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Deletion of the PPARδ gene exacerbates high-fat diet-induced nonalcoholic fatty liver disease in mice through the gut-liver axis

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ABSTRACT

Gut microbiota dysbiosis is an essential factor contributing to non-alcoholic fatty liver disease (NAFLD), in which the gut-liver axis plays a crucial role. Peroxisome proliferator-activated receptor δ (PPARδ) is considered a new direction for the research on NAFLD due to its positive regulation of glucose and lipid metabolism. Our experiment aimed to investigate the effect of PPARδ gene deletion on gut microbiota and NAFLD through the gut-liver axis. *PPARδ-/-* mice and wild-type mice were randomly divided into high-fat diet (HFD) groups and normal diet groups. In each group, six mice were sacrificed at weeks 4, 8, and 12. Metabolic indicators and inflammation indicators were measured, and the degree of liver steatosis and the ileum mucosa integrity were evaluated. Additionally, fecal samples were subjected to 16S rDNA gene sequencing and analysis of gut microbiota. Deletion of the PPARδ gene exhibited exacerbated effects on HFD-induced NAFLD and displayed more severe liver inflammation and intestinal mucosal barrier injuries. The HFD reduced the abundance of short-chain fatty acid (SCFA)-producing bacteria and increased the abundance of intestinal endotoxin-rich bacteria in mice. Deletion of the PPARδ gene exacerbated this trend, resulting in decreased abundances of *norank_f_Eubacterium_coprostanoligenes_group* and *Alloprevotella* and increased abundances of *Acidibacter*, *unclassified_f_Comamonadaceae*, *unclassified_c_Alphaproteobacteria*, *unclassified_f_Beijerinckiaceae*, *unclassified_f_Caulobacteraceae*, *unclassified_c_Bacteroidia* and *Bosea*. Spearman's correlation analysis found *Lachnoclostridium*, *unclassified_f_Rhizobiaceae, Allobaculum, Acinetobacter, Romboutsia, norank_f_Muribaculaceae* and *Dubosiella* showed some correlations with metabolic indicators, inflammation indicators, NAS and occludin. Deletion of the PPARδ gene exacerbated HFD-induced gut microbiota dysbiosis and affected NAFLD through the gut-liver axis.

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Introduction

NAFLD is the most common cause of liver enzyme abnormalities and chronic liver disease in developed regions such as Europe and the United States (1,2), and NAFLD has the highest incidence among liver diseases in China(3). The pathogenesis of NAFLD is complex. With the introduction of the concept of the gut-liver axis, it is widely realized that excessive growth of intestinal bacteria, impaired intestinal mucosal barrier, and metabolic endotoxemia plays a vital role in NAFLD development(4).

As a complex disorder, NAFLD involves many factors, and no specific drug exists to treat it. Existing drugs, such as insulin sensitizers, lipid-lowering drugs, and hepatoprotective drugs, cannot completely reverse the progression of NAFLD(5). So finding novel treatment medicines for NAFLD is very necessary. PPARδ is expressed in various tissues and plays a crucial role in insulin resistance, lipid metabolism and energy metabolism(6). Tanaka et al. found the PPARδ agonist GW501516 can reduce the levels of blood glucose and blood insulin as well as liver fat deposition and can be used to treat obesity with insulin resistance caused by an HFD(7). Currently, PPARδ is considered a new potential therapeutic target for the treatment of NAFLD due to its positive regulation of glucose and lipid metabolism(8). Studies on PPARδ have mainly focused on diabetes, abnormal lipid metabolism, and metabolic syndrome, but few on the gut microbiota in NAFLD. There needs to be more investigation to clarify the interactions among NAFLD, gut microbiota, and PPARδ.

Therefore, our experiment used PPARδ knockout mice to generate the NAFLD model by feeding on a HFD. By comparing metabolic indicators, liver inflammation, fat accumulation, intestinal mucosal barrier and gut microbiota, we analyzed the effect of PPARδ gene deletion on the formation of NAFLD and explored the relationship among PPARδ, the gut microbiota, the gut-liver axis and NAFLD. Our research aimed to provide more evidence that PPARδ could become a new target in NAFLD treatment.

Materials and Methods

Animal studies

Twelve-week-old male C57BL/6 *PPARδ-/-* mice (The Jackson Laboratory, United States) were divided into the PHFD group (n=18) and PND group (n=18), and twelve-

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week-old male wild-type C57BL/6 mice (Laboratory Animal Center of Xi'an Jiaotong University, China) were also divided into the WHFD group (n=18) and WND group (n=18). The PHFD group and WHFD group consumed an HFD (Beijing Keao Xieli Feed Co., LTD, China) consisting of 40% lard, 20% fructose, 2% cholesterol and 38% standard chow diet for 12 weeks, while the PND group and WND group consumed a normal chow (Laboratory Animal Center of Xi'an Jiaotong University, China) consisting of 5% fat, 25% protein, and 70% carbohydrate. A 20°C temperature and specific pathogen-free conditions were maintained in the vivarium. Six mice from each group were euthanized by cervical dislocation at weeks 4, 8, and 12. Their plasma, tissue, and feces samples were collected in RNase-free tubes, then kept at −80°C.

All experiments and the protocol were approved by the Second Affiliated Hospital of Xi'an Jiaotong University (Approval number: 2021245), and all operations and handling procedures were conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals.

Biochemical assays and inflammation evaluation

After 6 hours of fasting, mice were anesthetized intraperitoneally with 1% pentobarbital sodium (0.1mL/20g). Blood samples taken from the retro-orbital sinus were centrifuged at 3000 r/min for 15 min to isolate the plasma. An automated biochemical analyzer(Olympus, Japan) was used to measure plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglyceride (TG), and total cholesterol (TC).

