

## Basic Study

# Effects of different diets on intestinal microbiota and nonalcoholic fatty liver disease development

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## Abstract

### AIM

To study the effects of different diets on intestinal microbiota and nonalcoholic fatty liver disease (NAFLD) development at the same caloric intake.

### METHODS

Thirty male Sprague-Dawley rats were randomized into five groups (six rats each). The control diet (CON) group and free high-fat diet (FFAT) group were allowed *ad libitum* access to a normal chow diet and a high-fat diet, respectively. The restrictive high-fat diet (RFAT) group, restrictive high-sugar diet (RSUG) group, and high-protein diet (PRO) group were fed a high-fat diet, a high-sugar diet, and a high-protein diet, respectively, in an isocaloric way. All rats were killed at 12 wk. Body weight, visceral fat index (visceral fat/body weight), liver index (liver/body weight), insulin resistance, portal lipopolysaccharide (LPS), serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), and liver triglycerides were measured. The intestinal microbiota in the different groups of rats was sequenced using high-throughput sequencing technology.

## RESULTS

The FFAT group had higher body weight, visceral fat index, liver index, peripheral insulin resistance, portal LPS, serum ALT, serum AST, and liver triglycerides compared with all other groups ( $P < 0.05$ ). Taking the same calories, the RFAT and RSUG groups demonstrated increased body weight, visceral fat index, peripheral insulin resistance and liver triglycerides compared with the PRO group ( $P < 0.05$ ). The RFAT group also showed increased portal LPS compared with the PRO group ( $P < 0.05$ ). Unweighted UniFrac principal coordinates analysis of the sequencing data revealed that the intestinal microbiota structures of the CON, FFAT, RSUG and PRO groups were roughly separated away from each other. Taxon-based analysis showed that, compared with the CON group, the FFAT group had an increased abundance of *Firmicutes*, *Roseburia* and *Oscillospira* bacteria, a higher ratio of *Firmicutes* to *Bacteroidetes*, and a decreased abundance of *Bacteroidetes*, *Bacteroides* and *Parabacteroides* bacteria ( $P < 0.05$ ). The RFAT group showed an increased abundance of *Firmicutes* and decreased abundance of *Parabacteroides* bacteria ( $P < 0.05$ ). The RSUG group showed an increased abundance of *Bacteroidetes* and *Sutterella* bacteria, higher ratio of *Bacteroidetes* to *Firmicutes*, and a decreased abundance of *Firmicutes* ( $P < 0.05$ ). The PRO group showed an increased abundance of *Bacteroidetes*, *Prevotella*, *Oscillospira* and *Sutterella* bacteria, and a decreased abundance of *Firmicutes* ( $P < 0.05$ ). Compared with the FFAT group, the RFAT group had an increased abundance of *Bacteroidetes*, higher ratio of *Bacteroidetes* to *Firmicutes*, and decreased abundance of *Firmicutes* and *Oscillospira* bacteria ( $P < 0.05$ ).

## CONCLUSION

Compared with the high-protein diet, the NAFLD-inducing effects of high-fat and high-sugar diets are independent from calories, and may be associated with changed intestinal microbiota.

**Key words:** Nonalcoholic fatty liver disease; High-fat diet; Restrictive high-fat diet; Restrictive high-sugar diet; High-protein diet; Intestinal microbiota

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**Core tip:** Diet plays an important role in development of nonalcoholic fatty liver disease (NAFLD), and can shape intestinal microbiota, which is closely linked to NAFLD. We studied the effects of high-fat, high-sugar and high-protein diets on intestinal microbiota and NAFLD development in an isocaloric way. NAFLD-inducing effects of high-fat and high-sugar diets, compared with high-protein diet, are independent from calories, and these diets can alter intestinal microbiota independently from calories. The effects of these diets on NAFLD development at the same caloric intake may be associated with changes in intestinal microbiota. These findings are meaningful for appropriate dietary

therapy for NAFLD.

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## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a chronic liver disease in which triglycerides accumulate within the hepatocytes of patients with minimal or no alcohol intake and without any other known cause. It comprises a wide spectrum of liver damage ranging from steatosis to nonalcoholic steatohepatitis (NASH), advanced fibrosis and cirrhosis<sup>[1,2]</sup>. With the increasing incidence of obesity and diabetes mellitus, NAFLD has been recognized as a burgeoning health burden which affects 10%-24% of the general population and 70% of obese patients<sup>[3,4]</sup>. Approximately 30%-40% of individuals with simple steatosis progress to NASH, and NASH can progress to cirrhosis, which is a major risk factor for hepatocellular carcinoma<sup>[5,6]</sup>. Studies also have reported that NAFLD is a strong independent risk factor for cardiovascular disease<sup>[7]</sup>.

Although many genetic and environmental factors contribute to the development of NAFLD, diet is an important environmental factor that can affect the development of NAFLD. High-fat diet is a widely-studied diet that can induce NAFLD<sup>[8]</sup> and is often used to induce animal models of the disease<sup>[9]</sup>. Recently, high-sugar diet, mainly as high-fructose diet, has been found to play an important role in the development of NAFLD<sup>[10-12]</sup>. However, unlike high-fat and high-sugar diets, high-protein diet can ameliorate NAFLD<sup>[13]</sup>. The results of these former studies suggest that different types of diets may have different effects on the development of NAFLD, and high-fat and high-sugar diets are NAFLD-inducing diets.

To understand the effects of diet on the development of NAFLD, the intestinal microbiota must be considered because it is the interface between diet and the liver. Germ-free mice are resistant to NAFLD induced by high-fat diet<sup>[14]</sup>. However, when the intestinal microbiota was introduced into germ-free mice, the mice showed a rapid increase in body fat content and liver triglycerides. When a high-fat diet induces NAFLD, it also causes dysbiosis of the intestinal microbiota<sup>[15]</sup>. Moreover, small bowel bacterial overgrowth is associated with NAFLD, and patients with NASH have a lower percentage of *Bacteroidetes*<sup>[16,17]</sup>. These studies show a close link between the intestinal microbiota and NAFLD. As diet can affect the development of NAFLD through

**Table 1** Percentage of energy and energy density of the four diets

	Normal	High protein	High sugar	High fat
Protein (casein)	19.2%	60.0%	19.2%	19.2%
Sugar (fructose)	10.5%	10.5%	60.0%	10.5%
Fat (lard)	16.5%	16.5%	16.5%	60.0%
Energy density (kcal/kg)	3810	3810	3810	5179

the intestinal microbiota, it may be useful for us to understand the relationship between diet, intestinal microbiota, and NAFLD in order to prevent or treat this disease.

