces symptoms that resemble those observed during opiate withdrawal (Gosnell and Levine, 2009).

Possible interactions between opioid abuse and organ senescence bring us to other pieces of research dealing with impaired oxide reductive processes, mostly involved in drug addicts, as well as in patients suffering from ALD and NAFLD. Cellular senescence is stress-responsive program limiting the proliferation of damaged cells and leading to stable cell cycle arrest. An increase in ROS levels has been demonstrated to be crucial to inducing and maintaining senescence process (Davalli et al., 2016). Hepatocyte senescence can represent a foremost mechanism inducing intracellular fat accumulation, fibrosis and inflammation, characteristics of NAFLD, and its more severe form, i.e., non-alcoholic steatohepatitis, due to secretion of senescence-associated inflammatory mediators (Engelmann and Tacke, 2022). Analyzing sub-sequential molecular events related to the injury toward hepatocytes, alcohol stimulates the activity of cytochrome P450 enzymes, which contribute to ROS production, and reduces the levels of the antioxidant system (Wu and Cederbaum, 2003). Consequently, ethanol-induced oxidative stress causes DNA damage and defective DNA repair, inhibiting a main DNA repair factor that is 53BP1. The improperly repaired DNA damage further activates cell cycle checkpoint proteins, such as p53 and p16INK4α, and leads up to the onset of premature senescence (Romero et al., 2016). Finally, senescence markers, such as the transcription factors E2F1 and ID1 and the insulin-like growth factor binding protein-3, were significantly altered in ethanol-fed mouse liver specimens compared to controls (Meng et al., 2017). On the other hand, opioids increase the levels of ROS and decrease the activity of superoxide dismutase, catalase, and glutathione peroxidase that function as enzymatic antioxidants. Moreover, opioids augment the risk of vitamin deficiency and modify gene expression of target cells through ROS production (Zahmatkesh et al., 2017). To make matter worse, many opiate and alcohol addicts present with calcium and magnesium deficiencies due to poor diet and inadequate intake of calcium (Nabipour et al., 2014). Recently, there are many literature data indicating a key involvement of calcium signaling in cellular senescence (Martin and Bernard, 2018), as well as of magnesium (Killilea and Maier, 2008). Conclusively, production of ROS/nitrogen species can contribute to degenerative diseases and organ dysfunction, likely also of liver, occurring in morphine addicted or morphine-treated patient (Skrabalova et al., 2013). To prove the importance of these biochemical pathways, a recent research enrolling 1,602 patients with SUD compared with 2,858 non-SUD patients showed that there is an acceleration of age-related and degenerative pathologies in many tissues, evaluated by the higher levels of serum glucose, HbA1c elevation, fructosamine, concentrations, and microalbumin levels, all of them being biomarkers of age (Reece, 2013). In line with the centrality of oxidation–reduction process involved in drug abuse, a systematic review of the RCTs present into literature until 2017 showed the safety profile of N-acetylcysteine and its favorable tolerability, in addition to being an over-the-counter medication with an interesting potential clinical use for craving in SUDs (Duailibi et al., 2017). This is to cast a fresh light on therapeutical approach to SUD. Paradoxically, researchers have found that a preconditioning of one potent, short-acting synthetic opioid analgesic drug that is remifentanil could protect against hypoxia-induced cellular senescence (Lewinska et al., 2020).

How does constipation contribute to opioid toxicity and what is the link between opioid-induced constipation and infections?

Now, it is required to deepen how other deleterious consequences of opioid abuse can modify the hepatocyte integrity adding further damage to the preexisting alcohol or metabolic one. That opioids have a poor side effect profile which is ascertained. Indeed, the incidence of opioid-induced constipation is consistently high, varying from 15 to 81% (Lang-Illievich and Bornemann-Cimenti, 2019). Administration of opiates produces a rapid increase in release of ADH, (Van Vugt and Meites, 1980), as well as opioid use can lead to a reduction of fluid intake (Mallappallil et al., 2017). Fluid restriction increases constipation (Arnaud, 2003). Opioid-induced bowel dysfunction, manifesting as constipation, is mediated by peripheral μ-opioid receptors with resultant altered gastrointestinal motility (Lee and Hasler, 2016). Among factors impacting on gut motility, and thus constipation, beyond the action of immune and nervous system, the metabolism of bile acids, and secretion of mucus, the gastrointestinal microbiota is relevant. In fact, modifying the gut luminal environment, i.e., decreased numbers of *bifidobacteria* and *lactobacilli* in stool sample, with some probiotics, the motility and secretion in the gut are improved (Dimidi et al., 2017). To try to find a link between constipation from opioids and the possible underlying NAFLD of these subjects, recent observations hypothesize that *Lactobacillus* decreases intestinal lipid absorption, consequently protecting against diet-induced steatosis *in vivo* (Jang et al., 2019). Researchers have seen that opioids can lead to a dehydration status (Mallappallil et al., 2017), but it should not be overlooked the fact that also ethanol ingestion affects the hypothalamo-neurohypophysial system resulting in increased diuresis, dehydration, and hyperosmolality, thus summing up the effects of dehydration to altered microbiome (Madeira et al., 1993). Accordingly, recent pieces of research indicate that drinking water may be an important factor in shaping the human intestinal flora (Vanhaecke et al., 2022). In fact, euhydration and dehydration states determine changes in microbiota community and the immune response (Lukito, 2021). To get a further glimpse on microbiota, it is necessary to highlight that many infections of immunocompromised patients, such as alcohol addicts (Szabo and Saha, 2015), originate from the gastrointestinal tract, with alteration of the whole flora (Taur and Pamer, 2013). In fact, bloodstream infections are a frequently observed complication in liver cirrhosis patients of alcoholic origin (Xie et al., 2017). Stressing that concentrations of *Bifidobacterium* and *Lactobacillus* are significantly lower in constipated patients, potentially pathogenic bacteria and/or fungi could be increased (Khalif et al., 2005). In fact, the link between opioid-induced constipation and infection is represented by the frequent association of constipation with urinary tract infection (UTI) and upper urinary tract dilatation (Naseri et al., 2022). That gastrointestinal tract is one of the main anatomical regions implicated in the pathophysiology of UTIs, which is proved by the finding that intestinal dysbiosis may upregulate the expression of serotonin transporter involved in regulating gastrointestinal motility and contribute to the development of chronic constipation, supporting a never-ending vicious circle (Cao et al., 2017). To support the connection between drug abuse and constipation,

TABLE 3 Metabolic dysfunctions in opioid addicts.

Authors	Findings from the study	Reference number (year)
Rezaei et al.	Systematic review showing that opioids ↑ serum triglycerides and ↓ blood glucose and low-density lipoproteins.	Rezae and Rezaei (2021)
Ojo et al.	Meta-analysis showing that levels of total cholesterol were ↓ in diabetic patients, abusing of opium. Nutritional deficiencies, weight loss, and lipid dysregulation, due to liver dysfunction, may explain the findings.	Ojo et al. (2019)
Passariello et al.	Chronic heroin administration produced a state of fasting hyperinsulinemia even in the absence of obesity.	Passariello et al. (1983)
Passariello et al.	Alteration of glucose metabolism evidenced similarities between opiate addicts and non-insulin dependent diabetics.	Ceriello et al. (1987)
Gosnell et al.	Preference/consumption of sweet substances often parallels the self-administration of several drugs of abuse, and the termination of intermittent access to sweet substances produces symptoms that resemble those observed during opiate withdrawal.	Gosnell and Levine, (2009)
Zahmatkesh et al.	Opioids ↑ the levels of ROS and ↓ those of the activity of superoxide dismutase, catalase, and glutathione peroxidase that function as enzymatic antioxidants. Furthermore, they ↑ the risk of vitamin deficiency and modify gene expression of target cells through ROS production.	Zahmatkesh et al. (2017)
Nabipour et al.	Many opiate and alcohol addicts present with ↓ Ca and ↓ Mg due to poor diet and inadequate intake of *Ca.*	Nabipour et al. (2014)
Skrabalova et al.	Production of ROS/nitrogen species can contribute to degenerative diseases and organ dysfunction, likely of liver, occurring in morphine abusers or morphine-treated patients.	Skrabalova et al. (2013)
Reece et al.	1,602 patients with SUD compared with 2,858 non-SUD patients showed an acceleration of age-related and degenerative pathologies in many tissues, evaluated by the higher levels of serum glucose, HbA1c elevation, fructosamine, concentrations, and microalbumin levels.	Reece et al. (2013)
Duailibi et al.	A systematic review of the RCTs present into literature until 2017 showed the safety profile of N-acetylcysteine and its favorable tolerability, in addition to being an over-the-counter medication with an interesting potential clinical use for craving in SUDs.	Duailibi et al. (2017)
Lewinska et al.	Paradoxically, researchers have found that a preconditioning of one potent, short-acting synthetic opioid analgesic drug that is remifentanil could protect against hypoxia-induced cellular senescence.	Lewinska et al. (2020)
Dimidi et al.	Playing gastrointestinal microbiota a primary role in constipation, modification of the gut luminal environment, with certain probiotic ends up in improvement of the motility and secretion in the gut, resulting in *bifidobacteria* and *lactobacilli.*	Dimidi et al. (2017)
Jang et al.	A link between constipation from opioids and the underlying NAFLD is hypothesized by the fact that *Lactobacillus* decreases intestinal lipid absorption, consequently protecting against diet-induced steatosis *in vivo.*	Jang et al. (2019)
Mallappallil et al.	Opioid use can lead to a reduced fluid intake.	Mallappallil et al. (2017)
Van Vugt et al.	Administration of opiates produces a rapid increase in release of ADH.	Van Vugt and Meites (1980)

intriguingly, opioid use disorder may lead to the development of primary hypothyroidism (Pezeshki et al., 2023), disease in which constipation is frequently observed (Pustorino et al., 2004). Coming back to infections, it should be highlighted that fentanyl fundamentally exerts a relevant immunosuppression (Sacerdote, 2008). Because each functional compartment of the immune system plays a specialized role in host defense, defects in specific functions lead to increased susceptibility to specific pathogens (Dropulic and Lederman, 2016). In addition, use of both opioids and other substances through IV administration places individuals at increased risks of infectious diseases ranging from invasive bacterial and fungal infections to human immunodeficiency virus and viral hepatitis (Marks et al., 2022). In this context, it has been evidenced how fungal products (beta-glucans and candidalysin) activate the host's immune system to exacerbate liver and biliary diseases (Hartmann and Schnabl, 2023).

The role of cytochrome P450 enzymes

To give a broader view of the mechanisms underlying the possible liver damage, the metabolic pathways of opioids should be accentuated. Specifically, could liver microsomes exercise a significant part in inducing opioid hepatotoxicity, independently from the absorption and excretion of drugs? This has naturally led to deep dispute within the field. In human liver microsomes, there were no statistically significant differences in CYP activity as a function of age, gender, or ethnicity with *CYP3A4* activity being slightly greater in women than men (Parkinson et al., 2004).

Utilizing a proteomics approach to quantify the protein expression of *CYP3A4*, the results suggest that NAFLD and diabetes mellitus are associated with the decreased hepatic *CYP3A4* activity (Jamwal et al., 2018). In keeping with the previous findings, recent results demonstrate that the transcription repression of the *CYP3A4* activity in turn promotes liver fat excess (Huang et al., 2019), thus worsening NAFLD. Consequently, changes in definite cytochrome P450 enzymes, as those previously evidenced in livers of patients with steatosis, but also *in vivo* models of steatosis (in experimental animals) and *in vitro* models of fat-overloaded cells, pose major impact on drug-induced hepatotoxicity (Gómez-Lechón et al., 2009), on the basis that *CYP 3A4* is the main responsible for the metabolism of fentanyl (Figure 2) (Tateishi et al., 1996). Among the various factors that may influence the pharmacological response to opioids, genetic polymorphisms have generated some interest. A recent meta-analysis including 59 studies showed that *CYP3A4*1G* carriers consumed less opioids than homozygous *CYP3A4*1/*1* patients during the first 24 h of the postoperative course (Ren et al., 2015). Using a preclinical model, the authors evidenced that chronically fentanyl-treated animals (male Brown Norway X F344 rats aged 12, 18, 24, and 30 months) lost both fat and lean mass during early and late drug administration, as well as during early withdrawal, independently from aging (Mitzelfelt et al., 2011). To evaluate the link between *CYP3A4* polymorphisms and drug addiction risk, the authors found that in the Chinese Han population, rs4646440 and rs4646437 were associated with decreased risk of drug addiction in co-dominant and recessive models, while rs3735451 and rs4646437 were associated with drug addiction risk in the middle-aged and elderly people. Finally, rs3735451, rs4646440, and rs4646437 had strong relationship with decreased risk of drug addiction for men (Wang et al., 2019). Considering the high inter-subject variability of CYP 450, the activity of this superfamily of enzymes has been largely attributed to gene polymorphism. Indeed, this interpretation has quite completely changed following the rapid development in epigenetics that has revealed another aspect of regulatory mechanism of drug-related genes (Tang and Chen, 2015). Among the many epigenetic factors, affecting CYP expression, beyond histone modification, non-coding (nc) RNA regulation was particularly deepened. Of the ncRNA classes, miRNAs play a key role (Nakano and Nakajima, 2018). Numerous "stressors" from various sources, including food and alcohol (again the link with liver diseases), tobacco, air pollution, some pharmaceuticals, and commercial products, have been shown to alter the expression of miRNAs (Marrone et al., 2014). Furthermore, exposure to DNA methylating agents may lead to hyper-methylation of CYPs genes by DNA methyltransferases and inhibition of CYP expression. Consequently, also DNA methylation potentially contributes to the inter-individual expression of *CYP3A4 in* drug metabolism and associated adverse drug effects (Kacevska et al., 2012). It is essential to underline that chronic alcohol consumption, the cause of ALD, leads to enhanced activity of another member of the CYP family, i.e., *CYP2E1*, in the smooth endoplasmic reticulum (Leung and Nieto, 2013). Consequently, this difference could have potential major implications for the risk of drug toxicity.

Will opioid use contribute to liver damage within the context of the viral infection?

New cases of acute hepatitis C virus (HCV) have increased rapidly in the US since 2010 and have most often been associated with injection drug use. Similarly, a recent national boost in acute hepatitis B (HBV) has coincided with an epidemic of opioid abuse (Centers for Disease Control and Prevention, 2017). As a consequence, there is a significant rise in the proportion of organ donors who died due to overdose, but a better use of donors in the light of the risk of disease transmission is needed (Verna et al., 2019). However, there is evidence that opioid agonist therapy is associated with increased odds of HCV treatment initiation among people who use drugs (Bartlett et al., 2022). Exogenous opioids, such as morphine and fentanyl, have been found to impair the function of macrophages, natural killer cells, and T cells and weaken the gut barrier *in vitro* and in animal studies (Plein and Rittner, 2018). The opioid-induced immunosuppression, coupled with the release of cytokines, hormones, and bacterial products from intestinal tract, could explain from a hand the lack/slowness of viral clearance and on the other hand the ongoing liver injury. Drug users who are successfully treated for HCV infection can become reinfected if they inject unsafely (Proust et al., 2000). That said, fentanyl kinetics, studied in patients with cirrhosis, showed that its elimination half-life is not primarily influenced by the rate at which it is metabolized in the liver (Haberer et al., 1982). Surprisingly, HCV infection impacts gut–liver axis by altering the composition of gut microbiota (dysbiosis), characterized by a loss of microbial diversity and the expansion of potential pathogens (Marascio et al., 2022).

Furthermore, recent findings provide evidence of alterations of the gut microbiome and some related metabolites in patients with both immunotolerant or immuno-active phase of HBV infection (Li et al., 2022). By these findings, it is likely that the aforementioned diseases could impact on pharmacokinetics of opioids.

Concerning another viral infection, in one cross-sectional study, deaths due to opioid toxicity increased substantially during the COVID-19 pandemic (Mamdani et al., 2023). Intriguingly, although most patients with COVID-19 have a mild increase in transaminases, the highest rate of liver damage is in adult patients (Sadeghi Dousari et al., 2023). Obviously, the liver damage induced by this viral infection could be worsened by a contextual drug toxicity, mainly in patients with long-term COVID-19, whose laboratory data show ALT and AST levels persistently elevated (de Lima et al., 2023). An important point is that patients on opioid overdosing can present rhabdomyolysis but are often not diagnosed (Babak et al., 2017). Rhabdomyolysis could mimic liver damage, presenting with a marked transaminases elevation (Lim, 2020), and causes acute kidney injury (Hebert et al., 2022). Finally, also fentanyl overdose creates the same renal damage (Mallappallil et al., 2017).

