ASSESSMENT OF INTESTINAL MICROBIOTA IN NON-ALCOHOLIC
FATTY LIVER DISEASE

by

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University of Toronto

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FATTY LIVER DISEASE

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Master of Science Thesis
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ABSTRACT
Non-alcoholic fatty liver disease (NAFLD) includes simple hepatic steatosis (SS) and non-alcoholic steatohepatitis (NASH). NAFLD is tightly linked to obesity and is thought to be secondary to various noxious signals, some of which may originate from the intestinal microbiota (IM). Despite a growing body of evidence supporting a link between obesity and altered IM, there are no studies assessing the IM of patients with NAFLD. In this cross-sectional study we aimed at comparing fecal levels of total bacteria, Bacteroidetes, C. coccoides, C. leptum, Bifidobacteria, E. coli, and Archaea between healthy controls (HC) and patients with SS or NASH. We found higher C. coccoides levels in NASH compared to SS and lower percentage Bacteroidetes in NASH compared to SS and HC. Controlling for body mass index and fat intake we found an association between presence of NASH and percentage
Bacteroidetes. The latter inversely correlated with insulin resistance.
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<tr>
<td>ALP</td>
<td>Alkaline Phosphatase</td>
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<tr>
<td>ALT</td>
<td>Alanine Transaminase</td>
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<tr>
<td>ANCOVA</td>
<td>Analysis of Covariance</td>
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<tr>
<td>AST</td>
<td>Aspartate Transaminase</td>
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<tr>
<td>BMI</td>
<td>Body Mass Index</td>
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<tr>
<td>BMR</td>
<td>Basal Metabolic Rate</td>
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<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>ChREBP</td>
<td>Carbohydrate Responsive Element Binding Protein</td>
</tr>
<tr>
<td>CpG</td>
<td>cytosine phosphate guanine</td>
</tr>
<tr>
<td>DM2</td>
<td>Diabetes Mellitus type 2</td>
</tr>
<tr>
<td>DNL</td>
<td><em>De Novo</em> Lipogenesis</td>
</tr>
<tr>
<td>EER</td>
<td>Estimated Energy Requirements</td>
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<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<tr>
<td>FA</td>
<td>Fatty Acid</td>
</tr>
<tr>
<td>FAS</td>
<td>Fatty Acid Synthase</td>
</tr>
<tr>
<td>FFA</td>
<td>Free Fatty Acid</td>
</tr>
<tr>
<td>FIAF</td>
<td>Fasting-Induced Adipose Factor</td>
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<tr>
<td>FOS</td>
<td>Fructo-oligosaccharide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>------------------------------------------</td>
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<tr>
<td>FR</td>
<td>Food Records</td>
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<tr>
<td>GGT</td>
<td>Gamma Glutamyl Transferase</td>
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<tr>
<td>GLP</td>
<td>Glucagon-Like Peptide</td>
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<tr>
<td>Gpr</td>
<td>G-protein-coupled receptor</td>
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<tr>
<td>HbA1c</td>
<td>Glycated hemoglobin</td>
</tr>
<tr>
<td>HC</td>
<td>Healthy Controls</td>
</tr>
<tr>
<td>HDL</td>
<td>High Density Lipoprotein</td>
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<td>HOMA</td>
<td>Homeostatic Model Assessment</td>
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<tr>
<td>HSC</td>
<td>Hepatic Stellate Cell</td>
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<tr>
<td>IQR</td>
<td>Interquartal Range</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IM</td>
<td>Intestinal Microbiota</td>
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<tr>
<td>IR</td>
<td>Insulin Resistance</td>
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<tr>
<td>IRS</td>
<td>Insulin Receptor Substrate</td>
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<tr>
<td>LPL</td>
<td>Lipoprotein Lipase</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MM</td>
<td>Master Mix</td>
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<tr>
<td>MMP</td>
<td>Matrix Metalloproteinases</td>
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<tr>
<td>NAFLD</td>
<td>Non-Alcoholic Fatty Liver Disease</td>
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<td>NASH</td>
<td>Non-Alcoholic Steatohepatitis</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>PA</td>
<td>Physical Activity</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PYY</td>
<td>Peptide YY</td>
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<tr>
<td>SCFA</td>
<td>Short Chain Fatty Acids</td>
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<td>SIBO</td>
<td>Small Intestinal Bacterial Overgrowth</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor Of Cytokine Signaling</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide Dismutases</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol Regulatory Element Binding Protein</td>
</tr>
<tr>
<td>SS</td>
<td>Simple Steatosis</td>
</tr>
<tr>
<td>TG</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>TGF</td>
<td>Tumor Growth Factor</td>
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<tr>
<td>TIMP</td>
<td>Tissue Inhibitor of Metalloproteinases</td>
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<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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<tr>
<td>UHN</td>
<td>University Health Network</td>
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<tr>
<td>VLDL</td>
<td>Very Low Density Lipoprotein</td>
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<td>WT</td>
<td>Wild Type</td>
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LIST OF PUBLICATIONS AND ABSTRACTS RELATIVE TO THIS THESIS

The following abstracts and publications are products of work related to this thesis:

PUBLICATIONS


ABSTRACTS

**Mouzaki M**, Comelli E, Arendt B, Fischer S, Allard J. Intestinal Microflora Is Altered in Patients with Non-Alcoholic Fatty Liver Disease as Compared to Healthy Controls. Oral Presentation at the annual University of Toronto GI Research Day held in Toronto, June 2012.

CONTRIBUTIONS

Drs Allard and Comelli conceived this research project and along with Dr. Kamath reviewed and corrected this thesis. Subjects were recruited by myself, with Dr. Arendt and Julia Bonengel’s assistance. I was also the contact person for the study subjects when questions arose. The hepatologists and transplant surgeons at the University Health Network (UHN), University of Toronto, obtained the liver biopsy samples. Dr. Fischer provided the interpretation of the pathology of these samples. Blood samples were obtained and reported by the laboratory medicine staff at UHN. The stool analyses for the pilot project were performed by Angela Wang (Dr. Comelli’s laboratory, University of Toronto; qPCR) and the late Dr. Dennis Krause (University of Manitoba; 16S rRNA pyrosequencing). I performed the stool analyses for the current study with the guidance of Natasha Singh, Wen Su and Kervan Rivera-Rufner, under the supervision of Dr. Comelli. The standard curves had been created by Wen Su. Julia Bonengel, Dr. Arendt and myself input the dietary data in the computer program. I collected the data, performed the statistical analyses (apart from the sample size calculation that had been done by Dr. Arendt) and wrote this thesis.
1.0 INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is tightly linked to obesity and is thought to be the hepatic manifestation of the metabolic syndrome. Liver involvement in NAFLD ranges from simple steatosis (SS) to steatosis with inflammation (non alcoholic steatohepatitis; NASH) and potentially even fibrosis or cirrhosis [1]. The pathogenesis of this condition is not entirely understood; however various steatotic, inflammatory and pro-fibrotic signals originating from the diet, adipose tissue or the immune system have been shown to play a role [2]. In addition, the intestinal microbiota (IM) have also been associated with the development of various components of NAFLD [3].

The intestinal lumen hosts trillions of microorganisms that carry 150 fold more genes compared to the host, collectively referred to as the microbiome [4-6]. Microbial gene expression, as well as bacterial translocation from the intestine to the circulation, affects appetite, caloric salvage from the diet, regulation of host gene expression, as well as homeostasis of the immune system and the profibrotic mechanisms in the liver [7, 8]. These effects contribute to the development of obesity with accompanying hepatic steatosis, as well as systemic and hepatic inflammation and fibrosis.

Observational studies in humans provide evidence that the IM of obese patients is different to that of their leaner counterparts. This finding suggests that there may be differences in the IM of patients with NAFLD. This has not been studied and may provide answers regarding the pathophysiology of this condition.
Based on the literature, the current study aimed to compare the IM of patients with NAFLD to that of healthy controls (HC), while taking into account the potential confounding effects of various factors, such as dietary intake, physical activity levels and body mass index (BMI). Specifically, levels of Bacteroidetes, Firmicutes (the sum of *Clostridium coccoides* and *Clostridium leptum*), Bifidobacteria, *Escherichia coli*, Archaea and total bacterial counts were measured using quantitative Real-Time Polymerase Chain Reaction (qPCR) and were compared across 3 groups of subjects: HC, SS and NASH.
2.0 REVIEW OF THE LITERATURE

NAFLD is the most common cause of liver disease worldwide [1]. It is challenging to determine the true prevalence of NAFLD, as the gold standard for diagnosis is a liver biopsy, which is too invasive for population-wide studies. Biochemistry, imaging studies and autopsy reports have been used to estimate the burden of NAFLD around the world. A recent systematic review showed that the prevalence of NAFLD is variable and ranges from 3-50% depending on the diagnostic modality used and the geographical location of the population studied [9]. Fatty liver is tightly linked to obesity as evidenced by a much higher prevalence of this condition in obese patients, approaching 98% in some cohorts [9]. In contrast to SS, which does not seem to progress in the majority of cases, NASH has a more aggressive course with 15% of patients progressing to cirrhosis [10, 11].

Apart from the definite benefit of weight loss, as well as the partial effectiveness of vitamin E and pioglitazone, no other medical interventions have been successful in treating NASH to date [11-13]. Taking into consideration that in the United States alone, NASH affects 3-5% of the population (translating into 1.2 million people), the aforementioned risk of disease progression and the therapeutic challenge this disease poses, it is clear that research targeted at understanding its pathophysiology and developing new, effective treatment strategies is needed.
2.1 **PATHOPHYSIOLOGY OF NAFLD:**

The pathophysiology of NAFLD is complex. It is currently thought that the development of steatosis, inflammation and hepatocellular injury is secondary to multiple ‘hits’ arising from adipocytes, immune cells, dietary factors and the gastrointestinal tract, as shown in Figure 1 [2].
**Figure 1:** Proposed multiple hits in the pathogenesis of NAFLD [2]

FA: Fatty Acids; IR: Insulin Resistance; TLR: Toll Like Receptor, TNF: Tumor Necrosis Factor; IL: Interleukin

Steatosis results from the accumulation of triglycerides in the liver. These are primarily released from the adipose tissue after lipolysis but can also originate from the diet, *de novo* lipogenesis, decreased FA oxidation or Very Low Density Lipoprotein (VLDL) export from the liver. IR enhances lipolysis in the adipose tissue. Dietary constituents that contribute to steatosis are FA and fructose. Inflammation may arise from both intestinal (e.g. FA) but also adipose tissue-derived signals (e.g. TNF-α, IL-6).
2.1.1 Pathogenesis of steatosis:

Steatosis results from increased hepatic uptake of lipids from the periphery, increased *de novo* lipogenesis (DNL), decreased β-oxidation or decreased export of very low-density lipoproteins (VLDL) from the liver. In patients with NAFLD, the majority of intrahepatic fat (59%) originates from adipose tissue [14]. Insulin resistance (IR), frequently seen in obese patients with hepatic steatosis, enhances the flow of fatty acids (FA) from adipocytes to hepatocytes. This is partly mediated via increased lipolysis and decreased triglyceride (TG) storage within adipocytes seen in the setting of IR [14]. Apart from redistribution of endogenous lipids, excess dietary fat consumption also contributes to delivery of TG to the liver. Fifteen percent of intrahepatic fat originates from the diet [14]. Therefore, it is important to assess the diet when investigating factors that may contribute to the pathogenesis of NAFLD.

In addition to hepatic lipid uptake, imbalanced rates of DNL and β-oxidation contribute to steatosis as well [2]. This may be secondary to dietary, hormonal or other signals. FA up-regulate the expression of FA synthase (FAS), the final enzyme in DNL [15]. In patients with NAFLD, the hepatic mRNA of FAS and its transcriptional regulator Sterol Regulatory Element Binding Protein (SREBP) are increased [15]. Excessive fructose consumption, described in epidemiological studies of patients with NAFLD, provides substrates (e.g. citrate) for DNL and alters expression of genes that regulate both DNL and β-oxidation [16]. Fructose also stimulates IR via phosphorylation of insulin receptors [16]. Hepatic IR is, in turn, characterized by increased DNL and
decreased FA oxidation [17]. In addition, obese patients with NAFLD have decreased adiponectin levels [18]. This adipocytokine normally suppresses SREBP-1 and enhances carnitine palmitoyl-transferase expression, decreasing DNL and increasing β-oxidation, respectively[19]. Hence, low adiponectin levels may contribute to steatosis.

The initial hepatic response to lipid accumulation is to up-regulate TG secretion via VLDL export [20]. This process reaches a plateau when the lipid content of the liver surpasses 10% [20]. Once the rate of TG accumulation exceeds that of VLDL export there is progressive accrual of hepatic fat, as seen in patients with NAFLD. Accumulation of fat, particularly FA, can lead to hepatocellular injury and inflammation.

2.1.2 Pathogenesis of hepatocellular injury and inflammation:

Hepatocellular injury is secondary to the deleterious effect of toxic metabolites, such as FA, pro-inflammatory cytokines and immune cells, as well as gut-derived signals that are discussed below.

