Dissertation

Role of the relationships between genetic background, diet and microbiome in susceptibility to liver steatosis

submitted by

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Declaration

I hereby declare that this dissertation is my own original work and that I have fully acknowledged by name all of those individuals and organizations' that have Contributed to the research for this dissertation. Due acknowledgment has been made in the text to all other material used. Throughout this dissertation and in all related publications I followed the "Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz".

September 15th, 2019

Parts of this thesis are based on the following original publications that are referred to in the text wherever required, with permission from all co-authors:

1. *The links between the gut microbiome and non-alcoholic fatty liver disease (NAFLD). Safari Z, Gérard P. (2019). Cell Mol Life Sci. doi: 10.1007/s00018-019-03011-w.*

2. *One-week chow diet administration is effective to reverse steatosis and gut microbiota dysbiosis in high fat fed mice. Safari Z, Monnoye M, Abuja P.M, Mariadassou M, Kashofer K, Gérard P, and Zatloukal K. (2019). Nutrition Research (In Press).*

3. *Changes in the gut microbiota do not alter the different responses to high fat diet in A/J and C57BL/6J mice. Safari Z, Bruneau A, Monnoye M, Mariadassou M, Zatloukal K, Gérard P. (2019). Submitted.*

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This thesis is dedicated to

my beloved parents

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Contents

List of abbreviations

AMPK	AMP-Activated Protein Kinase
ANGPTL4	Angiopoietin-Like 4
BAs	Bile Acids
CSS	Chromosome Substituted Strain
CV	Conventional
FFA	Free Fatty Acids
FMT	Faecal Microbiome Transplantation
FOS	Fructooligosaccharide
FXR	Farnesoid X Receptor
GF	Germ-Free
GI	Gastrointestinal
GLP	Glucagon-Like Peptide
GMT	Gut Microbiome Transplantation
HBV	Hepatitis B Virus
HDL	High Density Lipoprotein
HFD	High-Fat Diet
LPS	Lipopolysaccharides
MCDD	Methionine Choline Deficient Diet
MetS	Metabolic Syndrome
NAFLD	Non-Alcoholic Fatty Liver Disease
NASH	Non-Alcoholic Steatohepatitis
OTU	Operational Taxonomic Unit
PAMPs	Pathogen-Associated Molecular Patterns
PCR	Polymerase Chain Reaction
PEMT	Phosphatidylethanolamine Methyltransferase
SCFA	Short-Chain Fatty Acid
TG	Triglyceride
TJ	Tight Junction

Zusammenfassung

Die nichtalkoholischen Fettlebererkrankungen (NAFLD) umfassen die einfache Steatose bis hin zu einer progressiveren Steatose mit assoziierter Hepatitis, Fibrose, Zirrhose und in einigen Fällen einem hepatozellulären Karzinom.

Die Darmflora (oder das Darmmikrobiom) ist normalerweise an der Energiegewinnung im Darm beteiligt, wobei sie eine zunehmende Rolle in der Pathogenese von Fettleibigkeit und NAFLD spielt. Daher sind die intestinalen Mikrobiotapopulationen mögliche therapeutische Ziele bei der Behandlung von NAFLD.

NAFLD resultiert aus komplexen Wechselwirkungen zwischen Gen- und Umweltfaktoren, einschließlich der Darmflora. Um diese Wechselwirkungen zu untersuchen, entschieden wir uns zunächst für zwei häufig verwendete Inzuchtstämme von Mäusen, NAFLD-anfällige C57B1/6J- und NAFLD-resistente A/J-Mäuse, sowie vier Kreuzungslinien. Diese abgeleiteten Stämme enthalten ein spezifisches Chromosom der A/J-Linie im genetischen Hintergrund von C57B1/6J und werden als Chromosomen-substituierte Stämme (CSS) bezeichnet. Die von uns ausgewählten CSS haben zuvor entweder eine hohe (CSS-1 und CSS-18) oder niedrige (CSS-8 und CSS-10) Fettakkumulation nach Fütterung mit einer fettreichen Diät in der Leber gezeigt. Im ersten Teil dieses Projekts zeigten die Analyse der metabolischen Parameter und der Darmflora in den Gründerstämmen sowie der ausgewählten CSS-Stämme Wechselwirkungen zwischen Ernährung, Leberphänotyp und metabolischen Parametern auf. Die Zusammensetzung des Mikrobioms unterschied sich zwischen CSS und den Gründerstämmen, obwohl alle CSS den genetischen Hintergrund von C57Bl/6J hatten. Außerdem fanden wir auch *Verrucomicrobiaceae* (ein mit Stoffwechselgesundheit assoziiertes Bakterium) in A/J und in allen CSS, die ein Chromosom von A/J enthielten, unabhängig von ihrem Leberphänotyp. Wir konnten keinen starken Zusammenhang zwischen Mikrobiom und metabolischen Parametern bezüglich des Schweregrades der Steatose in diesen CSS beobachten. Basierend auf diesen Ergebnissen ziehen wir den Schluss, dass für die Kontrolle des Krankheitsphänotyps der genetische Effekt stärker ist als der Einfluss von Diät oder dem Mikrobiom.

Im zweiten Teil dieser Arbeit beobachteten wir, dass eine achtwöchige Behandlung von Mäusen mit fettreicher Ernährung (HFD) in Verbindung mit einem gestörten Mikrobiom eine ausgeprägte Lebersteatose auslöste. Interessanterweise reichten bereits 7 Tage Standardfutter („Chow-Diät“, CD) aus, um die Leber wieder in einen normalen Zustand zu bringen, und das Mikrobiom dementsprechend zu einem nahezu ursprünglichen Muster zurückkehrte. Das Vorkommen einiger Bakterien, einschließlich *Prevotella*, *Parabacteroides*, *Lactobacillus* und *Allobaculum*, war bei einer Ernährungsumstellung von fettreich (HFD) auf normal (CD) reversibel. Fettreiche Ernährung (HFD)

verursachte eine Fettablagerung (Steatose) in der Leber, die mit den Veränderungen des Darmmikrobioms korrelierte. Diese Korrelation legt nahe, dass Änderungen in der Darmflora zu den metabolischen Auswirkungen fettreicher Ernährung beitragen und dass die Wiederherstellung der normalen Darmflora zu einer Verbesserung des Leberphänotyps führen kann.

Im dritten Teil des Projekts beobachteten wir, dass A/J- und C57Bl/6J-Mäuse eine unterschiedliche Mikrobiomstruktur und -zusammensetzung aufweisen. Außerdem zeigte sich, dass bei einigen Stoffwechselparametern sowie einigen Bakterienarten, A/J und C57Bl/6J unterschiedlich auf die Behandlung mit fettreicher Ernährung und Antibiotika reagieren. Im Weiteren führten wir einen fäkalen Mikrobiotaaustausch zwischen A/J- und C57B1/6J-Mäusestämmen durch. Dabei wurde die Spenderdarmflora aus einem anderen Stamm zugeführt, etabliert und dadurch die Darmflora der Empfänger verändert. Dies legt nahe, dass der genetische Einfluss auf den Leberphänotyp stärker ist als der des Darmmikrobioms.

Schlussfolgerung: Wir fanden sowohl in CSS als auch in ihren Gründerstämmen C57B1/6J und A/J unterschiedliche Mikrobiomzusammensetzungen in genetisch unterschiedlichen Mäusen mit unterschiedlicher Anfälligkeit für Steatose. Außerdem beobachteten wir, dass fettreiche Ernährung die Darmflora verändert und Steatose auslöst sowie dass die Umstellung der Ernährung von fettreich (HFD) auf normal (CD) in nur 7 Tagen sowohl die Hepatosteatose als auch die Zusammensetzung der Darmflora umkehren kann. Daraus schließen wir, dass Änderungen im Mikrobiom zu Ernährungseffekten beitragen können. Insgesamt zeigen diese Ergebnisse, dass Mikrobiom, Genetik und Ernährung auf verschiedenen Ebenen miteinander interagieren und so die Entwicklung oder Verschlechterung von NAFLD beeinflussen.

Abstract

Non-alcoholic fatty liver disease (NAFLD) ranges from simple steatosis to more progressive steatosis with associated hepatitis, fibrosis, cirrhosis, and in some cases hepatocellular carcinoma.

Gut microbiota is normally involved in the intestinal energy harvest and its role has been increasingly established in the pathogenesis of obesity and NAFLD. Therefore, the intestinal microbiota populations are potential therapeutic targets in the management of NAFLD.

NAFLD results from complex interactions between genetic and environmental factors, including the gut microbiota. To dissect these interactions, firstly, we utilized two commonly used inbred strains of mice, NAFLD-prone C57Bl/6J and NAFLD-resistant A/J mice as well as four derivative lines generated by crossing. These strains harbour one specific chromosome from A/J in a C57Bl/6J genetic background and are termed as Chromosome Substituted Strains (CSSs). These selected CSSs have previously shown either a high level (CSS-1 and CSS-18) or low level (CSS-8 and CSS-10) of fat accumulation in the liver. In the first part of this project, analysis of metabolic parameters and gut microbiota in the founder strains, as well as the selected CSS strains, revealed some interactions between diet, liver phenotype and metabolic parameters. The microbiome composition was different between CSSs and the founder strains, although the CSSs had all C57Bl/6J genetic background. We also observed *Verrucomicrobiaceae* (a bacterium associated with metabolic health) in A/J and all the CSSs harbouring one chromosome from A/J regardless of their liver phenotype. We did not observe a strong link between the microbiome and metabolic parameters concerning the severity of steatosis in these CSSs. Based on these findings we conclude that the genetic effect is stronger than the effect of diet or microbiome in controlling the disease phenotype.

In the second part, we found that 8 weeks of High Fat Diet (HFD) treatment induced marked liver steatosis in mice together with a perturbed microbiome. Interestingly, only 7 days of chow diet was enough to recover the liver to a normal status while the microbiome was accordingly reshaped to a close to initial pattern. The abundance of some of the bacteria including *Prevotella*, *Parabacteroides*, *Lactobacillus*, *Allobaculum* was reversible upon diet change from HFD to CD. The HFD caused fat deposition (steatosis) in the liver which correlated with the gut microbiome changes. This correlation 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 the third part, we observed that A/J and C57Bl/6J mice have different microbiome structure and composition. We further demonstrated that for some metabolic parameters as well as some bacterial species A/J and C57Bl/6J respond differently to HFD and antibiotic treatments. We also performed

microbiota exchange between A/J and C57Bl/6J mice strains. The donor gut microbiota from one strain was introduced, established, and changed the gut microbiota of the recipients from the other strain.

The differences in the microbiome of A/J and C57Bl/6J under different treatments did not correlated with their different phenotypes as microbiome exchange by faecal microbiome transplantation did not lead to clear phenotypical changes in these two strains. This suggests that the effect of genetics on liver phenotype is stronger than the one from the gut microbiome.

Conclusion: We found different microbiome composition in genetically different mice with different susceptibilities to steatosis including CSSs as well as in their founder strains i.e. C57Bl/6J and A/J. We also found that HFD changes microbiota and trigger steatosis and diet switching from HFD to chow in only 7 days can reverse both hepatosteatosis and gut microbiota composition. We conclude that microbiota changes can contribute to diet effects as its changes correlated with phenotype. Altogether, these findings show that microbiome, genetics and diet interact with each other in different levels and influence NAFLD development or aggravation.

1. General introduction

(Note: Part of the introduction is adopted from Safari et al. [1])

1.1. NAFLD

Non-alcoholic fatty liver disease (NAFLD) covers a broad spectrum of liver disease ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), through to advanced fibrosis and cirrhosis. NAFLD is shown to be correlated with an increased risk of mortality because it is strongly associated with type 2 diabetes (T2DM) and other features of the metabolic syndrome [2]. Furthermore, NASH, especially in the context of liver fibrosis or cirrhosis, is correlated with enhanced liver-related mortality and the development of hepatocellular carcinoma (HCC) [2-4]. Following the global rise in the NAFLD prevalence, NASH became the third cause for liver transplantation in the United States, and current reports suggest that NAFLD is a major factor linked to the high frequency of HCC in Western countries [1, 5, 6].

NAFLD prevalence in the world is currently about 24%1. It is one of the most common diseases in all continents, but the highest rates observed are from South America (31%) and the Middle East (32%), followed by Asia (27%), the USA (24%) and Europe (23%), however, NAFLD is less common in Africa (14%) [2].

The lower incidence of NAFLD in African-Americans in comparison to Hispanic-Americans is interesting because higher rates of obesity and hypertension were reported in African-American people [7]. These inconsistencies can be explained by differences in lifestyle, altered microbiota, prevalence of metabolic syndrome in the population, and genetic background, such as variations within the PNPLA3 (patatin-like phospholipase domain-containing 3) gene, which is shown to increase the predisposition to fat deposition in the liver, resulting NAFLD [8].

NAFLD is characterized by hepatic steatosis, defined as accumulation of fat (triglyceride) in more than 5% of hepatocytes in the absence of other causes of steatosis including congenital errors of metabolism or excess alcohol consumption. Simple steatosis is identified by accumulation of lipid in the liver without inflammation and often involves a relatively favourable clinical course [9]. While NASH, which is found in about 25%–40% of NAFLD patients [10-12] involves hepatocellular injury and liver inflammation and is an important risk factor for cirrhosis and hepatocellular carcinoma (HCC) [11, 13]. Alterations found in NASH are similar to those of alcoholic steatohepatitis (ASH), including steatosis, inflammation, hepatocyte ballooning (a type of hepatocyte injury), Mallory-Denk bodies, and ultimately fibrosis within the lobules [14].

Diet is assumed as an independent risk factor for NAFLD development especially a diet high in fats [15]. Studies performed on manipulation of dietary macronutrients and energy restriction, namely, restriction of carbohydrates, fat, or enrichment with monounsaturated fatty acids showed that dietary changes can improve metabolic syndrome [16]. Diets that imitate a Westernized pattern, such as those high in red meat consumption, refined grains, pastries, and sugar-laden beverages are coupled with a greater risk for the development of metabolic syndrome and subsequent NAFLD [1, 15].

Many patients with NAFLD suffer from obesity and have related diseases such as insulin resistance (IR) that plays a vital role in metabolic syndrome [17, 18]. Thus, NAFLD is also considered to be a hepatic indicator of metabolic syndrome which includes a group of complex conditions such as obesity, hypertriglyceridemia, hypertension, hyperglycaemia, and low HDL (high-density lipoprotein) which are prognostic risk factors of stroke, cardiovascular disease and diabetes, [19, 20].

Day and James (Day and James 1998) proposed the two-hit model to explain NAFLD development. The first hit is majorly related to the caloric excess leading to accumulation of additional lipid in liver cells. The disease does not proceed unless other cellular events or second hits appear. The second hits trigger inflammation, cell death and fibrosis, that are all NASH symptoms. Inflammation, cell damage and advanced fibrosis is linked to oxidative stress, mitochondrial dysfunction, deregulated cytokine signalling and IR.

To date, different mechanisms were described for the additional fat deposition in the hepatocytes. It is understood that metabolic syndrome deregulates the lipid metabolism and thus lead to excessive consumption of fats and carbohydrates.

Also, the increased level of Free Fatty Acids (FFA) leads to fat deposition. There are other reasons for impaired liver function such as bypass operation, protein-calorie malnutrition and or parenteral nutrition under which lipoprotein synthesis is perturbed [21, 22]. The elevated level of Triglyceride (TG) and FFA in the liver lead to IR. Many of NAFLD patients and even normal obese patients with a normal blood glucose level are affected by IR.

Additionally, the imbalances in adiponectin (an adipocyte-derived anti-inflammatory mediator) influence NASH development through their impact on hepatic fat accumulation and IR. [1, 23].

Furthermore, other factors such as Oxidative Stress (OS), impaired mitochondria function, deregulated hepatic lipid partitioning, FFA-induced hepatotoxicity and elevated liver cholesterol can cause inflammation and later lead to fibrosis [24, 25]. The interaction of these factors leads to advanced liver damages. It was proposed that the additional fat in the adipose tissues provokes a series of inflammatory signals which is identified by increased cytokine level from adipocytes as well as

leukocytes [26, 27]. These phenomena result in IR development in adipocytes and more FFAs are released. The cytokines and FFAs activate serine or threonine kinases and these kinases phosphorylate important signalling proteins including insulin receptor (IRS1 & IRS2) and thus IR is induced in liver and muscle. [28]. IR in the liver results in excess production of glucose and thus hyperglycaemia develops.

On the other hand, Insulin induces SREBP-1c (lipogenic transcription factor) gene and subsequently cause increased TG in plasma, increased VLDL level and hepato-steatosis [29].

Therefore, there is an association between the degree of inflammation in the adipose tissue, IR and the amount of liver fat [30]. Human studies also disclosed a link between the liver fat content and different factors of metabolic syndrome [31].

On the contrary, alterations in the liver metabolism can also cause a series of events leading to IR and inflammation. For example, inactivating the insulin receptor in the liver leads to hepatic IR, increased predisposition to atherosclerosis and dyslipidaemia in mice [32].

Besides, the activation of the NF-kB pathway in the liver increases cytokine production and IR in the liver as well as in peripheral tissues [33]. On the other hand, blocking NF-kB pathway using IkB super-repressor in the liver cells prevents the production of cytokines induced by High Fat Diet (HFD) and thus prevents obesity [33].

1.1.1. NAFLD models

Dietary models such as high-fat diet (HFD), high fructose diet and methionine-choline-deficient diet (MCDD), result in NAFLD development and progression to steatosis and IR in rodents but with no sign of inflammation [34].

The choline-deficient diet affects the β-oxidation and causes reduced production of VLDL leading pile-up of the cholesterol and FAs in the liver and inflammation with little or no IR [35, 36].

Steatosis and the impaired metabolic parameters such as weight gain, hyperlipidaemia and IR can be stimulated by high-fructose diet [37].

Genetic NAFLD models include mutations correlated with NAFLD predisposition, such as the ones reported for the PNPLA3 gene [38]. There are phenotypic differences between PNPLA3 related mouse models (induced expression/insertion, deletion and transgenic), thus, one should be cautious when applying genetic models to explain mechanisms involved in human disease [39].

About 45–75% of the animals' total calorie intake in HFD-fed animal models is taken from fat. HFD mimics the classical symptoms present in human metabolic syndrome. Unlike other animal models,

animals treated with an HFD mimic both the histopathology and pathogenesis of human NAFLD, as they have the characteristic features observed in human NAFLD patients, including obesity and IR [40]. However, the severity of these manifestations is different depending on different factors such as rodent strain [41, 42].

1.2. Microbiome

The gut harbours ~10^{13}–10^{14} bacteria, which is assumed to encode a number of genes >100 times as in the human genome [43].

Trillions of the microbes that inhabit in the human body, including bacteria, archaea, viruses, and eukaryotic microbes, live along the length of the gastrointestinal (GI) tract. At different sites of the GI tract, there are different compositions and amounts of bacteria per gram content, including in the stomach and duodenum (10-10^3), the small intestine (10^4-10^7) and the large intestine (10^{11} and 10^{12}), where the highest level of bacteria are found [44].

Despite the wide microbial variety in humans, only 4 main phyla dominate: Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. Nearly 90% of the microbes come from the Firmicutes and Bacteroidetes phyla [43] and each has special roles. The Major contributors of Firmicutes are as follows: *Lachnospiraceae*, which are primarily composed of microbes from the genera *Blautia*, *Clostridium*, *Coprococcus*, *Eubacterium*, *Roseburia*, and *Ruminococcaceae*, which are composed mostly of the genera *Faecalibacterium*, *Oscillospira*, and *Ruminococcus*. Important contributors to the Bacteroidetes phylum include *Bacteroides*, *Parabacteroides, Porphyromonas*, *Prevotellaceae* (*Prevotella*), and *Rikenellaceae* (*Alistipes*). The most important Actinobacteria are *Bifidobacteriaceae* (*Bifidobacterium*), and the most prevalent Proteobacteria are the *Enterobacteriaceae* (*Escherichia*) [45, 46]. Although the functions of most Firmicutes are not still clear, there are studies that indicating that some members of this phylum are butyrate-producing bacteria that boost energy extraction from the diet and also have a lot of beneficial effects [47, 48]. On the other hand, members of the phylum Bacteroidetes take part in carbohydrate metabolism via producing enzymes including glycosyltransferases, glycoside hydrolases, and polysaccharide lyases [44]. Understanding the functions coupled with the microbial community is essential because changes in the gut microbiota have been linked with host diseases, including obesity, type 2 diabetes, and NAFLD [49-51]. In patients with NAFLD, the abundance [52] and structure of the gut microbiome is altered (termed as dysbiosis) [46, 53]. The importance of the gut microbiome in the prevention and treatment of NAFLD is underlined by the study of Le Roy et al (performed at INRA). They selected two donor C57BL/6J based on their responses to an HFD. Germ-free mice were inoculated with the gut microbiota from either responsive or nonresponsive C57BL/6J to HFD and then treated with the same HFD. They observed

that mice colonized with the microbiome from responders developed hepatic macrovesicular steatosis indicating that differences in microbiota composition can determine response to an HFD in mice and lead to the NAFLD development [54]. Furthermore, the alteration in microbiota can change the disease progression [55-57]. Altogether, these findings suggest that the microbiome plays a critical role in NAFLD development [1].

Moreover, important pathogens such as *Escherichia coli* (*E. coli*), *Campylobacter jejuni*, *Salmonella enterica*, *Vibrio cholerae*, and *Bacteroides fragilis*, normally at very low levels (<0.1% gut microbiome) live in human colon [58-60]. The combination of a low abundance of pathogens and a high abundance of key genera including *Bacteroides*, *Prevotella* and *Ruminococcus* indicates a healthy state for gut microbiota [61]. There are also axial differences in the composition of gut microbiome from the lumen to the mucosal surface of the intestine. The most prevalent luminal microbial genera include *Bacteroides*, *Streptococcus*, *Bifidobacterium*, *Enterobacteriaceae*, *Enterococcus*, *Clostridium*, *Ruminococcus* and *Lactobacillus*; however, *Clostridium*, *Lactobacillus*, *Enterococcus* and *Akkermansia* are more frequent in the mucus layer as well as epithelial crypts of the small intestine [62]. Many factors can affect the composition and function of the gut microbiome. These factors include diet, genetics, mode of delivery at birth, geographic location, and exposure to medical treatments [63, 64]. Therefore, the gut microbiome composition is specific to each individual and in addition to these influencing factors, it also changes with age over the course of a lifetime. Besides, the gut microbiota impacts the metabolic phenotype of the host is involved in food and drug metabolism and enhances the immune system [1, 65].

One of the early studies on the link between the gut microbiome and host genetics exhibited the composition of the gut microbiota in different mice strains during a course of antibiotics. They found differences in the bacterial communities, indicating that the establishment of the gut microbiome does not happen by chance but is influenced by various host-derived factors [66]. Kovacs et al. studied some specific inbred mouse strains to find the role played by host genotype on the composition of the gut microbiota [67]. They demonstrated that genetic background is a strong contributing factor in determining the mouse intestinal microbiota. Thus, remarkable correlations were disclosed between eighteen host quantitative trait loci and the abundance of some of the particular microbial taxa [68].

The microbiota from family members is more similar to each other than from unrelated individuals [69, 70] which increases the possibility that genetic factors affect the microbiome composition. This is in agreement with a study correlating genetic loci in mice to the abundance of gut bacteria [68]. It is important to note that studies that did not observe significant genotype effects on microbiome diversity [47, 71] were not considering environmental conditions and might have been underpowered. Recent powerful studies have demonstrated that the *Christensenellaceae* family is a heritable taxon

which forms a co-occurrence network with other heritable taxa and is highly prevalent in individuals with a low body mass index (BMI) [72]. Adding *Christensenellaceae* to an obese-associated microbiome caused decreased weight gain in the recipient mice [72]. Likewise, nucleotide-binding oligomerization domain 2 (NOD2) risk allele is significantly correlated with intestinal bowel disease and an increased relative abundance of Enterobacteriaceae [73].

Furthermore, some studies found that changes including mutations in single host genes, i.e., APOA1, NOD2, Mediterranean Fever, and FUT2, impact the gut microbiota either by altering its composition or reducing bacterial diversity [74-76]. However, there are inconsistencies among human studies, as a general approach (e.g., using twins) did not reveal remarkable genotype effects on microbiome diversity [71, 74]. Therefore, unbiased approaches are necessary to study the heritability of the human gut microbiome. In overall, the gut microbiota and host genetics profoundly interact with each other, and it is hypothesized that changes in the gut microbiota content could supplement the specific genetic makeup of an individual.

In healthy conditions, the host and gut microbiome benefit from each other in a state referred to as eubiosis. Conversely, a perturbation in the microbiome structure or function that results from an abnormal ratio of commensal and pathogenic bacterial species is referred to as dysbiosis [1].

Comparisons between the gut microbiota composition of healthy individuals and of patients suffering from diverse pathologies showed a possible direct correlation between dysbiosis and inflammatory and metabolic disorders such as cardiovascular disease [77], obesity [78, 79], diabetes [49, 80], metabolic syndrome [81, 82] and liver diseases such as NAFLD [54, 83].

1.3. Microbiota and NAFLD

Germ-free (GF) mice have been used to define the consequences of the absence of gut microbiota and therefore to establish the host physiological functions that are influenced by these bacteria. Among these functions, it has been shown using these animal models that the gut microbiota has a role in obesity and related metabolic diseases [84]. The causative relationship between gut bacteria and obesity was further studied using microbiota transfer. For the first time, Bäckhed et al. demonstrated that transferring the normal caecum microbiota of conventional mice to GF C57BL/6 mice caused more fat deposition and IR in the body despite decreased food intake [85]. Turnbaugh et al. in line with previous findings, demonstrated that the obese microbiome helps to harvest more energy from the diet and that this trait is transferable by faecal microbiota transplantation (FMT) [79].

The comparison of GF and CV mice were used to investigate the role played by the microbiota in liver diseases. The altered expression of several important hepatic genes including CAR (constitutive

androstane receptor) was found between GF and CV mice. Furthermore, the absence of gut microbiota in GF mice results in increased accumulation of CAR ligands, including bilirubin, bile acids and steroid hormones, that cause altered liver xenobiotic metabolism favouring NAFLD development [86]. Comparisons of GF and CV mice also showed that the intestinal microbiota protects against fibrosis in mice [87] and could determine the susceptibility to the liver injury [88].

In summary, under different treatment conditions, the absence or presence of bacteria results in variable phenotypes associated with metabolic features and obesity as well as NAFLD. Altered hepatic gene expression, declined cytokine production, dyslipidaemia, reduced calorie usage, enhanced lipid excretion, reduced insulin resistance and altered susceptibility to induced liver injuries were demonstrated in GF mice. Taken together, these studies showed that GF conditions influence the progression and/or incidence of disease, which signifies the role of the microbiome in liver disease development.

Gut microbiota transplantation in GF mice has also been used to study the causality between microbiota composition and susceptibility to NAFLD [1]. Le Roy et al. was the first to show that the gut microbiota composition determines NAFLD development in C57BL/6 mice. By inoculating GF mice with the gut microbiota from responsive and non-responsive C57BL/6 mice to HFD, Le Roy et al. showed that the susceptibility to develop NAFLD phenotypes, including hyperglycaemia and steatosis, is transmissible by the gut microbiota. They also found that the gut microbiota affects the lipid metabolism in the liver, independent of obesity. [54]. Henao-Mejia et al. studied inflammasome-deficient mouse models (lacking pro-inflammatory multi-protein complexes), to assess the possible role of the microbiome in NAFLD [83]. They demonstrated that the gut microbiota changes due to NLRP6 and NLRP3 inflammasome deficiency were linked with aggravated hepatic steatosis and increased TNF-α expression. In addition, this phenotype was transferable by co-housing the inflammasome-deficient mice with the wild-type mice, implicating the inflammasome-mediated dysbiosis in NASH progression [83]. This evidence provides insight and increased insight into the role of the gut microbiome in the NAFLD development and progression as well as the mechanisms involved. These results also raise the question whether the gut microbiota plays a role in NAFLD in humans and which bacteria are involved.

1.3.1. Dysbiosis

The healthy intestine is normally colonized by a wide range of bacteria, including more than 1000 species. These bacteria are in a homeostatic balance with their host and contribute to the maintenance of a healthy state. Dysbiosis occurs if the intestinal bacterial homeostasis is perturbed. Any changes or imbalances in bacterial content or their metabolic functions, or any alterations in bacterial distribution

within the gut, that are correlated with a disease state is termed as dysbiosis [1]. There is a rising number of studies disclosing the link of the gut microbiota dysbiosis with both intestinal (irritable bowel syndrome, inflammatory bowel disease, etc.) and non-intestinal disorders (metabolic syndrome, cancers, brain diseases, etc.). Dysbiosis was shown to be associated with NAFLD [46, 52, 53] and its severity [55, 57, 89-91] in several human and animal studies. It was shown by Spencer et al. that, during choline depletion, different levels of Erysipelotrichia and Gammaproteobacteria in different individuals were correlated with changes in the hepatic fat accumulation in each subject. They revealed that augmented numbers of Erysipelotrichia at baseline were correlated with an increased risk of NAFLD development; whereas higher levels of Gammaproteobacteria at baseline were associated with a lower risk of NAFLD development [92]. In a study comparing the gut microbiome in NAFLD patients and lean subjects, gram-negative bacteria were observed to be increased in NAFLD patients, with up to 24% reduced Firmicutes and 20% elevated Bacteroidetes in patients relative to healthy non-obese adult individual levels. This reduction of Firmicutes included bacteria including the SCFAs-producing *Lachnospiraceae*, *Lactobacillaceae*, and 7α-dehydroxylating *Ruminococcaceae*, and the increase in opportunistic pathogenic bacteria that produce LPS was also found in patients with NAFLD [93]. Increases in gram-negative bacteria were also observed to be correlated with NAFLD in children. Indeed, Michail et al. [94], using 16S rRNA gene analysis, identified microbial changes in obese NAFLD children compared to obese children without NAFLD and lean healthy children. Higher levels of Epsilonproteobacteria and Gammaproteobacteria were found in children with NAFLD compared to in healthy lean and obese children. Moreover, children with NAFLD also showed increased levels of *Prevotella* than did healthy controls. However, the results can be contradictory, as Raman et al. observed increased bacteria belonging to the phylum Firmicutes (such as *Dorea, Lactobacillus*, *Roseburia* and *Robinsoniella*) in NAFLD subjects compared with controls [95]. They also observed a non-significant underrepresentation of *Ruminococcus* in NAFLD patients compared to healthy controls, which was in agreement with the observation of Wang et al. [93]. In contrast, Jiang et al. and Del Chierco et al. [96, 97] observed increased levels of *Dorea* and *Ruminococcus* in NAFLD cases.

Currently, the gut microbiome composition was characterized using whole-genome shotgun sequencing of DNA extracted from stool samples for differentiating between mild or moderate NAFLD and exacerbated fibrosis [98]. Firmicutes and Proteobacteria showed a different amount in the mentioned groups. Firmicutes was more frequent in mild or moderate NAFLD, whereas Proteobacteria was prevalent in fibrosis cases. At the species level, *Bacteroides vulgatus* was more frequent in mild or moderate NAFLD as well as in advanced fibrosis. *Eubacterium rectale* was found in high numbers in mild or moderate NAFLD, whereas *Escherichia coli* was more prevalent in advanced fibrosis. *Ruminococcus obeum*, and *E. rectale* were significantly less abundant in advanced fibrosis compared to mild/moderate NAFLD group. Finally, these authors created a random forest classification model

based on microbiome analysis that had a robust diagnostic precision (AUC 0.936) for characterisation of advanced fibrosis [98]. Likewise, Boursier et al. showed the link of gut microbiota to the level of disease aggravation from NAFLD to NASH. They found associations between enhanced levels of Bacteroides and NASH and between increased *Ruminococcus* and fibrosis development [99].

Zhu et al. observed a link between the amount of the endogenous ethanol produced in the gut and NASH in obese paediatric patients [46]. Accordingly, ethanol producing bacteria belonging to Proteobacteria/Enterobacteriaceae/Escherichia did not show a difference between healthy and obese microbiomes but were considerably increased in the gut microbiome of NASH patients. This higher prevalence of alcohol-producing bacteria in the microbiome of NASH patients was correlated with elevated ethanol concentration in the blood. Based on these findings about the role of alcohol metabolism in oxidative stress and thus in hepatic inflammation, alcohol-producing microbiota may be involved in NASH development [46]. Differences among other members of the gut microbiota have been demonstrated in patients with fatty liver disease and healthy controls. For instance, NASH patients had low amounts of *Anaerosporobacter* and *Faecalibacterium* but increased amounts of *Allisonella* and *Parabacteroides* [100]. Similarly, Mouzaki et al. have reported lower levels of Bacteroidetes in obese individuals with NASH compared to healthy controls, but they did not observe any differences between simple steatosis and healthy control microbiome [53].

Overall, these human studies reveal detectable differences in the microbiome between healthy individuals and NAFLD or NASH patients [1]. Nevertheless, owing to factors such as the variability of study design, methods, and clinical endpoints, the interpretation of these differences in association with the liver diseases is challenging and requires further studies to define the liver disease-associated dysbiosis.

Table 1 summarizes the human studies that demonstrate a link between dysbiosis and NAFLD and provides details regarding the specific bacterial groups identified.

Table 1: Comparison of microbiota in healthy subjects vs patients suffering from different liver diseases using ● qPCR or ◊16S rRNA sequencing. This table is taken from Safari et al. [1] with permission of *Springer Nature*.

	Disease	Phylum	Family	Genus	Population/ technique
1	HBV cirrhotic patients vs healthy subjects		*Enterobacteriaceae* ↗ *Firmicutes*↘	*Bacteroides–Prevotella*↘ *Enterococcus faecalis* ↘ *Faecalibacterium prausnitzii* ↘ *Clostridium clusters XI*↘ *clusters XIV*↘ *Lactic acid bacteria*↘ *(including Lactobacillus, Pediococcus, Leuconostoc, and Weissella)* *Bifidobacterium* ↘	Healthy (n = 32), HBV cirrhosis (n = 31) [101] ●
2	Cirrhotic patients vs healthy subjects	*Bacteroidetes* ↘ *Proteobacteria*↗ *Fusobacteria*↗	*Bacteroidaceae* ↘ *Streptococcaceae*↗ *Lachnospiraceae* ↘ *Veillonellaceae* ↗ *Enterobacteriaceae*↗ *Pasteurellaceae*↗	*Enterococcus faecalis* ↗ *Clostridium clusters XI*↗ *Fusobacteriaceae*↗	Healthy (n = 24), HBV cirrhosis (n =24), Alcoholic cirrhosis (n = 12) [102]◊
	Alcoholic cirrhotic patients vs healthy subjects		*Prevotellaceae*↗		
	Alcoholic cirrhotic patients vs HBV cirrhosis patients		*Prevotellaceae*↗		
3	HBV cirrhotic patients vs healthy subjects			*Bifidobacterium catenulatum group* ↘	Healthy (n = 15), HBV cirrhosis (n = 16) [103] ●
4	HBV cirrhotic patients vs healthy subjects			*Lactobacillus acidophilus*↘ *Lactobacillus rhamnosus*↘ *Lactobacillus reuteri*↘ *Lactobacillus gasseri*↗	Healthy (n = 38), HBV cirrhosis (n = 61) [104] ●

5	Cirrhotic patients vs healthy subjects		*Leuconostocaceae* ↗ *Lactobacillaceae* ↗ *Enterobacteriaceae*↗ *Alcaligenaceae*↗ *Fusobacteriaceae*↗ *Lachnospiraceae* ↘ *Ruminococcaceae* ↘ *Clostridium-Incertae sedis-XIV* ↘		Healthy (n = 10), Cirrhosis (n = 25) [105] ◊●
6	Mucosal samples - cirrhotic patients vs healthy subjects			*Clostridium*↗ *Dorea* ↘ *Subdoligranulum* ↘ *Acidaminococcus*↗ *Enterococcus*↗ *Burkholderia*↗ *Ralstonia* ↗ *Proteus*↗	Healthy (n = 17), Cirrhosis (n = 36) [106]◊
7	NASH patients vs healthy subjects			*Faecalibacterium*↘ *Anaerosporobacter* ↘ *Parabacteroides*↗ *Allisonella*↗	NASH (n=16), controls (n=22) [100] ◊
	NASH/obese vs healthy children	*Actinobacteria*↘ *Bacteroidetes*↗ *Firmicutes*↘ *Proteobacteria*↗	*Bifidobacteriaceae*↘ *Prevotellaceae*↗ *Rikenellaceae*↘ *Lachnospiraceae*↘ *Ruminococcaceae*↘	*Bifidobacterium*↘ *Prevotella*↗ *Alistipes* ↘ *Blautia* ↘ *Escherichia coli*↗	Healthy (n = 16), obese (n = 25), NASH (n = 22) [46] ◊
8	NAFLD patients vs healthy subjects		*Lactobacillaceae*↗ *Lachnospiraceae*↗ *Ruminococcaceae* ↘	*Lactobacillus*↗ *Robinsoniella*↗ *Roseburia* ↗ *Dorea* ↗ *Oscillibacter*↘	Healthy (n = 30), NAFLD (n = 30) [95] ◊
9	NAFLD patients vs healthy controls			*Alistipes*↘ *Prevotella*↘ *Escherichia coli*↗ *Odoribacter*↘ *Lactobacillus*↗	Healthy(n=32), NAFLD (n=53) [97] ◊

				Oscillibacter↘ *Anaerobacter*↗ *Clostridium XI*↗ *Streptococcus*↗ *Flavonifractor*↘	
10	NAFLD children vs Healthy/Obese children with no NAFLD		*Gammaproteobacteria (class)*↗	*Prevotella*↗	Healthy (n = 26), NAFLD (n = 13), Obese (n = 11) [94] ◊
11	Significant fibrosis vs Mild fibrosis		*Bacteroidaceae*↗ *Prevotellaceae*↘	*Ruminococcus*↗ *Bacteroides*↗ *Prevotella*↘	NASH (n=35), No NASH (n=22) [99] ◊
	NASH vs no NASH (NAFLD)		*Bacteroidaceae*↗ *Prevotellaceae*↘	*Bacteroides*↗ *Prevotella*↘	
12	Paediatric NAFLD, NASH, or obesity vs healthy	*Actinobacteria*↗ *Bacteroidetes*↘	*Rikenellaceae*↘	*Ruminococcus*↗ *Blautia*↗ *Dorea*↗ *Bradyrhizobium*↗ *Anaerococcus*↗ *Peptoniphilus*↗ *Propionibacterium-acnes*↗ *Oscillospira*↘	Paediatric NAFLD, NASH, or obese (n = 61); healthy (n = 54 [96] ◊

1.3.2. Mechanisms

Microbiota can improve or worsen NAFLD through several mechanisms, such as changing the permeability of the intestine, altering the amount of energy absorbed from diet, changing the expression of genes in the *de novo* lipogenesis and choline and bile acid metabolic signalling pathways, producing ethanol in the intestine and co-operating with the innate immunity (Figure 1). However, the associations between these parameters and NAFLD development or progression is still under debate [1]. These parameters are depicted here.

