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► To cite this version:

Zahra Safari, Magali Monnoye, Peter M. Abuja, Mahendra Mariadassou, Karl Kashofer, et al.. Steatosis and gut microbiota dysbiosis induced by high-fat diet are reversed by 1-week chow diet administration. *Nutrients*, 2019, 71, pp.72-88. 10.1016/j.nutres.2019.09.004 . hal-02503316

HAL Id: hal-02503316

<https://hal.science/hal-02503316>

Submitted on 9 Mar 2020

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Steatosis and gut microbiota dysbiosis induced by high-fat diet are reversed by 1-week chow diet administration

Zahra Safari^{a,b}, Magali Monnoye^b, Peter M. Abuja^a, Mahendra Mariadassou^c, Karl Kashofer^a, Philippe Gérard^{b,*}, Kurt Zatloukal^{a,**}

^a Institute of Pathology, Medical University of Graz, 8010 Graz, Austria

^b Micalis Institute, INRA, AgroParisTech, Université Paris-Saclay, 78350 Jouy-en-Josas, France

^c MaIAGE, UR1404, INRA, 78350 Jouy-en-Josas, France

ARTICLE INFO

Article history:

Received 24 April 2019

Revised 26 August 2019

Accepted 13 September 2019

Keywords:

Reversibility

Microbiome

Liver steatosis

NAFLD

High-fat diet

Diet switch

ABSTRACT

Many studies have recently shown that diet and its impact on gut microbiota are closely related to obesity and metabolic diseases including nonalcoholic fatty liver disease. Gut microbiota may be an important intermediate link, causing gastrointestinal and metabolic diseases under the influence of changes in diet and genetic predisposition. The aim of this study was to assess the reversibility of liver phenotype in parallel with exploring the resilience of the mice gut microbiota by switching high-fat diet (HFD) to chow diet (CD). Mice were fed an HF for 8 weeks. A part of the mice was euthanized, whereas the rest were then fed a CD. These mice were euthanized after 3 and 7 days of feeding with CD, respectively. Gut microbiota composition, serum parameters, and liver morphology were assessed. Eight weeks of HFD treatment induced marked liver steatosis in mice with a perturbed microbiome. Interestingly, only 7 days of CD was enough to recover the liver to a normal status, whereas the microbiome was accordingly reshaped to a close to initial pattern. The abundance of some of the bacteria including *Prevotella*, *Parabacteroides*, *Lactobacillus*, and *Allobaculum* was reversible upon diet change from HFD to CD. This suggests that microbiome modifications contribute to the metabolic effects of HFD feeding and that restoration of a normal microbiota may lead to improvement of the liver phenotype. In conclusion, we found that steatosis and gut microbiota dysbiosis induced by 8 weeks of high-fat diet can be reversed by 1 week of chow diet administration, and we identified gut bacteria associated with the metabolic phenotype.

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Abbreviations: CD, chow diet; H&E, hematoxylin and eosin; HFD, high-fat diet; NAFLD, nonalcoholic fatty liver disease; TG, triglyceride.

* Corresponding author. Tel.: +33 1 34 65 24 28.

E-mail addresses: philippe.gerard@inra.fr (P. Gérard), kurt.zatloukal@medunigraz.at (K. Zatloukal).

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) comprises a spectrum of disease ranging from simple and usually nonprogressive steatosis to nonalcoholic steatohepatitis (NASH) [1,2]. Obesity and insulin resistance have been identified as major NAFLD risk factors [3]. NAFLD is associated with altered hepatic lipid metabolism leading to fat deposition and de novo lipid synthesis in the liver. In recent years, evidences suggest an

<https://doi.org/10.1016/j.nutres.2019.09.004>

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involvement of the microbiota in NAFLD development [4]. In an early study, Drenick et al [5] observed hepatic steatosis development in patients undergoing gastric bypass surgery that coincided with bacterial overgrowth. Subsequently, small intestinal bacterial overgrowth has been shown to be more prevalent in patients with NASH than in healthy controls [6]. Later, Henao-Mejia et al [7] provided evidence that modulation of the intestinal microbiota through multiple inflammasome components is a critical determinant of NAFLD and NASH progression, whereas it has been demonstrated the gut microbiota protects against steatohepatitis in the methionine-choline-deficient model [8]. Using microbiota transplants strategy, we have further revealed that the propensity to develop NAFLD directly depends on gut microbiota composition, independently of obesity [9]. Altogether, these findings suggest that high-fat diet (HFD) alone is not sufficient to cause obesity or NAFLD development and that the gut microbiota contributes to these diet-induced metabolic disorders.

Dietary lipids have been linked to the development of hepatic steatosis and NASH in both animal models and humans; however, their role in pathophysiology of NAFLD remains unclear [10–12]. In the liver, an HFD leads to exaggerated free fatty acid levels, which induce hepatic insulin resistance, decreased fatty acid oxidation, and de novo lipogenesis in hepatocytes, causing weight gain and hepatic steatosis [13]. Lieber et al [14] used an HFD in male Sprague-Dawley rats and demonstrated the development of steatosis within 3 weeks associated with insulin resistance and increased markers of fibrogenesis. Similar results were reported subsequently in male C57BL/6 mice fed with an HFD for up to 16 weeks. HFD-fed mice demonstrated an increase in body weight, steatosis development, hepatocyte ballooning, augmented fasting serum glucose levels, and reduced adiponectin levels, suggesting hyperglycemia and insulin resistance [15]. Therefore, HFD treatments of rodents are commonly used as obesity and NAFLD models and are considered closer to human pathologies than models of gene inactivation. This is important because of the growing obesity epidemic [16] and the importance of overnutrition in the development of diseases, such as the metabolic syndrome and NAFLD [17–19].

Dietary changes have also been demonstrated to be central drivers of microbiome composition and function, and mice fed HFD have different microbiota composition from those that have been fed control or balanced diets [20,21]. Moreover, feeding different types of fat results in very different compositions of gut microbiota in mice. Similarly, the high-fat/high-sugar diet associated with Western lifestyle has altered the composition and metabolic activity of the human gut microbiome [22]. Such diet-induced changes to gut-associated microbial communities are now suspected of contributing to growing epidemics of chronic illness in the developed world, including obesity [23,24] and NAFLD [7,9,25].

Animal and human studies have shown the reversibility of NAFLD [26–28]. Indeed, in mild forms, fatty liver can be a reversible condition that could be improved with lifestyle modifications such as weight loss, diet changes, and increased physical activity. It was also observed that when obese humans changed their diet to restrict fat or carbohydrate, their gut microbiota shifted from an “obese” to a “lean” microbiome composition [23]. However, Thaiss et al [29] have recently discovered that although many of the

structural alterations of the microbiome are reversible upon weight normalization after obesity, certain elements of the obesity-associated composition and function persist after dieting even when many of the metabolic parameters have already normalized to preobesity levels.

Therefore, we propose the hypothesis that the effect of diet may be partly mediated through the intestinal microbiome whose products are transported into the liver and from there are transmitted to the rest of the body contributing to disease (or health) [30,31]. This implies that targeting the gut bacteria to restore a balanced microbiome could lead to disease improvement. The understanding of this complexity is changing nutrition science particularly related to liver diseases, and it is of considerable interest to decipher the reversibility of both NAFLD and gut microbiome dysbiosis and how they are related. Therefore, the objective of this study was to investigate to what extent the HF-induced steatosis and dysbiosis can be reversed by a chow diet and to identify which gut bacteria could be involved in disease development.

2. Methods and materials

2.1. Animal experiments

The experiments were carried out with male C57BL/6J mice obtained from Charles River (Sulzfeld, Germany) at the age of 8 weeks. The animals were housed 4 per cage under controlled conditions of temperature (set point 21°C) and air humidity (set point 50%) and under a 12-hour light/dark cycle. The bedding of the cages was mixed, and mice were allowed to acclimate for 2 weeks prior to start of the experiment while being fed a CD. Twelve mice were used for the study. HFD (E15741-347 γ -irradiated 25 kGy) and chow diet (control diet, CD; V1534-703 γ -irradiated 25 kGy) used in this study were

Table 1 – Ingredient composition of the CD

Ingredient	Amount (g/kg)
Wheat meal	220.00
Wheat middlings	100.00
Wheat feed	180.00
Barley	160.00
Oat hulls and bran	31.00
Soybeans/soybean meal (heat treated/toasted)	215.00
Soybeans concentrate (heat treated/toasted)	12.50
L-Lysine HCl	1.00
DL-Methionine	0.30
Vitamin and trace element premix with cereals as carrier	10.00
Choline chloride	3.20
Salt (NaCl)	6.00
Calcium phosphate, monobasic	10.00
Calcium carbonate	12.50
Calcium propionate	5.00
Sorbic acid	0.50
Sugar beet pulp	33.00

For vitamin mix and trace element composition, see Table 4. The kJ percentages for fat, protein, and carbohydrate were 9, 24, and 67, respectively.