Accumulation of FA in the liver can either exert direct hepatotoxic effects or cause indirect damage by oxidative stress induction. Hepatic TG synthesis, once thought to be the cause of hepatocellular injury, is now considered protective as it removes the truly lipotoxic free FA via esterification into TG. This was shown clearly in an animal model of NAFLD where feeding a high fat diet triggered hepatocellular damage when the ability to esterify FFA into TG was impaired via inhibition of diacylglycerol acyltransferase-2 (the last enzyme of the TG synthesis pathway) [21]. FA-induced lipotoxicity has been linked to the development of endoplasmic reticulum (ER) stress
within hepatocytes [22]. FA can also trigger the innate immune system by directly activating Toll-Like Receptor (TLR)-4 on the surface of macrophages, as well as hepatocytes and adipocytes, leading to up-regulation of cytokine synthesis and subsequently exerting local and systemic inflammatory effects [23, 24]. Both ER stress and TLR-4 activation impair insulin signaling in the liver, contributing to IR [25]. IR, frequently seen in patients with NAFLD, augments lipotoxicity by increasing the FA flux into the liver triggering a vicious cycle (Figure 2) [26].
**Figure 2:** Mechanisms of lipotoxicity

ER stress ↓ Hepatotoxicity

FATTY ACIDS in the liver

Insulin Resistance

TLR-4 activation ↓ Inflammatory cytokines

ER: Endoplasmic Reticulum; TLR: Toll Like Receptor

Hepatic fatty acids activate TLR-4 on the surface of Kupffer cells leading to the production of inflammatory cytokines. They also trigger ER stress and subsequent hepatocellular injury. Both inflammation and ER stress promote insulin resistance that leads to further fatty acid transfer from the adipose tissue to the liver.
Apart from direct hepatotoxic effects, FA also cause cellular damage through their metabolism. Abundance of free FA in the liver leads to increased lipid peroxidation with subsequent production of reactive oxygen species (ROS) and development of oxidative stress. The deleterious effects of this process are shown in animal models where diet-induced NASH can be prevented by antioxidants [27]. Patients with NAFLD are not only exposed to FA-induced oxidative stress but have impaired antioxidant mechanisms in part due to lower adiponectin levels as well [28]. In healthy subjects this adipocytokine stimulates the expression of antioxidant enzymes, such as superoxide dismutases (SOD; e.g. Cu - Zn SOD or Mn SOD), and hence hypo-adiponectemia, seen in obese patients, leads to impaired antioxidant mechanisms [28].

Besides direct lipotoxicity and oxidative stress induction, increased secretion of inflammatory cytokines, such as Tumor Necrosis Factor (TNF)-α and interleukin (IL)-6, from the visceral adipose tissue, also contributes to the hepatocellular injury seen in NAFLD [2, 29]. Adipocyte secretion of these cytokines is directly proportional to visceral adipose tissue mass and is particularly important in NAFLD because it does not only trigger hepatic inflammation but also contributes to hepatic IR [30, 31]. Circulating TNF-α enhances peripheral IR via serine phosphorylation of insulin receptor substrate (IRS)-1 and IL-6 contributes to IR via suppressor of cytokine signaling (SOCS)-3-mediated degradation of IRS-1 [24, 32, 33]. TNF-α and IL-6 contribute to inflammation via induction of chemotaxis of innate immune system cells, up-regulated expression of NF-κB-induced inflammatory proteins, as well as induction of apoptosis [34, 35]. Inflammatory cytokines can be expressed from both adipocytes and hepatocytes in
response to gut-derived signals. An example is bacterial lipopolysaccharide (LPS; endotoxin) that will be discussed below [36].

In addition to FA and cytokines, immune cells such as macrophages are involved in the inflammatory process [37]. Adiponectin maintains an anti-inflammatory equilibrium between the pro-inflammatory, ‘classically activated’ [M1] and the anti-inflammatory, ‘alternatively activated’ [M2] macrophages found in adipose tissue. Hypoadiponectemia leads to predominance of pro-inflammatory macrophages and further increase of circulating cytokines such as TNF-α [38]. Macrophage activation also occurs in the liver, where Kupffer cells cross-talk with B- or T-cells that then cause hepatocellular damage [37]. FA and circulating cytokines can activate Kupffer cells, which, in turn, release more cytokines, enhancing hepatic IR as well as local and systemic inflammation [39].

2.1.3 Pathogenesis of fibrosis:

The presence of fibrosis is not required for the diagnosis of NASH; however, it is the most important determinant of hepatic outcome in these patients [40]. Hepatic stellate cell (HSC) activation is the cardinal event in fibrosis development and in the setting of NAFLD it can be secondary to either pro-inflammatory or steatotic signals [41]. Following activation, HSC adopt a different phenotype similar to myofibroblasts with subsequent expression of proteins involved in extracellular matrix deposition (e.g. Matrix Metalloproteinases [MMP], collagen 1 and Tissue Inhibitor of Metalloproteinases [TIMP]) resulting ultimately in the development of fibrosis.
HSC can be stimulated by steatotic hepatocytes, hepatocellular debris released post-apoptosis, adipocytokines, as well as macrophages (including Kupffer cells) [17, 41-43]. The renin-angiotensin system appears to be involved in fibrogenesis as well [44]. Wobser et al. revealed the potential for steatosis-induced fibrogenesis by showing activation of HSC using culture medium retrieved from steatotic hepatocytes [45]. Oxidative stress, frequently seen in lipid-filled hepatocytes, has been implicated in HSC-induced fibrogenesis as well [46]. Activation of HSC also occurs by macrophages and Kupffer cells via Transforming Growth Factor (TGF)-β secretion [41]. Leptin, an adipocytokine whose excretion is up regulated in obesity, promotes TGF-β secretion from Kupffer cells but also increases expression of TIMP and decreases that of MMP leading to a net extracellular matrix deposition [17, 43]. Adiponectin has an opposite effect by suppressing the pro-fibrotic potential of leptin and also by impairing activation and proliferation of quiescent HSC [17, 47]. In addition, adiponectin controls the exaggerated activation of the renin-angiotensin system seen in the livers of patients with NAFLD [48]. Angiotensin-II produced by activated HSC leads to proliferation of HSC, expression of inflammatory cytokines and enhanced collagen synthesis; medications blocking the renin-angiotensin system have been shown to attenuate fibrosis [17]. Lastly, signals derived from the intestinal microbiota (IM) contribute to fibrosis, as will be discussed below. To conclude, both steatosis and inflammation predispose to hepatic fibrosis, the presence of which determines long-term outcomes in patients with NAFLD [40].
2.2 INTESTINAL MICROBIOTA

The IM is composed of bacteria, Archaea and viruses [49]. Despite significant inter-individual variations in the IM of humans, the dominating phyla are Bacteroidetes and Firmicutes [50-52]. The main phyla and some representative genera found in the human IM are shown in Table 1 [53].
**Table 1:** Main phyla in the human IM [53]

<table>
<thead>
<tr>
<th>Phyla</th>
<th>Representative genera</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
</tr>
<tr>
<td>Firmicutes</td>
<td><em>Clostridium, Lactobacillus, Enterococcus, Ruminococcus, Peptostreptococcus</em></td>
</tr>
<tr>
<td>Bacteroides</td>
<td><em>Bacteroides, Prevotella</em></td>
</tr>
<tr>
<td>Actinobacteria</td>
<td><em>Bifidobacterium</em></td>
</tr>
<tr>
<td>Proteobacteria</td>
<td><em>Escherichia, Desulfovibrio, Helicobacter</em></td>
</tr>
<tr>
<td>Verrucomicrobia</td>
<td></td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td></td>
</tr>
<tr>
<td>Synergistes</td>
<td>No known genera</td>
</tr>
<tr>
<td><strong>Archaea</strong></td>
<td></td>
</tr>
<tr>
<td>Euryarchaeota</td>
<td><em>Methanobrevibacter</em></td>
</tr>
</tbody>
</table>
Humans are colonized by 100 trillion bacterial cells, which host a significant number of genes collectively known as the microbiome [5, 50]. Expression of these genes plays a paramount role in health as it can affect metabolic and inflammatory pathways of the host [6]. Microorganisms are distributed in phylogenetic trees; from a functional perspective, recent metagenomic studies initially suggested that the human IM could be divided into 3 enterotypes based on similarities in enrichment with genes encoding for proteins involved in various metabolic pathways [49]. Further research failed to support such a notion [54].

Changes in the fecal microbiota composition may predispose to disease states and this condition is called dysbiosis [6, 55]. Diet, medications as well as gastrointestinal anatomy and motility determine the IM composition [6]. Alterations in any of these factors can lead to dysbiosis with the potential for subsequent development of inflammatory, autoimmune or other conditions [6].

Dietary alterations rapidly affect the IM composition. Animals fed a high fat diet were found to have a decrease in fecal *E. rectale, C. coccoides* and *Bifidobacteria* spp. [56]. In addition, diet high in saturated fat has been associated with an increase in *Bilophila wadsworthia*, a member of the Proteobacteria phylum [57]. In humans, Wu et al. showed that a fat-based diet leads to a disproportionate increase in *Bacteroides*, whereas a carbohydrate-dominated diet is associated with an increase in the *Prevotella* genus in the stool [58]. Brinkworth et al. placed 121 obese and overweight adults on either low-fat or low-carbohydrate diets for 8 weeks and showed that the latter led to a decrease in *Bifidobacteria* without changing the concentration of *Lactobacilli* [59]. Diet-induced selection of different bacteria is likely secondary to metabolic advantages
offered by their microbiome. Bacteria expressing genes that enable them to retrieve more energy from the metabolism of carbohydrates have a survival advantage in the setting of carbohydrate-predominant diets, whereas feeding a fat-based diet supports the selection of bacteria that utilize fat for energy [52].

Medications, such as antibiotics, rapidly alter the composition of the IM [60-62]. Certain bacterial strains do not recover until months after antibiotic use allowing for non-commensal, pathogenic bacteria to colonize the intestinal tract [6, 60]. Apart from antibiotics, probiotics and prebiotics can also alter the IM balance. Probiotics are ‘live microorganisms, which when administered in adequate amounts confer a health benefit to the host’ [63]. Prebiotics are defined as ‘non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth, activity or both of one to a limited number of bacteria in the colon and thus improve host health’ [64]. Via their effect on IM composition, both pro- and prebiotics influence metabolic and inflammatory cascades in the host and hence could prove helpful in the setting of NAFLD [3].

Apart from diet, medications and pre- or probiotics, the IM composition is also dependent upon other factors such as intestinal motility, age and genetic background of the host as discussed below [65-67].
2.2.1 IM and metabolism

Observational studies in humans reveal associations between IM composition and obesity, but the data on NAFLD and IM are scarce. A large body of the literature linking IM to nutrition metabolism is based on animal studies. The results of human studies in the field have not been consistent, as discussed in this section and summarized in Table 2.
Table 2: Studies comparing the IM of obese to that of lean adults

<table>
<thead>
<tr>
<th>Study</th>
<th>N (obese vs. lean)</th>
<th>Obese-predominant bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ley et al. [68]</td>
<td>12 vs. 2</td>
<td>↑Firmicutes/Bacteroidetes</td>
</tr>
<tr>
<td>Turnbaugh et al. [69]</td>
<td>14 vs. 140</td>
<td>↓Bacteroidetes, ↑Actinobacteria</td>
</tr>
<tr>
<td>Duncan et al. [70]</td>
<td>23 vs. 14</td>
<td>No difference in Bacteroidetes or Firmicutes</td>
</tr>
<tr>
<td>Jumpertz et al. [71]</td>
<td>9 vs. 12</td>
<td>No difference in <em>Bacteroides</em>, Firmicutes or Actinobacteria</td>
</tr>
<tr>
<td>Schwiertz et al. [72]</td>
<td>68 vs. 33</td>
<td>↓Firmicutes/Bacteroidetes</td>
</tr>
<tr>
<td>Wu et al. [73]</td>
<td>16 vs. 12</td>
<td>No differences at phylum level; at genus level: DM2 associated with ↑<em>Bacteroides, Bifidobacteria, E.coli</em></td>
</tr>
</tbody>
</table>

DM2: Diabetes Mellitus type 2
Ley et al. showed a higher Firmicutes to Bacteroides ratio in obese compared to lean adults, a finding that was reversible with weight loss [68]. Turnbaugh et al. compared the IM of obese and lean twins and revealed that obesity is associated with decreased microbial diversity, decreased Bacteroidetes and increased Actinobacteria; Firmicutes were not different compared to the lean group [69]. Weight loss in 18 unrelated patients from this group was associated with an increase in Bacteroidetes. Contrary to previous reports, Duncan et al. failed to show a difference in fecal Bacteroidetes between obese and lean adults [70]. Weight loss in this cohort was associated with an increase in butyrate-producing Firmicutes but Bacteroidetes remained unaffected. Another small study showed no difference in Firmicutes, Bacteroidetes and Actinobacteria between lean and obese adults [71]. Lastly, in contrast to most previous results, Schwiertz et al. revealed a relative abundance of fecal Firmicutes compared to Bacteroidetes of lean compared to obese subjects [72]. The inconsistency in the results of these studies may be secondary to sample size, environmental (such as dietary) or methodological differences.

In addition to the aforementioned studies, others have assessed the IM of patients with type 2 diabetes mellitus (DM2) specifically, a condition associated with significant metabolic imbalances as well as inflammation. Wu et al. compared the IM of 16 patients with DM2 to that of 12 healthy volunteers [73]. At the phylum level there were no differences between the groups with Bacteroidetes and Firmicutes being the predominant bacteria regardless of the presence of DM2. At a genus level though, the distribution of microorganisms was different between the groups, with diabetics having a relative abundance of *Bacteroides, Bifidobacteria, Lactobacillus* and *E. coli* in their
stool. The aforementioned IM differences seen in the setting of obesity and DM2 are of relevance in NAFLD, as it is strongly linked to both conditions.

Besides cross-sectional assessments of the IM, other studies have correlated the IM composition with weight changes during dynamic states, such as pregnancy or dieting. Collado et al. collected stool during the first and third trimesters of pregnancy of 18 overweight and 36 normal weight women [74]. Higher BMI was associated with increased pre-pregnancy *Bacteroides* and *S. aureus* and higher *Clostridium* spp. at the third trimester. Women with normal weight gains during pregnancy had higher fecal *Bifidobacteria* during the third trimester and *B. fragilis* correlated with overall weight gain. In another study, Santacruz et al. placed 36 teenagers on energy-restricted diets and exercise for 10 weeks [75]. At the end of the intervention, 23 patients lost more than 4kg (high weight loss group; median loss: 7.5%) and the rest had lost less than 2kg (low weight loss group; median loss: 1.3%). The latter group did not have any change in IM post-intervention whereas the former did, with *B. fragilis, Lactobacillus* and *Bifidobacteria/C. coccoides* ratio having increased and *C. coccoides* and *B. longum* having decreased. At baseline, the high weight loss group had elevated *B. fragilis, C. leptum* and total bacterial counts and lower lactobacilli and *C. coccoides*; indicating that IM may contribute to the response to lifestyle interventions such as dieting. Apart from the effects of dieting on IM, other means of weight loss, such as bariatric surgery, have been investigated as well.

