

# World Journal of *Gastroenterology*

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## Basic Study

**Dynamic alterations in the gut microbiota and metabolome during the development of methionine-choline-deficient diet-induced nonalcoholic steatohepatitis**

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**Author contributions:** Ye JZ, Li YT and Wu WR have contributed equally to this work. Ye JZ, Li YT, Wu WR and Li LJ conceived and designed the experiments; Ye JZ, Li YT and Wu WR performed the experiments; Ye JZ, Shi D, Fang DQ, Yang LY and Bian XY analyzed the data; Wu JJ, Wang Q and Jiang XW contributed analysis tools; Ye JZ, Li YT and Wu WR wrote the manuscript; Ye JZ and Peng CG conducted a critical revision of the manuscript for important intellectual content; Ye WC, Xia PC and Li LJ reviewed the manuscript; all authors have approved the final version of the manuscript.

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## Abstract

### AIM

To investigate changes in gut microbiota and metabolism during nonalcoholic steatohepatitis (NASH) development in mice fed a methionine-choline-deficient (MCD) diet.

### METHODS

Twenty-four male C57BL/6J mice were equally divided into four groups and fed a methionine-choline-sufficient diet for 2 wk (Control 2w group,  $n = 6$ ) or 4 wk (Control 4w group,  $n = 6$ ) or the MCD diet for 2 wk (MCD 2w group,  $n = 6$ ) or 4 wk (MCD 4w group,  $n = 6$ ). Liver injury, fibrosis, and intestinal barrier function were evaluated after 2 and 4 wk of feeding. The fecal microbiome and metabolome were studied using 16s rRNA deep sequencing and gas chromatography-mass spectrometry.

### RESULTS

The mice fed the MCD diet presented with simple hepatic steatosis and slight intestinal barrier deterioration after 2 wk. After 4 wk of feeding with the MCD diet, however, the mice developed prominent NASH with liver fibrosis, and the intestinal barrier was more impaired. Compared with the control diet, the MCD diet induced gradual gut microbiota dysbiosis, as evidenced by a marked decrease in the abundance of *Alistipes* and the (*Eubacterium*) *coprostanoligenes* group ( $P < 0.001$  and  $P < 0.05$ , respectively) and a significant increase in Ruminococcaceae UCG 014 abundance ( $P < 0.05$ ) after 2 wk. At 4 wk, the MCD diet significantly reduced the promising probiotic *Bifidobacterium* levels and markedly promoted *Bacteroides* abundance ( $P < 0.05$ , and  $P < 0.01$ , respectively). The fecal metabolomic profile was also substantially altered by the MCD diet: At 2 wk, arachidic acid, hexadecane, palmitic acid, and tetracosane were selected as potential biomarkers that were significantly different in the corresponding control group, and at 4 wk, cholic acid, cholesterol, arachidic acid, tetracosane, and stearic acid were selected.

### CONCLUSION

The MCD diet induced persistent alterations in the gut microbiota and metabolome.

**Key words:** Nonalcoholic steatohepatitis; Methionine-choline deficient diet; Gut microbiota; Metabolome; Nonalcoholic fatty liver disease

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**Core tip:** Nonalcoholic steatohepatitis (NASH) is increasingly prevalent as a remarkable problem worldwide. Increased evidence indicates the critical role of gut microbiota in NASH progression. We aimed to investigate the dynamic alterations in the gut microbiota and the related metabolites during NASH development in mice fed a methionine-choline-deficient (MCD) diet. We for the first time find that the MCD diet may induce persistent gut microbiota and metabolome deterioration.

Ye JZ, Li YT, Wu WR, Shi D, Fang DQ, Yang LY, Bian XY, Wu JJ, Wang Q, Jiang XW, Peng CG, Ye WC, Xia PC, Li LJ. Dynamic alterations in the gut microbiota and metabolome during the development of methionine-choline-deficient diet-induced nonalcoholic steatohepatitis. *World J Gastroenterol* 2018; 24(23): 2468-2481 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i23/2468.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i23.2468>

## INTRODUCTION

The prevalence of nonalcoholic fatty liver disease (NAFLD), the most common chronic liver disease worldwide, is increasing, encompassing a pathological spectrum from simple steatosis to nonalcoholic steatohepatitis (NASH)<sup>[1]</sup>. NASH is a liver characteristic of metabolic syndrome, accounting for lipid accumulation and hepatic inflammation<sup>[2]</sup> and is projected to be the main indication for liver transplantation in the next decade<sup>[3]</sup>. NASH is commonly associated with type 2 diabetes, cardiovascular disease, and end-stage kidney disease<sup>[4,5]</sup>, and no approved pharmacological therapies currently exist for NASH<sup>[6]</sup>.

Recently, compelling evidence has indicated the critical role of the gut microbiota in the pathogenesis and progression of NAFLD to NASH<sup>[7]</sup>. Normally, the commensal gut microbiota and the host maintain a beneficial symbiotic relationship. The liver may function as a vascular firewall, mediating mutualism between the host and the intestinal commensal bacteria<sup>[8]</sup>. This intimate connection between the gastrointestinal tract and liver defines the term gut-liver axis. However, gut microbiota dysbiosis increases the influx of harmful substances, such as lipopolysaccharide (LPS), ethanol, and bacterial DNA, into the liver through portal vein circulation and promotes NASH development<sup>[9,10]</sup>. Currently, early and noninvasive diagnosis of NASH with high sensitivity and specificity remains challenging. Metabolomics, along with other "omics" technologies, helps provide a detailed understanding of biochemical events using the systems biology approach and facilitates early diagnosis and exploitation of treatment strategies<sup>[11]</sup>.

Despite the importance of the gut microbiota and metabolome in NASH, detailed information regarding their role during NASH development is limited. The

purpose of the present study was to investigate dynamic alterations in the gut microbiota and metabolome during the development of methionine-choline-deficient (MCD) diet-induced NASH in a mouse model.

## MATERIALS AND METHODS

### **Animals and treatments**

The animal protocol was designed to minimize the animals' pain or discomfort and was approved by the Animal Care Committee of Zhejiang University School of Medicine (Permit number: 2017-591). Male C57BL/6J mice (8 wk old) were purchased from SLAC Laboratory (Shanghai, China). After their arrival, the mice were acclimatized for 1 wk in a specific pathogen-free environment (23 °C, 12 h/12 h light/dark, 50% humidity, and *ad libitum* access to food and water) prior to experimentation. The mice were fed a methionine-choline-sufficient diet (Research Diet, New Brunswick, United States) for 2 wk (the Control 2w group,  $n = 6$ ) or 4 wk (the Control 4w group,  $n = 6$ ). Alternatively, the mice were fed an MCD diet (Research Diet) for 2 wk (the MCD 2w group,  $n = 6$ ) or 4 wk (the MCD 4w group,  $n = 6$ ). The diet specifications are summarized in Supplementary Table 1. After 2 or 4 wk on the diet, the mice were euthanized by an intraperitoneal injection of 4% chloral hydrate (with 1 mg/100 mL of atropine, to inhibit respiratory secretions) for tissue collection.