High-fat and high-sugar diets are associated with hyperphagia; however, high-protein diet can reduce caloric intake<sup>[18-20]</sup>. In order to understand the effects of different diets on the development of NAFLD more clearly, we restricted the caloric intake of rats in the high-fat and high-sugar diet groups to the same levels as rats in the high-protein diet group, to exclude caloric intake as a confounder. Given the important role that the intestinal microbiota plays in the pathogenesis of NAFLD, and that the intestinal microbiota is greatly influenced by diet, we examined the dietary effects on the intestinal microbiota.

## MATERIALS AND METHODS

### Animals, diets and experimental design

Thirty 5-wk-old male Sprague-Dawley rats (Hunan SJA Laboratory Animal Co. Ltd., China) were housed at a regulated temperature ( $21 \pm 1.6^\circ\text{C}$ ), humidity ( $45\% \pm 10\%$ ), and an alternating 12-h light and dark cycle. After 1 wk of acclimation, rats were randomized into five groups (six rats each). The control diet (CON) group was allowed *ad libitum* access to a normal chow diet and the free high-fat diet (FFAT) group was allowed *ad libitum* access to a high-fat diet. The restrictive high-fat diet (RFAT) group, restrictive high-sugar diet (RSUG) group and high-protein diet (PRO) group had access to a high-fat diet, a high-sugar diet and a high-protein diet, respectively. The caloric intake of the RFAT and RSUG groups was calibrated to the same caloric intake as the PRO group. The energy density and percentage of energy derived from protein, sugar and fat in the different diets are shown in Table 1. Dietary foods were irradiated by  $\text{Co}^{60}$  and stored at  $4^\circ\text{C}$ , protected from air. Fresh diets were administered daily and any remaining food was weighed and discarded. Food intake was measured every day. The study was approved by the Animal Ethics Committee of Central South University and all efforts were made to minimize animal suffering.

### Oral glucose tolerance test

Two days before the rats were sacrificed, Oral glucose tolerance test (OGTT) was performed. Briefly, 12-h

fasted rats were administered 2 g/kg glucose orally. Blood samples were taken from the tail to measure blood glucose levels before and 30, 60, 90 and 120 min after glucose administration by using an OneTouch-UltraSmart Blood Glucose Monitoring System (LifeScan, Milpitas, United States).

### Liver index and visceral fat index assay

After the rats were killed, the livers were isolated and weighed. Liver index was calculated as the ratio of liver to body weight. Mesenteric, retroperitoneal, and epididymal fat was isolated and weighed as visceral fat mass. The visceral fat index was calculated as the ratio of visceral fat to body weight.

### Biochemical analysis

At the end of the experiment, all the animals were killed after a 12-h overnight fast. Blood samples were collected *via* cardiac puncture and centrifuged to obtain serum. The concentrations of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by standard procedures. The level of serum insulin was assayed with an ELISA kit (Mercodia, Uppsala, Sweden). Insulin resistance (IR) was determined by the homeostasis model assessment (HOMA) formula:  $[\text{HOMA-IR} = \text{fasting insulin } (\mu\text{U/mL}) \times \text{plasma glucose (mmol/L)} / 22.5]$ <sup>[21]</sup>. One milliliter of portal blood was collected into an apyrogenic tube for the lipopolysaccharide (LPS) assay. The level of portal LPS was measured with a chromogenic limulus amoebocyte lysate test kit (Bokang, Zhanjiang, China).

### Liver lipid measurement

Liver lipids were extracted using a modified chloroform/methanol method<sup>[22,23]</sup>. A small fragment of snap-frozen liver tissue was pulverized to a fine powder in liquid nitrogen. Approximately 50 mg of liver tissue was extracted twice in 1 mL of 2:1 (v/v) chloroform:methanol solution. The organic extracts were air dried and resuspended in 1 mL of 2% Triton X-100 solution. Liver triglycerides were measured colorimetrically using a Triglyceride Reagent Kit (Dongou, Wenzhou, China). Frozen sections stained with Oil Red O were also used for liver lipid detection.

### High-throughput sequencing of bacterial 16S rRNA gene and bioinformatic analysis

Before the rats were killed, fecal samples were collected and stored at  $-80^\circ\text{C}$ . 16S rRNA genes of V4 hypervariable region were amplified using specific primers (515F: 5'-GTGCCAGCMGCCGCGGTAA-3', 806R: 5'-GGACTACHVGGGTWTCTAAT-3') with the barcode. All polymerase chain reactions (PCRs) were carried out with Phusion High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, United States). PCR products were mixed in equidensity ratios. The mixture of PCR products was purified using a Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany). Sequencing

libraries were generated using the TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, United States). Lastly, the library was sequenced on an Illumina HiSeq2500 platform and 250 bp paired-end reads were generated.

QIIME pipeline (1.9.1) was used to process and analyze the 16S raw data for all samples<sup>[24]</sup>. Paired-end reads were merged using `join_paired_ends.py` script, and then the raw reads were demultiplexed and quality filtered (at Phred  $\geq$  Q20) using `split_libraries_fastq.py` script. The high quality reads were clustered into operational taxonomic units (OTUs) at 97% similarity using `pick_open_reference_otus.py` script. This script also picked a representative sequence for each OTU, and all representative sequences were taxonomically classified using the Greengene database `gg_13_8`. Sequence alignment was conducted using the PyNAST method and a phylogenetic tree was constructed. The phylogenetic tree was then used for Unweighted UniFrac principal coordinates analysis (PCoA). The chimera sequences were removed with `pick_open_reference_otus.py` script. To remove sampling depth heterogeneity, OTU abundance information was normalized using a standard sequence number corresponding to the sample with the least sequences. Subsequent analyses were all performed based on these normalized data.

### Statistical analysis

Data are presented as mean  $\pm$  SE. The differences in quantitative data were statistically analyzed using one-way analysis of variance. When differences were significant, *post hoc* comparisons were made using a Bonferroni multiple-comparison test. The relative abundance of different phyla and genera was compared between groups using the Mann-Whitney test. SPSS version 20.0 software was used for statistical analyses of the data. The results were considered significant at  $P < 0.05$ .

