Treatment by cefotaxime with high-fructose diet inducing non-alcoholic fatty liver disease and gut microbiota dysbiosis in mice

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Abstract: High-fructose diet is the main cause of the metabolic syndrome and induces host gut microbial dysbiosis and related obesity and nonalcoholic fatty liver disease (NAFLD). Several antibiotic treatments could prevent endotoxemia and fatty liver. However, other studies demonstrated that a high-fructose diet could affect the gut bacterial dysbiosis and induce fatty liver. This study was performed to partially modify the gut microbial composition by a single cefotaxime treatment, which might influence the fructose-induced NAFLD severity. Male C57BL/6J Narl mice were divided into four groups: vehicle/control diet (VE-CD, 5010 chow), vehicle/high-fructose diet (VE-FD, 30% fructose), antibiotic (cefotaxime (CF))/CD, and CF/FD (n = 8 in each group) (treatment for 16 weeks). The NAFLD-related symptoms, including body weight gain, hepatic steatosis severity, epididymal white adipose tissue hypertrophy, and insulin resistance occurrence, were observed only in the CF-FD group. The increased protein expression of hepatic lipogenesis was observed in the CF-FD group, but lipolysis protein expression was no difference. CF-FD exhibited significantly reduced microbial diversity and microbial composition. Increased abundances of Lachnospiraceae and Clostridiales XIII were observed in the feces of CF-FD, compared to VE-FD. This novel model reveals that particular antibiotics such as cefotaxime may affect the gut microbiota exacerbating the hepatic steatosis by the high-fructose diet.

Keywords: High-fructose diet; nonalcoholic fatty liver disease; cefotaxime; gut microbiota dysbiosis

1. Introduction

Fructose is a simple ketonic monosaccharide present in various plants, particularly fruits. Owing to its high relative sweetness, fructose is the sweetest structure among all naturally occurring carbohydrates. Therefore, fructose is the most commonly added sugar in sweetened beverages, usually in the form of high-fructose corn syrup, a mixture of fructose and glucose monosaccharides. A high fructose consumption is considered an indicator of
Western diets and induces metabolic syndrome in gut microbial dysbiosis. In an epidemiological study, data from the US population have been analyzed, collected from 1988 to 1994, for 21,483 adults and children. The fructose consumption was increased to 54.7 g/d (10.2% of the total caloric intake), compared to 37 g/d (8% of the total intake) in 1977–1978. The consumption was highest among adolescents (12–18 years), 72.8 g/d (12.1% of the total calories). Over 10% of the Americans’ daily calories originated from fructose [1]. Furthermore, the high fructose consumption is linked to metabolic syndrome, type 2 diabetes mellitus (T2DM), and nonalcoholic fatty liver disease (NAFLD)[2-4].

Ingredients in the diet can have both direct and indirect effects. The indirect effects are considered to be mediated by the gut microbiome. Digested food influences the community, structure, and function of the gut bacteria, which can improve or deteriorate the host's health. Fructose consumption is specifically linked to gut microbial dysbiosis [5, 6], which suggests that the fatty liver might be mediated by some gut microbiome [4, 7, 8]. The key bacterial products involved in the pathogenesis of NAFLD are lipopolysaccharides (LPSs) [9]. LPS (also referred to as endotoxin) is derived from Gram-negative bacteria. It significantly contributes to inflammation-related processes and insulin resistance [10]. It is able to cross the gastrointestinal mucosa through leaky tight junctions or infiltrating chylomicrons [11]. Recent studies have connected NAFLD to disturbances in the gut microbial environment. The microbial compositions differed between healthy individuals and patients with NAFLD [12].

Cefotaxime (third-generation antibiotic) is a β-lactam antibiotic. It is a broad-spectrum antibiotic with activity against numerous Gram-positive and -negative bacteria. β-lactams inhibit the bacterial cell wall synthesis by the final transpeptidation step of peptidoglycan synthesis in bacterial cell walls, thus inhibiting the cell wall biosynthesis. Bacteria eventually lyse owing to the ongoing activity of cell wall autolytic enzymes in the absence of cell wall assembly. Notable organisms against which cefotaxime is not active include Pseudomonas and Enterococcus. It has a modest activity against the anaerobic Bacteroides fragilis [13]. These results indicate that cefotaxime did not fully deplete gut microbiota. The effect of remnants’ gut microbiota in fructose-induced NAFLD is still unclear.