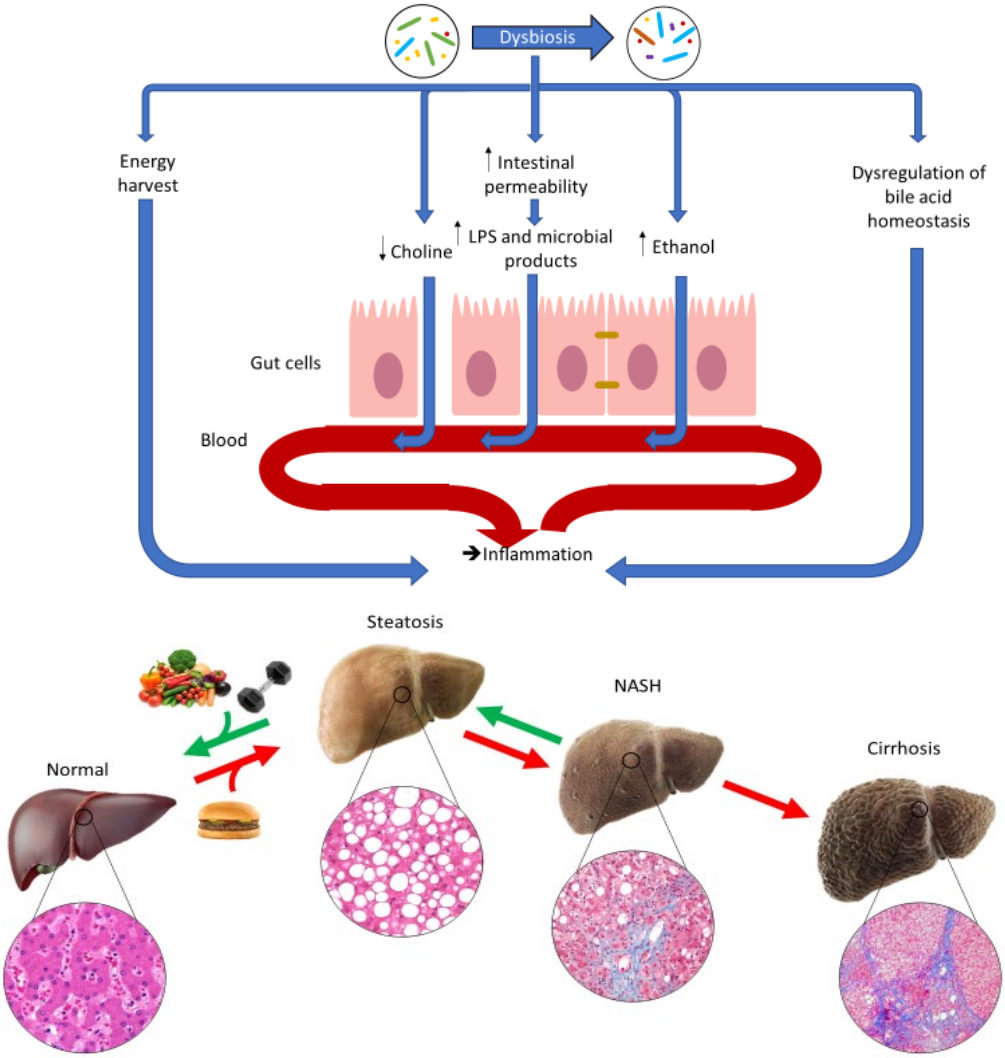


Figure 1. The mechanisms linking the microbiome to NAFLD development. Perturbation in the intestinal microbiota composition or function can result in increased gut permeability and facilitation of the passage of LPS and other inflammatory factors to the blood, decreased choline availability, changes in bile acid composition and increased ethanol production in the intestine. These factors and metabolites together with dietary lipids can result in liver steatosis, inflammation and, eventually, NASH development. This figure and figure legend are reproduced from Safari et al. [1] with permission of *Springer Nature*.

Increased Permeability

One of the main issues in NAFLD development and progression is gut permeability, which may be mediated by the microbiome (Figure 2) [1]. Several factors, including the mucus layer, antimicrobial peptides and the network of tight junction (TJ) proteins cooperate to preserve the function of gut barrier. Intestinal permeability has been associated with NAFLD severity; as Giorgio et al. revealed that there is higher intestinal permeability in children with steatohepatitis versus those with steatosis [107].

About 39.1% of the patients involved in a meta-analysis with 128 NAFLD cases revealed enhanced intestinal permeability based on the urinary excretion of a measured compound, in comparison to only 6.8% of healthy controls. Approximately 49.2% of NASH subjects had increased intestinal permeability [108]. This increased gut permeability could be owing to a weaker TJ protein network, as diminished expression of one of major TJ proteins, ZO-1 (zona occludens), was found in the intestinal mucosa of the patients with NAFLD [109]. The changed function of the gut barrier could cause the passage of proinflammatory molecules, and several human studies revealed that the later stages of NAFLD (with or without fibrosis initiation) are often correlated with augmented bacterial endotoxin levels in the blood [110-112]. Verdam et al. [113] found higher levels of plasma antibodies against LPS in NASH patients against healthy controls, and this effect was increased with increasing severity of liver disease.

In animal studies, treatment with both an HFD and high sucrose diet led in higher levels of LPS; lower expression of occludin, which is an important intestinal TJ protein; and higher fat depositions in rats [114]. LPS (produced by gram-negative bacteria) are known to influence the development of metabolic features and IR through TLR4-dependent activation of the NF-KB pathway. LPS can cross the gastrointestinal epithelium via leaky TJ or infiltrating chylomicrons [115]. Infusion of low doses of LPS in genetically obese mice resulted in steatohepatitis development [116] by enhancing the production of proinflammatory cytokines. LPS injections in mice also mimic HFD effects such as weight gain, IR, and NAFLD development. Moreover, mice deficient in TLR-4, are not only resistant to LPS-induced obesity and NAFLD but are also resistant to HFD-induced obesity and NAFLD [117], as well as NAFLD and NASH in various rodent models [118-120], demonstrating the essential role of the TLR4-NF-KB pathway in NAFLD pathophysiology. Similarly, inflammasome-deficient mice demonstrated exacerbated steatosis and inflammation in the liver due to TLR-4 and TLR-9 activation via their changed gut microbiota. [83]. The activation of the TLR-9 signalling pathway induces IL-1β production by Kupffer cells, resulting in hepatic steatosis, inflammation, and fibrosis [121].

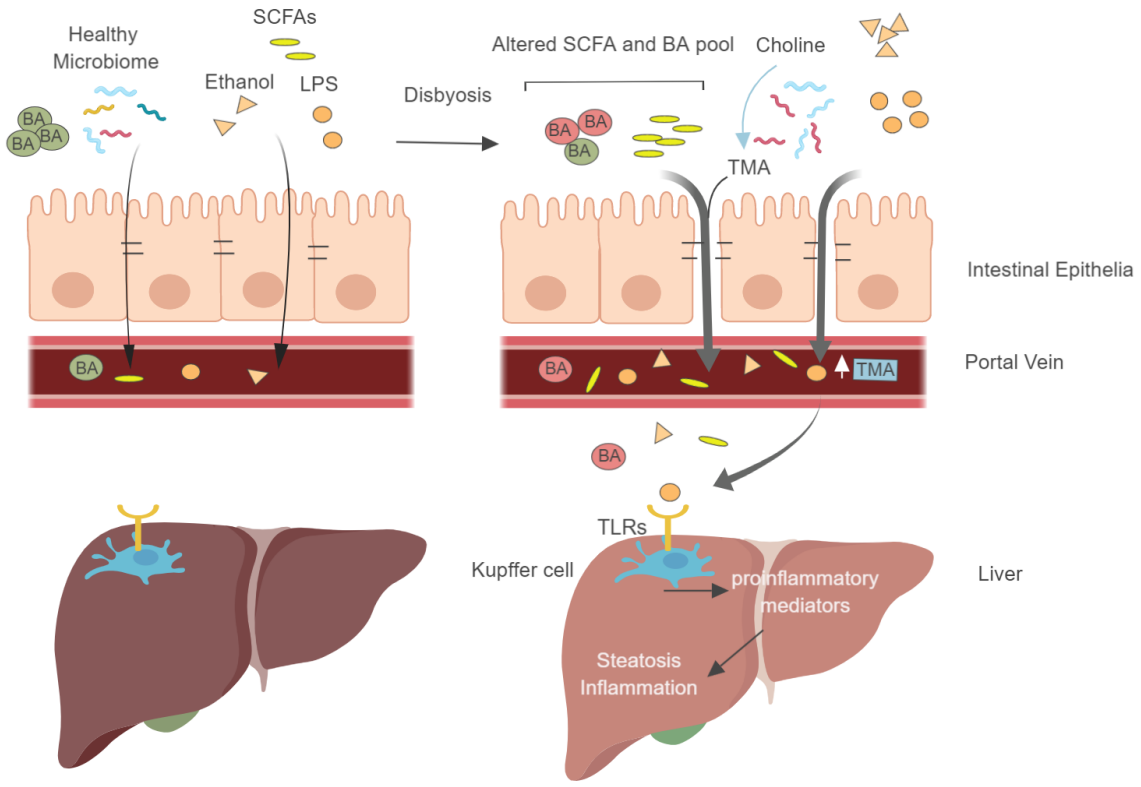


Figure 2. Schematic view of the role of the microbiome in gut permeability and NAFLD development. On the left side, the gut-liver axis components are operating normally; on the right side, NAFLD status is shown. The dysbiotic microbiome, together with the changed intestinal barrier due to the malfunction of the tight junctions, facilitates the translocation of some bacterial products into the portal vein. These bacterial products interact with Toll-like receptors (TLRs) on the surface of the hepatic cells, which leads to inflammation and NAFLD development. This figure and figure legend are reproduced from Safari et al. [1] with permission of *Springer Nature*.

Increased energy harvest

NAFLD is one of the comorbidities of obesity, and the gut microbiota was revealed to be involved in its development. Indeed, the gut microbiota is a key regulator of energy harvest from dietary food and can lead to increased fat deposition via different mechanisms, including the gut epithelium development [122, 123] by increasing the density of small intestinal villi and influencing gut physiology and motility via producing SCFAs that interact with G protein-coupled receptors (GPCRs) [124]. Bacterial enzymes extract calories from indigestible polysaccharides in the diet [123]. Lastly, it was shown that enteric bacteria decrease the synthesis and secretion of angiopoietin-like 4 protein in the small intestinal, leading to am augmented activity of lipoprotein lipase and increased liver fat deposition [85, 125].

Choline metabolism regulation

Dietary choline is crucial for VLDL production and hepatic lipid transfer. Hence, diets lacking choline are usually used to stimulate NAFLD in animal models. These diets cause decreased VLDL levels and beta-oxidation, leading to cholesterol and FAs deposition, oxidative stress and alterations in cytokines and adipokines, slight inflammation and fibrosis in the liver [96, 126, 127]. The gut microbiota influences the conversion of choline to dimethylamine (DMA) and trimethylamine (TMA) [128], which can lead to choline deficiency and finally NAFLD. Indeed, Dumas et al. analysed urinary metabolites in different mice strains fed HFD. They observed that in strain 129S6, the conversion of choline into methylamines by the gut microbiota decreased the bioavailability of choline and mirrored the effect of choline-deficient diets, leading to NAFLD and IR [129].

In humans, the effect of a choline-deficient diet on the gut microbiome composition and the consequences for NAFLD development was studied by Spencer et al. [92]. Patients received 10 days of a normal diet and then 42 days of a choline-depleted diet, which finally alters Gammaproteobacteria and Erysipelotrichia frequencies. Interestingly, the baseline levels of these taxa combined with a polymorphism in N-methyltransferase (PEMT), a vital enzyme in the metabolism of choline, could predict the predisposition of individuals to fatty liver disease stimulated by a choline-deficient diet. [92].

Bile Acids

Bile acids are saturated, hydroxylated C24 cyclopentanephenanthrene sterols that are essential for the absorption of lipids in the GI tract. Primary bile acids are made from cholesterol in the liver. They are conjugated to either taurine or glycine with an amide bond at the C24 carboxyl [130, 131]. Later, the gut microbiota converts the primary bile acids to more than twenty different secondary bile acids [130]. The bile acids also play a role as signalling molecules in their own metabolism, as well as energy, glucose, and lipoprotein metabolism through Farnesoid X receptor (FXR) and G-protein–coupled bile acid receptor 1 (TGR5) [97]. Therefore, the contribution of the gut microbiota in bile acid metabolism can improve health or lead to diseases depending on the structure and amount of the secondary bile acids that are produced [1]. FXR and TGR5 showed different affinities for individual bile acids: the stronger natural FXR agonists are CDCA>DCA>CA>LCA, whereas Tα-, TβMCA and UDCA function as antagonists. In a similar manner, bile acids also activate TGR5 with different potencies (LCA>DCA>CDCA>CA). This suggests that the changes in the composition of bile acids caused by gut microbiota dysbiosis may influence host metabolism by modifying these signals. In addition, it is well known that the secondary bile acids generated by the gut microbiota are usually observed in peripheral tissues, such as liver, heart and kidney, underlining their possible influence on the homeostasis in

mammalians [132]. The gut-liver axis has a crucial role in bile acid metabolism. The gut microbiota impacts bile acid production, pool size and structure as well as the enterohepatic circulation of bile acids, while bile acids control the gut microbiota size and content. Altogether, these mutual relationships between bile acids and the gut microbiota strongly influence host metabolism as well as metabolic diseases [133-135].

Indeed, conventional (CV) mice displayed a drop in tauro-conjugates (FXR antagonists) level compared to GF mice, but the CV mice, retained levels of the more toxic cholic acid [136]. Bile acid and fatty acid production can be prevented by activation of FXR via specific agonists and this also causes increases glucose and insulin sensitivity in obese and diabetic mice. Specific FXR activation also improves primary biliary cirrhosis and NASH through the reduction of the bile acid pool [137, 138]. This was shown using natural ligands (CA or CDCA), a semi-synthetic derivative of CDCA (obeticholic acid [OCA]), and synthetic non-steroidal molecules (GW4064 and WAY-362450). OCA has demonstrated great potential in the treatment of several hepatic diseases [139] and has now entered to phase II and III clinical studies. However, OCA treatment was reported to induce side effects such as pruritus, ascites or jaundice, implicating the complexity of the host response to FXR activation. The importance of bile acid-microbiota interactions in NAFLD was further studied by Janssen et al. who modulated microbiota by guar gum. Supplementing the mouse diet with this fermentable dietary fibre increased inflammation in the liver and fibrosis and strikingly augmented plasma and hepatic bile acid levels, while it declined adipose tissue mass and inflammation. Gut bacteria destruction using oral antibiotics reduced portal secondary bile acid levels and protected against NAFLD [140]. Therefore, it was speculated the causal correlation between changes in the gut microbiota and hepatic inflammation and fibrosis is probably through alterations of bile acids [1].

Overall, these studies show that the interactions between bile acids and intestinal microbes play indispensable roles in host metabolism [141] and metabolism-related diseases, including NAFLD.

Ethanol production

The fermentation of carbohydrates by intestinal bacteria leads to endogenous ethanol production that could cause NAFLD [46]. In a study carried out on obese mice, ethanol was found in exhaled breath, though the mice did not consume any alcohol [142]. Children with NASH were shown to have increased blood ethanol concentrations versus to healthy individuals or children with NAFLD, implying that endogenous ethanol production may contribute to exacerbated liver injuries by triggering inflammatory signals [46].

1.3.3. Therapeutic potential of the gut microbiota

The gut microbiota can be modified through antibiotics, prebiotics, probiotics, or synbiotics which are the combination of both prebiotics and probiotics. These modulators can affect the microbiome through the following different mechanisms, all of which impact susceptibility to NAFLD [143-145]: exerting anti-inflammatory effects by inhibition or elimination of invading bacteria or their products, reducing energy salvage, increasing Angplt4 production, improving the epithelial barrier function, decreasing the amount of ethanol produced by the gut microbiota, and influencing bile acid and choline metabolic signalling.

Antibiotics

Antibiotics must be used with caution because they may destroy important species associated with healthy status and lead to the appearance of antibiotic-resistant strains [146]. There are studies assessing the impact of antibiotic treatment of NAFLD in humans as well as in animal models. Six months of treatment with different norfloxacin and neomycin antibiotics reduced small intestinal bacterial overgrowth and boosted the liver function of patients with liver cirrhosis [147]. Moreover, chronic oral use of antibiotics was reported to suppress the gut bacteria, decrease the amount of portal secondary bile acid, and attenuate inflammation in the liver as well as fibrosis [140] in a NAFLD mouse model. Furthermore, the combined use of neomycin, bacitracin and streptomycin for four months was correlated with decreased level of liver triglycerides, lipid deposition and serum ceramide production in mice [97]. Also, the use of the antibiotics polymyxin B and neomycin in mice treated with a high fructose diet caused reduced fat deposition in the liver [148, 149].

In summary, the destroying or altering the gut microbiota caused by antibiotics appears to reduce susceptibility to fatty liver disease [1]. However, the risk of antibiotic resistance makes antibiotic difficult to use as a therapeutic strategy; thus, discovering new techniques to modulate the gut microbiota is needed to improve NAFLD.

Prebiotics

Prebiotics are poorly digested food ingredients that help the growth of beneficial microorganisms in the intestines and therefore positively alter the gut microbiota [150]. They make gut-mediated changes in luminal and peripheral metabolism including decreased bacterial hepatotoxins, increased intestinal epithelial barrier, reduced inflammation, reduced *de novo* lipogenesis, modified appetite and satiety, and enhanced glycaemic control, and all these effects could lead to NAFLD improvement. Prebiotics promote the bacterial production of SCFAs and the growth of indigenous *Bifidobacteria* and *Lactobacilli* as well as other beneficial bacterial species or reduce luminal pH and thus inhibit the

growth of pathogens [151]. Prebiotics also stimulate GLP-2 (gut trophic hormone), which can control endotoxin translocation via increased expression of epithelial TJ proteins and enhanced gut barrier function [81]. Hence, prebiotic treatment has been correlated with reduced levels of serum endotoxin [152]. It was observed that treatment the NASH subjects with 16 g/day of oligofructose (inulin-type fructans) in the diet for 8 weeks significantly decreased hepatic inflammatory markers [153].

Several promising animal studies also consider prebiotics as an effective dietary treatment for NAFLD [1]. In rodents, prebiotics reduced plasma lipid levels and hepatic triglyceride concentration [154-156]. These reductions might be due to the reduction of *de novo* fatty acid synthesis through decreased gene expression of enzymes in the lipogenesis pathway [155, 157-159]. Prebiotics were observed to improve the metabolic and HFD induced liver disorders. For instance, an HFD diet enriched with fungal chitin-glucan (CG) reduced TG in the liver in comparison to HFD alone. CG treatment also remarkably decreased HFD-induced body fat growth, body weight gain, and blood glucose and cholesterol level increases, regardless of caloric intake. These improvements were associated with *Clostridia cluster XIVa* gut bacteria and were not affected by incretin GLP-1 hormone [160]. Likewise adding fructooligosaccharides (FOS) to the diet, in a NAFLD mouse model, decreased hepatic TG by altering microbiota structure. This effect was linked with stimulated fatty acid oxidation via peroxisome proliferator-activated receptor alpha (PPAR-alpha) and augmented cholesterol deposition by inhibiting SREBPs (sterol regulatory element binding proteins) [152].

Probiotics

Probiotics are live bacteria or yeast that are beneficial to the host when used in sufficient quantities. Although it is not yet known how probiotics function, many animal studies as well human trials have shown NAFLD improvement following probiotic administration. As an instance, Vajro et al. [161] treated 20 obese NAFLD children with a diet enriched with *Lactobacillus rhamnosus* strain GG (LGG) or placebo for 8 weeks. They observed a drop in alanine aminotransferase and antipeptidoglycan-polysaccharide antibodies in the probiotic group compared with the group treated with the placebo only, regardless of BMI. This signifies the use of probiotics as a therapeutic means in obese children affected with NAFLD. Also, Famouri et al. studied the probiotic capsule (consisting of 4 probiotic strains) for 12 weeks in children with NAFLD and revealed diminished liver enzymes, cholesterol and TG level and improved sonography grade after probiotic intervention compared to the placebo group [162]. Alisi et al. treated NAFLD children with VSL#3 probiotic supplement (a mixture of 8 different lactic acid–producing bacteria) [163]. They showed that using VSL#3 for 4 months increases GLP- 1 levels and therefore improves fatty liver and BMI. Nevertheless, VSL#3 supplementation was recently observed to raise adiposity in obese Latino adolescents with no improvement in the liver fat amount [164], indicating that the efficacy of this probiotic cocktail may be highly variable. Aller et al.

carried out a 12-week double-blind experiment to assess the effects of daily consumption of a probiotic tablet containing 500 million *Lactobacillus bulgaricus* and *Streptococcus thermophilus* on adult NAFLD patients. There were no changes in the cardiovascular risk factors and anthropometric parameters between the treated and control groups; however, treatment with probiotics resulted in a remarkable improvement in aminotransferase levels [165]. In another study, probiotics could be beneficial in the context of other liver diseases; an interesting meta-analysis showed that patients who received probiotics prior to liver transplantation had substantially decreased rates of infections and hospitalization period [166].

In mouse models of NAFLD, a larger panel of probiotic strains was assessed which were also examined in humans. For instance, it was disclosed that the using *Lactobacillus casei* strain (LcS) as a supplement inhibited the methionine-choline-deficient (MCD) diet-induced development of NASH by reducing serum LPS concentrations [167]. Hence, altering the gut microbiome using LcS administration may be beneficial for regulating TJ proteins, maintaining gut barrier integrity, and finally improving hepatic inflammation. Similarly, there reports showing that VSL#3 reduced fat deposits and inflammatory and oxidative liver damage and reduced serum levels of alanine aminotransferase. [132, 168, 169]. Cano et al. [170] treated mice with *Bifidobacterium pseudocatenulatum CECT 7765* and observed improvements in the immunological and metabolic dysfunctions linked with HFD-induced obesity. Moreover, *Bifidobacteria* administration led to reduced IR, fat accumulation, and serum inflammatory markers in comparison to the mice fed an HFD without probiotics. However, normal chow-fed mice with or without probiotics did not show any differences in metabolic and liver parameters. Similarly, improved immune defence mechanisms in macrophages and dendritic cells and decreased gut inflammatory signals were revealed after oral consumption of *Bacteroides uniformis CECT 7771* in HFD-fed mice, and they showed less hepatic fat accumulation than did control mice [171]. Furthermore, *Akkermansia muciniphila* (associated with anti-obesity properties), was shown to improve liver injury in C57BL/6 mice by improving inflammation and hepatocellular death [172]. LGG treatment in a fructose-enriched diet mouse model of NAFLD, changed the gut microbiota leading in decreased hepatic expression of the genes that function in the lipogenesis pathway and improved liver steatosis. This treatment also resulted in decreased expression of the proinflammatory cytokines such as TNF-α, IL- 1β and IL-8R in the liver [173].

These studies demonstrated that using probiotic might be efficient against NAFLD development/progression. However, only a few bacterial cocktails were shown to be efficient and to slightly improve some of the parameters linked with the disease.

Synbiotics

Synbiotics (mixture of probiotics and prebiotics) were shown to enhance the survival and colonization of diet derived-microbial communities in the intestine by stimulating the growth or metabolism of specific health-promoting bacteria. A few studies have evaluated the effects of synbiotics on NAFLD subjects [1]. However, it was shown that synbiotic supplementation (FOS and probiotic strains) together with lifestyle modifications for 28 weeks is more beneficial for NAFLD treatment than lifestyle modifications alone. In addition, this synbiotic supplementation decreased inflammatory signals and reduced waist-to-hip ratio and BMI. These effects were observed at week 14 and persisted until the end of the treatment [55]. Similarly, Malaguarnera et al. [174] indicated that administration of *Bifidobacterium longum* with FOS in the diet together with lifestyle modification for 24 weeks significantly reduced hepatic fat deposition and the NASH activity index when compared to lifestyle modification alone. Safavi et al. [175] observed that synbiotic supplementation in obese children resulted in a considerable improvement in all blood lipid parameters after 8 weeks of treatment. Conversely, Ipar et al. [176] showed that synbiotic supplementation improves total and LDL (low-density lipoprotein) cholesterol levels remarkably but it does not affect the triglyceride levels.

In summary, the above-mentioned findings show the beneficial effect of prebiotics, probiotics and synbiotics on fatty liver symptoms, through improvement of gut barrier function.

Diet

Diet composition is an important driver of the gut microbiota structure [152, 173, 177-180], and the role of diet in shaping the structure of gut microbiota is even stronger than that of genetic factors [178]. Therefore, we questioned whether the effects of diet on NAFLD development are due, at least partially, to changes in the gut microbiota composition.

Zeng et al. [180] fed C57BL/6 mice with a fatty liver an HFD for 10 weeks to study whether the NAFLD phenotype is linked with microbiome changes [180]. They found an increased amount of DNA from *L. gasseri* and/or *L. taiwanensis* (from the *Lactobacillus acidophilus* species group) in the HFD than in the low-fat diet groups. Most of these bacteria were bile acid resistant; therefore, the authors speculated that the increase in *Lactobacillus* species induced by HFD could impact lipid metabolism through the changing bile acid metabolism, and thus contribute to NAFLD development. Conversely, diet can be used to rebuild a healthy microbiota to improve NAFLD. A study indicated that supplementation of a Chinese herbal formula (CHF) in HFD-induced NAFLD rat models improved NAFLD and resulted in decreased levels of *Escherichia*/*Shigella* and other LPS-containing bacteria that may harm the gut barrier and trigger a low-grade chronic inflammatory state [181]. The CHF supplementation also augmented *Collinsella* abundance [181], which may affect human epithelial cell proliferation, and

improve intestinal barrier integrity through SCFA production [182]. Finally, the Mediterranean diet, rich in polyunsaturated fats, polyphenols, carotenoids and vitamins, all of which have antioxidant and anti-inflammatory effects, was shown to be efficient in declining the risk of developing metabolic syndrome through protecting the integrity of the gut barrier and the decreasing endotoxaemia [183-185]

Thus, the gut microbiota may be associated with the adverse effect of HFD on NAFLD and the alteration of gut microbiota composition through diet could be an effective strategy to improve liver pathology.

Faecal microbiota transplantation (FMT)

Transfer of faecal material containing bacteria from a healthy donor to a diseased patient in order to re-establish a balanced gut microbiota composition is called FMT [1]. FMT has been reported to be effective to cure *Clostridium difficile* infection, and its use in a wide range of non-gastrointestinal disorders such as metabolic disorders proved to be useful [186, 187].

Currently, it was shown that after 8 weeks of FMT, mice had remarkably declined intrahepatic lipid deposition and levels of transaminases in the serum, along with a decreased degree of lobular inflammation and hepatocyte ballooning. This implies a positive effect of FMT in HFD-induced metabolic disorders [188].

To our knowledge, no FMT studies have been published so far in association with NAFLD. Nevertheless, the immense interest in FMT and its potential in liver diseases is revealed in several ongoing trials.

1.4. Mice strains used in this study

In this study, we used A/J and C57Bl/6J (C57) mice which are resistant and sensitive, respectively, to HFD-induced hepatosteatosis and obesity even though their food intake is the same [189, 190]. Also, they exhibit distinct inflammatory responses [191], and weight gain [192, 193]. Male C57BL/6 (C57) mice upon HFD develop hepatic steatosis verified by high level of liver TG, IR hepatocyte ballooning, Mallory bodies, higher fasting serum glucose levels, and decreased adiponectin levels, suggesting hyperglycaemia and IR [41].

We also used Chromosome Substituted Strains (CSSs) which are constructed by crossing A/J and C57 mice. Nadeau, Lander, and co-workers developed the first set of CSSs with the A/J strain as a chromosome donor and C57BL/6J (hereafter C57) as the recipient strain [194].

To simplify gene identification, animal models have been used because better control of environmental exposures and genetic background allows the effect of particular dietary nutrients on

disease induction, progression and severity to be studied. Also, gene discovery, as well as gene-gene and gene-environment interactions, can be identified efficiently.

The CSSs serves as interesting model system for stratifying the complex phenotype of steatosis over several distinct chromosomes [195]. Since it is known which chromosomes are playing a pivotal role in rendering susceptibility to steatosis, it is of interest to study the role of genetics and microbiome together in NAFLD development.

The susceptibility of different CSSs in this panel to HFD and developing steatosis was previously studied in our laboratory by Dr Kashofer (data unpublished). Therefore, we have selected mouse strains that show particularly high sensitivity towards agents involved in simple steatosis.

The complete panel of mouse chromosome substitution strains (CSSs) was developed [194] starting with two parental strains known to differ markedly in their predisposition to diet-induced obesity [189], atherosclerosis [196], iron metabolism [197] and many other traits (Mouse Phenome Database, The Jackson Laboratory, Bar Harbor, ME). The CSS panel consists of 21 inbred strains of mice, each with a single A/J-derived chromosome (Chr) with the remainder of the genome derived from strain C57BL/6J (C57) (Figure 3).

CSSs provide a special opportunity to characterize the architecture of complex traits in model organisms.

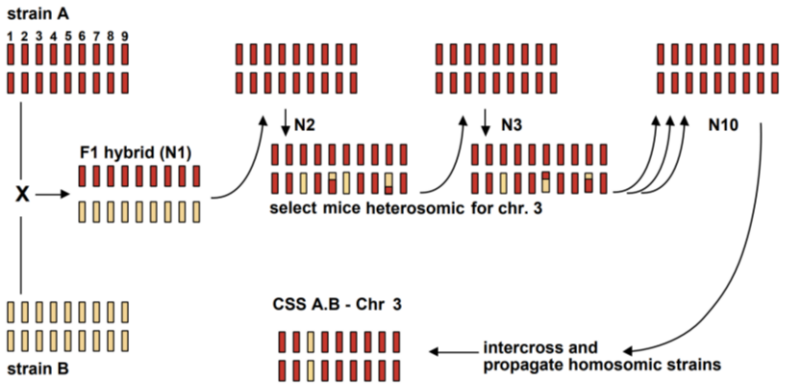


Figure 3. Strategy for constructing CSSs. The first step requires making hybrids between strains A and B, which are then backcrossed to host strain A. Progeny with a non-recombinant chromosome derived from donor strain B, in this case, chromosome 3, are identified in this and subsequent backcrosses. These mice are backcrossed to host strain A at each generation. At the tenth backcross generation (N11, counting the F1 hybrids as the N1 generation), males and females with the non-recombinant chromosome derived from donor strain B are intercrossed. Progeny of this intercross that are homozygous (homosomic) for chromosome 3 are used to propagate the homosomic strain. Special considerations apply to the X and Y chromosomes. A Y chromosome CSS is made by selecting males for backcrossing to the host strain at each backcross generation16–18. To ensure that the Y chromosome is not unintentionally transferred from the donor to the host strain, a heterosomic carrier

female is used for at least one backcross. Similarly, to eliminate the X chromosome derived from the donor strain, at least one backcross requires a male that is heterosomic for the desired autosome and has an intact X chromosome from the host strain. This figure and figure legend are reproduced from Nadeau et al. [195] with permission of *Springer Nature*.

1.5. Hypothesis and aims of the thesis

Recent studies have highlighted diet, genetic and microbial factors in the pathogenicity of NAFLD. Particularly, ethnic studies suggested that there is a difference between the way fat is stored in the organs in different ethnic groups, as African-Americans appear to store fat to a larger extent subcutaneously compared to the obese Hispanics who tend to deposit fat in the liver and visceral adipose tissue depots [7]. Therefore, according to the reports, not every person exposed to the same environmental and lifestyle risks develops steatosis. This was also confirmed by a previous study performed in our laboratory at the Medical University of Graz by Dr Karl Kashofer who showed that A/J, C57 and chromosome substituted mice fed with HFD and kept under the same conditions, developed different phenotypes of steatosis. Hence, it is assumed that the different genetic makeup of these CSSs mice is responsible for susceptibility or resistance to steatosis.

Moreover, there are several studies suggesting a strong interaction between the gut microbiota and the liver. Based on the anatomical position, the liver receives 70% of its blood supply from the intestine through the portal vein, thus it is the first target organ that interacts with microbial metabolites and the most exposed one to gut-derived toxic factors, such as bacteria and bacterial components [198].

Our hypothesis is that the gut microbiome and diet, together with genetics, contributes to the different susceptibility to fatty liver disease.

Hence, the aim of this thesis was to investigate the interaction of the genetic background, diet and microbiome in the development of fatty liver disease. This project was performed in different studies to pursue the following aims:

Specific Aim 1

Identifying the possible correlation between gut microbiota and the development of steatosis in particular CSS (Chromosome Substituted Strains) mice with variant degrees of steatosis under the HFD.

Specific Aim 2

Studying the reversibility of the liver phenotype and resilience of the gut microbiome in response to the diet switching from high fat to normal diet in C57 mice.

Specific Aim 3

Studying the effect of different treatments including HFD, antibiotics and microbiome exchange between C57 and A/J (steatosis susceptible and resistant) mice on the severity of NAFLD.

2.Method and materials

2.1. Materials

2.1.1. General technical equipment

PCR machine: Gene Amp PCR System 9700 PE, Applied Biosystems, Foster City, CA, USA

Thermo block: Thermomixer comfort, Eppendorf, Hamburg, Germany

Vortex: Vortex-Genie 2, USA

Water bath: mgw, Lauda RMS Brinkmann, USA

Incubators: 37°C and 65°C, Heraeus, Germany

Spectrophotometers:

- Smartspec 3000, BioRad, USA
- NanoDrop 2000, Thermo Fisher Scientific, USA

Shaker: Certomat H and U, Braun, Germany

Gel electrophoresis instruments: Sub-cell GT, BIO-Rad, Vienna, Austria

Gel imaging and documentation system: FlurChrom 5500, Alpha innotech, Germany

Centrifuges:

- Biofuge fresco, Heraeus, Buckinghamshire, United Kingdom
- Megafuge 2.0R, Heraeus, Germany
- Sorvall RC-5B, refrigerated superspeed centrifuge, Sorvall, Vienna, Austria

Specialized Instruments and resources used in animal dissections and sample storage:

- Surgical tools (knives, forceps and tweezers), VWR International, Vienna, Austria
- Surgical blades, VWR international, Vienna, Austria
- Syringes, VWR international, Vienna, Austria
- Paraffin cassettes, VWR international, Vienna, Austria
- Cryotube, VWR international, Vienna, Austria
- Accu-Check glucometer, Roche Diagnostics, Switzerland
- MagNA Lyser, Roche, Switzerland

2.1.2. Microscopes

- Light optical microscope: Nikon, Eclipse E 600 with 10x, 20x, 40x, 60x objectives, Nikon, Japan
- Digital Camera: Canon S3, Canon, Japan
- Confocal Laser Scanning Microscope: Zeiss LSM 510, Zeiss, Germany

2.1.3. Plasticware

- 1.5 mL to 15 ml tubes, Corning, Eppendorf and Sarstedt, Germany
- PCR-soft strips (0.2ml), Biozym, Germany
- 6 well plates and 75 cm^2 vented culture flasks, Costar, Germany

2.1.4. Chemicals, reagents and buffered solutions

Table 2: Chemicals, Reagents and Buffered solutions.