Table 2 – Ingredient composition of the HFD

Ingredient	Amount (g/kg)
Casein	277.00
Maltodextrin	156.00
Sucrose	84.90
Cellulose powder	60.00
L-Cystine	3.50
Vitamin premix	10.00
Mineral and trace element premix	60.00
Choline chloride (50% choline)	2.50
Butylated hydroxytoluene	0.10
Beef tallow, premier Jus	315.00
Soybean oil	31.00

For vitamin mix and trace element composition, see Table 4. The kJ percentages for fat, protein, and carbohydrate were 60, 20, and 20, respectively.

Table 3 – Mineral, fatty acid, and amino acid composition of the CD and HFD

Minerals	CD	HFD
	g/kg diet	
Calcium	10.0	9.2
Phosphorus	7.0	6.4
Sodium	2.4	2.0
Magnesium	2.2	2.3
Potassium	9.2	9.7
Fatty acids	CD	HFD
	g/kg diet	
12:0	–	0.4
14:0	0.1	11.8
16:0	4.5	82.7
17:0	–	3.8
18:0	0.9	60.6
20:0	0.1	0.4
16:1	0.1	1.33
18:1	6.2	122.9
18:2	17.6	25.3
18:3	2.3	3.4
Amino acids	CD	HFD
	g/kg diet	
Lysine	11.0	20.2
Methionine	3.8	8.6
Cystine	3.5	4.5
Threonine	7.2	10.7
Tryptophan	2.5	3.3
Arginine	11.9	9.5
Histidine	4.9	7.4
Valine	9.2	17.0
Isoleucine	7.9	13.8
Leucine	13.9	24.2
Phenylalanine	8.9	12.7
Glycine	8.9	5.2
Glutamic acid	42.2	55.0
Aspartic acid	18.4	18.2
Proline	13.1	28.0
Serine	10.1	14.6
Alanine	8.7	8.1

Table 4 – Vitamin and trace element composition in the CD and HFD

Vitamins	CD	HFD
	per kg diet	
Vitamin A	25 000 IU	15 000 IU
Vitamin D3	1500 IU	1500 IU
Vitamin E	135 mg	150 mg
Vitamin K (as MNB)	20 mg	20 mg
Thiamine (B1)	86 mg	25 mg
Riboflavin (B2)	32 mg	16 mg
Pyridoxine (B6)	31 mg	16 mg
Cobalamin (B12)	150 µg	30 µg
Nicotinic acid	153 mg	47 mg
Pantothenic acid	59 mg	55 mg
Folic acid	10 mg	16 mg
Biotin	710 µg	300 µg
Choline	1370 mg	920 mg
Trace elements	CD	HFD
	mg/kg diet	
Iron	186	168
Manganese	68	95
Zinc	91	65
Copper	15	13
Iodine	2.1	1.2
Selenium	0.3	0.2

purchased from SSNIFF (Soest, Germany). Diet ingredient compositions of the diets are given in Tables 1–4. Throughout the study, mice were weighed once weekly. All mice were first fed HFD for 8 weeks. Four mice were immediately euthanized at the end of the eighth week of HFD (HF group). The remaining mice were switched to the CD. From these mice, 4 were euthanized after 3 days of feeding CD (3d-CD group), and the remaining 4 mice were euthanized after 1 week of feeding CD (7d-CD group). Stool pellets were collected before HFD treatment and at the end of experiment for each group.

The blood was taken via cardiac puncture while the mice were under deep anesthesia using 1%–1.5% isoflurane in the oxygen via anesthesia machine (EZ-SA800 Single Animal System; Elst, Plexx, Netherland) and then euthanized by cervical dislocation. Serum parameters were measured in an automatic chemistry analyzer (Hitachi, Roche, Vienna, Austria). Livers were immediately removed and cut into pieces which were (1) snap frozen in methylbutane precooled with liquid nitrogen for staining, (2) fixed in 10% buffered formalin for histological/immunohistochemical analysis, or (3) placed in liquid nitrogen for triglyceride (TG) measurement. Stool pellets were taken individually from each mouse and stored immediately at –80°C until used. Animal experiments were approved by the federal animal care committee and conducted in compliance with the Austrian Law for Welfare of Laboratory Animals. (license no: BMWF-66.010/0017-II/3b/2014).

2.2. Liver histopathology

2.2.1. Hematoxylin and eosin staining

Hematoxylin and eosin (H&E) staining was performed on 5-µm-thick formalin-fixed paraffin-embedded liver sections. Paraffin

was removed from formalin-fixed paraffin-embedded liver sections by incubating at 65°C for 30 minutes followed by incubation in xylene for 15 minutes. The slides were rehydrated for 2 minutes each with decreasing concentrations of ethanol (90%, 70%, and 50%). After a brief wash with dd H₂O, the liver sections were stained with hematoxylin for 2 minutes followed by 30-second staining with eosin. Thereafter, the liver sections were washed with warm tap water for 2 minutes and mounted with Entellan (Merck, Guyancourt, France).

2.2.2. Liver scoring

The liver sections were analyzed on 5 fields (objective magnification 20×) per section for 3 criteria—steatosis, inflammation, and swelling of the cells—using the Brunt scoring [32]. Hepatic steatosis was assessed with a score ranging from 0 to 3 according to the accumulation of lipids in hepatocytes. A score of 0 was assigned to sections where lipids represented less than 5% of the cell, and a score of 3 was assigned to those where lipid accumulation occupied more than 66% of the cell surface. The inflammation of the tissue was also assessed with a score ranging from 0 to 3. These ratings were attributed according to the number of inflammatory foci observed on a field. If there was none, the score was 0; when there were more than 4, the score was 3. Finally, a score between 0 and 2 was assigned according to the ballooning of the cells. When the cell had a normal size for a hepatocyte, the score was 0; if the shape changed and showed ballooning, particularly, if ballooning were repeated within the same field, then the score was 3 [32].

2.2.3. Hepatic TG content

About 50 mg of frozen liver was homogenized in chloroform-methanol (2:1) to extract total lipids as previously described [33]. The organic extract was dried and reconstituted in isopropanol. Triglycerides were quantified using Triglycerides Colorimetric Assay kit (10010303; Cayman Chemical Company, Ann Arbor, MI, USA) and expressed as mg/g liver.

2.3. Microbiome analysis

2.3.1. DNA isolation and polymerase chain reaction amplification

Bacterial DNA was extracted using Maxwell RSC Blood DNA Kit (Promega, Mannheim, Germany) according to the manufacturer's instructions with minor modifications. Stool samples were homogenized on a MagNA Lyser Instrument using the Lysis Buffer and MagNA Lyser Green Beads (Roche Diagnostics GmbH, Mannheim, Germany). After homogenization, 2.5 mg/mL Lysozyme (Roth GmbH, Karlsruhe, Germany) was added to the samples and kept for 30 minutes at 37°C followed by digestion with 1 mg/mL Proteinase K for 60 minutes at 56°C. Enzyme activity was inactivated for 10 minutes at 95°C, and 600 μ L of lysate was used for the DNA isolation in the Maxwell RSC. DNA concentration was measured using Picogreen fluorescence. The hypervariable V4 region of the bacterial 16S rRNA gene was amplified with polymerase chain reaction (PCR) from 20 ng DNA using oligonucleotide primers 16s_515_S3_fwd: GATTGCCAGCAGCCGGTAA and 16s_806_S2_rev: GGACTACCAGGTATCTAAT. This 16S rDNA region was selected because of its high domain specificity and broad coverage of gastrointestinal bacteria compared with other variable regions [34]. Bacterial 16S rRNA was amplified using

Mastermix 16s Complete PCR Kit (Molzym, Bremen, Germany). The first PCR product was exposed to a second PCR with primers fusing the 16s primer sequence to the A and P adapters required for Ion Torrent sequencing while additionally including a molecular barcode sequence to allow multiplexing of up to 96 samples at the same time. PCR products were separated by electrophoresis in agarose gel, and the band of the expected length (350 nt) was removed from the gel and purified using the QiaQick (Qiagen, Hilden, Germany) gel extraction system. Then, DNA concentration of the final PCR product was measured by Picogreen fluorescence.

2.3.2. Sequencing

Amplicons were pooled equimolarly and amplified with emulsion PCR using the Ion Torrent One Touch 2.0 Kit according to manufacturer's instructions. Then, the beads were purified on Ion ES station transferred to Ion Torrent 318 chips for sequencing. Sequencing was performed on Ion Torrent PGM using the Ion 400BP Sequencing Kit running for 1000 flows (all reagents from Thermo Fisher Scientific, Waltham, MA, USA). Sequences were sorted by barcode and transferred to the Torrent Suite server. Unmapped bam files were then used as input for bioinformatic analysis.

2.3.3. Bioinformatics and phylogenetic analysis

All sequences were trimmed by a sliding window quality filter with a width of 20 nt and a cutoff of Q20. Reads mapping to the human genome and reads shorter than 100 nucleotides were removed using deconseq [35]. The resulting reads were subjected to error correction using the Acacia tool [36], leading to error correction of 10%-20% of reads. Then, PCR chimeras were removed by usearch algorithm in de novo and reference-based settings [37]. The final sequence files were analyzed by QIIME 1.8 workflow scripts [38]. OTU search was performed using the parallel_pick_open_reference_otus workflow script and the green genes 13_8 reference database.