## RESULTS

### Caloric intake, body weight, and liver index

Figure 1A shows the mean caloric intake of the different groups of rats. The FFAT group, which was fed a diet containing 60% of calories from fat, consumed more calories than the other groups. The RFAT, RSUG and PRO groups, which were restricted to the same caloric intake, consumed fewer calories than the CON group. Figure 1B shows the body weight of each group of rats over time. At the end of the experiment, the FFAT group had the highest body weight, visceral fat index, and liver index among all the groups ( $P < 0.05$ , Figure 1C-E). The RFAT and RSUG groups displayed no significant differences in body weight and visceral fat index compared with the CON group ( $P > 0.05$ ); however, the body weight and visceral fat index of

these two groups were higher than those in the PRO group ( $P < 0.05$ , Figure 1C and D). Compared with the CON group, the PRO group showed a decrease in body weight ( $P < 0.05$ ) but not visceral fat index ( $P > 0.05$ ). There were no significant differences in liver index among the other groups ( $P > 0.05$ , Figure 1E).

### Fasting serum insulin, IR, and OGTT

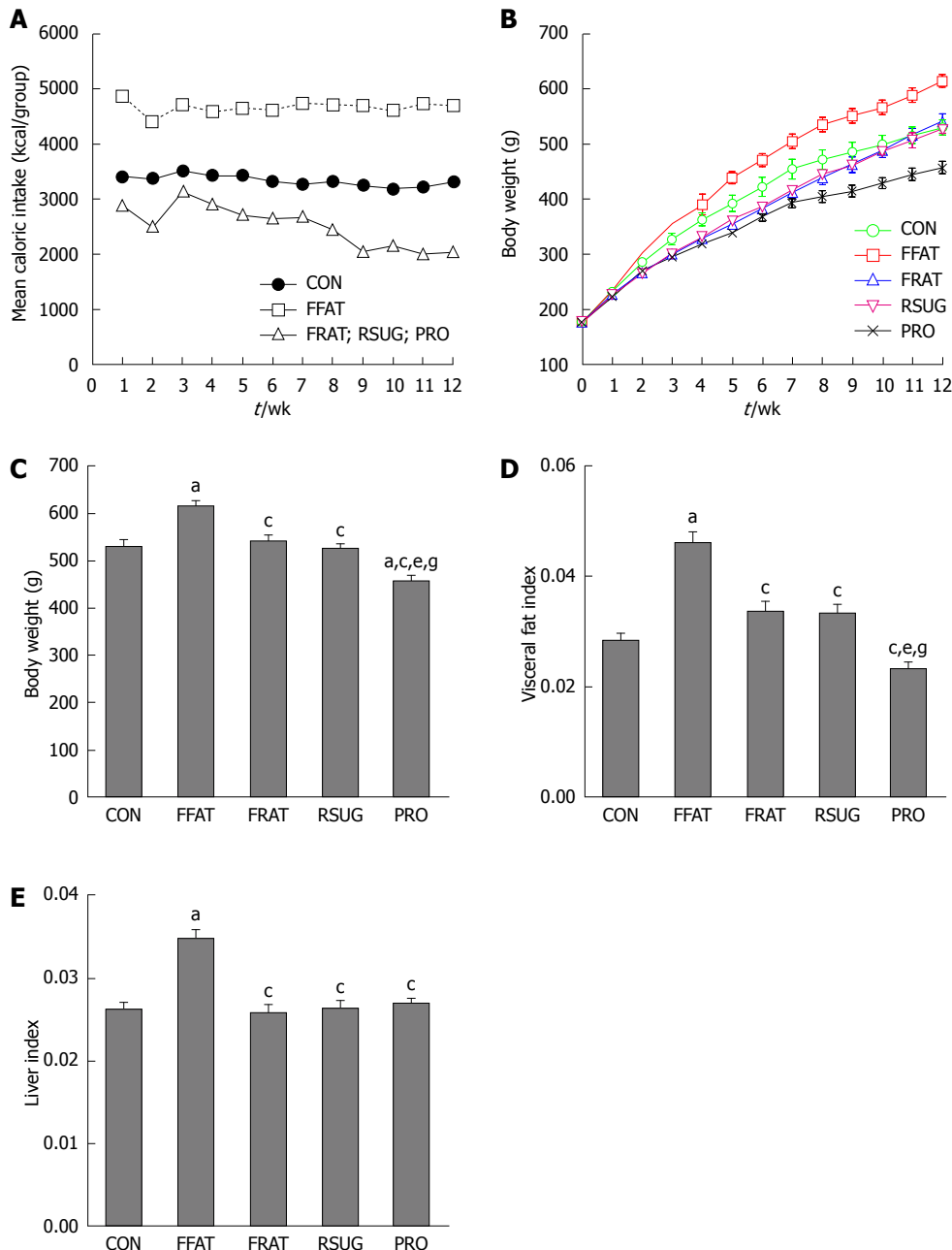
There were no significant differences in fasting blood glucose levels among the different groups (data not shown). However, compared with other groups, the FFAT group exhibited elevated fasting serum insulin and HOMA-IR values ( $P < 0.05$ ; Figure 2A and B). Although the RFAT, RSUG and PRO groups showed no significant differences in fasting serum insulin and HOMA-IR values compared with the CON group ( $P > 0.05$ ), the PRO group showed decreased fasting serum insulin and HOMA-IR values compared with the RFAT and RSUG groups ( $P < 0.05$ ; Figure 2A and B). The OGTT confirmed the impaired glucose tolerance of the FFAT group (Figure 2C and D). The area under the curve (AUC) of the OGTT showed that the PRO group had decreased AUC<sub>OGTT</sub> when compared with the RFAT and RSUG groups ( $P < 0.05$ ), but these three groups showed no significant differences in AUC<sub>OGTT</sub> values when compared with the CON group ( $P > 0.05$ ; Figure 2D).

### Portal LPS and liver function

At the end of the trial, portal LPS was measured. The FFAT group had higher levels of portal LPS compared with the other groups ( $P < 0.05$ ; Figure 3A). Taking the same calories, the RFAT group showed higher levels of portal LPS than the PRO group ( $P < 0.05$ ; Figure 3A). Serum ALT and AST are biomarkers of liver function, which can reflect liver injury. Only the FFAT group had higher serum ALT and AST than the other groups ( $P < 0.05$ ), and other groups had the same level of serum ALT and AST (Figure 3B and C).

### Liver triglycerides

The results of oil red O staining showed that the FFAT group had obvious accumulation of triglycerides in the liver (Figure 4A). However, the RFAT and RSUG groups had significantly less triglyceride accumulation in the liver than the FFAT group. The CON and PRO groups had no detectable triglyceride accumulation in the liver by oil red O staining. Colorimetric measurement confirmed the oil red O staining findings. Using colorimetric measurement, the FFAT group also showed increased liver triglyceride levels compared with the other groups ( $P < 0.05$ ; Figure 4B). Among the three isocaloric groups, the level of liver triglycerides was higher in the RFAT and RSUG groups than in the PRO group ( $P < 0.05$ ); however, when compared with the CON group, no significant differences were found ( $P > 0.05$ ).



