

Original Article

Gut microbiota and bile acids changes in MASLD mice model with hepatic *PLD1* knockout

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Abstract

Hepatocyte phospholipase D1 (*PLD1*) knockout alleviates metabolic dysfunction-associated steatotic liver disease (MASLD) in mice, but the underlying mechanism is largely unknown. In this study, the mice are divided into four groups: Con (wild-type mice with normal control diet), HFHC (wild-type mice with high-fat diet), Con_KO (hepatocyte *PLD1*-knockout mice with normal control diet), and HFHC_KO (hepatocyte *PLD1*-knockout mice with high-fat diet). Intestinal contents of mice are analyzed via metagenomics and metabolomics, and the liver bile acids are assessed by mass spectrometry imaging. The results show that at the phylum level the abundance of Bacillota in the intestines of MASLD model mice is significantly increased, whereas that of Bacteroidota significantly is decreased. However, after the deletion of hepatocyte *PLD1*, Pseudomonadota and Candidatus Bathyarchaeota are significantly decreased in the MASLD model mice. At the species level, compared with that in the Con group, the abundance of *Faecalibaculum rodentium* is significantly increased in the HFHC group, whereas hepatocyte *PLD1* knockout causes the abundances of *Desulfovibrionaceae bacterium* LT0009 and *Lachnospiraceae bacterium* 10-1 to be significantly decreased. In terms of intestinal bile acids, the levels of two bile acids (hyodeoxycholic acid and glycolithocholic acid) differ between the HFHC_KO group and the HFHC group. Association analysis shows that *Faecalibaculum* co-occurs with DCA, β MCA, Ω MCA and α MCA, while probiotic *Bacteroides uniformis* is significantly correlated with UDCA, 12-KetoLCA, and 7-KetoLCA. Finally, mass spectrometry imaging reveals that the TCA and TDCA contents in the liver are significantly decreased after *PLD1* knockout in hepatocytes. These findings demonstrate that hepatocyte *PLD1* knockout alters the gut microbiota and bile acids profiles, suggesting that *PLD1* deficiency may modulate MASLD progression by changing intestinal microbiota-bile acid homeostasis.

Key words bile acid, dysfunction-associated steatotic liver disease, gut microbiota, metagenomics, phospholipase D1

Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD) has emerged as the most prevalent chronic liver disease, and with the transformation of modern lifestyles and the increasing prevalence of metabolic syndrome, such as obesity, the prevalence of MASLD is likely to continue rising. MASLD is associated with an increased risk of cardiovascular events, extrahepatic malignancy, liver failure, and hepatocellular carcinoma [1–3]. Nevertheless, to date, there remains a lack of clear and reliable therapeutic approaches for MASLD in clinical practice.

An increasing number of researchers are now exploring the interaction between the gut-liver axis and MASLD, using the gut

microbiota as a key entry point to search for interventions for liver lesions in MASLD. Previous studies have demonstrated that the structure of the gut microbiota in MASLD patients often undergoes significant alterations, and the most typical feature is an imbalance in the ratio of Bacillota to Bacteroidetes, which is closely associated with the development of hepatic steatosis and obesity, strongly indicating the potential role of dysbiosis of the gut microbiota in the pathogenesis of MASLD [4]. In patients with metabolic dysfunction-associated steatohepatitis (MASH), the abundance of *Bacteroides* and *Ruminococcus* is significantly increased, whereas that of *Prevotella* is decreased [5]. In addition, animal studies have provided compelling evidence for the potential role of the gut

microbiota in MASLD [6]. Studies have shown that the ingestion of the intestinal microbiota from MASLD mice by normal mice can cause the transmission of MASLD-related disease symptoms [7].

Phospholipase D1 (PLD1), an isoenzyme of phospholipase D (PLD), has a unique biological function and can decompose phosphatidylcholine into phosphatidic acid. As an important lipid second messenger, phosphatidic acid is widely involved in the regulation of intracellular signaling pathways [8]. PLD1 is ubiquitously expressed on the surface of mammalian cells [8] and participates in lipid metabolism by regulating the accumulation of lipid droplets [9,10]. In our prior studies, PLD1 was found to be significantly upregulated in the livers of MASLD mice, and the disease was markedly alleviated when hepatic PLD1 was absent [11]. Although previous studies have highlighted the close relationship between the gut microbiota and MASLD, there is currently no evidence regarding the impact of hepatic PLD1 on the gut microbiota.

In this study, we investigated the effects of hepatic PLD1 on the gut microbiota and bile acid metabolism in high-fat diet-induced MASLD mice and investigated whether these effects could explain the remission of MASH disease caused by hepatic *PLD1* knockout. We conducted a series of rigorous experiments. Specifically, the intestinal contents of the mice were subjected to metagenomic and untargeted metabolomics sequencing, while liver tissue was subjected to bile acid metabolomic mass spectrometry imaging analysis. The results showed that in MASLD mice, the abundance of Bacillota was significantly increased, whereas that of Bacteroidetes was significantly decreased. However, when hepatic PLD1 was absent, the abundances of Pseudomonadota and Candidatus Bathyarchaeota were significantly decreased. Moreover, corresponding changes in bile acid levels were observed, and a certain correlation was found between the microbiota and bile acid levels.

Materials and Methods

Animal model

Hepatocyte *PLD1*-knockout mice were generated at the Shanghai Model Organisms Center, Inc. (Shanghai, China). All animals were housed in a pathogen-free, temperature-controlled facility at Peking University People's Hospital with a 12-h light/dark cycle. All animal experiment procedures were approved by the Institutional Animal Care and Ethics Committee (animal ethics approval number: 2024PHE122). For the experiment, 8–9-week-old hepatocyte *PLD1*-knockout mice and wild-type mice were provided either a normal control diet or a high-fat diet rich in fat and cholesterol (western diet composition: 50% sugar, 20% protein, 21% fat, and 1% cholesterol; sourced from Research Diets (New Brunswick, USA)). After 16 weeks of feeding, mouse feces were collected in a sterile environment, and these mice were subsequently sacrificed to obtain liver tissues.