Categories	Solutions/buffers/kits	Recipe	Company	Storage Conditions
General	1 M TRIS, pH 7-8	121.1 g TRIS base		RT
Usage		1 L dd H_2O		
		pH adjustments with conc. HCl		
	0.5 M EDTA, pH 8.0	93.05 g of EDTA		RT
		500 ml dd H_2O		
		pH adjustments with 5M NaOH		
	5 M NaOH	100 g NaOH		RT
		500 ml dd H_2O		
	5 M NaCl	292.2 g NaCl		RT
		1 L dd H_2O		
	3 M KCl	93.2 g of KCl		RT
		500 ml dd H_2O		
	PBS pH 7.2 - 7.3	1.4625 g $Na_2HPO_4.H_2O$		RT
		0.2450 g KH_2PO_4		
		8 g NaCl		
		500 ml dd H_2O		
	TE buffer	5 ml 1M TRIS, pH 8.0		RT
		1ml 0.5M EDTA, pH 8.0		

		500 ml ddH20		
	10 % Triton-X-100	5 ml Triton-X-100		RT
		50 ml ddH_20		
Agarose gel electrophoresis	20X Tris-Borate buffer (TBE) buffer	216 g Tris		RT
		110 g Boric acid		
		40 ml 0.5 M EDTA, pH 8.0		
	5 X gel loading buffer		Fermentas	4^0C
	Gene Ruler 50bp ladder		Fermentas	4^0C
	Gene Ruler 1Kb ladder		Fermentas	4^0C
	MIDORI Green Advance DNA stain		NIPPON Genetics Co, Ltd	4^0C
	Standard Agarose		Eurobio	RT
	Seakem ME Agarose for gel electro-phoresis		Cambrex	RT
	1kb DNA Ladder		BioLabs	4^0C
RNA extraction	TRIZOL reagent		Invitrogen	4^0C

2.1.5. Enzymes and primer

Table 3 : Enzymes and primer.

Purpose	Forward or Reverse (F/R)	Identifier	Company
qPCR primers-MUG		Sequence	Company
HSL4	F	CCTTCATGTCTCCTCTGCTG	TaqMan
HSL4	R	CCGAGTCATCTAGCATGGG	TaqMan
PPARg1	F	CCAAGAATACCAAAGTGCGA	TaqMan
PPARg1	R	AATGGCCATGAGGGAGTTAG	TaqMan
ATGL2	F	AAAGAGCAGACGGGTAGCAT	TaqMan

ATGL2	R	GACAGTCTGGAAGGCAGATG	TaqMan
FAS1	F	GAGGACACTCAAGTGGCTGA	TaqMan
FAS1	R	GTGAGGTTGCTGTCGTCTGT	TaqMan
ACCa1	F	TCATGAACACCCAGAGCATT	TaqMan
ACCa1	R	TAGTGGCCGTTCTGAAACTG	TaqMan
SREBP1c2	F	GCACTGAAGCAAAGCTGAAT	TaqMan
SREBP1c2	R	TGCAAGAAGCGGATGTAGTC	TaqMan
ApoB 100	F	CAACACCAGCAGAGACCACT	TaqMan
ApoB 100	R	TTCTCCAGATCCTTGCACAC	TaqMan
Mlxipl	F	GAAGTTCTGGGTGTTCAGCA	TaqMan
Mlxipl	R	GGAGAGCAGGTAGGGAACAG	TaqMan
Perilipin2	F	AGAGAAGCATCGGCTACGAC	TaqMan
Perilipin2	R	GCGATAGCCAGAGTACGTGA	TaqMan
DGAT1	F	GCATTTCAGATTGAGAAGCG	TaqMan
DGAT1	R	CTGGGAAGCAGATGATTGTG	TaqMan
18S	F	AGTCCCTGCCCTTTGTACACA	TaqMan
18S	R	CGATCCGAGGGCCTCACTA	TaqMan
qPCR primers-INRA		Gene Bank accession n°	ThermoFisher Assay ID
Actb	F/R	EF043617	Mm04394036_g1
Gapgh	F/R	NM_001289726	Mm99999915_g1
Tlr4	F/R	NM_021297	Mm00445273_m1
Tnfα	F/R	NM_001278601	Mm00443258_m1
Il6	F/R	NM_031168	Mm00446190_m1
Reg3γ	F/R	NM_011260	Mm01181783_g1
Tjp1	F/R	NM_001163574	Mm00493699_m1
Ocln	F/R	NM_008756	Mm00500912_m1

Glp2r	F/R	NM_175681	Mm00558835_m1
Acaca	F/R	NM_133360	Mm01304257_m1
Fas	F/R	NM_007987	Mm01204974_m1
Srebf1	F/R	NM_011480	Mm00550338_m1
Pparγ	F/R	NM_011146	Mm00440940_m1
Scd1	F/R	NM_009127	Mm00772290_m1
Cd36	F/R	NM_001159555	Mm00432403_m1
Ffar2	F/R	NM_001168509	Mm02620654_s1
Ffar3	F/R	NM_001033316	Mm02621638_s1
Nr1h2	F/R	NM_001285517	Mm00437265_g1
Nr1h4	F/R	NM_001163504	Mm00436425_m1
Gcg	F/R	NM_008100	Mm01269055_m1
Timp1	F/R	NM_001044384	Mm01341361_m1
Nfkb1	F/R	NM_008689	Mm00476361_m1
16srRNA Analysis		Sequence	Company
V3–V4 region-MUG			
16s_515_S3_fwd	F	GATTGCCAGCAGCCGCGGTAA	MUG
16s_806_S2_rev	R	GGACTACCAGGGTATCTAAT	MUG
V3-V4 region-INRA			
16S rRNA	F	TACGGRAGGCAGCAG	MolTaq (Molzym, Plaisir, France)
16S rRNA	R	ATCTTACCAGGGTATCTAATCCT	MolTaq

2.2. Methods

Note: The methods used at the Medical University of Graz (MUG) Graz, Austria or Institut National de la Recherche Agronomique (INRA), Jouy-en-Josas, France are distinguished by mentioning the abbreviation of the lab at the beginning of each method.

2.2.1. Animal experiments

MUG: Animal experiments were approved by the local animal care committee and conducted in compliance with the Austrian Law for Welfare of Laboratory Animals (BMWF-66.010/0017-II/3b/2014 & BMWFW-66.010/0103-WF/V/3b/2016). All the mice were fed normal chow for the first two weeks of acclimatisation and based on the experiment design received HFD (E15741-347 γ-irradiated 25 kGy) and chow diet (control diet, CD; V1534-703 γ-irradiated 25 kG) used in this study were purchased from SSNIFF® (Soest, Germany). Blood was collected by an intracardiac puncture under deep anaesthesia and serum parameters were measured in an automatic chemistry analyser (Hitachi, Roche, Vienna, Austria). Finally, mice were sacrificed by cervical dislocation. Livers were immediately removed and cut into pieces which were (i) snap frozen in methylbutane precooled with liquid nitrogen for immunofluorescence staining; (ii) fixed in formalin for histological/immunohistochemical analysis, or (iii) placed in liquid nitrogen for biochemical/RNA expression analyses.

INRA: Experiments were performed according to the European Guidelines for the Care and Use of Laboratory Animals and approved by the French Veterinary Authorities (Authorization number 78–60). All conventional male C57BL/6J and A/J mice were purchased at 6 weeks of age from the Jackson Laboratory (USA). Upon arrival, mice were kept under a 12h light/dark cycle for 1 week. All mice were given autoclaved water and γ-irradiated chow (45 kGy) or an HFD (25 kGy, 60% energy from fat, D12492; Research Diets, New Brunswick, NJ). Body weight was monitored weekly during the experiment.

After one week of acclimatization, 7-week-old normal chow-fed C57 and A/J mice were gavaged twice a day drinking water (placebo) or 0.25 ml of a cocktail of antibiotic including 1mg/ml of Ampicillin, 1mg/ml of Colistin and 45µg/ml of Vancomycin for 5 days by gavage (previously set up in our lab at INRA).

There are four groups in this study. Each group is composed of 12 A/J and 12 C57BL/6J mice. The first one, which corresponds to the control group (HFD), received water by gavage for 5 days to be in the same stress condition as the groups receiving antibiotics. The second group (AB) received antibiotics by gavage for 5 days. The third group (Same) after antibiotic treatment received a microbiota transfer from a mouse of the same strain (A/J to A/J or C57BL/6J to C57BL/6J). In the fourth group (Rev) after

antibiotic treatment microbiota is transferred from one strain to another (A/J to C57BL/6J or conversely). Then, all the groups were challenged with HFD (60% fat by calories) for 7 weeks.

Microbiome transfer was performed by resuspending the Faecal pellets from each donor 0.1g per strain into 9 ml of sterile water. The dilution is given at the same time by gavage at the rate of 0.2 ml/mouse.

After the approval of the result of the qPCR showing the sterilization of the gut, all the mice were fed by a sterilized HFD and water were ad libitum. Oral glucose tolerance tests were performed after 7 weeks feeding and all mice were sacrificed immediately (see below) after the completion of the oral glucose tolerance test.

2.2.1.1. Sample collection

MUG: The blood was taken via cardiac puncture while the mice were under deep anaesthesia using 1-1,5% Isoflurane in the Oxygen via anaesthesia machine (Plexx, Netherland, EZ-SA800 Single Animal System) and then sacrificed by cervical dislocation. The liver was harvested and fixed in 10% neutral-buffered formalin, while the remaining liver tissue was snap frozen in liquid N_2 and stored at −80°C for further analysis.

INRA: Mice were sacrificed after OGTT test by decapitation at the end of each study. Blood was collected from the cheek and transferred to EDTA coated tubes and plasma samples were stored at −80 °C. Tissues were flash frozen in liquid nitrogen and stored at −80 °C. After exsanguination, mice were killed by cervical dislocation. Subcutaneous adipose tissue depots, intestines, cecum, cecum content, ileum, colon and liver were precisely dissected, weighed and immediately immersed in liquid nitrogen followed by storage at −80°C for further analysis.

2.2.1.3. OGTT test

INRA: The OGTT was performed after 8h food deprivation at the end of HFD feeding. Blood glucose from tail vein was analysed using Accu-Check glucometer (Roche Diagnostics), before (time 0 min) and at 15, 30, 60, and 120 min after the animals were given a glucose solution at 2 g/kg body weight (BW) by oral gavage. Samples collected at 0, 15, and 120 min were put into chilled EDTA-coated tubes for insulin determination. The remaining blood was then centrifuged at 10 000 g for 10 min. Plasma was aliquoted and frozen at −80°C until analysis

2.2.2. Histological staining

2.2.2.1. Haematoxylin and eosin (H&E) staining

MUG and INRA: H&E staining was performed on 4µm thick formaldehyde fixed paraffin embedded (FFPE) liver sections. Paraffin was removed from FFPE liver sections by incubating at 65^0C for 20 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 H_2O, the liver sections were incubated with haematoxylin for 2 minutes followed by 30 seconds incubation with eosin. Thereafter, the liver sections were washed with warm tap water for 2 minutes and mounted with entellan.

2.2.2.2. Liver steatosis scoring:

MUG and INRA: virtual slides were made by using the Pannoramic Scan 150 (3DHISTECH Ltd., Hungary) and examined in a blinded manner by a semi-quantitative scoring system. Briefly, steatosis (0–3 points), lobular inflammation (0–3) and ballooning (0–2) of hepatocytes were evaluated. Points were summed up to obtain a total fatty liver activity score, which ranged from 0 (no pathology) to 8 (severe disease) [199].

2.2.2.3. Hepatic triglycerides content

MUG: About 50-70mg of frozen liver from receiver mice were homogenised in chloroform-methanol (2:1) in order to extract total lipids according to the methodology of Folch et al. [200]. The organic extract was dried and reconstituted in isopropanol. The triglyceride content was measured with a Triglycerides Colorimetric Assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions and expressed in and expressed as mg/g liver.

INRA: As described above. The triglyceride content was measured with a triglyceride's determination kit (Sigma-Aldrich, Saint-Louis, Missouri, USA) according to the manufacturer's instructions.

2.2.3. Serum insulin

INRA: In this experiment, a dosage by the sandwich Elisa method was performed using "ELISA kit – Mouse insulin" (Merck Milipore™) following their instructions. The 96 well plates, coated with a quantity of anti-rat antibody, was washed 3 times using the Wash Buffer 300 M L. Then, the Assay Buffer was added to the wells containing the whites and samples, while in the wells of the standard, blanks and quality controls, sodium azide solution were added. After the addition of the standard (0.2 ng/ML, 0.5 ng/ml, 1 ng/ml, 2 ng/ml, 5 ng/ml and 10 ng/ml), quality control and mouse serum samples, in their respective wells, the detection antibody was added. After leaving the plate agitated for 2h at

room temperature and after three washes at the Wash Buffer, the enzyme was added. The plate was stirred for 30 minutes and then washed for six times. Then the substrate was inserted into the wells and then the plate was left agitated for 15 minutes before adding the stop solution. Finally, the absorbance was read at 450 nm and 590nm on a plate reader TECAN infinite 200.

2.2.4. Serum leptin

INRA: Determination of leptin was done by an assay based on sandwich Elisa method using "ELISA kit Mouse Leptin" (Merck Milipore™) following the supplier's instructions. Thus, the 96 well plate, covered with a small quantity of Antiserum, was washed 3 times using the Wash Buffer. Then, Assay Buffer was added to all wells and sodium azide solution only in wells of the standard, blanks and quality controls. The standards (0.23 ng/ML, 0.47 ng/ml, 0.94 ng/ml, 1.88 ng/ml, 3.75 ng/ml, 7.5 ng/ml, 15 ng/ml and 30 ng/ml) were made using a 30ng/ml mouse leptin standard and Assay Buffer. After adding the standard, the quality controls and mouse serum samples were loaded in their respective wells. Then antiserum was added. After leaving the plate agitating for 2h at room temperature, it was washed with the Wash Buffer. The detection antibody was then added to all the wells and the plate was placed on agitator for 1h. After wash, the enzyme solution was placed in the wells and then the plate was stirred for 30 minutes. After rinsing, the substrate was inserted into the wells and the plate was agitated for 15 minutes. Finally, the Stop solution was added, and the absorbance was read at 450 nm and 590nm on a plate reader TECAN infinite 200.

2.2.5. Serum parameters

MUG: Serum was analyzed at Kinderklinik, Medical University of Graz, using automatic chemistry analyzer (Hitachi, Roche, Switzerland).

INRA: Serum parameters were analyzed by the biochemistry platform of the Paris Diderot University.

2.2.6. Liver genes expression

MUG: Livers were homogenised using MagNA Lyser (Roche) for 30 seconds at 7500 rpm and RNA was extracted with TRIZOL reagent (Life Technologies) following manufacturer's instructions. 2 µg of RNA was transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Qiagen, Germany).

To determine the expression of selected genes, quantitative real-time qPCR was performed using QIAGEN's real-time PCR cycler and cDNA was amplified with Power SYBR® Green PCR Master Mix (Qiagen, Germany).

qPCR: initial activation step: 5 min at 95**:** two-step cycling: Denaturation 5s at 95 combined annealing/extension 10s at 60 for 35 to 40 cycles. qPCR machine: Primers list are found in Table 3.

Samples were analysed in triplicates using specific primers (primer table) and 18S was used as an internal control. Relative expression levels were computed as a difference in the amount of the gene of interest and the internal control. Results are presented as mean with standard deviation.

INRA: As described elsewhere [201], total RNA of the selected genes for liver and ileum (Table 4) was extracted using the RNeasy Mini kit (Qiagen). RIN (RNA integrity number) values were assessed with an Agilent 2100 Bioanalyzer using the RNA 6000 Nano Kit. Total RNA (10 µg) was reverse transcribed using random primers and a High-Capacity Complementary DNA Reverse Transcription Kit (Applied Biosystems). Pre-amplification of cDNA was then performed using the TaqMan® PreAmp Master Mix (Applied Biosystems). The final cDNA samples were stored at -20°C until RT-qPCR was performed using the TaqMan® Gene Expression Technology (Applied Biosystems). The primers used are found in Table 3. DNA was amplified using the StepOne Plus Real-Time PCR system (Applied Biosystems). Data were recorded by the manufacturer's software and the RQ Manager Analysis Software (Applied Biosystems) was used to determine Ct values. GAPDH was identified as the least variable housekeeping gene and was chosen to normalize data in this study. Relative quantification of gene expression was calculated by means of ddCt values (2- [(Ct (target gene) – Ct (GAPDH)) treated – (Ct target gene – Ct GAPDH) untreated]).

Table 4 : List of genes quantified by PCR in real time according to the tissue.

Functional group	Gene symbol		Liver	Ileum
Endotoxin	*TLR4*		√	√
Inflammatory tone	*TNFα*		√	√
	IL6		√	√
	Reg3γ			√
Gut permeability	*ZO1*	*Tjp1*		√
	OCCLUDIN	*Ocln*		√
	GLP-2r			√
Lipid metabolism	*ACC1*	*Acaca*	√	
	FAS		√	
	SREBP1		√	
	PPARγ	*Pparg*	√	
	SCD1		√	
	CD36			√
	GPR43	*FFAR2*		√
	GPR41	*FFAR3*		√
	LXR		√	
	FXR		√	
Appetite-regulating peptides	*GLP1 (7-36)*	*gcg*		√
	Ghrelin	*Ghrl*		
	leptin（Ob(Lep) gene ）			
Fibrosis/fibrinolysis	*TIMP1*		√	
Nuclear factor kB Signaling	*NF-κB*		√	√
Endogenous Genes				
endogenous control assays				
18S	Eukaryotic 18S rRNA			
ACTB	Actin, Beta, cytoplasmic			
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase			

2.2.7. Microbiome analysis

MUG: *DNA Isolation and PCR Amplification*

As described elsewhere [202], bacterial DNA was extracted with the Maxwell RSC Blood DNA Kit (Promega, Mannheim, Germany) according to the manufacturer's instructions with slight modifications. Using the Lysis Buffer stool samples were homogenized on a MagNA Lyser Instrument using MagNA Lyser Green Beads (Roche Diagnostics GmbH, Mannheim, Germany). After homogenization samples were treated with 2,5mg/ml Lysozyme (Roth GmbH, Karlsruhe, Germany) for 30 min at 37 °C followed by digestion with 1mg/ml Proteinase K for 60min at 56°C. Enzyme activity was inactivated for 10 min at 95°C and 600 µl of lysate was used for the DNA isolation in the Maxwell RSC.

DNA concentration was measured by Picogreen fluorescence. The variable V3–V4 region of the bacterial 16S rRNA gene was amplified with PCR from 20ng DNA using oligonucleotide primers 16s_515_S3_fwd: GATTGCCAGCAGCCGCGGTAA and 16s_806_S2_rev: GGACTACCAGGGTATCTAAT. Bacterial 16S rRNA was amplified with the Mastermix 16s Complete PCR Kit (Molzym, Bremen, Germany). The first PCR reaction product was subjected to a second round of PCR with primers fusing the 16s primer sequence to the A and P adapters necessary for Ion Torrent sequencing while additionally including a molecular barcode sequence to allow multiplexing of up to 96 samples simultaneously. PCR products were subjected to agarose gel electrophoresis and the band of the expected length (350nt) was excised from the gel and purified using the QiaQick (Qiagen, Hilden, Germany) gel extraction system. DNA concentration of the final PCR product was measured by Picogreen fluorescence.

Sequencing

As described elsewhere [202], amplicons from the samples were pooled equimolarly and subjected to emulsion PCR using the Ion Torrent One Touch 2.0 Kit according to manufacturer's protocols. After emulsion PCR the beads were purified on Ion ES station and loaded onto Ion Torrent 318 chips for sequencing. Sequencing reactions were performed on Ion Torrent PGM using the Ion 400BP Sequencing Kit running for 1000 flows (all reagents from Thermo Fisher Scientific, MA, USA). Sequences were split by barcode and transferred to the Torrent Suite server. Unmapped bam files were used as input for bioinformatics.

Bioinformatics and Phylogenic Analysis

As described elsewhere [202], all sequences were initially trimmed by a sliding window quality filter with a width of 20nt and a cutoff of Q20. Reads shorter than 100 nucleotides and reads mapping to the human genome were removed using deconseq [203]. The resulting reads were subjected to error correction using the Acacia tool [204] leading to error correction of 10-20% of reads. Subsequently, PCR chimeras were removed by search algorithm in de-novo and reference based settings [205]. The final sequence files were then analyzed by QIIME 1.8 workflow scripts [206]. OTU search was performed using the parallel_pick_open_reference_otus workflow script and the greengenes 13_8 reference database.

Statistical analysis and visualization

As described elsewhere [202], OTUs were visualized as OTU tables, bar charts and PCOA plots using the Qiime core microbiome script. Additionally, groupings supplied in the mapping file were tested for statistical significance using the QIIME implementation of the Adonis test and significance of individual

bacterial strains was determined by Kruskal-Wallis test. Lefse [207] analysis was performed to detect statistically relevant strains in several of the study groupings.

INRA: *DNA isolation*

Metagenomic DNA was obtained from faecal content of fasted SPF mice after mechanical lysis followed by purification according to a published protocol [208] using GNOME® DNA Kit (MP Biomedicals™). DNA Extraction was performed by Aurelia Bruneau, technician, at INRA.

amplification of DNA of interest was achieved through "antisense" (GGAGTTCAGACGTGTGCTCTTCCGATCTTGARTTTMCTTAACYGCCCC) and "Sens" (CTTTCCCTACACGACGCTCTTCCGATCTGTGCCAGCMGCCGCGGTAA) primers. Indeed, in each well of a plate 96 wells 36 M L of DNA, containing 20 ng of DNA, and 13.7 µ L of Mix (10 M L of Buffer 5x, 1 M L of dNTPs, 1.25 µ L of Primer R and F and 0.2 µ L of ATQ polymerase) were added. For this purpose, the "KAPA2G Robust polymerase PCR" Kit (Kapa Biosystems ™) was used. The plate was then placed in the Thermo-Hybaid ™ using the following steps: 95 °c/5min then 40 cycles at 95 °c/30sec – 65 °c/30sec – 72 °c/60sec, and finally 72 °c/10min. In order to check the quality of the Amplicon (fragment size and amount of DNA), a 1% agarose gel containing Midori dye and a ladder was applied. DNA samples were then sent to the GeT-PlaGe sequencing platform of GenoToul (INRA, Toulouse) for sequencing,

16S rRNA Sequencing

V3-V4 region of the 16S rRNA genes was amplified using MolTaq (Molzym, Plaisir, France) and the primers V3F: TACGGRAGGCAGCAG and V4R: ATCTTACCAGGGTATCTAATCCT. Purified amplicons were sequenced using the Miseq sequencing technology (Illumina) at the GeT-PLaGe platform (Toulouse, France). Paired-end reads obtained from MiSeq sequencing were analysed using the Galaxy-supported pipeline named FROGS (Find, Rapidly, OTUs with Galaxy Solution). For the pre-processing, reads with length ≥ 380 bp were kept. The clustering and chimaera removal tools followed the guidelines of FROGS [209]. Assignation was performed using SILVA 16 S. OTUs with abundances lower than 0.005% were removed from the analysis [210].

2.2.8. Statistical analysis

Statistical analysis for each result normality was tested by D'Agostino-Pearson and Shapiro-Wilk. If the results followed the normal law, a parametric test of ANOVA (Tukey's multiple comparison tests) was used, otherwise, the non-parametric test, Kruskal-Wallis, was used. A t-test was applied for comparing two groups. For statistical analysis, R 3.5 software using the package Bioconductor 3.8 and GraphPad Prism 6 were used.

3. Identifying the possible correlation between gut microbiota in the development of steatosis in four CSS mice with a variant degree of steatosis under the HFD

3.1. Background

Chromosome substitution strains (CSSs) are a unique paradigm for this survey because they enable statistically independent, powerful tests for the phenotypic effects of each chromosome on a uniform inbred genetic background.

As known from the literature, CSS-10 develops a so-called “lean phenotype” which is characterized by lower body weight and our previous research also confirmed that CSS-10 and CSS-8 develop less steatosis of the liver than C57 founder strain (Figure 4). In contrast, opposite phenotypes were observed in the CSS-1 and to a lesser degree in CSS-18. These animals were generally larger than the founder strains and developed extensive steatosis of the liver upon HFD. This establishes that genes on the chromosome 1 of A/J and C57 animals work together in producing a strong phenotype which is not seen in either founder strain. This unexpected result is of high relevance as it establishes that CSSs do not always exhibit phenotypes which are intermediates of the founder strains but can also show new ones which must result from a complex interaction of genes on the exchanged chromosome with the host genome.

Furthermore, evidence for the role of the gut microbiota in energy storage and the subsequent development of obesity and some of its related diseases, particularly NAFLD is now well established. It has been suggested that the gut microbiome is involved in gut permeability, low-grade inflammation and immune balance. Molecular mechanisms by which the microbiome can cause NAFLD or its progression toward non-alcoholic steatohepatitis include modulating dietary choline metabolism, regulating bile acid metabolism and producing endogenous ethanol [211].

Therefore, based on these observations, the CSSs could be a powerful model for studying steatosis and to determine whether gut microbiome could play a role as a modulator of this disease.

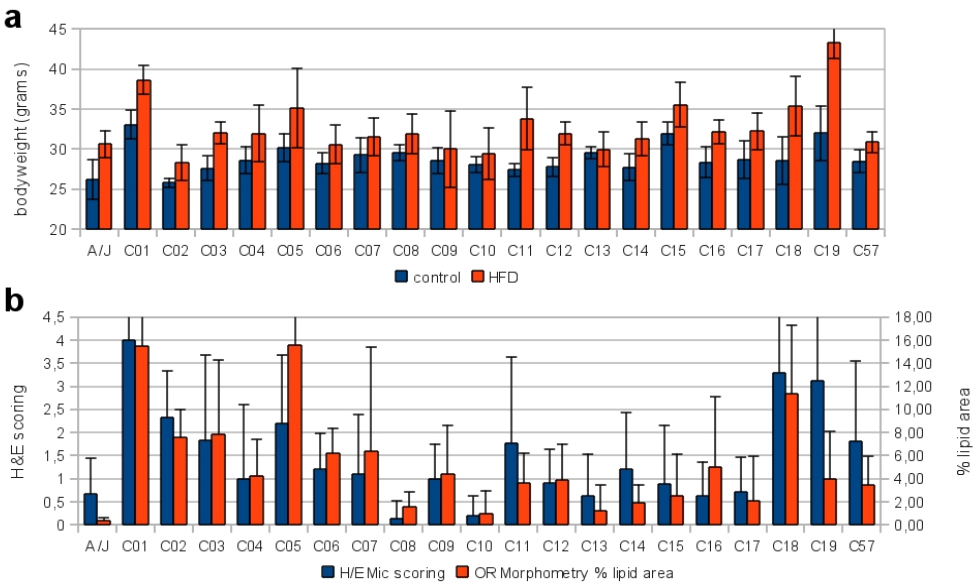


Figure 4. Weight gain upon excess calorie uptake is present in both founder strains and all CSSs, albeit in different degrees **a.** Steatosis, as measured by H&E scoring or by morphometric evaluation of the fatty area in oil red stained cryosections was influenced to a higher degree than body weight **b.** The mice all received HFD. It became evident that the CSS-1 and CSS-18 accumulate more lipids in the liver tissue than any of the founder strains. This figure was produced by Karl Kashofer at Medical University of Graz (unpublished data).

In this study, 6 different mouse strains (n=5 per group) including C57, A/J, CSS-1, CSS-18, CSS-8 and CSS-10 were fed HFD for 8 weeks (after 2 weeks of acclimatization) and then sacrificed at the end of study. Liver and serum parameters were studied for NAFLD assessment (Figure 5) Mice stool pellets were collected from individual mice at the beginning and at the end of the study for 16S rRNA sequencing.

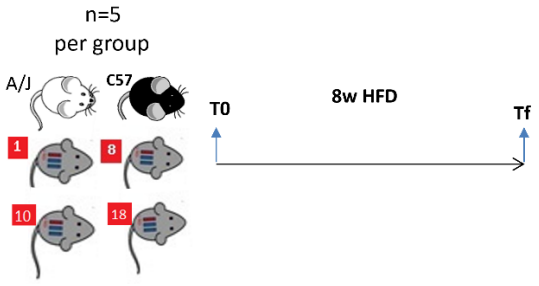


Figure 5. Experiment design: A/J, C57 and four Chromosome Substituted Strains (CSSs) constructed using these two strains, which are susceptible (CSS-1 and CSS-18) or resistant (CSS-10 and CSS-8), respectively, to hepatosteatosis under HFD are used in this study. All the mice strains were challenged with HFD for 8 weeks. In each group n=5 mice.

3.2. Results

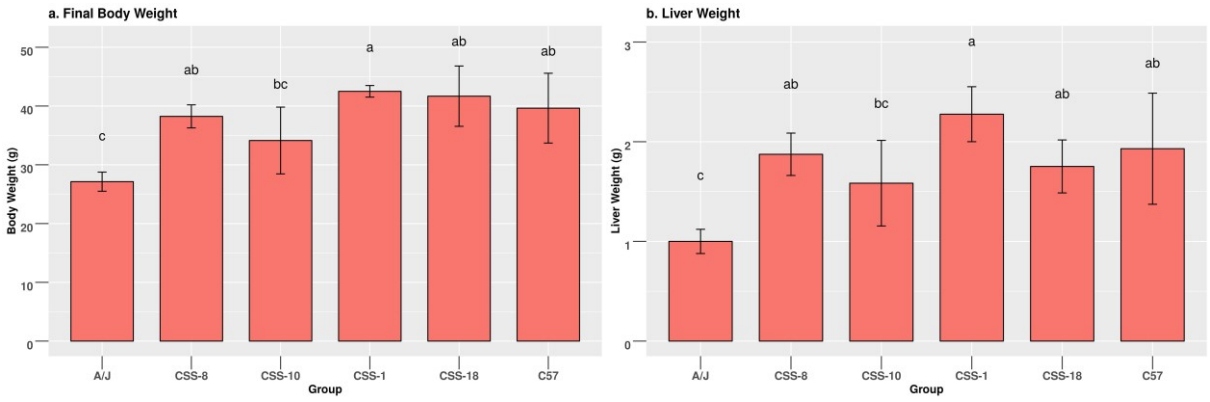
3.2.1. NAFLD phenotype

We studied some of the metabolic parameters, such as body weight, liver weight and liver/body weight ratio in the selected CSSs as well as A/J and C57 strains after 8 weeks of treatment with HFD.

The final weight after 8 weeks HFD treatment in A/J group showed a significant difference compared to C57 (Figure 6). The weight for CSS-10 group showed significantly lower weight compared to CSS-18 group. The CSS-8, CSS-1 and CSS-18 showed remarkably higher weight than their donor A/J strain (Figure 6a). The liver weight is significantly higher in C57 mice compared to A/J mice. Also, the CSS-10 showed significantly lower liver weight compared to CSS-1 (Figure 6b). Liver to body weight ratio was significantly lower in A/J group compared to CSS-1 and CSS-8 groups (Figure 6c).

Furthermore, we measured the TG level in the liver tissue and assessed the amount of fat accumulation in the liver by scoring H&E stained liver sections under the microscope.

The analysis for the amount of triglyceride (TG) in the liver tissue indicated a significant difference in the TG level between C57 and A/J mice (Figure 7a). In addition, the CSSs with fatty liver phenotype i.e. CSS-1 and CSS-18 showed a remarkably higher TG level in hepatocytes compared to the CSSs with less fat deposition in the liver i.e. CSS-8 and CSS-10. The liver fat scoring revealed that C57, CSS-18 and to a lower level CSS-1 had a significantly higher amount of fat in the liver compared to A/J and CSS-10 mice (Figure 7b). Among the CSS strains the most susceptible strains to HFD was CSS-18 which showed an equal amount of fat in the liver to C57 and the resistant one to HFD was CSS-10 with a low amount of fat but still higher than that in A/J. In Figure 8 the high and low level of fat accumulation in the liver are evident in the H&E stained liver sections of CSS-1 and CSS-10.



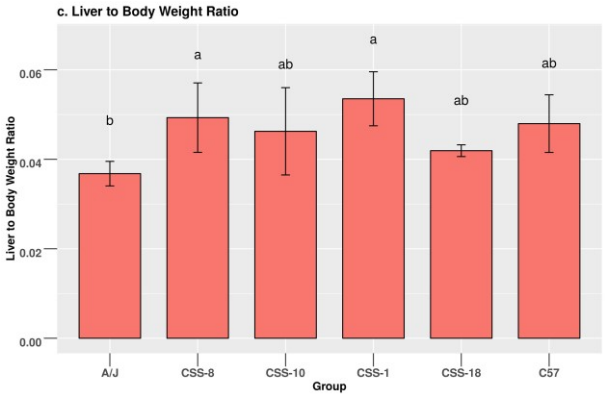


Figure 6. The metabolomic parameters measured in different CSSs and their founder strains i.e. A/J and C57 after 8 weeks of HFD treatment; **a.** final body weight, **b.** liver weight, **c.** liver/body weight ratio. Groups were compared using Tukey's multiple comparison test. p-values were calculated at a 95% confidence level. Each group contains n=5 mice.

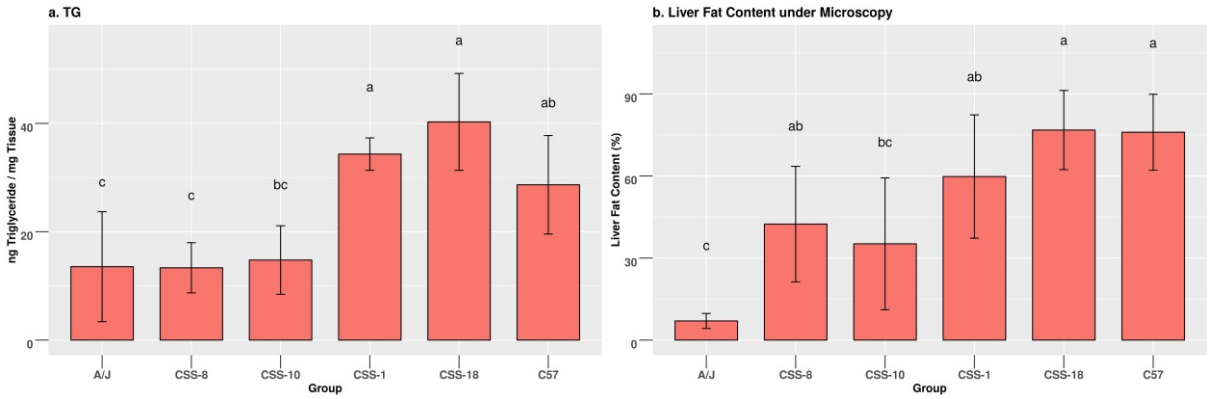


Figure 7. Assessment of the lipid amount in the liver. **a.** Quantification of liver TG levels per tissue weight (mg) after 8 weeks of HFD in different strains, **b.** Liver scoring. Histological grading and staging system for liver steatosis and necrosis were based on the study by Tiniakos et al. [199]. Groups were compared using Tukey's multiple comparison test. p-values were calculated at a 95% confidence level. Each group contains n=5 mice.

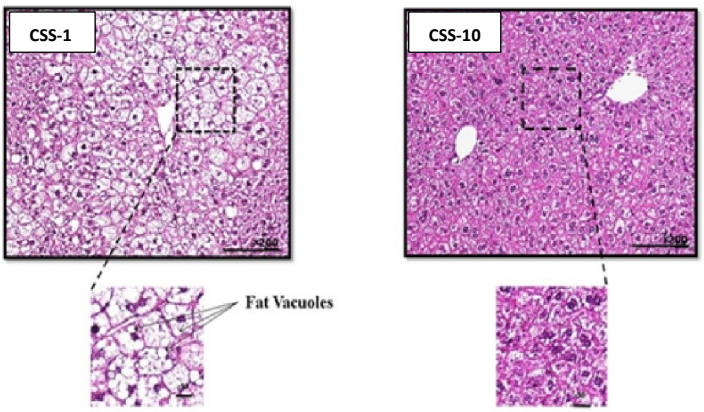


Figure 8. Different amount of fat accumulation in the hepatocytes of CSS-1 vs CSS-10. Microscopic magnitude x20.