2.4. Statistical analyses

Using the QIIME core microbiome script, OTUs were visualized as OTU tables, bar charts, and PCOA plots. Additionally, groupings supplied in the mapping file were tested for statistical significance using the QIIME implementation of the Adonis test. Significance of individual bacterial strains was determined by Kruskal-Wallis test. Multiple testing corrections (false discovery rate) were used to avoid false positives (significance threshold = 0.05). For statistical analysis, R 3.5 software using the package Bioconductor 3.8 and GraphPad Prism 6 were used.

3. Results

To investigate the effects of HFD on microbiome and liver phenotype, the following experimental setup was used: twelve 8-week-old male C57BL/6 J mice were fed HFD for 8 weeks. Four mice were then euthanized, and livers were isolated (termed as HF). The remaining mice were switched to CD and euthanized after 3 (n = 4) and 7 days (n = 4) (termed as 3d-CD and 7d-CD, respectively) (Fig. 1A). The mice (n = 12)

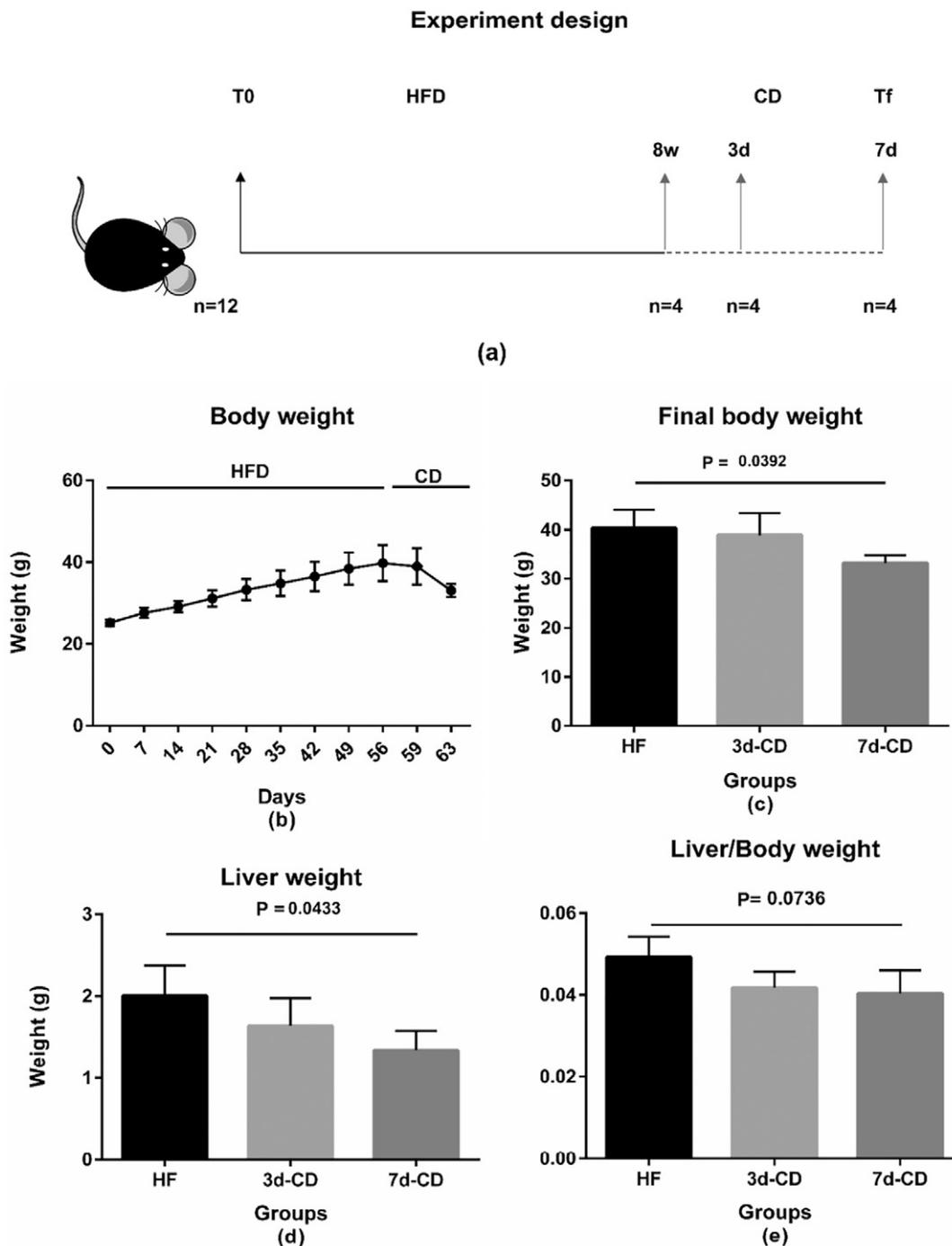


Fig. 1 – Experimental design and body and liver weights. (A) 12 mice received HFD for 8 weeks. Four mice were euthanized at the end of the HFD period (HF). The remaining mice were switched to a CD for additional 3 (3d-CD) and 7 days (7d-CD), respectively. (B) Body weight curve, (C) final body weight, (D) liver weight, and (E) liver to body weight ratio in the 3 studied groups. Values are means with error bars using SD. Groups were compared using Tukey multiple comparison test. P values were calculated at a 95% confidence level. Each group contains n = 4 mice.

displayed significant weight gain during the 8-week HFD period (Fig. 1B). The average of the weight gain for all the mice after 8 weeks of HFD was 14.43 g. Switching to CD led to an average decrease in body weight of 1.57 g at 3 days and 3.03 g at 7 days, the latter being significant ($P = .0392$) (Fig. 1C). The

liver weight in the 7d-CD group also showed significantly lower value compared to the HF group ($P = .0433$) (Fig. 1D). Moreover, the liver to body ratio in the same group also showed a trend to be lower as compared to the HF group ($P = .0736$) (Fig. 1E).

3.1. CD reverses the HFD-induced steatosis

Histology and liver scoring showed evidence of steatosis in the HF and 3d-CD groups, whereas steatosis was absent in the 7d-CD group. No inflammation and ballooning were observed in the liver of any mouse (Fig. 2A and C). The mice with 1 week of CD showed significantly lower fat content in the hepatocytes, indicating that 7 days of CD is necessary and sufficient to reverse 8-week-HFD-induced steatosis in C57BL/6 J mice (Fig. 2A). The triglyceride assay also confirmed the significant difference in the fat deposited in the liver between the HF group (7.237 ng/mg tissue) and the group additionally treated for 1 week with CD (7d-CD) (2.990 ng/mg tissue) ($P = .0132$) (Fig. 2B). A significant difference in the TG levels was also revealed between the 3d-CD (6.457 ng/mg tissue) group and 7d-CD group ($P = .0372$), further supporting that 7 days of CD is needed to reverse hepatic triglyceride accumulation (Fig. 2B).

Based on these results, we chose to compare 7d-CD with HF to further investigate the capacity of CD to reverse the effects of HFD feeding. Therefore, the following figures will present results obtained in mice euthanized after 8 weeks of HFD (HF group) and in mice euthanized after 8 weeks of HFD followed by 7 days of CD (7d-CD group).

To better define the ability of 7 days of CD to reverse the adverse metabolic effects of HFD, we assayed serum parameters including albumin, alkaline phosphatase, triglyceride, lactate dehydrogenase, aspartate aminotransferase, alanine transaminase, glucose, and cholesterol. Among these parameters, glucose and cholesterol were found to be significantly reduced in 7d-CD vs HF group ($P < .0001$ and $P = .0216$, respectively) (Table 5). In addition, the ALT level showed a trend for being decreased in 7d-CD vs HF group ($P = .154$). No differences were found between the 2 groups for the other parameters measured (Table 5).

3.2. CD reverses the HFD-induced dysbiosis

Replacing HFD with CD for 1 week normalized body weight and reversed the hepatic steatosis observed in HF group mice. To decipher if these changes in mice phenotype were associated with modifications of the microbiome, the stool pellets from mice of both groups (HF and 7d-CD) were taken before HFD treatment (T0) and at the end of experiment for each group (after 8 weeks of HFD for HF group [HF-Tf] and after 8 weeks of HFD followed by 1 week of CD for 7d-CD group [7d-CD-Tf]) and analyzed using 16S rRNA sequencing.