Recent studies have elucidated the role of the gut microbiota in the regulation of energy homeostasis and revealed its association with numerous metabolic diseases such as diabetes, obesity, and NAFLD [14-16]. Thus, this study was carried out to determine the effects of cefotaxime on the severity of fructose-diet-induced fatty liver disease to clarify the involvement of gut microbiota in these mechanisms. We focused on the gut microbiota differences of diversity and compositions, which may have a regulatory role for physiological activities, using a novel animal model.

2. Materials and Methods

2.1. Animals and Experimental Design

Forty specific-pathogen-free C57BL/6J Narl male mice were used in this study (7–8 weeks old). The mice were obtained from the National Laboratory Animal Center (Taipei, Taiwan). They were maintained in a vinyl isolator in a room at a constant temperature (21 ± 1 °C) and humidity of 55–65% with a 12-h/12-h light/dark schedule. The mice were maintained on a normal chow diet and were given sterile water to drink ad libitum for an acclimation period of seven days. The mice were then divided into four groups (n = 10 for each): vehicle + chow diet (VE-CD), cefotaxime + chow diet (CF-CD), vehicle + high-fructose diet (70% of calories, D1707R) (VE-FD), and cefotaxime + high-fructose diet (CF-FD). The mice were administered by oral gavage of vehicle (saline) or antibiotic treatment (cefotaxime: 300 mg/kg) for 10 days and were then fed with either a standard chow or high-fructose diet. The mice were finally sacrificed with 95% CO2 asphyxiation and blood was withdrawn by cardiac puncture. The livers were weighed and the body weight was determined for standardization.
A part of the liver and epididymal fat pad tissues were quickly removed and fixed in 10% neutral buffered formalin for 24 h before being processed for a histopathologic analysis and immunohistochemistry staining. A part of the liver tissues used for Oil Red O staining was fixed with a tissue-embedding medium compound (Tissue-Tek O.C.T. Compound, Sakura Finetek, Torrance, CA), rapidly frozen in a hexane environment with dry ice and acetone, and stored at -80 °C frozen. Several portions of the liver tissue were quickly removed, snap-frozen in liquid N₂, and stored at -80 °C. Frozen tissues were used for liver triglyceride, protein, and messenger ribonucleic acid (mRNA) analyses.

2.2. Antibiotic Treatment

The cefotaxime treatment was administered in saline by daily oral gavage (300 mg/kg) for 10 days. Cefotaxime was chosen because it is a broad-spectrum antibiotic with activity against numerous Gram-positive and -negative bacteria. Considering its broad spectrum of activity, cefotaxime is used for various infections. In addition, the absorption of cefotaxime is poorly and thus have no systemic effects.

2.3. Ethics Statement

All procedures were performed in an animal facility accredited by the Association for Assessment and Accreditation for Laboratory Animal Care, with the approval of the Institutional Animal Care and Use Committee at the National Laboratory Animal Center, who approved the mouse experiments with the approval number IACUC2015M02. The methods applied in this study were carried out in accordance with the approved guidelines.

2.4. Clinical Biochemistry

After 16 weeks, the mice were fasted for 6 h and sacrificed by CO₂ asphyxiation. Blood was collected into endotoxin-free microfuge tubes by cardiac puncture and allowed to clot. The blood samples were centrifuged at 3,000 g for 10 min. The serum was collected and frozen at -80 °C until a biochemical analysis. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose (GLU), total cholesterol (T-CHO), triglycerides (TG), high-density lipoprotein (HDL), and non-HDL levels were determined using an automated chemistry analyzer (HITACHI 7080, Hitachi, Tokyo, Japan).

2.5. Liver Triglyceride Determination

All liver samples weighed approximately 50 mg and were homogeneous. Liver triglyceride levels were determined in liver tissues from each sample using a triglyceride colorimetric assay kit (Cayman, Ann Arbor, MI). The assays were performed in accordance with the manufacturer’s protocol.