Discussion

Surfing the literature, the answer to the initial question whether there is evidence of liver toxicity by opioids, synthetic opioids and mainly by fentanyl is positive. However, many aspects remain unsolved with an unmet need to deepen the topic, in relation to the ways in inducing liver damage, and the timing of use/abuse of these drugs, theme that is subject of much controversy, i.e., pain control. Our main aim, addressing an important question in the light of skyrocketing increase of obesity and alcohol addiction, especially among youngsters, was digging into the results of some studies to draw the evidence that the co-presence of NAFLD and ALF, likely mediated by gut flora dysbiosis, could impact on the onset and progression of these very widespread diseases. Placing this review within the context of previous studies, the authors ought to report

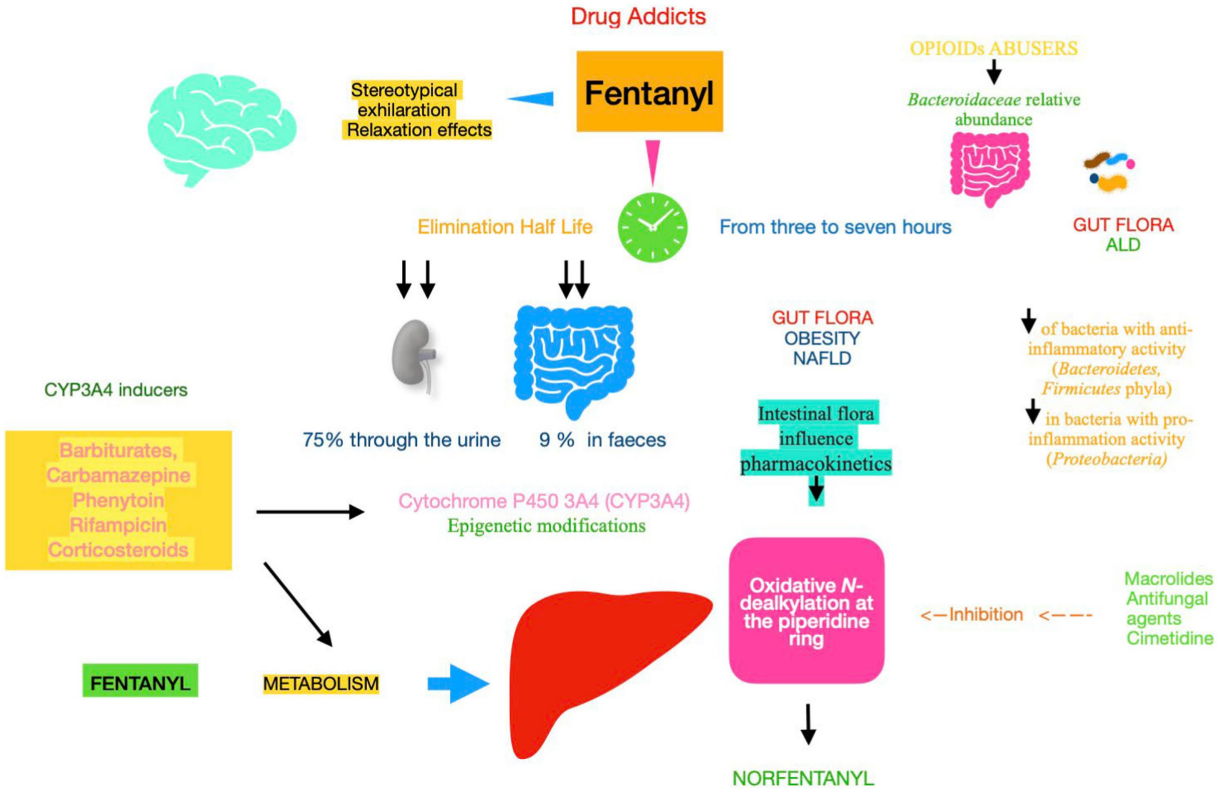

FIGURE 2
Pharmacokinetics and metabolism of fentanyl Fentanyl metabolism and elimination (half-life), both of them, play an important role in determining drug pharmacokinetics and assessing their efficacy and its adverse effects. Gut microbiota plays a key role during enterohepatic circulation along the drug absorption phase and mainly through various microbial enzymatic reactions in the gut. Fentanyl is 99% N-dealkylated to norfentanyl by cytochrome P450. Utilizing a proteomics approach to quantify the protein expression of CYP3A4 and related enzymes, the results suggest that NAFLD is associated with the decreased hepatic CYP3A4 activity. Epigenetic changes in CYP genes would lead to inter-individual differences in drug responses. Understanding epigenetic mechanisms can help reduce toxicity for drugs metabolized by CYP enzymes. NAFLD, non-alcoholic fatty liver disease; ALD, alcoholic liver disease; CYP, cytochrome P450 enzyme.

that data from literature emphasize the main role of gut microbiome in all the three pathologies, such as opioid addiction and both NAFLD and ALD, suggesting that there could be an additive effect in inducing hepatic injury. First, it is intriguing to look at how the results of the selected studies could be important to the audience. The study of the intestinal microbiota reveals that it is constantly evolving in response to host as age, nutrition (the role of rich-calorie foods in obesity), lifestyle (alcoholic beverage abuse), hormonal changes, inherited genes, epigenetic modifications, and underlying disease (NAFLD and ALD as possible examples) are major determinants of the human microbiome at any given point in time (Ogunrinola et al., 2020). As correct hypothesis, a balanced microbiota has a fundamental role in health maintenance. However, gut dysbiosis is a key aspect in linking prior and, in most cases, evolving hepatic diseases with opioid addiction. In fact, alterations in the intestinal microbiota can impact drug metabolism, favoring more toxic metabolites, ultimately injuring liver tissues (hepatotoxicity), and in turn worsening the preexisting alcohol or metabolic liver damage. On the other hand, altered drug metabolism enzyme expression has been found in rodent and human samples of NAFLD (Zhu et al., 2023), with the result that underlying metabolic-induced damage could give place to or worsen liver toxicity by opioids. Nevertheless, without lessening the importance of gut flora, the role of microsomal enzymes, specifically CYP 450, should not overlooked, mainly concerning the genetic expression and epigenetic modifications, as presented in interesting studies. Summing up the aforementioned evidence concerning the opioid-related liver toxicity, there is substantial and compelling support for the hypothesis that alcoholic beverages, foods rich in sugar and fat, and illicit drug use, being pleasurable feelings associated with rewards, all of them conduct a determinant contribution. Research shows that there is a link between substance abuse and obesity, as well as obesity-associated diseases, in brain functioning. In keeping with this evidence, current literature emphasizes the role of melanin-concentrating hormone (MCH), a neuropeptide primarily transcribed in the lateral hypothalamus, in mediating the intake and reinforcement of commonly abused drugs (Morganstern et al., 2020). By the same accord, the opioid antagonist naltrexone blocks the endogenous opioid-mediated inhibition of anorexigenic pro-opiomelanocortin (POMC) neurons, leading to sustained POMC stimulation, thus acting on central appetite and reward region (Walmsley and Sumithran, 2023). Fascinating findings evidence that central MCH directly controls hepatic and adipocyte metabolism through different pathways (Imbernon et al., 2013). Furthermore, intracerebroventricular infusion of MCH caused induction of hepatic steatosis and increase in body weight (prominent aspect) in high-fat diet-fed wild-type mice (Kawata et al., 2017). It is emerging that alcohol excess, apart its direct hepatotoxicity, is of paramount importance in leading to weight gain, mainly when associated with illicit drug

dependence and negative eating behaviors especially in young and very young people (Tarantino et al., 2022). The high prevalence of NAFLD all over the world is associated with the pandemic of obesity. This explosion is testified by an updated analysis finding that mean BMI increased from 23.0 (95% CI, 22.8–23.2) in 1976–1980 to 27.5 (95% CI, 25.5–29.4) in 2017–2018, and the prevalence of obesity increased from 5.5% (95% CI, 4.3–7.0%) to 32.6% (95% CI, 22.1–45.2%) (Ellison-Barnes et al., 2021). In this scenario, the gut flora modification ascribed to NAFLD develops an additive role (Hrncir et al., 2021). To reinforce the pivotal role of microbiota, similar downstream signaling events are involved in the onset and progression of ALD and NAFLD (Fleming et al., 2020). A large proportion of street-purchased opioids includes fentanyl, and the drug has also become an adulterant within the stimulant supply (Tarantino and Citro, 2024). To stem this tide, also the hepatologists' awareness is imperative to increase, coupled with other specialists, beyond educational programs (Office of Addiction Services and Supports, 2023). Limitations to this review consist in the paucity of specific data of literature concerning the models of liver toxicity by opioids that does not permit deepening the sub-sequential molecular processes displayed by the use/abuse of these drugs. Still, a drawback is the lack of clinical trials, utilizing robust methodologies and advanced statistical analysis, in patients contextually suffering from NAFLD and drug abuse, as well as studies addressing both alcohol and drug dependence, focusing on the possible liver damage. It needs to be emphasized, as final observation, that authors do not know for sure whether the modifications of the microbiota are cause, effect, or side effect of opioid addiction in the context of other hepatic diseases.

Future directions

The authors would suggest some other field of research, starting from studies documenting that endogenous and exogenous opioids not only induce analgesic effects by regulating pre- and post-synaptic sensory neurons but also interact with opioid receptors present in the immune system, resulting in immune modulation (Ninković and Roy, 2013). Specifically, the release and diffusion of neurotransmitters favor signaling through lymphocyte cell surface receptors, which regulates the immune response (Sacerdote et al., 2008). Interesting findings suggest that the immune system abnormalities in heroin addicted patients can be restored to almost normal values by controlled treatment with methadone and buprenorphine (Oshaghi et al., 2023). Fascinating enough, opioids can favorably harmonize the neurotransmitter systems controlling mood and are suggested in the treatment of psychiatric disorders (Kawata et al., 2017). Finally, the wide spreading use of antibiotics (English and Gaur, 2010) has serious effects on the host through the gut microbiome and can affect various functions including immune regulation, metabolic activities, and thus overall health (Patangia et al., 2022). It would be captivating to establish whether antibiotic-disrupted gut flora contribute to liver toxicity by opioids and whether phage therapy could be proposed as a clinical alternative to restore intestinal eubiosis, due to its immunomodulatory and bactericidal effect against bacterial pathogens (Gutiérrez and Domingo-Calap, 2020), and consequently reduce liver damage in these addicts. Basing on the finding that magnesium reduces the intensity of addiction to opioids (Magnesium in the Central Nervous Systems, 2023), another interesting approach could be evaluating the use of this essential mineral in trying to alleviate the morphine-induced tolerance and withdrawal symptoms in humans, as was found in mice with administration of lamotrigine or magnesium sulfate or their combination (Habibi-Asl et al., 2009). Still, the main role of the epigenetic changes in CYP genes, leading to inter-individual differences in drug responses, should be stressed to decrease adverse drug reactions for drugs metabolized by CYP enzymes. Specifically, new studies are needed to develop CYP-based pharmacoepigenetics to guide clinical applications for precision medicine with reduced risk of toxicity (Jin and Zhong, 2023). As significant subject, extracellular vesicles may start providing information about mechanisms and pathogenesis in substance use disorders, consenting to potential therapeutic options being probed (Odegaard et al., 2020). Finally, to raise awareness of this nationwide epidemic, educational presentations for students in all age ranges, from elementary school through graduate school, are supposed to be implemented.

Conclusion

The "take-home" message that the authors want our readers to leave with is that there is proof of a close link between key topics such as gut microbiome, liver metabolism, and inflammation in hepatic toxicity in opioid addicts. On this basis, the preexisting liver diseases, such as ALD or NAFLD, could worsen liver toxicity from opioid abuse, as well as opioid consume could bring further damage to the aforementioned liver diseases with complex and difficult to fully address mechanisms. Finally, the interdisciplinary nature of the topic, linking addiction medicine, hepatology, and microbiology, also makes it an interesting and relevant area of study to promote the initial and deserving interest of scientists toward this issue, unfortunately lost in time.

Author contributions

GT: Conceptualization, Investigation, Supervision, Methodology, Writing – original draft. MC: Conceptualization, Investigation, Supervision, Writing – review & editing. VC: Conceptualization, Investigation, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Acharya, C., Betrapally, N. S., Gillevet, P. M., Sterling, R. K., Akbarali, H., White, M. B., et al. (2017). Chronic opioid use is associated with altered gut microbiota and predicts readmissions in patients with cirrhosis. *Aliment. Pharmacol. Ther.* 45, 319–331. doi: 10.1111/apt.13858

Alkhouri, N., Almomani, A., Le, P., Payne, J. Y., Asaad, I., Sakkal, C., et al. (2022). The prevalence of alcoholic and nonalcoholic fatty liver disease in adolescents and young adults in the United States: analysis of the NHANES database. *BMC Gastroenterol.* 22:366. doi: 10.1186/s12876-022-02430-7

American Psychiatric Association (2013). Diagnostic and statistical manual of mental disorders. *5th* Edn. Arlington, VA: American Psychiatric Association.

Arnaud, M. J. (2003). Mild dehydration: a risk factor of constipation? *Eur. J. Clin. Nutr.* 57, S88–S95. doi: 10.1038/sj.ejcn.1601907

Babak, K., Mohammad, A., Mazaher, G., Samaneh, A., and Fatemeh, T. (2017). Clinical and laboratory findings of rhabdomyolysis in opioid overdose patients in the intensive care unit of a poisoning center in 2014 in Iran. *Epidemiol. Health* 39:e2017050. doi: 10.4178/epih.e2017050

Baden, J. M., Kundomal, Y. R., Luttropp, M. E. Jr., Maze, M., and Kosek, J. C. (1985). Effects of volatile anesthetics or fentanyl on hepatic function in cirrhotic rats. *Anesth. Analg.* 64, 1183–1188. doi: 10.1213/00000539-198512000-00009

Baik, J. H. (2013). Dopamine signaling in reward-related behaviors. *Front. Neural Circuits* 7:152. doi: 10.3389/fncir.2013.00152

Bajaj, J. S. (2019). Alcohol, liver disease and the gut microbiota. *Nat. Rev. Gastroenterol. Hepatol.* 16, 235–246. doi: 10.1038/s41575-018-0099-1

Banerjee, S., Sindberg, G., Wang, F., Meng, J., Sharma, U., Zhang, L., et al. (2016). Opioid-induced gut microbial disruption and bile dysregulation leads to gut barrier compromise and sustained systemic inflammation. *Mucosal Immunol.* 9, 1418–1428. doi: 10.1038/mi.2016.9

Bartlett, S. R., Wong, S., Yu, A., Pearce, M., MacIsaac, J., Nouch, S., et al. (2022). The impact of current opioid agonist therapy on hepatitis C virus treatment initiation among people who use drugs from the direct-acting antiviral (DAA) era: a population-based study. *Clin. Infect. Dis.* 74, 575–583. doi: 10.1093/cid/ciab546

Bedogni, G., Gastaldelli, A., Manco, M., De Col, A., Agosti, F., Tiribelli, C., et al. (2012). Relationship between fatty liver and glucose metabolism: a cross-sectional study in 571 obese children. *Nutr. Metab. Cardiovasc. Dis.* 22, 120–126. doi: 10.1016/j.numecd.2010.05.003

Cao, H., Liu, X., An, Y., Zhou, G., Liu, Y., Xu, M., et al. (2017). Dysbiosis contributes to chronic constipation development via regulation of serotonin transporter in the intestine. *Sci. Rep.* 7:10322. doi: 10.1038/s41598-017-10835-8

Centers for Disease Control and Prevention. (2017). *Surveillance for Viral Hepatitis – United States Report*. Available at: https://archive.cdc.gov/#/details?url=https://www.cdc.gov/hepatitis/statistics/2017surveillance/index.htm Accessed September, 2024).

Centre for Disease Control and prevention. (2023). Available at: https://www.cdc.gov/stopoverdose/fentanyl/index.html (Accessed April, 2023).