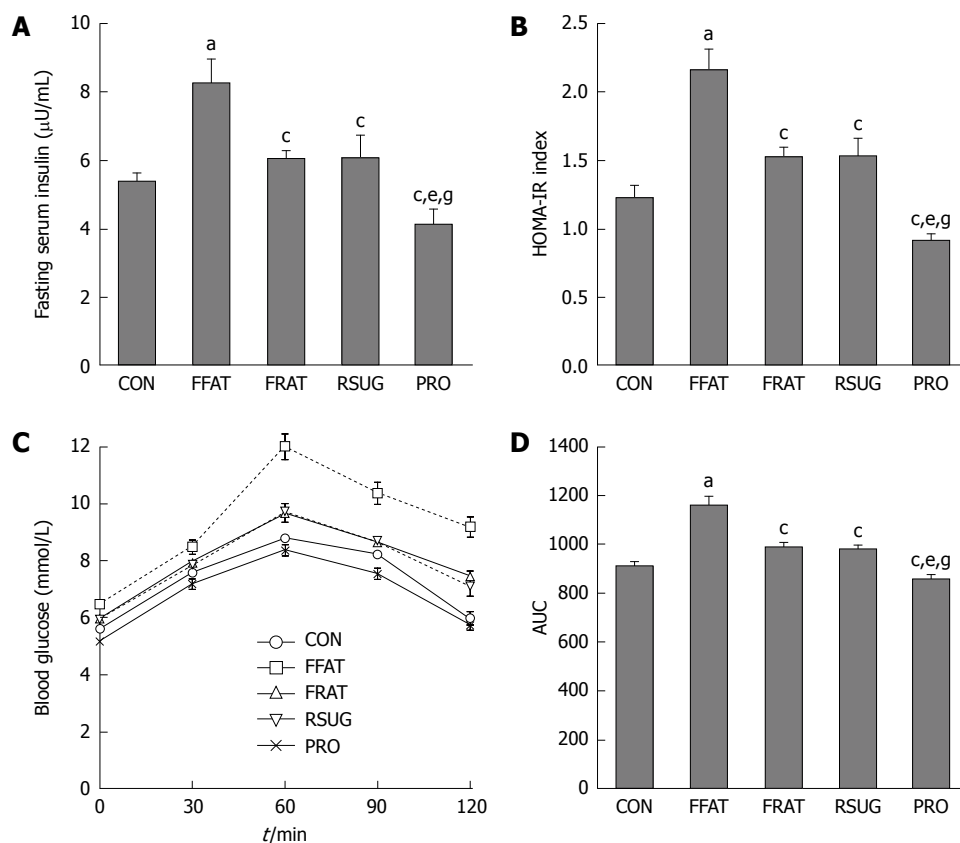
**Figure 1** Caloric intake, body weight, visceral fat index, and liver index in rats. A: Mean caloric intake; B: Body weight at different experimental periods; C: Body weight at 12 wk; D: Visceral fat index, calculated as visceral fat weight/body weight; E: Liver index, calculated as liver weight/body weight. Differences were denoted as follows: <sup>a</sup> $P < 0.05$  vs CON group rats, <sup>c</sup> $P < 0.05$  vs FFAT group rats, <sup>e</sup> $P < 0.05$  vs RFAT group rats, <sup>g</sup> $P < 0.05$  vs restrictive high-sugar diet (RSUG) group rats.

### Intestinal bacterial composition

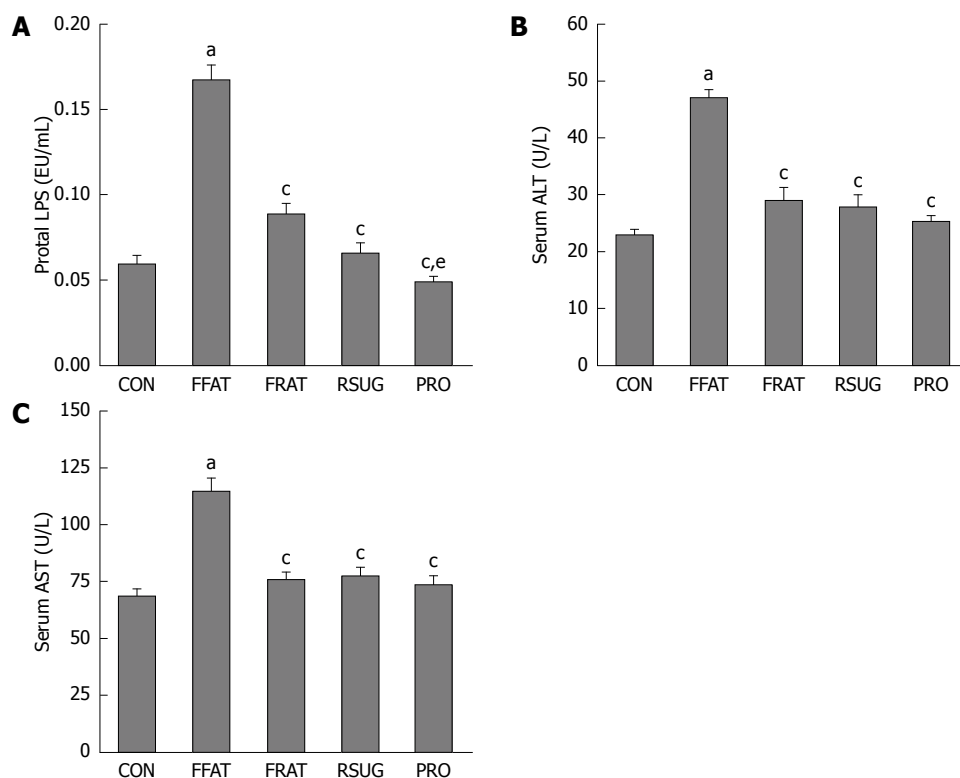
A total of 1705045 high-quality reads (average of 56 835 sequences per sample) were delineated into OTUs at the 97% similarity level. Rarefaction curves revealed that all the samples had a similar pattern, which indicates that some new OTU would be expected with additional sequencing (Figure 5A). However, the good-coverage index, an indicator of sequencing depth, of all samples ranged from 0.968 to 0.977. Combining the results of the rarefaction curves and the good-coverage index, we demonstrated that the sequencing depth in the present study was sufficient to reflect the bacterial composition of different group samples.