Liver proinflammatory bacterially derived factors LPS, and inflammatory cytokines TNF-α, IL-6 and IFN-γ, were all measured by ELISA kits (Beijing Dakewe Biotechnology Co., Ltd, China). At 0 to 4°C, liver homogenate was diluted 1:10 (w/v) in PBS buffer and homogenized by tissue homogenizer. ELISA kit's instructions were followed for the processing of liver tissue samples.

Tissue histological analysis

For pathological analysis, the liver and ileum tissue were fixed in 4 percent paraformaldehyde, embedded in paraffin, sectioned at a thickness of 4 μm, and stained with hematoxylin-eosin. A pathologist who was blinded to the experiments determined the NAFLD activity score (NAS) based on the four histological characteristics: steatosis (0-3), lobular inflammation (0-2), hepatocellular ballooning (0-2), and fibrosis (0-4). Oil red O was used to detect lipids in frozen liver sections. Immunohistochemistry staining of paraffin-embedded ileum sections was conducted to determine the expression of occludin (Abcam, United States).

Real-time qPCR

Total RNA was extracted using TRIzol (Takara, Dalian, China) and reverse-transcribed into complementary DNA using PrimeScript RT Master Mix (Takara, Dalian, China). Thereafter, real-time qPCR was performed using SYBR Premix Ex Taq II (Takara, Dalian, China). Primer sequences for occludin (F: 5'—TCAACGGCACAGTCAAGG—3', R: 5'—ACTCCACGACATACTCAGC—3') and GAPDH (F: 5'—TTTGCTGTGAAAACCCGAAGA—3', R: 5'—ACTGTCAACTCTTTCCGCATA—3') were synthesized by Beijing AuGCT DNA-SYN Biotechnology Co., Ltd. (Beijing, China).

Fecal sample collection

The Stool Genomic DNA Kit (Beijing ComWin Biotech Co., Ltd., China) was utilized to extract fecal DNA in accordance with the instructions. Amplification of the V4 region (520F: 5-AYTGGGYDTAAAGNG-3, 802R: 5-TACNVGGGTATCTAATCC-3) of 16S rRNA was carried out by PCR. The amplified DNA was purified with an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, United States), and quantified with a Quantus™ Fluorometer (Promega, United States). Purified amplicons were paired-end sequenced on an Illumina MiSeq PE300 platform in Shanghai Personalbio Co., Ltd. The resulting sequences were merged using FLASH (v1.2.11) and quality filtered using Fastp (0.19.6). Then, the high-quality sequences were denoised using the DADA2 plugin in the QIIME 2 (version 2020.2) pipeline with the suggested parameters. Using the Naive Bayes consensus taxonomy classifier implemented in QIIME 2 and the SILVA 16S rRNA database (v138), amplicon sequence variants taxonomy was determined. The Majorbio Cloud Platform's free online platform (cloud.majorbio.com) was utilized to analyze the 16S rRNA microbiome sequencing data.

Statistical analysis

Data were analyzed using SPSS 23.0 data statistics software and expressed as the mean ± SEM. The differences were examined using Student's t-test or the Wilcoxon rank-sum test between the two groups. $p < 0.05$ were considered statistically significant.

Results

Deletion of the PPARδ gene exacerbated gut microbiota imbalance caused by HFD

We found that in comparison to the ND group, the changing trends in the gut microbiota of the PHFD group and WHFD group were approximately consistent at the phylum level (Figure 1A), with both groups showing a reduced abundance of *Bacteroides* (22.56% vs 66.82%, *P*=0.00172; 18.90% vs 40.01%, *P*=0.01365) and an increased ratio of *Firmicutes*/*Bacteroides* (1.47 vs 0.78, *P*=0.01891; 2.43 vs 0.35, *P*=0.00987) (Figure 1B). Yet, the abundance of *Proteobacteria* in the PHFD group increased remarkably (30.77% vs. 13.21%, *P*=0.00453), while the abundance of *Firmicutes* rose considerably in the WHFD group (50.42% vs. 22.09%, *P*=0.01608) (Figure 1B).

From phylum to genus, the specificity of bacteria in each group gradually increased (Figure 1C). After 12 weeks of an HFD, compared to the ND group, there were decreases in the abundances of *Dubosiella*, *norank_f__Muribaculaceae*, *Parasutterella* and *Allobaculum*, and increases in the abundances of *Blautia* in the WHFD group (Figure 1E), while in the PHFD group, there were increases in the abundances of *Corynebacterium*, *unclassified_c__Actinobacteria*, *Romboutsia*, *unclassified_c__Clostridia*, *Acinetobacter*, *Acidibacter*, *unclassified_c__Alphaproteobacteria*, *unclassified_f__Sphingomonadaceae, unclassified_f__Beijerinckiaceae* and *unclassified_f__Rhizobiaceae*, and reductions in the abundances of *Paraprevotella* and *norank_f__Muribaculaceae* (Figure 1F). Moreover, compared to the WHFD group, the abundances of *norank_f__Eubacterium_coprostanoligenes_group* and *Alloprevotella* were decreased in the PHFD group, and the abundances of *Acidibacter*, *unclassified_f__Co-*