Ceriello, A., Giugliano, D., Passariello, N., Quatraro, A., Dello Russo, P., Torella, R., et al. (1987). Impaired glucose metabolism in heroin and methadone users. *Horm. Metab. Res.* 19, 430–433. doi: 10.1055/s-2007-1011844

Chau, D. L., Walker, V., Pai, L., and Cho, L. M. (2008). Opiates and elderly: use and side effects. *Clin. Interv. Aging* 3, 273–278. doi: 10.2147/cia.s1847

Comer, S. D., and Cahill, C. M. (2019). Fentanyl: receptor pharmacology, abuse potential, and implications for treatment. *Neurosci. Biobehav. Rev.* 106, 49–57. doi: 10.1016/j.neubiorev.2018.12.005

Davalli, P., Mitic, T., Caporali, A., and Lauriola, A. (2016). Cell senescence, and novel molecular mechanisms in aging and age-related diseases. *Oxidative Med. Cell. Longev.* 2016:3565127. doi: 10.1155/2016/3565127D'Arca D. Ros

de Lima, I. C., de Menezes, D. C., Uesugi, J. H. E., Bichara, C. N. C., da Costa Vasconcelos, P. F., Quaresma, J. A. S., et al. (2023). Liver function in patients with long-term coronavirus disease 2019 of up to 20 months: a cross-sectional study. *Int. J. Environ. Res. Public Health* 20:5281. doi: 10.3390/ijerph20075281

Dhurjad, P., Dhavaliker, C., Gupta, K., and Sonti, R. (2022). Exploring drug metabolism by the gut microbiota: modes of metabolism and experimental approaches. *Drug Metab. Dispos.* 50, 224–234. doi: 10.1124/dmd.121.000669

Dimidi, E., Christodoulides, S., Scott, S. M., and Whelan, K. (2017). Mechanisms of action of probiotics and the gastrointestinal microbiota on gut motility and constipation. *Adv. Nutr.* 8, 484–494. doi: 10.3945/an.116.014407

Dolkar, T., Hamad, A. M., Han, M. M., Thu, M. B., and Gayam, V. R. (2022). Cocaine and opioid-induced acute liver injury: a rare case report. *Cureus* 14:e23630. doi: 10.7759/cureus.23630

Dropulic, L. K., and Lederman, H. M. (2016). Overview of infections in the immunocompromised host. *Microbiol Spectr.* 4:16. doi: 10.1128/microbiolspec.DMIH2-0026-2016

Duailibi, M. S., Cordeiro, Q., Brietzke, E., Ribeiro, M., LaRowe, S., Berk, M., et al. (2017). N-acetylcysteine in the treatment of craving in substance use disorders: systematic review and meta-analysis. *Am. J. Addict.* 26, 660–666. doi: 10.1111/ajad.12620

Dubinkina, V. B., Tyakht, A. V., Odintsova, V. Y., Yarygin, K. S., Kovarsky, B. A., Pavlenko, A. V., et al. (2017). Links of gut microbiota composition with alcohol dependence syndrome and alcoholic liver disease. *Microbiome* 5:141. doi: 10.1186/s40168-017-0359-2

Ellison-Barnes, A., Johnson, S., and Gudzune, K. (2021). Trends in obesity prevalence among adults aged 18 through 25 years, 1976-2018. *JAMA* 326, 2073–2074. doi: 10.1001/jama.2021.16685

Engelmann, C., and Tacke, F. (2022). The potential role of cellular senescence in non-alcoholic fatty liver disease. *Int. J. Mol. Sci.* 23:652. doi: 10.3390/ijms23020652

English, B. K., and Gaur, A. H. (2010). The use and abuse of antibiotics and the development of antibiotic resistance. *Adv. Exp. Med. Biol.* 659, 73–82. doi: 10.1007/978-1-4419-0981-7_6

Fassoulaki, A., Andreopoulou, K., Williams, G., and Pateras, C. (1986). The effect of single and repeated doses of thiopentone and fentanyl on liver function in the rat. *Anaesth. Intensive Care* 14, 145–147. doi: 10.1177/0310057X8601400208

Fassoulaki, A., Eger, E. I. 2nd, Johnson, B. H., Ferrell, L. D., Smuckler, E. A., Harper, M. H., et al. (1984). Nitrous oxide, too, is hepatotoxic in rats. *Anesth. Analg.* 63, 1076–1080. doi: 10.1213/00000539-198412000-00005

Fassoulaki, A., Kaniaris, P., and Varonos, D. D. (1984). Do general anaesthetics perturb lipid membranes? The role of cholesterol. *Br. J. Anaesth.* 56, 1045–1049. doi: 10.1093/bja/56.9.1045

Fleming, T., Barker, A., Ivsins, A., Vakharia, S., and McNeil, R. (2020). Stimulant safe supply: a potential opportunity to respond to the overdose epidemic. *Harm Reduct. J.* 17:6. doi: 10.1186/s12954-019-0351-1

Fu, X., Dong, B., Tian, Y., Lefebvre, P., Meng, Z., Wang, X., et al. (2015). MicroRNA-26a regulates insulin sensitivity and metabolism of glucose and lipids. *J. Clin. Invest.* 125, 2497–2509. doi: 10.1172/JCI75438

Gómez-Lechón, M. J., Jover, R., and Donato, M. T. (2009). Cytochrome p450 and steatosis. *Curr. Drug Metab.* 10, 692–699. doi: 10.2174/138920009789895543

Gosnell, B. A., and Levine, A. S. (2009). Reward systems and food intake: role of opioids. *Int. J. Obes.* 33, S54–S58. doi: 10.1038/ijo.2009.73

Grattagliano, I., Caraceni, P., Portincasa, P., Domenicali, M., Palmieri, V. O., Trevisani, F., et al. (2003). Adaptation of subcellular glutathione detoxification system to stress conditions in choline-deficient diet induced rat fatty liver. *Cell Biol. Toxicol.* 19, 355–366. doi: 10.1023/b:cbto.0000013341.73139.fc

Grimm, S. L., Mendez, E. F., Stertz, L., Meyer, T. D., Fries, G. R., Gandhi, T., et al. (2023). MicroRNA-mRNA networks are dysregulated in opioid use disorder postmortem brain: further evidence for opioid-induced neurovascular alterations. *Front. Psych.* 13:1025346. doi: 10.3389/fpsyt.2022.1025346

Gutiérrez, B., and Domingo-Calap, P. (2020). Phage therapy in gastrointestinal diseases. *Microorganisms* 8:1420. doi: 10.3390/microorganisms8091420

Haberer, J. P., Schoeffler, P., Couderc, E., and Duvaldestin, P. (1982). Fentanyl pharmacokinetics in anaesthetized patients with cirrhosis. *Br. J. Anaesth.* 54, 1267–1270. doi: 10.1093/bja/54.12.1267

Habibi-Asl, B., Hassanzadeh, K., Vafai, H., and Mohammadi, S. (2009). Development of morphine induced tolerance and withdrawal symptoms is attenuated by lamotrigine and magnesium sulfate in mice. *Pak. J. Biol. Sci.* 12, 798–803. doi: 10.3923/pjbs.2009.798.803

Hartmann, P., and Schnabl, B. (2023). Fungal infections and the fungal microbiome in hepatobiliary disorders. *J. Hepatol.* 78, 836–851. doi: 10.1016/j.jhep.2022.12.006

He, Q., Li, F., Li, J., Li, R., Zhan, G., Li, G., et al. (2017). MicroRNA-26a-interleukin (IL)-6-IL-17 axis regulates the development of non-alcoholic fatty liver disease in a murine model. *Clin. Exp. Immunol.* 187, 174–184. doi: 10.1111/cei.12838

Hebert, J. F., Burfeind, K. G., Malinoski, D., and Hutchens, M. P. (2022). Molecular mechanisms of rhabdomyolysis-induced kidney injury: from bench to bedside. *Kidney Int. Rep.* 8, 17–29. doi: 10.1016/j.ekir.2022.09.026

Hrncir, T., Hrncirova, L., Kverka, M., Hromadka, R., Machova, V., Trckova, E., et al. (2021). Gut microbiota and NAFLD: Pathogenetic mechanisms, microbiota signatures, and therapeutic interventions. *Microorganisms* 9:957. doi: 10.3390/microorganisms9050957

Huang, Z., Wang, M., Liu, L., Peng, J., Guo, C., Chen, X., et al. (2019). Transcriptional repression of CYP3A4 by increased miR-200a-3p and miR-150-5p promotes steatosis *in vitro*. *Front. Genet.* 10:484. doi: 10.3389/fgene.2019.00484

Imbernon, M., Beiroa, D., Vázquez, M. J., Morgan, D. A., Veyrat-Durebex, C., Porteiro, B., et al. (2013). Central melanin-concentrating hormone influences liver and adipose metabolism via specific hypothalamic nuclei and efferent autonomic/JNK1 pathways. *Gastroenterology* 144, 636–649.e6. doi: 10.1053/j.gastro.2012.10.051

Jamwal, R., de la Monte, S. M., Ogasawara, K., Adusumalli, S., Barlock, B. B., and Akhlaghi, F. (2018). Nonalcoholic fatty liver disease and diabetes are associated with decreased CYP3A4 protein expression and activity in human liver. *Mol. Pharm.* 15, 2621–2632. doi: 10.1021/acs.molpharmaceut.8b00159

Jang, H. R., Park, H. J., Kang, D., Chung, H., Nam, M. H., Lee, Y., et al. (2019). A protective mechanism of probiotic Lactobacillus against hepatic steatosis via reducing host intestinal fatty acid absorption. *Exp. Mol. Med.* 51, 1–14. doi: 10.1038/s12276-019-0293-4

Jin, J., and Zhong, X. B. (2023). Epigenetic mechanisms contribute to Intraindividual variations of drug metabolism mediated by cytochrome P450 enzymes. *Drug Metab. Dispos.* 51, 672–684. doi: 10.1124/dmd.122.001007

Johnson, F., and Wardle, J. (2014). Variety, palatability, and obesity. *Adv. Nutr.* 5, 851–859. doi: 10.3945/an.114.007120

Jones, A. (2019). Alcohol, its absorption, distribution, metabolism, and excretion in the body and pharmacokinetic calculations. Wiley interdisciplinary reviews. *Forensic Sci.* 1:e1340. doi: 10.1002/wfs2.1340

Kacevska, M., Ivanov, M., Wyss, A., Kasela, S., Milani, L., Rane, A., et al. (2012). DNA methylation dynamics in the hepatic CYP3A4 gene promoter. *Biochimie* 94, 2338–2344. doi: 10.1016/j.biochi.2012.07.013

Kawata, Y., Okuda, S., Hotta, N., Igawa, H., Takahashi, M., Ikoma, M., et al. (2017). A novel and selective melanin-concentrating hormone receptor 1 antagonist ameliorates obesity and hepatic steatosis in diet-induced obese rodent models. *Eur. J. Pharmacol.* 796, 45–53. doi: 10.1016/j.ejphar.2016.12.018

Khalif, I. L., Quigley, E. M., Konovitch, E. A., and Maximova, I. D. (2005). Alterations in the colonic flora and intestinal permeability and evidence of immune activation in chronic constipation. *Dig. Liver Dis.* 37, 838–849. doi: 10.1016/j.dld.2005.06.008

Killilea, D. W., and Ames, B. N. (2008). Magnesium deficiency accelerates cellular senescence in cultured human fibroblasts. *Proc. Natl. Acad. Sci.* U.S.A. 105, 5768–73. doi: 10.1073/pnas.0712401105

Kokubun, H., Ebinuma, K., Matoba, M., Takayanagi, R., Yamada, Y., and Yago, K. (2012). Population pharmacokinetics of transdermal fentanyl in patients with cancer-related pain. *J. Pain Palliat. Care Pharmacother.* 26, 98–104. doi: 10.3109/15360288.2012.679725

Lang-Illievich, K., and Bornemann-Cimenti, H. (2019). Opioid-induced constipation: a narrative review of therapeutic options in clinical management. *Korean J. Pain* 32, 69–78. doi: 10.3344/kjp.2019.32.2.69

Lee, A. A., and Hasler, W. L. (2016). Opioids and GI motility-friend or foe? *Curr. Treat. Options Gastroenterol.* 14, 478–494. doi: 10.1007/s11938-016-0112-0

Leung, T. M., and Nieto, N. (2013). CYP2E1 and oxidant stress in alcoholic and non-alcoholic fatty liver disease. *J. Hepatol.* 58, 395–398. doi: 10.1016/j.jhep.2012.08.018

Lewinska, A., Adamczyk-Grochala, J., Bloniarz, D., Horeczy, B., Zurek, S., Kurowicki, A., et al. (2020). Remifentanil preconditioning protects against hypoxia-induced senescence and necroptosis in human cardiac myocytes *in vitro*. *Aging (Albany NY)* 12, 13924–13938. doi: 10.18632/aging.103604

Li, Y. N., Kang, N. L., Jiang, J. J., Zhu, Y. Y., Liu, Y. R., Zeng, D. W., et al. (2022). Gut microbiota of hepatitis B virus-infected patients in the immune-tolerant and immune-active phases and their implications in metabolite changes. *World J. Gastroenterol.* 28, 5188–5202. doi: 10.3748/wjg.v28.i35.5188

Lim, A. K. (2020). Abnormal liver function tests associated with severe rhabdomyolysis. *World J. Gastroenterol.* 26, 1020–1028. doi: 10.3748/wjg.v26.i10.1020

Liu, Z., Huang, H., Zeng, Y., Chen, Y., and Xu, C. (2022). Association between ultra-processed foods consumption and risk of non-alcoholic fatty liver disease: a population-based analysis of NHANES 2011-2018. *Br. J. Nutr.* 130, 996–1004. doi: 10.1017/S0007114522003956

Liu, D. Q., Zhou, Y. Q., and Gao, F. (2019). Targeting cytokines for morphine tolerance: a narrative review. *Curr. Neuropharmacol.* 17, 366–376. doi: 10.2174/1570159X15666171128144441

Lukito, W. (2021). Current evidence in water and hydration science. *Ann. Nutr. Metab.* 77, 1–6. doi: 10.1159/000521769

Madeira, M. D., Sousa, N., Lieberman, A. R., and Paula-Barbosa, M. M. (1993). Effects of chronic alcohol consumption and of dehydration on the supraoptic nucleus of adult male and female rats. *Neuroscience* 56, 657–672. doi: 10.1016/0306-4522(93)90363-k

Magnesium in the Central Nervous Systems. (2023). Available at: https://www.ncbi.nlm.nih.gov/books/NBK507260/ (Accessed June, 2023).

Mallappallil, M., Sabu, J., Friedman, E. A., and Salifu, M. (2017). What do we know about opioids and the kidney? *Int. J. Mol. Sci.* 18:223. doi: 10.3390/ijms18010223

Mamdani, M., Paterson, J. M., and Juurlink, D. N. (2023). Trends in opioid toxicity-related deaths in the US before and after the start of the COVID-19 pandemic, 2011-2021. *JAMA Netw. Open* 6:e2322303. doi: 10.1001/jamanetworkopen.2023.22303

Marascio, N., De Caro, C., Quirino, A., Mazzitelli, M., Russo, E., Torti, C., et al. (2022). The role of the microbiota gut-liver Axis during HCV chronic infection: a schematic overview. *J. Clin. Med.* 11:5936. doi: 10.3390/jcm11195936

Marks, L. R., Nolan, N. S., Liang, S. Y., Durkin, M. J., and Weimer, M. B. (2022). Infectious complications of injection drug use. *Med. Clin. North Am.* 106, 187–200. doi: 10.1016/j.mcna.2021.08.006

Marrone, A. K., Beland, F. A., and Pogribny, I. P. (2014). Noncoding RNA response to xenobiotic exposure: an indicator of toxicity and carcinogenicity. *Expert Opin. Drug Metab. Toxicol.* 10, 1409–1422. doi: 10.1517/17425255.2014.954312

Martin, N., and Bernard, D. (2018). Calcium signaling and cellular senescence. *Cell Calcium* 70, 16–23. doi: 10.1016/j.ceca.2017.04.001

Meijnikman, A. S., Davids, M., Herrema, H., Aydin, O., Tremaroli, V., Rios-Morales, M., et al. (2022). Microbiome-derived ethanol in nonalcoholic fatty liver disease. *Nat. Med.* 28, 2100–2106. doi: 10.1038/s41591-022-02016-6

Meng, J., Banerjee, S., Li, D., Sindberg, G. M., Wang, F., Ma, J., et al. (2015). Opioid exacerbation of gram-positive sepsis, induced by gut microbial modulation, is rescued by IL-17A neutralization. *Sci. Rep.* 5:10918. doi: 10.1038/srep10918

Meng, F., Lorenzo, S. R., Francis, H., Glaser, S., and Alpini, G. (2017). Characterization of cellular senescence mechanisms in alcoholic liver injury. *FASEB J.* 31:804.3. doi: 10.1096/fasebj.31.1_supplement.804.3

Meng, J., Yu, H., Ma, J., Wang, J., Banerjee, S., Charboneau, R., et al. (2013). Morphine induces bacterial translocation in mice by compromising intestinal barrier function in a TLR-dependent manner. *PLoS One* 8:e54040. doi: 10.1371/journal.pone.0054040

Minimum Alveolar Concentration. (2023). Available at: https://www.sciencedirect.com/topics/medicine-and-dentistry/minimum-alveolar-concentration (Accessed April, 2023).

