Int J Clin Exp Med 2019;12(5):4794-4805
www.ijcem.com /ISSN:1940-5901/IJCEM0076472

Original Article

The metabolic profiles and gut microbiota alteration of rats with high fat diet-induced NAFLD

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Received March 21, 2018; Accepted October 25, 2018; Epub May 15, 2019; Published May 30, 2019

Abstract: Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease widespread. Increasing evidence suggest that the alterations in diet composition is associated with the epidemic of NAFLD. However, how dietary fat exposure influences the progression of NAFLD is smeared. In the present study, we aimed to explore the effects of dietary fat on NAFLD via metabolic profiles and gut microbiota. Male Wistar rats were placed on either normal chow diet (NCD) or high fat diet (HFD) for eight weeks. Liver histopathology and biochemical examinations of serum and liver were determined. GC/MS were used to elucidate the metabolic profiles of the serum, urine, liver and feces, and 16S rDNA sequencing were applied to determine the structure of gut microbiota. Typical NAFLD phenotypes in rats were identified with significant hepatic steatosis and dyslipidemia upon high dietary fat exposure. Metabolomics and microbiota analysis inferred that HFD disturbed energy metabolism and amino acid homeostasis, suppressed fatty acids oxidation, enhanced triglyceride synthesis, and promoted insulin resistance and inflammation. Our data demonstrated that dietary fat played a pivotal role in NAFLD development, and was related to glucose metabolism, lipid metabolism, amino acid metabolism and inflammation.

Keywords: Metabolomics, gut microbiota, high fat diet, nonalcoholic fatty liver disease

Introduction

Nonalcoholic fatty liver disease (NAFLD) is characterized by hepatic fat accumulation and encompasses a wide spectrum of disorders including simple steatosis, non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis and even hepatocellular carcinoma [1]. The prevalence of NAFLD is increasing with the epidemic of obesity, type 2 diabetes, cardiovascular disease and metabolic syndrome and currently affects approximately 20%-50% of population worldwide [2, 3].

Excessive energy intake, especially high dietary fat, together with insufficient exercise and genetic susceptibility, are considered to be important contributors in NAFLD development [4]. As one of the most important factors for daily nutrient acquisition, dietary components play significant role [5, 6]. Although high fat diet (HFD) is believed to induce NAFLD phenotype featured by increasing liver lipids and plasma insulin levels, inflammatory responses, and the homeostasis of cellular metabolism derangement [7], the underlying mechanisms are not fully elucidated.

Therefore, it is of great importance to select appropriate technologies which could clarify the relationships between dietary fat intake and NAFLD. Metabolomics, focusing on metabolic profiles from various types of samples, considered to be essential for understanding the biological mechanisms of complex diseases in recent years [8]. It could also provide a new vision of the connection between a particular nutrient intake or dietary pattern and the corresponding metabolic profiles [9]. Thus, we proposed that using integrated metabolomics technology to document the global changes in serum, liver, urine and feces might provide comprehensive information of NAFLD pathogenesis. Gut microbiota controls host metabolic regulations through affecting a wide variety of

biological processes, and the dysregulation of gut microbiota is closely associated with metabolic disorders such as obesity and NAFLD [10, 11]. Moreover, diet is reported to modulate composition of gut microbiota, and alterations of *Bacteroidetes* and *Firmicutes* in mice are reported to be involved in NAFLD pathogenesis [12]. Indeed, gut microbiota modulates a number of risk factors of NAFLD, such as obesity, insulin resistance, choline metabolism and inflammation [13].

In this study, to get better understanding of how HFD affect NAFLD development, we applied gas chromatography/mass spectrometry (GC/MS)-based approach to profile the serum, liver, urine and feces of HFD and normal chow diet (NCD) fed rats, and the 16S rDNA sequencing to analyze the specific bacteria in feces. Our data suggest that alteration of metabolites and gut bacteria might contribute to the development of NAFLD.

Materials and methods

Experimental design

Male Wistar rats (8 weeks old, 200 ± 20 g; Shanghai SLAC Laboratory Animal Co., Ltd, Shanghai, China) were placed in a room with a controlled temperature (25 ± 2°C), relative humidity (60 ± 5%), and a standard 12 h/12 h light-dark cycle. After 1 week adaptation, rats were randomly divided into two groups (n = 8 per group). Normal group rats were fed with NCD and NAFLD group rats were fed with HFD, in which 10 percent lard, 20 percent sucrose, 2 percent cholesterol and 1 percent bile salt were added into NCD. Water was supplied *ad libitum* throughout the study. After eight weeks feeding, six rats from each group were held in individual metabolic cages for 24 h to collect urine and feces. After an overnight fasting, the rats were intraperitoneally injected with sodium pentobarbital (0.5 mL/100 g body weight) for anesthesia. Blood were obtained via the abdominal aorta, and the serum was separated by centrifuged for 15 min at 3000 rpm. Liver specimens were rapidly removed, weighted, washed with pre-cooled normal saline, fixed in 4% paraformaldehyde or stored at 80°C after snap-frozen in liquid nitrogen.

Animal breeding, care and all protocols were approved by Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine.

Biochemical analysis of serum and liver tissue

Serum triacylglycerol (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), free fatty acid (FFA), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (TBIL), direct bilirubin (DBIL), total bile acid (TBA), and glucose (GLU) were measured by an automated biochemical analyzer.

Hepatic TG and TC were analyzed using assay kits (Dongou Technologies Inc., Zhejiang, China) according to the manufacturer's instructions and read with UV-mini1240 spectrophotometer (Shimadzu, Kyoto, Japan).

Liver histological analysis

Paraformaldehyde-fixed paraffin sections of the liver were stained with hematoxylin-eosin (H&E) for pathological analysis. Frozen samples were sectioned and stained with Oil Red O to detect the lipid droplets. Representative photomicrographs were captured at 200 magnification using a system incorporated in a Nikon Eclipse 50i microscope.

Metabolomic analysis

The process of sample preparation, GC/MS analysis, data processing, bioinformatics and statistical analysis were conducted as our previous work [14]. In brief, the serum, liver, urine and feces were prepared, added with internal standards, dried under gentle nitrogen stream, incubated with methoxylamine hydrochloride in anhydrous pyridine, derivatized with BSTFA (with 1% TMCS) and then splitless injected performing on an Agilent 7890A series gas chromatograph coupled to a HP-5MS column (30 m × 0.25 mm, 0.25 film thickness) and an Agilent 5975C inert MSD detector. The GC/MS data were processed with DataBridge (Perkin-Elmer, USA), and multivariate statistical analysis was applied with SIMCA-P 11.0 software (Umetrics AB, Umeå, Sweden) to perform principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA). The differential metabolites and metabolic pathways were determined using the Golm Metabolome and KEGG Database.

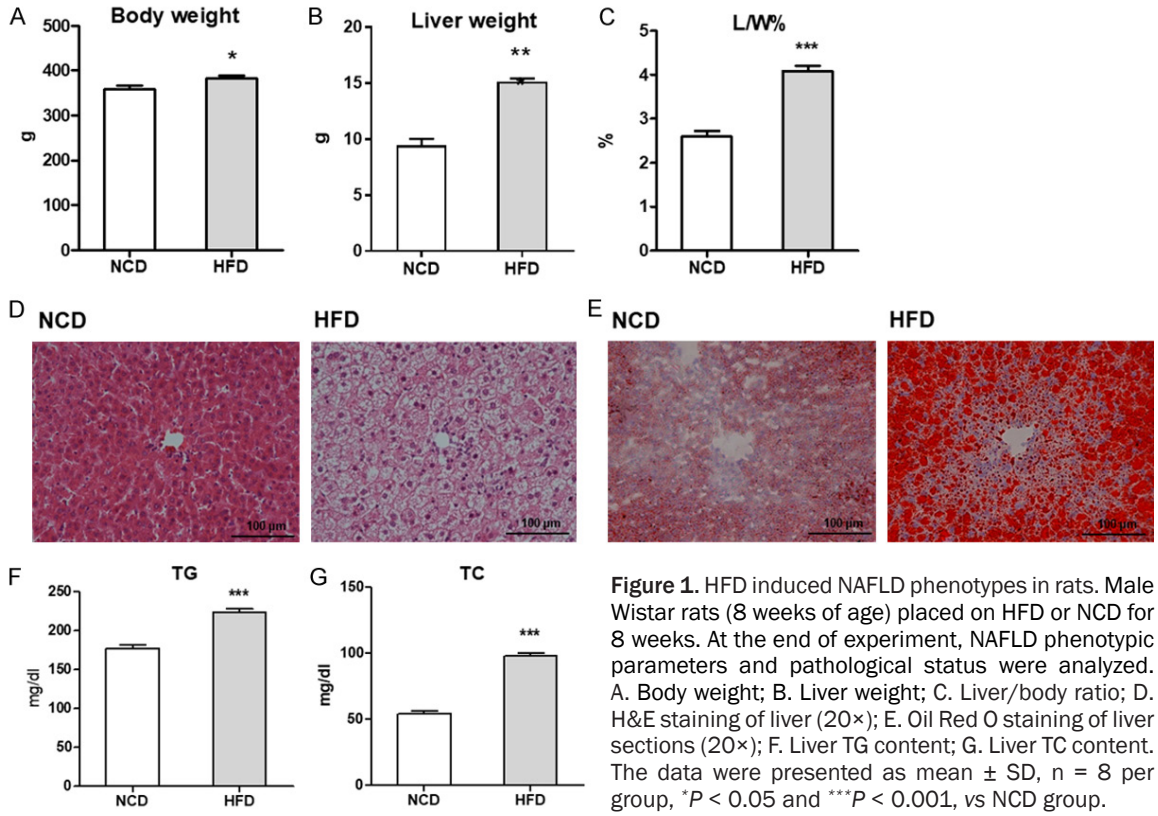

Figure 1. HFD induced NAFLD phenotypes in rats. Male Wistar rats (8 weeks of age) placed on HFD or NCD for 8 weeks. At the end of experiment, NAFLD phenotypic parameters and pathological status were analyzed. A. Body weight; B. Liver weight; C. Liver/body ratio; D. H&E staining of liver (20×); E. Oil Red O staining of liver sections (20×); F. Liver TG content; G. Liver TC content. The data were presented as mean ± SD, n = 8 per group, $^{*}P < 0.05$ and $^{***}P < 0.001$, *vs* NCD group.