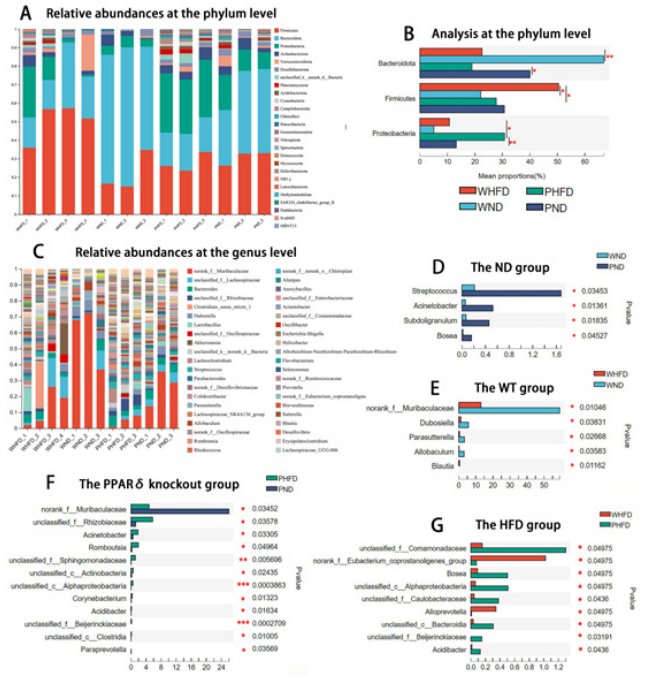

Figure 1. A: Relative abundances of various bacteria at the phylum level. B: Analysis at the phylum level. C: Relative abundances of various bacteria at the genus level. D-G: Analysis of the ND group (D), WT group (E), *PPARδ-/-* group (F) and HFD group (G) at the genus level. **P<0.05, **P<0.01, ***P<0.001*.

mamonadaceae, *unclassified_c__Alphaproteobacteria*, *unclassified_f__Beijerinckiaceae*, *unclassified_f__Caulobacteraceae*, *unclassified_c__Bacteroidia* and *Bosea* were increased (Figure 1G).

According to the results of the nonmetric multidimensional scaling (NMDS) and principal coordinates analysis (PCoA), the deletion of the PPARδ gene and HFD consumption changed the overall gut microbiota composition. The NMDS analysis based on the UniFrac distance separated the ND group from the HFD group. However, two samples of the WHFD group were far away from the HFD group, which may be considered an individual difference factor (Figure 2A). In the PCoA, PC1 was the main coordinate, which accounted for 61.35%, and predominantly reflected the effect of diet because PC1 separated samples fed an HFD from those fed an ND (Figure 2B). The HFD group was farther from the ND group, indicating that the gut microbiota was quite different among these groups, which might be caused by the different diets; The PHFD group was right next to the WHFD group, showing the HFD had similar effects on the two groups, but two samples from the WHFD group were slightly farther away from the PHFD group, showing that there was a certain degree of confounding in the samples, which was consistent with the NMDS analysis results.

Furthermore, the community heatmap showed that dietary factors were the most significant factors affecting gut microbiota (Figure 2C). Spearman's Correlation Heatmap displayed at the genus level the *unclassified_f__Rhizobiaceae*, *unclassified_k__norank_d__Bacteria* and *Acinetobacter* was significantly positively correlated with body weight, liver index, NAS, ALT, AST, TG, TC, LPS, TNF-α, IL-6 and IFN-γ, and negatively correlated with occludin. *Lachnoclostridium* displayed the same trend, showing a negative correlation with occludin, and positive correlations with NAS, TG, LPS, and TNF-α. *Romboutsia* also exhibited positive correlations with body weight, NAS, TC and LPS. In contrast, *norank_f__Muribaculaceae* was considerably linked negatively with body weight, liver index, NAS, ALT, AST, TG, TC, LPS, TNF-α, IL-6 and IFN-γ, and positively with occludin. *Allobaculum* showed a similar trend, except for the TNF-α, ALT, AST, TC and liver index, and *Dubosiella* also showed a negative correlation with TG (Figure 2D).

Deletion of the PPARδ gene exacerbated obesity, hepatic steatosis and lipid metabolic disturbance induced by a HFD

PPARδ knockout mice gained weight rapidly and obviously after being fed an HFD, and their body weight was considerably greater than the PND group from the 2nd week and greater than the WHFD group from the 6th week. This observation confirmed that a lack of PPARδ can accelerate and exacerbate the weight gain caused by an HFD. The HFD group displayed increased levels of liver index and metabolism indices, and importantly, the PHFD group showed a substantial worsening in whole body and liver metabolism indices in comparison with the WHFD group at the same time point, including body weight, liver index, TC, TG, ALT and AST (Figure 3C-H).

The liver specimens in the HFD group were paler and larger. Histopathology showed that the hepatic lobules were intact and clear in the ND group: hepatocytes were distributed regularly, and no lipid droplets were observed in hepatocytes (Figure 3A). Conversely, in the HFD group, there were clear lipid droplets and hepatocellular ballooning. Importantly, we observed that the PHFD group developed NAFLD in a shorter period and developed more seriously, and progressed faster than the WHFD group at the same time point (Figures 3A and B). Moreover, the PHFD group's NAS was higher than the WHFD group (Table 1). Inflammatory infiltration and punctate necrosis were both observed in the PHFD group at the 12th week, and the NAS was >4, which is regarded as the diagnosis of nonalcoholic steatohepatitis (NASH), while mice in the WHFD group were only diagnosed with simple fatty liver at the same time point.