### **Sample collection**

Fecal samples were collected from all mice upon defecation and were stored at -80 °C for further procedures. Blood samples were centrifuged (3000 rpm, 15 min) for serum separation, and all serum aliquots were stored in a -80 °C freezer. Liver and colon samples were fixed in either neutral-buffered formalin or optimal cutting temperature (OCT)-embedding media for histological staining or were snap-frozen in liquid nitrogen and kept in a -80 °C freezer for further procedures.

### **Histopathological evaluation of liver tissue**

Paraffin-embedded liver sections were stained with hematoxylin-eosin (HE) or Masson's trichrome to detect liver injury or fibrosis. The stained sections were scanned using a NanoZoomer Digital Pathology system (Hamamatsu Photonics, KK, Japan), which digitally scanned the sections into a particular image format for further assessment. The HE-stained sections were scored in accordance with the NAFLD activity score (NAS) system (score 0-2: Not NASH, 3-4: Borderline NASH, and 5-8: NASH)<sup>[12]</sup>. Six fields from each section were selected and analyzed at 200 × magnification. The Masson's trichrome-stained sections were analyzed to quantify fibrosis using Image-Pro Plus software (version 6.0, Media Cybernetics, Rockville, United States) as previously detailed<sup>[13]</sup>. For each section, the blue area (collagen) was normalized to the red area (hepatocyte). The fibrosis index (%) was calculated as a percent of

the total tissue region and represented the average of six randomly selected fields from each section.

### **Oil red O staining and liver triglyceride assay**

Frozen liver sections fixed in OCT (10 μm) were stained with Oil red O (Sigma-Aldrich, St. Louis, MO, United States). Images were captured using the abovementioned NanoZoomer Digital Pathology system. To quantify intrahepatic lipid accumulation, the mean optical density of the red intensity was assessed using Image-Pro Plus software.

The liver triglyceride (TG) content was determined using a commercial kit from Applygen Technologies Inc. (Beijing, China) according to the manufacturer's protocols, and the final TG concentrations were normalized to the corresponding protein content.

### **Immunohistochemistry and immunofluorescence staining**

Paraffin-embedded liver sections were stained for F4/80 (anti-active macrophage) (Abcam, Cambridge, United Kingdom) and α-SMA (fibrosis hallmark) with immunohistochemistry (IHC) staining procedures as previously detailed<sup>[14]</sup>. Briefly, liver sections were incubated with a specific primary antibody, followed by incubation with horseradish peroxidase (HRP)-linked secondary antibody (Dako, Glostrup, Denmark) and 3,3'-diaminobenzidine; the sections were then scanned with the NanoZoomer Digital Pathology system. Image-Pro Plus software was used to count F4/80+ cells and quantitatively analyze the staining intensity of α-SMA as previously described<sup>[13]</sup>. Six fields of view were randomly selected in each section.

Likewise, paraffin-embedded colon sections were stained for Zonula occludens-1 (ZO-1) (intestinal barrier hallmark) (Proteintech, Rosemont, IL, United States) with standard immunofluorescence staining procedures as previously detailed<sup>[15]</sup>. Briefly, sections were incubated with the rabbit polyclonal ZO-1 antibody, followed by incubation with Texas Red-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA, United States) and 4',6-diamino-2-phenyl indole (DAPI), and images were captured using a Zeiss LSM T-PMT confocal microscope (Zeiss, Jena, Germany).

### **Microbial community analysis of fecal samples**

Total DNA was extracted from fecal samples with a QIAamp fast DNA stool mini kit (Qiagen, Valencia, CA, United States) following the manufacturer's handbook. After determining the DNA concentration and integrity, an amplicon sequencing library was constructed based on the PCR-amplified V3-V4 variable regions of 16S rDNA. Then, the qualified libraries were paired-end sequenced on an Illumina MiSeq platform according to manufacturer's procedures. Raw sequencing data were subjected to filtration using Trimmomatic, FLASH, and QIIME software. Then, clean reads were clustered into operational taxonomic units (OTUs) using UPARSE

**Table 1** Effects of the methionine-choline-deficient diet on nonalcoholic fatty liver disease activity score, alanine aminotransferase, aspartate aminotransferase, and hepatic triglycerides levels

	Control 2w	MCD 2w	Control 4w	MCD 4w
NAS	0.17 ± 0.17	1.17 ± 0.17 <sup>a</sup>	0.33 ± 0.21	4.83 ± 0.31 <sup>c,f,j</sup>
Steatosis	0.00 ± 0.00	1.17 ± 0.17 <sup>b</sup>	0.33 ± 0.21	2.50 ± 0.22 <sup>c,e,j</sup>
Inflammation	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.17 ± 0.17 <sup>b,e,i</sup>
Ballooning	0.17 ± 0.17	0.00 ± 0.00	0.00 ± 0.00	1.17 ± 0.31
TG (nmol/mg protein)	179.41 ± 21.72	592.88 ± 55.84 <sup>b</sup>	204.45 ± 6.76 <sup>e</sup>	731.60 ± 113.46 <sup>a,h</sup>
ALT (U/L)	19.33 ± 0.88	413.83 ± 81.77 <sup>a</sup>	34.83 ± 2.64 <sup>b,d</sup>	974.17 ± 163.00 <sup>a,h</sup>
AST (U/L)	105.50 ± 10.55	327.33 ± 39.92 <sup>a</sup>	109.83 ± 11.91 <sup>d</sup>	749.677 ± 92.34 <sup>b,d,i</sup>

<sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 and <sup>c</sup>*P* < 0.001 vs Control 2w group, <sup>d</sup>*P* < 0.05, <sup>e</sup>*P* < 0.01 and <sup>f</sup>*P* < 0.001 vs MCD 2w group, <sup>h</sup>*P* < 0.05, <sup>i</sup>*P* < 0.01 and <sup>j</sup>*P* < 0.001 vs Control 4w group. The data are shown as the means ± SEM of 6 mice per group. MCD: Methionine-choline-deficient; NAS: NAFLD activity score; NAFLD: Nonalcoholic fatty liver disease; TG: Triglycerides; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

software with a 97% threshold. The representative read from each OTU was selected using the QIIME package<sup>[16]</sup>. Representative OTU sequences were annotated and taxonomically classified using Ribosomal Database Project (RDP) Classifier v.2.2, trained on the Silva database version 123<sup>[17]</sup>. The linear discriminant analysis (LDA) effect size (LEfSe) method (<http://huttenhower.sph.harvard.edu/galaxy/>) was applied to differentiate taxa with statistical significance and biological relevance<sup>[18]</sup>.