2.6. Endotoxin Assay

Serum specimens were collected in a nonpyrogenic tube. The serum was assayed for endotoxin using a commercially available pyrochrome limulus amebocyte lysate kit (Associates of Cape Cod, Falmouth, MA) according to the manufacturer’s instructions.

2.7. Adipokine Assay

The adipokine level responses (IL-6, TNF-α, insulin, leptin, and resistin) from serum were detected using a high-sensitivity mouse serum adipokine immunoassay kit (Millipore, Billerica, MA). The assays were performed in accordance with the manufacturer’s protocol using Luminex Technology.

2.8. Oil Red O Stain

A fresh liver tissue was embedded in an optimal cutting temperature (OCT) compound (Tissue-Tek O.C.T. Compound, Sakura Finetek, Torrance, CA, USA). The tissue was sectioned at 4 μm using a universal microtome cryostat (Leica CM3050S, Leica Microsystems, Nussloch GmbH, Germany) and processed for a fat accumulation examination by Oil Red O staining. The liver tissue was fixed with 10% neutral buffered formalin for 10 min, stained with a fresh 60% Oil Red O working solution for 7 min, and counterstained with hematoxylin for 30 s.

2.9. Insulin Resistance Indicator: Homeostasis Model Assessment of Insulin Resistance (HOMA-IR)

HOMA-IR was calculated as (fasting glucose level × fasting insulin level)/22.5.
2.10. Histopathologic Evaluation

Tissue samples from the livers were saved, fixed in 10% neutral buffered formalin for one day, dehydrated, embedded in paraffin, cut into 4-μm sections, and stained with hematoxylin–eosin (H&E) for a histological examination. The hepatic steatosis score was evaluated as described by Dixon et al. [17]. The slides were also incubated with a 0.1% Sirius red solution dissolved in an aqueous saturated picric acid for 1 h, washed in acidified water (0.1N hydrogen chloride), dehydrated, and mounted with DPX Mounting for a fibrosis evaluation. The collagen components were stained with Sirius-Red-stained for liver fibrosis score, the stage of fibrosis was scored based on the 5-point scale proposed by Kleiner et al[18]. All slides were reviewed by a veterinarian.

2.11. Immunohistochemistry Staining for CD68

Liver sections (4 μm) were deparaffinized and endogenous peroxidases were blocked by 3% hydrogen peroxide. For antigen retrieval, the slides were submerged in a 10-mM citrate buffer (pH = 6.0) until boiling. The sections were incubated at 4 °C overnight with the primary anti-CD68 antibody (ab125212, Abcam). The slides were subsequently treated with a PictureTM HRP Polymer conjugate (87-8963, Invitrogen) at room temperature for 20 min. The horseradish peroxidase localization was visualized using a 3-amino-9-ethylcarbazole substrate-chromogen kit (K3461, Dako).

2.12. Western Blot Analysis

To detect protein expression, the liver tissues’ protein was extracted using T-PER™ Tissue Protein Extraction Reagent (Pierce, Rockford, IL). The lysates were centrifuged at 10,000g for 15 min at 4 °C. The supernatants were collected. Equal amounts of protein were loaded for 4–12% bis-tris sodium dodecyl sulfate polyacrylamide gel electrophoresis for each sample and proteins were transferred onto nitrocellulose membranes by electrophotography. The membranes were incubated with primary antibodies for fatty acid synthase (FAS), acetyl coenzyme A carboxylase phosphorylation (pACC) (Cell Signaling Technology, Beverly, MA, USA), and GAPDH (Santa Cruz, CA, USA) overnight at 4 °C. The membranes were further incubated with secondary antibodies for 1 h at room temperature. Proteins were detected using an enhanced chemiluminescence reagent (Millipore, Billerica, MA). The amount of protein expression was corrected using a GAPDH internal control. The protein expression was quantified using the open-source software ImageJ (version 1.51).