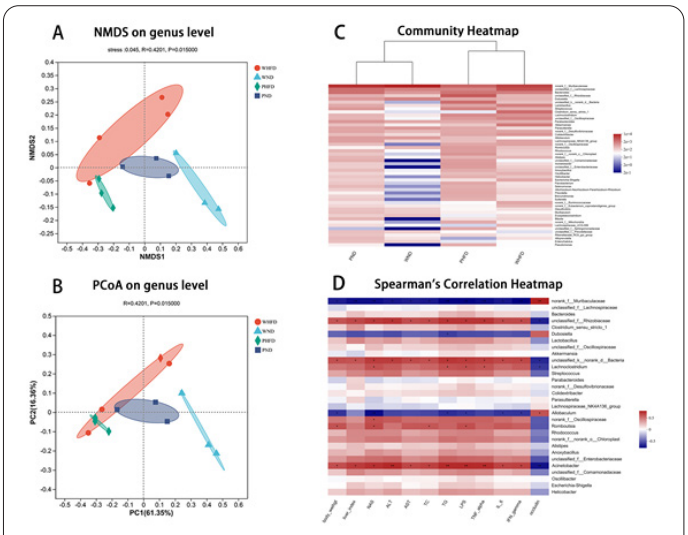

Figure 2. A-B: NMDS (A) showed the differences in the bacterial communities, and PCoA (B) showed their similarity. C: Hierarchical cluster analysis displayed that the ND groups and HFD groups clustered first and then grouped. D: The correlations between gut microbiota and metabolic indicators in mice (G). **P<0.05, **P<0.01.*

Table 1. The NAFLD activity score in the HFD group.

Groups	NAS		
	4 w	8 w	12 w
PHFD group	1.50±0.55*	3.33±0.52**	4.67±1.03**
WHFD group	0.83±0.41	1.83±0.75	3.00±0.63
t or t'	2.39	4.025	3.371
P	0.038	0.002	0.007

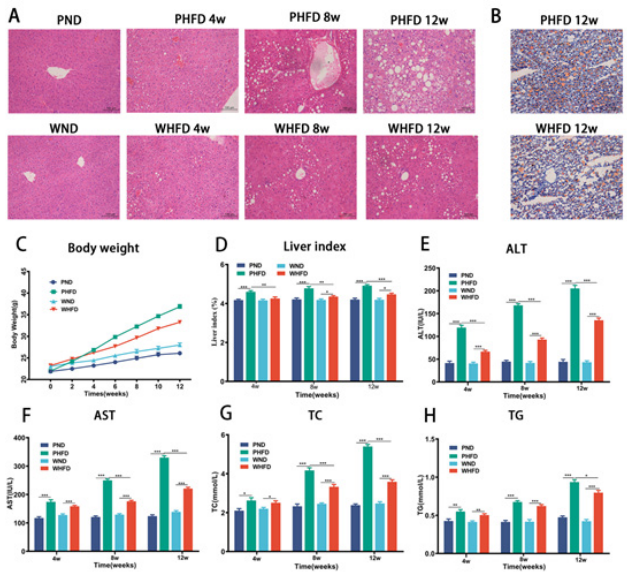

Figure 3. A: Histopathology of the liver by HE staining at the 4th, 8th and 12th weeks (original magnification, ×200). B: Histopathological changes in liver tissues by oil red O staining at the 12th week in the HFD group (×400). C: Body weight changes at 12 weeks. D: Liver index=liver weight (g)/body weight (g) × 100%. E-F: Serum concentrations of ALT (E) and AST (F), which represent liver function. G-H: Serum concentrations of TC (G) and TG (H), which represent lipid metabolism. **P<0.05, **P<0.01, ***P<0.001*.

Deletion of the PPARδ gene exacerbated intestinal mucosal barrier injuries and liver inflammation in HFD-fed mice

The intestinal mucosal barrier was associated with the villi's structure and the ileum's tight junctions. The microvilli on the mucosa of the terminal ileum were arranged in an orderly manner in the ND group, and the connection structure between adjacent cells was clear. In contrast, HE staining revealed varying degrees of injury to the ileal mucosa in the HFD group, and the PHFD group had more severe damage than the WHFD group at the same time point (Figure 4A). Immunohistochemistry for occludin revealed the same results. Occludin was less abundant in the HFD group and was gradually reduced with increasing feeding time (Figure 4B). The PHFD group also had more reduced occludin mRNA levels compared to the WHFD group (Figure 4C). It suggested that in HFD-fed mice, PPARδ gene deletion exacerbated intestinal mucosal barrier injuries. Furthermore, the HFD group displayed higher levels of pro-inflammatory bacterially derived factors and pro-inflammatory cytokines in the liver in the 12th week, including LPS, TNF-α, IL-6, and IFN-γ, and the levels in the PHFD group increased to a greater extent than the levels of the WHFD group (Figure 4D).

Discussion

In our experiment, there was no significant difference in transaminase, blood lipids or liver pathology between the *PPARδ-/-* mice and the WT mice when they were both provided an ND, except for the slightly lighter body weight in the PPARδ knockout mice. This finding was consistent with prior studies showing that the lighter body weight of PPARδ knockout mice was due to their lower gonadal fat storage(9). With the prolongation of HFD feeding, the degree of steatosis in the PHFD group rapidly worsened and progressed to NASH at a faster rate. Liver damage and abnormal lipid metabolism were observed in PPARδ gene knockout mice after an HFD and continued to be exacerbated with the prolongation of HFD feeding; in addition, these effects were more serious than those in the WHFD group at the same time point. Our experiment confirmed that deletion of the PPARδ gene exacerbated lipid metabolic disorders induced by an HFD and significantly promoted the progression of NAFLD.