#### Metabolomic profiling analysis of fecal samples

Metabolomic profiling analysis was performed as previously described<sup>[19]</sup> with slight modification. Briefly, fecal metabolites were extracted by mixing 15 mg of feces with 800 µL of ice-cold methanol. After homogenization and centrifugation, the supernatant was transferred into an Eppendorf tube containing 20 µL of 1 mg/mL heptadecanoic acid as the internal standard. Then, the sample was dried using a nitrogen stream (Aosheng, Hangzhou, China). The residue was reconstituted in 50 µL of 15 mg/mL methoxylamine hydrochloride in anhydrous pyridine and was incubated at 37 °C for 24 h. Then, 50 µL of *N,O*-bistrifluoroacetamide (BSTFA) [with 1% trimethylsilyl chloride (TMCS)] (Sigma-Aldrich, St. Louis, MO, United States) was added to the mixture, and the sample was incubated at 70 °C for 120 min. Metabolomic analysis was performed with a gas chromatography-mass spectrometry (GC-MS) on an Agilent 7890A GC system coupled to an Agilent 5975C inert mass selective detector (MSD) system (Agilent Technologies, Santa Clara, CA, United States). For data analysis, ChemStation software (version E.02.02.1431), and ChromaTOF software (version 4.34, LECO, St. Joseph, MI, United States) were used. Metabolites were identified by the National Institute of Standards and Technology (NIST) and Fiehn databases. Principal component analysis (PCA) and orthogonal partial least-squares-discriminant analysis (OPLS-DA) were performed to visualize metabolic differences among the experimental groups. Differential metabolites were selected according to the statistically significant VIP values obtained from the OPLS-DA model, and the *P* values from two-tailed Student's *t*-tests on the

normalized peak areas; metabolites with VIP values > 1 and *P* values < 0.05 were included.

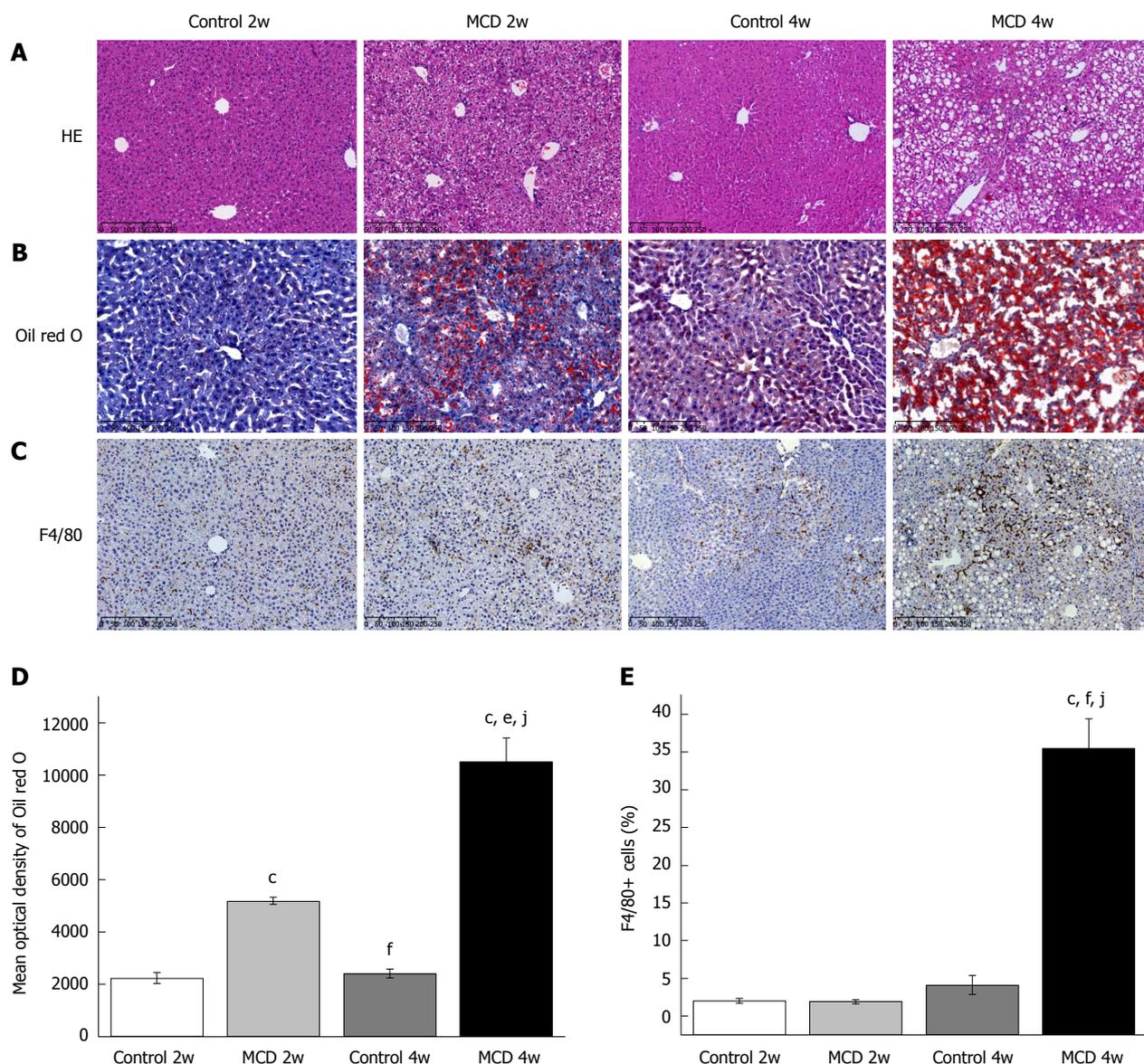
#### Statistical analysis

The data are shown as the means ± SEM, and the Kolmogorov-Smirnov test was performed to assess data normality. For most data, one-way ANOVA with Tukey's post hoc test was used to determine the significance between the groups. The Wilcoxon rank sum test was used to evaluate alpha diversity and principal coordinates between the different cohorts in the 16s sequencing analysis. Analysis of similarities (ANOSIM) was performed to test for microbial community clustering according to unweighted UniFrac distance matrices. *P* values < 0.05 were considered significant. The data were analyzed using SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL, United States).

## RESULTS

### MCD diet resulted in gradual liver injury and intestinal barrier impairment

After 2 wk, the mice fed the MCD diet developed simple hepatic steatosis: the liver showed predominantly microvesicular fat accumulation without lobular inflammation and ballooning (Figure 1A, Table 1). Hepatic steatosis was significantly increased in the MCD 2w group compared with that in the Control 2w group, as further indicated by Oil red O staining and hepatic TG quantification (Figure 1B and D, Table 1). No significant difference was found in the number of F4/80+ cells between the MCD 2w group and the Control 2w group (Figure 1C and E). Compared with the Control 2w group, the MCD 2w group presented significantly increased serum alanine aminotransferase (ALT) (*P* < 0.01) and aspartate aminotransferase (AST) (*P* < 0.01) levels (Table 1). Evidence of liver fibrosis was not found, as shown by Masson's trichrome and α-SMA immunohistochemical staining (Figure 2). Intestinal barrier destruction is associated with NAFLD progression<sup>[10]</sup>; therefore, we detected tight junction protein expression in the colon, where the most abundant gut microbiota reside<sup>[20]</sup>. ZO-1 immunostaining revealed that the colon tissues of the mice in the MCD 2w group exhibited



**Figure 1** Methionine-choline-deficient diet induced hepatic injury, fat accumulation, and macrophage infiltration. A-C: Representative liver histology assessed by HE staining (A), Oil red O staining (B), and macrophages (F4/80) staining (C). Scale bar: 250  $\mu$ m. D: Representative staining intensities of Oil red O as designated by mean optical density. E: Percentage of F4/80 positive cells. The data are given as the means  $\pm$  SEM.  $n = 6$  per group. <sup>c</sup> $P < 0.001$  vs Control 2w group, <sup>e</sup> $P < 0.01$  and <sup>f</sup> $P < 0.001$  vs MCD 2w group, <sup>j</sup> $P < 0.001$  vs Control 4w group by post hoc ANOVA one-way statistical analysis. MCD: Methionine-choline-deficient.