Metagenomic analysis

Total genomic DNA was extracted from 0.5 g of stool with a FastPure Stool DNA Isolation Kit (magnetic bead) (MJYH, Shanghai, China) according to the manufacturer's instructions. The concentration and purity of the extracted DNA were assessed via SynergyHTX and NanoDrop2000, respectively. DNA quality was verified via 1% agarose gel electrophoresis. For paired-end library construction, the DNA extract was fragmented to an average size of approximately 400 bp via a Covaris M220 (Gene Company Limited,

Hong Kong, China). The paired-end library was generated with the NEXTFLEX Rapid DNA-Seq Kit (Bioo Scientific, Austin, USA). Sequencing was conducted on an Illumina NovaSeq™ X Plus (Illumina Inc., San Diego, USA) at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China), employing the NovaSeq X Series 25B Reagent Kit following the manufacturer's protocols (www.illumina.com). Metagenomic sequencing data were analyzed on the Majorbio Cloud Platform (www.majorbio.com).

Bile acid metabolomic analysis

The 47 bile acid standards were weighed accurately and prepared as single standard master mixtures in methanol. The working standard solution was prepared by measuring an appropriate amount of each master batch and diluting it with 50% acetonitrile to a suitable concentration. The isotope standards (CDCA-D4, CA-D4) were weighed accurately and prepared as a single master batch with methanol. The working standard solution was added to the internal standard solution at a volume ratio of 1:1 and mixed well to obtain solutions with different concentrations.

Dry the fecal specimens and take 100 mg from each sample. Fifty microlitres of internal standard working solution (200 ng/mL) and 350 µL of extraction solution (methanol) were added, vortexed and mixed for 30 s, sonicated at low temperature for 30 min (5°C, 40 kHz), stored at –20°C for 30 min, centrifuged at 4°C for 15 min at 13,000 g, the supernatant were collected for subsequent LC-MS/MS analysis.

The supernatant was blown dry with nitrogen, 100 µL of 50% acetonitrile was added to redissolve it, the mixture was vortexed for 30 s, sonicated at 5°C (40 kHz) for 10 min, and centrifuged at 4°C for 15 min at 13,000 g, after which the supernatant was used for LC-MS/MS analysis.

The LC-MS/MS analysis was performed on an ExionLC AD system coupled with a QTRAP® 6500+ mass spectrometer (Sciex, Boston, USA) at Majorbio Bio-Pharm Technology (Shanghai, China). The samples were separated via a Waters BEH C18 column (150 × 2.1 mm, 1.7 µm) maintained at 50°C. The mobile phases consisted of solvent A (water with 0.025% formic acid and 10 mM ammonium acetate) and solvent B (acetonitrile:methanol, 9:1 v/v), which were delivered at 0.4 mL/min. The gradient program was as follows: 0–0.5 min, 5%–10% B; 0.5–1.0 min, 10%–20% B; 1.0–2.0 min, 20%–28% B; 2.0–8.0 min, isocratic at 28% B; 8.0–11.0 min, 28%–33% B; 11.0–20.0 min, 33%–45% B; 20.0–23.0 min, 45%–50% B; 23.0–29.0 min, 50%–80% B; 29.0–29.01 min, 80%–100% B; 29.01–31.0 min, isocratic at 100% B; 31.0–31.01 min, 100%–5% B; and 31.01–33.0 min, isocratic at 5% B for system equilibration. The samples were stored at 4°C during analysis. Mass spectrometric data were collected via an ESI source in negative ion mode on a SCIEX QTRAP 6500+ with the following optimized parameters: CUR 35, CAD medium, IS-4500 V, TEM 550°C, GS1 50, and GS2 50. The raw data were imported into Sciex OS software, and metabolite concentrations were calculated via linear regression standard curves.

Mass spectrometry imaging

Mass spectrometry imaging was carried out with a Shimadzu instrument. The chemicals used were from Sigma-Aldrich (St Louis, USA): α -cyano-4-hydroxycinnamic acid (CHCA, 97%), 1,5-diaminonaphthalene (DAN, 97%), and Girard's reagent T (GIT, 98%). The preparation of liver sections was performed according to a

standard procedure. Livers were sectioned at -20°C and mounted on ITO slides (South China Technology Co., Ltd., Shenzhen, China) with a conductive layer thickness of 1200 nm. Finally, the sections were subjected to laser irradiation.

For the analysis of cinnamaldehyde and methoxy-cinnamaldehyde with air pressure MALDI mass spectrometry imaging (AP-MALDI MSI), they were first derivatized with GIT and then subjected to CHCA as the matrix. GIT and CHCA were prepared at 10.0 mg/mL in 20% acetic acid and 50% acetonitrile aqueous solutions (0.1% TFA), respectively. The samples were subjected to GIT derivatization for 80 min at room temperature, after which CHCA was sprayed onto the sample surface. Both the GIT and CHCA were sprayed with an iMLayer AERO automatic sprayer (Shimadzu, Kyoto, Japan). The common spraying parameters were as follows: layers, 20; pumping pressure, 0.1 MPa; spraying pressure, 0.2 MPa; drying time, 1 s; washing frequency, 1 layer/wash; and scan pitch, 1 mm. The flow rate, nozzle distance, and stage speed were different: 0.015 and 0.06 mL/min, 9 and 5 cm, and 120 and 70 mm/s for the GIT and CHCA, respectively. The remaining compounds were detected in negative ion mode with DAN as the matrix. DAN was vapor-deposited onto the sample surface with an iMLayer™ matrix vapor deposition system (Shimadzu). The deposition parameters were as follows: temperature, 180°C ; vacuum, 2×10^{-2} Pa; and time, 5 min.