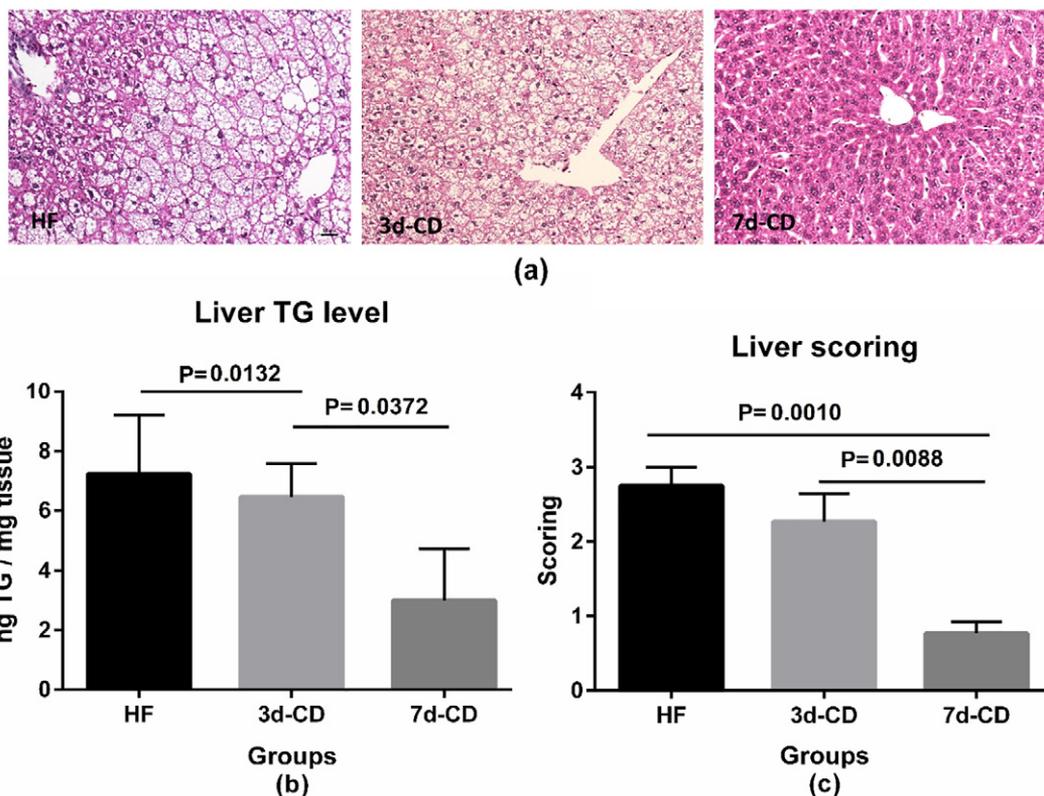


Fig. 2 – (A) Representative H&E staining of liver sections (5 μm , 20 \times magnification). The histology of the liver stained with H&E technique showed that the liver of the mice treated with control diet for 7 days after 8 weeks of HFD treatment (7d-CD) had less fat and displayed a normal status compared to HF (fed with HFD) and 3d-CD (fed with HFD plus 3 days of control diet) groups. (B) Quantification of liver TG levels per tissue weight (mg) in HF, 3d-CD, and 7d-CD groups. (C) Liver scoring. Histological grading and staging system for liver steatosis was based on the Brunt scoring described by Tiniakos et al [32]. Values are means with error bars using SD. Groups were compared using Tukey multiple comparison test. P values were calculated at a 95% confidence level. Each group contains $n = 4$ mice.

Table 5 – Serum parameters measured in HF (fed with HFD for 8 weeks) vs 7d-CD (fed with HFD for 8 weeks plus 1 week of control diet) groups

Groups	Alanine aminotransferase (U/L)			Alkaline phosphatase (U/L)		
	Mean	SD	P value	Mean	SD	P value
HF	20.5000	8.7369	.1548	22.2500	4.2720	.4224
7d-CD	12.5000	4.5092		18.5000	7.5939	
Groups	Aspartate aminotransferase (U/L)			Serum albumin (g/dL)		
	Mean	SD	P value	Mean	SD	P value
HF	70.0000	58.3267	.6437	1.1250	0.0500	.0871
7d-CD	55.0000	19.9332		0.9250	0.1893	
Groups	Serum cholesterol (mg/dL)			Serum glucose (mg/dL)		
	Mean	SD	P value	Mean	SD	P value
HF	57.5000	1.9149	<.0001	86.0000	5.4772	.0216
7d-CD	30.5000	2.3805		67.7500	10.5000	
Groups	Lactate dehydrogenase (U/L)			Serum triglyceride (mg/dL)		
	Mean	SD	P value	Mean	SD	P value
HF	264.7500	170.6778	.6119	23.5000	5.1962	.3679
7d-CD	213.7500	84.9799		20.7500	2.2174	

Data are presented as means with standard deviation; n = 4 per group. For comparing the groups, an unpaired t test was used. P values were calculated at a 95% confidence level.

Rarefaction curves suggested that richness was lower at the end of the experiment in both HF and 7d-CD groups compared to T0, indicating that HFD reduced the bacterial richness which was not restored by 1 week of CD (Fig. 3). Multidimensional scaling analysis first showed that before HFD treatment (T0), all mice

clustered together, indicating similar gut microbiota composition (Fig. 4). HFD treatment greatly modified the microbiota composition leading to a separate cluster (HF-Tf). Finally, 7 days of CD led to a new cluster (7d-CD-Tf) closer but slightly different from the original one. These results indicate that HFD strongly affects

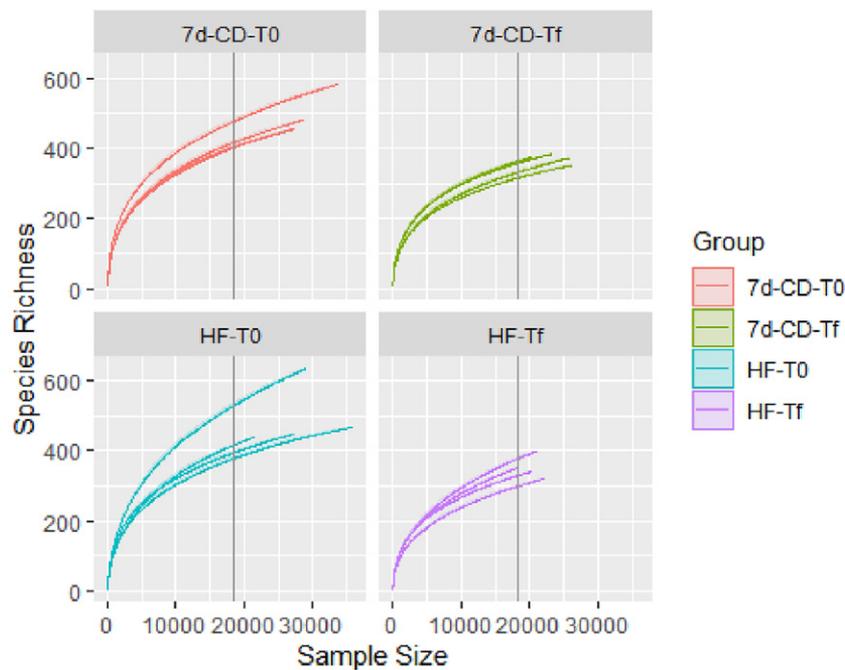


Fig. 3 – Rarefaction curves used to estimate richness in HF (fed with HFD for 8 weeks) vs 7d-CD (fed with HFD for 8 weeks plus 1 week of control diet) group at T0 (before HFD treatment) and Tf (after HFD or after HFD and control diet).

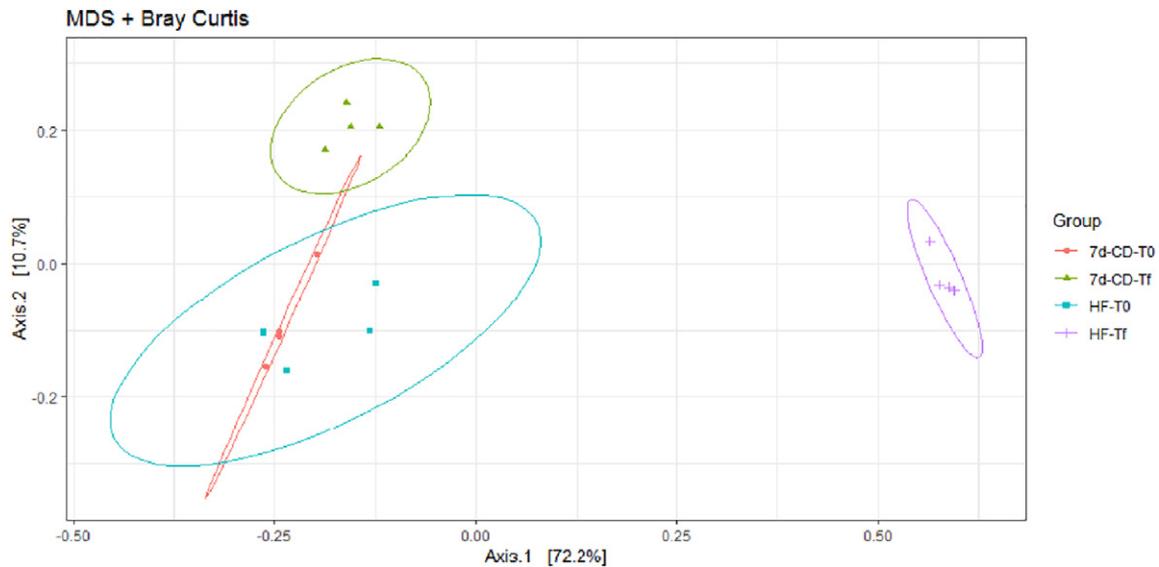


Fig. 4 – β -Diversity analysis via nonmetric multidimensional scaling of Bray-Curtis Dissimilarity Indices. Each dot represents an animal, projected onto first (horizontal axis) and second (vertical axis) variables. The ellipse determines the 95% confidence interval.

gut microbiota composition, which is partially restored by 1 week of CD.