Microbiota analysis

The fecal DNA extraction, pyrosequencing, and bioinformatics and statistical analysis were described in details as our previous work [14]. In brief, the samples of fecal DNA were extracted using the QIAamp DNA stool mini kit together with the protocol for "Isolation of DNA from stool for human DNA analysis", PCR amplified for the V3 hypervariable regions of the 16S rDNA gene on an Eppendorf thermocyclera, quantified with fluorometric quantification method that used dsDNA binding dyes and secondary analyzed using the MiSeq system with MiSeq Reporter (MSR) software. Operational taxonomic units (OTUs) clustering, principal coordinate analysis (PCoA) and nonmetric multidimensional scaling (NMDS) were performed by the Mothur program. OTUs were used to analyze rarefaction curve and Shannon diversity index, and generate heatmap by R software.

Statistical analysis

SPSS 17.0 statistical software (SPSS, Chicago, IL, USA) was used for data analysis. Student's *t* test was applied to show the difference between groups. The data were presented as mean ± standard deviation (SD), the criterion used for statistical significance was $P < 0.05$.

Results

HFD induced NAFLD phenotype in Wistar rats

Compared with NCD rats, HFD rats demonstrated significantly increased body weight (obesity), liver weight and liver/body ratio (**Figure 1A-C**). Histological examination of liver tissues showed that HFD caused notable steatosis and lipid droplets accumulation, which evidenced by H&E (**Figure 1D**) and oil red O (**Figure 1E**) staining, respectively. Meanwhile, biochemistry analysis confirmed the significantly increased hepatic TG (**Figure 1F**) and TC (**Figure 1G**) content in rats fed with HFD, which was consistent with the histological change.

To evaluate whole-body glucose and lipid metabolism, we next measured serum biochemistry parameters in the rats. HFD-fed rats had significantly higher serum TC and LDL-c levels whereas lower HDL-c levels than NCD-fed rats. A trend towards higher TG and FFA in serum

Table 1. Serum phenotypic parameters

Items	NCD rats (n = 8)	HFD rats (n = 8)
TG (mmol/l)	0.60 ± 0.18	0.70 ± 0.26
TC (mmol/l)	1.12 ± 0.19	2.49 ± 0.36***
HDL (mmol/l)	0.78 ± 0.06	0.60 ± 0.07***
LDL (mmol/l)	0.18 ± 0.04	1.38 ± 0.21***
FFA (mmol/l)	0.37 ± 0.08	0.43 ± 0.07
ALT (mmol/l)	20.3 ± 2.8	42.2 ± 12.6***
AST (mmol/l)	109.2 ± 6.6	151.2 ± 41.1*
ALP (mmol/l)	56.0 ± 7.6	89.3 ± 16.9***
TBIL (mmol/l)	0.7 ± 0.1	0.6 ± 0.5
DBIL (mmol/l)	0.1 ± 0.1	0.2 ± 0.1*
TBA (mmol/l)	4.5 ± 1.4	5.4 ± 1.9
GLU (mmol/l)	8.82 ± 1.63	9.29 ± 2.02

Data are presented as Mean ± SD. *$P < 0.05$ and ***$P < 0.001$, compared with NCD group.

was also observed in HFD-fed group, although there was no statistical difference (**Table 1**). These results indicated that 8-week HFD feeding caused severe hyperlipidemia. Compared with NCD rats, serum ALT and AST levels significantly increased in HFD rats, indicating possible liver damage. In addition, serum ALP was also elevated in HFD rats (**Table 1**). However, the levels of TBIL, DBIL, TBA, GLU were not statistically different between the two groups (**Table 1**).

HFD caused alteration of metabolomic profiles

We further analyzed metabolomic profiles in serum, liver, urine and feces. The distribution of all samples was represented by the score plot of PCA. The profiles of all samples (serum, liver, feces, and urine) in the two groups were scattered and could be completely separated (Figure S1A-D), (A) R^2X = 0.61, Q^2 = 0.176; (B) R^2X = 0.663, Q^2 = 0.245; (C) R^2X = 0.623, Q^2 = 0.265; (D) R^2X = 0.664, Q^2 = 0.278. Since parameters of the PCA model (R^2X and Q^2) more than 50% predict good clustering result, the available data indicated that the PCA model is not suitable for the clustering. PLS-DA is a multivariate classification method, which seeks directions that are optimal for discrimination and correlation with the response in comparison with PCA which focus only on high variance [15].

Despite the different types of samples, HFD and NCD groups were clearly separated (Figure S1A-D). The evaluation parameters of PLS-DA models, R^2X, R^2Y and Q^2, were 0.398, 0.983 and 0.617 in serum, 0.438, 0.987 and 0.884 in liver, 0.53, 0.999 and 0.887 in urine, and 0.599, 0.996 and 0.913 in feces, respectively. Moreover, the PLS-DA scores plot for the first two PCs of HFD and NCD groups were located in different clusters.

Heat map and Pearson's correlation analysis were applied to systematically screen the characteristic metabolites. The correlations were demonstrated in different colors (Figure S2A, S2C, S2E, S2G). To better visualize the differences between the two groups, we constructed a heat map of metabolite abundances for the confidently identified metabolites (Figure S2B, S2D, S2F, S2H). The two groups showed quite different metabolic profiles in serum (15 metabolites increased and 16 metabolites decreased) (Table S1), liver (20 metabolites increased and 16 metabolites decreased) (Table S2), urine (41 metabolites increased and 15 metabolites decreased) (Table S3), and feces (16 metabolites increased and 27 metabolites decreased) (Table S4). These differentially expressed metabolites were mainly distributed in glucose, fatty acid, amino acid, and sterols related pathways.

Abnormalities of glucose metabolism in HFD rats

Among the differentially expressed metabolites, 5 metabolites (pyruvic acid, gluconic acid, citric acid, succinic acid and glyceric acid) were associated with glucose metabolism. Compared with NCD group, there was a significantly increase of pyruvic acid in liver and urine of HFD-fed rats, whereas a decrease in serum and feces (**Figure 2A**). HFD feeding induced a significant increase in gluconic acid level in serum, liver and urine (**Figure 2B**). The level of citric acid was decreased in serum, but increased in urine of HFD rats (**Figure 2C**), and succinic acid and glyceric acid levels were increased in serum and urine of HFD rats (**Figure 2D**, **2E**). These alterations indicated that tricarboxylic acid (TCA) cycle and energy metabolism homeostasis were both affected by HFD feeding.

Abnormalities of lipids metabolism in HFD rats

Fatty acid associated metabolites (oleic acid, 2-hydroxybutyric acid and arachidonic acid)

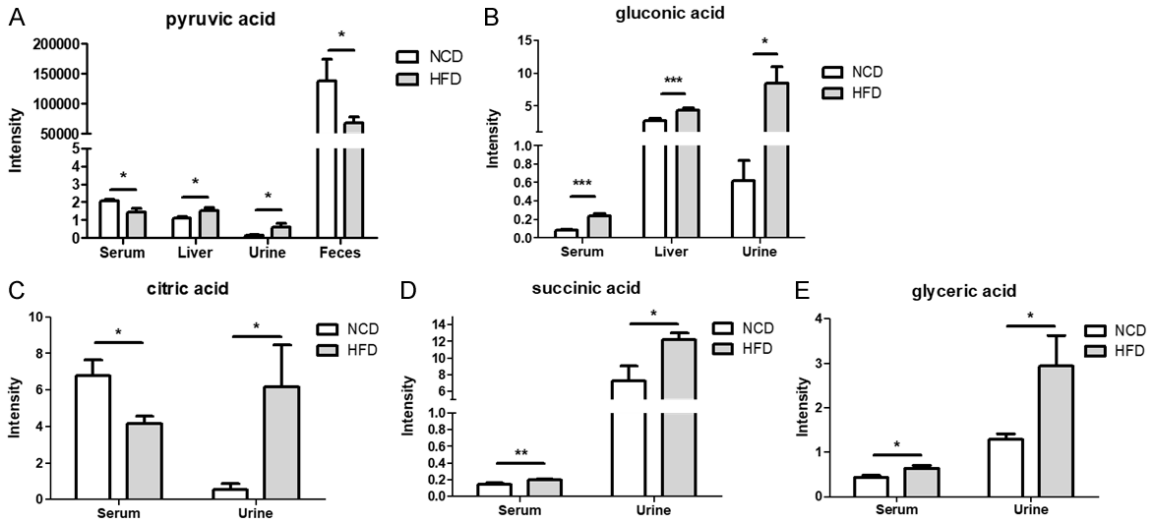

Figure 2. Differentially expressed metabolites in glucose metabolism. A. Pyruvic acid; B. Gluconic acid; C. Citric acid; D. Succinic acid; E. Glyceric acid. The data were presented as mean ± SD, n = 8 per group, $^{*}P < 0.05$ and $^{***}P < 0.001$, *vs* NCD group.

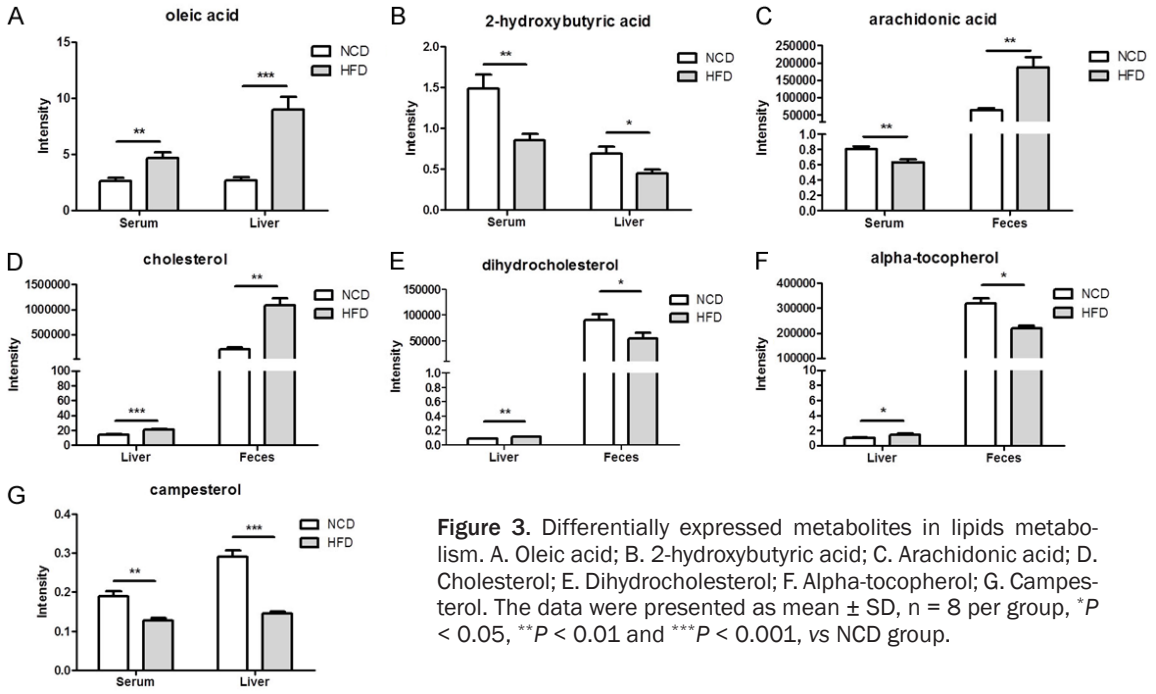

Figure 3. Differentially expressed metabolites in lipids metabolism. A. Oleic acid; B. 2-hydroxybutyric acid; C. Arachidonic acid; D. Cholesterol; E. Dihydrocholesterol; F. Alpha-tocopherol; G. Campesterol. The data were presented as mean ± SD, n = 8 per group, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, *vs* NCD group.

were affected by HFD. Compared with NCD group, HFD-fed rats showed significantly increase of oleic acid in serum and liver (**Figure 3A**). HFD feeding induced a significant decrease in 2-hydroxybutyric acid level in serum and liver (**Figure 3B**). The level of arachidonic acid was decreased in serum, whereas increased in feces of HFD rats (**Figure 3C**). These alterations suggested that fatty-acid oxidation and synthesis dysregulated upon HFD feeding.