Mass spectrometry imaging was performed on an iMScope QT (Shimadzu) instrument equipped with an optical microscope, an AP-MALDI source, and a quadrupole time-of-flight mass spectrometer (LCMS-9030). Statistical analysis and image reconstruction were carried out with IMAGEREVEAL™ MS (Shimadzu). Normalization was performed for each sample with the TIC as the denominator. The minimum threshold value was set as 0% to avoid missing any possible active ingredients. The average value of the weighted mass peak area of each region of interest (ROI, generally the whole sample) was used as an indicator of the relative content of each active ingredient.

Statistical analysis

Statistical analysis was performed via GraphPad Prism version 10.1.2 (GraphPad Software, San Diego, USA), and the data are expressed as the mean $\pm$ SEM. Differences between two groups were compared via Student's *t* test for normal distribution and the Mann-Whitney test for abnormal distribution. Statistical significance was set at $P < 0.05$.

Results

A high-fat diet significantly alters the diversity of the intestinal microbiota in mice

In our previous study, hepatocyte *PLD1* knockout was found to mitigate high-fat diet-induced MASLD in mice. To investigate the impact of hepatocyte *PLD1* knockout on the intestinal microbiota and bile acid metabolism, hepatocyte *PLD1*-knockout mice and wild-type mice were randomly assigned to two groups and fed with either a high-fat diet or a normal control diet. The mice were divided into four groups: Con (wild-type mice with normal control diet), HFHC (wild-type mice with high-fat diet), Con_KO (hepatocyte *PLD1*-knockout mice with normal control diet), and HFHC_KO (hepatocyte *PLD1*-knockout mice with high-fat diet). Metagenomic sequencing of the intestinal microbiota was performed after we collected the intestinal contents from the mice. The results

demonstrated that the differences across groups were generally greater than the differences within groups (Figure 1A). Compared with those fed a normal diet, mice fed a high-fat diet presented significantly different intestinal microbiota distributions, while minimal differences were observed between the Con group and Con_KO group, as well as between the HFHC group and the HFHC_KO group (Figure 1B,C). In terms of microbial diversity, the Shannon index and Chao index of the intestinal microbiota in the HFHC and HFHC_KO groups were significantly lower than those in the other two groups, while there was no significant difference between the HFHC and HFHC_KO groups (Figure 1D). Further analysis of the gut microbial distribution at the phylum level revealed that Bacillota and Bacteroidota were predominant in all the groups (Figure 1E). Therefore, a high-fat diet had a more pronounced effect on the diversity of the gut microbiota than did hepatic *PLD1* in mice.

Knocking out hepatocyte *PLD1* leads to significant alterations in the abundance of certain bacterial species

At the phylum level, compared with the mice fed with a normal diet, those fed a high-fat diet presented a notable increase in the abundance of Bacillota and a significant decrease in Bacteroidota. However, when the HFHC group was compared with the HFHC_KO group, no significant differences were observed in these two dominant phyla (Figure 2A,C). Additionally, the levels of Pseudomonadota and Candidatus Bathyarchaeota were significantly lower in the HFHC_KO group than in the HFHC group (Figure 2B). Further analysis at the species level revealed that, compared with the Con mice, HFHC mice presented a significant increase in *Faecalibaculum rodentium*, along with notable changes in other low-abundance species. Meanwhile, compared with the HFHC group, the HFHC_KO group presented significant decreases in *Desulfovibrionaceae bacterium* LT0009 and *Lachnospiraceae bacterium* 10-1 (Figure 2D,E). By intersecting the differentially expressed species between the Con group and the HFHC group and between the HFHC group and the HFHC_KO group, a total of 18 differentially expressed species were identified. Among these 18 species, *Desulfovibrionaceae bacterium* LT0009 and *Lachnospiraceae bacterium* 10-1 had the highest abundances (Figure 2F and Table 1). Previous studies have indicated that *Lachnospiraceae* can produce long-chain fatty acids (elaidate) by decomposing high-fat foods and that elaidate can damage the intestinal barrier and cause metabolic disorders such as obesity. Although it has little effect on diversity, hepatic *PLD1* knockout indeed led to significant changes in the abundance of *Desulfovibrionaceae bacterium* LT0009 and *Lachnospiraceae bacterium* 10-1.

Hepatocyte *PLD1* knockout changes the gut microbiome metabolism of mice

Initially, our findings revealed that the intestinal microorganisms across all groups of mice were predominantly enriched within metabolic pathways (Figure 3A,B). Cluster analysis of the enriched metabolic pathways revealed that the metabolic environment in the gut of mice fed with a high-fat diet was markedly different from that of mice fed with a normal control diet, whereas the effect of gene knockout was relatively subtle (Figure 3C). Further investigation through bile acid metabolomics sequencing analysis of the intestinal contents revealed that when the HFHC group was compared with the Con group, a total of 23 bile acids exhibited