Unweighted UniFrac PCoA revealed that despite inter-individual variation, the intestinal microbiota structures of the CON, FFAT, RSUG and PRO groups were roughly separated away from each other (Figure 5B). However, the intestinal microbiota structure of the RFAT group was not well separated.

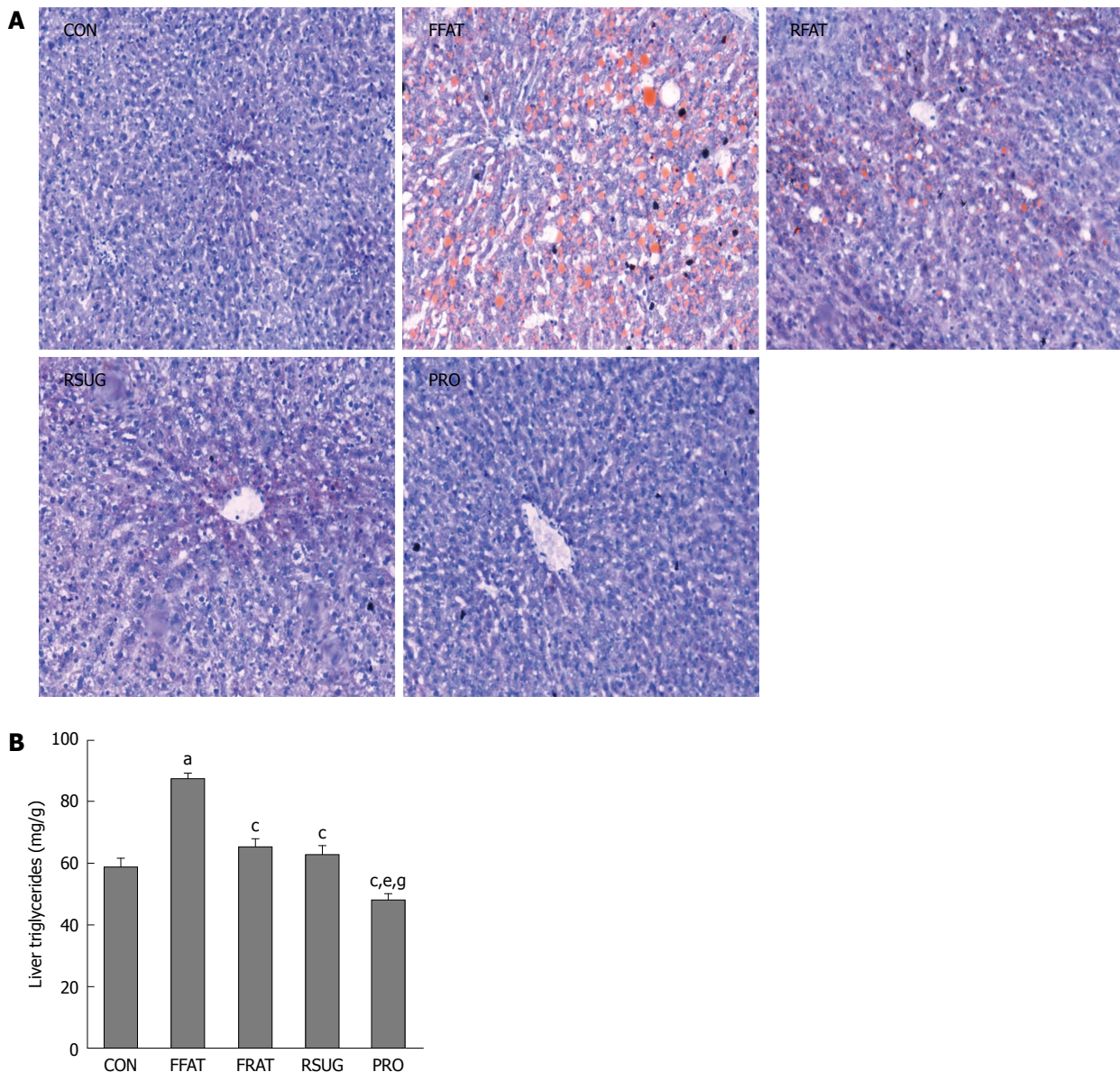
For taxon-based analysis, a total of 15 phyla were detected and the most abundant phyla included *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Tenericutes* (Figure 5C). Compared with the CON group samples, the FFAT group samples showed an increased abundance of *Firmicutes*, decreased abundance of *Bacteroidetes*, and lower ratio of *Bacteroidetes* to



**Figure 2** Fasting serum insulin, insulin resistance, and oral glucose tolerance test in rats. A: Fasting serum insulin; B: HOMA-IR, calculated according to the formula fasting insulin (μU/mL) × fasting glucose (mmol/L)/22.5; C: Oral glucose tolerance test (OGTT); D: Area under the curve (AUC) of OGTT. Differences were denoted as follows: <sup>a</sup>*P* < 0.05 vs CON group rats, <sup>c</sup>*P* < 0.05 vs free high-fat diet (FFAT) group rats, <sup>e</sup>*P* < 0.05 vs RFAT group rats, <sup>g</sup>*P* < 0.05 vs restrictive high-sugar diet (RSUG) group rats.



**Figure 3** Portal lipopolysaccharide and liver function in rats. A: Portal LPS; B: Serum ALT; C: Serum AST. Values are expressed as mean ± SE. Differences were denoted as follows: <sup>a</sup>*P* < 0.05 vs CON group rats, <sup>c</sup>*P* < 0.05 vs FFAT group rats, <sup>e</sup>*P* < 0.05 vs RFAT group rats. LPS: Lipopolysaccharide; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