Moreover, sterols associated metabolites (cholesterol, dihydrocholesterol, alpha-tocopherol and campesterol) were affected by HFD as well. Compared with NCD group, there were significantly increase of cholesterol in liver and feces of HFD-fed rats (**Figure 3D**). HFD feeding induced a significant increase in dihydrocholesterol and alpha-tocopherol in liver, whereas decreased in feces of HFD rats (**Figure 3E**, **3F**). The level of campesterol was decreased in

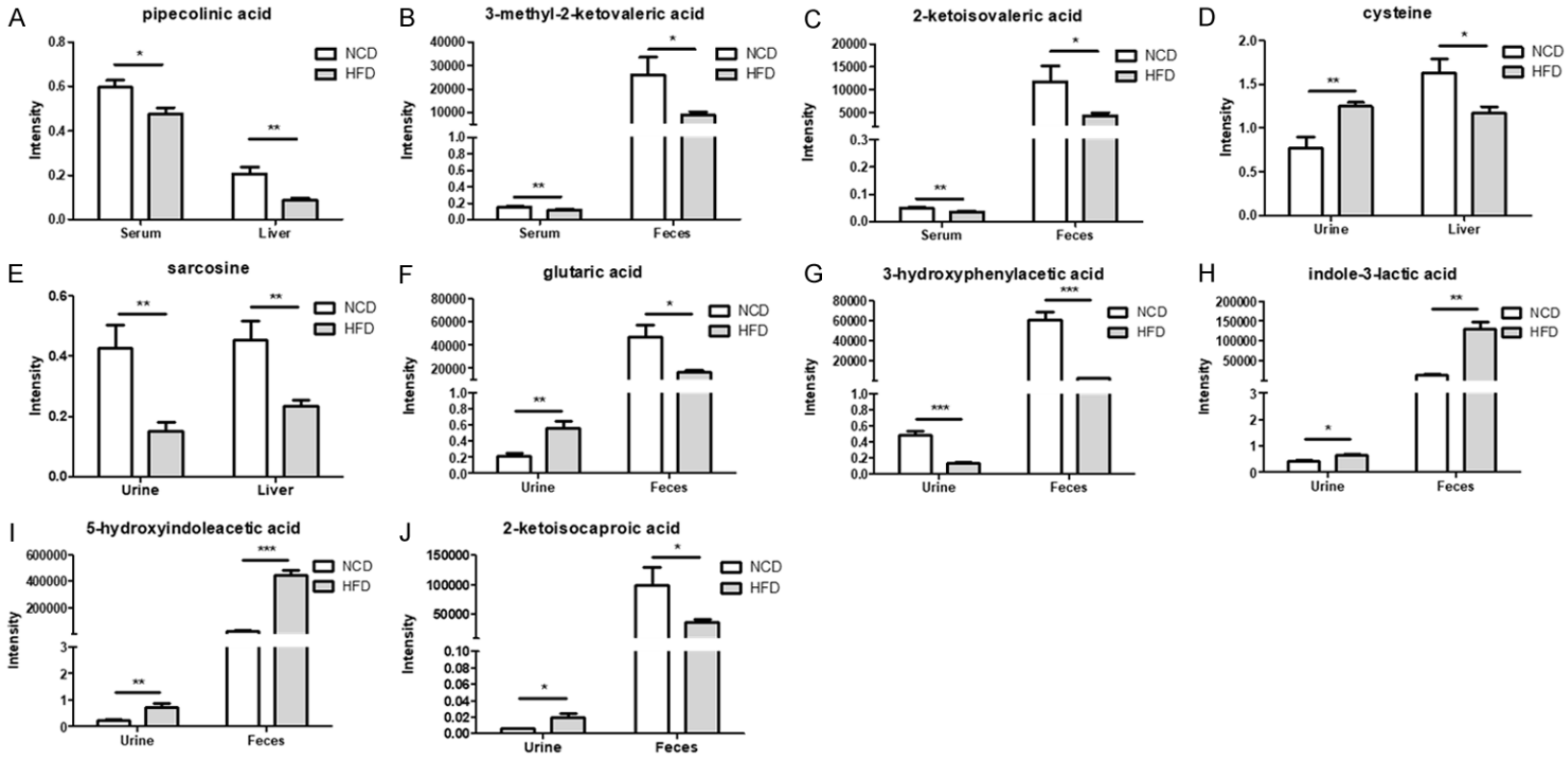

Figure 4. Differentially expressed metabolites in amino acid metabolism. A. Pipecolinic acid; B. 3-methyl-2-ketovaleric acid; C. 2-ketoisovaleric acid; D. Cysteine; E. Sarcosine; F. Glutaric acid; G. 3-hydroxyphenylacetic acid; H. Indole-3-lactic acid; I. 5-hydroxyindoleacetic acid; J. 2-ketoisocaproic acid. The data were presented as mean ± SD, n = 8 per group, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, *vs* NCD group.

serum and liver of HFD rats (**Figure 3G**). These alterations suggested that absorption and synthesis of sterols was influenced by HFD feeding.

Abnormalities of amino acid metabolism in HFD rats

10 metabolites associated with amino acid (pipecolinic acid, 3-methyl-2-ketovaleric acid, 2-ketoisovaleric acid, cysteine, sarcosine, glutaric acid, 3-hydroxyphenylacetic acid, indole-3-lactic acid, 5-hydroxyindoleacetic acid and 2-ketoisocaproic acid) were affected by HFD. Compared with NCD rats, there was a significantly decrease of pipecolinic acid in serum and liver of the HFD-fed rats (**Figure 4A**). HFD feeding induced significant decrease of 3-methyl-2-ketovaleric acid and 2-ketoisovaleric acid in serum and feces (**Figure 4B**, **4C**). The level of cysteine was significantly increased in urine of HFD rats, whereas was decreased in liver of HFD rats (**Figure 4D**). The level of sarcosine was significantly decreased in urine and liver of HFD rats (**Figure 4E**). Glutaric acid was increased in urine but decreased in feces of HFD rats (**Figure 4F**), whereas the level of 3-hydroxyphenylacetic acid was decreased in urine and feces of HFD rats (**Figure 4G**). HFD feeding induced significant increase of indole-3-lactic acid and 5-hydroxyindoleacetic acid in urine and feces (**Figure 4H**, **4I**). The level of 2-ketoisocaproic acid was increased in urine but decreased in feces of HFD rats (**Figure 4J**). These alterations suggested that amino acid metabolism homeostasis was disturbed by HFD feeding.

HFD distorted gut microbiota and reduced bacterial diversity

For the bacterial community analysis of the 12 samples, we obtained a total of 254063 valid sequences (80.8% of the total) and 34866 OTUs. While rarefaction curve was not plateaued with the current sequencing, Shannon diversity curve suggested that most diversity of all samples had been captured at this sequencing depth (Figure S3A, S3B). The gut microbiota from NCD and HFD groups could be separated by PCoA based on the relative abundance of OTUs (Figure S3C), and NMDS further confirmed the effect (Figure S3D).

We detected 8272 OTUs through NCD and HFD groups, and finally identified 50 genus-level OTUs as key variables. The relative abundance of each sample was calculated, and the heat map was protracted to show the genus level clustering (**Figure 5**). At the family level, the most abundant genus-level taxa were: *Bacteroidaceae* (12 OTUs), *Prevotellaceae* (5 OTUs), *Enterobacteriaceae* (5 OTUs), S24-7 (5 OTUs), *Paraprevotellaceae* (4 OTUs), *Ruminococcaceae* (4 OTUs), *Lachnospiraceae* (3 OTUs), *Lachnospiraceae* (2 OTUs), *Verrucomicrobiaceae* (2 OTUs), and *Desulfovibrionaceae* (2 OTUs). At the genus level, 11 OTUs were enriched in the gut microbiota of HFD rats, which were related to glusoce metabolism - Oscillospira (2 OTUs), lipids metabolism - *Bacteroides* (7 OTUs) and *Akkermansia* (1 OTU), and inflammation - *Prevotella* (1 OTUs) compared with NCD rats. 21 OTUs were eliminated or decreased in the gut microbiota of HFD rats, mainly related to glucose metabolism - *Oscillospira* (1 OTU), *Blautia* (1 OTU), *Roseburia* (1 OTU) and *Phascolarctobacterium* (1 OTU), lipids metabolism - *Bacteroides* (5 OTUs), *Clostridium* (1 OTU), and inflammation - *Prevotella* (7 OTUs) compared with NCD rats. These alterations indicated that HFD disturbed the gut microbiota and could result in the impaired glucose and lipids metabolism, and even inflammation.

Discussion

In the present study, we observed that 8-week HFD feeding could induce NAFLD in rats, and our GC/MS analysis combined with 16S rDNA sequencing method identified the altered metabolites and microbiota, implying that the changes of serum, hepatic, urinary and fecal metabolome, as well as the gut-microbiotal composition were associated with the development of NAFLD.