Mitzelfelt, J. D., Dupree, J. P., Seo, D. O., Carter, C. S., and Morgan, D. (2011). Effects of chronic fentanyl administration on physical performance of aged rats. *Exp. Gerontol.* 46, 65–72. doi: 10.1016/j.exger.2010.10.004

Morganstern, I., Gulati, G., and Leibowitz, S. F. (2020). Role of melanin-concentrating hormone in drug use disorders. *Brain Res.* 1741:146872. doi: 10.1016/j.brainres.2020.146872

Mutlu, E. A., Gillevet, P. M., Rangwala, H., Sikaroodi, M., Naqvi, A., Engen, P. A., et al. (2012). Colonic microbiome is altered in alcoholism. *Am. J. Physiol. Gastrointest. Liver Physiol.* 302, G966–G978. doi: 10.1152/ajpgi.00380.2011

Mysonhimer, A. R., Cannavale, C. N., Bailey, M. A., Khan, N. A., and Holscher, H. D. (2023). Prebiotic consumption alters microbiota but not biological markers of stress and inflammation or mental health symptoms in healthy adults: a randomized, controlled, crossover trial. *J. Nutr.* 153, 1283–1296. doi: 10.1016/j.tjnut.2023.02.015

Nabipour, S., Ayu Said, M., and Hussain Habil, M. (2014). Burden and nutritional deficiencies in opiate addiction- systematic review article. *Iran. J. Public Health* 43, 1022–1032

Nagamatsu, K., Ohno, Y., Ikebuchi, H., Takahashi, A., Terao, T., and Takanaka, A. (1986). Morphine metabolism in isolated rat hepatocytes and its implications for hepatotoxicity. *Biochem. Pharmacol.* 35, 3543–3548. doi: 10.1016/0006-2952(86)90624-6

Nakano, M., and Nakajima, M. (2018). Current knowledge of microRNA-mediated regulation of drug metabolism in humans. *Expert Opin. Drug Metab. Toxicol.* 14, 493–504. doi: 10.1080/17425255.2018.1472237

Naseri, M., Aliashrafzadeh, H., Otadi, M., Ebrahimzadeh, F., Badfar, I., Alipourfard, E., et al. (2022). Human responses in public health emergencies for infectious disease control: An overview of controlled topologies for biomedical applications. *Contrast. Media. Mol. Imaging.* 2022:6324462. doi: 10.1155/2022/6324462

National Institute on Drug Abuse. (2023). Available at: https://nida.nih.gov/research-topics/trends-statistics/overdose-death-rates (Accessed April, 2023).

Niemelä, O. (1999). Aldehyde-protein adducts in the liver as a result of ethanol-induced oxidative stress. *Front. Biosci.* 4, D506–D513. doi: 10.2741/niemela

Niemelä, O., Parkkila, S., Juvonen, R. O., Viitala, K., Gelboin, H. V., and Pasanen, M. (2000). Cytochromes P450 2A6, 2E1, and 3A and production of protein-aldehyde adducts in the liver of patients with alcoholic and non-alcoholic liver diseases. *J. Hepatol.* 33, 893–901. doi: 10.1016/s0168-8278(00)80120-8

Niemelä, O., Parkkila, S., Pasanen, M., Iimuro, Y., Bradford, B., and Thurman, R. G. (1998). Early alcoholic liver injury: formation of protein adducts with acetaldehyde and lipid peroxidation products, and expression of CYP2E1 and CYP3A. *Alcohol. Clin. Exp. Res.* 22, 2118–2124. doi: 10.1111/j.1530-0277.1998.tb05925.x

Ninković, J., and Roy, S. (2013). Role of the mu-opioid receptor in opioid modulation of immune function. *Amino Acids* 45, 9–24. doi: 10.1007/s00726-011-1163-0

Odegaard, K. E., Chand, S., Wheeler, S., Tiwari, S., Flores, A., Hernandez, J., et al. (2020). Role of extracellular vesicles in substance abuse and HIVRelated neurological pathologies. *Int. J. Mol. Sci.* 21:6765. doi: 10.3390/ijms21186765

Office of Addiction Services and Supports. (2023). Available at: https://oasas.ny.gov/prevent-overdose-campaign (Accessed April, 2023).

Ogunrinola, G. A., Oyewale, J. O., Oshamika, O. O., and Olasehinde, G. I. (2020). The human microbiome and its impacts on health. *Int. J. Microbiol.* 2020, 8045646–8045647. doi: 10.1155/2020/8045646

Ojo, O., Wang, X. H., Ojo, O. O., and Ibe, J. (2019). The impact of opium abuse on lipid profile in patients with diabetes: a systematic review and Meta-analysis. *Int. J. Environ. Res. Public Health* 16:4795. doi: 10.3390/ijerph16234795

Oshaghi, M., Kourosh-Arami, M., and Roozbehkia, M. (2023). Role of neurotransmitters in immune-mediated inflammatory disorders: a crosstalk between the nervous and immune systems. *Neurol. Sci.* 44, 99–113. doi: 10.1007/s10072-022-06413-0

Parkinson, A., Mudra, D. R., Johnson, C., Dwyer, A., and Carroll, K. M. (2004). The effects of gender, age, ethnicity, and liver cirrhosis on cytochrome P450 enzyme activity in human liver microsomes and inducibility in cultured human hepatocytes. *Toxicol. Appl. Pharmacol.* 199, 193–209. doi: 10.1016/j.taap.2004.01.010

Passariello, N., Giugliano, D., Quatraro, A., Consoli, G., Sgambato, S., Torella, R., et al. (1983). Glucose tolerance and hormonal responses in heroin addicts. A possible role for endogenous opiates in the pathogenesis of non-insulin-dependent diabetes. *Metabolism* 32, 1163–1165. doi: 10.1016/0026-0495(83)90065-3

Patangia, D. V., Anthony Ryan, C., Dempsey, E., Paul Ross, R., and Stanton, C. (2022). Impact of antibiotics on the human microbiome and consequences for host health. *Microbiology* 11:e1260. doi: 10.1002/mbo3.1260

Peacock, A., Leung, J., Larney, S., Colledge, S., Hickman, M., Rehm, J., et al. (2018). Global statistics on alcohol, tobacco and illicit drug use: 2017 status report. *Addiction* 113, 1905–1926. doi: 10.1111/add.14234

Pei, K., Gui, T., Kan, D., Feng, H., Jin, Y., Yang, Y., et al. (2020). An overview of lipid metabolism and nonalcoholic fatty liver disease. *Biomed. Res. Int.* 2020, 4020249–4020212. doi: 10.1155/2020/4020249

Pezeshki, B., Pourmontaseri, H., Homayounfar, R., Moghaddam, M. T., and Dehghan, A. (2023). Thyroid function and opium use disorder: a cross-sectional study on the Fasa adults cohort study (FACS), 2017. *BMC Endocr. Disord.* 23:264. doi: 10.1186/s12902-023-01467-3

Plein, L. M., and Rittner, H. L. (2018). Opioids and the immune system - friend or foe. *Br. J. Pharmacol.* 175, 2717–2725. doi: 10.1111/bph.13750

Proust, B., Dubois, F., Bacq, Y., Le Pogam, S., Rogez, S., Levillain, R., et al. (2000). Two successive hepatitis C virus infections in an intravenous drug user. *J. Clin. Microbiol.* 38, 3125–3127. doi: 10.1128/JCM.38.8.3125-3127.2000

Pustorino, S., Foti, M., Calipari, G., Pustorino, E., Ferraro, R., Guerrisi, O., et al. (2004). Interazioni funzionali tra tiroide e motilità gastrointestinale [Thyroid-intestinal motility interactions summary]. *Minerva Gastroenterol. Dietol.* 50, 305–315

Reece, A. S. (2013). Manifold implications of forgotten hyperglycemia in clinical opiate dependence. *Drug Chem. Toxicol.* 36, 55–66. doi: 10.3109/01480545.2011.649286

Ren, Z. Y., Xu, X. Q., Bao, Y. P., He, J., Shi, L., Deng, J. H., et al. (2015). The impact of genetic variation on sensitivity to opioid analgesics in patients with postoperative pain: a systematic review and meta-analysis. *Pain Physician* 18, 131–152

Rezaei, A., and Rezaei, M. (2021). Effects of opioids and psychoactive drugs on blood lipid and glucose indices; a systematic review of population-based evidences. *Endocr. Metab. Immune Disord. Drug Targets* 21, 2181–2190. doi: 10.2174/1871530321666210809155928

Romero, A. M., Palanca, A., Ruiz-Soto, M., Llorca, J., Marín, M. P., Renau-Piqueras, J., et al. (2016). Chronic alcohol exposure decreases 53BP1 protein Levels leading to a defective DNA repair in cultured primary cortical neurons. *Neurotox. Res.* 29, 69–79. doi: 10.1007/s12640-015-9554-8

Sacerdote, P. (2008). Opioid-induced immunosuppression. *Curr. Opin. Support. Palliat. Care* 2, 14–18. doi: 10.1097/SPC.0b013e3282f5272e

Sacerdote, P., Franchi, S., Gerra, G., Leccese, V., Panerai, A. E., and Somaini, L. (2008). Buprenorphine and methadone maintenance treatment of heroin addicts preserves immune function. *Brain Behav. Immun.* 22, 606–613. doi: 10.1016/j.bbi.2007.12.013

Sadeghi Dousari, A., Hosseininasab, S. S., Sadeghi Dousari, F., Fuladvandi, M., and Satarzadeh, N. (2023). The impact of COVID-19 on liver injury in various age. *World J. Virol.* 12, 91–99. doi: 10.5501/wjv.v12.i2.91

Schulzke, J. D., Ploeger, S., Amasheh, M., Fromm, A., Zeissig, S., Troeger, H., et al. (2009). Epithelial tight junctions in intestinal inflammation. *Ann. N. Y. Acad. Sci.* 1165, 294–300. doi: 10.1111/j.1749-6632.2009.04062.x

Schutijser, B. C. F. M., Jongerden, I., Klopotowska, J. E., Moesker, M., Langelaan, M., Wagner, C., et al. (2020). Nature of adverse events with opioids in hospitalised patients: a post-hoc analysis of three patient record review studies. *BMJ Open* 10:e038037. doi: 10.1136/bmjopen-2020-038037

Schwenger, K. J., Clermont-Dejean, N., and Allard, J. P. (2019). The role of the gut microbiome in chronic liver disease: the clinical evidence revised. *JHEP Rep.* 1, 214–226. doi: 10.1016/j.jhepr.2019.04.004

Shingu, K., Eger, E. I. 2nd, Johnson, B. H., Lurz, F. W., and Hickey, R. F. (1983). MAC values of thiopental and fentanyl in rats. *Anesth. Analg.* 62, 151–154. doi: 10.1213/00000539-198302000-00005

Skrabalova, J., Drastichova, Z., and Novotny, J. (2013). Morphine as a potential oxidative stress-causing agent. *Mini Rev. Org. Chem.* 10, 367–372. doi: 10.2174/1570193X113106660031

Smith, H. S. (2009). Opioid metabolism. *Mayo Clin. Proc.* 84, 613–624. doi: 10.1016/S0025-6196(11)60750-7

Swanson, L. W. (2000). Cerebral hemisphere regulation of motivated behavior. *Brain Res.* 886, 113–164. doi: 10.1016/s0006-8993(00)02905-x

Szabo, G., and Saha, B. (2015). Alcohol's effect on host defense. *Alcohol Res.* 37, 159–170

Tang, X., and Chen, S. (2015). Epigenetic regulation of cytochrome P450 enzymes and clinical implication. *Curr. Drug Metab.* 16, 86–96. doi: 10.2174/1389200216021507 13114159

Tarantino, G., Cataldi, M., and Citro, V. (2022). Could alcohol abuse and dependence on junk foods inducing obesity and/or illicit drug use represent danger to liver in young people with altered psychological/relational spheres or emotional problems? *Int. J. Mol. Sci.* 23:10406. doi: 10.3390/ijms231810406

Tarantino, G., and Citro, V. (2024). What are the common downstream molecular events between alcoholic and nonalcoholic fatty liver? *Lipids Health Dis.* 23:41. doi: 10.1186/s12944-024-02031-1

Tateishi, T., Krivoruk, Y., Ueng, Y. F., Wood, A. J., Guengerich, F. P., and Wood, M. (1996). Identification of human liver cytochrome P-450 3A4 as the enzyme responsible for fentanyl and sufentanil N-dealkylation. *Anesth. Analg.* 82, 167–172. doi: 10.1097/00000539-199601000-00031

Taur, Y., and Pamer, E. G. (2013). The intestinal microbiota and susceptibility to infection in immunocompromised patients. *Curr. Opin. Infect. Dis.* 26, 332–337. doi: 10.1097/QCO.0b013e3283630dd3

Van Vugt, D. A., and Meites, J. (1980). Influence of endogenous opiates on anterior pituitary function. *Fed. Proc.* 39, 2533–2538

Vanhaecke, T., Bretin, O., Poirel, M., and Tap, J. (2022). Drinking water source and intake are associated with distinct gut microbiota signatures in US and UK populations. *J. Nutr.* 152, 171–182. doi: 10.1093/jn/nxab312

Verna, E. C., Schluger, A., and Brown, R. S. (2019). Opioid epidemic and liver disease. *JHEP Rep.* 1, 240–255. doi: 10.1016/j.jhepr.2019.06.006

Walmsley, R., and Sumithran, P. (2023). Current and emerging medications for the management of obesity in adults. *Med. J. Aust.* 218, 276–283. doi: 10.5694/mja2.51871

Wang, L., Bai, M., Jin, T., Zheng, J., Wang, Y., He, Y., et al. (2019). Effects of *CYP3A4* polymorphisms on drug addiction risk among the Chinese Han population. *Front. Public Health* 7:315. doi: 10.3389/fpubh.2019.00315

Wilson, N., Kariisa, M., Seth, P., Smith, H. 4th, and Davis, N. L. (2020). Drug and opioid-involved overdose deaths - United States, 2017-2018. *MMWR Morb. Mortal Wkly. Rep.* 69, 290–297. doi: 10.15585/mmwr.mm6911a4

Wu, D., and Cederbaum, A. I. (2003). Alcohol, oxidative stress, and free radical damage. *Alcohol Res. Health* 27, 277–284

Xie, Y., Tu, B., Xu, Z., Zhang, X., Bi, J., Zhao, M., et al. (2017). Bacterial distributions and prognosis of bloodstream infections in patients with liver cirrhosis. *Sci. Rep.* 7:11482. doi: 10.1038/s41598-017-11587-1

Xu, Y., Liu, X., Liu, X., Chen, D., Wang, M., Jiang, X., et al. (2021). The roles of the gut microbiota and chronic low-grade inflammation in older adults with frailty. *Front. Cell. Infect. Microbiol.* 11:675414. doi: 10.3389/fcimb.2021.675414

Xu, H., Tian, Y., Tang, D., Zou, S., Liu, G., Song, J., et al. (2021). An endoplasmic reticulum stress-MicroRNA-26a feedback circuit in NAFLD. *Hepatology* 73, 1327–1345. doi: 10.1002/hep.31428

Yu, T., Wang, P., Wu, Y., Zhong, J., Chen, Q., Wang, D., et al. (2022). MiR-26a reduces inflammatory responses via inhibition of PGE2 production by targeting COX-2. *Inflammation* 45, 1484–1495. doi: 10.1007/s10753-022-01631-2

Zahmatkesh, M., Kadkhodaee, M., Salarian, A., Seifi, B., and Adeli, S. (2017). Impact of opioids on oxidative status and related signaling pathways: An integrated view. *J. Opioid Manag.* 13, 241–251. doi: 10.5055/jom.2017.0392

Zhang, Q., Liu, Y., Yuan, Y., Yao, F., Zhang, H., Zhao, C., et al. (2022). miR-26a-5p protects against drug-induced liver injury via targeting bid. *Toxicol. Mech. Methods* 32, 325–332. doi: 10.1080/15376516.2021.2003919

Zhang, J., Zhang, J., and Wang, R. (2018). Gut microbiota modulates drug pharmacokinetics. *Drug Metab. Rev.* 50, 357–368. doi: 10.1080/03602532.2018.1497647

Zhu, Y., Chen, L., He, Y., Qin, L., Tan, D., Bai, Z., et al. (2023). The alteration of drug metabolism enzymes and pharmacokinetic parameters in nonalcoholic fatty liver disease: current animal models and clinical practice. *Drug Metab. Rev.* 55, 163–180. doi: 10.1080/03602532.2023.2202359

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Genetically predicted immune cells mediate the association between gut microbiota and autoimmune liver diseases

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Background: Increasing evidence suggests an association between gut microbiota and Autoimmune Liver Diseases (AILDs). However, causal inference remains controversial due to confounding bias in observational studies. Additionally, there is currently no clear evidence indicating that immune cells act as intermediate phenotypes in the pathogenesis of AILDs. This study utilizes the Mendelian Randomization (MR) method to investigate the causal relationships among gut microbiota, immune cells, and AILDs.