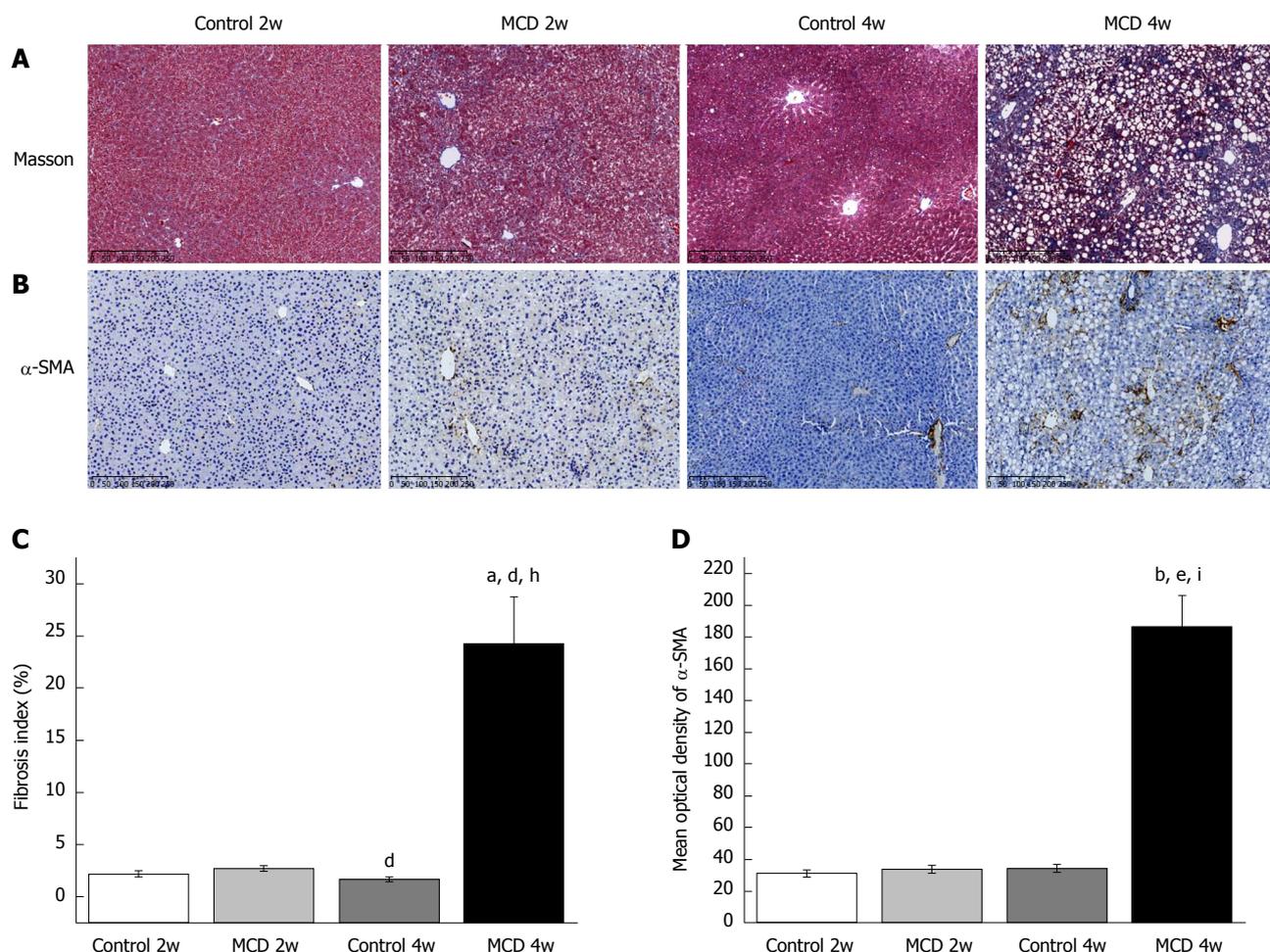
increased disruption and disorganization on the apical surface and in the crypts (Figure 3).

As expected, the mice in the MCD 4w group developed prominent NASH, as evidenced by major hepatic steatosis with lobular inflammation and ballooning hepatocytes in the liver (Figure 1A, Table 1). Oil red O staining and hepatic TG quantification also revealed that fat accumulation was significantly increased in the livers of the mice fed the MCD diet for 4 wk (the MCD 4w group) compared with that in the mice fed the control diet for 4 wk (the Control 4w group) (Figure 1B and D, Table 1). F4/80+ cell infiltration was significantly increased in the MCD 4w group compared with that in the Control 4w group (Figure 1C and E). In addition, we found that the mice in the MCD 4w group exhibited indications of liver fibrosis, including periportal and interstitial collagen deposition (Figure 2A

and C). Immunohistochemical analysis further confirmed our result:  $\alpha$ -SMA protein expression was significantly up-regulated in the MCD 4w group compared with that in the Control 4w group (Figure 2B and D). Intestinal barrier function was further impaired in the mice fed the MCD diet for 4 wk compared with that in the mice fed the control diet, as revealed by ZO-1 immunostaining (Figure 3).