Using a Venn diagram, we compared the OTUs shared between by the mice before (T0) ($n = 8$) and after treatment (HF-Tf [$n = 4$] and 7d-CD-Tf [$n = 4$]). The results confirmed β -diversity analysis which showed that bacterial community from 7d-CD-Tf is closer to initial bacterial community than the one from HF-Tf. Indeed, 268 OTUs were shared between baseline (T0) and 7d-CD-Tf microbiomes, whereas only 183 OTUs were shared between baseline (T0) and HF-Tf microbiomes (Fig. 5). Interestingly, 151

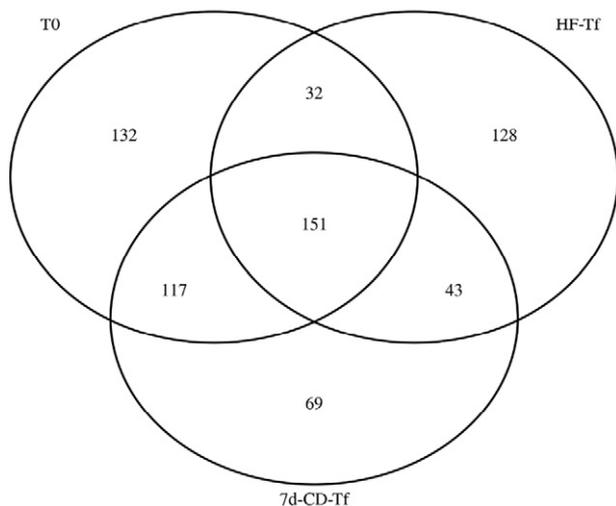


Fig. 5 – Venn diagram based on OTUs distribution (at 3% dissimilarity) showing the OTUs shared by the mice before (T0) ($n = 8$) and after treatment (HF-Tf [$n = 4$] and 7d-CD Tf [$n = 4$]).

OTUs were present in the 3 compared microbiomes, indicating that the presence of these bacterial species was not affected by the changes in diet (Fig. 5).

The 16S rRNA gene sequencing of mice fecal samples revealed that the observed species and the richness (Chao index) were reduced in both HF and 7d-CD groups, further indicating that diversity was decreased by HFD and not restored by 1 week of CD (Fig. 6). However, Shannon diversity index was found higher in 7d-CD-Hf compared to HF-Tf, indicating a less biased community structure (more equal abundances of the bacterial species) after 7 days of CD (Fig. 6).

To further assess the effects of the diet treatments on microbiota, we analyzed microbiota composition in mice before (HF-T0) and after (HF-Tf) HFD, and before (7d-CD-T0) and after (7d-CD-Tf) HFD + 1 week of CD. In all mice groups, the most abundant phyla include Bacteroidetes, Deferribacteres, Firmicutes, Tenericutes, and Verrucomicrobia (Fig. 7). The population of Bacteroidetes was decreased by the HFD treatment, whereas it was found to be increased in 7d-CD mice at the end of the experiment (Figs. 7 and 8). HFD treatment also led to increased populations of Firmicutes and Actinobacteria which were not found in 7d-CD mice. Accordingly, the Firmicutes to Bacteroidetes ratio was found to be significantly higher in HF-Tf group compared to other studied groups. Tenericutes almost disappeared following HFD treatment, whereas its abundance was similar in 7d-CD group before and after treatment (Figs. 7 and 8). These results indicate that, at the phylum level, 1 week of CD is sufficient to markedly abolish HFD-induced microbiota changes.

At the family level, Porphyromonadaceae, Clostridiaceae, Lactobacillaceae, and Erysipelotrichaceae were highly increased by the HFD treatment, whereas their abundance was similar in 7d-CD group before and after treatment (Figs. 9 and 10), indicating that 1 week of CD is sufficient to abolish the impact of 8 weeks of HFD on these bacterial populations.

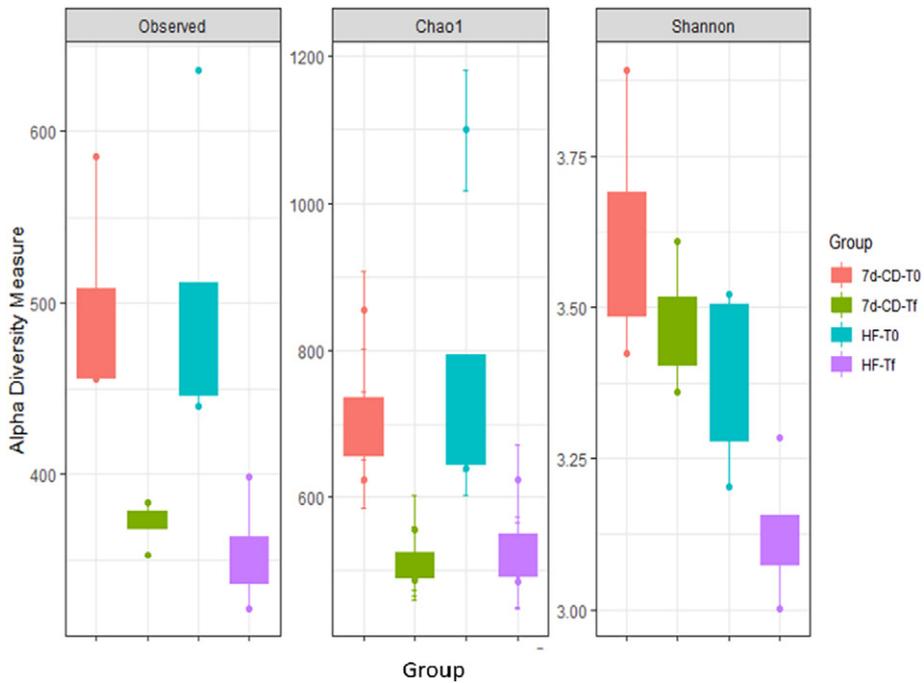


Fig. 6 – α -Diversity indices in fecal samples of mice before and after treatment in 7d-CD and HF groups. Chao1 estimates the number of species, whereas Shannon estimates the effective number of species. Each group contains n = 4 mice.

Similarly, Prevotellaceae disappeared after HFD treatment, whereas 1 week of CD restored its population ($P < .0001$). Conversely, Anaeroplasmataceae populations disappeared following HFD treatment, which was only slightly corrected

by 1 week of CD, indicating an incomplete restoration of the baseline microbiota (Fig. 10).

At the genus level, *Parabacteroides*, *Allobaculum*, *Lactobacillus*, and an unknown genus in the Erysipelotrichaceae family were

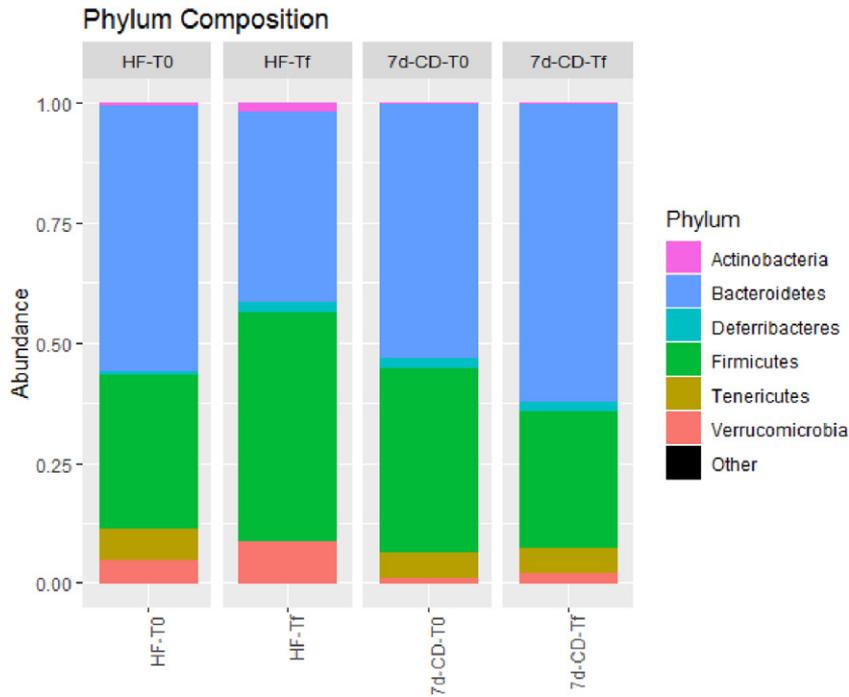


Fig. 7 – Microbiota composition at the phylum level (relative abundance) in HF and 7d-CD groups at T0 and Tf. Each group contains n = 4 mice.