Furet et al. performed real-time PCR to assess the effect of Roux-en-Y Gastric Bypass (RYGP) surgery on bacterial populations in the gut of 27 obese patients, 7 of whom had DM2 [76]. At baseline, obese patients had lower *Bacteroides/Prevotella* and
patients with DM2 had lower *F. prausnitzii* levels. Six months later, all patients had increased fecal *Bacteroides/Prevotella, E. coli* and decreased *Bifidobacteria*, all of which correlated with BMI, fat mass and leptin levels. *Lactobacilli* were also decreased after weight loss. Diabetics were found to have higher *F. prausnitzii* after weight loss, which correlated negatively with IL-6 and C-reactive protein levels. In a smaller study, Zhang et al. compared the IM of 3 healthy subjects to that of 3 obese and 3 post-gastric bypass patients [77]. Obese patients had more methane producing Archaea in their stool, whereas gastric bypass was associated with lower Firmicutes and higher Gammaproteobacteria compared to the other groups. Overall, there is an association between weight loss and changes in IM in humans that needs to be investigated further.
2.3 IM and NAFLD

Observations linking fecal microorganisms to weight and obesity have drawn interest on the potential contribution of the IM in the development of NAFLD. Animal models have aided in shedding light on mechanisms via which intestinal bacteria could trigger hepatic steatosis, systemic inflammation and even fibrosis.

2.3.1 IM and steatosis

*Animal studies*

As mentioned above, steatosis is the result of increased hepatic lipid synthesis or uptake from the periphery (diet or adipose tissue), decreased metabolism (β-oxidation) or export (in the form of VLDL) from the liver as well as inflammation. The IM has the potential to affect most of these pathways.

2.3.1.1 Role of IM in appetite regulation

Appetite dictates dietary intake and is affected by intestinal bacteria [56]. It is regulated, in part, by the autonomic nervous system (vagus nerve), as well as various hormones and neuropeptides, such as the orexigenic ghrelin and the anorexigenic peptide YY (PYY), glucagon-like peptide (GLP)-1 and leptin [78]. Prebiotic supplementation alters IM composition and interferes with appetite signals in various
animal studies. For instance, Reimer et al. showed an increased GLP-1 response after a glucose load in rats fed a high fiber diet [79]. Similarly, Cani et al. supplemented rats for 3 weeks with fructans and demonstrated decreased food intake and loss of epididymal fat mass, which were associated with increased GLP-1 levels, as well as attenuated ghrelin secretion in the fasting state [80]. In an analogous experiment, various amounts of dietary fructans led to a dose-dependent increase in PYY and decrease in ghrelin levels of obese rats [81]. Ghrelin correlated with Bacteroides levels in the stool of these mice.

In addition to hormones and peptides involved in appetite regulation, the IM also affect regulators of appetite in the central and peripheral nervous system. More specifically, endotoxin from the cell wall of Gram-negative bacteria has been proven to alter expression of genes at the level of the hypothalamus and pituitary as well as inducing afferent vagus nerve activation, all of which can contribute to altered satiety signaling [82, 83]. In summary, the IM may contribute to the development of hepatic steatosis via impaired appetite regulation that leads to increased dietary intake.

2.3.1.2 Role of IM in energy salvage from diet

Apart from increased food intake, the IM can lead to obesity and fatty liver by allowing more efficient fermentation of dietary components. Turnbaugh et al. showed that obese mice extract energy from the diet more efficiently than lean mice [52]. After confirming differences in IM between genetically obese (ob/ob) and wild type (WT) mice (obese mice had higher Firmicutes/Bacteroidetes, as well as a relative abundance
of Archaea), Turnbaugh et al. found the microbiome of ob/ob mice to be enriched in
genes involved in the metabolism of indigestible carbohydrates. The energy remaining
in the stool of these mice was decreased, whereas the concentration of the short chain
fatty acids (SCFA) butyrate and acetate (synthesized via fermentation of indigestible
carbohydrates) was increased. Fecal transplantation from ob/ob to lean WT mice led to
weight gain despite unchanged dietary intake in the latter group. This study shows that
differences in the IM of obese vs. lean mice are accompanied by altered dietary
fermentation potential, increased energy extraction from the diet and SCFA production.
SCFA, such as acetate, provide substrates for hepatic DNL, which along with increased
energy salvage from the diet may contribute to obesity and NAFLD as discussed below
[84].

The potential for fermentation of indigestible nutrients by certain bacteria
appears to be enhanced by the presence of other microorganisms, such as Archaea. In
an effort to investigate the role of Archaea in nutrient digestion, Samuel et al.
conventionalized germ-free mice with Bacteroides thetaiotaomicron with or without
Methagenobacteria smithii (the most prominent Archaea in stool) [85]. The addition of
the latter increased B. thetaiotaomicron-induced fermentation of fructans to acetate and
led to increased adiposity. Taking into consideration the aforementioned finding by
Turnbaugh et al. that ob/ob mice have an increased relative abundance of Archaea in
their stool, it appears that, at least in mice, the obese phenotype is not only dependent
on relative concentrations of single bacteria but it is also affected by metabolic cross-
talk between various microorganisms.
2.3.1.3 Role of IM in regulating host gene expression

Apart from increased caloric salvage from the diet, bacteria can also modulate host gene expression. Backhed et al. assessed the expression of genes involved in hepatic lipid handling in germ-free mice, which were conventionalized with IM from their normal counterparts [86]. Conventionalization led to weight gain, IR and hepatic steatosis despite a decrease in overall dietary intake. The expression of genes regulating DNL such as Carbohydrate-Responsive Element Binding Protein (ChREBP) and SREBP increased post-conventionalization. In addition, these mice have elevated levels of products of extensive carbohydrate fermentation in the portal blood, as well as monosaccharides, supporting the theory that enhanced energy harvest from the diet contributes to metabolic changes. Monosaccharides can also be used for DNL in the liver, which along with increased expression of genes involved in DNL suggests they could have played a role in the development of steatosis.

Conventionalization of germ-free mice was also associated with increased lipoprotein lipase (LPL) activity, potentially contributing to increased flux of FA in the liver. This was thought to be at least in part, due to bacterial suppression of intestinal synthesis of Fasting-Induced Adipose Factor (fiaf; also known as angiopointin-like protein 4), a known inhibitor of LPL [84, 86]. In summary, this study implicated the IM with increased energy extraction from the diet, enhanced FA delivery to the liver and stimulated DNL, all of which are linked to the development of obesity and hepatic steatosis.
The IM can also impair DNL by fermenting indigestible carbohydrates and leading to the production of SCFA, such as propionate [87]. As mentioned above, acetate is a SCFA that once it reaches the liver via the portal vein, is converted to acetyl-coenzyme A and enters the DNL pathway. Conversely, propionate has been shown in animal studies to attenuate rates of lipogenesis postulated to be secondary to competition with acetate for entry in the hepatocyte [87-89]. Prebiotic use leading to higher propionate to acetate ratios in the portal vein decreases DNL and hepatic steatosis in animal studies [89].

Apart from microbiota-induced DNL as a mechanism for intrahepatic fat accumulation, Backhéd et al. looked at the role of IM in expression of genes involved in β-oxidation as a steatotic trigger [84]. Germ-free and conventionalized mice were placed on a Western diet, which led to significant weight gains in the latter but normal gains in the former group, despite similar dietary intakes. Contrary to previous reports, energy harvest from the diet was the same between the groups. Germ-free mice however, had increased AMP-activated Protein Kinase activation in the liver and gastrocnemius muscle suggesting that enhanced β-oxidation could be contributing to the leaner phenotype. Also, these mice had elevated peripheral TG levels, thought to be secondary to the lack of fiaf inhibition in the germ-free setting. Fiaf knockout models showed that this enzyme is associated with expression of AMPK-independent genes involved in β-oxidation [84]. Overall, this study revealed that gut bacteria regulate hepatic and muscular β-oxidation as well as LPL activity via AMPK and Fiaf, respectively.

Altered expression of genes involved in lipid handling in the liver has also been linked to insulin sensitivity. Membrez et al. assessed the effect of broad-spectrum
antibiotic treatment on IR of genetically obese (ob/ob) as well as diet-induced obese mice [90]. Ampicillin and norfloxacin use for 2 weeks led to increased expression of genes involved in β-oxidation and decreased expression of those regulating DNL. This finding was associated with decreased hepatic steatosis in ob/ob mice as well as decreased IR as measured by glucose tolerance. Lastly, antibiotic use in these mice attenuated circulating endotoxin levels, linking inflammation to steatosis.

### 2.3.1.4 IM involvement in inflammation-driven steatosis

Until recently, it was thought that inflammation is secondary to steatosis in the setting of NAFLD; however, more current evidence supports the notion that inflammatory signals precede hepatic lipid accumulation and that the IM is involved in this process [2]. Cani et al. infused mice with LPS and caused weight gain, hepatic fat accumulation and IR, in a cluster of differentiation (CD)-14-dependent fashion [91]. CD-14 is the co-receptor that, along with TLR-4, recognizes LPS on the cell surface. The metabolic alterations seen in mice exposed to LPS were similar to those induced by feeding a high-fat diet. Interestingly, this diet led to increased proportion of LPS-containing bacteria in the gut with subsequent weight gain and IR. The mechanism of action of LPS appears to be TLR-4 dependent and, as in the case of FA-induced TLR-4 activation, LPS triggers inflammation but also IR, via TLR-4 activation [23, 56, 91]. In another experiment, Cani et al. reversed the endotoxemia caused by a high fat diet by adding prebiotics to the diet of mice [92]. This intervention led to an increase in fecal Bifidobacteria counts, decreased endotoxemia and improved insulin sensitivity,
suggesting that the IM also exerts beneficial metabolic and immunologic effects. Lastly, the link between immunity and steatosis was shown when human and animal cells derived from the pancreatic islets of Langerhans were exposed to LPS [93]. Twenty-four hours later, these cells have decreased synthesis of insulin via a mechanism dependent on TLR-4 activation. Previous studies had demonstrated worsening IR with exposure to endotoxin, which is associated with increased circulating insulin levels; in this study however, cell treatment with LPS led to decreased insulin, which may have been secondary either to the short duration of exposure or the in vitro nature of the study. Further research is needed to elucidate the role of LPS, as well as other bacterial cell wall components, in the development of IR and hepatic steatosis.

In summary, animal studies have shown that the IM can contribute to obesity and hepatic steatosis by deregulating satiety signaling, increasing the efficiency of nutrient handling, providing more substrates for DNL, altering expression of genes involved in lipid synthesis and oxidation, affecting insulin sensitivity, as well as inducing steatotic pathways via inflammatory signaling. These findings provide support for investigating this issue further in humans.

Human studies

Research in humans has attempted to replicate the results of animal studies and there appears to be preliminary evidence linking the IM with the development of obesity and hepatic steatosis. Similarly to animal data, intestinal bacteria appear to participate in appetite regulation of humans, as shown by decreased sensation of
hunger and increased GLP-1 and PYY levels in healthy adults supplemented with prebiotics [94]. Further research is needed to elucidate the exact role of IM in appetite regulation of humans, especially in the setting of obesity.

2.3.1.5 Role of IM in energy salvage from the diet of humans

In terms of the role of the IM in caloric extraction from the diet, results of human studies mirror those observed using animal models. Turnbaugh et al. assessed the microbiome of 18 adults and showed that subjects with taxonomically similar IM profiles had similar metabolic profiles [69]. Specifically, Bacteroidetes carried an increased genetic potential to ferment carbohydrates, whereas Firmicutes were enriched with genes encoding for proteins involved in nutrient transport. The microbiome of obese adults was characterized by abundance in phosphotransferase-encoding genes. The latter encode for proteins that participate in carbohydrate metabolism and, hence, this finding may be secondary to a relatively increased consumption of carbohydrates in the setting of obesity. Actinobacteria carry the majority (75%) of genes associated with an obese metabolic profile with Firmicutes providing the rest (25%). Bacteroidetes provide 45% of the lean metabolic potential in this cohort. In another study, the IM composition of 12 lean and 9 obese adults, who had been placed on 2 calorically distinct diets (2400 kcal/day vs. 3400 kcal/day) in a randomized cross-over design, was found to change rapidly with dietary interventions [71]. Overfeeding led to an increase in Firmicutes and a decrease in Bacteroidetes, with associated changes in energy salvage from the diet. A 20% increase in Firmicutes was
accompanied by an increase in energy harvest by 150 kcal and a similar decrease in Bacteroidetes was associated with an isocaloric decrease in energy harvest from the diet.

2.3.1.6 NAFLD, choline deficiency and IM in humans

In addition to the aforementioned observational studies in humans, Spencer et al. provided evidence linking bacterial microorganisms to steatosis in an interventional study where hepatic fat deposition was induced via a choline-deficient diet [95]. This effect could have been secondary to the role of choline in VLDL packaging and release from the liver; however, this diet also led to IM alterations (which primarily involved Gammaproteobacteria) that could have contributed to the steatotic phenotype. Research aimed at revealing causation, rather than simply associations, is needed to link choline-deficiency-induced IM changes to the development of fatty liver.