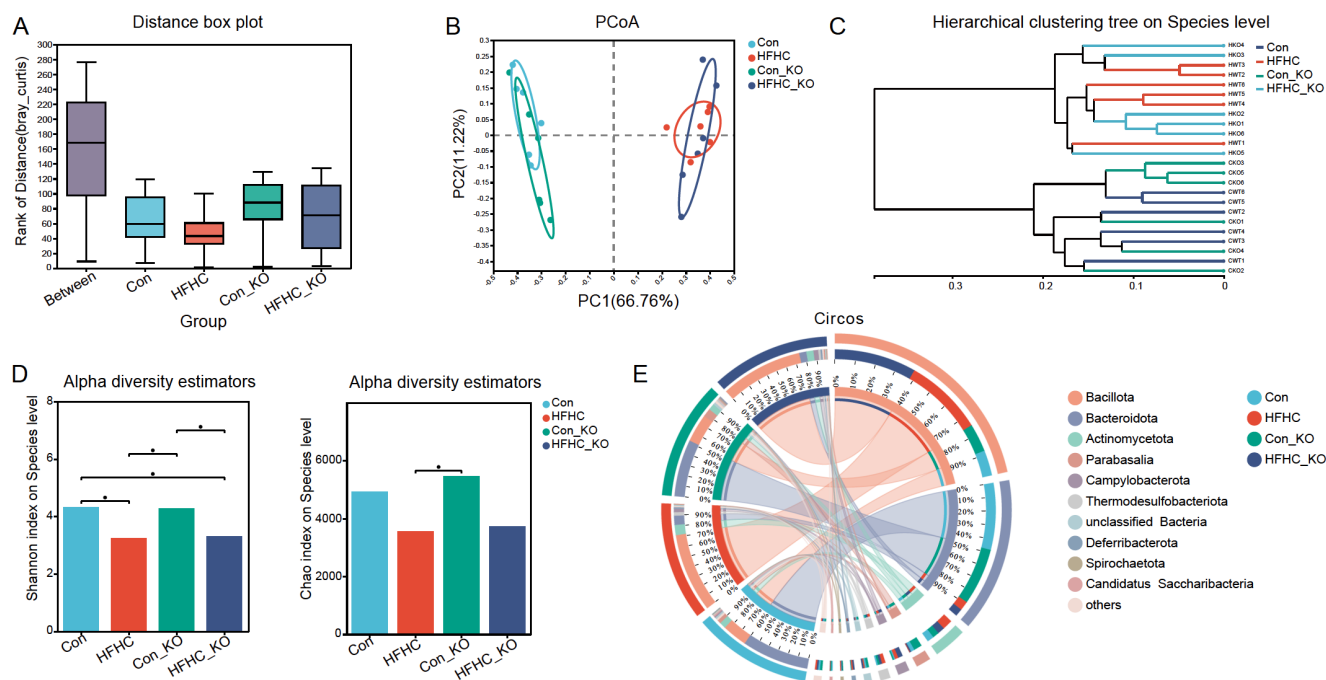


Figure 1. A high-fat diet changes the diversity of the intestinal flora in mice Overall status of intestinal microbiota diversity in each group of mice. (A) A box plot was used to show the differences between groups and within groups. The ordinate represents the distance value. The boxes “Between” refer to differences between groups, whereas the others represent differences within each group. (B) The similarities and differences in the communities between groups were demonstrated via two-dimensional visual scatter plots, and the degree of agglomeration and dispersion of samples was reflected by the distance between samples. (C) A sample-level clustering tree was used to show the similarities and differences in species composition among the samples. (D) Differences in the Chao1 index and Shannon index among the different groups. (E) Circos sample-species diagrams were used to show the distribution of species present in different groups. On one side of the graph are the groups, and on the other side are the dominant species. Data are presented as the mean $\pm$ SEM, $n = 6$ mice per group. * $P < 0.05$.

significant changes. In contrast, only two bile acids, namely, hydoxycholeic acid (HDCA) and glycincholeic acid (GLCA), were significantly altered in the HFHC_KO group compared with the HFHC group. (Figure 3D,E). Intriguingly, these two bile acids did not coincide with the previously mentioned 23 bile acids. Remarkably, we observed a high degree of consistency between the metagenomic data and the bile acid metabolome data (Figure 3F). Collectively, at the metabolic level, the hepatic knockout of *PLD1* significantly modified the composition of bile acids in the gut, with HDCA and GLCA being prime examples of such alterations.

Bile acid levels in the gut and liver are correlated with bacterial abundance in the gut

To further explore these relationships, we performed co-occurrence probability analysis with the top 50 bacterial genera and all 46 measured bile acids, and the results revealed that DCA, β MCA, Ω MCA, and α MCA exhibited a particularly high tendency to co-occurred with all bacterial genera. Among the bacteria, *Faecalibaculum* showed the maximum co-occurrence probability with all four bile acids (Figure 4A). Previous studies have confirmed that *Bacteroides uniformis* has beneficial effects on liver metabolic diseases. In the present study, *B. uniformis* was significantly correlated with UDCA, 12-ketoLCA and 7-ketoLCA bile acids; however, it is likely that other bacterial species may also be involved in the metabolism of these bile acids (Figure 4B–E). In addition, we also employed mass spectrometry imaging to analyze liver tissues from the four groups of mice. The results indicated that the contents of TCA and TDCA in the liver of the HFHC group were

significantly greater than those in the Con group, whereas the contents of TCA and TDCA in the HFHC_KO group were significantly lower than those in the HFHC group (Figure 4F–H). This discrepancy from the changes observed in the intestinal contents may be attributed to the enterohepatic circulation and the distinct microenvironmental characteristics of the liver and intestine. In conclusion, our findings demonstrate a correlation between bile acid levels and the abundance of microbiota; although the causal relationship remains to be elucidated, these results suggest a potential mechanism by which *PLD1* may influence bile acid metabolism through its effects on the gut microbiota.