Among the alternations of dietary fat and glucose metabolism, energy metabolism was disturbed prominently after high fat intake. The liver is an essential metabolic organ for glucose homeostasis. In healthy subjects, glucose is metabolized into pyruvate through glycolysis in the cytoplasm, and pyruvate is completely oxidized to generate adenosine triphosphate (ATP) through the TCA cycle and oxidative phosphorylation in the mitochondria [16]. HFD-induced NAFLD rats demonstrated an abnormal energy homeostasis by depleting TCA cycle related metabolites (pyruvate and citrate) and elevating pentose phosphate pathway related metabolites (gluconic acid and glyceric acid) in se-

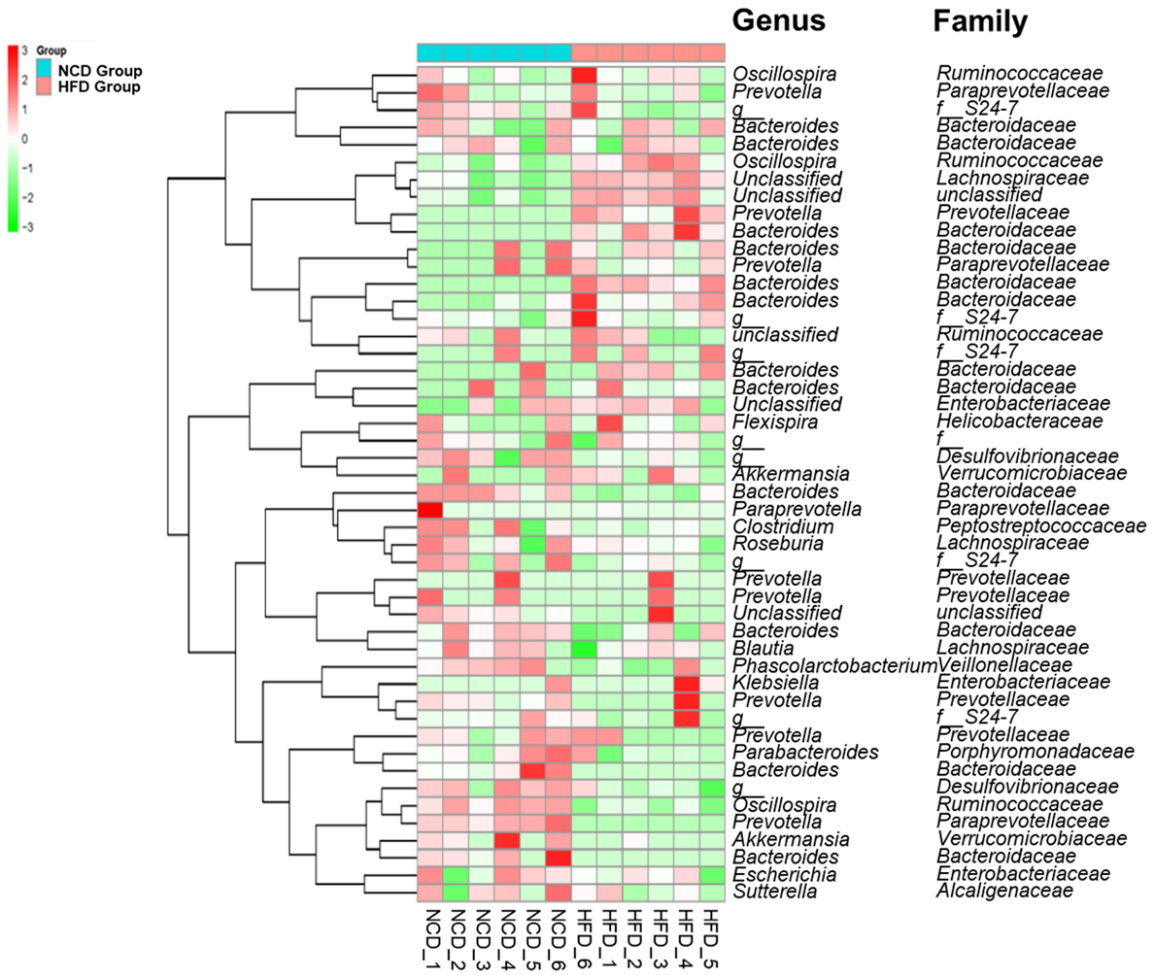

Figure 5. The structure of micorbiota in the feces. Heat map of key OTUs indicating genus-level changes between groups. The relative abundance of each genus was indicated by a gradient of color from green (low abundance) to red (high abundance). Complete linkage clustering of samples was based on the genus composition and abundance.

rum. Interestingly, the levels of all metabolites involved in TCA cycle remained high in urine, including pyruvic acid, citric acid and succinic acid. A likely explanation could be that rats under HFD have a delay in glucose clearance, possibly due to peripheral insulin resistance that results from higher levels of circulating free fatty acids.

Gut microbiota and its co-metabolites also presented to have an influence on the regulation of energy homeostasis. Recent evidences have demonstrated the correlation between short-chain fatty acids (SCFAs)-producing bacteria and metabolic diseases. SCFAs-producing bacteria are able to utilize host's non-absorbed carbohydrates such as glycans and fibers, and then secrete SCFAs such as acetate, propionate, butyrate, isobutyrate [17, 18]. Their health-promoting functions mainly involved in producing nutrients for the host and providing energy for the colonic epithelium [19], thus making decreased SCFAs a vital causative factor for NAFLD [20]. Our study showed significant decreases in the SCFAs-producing genus of *Phascolarctobacterium*, *Roseburia*, *Blautia* and *Oscillospira* in HFD-induced NAFLD rats, indicating that HFD could suppress host energy metabolism by affecting the structure of SCFAs-producing bacteria in the intestine.

Another important observation of this study was the metabolites related to fatty acids and sterols. Besides histological and biochemical evidences of lipid accumulation in liver, dietary fat also has a marked impact on the composi-

tion of polyunsaturated fatty acid (PUFA) (mainly arachidonic acid) and monounsaturated fatty acid (MUFA) (mainly oleic acid) in serum and liver. The depletion of PUFA probably implied a reduction in fatty acid oxidation and excessive triglyceride synthesis in hepatocytes. In particular, the increased utilization of arachidonic acid (AA), which is the precursor of pro-inflammatory metabolites such as hydroxyeicosatetraenoic acids (HETEs), could accelerate the progression of hepatotoxicity [21]. The increased level in MUFA was associated with the severity of the hepatic lesion [22]. Our data was in accordance with these studies [23], indicating dietary fat in suppressing fatty acid oxidation, promoting triglyceride synthesis and hepatotoxicity.

Sterol lipids, including cholesterol and other plant sterols (e.g., campesterol), originate either from endogenous synthesis or from diet. Several studies have indicated that plant sterols could be routinely used as markers of cholesterol absorption [24, 25], and therefore the increased cholesterol and decreased campesterol levels in serum or liver probably demonstrated an enhancement of cholesterol absorption and synthesis. In addition, taurine has many important biological roles such as conjugation of cholesterol and bile acids and antioxidation [26]. It has been reported that taurine as a metabolite could increase fatty acid oxidation [27]. In line with this, the decreased level of taurine also implied the suppression of fatty acid oxidation in NAFLD.

Bile acids are steroid acids that are synthesized in hepatocytes from cholesterol and secreted into the intestinal tract, which are then mainly translated into different forms by anaerobic bacteria of *Bacteroides* and *Clostridium* and play a major role in promoting the metabolism of dietary fat and the absorption of cholesterol [28]. The decrease of *Bacteroides* and *Clostridium* attenuated cholesterol absorption in NAFLD rats. In addition, we also observed reductions of *Akkermansia* in model rats, whose amount correlate negatively with the total body fat content, and may contribute to fat accumulation [29].

An imbalance of amino acid metabolism associated with the long-term dietary fat intervention contained branched chain amino acids (BCAAs), aromatic amino acids (AAAs), lysine and serine. Of note, BCAAs (valine, leucine and isoleucine) metabolism is associated with insulin resistance and abnormal glucose tolerance [30]. As degradation products of BCAAs, the depleted levels of 2-ketoisovaleric acid and 3-methyl-2-ketovaleric acid in serum and increased 2-ketoisocaproic acid in urine indicated a significant hepatic BCAAs accumulation, which may contribute to hepatic mitochondrial dysfunction in NAFLD [31]. AAA (e.g., phenylalanine and tyrosine) exhibited a strong connection with hepatic lipid metabolism and inflammation [32]. 3-hydroxyphenylacetic acid and indole-3-acetic acid, the major products of phenylalanine and tryptophan, could be effectively metabolized by the genus of *Bacteroides* and *Clostridium* [33]. In line with this, the decrease of the above metabolites in urine and bacteria in feces showed increased sensitivity to hepatic steatosis. Because the liver is a critical organ for amino acid homeostasis, the imbalance could be a consequence of abnormal liver function.

In addition, inflammation related metabolites and bacterium have been observed in our study. Recent investigation has shown that certain naturally occurring purines can exert anti-inflammatory properties [34]. Nucleoside adenosine is one of the best-characterized purines and has been thought to influence nearly all aspects of an immune response [35]. Among them, inosine can play a key role in a variety of inflammatory diseases by inhibiting the release of free radicals and pro-inflammatory cytokines from immune cells [36]. However, the anti-inflammatory effect of inosine was partly abolished under dietary fat intervention. Moreover, the intestinal bacterium *Prevotella* was reported to thrive in a pro-inflammatory environment and might even increase inflammation [37]. *Parabacteroides*, a candidate anti-inflammatory bacterium [38], was observed decreased in HFD group, which might be related to low grade inflammation presents in metabolic diseases including NAFLD.