2.3.1.7 Role of IM in the development of insulin resistance in humans

Besides dietary factors, there seems to be cross talk between IM and metabolic hormones. Insulin affects glucose and lipid homeostasis and IR plays an important role in the pathogenesis of NAFLD. As in animals, bacterial LPS triggers systemic IR in humans, as shown by Mehta et al. who induced IR in healthy subjects by infusing endotoxin parenterally [96]. This change was associated with decreased insulin
receptor substrate (IRS)-1 expression. IR is the cardinal feature of DM2. Creely et al. measured plasma endotoxin levels of patients with DM2 and found it increased by 76% compared to non-diabetic controls [97]. LPS levels correlated with insulin levels in this cohort. Metabolic endotoxemia has also been confirmed in patients with advanced NAFLD [98-100]. Overall, there is preliminary evidence from human studies that IM-induced endotoxemia contributes to IR and may contribute to the development of hepatic steatosis and potentially even inflammation.

To conclude, there is preliminary evidence from both animal and human studies linking the IM to the development of hepatic steatosis. These microorganisms may contribute to steatosis by altering appetite regulation, increasing caloric salvage from the diet, modifying host gene expression and triggering inflammation that subsequently promotes steatosis. Further studies in humans are needed to support the current evidence.

### 2.3.2 IM and steatohepatitis

Hepatic inflammation seen in the setting of NASH can be triggered by bacterial particles, cytokines or immune cells. Oxidative stress has also been implicated in this process and may be affected by the composition of the IM.
**Animal studies**

2.3.2.1 **Role of intestinal permeability in NASH**

The strong link between endotoxemia and the development of DM2 and NAFLD raises the question whether increased intestinal permeability is affected by intestinal bacteria and if it could play a role in the pathogenesis of steatohepatitis. In mice, a high-fat diet led to decreased levels of *Lactobacillus* in the colon and enhanced intestinal permeability, a finding associated with increased concentration of activated macrophages, as well as inflammatory cytokines within the mesenteric adipose tissue [101]. Increased intestinal permeability was also reported in genetically obese (*ob/ob*) mice [102]. Prebiotics led to an increase in Bifidobacteria counts in the stool of these mice, enhanced secretion of the entero-endocrine hormone Glucagon Like Peptide (GLP)-2 and decreased intestinal permeability, which were associated with attenuated endotoxemia and hepatic inflammation [102]. The decrease in intestinal permeability was GLP-2-dependent and mediated in part by increased expression of genes encoding for intercellular tight-junction proteins.

Apart from GLP-2, other molecules derived from IM metabolism may affect the gut barrier. An example is increased intestinal synthesis of ethanol reported in obese mice, which can be decreased by the use of antibiotics [103]. Ethanol is metabolized to acetaldehyde by colonic bacteria, increasing intestinal permeability via mechanisms that are currently unknown [104].
2.3.2.2 Role of TLR activation in NASH

Increased intestinal permeability may lead to endotoxemia resulting from translocation of LPS from the cell wall of Gram-negative bacteria that reside in the intestine. LPS is then recognized by TLR-4, a membrane-bound antigen receptor expressed in various cells including hepatocytes and immune cells. This can lead to increased number of activated macrophages in the liver and up-regulated expression of inflammatory cytokines, such as IL-1β and TNF-α, in hepatic macrophages and endothelial cells [105]. The role of TLR-4 in the pathogenesis of steatohepatitis has also been supported by studies revealing attenuated inflammation and steatosis in TLR-4-deficient mice fed a steatohepatitis-inducing diet [106].

Apart from TLR-4, other TLR have been implicated in the development of hepatic inflammation, which along with their bacterial agonists are summarized in Table 3.
Table 3: TLRs, their bacterial agonists and published associations with NAFLD

<table>
<thead>
<tr>
<th>Type of TLR</th>
<th>Bacterial TLR agonists</th>
<th>Associations with NAFLD</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR-1,2,6 heterodimerize</td>
<td>Gram-positive bacteria-derived lipoprotein</td>
<td>Increased hepatic expression of these TLR in animal models of fructose-induced NASH.</td>
<td>[107]</td>
</tr>
<tr>
<td>TLR-3 (intracellular)</td>
<td>Double stranded RNA</td>
<td>As above.</td>
<td>[107]</td>
</tr>
<tr>
<td>TLR-4 (membrane-bound)</td>
<td>Gram-negative bacteria-derived lipopolysaccharide (LPS)</td>
<td>As above and also attenuated hepatic steatosis in TLR-4 KO mice fed a NASH-inducing diet. TLR-4 activation on HSC leads to fibronectin production and down-regulation of the TGF-β pseudoreceptor Bambi promoting fibrosis.</td>
<td>[107]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[106]</td>
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<tr>
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<td>[108]</td>
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<td></td>
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<td>[7,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>109]</td>
</tr>
<tr>
<td>TLR-5</td>
<td>Flagellin</td>
<td>TLR-5 KO mice are hyperphagic and have altered IM composition.</td>
<td>[66]</td>
</tr>
<tr>
<td>TLR-9 (intracellular)</td>
<td>Nucleic acid CpG motifs containing unmethylated DNA</td>
<td>TLR-9 KO mice on NASH-inducing diet have ↓hepatic steatosis, inflammation and fibrosis. Increased expression of TLR in animal models of fructose-induced NASH. TLR-9 activation on Kupffer cells leads to IL-1β up-regulation and fibrosis.</td>
<td>[110]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[107]</td>
</tr>
</tbody>
</table>

TLR: Toll-Like Receptor; CpG: cytosine phosphate guanine; KO: knock-out
2.3.2.3 Role of SCFA in NASH

In addition to bacterial translocation and recognition from TLRs, IM can affect the immune system via their role in intestinal SCFA synthesis. SCFA are trophic for the colonocytes (butyrate), but can also exert systemic anti-inflammatory effects by interacting with their receptors (e.g. G-protein-coupled receptor 43; Gpr43) [111]. Maslowski et al. demonstrated that Gpr43−/− mice have exaggerated or prolonged immune responses to various inflammatory processes. A similar picture is seen in germ-free mice, which have little or no fecal SCFA content. It appears that the interaction between SCFA and their receptors is indispensable for the homeostasis of the immune system and, hence, lack of dietary fiber or the microbiota responsible for SCFA synthesis contributes to systemic inflammation [111].

2.3.2.4 IM, inflammasome and NASH

A very distinct process via which the IM was recently shown to contribute to hepatic inflammation is that of inflammasome-mediated dysbiosis [112]. The inflammasome is a multi-protein complex that senses internal or external ‘danger signals’ and subsequently leads to the activation of pro-inflammatory cytokines [113]. Both LPS and palmitate activates the inflammasome in hepatocytes of livers with steatohepatitis (but not those with simple steatosis) [114]. Henao-Mejia et al. provided a link between the inflammasome and the IM by showing that deficiency of host
inflammasome responses alters the composition of the IM allowing for subsequent cellular influx of bacterial components that then activate TLR-4 and TLR-9 receptors and result in systemic inflammation [112].

2.3.2.5 IM, oxidative stress and NASH

A final link between hepatic inflammation and IM is provided by studies assessing the effect of intestinal bacteria on intrahepatic oxidative stress. Intestinal bacteria participate in the resolution of oxidative stress. For instance, the addition of Bifidobacteria to the diet of mice fed thermally-oxidized soybean oil led to attenuation of hepatic oxidative stress markers and increased anti-oxidant potential [115]. In another study, *Lactobacillus* species protected against aflatoxin-induced hepatic oxidative stress in rats [116]. Addition of the prebiotic fructooligosaccharide into the diet of mice exposed to d-galactose led to increased fecal Bifidobacteria counts, which was associated with increased hepatocellular antioxidant potential and protection from d-galactose-induced hepatopathy and steatosis [117].
**Human studies**

2.3.2.6 Role of intestinal permeability in human NASH

Miele et al. compared the intestinal permeability of patients with NAFLD to that of healthy controls and subjects with untreated celiac disease, via duodenal expression of tight junction zona occludens-1 [98]. The NAFLD group had increased intestinal permeability compared to controls, as well as higher prevalence of small intestinal bacterial overgrowth (SIBO), both of which correlated with the degree of hepatic steatosis but, interestingly, not with inflammation. Contrary to these findings, Wigg et al. using a dual lactulose-rhamnose sugar test found no difference in intestinal permeability or endotoxin levels between patients with NASH and healthy controls, despite an increased prevalence of SIBO in the former group [118]. Another study, using glucose hydrogen breath tests in 146 obese patients with NAFLD showed an increased prevalence of SIBO compared to healthy controls and an association between bacterial overgrowth and severe hepatic steatosis (but not inflammation) [99]. Lastly, there is evidence supporting the notion that ethanol leads to impaired gut barrier function as seen in patients with alcoholic liver disease [119]. Higher ethanol levels are found in expired breath samples of obese women compared to their lean counterparts [120]. SIBO, which is more prevalent in the setting of obesity, has been associated with detectable ethanol levels in humans, likely secondary to extensive bacterial fermentation of carbohydrates [121].
These observational data linking IM to intestinal permeability led to further research aimed at identifying structural changes that the microbiota induces on the gut barrier. Karczewski et al. infused *L. plantarum* in the duodenum of healthy adults and demonstrated increased expression of zonula occludens (ZO)-1 and occludin between epithelial cells [122]. In vitro testing revealed that *L. plantarum* increased occludin expression via a TLR-2 dependent pathway.

2.3.2.7 Interventional studies of probiotics in human NAFLD

Despite the paucity of research directly assessing the IM of patients with NAFLD, a few interventional studies have looked at the effect of probiotics in the management of this condition. These studies are few and characterized by small sample sizes and lack of histological follow-up in all but one. Aller et al. performed a 3 month long randomized control trial (RCT) of *L. bulgaricus* and *S. thermophilus* supplementation in 30 adults with biopsy-proven NAFLD and showed significant decreases in transaminases and GGT levels [123]. In another study, VSL#3 supplementation (containing *S. thermophilus* and various strains of *Lactobacillus* and *Bifidobacteria*) of 22 adults with histologically confirmed NAFLD for 3 months led to decreased transaminases, as well as decreased serum lipid peroxidation markers such as malondialdehyde and 4-hydroxynonenal, without altering BMI [124]. In a pediatric study by Vajro et al. supplementation of children with ultrasound-confirmed NAFLD with *L. rhamnosus* strain GG led to a decrease in ALT without altering BMI, imaging-determined steatosis or markers of inflammation such as TNF-α [125].
Malaguarnera et al. performed the only study where the effects of synbiotics were assessed via repeat liver biopsy [126]. In this study, 66 adults with NASH were randomized to lifestyle interventions alone versus lifestyle interventions plus \textit{B. longum} and fructooligosaccharide (FOS) supplementation for 24 weeks. Both arms were successful in improving the metabolic profile of the patients; however, the addition of pre- and probiotics led to further decreases in glucose, cholesterol, IR, CRP, TNF-\(\alpha\), transaminases as well as histologically confirmed steatosis and inflammation. Overall, a histological response was seen in 67\% of the biopsies. Lack of data on potential differences in the IM of patients with NAFLD compared to healthy subjects does not allow for selection of optimal probiotics for the management of NAFLD. Nevertheless, the findings do support the notion that the IM could well be involved in the development of human NAFLD.

To conclude, current evidence supports the role of IM in the development of inflammation within the liver. Inflammation appears to be secondary to direct toxicity of the FFA, immune system activation or dysregulation induced by the translocation of bacteria and their cellular components, such as endotoxin, into the circulation.

\subsection*{2.3.3 IM and fibrosis}

Little is known regarding the role of the IM in the development of fibrosis in the setting of NAFLD. Recently described mechanisms via which intestinally derived bacteria could trigger fibrosis implicate TLRs in this process [7].
Animal studies

2.3.3.1 Role of TLR activation in fibrosis

TLRs are expressed on all major cell types in the liver, including Kupffer cells and HSC, allowing for TLR agonists such as LPS and bacterial DNA, to activate these cells [108]. Kupffer cell activation within the liver leads to the production of profibrogenic cytokines, such as TGF-β, that indirectly stimulate quiescent HSC to differentiate into myofibroblasts [7]. The latter also occurs via direct recognition of bacterial components by TLRs on HSC. Myofibroblasts are responsible for the formation of extracellular matrix that characterizes fibrosis development [7]. Of all the TLRs expressed by cells located in the liver, TLR-4 and TLR-9 have been implicated in fibrosis development (Table 3).

2.3.3.2 Effects of probiotics on fibrosis

In addition to in vitro studies addressing the role of intestinal bacteria in fibrosis signaling, both animal and human studies of probiotic supplementation provide additional evidence linking IM and fibrosis in the setting of NAFLD. For example, Velayudham et al. supplemented C57BL/6 mice on a NASH-inducing methionine-choline deficient diet with VSL#3 [127]. Addition of this probiotic led to attenuation of fibrosis development as well as decreased expression of collagen and α-smooth muscle
actin. TGF-β levels remained the same; however, the expression of TGF-β pseudoreceptor Bambi, which attenuates the profibrogenic signaling, was increased with the use of VLS#3.

**Human studies**

The only human study addressing the role of pre- and probiotics in fibrosis progression is that of Malaguarnera et al. where 66 adults with NASH were randomly assigned to diet and exercise alone versus diet, exercise and *B. longum* with fructooligosaccharide supplementation [126]. The synbiotic improved fibrosis in 70% of patients suggesting that the IM is responsible, at least in part, for fibrosis development.

To conclude, the IM appears to induce intrahepatic fibrosis primarily via TLR activation. Further research is now needed to fully elucidate the role of the intestinal microorganisms in the development and progression of fibrosis.