Discussion

Upon exposure to a high-fat diet, the gut microbiota composition and bile acid profiles undergo profound transformations. In previous investigations, remarkable shifts in intestinal microbes were consistently observed in MASLD disease. Animal studies have provided compelling evidence that inoculating normal mice with the gut microbiota from MASLD mice can trigger the manifestation of MASLD, strongly suggesting a potential causal link between the gut microbiota and the pathogenesis of MASLD [12]. Additionally, several studies have revealed a positive correlation between *Bacteroides* or *Ruminococcus* and the severity of MASLD in human patients [5]. While species-specific disparities between mouse and human gut microbiota pose challenges for translational research, fecal microbiota transplantation (FMT) has emerged as a valuable tool. FMT from patients with MASH into germ-free mice has successfully recapitulated key features of MASH, including hepatic

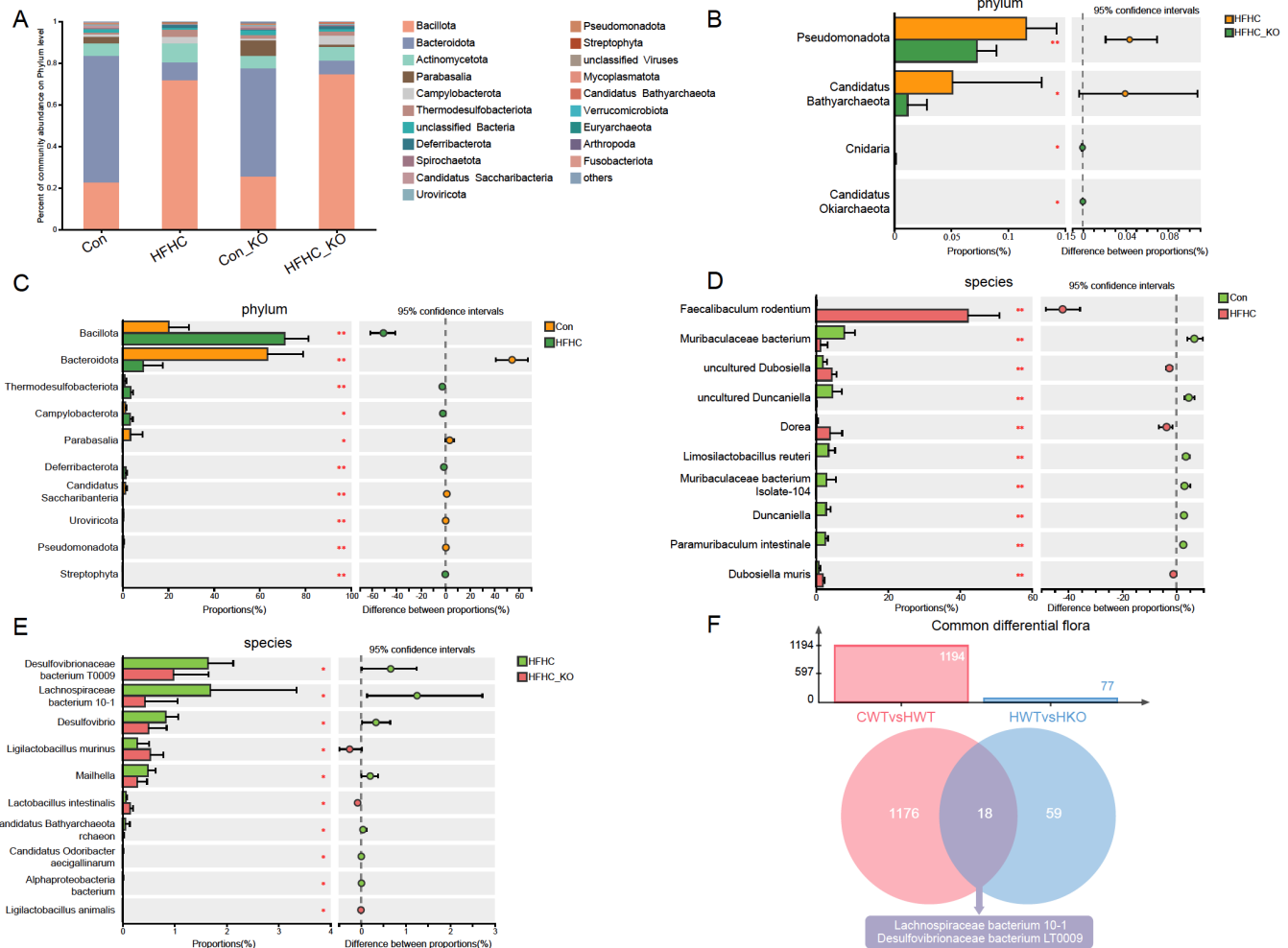


Figure 2. Liver *PLD1* knockout results in a significant change in the abundance of a subset of bacteria (A) The community bar chart showing the species composition of the top 20 most abundant species in all the samples and the proportions of different species, and other low-abundance species are classified as others. (B) The bar chart showing the difference in the mean relative abundance of the different phyla between the HFHC and HFHC_KO groups. (C) The bar chart showing the difference in the mean relative abundance of the different phyla between the Con group and the HFHC group. (D) The bar chart showing the difference in the mean relative abundance of the different species between the Con group and the HFHC group. (E) Bar chart showing the differences in the mean relative abundances of the different species between the HFHC and HFHC_KO groups. (F) Eighteen species were obtained by intersection of Con vs. HFHC mice and HFHC vs. HFHC_KO mice. Data are presented as the mean $\pm$ SEM, $n = 6$ mice per group. * $P < 0.05$, ** $P < 0.01$.

steatosis and inflammation. These pathological changes are further exacerbated by high-fat diet consumption [13].

In the present study, metagenomic analysis revealed that high-fat diet intervention elicited profound changes in the gut microbiota composition of mice, such as a decrease in Bacteroidota and an increase in Bacillota, in which *Faecalibaculum rodentium* increased. Intriguingly, compared with high-fat diet exposure, liver-specific knockout of *PLD1* induced less dramatic changes in the gut microbiota, particularly in terms of microbiota diversity. This phenomenon may be attributed to the fact that genetic alterations affect only hepatic cells, exerting a limited effect on the intestinal environment.