In conclusion, applying metabolomics technology combined with 16S rDNA sequencing approach as integrative assessment tools, we identified the metabolomics profiles and alteration of gut microbiota in NAFLD development, and found that dietary fat exposure were inferred to cause metabolism dysfunction in

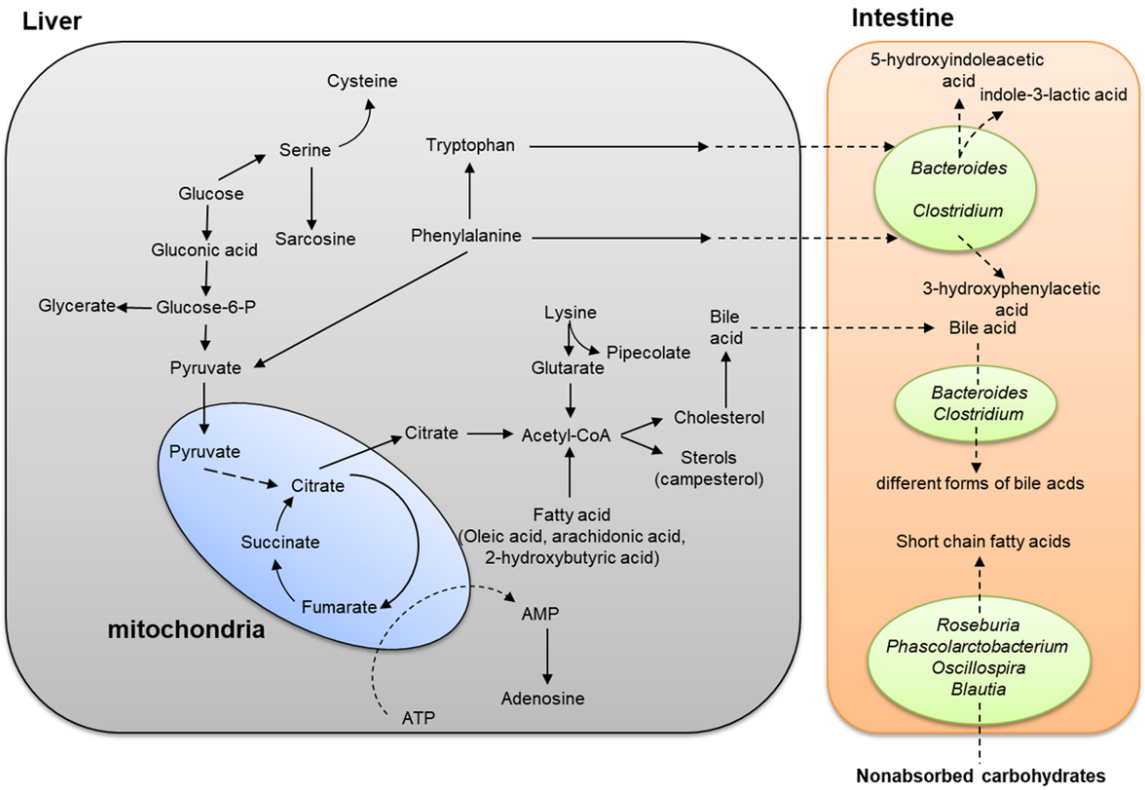

Figure 6. Disturbed metabolic pathways in HFD-induced NAFLD rats. Dietary fat exposure were inferred to cause metabolism dysfunction in different types of sample and disturbances in the intestinal microbiota due to the abnormal energy metabolism and amino acid homeostasis, suppressed fatty acids oxidation, enhanced triglyceride synthesis, hepatic mitochondrial dysfunction, insulin resistance and inflammation in NAFLD.

NAFLD (**Figure 6**). This provided insight into the opportunities involved in identifying characteristic profiling of dietary intervention, which may contribute to a better understanding in NAFLD pathogenesis. However, further studies are still needed to get novel targets for therapy and prevention of NAFLD.

Acknowledgements

We thank Dr. Xiangbing Shu and Hanchen Xu for the help in animal feeding, and our appreciation also extends to Dr. Haiyan Song for her supporting in ordering experimental reagents and materials. This study was supported by the National Nature Science Foundation of China (grant No. 81620108030, 81774084), Shanghai Rising-Star Program (grant No. 17QA140-4000).

Disclosure of conflict of interest

None.

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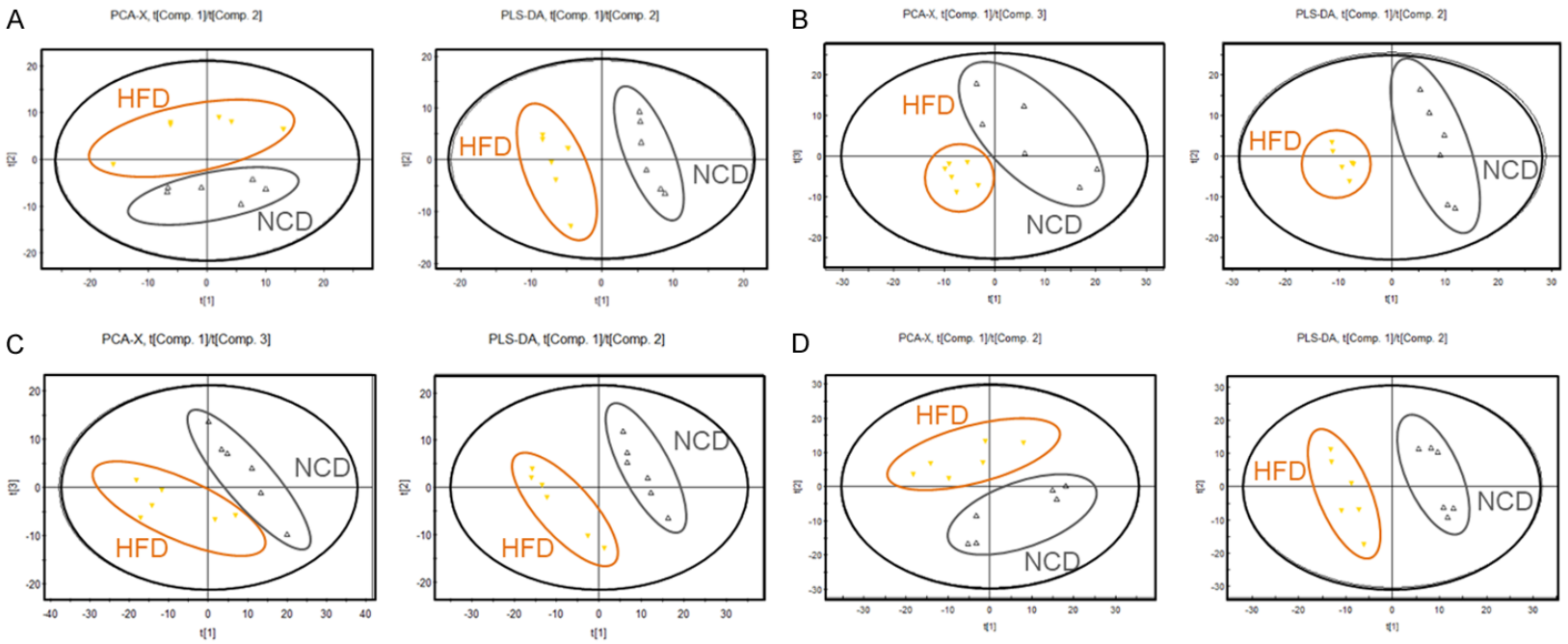

Figure S1. PCA and PLS-DA score plots of rats. The samples were analyzed by GC/MS method, and the PCA and PLS-DA score plots of (A) serum, (B) liver, (C) urine, and (D) feces were demonstrated.

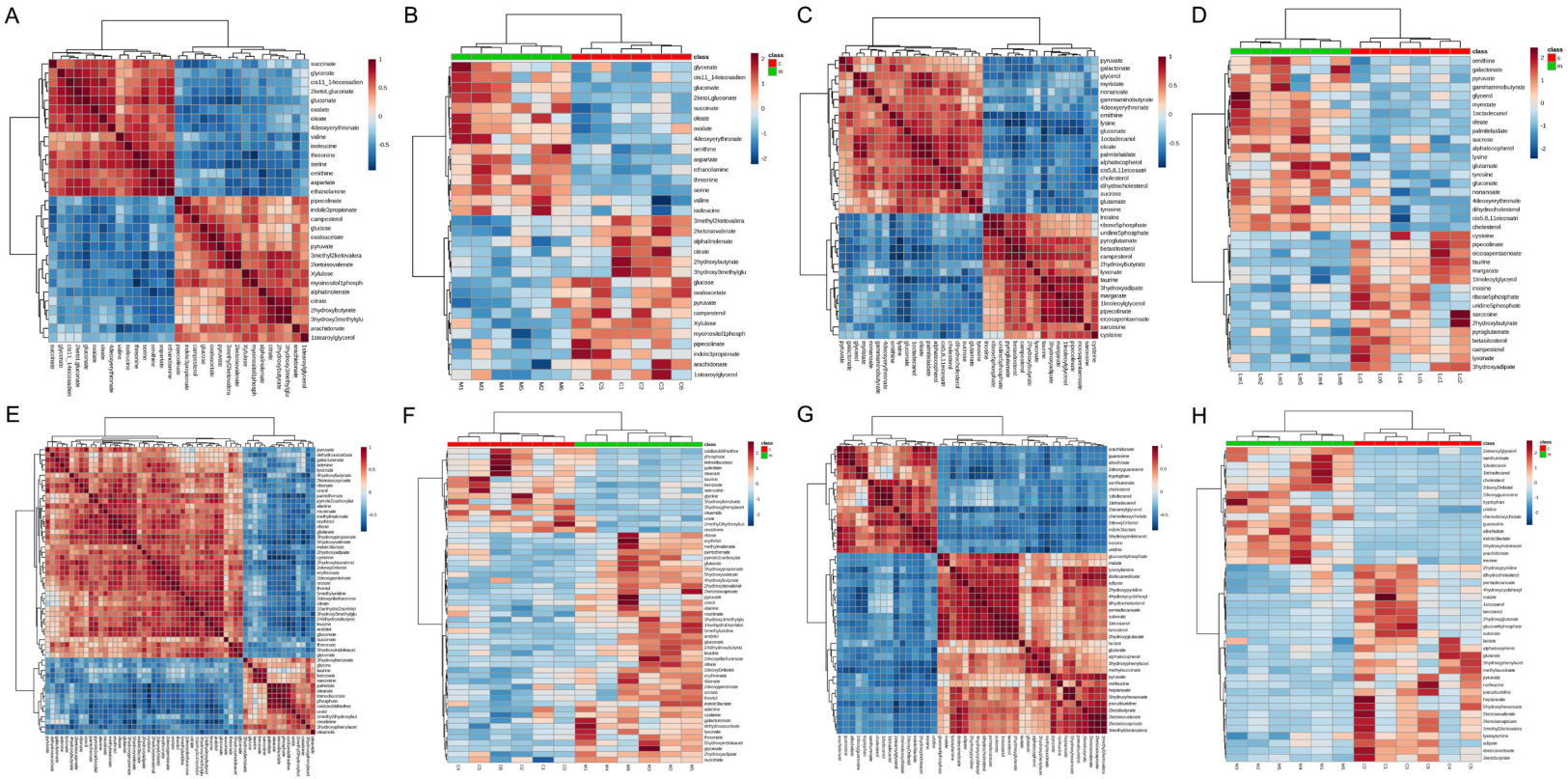

Figure S2. Pearson's correlations and clustering of metabolites. A. Pearson's correlations of the 31 metabolites determined from serum samples; B. Heat map showing the fold change (FC) of 31 metabolites in serum; C. Pearson's correlations of the 36 metabolites determined from liver samples; D. Heat map showing the FC of 36 metabolites in liver; E. Pearson's correlations of the 56 metabolites determined from urine samples; F. Heat map showing the FC of 56 metabolites in urine; G. Pearson's correlations of the 43 metabolites determined from urine samples; H. Heat map showing the FC of 43 metabolites in urine. Shades of blue represent FC decrease while red represent FC increase.