In summary, research to date provides preliminary evidence that there may be a role of the IM in the pathogenesis of NAFLD; however, most of the studies have been done in animals and the human studies undertaken to date have focused on obesity rather than NAFLD alone. There are currently no published studies assessing the IM composition of patients with various stages of NAFLD, while controlling for the potential confounding effects of obesity.
3.0 **PILOT DATA**

Due to the paucity of data assessing the IM of patients with NAFLD and prior to the initiation of this study a pilot project was launched at the University Health Network (UHN) in collaboration with the University of Manitoba. For this project, 8 HC and 16 patients with NAFLD (5 with SS and 11 with NASH) were included. The HD were staff that worked at UHN, who had no evidence of liver disease and met the inclusion and exclusion criteria of the current study (see below) but did not have a diagnostic liver biopsy. Patients with NAFLD were recruited from the hepatology clinics at UHN. They were diagnosed with NAFLD after having undergone various tests to investigate the underlying cause of elevated transaminases or fatty infiltration of the liver. Both groups provided a stool sample that was collected and stored as described in the Methods section below. Quantitative PCR was done at UHN to determine the fecal Bifidobacteria counts. In addition, samples were sent to the University of Manitoba (Dr. Denis Krause) where pyrosequencing was performed. The results are summarized in **Table 4** and **Figure 3**.

The two groups (HC and NAFLD) were not different for age or gender distribution; however, patients with NAFLD had higher BMI. The results of the quantitative PCR revealed that patients with NAFLD had lower fecal Bifidobacteria counts compared to HC. Pyrosequencing showed that Bacteroidetes, Firmicutes and Proteobacteria were correlated with NASH and SS, but not HC. Verrucomicrobia, Actinobacteria and Cyanobacteria correlated more with HC. In addition, considering that the larger the circle the more variation there is in a particular group it is shown in
**Figure 3** that the IM of patients with SS varies more than that of subjects with NASH or HC. Taken together with the aforementioned literature, these results led to the current study hypothesis.
**Table 4:** Clinical characteristics of pilot project cohort

<table>
<thead>
<tr>
<th>Variables</th>
<th>NAFLD (n=16)</th>
<th>Controls (n=8)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>49.4 (11.7)</td>
<td>46.9 (5.2)</td>
<td>0.46</td>
</tr>
<tr>
<td>Gender (%)</td>
<td>44%</td>
<td>37%</td>
<td>0.08</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>87.7 (11.5)</td>
<td>72.4 (14)</td>
<td>0.02</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>31.2 (4)</td>
<td>24.7 (3.4)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Steatosis %</td>
<td>37.7 (20.5)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Data expressed as mean with (SD). BMI: Body Mass Index; N/A: non-applicable
**Figure 3:** Analyses of the fecal IM of 8 HC and 16 NAFLD patients using 454 Pyrosequencing®

The circles group NASH, SS and controls by the similarity in their IM. The straight lines indicate which phyla are most correlated with those groups. The size of the circle correlates with the variability in IM in each group. PCA: Principal Component Analysis.
4.0 AIMS AND HYPOTHESES

Aims:
1. The first aim of this thesis was to quantify and compare fecal Bifidobacteria between patients with SS, NASH and healthy subjects.

   PRIMARY HYPOTHESIS: Fecal Bifidobacteria counts of patients with NASH will be lower compared to those with SS or the HC, while patients with SS will have lower fecal Bifidobacteria counts compared to HC.

2. The second aim was to compare the ratio of fecal Firmicutes (measured as the sum of *C. leptum* and *C. coccoides*) to Bacteroidetes between patients with SS, NASH and healthy subjects.

   SECONDARY HYPOTHESIS: Patients with NASH will have a higher Firmicutes to Bacteroidetes ratio in their stool compared to those with SS or the HC.

3. The third aim was to compare the fecal concentration of other microbes: total bacteria, *C. coccoides, C. leptum, E. coli*, and Archaea, as well the ratio of these bacteria to total bacterial counts between patients with SS, NASH and healthy subjects.
4. The fourth aim was to measure and compare dietary intake and activity levels of subjects in all groups and then determine if there was a correlation between dietary intake and IM composition.

**SECONDARY HYPOTHESIS:** Patients with NASH consume a higher percentage of fat and/or carbohydrates in comparison to those with SS or the HC.
5.0 MATERIALS AND METHODS

5.1 Study design:

This was a cross-sectional study performed at a single institution from July 2010 to April 2012. Research Ethics Board approval had been obtained by UHN, as well as the University of Toronto prior to the initiation of this research project.

Adult subjects followed at UHN were recruited into this study. Patients with a clinical suspicion of NAFLD (usually due to elevated transaminases in the setting of obesity) were initially assessed by hepatologists at UHN following standard medical practices. Once NAFLD was diagnosed and all other causes of liver disease had been excluded, patients were advised by their physician to lose weight and increase activity level. No specific dietary intervention was recommended. Patients who at their follow-up visit (usually 6 months later) had not lost weight were then scheduled for a liver biopsy to investigate the severity of NAFLD. During that visit, the aims of this study were explained and if they showed interest, they were recruited (study visit day #1). At the same time subjects were instructed on how to collect and transport a stool sample (Appendix 1). In addition, they were shown how to complete 7-day dietary food records (FR) and 7-day activity logs (see details below). They were asked to return the stool sample and the dietary/activity data the morning of their liver biopsy (study visit day #2). During that day a blood sample was taken and sent directly to the laboratory at UHN for standard metabolic, nutritional and hepatic parameters, as explained below.
Subjects undergoing candidacy assessment for liver donation by the Healthy Living Donor Liver Transplant Program of Toronto General Hospital were contacted to participate as healthy controls. When these subjects approached the liver transplant program regarding their interest in hepatic donation, they were asked (by the transplant team) if they would be interested in participating in this study. If they showed interest, an appointment was booked and during that visit (study visit day #1), the purpose and details of the study were discussed. Those who agreed to participate were then recruited and were given the same instructions for stool sample and diet/activity data collection as NAFLD patients. These were returned on the week prior to the donation, on a day that was convenient for study participants (study visit day #2). During that day, blood samples were collected similarly to the protocols for NAFLD patients. Liver histology for these healthy controls was collected either during a pre-donation biopsy done to verify the healthy state of the liver or during the hepatectomy.

Inclusion criteria for this study were age > 18 years and NAFLD or healthy liver as confirmed by a recent liver biopsy (within one month from recruitment). Exclusion criteria were presence of liver disease other than NAFLD, anticipated need for liver transplantation within a year or complications of end-stage liver disease such as variceal bleeding, ascites or jaundice; concurrent medical illnesses, abnormal coagulation or other reasons contraindicating a liver biopsy; use of medications known to precipitate steatohepatitis within 6 months prior to study entry, consumption of more than 20 g of alcohol per day, use of antibiotics, pre- or probiotics in the preceding 6 months, use of supplements (such as vitamin E or C) for the treatment of NAFLD;
chronic gastrointestinal diseases, previous gastrointestinal surgery modifying the
anatomy or motility; pregnancy or lactating state.

5.2 Clinical data:

During study visit day #1 patients provided information regarding medication
(and other herbal / natural supplement) use, alcohol consumption and smoking history.
They also discussed their past medical history and in addition, data on ethnic
background, previous abdominal surgeries and ownership of pets were collected.
During the same visit, subjects were weighed using a Health-o-meter scale (Continental
Scale Corp, INC; Beford Heights, OH) and had a height measurement using a
stadiometer attached to the scale. BMI was calculated as the ratio of the weight (kg)
divided by the height (m) squared.
5.3 Nutritional and Activity assessment:

During study visit day #1, the subjects were instructed on how to complete the 7-day FR. They were asked to complete it around the time of the liver biopsy (or liver donation) and during the week preceding the collection of the stool sample. Subjects were advised to continue eating regular meals and itemize their food intake using the 2D Food Portion Visual chart (Nutrition Consulting Enterprises, Framingham, MA) to estimate portion sizes. Data were analyzed using Diet Analysis Plus Version 7.0.1 (Thomson Wadsworth, Stamford, CT) [128].

Daily activity of participants was recorded on a daily basis for 7 consecutive days. Subjects were instructed to list the type of activity they were involved with, the duration and the level of difficulty (mild, moderate, strenuous, very strenuous) based on examples provided to them (Appendix 2). Units of exercise were used to estimate daily activity levels as follows: 1 unit = 30min mild, 20 min moderate, 10 min strenuous, or 5 min very strenuous activity. This is a validated tool used for the assessment of activity in other published studies [129].

Basal Metabolic Rate (BMR) was calculated based on the Harris-Benedict equation (men: BMR = 66.5 + (13.75 x weight in kg) + (5.003 x height in cm) - (6.755 x age in years); women: BMR = 655.1 + (9.563 x weight in kg) + (1.850 x height in cm) - (4.676 x age in years) and Estimated Energy Expenditure (EER) calculated based on Health Canada Guidelines [men: EER =662-(9.53*age) +PA*[(15.91*weight in kg) + (539.6* height in m/100)]; women: EER =354-(6.91*age)+PA*[(9.36*weight in kg)+


(726*height in m/100)), where PA stands for physical activity [130]. The physical activity coefficients used are shown in Table 5.
Table 5: Physical Activity Coefficients used for Estimated Energy Expenditure Calculations


<table>
<thead>
<tr>
<th></th>
<th>Sedentary</th>
<th>Low Active</th>
<th>Active</th>
<th>Very Active</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typical daily</td>
<td>Typical daily living activities</td>
<td>Typical daily living activities</td>
<td>Typical daily living activities</td>
<td>Typical daily living activities PLUS</td>
</tr>
<tr>
<td>living activities (e.g. household tasks, walking to the bus)</td>
<td>PLUS 30-60 minutes of daily moderate activity (e.g. walking at 5-7 km/h)</td>
<td>PLUS at least 60 minutes of daily moderate activity</td>
<td>PLUS at least 60 minutes of daily moderate activity PLUS an additional 60 minutes of vigorous activity or 120 minutes of moderate activity</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Men &gt;19yo</th>
<th>Low Active</th>
<th>Active</th>
<th>Very Active</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedentary</td>
<td>1.00</td>
<td>1.11</td>
<td>1.25</td>
<td>1.48</td>
</tr>
<tr>
<td>Low Active</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Very Active</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men &gt;19yo</td>
<td>1.00</td>
<td>1.12</td>
<td>1.27</td>
<td>1.45</td>
</tr>
<tr>
<td>Women &gt;19yo</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.4 Biochemistry:

Fasting blood tests assessing the liver (AST, ALT, GGT, ALP, albumin) as well as carbohydrate (glucose, insulin, C-peptide) and lipid metabolism (triglycerides, total cholesterol, low density lipoprotein [LDL], high density lipoprotein [HDL]) were done on study visit day #2. Blood was drawn and analyzed by the Laboratory Medicine Program at the UHN. Fasting plasma glucose and insulin were measured by the enzymatic hexokinase method and radioimmunoassay, respectively. Liver enzymes in plasma as well as serum triglycerides, total cholesterol and HDL were measured using the Architect c8000 system (Abbott Laboratories). LDL was calculated as total - HDL cholesterol.

IR was calculated using the Homeostasis Model Assessment (HOMA)-IR [131]. HOMA-IR is the product of fasting glucose (mmol/L) and insulin (mU/L) divided by 22.5.
5.5 Histology:

Liver samples obtained by hepatologists via percutaneous liver biopsy (or the transplant surgeons during hepatectomy) were preserved in neutral buffered formalin and sent to the Pathology department. A pathologist blinded to the study group, assessed liver biopsies for the presence of steatosis, inflammation and fibrosis. Presence of NASH was determined using the Brunt scoring system, which is a validated and reproducible tool for the diagnosis of this condition [1, 132]. With this scoring system patients are diagnosed as having mild NASH (grade 1) if there is up to 66% steatotic hepatocytes with occasional ballooning degeneration at zone 3, with no or mild chronic portal inflammation; moderate NASH (grade 2) with steatosis of any degree and with obvious hepatocyte ballooning, mild to moderate chronic portal inflammation and possibly zone 3 pericellular fibrosis; and severe NASH (grade 3) if there is panacinar steatosis, obvious ballooning, intra-acinar inflammation with or without mild or moderate chronic portal inflammation [132].
5.6 Stool sample collection and handling:

At study visit day #1, participants received a stool collection kit, including a plastic collection/storage container with a tightly closing lid, an insulated bag and cooling elements and were instructed how to collect stool samples. Within 24 hours of the next appointment, subjects were asked to collect one sample that they would then freeze immediately in their home freezer. The frozen sample was transferred in the insulated bag with cooling elements to their second appointment at the hospital, where it was collected and then stored at -80°C until analysis. This method of stool collection was previously used successfully in a study aimed at establishing a human gut microbial gene catalogue [4].