Methods: Initially, we conducted a two-sample MR analysis to predict the causal relationships among 412 gut microbiota, 731 immune phenotypes, and AILDs. Subsequently, a series of sensitivity analyses were performed to validate the initial MR results and reverse MR analysis was conducted to exclude reverse causality. Finally, a two-step MR analysis was utilized to quantify the proportion of the impact of gut microbiota on AILDs mediated by immune cells.

Results: Following rigorous MR analysis, our findings indicate that increased involvement of the gut microbiome in the *superpathway of L-tryptophan biosynthesis* is positively associated with an elevated risk of Autoimmune Hepatitis (AIH). The effect is partially mediated by the *CD14+ CD16+ monocyte Absolute Count*, which accounts for 17.47% of the total effect. Moreover, the *species Ruminococcus obeum* appears to mediate the development of Primary Sclerosing Cholangitis (PSC) through *CD62L-CD86+ myeloid Dendritic Cell %Dendritic Cell*, contributing to 32.47% of the total observed effect.

Conclusion: Our study highlights the potential mediating mechanisms of immune cells in the causal relationship between the gut microbiome and AILDs. These insights provide a foundation for developing preventive strategies for AILDs in clinical practice.

KEYWORDS

Mendelian randomization, autoimmune hepatitis, primary biliary cholangitis, primary sclerosing cholangitis, immune cells, gut microbiota

1 Introduction

Autoimmune Liver Diseases (AILDs) represent a spectrum of disorders characterized by the immune system's persistent and progressive assault on hepatic cells. This category encompasses Autoimmune Hepatitis (AIH), Primary Biliary Cholangitis (PBC), and Primary Sclerosing Cholangitis (PSC) (Fischer and Goltz, 2020). Individuals afflicted with AILDs commonly experience symptoms like fatigue, jaundice, and abdominal discomfort, which may progress to severe complications such as cirrhosis and liver failure if not adequately managed (Muratori et al., 2023). The incidence of AILDs has historically been low worldwide, but it has been gradually increasing in recent years, presenting substantial challenges to both affected individuals and healthcare infrastructure. Moreover, these diseases are frequently associated with extraliver conditions, including arthralgia and thyroiditis, affecting a considerable proportion of patients (Mihai et al., 2024). This widespread impact underscores the urgent need for refined diagnostic approaches and effective treatment strategies in the management of AILDs (Kardashian et al., 2023).

Increasing research emphasizes the critical role of the gut microbiome in the development of AILDs via the gut-liver axis (Qian et al., 2023). This involves mechanisms such as gut barrier dysfunction, immune regulation, metabolic products, and inflammatory factors. With advances in immunology, it has also been recognized that the imbalance of gut microbiota and its metabolic products are key signals inducing immune responses in the liver tissue of AILDs (Schneider et al., 2023; Zheng et al., 2021). Studies suggested that Pien Tze Huang (PTH) stimulates beneficial bacteria, increasing Regulatory T cell (Treg)/myeloid Regulatory T cell (mTreg) cells and Interleukin (IL)-10 production, inhibiting Toll-like receptor (TLR)4/Nuclear Factor-κB (NF-κB) and C-X-C motif chemokine ligand 16 (CXCL16)/C-X-C motif chemokine receptor 6 (CXCR6) signaling pathways, and reducing liver pathology in AIH mice (Oldereid et al., 2024). In PBC mouse models, early gut microbiome changes induce Bile duct Epithelial Cell (BEC) apoptosis via TLR2, recruiting CD8 T cells and causing a vicious cycle (Wang et al., 2022). Quraishi et al. discovered reduced gut microbiota diversity in PSC patients, potentially leading to abnormal homing of gut-specific lymphocytes and gut permeability, causing mucosal immune dysregulation (Quraishi et al., 2017). However, current research primarily focuses on animal experiments and small-scale clinical studies aimed at elucidating the potential correlation between gut microbiota and AILDs. These studies rarely form a coherent pathological mechanism, and direct evidence of the critical role of immune cells in the pathogenesis of AILDs is still lacking.

Mendelian Randomization (MR) is a method used in epidemiology and genetics to assess the causal impact of exposures on outcomes, offering significant advantages in determining causality and directionality (Larsson and Burgess, 2021). We obtained the latest summary statistics for 412 gut microbiota and 731 immune cells from large public genome-wide association study (GWAS) consortia to perform two-sample and mediation MR analyses. This approach evaluates the causal relationships between the gut microbiome and AILDs and explores the potential mediating role of immune cells. These insights provide valuable understanding for future genetic and therapeutic research related to the gut microbiome and AILDs.

2 Materials and methods

2.1 Study design

We conducted a two-sample and mediation MR study to explore the causal relationships between gut microbiota, immune cells, and AILDs, as displayed in Figure 1. When conducting MR analyses, instrumental variables (IVs) must satisfy three core principles: (i) genetic variants used as IVs must be significantly associated with gut microbiota or immune cells; (ii) genetic variants must not be influenced by other confounding factors; and (iii) genetic variants must affect AILDs only through gut microbiota or immune cells.

2.2 Data sources

The summary statistics for gut microbiota were obtained from the Dutch microbial GWAS, which included metagenomic sequencing data from 7,738 individuals in the northern Netherlands L (Lopera-Maya et al., 2022). This GWAS explored the relationship between host genetics and the gut microbiome by analyzing 207 microbial taxa and 205 functional pathways.

The genetic dataset analyzing 731 distinct immunophenotypes from 3,757 Sardinians was sourced from a GWAS (accession numbers GCST0001391 to GCST0002121) (Orrù et al., 2020). This dataset includes 539 immune traits and 192 relative count levels, encompassing morphological parameters, median fluorescence intensities of surface antigens, and cell counts. These immunophenotypes elucidate the complex genetic regulation of immune cells at the cell-subtype level in autoimmune diseases.

The dataset for AIH was sourced from a GWAS (GCST90018785) and includes 821 cases and 484,413 controls, all of European ancestry (Sakaue et al., 2021). We acquired genetic data for PBC from the FinnGen R10 database, comprising 609 cases of PBC out of a total of 306,504 participants. The summary statistics data are accessible through the following Google Cloud Storage link: https://r10.finngen.fi/. This dataset provides a comprehensive genetic insight into PBC, aiming to identify associated genetic variants. The PSC dataset, obtained by Sun-Gou Ji and colleagues from the International PSC Study Group, represents the largest PSC GWAS dataset to date (Ji et al., 2017). In addition, it focuses on the genetic determinants associated with PSC, comprising 2,871 cases and 12,019 controls. Moreover, the analysis has been adjusted for potential confounders such as gender, age, and genotyping batches. All original data in this study were sourced from publicly available databases and previous studies, all of which had received ethical approval.

2.3 Instrumental variables selection

To obtain a sufficient number of Single Nucleotide Polymorphisms (SNPs) related to the gut microbiota, we set the selection criteria for IVs at $p < 1 \times 10^{-5}$. This threshold is consistent with similar criteria utilized in published studies (Qu et al., 2024). Given the sufficient number of SNPs for immune cell phenotypes, we applied a more stringent threshold of $p < 5 \times 10^{-8}$ to select SNPs closely associated with them. Additional screening criteria included the following: First, screening for independent SNPs was conducted through Linkage

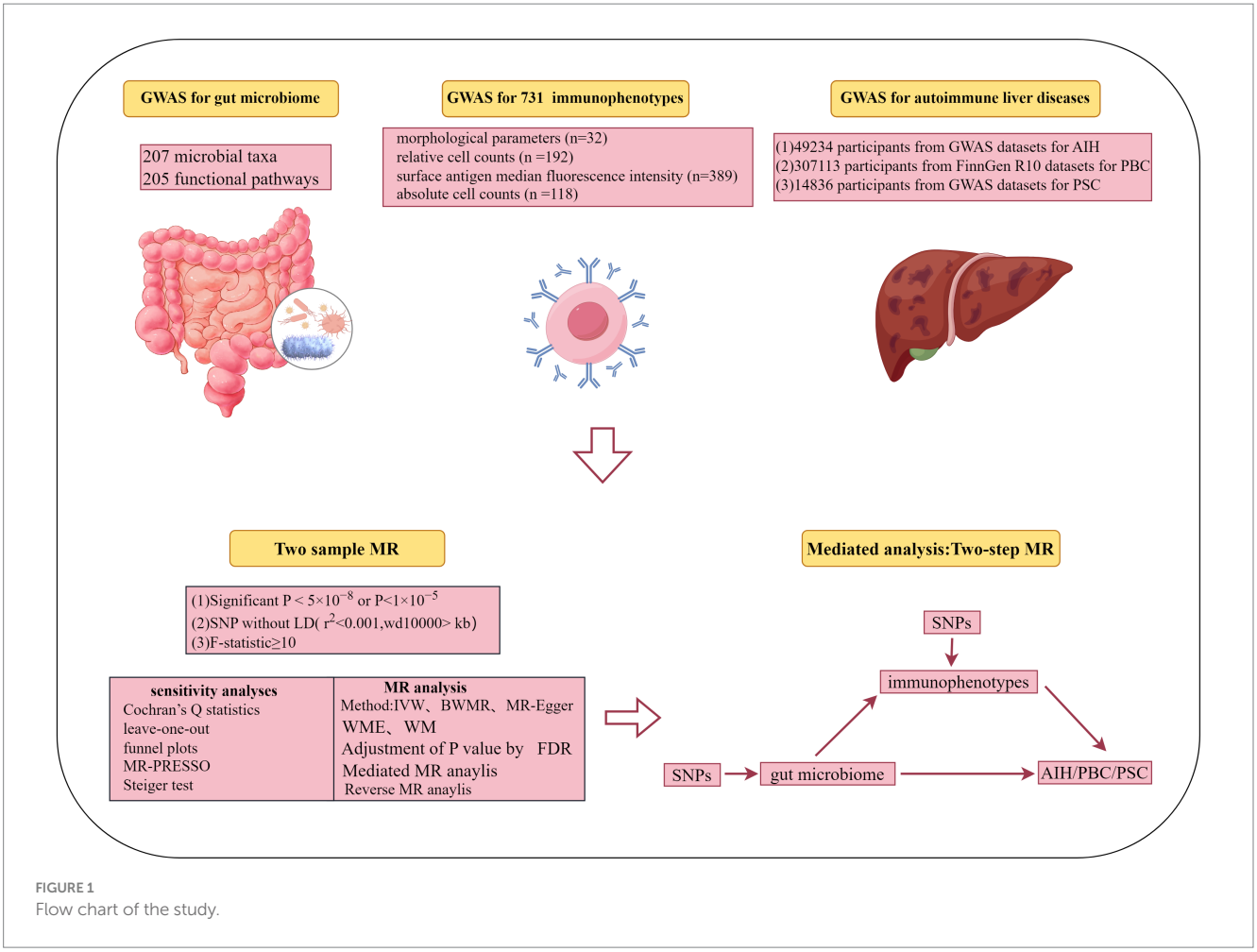

FIGURE 1
Flow chart of the study.

Disequilibrium (LD) analyses, utilizing a threshold of $r^2 < 0.001$ and a physical distance of 10,000 kb (Palmer et al., 2012; Levin et al., 2020). Second, to reduce weak instrument bias and ensure a strong correlation with exposure, SNPs with an F-statistic greater than 10 were selected as IVs (Gill et al., 2019).

2.4 Statistical analysis strategy

In this study, we employed five distinct methods to assess the bidirectional causal relationships between the gut microbiome, immune cells, and AILDs. These methods included Inversevariance Weighted (IVW) (Burgess et al., 2013), Bayesian Weighted Mendelian Randomization (BWMR), MR-Egger, Weighted Median (WME), and Weighted Mode (WM) (Burgess et al., 2015; Bowden et al., 2015). The IVW method served as our primary approach, offering high efficiency and accuracy, simplicity, the ability to reduce the impact of individual SNP bias, and broad applicability. It is widely used in epidemiological research. Note that results were considered statistically significant when the IVW *p*-value was less than 0.05. At the same time, BWMR offered the advantage of addressing pleiotropy and weak instrument biases through Bayesian weighting while enhancing analysis efficiency with an advanced Variational Expectation Maximization algorithm and was, therefore, used as a supplementary method to IVW (Bowden et al., 2016). Additionally, to control for potential false positives in large-scale omics studies, we applied the False Discovery Rate (FDR) to manage statistical bias resulting from multiple comparisons while maintaining statistical power (Ma et al., 2024). Notably, associations were considered significant when FDR < 0.05.

To address potential issues such as pleiotropy and heterogeneity in our two-sample MR analyses, we conducted a series of sensitivity analyses. Heterogeneity, which may arise due to variations in experimental conditions, selected populations, and SNPs, was assessed using Cochran's Q statistic, with a *p*-value >0.05 indicating no significant heterogeneity (Zhao et al., 2020). The MR-Egger intercept method was employed to evaluate pleiotropy, with a *p*-value >0.05 suggesting the absence of pleiotropy (Burgess et al., 2015). Additionally, the MR-PRESSO method was used to filter out SNPs that might introduce bias, thereby enhancing the reliability of causal inferences (Verbanck et al., 2018). Finally, leave-one-out analysis and funnel plots were utilized to ensure the consistency of the results. To further enhance the robustness of the IVs extracted, we employed the Steiger test method to mitigate reverse causation (Hemani et al., 2017). It is worth mentioning that, when the number of available SNPs was less than three, limiting the scope of comprehensive MR analysis, these results were excluded from the analysis to ensure robustness and reliability of the findings. This approach was taken to maintain the integrity of the MR analysis and to focus on results derived from a sufficient number of SNPs for meaningful causal inference. Additionally, to further mitigate the interference of reverse causality

and ensure the reliability of our research findings, we used AILDs as the exposure. Significantly associated SNPs ($p < 5 \times 10^{-8}$) with AILDs were selected as IVs. Thus, we conducted reverse MR studies using gut microbiota or immune cells as the outcomes.

Exploration of the potential mediating mechanisms of immune cells was conducted through a two-step MR mediation analysis. Initially, univariable MR analysis was employed to estimate the effect of exposure on the mediator, obtaining β1. Subsequently, multivariable MR analysis was used to estimate the effect of each mediator on the outcome, obtaining β2. Meanwhile, the indirect effect was estimated by multiplying these two regression estimates (β1 × β2) (Carter et al., 2021). This methodology enabled the determination of the proportion of mediation by immune cells in the causal relationship between gut microbiota and AILDs. All analyses were conducted using "TwoSampleMR," "MR-PRESSO," and "frostplot," a package in the R software (R version 4.3.2).

3 Results

3.1 Instrument variables included in analysis

Detailed information on the final SNPs meeting the requirements of our study for each bacterial trait or functional pathway is provided in Supplementary Table S1. All SNPs used in our analysis had *F*-values exceeding 10.

3.2 MR and sensitivity analysis of gut microbiota and AILDs

3.2.1 AIH

Through rigorous MR analysis, using the IVW method as our primary analytical strategy, we identified 16 gut microbial features associated with AIH, including 8 microbial taxa and 8 functional pathways (Figure 2; Supplementary Table S2). However, after FDR correction, no gut microbiota were significantly associated. *Superpathway of heme biosynthesis from glutamate* (OR = 0.552, $p < 0.001$), *superpathway of polyamine biosynthesis I* (OR = 0.557, $p = 0.014$), *Species.Roseburia unclassified* (OR = 0.788, $p = 0.025$) were the top three features negatively associated with AIH. Conversely, *Order.Actinomycetales* (OR = 1.533, $p = 0.029$), *superpathway of L-tryptophan biosynthesis* (OR = 1.380, $p = 0.006$), *L-arginine degradation II* (*AST pathway*) (OR = 1.490, $p = 0.006$) were positively associated with AIH.