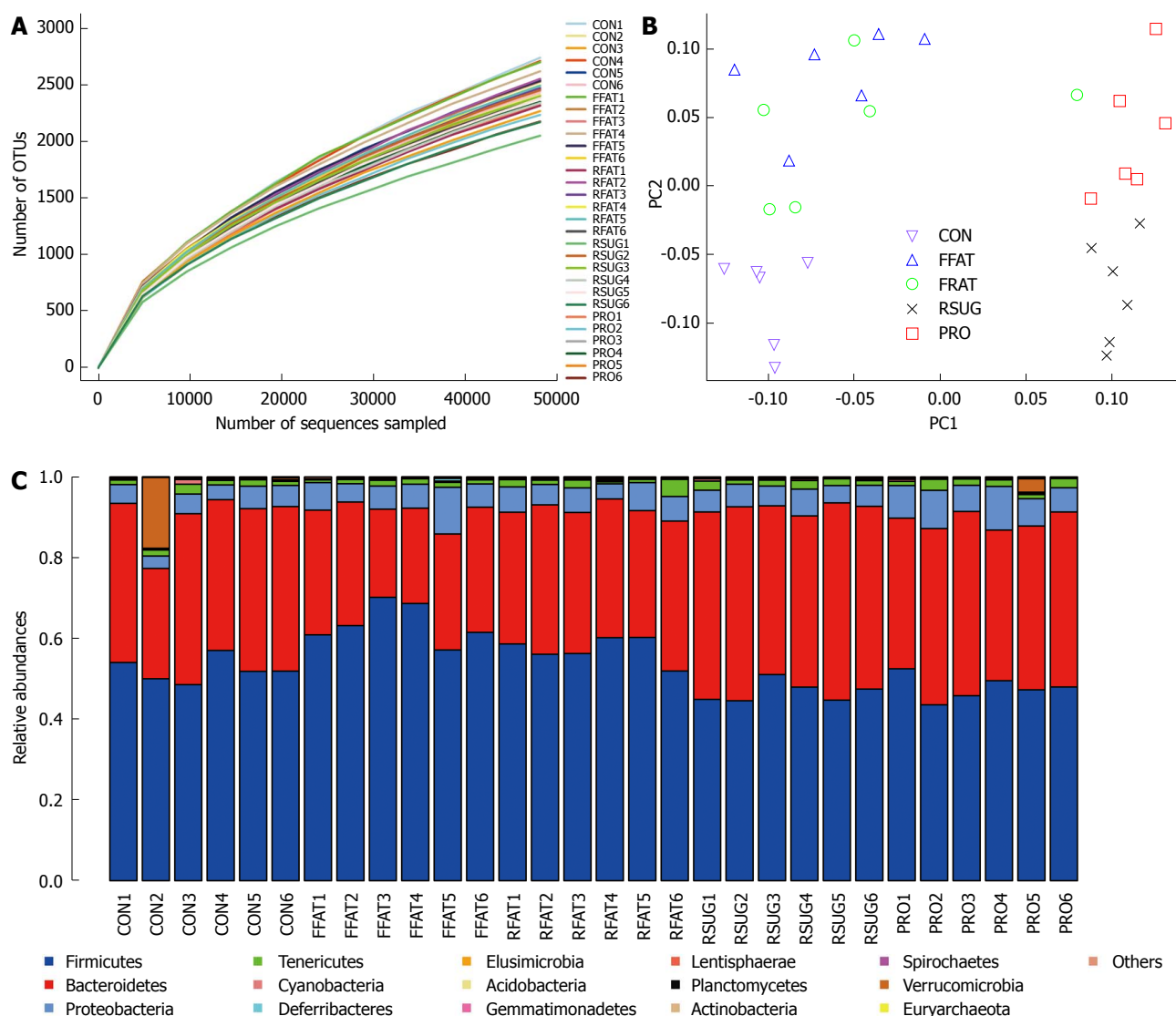


**Figure 4** Liver triglycerides in rats. A: Liver sections stained with oil red O; representative photomicrographs were captured at magnification  $\times 200$ ; B: Liver triglycerides. Differences were denoted as follows: <sup>a</sup> $P < 0.05$  vs CON group rats, <sup>b</sup> $P < 0.05$  vs FFAT group rats, <sup>c</sup> $P < 0.05$  vs RFAT group rats, <sup>d</sup> $P < 0.05$  vs RSUG group rats. CON: Control diet; FFAT: Free high-fat diet; RSUG: Restrictive high-sugar diet; RFAT: Restrictive high-fat diet.

*Firmicutes* ( $P < 0.05$ ; Figure 6A). The RFAT group samples showed only an increased abundance of *Firmicutes* ( $P < 0.05$ ; Figure 6A). The RSUG group samples showed an increased abundance of *Bacteroidetes*, higher ratio of *Bacteroidetes* to *Firmicutes*, and decreased abundance of *Firmicutes* ( $P < 0.05$ ; Figure 6A). The PRO group samples showed an increased abundance of *Bacteroidetes* and decreased abundance of *Firmicutes* ( $P < 0.05$ ; Figure 6A). The FFAT and RFAT groups had the same high-fat diet, and the only difference between these two groups was the total caloric intake. However, compared with the FFAT group samples, the RFAT group samples showed an increased abundance of *Bacteroidetes*, higher ratio of *Bacteroidetes* to *Firmicutes*, and decreased abundance of *Firmicutes* ( $P$

$< 0.05$ ; Figure 6A).

At the genus level, a total of 109 genera were found, and we only selected the genera with a relative abundance  $> 1\%$  for analysis. Compared with the CON group samples, the FFAT group samples showed an increased abundance of *Roseburia* and *Oscillospira*, and a decreased abundance of *Bacteroides* and *Parabacterioides* ( $P < 0.05$ ; Figure 6B). The RFAT group samples showed a decreased abundance of *Parabacterioides* ( $P < 0.05$ ; Figure 6B); the RSUG group samples showed an increased abundance of *Sutterella* ( $P < 0.05$ ; Figure 6B); the PRO group samples showed an increased abundance of *Prevotella*, *Oscillospira*, and *Sutterella* ( $P < 0.05$ ; Figure 6B). Compared with the FFAT group samples, the RFAT group samples showed a decreased



**Figure 5 Bacterial composition of different group samples.** A: Rarefaction curves (at a 97% similarity level); B: Unweighted UniFrac PCoA; C: Intestinal microbiota composition at the phylum level. FFAT: Free high-fat diet; RSUG: Restrictive high-sugar diet; RFAT: Restrictive high-fat diet; CON: Control diet.