5.6.1 Homogenization:

Once all the samples were collected the homogenization process began. Each sample was transferred into a masticator bag and placed in room temperature until a smooth consistency was obtained (1-3 hours, depending on stool size). Most of the air was removed from the bag and the sample placed into a masticator blender for 2 minutes. Homogenization was then completed by manual manipulation of the sample for another minute. Finally, a corner of the masticator bag was cut and the homogenized sample transferred to a microtube, which was then placed on dry ice until weighing; the remainder of the sample transferred to larger tubes and stored at -80°C for future use. This method of homogenization has been previously used in studies measuring fecal propionate concentrations [133].
\textbf{5.6.2 Fecal DNA extraction:}

DNA was extracted from 0.1 g of feces using the E.Z.N.A.\textsuperscript{TM} stool DNA Isolation Kit (Omega; Norcross, GA), following the protocol provided by the manufacturer that was modified to include a lysozyme digestion step (20 mg/ml and incubation at 37°C for 30 minutes) prior to the addition of the buffers. In detail, 0.1 g of feces were placed in a 1.5 mL centrifuge tube. A precision balance (Monobloc weighing technology; Mettler Toledo INC., USA) was used to weigh stool samples. Two hundred mg of glass beads and 540 uL of SLB were added to the tube. The mixture was placed onto a vortex for 13 minutes and then 20 uL of lysozyme added. The solution was then incubated at 37°C for 30 minutes. Sixty uL of DS buffer and 20 uL of Proteinase K were added and the mixture incubated at 70°C, 300 rpm for 13 minutes, with 10-second vortex half way. Incubation at 95°C for 5 minutes followed and subsequently 200 uL of SP2 buffer was added. The sample was then placed on the vortex for 30 seconds and on ice for 5 minutes. Centrifugation at 21000 x g rcf for 5 minutes followed. The supernatant was transferred into new 1.5 mL centrifuge tubes and 200 uL of HTR reagent was added. The mixture was placed onto a vortex for 10 minutes, incubated at room temperature for 2 minutes and then centrifuged for 2 minutes at 21000 x g. The supernatant was transferred to 2 mL centrifuge tubes and 250 uL of BL buffer and 250 uL of absolute ethanol were added. After a 10 second vortex, 900 uL of the mixture was transferred onto a DNA column that had been prepared by adding the DNA column into collection tubes, adding 100 uL of equilibration buffer into the DNA column, incubating in room temperature for 4 minutes and centrifuging for 1 minute. Once a sample was added onto the DNA column,
it was centrifuged for 1 minute. The flow-through was discarded and the DNA column placed into a new 2 mL collection tube. Five hundred μL of VHB buffer was added onto the DNA column and a 30 second centrifuge at 21000 x g followed. DNA wash buffer (700 μL) was added to the DNA column, which was then centrifuged for 1 minute and the flow-through discarded. The DNA column was then transferred to another 2 mL collection tube and the DNA wash buffer step repeated. The DNA column was then added to a new 2 mL collection tube and centrifuged for 1 minute. The flow-through was discarded a repeated centrifugation followed, with the DNA column cap open to allow for ethanol evaporation. The DNA column was then placed into a new 1.5 mL microcentrifuge tube and 100 μL of distilled water at 65 °C were added. The sample was then incubated for 5 minutes and centrifuged for 1 minute. The DNA column was then discarded and the flow-through stored at -20 °C until measurement of DNA concentration.

DNA concentration and purity were measured using ThermoScientific Nanodrop 1000 Spectrophotometer (ThermoScientific, Rockford, IL). DNA samples were subsequently stored at -20°C until further analyses.

5.6.3 Real-time PCR:

Microbial groups of interest: Bifidobacteria, Bacteroidetes, *C. leptum, C. coccoides*, *E. coli*, as well as total bacteria and Archaea, were analyzed by quantitative real-time PCR. Assays were run in triplicates in a 384 well optical plate using 7900HT thermocycler from Applied Biosystems (Foster City, CA) under default thermocycling conditions. Fifty nanograms of extracted DNA together with TaqMan Gene Expression
Master Mix (MM) and specific TaqMan primers (Applied Biosystems) were used for detection and amplification of microbial genes. Custom-made TaqMan primers for total bacteria, *C. coccoides*, *C. leptum*, Bacteroidetes, Bifidobacteria and Archaea were used [134, 135]. Real-time PCR for *E. coli* was done using SYBR Green Gene Expression MM (Applied Biosystems) and the specific forward and reverse primer [134]. The ramping profile for SYBR Green amplifications was similar, except for the following alterations: the first cycle was deleted and a dissociation stage was added in the end. Number of cells of each microorganism in fecal samples was calculated by interpolation from standard curves (Table 6).
**Table 6:** Standard curves used in the interpretation of the qPCR results

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Standard curve</th>
<th>Upper &amp; lower detection limit (Ct)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bacteria</td>
<td>$y = -3.304x + 35.619$</td>
<td>14.39 - 25.80</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>$y = -3.4824x + 35.052$</td>
<td>10.34 - 31.21</td>
</tr>
<tr>
<td><em>C. leptum</em></td>
<td>$y = -3.3033x + 38.891$</td>
<td>14.89 - 31.31</td>
</tr>
<tr>
<td><em>C. coccoides</em></td>
<td>$y = -3.3236x + 37.013$</td>
<td>15.66 - 35.42</td>
</tr>
<tr>
<td>Bifidobacteria</td>
<td>$y = -3.453x + 35.25$</td>
<td>12.06 to 36.33</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>$y = -3.3054x + 30.669$</td>
<td>10.14 - 29.74</td>
</tr>
<tr>
<td>Archaea</td>
<td>$y = -3.3095x + 40.014$</td>
<td>5.76 - 32.12</td>
</tr>
</tbody>
</table>
5.7 **Statistical analyses:**

The sample size calculation was based on the data by Ley et al. as well as our pilot study [68]. Calculations for the main variables (Bifidobacteria and Firmicutes / Bacteroidetes) were based on data from obese and lean subjects, as information on patients with NAFLD was not available. Therefore, assuming estimates of lean cases corresponded to HC, estimates of obese cases corresponded to patients with NASH and estimates for SS would fall in the mid-point of these two values. SD estimates were allocated in the same manner. Alpha was set to 5%. Analysis of variance was performed prior to the study and based on the pilot project, as well as data from Ley et al., in order to demonstrate differences in Bifidobacteria, Firmicutes and Bacteroidetes with a power of 0.8 30 subjects would need to be recruited in each group [68].

Results of the fecal analyses were expressed as log_{10} cells/g of wet feces and, as they were not normally distributed, non-parametric tests were used for statistical analyses (Kruskal-Wallis for 3 group-comparisons; Stata version 12 and GraphPad Prism version 4.0). High and low outliers were defined as numbers higher than the third quartile plus 1.5 times the interquartile range (IQR) and lower than the first quartile minus 1.5 times the IQR, respectively [136]. Spearman correlation coefficients were used to assess bivariate relationships between variables. Analysis of covariance (ANCOVA) was used to investigate the associations between IM and liver disease state after controlling for variables that were significant at the univariate level. Statistically significant α was considered any value lower than 0.05.
6.0 **RESULTS**

6.1 Subjects

At the time of completion of this thesis a total of 50 patients had been enrolled: 17 HC, 11 SS and 22 NASH. Three of the SS patients had hepatic steatosis after medical investigations for the assessment of candidacy for hepatic donation and hence were transferred from the HC to the SS group to form the final three study groups. Demographic and laboratory data are summarized in Table 7. Patients with NASH and SS were older, compared to HC. The distribution of males and females in each group was not found to be statistically different.

Information on ethnicity, history of abdominal surgery and pet ownership was available for 7 HC, 2 patients with SS and 8 with NASH. The small numbers did not allow for statistical comparisons of these variables. Of the HC, 72% were Caucasians, 14% Asians and 14% of mixed race. Fifty percent of patients with SS were Caucasians and 50% Asians, while those with NASH were 38% Caucasians, 25% Asians, 12% Hispanics and 25% of mixed ethnicity. None of the HC or SS patients had had a history of abdominal surgery and only 1 patient with NASH had a history of 2 cesarean sections. Seventy-one percent of HC had pets, compared to 50% of patients with SS and NASH.

As expected, patients with NASH had higher BMI compared to controls. ALT was higher in NASH and SS compared to HC and AST was higher in NASH compared to HC. No differences were found in ALP, glucose, HbA1c, cholesterol and triglyceride levels.
among the three groups. Patients with NASH had higher HOMA-IR and hence, higher IR in comparison to HC. All patients had normal liver synthetic function as determined by normal albumin and INR levels.

Liver biopsy results revealed that the median steatosis for the SS group was 12.5% (range: 5-35%) and for the NASH group 40% (range: 5-90%). As expected the majority of NASH patients had ballooning degeneration (95%) whereas 80% had a variable degree of fibrosis. Twenty percent of the patients with SS were reported as having fibrosis as well, which may have been secondary to the location where the sample was taken from, as fibrosis is not supposed to be a feature of SS.
Table 7: Demographics and laboratory results of the study population

<table>
<thead>
<tr>
<th>Variables</th>
<th>HC (n=17)</th>
<th>SS (n=11)</th>
<th>NASH (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>36 (23-58) a,b</td>
<td>48 (22-55) b</td>
<td>47 (29-68) a</td>
</tr>
<tr>
<td><strong>Gender (%male)</strong></td>
<td>59</td>
<td>36</td>
<td>45</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>26.0 (18.8-30.5) a</td>
<td>28.6 (23.5-44.2)</td>
<td>32.4 (24.2-49.5) a</td>
</tr>
<tr>
<td><strong>ALT (IU/L)</strong></td>
<td>17 (6-41) a,b</td>
<td>32 (14-54) b</td>
<td>69 (22-168) a</td>
</tr>
<tr>
<td><strong>AST (IU/L)</strong></td>
<td>22 (12-31) a</td>
<td>21 (16-40)</td>
<td>44 (18-114) a</td>
</tr>
<tr>
<td><strong>ALP (IU/L)</strong></td>
<td>65 (49-98)</td>
<td>64 (42-105)</td>
<td>77 (37-114)</td>
</tr>
<tr>
<td>**Glucose (mmol/L)</td>
<td>5.0 (4.1-6.5)</td>
<td>5.1 (4.6-6.5)</td>
<td>5.8 (4.1-7.6)</td>
</tr>
<tr>
<td>**Insulin (pmol / L)</td>
<td>21 (15-61)</td>
<td>33 (15-465)</td>
<td>97 (29-437)</td>
</tr>
<tr>
<td><strong>HbA1c</strong></td>
<td>0.054 (0.046-0.057)</td>
<td>0.055 (0.050-0.072)</td>
<td>0.061 (0.050-0.074)</td>
</tr>
<tr>
<td><strong>HOMA-IR</strong></td>
<td>0.60 (0.5-2.0) a</td>
<td>2.15 (0.5-13.7)</td>
<td>4.15 (1.0-34.6) a</td>
</tr>
<tr>
<td><strong>Triglycerides</strong></td>
<td>0.94 (0.58-3.32)</td>
<td>1.10 (0.62-3.97)</td>
<td>1.58 (0.28-3.29)</td>
</tr>
<tr>
<td><strong>Cholesterol</strong></td>
<td>4.40 (2.78-7.46)</td>
<td>5.05 (3.75-6.88)</td>
<td>4.56 (2.65-6.94)</td>
</tr>
</tbody>
</table>

*p=0.003; *p=0.001; **p<0.001

Values are expressed as medians (range). For each comparison, the same letter indicates the groups between which the statistical difference was significant.
6.2 Dietary Intake

Dietary data are summarized in Table 8. The total number of calories, expressed as total daily intake, was similar among the three groups. Adjusting caloric intake for weight (total kcal/day divided by weight), revealed that HC were consuming more calories per kilogram compared to patients with NASH. The BMR and EER were similar among all groups, but the reported dietary intake was lower than the EER in all 3 groups indicating potential dietary under-reporting or activity over-reporting.

There was no difference in daily intake of protein, carbohydrate and fat. There was a trend towards increased percentage fat intake (calculated as the ratio of percentage calories consumed from fat to total calories consumed per day) in HC, but the percentage carbohydrate intake (calculated similarly to fat) was not different between the groups. When dividing the percentage fat intake by BMR to adjust for factors such as age, the HC group was found to consume more energy from fat compared to patients with SS and NASH.
### Table 8: Dietary data and calculations

<table>
<thead>
<tr>
<th>Variables</th>
<th>HC (n=17)</th>
<th>SS (n=11)</th>
<th>NASH (n=22)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BMR (kcal/day)</strong></td>
<td>1541 (1223-2145)</td>
<td>1655 (1252-2224)</td>
<td>1635 (1329-2052)</td>
<td>0.99</td>
</tr>
<tr>
<td><strong>EER (kcal/day)</strong></td>
<td>2683 (1675-4115)</td>
<td>2639 (2042-3681)</td>
<td>2418 (1768-3899)</td>
<td>0.62</td>
</tr>
<tr>
<td><strong>Intake (kcal/day)</strong></td>
<td>2094 (1103-2796)</td>
<td>1715 (1248-2348)</td>
<td>1617 (1025-2653)</td>
<td>0.23</td>
</tr>
<tr>
<td>% fat intake</td>
<td>37 (29-45)</td>
<td>33 (22-39)</td>
<td>34 (18-43)</td>
<td>0.06</td>
</tr>
<tr>
<td>% carbohydrate intake</td>
<td>49 (4-57)</td>
<td>53 (39-74)</td>
<td>47 (4-65)</td>
<td>0.42</td>
</tr>
<tr>
<td><strong>Weight-adjusted intake</strong></td>
<td>26.9 (13.8-64.1)</td>
<td>21.7 (15.3-27.9)</td>
<td>17.4 (10.7-31.6)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>(kcal/kg/day)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intake/BMR</td>
<td>1.18 (0.6-2.3)</td>
<td>1.15 (0.7-1.2)</td>
<td>0.88 (0.6-1.6)</td>
<td>0.40</td>
</tr>
<tr>
<td>% fat intake/BMR</td>
<td>0.023 (0.016-0.035)</td>
<td>0.019 (0.012-0.47)</td>
<td>0.020 (0.013-0.031)</td>
<td>0.04</td>
</tr>
</tbody>
</table>

The values are expressed as medians (range). For each comparison, the same letter indicates the groups between which the statistical difference was significant.
6.3 Intestinal microbiota

The median duration of time that samples were kept in the -80°C freezer was not different between the three groups: 20 months for the HC, 23 months for the SS and 18 months for the NASH group (IQR: 8, 13, 15, respectively; p=0.24).

There were no statistically significant differences between the groups for Bifidobacteria, Bacteroidetes, *C. leptum*, *E. coli* and total bacteria, as shown in Figures 4-8. Patients with NASH had higher *C. coccoides* levels in their stool when compared to those with SS (Figure 9).

There were no differences in the Firmicutes to Bacteroidetes ratio between the groups (Figure 10). Apart from direct comparisons of fecal bacterial counts, the ratio of each IM of interest to the total bacteria was also compared between HC and patients with, SS and NASH. The results were significant for percentage Bacteroidetes, which was lower in patients with NASH compared to both SS and HC (Figure 11). Archaea were only measurable in 5 HC, 2 SS and 2 NASH, which limited the statistical power for any comparisons between the groups (Figure 12).