Table S1. Endogenous metabolites of the serum from NCD group and HFD group

Metabolites	Formula	Mol Weight	VIP	*p*-value[a]	FC	HMDB	KEGG	Pathway (KEGG)
Oxaloacetic acid	$C_4H_4O_5$	132.0716	1.69	8.90E-03	1.28	HMDB00223	C00036	Glycolysis/Gluconeogenesis; Citrate cycle (TCA cycle)
Glucose	$C_6H_{12}O_6$	180.1559	1.73	6.94E-03	0.19	HMDB00122	C00031	Glycolysis/Gluconeogenesis; Pentose phosphate pathway
Pyruvic acid	$C_3H_4O_3$	88.0621	1.58	1.77E-02	0.51	HMDB00243	C00022	Glycolysis/Gluconeogenesis; Citrate cycle (TCA cycle); Pentose phosphate pathway
Citric acid	$C_6H_8O_7$	192.1235	1.57	2.06E-02	0.70	HMDB00094	C00158	Citrate cycle (TCA cycle); Alanine, aspartate and glutamate metabolism
Succinic acid	$C_4H_6O_4$	118.088	1.92	1.70E-03	-0.43	HMDB00254	C00042	Citrate cycle (TCA cycle); Alanine, aspartate and glutamate metabolism
Xylulose	$C_5H_{10}O_5$	150.1299	1.89	1.71E-03	0.19	HMDB01644	C00310	Pentose and glucuronate interconversions
Gluconic acid	$C_6H_{12}O_7$	196.1553	2.09	1.30E-04	-1.46	HMDB00625	C00257	Pentose phosphate pathway
Glyceric acid	C3H6O4	106.0773	1.55	2.02E-02	-0.58	HMDB00139	C00258	Pentose phosphate pathway; Glycine, serine and threonine metabolism; Glycerolipid metabolism
Threonine	$C_3H_7NO_2$	89.0932	2.16	2.74E-05	-0.58	HMDB00167	C00188	Glycine, serine and threonine metabolism
Serine	$C_2H_5NO_2$	75.0666	2.26	6.81E-07	-0.37	HMDB00187	C00065	Glycine, serine and threonine metabolism; Cysteine and methionine metabolism
Valine	$C_5H_{11}NO_2$	117.1463	1.61	1.45E-02	-0.13	HMDB00883	C00183	Valine, leucine and isoleucine degradation
3-methyl-2-ketovaleric acid			1.92	1.34E-03	0.58	HMDB00491	C03465	Valine, leucine and isoleucine degradation
2-ketoisovaleric acid	$C_5H_8O_3$	116.1152	1.79	4.71E-03	0.45	HMDB00019	C00141	Valine, leucine and isoleucine degradation
Isoleucine	$C_6H_{13}NO_2$	131.1729	1.69	8.67E-03	-0.22	HMDB00172	C00407	Valine, leucine and isoleucine degradation
Oleic acid	$C_{18}H_{34}O_2$	282.4614	1.81	3.61E-03	-0.84	HMDB00207	C00712	Long chain fatty acid
Arachidonic acid	$C_{20}H_{32}O_2$	304.4669	1.70	7.91E-03	0.34	HMDB01043	C00219	Long chain fatty acid
Cis-11,14-Eicosadienoic acid	$C_{20}H_{36}O_2$	308.4986	1.67	1.00E-02	-0.41	HMDB05060	C16525	Long chain fatty acid
Alpha-Linolenic acid	$C_{18}H_{30}O_2$	278.4296	1.66	1.05E-02	0.21	HMDB01388	C06427	Long chain fatty acid
2-hydroxybutyric acid	$C_4H_8O_3$	104.1045	1.70	8.62E-03	0.79	HMDB00008	C05984	Fatty acid, monohydroxy
1-stearoylglycerol	$C_{21}H_{42}O_4$	358.5558	1.41	4.04E-02	0.21	HMDB31075		Lipid
Aspartic acid	$C_4H_7NO_4$	133.1027	1.87	2.12E-03	-0.85	HMDB00191	C00049	Alanine, aspartate and glutamate metabolism; Glycine, serine and threonine metabolism; Cysteine and methionine metabolism
Ornithine	$C_5H_{12}N_2O_2$	132.161	1.83	2.99E-03	-0.96	HMDB00214	C00077	Arginine and proline metabolism
Pipecolinic acid	$C_6H_{11}NO_2$	129.157	1.55	2.13E-02	0.33	HMDB00070	C00408	Lysine degradation
Oxalic acid	$C_2H_2O_4$	90.0349	1.75	5.74E-03	-0.46	HMDB02329	C00209	Microbial metabolism in diverse environments; Chloroalkane and chloroalkene degradation; Glyoxylate and dicarboxylate metabolism
Ethanolamine	C_2H_7NO	61.0831	2.02	3.73E-04	-0.42	HMDB00149	C00189	Phosphonate and phosphinate metabolism; Glycerophospholipid metabolism
Myo-inositol-1-phosphate	$C_6H_{13}O_9P$	260.1358	2.02	3.72E-04	0.38	HMDB00213	C04006	Inositol phosphate metabolism
Campesterol	$C_{28}H_{48}O$	400.6801	1.92	1.56E-03	0.58	HMDB02869	C01789	A dietary phytosterol found in plants, lowering cholesterol
4-deoxyerythronic acid	$C_4H_8O_4$	120.1039	1.84	2.74E-03	-0.58	HMDB00498	-	-
2-Keto-l-gluconic acid	$C_6H_{10}O_7$	194.1394	1.80	3.98E-03	-0.43	HMDB11732	C15673	-
Indole-3-propionic acid	$C_{11}H_{11}NO_2$	189.2105	1.67	1.20E-02	1.98	HMDB02302	-	-
3-hydroxy-3-methylglutaric acid	$C_6H_{10}O_5$	162.1406	1.44	3.82E-02	0.56	HMDB00355	C03761	-

Note: [a]Comparison of differential metabolites between NCD group and HFD group with a Student's *t* test; FC: fold change. VIP was obtained from PLS-DA with a threshold of 1.0. The positive and negative values of FC indicate a respective increased or decreased concentration of each metabolite in the NCD group versus HFD group (NCD/HFD).

Table S2. Endogenous metabolites of the liver from NCD group and HFD group

Metabolites	Formula	Mol Weight	VIP	*p*-value	FC	HMDB	KEGG	Pathway (KEGG)
Pyruvic acid	$C_3H_4O_3$	88.0621	1.26	4.01E-02	-0.47	HMDB00243	C00022	Glycolysis/Gluconeogenesis; Citrate cycle (TCA cycle); Pentose phosphate pathway
Gluconic acid	$C_6H_{12}O_7$	196.1553	1.73	8.53E-04	-0.65	HMDB00625	C00257	Pentose phosphate pathway
Ribose-5-phosphate	$C_5H_{11}O_8P$	230.1098	1.36	2.28E-02	0.88	HMDB01548	C00117	Pentose phosphate pathway
Gama-aminobutyric acid	$C_4H_9NO_2$	103.1198	1.35	2.23E-02	-0.62	HMDB00112	C00334	Alanine, aspartate and glutamate metabolism; Arginine and proline metabolism; beta-Alanine metabolism
Glutamic acid	$C_5H_9NO_4$	147.1293	1.53	6.75E-03	-2.27	HMDB00148	C00025	Alanine, aspartate and glutamate metabolism; Arginine and proline metabolism; Taurine and hypotaurine metabolism
Ornithine	$C_5H_{12}N_2O_2$	132.161	1.67	1.74E-03	-0.50	HMDB00214	C00077	Arginine and proline metabolism
Galactonic acid	$C_6H_{12}O_7$	196.1553	1.34	2.43E-02	-0.73	HMDB00565	C00880	Galactose metabolism
Glycerol	$C_3H_8O_3$	92.0938	1.40	1.71E-02	-0.64	HMDB00131	C00116	Galactose metabolism; Glycerolipid metabolism
Pyroglutamic acid	$C_5H_7NO_3$	129.114	1.74	7.26E-04	0.42	HMDB00267	C01879	Glutathione metabolism
Sarcosine	$C_3H_7NO_2$	89.0932	1.49	9.00E-03	0.95	HMDB00271	C00213	Glycine, serine and threonine metabolism
Cysteine	$C_3H_7NO_2S$	121.1582	1.37	2.34E-02	0.48	HMDB00574	C00097	Glycine, serine and threonine metabolism; Cysteine and methionine metabolism; Taurine and hypotaurine metabolism
Pipecolinic acid	$C_6H_{11}NO_2$	129.157	1.59	4.19E-03	1.21	HMDB00070	C00408	Lysine degradation
Lysine	$C_6H_{14}N_2O_2$	146.1876	1.83	1.89E-04	-0.62	HMDB00182	C00047	Lysine degradation; Lysine biosynthesis; Biotin metabolism
Nonanoic acid	$C_9H_{18}O_2$	158.238	1.32	2.76E-02	-0.30	HMDB00847	C01601	Fatty acid
2-hydroxybutyric acid	$C_4H_8O_3$	104.1045	1.31	2.99E-02	0.64	HMDB00008	C05984	Fatty acid, monohydroxy
Cis-5,8,11-eicosatrienoic acid	$C_{20}H_{32}O_2$	304.4669	1.55	6.63E-03	-0.48	HMDB10378	C00219	Long chain fatty acid
Eicosapentaenoic acid	$C_{20}H_{30}O_2$	302.451	1.72	1.09E-03	1.12	HMDB01999	C06428	Long chain fatty acid
Margaric acid	$C_{18}H_{30}O_2$	278.4296	1.70	1.31E-03	0.54	HMDB02259	C06427	Long chain fatty acid
Myristic acid	$C_{14}H_{28}O_2$	228.3709	1.36	2.19E-02	-0.82	HMDB00806	C06424	Long chain fatty acid
Oleic acid	$C_{18}H_{34}O_2$	282.4614	1.79	3.25E-04	-1.74	HMDB00207	C00712	Long chain fatty acid
1-linoleoylglycerol			1.59	3.93E-03	0.88	-	-	Lipid
Inosine	$C_{10}H_{12}N_4O_5$	268.2261	1.28	3.53E-02	0.35	HMDB00195	C00294	Purine metabolism
Uridine-5'-phosphate	$C_9H_{13}N_2O_9P$	324.1813	1.43	1.53E-02	1.45	HMDB00288	C00105	Pyrimidine metabolism
Sucrose	$C_{12}H_{22}O_{11}$	342.2965	1.53	6.98E-03	-0.78	HMDB00258	C00089	Starch and sucrose metabolism; Galactose metabolism
Beta-sitosterol	$C_{29}H_{50}O$	414.7067	1.88	5.97E-05	1.20	HMDB00852	C01753	Steroid biosynthesis
Cholesterol	$C_{27}H_{46}O$	386.6535	1.79	4.24E-04	-0.54	HMDB00067	C00187	Steroid biosynthesis; Steroid degradation
Taurine	$C_2H_7NO_3S$	125.1469	1.93	1.78E-05	4.54	HMDB00251	C00245	Taurine and hypotaurine metabolism
Tyrosine	$C_9H_{11}NO_3$	181.1885	1.66	2.23E-03	-0.74	HMDB00158	C00082	Tyrosine metabolism; Phenylalanine metabolism
Alpha-tocopherol	$C_{29}H_{50}O_2$	430.7061	1.45	1.28E-02	-0.57	HMDB01893	C02477	Vitamin digestion and absorption; antioxidants
Campesterol	$C_{28}H_{48}O$	400.6801	1.94	9.70E-06	1.00	HMDB02869	C01789	A dietary phytosterol found in plants, lowering cholesterol
Lyxonic acid			1.41	1.61E-02	0.23	-	-	A sugar acid
1-octadecanol	$C_{18}H_{38}O$	270.4937	1.94	1.17E-05	-0.54	HMDB02350	-	-
3-hydroxyadipic acid	$C_6H_{10}O_5$	162.1406	1.63	2.80E-03	1.45	HMDB00345	-	The oxidation of 3-hydroxy fatty acid
4-deoxyerythronic acid	$C_4H_8O_4$	120.1039	1.30	3.22E-02	-0.40	HMDB00498	-	-
Dihydrocholesterol	$C_{27}H_{48}O$	388.6694	1.62	3.20E-03	-0.42	HMDB00908	-	-
Palmitelaidic acid	$C_{16}H_{30}O_2$	254.4082	1.73	7.85E-04	-1.49	HMDB12328	-	Long chain fatty acid