2.13. Analysis of the Gut Microbiota Composition

Stool samples were frozen and stored at 80 °C. DNA was extracted using the QIAamp Fast DNA Stool Mini Kit (#51604, QIAGEN). For each stool sample, amplicon sequencing was carried out using polymerase chain reaction amplification of the V3 and V4 regions of 16S ribosomal DNA (rDNA) of bacterial genomes and sequencing on a MiSeq platform (Illumina, San Diego, CA, USA). Raw data were filtered, and then clustered to form operational taxonomic units (OTUs) at a similarity of 97% using the QIIME2 pipeline. Each of these OTUs was annotated as a taxonomic unit using the SILVA database. The software R was used to calculate the α-diversity and β-diversity. The abundance of annotated OTUs was submitted to the LEfSe online tool.

2.14. Statistical Analysis

All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). The results are presented as mean ± standard deviation. Differences between groups were analyzed using the Mann–Whitney U test or one-way analysis of variance for multiple comparisons. The statistical significance was set at P < 0.05.

3. Results

3.1. Mice Treated with Cefotaxime: Body, Liver, and Fat Pad Weight Increases Under High-Fructose Feeding

The CF-FD group gained more body weight than the VE-FD group and developed obesity. As shown in Table 1, the body weight at the end of the dietary intervention was 35.2 ± 3.4 g for the CF-FD group and 26.1 ± 1.7 g for the VE-FD group (p < 0.05). The body
weight with body weight gain percent of the CF-FD group was significantly more increased compared to that of the VE-FD group from week 2 to week 16 (Figure 1). Moreover, the liver weight was significantly higher in the CF-FD group than in the VE-FD group. The masses of epididymal fat, subcutaneous fat, perirenal fat, and total fat were significantly larger in the CF-FD group than in the VE-FD group. The terminal body weight, liver weight, epididymal fat weight, subcutaneous fat weight, perirenal fat weight, and total fat weight were not significantly different between the CF-CD and VE-CD groups (Table 1). The daily food intake converted to calories was significantly higher in the CD groups than in the FD groups (Figure 1D).

![Figure 1. The effect of high fructose feeding under Cefotaxime treatment on the body weight and food intake.](image)

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<tr>
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<tr>
<td>Body weight (g)</td>
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<td>Liver (g)</td>
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<tr>
<td>Total fat (g)</td>
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CF: Cefotaxime; VE: vehicle; CD: control diet; FD: high fructose diet. Results are expressed as mean ± SD. *p<0.05 compared to the VE-FD group; # p <0.05 compared to the VE-CD group.
3.2. Cefotaxime Treatment Inducing Higher Insulin Resistance Parameters Under High-Fructose Feeding

In the CF-FD group, the serum insulin level was significantly increased than other groups. The serum insulin levels were increased more than 2.2 times in the CF-FD mice compared to those in the VE-FD mice (Figure 2A). The serum glucose level in the CF-FD group was significantly higher than that in the VE-FD group (fasting serum glucose: 352.0 ± 40.0 mg/dL in the CF-FD vs. 275.7 ± 23.1 mg/dL in the VE-FD group) (Figure 2B). HOMA-IR was significantly higher in the CF-FD group than in the VE-FD group (Figure 2C). Among the levels of the four types of adipokines, only the leptin levels were significantly increased. Compared to the VE-FD group, the serum leptin level was increased more than 8.7 times in the CF-FD group (Figure 2D). The serum level of resistin was not different between the CF-FD and VE-FD groups (Figure 2E).