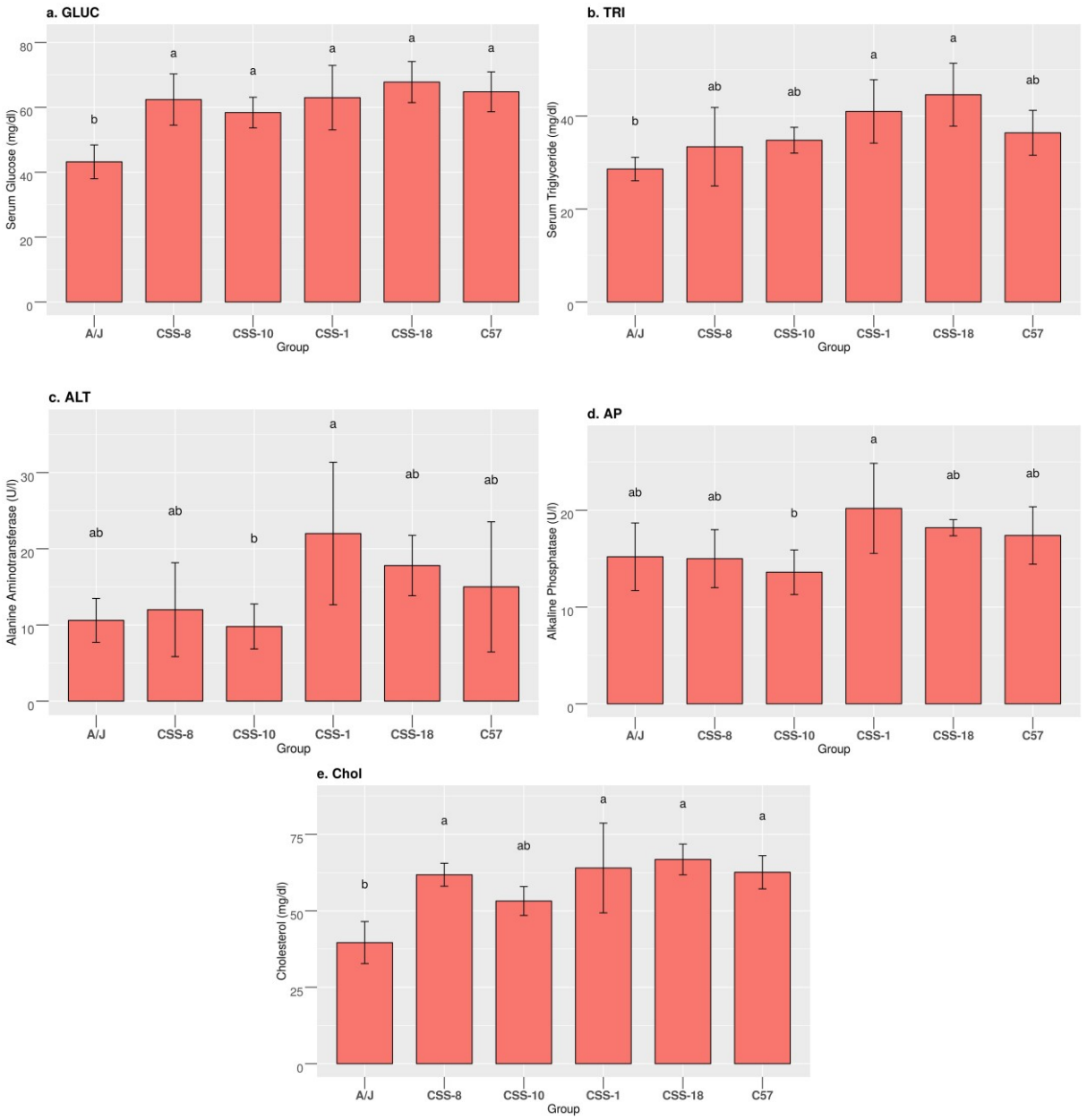


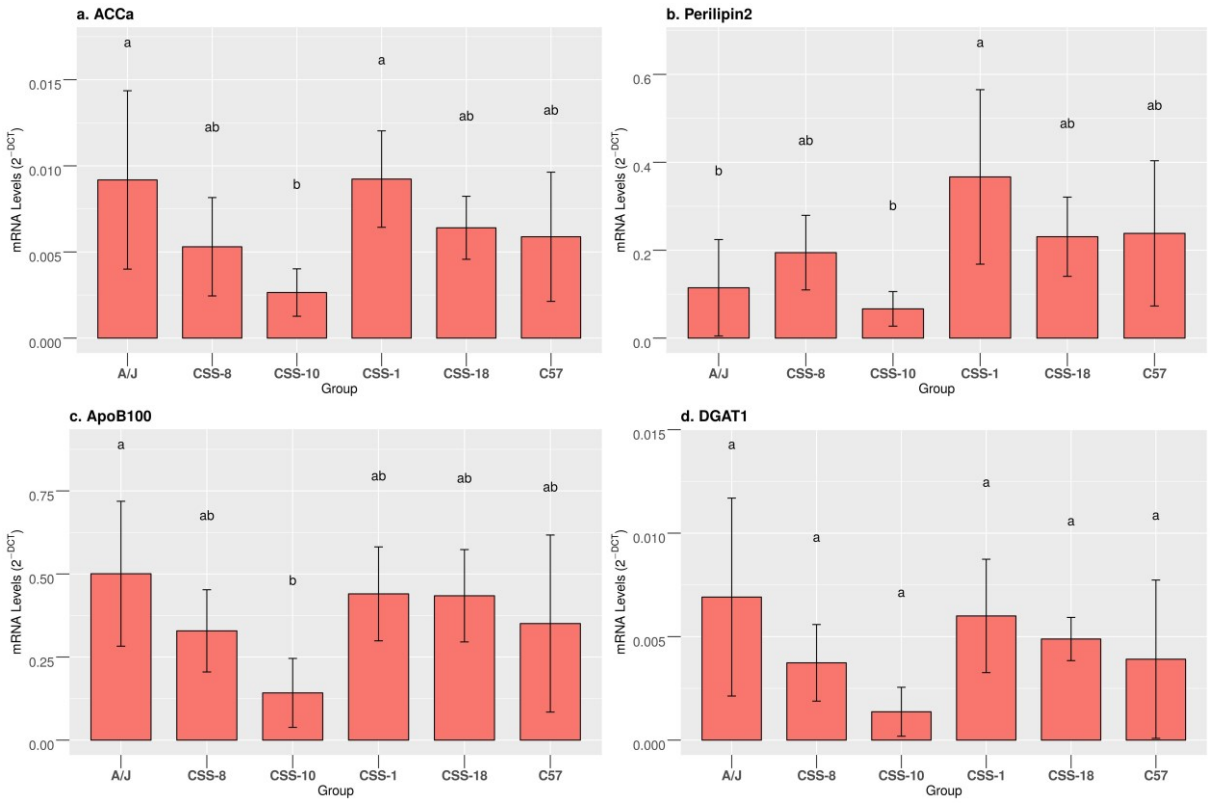
Figure 9. The metabolic parameters in the mice serum in the selected CSSs as well as A/J and C57 strains after 8 weeks of treatment with HFD. Groups were compared using Tukey's multiple comparison test. p-values were calculated at a 95% confidence level. Each group contains n=5 mice.

All the CSSs (CSS-8, CSS-10, CSS-1, CSS-18) and the C57 mice showed a significantly higher level of glucose in their serum than A/J mice (Figure 9a). Serum TG is only significantly higher in the CSSs with fatty liver (CSS-1 and CSS-18) in comparison to A/J mice (Figure 9b). ALT and AP enzymes were only significantly higher in CSS-1 versus CSS-10 (Figure 9c and Figure 9d). Serum cholesterol level showed a significantly lower level in A/J mice compared to CSS-1, CSS-18, CSS-8 and C57 groups (Figure 9e). CSS-10 revealed a reduced tendency in cholesterol level (p=0.0918) compared to CSS-1 but still higher than in A/J mice (Figure 9e).

To study the fat metabolism at the gene level in the CSSs, from the numerous publications, we selected 10 key genes which are involved in lipid metabolism. The list of these genes with their function can be found in Table 5.

Table 5: The list of key genes involved in fat metabolism that were analysed in this study.

ACCa (Acetyl Coenzyme A Carboxylase alpha)	Regulating fatty acid synthesis
Perilipin2	Formation of lipid droplets
APOB100 (Apolipoprotein B)	The primary protein in low-density lipoprotein (LDL) cholesterol
HSL (Hormone Sensitive Lipase)	Lipolysis in the liver and can mobilize hepatic triglycerides
FAS (Fatty Acid Synthase)	Lipid synthesis
DGAT1 (Diacylglycerol Acyltransferase 1)	TG synthesis
PPARg (Peroxisome Proliferator-activated Receptor gamma)	Lipid metabolism
ChREBP/MLXIPL (Carbohydrate-Response Element Binding Protein)	Regulating lipogenic genes and fat storage
ATGL (Adipose Triglyceride Lipase)	Lipolysis in the liver and can mobilize hepatic triglycerides
SREBP1c (Sterol Regulatory Element-Binding Protein)	Lipid metabolism



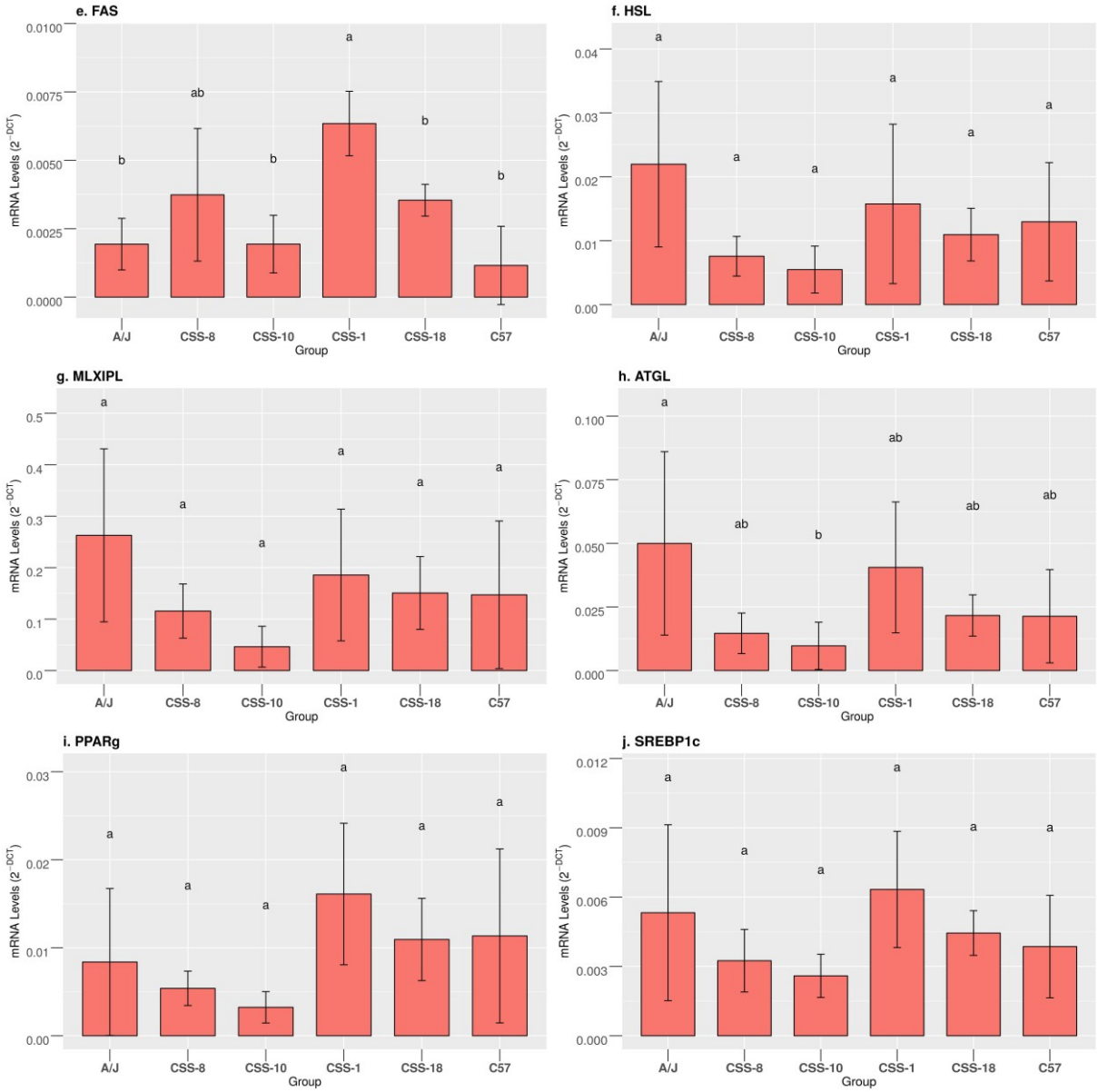


Figure 10. Expression of different genes involved in fat metabolism in different groups after 8 weeks of treatment with HFD. Groups were compared using Tukey's multiple comparison test. p-values were calculated at a 95% confidence level. Each group contains n=5 mice.

Among all these genes only PParg and Perilipin2 seemed to show an expression level corresponding to the hepatic fat accumulation level in A/J, C57 and the CSSs made from these two strains.

A high expression level in CSS-1 versus a low expression level in CSS-10 was observed in almost all of the genes but these findings were not comparable with that of the founder strains (A/J and C57) in terms of the amount of fat deposition in the liver and the previous findings (Figure 10). The expression of these genes in CSS-8 and CSS-18 were not concordant with their liver phenotype. This data demonstrates that CSS-1 and CSS-10 could be a more reliable and less heterogeneous model for further gene studies.

3.2.2. Different microbiome content in CSSs versus founder strains

The Chao1 index showed close microbiome richness for the founder strains (A/J and C57) after HFD (Figure 11). The CSS-1, CSS-8 and CSS-10 showed lower richness compared to the parental strains and CSS-18 showed a higher level of richness compared to the founder strains and other CSSs.

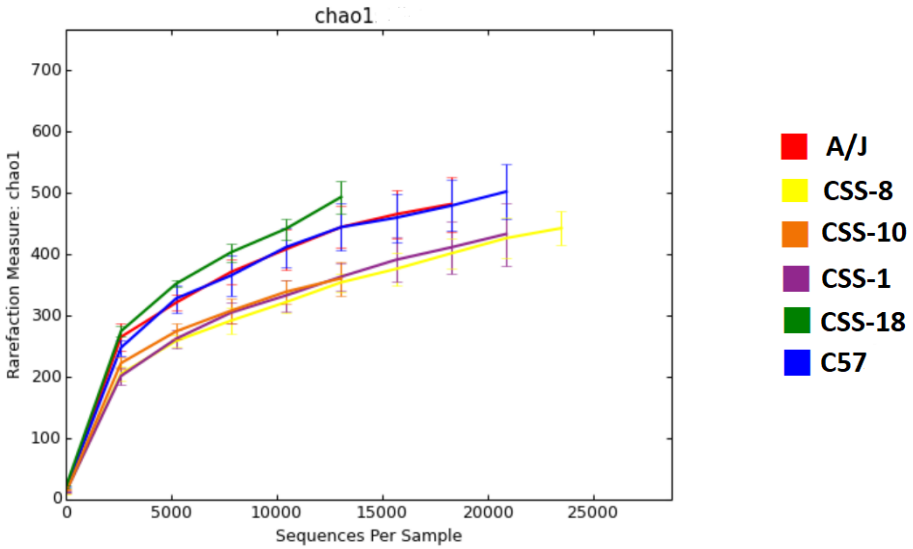


Figure 11. Rarefaction curves were used to estimate richness between different mice after HFD treatment. X indicates the number of sequences and Y shows the number of OTUs.

PCO plot shows different composition between the CSSs and each of the founder strains (A/J and C57), suggesting that microbiome is strongly influenced by the host's genetics (Figure 12). Although the genetic background of CSS-1, CSS8 and CSS-18 is similar to C57 except for one chromosome, the microbiome showed completely different composition compared to C57. However, CSS-10 mice appear to have a microbiome close to that of C57 mice (Figure 12).

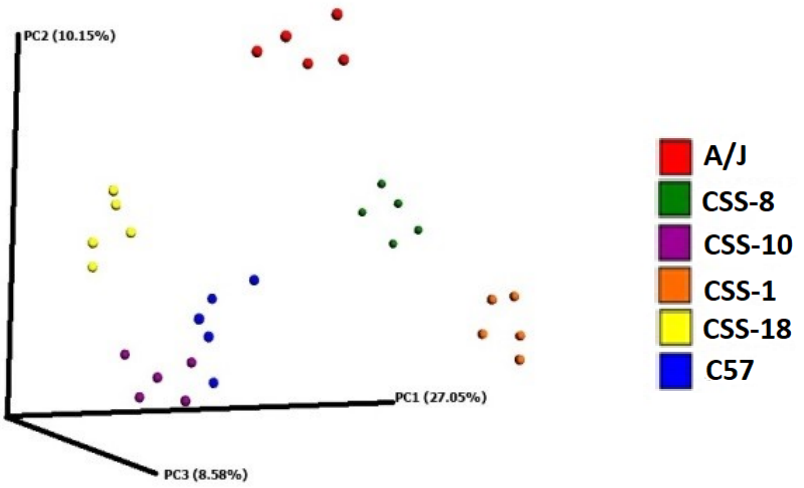


Figure 12. A/J, C57 and different CSS mice exhibit different microbial composition.

At the Phylum level *Verrucomicrobia* was one of the differentially represented bacteria between the six studied groups. It was more prevalent in CSSs and A/J groups than in group (Figure 13).

Similarly, under this phylum, higher levels of *Verrucomicrobiaceae* family was observed in all the CCSs (CSS-1, CSS-18, CSS-8, CSS-10) as well as the donor strain A/J, while C57 mice had significantly lower amount of this family in their microbiome (Figure 14).

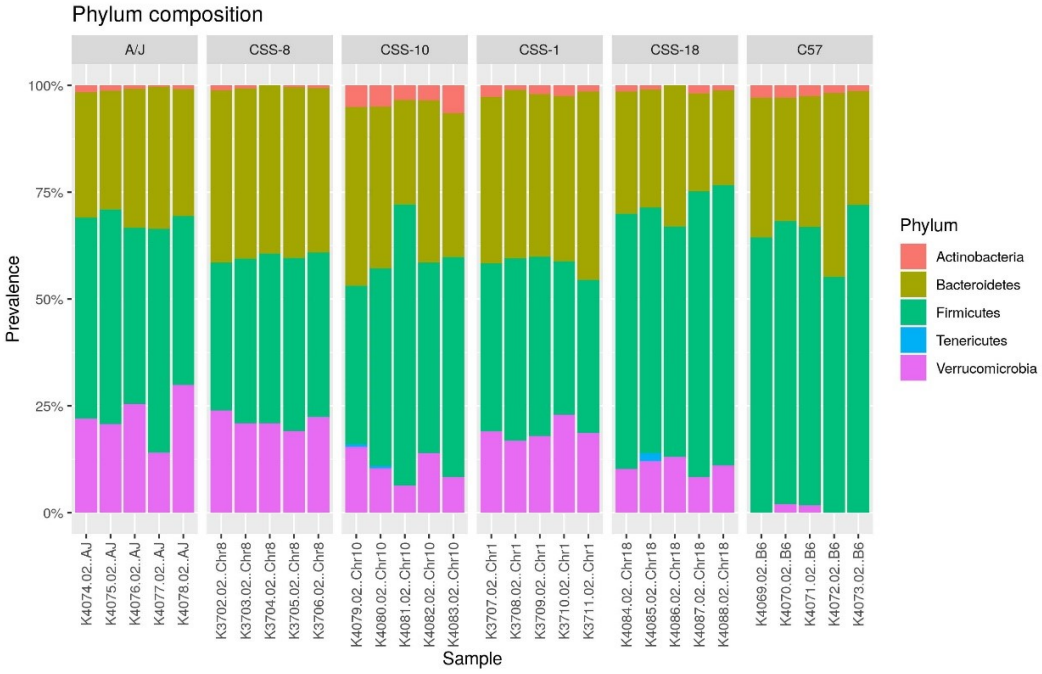


Figure 13. Microbiome composition of different mice strains at the phylum level after HFD treatment.

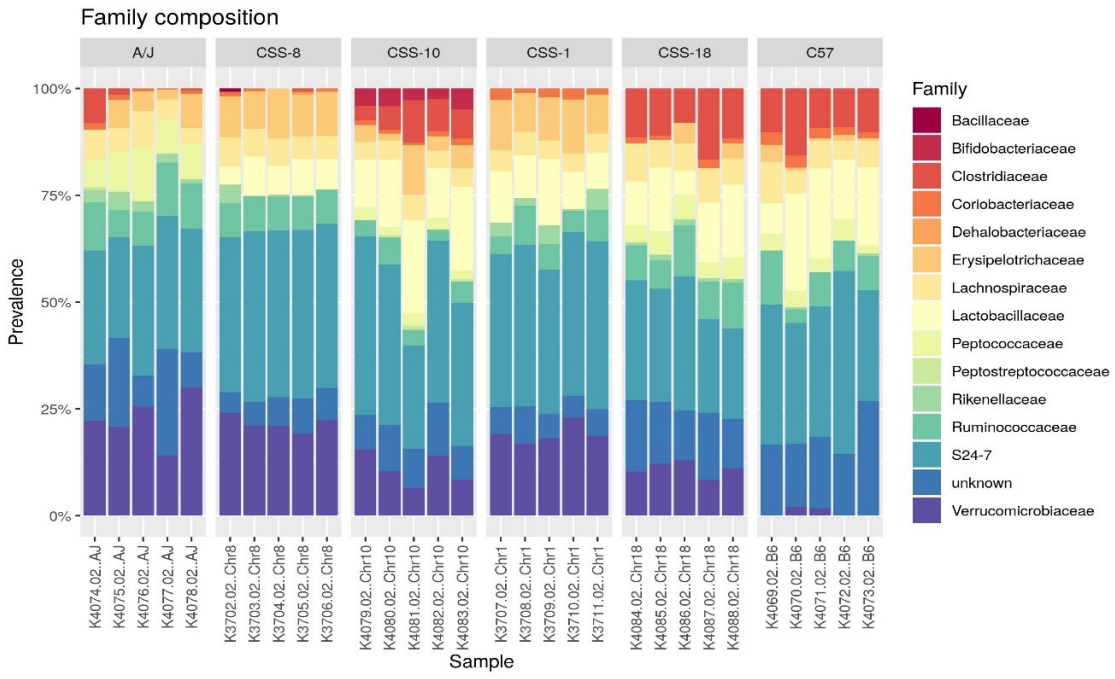


Figure 14. Microbiome composition of different mice strains at the family level after HFD treatment.

From other differentially presented families only *Lactobacillaceae*, *Clostridiaceae* and *Coriobacteriacea* were more prevalent in C57 than in A/J mice. Conversely, *Peptococcaceae* was more abundant in A/J than C57 mice (Figure 15). The abundance of these families was variable in CSSs mice and did not correlate to their liver phenotype.

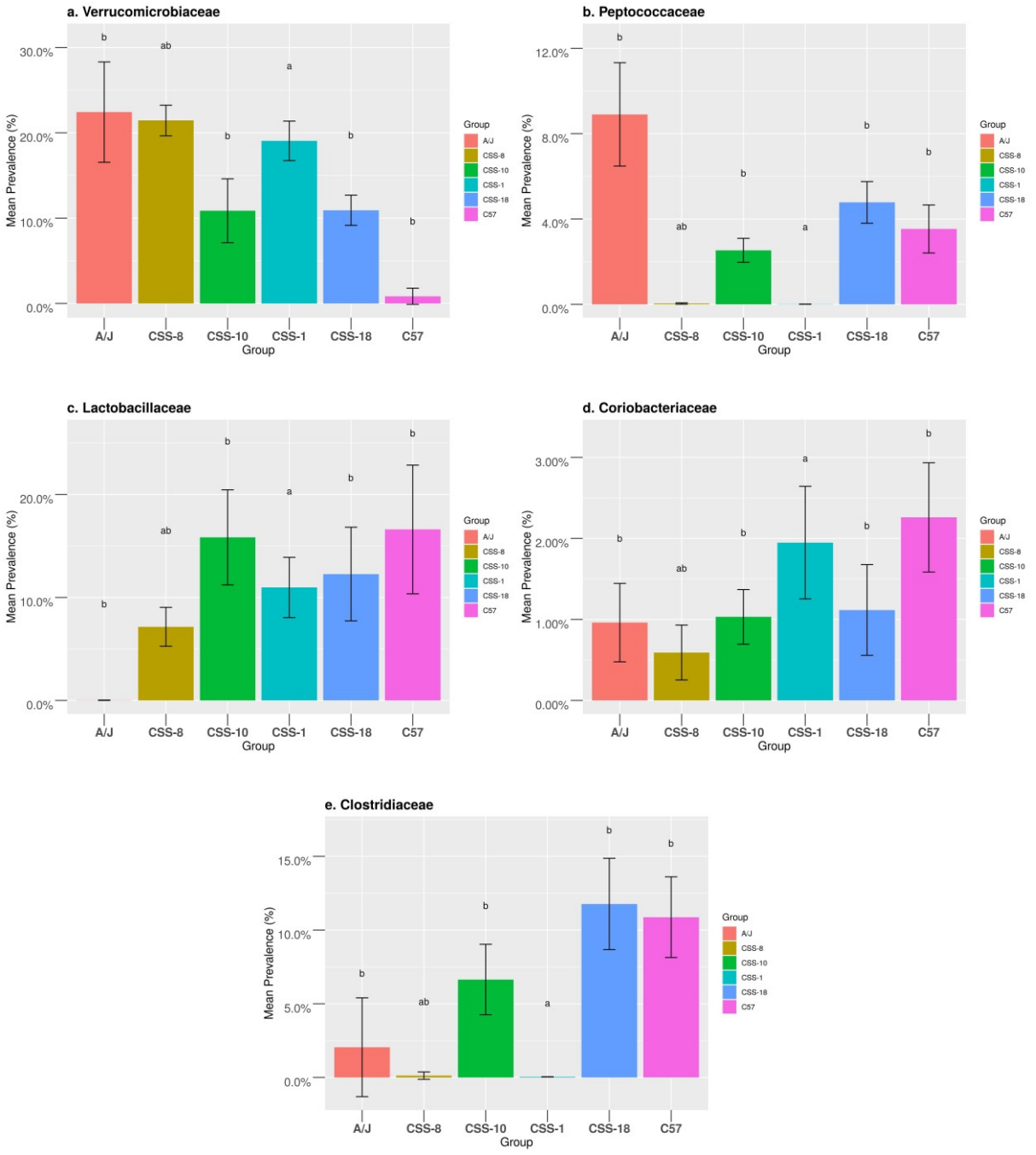


Figure 15. Abundance of the bacterial families in the different groups. Groups were compared using Tukey's multiple comparison test. p-values were calculated at a 95% confidence level. Each group contains n=5 mice.

3.2.3. Correlation between genetic background and microbiome in NAFLD development

Our results confirmed the previous findings of our laboratory showing a high level of hepatic fat deposition in CSS-1 and CSS-18 groups and low level of fat deposition in CSS-8 and CSS-10 groups according to liver microscopy and TG results. In the other hand, the microbiome data showed different gut microbiome composition in CSSs mice compared to A/J and C57 groups which emphasis on the role of genetics on microbiome content. However, we did not observe any link between steatosis phenotype in different CSSs and their microbiome composition.

3.3. Discussion

In this work, we studied the liver phenotype in parallel with the gut microbiome in some of the CSSs mice showing extreme phenotypes including CSS-1, CSS-18, CSS-8 and CSS-10 and their founder strains, A/J and C57, under HFD.

C57BL/6 and A/J are two different isogenic mouse strains, commonly used in biomedical research. In addition to the different genetic backgrounds, these two mouse strains show different inflammatory responses [212], complement system activation (A/J mice are C5-deficient) [213], drug metabolism [214] and different weight gain [189].

Our study is in line with the studies showing a different weight gain between A/J and C57 [189], while Gallou-Kabani et al. reported a similar weight gain and hyperleptinemia in both strains and sexes of A/J and C57 [215].

We also found a significantly higher level of glucose in the serum of all the CSSs (CSS-8, CSS-10, CSS-1, CSS-18) and the C57 mice compared to A/J mice.

The liver pathological parameters such as liver scoring for fat accumulation and liver TG were higher in the CSS-1 and CSS-18 groups and lower in CSS-8 and CSS-10 groups as previously observed in our laboratory at the Medical University of Graz.

Serum TG was only significantly higher in the CSSs with fatty liver (CSS-1 and CSS-18) in comparison to A/J mice. AST and AP enzymes were only significantly higher in CSS-1 versus CSS-10. All these parameters confirm the results produced in our laboratory by Dr Karl Kashofer suggesting a different susceptibility of HFD fed C57 and A/J to steatosis and susceptible phenotype for CSS-1 and CSS-18 as well as a resistance to steatosis for CSS-8 and CSS-10.

The higher susceptibility of C57 mice and the resistance of A/J mice to obesity, inflammation, as well as liver injuries were previously shown elsewhere [189, 216]. The CSSs constructed by only one chromosome from A/J in a C57 genetic background show variant liver phenotype leading to the genetic role played by the genes on the targeted chromosomes.

To further discover this, we chose some candidate genes involved in fat metabolism in the liver and thus in steatosis development. However, the results showed variant gene expression levels except for PPARg and Perilipin genes. These two genes were highly expressed in C57 versus A/J mice. Furthermore, the CSS-1 and CSS-10 had a significantly higher and lower level of expression, respectively, for the mentioned genes compared to other groups which are consistent with the high and low amount of fat accumulation observed in their liver.

It was shown that an HFD amplifies the expression of Perilipin2 through activation of peroxisome proliferator-activated receptor γ (PPARg) and thus leads to fatty liver development [217]. In mice, PPARg is located on chromosome 6 and Perilipin 2 is located on chromosome 4 which does not correspond to the CSSs mice we studied. Therefore, other multiple gene interactions could explain this different response to HFD rather than a single chromosome change.

Since the liver phenotype is in accordance with the expression of some of the genes and not the others, we speculated that there are some underlying mechanisms or compensatory pathways which cause these genes alterations, and this needs further studies.

In fact, a group of genes interact with each other to cause or lead to a disease such as steatosis. In addition, receiving one chromosome from another strain could also affect the normal expression of the other genes.

In the microbiome part of our study, we observed a different microbiome for C57, and A/J strains as observed by another study [218]. We also found different microbiome composition for different CSSs which differ in only one chromosome compared to C57.

Interestingly, we observed *Verrucomicrobiaceae* and its subspecies *Akkermansia*, in all the CCSs (CSS-1, CSS-18, CSS-8, CSS-10) as well as the donor strain A/J but not in C57.

Akkermansia was reported to decrease endotoxemia and improve immunological disorders and maintain the gut barrier integrity [219]. *Akkermansia* was found to be negatively associated with several pro-inflammatory cytokine levels and positively correlated with the gut barrier marker ZO-1. It was also found that *Akkermansia* protects against immune-mediated liver injury in a mouse model by decreasing inflammation and hepatocellular death[172]. However, in our findings, *Akkermansia* is prevalent in all the CSSs with one chromosome from A/J, regardless of their liver phenotype which might suggest a stronger role for genetics on the microbiome content.

We could not see a link between microbiome data and the steatosis severity in the studied CSSs. However, there are studies showing a link between Genetics and gut microbiome composition [67, 220, 221].

For example, Kovacs found that genetic background significantly impacts the microbiota composition and is a stronger determinant than gender [67]. They used DNA fingerprinting method to study the gut microbiota of eight different recombinant inbred mouse lines, whose level of genetic diversity reflects that of a natural human population. Analyses based on automated ribosomal internal transcribed spacer analysis demonstrated a significant higher similarity of the gut microbiota composition within mouse lines than between them or within same-gender groups. They suggested that genetic

polymorphisms determine the intestinal microbiota of mammals and eventually could affect host susceptibility to diseases [67].

Campbell et al. characterized the impact of host genetics and environment on cecum microbiome in 10 genetically different, inbred mouse strains [220]. They characterized bacterial phylotypes that appear to be discriminative and strain-specific to each mouse line used in this study. Cohabitation of different strains of mice demonstrated an interaction of host genetic and environmental factors in the determination of gut bacterial groups, in which bacterial communities became more similar but kept strain specificity.

Ussar et al. studied three commonly used inbred strains of mice-obesity/diabetes-prone C57Bl/6J mice, obesity/diabetes-resistant 129S1/SvImJ from Jackson Laboratory, and obesity-prone but diabetes-resistant 129S6/SvEvTac from Taconic. They also used three derivative lines created by breeding these strains in a new, common environment [222]. They demonstrated strong interactions between microbiota, diet, breeding site, and metabolic phenotype by analysing metabolic parameters and gut microbiota in all strains and their environmentally normalized derivatives. Strain-dependent and strain-independent links were observed between specific microbiota and phenotypes, some of which could be transferred to germ-free recipient animals by FMT. Environmental reprogramming of microbiota caused 129S6/SvEvTac to be obesity resistant. They concluded that the development of obesity/metabolic syndrome is the result of interactions between gut microbiota, host genetics, and diet. Hence, in permissive genetic backgrounds, environmental reprograming of microbiota can improve or worsen the development of metabolic syndrome [222].

Hildebrand et al. investigated the variability of the gut microbiota in five common lab mouse strains by 16S rDNA pyrosequencing [221]. They found initial evidence for richness-driven, strain-independent murine enterotypes that show a remarkable resemblance to those in human. They also found that genetic distance associates positively to microbiota distance so that genetically similar strains have more similar microbiota than genetically distant ones [221].

Our study has some limitations which should be pointed out. With a chromosome level study, it is complicated to distinguish the interaction of the genes leading to steatosis and study the related microbiome alterations possibly involved in the incidence or aggravation of these diseases.

One of our study limitations was the number of samples and lack of control groups with a chow diet treatment. Secondly, we only studied the microbiome content on the extreme phenotypes in terms of showing severe or low-level hepatic steatosis. However, studying microbiome composition for the whole CSS panel with 19 strains could give a more comprehensive view over each chromosome leading

to better identification of the founder strains and their different response to HFD treatment and consequently steatosis development.

4. Studying the reversibility of the liver phenotype and resilience of the gut microbiome in response to the diet switching from high fat to normal diet in C57 mice

(Note: Part of the introduction is adopted from Safari et al. [223])

4.1. Background

NAFLD is a spectrum disease ranging from the benign and usually non-progressive simple steatosis to non-alcoholic steatohepatitis (NASH). NASH is found in 30% of NAFLD patients and is described as the beginning stages of inflammation and lobular ballooning resulting from persistent hepatic injury [224-226]. Therefore, NAFLD is associated with high morbidity. Obesity and insulin resistance have been identified as NAFLD risk factors [225, 226].

NAFLD is characterized by fat deposition and *de novo* lipid synthesis in the liver. The best way to stop disease aggravation is a low-carbohydrate diet. However, the mechanisms underlying the successful intervention are poorly understood because in most of the studies NAFLD is confounded by concomitant weight loss.

In recent years, evidence suggested an involvement of the microbiota in NAFLD development. In an early study, Drenick et al. [227] observed hepatic steatosis development in patients undergoing gastric bypass surgery that coincided with bacterial overgrowth. The patients that were treated with the antibiotic metronidazole showed a regression in hepatic steatosis, indicating an important role of the gut microbiota in fatty liver development. Besides, bacterial overgrowth in the small intestinal has been shown to be more frequent in patients with NASH versus to healthy individuals [52, 228]. Later, Henao-Mejia et al. have provided evidence that modulation of the intestinal microbiota through multiple inflammasome components is a critical determinant of NAFLD and NASH progression [83].

The group of Dr Philippe Gérard at INRA, Jouy-en-Josas, has previously revealed that differences in microbiota composition can determine response to HFD in mice. Using microbiota transplants strategy, they found that the propensity to develop NAFLD directly depends on gut microbiota composition, independently of obesity [54].

Also, animal studies showed that germ-free (GF) mice are resistant to obesity when consuming a high-fat, high-carbohydrate Western diet compared to conventional mice [85, 125, 229].

Altogether, these findings suggest that HFD alone is not sufficient to cause obesity and NAFLD and that the gut microbiota contributes to these diet induced metabolic disorders [223].

Dietary fat and cholesterol have been correlated to the incidence of hepatic steatosis and NASH in both animal models and humans, however, the etiology of NAFLD remains unclear [230-232].

In the liver, a 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 [233, 234]. Specifically, excessive saturated fat consumption increases hepatocyte endoplasmic reticulum stress and hepatic steatosis [235].

Lieber et al. used HFD, in male Sprague–Dawley rats and demonstrated the development of steatosis within three weeks correlated with IR and increased markers of fibrogenesis [42]. 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 [40]. Therefore, HFD treatment in rodents are commonly used as obesity and NAFLD models and are considered more relevant to human pathologies than models of gene inactivation, given the growing obesity epidemic [236] and the importance of overnutrition in the development of diseases such as metabolic syndrome and NAFLD [237-239].

NAFLD reversibility

The liver normally repairs itself by renewing liver cells when the old ones are damaged. Furthermore, 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. In many cases, fatty liver has no symptoms. Different animal or human studies have shown the reversibility of NAFLD [240-242].

Researchers have been successful in reducing the risk of NAFLD development when using a diet modification approach. For instance, a Mediterranean diet, rich in polyunsaturated fats, polyphenols, carotenoids and vitamins, with their anti-inflammatory and anti-oxidant effects, were shown to be effective in reducing the risk of metabolic syndrome [183].