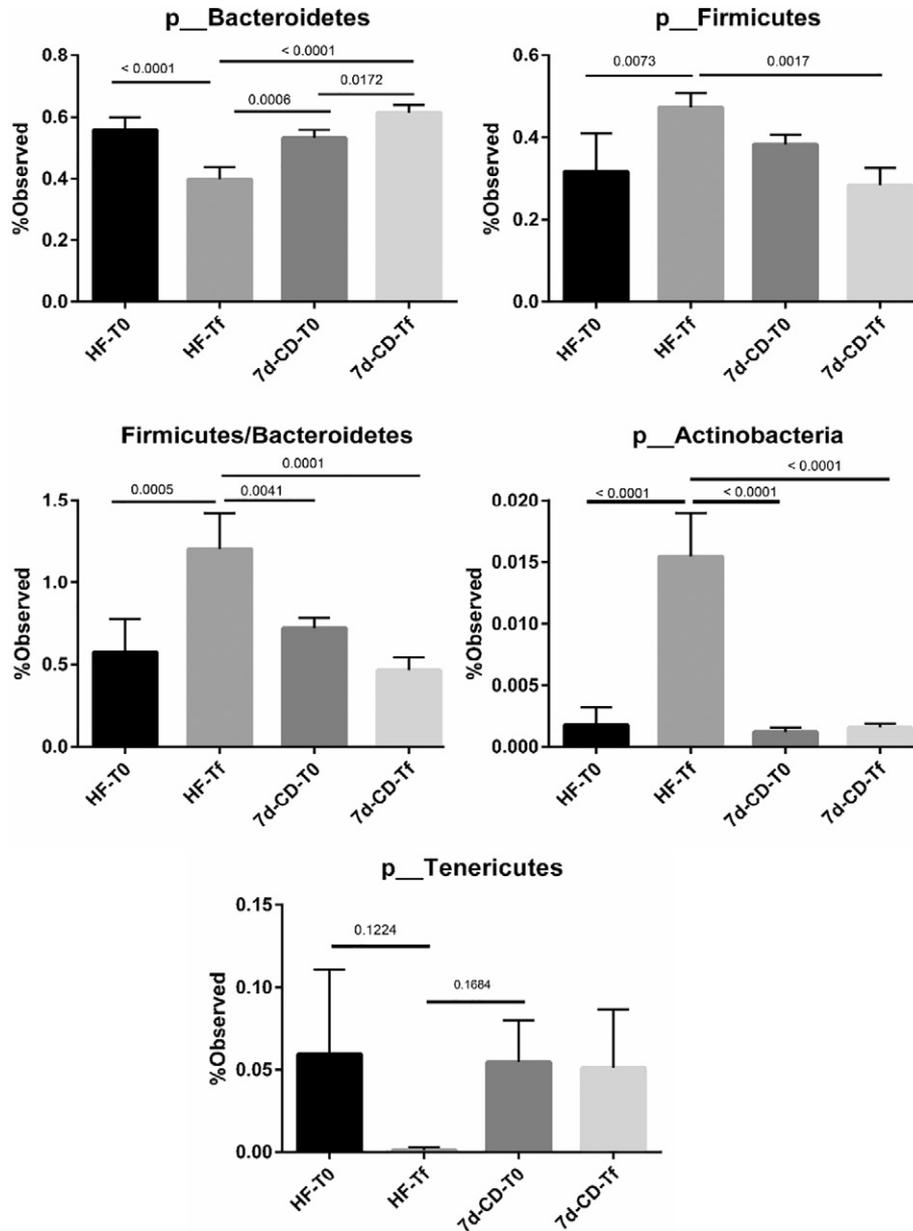


Fig. 8 – Impact of diet treatments on the relative abundances of phyla. Groups were compared using Tukey multiple comparison test. P values were calculated at a 95% confidence level. Each group contains n = 4 mice.

boosted by HFD treatment. These increases were not found in 7d-CD-Tf, suggesting that 1 week of CD completely abolished this effect (Fig. 11). HFD also caused the eradication of *Prevotella* and *Anaeroplasmata*, and the CD led to restoration of *Prevotella* only, further indicating that 1 week of CD led to a marked but incomplete restoration of the baseline microbiota.

4. Discussion

In the experimental setup used in this study, mice were fed HFD for 8 weeks followed by a change to CD to first describe how HFD affects body weight gain, liver phenotype, and gut microbiota composition in mice and then to evaluate the reversibility of

these HFD-induced effects. We first evaluated the effects of HFD on host parameters and found that 8 weeks of HFD induced obesity and steatosis in mice. In line with our results, Zeng et al [31] studied C57BL/6 mice fed an HF (45% energy) or low-fat (10% energy) diet for 10 weeks. The HFD-fed mice increased their body weight by 34% compared to the low-fat-fed mice, and the liver of these mice also exhibited a dramatic increase in the number of lipid droplets. Then we aimed to investigate if these HF-induced metabolic disorders are reversible after switching HF to CD. In our study, 3 days of CD after 8 weeks of HFD treatment was not able to reverse the fatty liver phenotype and weight gain due to HFD treatment. Conversely, the liver recovers and shows essentially normal phenotype after 7 days of CD. One week of CD following 8 weeks of HFD led to reduced

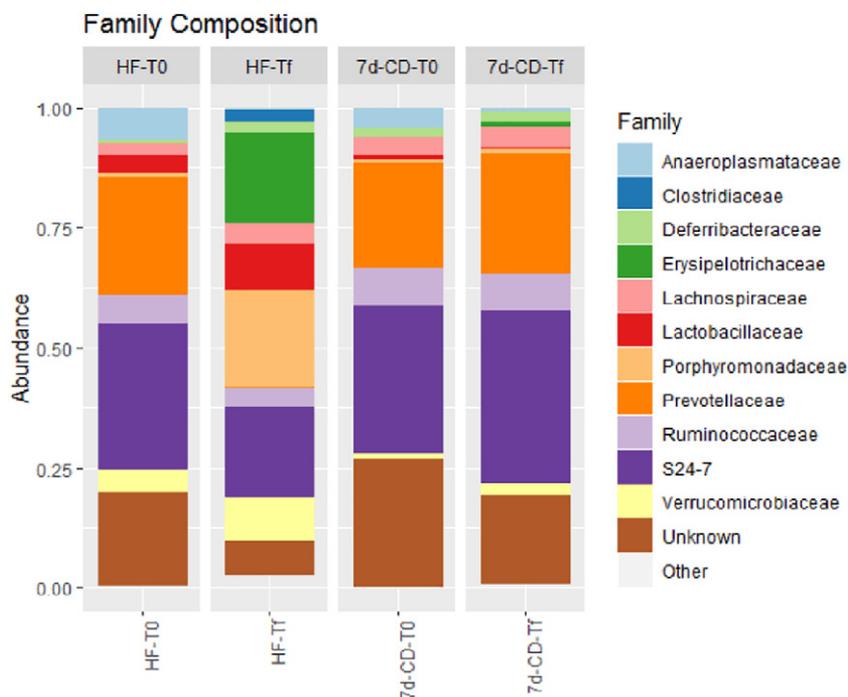


Fig. 9 – Microbiota composition at the family level (relative abundance) in HF and 7d-CD groups at T0 and Tf. Each group contains n = 4 mice.

amount of fat deposition in hepatocytes which was also confirmed by TG assay and scoring. Kowalski et al [27] used HFD (42% energy from fat and 20% from sucrose) for 56 days followed by 9 days of ad libitum CD. Glucose intolerance and hyperinsulinemia were completely normalized, whereas fat mass and liver triglycerides showed an intermediate phenotype. The diet reversal also resulted in significant reductions in hepatic de novo lipogenesis when compared to the high-fat, high-sucrose diet groups. Altogether, these results and ours indicate that the metabolic and hepatic disorders induced by HFD are mainly reversible with a minimum of 1 week of CD being necessary to recover close to initial levels.

Together with effects on host metabolic parameters, HFD is responsible for rapid changes in microbiome composition. Indeed, it was observed that switching from a low-fat, plant polysaccharide-rich diet to a high-fat, high-sugar Western diet shifted the structure of the microbiota within a single day, changed the representation of metabolic pathways in the microbiome, and altered microbiome gene expression [22]. However, the shifts in response to daily variation in the diet are shown to be at the genus and species level rather than at the phylum level, and long-term diet has been proven to be the primary driver in determining one's gut microbiota [39]. Moreover, different types of high-fat diet, with different percentages of saturated and polyunsaturated fatty acids, were shown to have different effects on the gut microbiota [40]. Carmody et al [41] studied the effect of dietary perturbations on the gut microbiota of 5 inbred mouse strains, showing that consumption of a high-fat, high-sugar diet reproducibly changed the gut microbiota in spite of the differences in host genotype. Particularly, the gut microbiota displayed a linear dose-dependent response to dietary

perturbations, and each diet-responsive bacterial group took an average of 3.5 days to reach a new steady state [41]. In our study, the gut microbiota community of the HF group at 8 weeks clustered separately from that of initial microbiome before HF feeding, indicating a broad effect of HF on gut microbiota composition. Moreover, both diversity and richness of the microbiome were decreased by the HFD treatment in accordance with previous studies [22,41].