Note: [a]Comparison of differential metabolites between NCD group and HFD group with a Student's *t* test; FC: fold change. VIP was obtained from PLS-DA with a threshold of 1.0. The positive and negative values of FC indicate a respective increased or decreased concentration of each metabolite in the NCD group versus HFD group (NCD/HFD).

Table S3. Endogenous metabolites of the urine from NCD group and HFD group

Metabolites	Formula	Mol Weight	VIP	*p*-value	FC	HMDB	KEGG	Pathway (KEGG)
Pyruvic acid	$C_3H_4O_3$	88.0621	1.24	4.46E-02	-2.06	HMDB00243	C00022	Glycolysis
Gluconic acid	$C_6H_{12}O_7$	196.1553	1.30	1.10E-02	-3.77	HMDB00625	C00257	Pentose phosphate pathway
Ribose	$C_5H_{10}O_5$	150.1299	1.23	1.56E-02	-0.68	HMDB00283	C00121	Pentose phosphate pathway
Glyceric acid	$C_3H_6O_4$	106.0773	1.09	3.88E-02	-1.20	HMDB00139	C00258	Pentose phosphate pathway; Glycine, serine and threonine metabolism
Citric acid	$C_6H_8O_7$	192.1235	1.13	3.70E-02	-3.45	HMDB00094	C00158	Citrate cycle (TCA cycle)
Succinic acid	$C_4H_6O_4$	118.088	1.29	3.45E-02	-0.76	HMDB00254	C00042	Citrate cycle (TCA cycle); Phenylalanine metabolism
Creatinine	$C_4H_7N_3O$	113.1179	1.16	2.59E-02	0.83	HMDB00562	C00791	Arginine and proline metabolism
Pyrrole-2-carboxylic acid	$C_5H_5NO_2$	111.0987	1.07	4.78E-02	-0.47	HMDB04230	C05942	Arginine and proline metabolism
Dehydroascorbic acid	-	-	1.72	1.11E-02	-0.68	HMDB01264	C00425	Ascorbate and aldarate metabolism
Threonic acid	$C_4H_8O_5$	136.1033	1.35	1.64E-02	-0.80	HMDB00943	C01620	Ascorbate and aldarate metabolism
3-hydroxybenzoic acid	$C_7H_6O_3$	138.1207	1.36	1.45E-02	2.07	HMDB02466	C00587	Benzoate degradation
Pantothenic acid	$C_9H_{17}NO_5$	219.235	1.14	4.07E-02	-0.78	HMDB00210	C00864	Beta-Alanine metabolism; Pantothenate and CoA biosynthesis
Uracil	$C_4H_4N_2O_2$	112.0868	1.10	4.16E-02	-0.74	HMDB00300	C00106	Beta-Alanine metabolism; Pyrimidine metabolism
3-hydroxypropionic acid	$C_3H_6O_3$	90.0779	1.60	2.32E-04	-2.37	HMDB00700	C01013	Beta-Alanine metabolism; Propanoate metabolism
4-hydroxybutyric acid	$C_4H_8O_3$	104.1045	1.38	7.12E-03	-1.98	HMDB00710	C00989	Butanoate metabolism
Alanine	$C_3H_7NO_2$	89.0932	1.39	6.54E-03	-1.33	METPA0179	C01401	Alanine, aspartate and glutamate metabolism; Cyanoamino acid metabolism
Cysteine	-	-	1.37	4.82E-03	-0.69	METPA0075	C00736	Cysteine and methionine metabolism
Sarcosine	$C_3H_7NO_2$	89.0932	1.55	6.32E-03	1.48	HMDB00271	C00213	Glycine, serine and threonine metabolism
Glycine	$C_2H_5NO_2$	75.0666	1.33	2.68E-02	0.99	HMDB00123	C00037	Glycine, serine and threonine metabolism; Cyanoamino acid metabolism
Palmitic acid	$C_{16}H_{32}O2$	256.4241	1.22	1.69E-02	1.40	HMDB00220	C00249	Long chain fatty acid
Stearic acid	$C_{18}H_{36}O2$	284.4772	1.31	8.59E-03	1.34	HMDB00827	C01530	Long chain fatty acid
Glutaric acid	$C_5H_8O_4$	132.1146	1.33	6.97E-03	-1.35	HMDB00661	C00489	Lysine degradation
Nicotinic acid	$C_6H_5NO_2$	123.1094	1.14	2.96E-02	-0.54	HMDB01488	C00253	Nicotinate and nicotinamide metabolism
Arabitol	$C_5H_{12}O_5$	152.1458	1.31	1.12E-02	-1.95	HMDB00568	C01904	Pentose and glucuronate interconversions
Benzoic acid	$C_7H_6O_2$	122.1213	1.69	3.73E-03	1.45	HMDB01870	C00180	Phenylalanine metabolism
3-hydroxyphenylacetic acid	$C_8H_8O_3$	152.1473	1.83	4.57E-05	1.86	HMDB00440	C05593	Phenylalanine metabolism
Indole-3-lactic acid	$C_{11}H_{11}NO_3$	205.2099	1.22	2.01E-02	-0.59	HMDB00671	C02043	Tryptophan metabolism
5-hydroxyindoleacetic acid	$C_{10}H_9NO_3$	191.1834	1.52	2.46E-03	-1.72	HMDB00763	C05635	Tryptophan metabolism
Leucine	$C_6H_{13}NO_2$	131.1729	1.39	3.75E-03	-1.04	HMDB00687	C00123	Valine, leucine and isoleucine degradation
2-ketoisocaproic acid	$C_6H_{10}O_3$	130.1418	1.30	1.04E-02	-1.68	HMDB00695	C00233	Valine, leucine and isoleucine degradation
Methylmalonic acid	$C_4H_6O_4$	118.088	1.12	4.23E-02	-0.84	HMDB00202	C02170	Propanoate metabolism; Valine, leucine and isoleucine degradation
Adenine	$C_5H_5N_5$	135.1267	1.30	1.57E-02	-0.48	HMDB00034	C00147	Purine metabolism
Uric acid	$C_5H_4N_4O3$	168.1103	1.25	1.40E-02	0.83	HMDB00289	C00366	Purine metabolism
Orotic acid	$C_5H_4N_2O_4$	156.0963	1.15	4.26E-02	-0.43	HMDB00226	C00295	Pyrimidine metabolism
2-hydroxyisovaleric acid	$C_5H_{10}O_3$	118.1311	1.58	3.29E-04	-1.61	HMDB00407	-	Synthesis and degradation of ketone bodies Valine, leucine and isoleucine degradation
Taurine	$C_2H_7NO_3S$	125.1469	1.48	3.67E-02	1.19	HMDB00251	C00245	Taurine and hypotaurine metabolism; Tyrosine metabolism
Phosphate	H_3PO_4	97.9952	1.12	4.66E-02	1.16	HMDB01429	C00009	-
Erythritol	$C_4H_{10}O_4$	122.1198	1.24	1.50E-02	-0.82	HMDB02994	C00503	-
Oxidized dithiothreitol	$C_4H_8O_2S_2$	152.235	1.25	1.66E-02	1.07	HMDB59664	C01119	-

Ribonic acid	$C_5H_{10}O_6$	166.1293	1.30	1.10E-02	-0.45	HMDB00867	C01685	-
2-hydroxyadipic acid	$C_6H_{10}O_5$	162.1406	1.51	1.78E-03	-0.63	HMDB00321	C02360	-
3-hydroxy-3-methylglutaric acid	$C_6H_{10}O_5$	162.1406	1.34	6.34E-03	-0.96	HMDB00355	C03761	-
1,5-anhydro-D-sorbitol	$C_6H_{12}O_5$	164.1565	1.44	2.00E-03	-2.05	HMDB02712	C07326	-
Galacturonic acid	$C_6H_{10}O_7$	194.1394	1.38	2.58E-02	-0.60	HMDB02545	C08348	-
Threitol	$C_4H_{10}O_4$	122.1198	1.17	4.38E-02	-0.31	HMDB04136	C16884	-
2-deoxy-D-ribotol	$C_8H_{16}O$	128.212	1.54	6.25E-04	-0.80	HMDB31295	C17145	-
Oleamide	$C_{18}H_{35}NO$	281.4766	1.47	3.56E-03	5.38	HMDB02117	C19670	-
Iminodiacetic acid	$C_4H_7NO_4$	133.1027	1.08	4.68E-02	0.76	HMDB11753	C19911	-

Note: [a]Comparison of differential metabolites between NCD group and HFD group with a Student's *t* test; FC: fold change. VIP was obtained from PLS-DA with a threshold of 1.0. The positive and negative values of FC indicate a respective increased or decreased concentration of each metabolite in the NCD group versus HFD group (NCD/HFD).