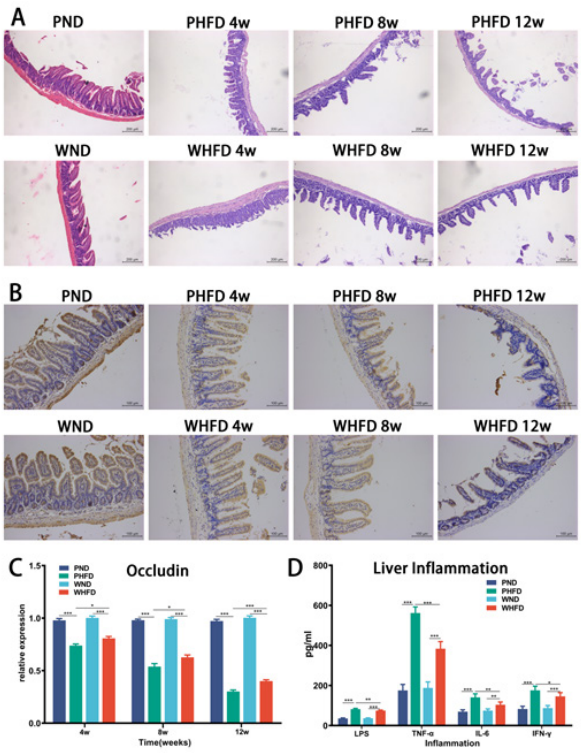

Figure 4. A: HE staining of the ileum showing that PPARδ gene deletion exacerbated mucosal damage caused by HFD; B: Immunohistochemistry for occludin showing that HFD reduced the expression of occludin, which indicated injuries to the tight junction of the ileum; C: Occludin mRNA expression in the ileum at weeks 4, 8, and 12. D: Concentrations of the proinflammatory bacterially derived factor and proinflammatory cytokines in liver homogenates. **P*<0.05, ***P*<0.01, ****P*<0.001.

The gut-liver axis is a meaningful way to investigate the mechanism of NAFLD(10). The intestinal mucosal mechanical barrier can protect the liver from harmful substances such as intestinal endotoxin. NAFLD-associated gut microbiota dysbiosis generated by long-term HFD intake can disrupt intestinal barrier function by lowering occludin expression(11). Intestinal mucosal barrier injuries promote the entry of endotoxins into the liver, consequently stimulating the release of inflammatory cytokines and, as a result, triggering liver steatosis(10). In our experiment, the PPARδ gene deletion exacerbated the intestinal mucosal damage caused by an HFD and further exacerbated the liver inflammation, confirming that deletion of the PPARδ gene promoted the development of NAFLD through the gut-hepatic axis.

The gut microbiota was an essential part of the gut-hepatic axis. Consistent with prior research(12), we found that an HFD could generally cause a significant rise in bacteria of the phylum *Firmicutes* and *Proteobacteria* and simultaneously suppress the phylum *Bacteroides*. The phylum *Bacteroides* is the predominant helpful bacteria in the human gut. Ley et al. found that obese patients had a remarkable increase in *Firmicutes*, while *Bacteroides* decreased by 50%(13), and after their body weight was reduced through diet control, the abundances of *Bacteroides* increased and *Firmicutes* decreased, suggesting that the increase in *Firmicutes* could produce relatively complete energy metabolism, increase energy absorption in the body, increase the production and accumulation of fat, and consequently promote obesity and even NAFLD. Many studies found that the phylum *Proteobacteria* was increased in NAFLD patients(14). *Proteobacteria* belong to gram-negative Bacillus, which can produce endotoxins and promote liver inflammation and NAFLD(10). In our experiment, the consistency of the changes in the *Firmicutes*/*Bacteroides* ratio indicated that dietary factors played a major role in gut microbiota modifications. Still, the increase of *Proteobacteria* in the PHFD group also showed that genotype played a meaningful role in the progression of NAFLD.

From phylum to genus, the specificity of bacteria gradually increased in the HFD group, and the groups differed significantly. Compared to the ND group, the abundance of bacteria that are beneficial for health was reduced in the HFD group, and further, the deletion of the PPARδ gene exacerbated this trend. Consistent with our conclusion, many studies observed the abundances of *Paraprevotella*, *Allobaculum*, *Parasutterella*, *Dubosiella, Muribaculaceae* and *Alloprevotella* showed a negative correlation with blood lipids and NAFLD(15-18), suggesting that these bacteria might be beneficial to lipid metabolism and NAFLD. *Muribaculaceae, Paraprevotella* and *Alloprevotella* belong to the class *Bacteroidia*, which includes butyrate-producing bacteria, and their abundance showed a positive correlation with butyric acid(19,20). *Dubosiella* and *Allobaculum* both belong to the family *Erysipelotrichaceae*, which are also SCFAs producers(16,21). In the intestine, gut bacteria ferment nondigestible polysaccharides to produce SCFAs. SCFAs can stimulate glucagon secretion(22). SCFA/GPR43 signaling suppresses inflammation and improves gut permeability, which minimizes the liver damage caused by LPS and, as a result, reduces insulin resistance, liver inflammation and liver steatosis(23). SCFAs also participate in enterohepatic circulation by affecting bile acid metabolism and competitively inhibiting pathogenic bacterial growth(24). In addition, *Dubosiella* was not only connected with SCFAs, but also showed a positive connection with the 4-hydroxyphenylacetic acid, metabolites dimethyl fumarate, prostaglandin, lactitol, and cafestol(25), the reduction of which might lead detrimental consequences on the liver health.

For helpful bacteria *Eubacterium coprostanoligenes*, Wan Y et al. found that some species in the *Eubacterium group* which might produce SCFAs were reduced under the condition of HFD(26), implying a possible role for *norank_f__Eubacterium_coprostanoligenes_group* in the prevention of NAFLD through SCFAs. *Eubacterium coprostanoligenes* is also a coprostanol-producing eubacterium and can generate coprostanol(27), which cannot be reabsorbed and is excreted. Consequently, this transformation efficiently lowers cholesterol from circulation. Up to 50% of the steroids in human feces are composed of coprostanol, which means that people who have this intestinal coprostanol-producing eubacterium will absorb half as much of the cholesterol as others after high-cholesterol diet consumption(27). Moreover, Wei W et al. found that *Eubacterium coprostanoligenes* had a positive correlation with fecal sphingosine levels(28). The reduction in serum sphingosine could induce dyslipidemia(28). Therefore, the decrease of *norank_f__Eubacterium_coprostanoligenes_group* in the PHFD group might result in the reduction in SCFAs, coprostanol, and sphingosine, which might then induce dyslipidemia and NAFLD.