Human populations with a modern Western diet or a rural diet showed distinct gut microbiota structures [42], and we may speculate that these changes in gut microbiota could contribute to the higher incidence of metabolic diseases including obesity and NAFLD occurring in Western populations. Therefore, it is of primary importance to assess the capability of a normal CD to restore a balanced gut microbiota that can contribute to acquiring a healthy status. The diversity of bacteria in HF group is decreased in our study as previously found by others. A decreased diversity of the microbiota has also been observed in obese individuals, where it is correlated to the reduction in insulin sensitivity and the stimulation of inflammation [43,44]. Regarding liver diseases, alterations such as lower diversity and a phylum-level change in the fecal microbiome has been demonstrated in NAFLD [45,46]. Thus, our finding of a general dysbiosis characterized by a decreased diversity in mice administered an HFD for 8 weeks raises the possibility that this imbalanced microbial ecology plays a role in increasing the susceptibility to liver diseases. This is in line with other studies showing that the changes in gut microbiota induced by HFD vs CD may act as an important mediator in the etiology of obesity and related metabolic diseases, probably via disrupting regulation of lipid metabolism and inducing low-grade inflammation [47-49].

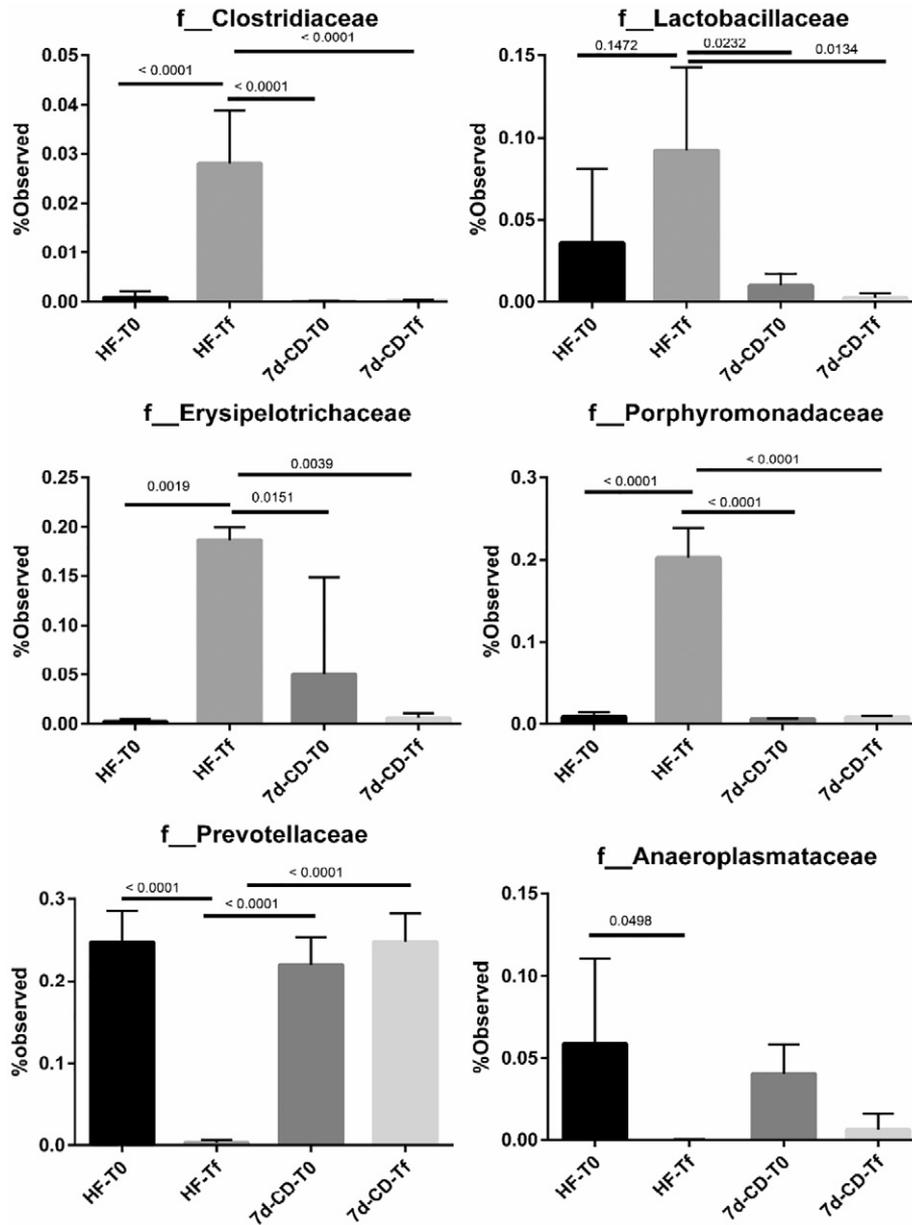


Fig. 10 – Impact of diet treatments on the relative abundances of bacterial families. Groups were compared using Tukey multiple comparison test. P values were calculated at a 95% confidence level. Each group contains n = 4 mice.

In our study, the abundance of Actinobacteria, Firmicutes, Clostridiaceae, Erysipelotrichaceae, Porphyromonadaceae, *Parabacteroides*, and *Allobaculum* increased significantly after HFD treatment for 8 weeks. Concurrently, Bacteroidetes, Prevotellaceae, and *Prevotella* were significantly underrepresented at the end of HFD treatment. Consistent with these findings, we have previously observed increased *Allobaculum* (a genus in Erysipelotrichaceae) after HFD consumption [9]. Moreover, decreased *Prevotella* [50] and increased *Allobaculum* numbers in HFD-fed mice were also reported by other studies [50,51]. HFD feeding modulates the gut microbiota composition by decreasing the prevalence of specific gut barrier-protecting bacteria and increasing the prevalence of

opportunistic pathogens that can release free antigens such as lipopolysaccharides. This imbalance may be associated with a higher gut permeability, leading to higher plasma levels of endotoxin and inducing inflammation and eventually the development of metabolic disorders, a phenomenon termed *metabolic endotoxemia* [48,49].

Structural resilience is the ability of a complex system to recover to its normal state after removal of the perturbation. Microbiome reversibility has been particularly studied in the context of antibiotic treatments. As an example, after disruption by antibiotics, the structure of the gut microbiota began to return to its initial state by 1 week after the end of each antibiotic course but did not reach complete recovery