#### MCD diet induced gradual gut microbiota dysbiosis

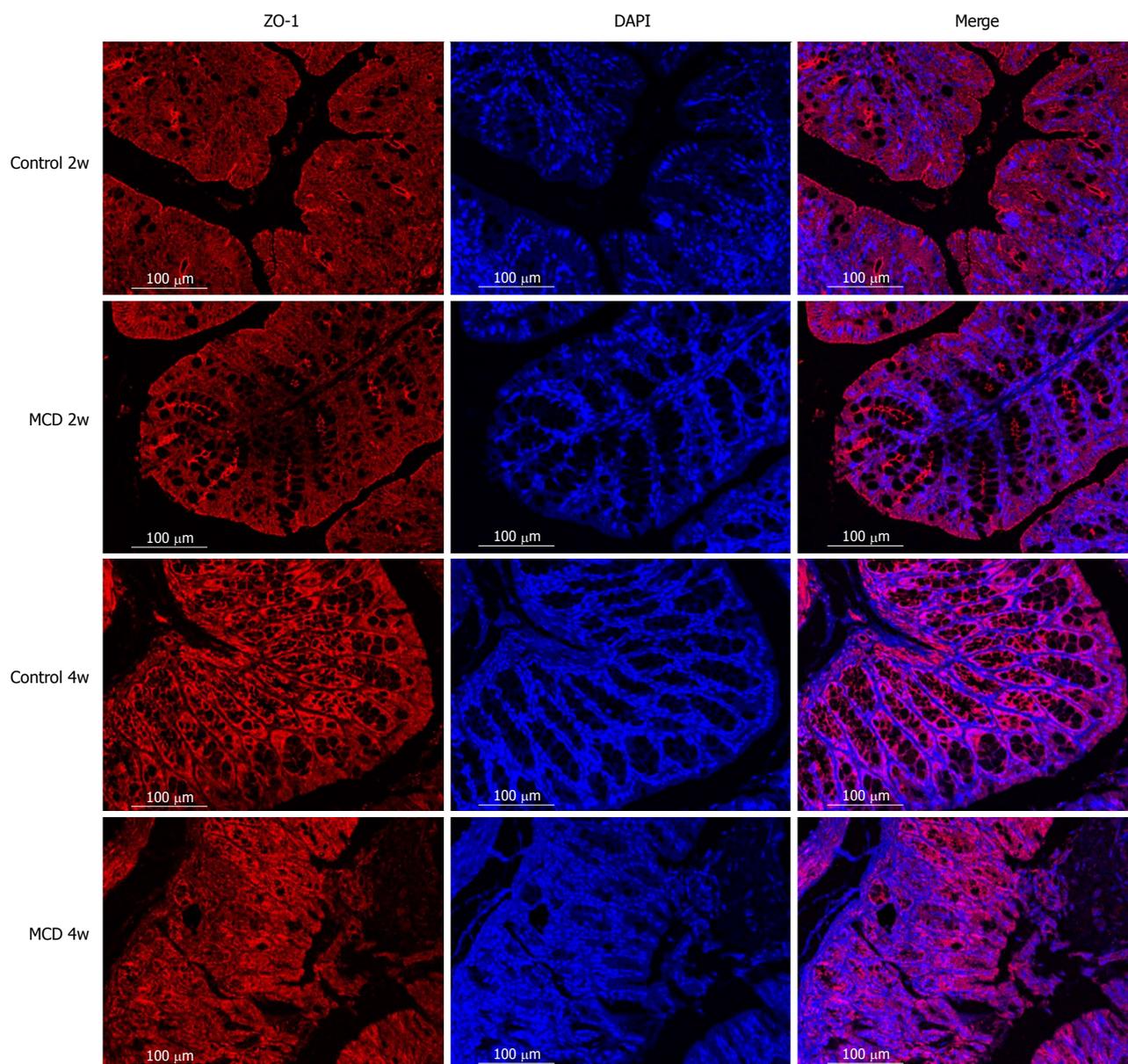
The MCD diet clearly altered the gut microbiota configuration. No significant differences were observed in alpha diversity between the control and MCD groups after 2 and 4 wk of treatment, as estimated by the Chao1, Shannon, and Simpson indices (data not shown). However, these two groups were clearly separated into different clusters at 2 wk (ANOSIM,  $P < 0.01$ ,  $r =$



**Figure 2** Methionine-choline-deficient diet induced liver fibrosis after 4 wk of feeding. A-B: Representative liver histology assessed by Masson's trichrome staining (A) and  $\alpha$ -SMA staining (B). Scale bar: 250  $\mu$ m. C: Percent of Masson's trichrome-stained area as indicated by the fibrosis index (%). D: Representative staining intensities of  $\alpha$ -SMA as designated by the mean optical density. The data are given as the means  $\pm$  SEM.  $n = 6$  per group. <sup>a</sup> $P < 0.05$  and <sup>b</sup> $P < 0.01$  vs Control 2w group, <sup>d</sup> $P < 0.05$  and <sup>e</sup> $P < 0.01$  vs MCD 2w group, <sup>h</sup> $P < 0.05$  and <sup>i</sup> $P < 0.01$  vs Control 4w group by post hoc ANOVA one-way statistical analysis. MCD: Methionine-choline-deficient.

0.6352) and at 4 wk (ANOSIM,  $P < 0.01$ ,  $r = 0.9074$ ) in the unweighted UniFrac principal coordinate analysis (PCoA), which was performed to calculate the beta-diversity values (Figure 4A). The most abundant taxa at the phylum, family, and genus levels are shown in Figure 4B-D. At the phylum level, Tenericutes was increasingly more abundant in the fecal microbiota of the MCD group compared with that of the control group at 2 and 4 wk ( $P < 0.05$ , and  $P < 0.01$ , respectively), while Verrucomicrobia was consistently less abundant in the MCD group at 2 and 4 wk ( $P < 0.05$ , and  $P < 0.05$ , respectively). Compared with the control group, the MCD group had a significantly higher abundance of Firmicutes and a significantly reduced abundance of Proteobacteria at 2 wk ( $P < 0.001$ , and  $P < 0.05$ , respectively). At 4 wk, the abundance of Actinobacteria was significantly lower in the MCD group than that in the corresponding control group ( $P < 0.01$ ). At the family level, the relative abundance levels of Rikenellaceae, Desulfovibrionaceae, and Verrucomicrobiaceae were persistently reduced in the MCD group compared with those in the control

group at 2 wk ( $P < 0.001$ ,  $P < 0.05$ , and  $P < 0.05$  respectively) and at 4 wk ( $P < 0.05$ ,  $P < 0.05$ , and  $P < 0.05$  respectively). The relative abundance of the Bacteroidales S24-7 group was significantly lower in the MCD group than that in the control group at 2 wk ( $P < 0.01$ ), while Ruminococcaceae was significantly higher in the MCD group ( $P < 0.05$ ). Compared with the control group, the MCD group had significantly higher abundance levels of Bacteroidaceae and Enterobacteriaceae at 4 wk ( $P < 0.01$ , and  $P < 0.05$  respectively) and a significantly reduced abundance of Bifidobacteriaceae ( $P < 0.05$ ). At the genus level, the abundance of the Rikenellaceae RC9 gut group was significantly reduced in the MCD group compared with that in the control group at 2 and 4 wk ( $P < 0.001$ , and  $P < 0.05$ , respectively). Compared with the control group, the MCD group presented with marked decreases in the abundance levels of Alistipes and (Eubacterium) coprostanoligenes at 2 wk ( $P < 0.001$ , and  $P < 0.05$ , respectively) and a significant increase in Ruminococcaceae UCG 014 abundance ( $P < 0.05$ ). However, at 4 wk, the MCD diet significantly reduced the



**Figure 3** Methionine-choline-deficient diet resulted in gradual intestinal barrier impairment. Representative colon histology was assessed by ZO-1 immunofluorescence staining. Scale bar: 100  $\mu\text{m}$ . The data are given as the means  $\pm$  SEM. MCD: Methionine-choline-deficient.