At the same time, the abundance of harmful bacteria was increased in the HFD group, and the deletion of the PPARδ gene also exacerbated this trend. The conditional pathogen *Corynebacterium* belongs to *Actinobacteria*. Chen L found that compared to levels in healthy adults, *Actinobacteria* was remarkably higher in NAFLD patients and has positive correlations with the fatty liver degree and abnormal liver function(29). Kordy K found that NASH was connected with a marked decrease in *Corynebacterium*(30). Li R reported that *Corynebacterium* was remarkably positively associated with triglyceride, cholesterol, IL-6 and TNF-α(31). These evidences were consistent with our conclusion that *Actinobacteria* and *Corynebacterium* might accelerate the progression of NAFLD. Another harmful bacteria *Clostridia* is a large and important bacterial group in the phylum *Firmicutes*. Etxeberria U et al. detected a positive connection between the percentage of animal visceral fat tissue and *Clostridia* levels(32), and Sookoian S et al. found that increased abundances of the classes *Clostridia* and *Actinobacteria* were associated with NASH among morbidly obese patients(33). *Romboutsia* and *Blautia* both belong to the class *Clostridia*, and their abundances were markedly enriched in humans with NAFLD(34,35). Furthermore, patients with NASH had more abundant *Blautia* than those with NAFLD(35). Elevated abundances of *Romboutsia* and *Blautia* were also observed to be positively correlated with TG, TC and inflammatory markers such as TNF-α(36). This was consistent with our conclusion, although *Blautia* was also discovered to be one of the SCFA producers that may be beneficial to NAFLD. It was inferred that the increase in *Blautia* in the NAFLD or NASH group might be a compensatory increase to supplement the reduction in other SFCA-producing bacteria, or it might be a compensatory increase caused by a high-calorie diet(33). In addition, although

no differences in *Lachnoclostridium* which also belongs to the class *Clostridia* were discovered among the four groups in our experiment, Spearman's correlation analysis showed *Lachnoclostridium* had positive correlations with NAS, TG, LPS and TNF-α and a negative correlation with occludin. Perhaps this is due to the positive correlation between *Lachnoclostridium* and the pathological changes of NAFLD(37).

Of the increased harmful bacteria in the HFD group, the largest number came from *Proteobacteria*. *Acinetobacter* bacteria are considered harmful bacteria and belong to the class *Gammaproteobacteria*, the same as *Acidibacter*; *Sphingomonadaceae*, *Beijerinckiaceae*, *Rhizobiaceae*, *Caulobacteraceae* and *Bosea* belong to the class *Alphaproteobacteria*; and *Comamonadaceae* belongs to the class *Betaproteobacteria*. They all belong to the phylum *Proteobacteria*, and were observed to be overrepresented in a HFD setting(33). Many studies have confirmed that NAFLD was connected to *Proteobacteria*: *Gammaproteobacteria* was increased in the intestine of children with NAFLD(38); *Gammaproteobacteria* and *Alphaproteobacteria* were overrepresented in the non-morbidly obese NAFLD group(33); as a conditioned pathogen with a proinflammatory effect, *Acinetobacter* was increased in a high-calorie diet and associated with obesity(39). All the bacteria of *Proteobacteria* are gram-negative bacilli. The gram-negative bacteria's outer membrane contains LPS, which is an endotoxin responsible for triggering inflammation when it enters the circulation. Compared to LPS derived from the phylum *Bacteroidetes*, LPS derived from the phylum *Proteobacteria* has 1,000 times more endotoxin activity(40). Cell experiments found that LPS derived from *Acinetobacter baumannii* has a similar effect on the expression of MIP-2 as that of LPS prepared from *Escherichia coli*, which also increases TNF-α and IL-6 levels(41). Long-term consumption of an HFD can cause gut dysbiosis, which may lead to an increase in gram-negative bacteria endotoxin generation, such as LPS. LPS disrupts intracellular tight junctions in the gut, whereby LPS generated in the intestine enters the liver, subsequently activating Kupffer cells to discharge inflammatory cytokines, and further triggering dysfunctional lipid metabolism and even NAFLD. Moreover, the increase of *Comamonadaceae* and *Caulobacteraceae* can lead to reduced secretion of leptin and ghrelin, both of which were related to an increase in feed intake(42). Projecting these findings to our experiment, an increase in *unclassified_f__Comamonadaceae* and *unclassified_f__Caulobacteraceae* in the PHFD group might contribute to physiologic responses that reduce satiety in the mice, which could increase food intake and contribute to obesity. In addition, host inflammation was demonstrated to foster the increase of oxygen-resistant bacteria like *Enterobacteriaceae* (*Gammaproteobacteria*)(43). The PHFD group displayed more serious liver inflammation, which may, in turn, promote the growth of intestinal *Proteobacteria*, in a vicious cycle.