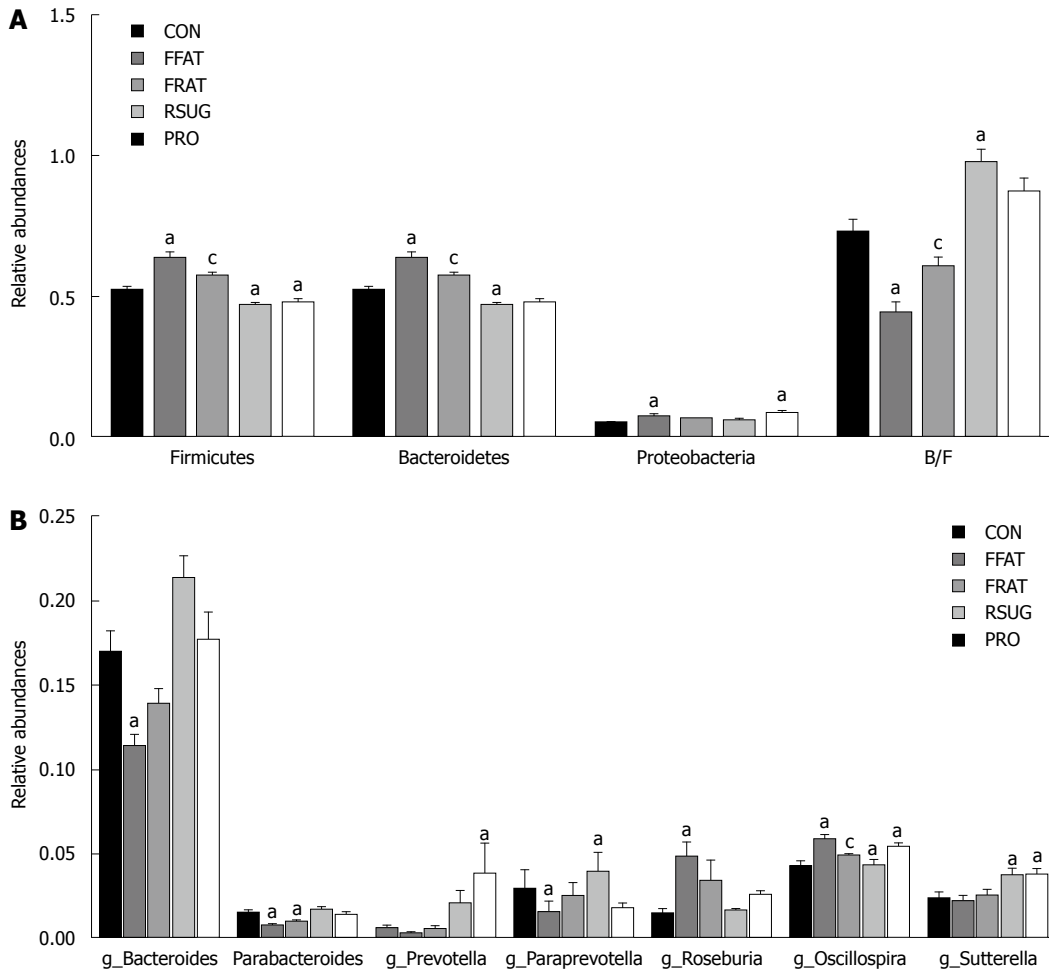
abundance of *Oscillospira* ( $P < 0.05$ ; Figure 6B).

## DISCUSSION

We investigated the effects of different diets on the development of NAFLD, excluding caloric intake as a confounder. We demonstrated that high-fat and high-sugar diets in rats increased body weight, IR, and liver triglycerides than a high-protein diet, and that these effects were independent of caloric intake. We also found that different diets changed the intestinal microbiota composition independently from caloric intake. The different effects of these diets on NAFLD development, at the same caloric intake, may be associated with changes in the intestinal microbiota.

Obesity and IR are recognized as risk factors for NAFLD<sup>[25,26]</sup>. In most cases, NAFLD is a complication of obesity<sup>[4]</sup>. Peripheral IR may cause accumulation of liver triglycerides *via* three main mechanisms: increased peripheral lipolysis (which increases free fatty

acids in the liver), hyperinsulinemia (which stimulates activity of the key lipogenic transcription factors) and hyperglycemia (which increases glucose concentration in the liver and provides substrates for lipogenesis)<sup>[27]</sup>. Free availability of a high-fat or high-sugar diet can cause rodent obesity, IR, and accumulation of liver triglycerides<sup>[28,29]</sup>. However, free availability of a high-protein diet has the opposite effect<sup>[14]</sup>. In the present study, we restricted the caloric intake of these three diets to the same level, and found that high-fat and high-sugar diets caused experimental rats to have greater body weight, peripheral IR, and accumulation of liver triglycerides than a high-protein diet. This suggests that, compared with a high-protein diet, the NAFLD-inducing effects of high-fat and high-sugar diets are independent from caloric intake. The results of the present study showed no significant difference in body weight, peripheral IR, and accumulation of liver triglycerides between the RFAT and CON groups. However, de Meijer *et al.*<sup>[30]</sup> found that mice with a



**Figure 6 Comparison of relative abundance at the phylum and genus levels.** A: Comparison of relative abundance at the phylum level; B: Comparison of relative abundance at the genus level. <sup>a</sup> $P < 0.05$  vs CON group rats; <sup>c</sup> $P < 0.05$ , FFAT group rats vs RFAT group rats. B/F: Ratio of *Bacteroidetes* to *Firmicutes*; FFAT: Free high-fat diet; RSUG: Restrictive high-sugar diet; RFAT: Restrictive high-fat diet; CON: Control diet.

restrictive high-fat diet showed higher body weight, peripheral IR, and accumulation of liver triglycerides than the control diet mice. The main reason for this discrepancy may be the different restrictive levels that we chose. The study by de Meijer *et al.*<sup>[30]</sup> restricted the caloric intake of the high-fat diet group to the same level as the control diet group; however, we restricted the caloric intake of the high-fat diet group to the same level as the high-protein diet group, which consumed fewer calories than the control diet group. This discrepancy may reflect the role that calories play in the development of NAFLD. Compared with the FFAT group, the RFAT group showed decreased body weight, peripheral IR, and accumulation of liver triglycerides. This confirms the role of calories in the development of NAFLD. We conclude that dietary composition and caloric intake are two independent factors that can affect the development of NAFLD.

LPS is a constituent of Gram-negative bacteria and can trigger the secretion of proinflammatory cytokines, such as tumor necrosis factor- $\alpha$ , when it binds to Toll-like receptor (TLR) 4 on the membrane surface of monocytes, macrophages, adipocytes and hepatocytes.

After continuous subcutaneous infusion of LPS for 4 wk, wild-type mice exhibited obesity, adipose tissue weight gain, steatosis, and fasting hyperglycemia<sup>[31]</sup>. However, mice mutant for CD14 (a key multifunctional receptor that mediates the combination of LPS and TLR4) resisted most of the LPS-induced features of metabolic diseases. Therefore, LPS is considered a triggering factor in the development of metabolic disorders. In NAFLD patients, elevated LPS levels have been observed<sup>[32]</sup>. However, the relationship between different diets and LPS is unclear. In the present study, with the same caloric intake, rats fed a high-fat diet showed higher portal LPS levels than those fed a high-protein diet, suggesting that compared with a high-protein diet, a high-fat diet can elevate portal LPS level independently from caloric intake. Unexpectedly, although the RFAT group showed increased portal LPS levels, serum ALT and AST levels in the RFAT and PRO groups did not differ significantly. A possible explanatory hypothesis is that there is a threshold level of LPS concentration in the liver that induces obvious injury, and the liver can handle the elevated LPS which does not reach the threshold level. The higher levels of

portal LPS and serum ALT and AST in the FFAT group compared with the RFAT group partly confirm this hypothesis.