Dietary changes have been demonstrated to be central drivers of microbiome composition and function, and markedly affect the microbiome within days of initiation [243, 244].

Diet plays an important role in the colonization, maturation and stability of the gut microbiome [223]. There is rising concern that current lifestyle innovations, particularly, the high-fat/high-sugar ‘Western’ diet, have altered the genetic composition and metabolic activity of the human gut microbiome [79]. 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 [47, 245] and NAFLD [54, 83].

Some fat components are indigestible where they can affect the microbial composition while passing through the colon and are then excreted in faeces. Subsequently, the consumption of high-fat foods tends to induce substantial changes in the composition of GI tract microbiota [246, 247]. Accordingly, mice fed on HFD have different microbiota composition from those that have been fed control or balanced diets [243, 248].

Both animal and human experiments have demonstrated that switching the diet can rapidly alter the gut microbiome structure [249, 250] and that feeding different types of fat result in very different composition of gut microbiota. For instance, mice fed a high-lard diet for 11 weeks developed signs of metabolic disease, while mice fed fish oil remained healthy [251]. 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 [245].

Furthermore, Thaiss et al. 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 the majority of metabolic parameters have already normalized to preobesity levels [252].

These studies are confirming a very important concept, namely that the effect of diet may be mediated through the intestinal microbiome whose products flow into the liver and from there are transmitted to the rest of the body contributing to disease (or health) [180, 253]. The understanding of this complexity is changing the nature of nutrition science particularly related to liver diseases. Therefore, the objective of this study is to find out whether a normal diet can reverse both steatosis and dysbiosis in the gut microbiota [223].

4.2. Results

To study the effects of HFD on microbiome and liver phenotype we used twelve 8-week-old male C57BL/6 J mice (Charles River, Sulzfeld, Germany) and fed them HFD (ssniff®, R/M D12492, Soest, Germany) for 8 weeks. Four mice were then sacrificed (termed as HF). The diet for the remaining mice was switched to chow and they were sacrificed after 3 (n=4) and 7 days (n=4) (termed as 3d-CD and 7d-CD), respectively. The mice exhibited significant weight gain during the 8-week HFD period (Figure 16b). After 8 weeks of HFD, the average of the final weight for all the mice was 14.43 g. Switching to a chow diet resulted in a decrease in body weight of 1.57 g at 3d, and 3.03 g at 7d, the latter being significant (p=0.0392) (Figure 16a and Figure 16b). The liver weight in the 7d-CD group also revealed a significantly lower value in comparison to HF group (p=0.0433) (Figure 16c). Moreover, the liver to body ratio in the same group also demonstrated a decreased tendency compared to the HF group (p=0.0736) (Figure 16d).

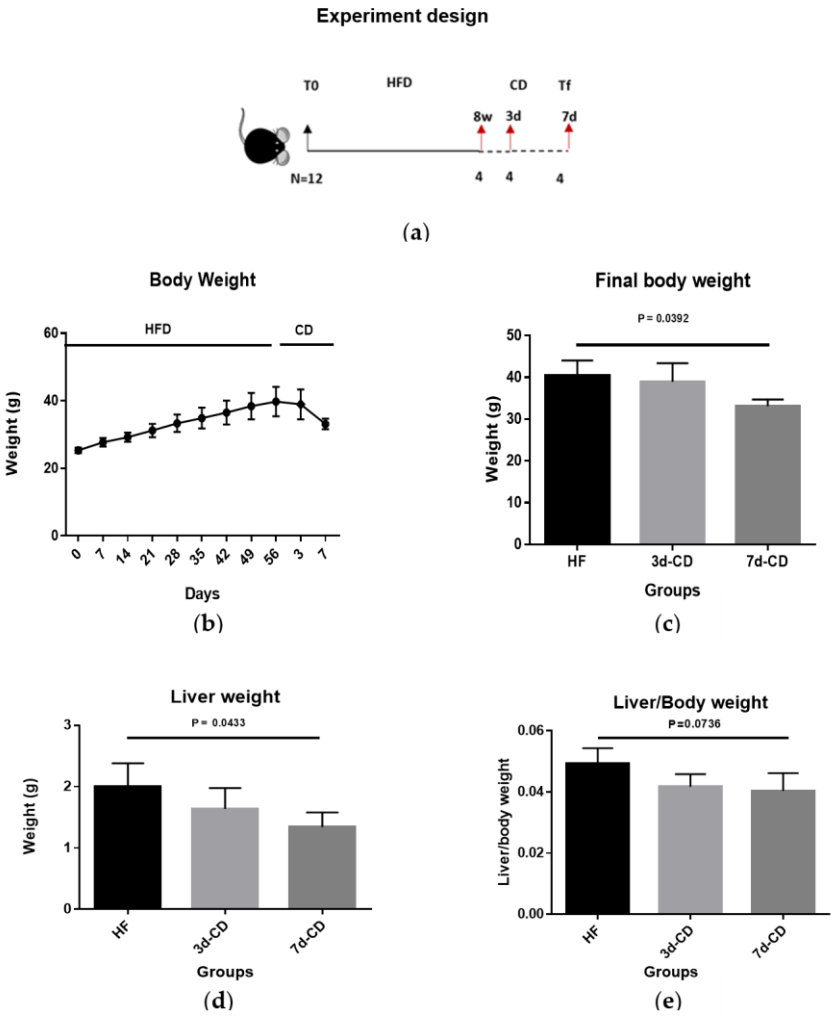


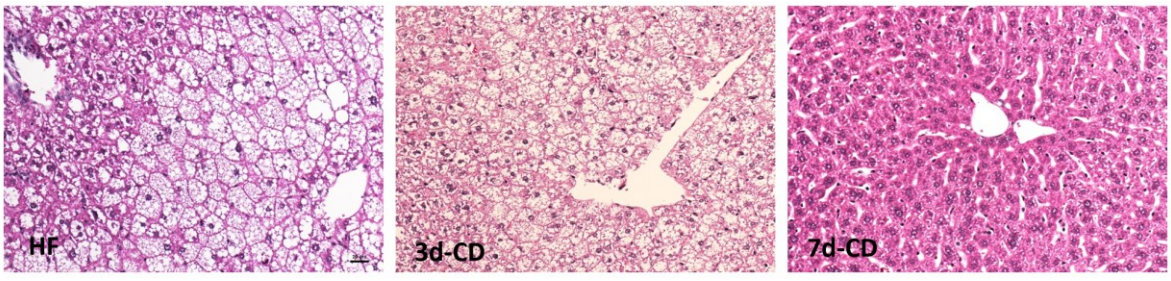
Figure 16. This figure includes the experiment design and the results for body and liver weight: **a.** Twelve mice were divided into three groups receiving High Fat Diet (HFD), the experiments stopped

for the first group after 8 weeks HFD, the remaining mice were switched to a Control Diet (CD) for additional for 3 and 7 days, respectively; **b.** Body weight curve; **c.** Final body weight; **d.** Liver weight; **e.** Liver to body weight ratio in the three studied groups. Groups are compared using Tukey's multiple comparison tests and error bars represent standard deviation (SD). This figure and figure legend are reproduced from Safari et al. [223] with permission of *Elsevier*.

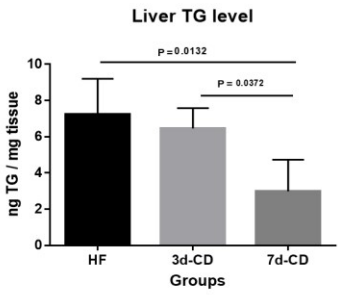
4.2.1. Chow diet reverses the HFD-induced steatosis

The HFD caused a remarkable increase in the liver weight, as well as in the amount of lipid deposition in HF mice (Figure 16d and Figure 17). Three days of chow did not improve the fatty liver status in the mice (Figure 17a). However, the mice with one week of chow diet showed a lower amount of fat in the hepatocytes implying that 7d of chow diet is necessary and sufficient to reverse HFD induced steatosis in C57BL/6 J mice after 8 weeks of treatment with HFD (Figure 17a). The triglyceride assays also confirmed the significant difference in the fat deposited in the liver between HF group (7.237ng/mg tissue) and the group additionally treated with 1 week of chow (7d-CD) (2.990 ng/mg tissue) (p=0.0132) (Figure 17b). A significant difference in the TG level was also revealed between 3d-CD (6.457 ng/mg tissue) and 7d-CD (p=0.0372) group further demonstrating that 7 d of chow diet are needed to reverse triglyceride accumulation (Figure 17b).

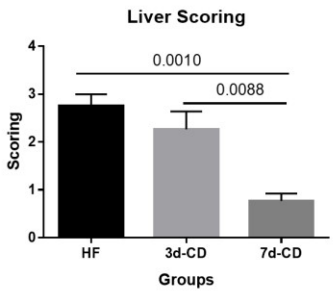
Based on these findings, we chose to compare 7d-CD with HF to further investigate the capacity of CD to reverse the impacts of HF feeding on the metabolic parameters.



(a)



(b)



(c)

Figure 17. a. H&E staining of liver sections (5 µm, 20x magnification). The histology of the liver stained with H&E technique showed that the liver of the mice treated with CD for 7 days (7d-CD) had less fat and displayed a normal status compared to HF group; **b**. Quantification of liver TG levels per tissue

weight (mg) in HF, 3d-CD, 7d-CD groups; **c**. Liver scoring. Histological grading and staging system for liver steatosis was based on the study by Tiniakos et al [199]. This figure and figure legend are reproduced from Safari et al. [223] with permission of *Elsevier*.

To better study the ability of 7d of CD to reverse the adverse metabolic effects of HFD, we measured serum parameters including TRI (Triglyceride), LDH (lactate dehydrogenase), Albumin (ALB), APS (alkaline phosphatase), AST (aminotransferase), ALT (alanine transaminase), glucose and cholesterol. Glucose and cholesterol were found to be significantly reduced in 7d-CD vs HF group ($p<0.0001$ and $p= 0.0216$, respectively) (Figure 18). In addition, the ALT level showed a decreased tendency in 7d-CD vs HF group ($p=0.154$) (Data not shown).

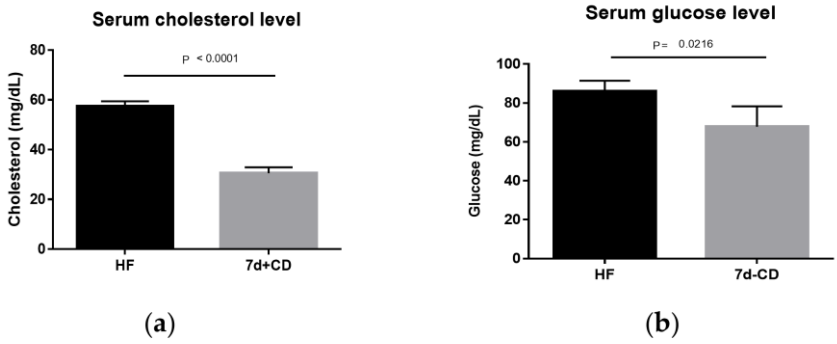


Figure 18. a. Serum cholesterol; **b.** serum glucose levels after 8 weeks of HFD feeding (HF) and one week of CD feeding (7d-CD). Data are presented as mean with standard deviation; n=4 mice/group. For comparing the groups, t-test was used. This figure and figure legend are reproduced from Safari et al. [223] with permission of *Elsevier*.

4.2.2. Chow diet reverses the HFD-regulated microbiome

Replacing HFD with chow for one-week normalized body weight and reversed the hepatosteatosis in HF mice. To decipher if these changes in mice phenotype were correlated with modifications of the microbiome, the stool pellets from both groups (HF and 7d-CD) were taken before HFD treatment, after 8 weeks of HF, and after 1 week of chow diet and analyzed using 16s rRNA sequencing.

Before HF treatment (T0) all mice clustered together in the PCO plot indicating similar gut microbiota composition (Figure 19). HF treatment remarkably changed the microbiota composition leading to a separate cluster. Finally, 7d of CD led to a new cluster closer but different from the original one. These results revealed that HF strongly affects gut microbiota composition, which is only partially reestablished by one week of chow diet.

According to the Venn diagram, 183 OTUs were observed both in T0 and HF groups. In addition, 171 new OTUs are grown after HF and only 43 OTUs remain after CD. 151 OTUs were found in both HF and

7d-CD groups at T0 and Tf. 69 OTUs are only observed in the 7d-CD group and 128 OTUs only in HF group (Figure 20).

Furthermore, the diversity of the bacteria seems to be reduced by HF diet and restored by one week of CD. However, the bacterial richness was decreased in both HF and 7d-CD and was not affected by the diet change [223].

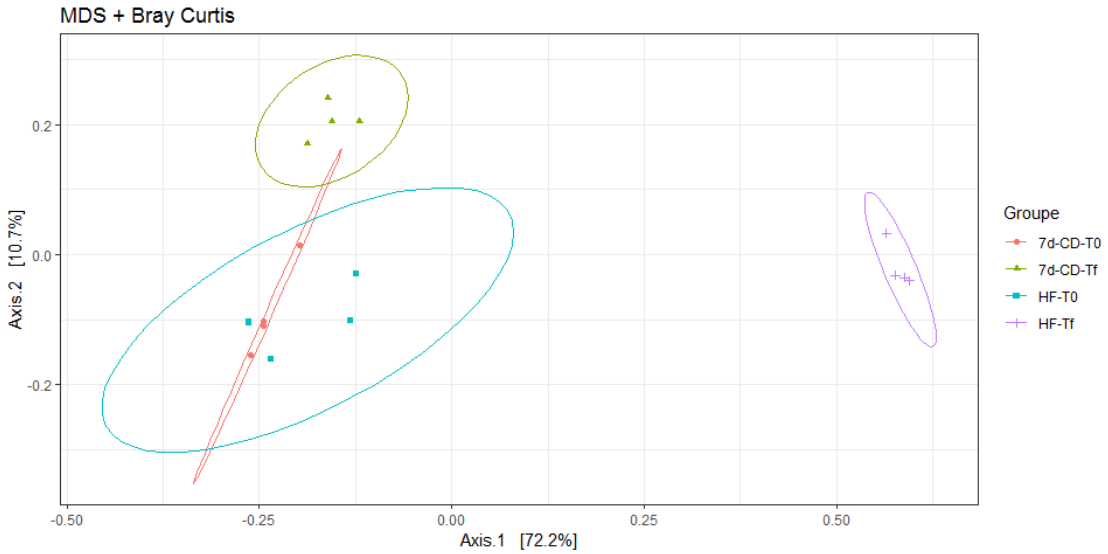


Figure 19. β-diversity analysis via non-metric multidimensional scaling (NMDS) of Bray–Curtis Dissimilarity Indices. Each dot represents an animal, projected onto first (horizontal axis) and second (vertical axis) variables. The ellipse determines the 95% confidence interval. HF: mice fed with high fat diet for eight weeks, 7d-CD: mice fed with high fat diet for eight weeks plus one week of control diet, T0: before high fat diet treatment, Tf; after high fat diet or after high fat diet and control diet. This figure and figure legend are reproduced from Safari et al. [223] with permission of *Elsevier*.

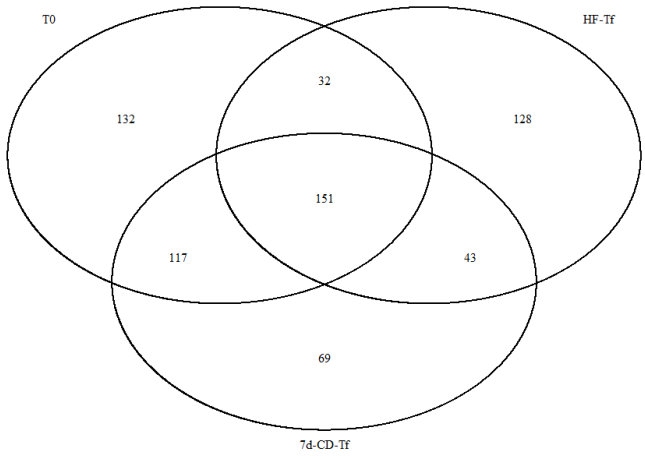


Figure 20. Venn diagram based on OTUs distribution (at 3 % dissimilarity) showing the OTUs shared by the mice before (T0) (n=8) and after treatment (HF-Tf (n=4) and 7d-CD Tf (n=4)). HF: mice fed with high fat diet for eight weeks, 7d-CD: mice fed with high fat diet for eight weeks plus one week of control

diet, T0: before high fat diet treatment, Tf; after high fat diet or after high fat diet and control diet. This figure and figure legend are reproduced from Safari et al. [223] with permission of *Elsevier*.

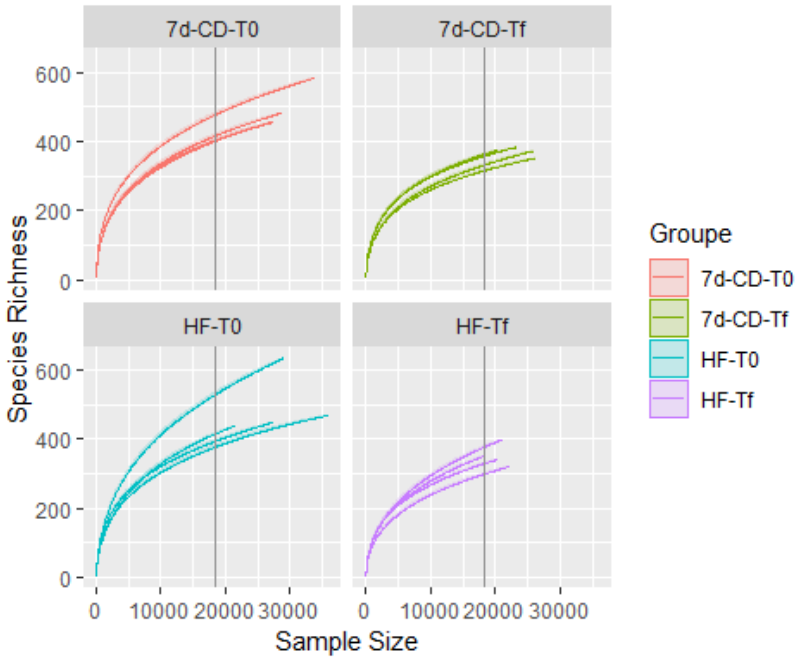


Figure 21. Rarefaction curves used to estimate richness in HF (fed with high fat diet for eight weeks) vs. 7d-CD (fed with high fat diet for eight weeks plus one week of control diet) group at T0 (before HFD treatment) and Tf (after high fat diet or after high fat diet and control diet). This figure and figure legend are reproduced from Safari et al. [223] with permission of *Elsevier*.

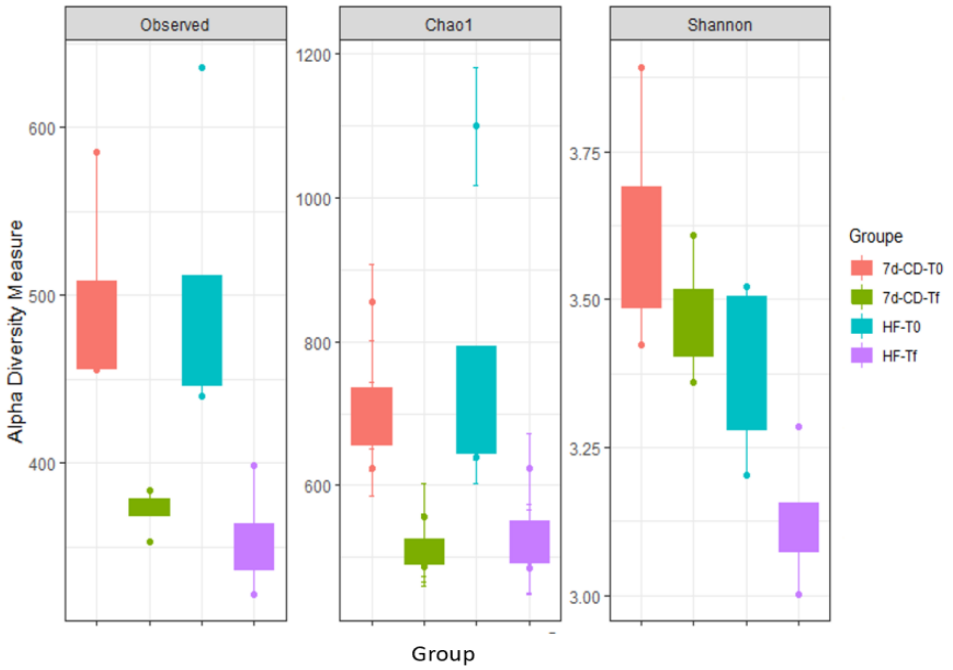


Figure 22. α-diversity indices in faecal samples of mice before and after treatment in 7d-CD and HF groups. Chao1 estimates the number of species, whereas Shannon estimates the effective number of species. HF: mice fed with high fat diet for eight weeks, 7d-CD: mice fed with high fat diet for eight

weeks plus one week of control diet, T0: before high fat diet treatment, Tf: after high fat diet or after high fat diet and control diet. Each group contains n=4 mice. This figure and figure legend are reproduced from Safari et al. [223] with permission of *Elsevier*.

The 16S rRNA gene sequencing of mice faecal samples revealed that the observed species and the richness (Chao index) were reduced in both HF and 7d-CD group, while observed species are less in HF group compared to 7d-CD group (Figure 22). The diversity of bacteria in the 7d-CD group is almost back to the level at T0, while HF group showed a decreased diversity compared to T0 as well as 7d-CD group (Figure 22).

At the Phylum level, the prevalence of Bacteroidetes was decreased by the HF treatment while it was increased in 7d-CD mice at the end of the experiment (Figure 23). HF treatment also led to an increased frequency of Firmicutes and Actinobacteria which was not found in 7d-CD mice (Figure 24). Accordingly, the ratio Firmicutes/Bacteroidetes was found significantly higher in HF group compared to other studied groups (Figure 24). Tenericutes showed a reducing trend from T0 to HF and then reappeared after 1 week feeding with CD. These results revealed that, at the phylum level, one week of chow diet is sufficient to abolish HF-induced microbiota changes (Figure 24).

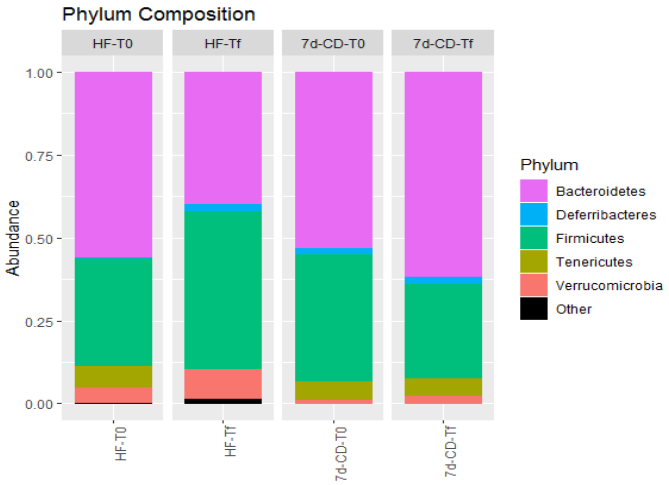


Figure 23. Microbiota composition at the phylum level (relative abundance) in HF and 7d-CD groups at T0 and Tf. HF: mice fed with high fat diet for eight weeks, 7d-CD: mice fed with high fat diet for eight weeks plus one week of control diet, T0: before high fat diet treatment, Tf; after high fat diet or after high fat diet and control diet. This figure and figure legend are reproduced from Safari et al. [223] with permission of *Elsevier*.

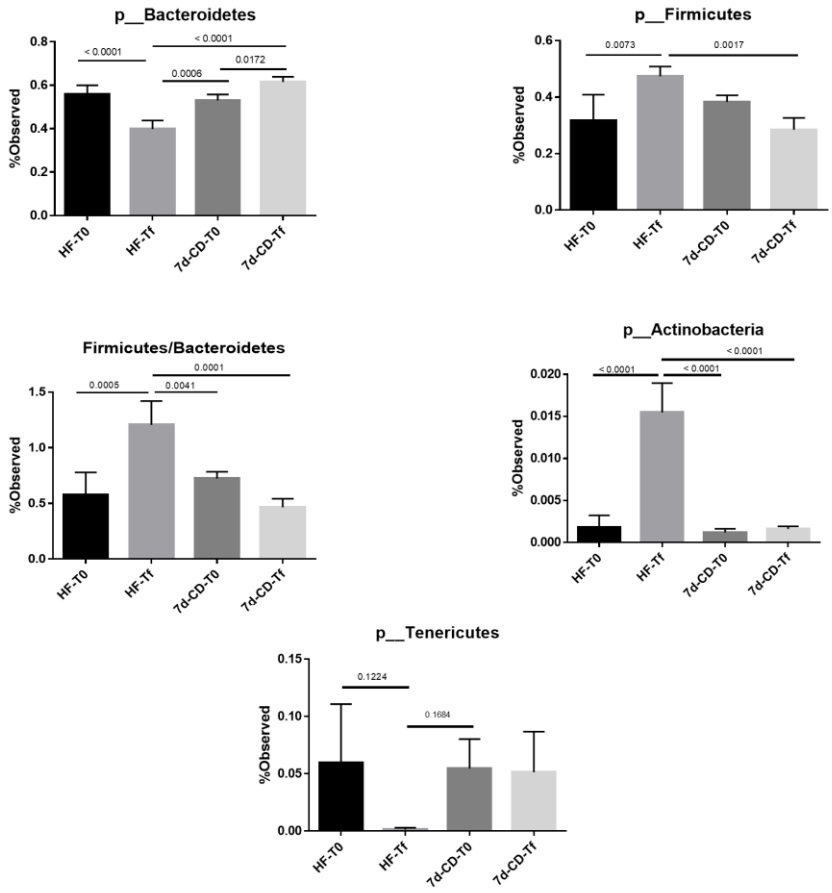


Figure 24. Impact of diet treatments on the relative abundances of phyla. HF: mice fed with high fat diet for eight weeks, 7d-CD: mice fed with high fat diet for eight weeks plus one week of control diet, T0: before high fat diet treatment, Tf; after high fat diet or after high fat diet and control diet. Groups were compared using Tukey's multiple comparison test. p-values were calculated at a 95% confidence level. Each group contains n=4 mice. This figure and figure legend are reproduced from Safari et al. [223] with permission of *Elsevier*.

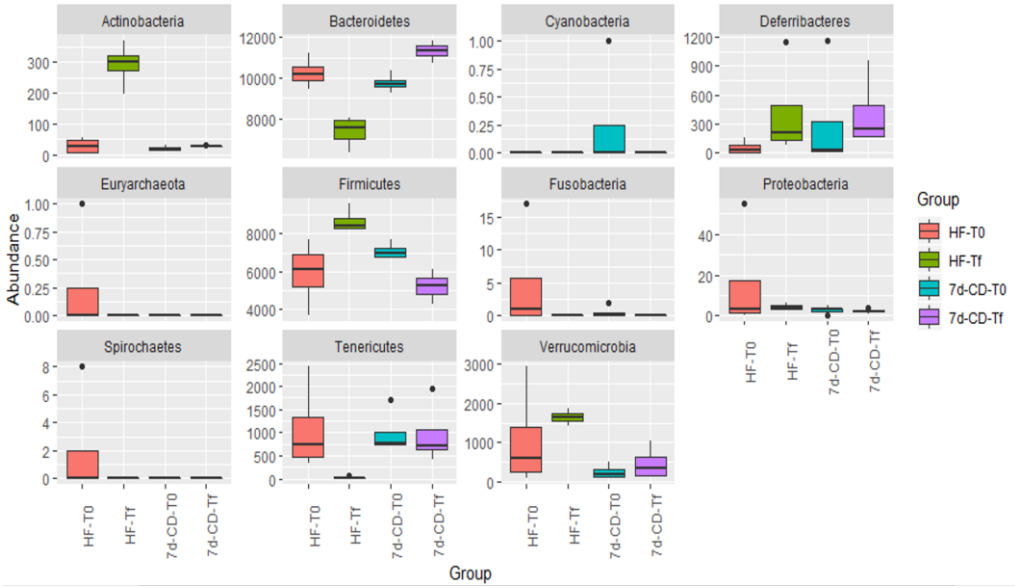


Figure 25. The 5 phyla of interest; *Bacteroidetes*, *Deferribacteres*, *Firmicutes*, *Tenericutes* and *Verrucomicrobia* are shown in this figure. This figure and figure legend are reproduced from Safari et al. [223] with permission of *Elsevier*.

At the family level, *Porphyromonadaceae, Clostridiaceae, Lactobacillaceae and Erysipelotrichaceae* were highly increased by the HF treatment, which was not observed after one week of chow diet (7d-CD-Tf) (Figure 26). In contrast, *Prevotellaceae* disappeared after HF treatment while one week of CD restored this family ($p<0.0001$) (Figure 27). *Anaeroplasmataceae* also vanished following HF treatment but was only slightly restored by the one-week CD period (Figure 27).

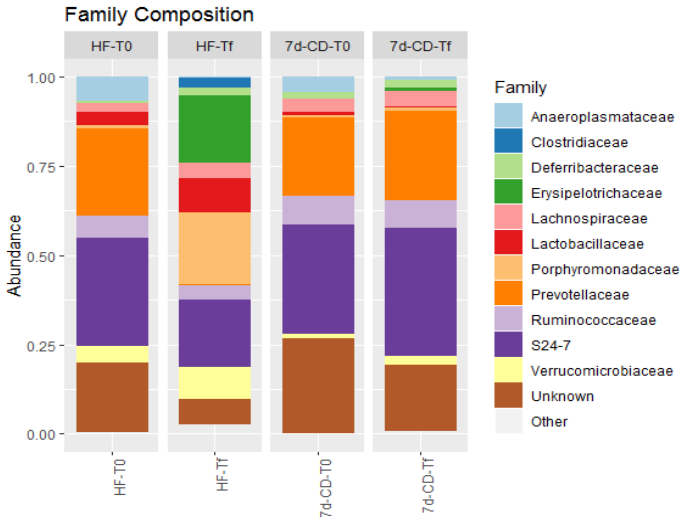


Figure 26. Microbiota composition at the family level (relative abundance) in HF and 7d-CD groups at T0 and Tf. HF: mice fed with high fat diet for eight weeks, 7d-CD: mice fed with high fat diet for eight weeks plus one week of control diet, T0: before high fat diet treatment, Tf; after high fat diet or after high fat diet and control diet. Each group contains n=4 mice. This figure and figure legend are reproduced from Safari et al. [223] with permission of *Elsevier*.

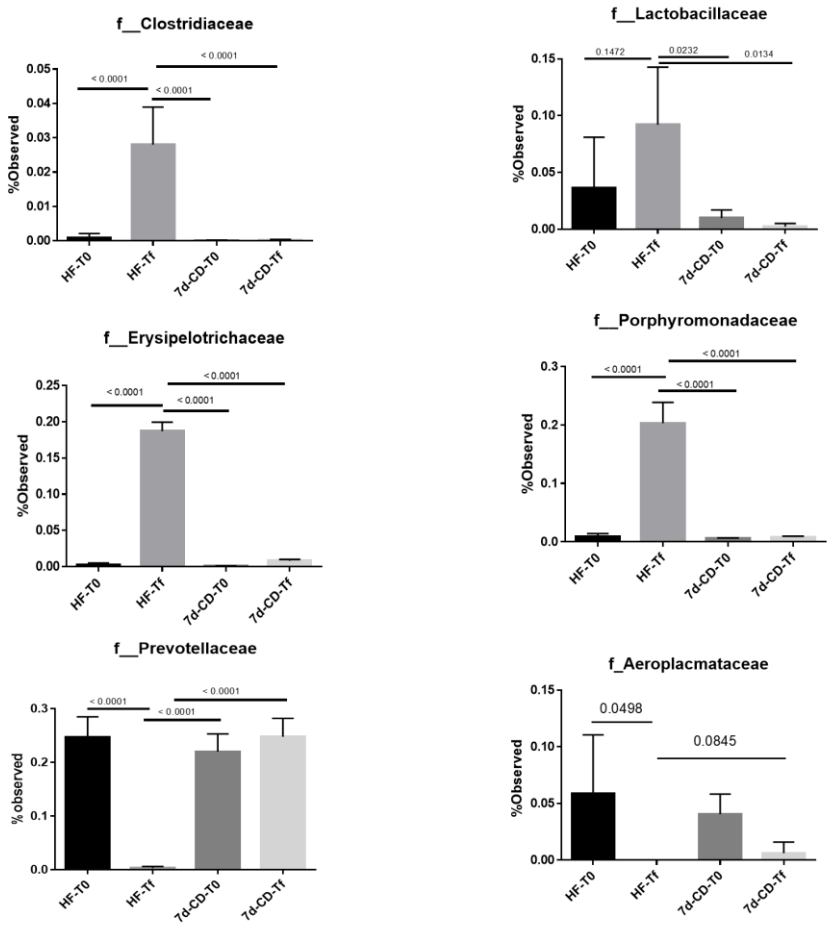


Figure 27. Impact of diet treatments on the relative abundances of bacterial families. HF: mice fed with high fat diet for eight weeks, 7d-CD: mice fed with high fat diet for eight weeks plus one week of control diet, T0: before high fat diet treatment, Tf; after high fat diet or after high fat diet and control diet. Groups were compared using Tukey's multiple comparison test. p-values were calculated at a 95% confidence level. Each group contains n=4 mice. This figure and figure legend are reproduced from Safari et al. [223] with permission of *Elsevier*.

At the genus level, *Parabacteroides*, *Allobaculum*, *Lactobacillus* and an unknown genus in *Erysipelotrichaceae* family were enhanced by HFD treatment which was later completely eradicated by one week of CD diet (Figure 28). In agreement with family-level results, HFD caused the eradication of *Prevotella and Anaeroplasma*, and the chow diet only led to the restoration of *Prevotella*.

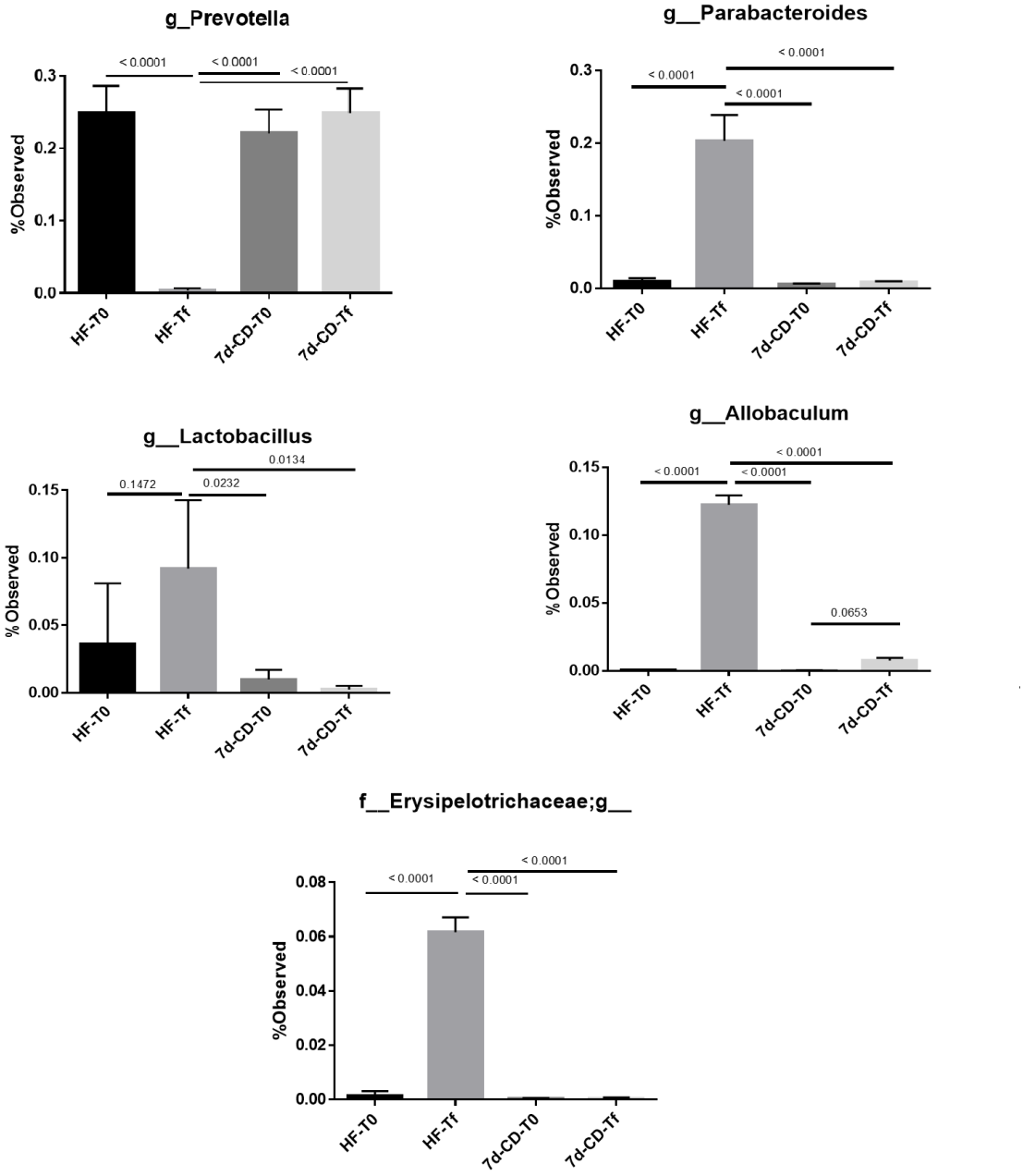


Figure 28. Impact of diet treatments on the relative abundances of bacterial genera. HF: mice fed with high fat diet for eight weeks, 7d-CD: mice fed with high fat diet for eight weeks plus one week of control diet, T0: before high fat diet treatment, Tf: after high fat diet or after high fat diet and control diet. Groups were compared using Tukey's multiple comparison test. p-values were calculated at a 95% confidence level. Each group contains n=4 mice. This figure and figure legend are reproduced from Safari et al. [223] with permission of *Elsevier*.