In summary, our experiment confirmed that under the induction of an HFD, mice lacking the PPARδ gene developed more serious gut dysbiosis, which was manifested as a large increase in intestinal LPS-rich bacteria and a significant reduction in SCFA-producing bacteria. As a result, intestinal mucosal barrier damage occurred, and more endotoxins were produced and entered the liver, which exacerbated liver inflammation and lipid metabolism disorders and finally promoted the development of NAFLD. Our experiment confirmed that PPARδ shows a significant effect on NAFLD through the gut-liver axis. However, there were also some limitations in our research. Due to the small number of experimental mice, variances existed in individual characteristics, which led to the failure to fully reveal the actual situation of the gut microbiota. In addition, due to conditions and other factors, we discussed the gut-liver axis very preliminarily and did not study the bacterial metabolites and specific molecular mechanisms in depth. Therefore, we plan to further complete the study of PPARδ-related bacterial metabolites and the molecular mechanisms in future research, to expand the sample size of mice, and to strictly control the sampling environment to obtain more accurate experimental results.

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References

1. Younossi ZM, Stepanova M, Younossi Y, Golabi P, Mishra A, Rafiq N, et al. Epidemiology of chronic liver diseases in the USA in the past three decades. Gut 2020; 69(3):564-568.
2. Cholongitas E, Pavlopoulou I, Papatheodoridi M, Markakis GE, Bouras E, Haidich AB, et al. Epidemiology of nonalcoholic fatty liver disease in Europe: a systematic review and meta-analysis. Ann Gastroenterol 2021; 34(3):404-414.
3. Zhou J, Zhou F, Wang W, Zhang XJ, Ji YX, Zhang P, et al. Epidemiological Features of NAFLD From 1999 to 2018 in China. Hepatology 2020;71(5):1851-1864.
4. Bakhshimoghaddam F, Alizadeh M. Contribution of gut microbiota to nonalcoholic fatty liver disease: Pathways of mechanisms. Clin Nutr ESPEN 2021;44:61-68.
5. Zhang CH, Zhou BG, Sheng JQ, Chen Y, Cao YQ, Chen C. Molecular mechanisms of hepatic insulin resistance in nonalcoholic fatty liver disease and potential treatment strategies. Pharmacol Res 2020;159:104984.
6. Bojic LA, Telford DE, Fullerton MD, Ford RJ, Sutherland BG, Edwards JY, et al. PPARδ activation attenuates hepatic steatosis in Ldlr-/- mice by enhanced fat oxidation, reduced lipogenesis, and improved insulin sensitivity. J Lipid Res. 2014; 55(7): 1254-1266.
7. Tanaka T, Yamamoto J, Iwasaki S, et al. Activation of peroxisome proliferator-activated receptor delta induces fatty acid beta-oxidation in skeletal muscle and attenuates metabolic syndrome. Proc