Table S4. Endogenous metabolites of the feces from NCD group and HFD group

Metabolites	Formula	Mol Weight	VIP	*p*-value	FC	HMDB	KEGG	Pathway (KEGG)
Glucose-6-phosphate	$C_6H_{13}O_9P$	260.1358	1.26	3.23E-02	4.00	HMDB01401	C00092	Glycolysis/Gluconeogenesis
Pyruvic acid	$C_3H_4O_3$	88.0621	1.39	1.41E-02	1.21	HMDB00243	C00022	Glycolysis/Gluconeogenesis; Citrate cycle (TCA cycle); Pentose phosphate pathway
Malic acid	$C_4H_6O_5$	134.0874	1.21	4.02E-02	2.44	HMDB00156	C00149	Citrate cycle (TCA cycle)
Dodecanedioic acid	$C_{12}H_{22}O_4$	230.3007	1.42	1.15E-02	1.67	HMDB00623	C02678	Fatty acid
Suberic acid	$C_8H_{14}O_4$	174.1944	1.36	1.79E-02	1.33	HMDB00893	C08278	Fatty acid
Heptanoic acid	$C_7H_{14}O_2$	130.1849	1.20	4.29E-02	2.96	HMDB00666	C17714	Fatty acid
Glutaric acid	$C_5H_8O_4$	132.1146	1.40	1.31E-02	1.69	HMDB00661	C00489	Fatty acid degradation; Lysine degradation
Norleucine	$C_6H_{13}NO_2$	131.1729	1.40	1.70E-02	2.16	HMDB01645	C01933	Fatty acid,monoamino
5-hydroxyhexanoic acid	$C_6H_{12}O_3$	132.1577	1.29	2.68E-02	2.01	HMDB00525	-	Fatty acid, monohydroxy
1-dodecanol	$C_{12}H_{26}O$	186.3342	1.50	7.21E-03	-4.79	HMDB11626	C02277	Fatty alcohol
1-eicosanol	$C_{20}H_{42}O$	298.5469	1.35	1.89E-02	1.75	HMDB11619	-	Fatty alcohol
2-stearoylglycerol	$C_{21}H_{42}O_4$	358.5558	1.79	1.68E-04	-1.45	HMDB31075	-	Lipid
Arachidonic acid	$C_{20}H_{32}O_2$	304.4669	1.45	8.59E-03	-1.30	HMDB01043	C00219	Long chain fatty acid
Pentadecanoic acid	$C_{15}H_{30}O_2$	242.3975	1.22	4.17E-02	1.01	HMDB00826	C16537	Long chain fatty acid
1-tetradecanol	$C_{14}H_{30}O$	214.3874	1.41	1.50E-02	-3.71	HMDB11638	-	Long chain fatty acid
Inosine	$C_{10}H_{12}N_4O_5$	268.2261	1.57	3.00E-03	-1.28	HMDB00195	C00294	Purine metabolism
2'-deoxyguanosine	$C_{10}H_{13}N_5O4$	267.2413	1.23	3.69E-02	-1.87	HMDB00085	C00330	Purine metabolism
Guanosine	$C_{10}H_{13}N_5O_5$	283.2407	1.45	8.96E-03	-1.84	HMDB00133	C00387	Purine metabolism
Uridine	$C_9H_{12}N_2O_6$	244.2014	1.73	3.96E-04	-1.55	HMDB00296	C00299	Pyrimidine metabolism
Lactic acid	$C_3H_6O_3$	90.0779	1.31	2.48E-02	0.76	HMDB00190	C00186	Pyruvate metabolism
Lanosterol	$C_{30}H_{50}O$	426.7174	1.37	1.71E-02	1.67	HMDB01251	C01724	Steroid biosynthesis
Cholesterol	$C_{27}H_{46}O$	386.6535	1.57	3.41E-03	-2.15	HMDB00067	C00187	Steroid biosynthesis; Steroid degradation
Indole-3-lactic acid	$C_{11}H_{11}NO_3$	205.2099	1.65	1.26E-03	-2.95	HMDB00671	C02043	Tryptophan metabolism
Xanthurenic acid	$C_{10}H_7NO_4$	205.1669	1.46	9.12E-03	-3.84	HMDB00881	C02470	Tryptophan metabolism
5-hydroxyindoleacetic acid	$C_{10}H_9NO_3$	191.1834	1.87	1.59E-05	-4.10	HMDB00763	C05635	Tryptophan metabolism
Tryptophan	$C_{11}H_{12}N_2O_2$	204.2252	1.29	2.63E-02	-3.50	HMDB00929	C00078	Tryptophan metabolism; Glycine, serine and threonine metabolism

3-hydroxyphenylacetic acid	$C_8H_8O_3$	152.1473	1.86	3.97E-05	4.91	HMDB00440	C05593	Tyrosine metabolism; Phenylalanine metabolism
2-ketoisocaproic acid	$C_6H_{10}O_3$	130.1418	1.31	2.22E-02	1.62	HMDB00695	C00233	Valine, leucine and isoleucine degradation
3-methyl-2-ketovaleric acid			1.24	3.30E-02	1.63	HMDB00491	C03465	Valine, leucine and isoleucine degradation
2-ketoisovaleric acid	$C_5H_8O_3$	116.1152	1.22	3.78E-02	1.59	HMDB00019	C00141	Valine, leucine and isoleucine degradation
Alpha-tocopherol	$C_{29}H_{50}O_2$	430.7061	1.40	1.27E-02	0.82	HMDB01893	C02477	Vitamin digestion and absorption
Chenodeoxycholic acid	$C_{24}H_{40}O_4$	392.572	1.67	9.72E-04	-2.79	HMDB00518	C02528	Bile acid biosynthesis
Adipic acid	$C_6H_{10}O_4$	146.1412	1.22	4.29E-02	0.90	HMDB00448	C06104	Caprolactam degradation
2-hydroxyglutaric acid	$C_5H_8O_5$	148.114	1.24	3.72E-02	1.34	HMDB00694	C03196	-
Methylsuccinic acid	$C_5H_8O_4$	132.1146	1.66	1.18E-03	1.11	HMDB01844	C08645	-
2-deoxy-D-ribotol	$C_5H_{12}O_4$	136.1464	1.74	3.43E-04	-1.37	HMDB33919	-	-
2-hydroxypyridine			1.52	5.20E-03	0.57	-	-	-
2-ketobutyric acid	$C_4H_6O_3$	102.0886	1.47	7.78E-03	1.45	HMDB00005	-	-
4-Hydroxycyclohexylacetic acid	$C_8H_{14}O_3$	158.195	1.28	3.01E-02	0.82	HMDB00451	-	-
Allocholic acid	$C_{24}H_{40}O_5$	408.5714	1.50	6.10E-03	-5.73	HMDB00505	-	-
Dihydrocholesterol	$C_{27}H_{48}O$	388.6694	1.21	4.42E-02	0.95	HMDB00908	-	-
Lyxosylamine			1.25	3.44E-02	0.66	-	-	-
Pseudo uridine			1.63	1.74E-03	1.56	-	-	-

Note: [a]Comparison of differential metabolites between NCD group and HFD group with a Student's *t* test; FC: fold change. VIP was obtained from PLS-DA with a threshold of 1.0. The positive and negative values of FC indicate a respective increased or decreased concentration of each metabolite in the NCD group versus HFD group (NCD/HFD).

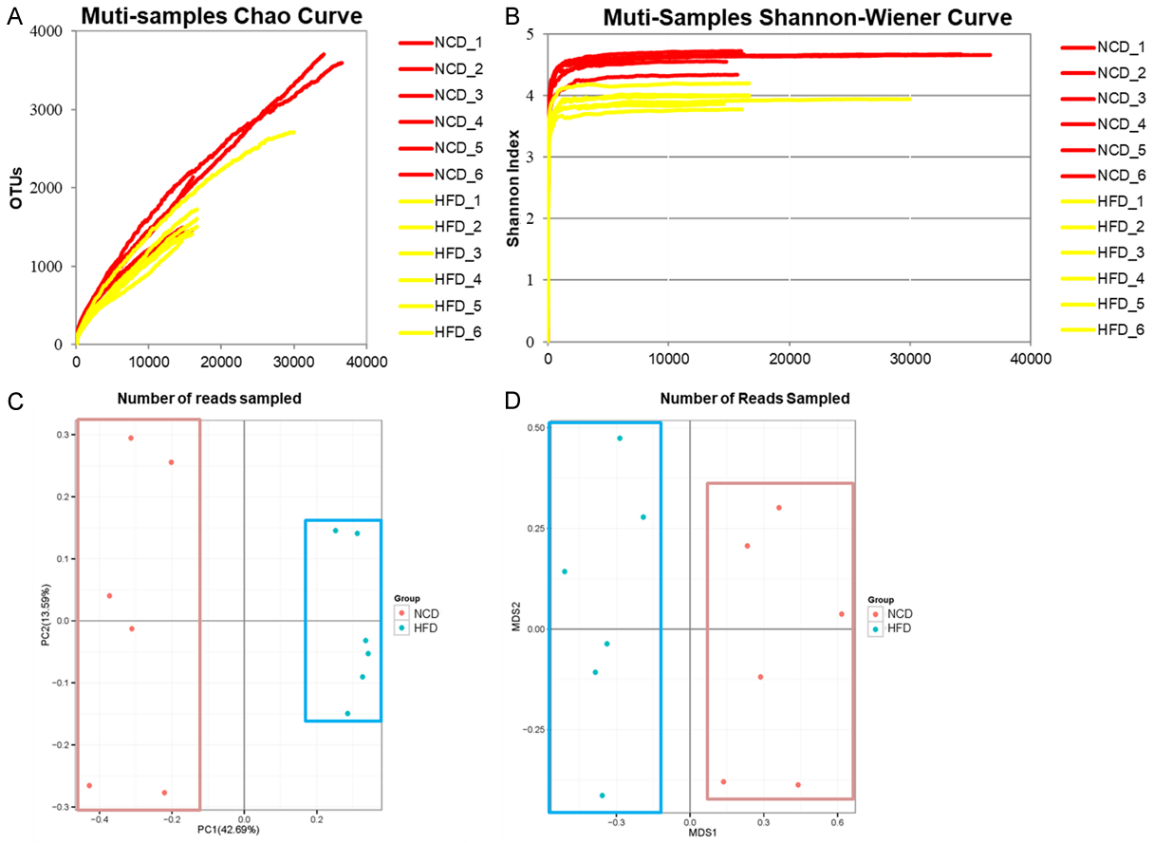

Figure S3. Evaluation of α-diversity and β-diversity in samples between NCD group and HFD group in the pyrosequencing run. A. Rarefaction curves of sequencing samples; B. Shannon diversity index curves of sequencing samples. C. PCoA plot of NCD group and HFD group; D. NMDS plot of NCD group and HFD group. Samples were grouped by different colors and the percent of variation was explained by each axis. The closer the distance of samples was, the more similar the sample composition.