3.3. Cefotaxime Treatment Inducing Hyperlipidemia and Higher Hepatic Lipid Accumulation Under High-Fructose Feeding

We confirmed the development of hepatic steatosis in H&E-stained liver sections and visualized them by Oil Red O staining (Figure 3A). The hepatic steatosis score of the CF-FD group corresponded to moderately macrovesicular and microvesicular steatoses, enhanced in the pericentral area and extended to the entire liver (Figure 3B). In contrast, no steatosis was observed in the other three groups. The Oil Red O staining revealed that the hepatic lipid accumulation was considerably higher in the CF-FD group than in the VE-FD group. Compared to the VE-FD group, the CF-FD group exhibited an exacerbated degree of fatty liver, measured by the liver triglyceride content. The liver TG assay indicated that the CF-FD group exhibited 1.5 times higher value than that of the VE-FD group (312.8 ± 62.6 mg/g in CF-FD vs. 211.5 ± 54.1 mg/g in VE-FD). There were no significant difference between the CF-CD and VE-CD groups (Figure 3C). Although the serum level of TG did not increase in CF-FD, the T-CHO, non-HDL-C, and HDL-C levels were significantly higher in the CF-FD group than in the VE-FD group (T-CHO: 221.1 ± 16.2 mg/dL in CF-FD vs. 135.9 ± 10.7 mg/dL in VE-FD) (Figure 3D–G).
3.4. Cefotaxime Treatment with High-Fructose Diet Inducing Hepatic Inflammation without Fibrosis

The histological and immunohistochemical staining showed that mildly hepatic focal necrosis was observed only in the CF-FD group (Figure 4A). In addition, the liver sections of the CF-FD group exhibited a slightly higher inflammatory cell infiltration than that of the VE-FD group. The immunohistochemical staining showed that the number of CD68-positive cells was higher in the CF-FD group than in the VE-FD group (Figure 4B). The serum levels of ALT and AST were considerably higher in the CF-FD group than in the VE-FD group (ALT: 124.3 ± 93.6 U/L in CF-FD vs. 31.4 ± 12.5 in VE-FD) (Figure 4C,D). In the CF-FD group, the serum IL-6, TNF-α, and LPS levels were not significantly different from those in the VE-FD group (Figure 4E–G). The Fibrosis score revealed that no fibrosis was found in each group (Supplementary Table S1).
3.5. Cefotaxime Treatment with High-Fructose Diet Increasing the Lipogenesis Protein Expression

To elucidate the underlying mechanisms for the fatty liver exacerbation in the CF-FD group, we evaluated the hepatic expression of lipogenesis and lipolysis enzyme by western blotting (Figure 5A). Compared to the VE-FD mice, the protein expression level of hepatic FAS was significantly higher in the CF-FD group (Figure 5B). The protein expression levels of hepatic phospho-ACC were not significantly different between the CF-FD and VE-FD groups (Figure 5C).
3.6. Gut Microbiota Analysis

We analyzed the gut microbiota composition using the 16S rRNA genes in mice fed with high-fructose diet under the cefotaxime treatment. The Shannon index indicated that the bacterial diversity of the CF-FD group was significantly lower than that of the VE-FD group (Figure 6A). The total number of OTUs showed that the species richness of the CF-FD group was significantly lower than those of the other groups. However, the total number showed the absence of significant differences between the CF-CD, VE-CD, and VE-FD groups (Figure 6B). A principal coordinate analysis showed that the mice clustered into relatively distinct groups based on the different diet and cefotaxime treatment conditions according to the distances between the mouse samples. The results were clearly separated into four districts (Figure 6C). These results suggest that the effects of the high-fructose diet and cefotaxime on the gut microbiota might be important factors in this study. The phyla observed in this study were Actinobacteria, Bacteroidetes, Deferribacteres, Firmicutes, Proteobacteria, Patescibacteria, Tenericutes, and Verrucomicrobia. The gut microbiota composition exhibited almost 100% of Firmicutes in the CF-CD and CF-FD groups. However, Firmicutes (47–67%) and Bacteroidetes (48–24%) dominated in the VE-CD and VE-FD groups (Figure 6D). The differential enrichment of specific bacteria at the family level was observed in both cladograms and histograms based on Linear discriminant analysis (LDA) score > 2 for pairwise comparisons. At the family level, the abundance of Lachnospiraceae and Clostridiales XIII was higher in the CF-FD group than in the VE-FD group, while that of Muribaculaceae and Tannerellaceae was higher in the VE-FD group (Figure 7A). The numbers of genera that differed for VE-FD and CF-FD were 19 and 13,
respectively. Among the genera, all 12 belonging to the class level of Clostridia were increased in CF-FD compared to VE-FD (Figure 7B). The other group analysis and comparison results are shown in Supplementary data (Supplementary Figure S2).