Natl Acad Sci U S A. 2003;100(26):15924-15929.
8. Chen J, Montagner A, Tan NS, Wahli W. Insights into the Role of PPARβ/δ in NAFLD. Int J Mol Sci. 2018;19(7):1893. Published 2018 Jun 27.
9. Peters JM, Lee SS, Li W, Ward JM, Gavrilova O, Everett C, et al. Growth, adipose, brain, and skin alterations resulting from targeted disruption of the mouse peroxisome proliferator-activated receptor beta(delta). Mol Cell Biol 2000;20(14):5119-5128.
10. Martín-Mateos R, Albillos A. The Role of the Gut-Liver Axis in Metabolic Dysfunction-Associated Fatty Liver Disease. Front Immunol 2021;12:660179.
11. Nakanishi T, Fukui H, Wang X, Nishiumi S, Yokota H, Makizaki Y, et al. Effect of a High-Fat Diet on the Small-Intestinal Environment and Mucosal Integrity in the Gut-Liver Axis. Cells 2021;10(11):3168.
12. Velázquez KT, Enos RT, Bader JE, Sougiannis AT, Carson MS, Chatzistamou I, et al. Prolonged high-fat-diet feeding promotes non-alcoholic fatty liver disease and alters gut microbiota in mice. World J Hepatol 2019;11(8):619-637.
13. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. Nature 2006;444(7122):1022-1023.
14. Özkul C, Yalınay M, Karakan T, Yılmaz G. Determination of certain bacterial groups in gut microbiota and endotoxin levels in patients with nonalcoholic steatohepatitis. Turk J Gastroenterol 2017;28(5):361-369.
15. Mu H, Zhou Q, Yang R, Zeng J, Li X, Zhang R, et al. Naringin Attenuates High Fat Diet Induced Non-alcoholic Fatty Liver Disease and Gut Bacterial Dysbiosis in Mice. Front Microbiol 2020;11:585066.
16. Bai J , Zhu Y , Dong Y . Modulation of gut microbiota and gut-generated metabolites by bitter melon results in improvement in the metabolic status in high fat diet-induced obese rats. Journal of Functional Foods 2018; 41:127-134.
17. Cao W, Chin Y, Chen X, Mi Y, Xue C, Wang Y, et al. The role of gut microbiota in the resistance to obesity in mice fed a high fat diet. Int J Food Sci Nutr 2020;71(4):453-463.
18. Tong AJ, Hu RK, Wu LX, Lv XC, Li X, Zhao LN, et al. Ganoderma polysaccharide and chitosan synergistically ameliorate lipid metabolic disorders and modulate gut microbiota composition in high fat diet-fed golden hamsters. J Food Biochem 2020;44(1):e13109.
19. Fei Y, Wang Y, Pang Y, Wang W, Zhu D, Xie M, et al. Xylooligosaccharide Modulates Gut Microbiota and Alleviates Colonic Inflammation Caused by High Fat Diet Induced Obesity. Front Physiol 2020;10:1601.
20. Lagkouvardos I, Lesker TR, Hitch TCA, Gálvez EJC, Smit N, Neuhaus K, et al. Sequence and cultivation study of Muribaculaceae reveals novel species, host preference, and functional potential of this yet undescribed family. Microbiome 2019;7(1):28.
21. Ye X, Liu Y, Hu J, Gao Y, Ma Y, Wen D. Chlorogenic Acid-Induced Gut Microbiota Improves Metabolic Endotoxemia. Front Endocrinol (Lausanne) 2021;12:762691.
22. Canfora EE, Jocken JW, Blaak EE. Short-chain fatty acids in control of body weight and insulin sensitivity. Nat Rev Endocrinol 2015;11(10):577-591.
23. Fukui H. Role of Gut Dysbiosis in Liver Diseases: What Have We Learned So Far? Diseases 2019;7(4):58.
24. Ye X, Shen S, Xu Z, Zhuang Q, Xu J, Wang J, et al. Sodium butyrate alleviates cholesterol gallstones by regulating bile acid metabolism. Eur J Pharmacol 2021 Oct 5;908:174341.
25. Zhang Z , Chen X , Cui B . Modulation of the fecal microbiome and metabolome by resistant dextrin ameliorates hepatic steatosis and mitochondrial abnormalities in mice. Food Funct 2021;12(10):4504-4518.
26. Wan Y, Tong W, Zhou R, Li J, Yuan J, Wang F, et al. Habitual animal fat consumption in shaping gut microbiota and microbial metabolites. Food Funct 2019;10(12):7973-7982.
27. Koppel N, Maini Rekdal V, Balskus EP. Chemical transformation of xenobiotics by the human gut microbiota. Science 2017;356(6344):eaag2770.
28. Wei W, Jiang W, Tian Z, Wu H, Ning H, Yan G, et al. Streptococcus and g. Eubacterium_coprostanoligenes_group combined with sphingosine to modulate the serum dyslipidemia in high-fat diet mice. Clin Nutr 2021;40(6):4234-4245.
29. Del Chierico F, Nobili V, Vernocchi P, Russo A, De Stefanis C, Gnani D,et al. Gut microbiota profiling of pediatric nonalcoholic fatty liver disease and obese patients unveiled by an integrated meta-omics-based approach. Hepatology 2017;65(2):451-464.
30. Kordy K, Li F, Lee DJ, Kinchen JM, Jew MH, La Rocque ME, et al. Metabolomic Predictors of Non-alcoholic Steatohepatitis and Advanced Fibrosis in Children. Front Microbiol 2021;12:713234.
31. Li R, Yao Y, Gao P, Bu S. The Therapeutic Efficacy of Curcumin vs. Metformin in Modulating the Gut Microbiota in NAFLD Rats: A Comparative Study. Front Microbiol 2021;11:555293.
32. Etxeberria U, Arias N, Boqué N, Macarulla MT, Portillo MP, Milagro FI, et al. Shifts in microbiota species and fermentation products in a dietary model enriched in fat and sucrose. Beneficial Microbes 2015; 6(1):97-111.
33. Sookoian S, Salatino A, Castaño GO, Landa MS, Fijalkowky C, Garaycoechea M, et al. Intrahepatic bacterial metataxonomic signature in non-alcoholic fatty liver disease. Gut 2020;69(8):1483-1491.
34. Chen F, Esmaili S, Rogers GB, Bugianesi E, Petta S, Marchesini G, et al. Lean NAFLD: A Distinct Entity Shaped by Differential Metabolic Adaptation. Hepatology 2020;71(4):1213-1227.
35. Shen F, Zheng RD, Sun XQ, Ding WJ, Wang XY, Fan JG. Gut microbiota dysbiosis in patients with non-alcoholic fatty liver disease. Hepatobiliary Pancreat Dis Int 2017;16(4):375-381.
36. Duan R, Huang K, Guan X, Li S, Xia J, Shen M, et al. Tectorigenin ameliorated high-fat diet-induced nonalcoholic fatty liver disease through anti-inflammation and modulating gut microbiota in mice. Food Chem Toxicol 2022;164:112948.
37. Cui H, Li Y, Wang Y, Jin L, Yang L, Wang L,et al. Da-Chai-Hu Decoction Ameliorates High Fat Diet-Induced Nonalcoholic Fatty Liver Disease Through Remodeling the Gut Microbiota and Modulating the Serum Metabolism. Front Pharmacol 2020;11:584090.
38. Michail S, Lin M, Frey MR, Fanter R, Paliy O, Hilbush B, et al. Altered gut microbial energy and metabolism in children with non-alcoholic fatty liver disease. FEMS Microbiol Ecol 2015;91(2):1-9.
39. Kong C, Gao R, Yan X, Huang L, Qin H. Probiotics improve gut microbiota dysbiosis in obese mice fed a high-fat or high-sucrose diet. Nutrition 2019;60:175-184.
40. Tang W, Yao X, Xia F, Yang M, Chen Z, Zhou B, et al. Modulation of the Gut Microbiota in Rats by Hugan Qingzhi Tablets during the Treatment of High-Fat-Diet-Induced Nonalcoholic Fatty Liver Disease. Oxid Med Cell Longev 2018;2018:7261619.
41. Unno Y, Sato Y, Nishida S, Nakano A, Nakano R, Ubagai T, et al. Acinetobacter baumannii Lipopolysaccharide Influences Adipokine Expression in 3T3-L1 Adipocytes. Mediators Inflamm 2017;2017:9039302.
42. Yin J, Han H, Li Y, Liu Z, Zhao Y, Fang R, et al. Lysine Restriction Affects Feed Intake and Amino Acid Metabolism via Gut Microbiome in Piglets. Cell Physiol Biochem 2017;44(5):1749-1761.
43. Lupp C, Robertson ML, Wickham ME, Sekirov I, Champion OL, Gaynor EC, et al. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteria-

ceae. Cell Host Microbe 2007;2(3):204.