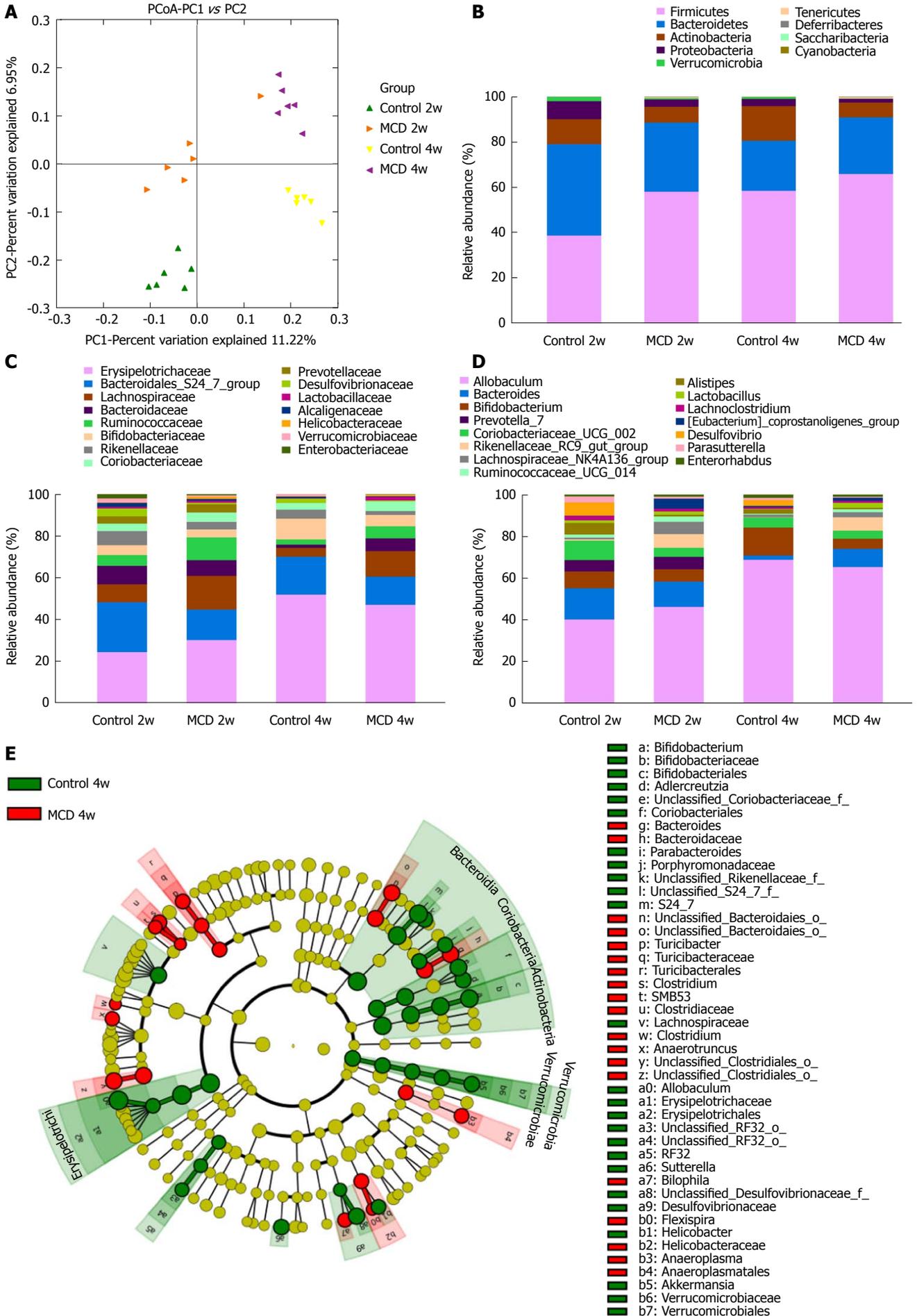
abundance of the promising probiotic *Bifidobacterium* and markedly promoted *Bacteroides* abundance ( $P < 0.05$ , and  $P < 0.01$ , respectively).

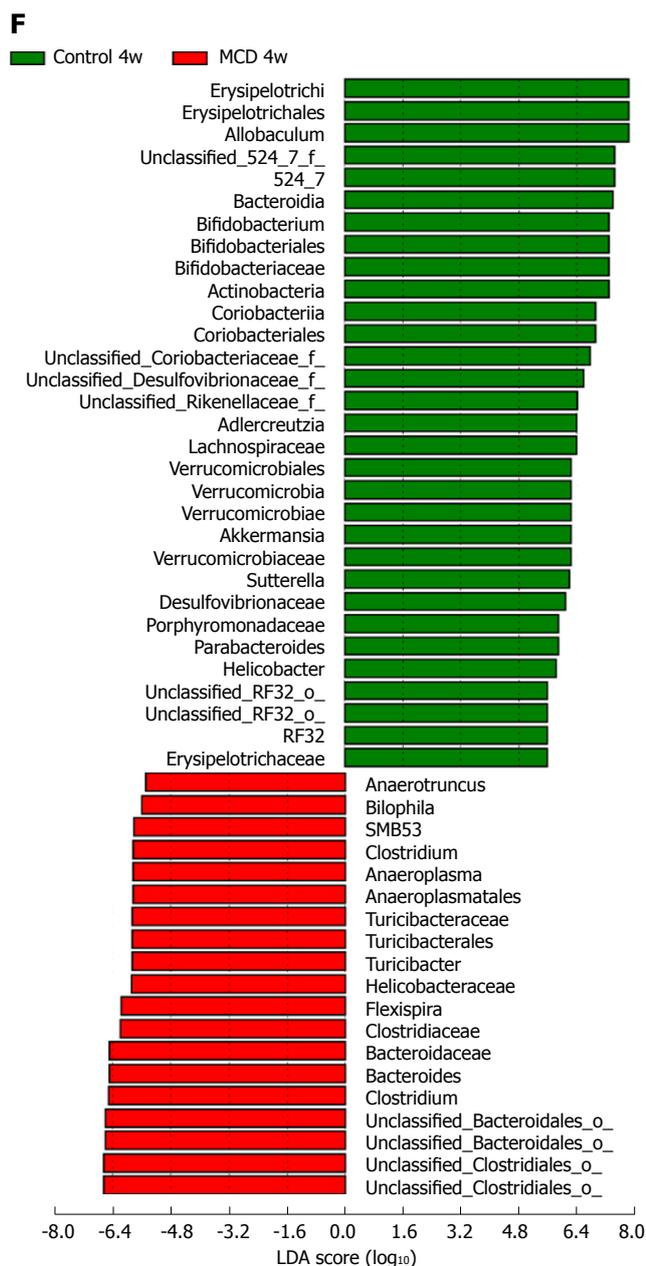
To characterize further the distinguishing phylotypes in the gut microbiota of the two groups, LfSe analysis was performed. No significant differences were found between the MCD 2w group and Control 2w group. At 4 wk, however, we found that the MCD diet increased the abundance levels of *Anaerotruncus*, *Bilophila*, *SMB53*, *Clostridium*, *Anaeroplasma*, *Turicibacter*, *Helicobacteraceae*, *Flexispira*, and *Bacteroides* [LDA score ( $-\log_{10}$ )  $> 4.8$ ] and decreased the abundance levels of *Allobaculum*, *S24-7*, *Bifidobacterium*, *Adlercreutzia*, *Lachnospiraceae*, *Akkermansia*, *Sutterella*, *Desulfovibrionaceae*, *Porphyromonadaceae*, *Parabacteroides*, and *Erysipelotrichaceae* [LDA score ( $\log_{10}$ )  $> 4.8$ ] (Figure 4E and F).

### **MCD diet altered the fecal metabolomic profile during NASH progression**

Using an untargeted strategy, we studied the fecal metabolome associated with functional characteristics of the gut microbiome, and a total of 322 metabolites were ultimately identified and quantified.