3.2.2 PBC

We identified 14 gut microbial features associated with PBC, including 9 microbial taxa and 5 functional pathways (detailed results are displayed in Figure 3; Supplementary Table S3). However, after FDR correction, no gut microbiota were significantly associated. *Genus.Parabacteroides* (OR = 0.562, $p = 0.009$), *Genus.Gordonibacter* (OR = 0.739, $p = 0.022$), *Species.Gordonibacter pamelaeae*: (OR = 0.740, $p = 0.022$) were the top three features negatively associated with PBC. Conversely, *Species.Odoribacter splanchnicus* (OR = 1.779, $p = 0.001$), *preQ$_0$ biosynthesis* (OR = 1.679, $p = 0.013$), and other features were positively associated with PBC.

3.2.3 PSC

Using the same method, we identified 19 gut microbial features associated with PSC, including 9 microbial taxa and 10 functional pathways (detailed results are illustrated in Figure 4; Supplementary Table S4). However, after FDR correction, no gut microbiota was significantly associated. *Lactose and galactose degradation I*: (OR = 0.651, $p = 0.002$), *Family.Sutterellaceae*: (OR = 0.505, $p = 0.003$), *Species.Eubacterium siraeum*: (OR = 0.545, $p = 0.003$) were the top three features negatively associated with PSC. Conversely, *Species.Lachnospiraceae bacterium_7_1_58FAA*: (OR = 1.490, $p = 0.008$), *Species.*

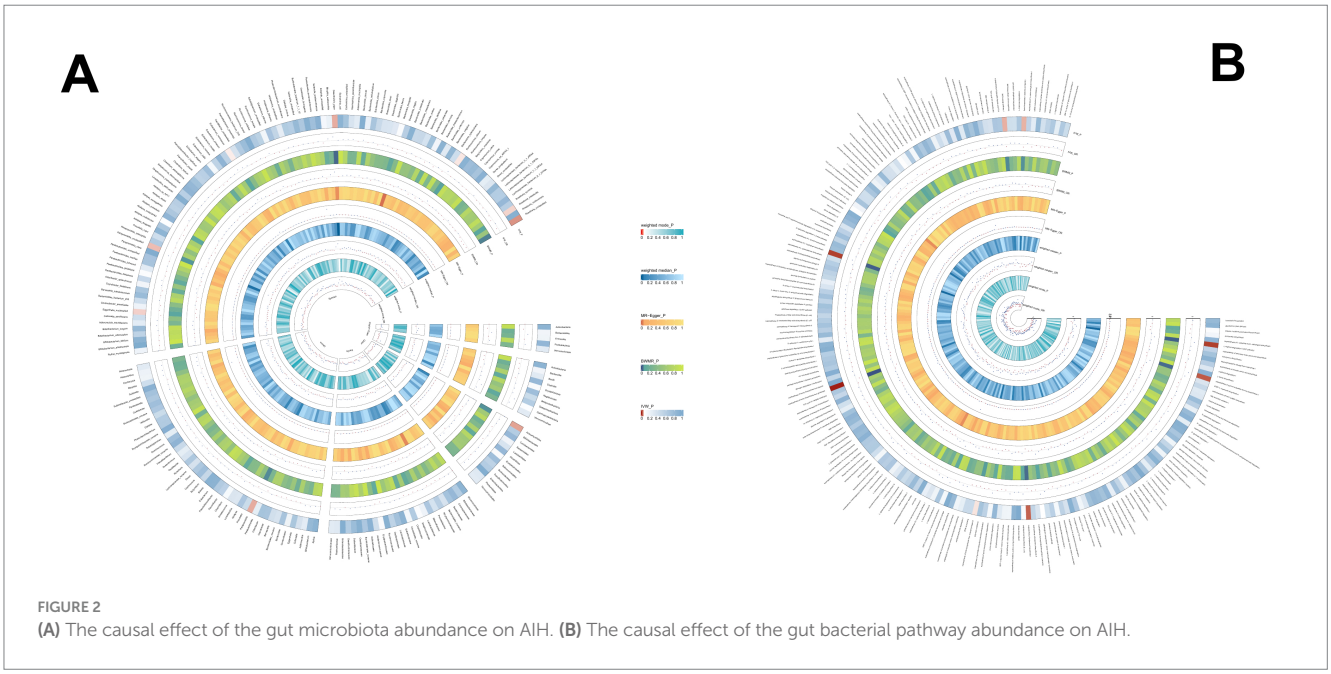

FIGURE 2
(A) The causal effect of the gut microbiota abundance on AIH. **(B)** The causal effect of the gut bacterial pathway abundance on AIH.

FIGURE 3
(A) The causal effect of the gut microbiota abundance on PBC. **(B)** The causal effect of the gut bacterial pathway abundance on PBC.

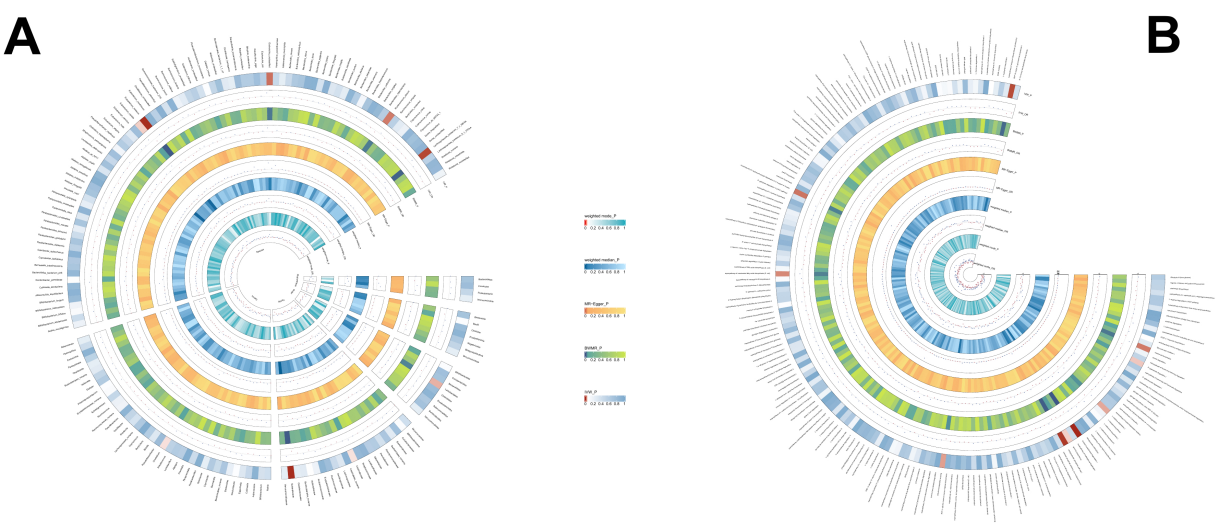

FIGURE 4
(A) The causal effect of the gut microbiota abundance on PSC. **(B)** The causal effect of the gut bacterial pathway abundance on PSC.

Escherichia unclassified (OR = 1.500, $p = 0.017$), *mixed acid fermentation pathway* (OR = 1.864, $p = 0.020$), and other features were positively associated with PSC.

3.3 MR and sensitivity analysis of immune cells and AILDs

3.3.1 AIH

We discovered that 27 immune cell phenotypes significantly affected AIH, as evidenced by an IVW *p*-value of less than 0.05 (including 8cDC, 6TBNK, 4Monocyte, 4Treg, 3Maturation stages of T cell, 2B cell) (Figure 5; Supplementary Table S5). By FDR correction (PFDR <0.05), we discovered one immunophenotype to be protective against AIH: *HLA DR+ Natural Killer %Natural Killer* (OR = 0.703, 95%CI 0.587 ~ 0.842, $p = 0.000134$, PFDR = 0.028). In addition, we discovered that there are two immunophenotypes that have dangerous effects on AIH:*CD28-CD8+ T cell %CD8+ T cell* (OR = 2.203, 95%CI 1.457 ~ 3.331, $p = 0.00018$, PFDR = 0.028), *CD28-CD8+ T cell Absolute Count* (OR = 2.354, 95%CI 1.587 ~ 3.493, $p = 2.107 \times 10^{-5}$, PFDR = 0.0097).

3.3.2 PBC

Based on the IVW method, the results indicate that 24 immune cell phenotypes significantly influence PBC. This includes 2B cell, 4cDC, 4Maturation stages of T cell, 4Monocyte, 4Myeloid cell, 3TBNK, 3Treg (Figure 6; Supplementary Table S6). By FDR correction (PFDR <0.05), we discovered three immunophenotypes to be protective against PBC: *Terminally Differentiated CD4+ T cell %T cell* (OR = 0.498, 95% CI 0.353 ~ 0.703, $p = 7.331 \times 10^{-5}$, PFDR = 0.0108). *HLA DR on CD14-CD16+ monocyte* (OR = 0.700,

10.3389/fmicb.2024.1442506

outcome	Panel	exposure	nsnp	b	se	PFDR	OR(95%CI)
AIH	B cell	BAFF-R on CD24+ CD27+ B cell	10	-0.105	0.054	0.636	0.900(0.811-1.000)
		CD38 on IgD+ CD38dim B cell	4	-0.226	0.115	0.636	0.798(0.637-0.999)
	cDC	CD62L- Dendritic Cell Absolute Count	5	0.267	0.103	0.183	1.306(1.067-1.598)
		CD86+ myeloid Dendritic Cell Absolute Count	5	0.258	0.095	0.148	1.294(1.075-1.558)
		CD86+ myeloid Dendritic Cell %Dendritic Cell	4	0.252	0.102	0.231	1.286(1.053-1.572)
		CD62L- myeloid Dendritic Cell Absolute Count	6	0.266	0.090	0.125	1.305(1.095-1.556)
		CD62L- myeloid Dendritic Cell %Dendritic Cell	7	0.276	0.089	0.115	1.318(1.106-1.570)
		CD62L- CD86+ myeloid Dendritic Cell Absolute Count	6	0.243	0.089	0.148	1.274(1.070-1.517)
		CD62L- CD86+ myeloid Dendritic Cell %Dendritic Cell	6	0.250	0.092	0.148	1.284(1.073-1.538)
		CD80 on myeloid Dendritic Cell	4	0.393	0.179	0.389	1.481(1.043-2.103)
	Maturation stages of T cell	Naive CD4+ T cell %CD4+ T cell	4	0.358	0.161	0.381	1.431(1.045-1.960)
		Effector Memory CD4+ T cell %T cell	5	-0.410	0.169	0.244	0.664(0.477-0.925)
		Terminally Differentiated CD4-CD8- T cell Absolute Count	3	0.697	0.315	0.388	2.009(1.083-3.726)
	Monocyte	CD14+ CD16+ monocyte Absolute Count	3	-0.361	0.111	0.089	0.697(0.561-0.865)
		CD14+ CD16+ monocyte %monocyte	3	-0.334	0.103	0.089	0.716(0.585-0.876)
		CD16 on CD14- CD16+ monocyte	9	-0.190	0.057	0.089	0.827(0.739-0.925)
		CD16 on CD14+ CD16+ monocyte	5	-0.241	0.084	0.137	0.786(0.667-0.926)
	TBNK	HLA DR+ Natural Killer Absolute Count	4	-0.308	0.101	0.117	0.735(0.603-0.896)
		HLA DR+ Natural Killer %Natural Killer	7	-0.353	0.092	0.028	0.703(0.587-0.842)
		HLA DR+ Natural Killer %CD3- lymphocyte	5	-0.366	0.122	0.124	0.694(0.546-0.881)
		FSC-A on Natural Killer	3	-0.310	0.155	0.615	0.733(0.541-0.994)
		SSC-A on HLA DR+ Natural Killer	3	-0.471	0.173	0.148	0.624(0.445-0.876)
		SSC-A on CD4+ T cell	4	-0.473	0.169	0.145	0.623(0.448-0.867)
	Treg	CD28- CD8dim T cell %T cell	3	0.478	0.179	0.161	1.613(1.135-2.292)
		CD28- CD8+ T cell %CD8+ T cell	3	0.790	0.211	0.028	2.203(1.457-3.331)
		CD28- CD8+ T cell Absolute Count	3	0.856	0.201	0.010	2.354(1.587-3.493)
		CD28 on CD28+ CD45RA+ CD8+ T cell	3	0.409	0.140	0.137	1.505(1.143-1.981)

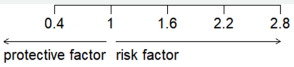

FIGURE 5
Forest plot illustrates the positive IVW-MR results of causal links between immune cells and AIH.

outcome	Panel	exposure	nsnp	b	se	PFDR	OR(95%CI)
PBC	B cell	CD20 on B cell	4	0.224	0.224	0.502	1.405(1.012-1.949)
		CD20 on IgD- CD27- B cell	4	-0.201	-0.201	0.341	1.952(1.146-3.325)
	cDC	CD62L- Dendritic Cell %Dendritic Cell	4	-0.698	-0.698	0.475	1.251(1.012-1.547)
		CCR2 on CD62L+ myeloid Dendritic Cell	3	0.372	0.372	0.351	1.355(1.060-1.730)
		SSC-A on plasmacytoid Dendritic Cell	5	0.593	0.593	0.341	1.587(1.101-2.289)
		SSC-A on monocyte	8	0.650	0.650	0.452	0.842(0.722-0.981)
	Maturation stages of T cell	Effector Memory CD4+ T cell %T cell	3	-0.308	-0.308	0.522	0.543(0.297-0.991)
		Terminally Differentiated CD4+ T cell %T cell	4	-0.258	-0.258	0.011	0.498(0.353-0.703)
		Effector Memory CD8+ T cell %CD8+ T cell	4	0.381	0.381	0.452	1.450(1.040-2.023)
		CD45RA+ CD8+ T cell %T cell	3	0.340	0.340	0.522	1.809(1.007-3.250)
	Monocyte	CD14- CD16- Absolute Count	3	-0.255	-0.255	0.341	1.916(1.149-3.197)
		HLA DR on CD14- CD16+ monocyte	8	0.303	0.303	0.026	0.700(0.577-0.849)
		HLA DR on CD14+ CD16+ monocyte	5	0.462	0.462	0.000	0.635(0.536-0.753)
		HLA DR on monocyte	3	-0.172	-0.172	0.341	0.775(0.636-0.944)
	Myeloid cell	CD33dim HLA DR+ CD11b+ %CD33dim HLA DR+	3	-0.596	-0.596	0.475	0.841(0.713-0.991)
		CD33dim HLA DR+ CD11b- %CD33dim HLA DR+	3	0.736	0.736	0.475	1.191(1.009-1.405)
		HLA DR on CD33dim HLA DR+ CD11b+	4	0.569	0.569	0.452	0.705(0.516-0.963)
		HLA DR on CD33dim HLA DR+ CD11b-	5	1.074	1.074	0.475	0.740(0.559-0.980)
	TBNK	B cell %CD3- lymphocyte	3	-1.014	-1.014	0.452	0.735(0.559-0.966)
		HLA DR+ Natural Killer %Natural Killer	6	0.463	0.463	0.341	0.773(0.630-0.949)
		SSC-A on HLA DR+ Natural Killer	3	0.883	0.883	0.457	0.739(0.561-0.973)
	Treg	CD39+ resting CD4 regulatory T cell Absolute Count	10	0.554	0.554	0.402	0.818(0.692-0.966)
		Activated & resting CD4 regulatory T cell %CD4+ T cell	3	-0.208	-0.208	0.508	0.673(0.457-0.989)
		CD28+ CD45RA- CD8+ T cell %T cell	3	-0.281	-0.281	0.051	1.463(1.169-1.831)

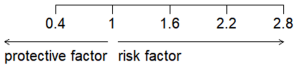

FIGURE 6
Forest plot shows the positive IVW-MR results of causal links between immune cell and PBC.

95% CI 0.577 ~ 0.849, $p = 0.00029$, PFDR = 0.026). *HLA DR on CD14+ CD16+ monocyte* (OR = 0.635, 95% CI 0.536 ~ 0.753, $p = 1.700 \times 10^{-7}$, PFDR = 7.513×10^{-5}). However, no immnophenotypes were found to be positively associated with PBC.