Fig. 6. Diversity and similarity of gut microbiota under cefotaxime and high fructose diets. Bacterial diversity estimate by (A) Shannon index and species richness estimates by (B) Chao1 (C)Total number (D) PCoA plot with UniFrac distances (E) Relative abundance of gut microbiota
Fig 7. Comparison of gut microbiota composition under cefotaxime treatment and high fructose feeding. (A) Taxonomic representation of statistically and biologically consistent differences between VE-FD and CF-FD group. Differences are represented by the color of the most abundant class (red indicates CF-FD, and green indicates VE-FD). (B) Histogram of the LDA scores for differentially abundant between CF-FD and VE-FD. The cladogram was calculated by LEfSe, a metagenome analysis of abundant taxons of OTUs, Only taxa meeting an LDA significant threshold > 2 and p <
4. Discussion

This study shows that cefotaxime (third-generation antibiotic) could induce composition changes in gut microbiota, which further affects the physiological and metabolic profiles of liver lipogenesis under the high-fructose consumption. The synergistic cefotaxime high-fructose diet exacerbated the body weight gain, liver fat accumulation, and insulin-resistance effects and partially modified the gut microbiota composition. The data, for the first time, demonstrate that gut microbiota has a crucial role to mediate the NAFLD by a single cefotaxime pretreatment with the high-fructose diet. Moreover, various lipogenic effects, including the blood glucose level and serum cholesterol level, were up-regulated. Therefore, the cefotaxime treatment largely facilitates lipid metabolism regulatory disorders and NAFLD in gut microbiota dysbiosis.

Recent studies revealed that the final body weights of animals after 8-week or 12-week high-fructose diet feeding were not statistically different [6, 19]. In the CF-CD, VE-CD, and VE-FD groups, the body weight did not largely increase during the 16-week experimental period. Moreover, the body weight of the VE-FD group was consistent with the study by Dai and McNeill, who reported absence of increase in the body mass with the increase in the consumption of fructose, even after 14 weeks [20]. Similarly, another study using a 60% fructose diet demonstrated the absence of association with the insulin resistance during chronic ingestion of fructose in Sprague–Dawley rats [19]. Notably, we demonstrated that the total fat mass and epididymal and retroperitoneal deposits were significantly larger in CF-FD, which shows that cefotaxime may contribute and synergistic to high fructose diet-induced adipogenesis in liver and fat tissue.

A third-generation broad-spectrum oral cephalosporin (cefdinir) might alter the gut microbiota (particularly Gram-negative bacteria) to ameliorate high-fructose-diet-induced diabetes, according to a rat study [21]. Moreover, the intestinal microflora across the subjects with type-2 diabetes was relatively enriched with Gram-negative bacteria, belonging to the phyla Bacteroidetes and Proteobacteria. These alterations were related to a reduction in body weight gain and improvement in the inflammatory and metabolic health of the host. In this study, almost 100% of Firmicutes were present in the CF-FD group, compared to the other groups, which was related to the increased weight gain, NAFLD exacerbation, and insulin resistance. The cefotaxime with fructose diets caused a severe NAFLD, correlated with the increased abundance of Lachnospiraceae and Clostridiales XIII in the gut microbiota composition. Our results are inconsistent with those of Di Luccia et al., who reported that Coprococcus and Ruminococcus for members of the Coriobacteria family were rescued by an antibiotic mix (ampicillin + neomycin) treatment [22]. Bier et al. reported that a treatment with the same antibiotic mix could not ameliorate the metabolic changes in rats exhibiting dysbiosis after consuming a high-fructose diet [19].

One of the most striking observations following the cefotaxime treatment was the enhanced hepatic steatosis and hypertrophy of white adipocytes in different fat pads in the high-fructose-diet-fed group compared to the other groups. The histological characteristics of the present mouse model, such as the microvesicular and macrovesicular steatosis, were similar to those of human NAFLD [23]. Moreover, the number of CD68-positive cells and serum levels of ALT were higher in the CF-FD group than in the other groups. These results clarify that the combination of cefotaxime with high-fructose-diet might cause a slightly inflammation in the liver. In this study, although the serum levels of IL-6 and TNF-α did not differ between the groups. These findings might suggested that the high fructose intake and CF treatment only trigger hepatic steatosis and mimic local inflammation.