The PCA model was established ( $R^2X = 0.526$ ,  $Q^2 = 0.223$ ), corresponding to the four groups (the Control 2w group, MCD 2w group, Control 4w group, and MCD 4w group) (Figure 5A), and the score plot showed a clustering tendency in the first predictive principal component (X axis) and second predictive principal component (Y axis). Then, an OPLS-DA model was constructed. As depicted in Figure 5B, the score plot in the direction of the X axis (the first predictive principal component) and Y axis (the first orthogonal principal





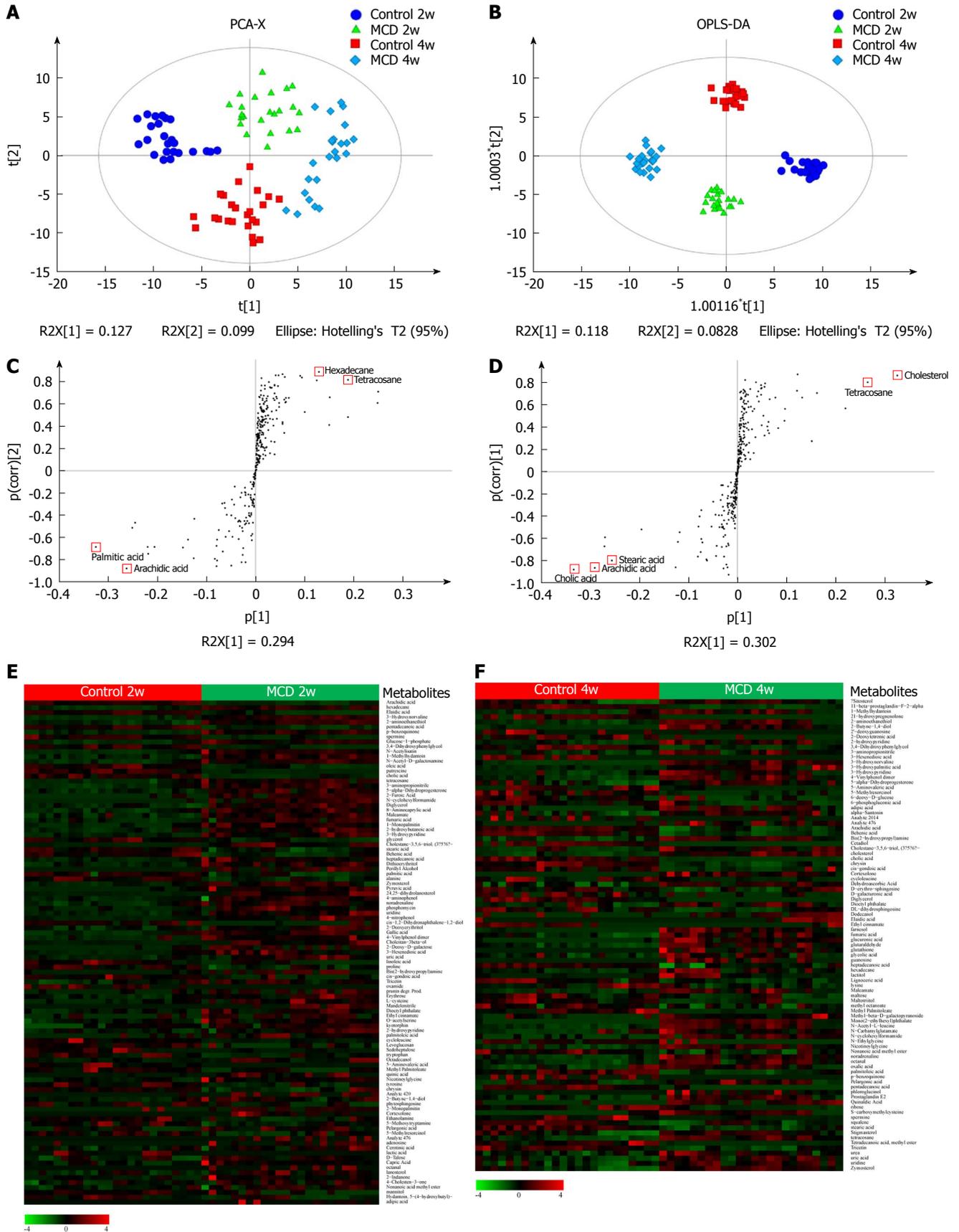
**Figure 4 Methionine-choline-deficient diet induced gut microbiota dysbiosis.** A: PCoA plot of the microbiota based on unweighted UniFrac metric. Each symbol represents one sample ( $n = 6$  per group). B-D: Top most abundant taxa at the phylum (B), family (C), and genus (D) levels. E: LEfSe cladogram represented taxon enriched in the Control 4w group (green) and in the MCD 4w group (red). Rings from the inside out represented taxonomic levels from phylum to genus. Sizes of the circles indicate the relative abundance levels of the taxa. F: Discriminative biomarkers with an LDA score  $> 4.8$ . MCD: Methionine-choline-deficient.

component) showed a significant separation in the metabolomics datasets among the four groups. The explained variance,  $R^2$ , was 0.968. The cross-validated predictive ability  $Q^2$  was 0.914, indicating that a random fecal GC-MS spectrum discriminates among the four groups 91.4% of the time. Therefore, these results indicate the distinct clustering of the fecal metabolomic profiles induced by the MCD diet over time. Characteristic metabolites that had been significantly modified by the MCD diet were further identified according to the OPLS-DA model, with VIP values  $> 1$  and  $P$  values  $< 0.05$  (Figure 5E and F, and Supplementary Tables 2 and 3). Ultimately, 103 and 93 metabolites were selected at 2 and 4 wk, respectively, most of which were mainly associated with

pathways involved in lipid, amino acid, carbohydrate, nucleotide, cofactors, and vitamin metabolism. S-plots were used to identify further potential biomarkers among the metabolites. As shown in Figure 5C and D, arachidic acid, hexadecane, palmitic acid, and tetracosane at 2 wk and cholic acid, cholesterol, arachidic acid, tetracosane, and stearic acid at 4 wk were the farthest from the origin and were selected as potential biomarkers due to their marked contribution to the separation between the control and MCD groups.

## DISCUSSION

To the best of our knowledge, this is the first study to



**Figure 5** Methionine-choline-deficient diet altered the fecal metabolomic profile during nonalcoholic steatohepatitis progression. A: 4-state model of a PCA plot comparing between the Control 2w (circle), MCD 2w (triangle), Control 4w (square), and MCD 4w groups (diamond). B: OPLS-DA score scatter plot comparing the Control 2w (circle), MCD 2w (triangle), Control 4w (square), and MCD 4w groups (diamond). Each symbol represents one sample ( $n = 24$  per group, with a quadruplicate technical replicates of 6 samples per group). C-D: S-plot constructed from the OPLS-DA model at 2 wk (C) and 4 wk (D). The selected metabolites (red triangles) are located in the upper far right and lower far left with considerable potential as biomarkers and marked discrimination between the groups. E-F: Euclidean distance hierarchical clustering analysis visualizing the different intensity levels of characteristic metabolites at 2 wk (E) and 4 wk (F). PCA: Principal component analysis; MCD: Methionine-choline-deficient; OPLS-DA: Orthogonal partial least-squares-discriminant analysis.

demonstrate dynamic alterations in the gut microbiota and metabolome in an experimental model of MCD diet-induced steatohepatitis: We sought to determine the key microbiota and metabolites involved in NASH progression over time.