3.3.3 PSC

The results revealed that 26 immune cell phenotypes significantly influence PSC, as indicated by the IVW method. This includes 16Treg, 6cDC, 2TBNK, 1Monocyte, 1Maturation stages of T cell (Figure 7;

10.3389/fmicb.2024.1442506

outcome	Panel	exposure	nsnp	b	se	PFDR	OR(95%CI)
PSC	cDC	CD62L- Dendritic Cell Absolute Count	3	0.278	0.130	0.257	1.321(1.023-1.705)
		CD86+ myeloid Dendritic Cell Absolute Count	3	0.277	0.130	0.257	1.319(1.023-1.700)
		CD62L- myeloid Dendritic Cell Absolute Count	4	0.282	0.114	0.257	1.326(1.061-1.657)
		CD62L- myeloid Dendritic Cell %Dendritic Cell	4	0.273	0.115	0.257	1.314(1.050-1.645)
		CD62L- CD86+ myeloid Dendritic Cell Absolute Count	3	0.264	0.119	0.257	1.302(1.031-1.645)
		CD62L- CD86+ myeloid Dendritic Cell %Dendritic Cell	3	0.293	0.123	0.257	1.341(1.053-1.708)
	Maturation stages of T cell	CD45RA on naive CD4+ T cell	7	0.075	0.037	0.272	1.078(1.002-1.160)
	Monocyte	CD16 on CD14- CD16+ monocyte	4	-0.269	0.107	0.257	0.764(0.620-0.942)
	TBNK	HLA DR+ Natural Killer %Natural Killer	3	-0.314	0.138	0.257	0.731(0.557-0.958)
		FSC-A on CD4+ T cell	3	-0.805	0.156	0.000	0.447(0.329-0.607)
	Treg	CD39+ resting CD4 regulatory T cell Absolute Count	4	0.095	0.043	0.257	1.099(1.010-1.196)
		CD39+ resting CD4 regulatory T cell %CD4 regulatory T cell	4	0.066	0.029	0.257	1.069(1.010-1.130)
		Activated CD4 regulatory T cell %CD4 regulatory T cell	3	-0.112	0.055	0.267	0.894(0.804-0.995)
		CD39+ secreting CD4 regulatory T cell %CD4 regulatory T cell	5	0.061	0.027	0.257	1.062(1.007-1.121)
		CD39+ CD4+ T cell %T cell	4	0.058	0.028	0.257	1.060(1.004-1.119)
		CD39+ CD4+ T cell %CD4+ T cell	4	0.057	0.027	0.257	1.059(1.004-1.117)
		CD28+ CD45RA+ CD8dim T cell Absolute Count	3	0.096	0.033	0.234	1.101(1.032-1.173)
		CD39+ CD8+ T cell %T cell	3	0.058	0.028	0.257	1.060(1.004-1.120)
		CD39+ CD8+ T cell Absolute Count	4	0.063	0.027	0.257	1.065(1.009-1.123)
		CD3 on CD39+ activated CD4 regulatory T cell	3	-0.125	0.047	0.257	0.882(0.804-0.968)
		CD3 on secreting CD4 regulatory T cell	3	-0.101	0.046	0.257	0.904(0.826-0.990)
		CD3 on CD4 regulatory T cell	3	-0.100	0.046	0.257	0.905(0.827-0.989)
		CD25 on CD39+ CD4+ T cell	3	-0.089	0.044	0.274	0.915(0.839-0.998)
		CD39 on CD39+ activated CD4 regulatory T cell	4	0.056	0.023	0.257	1.057(1.010-1.107)
		CD39 on CD39+ secreting CD4 regulatory T cell	4	0.065	0.027	0.257	1.067(1.012-1.125)
		CD39 on CD39+ CD4+ T cell	3	0.057	0.027	0.257	1.058(1.004-1.115)

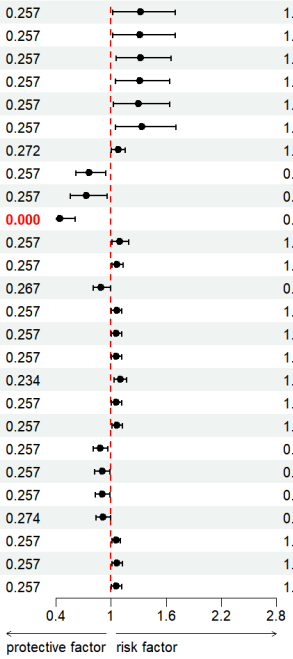

FIGURE 7
Forest plot shows the positive IVW-MR results of causal links between immune cell and PSC.

Supplementary Table S7). By FDR correction (PFDR <0.05), we discovered one immunophenotype to be protective against PSC: *FSC-A on CD4+ T cell* (OR = 0.447, 95%CI 0.329 ~ 0.607, $p = 2.530 \times 10^{-7}$, PFDR = 7.104×10^{-5}).

3.4 Reverse MR analysis

To investigate the causal relationship between the gut microbiome and AILDs further, we conducted a reverse MR analysis, treating AILDs as the exposure and the gut microbiome as the outcome. The results indicate a causal relationship between PBC and *Species.Odoribacter splanchnicus* (OR = 0.955, 95% CI 0.919–0.992, $p = 0.020$), and between PSC and *L histidine degradation I* (OR = 1.032, 95% CI 1.005–1.060, $p = 0.020$). These two gut microbiota will be excluded in subsequent mediation studies. However, no other significant causal relationships were observed, as detailed in Supplementary Tables S8–S10.

3.5 Mediation analysis result

To elucidate the potential mechanisms underlying the occurrence and development of AILDs, we carried out a mediation analysis to establish the causal pathways linking gut microbiota to AILDs through immune cells. This analysis builds on earlier findings that identified significant associations between certain gut microbiota and immune cells and AILDs, as detailed in our Supplementary Tables S11–S13. Initially, the causal relationships between specific gut microbiota and immune cells were assessed using two-sample MR. In the context of AIH, we identified 18 associations linking gut microbiota with immune cells. For PBC, we discovered 4 such associations, and for PSC, we detected 14 associations. MR mediation analysis revealed one immune cell type acting as a mediator in the pathway from gut microbiota to AIH. Notably, the *superpathway of L-tryptophan biosynthesis* to AIH appears to be mediated by the *CD14+ CD16+ monocyte Absolute Count*, with a mediation proportion of 17.47%. For PSC, one immunophenotype was identified as mediator. *Species. Ruminococcus obeum*'s influence on PSC may be mediated by the percentage of *CD62L-CD86+ myeloid Dendritic Cell*, accounting for 32.47% (Table 1).

4 Discussion

In this study, we used genetic prediction to investigate how the abundance of gut microbiota and their pathways influence the development of AILDs. Our data were further refined to the species level, offering a more in-depth analysis compared to previous research and facilitating the exploration of potential mediating mechanisms involving immune cells. The large sample size and multivariable summary data of both gut microbiota and immune cells were utilized in MR analysis, yielding numerous positive associations with AILDs. Some of these findings are consistent with existing observational studies, thereby reinforcing their conclusions. However, other results contradicted previous findings, likely due to racial differences, warranting further investigation. Additionally, some novel findings provide new perspectives for future research.

Evidently, conclusions consistent with previous observational studies are of particular interest to us. *Roseburia* can upregulate tight junctions and enhance mucin production, thereby maintaining the integrity of the intestinal barrier and preventing the translocation of toxins such as Lipopolysaccharides (LPS) from the gut to the liver (Wei et al., 2020). Additionally, it increases anti-inflammatory cytokines, such as IL-22,

TABLE 1 Results of two-step Mendelian randomization mediation analysis.

Exposure	Mediator	Outcome	X-M		M-Y		X-Y		Effect rate
			OR (95%CI)	*P*	OR (95%CI)	*P*	OR (95%CI)	*P*	
Superpathway of L-tryptophan biosynthesis	CD14+ CD16+ monocyte Absolute Count	AIH	0.856 (0.759,0.965)	0.011	0.700 (0.561,0.865)	0.001	1.380 (1.095,1.738)	0.006	17.47%
Species. Ruminococcus_ obeum	CD62L-CD86+ myeloid Dendritic Cell %Dendritic Cell	PSC	0.730 (0.615,0.868)	0.0003	1.341 (1.052,1.708)	0.018	0.753 (0.593,0.956)	0.020	32.47%

X, exposure; M, mediator; Y, outcome; AIH, Autoimmune Hepatitis; PSC, Primary Sclerosing Cholangitis.

effectively inhibiting the interaction between LPS and TLR4 and reducing hepatic inflammation pathways (Jiang et al., 2023), thus providing protection against AIH. By sequencing the highly variable V3-V4 region of the 16S rRNA gene in stool samples from patients with early-stage AIH and comparing these with samples from healthy individuals, we observed an AIH-associated enrichment of *Eubacterium* (Elsherbiny et al., 2020; Terziroli Beretta-Piccoli et al., 2022), which aligns with our study findings. The *species Eubacterium rectale* was also identified as a risk factor for PSC, yet it appears to be protective against PBC. In addition, we discovered that *Bacteroides* may reduce the risk of PBC. Evidence demonstrates that this bacterium can produce health-promoting phenylpropanoid derivatives and generate heparinase, facilitating the extraction of heparin and heparan sulfate from bacterial sources and categorizing it as a beneficial gut microorganism (Lv et al., 2016; Bohlmann et al., 2015). Similarly, fecal DNA sequencing in patients with PSC has revealed a significant reduction in the abundance of *Bacteroides* (Kummen et al., 2021). A reduction in *Ruminococcus* lowers Short-Chain Fatty Acids (SCFA) production, impairing intestinal barriers and potentially exacerbating PSC-related inflammation and immune responses through disrupted gut-liver interactions (Coufal et al., 2019). Prior research has identified the *Ruminococcaceae family* as protective against PBC. Our study advances this understanding, revealing that *Genus.Ruminococcaceae noname* and *Species. Ruminococcaceae bacterium D16* exhibit similar protective effects (Yang et al., 2024).

Gut bacterial pathway abundance refers to the presence and levels of specific metabolic and signaling pathways within the gut microbiome. Our research has discovered that the gut microbiota can play a protective role in AIH by participating in the key biosynthesis pathways of heme, polyamines, and riboflavin. Fibroblast growth factor 4 can increase the levels of heme oxygenase in ConA-induced AIH mice, thereby inhibiting ferroptosis in hepatocytes (Zheng et al., 2021). Tryptophan metabolites may serve as activators of the Aryl hydrocarbon Receptor (AhR), potentially inducing AIH-like pathology. Concurrently, the transcriptional activity of AhR is modulated by the Aryl Hydrocarbon Receptor Repressor (AHRR), which inhibits AhR signal transduction in Treg and Th17 cells, thereby facilitating the development of AIH (Xiang et al., 2024). Arginine residues at specific positions within the (Human Leukocyte Antigen)HLA-DRβ polypeptide are critical for determining genetic susceptibility to AIH. Additionally, studies demonstrated that the interaction between arginine and negatively charged amino acid residues on antigenic peptides facilitates salt bridge formation (Czaja and Donaldson, 2000). This enhances the stability and presentation of antigenic peptides to T-helper cells, thereby promoting immune activation and the continuation of the autoimmune response in AIH (Donaldson et al., 1994). The derivative of methionine, S-adenosyl-L-methionine (SAMe), exerts hepatoprotective effects through methylation and redox mechanisms. Notably, supplementation with SAMe significantly improves clinical symptoms in non-cirrhotic patients with PBC (Kilanczyk et al., 2020; Lai et al., 2022). Lipid A is a crucial component of the LPS discovered in the outer membrane of Gram-negative bacteria. It serves as the endotoxic component of LPS and plays a vital role in bacterial survival and virulence. Lipid IVA is a precursor in the biosynthesis of Lipid A and is critical for the proper formation of LPS (Paul et al., 2022). In a mouse model, knocking out L-histidine decarboxylase (HDC) led to reduced histamine levels, resulting in alleviated biliary damage and liver fibrosis. This suggests that histamine exacerbates PSC by intensifying inflammatory and fibrotic pathways (Kennedy et al., 2020).

Our study yielded findings that diverge from previous research. While earlier studies have suggested that *Ruminiclostridium 9* exerts a protective effect against AIH (Fu et al., 2023), and that the *Ruminococcaceae NK4A214* group increases the risk of AIH, our analysis did not confirm these associations. Notably, we identified *Ruminococcus obeum*, a member of the same family, as a potential risk factor for AIH. These discrepancies are likely attributable to differences in genetic backgrounds and interpopulation interactions across various regions. Geographic variations are a key determinant of gut microbiota diversity (Zhou et al., 2024). Immune homeostasis and genetic susceptibility are key mechanisms in the pathogenesis of AILDs (Tilg et al., 2022). In patients with PSC, there is a notable increase in naive-like CD4+ T cells in the liver compared to healthy liver tissue. Concurrently, another study indicates that reduced apoptosis in activated CD4+ T cells may play a role in the immunological dysregulation observed in PSC (Schoknecht et al., 2017; Poch et al., 2021). Our research suggests that FSC-A measurements on CD4+ T cells may act as a protective factor in PSC. The HLA genes are widely recognized as a genetic foundation for AILDs, albeit with some variations among different types. HLA-DR, a major histocompatibility complex class II molecule, participates in immune responses to extracellular pathogens by presenting antigens to helper T cells. Moreover, elevated expression of HLA-DR is usually linked to enhanced immune activation, which is essential for defending against pathogens. However, it can also lead to pathological autoimmune responses (Li et al., 2018; Higuchi et al., 2021). Our study indicates that HLA-DR expression on CD14-CD16+ monocytes and CD14+ CD16+ monocytes has a protective effect against PBC, while HLA-DR+ natural

killer cells offer protection against AIH. Conversely, CD4 expression on HLA-DR+ CD4+ T cells increases the risk of PBC.

Recent advances in immunology and microbiology have uncovered complex interactions between the gut microbiota and the immune system, underscoring their significance in AILDs (Li et al., 2021; Liwinski et al., 2022). Yet, there are currently limited observational studies on how the gut microbiota influences AILDs through immune-mediated pathways (Li et al., 2022). To our knowledge, this is the first MR study to utilize immune cells as mediators to explore their role in the relationship between the gut microbiome and AILDs. Our findings are largely based on genetic predictions, demonstrating that the abundance of the *superpathway of L-tryptophan biosynthesis* in the gut microbiome correlates positively with the risk of AIH. This association is linked to the activation of the AhR by tryptophan metabolites (Xiang et al., 2024), with *CD14+ CD16+ monocyte Absolute Count* partially mediating this process. Genetically, the morphological parameters of immune cells play a central role in immunoregulation (Longhi et al., 2021). PSC often co-occurs with inflammatory bowel disease (IBD) due to interactions within the liver-gut-immune axis, which may result from the abnormal expression of gut-homing molecules in the PSC liver, thereby facilitating the transport of CD8 memory T cells between the gut and liver (Bozward et al., 2021). Consequently, fecal microbiota transplantation, widely used in IBD patients, may offer a novel therapeutic approach for PSC. Regulatory Tregs are crucial for maintaining the balance between the immune response to self-antigens and tissue damage caused by immune activation (Jeffery et al., 2016). Our study found that *Species. Ruminococcus obeum* may protect against PSC through mediation by *CD62L-CD86+ myeloid Dendritic Cell %Dendritic Cell*,providing new perspectives for future therapeutic targets for PSC (Li et al., 2019). Nevertheless, further research is required to validate these pathways.

However, this study has some limitations. First, the initial research lacked specific information such as age and gender, which are particularly relevant, provided that the incidence of AILDs appears to be associated with these characteristics according to epidemiological findings. The absence of this information hindered further subgroup analysis. Second, most of the GWAS data in this study were derived from individuals of European descent, limiting the generalizability of the findings to other racial groups. Lastly, although the MR method effectively assesses the causal relationship between exposure factors and outcomes, the credibility of these mediating mechanisms requires further validation through experimental and clinical studies.

5 Conclusion

This comprehensive analysis provides a clearer understanding of the complex interactions between the gut microbiome, immune cells, and the pathogenesis of AILDs, offering a valuable foundation for future research into targeted immune therapies for AILD patients. Nonetheless, additional basic and clinical research is essential to support these insights.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

Ethical approval was not required for the study involving humans in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and the institutional requirements.