4.3. Discussion

In this study, mice were fed HFD for eight weeks followed by switching to a chow diet to first describe how HFD impacts the body weight, liver phenotype, and the gut microbiota composition in mice and to further evaluate the reversibility of these HFD-induced effects.

The metabolic parameters such as body weight, liver weight, serum glucose and cholesterol, liver TG were remarkably higher in HF than 7d-CD group ($p=0.0216$, $p<0.0001$) [223].

One of the dieting issues in the age of the obesity epidemic is the cycle of weight loss and regain known as the ‘‘yo-yo effect.’’ Thaiss et al. demonstrated that the microbiome plays a key role in this phenomenon and that simple dietary supplementations can reset the weight-rebound clock [252].

Thaiss et al. utilized mouse models of weight loss and recurrent obesity and found that obesity-induced alterations to the microbiome persist over long periods of time and enhance the rate of weight gain during a secondary metabolic challenge. In addition, low flavonoid levels in post-dieting mice were shown to be associated with the increased weight regain, whereas their therapeutic replenishment improved this susceptibility.

In accordance with our results, Zeng et al. studied on C57BL/6 mice, fed an high-fat (45% energy) or low-fat (LF) (10% energy) diet for 10 weeks [180]. The HFD-fed mice increased their body weight by 34% compared to the low-fat fed mice and the liver of the mice fed the HFD also demonstrated dramatic increases in the amount of lipid deposition, inflammatory cell infiltration and inducible nitric oxide synthase protein concentration.

A high frequency of *Parabacteroides, a* major producer of succinate, was found in our study which was shown to be positively correlated with body weight in our study. To our knowledge, the exact reason for decreased bacterial diversity in NAFLD remains vague. Lower diversity and a phylum-level change in the faecal microbiome has been demonstrated in diseases including NAFLD [105, 254-256] and obesity [47]. Such changes in the intestinal microbiome have been frequently reported for different liver diseases [254]. Thus, our finding of general dysbiosis in NAFLD raises the possibility that the compositional change could result in imbalanced microbial ecology, which might itself lead to increased susceptibility to liver diseases.

In our study 3 days of CD after 8 weeks of HFD treatment was not enough to change the fatty liver (steatosis) phenotype and weight loss in this group was not significant compared to the HF group. However, after 7 days of normal diet, the liver recovered and showed the normal phenotype [223].

Changing the diet from HFD to normal led to a reduced amount of fat deposition in hepatocytes within 7 days which is confirmed by TG result and scoring. Kowalski et al. [241] used HFD (42% energy from fat and 20% from sucrose) for 56 days followed by 9 days of ad libitum chow (HFD → CHOW). They evaluated the acute (7–9 days) hepatic effects of switching high-fat high-sucrose diet (HFD) fed obese mice back to a chow diet. Upon the switch, energy intake was decreased, leading to reductions in fat mass and hepatic triacyl- and diacylglycerol. However, these parameters were still elevated compared to chow-fed mice, thus demonstrating an intermediate phenotype. Nevertheless, glucose intolerance and hyperinsulinemia were completely normalized. The diet reversal resulted in marked reductions in hepatic de novo lipogenesis when compared to the chow and HF groups.

This rapid reduction in voluntary energy intake, as a result of HF mice being switched back to a chow diet, completely normalizes glucose intolerance, fasting hyperglycaemia and hyperinsulinemia after only 7 days was also observed in humans [240, 242].

The gut microbiota community of the HF group at 8 weeks clustered separately from that of the 7d-CD group as revealed by PCO plot and for 7d-CD group stayed close to the microbiome composition at T0. Consistently, the effect of an HFD on the gut microbiota has been studied extensively in animals and an HFD caused shifts in the diversity and taxa distribution of gut bacterial communities [243].

Previous studies in murine models as well as in humans have shown that different dietary composition can shape the divergent structure of gut microbiota [257-261]. For instance, human populations with a modern Western diet or a rural diet showed distinct gut microbiota structures [257] and we may speculate that these changes in gut microbiota could contribute to the higher prevalence of metabolic diseases including obesity and NAFLD occurring in western populations. Therefore, it is important to assess the capability of a normal chow diet to restore a balanced gut microbiota that can participate in healthy status.

Both diversity and richness analyses revealed that the composition of faecal microbiota in the HF group was distinctly different from that of the 7d-CD implying that the gut microbiota has responded to the diet change. However, liver parameters for the 3d-CD did not show a significant difference compared to the HF group.

This confirms the studies showing that the changes in overall structure of gut microbiota induced by HFD versus chow diet may act as an important mediator in the aetiology of obesity and related metabolic diseases, probably via impairing regulation of the host lipid metabolism [85, 245, 262] and triggering low-grade inflammation [117, 261, 263].

The diversity of bacteria in HF group is reduced in our study, the reduced diversity in microbiota composition in obese individuals is found to be linked with the reduction in insulin sensitivity and the stimulation of inflammation [248, 264].

Different formulations of HFD, with different percentages of saturated and polyunsaturated fatty acids, apparently have different impacts on the gut microbiota. Nevertheless, long-term diets were revealed to stimulate stronger changes in the microbiota than short-term ones [265, 266].

In our study, the frequency of Actinobacteria, Firmicutes, *Clostridiaceae*, *Erysipelotrichaceae*, *Porphyromonadaceae*, *Parabacteroides*, *Erysipelotrichaceae* and *Allubbaculum* augmented significantly after HFD treatment for eight weeks. However, *Bacteroidetes*, *Prevotellaceae* and *Prevotella* were significantly diminished at the end of HFD treatment [223].

In line with our findings, increased *Allobaculum* (a genus in *Erysipelotrichaceae*, previously identified as *Mollicutes*) level was also observed in our lab by Le Roy et al. in the group treated with HFD versus control group [54]. Moreover, declined frequency of *Prevotella* [267] and increased level of *Allobaculum* in HF fed mice was also observed by another study [250, 267].

HFD feeding alters the composition of gut microbiota by reducing the frequency of certain gut barrier-protecting bacteria and increasing the prevalence of opportunistic pathogens with the ability to produce free antigens such as lipopolysaccharides. This imbalance may be correlated with a higher gut permeability, resulting in higher plasma levels of endotoxin, higher levels of inflammation and eventually the development of metabolic disorders, a phenomenon termed as metabolic endotoxemia [261, 263].

In mice, changes can be induced in the gut microbiome after just one single day on a different diet [79]. Turnbaugh et al. constructed humanized mice from germ-free C57BL/6J mice. They exhibited that changing a low-fat, plant polysaccharide-rich diet with a high-fat, high-sugar "Western" diet shifts the microbiota structure in a single day changes the metabolic pathways as well as gene expression in the microbiome.

Structural resilience is the capacity of a complex system to recover to its normal state after the stress has been removed [250]. After certain perturbations such as antibiotic treatment, the gut microbiota may not entirely return to the baseline state [249]. Long-term HFD can affect the microbiome composition in the distal gut of mammals, which may be responsible for the development of obesity and associated metabolic disorders [257, 261, 263], but to what extent the HFD-impaired structure of the gut microbiota can be restored after returning to a balanced diet is still not clear.

Dethlefsen et al. destroyed the gut microbiome of the mice and observed that the structure of the gut microbiota returned to its initial state by 1 week after the end of each antibiotic course, but this recovery was not complete even after approximately 5 months [249].

After antibiotic treatment some members may be irreversibly omitted from the ecosystem, while diet only provides growth advantages to certain individuals and disadvantages to others, allowing complete revert to the normal state after the perturbation is removed. By switching to a more balanced diet, one can reverse HFD-induced changes in the gut microbiota structure, providing that the host initially had a healthy gut microbiota [250].

Zhang et al. investigated the dynamic changes of the gut microbiota in C57BL/6J mice fed with a 34.9% fat HFD for 12 weeks and then changed their diet to chow diet (CD) for 10 weeks.[250]. HFD feeding significantly impacted the richness and diversity of the bacterial community, which was reversible upon changing to a normal diet feeding. They observed an enhanced number of Firmicutes and Proteobacteria and decreased the number of Bacteroidetes in the group fed with HFD and their reversal after receiving a normal diet.

According to our data, HF diet caused increased frequency of Firmicutes ($p=0.0017$) and Firmicutes/Bacteroidetes ($p<0.0001$) and reduced Bacteroidetes ($p=0.0001$) compared with a 7d-CD group close to the initial level at T0 [223], which is in agreement with some other studies [250, 262, 268].

Our data also showed a significantly higher prevalence of *Actinobacteria* in HF group vs 7d-CD group ($p<0.0001$), suggesting reversibility of this phylum upon changing diet. Consistent with our findings, it was shown that *Actinobacteria* abundance is positively associated with an HFD and negatively associated with fiber intake [262, 266].

Within the family level, *Erysypelotrichaceae*, *Lactobacillaceae*, *Clostridiaceae*, and *Porphyromonadaceae* bloomed in HF group and decreased after treatment with chow diet ($p=0.0001$, $p=0.0134$, $p<0.0001$, $p<0.0001$, respectively) and their frequency returned to almost close to initial level at corresponding T0 groups [223]. Mice fed a high-fat or western diet showed also increased *Erysipelotrichaceae* elsewhere [79, 262].

Zhang and colleagues identified four different lineages within *Erysipelotrichaceae* to respond differently to diet or host health phenotypes [261], while some studies reported an increase of *Erysipelotrichaceae* in mice on an high-fat or western diet [79, 262, 267, 269] which confirms the results of our study.

In addition, our data revealed a significantly reduced level of hydrogen-producing *Prevotellaceae* ($p<0.0001$), a family in the phylum *Bacteroidetes* and repopulated again after normal diet in 7d-CD group which is in accordance with the findings of Heisel et al. [267], who used HFD contained 60% calories from fat compared to 18% for standard chow for 16 weeks on C57 mice for studying the effects of HFD on fungal and bacterial community structures in a mouse model.

However, human studies have shown a higher level of *Prevotellaceae* in obese individuals compared with lean subjects and with those after gastric bypass [270, 271]. Zhang et al. have also found obesity to be associated with an increase in the family *Prevotellaceae* before the surgery, and following surgery, *Prevotellaceae* was reduced to the level of lean individuals [272].

Within genus level, *Allobaculum* and *Parabacteroides* family were also prevalent in HF group and their number remarkably reduced after 7 days of treatment with the chow diet ($p<0.0001$, $p=0.0001$, $p=0.0134$, $p<0.0001$, respectively). On the contrary, *Prevotella* showed a decreased frequency in the HF-fed mice and its amount elevated in mice treated with chow diet ($p<0.0001$).

In human studies, a significantly higher frequency of *Lactobacillus* species (from the phylum Firmicutes) was also reported in obese patients against lean controls [273].

We found an augmented number of *Lactobacillus* in the HF group which was reduced after CD. *Lactobacillus* may be correlated with the production of volatile organic compounds such as acetate and ethanol [274], which may be critical for the pathogenesis of obesity and NAFLD.

Altogether, these results revealed that the interactions between western/HF diet and gut microbiota might be linked with NAFLD as changing the diet influences the liver phenotype as well the microbiome and lead them both to the initial status before HF treatment.

The intestinal microbiota has been widely studied at both phylogenetic and metagenomic levels in the context of metabolic disorders [178, 275, 276]. The novelty of the present work lies in the characterization of faecal microbial communities in the mice with diet change and their correlation with development and amelioration of the fatty liver disease.

Carmody et al., used more than 200 strains of mice to study whether changes in gut microbiota are mainly led by host genetics or by dietary factors [178]. Their findings demonstrated that a high-fat and high-sugar diet reproducibly change the gut microbiota despite differences in host genotype [178]. Notably, the gut microbiota showed a linear dose response to dietary perturbations and each diet-responsive bacterial group required an average of 3.5 days to reach a new steady state [178]. However, repeated dietary changes demonstrated that most microbiome alterations are reversible, while the richness of certain bacteria depends on prior consumption [178].

The family *Erysipelotrichaceae* is emerging as a group of bacteria that may impact host metabolism and inflammatory diseases [277, 278], and its closely related species have been associated with an HFD [79].

Zhu et al. identified the gut microbiomes of NASH, obese, and healthy children and adolescents. Ecological diversities (alpha and beta) were different among three groups, indicating a strong connection between gut microbiomes and liver health [46].

Furthermore, in human studies, Spencer and colleagues reported a positive link between *Erysipelotrichi* and the changes in the liver fat of the female subjects who received diets in which choline levels were manipulated [253].

Moreover, we found an increased number of genus *Lactobacillus* only in the HF group with fatty liver whose frequency decreased by changing the HFD to normal chow [223].

In accordance with our study, a high level of *Lactobacillus* species was revealed in faecal samples of the mice fed with HFD compared to mice fed a low-fat diet for 10 weeks. A positive association was noted between the frequency of L*actobacillus* DNA in the faecal samples and the severity of steatosis within mouse livers. The authors speculated that the *Lactobacillus* species could influence lipid metabolism through impacting the bile acid metabolism and thus contribute to the risk for fatty liver [180].

In our study, the *Parabacteroides* genus was abundant only in HF group with steatosis and returned to its normal status as T0 after CD. To our knowledge, the link between the frequency of this bacteria in response to diet change and liver status is not so far reported. This genus could be interesting to be studied in HFD associated metabolic disease such as NAFLD.

There are studies showing that rapid dynamic shifts (over 24-48 hours) can occur in the microbiota in response to dietary alterations. However, the shifts in response to daily variation in the diet are at the genus and species level, rather than at the phylum level. Moreover, the subject's microbiota tends to revert back to the state prior to the intervention, implying that a long-term diet is the main driver in shaping the gut microbiota [279].

5. Studying the effect of different treatments including HFD, antibiotic and microbiome exchange between C57 and A/J mice on the severity of NAFLD

Note: Part of the introduction is adopted from Safari et al. (submitted).

5.1. Background

Non-alcoholic fatty liver disease (NAFLD) is a chronic liver disorder which is prevalent in developed and developing countries [2].

A HFD is widely used to stimulate hepatic steatosis and NASH in animal models. However, variable results are obtained with regard to the degree of steatosis, inflammation and fibrosis. In fact, the results vary based on the type of the fat and its amount in the diet, the rodent strain and the duration of treatment [280].

Animal models of NAFLD/NASH provide essential information, not only for elucidating the pathogenesis of NAFLD/NASH but also for evaluating the therapeutic effects of various agents. Fat pile up in the liver is closely correlated with metabolic derangements related to obesity and insulin resistance [281].

Mouse genetics can be applied to investigate the relationships between diet, host genetics, and metabolic responses [222, 282, 283].

The C57BL/6J mouse strain (C57) is a good model mirroring human metabolic derangements that are found in obesity because when fed an HFD, these mice showed obesity, steatosis, hyperinsulinemia, hyperglycaemia, and hypertension, while they remained lean without metabolic abnormalities when fed a chow diet [284]. Although an increase in body weight can be noticed after 2 weeks, the increase is gradual and becomes noticeable after 4 weeks. After 16-20 weeks of HFD feeding, a mouse will typically demonstrate 20-30% increase in body weight when compared to a chow-fed mouse [285]. Different mice strains respond differently to HFD. For example, long-term HF diet loading was shown to induce obesity and insulin resistance in C57BL/6J mice [286, 287]. C57 and A/J mice are sensitive and resistant, respectively, to HFD-induced hepatosteatosis and obesity even though their food intake is the same [189, 288]. Also, they exhibit distinct inflammatory responses [212], and weight gain [289, 290].

Surwit et al. clearly show that the development of obesity and hyperglycemia in A/J and C57 is a complex interaction of genetic background and diet [189]. Their data clearly show that major genetic differences exist in how fat is handled in inbred strains. Genetic predisposition can be more important

in determining the degree of obesity in response to an HFD than caloric intake (Safari et al. submitted). In the C57 mouse, it must be considered a pathologic response to fat in the diet [189].

Similarly, Watson et al. fed A/J and C57 mice with low and HFD for 2, 10, and 16 weeks (277). They found that A/J deposit less fat compared to C57 despite having the same amount of food. They also demonstrated that the ability of A/J mice to resist diet-induced obesity is associated with a strain-specific increase in leptin, Uncoupling protein 1 (UCP1) and UCP2 expression in adipose tissue. These results indicate that the HFD diet does not compromise leptin-dependent regulation of adipocyte gene expression in A/J mice and suggest that maintenance of leptin responsiveness confers resistance to diet-induced obesity.

The low propensity of A/J mice to TG deposition has been previously observed by Surwit et al. [190] and Kondo et al. [291], where an upregulation for the genes linked to lipid metabolism in the small intestine ($NADP^+$-dependent cytosolic malic enzyme, carnitine palmitoyltransferase I, liver fatty acid binding protein and pyruvate dehydrogenase kinase-4) was reported in A/J mice and the same genes were downregulated in A/J. Therefore A/J is considered as obesity resistant and C57 as obesity-prone. [291]. Similarly, in a diabetes type II model [190], A/J mice were observed to be resistant whereas C57 mice were prone to IR. A/J mice are deficient in complement C5 component [213] implying that this protein may be important in lipid metabolism. Component C5, particularly its fragment C5a and its receptor C5aR, has a central role in the inflammatory response [213, 292]. In addition, the alternative C5a receptor, C5L2, which can also bind C3a and C3adesArg, is associated with TG synthesis and glucose capture by adipocytes [293]. Therefore C5aR and C5L2 both play a role under high-fat dietary and inflammatory conditions [216].

Together with their effects on host phenotype, HFDs have a profound impact on gut microbiota (Safari et al. submitted). In fact, there are a lot of evidence linking HFD-induced alterations to the gut microbiota in the obesity epidemic.

HFD feedings were correlated with decreased diversity in the gut microbiota [294, 295]. There are also studies showing that the gut microbiota is a signature of the metabolic phenotypes independent of differences in host genetic background and diet [296]. Those differences in the gut microbiota composition can affect the response to an HFD in mice [54]. Therefore, we investigated whether the differences in the susceptibility to develop metabolic disorders between different mouse strains are due to genetics only or in part to differences in gut microbiota (Safari et al. submitted).

Recently, the role of intestinal dysbiosis influenced by diet, antibiotic exposure and lifestyle factors as well as the genetic background was highlighted in association with Obesity, type 2 diabetes, and metabolic syndrome including NAFLD.

Mahana et al. Used C57BL/6 mice and treated them lifelong sub-therapeutic antibiotic treatment (STAT), or not (control), and fed them HFD [297]. They showed that antibiotic alterations in the murine gut microbiome increase the adiposity, IR, and liver disease correlated with HFD.

Vaahtovuo et al. indicated that modification of the gut microbiota by a course of antibiotics is followed by regeneration of the intestinal microbiota in mice depending on the genotype of the host [66]. They treated mice from eight strains with Ciprofloxacin and Clindamycin antibiotics for 1 week via drinking water to change the composition of the community. They observed a significant change in the intestinal microbiota. However, following a return to the normal diet, the community recovered remarkably quickly and 1 week after finishing antibiotic treatment the faecal CFA (bacterial cellular fatty acids) profiles were similar to those before treatment, demonstrating the community's ability to restore its original state. Recovery was also observed to be linked to the host genotype as the similarity indices between the strains with the same genetic background were regularly higher than those between the strains from different genetic backgrounds.

Furthermore, there are numerous studies on the possible evidence for the interaction between host genetics and gut bacteria [298].

Kreznar et al. demonstrated that the gut microbiota contributes to strain-specific susceptibility to diet-induced metabolic disease and identify links between microbial metabolism and insulin secretion [299]. He investigated the metabolic phenotypes and gut microbiota composition of the eight genetically distinct inbred mice in response to chronic consumption of two defined diets: a high-fat/high-sucrose diet (HF/HS) and a control diet. They found remarkable variation in diabetes-related phenotypes and gut microbiota composition as a function of host genotype and diet. Germ-free (GF) mice were colonized with microbiota derived from two founder strains that exhibited divergent metabotypes, C57BL/6J and CAST/EiJ and the transplanted animals were maintained on the HF/HS diet. These two mice showed different insulin secretion responses and susceptibility to diet-induced metabolic disease.

Complex dynamics of microbial communities underlie their essential roles in health and disease. To maintain or restore healthy states, we must better understand the nature and basis of stability in the gut microbiota, under normal and perturbed conditions (Safari et al. submitted).

colonization of germ-free (GF) mice with gut microbiota from responsive C57 mice to HFD [47, 54] led to liver steatosis in these GF mice implying casual effects of the microbiota. Similarly, changes in gut microbiota induced by antibiotics [300, 301], cohousing animals [83], or changing the breeding/rearing environment [222] can impact the response to HFD-induced obesity and IR [302].

Dumas et al. [303] tested the effects of dietary changes, i.e., switching from a 5% control low-fat diet (LFD) to a 40% HFD, on plasma and urine metabolic 1H NMR profiles in inbred mouse strain 129S6, documented for its susceptibility to IR or NAFLD [304], and in BALB/c strain, which exhibits evidence of resistance to these phenotypes.

Exactly how gut microbiota works together with host genetics and affect the metabolic phenotype is not yet clarified. However, several mechanisms were suggested including the effects of bacterially released LPSs [263], the metabolism of short-chain fatty acids and bile acids [305, 306], and the impact on the host's immune system [307].

In our current study, we aimed to find out the contribution of genetics, diet and microbiota on the metabolic response to HF, and the influence of genetics on microbiota changes.

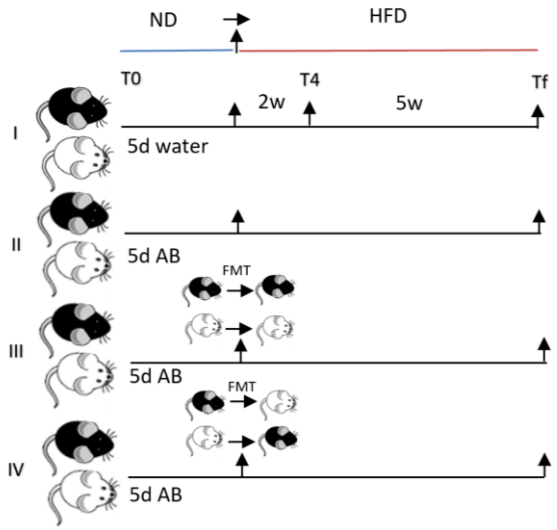


Figure 29. Study design; Study was performed on 4 groups each containing 12 A/J and 12 C57mice treated with 1) only with HFD, 2) AB and HFD, 3) AB, FMT from same strain and HFD, 3) AB, FMT from the opposite strain and HFD. ND; Normal diet, HFD; high-fat diet, T0; before HFD treatment, T4; 2 weeks of HFD treatment, Tf; 7 weeks of HFD treatment. d; day, AB: antibiotic treatment, FMT; faecal microbiota transplantation, white mouse; A/J, black mouse; C57. The figure and figure legend are taken from Safari et al. (submitted).

5.2. Results

Our study is composed of four groups. The first one, which corresponds to the control group (HFD), received water by gavage for 5 days. The second (AB), third (Same) and fourth (Rev) group received antibiotics by gavage for 5 days. The third group (Same) and the fourth group (Rev) also received a microbiota transfer from a mouse of the same strain (A/J to A/J or C57to C57) or the opposite strain (A/J to C57or conversely), respectively. All the groups were then treated with HFD for 7 weeks (Figure 29) (Safari et al. submitted).

HFD feeding increased body weight gain, in C57 mice about two folds more than in A/J mice under HF treatment (Figure 30b) and C57 mice showed significantly higher final weight compared to A/J mice (Figure 30a). AB treatment caused a non-significant reduction in the gained weight of C7 mice under AB treatment compared to other treatment groups for this strain, however the gained weight in A/J mice were not affected by AB treatments. FMT between the same and opposite strain in C57 caused more weight gain compared to AB group for this strain. Only A/J-Same group shows a tendency of increased weight gain compared to A/J-AB.

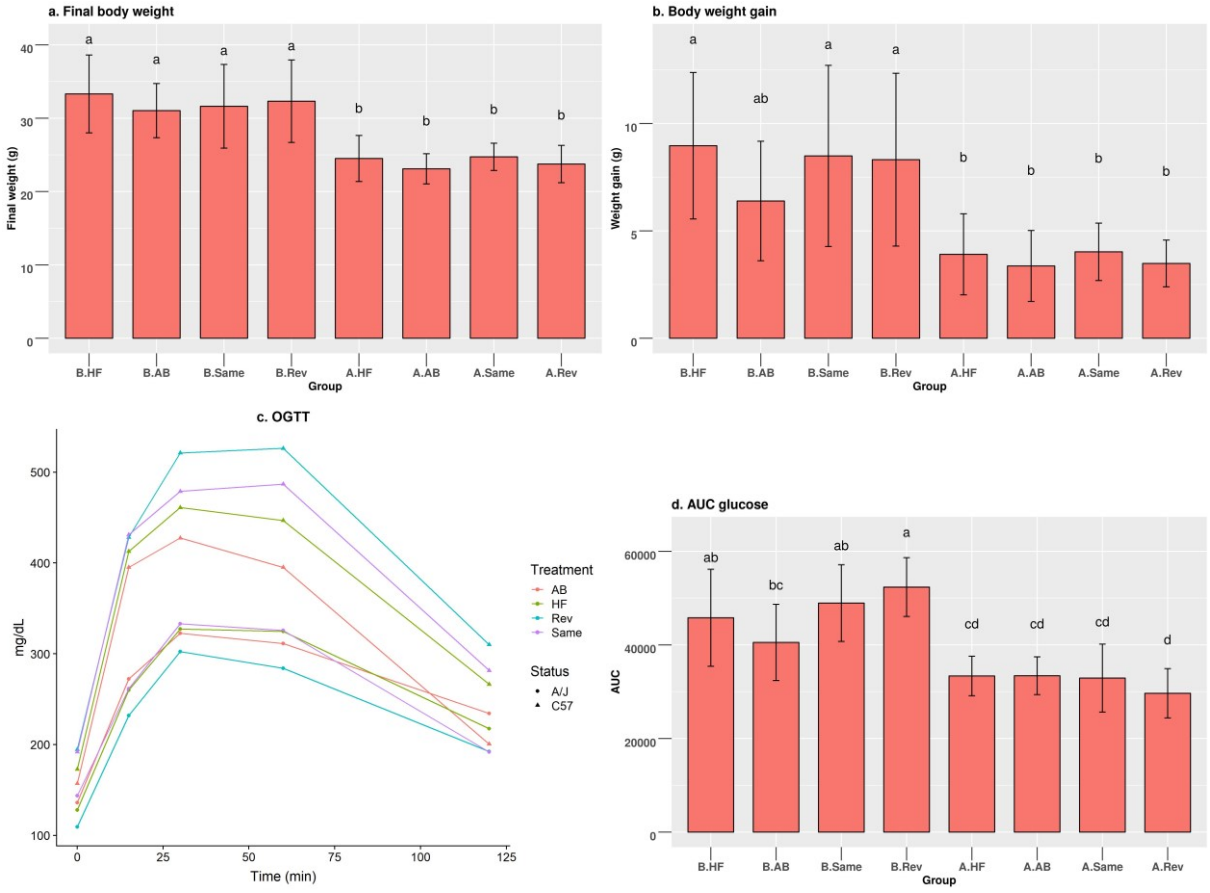


Figure 30. a. Body weight and **b.** Body weight gain in different groups/strains, **c.** Serum glucose levels within 120 min following acute glucose challenge **d.** Area under the curve (AUC). Mean ± SD, n = 6 mice per strain/group. Groups were compared using Tukey's multiple comparison test. p-values were

calculated at a 95% confidence level. Each group contains. The figure and figure legend are taken from Safari et al. (submitted).

Comparing the effect of the treatment in both strains, only the AB treatment was observed to affect the body weight gain.

In Oral Glucose Tolerance Test (OGTT) the C57 mice showed a higher level of glucose in their serum during different time points compared to A/J mice, under HFD (Figure 30c). The area under the curve (AUC) was significantly different only between the A/J and C57 indicating lower glucose tolerance in C57 mice (Figure 30d).

AB treatment tended to improve glucose tolerance in C57 mice but not in A/J (not significant). FMT from same or reverse strains did not affect the glucose tolerance in any of the strains indicating that changing the microbiome does not impact the differences in glucose tolerance due to the dominating effects of genetics between the two mice strains.

In Oral Glucose Tolerance Test (OGTT) the C57 mice showed higher level of glucose in their serum during different time points compared to A/J mice, under HFD. The area under the curve (AUC) was significantly different only between the A/J and C57 indicating lower glucose tolerance in C57 mice.
AB treatment tended to improve glucose tolerance in C57 mice but not in A/J (not significant). FMT from same or reverse strains did not affect the glucose tolerance in neither of the strains indicating that changing the microbiome does not impact the differences in glucose tolerance due to genetics between the two mice strains.

5.2.1. Steatosis phenotype

Liver weight was variable between the two strains, regardless of the treatment. Different treatments did not affect the liver weight significantly in both A/J and C57 strain. We did not observe any differences in the liver scoring for A/J vs C57 regardless of the treatment (Figure 31b).

The amount of fat deposited in the liver for C57 tends to be higher in C57-Same and C57-Rev group compared to A/J-Same and A/J-Rev and AB treatment caused a nonsignificant reduction in the amount of hepatic fat accumulation (Figure 31b).

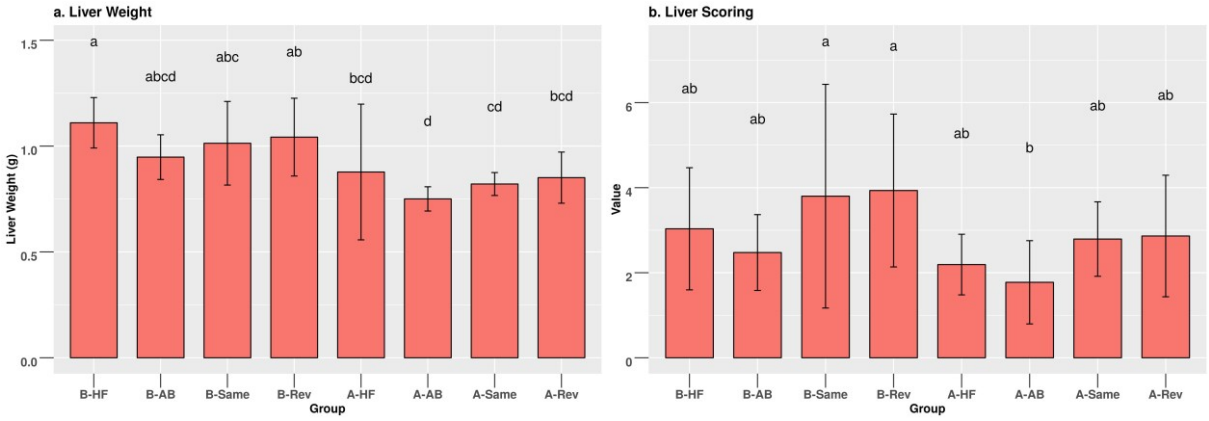


Figure 31. a. Liver weight and **b.** Liver scoring in different groups/strains (n=6 per group). Scoring was performed according to Tiniakos et al. [199]. Groups were compared using Tukey's multiple comparison test. p-values were calculated at a 95% confidence level. The figure and figure legend are taken from Safari et al. (submitted).

Comparing the TG proportion in the liver of mice, a marked difference between C57 and A/J mice regardless of the treatment group is observed(Figure 32). Indeed, we were able to show a significant difference ($p<0.05$) between group A/J-AB (14,69 mg TG/g liver) and C57-AB (27.6 mg TG/g liver). Although in the other groups the difference between the C57 and the A/J was visible, this difference was not significant.

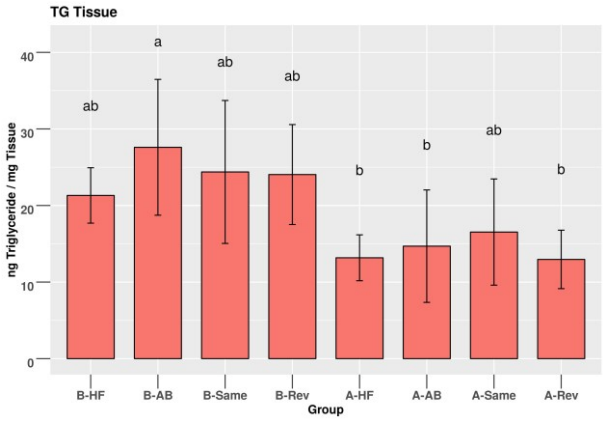


Figure 32. Evaluation of TG amount in the liver of C57 and A/J mice in different groups (n=6 per group). Groups were compared using Tukey's multiple comparison test. p-values were calculated at a 95% confidence level. The figure and figure legend are taken from Safari et al. (submitted).

5.2.2. Serum parameters

The serum HDL level was significantly higher in all C57 mice than in A/J mice. However, the mice of the same strain in different treatments did not show any differences. We observed no differences in the

serum TG level in either of the strains even if the AB treatment in A/J group tended to reduce in the serum TG level (Figure 33).

Alat enzyme was found higher in the serum of A/J mice-HF than C57-HF. This level was not affected by treatment in both mouse strains (Figure 33).

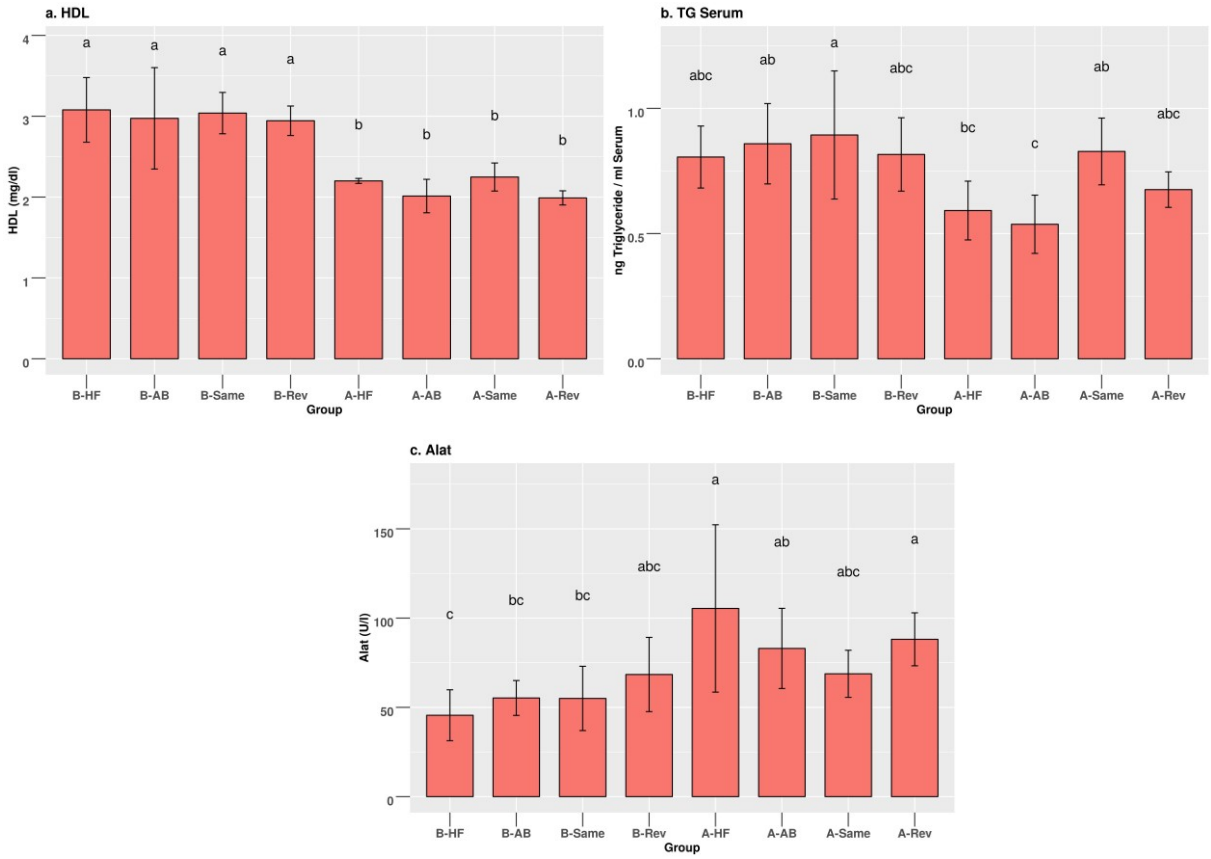


Figure 33. Analysis of the serum parameters in different strains/treatment groups (n=6 per group). Groups were compared using Tukey's multiple comparison test. p-values were calculated at a 95% confidence level. The figure and figure legend are taken from Safari et al. (submitted).