Adipokines are cytokines secreted by adipose tissues. Leptin is a hormone produced directly from adipocytes, associated with T2DM, characterized by insulin resistance. In case of obesity, humans and rodents exhibit high serum levels of leptin [24, 25], which indicates that serum leptin might be a predictive risk factor for insulin resistance. In this
study, the serum levels of insulin, resistin, glucose, and HOMA-IR were higher in the CF-FD group than in the other groups. Literature data indicate that, in rats fed with a fructose-rich diet, the development of metabolic syndrome directly correlates with variations in the gut concentrations of specific bacterial taxa [22]. In addition, the Coprococcus and Ruminococcus levels were increased by the fructose-rich diet. The correlation between their abundance and development of metabolic syndrome was demonstrated. Our finding is inconsistent with a previous report indicating that the above taxonomy was present in the VE-FD group.

Several studies suggest strong correlations between the gut microbiota and inflammatory cytokines and their contribution to the progression of NAFLD [23,27]. NAFLD/Nonalcoholic steatohepatitis is associated with increased levels of Gram-negative microbiomes and endotoxemia [26, 27]. We analyzed possible correlations between NAFLD and Gram-negative microbiomes, the source of LPS [28]. Our results showed that the LPS levels in the VE-CD group were lower than those of the other groups. Additionally, we observed an increased serum endotoxin level and increased incidence of endotoxemia in the VE-FD, CF-CD, and CF-FD groups. However, the further analysis of the data revealed no correlation with the concentration of serum endotoxin. Therefore, the CF-FD animal model might only slightly induce endotoxin production to contribute to hepatitis.

Previous studies demonstrated decreased Bacteroidetes and increased Firmicutes levels in NAFLD patients compared to healthy individuals, which suggests an association between the Bacteroidetes/Firmicutes ratio and NAFLD [14, 15, 29]. In the CF-CD and CF-FD groups, almost 100% of Firmicutes were observed. This gut microbiota composition was not consistent with that reported by Bier et al., who reported that a high-fructose diet versus the control diet led to significantly lower levels of Firmicutes and higher levels of Bacteroidetes, and consequently to a reduced Firmicutes/Bacteroidetes ratio [19]. However, in this study, Firmicutes (47–67%) and Bacteroidetes (48–24%) were dominant in the VE-CD and VE-FD groups. These changes in Bacteroidetes and Firmicutes abundances by fructose have also been detected in a recent study on Fischer F344 rats [30]. In this study, at the family level, the abundance of Lachnospiraceae and Clostridiales XIII was higher in the CF-FD group than in the VE-FD group. Lachnospiraceae and Clostridiales XIII are two families of the Clostridia class of Firmicutes. We considered that these two families contributed to the pathological characteristics under the high-fructose diet and led to significant differences in steatosis compared to the other groups.

In this study, we developed a simple strategy to induce NAFLD and gut microbiota dysbiosis through a single cefotaxime treatment with a high-fructose diet. This mouse model indicated that the gut microbiota modulation by cefotaxime under the high-fructose diet has an aggravative role in the NAFLD and insulin resistance. This novel animal model can be further applied in NAFLD drug development in the future.

**Supplementary Materials:** Table S1: Liver fibrosis score, Figure S1: Taxonomic representation of each groups. Differences are represented by the color of the most abundant class. The cladogram was calculated by LEfSe, a metagenome analysis of abundant taxons of OTUs, Only taxa meeting an LDA significant threshold > 2 and p < 0.05 are shown.

**Author Contributions:** Conceptualization, C.-C.C. and H.-L.C.; methodology, W.-C.H., Y.-C.W., C.-F.C., T.-J.L., S.-W.H. and J.-Y.L.; software, Y.-P.L. and Y.-H.C.; validation, W.-C.H. and S.-W.H.; writing—original draft preparation, Y.-P.L.; writing—review and editing, Y.-P.L., C.-C.C. and H.-L.C.; visualization, Y.-P.L.; supervision, W.-C.H., C.-C.C. and H.-L.C.. All authors have read and agreed to the published version of the manuscript.

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