Author contributions

JZ: Data curation, Writing – original draft, Conceptualization, Formal analysis, Writing – review & editing, Investigation, Visualization. YH: Conceptualization, Supervision, Writing – review & editing, Writing – original draft. JX: Conceptualization, Supervision, Writing – review & editing, Writing – original draft. HuS: Writing – review & editing, Supervision. QZ: Writing – review & editing, Writing – original draft, Conceptualization, Supervision. HaS: Conceptualization, Writing – review & editing, Supervision, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1442506/full#supplementary-material

References

Bohlmann, L., Chang, C. W., Beacham, I., and von Itzstein, M. (2015). Exploring bacterial heparinase II activities with defined substrates. *Chembiochem* 16, 1205–1211. doi: 10.1002/cbic.201500081

Bowden, J., Davey Smith, G., and Burgess, S. (2015). Mendelian randomization with invalid instruments: effect estimation and bias detection through egger regression. *Int. J. Epidemiol.* 44, 512–525. doi: 10.1093/ije/dyv080

Bowden, J., Davey Smith, G., Haycock, P. C., and Burgess, S. (2016). Consistent estimation in mendelian randomization with some invalid instruments using a weighted median estimator. *Genet. Epidemiol.* 40, 304–314. doi: 10.1002/gepi.21965

Bozward, A. G., Ronca, V., Osei-Bordom, D., and Oo, Y. H. (2021). Gut-liver immune traffic: deciphering immune-pathogenesis to underpin translational therapy. *Front. Immunol.* 12:711217. doi: 10.3389/fimmu.2021.711217

Burgess, S., Butterworth, A., and Thompson, S. G. (2013). Mendelian randomization analysis with multiple genetic variants using summarized data. *Genet. Epidemiol.* 37, 658–665. doi: 10.1002/gepi.21758

Burgess, S., Scott, R. A., Timpson, N. J., Davey Smith, G., and Thompson, S. G. (2015). Using published data in mendelian randomization: a blueprint for efficient identification of causal risk factors. *Eur. J. Epidemiol.* 30, 543–552. doi: 10.1007/s10654-015-0011-z

Carter, A. R., Sanderson, E., Hammerton, G., Richmond, R. C., Davey Smith, G., Heron, J., et al. (2021). Mendelian randomisation for mediation analysis: current methods and challenges for implementation. *Eur. J. Epidemiol.* 36, 465–478. doi: 10.1007/s10654-021-00757-1

Coufal, S., Galanova, N., Bajer, L., Gajdarova, Z., Schierova, D., Jiraskova Zakostelska, Z., et al. (2019). Inflammatory bowel disease types differ in markers of inflammation, gut barrier and in specific anti-bacterial response. *Cells* 8:719. doi: 10.3390/cells8070719

Czaja, A. J., and Donaldson, P. T. (2000). Genetic susceptibilities for immune expression and liver cell injury in autoimmune hepatitis. *Immunol. Rev.* 174, 250–259. doi: 10.1034/j.1600-0528.2002.017401.x

Donaldson, P., Doherty, D., Underhill, J., and Williams, R. (1994). The molecular genetics of autoimmune liver disease. *Hepatology* 20, 225–239. doi: 10.1016/0270-9139(94)90157-0

Elsherbiny, N. M., Rammadan, M., Hassan, E. A., Ali, M. E., el-Rehim, A. S. A., Abbas, W. A., et al. (2020). Autoimmune hepatitis: shifts in gut microbiota and metabolic pathways among Egyptian patients. *Microorganisms* 8:1011. doi: 10.3390/microorganisms8071011

Fischer, H. P., and Goltz, D. (2020). Autoimmune liver diseases. *Pathologie* 41, 444–456. doi: 10.1007/s00292-020-00807-7

Fu, Y., Li, J., Zhu, Y., Chen, C., Liu, J., Gu, S., et al. (2023). Causal effects of gut microbiome on autoimmune liver disease: a two-sample mendelian randomization study. *BMC Med. Genet.* 16:232. doi: 10.1186/s12920-023-01670-0

Gill, D., Efstathiadou, A., Cawood, K., Tzoulaki, I., and Dehghan, A. (2019). Education protects against coronary heart disease and stroke independently of cognitive function: evidence from mendelian randomization. *Int. J. Epidemiol.* 48, 1468–1477. doi: 10.1093/ije/dyz200

Hemani, G., Tilling, K., and Davey Smith, G. (2017). Orienting the causal relationship between imprecisely measured traits using GWAS summary data. *PLoS Genet.* 13:e1007081. doi: 10.1371/journal.pgen.1007081

Higuchi, T., Oka, S., Furukawa, H., Tohma, S., Yatsuhashi, H., and Migita, K. (2021). Genetic risk factors for autoimmune hepatitis: implications for phenotypic heterogeneity and biomarkers for drug response. *Hum. Genomics* 15:6. doi: 10.1186/s40246-020-00301-4

Jeffery, H. C., Braitch, M. K., Brown, S., and Oo, Y. H. (2016). Clinical potential of regulatory T cell therapy in liver diseases: an overview and current perspectives. *Front. Immunol.* 7:334. doi: 10.3389/fimmu.2016.00334

Ji, S. G., Juran, B. D., Mucha, S., Folseraas, T., Jostins, L., Melum, E., et al. (2017). Genome-wide association study of primary sclerosing cholangitis identifies new risk loci and quantifies the genetic relationship with inflammatory bowel disease. *Nat. Genet.* 49, 269–273. doi: 10.1038/ng.3745

Jiang, H., Fang, Y., Wang, Y., Li, T., Lin, H., Lin, J., et al. (2023). FGF4 improves hepatocytes ferroptosis in autoimmune hepatitis mice via activation of CISD3. *Int. Immunopharmacol.* 116:109762. doi: 10.1016/j.intimp.2023.109762

Kardashian, A., Serper, M., Terrault, N., and Nephew, L. D. (2023). Health disparities in chronic liver disease. *Hepatology* 77, 1382–1403. doi: 10.1002/hep.32743

Kennedy, L., Meadows, V., Demieville, J., Hargrove, L., Virani, S., Glaser, S., et al. (2020). Biliary damage and liver fibrosis are ameliorated in a novel mouse model lacking l-histidine decarboxylase/histamine signaling. *Lab. Investig.* 100, 837–848. doi: 10.1038/s41374-020-0405-8

Kilanczyk, E., Banales, J. M., Wunsch, E., Barbier, O., Avila, M. A., Mato, J. M., et al. (2020). S-adenosyl-L-methionine (SAMe) halts the autoimmune response in patients with primary biliary cholangitis (PBC) via antioxidant and S-glutathionylation processes in cholangiocytes. *Biochim. Biophys. Acta Mol. basis Dis.* 1866:165895. doi: 10.1016/j.bbadis.2020.165895

Kummen, M., Thingholm, L. B., Rühlemann, M. C., Holm, K., Hansen, S. H., Moitinho-Silva, L., et al. (2021). Altered gut microbial metabolism of essential nutrients in primary sclerosing cholangitis. *Gastroenterology* 160, 1784–1798.e0. doi: 10.1053/j.gastro.2020.12.058

Lai, H., Li, Y., Zhang, H., Hu, J., Liao, J., Su, Y., et al. (2022). exoRBase 2.0: an atlas of mRNA, lncRNA and circRNA in extracellular vesicles from human biofluids. *Nucleic Acids Res.* 50, D118–D128. doi: 10.1093/nar/gkab1085

Larsson, S. C., and Burgess, S. (2021). Causal role of high body mass index in multiple chronic diseases: a systematic review and meta-analysis of mendelian randomization studies. *BMC Med.* 19:320. doi: 10.1186/s12916-021-02188-x

Levin, M. G., Judy, R., Gill, D., Vujkovic, M., Verma, S. S., Bradford, Y., et al. (2020). Genetics of height and risk of atrial fibrillation: a mendelian randomization study. *PLoS Med.* 17:e1003288. doi: 10.1371/journal.pmed.1003288

Li, Z. J., Gou, H. Z., Zhang, Y. L., Song, X. J., and Zhang, L. (2022). Role of intestinal flora in primary sclerosing cholangitis and its potential therapeutic value. *World J. Gastroenterol.* 28, 6213–6229. doi: 10.3748/wjg.v28.i44.6213

Li, S., Li, Y., Chen, B., Zhao, J., Yu, S., Tang, Y., et al. (2018). exoRBase: a database of circRNA, lncRNA and mRNA in human blood exosomes. *Nucleic Acids Res.* 46, D106–D112. doi: 10.1093/nar/gkx891

Li, R., Mao, Z., Ye, X., and Zuo, T. (2021). Human gut microbiome and liver diseases: from correlation to causation. *Microorganisms* 9:1017. doi: 10.3390/microorganisms9051017

Li, Y., Zhao, J., Yu, S., Wang, Z., He, X., Su, Y., et al. (2019). Extracellular vesicles long RNA sequencing reveals abundant mRNA, circRNA, and lncRNA in human blood as potential biomarkers for cancer diagnosis. *Clin. Chem.* 6, 798–808. doi: 10.1373/clinchem

Liwinski, T., Heinemann, M., and Schramm, C. (2022). The intestinal and biliary microbiome in autoimmune liver disease-current evidence and concepts. *Semin. Immunopathol.* 44, 485–507. doi: 10.1007/s00281-022-00936-6

Longhi, M. S., Mieli-Vergani, G., and Vergani, D. (2021). Regulatory T cells in autoimmune hepatitis: an updated overview. *J. Autoimmun.* 119:102619. doi: 10.1016/j.jaut.2021.102619

Lopera-Maya, E. A., Kurilshikov, A., Van der Graaf, A., Hu, S., Andreu-Sánchez, S., Chen, L., et al. (2022). Effect of host genetics on the gut microbiome in 7,738 participants of the Dutch microbiome project. *Nat. Genet.* 54, 143–151. doi: 10.1038/s41588-021-00992-y

Lv, L. X., Fang, D. Q., Shi, D., Chen, D. Y., Yan, R., Zhu, Y. X., et al. (2016). Alterations and correlations of the gut microbiome, metabolism and immunity in patients with primary biliary cirrhosis. *Environ. Microbiol.* 18, 2272–2286. doi: 10.1111/1462-2920.13401

Ma, Z., Zhao, M., Zhao, H., and Qu, N. (2024). Causal role of immune cells in generalized anxiety disorder: mendelian randomization study. *Front. Immunol.* 14:1338083. doi: 10.3389/fimmu.2023.1338083

Mihai, I. R., Rezus, C., Burlui, M. A., Cardoneanu, A., Macovei, L. A., Richter, P., et al. (2024). Autoimmune liver diseases and rheumatoid arthritis—is there an Etiopathogenic link? *Int. J. Mol. Sci.* 25:3848. doi: 10.3390/ijms25073848

Muratori, L., Lohse, A. W., and Lenzi, M. (2023). Diagnosis and management of autoimmune hepatitis. *BMJ* 380:e070201. doi: 10.1136/bmj-2022-070201

Oldereid, T. S., Jiang, X., Nordhus, K. S., Ponzetta, A., Bjørnholt, J. V., Björkström, N. K., et al. (2024). Role of bacteria and microbial metabolites in immune modulation during early life. *Scand. J. Immunol.* 99:e13336. doi: 10.1111/sji.13336

Orrù, V., Steri, M., Sidore, C., Marongiu, M., Serra, V., Olla, S., et al. (2020). Complex genetic signatures in immune cells underlie autoimmunity and inform therapy. *Nat. Genet.* 52, 1036–1045. doi: 10.1038/s41588-020-0684-4

Palmer, T. M., Lawlor, D. A., Harbord, R. M., Sheehan, N. A., Tobias, J. H., Timpson, N. J., et al. (2012). Using multiple genetic variants as instrumental variables for modifiable risk factors. *Stat. Methods Med. Res.* 21, 223–242. doi: 10.1177/0962280210394459

Paul, B., Lewinska, M., and Andersen, J. B. (2022). Lipid alterations in chronic liver disease and liver cancer. *JHEP Rep.* 4:100479. doi: 10.1016/j.jhepr.2022.100479

Poch, T., Krause, J., Casar, C., Liwinski, T., Glau, L., Kaufmann, M., et al. (2021). Single-cell atlas of hepatic T cells reveals expansion of liver-resident naive-like CD4+ T cells in primary sclerosing cholangitis. *J. Hepatol.* 75, 414–423. doi: 10.1016/j.jhep

Qian, Q., He, W., Tang, R., and Ma, X. (2023). Implications of gut microbiota in autoimmune liver diseases. *Minerva Gastroenterol* 69, 95–106. doi: 10.23736/S2724-5985.21.02860-9

Qu, K., Li, M. X., Gan, L., Cui, Z. T., Li, J. J., Yang, R., et al. (2024). To analyze the relationship between gut microbiota, metabolites and migraine: a two-sample mendelian randomization study. *Front. Microbiol.* 15:1325047. doi: 10.3389/fmicb.2024.1325047

Quraishi, M. N., Sergeant, M., Kay, G., Iqbal, T., Chan, J., Constantinidou, C., et al. (2017). The gut-adherent microbiota of PSC-IBD is distinct to that of IBD. *Gut* 66, 386–388. doi: 10.1136/gutjnl-2016-311915

Sakaue, S., Kanai, M., Tanigawa, Y., Karjalainen, J., Kurki, M., Koshiba, S., et al. (2021). A cross-population atlas of genetic associations for 220 human phenotypes. *Nat. Genet.* 53, 1415–1424. doi: 10.1038/s41588-021-00931-x

Schneider, K. M., Kummen, M., Trivedi, P. J., and Hov, J. R. (2023). Role of microbiome in autoimmune liver diseases. *Hepatology* 27:506. doi: 10.1097/HEP.0000000000000506

Schoknecht, T., Schwinge, D., Stein, S., Weiler-Normann, C., Sebode, M., Mucha, S., et al. (2017). CD4+ T cells from patients with primary sclerosing cholangitis exhibit reduced apoptosis and down-regulation of proapoptotic Bim in peripheral blood. *J. Leukoc. Biol.* 101, 589–597. doi: 10.1189/jlb.5A1015-469R

Terziroli Beretta-Piccoli, B., Mieli-Vergani, G., and Vergani, D. (2022). HLA, gut microbiome and hepatic autoimmunity. *Front. Immunol.* 13:980768. doi: 10.3389/fimmu.2022.980768

Tilg, H., Adolph, T. E., and Trauner, M. (2022). Gut-liver axis: pathophysiological concepts and clinical implications. *Cell Metab.* 34, 1700–1718. doi: 10.1016/j.cmet.2022.09.017

Verbanck, M., Chen, C. Y., Neale, B., and Do, R. (2018). Detection of widespread horizontal pleiotropy in causal relationships inferred from mendelian randomization between complex traits and diseases. *Nat. Genet.* 50, 693–698. doi: 10.1038/s41588-018-0099-7

Wang, Y. W., Lin, C. I., Chen, H. W., Wu, J. C., and Chuang, Y. H. (2022). Apoptotic biliary epithelial cells and gut dysbiosis in the induction of murine primary biliary cholangitis. *J. Transl. Autoimmun.* 6:100182. doi: 10.1016/j.jtauto

Wei, Y., Li, Y., Yan, L., Sun, C., Miao, Q., Wang, Q., et al. (2020). Alterations of gut microbiome in autoimmune hepatitis. *Gut* 69, 569–577. doi: 10.1136/gutjnl-2018-317836

Xiang, X., Li, Q., Wan, J., Chen, C., Guo, M., He, Z., et al. (2024). The role of amino acid metabolism in autoimmune hepatitis. *Biomed. Pharmacother.* 173:116452. doi: 10.1016/j.biopha.2024.116452

Yang, J., Ma, G., Wang, K., Yang, H., Jiang, S., Fan, Q., et al. (2024). Causal associations between gut microbiota and cholestatic liver diseases: a mendelian randomization study. *Front. Med.* 11:1342119. doi: 10.3389/fmed.2024.1342119

Zhao, J., Ming, J., Hu, X., Chen, G., Liu, J., and Yang, C. (2020). Bayesian weighted mendelian randomization for causal inference based on summary statistics. *Bioinformatics* 36, 1501–1508. doi: 10.1093/bioinformatics/btz749

Zheng, Y., Ran, Y., Zhang, H., Wang, B., and Zhou, L. (2021). The microbiome in autoimmune liver diseases: metagenomic and metabolomic changes. *Front. Physiol.* 12:715852. doi: 10.3389/fphys

Zhou, J., Zhu, D., Xu, Y., Chen, C., and Wang, K. (2024). Genetically predicted gut microbiota mediate the association between plasma lipidomics and primary sclerosing cholangitis. *BMC Gastroenterol.* 24:158. doi: 10.1186/s12876-024-03246-3

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