5.2.3. Insulin and leptin

The insulin concentration tended to be higher in the serum of C57 (1.693 ng/ ML) than in A/J (0.912 ng/ML) mice (Figure 34a). Nevertheless, no significant difference was shown between the two mouse strains or between mice of the same strain on different treatments.

Leptin revealed a significant difference between the mice from A/J-Rev and C57-Rev groups (Figure 34b). Thus, the C57 mice that had the A/J mouse microbiota (C57-Rev) had an average of 22.55 ng/ml of leptin, whereas A/J mice with the microbiota of mouse C57 (A/J-Rev) showed 6.41 ng/ml (Figure 34b).

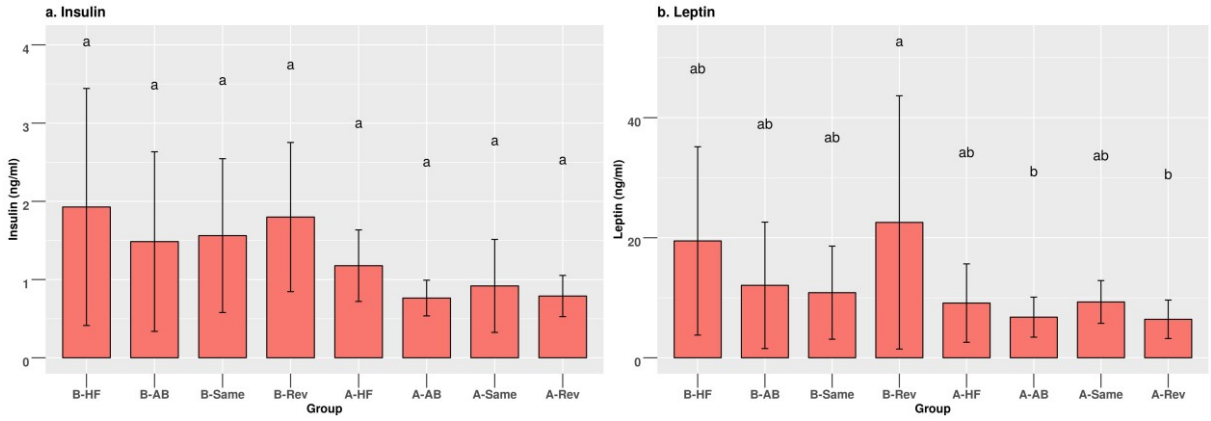


Figure 34. Quantification of **a.** insulin and **b.** leptin in the C57 and A/J mice in different groups (n=6 per group). Groups were compared using Tukey's multiple comparison test. p-values were calculated at a 95% confidence level. The figure and figure legend are taken from Safari et al. (submitted).

5.2.4. Gene expression

The gene expression pattern for some candidate genes involved in immune responses, intestinal permeability, lipid metabolism, peptides that regulate fibrosis, nuclear transcription factors and endotoxins were studied. Ten genes were selected for the ileum and nine genes for the liver (see Table 4Table 4).For studying the genes for the ileum, we used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene, whereas for liver genes, β-actin was used as the housekeeping gene. To analyse gene expression between the two mouse strains within different groups, A/J mice were used as a control. In contrast, to show differences in expression between the different groups, HF group was used as a control for AB group and AB group was used as a control for Same and Rev groups considering that always A/J mice were normalized with A/J mice and C57 mice with C57 mice.

Between the two strains, we were only able to identify differences for three genes (two genes in ileum and one in the liver) (Figure 35). For Tlr4 and Ffar2 in the ileum and Scd1 in the liver, we observed a significant difference between the A/J mice and C57 mice (Figure 35a,b). For the ileum Tlr4 gene, AB treatment had only influence on A/J mice. A/J-AB group showed differences with the other A/J groups; however, this difference is significant only compared to A/J-Rev group (Figure 35a). The microbiota exchange between the two strains seems to affect the Rev groups in both strains compared to the Same group. For ileum Ffar2 gene, the A/J-AB group showed a lower expression compared to other A/J groups (Figure 35b). C57 mice were not influenced by AB treatment. The microbiota exchange in both strains affected the expression level of this gene (not significant) in Rev compared to Same groups. The C57-AB group tends to have a lower expression for this gene compared to other C57 groups. The microbiome exchange tends to be higher in C57-Rev compared to C57-Same. A/J mice do not show any significant differences between various treatment groups (Figure 35).

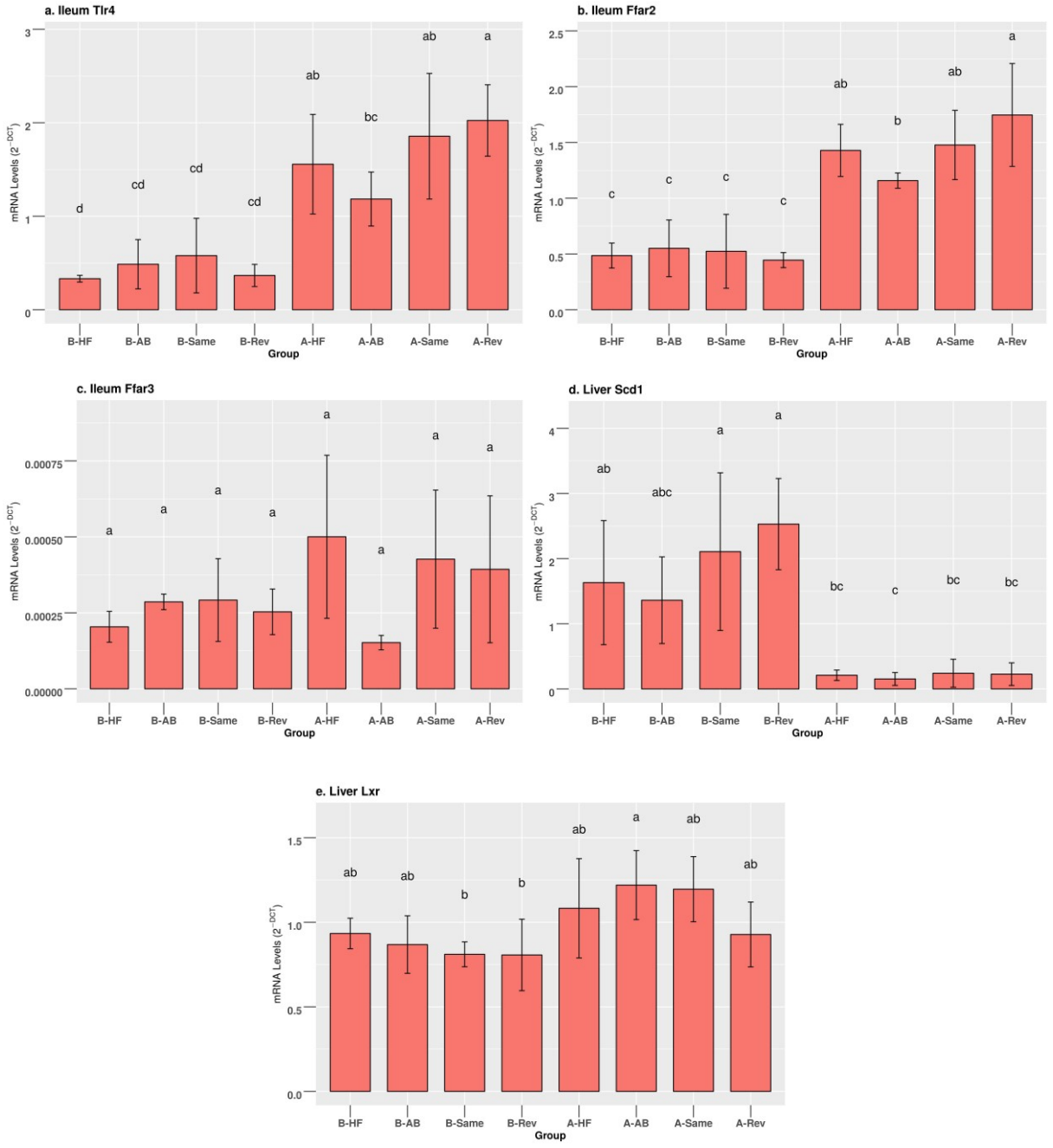


Figure 35. Different gene expression in Liver and Ileum of A/J and C57 mice under different treatments. Groups were compared using Tukey's multiple comparison test. p-values were calculated at a 95% confidence level.

5.2.5. Microbiome comparison between A/J and C57

Chao1 index indicated that community richness in C57 is about the same level as that of A/J indicating that the number of bacterial species is similar in the two mice strains (Figure 36). Conversely, the Shannon diversity index was lower in C57 than in A/J indicating a biased community structure dominated by a few bacterial species in C57 (Figure 36).

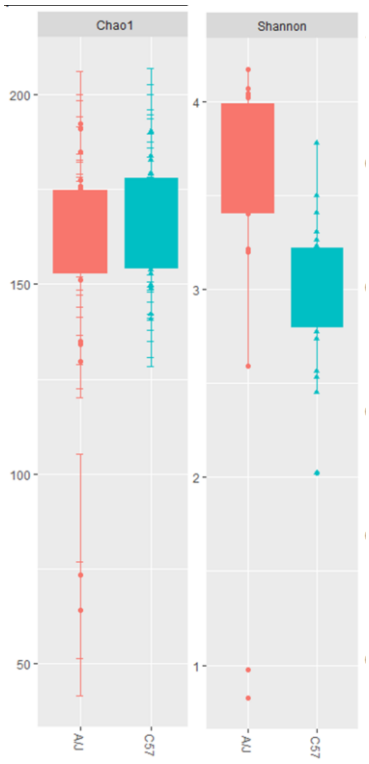


Figure 36. Box plots representing the comparison between the diversity indices for C57 and A/J groups. Results for the Chao1 index, where the estimated number of OTUs in the community is proportional to the total number of species. Results of the Shannon diversity index, where the box plot represents the richness estimator. Significant differences are defined at the 95% confidence level. Figure and figure legend are taken from Safari et al. (submitted).

Principal-component analysis of 16S rRNA sequence data of faecal contents exhibited clear differences in community structure between the two mice strains (Figure 37).

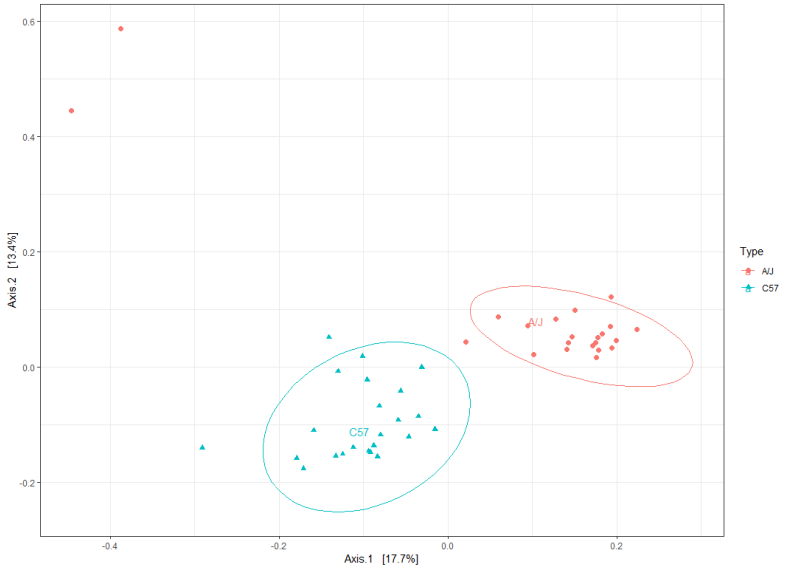


Figure 37. Unweighted UniFrac-based 3D PCoA plot constructed on all OTUs. A/J and C57 mice exhibit different microbial composition. Analysis of similarity (ANOSIM) with 999 permutations was used to detect the statistically significant differences in microbial community composition between A/J and C57. Figure and figure legend are taken from Safari et al. (submitted).

At the phylum level, the Bacteroidetes populations were higher in C57 mice than in A/J mice. Accordingly, the proportion of Firmicutes/Bacteroidetes was found higher in A/J mice than in C57 mice (Data not shown).

The *Bacteroidaceae* family was among the abundant families in both strains, but it was higher in C57 mice rather than A/J mice (Data not shown). Within the order *Bacteroidales*, Family S24-7 was also observed to be overrepresented in the gut microbiome of C57 mice in comparison to A/J mice (Data not shown).

The *Alcaligenaceae* family within *Burkholderiales* order from Proteobacteria phylum was also found more abundant in C57 mice than in A/J mice (Data not shown).

Conversely, the population of *Coriobacteriaceae* was found very low in C57 mice whereas it was one of the more abundant families in A/J mice. (Data not shown).

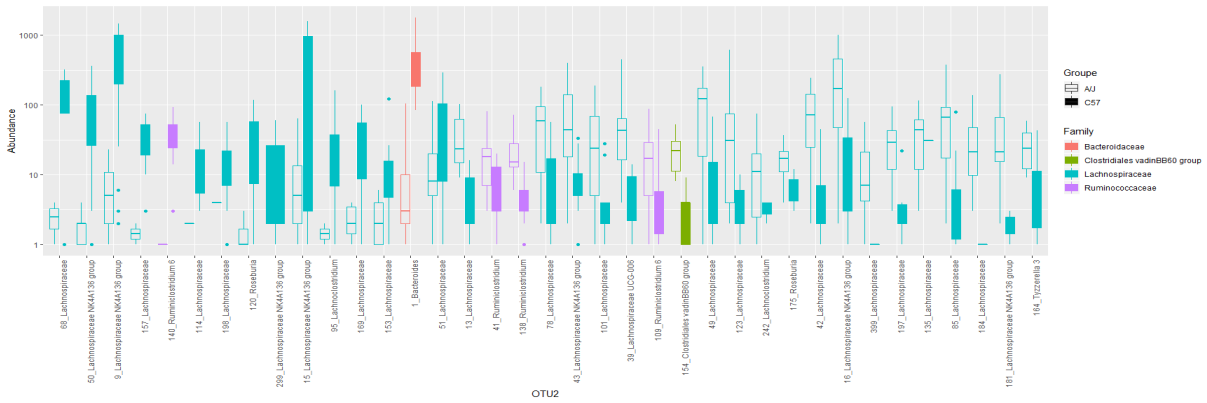


Figure 38. Log fold change of OTUs with differential abundances (p < 1e-4) in A/J (n = 23) and C57 (n = 23) mice. OTUs present in less than 25% of samples or with read count lower than 50 were filtered out. Differential abundance was tested using the negative binomial model implemented in DESeq2 and p-values corrected with the FDR procedure. Figure and figure legend are taken from Safari et al. (submitted).

53 OTUs were significantly different between C57 and A/J strain including 51 Firmicutes, 1 Bacteroidetes and 1 Proteobacteria. At the family level, out of these 53 OTUs, there are 13 *Ruminoccocaceae*, 1 *Alcaligenaceae* and one *vadinBB60 group* from *Clostridiales* and the rest are from *Lachnospiraceae* (Figure 38). 27 OTUs are more prevalent in C57 and 26 OTUs are more frequent in A/J mice (Figure 38).

5.2.6. Effect of HFD on the microbiome of A/J and C57

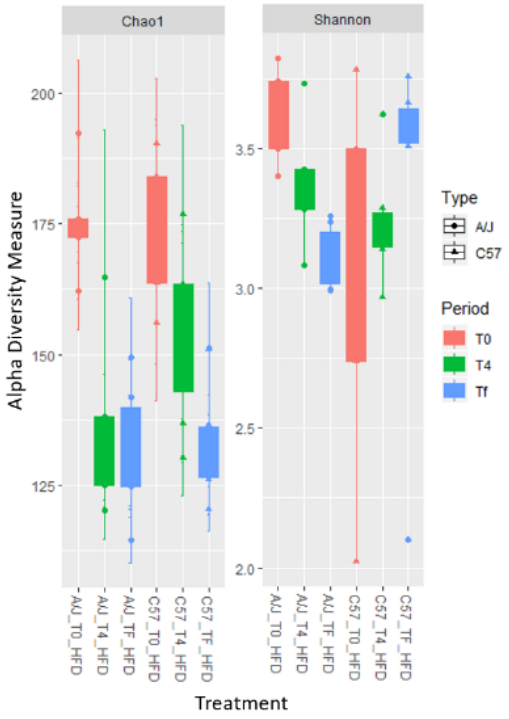


Figure 39. (a) Box plots representing a comparison between the Shannon and Chao1 diversity indices in A/J (n=16) and C57 (n=17) in response to HFD at different time phases. Time phase had a significant impact on Chao1 richness (p<1e-6, two-way anova) but not Shannon richness (p = 0.9, two-way anova). Mice strain had neither impact on Chao1 richness (p=0.23, two-way anova) nor on Shannon richness (p = 0.32, two-way anova. Figure and figure legend are taken from Safari et al. (submitted).

It was observed that the richness of microbiome in both C57 and A/J mice drops significantly after HFD treatment, which occurred quickly in A/J mice, while C57 mice tended to lose their bacteria richness slowly leading to a similar level of richness at the end of the experiment (Figure 39). Shannon diversity index at T0 was higher in A/J compared to that of C57 but decreased remarkably after HFD treatment. In contrast, the Shannon index for C57 increased after HFD treatment suggesting that HFD lead to an equal abundance of the bacterial species in C57 mice (Figure 39).

β-diversity indices were used to estimate the distance between samples from both strains in HFD groups and their shift under HFD treatment during the different time points (T0: before HFD treatment. T4: 2 weeks of HFD treatment, Tf: 7 weeks of HFD treatment) based on the evolutionary relationship of the sample's sequences and their abundance (Figure 40). Both strains clustered together before HFD treatment while they were separated after 2 weeks of HFD (T4) indicating that their composition shifted to different directions in response to HFD. Interestingly, T4 and Tf samples clustered together

for each strain showing that the microbiota composition hardly changed between 2 and 7 weeks of HFD (Figure 40).

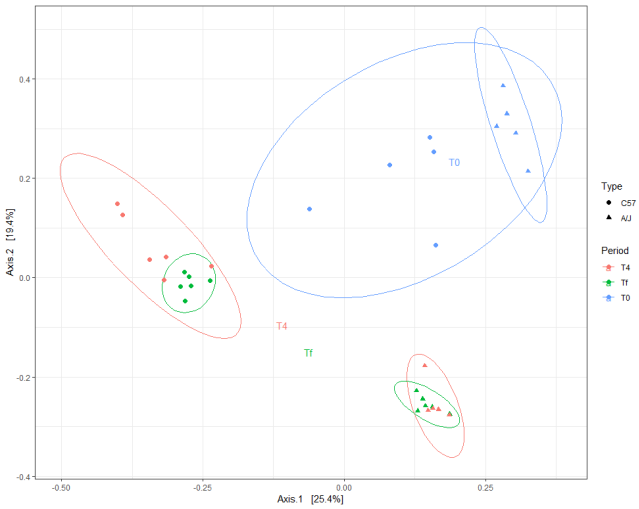


Figure 40. PCoA plot based on the Jaccard distance between samples. Microbial composition varies with both mice strain and time phase (p< 1e-3 for both, analysis of similarity test using the adonis function from the vegan package with 999 permutations). Figure and figure legend are taken from Safari et al. (submitted).

We first compared the effect of HFD in A/J and C57 at the phylum level. In C57 mice *Proteobacteria* appeared following HFD treatment. Conversely, this phylum was found at significantly lower numbers in A/J mice and did not change markedly under HFD treatment (Figure 41).

At T0, the amount of Bacteroidetes in A/J mice was also lower compared to C57 mice, while it increased gradually after HFD treatment and reached to a higher level at Tf (Figure 41). Conversely, the population of Bacteroidetes remained similar between T0 and T4 before a marked decrease at Tf (Figure 41).

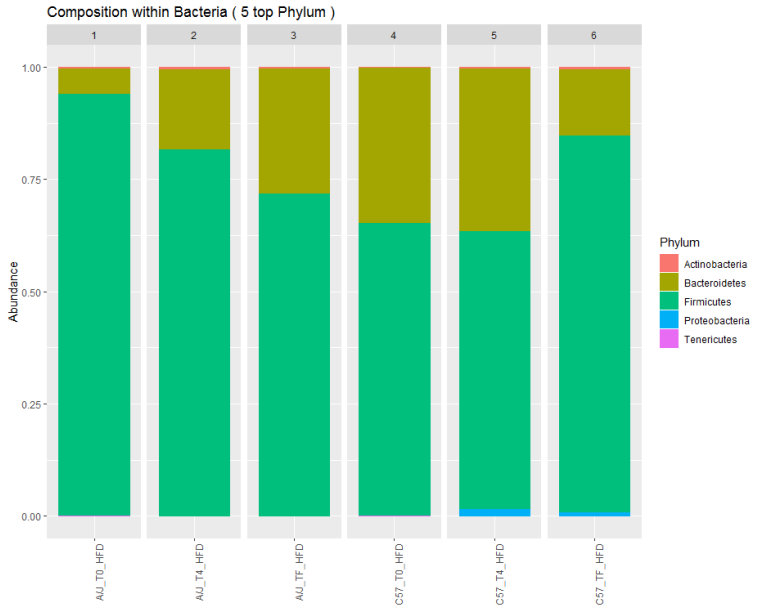


Figure 41. Phylum-level composition of the C57 (n=16) and A/J (n=17) strains during different time phases; before treatment (T0), 2 weeks after HFD treatment (T4), after 7 weeks of HFD treatment (Tf). Impact of HFD on the relative abundances of families. Figure and figure legend are taken from Safari et al. (submitted).

We also analysed the microbiome at the Family level. Only the families which were found significantly different between the two mice strains are shown in Figure 42.

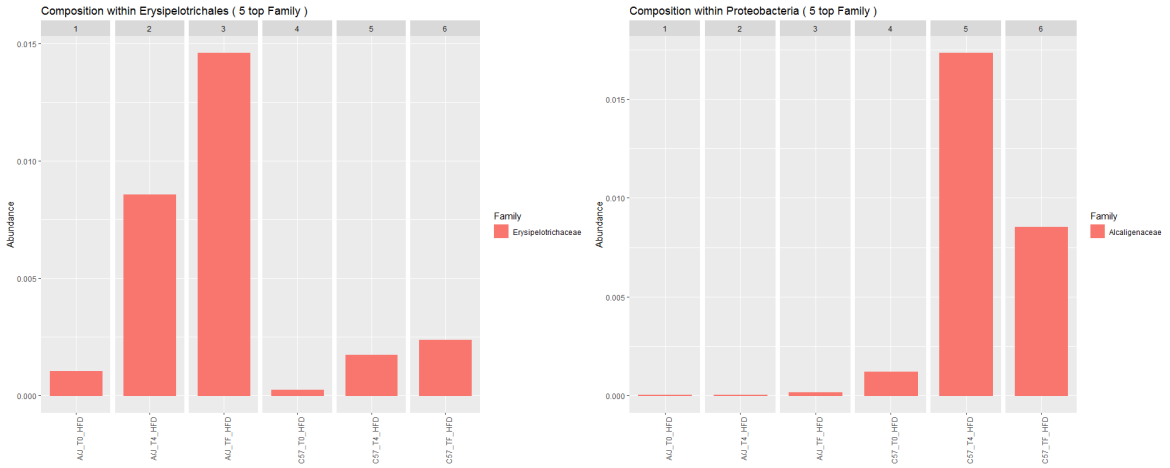


Figure 42. The impact of HFD on *Erysipelotrichaceae* and *Alcaligenaceae* frequency in C57 and A/J. Figure and figure legend are taken from Safari et al. (submitted).

Alcaligenaceae within Proteobacteria phylum was an abundant bacterium in C57 compared to A/J at T0. Moreover, the number of *Alcaligenaceae* increased significantly at T4 and with a slight reduction at Tf in C57 while its amount remained low during the whole HFD treatment in A/J mice (Figure 43).

Erysipelotrichaceae belonging to Firmicutes phylum was found more prevalent in A/J than in C57 mice at T0. In both mice strains, HFD triggered an increase in *Erysipelotrichaceae* populations which is markedly greater in A/J mice (Figure 43).

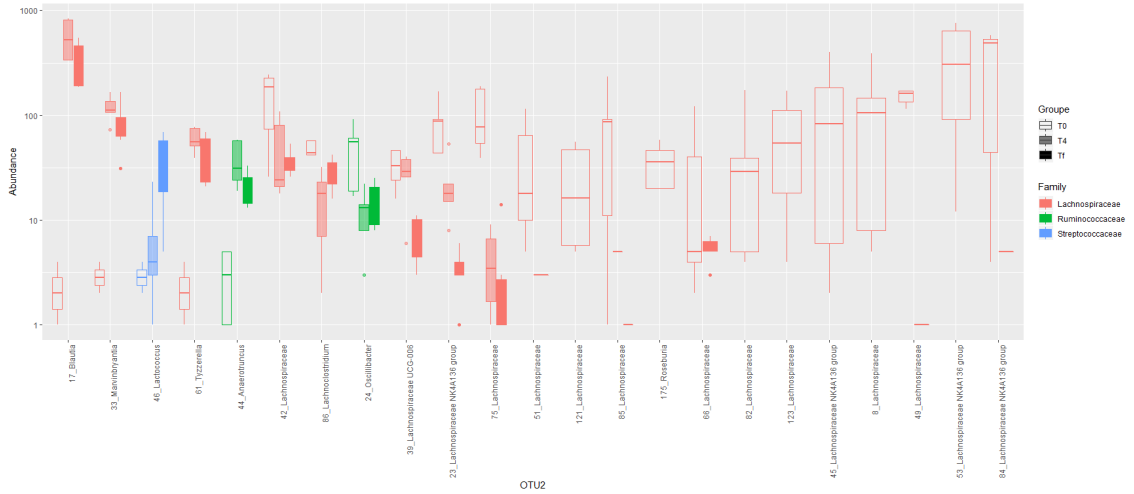


Figure 43. Abundance boxplots of OTUs whose abundance changed (p < 1e-4) in response to HFD in A/J mice. OTUs present in less than 3 of samples (out of 16) or with read count lower than 50 were filtered out. Differential abundance was tested using the negative binomial model implemented in DESeq2 and p-values corrected with the FDR procedure. Each OTU is referenced by its unique ID (e.g. 123) in the study. Figure and figure legend are taken from Safari et al. (submitted).

In A/J strain, 32 OTUs, all from Firmicutes phylum were differently expressed at different time points. Under HFD treatment, six OTUs (belonging to *Blautia*, *Marvinbryantia*, *Lactococcus*, *Tyzzerella*, and *Anaerotruncus* genera) were increased and 26 OTUs (belonging to *Oscillibacter*, *Lachnoclostridium*, *Lachnospiraceae*, *Lachnospiraceae VCG-006, Lachnospiraceae NK4A136* and *Roseburia genera*) decreased in this group (Figure 43).

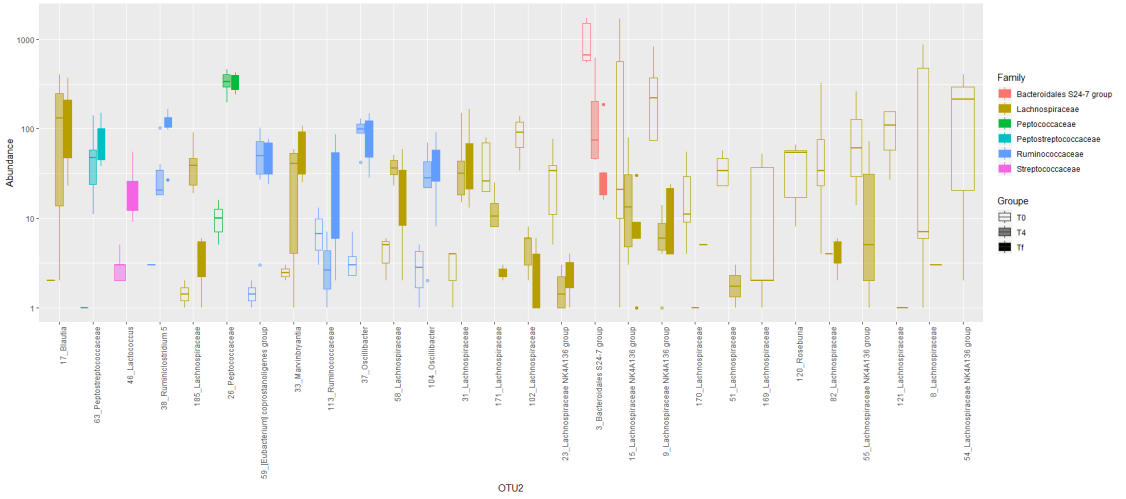


Figure 44. Abundance boxplots of OTUs whose abundance changed (p < 1e-4) in response to HFD (p < 1e-4) in C57 mice. OTUs present in less than 3 of samples (out of 16) or with read count lower than 50 were filtered out. Differential abundance was tested using the negative binomial model implemented

in DESeq2 and p-values corrected with the FDR procedure. Each OTU is referenced by its unique ID (e.g. 123) in the study. Figure and figure legend are taken from Safari et al. (submitted).

In C57 strain 39 OTUs (37 Firmicutes, one Actinobacteria and one Bacteroidetes) show significantly different frequency under HFD at different time points out of which 14 OTUs showing increase (belonging to *Blautia*, *Peptostreptococcaceae*, *Lactococcus*, *Ruminiclostridium*, *Peptoccocaceae*, *Eubacterium coprostanoligenes* group, *Marvinbryantia*, *Ruminococcaceae*, *Oscillibacter*, *Lachnospiraceae*) and 25 OTUs showed decrease (belonging to *Lachnospiraceae*, *Lachnospiraceae* NK4A136 group, *Bacteroidales* S24-7 group, *Roseburia*) (Figure 44).

Out of OTUs, 14 and 6 OTUs were increased under HFD in C57 and A/J, respectively. *Blautia*, *Lactococcus* and *Marvinbryantia* were common among increased bacteria of both strains. However, *Tyzzerella*, *Anaerotruncus* were only expanded in A/J mice and *Peptostreptococcaceae*, *Ruminiclostridium*, *Peptoccocaceae*, *Eubacterium coprostanoligenes group*, *Ruminococcaceae*, *Oscillibacter*, *Lachnospiraceae* were only raised in C57.

25 and 26 OTUs were decreased under HFD in C57 and A/J, respectively. *Lachnospiraceae*, *Lachnospiraceae NK4A136* and *Roseburia* were common among decreased bacteria in both strains. After HFD treatment, *Oscillibacter*, *Lachnoclostridium* and *Lachnospiraceae VCG-006* were diminished in A/J while *Bacteroidales S24-7 group* were reduced in C57 mice only.

5.2.7. Effect of AB on microbiome

Glucose level was improved after AB treatment in C57 mice but not in A/J mice (Figure 30c and Figure 30d). As expected, the richness of the bacteria dropped after AB treatment in both A/J and C57 strains. The Shannon diversity index was reduced in both mice strains after AB treatment but C57 recovered quickly and returned to a close to normal diversity (Figure 45).

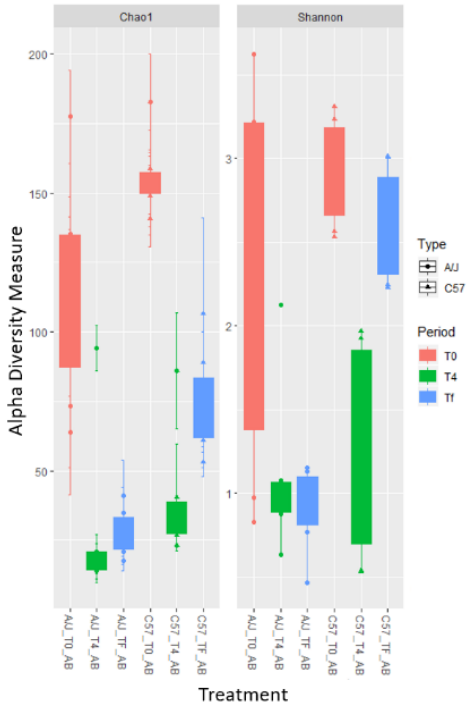


Figure 45. Richness (Chao1) and alpha diversity (Shannon) indices in A/J (n=18) and C57 (n=18) microbiota in response to AB treatment. AB treatment had a significant impact on both Chao1 richness (p<1e-10, two-way anova) and Shannon richness (p < 1e-4, two-way anova). Mice strain had an impact on both Chao1 richness (p = 0.001, two-way anova) and Shannon richness (p = 0.002, two-way anova). Figure and figure legend are taken from Safari et al. (submitted).

Antibiotic treatment altered intestinal microbiota and both strains are grouped separately at Tf (Figure 46). In the C57 strain, the richness and diversity at T4 are significantly lower versus those at Tf. However, the A/J mice show lower diversity at both T4 and Tf.

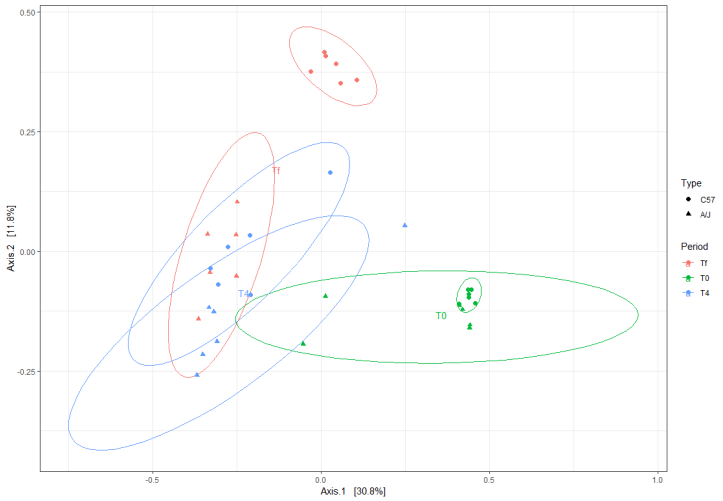


Figure 46. PCoA plot based on the Jaccard distance between samples. Microbial composition varies with both mice strain (p = 0.005) and time phase (p< 1e-3, analysis of similarity test using the adonis

function from the vegan package with 999 permutations). Figure and figure legend are taken from Safari et al. (submitted).

A majority of the bacterial clusters were highly decreased by the AB treatment and did not recover, particularly in A/J mice. Clusters 1,2,30 and 4 (belonging to *Bacteroides*, *Peptoclostridium*, *Blautia* and *Enterococcus* genera) were increased in both strains following AB treatment with a more pronounced effect in A/J mice. In C57, some of the clusters diminished by AB recovered at higher level at the end of the experiment (Tf) (Figure 47).

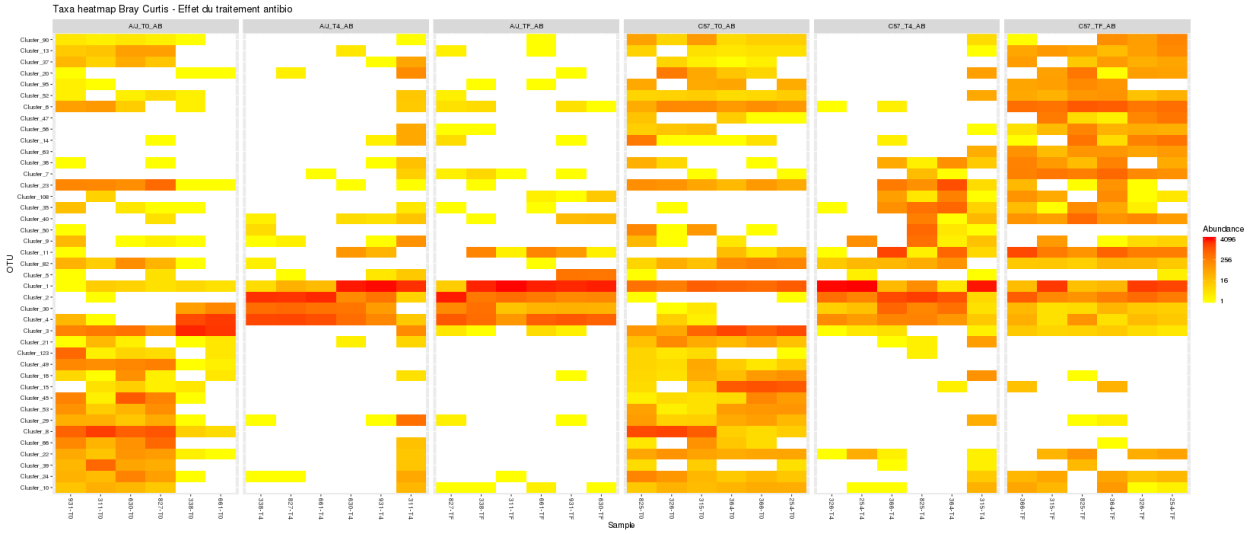


Figure 47. Heatmap, showing the impact of AB treatment on the microbiota composition in A/J and C57 mice. Figure and figure legend are taken from Safari et al. (submitted).