Subsequent to initial comparisons of IM between the groups, potential relationships between dietary intake and bacteria counts were explored. When assessing the whole cohort, there were no statistically significant correlations between total caloric intake, percentage fat or carbohydrate consumption and fecal Bacteroidetes, *C. leptum*, *C. coccoides*, Bifidobacteria or *E. coli* (p>0.05). Performing the same correlations for the NAFLD cohort only, there was a small but statistically
significant negative association between total daily caloric intake and fecal Bacteroidetes counts (Spearman r: -0.43, p=0.038).

Taking into consideration that BMI and percentage fat intake could be contributing to the association found between percentage Bacteroidetes and presence of NASH, linear regression was performed to control for these potential confounders. There was no interaction between BMI and percentage fat intake and ANCOVA was possible. ANCOVA revealed that, after controlling for BMI and percentage fat intake, there was a statistically significant association between percentage Bacteroidetes in stools and the presence of NASH (p= 0.027; 95% CI= -1.71 to -0.11). However, this was not the case with *C. coccoides*, which no longer associated with NASH once BMI and percentage fat intake were taken into account with ANCOVA (p>0.05).

To explain the lower percentage of Bacteroidetes in patients with NASH and based on literature supporting the theory that as Bacteroidetes die they release endotoxin, which in turn triggers IR [91], a Spearman rank correlation was performed between percentage Bacteroidetes and HOMA-IR of the NAFLD cohort. There was a statistically significant negative correlation: as the percentage Bacteroidetes decreased, IR increased (Figure 13).
**Figure 4:** Bifidobacteria load in stool samples from the three study groups

There were no statistically significant differences in median fecal Bifidobacteria counts between the groups (HC: n= 17; SS: n=11; NASH: n=22).
**Figure 5:** Bacteroidetes load in stool samples from the three study groups

![Graph showing Bacteroidetes load in stool samples from the three study groups.](image)

There are no statistically significant differences in median fecal Bacteroidetes counts between the groups (HC: n= 17; SS: n=11; NASH: n=22).
Figure 6: *C. leptum* load in stool samples from the three study groups

There are no statistically significant differences in median fecal *C. leptum* counts between the groups (HC: n=17; SS: n=11; NASH: n=20).
**Figure 7:** *E. coli* load in stool samples from the three study groups

There are no statistically significant differences in median fecal *E. coli* counts between the groups (HC: n= 17; SS: n=11; NASH: n=22).
**Figure 8:** Total bacteria load in stool samples from the three study groups

There are no statistically significant differences in total fecal bacteria counts (median) between the groups (HC: n= 17; SS: n=11; NASH: n=22).
Figure 9: *C. coccoides* load in stool samples from the three study groups.

Three NASH outliers have been removed from this comparison (HC: n = 17; SS: n = 11; NASH: n = 19). Patients with non-alcoholic steatohepatitis (NASH) have higher median fecal *C. coccoides* compared to patients with simple hepatic steatosis (SS).
**Figure 10:** Comparisons for Firmicutes to Bacteroidetes ratio between the groups

There are no statistically significant differences in the median Firmicutes (sum of *C. leptum* and *C. coccoides*) to Bacteroidetes ratio between the groups (HC: n= 17; SS: n=11; NASH: n=22).
**Figure 11:** Percentage Bacteroidetes (Bacteroidetes to total bacteria ratio) load in stool samples from the three study groups

Patients with non-alcoholic steatohepatitis (NASH) have lower median, fecal percentage Bacteroidetes compared to both patients with simple hepatic steatosis (SS) and healthy controls (HC; HC: n= 17; SS: n=11; NASH: n=22).
**Figure 12:** Archaea in stool samples of healthy controls and patients with NAFLD

Archaea were detectable in 5 healthy controls (HC) and 4 patients with non-alcoholic fatty liver disease (NAFLD; 2 with simple steatosis [SS] and 2 with non-alcoholic steatohepatitis [NASH]). The median difference between the groups was not statistically significant.
**Figure 13**: Negative correlation between percentage Bacteroidetes and HOMA-IR in patients with NAFLD

Spearman correlation $r = -0.49$; $p=0.002$; $n=33$
7.0 **DISCUSSION**

This is, to our knowledge, the first study aimed at assessing the IM of patients with NAFLD. The results demonstrated no differences in Bifidobacteria or Firmicutes to Bacteroidetes ratio between NASH, SS and HC. However, *C. coccoides* (member of Firmicutes phylum) was higher in NASH compared to SS, and percentage Bacteroidetes was lower in NASH compared to both SS and HC. The groups did not differ in *C. leptum*, *E. coli* or total bacteria counts, while few subjects overall had detectable Archaea. Adjusting for BMI and percentage fat intake, the negative association between percentage Bacteroidetes and presence of NASH remained, while the positive one between *C. coccoides* and NASH was no longer significant. In addition, percentage of Bacteroidetes inversely correlated with IR (HOMA-IR). Lastly, an inverse correlation was found between caloric intake and Bacteroidetes in the NAFLD cohort. This study, therefore, provides preliminary evidence for a link between IM composition and the presence of NASH.

The finding that fecal Bifidobacteria were not different between the groups was surprising considering previously published literature in the field of obesity, as well as the preliminary findings of the pilot project. The latter had revealed that patients with NAFLD have lower fecal Bifidobacteria counts compared to HC. One possible explanation for this discrepancy is the difference in the control groups that participated in the two projects. The HC of the pilot study may not have reflected the general population, as they were all UHN staff, who may follow an overall healthy lifestyle and are more knowledgeable in the area of nutrition than the public at large. The HC that
participated in this larger study were more representative of the general population as they came from various socioeconomic and ethnic backgrounds and did not share the same work environment.

Apart from the control groups, the inability to show difference in Bifidobacteria (as well as all other microbes assessed) may have been secondary to lack of statistical power. The sample size that had been calculated based on the results of the pilot study (n=30 for each group) had not been reached at the time of this thesis completion. Despite that, 50 subjects were recruited for this study, which is quite similar to the cohort size of other cross-sectional studies that have aimed at assessing the differences in the IM of obese versus lean adults, as shown in Table 2. The lack of difference may also be due to the presence of confounders, which are discussed below.

Another hypothesis of this study was that patients with NASH have a higher fecal Firmicutes to Bacteroidetes ratio compared to subjects with SS and HC. This hypothesis was based on human data in the field of obesity published by Ley et al.. The results of the current study failed to support this hypothesis, which is in line with other smaller projects that despite using different methods to characterize the IM, such as pyrosequencing or Fluorescent In Situ Hybridization (FISH), have also failed to show differences in Firmicutes and Bacteroidetes between lean and obese adults [70, 71]. In contrast, a larger study by Schwiertz et al. [72] that used qPCR for a cohort of 101 adults in order to measure the Firmicutes to Bacteroidetes ratio, found it to be decreased in obesity, which is the exact opposite of the results by Ley et al. [68]. Differences in environment, methodology and sample size, as well as intra-individual variability, could account for the contradicting results of these studies.
It was recently reported that the Firmicutes to Bacteroidetes ratio increases with the duration of sample storage in the -80°C [137]. The median duration of storage time was not different between the groups and, hence, is unlikely to have contributed to the findings.

*C. coccoides* belongs to the Firmicutes phylum and is one of the most abundant of the bacterial groups in the human IM [138]. In our study, this group was higher in patients with NASH compared to those with SS. Apart from the limited human data assessing the microbiome for Firmicutes as a whole, there are no mechanisms yet known via which *C. coccoides* could contribute to the development of NASH. In these studies, comparing the microbiome of obese to that of lean individuals has shown that Firmicutes provide one quarter of the obesogenic genetic potential, implicating them in the development of obesity and hence potentially even in NAFLD [69]. Another mechanism that could, at least in theory, link *C. coccoides* to NASH is that these species may be increasing to the detriment of other ‘protective’ microorganisms rendering the liver more susceptible to pro-inflammatory signals arising from the diet or the adipose tissue.

The association found between higher *C. coccoides* and NASH was no longer significant when BMI and percentage fat intake were taken into account. This is not surprising, as previously published literature revealed a link between *C. coccoides* and obesity. Specifically, Nadal et al. showed that lifestyle intervention-associated weight loss in 39 adolescents from Spain was accompanied by a decrease in *C. coccoides*, providing a link between BMI and this microorganism [139].
Along with Firmicutes, Bacteroidetes comprise the majority of the human IM [53, 69]. In this study, Bacteroidetes as percentage of total bacteria were lower in patients with NASH compared to both SS and HC. This finding is in agreement with previously published literature in the field of obesity demonstrating lower Bacteroidetes in patients with higher BMI [68, 69].

ANCOVA showed a BMI- and fat intake-independent association between percentage Bacteroidetes and presence of NASH. There are various theories to support an inverse correlation between this phylum and steatohepatitis. First, lower Bacteroidetes could contribute to increased appetite as shown in animal studies by Parnell et al., who supplemented mice with prebiotics and witnessed a decreased dietary intake associated with changes in expression of appetite-regulating peptides, such as an increase in PYY and GLP-1 precursor, as well as a decrease in ghrelin that directly correlated with Bacteroides levels [81].

The results of the current study in humans show similar energy intakes between the groups. Hence, the theory that lower Bacteroidetes increase appetite does not explain the association found with NASH. However, within the NALFD cohort, the reported daily caloric intake was inversely correlated with fecal Bacteroidetes counts, which may provide indirect evidence for a link between these bacteria and appetite regulation. Jumpertz et al. also reported an association between increased caloric intake in subjects with lower fecal Bacteroidetes in the literature [71]. Assessment of appetite regulating peptides in cross-sectional or interventional studies where the IM is altered with the use of pre- or probiotics would be useful in clarifying the exact role of gut bacteria in appetite control.
Bacteroides are propionate-producing bacteria and this SCFA is known to not only be anorexigenic, via increased leptin expression, but to also compete with acetate for entry into hepatocytes [139]. Acetate is a precursor for DNL and hence increased propionate aids in decreasing DNL rates in the liver. If this effect of Bacteroidetes truly played a significant role in the pathogenesis of NAFLD however, there should not have been differences in levels of these bacteria in the IM patients with SS and NASH. In addition, this theory does not explain the inflammation seen in patients with NAFLD.

Bacteroidetes carry roughly 45% of the lean metabolic potential as shown in a study by Turbaugh et al. that compared the microbiome of lean and obese adults [52]. Indirect support for these findings has also been provided by Jupertz et al., who showed that a 20% increase in fecal Bacteroidetes was associated with a 150 kcal decrease in energy harvest from the diet [71]. Decreased Bacteroidetes in the setting of NASH could then suggest a relative increase in genes with obesogenic potential that leads to increased caloric extraction from the diet and subsequent worsening of the metabolic profile. This theory explains obesity and steatosis, but not the hepatic inflammation that is characteristic in NAFLD.

Another possibility is that immune activation seen in the setting of NASH leads to alterations in the IM composition, as seen in the TLR-5 and TLR-9 knockout mouse models, where immune dysregulation leads to significant alterations in the IM [66, 110]. This hypothesis suggests a host to IM effect rather than the opposite and could well explain differences in the IM of patients with NASH compared to those observed in SS and HC.
The last and strongest theory supporting a link between Bacteroidetes and NASH is that death of these bacteria leads to the release of LPS from the bacterial cell wall, which subsequently activates steatotic, inflammatory and fibrotic signals via LPS-TLR-4 interaction on hepatocytes, immune cells and HSC [91, 102]. Patients with NAFLD may initially have similar Bacteroidetes levels as HC, but with time these bacteria die, releasing LPS and triggering various metabolic and inflammatory cascades that persist even after their number is decreased in the gut. It is not clear whether it is dietary, immune or other bacterial factors that trigger death of Bacteroidetes, but this theory explains all the features of NASH including steatosis, inflammation and fibrosis, as well as the documented finding of metabolic endotoxemia described in obese and diabetic adults, as well as patients with NAFLD [97, 98, 100]. Endotoxemia triggers IR, which is found in the majority of patients with NAFLD [91, 96]. The effects of endotoxin are not related to its half-life and, hence, in NAFLD the mere release of endotoxin may suffice for the activation of steatotic, pro-inflammatory and pro-fibrotic pathways [140].

To indirectly test this last hypothesis, a correlation was performed between the percentage Bacteroidetes and IR, as calculated with HOMA-IR. As shown in Figure 13, there was a statistically significant correlation between the two, such that as Bacteroidetes levels decrease, IR increases. Although LPS levels were not measured in this study to provide additional evidence in support of this theory, it would be of value to perform these types of measurements in the future.

*E. coli* counts and Archaea were not statistically different between the groups. The overall low abundance of *E. coli* in the stool and the fact that Archaea were only found in a small proportion of study subjects likely contributed to the difficulty in
detecting potential differences. It has been demonstrated that even though certain bacteria may not dominate the human IM quantitatively, they still may have functional dominance by carrying ‘unique’ genes that allow for metabolic and survival advantages that can affect the IM as a whole [49]. *E. coli*, for example, contain 90% of the genetic pool for the expression of proteins that participate in pilus assembly [49]. Some pili allow for prolonged intestinal colonization, whereas F-pili permit the transfer of plasmids to other bacteria [141]. The latter aids in exchanging genetic information that can render bacteria resistant to antibiotics and change their ability to ferment or transport various nutrients. This is an important example of the significant functional role that a fecal microorganism can have despite an overall low abundance.

Metagenomics rather than descriptive methods, such as qPCR and pyrosequencing, should be used to assess the function of these bacteria or Archaea.

In this cohort, patients with NASH were older than HC. This finding is not surprising as NAFLD is frequently diagnosed (and may even be more prevalent) in older individuals [142, 143]. This may be due to cumulative weight gain and visceral adipose tissue deposition over time, but may also be secondary to increased DNL rates in older patients [143, 144]. Also, HC participating in the Living Liver Donor program need to be relatively healthy in order to be able to donate a segment of their liver. Given that younger age frequently correlates with an overall healthier state, it is not too surprising that this group had a younger median age.