Mice fed the MCD diet developed simple hepatic steatosis at 2 wk and prominent NASH at 4 wk. Among the most abundant taxa, the prevalence of the Rikenellaceae RC9 gut group in the family *Rikenellaceae*, family *Desulfovibrionaceae*, and family *Verrucomicrobiaceae* in the phylum Verrucomicrobia was consistently decreased at 2 and 4 wk in the MCD-supplemented group compared with that in the control group, while the phylum Tenericutes was consistently increased in the MCD-supplemented group. The phylum Tenericutes was also increased in the mice fed a high-fat diet and is correlated with obesity-associated metabolic characteristics<sup>[21]</sup>. The Rikenellaceae RC9 gut group was previously shown to be decreased in rats with hypertriglyceridemia-related acute necrotizing pancreatitis and in cows receiving oil supplementation<sup>[22,23]</sup>, but the specific function of this taxa remains poorly understood. High *Rikenellaceae* abundance is reportedly associated with healthy metabolic states<sup>[24]</sup>, and one study reported the ability of some of the bacteria in the family *Rikenellaceae* to produce butyrate<sup>[25]</sup>. *Desulfovibrionaceae* are known as sulfate-reducing bacteria that produce hydrogen sulfide (H<sub>2</sub>S)<sup>[26]</sup>, which has its pros and cons in relation to gut health. Some studies have demonstrated that H<sub>2</sub>S functions as a mucosal barrier breaker and therefore leads to inflammation<sup>[27,28]</sup>. Regarding the advantages of H<sub>2</sub>S, previous studies have reported that H<sub>2</sub>S is a crucial mediator in gastrointestinal mucosal defense and repair and in reducing systemic inflammation<sup>[26,29]</sup>. *Verrucomicrobiaceae* abundance is positively correlated with gastrointestinal health and negatively correlated with gut inflammation<sup>[30]</sup>. Therefore, disrupted barrier homeostasis, as evidenced by the ZO-1 immunostaining results in this study, may be associated with a decrease in MCD-sensitive microbiota.

In this study, we found that *Bifidobacterium* in the family *Bifidobacteriaceae*, *Bacteroides* in the family *Bacteroidaceae*, and the family *Enterobacteriaceae* respond to the MCD diet in a time-dependent manner. The first of these taxa tended to decrease, while the latter two tended to increase after only 4 wk of feeding. Consistently, *Bifidobacterium* was significantly decreased in NASH subjects compared with that in healthy and obese subjects<sup>[10]</sup>. Various members of the genus *Bifidobacterium* have recently attracted substantial interest due to their multiple beneficial effects on the host, such as protection against enteropathogenic infection through acetate production<sup>[31]</sup>, promotion of antitumor immunity, and facilitation of programmed cell death protein 1 ligand 1 (PD-L1)-specific antibody therapy<sup>[32]</sup>. Although *Bacteroides* spp. are overall regarded as beneficial microorganisms<sup>[33]</sup>, they can also cause gut-related bacteremia in conditions of high portal venous pressure<sup>[34]</sup>.

*Enterobacteriaceae* include many potential pathogens and LPS-producing bacteria and are significantly increased in NASH patients<sup>[10]</sup>. Furthermore, as evidenced by LEfSe analysis, the MCD diet favored LPS-producing bacteria (*Bilophila*, and *Anaeroplasm*<sup>[35,36]</sup>), pro-inflammatory bacteria (*Anaerotruncus*, and *Turicibacter*<sup>[37,38]</sup>), and other opportunistic pathogens (*SMB53*, *Clostridium*, *Helicobacteraceae* and *Flexispira*<sup>[39-42]</sup>) after 4 wk of feeding and inhibited the promising probiotics *Sutterella* and *Akkermansia*<sup>[43,44]</sup>. Therefore, these data indicate an important role for the microbiota in NAFLD progression to NASH.

To link the gut microbiota with their functional states, an untargeted metabolomics analysis was integrated into this study. A core of the MCD diet-responsive signaling pathways were involved in lipid, nucleotide, cofactor, vitamin, carbohydrate, and amino acid metabolism. S-plots further identified potential biomarkers among the metabolites during NASH development. Arachidic acid under-representation and tetracosane over-representation were prominent features in the group with MCD diet-induced steatosis and NASH compared with the corresponding control groups at 2 and 4 wk. However, functional exploration of the association between arachidic acid and tetracosane with non-alcoholic fatty liver disease has not been well documented and warrants further studies. Kuroda *et al.*<sup>[45]</sup> reported that hexadecane causes nonspecific inflammation. Therefore, we speculated in this study that the increased hexadecane levels after the MCD diet treatment for 2 wk may contribute to disease progression, although we have not yet obtained direct evidence for this effect. Jiao *et al.*<sup>[46]</sup> found that the cholic acid level is increased in NASH patients. JianHua *et al.*<sup>[47]</sup> found that cholic acid is significantly decreased in humans with NASH, which is consistent with our study results in the MCD 4w group. Stearic acid is a potent anti-inflammatory lipid and may accelerate hepatic dysfunction recovery in a rat model of liver injury<sup>[48]</sup>. Our study found that the stearic acid level was significantly decreased in the NASH mice after the MCD diet treatment for 4 wk. Although our study found an association between altered gut microbiota and metabolism and NASH, a causative contribution of the gut microbiota and metabolism to NASH progression has not been sufficiently documented. Establishing a better understanding of the gut microbiota and metabolomics in this disease state will provide beneficial information for the treatment and prevention of NAFLD.

In conclusion, the MCD diet induced gut microbiota and metabolome deterioration. Fundamental observations of these alterations will provide new insight into NASH-associated intestinal disorder and gut-targeted therapies for NASH.

## ARTICLE HIGHLIGHTS

### Research background

The contributing role of the gut microbiota in the pathogenesis of nonalcoholic

steatohepatitis (NASH) has been extensively studied.

### Research motivation

Gut microbiota dysbiosis in NASH is mainly depicted as an endpoint, and little is known regarding the microbiota disturbances during NASH progression.

### Research objectives

Our goal was to investigate dynamic changes in the gut microbiota and its metabolism during the progression from simple hepatic steatosis to NASH in mice fed a methionine-choline-deficient (MCD) diet.

### Research methods

C57BL/6J mice were equally divided into four groups and fed either a methionine-choline-sufficient diet for 2 or 4 wk (the Control 2w group and Control 4w group, respectively) or the MCD diet for 2 or 4 wk (the MCD 2w group and MCD 4w group, respectively) ( $n = 6$  per group). Liver injury, fibrosis, intestinal barrier function, and the fecal microbiome and metabolome were studied.

### Research results

The mice fed with the MCD diet for 2 wk developed simple hepatic steatosis, which progressed to prominent NASH with liver fibrosis after 4 wk. Compared with the control diet, the MCD diet induced gradual intestinal barrier impairment and gut microbiota dysbiosis; the fecal metabolomic profile was also substantially altered by the MCD diet.

### Research conclusions

The MCD diet induced persistent alteration of the gut microbiota and metabolome.

### Research perspectives

We may have for the first time shown that an MCD diet induced persistent gut microbiota and metabolome deterioration. Fundamental observations of these alterations will provide new insight into NASH-associated intestinal disorder and gut-targeted therapies for NASH.

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