Notably, we found two species whose abundance changed significantly: *Lachnospiraceae bacterium 10-1* and *Desulfovibrionaceae bacterium LT0009*. However, owing to the limitations of bacterial isolation methods, their characteristics and effects have not been further studied. It has been reported that *Lachnospiraceae*

can act as a probiotic to protect the intestinal barrier through its metabolite butyrate, thereby improving metabolism in the body and liver [14–16]. However, other researchers have reported that the commensal species *Fusimonas intestini* can damage the intestinal barrier by decomposing high-fat foods to produce long-chain fatty acids—elaidate, causing metabolic disorders such as obesity [17]. With respect to *Desulfovibrio*, the *Desulfovibrio* genus has been shown to dominate when a high-fat diet impairs the gut microbiota composition and is associated with cancer progression [18].

Given that *PLD1* gene editing occurs exclusively in the liver and does not directly affect the gut microbiota, the following question arises: what mediates the connection between the two? We suspect that the most likely mediator is bile acids, since the enterohepatic circulation of bile acids is the most direct way for the gut to interact with the liver. Indeed, bile acids play a crucial role in enterohepatic circulation in MASLD. Bile acids generally have certain toxic effects on bacteria, yet under specific circumstances, they can promote

Table 1. The mean abundances of the 18 differentially changed species shown in Figure 2F

Species	Con-mean (%)	HFHC-mean (%)	HFHC_KO-mean (%)
<i>Lachnospira hominis</i>	0.001038	0	7.21×10 ⁻⁵
<i>Candidatus Arthromitus</i> sp. SFB-rat-Yit	0.000153	1.73×10 ⁻⁶	3.73×10 ⁻⁵
<i>Candidatus Arthromitus</i> sp. SFB-2	0.002180	1.52×10 ⁻⁵	0.000955
<i>Candidatus Arthromitus</i> sp. SFB-5	0.001595	2.32×10 ⁻⁵	0.000796
<i>Candidatus Arthromitus</i> sp. SFB-3	0.002991	4.00×10 ⁻⁵	0.001352
<i>Candidatus Arthromitus</i> sp. SFB-1	0.002832	1.74×10 ⁻⁵	0.001451
<i>Candidatus Arthromitus</i> sp. SFB-mouse	0.011666	0.000173	0.005278
<i>Candidatus Arthromitus</i> sp. SFB-mouse-NL	0.00656	8.10×10 ⁻⁵	0.002950
<i>Lachnospiraceae bacterium</i> 10-1	0.013489	1.577748	0.428812
<i>Unclassified Lactobacillaceae</i>	0.202593	0.005889	0.012727
<i>Pseudoflavonifractor capillosus</i>	0.002275	0.008396	0.003357
<i>Desulfovibrionaceae bacterium</i> LT0009	0.01374	1.551055	0.979544
<i>Mailhella</i> sp.	0.00644	0.464521	0.280950
<i>Bacteroides</i> sp. L10-4	0.008627	0.000767	0.002201
<i>Desulfovibrio</i> sp. An276	0.002947	7.87×10 ⁻⁵	2.58×10 ⁻⁶
<i>Lachnospiraceae bacterium</i> NSJ-143	0.000719	1.79×10 ⁻⁵	1.87×10 ⁻⁵
<i>Clostridium</i> sp. CAG:465	0.000519	4.37×10 ⁻⁵	0.000107
<i>Peptoniphilaceae bacterium</i>	3.01×10 ⁻⁵	0.000438	0.000806

The mean abundance of 18 bacterial species (with significant differences shown in Figure 2F) in fecal samples of Con group, HFHC group, and HFHC_KO group mice.