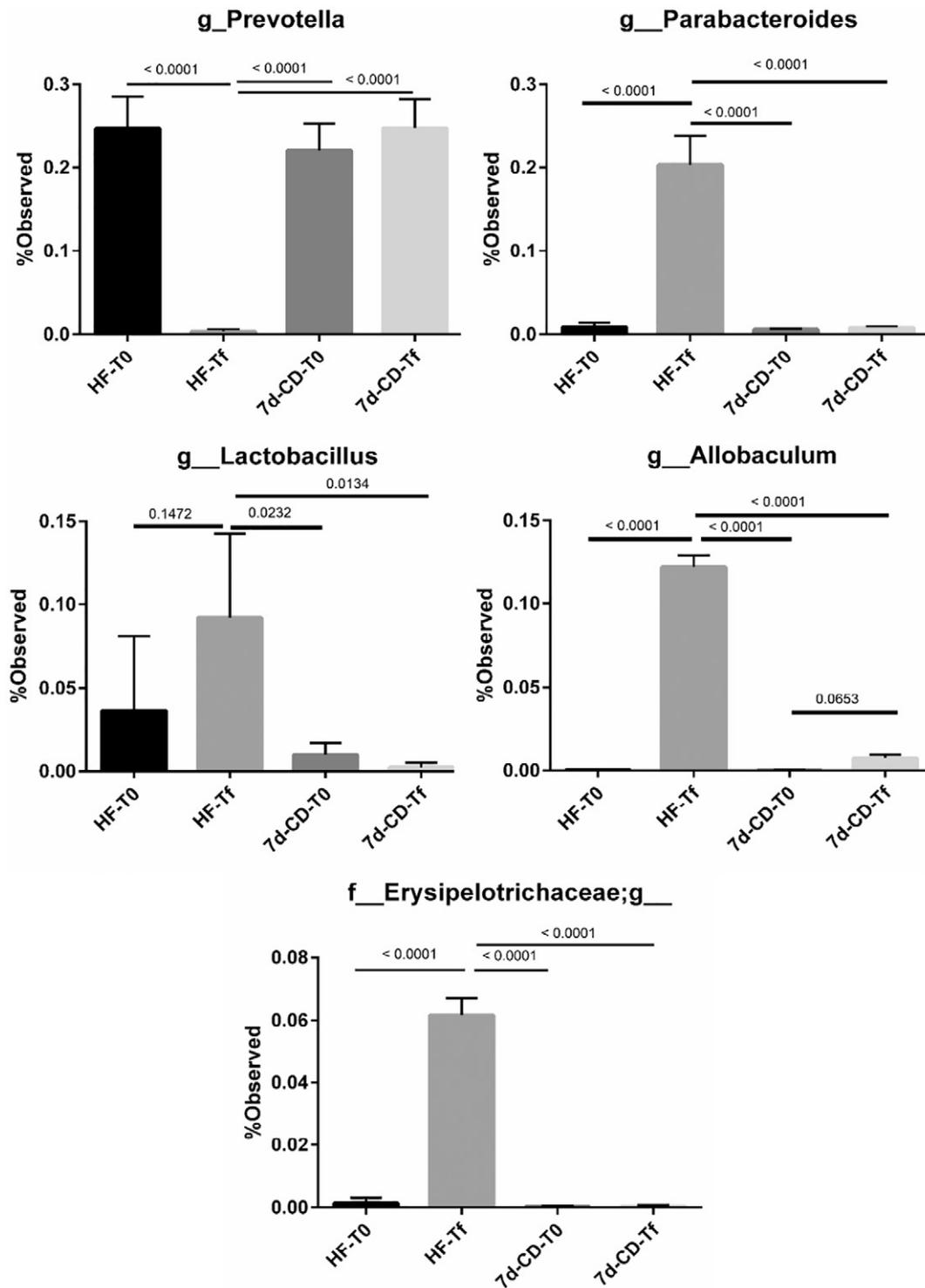


Fig. 11 – Impact of diet treatments on the relative abundances of bacterial genera. Groups were compared using Tukey multiple comparison test. P values were calculated at a 95% confidence level. Each group contains n = 4 mice.

even after 5 months [52]. However, it is not yet known to what extent the HFD-disrupted structure of the gut microbiota can be reversed after returning to a balanced diet. One of the dieting problems in the age of the obesity epidemic is the cycle of weight loss and regain known as the *yo-yo effect*. Thaiss et al [29] demonstrated that the microbiome plays a

key role in this phenomenon, as HF-induced changes to the microbiome persist over long periods of time and enhance the rate of weight gain during secondary HF challenge. Zhang et al [51] monitored the dynamic changes of the gut microbiota in C57BL/6J mice fed with a 34.9% fat HFD for 12 weeks and then reverted to CD for 10 weeks. HFD feeding significantly

influenced the richness and diversity of the bacterial community, which was reversible upon reverting to normal diet feeding. They observed increased number of Firmicutes and Proteobacteria and decreased number of Bacteroidetes in the group fed with HFD and their reversal after receiving normal diet. In our study, we assessed whether feeding our mice with CD for a shorter period (7 days) was able to reverse the effect of HFD (60% energy from fat) on the gut microbiota composition. Based on our findings, HFD caused increased frequency of Firmicutes and Firmicutes/Bacteroidetes and decreased Bacteroidetes, which are in agreement with some other studies [51,53,54]. Our findings also showed significantly higher amount of Actinobacteria in HF group vs 7d-CD group, indicating that this phylum is highly susceptible to diet changes and that its HFD-induced increase is reversible upon changing diet. This confirms the results of previous studies indicating that Actinobacteria abundance is positively associated with an HFD and negatively associated with fiber intake [40,54]. At the family level, we observed that Erysipelotrichaceae, Lactobacillaceae, Clostridiaceae, and Porphyromonadaceae bloomed in HF group and showed a significant drop in number after treatment with CD reaching close to initial level. Zhang et al [49] identified 4 different lineages within Erysipelotrichaceae that respond differently to diet or host health phenotypes, whereas some studies observed an increase of Erysipelotrichaceae in mice on HF or Western diet [22,50,54,55] in accordance with our results. In addition, our data revealed a significantly reduced level of hydrogen-producing Prevotellaceae following HF feeding and its repopulation after normal diet in 7d-CD group, which are in accordance with the findings of Heisel et al [50]. In contrast, Zhang et al [56] showed obesity to be associated with an increase of family Prevotellaceae prior to the surgery, and following surgery, Prevotellaceae was reduced to the level of lean individuals. Also, human studies have shown higher levels of Prevotellaceae in obese individuals compared with lean subjects and with those after gastric bypass [57,58]. Indeed, obesity is not systemically due to a lipid-rich diet, and gut microbiota composition in obese individuals results from many factors and does not always correlate with the effects of HF observed in animal models. This further suggests that this bacterial family does not play a role in metabolic phenotypes, as it has been found increased or decreased in humans and animals with obesity or steatosis. At the genus level, *Allobaculum* and *Parabacteroides* were also overrepresented in HF group and remarkably reduced after 7 days of treatment with the CD. Conversely, *Prevotella* showed a reduced frequency in HF-fed mice, and its amount was elevated in mice treated with CD. We also observed an increased number of *Lactobacillus* in HF group, which was reduced after CD. *Lactobacillus* may be associated with the production of volatile organic compounds such as acetate and ethanol [59], which may be involved in the pathogenesis of obesity and NAFLD. In human studies, a significantly higher level of *Lactobacillus* species (from the phylum Firmicutes) was also found in obese patients than in lean controls [60].

These results revealed that HF feeding highly impacts gut microbiome composition but that 7 days of CD is sufficient to reverse the majority of HF-induced microbiota changes. Because metabolic parameters were reversible in parallel, we

may postulate that gut microbiota changes contribute to the changes in liver and metabolic phenotype. Using gut microbiota transplant experiments, we have already shown that gut microbiota composition determines steatosis development in recipient mice [9]. Transplantation of microbiota taken after HFD treatment and after CD restoration could be used in the future to decipher if the associated microbiota changes are responsible for improvement of steatosis. The intestinal microbiota has been extensively studied at both the phylogenetic and metagenomic levels in the context of metabolic disorders [41,61,62]. The novelty of the present work lies in the investigation of the reversibility of HFD-induced dysbiosis and its association with development and improvement of the fatty liver disease. Among the bacterial groups that were found to change in parallel with steatosis in our study, the family Erysipelotrichaceae is emerging as a group of bacteria that may influence host metabolism and inflammatory diseases [63,64]. Interestingly, Spencer et al [30] showed a positive association of the Erysipelotrichi abundance with the changes in liver fat in women who were placed on diets in which choline levels were manipulated. We also found that *Lactobacillus* was associated with fatty liver and was found reversible upon diet changes. Accordingly, a positive correlation between the amount of *Lactobacillus* DNA in the fecal samples and the severity of steatosis within mouse livers was already observed. The authors speculated that the *Lactobacillus* species could influence lipid metabolism through effects on bile acid metabolism and thus contributed to the risk for fatty liver [31]. An increased population of *Parabacteroides*, a major producer of succinate, was shown to be positively correlated with body weight in our study. To our knowledge, the link between abundance of these bacteria in response to diet change and liver status has not been reported so far. This genus could be interesting to be studied in HFD-associated metabolic disease such as NAFLD.

Finally, several limitations of our study should be mentioned. First, the generalizability of our findings may be limited owing to the low number of animals, the absence of food intake monitoring, and the lack of mechanistic approach. Also, it would be of great interest to test whether similar results would be obtained with different types of HFD.

In conclusion, we found that HFD leads to marked liver steatosis and that 1 week of CD is sufficient to reverse steatosis. Furthermore, we observed that HFD changes microbiota and 1-week CD essentially reversed it. Together, these findings are consistent with the hypothesis that diet-induced changes to the gut microbiota may contribute to NAFLD. The question as to whether specific populations are responsible for weight gain and steatosis or are simply flourishing as a consequence of the diet being consumed (ie, are these populations a “cause” or an “effect”) remains a key one. More broadly, our results emphasize that a more comprehensive understanding of diet-related diseases will benefit from elucidating links between nutrition, microbiome, and NAFLD. The use of germ-free mice and mice conventionalized with known microbial species should help to reveal the influence of specific populations on host health and in turn reveal therapeutic targets in the fight against obesity and associated liver diseases.

Acknowledgment

This work was funded by the Förderung der wissenschaftlichen Forschung (FWF) (W 1226, DK Metabolic and Cardiovascular Disease) at the Medical University of Graz. We are grateful to the INRA MIGALE bioinformatics platform (<http://migale.jouy.inra.fr/>) for providing computational resources. We are also thankful to Ulrike Fackelmann from Medical University of Graz for technical assistance. The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results. The author contributions are as follows: conceptualization, KZ and ZS; methodology, KZ and ZS; investigation, ZS and PMA; software, MM, KK, and MM; writing – original draft, ZS; writing – review and editing, ZS, PG, and KZ; funding acquisition, KZ and ZS; resources, PG and KZ; supervision, PG and KZ.

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