The human intestine harbors > 2000 species of commensal bacteria that define the intestinal microbiota. This community consists of 10 times more bacteria than human cells and encodes 100-200-fold more genes than our own genome<sup>[33,34]</sup>. The intestinal microbiota provides the host with enhanced metabolic capabilities (fermentation of nondigestible dietary residue, production of vitamin K, and absorption of ions), protection against pathogens, education of the immune system, and modulation of gastrointestinal development. So the intestinal microbiota can be viewed as a "metabolic organ". This organ can affect host metabolic processes<sup>[35,36]</sup>. Although the composition of the intestinal microbiota is influenced by multiple factors, diet plays an important role in shaping it<sup>[37,38]</sup>. In the present study, Unweighted UniFrac PCoA results showed that the intestinal microbiota structures of the CON, FFAT, RSUG and PRO groups were roughly separated away from each other; however, the intestinal microbiota of the RFAT group was not well separated. However, taxon-based analysis found that intestinal microbiota composition of the RFAT, RSUG and PRO groups differed from that of the CON group. These results suggest that different diets can affect the composition of the intestinal microbiota independently from caloric intake. This important finding may be helpful in better understanding the effect of diet on the composition of the intestinal microbiota. In the present study, the RSUG group showed an increased abundance of *Bacteroidetes*. Some species of bacteria in this phylum, such as *Bacteroides thetaiotaomicron*, can encode adequate carbohydrate active enzymes for carbohydrate metabolism of food<sup>[39]</sup>. This enables the host to extract more energy from the diet, which will be deposited in the liver in the form of triglycerides. Unlike the RSUG group, the RFAT group showed an increased abundance of *Firmicutes*; however, how the bacteria in this phylum affect NAFLD development is not clear. The effect of a high-protein diet on the composition of the intestinal microbiota is not well studied. We found that the PRO group had an increased abundance of *Bacteroidetes* and *Sutterella* bacteria, and decreased abundance of *Firmicutes*. This change in intestinal microbiota was similar to that in the RSUG group; however, the PRO group also had an increased abundance of *Prevotella* and *Oscillospira*. Kovatcheva-Datchary *et al.*<sup>[40]</sup> found that *Prevotella* is associated with improvement in glucose metabolism. *Oscillospira* has never been cultured, so it is an enigmatic bacterial genus and little is known of its role in the intestinal tract. However, recent studies found that *Oscillospira* is positively associated with leanness, and is reduced in pediatric NASH<sup>[41,42]</sup>. We conclude that the beneficial effects of high-protein diet on NAFLD may be closely associated with *Prevotella* and *Oscillospira*, which requires further study. Changes in intestinal microbiota

are one mechanism by which diet affects NAFLD development, so we can conclude that the different effects of these diets on NAFLD development, at the same caloric intake, may be associated with changes in intestinal microbiota. However, the exact role that these different bacteria play in the development of NAFLD remains unclear and needs further study. Previous studies have found that high-fat diet reduces *Bacteroides* and increases *Firmicutes* and *Proteobacteria*. The ratio of *Bacteroidetes* to *Firmicutes* was also decreased by a high-fat diet<sup>[43,44]</sup>. The results of the present study confirmed these findings. Moreover, we found that the high-fat diet increased *Roseburia* and *Oscillospira* spp. However, Neyrinck *et al.*<sup>[45]</sup> have reported that the number of *Roseburia* spp. was decreased when mice were fed a high-fat diet. As host genotype is also an important factor that can affect the intestinal microbiota, the reason for this contrary result may be mainly due to the different animals that were used<sup>[21]</sup>. Taxon-based analysis also found that intestinal microbiota composition of the RFAT group was different from that of the FFAT group, suggesting that, except for different dietary composition, caloric intake is an independent factor that can shape the intestinal microbiota.

Because of the limitations of the bacterial 16S rRNA gene sequencing technique that we adopted, we could not classify sequences at the species level, and the relationship between intestinal bacteria and portal LPS levels was undefined. It should be pointed out that the results of the present study were obtained from rats and host genotype also can affect intestinal microbiota composition, so it may not be appropriate to apply our results directly to humans.

In conclusion, our present study found that compared with the high-protein diet, the NAFLD-inducing effect of the high-fat and high-sugar diets is independent from caloric intake. This helps in understanding the effects of diet on the development of NAFLD. In addition, the effects of diet on the intestinal microbiota in the present study extend our knowledge of the relationship between diet and the intestinal microbiota. In the future, we can manipulate the intestinal microbiota by diet to prevent or treat NAFLD. Overall, our findings shed some light on the desirability of dietary therapy for NAFLD.

## COMMENTS

### Background

Although several studies have elaborated the relationship between different diets and nonalcoholic fatty liver disease (NAFLD) development, those studies had the confounder of caloric intake. The intestinal microbiota is the interface between diet and the liver; however, the relationship between diet, intestinal microbiota and NAFLD is unclear.

### Research frontiers

Previous studies have shown that high-fat and high-sugar diets can induce NAFLD; however, a high-protein diet can ameliorate it. Diet is also an important factor that can shape the intestinal microbiota, and NAFLD patients are always

associated with changes in intestinal microbiota.

### Innovations and breakthroughs

To the best of the authors' knowledge, this is the first study to evaluate the effects of different diets on the intestinal microbiota and NAFLD development at the same caloric intake. This study suggests that compared with a high-protein diet, the NAFLD-inducing effect of high-fat and high-sugar diets is independent from caloric intake. The different effects of these diets on NAFLD development, at the same caloric intake, may be associated with changes in the intestinal microbiota.

### Applications

This study extends the authors' knowledge of the relationship between diet, intestinal microbiota, and NAFLD. It sheds some light on the desirability of dietary therapy for NAFLD.

### Terminology

Unweighted UniFrac principal coordinates analysis is a method used to discriminate the microbiota composition of the different groups based on evolutionary distance.

### Peer-review

In this paper, the authors examined the effects of different diets on NAFLD and intestinal microbiota. They found that high-fat diet and high-sugar diet but not high-protein diet induced NAFLD independently from calories. Also diet affects the microbiota.

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