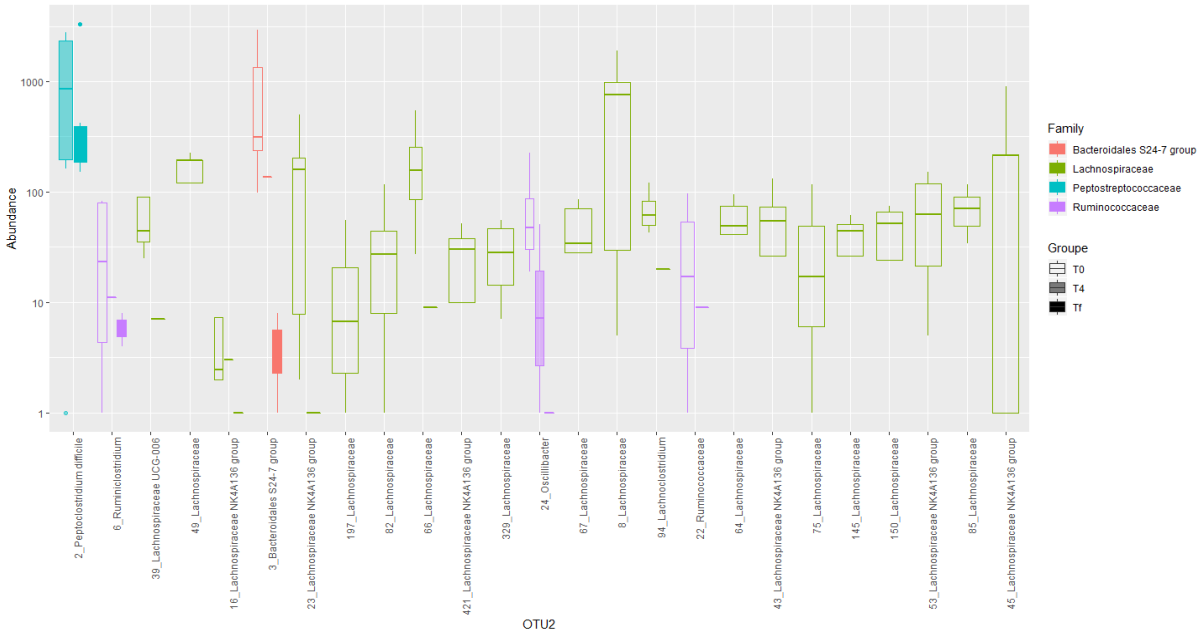


Figure 48. Abundance boxplots of OTUs whose abundance changed ($p < 1e\text{-}4$) in response to AB treatment ($p < 1e\text{-}4$) in A/J mice. OTUs present in less than 3 of samples (out of 16) or with read count

lower than 50 were filtered out. Differential abundance was tested using the negative binomial model implemented in DESeq2 and p-values corrected with the FDR procedure. Each OTU is referenced by its unique ID (e.g. 123) in the study. Figure and figure legend are taken from Safari et al. (submitted).

In A/J strain under AB treatment, 45 OTUs were significantly different between T0 and Tf. Interestingly, they all belong to Firmicutes except one from Bacteroidetes, including 26 *Lachnospiraceae*, 16 *Ruminococcaceae*, 1 *Clostridiales vadinBB60 group*, 1 *Peptostreptococcaceae* and 1 *Bacteroidales S24-7 group*. Only *Peptostreptococcaceae* was significantly increased at T4 and Tf compared to T0 and all other 44 significantly different OTUs showed decrease at T4 as well as Tf (Figure 48)

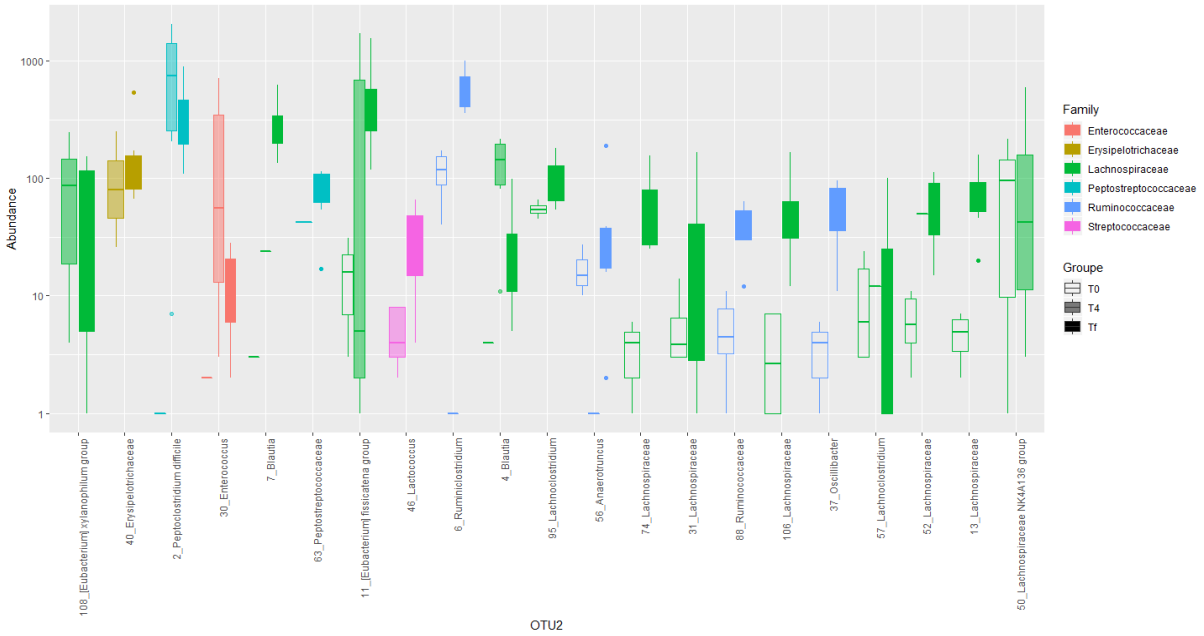


Figure 49. Abundance boxplots of OTUs whose abundance changed ($p < 1e-4$) in response to AB treatment ($p < 1e-4$) in C57 mice. OTUs present in less than 3 of samples (out of 16) or with read count lower than 50 were filtered out. Differential abundance was tested using the negative binomial model implemented in DESeq2 and p-values corrected with the FDR procedure. Each OTU is referenced by its unique ID (e.g. 123) in the study. Figure and figure legend are taken from Safari et al. (submitted).

23 OTUS (all from Firmicutes phylum) are significantly increased except *Lachnospiraceae NK4A136 group* which shows a decrease under AB treatment at Tf. In the family level, there were 13 *Lachnospiraceae*, 5 *Ruminococcaceae*, 1 *Streptococcaceae*, 2 *Peptostreptococcaceae*, 1 *Enterococcaceae*, 1 *Erysipelotrichaceae* that were significantly different in number between T0 and Tf (Figure 49).

5.2.8. Microbiome exchange after 7 weeks of HFD treatment

To compare the changes in the gut microbiota after the FMT between same (A/J-Same, C57-Same) and opposite strains (A/J-Rev, C57-Rev), a Venn diagram was used. In the experimental groups, 65 OTUs

were common to all the compared groups, and 4, 19, 8 and 10 OTUs were identified in A/J-Same, C57-Same, A/J-Rev and C57-Rev samples only, respectively (Figure 50).

A/J-Same shared more OTUS with C57-Rev than with A/J-Rev indicating effective transplant of A/J microbiota in C57 mice. Similarly, C57-Same shared more OTUS with A/J-Rev than with C57-Rev indicating that C57 microbiota has been adequately transplanted in A/J mice. However, our results clearly showed that not all bacterial species from one mouse strain were able to colonize the gut of the other mouse strain.

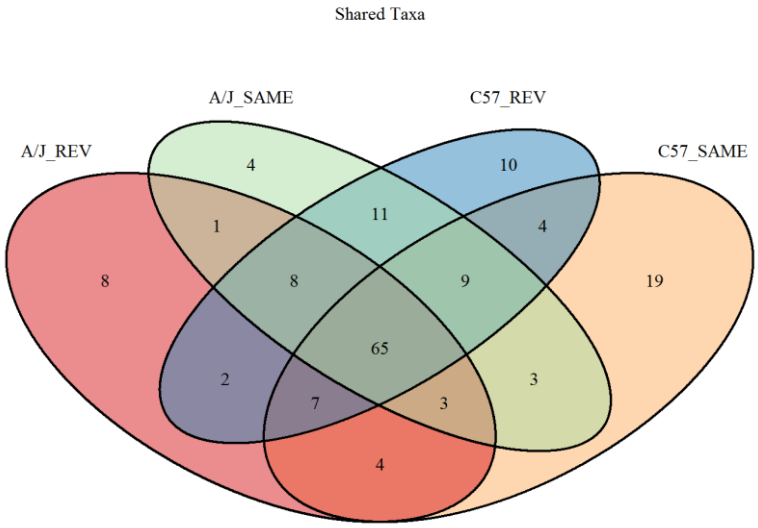


Figure 50. Venn diagrams highlighting the unique and shared OTUs in the different studied groups. Figure and figure legend are taken from Safari et al. (submitted).

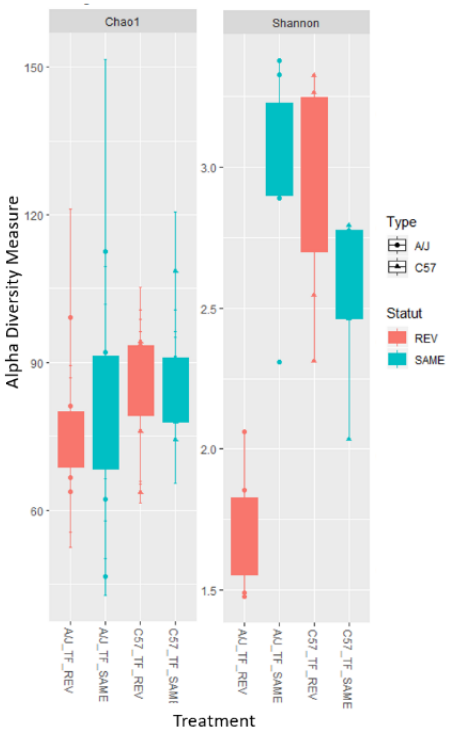


Figure 51. Richness (Chao1) and alpha diversity (Shannon) indices in A/J (n=12) and C57 (n=11) microbiota in response to microbiome exchange. Neither mice strain nor microbiome exchange had a significant impact on the Chao1 richness (two-way anova with interaction). In contrast, all of the mice strain, microbiome exchange and the interaction between them were significant on the Shannon richness ($p < 1e\text{-}5$ for all effects, two-way anova with interaction). Figure and figure legend are taken from Safari et al. (submitted).

The richness (Chao1) was not affected by gut microbiota transplants in C57 and A/J mice. Conversely, transplants of C57 microbiota lead to a marked decrease in Shannon index indicating that few bacterial species are dominant in these community. Coherently, mice associated with A/J microbiota (A/J-Same, C57-Rev) displayed elevated Shannon index. (Figure 51).

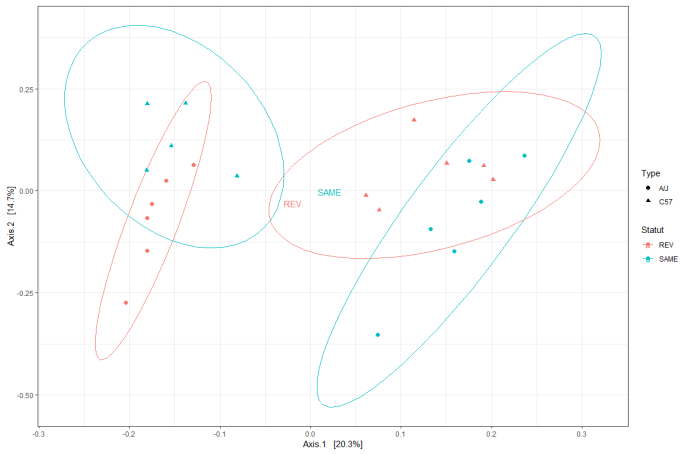


Figure 52. PCoA plot based on the Jaccard distance between samples. Microbial composition varies with both mice strain ($p = 0.002$), status (self versus exchange) ($p = 0.012$) and the interaction ($p <$

0.001, analysis of similarity test using the adonis function from the vegan package with 999 permutations). Figure and figure legend are taken from Safari et al. (submitted).

PCoA plots of the microbiota profiles further demonstrated the efficacy of the microbiota exchange. Indeed, A/J-Same and C57-Rev clustered together, as well as C57-Same with A/J Rev (Figure 52).

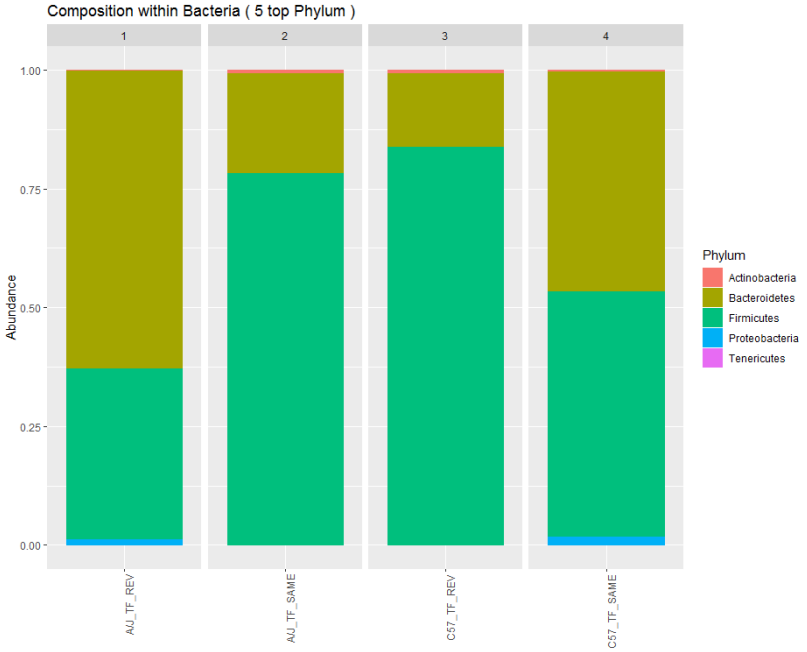


Figure 53. Phylum-level composition of the C57 (n=11) and A/J (n=12) strains after microbiome transfer, before treatment (T0), 2 weeks after HFD treatment (T4), after 7 weeks of HFD treatment (Tf). Impact of HFD on the relative abundances of families. Figure and figure legend are taken from Safari et al. (submitted).

At the phylum level, the microbiome exchange between the two strains appeared effective. Indeed, C57-Rev and A/J-Rev displayed a microbiota profile close to the one of the other strains (A/J-Same and C57-Same, respectively). Mainly, A/J-Rev mice showed higher prevalence of Bacteroidetes compared to the A/J mice receiving microbiome from A/J donor (A/J-Same) (Figure 53). A/J-Rev also represent Proteobacteria which is lacking in A/J-Same mice. Both characteristics i.e. higher level of Bacteroidetes and presence of proteobacteria could be transferred from C57-Same mice to the A/J mice (Figure 53).

On the other hand, C57-Rev group showed more or less the same pattern as A/J-Same group with a relatively lower level of Bacteroidetes and a higher level of Firmicutes compared to C57-Same (Figure 53).

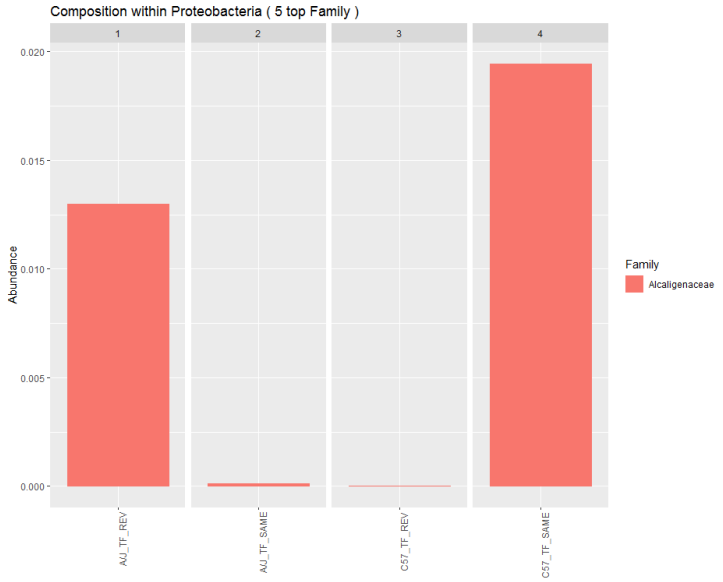


Figure 54. The effect of microbiome exchange between the two strains on *Alcaligenaceae* family. Figure and figure legend are taken from Safari et al. (submitted).

Before microbiota exchange, several bacterial families differed in proportion between C57 and A/J mice microbiota (data not shown). Among them, *Alcaligenaceae* are almost completely absent in A/J mice while they are part of the C57 microbiota. *Alcaligenaceae* family was effectively transferred from C57 to A/J strain. In contrast, the A/J-Same mice which showed a very low level of *Alcaligenaceae*, could transfer this characteristic to C57-Rev group (Figure 54).

5.3. Discussion

Microbiota in the gastrointestinal tract is seeded just after birth. Although the composition is influenced by both host genetics and environmental factors, the gut microbiome can be remodelled throughout life, depending on many factors, including diet, antibiotics, and some disease states [308, 309]. Multiple studies have established an important role of the gut microbiota in the pathogenesis of a variety of diseases, including inflammatory bowel diseases, obesity, and type 2 diabetes. Exactly how gut microbiota modify disease risk in each of these cases involves important interactions with the host's genetic background [222, 283].

Furthermore, studies have demonstrated that different strains of mice and mice from different suppliers exhibit different rates of obesity and diabetes when challenged with a high-fat diet [222, 310] highlighting the role of both genetics and environment in host metabolism. Moreover, transplant of different microbiota to genetically identical mice can change their phenotype including steatosis development [54]. Therefore, we can wonder if the gut microbiome of A/J and C57 mice contributes to their different responses to HFD.

Under HFD feeding body weight gain increased in C57 mice about two folds more than in A/J mice. This is in accordance with other studies showing higher weight gain after HFD for C57 mice compared to HFD fed A/J mice [189].Targeting the gut microbiota using antibiotics or faecal transplants did not affect the final weight of the mice in neither of the strains.

Whatever the treatments, C57 mice showed lower glucose tolerance compared to A/J mice. In the study performed by Surwit et al. on these two strains after HFD treatment (containing 35.8% fat) for six months, obese C57 mice showed clear-cut diabetes [311]. These results are also in line with the findings of Fraulob et al. on C57 mice fed with 60% high-fat chow for 16 weeks displaying greater mass gain and impairment of glucose clearance compared to the mice fed with standard chow (with10% fat) [289].

In contrast to previous reports, Gallou-Kabani et al. observed severely impaired glucose tolerance in A/J male mice. They also demonstrated that in contrast to C57 mice, which displayed overt type 2 diabetes, A/J mice of both sexes remained normoglycemic [215].

In our study, glucose tolerance was significantly different between the A/J and C57 strains in all the different treatment groups except AB group, indicating that the genetic of the strains is dominant to environmental changes such as HFD, or microbiome exchange. However, AB treatment slightly improved weight gain and glucose tolerance in C57 mice only suggesting that the gut microbiota may have a greater influence on this strain's phenotype (Safari et al. submitted). This is also in accordance

with the resistance of germ-free C57 mice to diet induced obesity and insulin resistance (Rabot et al, 2010). Coherently, C57 mice showed a reduction in inflammatory markers as well as improved insulin signalling and glucose metabolism when given antibiotics while obesity-resistant 129S1 and obesity-prone 129S6 mice were not affected by antibiotics treatment [312]. Remarkably, many of these changes were reproduced by the transfer of gut microbiota from antibiotic-treated donors to germ-free or germ-depleted C57 mice. Also, Ellekilde et al. suggested that peak in glucose concentrations in OGTT is the metabolic parameter mostly affected by the change in gut microbiota in C57 mice [313]. Altogether, these results indicate that glucose metabolism driven by an HFD can be modified by antibiotic-induced changes in gut microbiota but that these effects depend on important interactions with the host's genetic background (Safari et al. submitted).

In general, host genetic background is relatively strong determinant of faecal microbiome composition [67, 314] and thus the two genetically different strains studied here showed different microbiome composition.

We observed differences in the microbiome composition between A/J and C57 strain before any treatment. Particularly, Bacteroidetes is higher in C57 mice than A/J and conversely Firmicutes observed to be higher in A/J mice compared to C57 mice.

Furthermore, *Bacteroidaceae*, Bacteroidales S24-7 group and *Alcaligenaceae* were also found to be overrepresented in C57 in comparison to A/J mice. Conversely, *Coriobacteriaceae* revealed higher abundancy in A/J mice rather than C57 mice.

Coherently, Ericsson et al. studied the effects of supplier and genetic background on the composition of microbiome in C57 and A/J strains and observed more *Alcaligenaceae* in the C57 mice than A/J mice (both from Jax laboratory) at 3.5 and 24 weeks [315]. Interestingly, the Shannon diversity index was found higher in A/J strain compared to C57, indicating a more equal abundance of the different bacterial species.

Diet is considered as one of the most critical environmental factors shaping gut microbial structures [178, 257]. HFD feedings have been associated with modifications in the gut microbial profile as well as decreased diversity [294, 295]. In line with these studies, we found that HFD highly decreased the microbiome richness in both strains. Surprisingly, HFD also decreased the Shannon diversity in A/J mice while it increased it in C57 mice indicating that the effect of HFD on the microbiome depends on host genetics. Our results also revealed a different effect of HFD on microbiota composition in both mice strains. After HFD treatment, Bacteroidetes decreased in C57 and increased in A/J compared to its initial level. Accordingly, the Firmicutes level increased in C57 and reduced in A/J mice.

In addition, phylum Proteobacteria appeared only in C57 group and not A/J mice after HFD treatment. An increased prevalence of the bacterial phylum Proteobacteria was proposed as a marker for an unstable microbial community (dysbiosis) and a potential diagnostic criterion for disease [316]. Therefore, we can wonder whether this increase in Proteobacteria contribute to the metabolic disorders observed in C57 only in response to HFD feeding (Safari et al. submitted).

Erysipelotrichaceae number in both strains was increased by HFD both in C57 and A/J but the increment was significantly higher in C57 strain compared to its amount before the treatment. *Erysipelotrichaceae* have been repeatedly linked to the host lipid metabolism and associated with the dyslipidemic phenotypes Zhang and colleagues identified four different lineages within *Erysipelotrichaceae* to respond differently to diet or host health phenotypes [261], while Fleissner and colleagues observed an increase of *Erysipelotrichaceae* in mice on high-fat or western diet [269]. Spencer and colleagues showed that the abundance of *Erysipelotrichi* were positively associated with changes in liver fat in female subjects who were placed on diets in which choline levels were manipulated [253]. *Erysipelotrichaceae* have been also linked to lipidemic imbalances in mice and in a hamster model of hypercholesterolemia [261, 317]. We speculate that the high number of this bacteria after HFD in C57 mice may cause aggravated metabolic parameters such as higher body weight and glucose tolerance compared to A/J mice.

Alcaligenaceae was increased by HFD only in C57 mice. This bacterium could also be one of the responsible factors aggravating metabolic phenotype of C57 under HFD treatment as increased level of *Alcaligenaceae* in NASH and obese children versus to healthy controls was previously reported by Zhu et al. [46].

As expected, antibiotic treatment has significantly reduced Shannon community diversity indices, but the effect was found different in the two mice strains. Indeed, diversity remained low 8 weeks after antibiotic treatment in A/J while a diverse community, different from the initial one, recolonized C57 mice gut.

Regarding microbiota composition, *Alcaligenaceae* which is only seen in C57 mice could survive after AB treatment in this strain (Safari et al. submitted). This is in agreement with the study performed by Clarke et al. [318] who found higher proportion of this bacteria in vancomycin treated mice compared to lean and DIO (Diet Induced Obesity) groups.

Others have shown that, under some circumstances, antibiotic treatment can also decrease HFD-induced endotoxemia in obese mice, which may have beneficial effects on glucose metabolism [319]. However, long-term antibiotic treatment would probably not be an acceptable approach to treating obesity-associated insulin resistance and metabolic syndrome [320]. In fact, low-dose antibiotic treatment in humans in early life may actually increase the risk of metabolic dysfunction in

adulthood [277]. Likewise, short-term treatment of obese humans with metabolic syndrome with vancomycin appears to decrease, rather than increase, systemic insulin sensitivity [321]. This is consistent with our findings that the interaction between the gut microbiome and glucose level or insulin sensitivity is very complex and depends on multiple host factors as well as the environment.
In addition, short term antibiotic treatment applied in this study better relates to clinical situations in human and thus can be more practical.
Faecal microbiota transplantation has been previously shown to transfer a special phenotype from donor to the recipient animal [47, 54, 85]. For instance, Backhed et al. for the first time induced weight gain and increased insulin resistance in GF (C56BL/6) mice upon oral administration of faecal material from their conventional counterparts, despite a simultaneous reduction of food intake [85] Researchers attributed this to a more effective carbohydrate uptake (and subsequent lipolysis leading to increased body fat content) due to processing of nutrients by the microorganisms present. It was suggested that an altered gut microbial community, as a primary trigger, is causative rather than consequential by showing the transmissibility of the obese phenotype through faecal transplantation [47, 262]. Therefore, exchanging the microbiome between diet-induced metabolic syndrome susceptible and resistant strains might change the phenotypes of the recipient mice and give insights into the contribution of the microbiota in mouse physiology.

In our study, the microbiome exchange did not markedly affect the glucose tolerance in recipient mice. Moreover, we did not observe a significant change in weight of the A/J mice receiving microbiome from C57 mouse and vice versa compared with the mice that received microbiome from the same strain. This suggests that the genetic effect overcomes the potential influence of the microbiome and that differences in gut microbiome composition are not responsible for differences in susceptibility to metabolic disorders between A/J and C57 mice. We may also speculate that despite the microbiome exchange was globally successful as demonstrated by PCoA analysis, important specific bacterial species from one mouse strain were not able to colonize the other mouse strain (Safari et al. submitted). As an example, bacteria from the *Bacteroidales S24-7* group which were abundant in A/J mice did not colonize C57 mice. This highlights the importance of recipient genetics on gut microbiota shaping and may explain why microbiota exchange was not associated with changes in metabolic phenotypes in our study. This is an important point that may have clinical implications as faecal microbiota transplants (FMT) in humans are inevitably performed between subjects who are genetically different. Therefore, the inability of certain bacterial species to colonize recipient subject may limit the efficacy of FMT if these bacteria are responsible for the beneficial health effects. This suggests that microbiota composition should be deeply analysed in donor and recipient subjects to identify the bacterial species associated with efficacy or failure of therapeutic effects of FMT.

5.4. Conclusion

Here, we describe the microbiome diversity and composition of two mice strains with different susceptibilities to metabolic disorders. We found that resistant A/J and susceptible C57 mice harbour distinct microbial communities highlighting the impact of host genetics on microbiota shaping. Moreover, we showed that HFD and antibiotic treatment differently altered the microbiota in the two mice strains indicating that microbiota structure mainly results from genetic and environmental factors. Following antibiotic treatment, we were able to properly exchange the microbiome composition between the two strains indicating that the majority of the bacterial species from one mouse strain can colonize the other. This did not affect the metabolic phenotype of the recipient mice significantly suggesting that the gut microbiome does not contribute to differences in metabolic phenotypes between the two mice strains. However, we cannot rule out that the most relevant bacterial species in terms of health effects were not properly transferred from one mouse strain to the other. This uncomplete transfer of the gut microbiota should be taken into account when evaluating efficacy of FMT in humans (Safari et al. submitted).

6. Comprehensive discussion

Recent studies in both rodents and humans revealed that gut microbiota also contributes t metabolic diseases [322]. In fact, obese humans and rodents have less diverse gut communities than their lean counterparts [79, 264, 323]. Likewise, metagenomic studies have reported differences in the microbial gene pool identified in the gut microbiome of individuals with obesity [324], T2D [325, 326] or NAFLD [46]. Evidence for a causal link between the gut microbiota and metabolic dysfunctions has arisen from studies indicating that co-housing [83, 327] or antibiotic treatment [277, 320] can alter obesity and metabolic phenotype in rodent models. FMT from responder mice to HFD versus non-responder mice, obese versus lean humans and human twin pairs stably discordant for obesity into germ-free mouse recipients transmits donor adiposity and metabolic phenotypes [54, 328, 329]. Some of the effects of gastric bypass surgery on obesity and metabolic diseases have also been correlated to alterations in gut microbiota [330]. Although cross-sectional studies and short-term experiments evaluating effects of microbiota transfer have provided important insights into the role of gut microbiota in metabolic syndrome, further studies are required to explore the long-term nature of normal environmental changes and the complex interaction between host genetics, diet and the microbiota in the regulation of metabolism [222].

Using bacterial 16S rRNA sequencing and FMT to germ-free recipient mice, Ussar et al [222] exhibited both the original differences in phenotypes and their changes following environmental normalization and dietary challenge in three commonly-used inbred strains of mice; obesity/diabetes-prone C57Bl/6J mice, obesity/diabetes-resistant 129S1/SvImJ, from Jackson Laboratory and obesity-prone, but diabetes resistant 129S6/SvEvTac from Taconic-plus are closely associated with the gut microbiota. Hence, they suggested that the composition of the gut microbiota is highly dependent on diet, environmental history and host genetics [222].

Therefore, in the present work, we characterized interactions between host genetics, diet and the gut microbiota by conducting three different studies on A/J and C57 mouse strains which are resistant or susceptible, respectively, to steatosis under HFD treatment. We also studied a CSS panel which are constructed by crossing these two mice strains and keeping only mice that hold one certain chromosome from A/J and the rest of the chromosomes from C57 strain.

The aim of this project was to investigate the interaction of genetics, environmental changes such as diet change and gut microbiome in steatosis development.

We conducted this project in three studies following three aims: Firstly, we aimed to study the role of genetics per se in steatosis development utilizing the CSS mice. The whole panel of CSS mice with 19 CSSs was previously studied in our laboratory at the Institute of Pathology, Medical University of Graz

by Dr Karl Kashofer. It was found that CSSs show a different level of fat accumulation in their liver compared to their founder strains (A/J and C57). Therefore, we targeted two of the CSSs with high hepatic fat deposition (CSS-1 and CSS-18) and two CSSs with low amount of hepatic fat accumulation (CSS-8 and CSS-10) and fed them HFD for 8 weeks to reproduce the same observed phenotypes and particularly to investigate the role of genetics in steatosis development and its severity.

Our results showed a strong correlation between liver phenotype and a single chromosome indicating the role of genetics in the development of hepatic steatosis. Besides, with only changing one chromosome, microbiome of each of the CSSs showed different community compared to the founder strains which also confirmed the role of genetics on gut microbiome structure and composition. However, there was not a clear link between microbiome changes in each CSS and the amount of fat accumulated in their livers.

There are studies showing that genetics can affect NAFLD development. For instance, Romeo et al. [331] studied patients with and without NAFLD of various ethnicities including European, Hispanic and African-American. The liver fat was measured by proton magnetic resonance spectroscopy. One variant (rs738409), a G allele encoding I148M in *PNPLA3* gene was associated with an increased fat level in the liver across all the ethnicities. Furthermore, the variant was also associated with higher levels of ALT, histologic NAFLD including steatosis [332, 333].

Furthermore, the microbiome composition was reported to be influenced by the genetic background of the host.

One of the early studies on the interaction between host genetics and the gut microbiome reported the composition of the gut microbiota in different mice strains during a course of antibiotics. They observed differences in the bacterial communities, suggesting that the establishment of the gut microbiome does not occur by chance but is driven by various host-derived factors [334].

Kovacs et al. [67] studied several specific inbred mouse strains to understand the role of the host genotype in the composition of the gut microbiota. They found that genetic background is a strong determinant in shaping the mouse intestinal microbiota. Thus, considerable associations were revealed between eighteen host quantitative trait loci and the frequency of particular microbial taxa [68]. Moreover, several studies reported that changes such as mutations in single host genes, i.e., APOA1, NOD2, Mediterranean Fever, and FUT2, influence the gut microbiota either by changing its composition or reducing bacterial diversity [75, 261, 335, 336]

Furthermore, there are evidence showing the link between the microbiome and NAFLD development which we failed to observe in our first study. Therefore, we secondly, aimed to study the effect of diet

switch from HFD to a chow diet on the steatosis reversibility in parallel with microbiome changes on our C57 mouse models. Interestingly, we observed that with only 7 days of normal diet after 8 weeks of HFD treatment the liver recovers and gets back to a close to healthy status with less amount of fat deposition and microbiome too shows a composition similar to the initial microbiome before the HFD treatment.

These data are in line with the findings of Zhang et al. who studied 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 chow diet (CD) for 10 weeks.[250]. HFD feeding remarkably impacted the richness and diversity of the bacterial community, which was reversible upon switching to a normal diet. They observed an increased number of Firmicutes and Proteobacteria and a decreased number of Bacteroidetes in the group fed with HFD and their reversal after receiving a normal diet.

Our study also confirms the results obtained by Dethlefsen et al. who observed that after microbiome disruption by antibiotics, the structure of the gut microbiota began to revert to its initial state by one week after the end of each antibiotic course, but the recovery was not complete even after approximately 5 months [249].

Lastly, we used the faecal microbiome transplantation technique between C57 and A/J, steatosis susceptible and resistant strains, respectively, to investigate if the microbiome per se has a role in steatosis development. We also studied the strain-specific characteristics under normal condition, HF or AB treatment as well as cross microbiome transplantation between A/J and C57 mice.

We observed differences between A/J and C57 in some of the metabolic phenotypes and particularly they showed a different microbiome structure and composition.

Microbiome transfer between the strains was successful, however, microbiome exchange did not show a strong effect on the parameters associated with fatty liver diseases such as liver scoring or TG level, glucose, insulin and leptin as well as expression of some genes in the ileum and liver.

To date, most studies of gut microbiota transfer are performed in germ-free mice. Ellekilde et al. tried to use an antibiotic treatment approach in. C57BL/6 mice instead of using GF mice. They treated conventional mice with ampicillin before inoculation, at weaning or eight weeks of age with gut microbiota from lean or obese donors [313]. The clinical parameters and gut microbiota of the recipients were characterized one and six weeks after inoculation. The results revealed, that the gut microbiota recipients received the gut microbiota of the donors successfully. Six weeks after FMT, the differences persisted, however, alteration of the gut microbiota occurred within the groups during 7 weeks. The clinical parameters of the donor phenotype were partly transferred

from obese to lean mice, especially β cell hyperactivity in the obese recipients [337]. Thus, a successful inoculation of gut microbiota was not age dependent for the microbes to colonize. In addition, transferring different microbial contents to conventional antibiotic-treated mice was possible at least for a time period during which the microbiota may permanently alter important host functions [337].

Ussar et al revealed NAFLD results of complex interplays between genetic and environmental factors, including the gut microbial community. Consequently, when genetically identical mice were raised in different environments and challenged with HFD, there showed major differences in the composition of the gut microbiota that can affect the development of obesity, liver steatosis, IR and other components of the metabolic syndrome [222].

Indeed, in some genetic backgrounds (such as C57) environmental FMT can significantly ameliorate the development of features of the metabolic syndrome, whereas, in other backgrounds (such as A/J), strong genetic influences may be dominant.

We observed that in most of the metabolic phenotypes such as (body weight, OGTT, etc.) C57 mice responded completely or partially to the microbiome transplantation from a resistant strain (A/J). However, A/J mice remained more or less unaffected to the microbiome from C57. In humans, it could also be the case as due to different genetic backgrounds some individuals may be resistant and not respond to FMT while some others show altered phenotype after FMT.

7. Conclusion

We found different microbiome composition in chromosome substitutes strains (CSSs) as well as in their founder strains i.e. C57 and A/J. We also found that HF changes microbiota and trigger steatosis and that one week of CD is sufficient to reverse hepatosteatosis in mice. Therefore, we conclude that microbiota changes can contribute to diet effects as its changes correlated with phenotype. Furthermore, it is already known that A/J and C57 strains have different genetic background. In our study, we showed microbiome differences between these mice and showed that HFD changed some of the NAFLD phenotypes in these two strains. Altogether, these findings show that microbiome, genetics and diet interact with each other and lead to NAFLD development or aggravation.

The question as to whether specific populations are responsible for weight gain or are simply flourishing as a consequence of the diet being consumed (i.e., 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 GF 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.

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