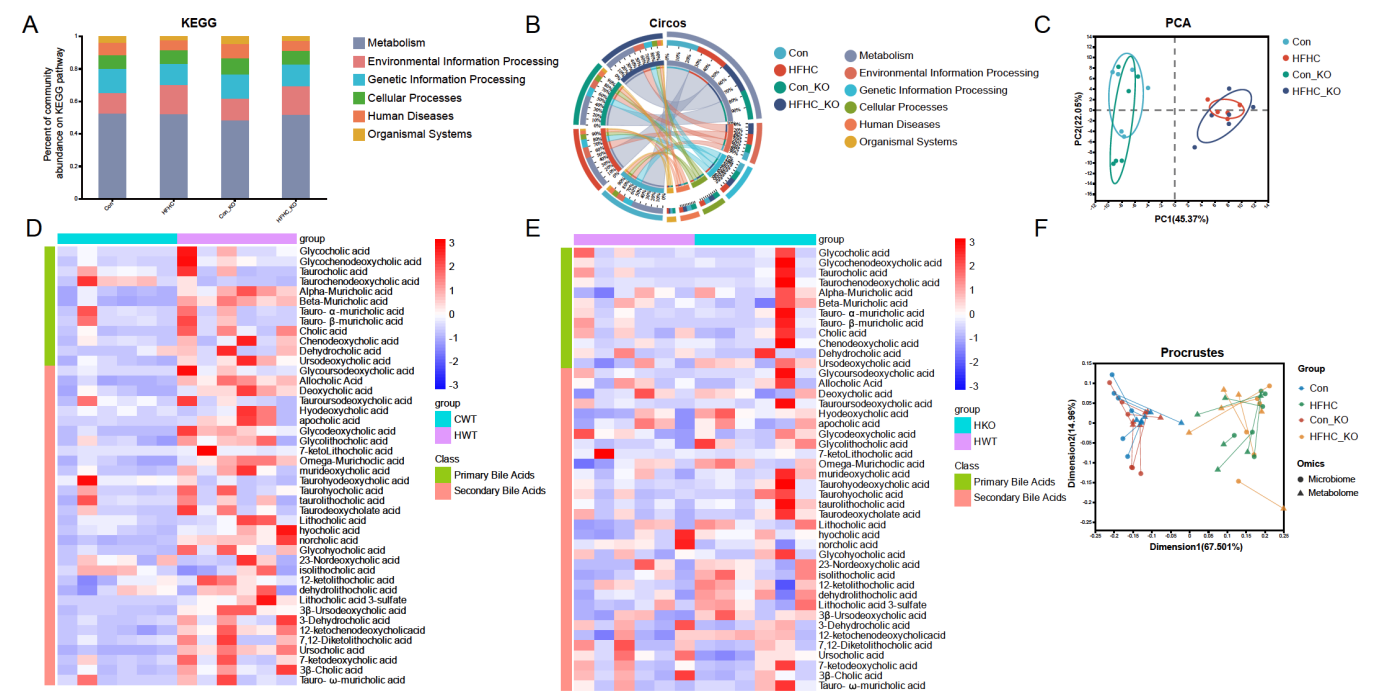


Figure 3. The high-fat diet significantly changes the gut microbiome metabolism of the mice (A) The KEGG bar chart showing the composition of KEGG functions and the proportions of different functions in each group. (B) Circos sample and function diagram was used to show the distribution of KEGG functions present in different groups. On one side of the graph are the samples and their groups, and on the other side are the KEGG function items. (C) Scatter plot showing the similarity and difference of the functions of different samples and reflecting the degree of convergence and dispersion of the functions of samples by the distance between samples (Euclidian distance algorithm). (D,E) Heatmap showing the distribution of bile acids in different groups of samples. (F) Procrustes analysis plot showing the consistency of samples across different datasets. Each line segment represents a sample, and the lines represent the Procrustes residuals of the two ordering configurations. The shorter the lines are, the greater the consistency between the two datasets. *n* = 6 mice per group.

microbial diversity. Emerging evidence from multiple studies indicates that the microbiota diversity in patients with cholestasis is reduced [19], and in mice, bile duct ligation leads to a decrease in microbial β diversity [20]. Although the underlying mechanism has

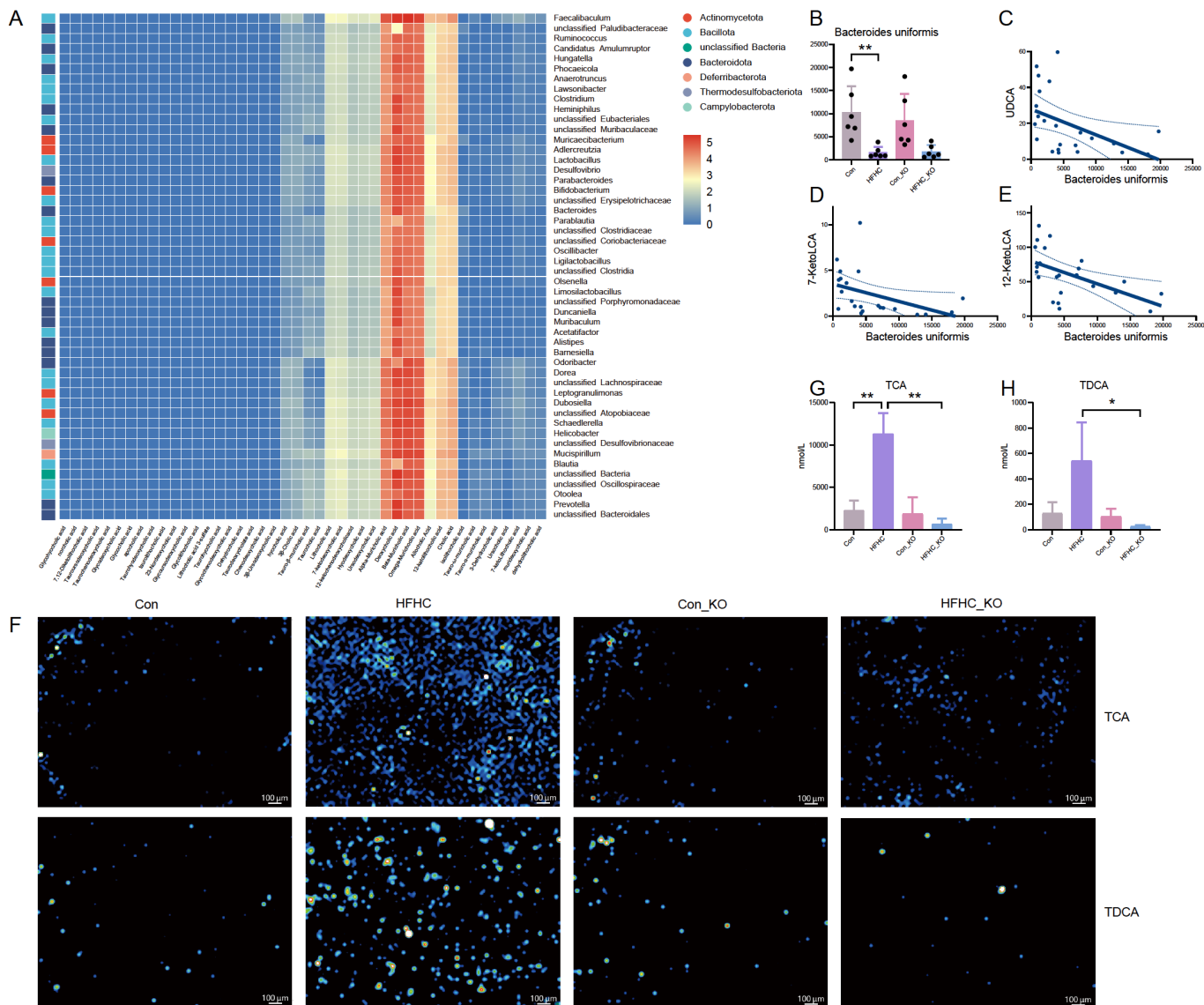


Figure 4. Bile acids in the intestinal contents and liver are correlated with bacterial abundance in the gut (A) Mmvec analysis diagram showing co-occurrence probabilities between microorganisms and bile acids. The microorganisms with the top 50 co-occurrence probabilities were selected for plotting. The color bar shows the logarithmic conditional probability between microorganisms and bile acids; the redder the color is, the greater the probability of their co-occurrence. (B) The histogram showing the abundances of *Bacteroides uniformis* in different groups. (C–E) Correlation plot showing the correlation between bile acids and *Bacteroides uniformis*. (F) The mass spectrogram shows the contents of TCA and TDCA in the different groups. (G,H) The histogram showing the contents of TCA and TDCA in different groups according to the F diagram. Data are presented as the mean $\pm$ SEM, $n = 6$ mice per group. * $P < 0.05$, ** $P < 0.01$.

not been fully elucidated, it is hypothesized that bile acid secretion may serve as an energy substrate to support the survival and proliferation of multiple microorganisms, thereby contributing to the maintenance of microbial diversity [21]. TUDCA mitigates disease progression in MASLD mice by reducing intestinal inflammation, restoring intestinal barrier integrity, reducing intestinal fat transport and regulating the intestinal microbiota composition [22]. As a newly discovered probiotic, *B. uniformis* reduces MASH by producing the modified bile acid 3-sucCA, which promotes the growth of *Akkermansia muciniphila* [23].

Therefore, we performed bile acid metabolomics analysis of the intestinal contents. We found that dietary conditions had a strong effect on bile acid levels, but we did not identify any bile acids that

could directly explain the phenomenon whereby *PLD1* knockout alleviated fatty liver induced by a high-fat diet. We found that the *Faecalibaculum* was significantly co-dominant with bile acids, including DCA, α MCA, β MCA and Ω MCA; however, the causal interplay among them remains undefined. Therefore, our subsequent research will employ strategies such as microbiota transplantation to systematically investigate the bidirectional relationship between bile acid profiles and the gut microbial community. This approach aims to investigate whether *Faecalibaculum* modulates bile acid metabolism or if these bile acids shape *Faecalibaculum* abundance, shedding light on the mechanistic linkages underlying their co-dominance. In addition, no significant correlations were detected between bile acids and the two

differentially abundant bacteria, *Desulfovibrionaceae bacterium* LT0009 and *Lachnospiraceae bacterium* 10–1. In addition, previous studies have demonstrated that *B. uniformis* exerts beneficial effects on liver metabolic diseases such as MASLD through *A. muciniphila* [23], and we found that it was significantly increased in our MASLD mouse model and was significantly correlated with three beneficial bile acids, UDCA, 12-ketoLCA, and 7-ketoLCA. However, these correlations were not positive. We suggest that other bacteria may also contribute to bile acid metabolism. The increase in *B. uniformis* may be an adaptive response of the intestinal ecosystem to counteract pathological damage.

Notably, our findings reveal a discrepancy in bile acid profiles between the intestine and liver, which is likely attributed to the different metabolic processes and regulatory mechanisms of bile acids in the intestines and the liver. In the liver, cholesterol is converted into primary bile acids through a series of enzymatic reactions initiated by the rate-limiting enzyme CYP7A1 [24]. Conversely, in the intestine, the presence of the gut microbiota drives the transformation of primary bile acids into secondary bile acids via bacterial bile salt hydrolase (BSH) and 7 α -hydroxylase activities. FXR is highly expressed in the gastrointestinal tract and serves as a critical bile acid sensor. It mediates feedback inhibition of hepatic bile acid synthesis, thereby maintaining exceedingly low bile acid concentrations in hepatocytes to prevent liver injury and cholestasis. Notably, different bile acids have significantly different efficacies in activating FXR, further influencing metabolic outcomes [24]. Collectively, these integrated mechanisms—including organ-specific metabolic transformations and FXR-mediated regulatory loops—give rise to the marked divergence in bile acid compositions between the liver and intestine.

If the intestinal microbiota alterations induced by *PLD1* knockout are attributed to changes in bile acid levels, how exactly do these bile acid modifications occur? We postulate two potential mechanisms. First, *PLD1* in the liver may regulate the expression of bile acid synthesis enzymes, such as CYP7A1 and CYP8B1, which are crucial for the biosynthesis of primary bile acids. Second, the knockout of *PLD1* may alter the activity of microbial enzymes involved in bile acid metabolism, especially 7 α -hydroxylase, which is necessary for the formation of secondary bile acids. To clarify which mechanism is operative, we plan to employ PCR analyses to simultaneously quantify the expressions of key bile acid metabolism enzymes in liver tissues and the intestinal microbiota after *PLD1* knockout in the liver. This method helps elucidate whether *PLD1* directly regulates bile acid metabolism through the liver enzymatic pathway or indirectly regulates bile acid metabolism through microbial metabolic activities.

Additionally, as this study is fundamentally correlational, it has certain limitations in exploring functions and mechanisms. In subsequent studies, this phenomenon can be further confirmed by overexpressing *PLD1*. In addition, *in vitro* studies using organoids can also be carried out to delve into the underlying mechanisms, whereas the animal model employed in this study was mice, whose microbiota composition is substantially different from that of humans. For further research on human MASLD, two potential approaches could be adopted: transplanting human intestinal contents into mice or conducting direct investigations in human patients.

In conclusion, the gut microbiota and bile acid content significantly changed after high-fat diet feeding. Hepatic *PLD1*

knockout may alter the composition of bile acid by disturbing intestinal microbiota homeostasis and subsequently influence the disease progression of MASLD.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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