While the IM of infants and elderly patients differs to that of adults, within the adult spectrum, it is unlikely that there are significant, age-dependent variations in the
IM composition [65]. For that reason, age was not considered as a confounder and was not included in the ANCOVA.

Differences in BMI between groups could exert a confounding effect on the findings. Considering the strong correlation between the presence of obesity and the development of NAFLD, it was not surprising to see in this cohort that patients with NASH had a higher BMI compared to HC. Despite this fact, it is important to note that the median BMI of the HC group was in the overweight range [145]. The proportion of subjects who were overweight in this group (59%) reflects, however, the Canadian population that is characterized by an overall prevalence of overweight and obesity of 53% [144]. This is unlikely to have influenced the results of our study, as every patient included in the HC group had a biopsy-proven unaffected (non-steatotic, non-inflamed) liver. Various previous studies have linked obesity to an altered IM but the aim of this study was to identify BMI-independent associations between various IM and the extent of hepatic involvement. Given the lower prevalence of NAFLD within normal-weight subjects and the fact that lean people are less likely to seek medical attention and undergo laboratory or imaging studies that would incidentally indicate NAFLD, it is challenging to match the groups for BMI. However, this confounding factor was adjusted for using a linear regression model.

Serum transaminase levels were higher in the NASH and/or SS groups compared to the HC. This was expected as the NAFLD diagnosis was established after a work-up that had been initiated because of incidental findings of elevated transaminases. As already mentioned, transaminase levels do not correlate with disease severity and there is a significant within patient variation in the levels with time. This was recently
shown with the TONIC and PIVENS trials, two of the largest randomized, controlled studies aimed at treating children with NAFLD and adults with NASH, respectively [12, 13]. Within these cohorts, the transaminases of patients assigned to the placebo groups were shown to vary significantly with time, despite the fact that their liver disease remained unchanged. Measuring transaminases has merely a supportive role in the assessment and diagnosis of NAFLD, and cannot differentiate healthy subjects from others with SS or NASH.

In terms of the metabolic profile of the study cohort, IR (as calculated with HOMA-IR) was higher in patients with NASH compared to HC. This finding is in agreement with a recently published report revealing a strong association between the presence of diabetes and findings of NASH and/or hepatic fibrosis [146]. IR is thought to contribute to the pathogenesis of NAFLD; however, the cross-sectional nature of the current study can neither support nor reject a causal relationship between IR and liver disease state. There were no statistically significant differences between fasting levels of glucose, insulin, HbA1c, triglycerides or cholesterol between the three study groups, which may simply be the result of decreased power of a limited sample size.

In this study, 7-day food records were used to assess dietary intake. Study participants were asked to quantify food intake based on the 2D portion charts that are thought to decrease variation in reporting. All subjects were instructed on how to complete food records and the recorded data assessed for accuracy and completion when returned to the researchers. The rationale for using this tool, rather than a 24-hour diet recall or a Food Frequency Questionnaire (FFQ), was that the IM is sensitive to variations in dietary intake and such alterations have been noted to occur as early as
24 hours from the implementation of a new diet [147]. In addition, 7-day food records are the most reliable tool for the assessment of macro- and micronutrient intake [148, 149] and they are open-ended to accomodate any dietary combination that subjects want to report. They do not depend on memory, so there is a lower likelihood of introducing recall bias compared to that seen when patients complete a FFQ. Also, the results of 24-hour diet recalls can be affected by individual day-to-day variability in intake, as the physiologic response to overeating one day is to decrease intake the following day and vice-versa, a phenomenon that could under- or overestimate overall energy intake [149].

Using 7-day food records, no difference was found in the average caloric intake between the three study groups. In order to correct for potential differences in energy requirements due to factors such as age, gender, weight and height the average daily caloric intake was divided by BMR and that ratio again found to not be different between the groups. However, the percentage fat intake divided by BMR revealed that HC consumed more calories from fat compared to patients with SS and NASH. BMR was calculated with the Harris-Benedict equation, which is commonly used in clinical practice [150]. When compared to indirect calorimetry, or other equations used to determine BMR, it has been shown to be fairly accurate [150, 151]. Garrel et al. compared it to indirect calorimetry and found that it tends to overestimate BMR in lean patients; that difference however, decreases as the subjects’ weight increases [151]. In addition, Weijs et al. reported that the Harris-Benedict equation is an accurate tool for predicting the BMR of women with a wide BMI range (18.5-50 kg/m²) [150]. Considering the equally wide range of BMI of our cohort, it appears that the Harris-
Benedict equation is an appropriate tool for comparisons of BMR between the groups. Despite efforts to correct caloric intake with BMR for differences, such as age, gender, weight and height between the groups, there is always a possibility of other factors affecting dietary data, such as under-reporting of intake, which may affect the results of this study.

Under reporting of intake is possible despite the use of a validated tool such as 7-day food records. It has been shown that under-reporting of overall intake is in direct correlation with BMI [152]. In our study, all 3 groups of participants reported a total caloric intake that was lower than their respective EER. None of the subjects had been losing weight during the months preceding their enrolment and hence that could provide indirect evidence of under-reporting in all three groups, assuming that the EER calculations were accurate.

EER was calculated according to Health Canada guidelines using objective data such as gender, age, weight and height, as well as subjective, patient-reported data on physical activity. These equations were developed using doubly labeled water, which is the gold standard for assessment of total energy expenditure, and hence widely used in clinical practice [153]. Study participants were asked to keep a record of activities over a span of 7 days in an effort to have average daily activity levels estimated as accurately as possible. Considering the differences between reported daily caloric intake and calculated EER in the setting of stable body weight, apart from dietary under-reporting one should consider the possibility of physical activity over-reporting. One way to resolve this problem would be to ask patients to carry a pedometer or keep them in a research unit over a period of one week in order to monitor levels of physical activity.
The latter would not have been a true representation of their lives and, hence, could also introduce other errors to the activity estimates.
8.0 **STRENGTHS AND LIMITATIONS**

This was a novel study, addressing the most prevalent microbial communities in patients with NAFLD (and those previously linked to obesity) in an attempt to identify BMI-independent associations between the IM and the presence of NASH. Such study has not been reported in the literature before and provides a stepping-stone for further research in the important field of NAFLD. That is of significance considering the current prevalence of NAFLD and the potential social and financial magnitude of its associated comorbidities.

This study has various strengths and limitations. A strength was the availability of liver biopsies that allowed to correctly assign subjects in each group. The presence or absence of elevation in transaminases does not correlate with the type of NAFLD (SS or NASH) nor does it indicate severity of hepatic involvement (e.g. presence or absence of fibrosis), which is why histology is necessary to adequately characterize the degree of hepatic involvement in the setting of NAFLD but also in otherwise healthy adults. That was clearly shown in the case of 3 patients that were considered healthy but after undergoing a liver donation operation were found to have SS on histology.

Another strength was the use of 7-day food records and activity logs, which even though they may have not been completely accurate, allowed for correcting for these potential confounding effects when comparing the IM between study groups.

One limitation of this study was the fact that we were unable to reach the sample size calculated a priori as necessary to have adequate power to show differences
between groups. Also, the cross-sectional nature of this study does not allow for the understanding of the pathophysiology. Nevertheless, the findings do provide the foundation for further research in this field. In addition, with the use of PCR we were only able to assess certain groups of bacteria rather than the IM as a whole. Pyrosequencing offers a broader description of differences in the IM at a phylum level between HC and patients with NAFLD. However, even pyrosequencing still cannot explain the functional complexity of the IM, as subtle differences in the relative proportions of certain species or sub-species may confer significantly different metabolic and inflammatory phenotypes between patients [75]. Considering the potential effects of bacteria in the regulation of human metabolism and inflammation, more advanced techniques comparing the groups at a microbiome level would have offered even more significant information in this field.
9.0 **CONCLUSIONS**

This is the first study aimed at identifying differences in the IM of patients with NAFLD compared to HC. The results show a diet- and BMI-independent negative association between percentage Bacteroidetes and the presence of NASH. These bacteria appear to be correlated with IR. There are no diet- and BMI-independent differences for Bifidobacteria, *C. leptum, C. coccoides, E. coli*, total bacterial counts or Archaea between patients with NASH, SS and HC.

The results of this research project, in relation to the study aims, were as follows:

- This study did not reveal any differences in fecal Bifidobacteria counts between subjects with NASH and those with SS or HC.

- The ratio of Firmicutes (expressed in the current study as the sum of *C. coccoides* and *C. leptum*) to Bacteroidetes was not higher in patients with NASH compared to those with SS and HC.

- Fecal concentration of *C. coccoides* was higher in patients with NASH compared to SS. Total bacteria, *C. leptum, E. coli* and Archaea, as well as the ratio of the aforementioned bacteria to total bacteria were not different between the three study groups.
• There was a trend for increased fat consumption by HC compared to patients with NASH and SS, as well as a statistically significant difference in percentage fat intake to BMR ratio, between HC and patients with NASH or SS. There were no differences in carbohydrate intake between the groups.

Future studies should investigate the IM composition of patients with NAFLD further, as it may serve as a potential therapeutic target.
10.0 FUTURE DIRECTIONS

Considering the results presented in this thesis, the next step is to measure and compare endotoxin levels between the groups and correlate these with the percentage Bacteroidetes, as well as with degree of IR. This would provide indirect support of the hypothesis that it is Bacteroidetes-derived endotoxin that triggers IR and inflammation seen in patients with NASH. The IM findings and LPS levels could also be correlated with adipocytokines, such as leptin and adiponectin, as well as TNF-α levels to provide further associations between microbiota and hormonal and inflammatory changes.

As a second step, it would be of value to perform 16S rRNA analysis on the stored stool samples, as this will allow for a broader understanding of the IM differences between the groups, at the phylum level. Considering that metabolic pathways of microbes, such as fermentation of indigestible plant carbohydrates, is affected by the IM composition at a sub-class level, pyrosequencing still will not suffice. At that point the next step would be to perform metabolomic studies that would allow for a more detailed understanding of the microbiome and its potential contribution in the development and progression of conditions such as SS and NASH.

Similar to the study by Arumugan et al. it would also be interesting to investigate the enterotypes of patients with NAFLD and to also try to isolate functional microbial molecules (rather than bacteria at the phylum level alone) that group these patients together [49]. This approach would allow for identification of proteins that may be contributing to either the development or progression of NAFLD, and thus shed light onto the pathogenesis of this complex condition.
NAFLD also affects the pediatric population and hence it would be important to investigate the IM composition of young children as well, as the role certain bacteria may be different at various ages.

Once the IM composition of patients with SS and NASH is described on a larger scale, at the phylum but also the species level, it would be interesting to investigate various environmental factors that may alter the IM early on in life and as such, predispose to the development of the metabolic syndrome and NAFLD. Case-control studies addressing the role of antibiotic use in childhood, or the effects of gastrointestinal illnesses or surgeries in the first years of life for example, may reveal early environmental ‘hits’ that not only exert long-term effects on the IM composition but potentially also alter microbial gene expression that eventually leads to the development of obesity or other inflammatory conditions.

Apart from addressing the IM of patients with NAFLD, it would be of importance to also compare the fecal concentration of other microbial-derived nutrients, such as SCFA. Animal studies have linked SCFA and their intestinal receptors to body weight, adiposity, as well as inflammatory pathways. To date, it is not yet clear what the role of SCFA is, in children or adults with NAFLD.

In addition to descriptive studies in humans, a different type of research can be used to better understand the mechanisms linking the IM to the development of NAFLD. Conventionalization of germ-free mice with fecal material retrieved from patients with NAFLD will aid in identifying the exact contribution of microorganisms in host gene expression and metabolism, while controlling for factors, such as genetic background, as well as other host-derived effects, such as immunity. These types of experiments can
also provide more information on the direct effects of dietary factors on the IM composition and subsequently the development of NAFLD. For example, one can compare the metabolic effects of IM transplanted in germ-free mice after having been retrieved from patients with NAFLD, who have been consuming a high fat versus a high carbohydrate diet. Conventionalization of germ-free mice with human feces can also aid in identifying the role of microorganisms in gut barrier maintenance. Assessing the expression of tight-junction proteins before and after conventionalization of these mice is a potential way of addressing this question.

Once enough information is collected regarding the IM composition and the microbiome of patients with NAFLD, as well as their potential metabolic effects, it will become evident whether altering the IM would aid in the attenuation of inflammation and steatosis found in patients with NASH. Pre- or probiotics aimed at increasing bacteria that compete with the pathogenic microorganisms could also provide a means of addressing intestinal dysbiosis. Lastly, fecal transplantation may be proven to be advantageous in managing NAFLD and can be studied in well-designed randomized trials, if future evidence further implicates the IM with the development of NAFLD. Vrieze et al. have already attempted at treating the metabolic syndrome with such an intervention and the results appear promising [154]. It is important to note, however, that unless these interventions are repeated at frequent time intervals, other environmental factors, including the diet, could limit efficacy in the long-term.

Given the complex pathogenesis of NAFLD, it is unlikely that altering the IM alone will be sufficient to treat this condition. However, it could become an important adjunct in the management of affected patients, which among other measures could
always include lifestyle interventions, such as weight loss and increased physical activity, as well as medical treatments, such as vitamin E or pioglitazone [155].
11.0 APPENDIX

APPENDIX 1

Subject Instructions for Stool Collection

You will be provided by a Plastic Buff Bag with:
1- Collection Bowl
2- Re-sealable plastic bag
3- Storage Ziploc container
4- Two ice bags
5- Cooler bag

Please follow these instructions carefully.

Important: As soon as you are at home, please put the ice bags in the freezer.

Collection Bowl and Package of Plastic Bag
You will note that the collection bowl has no bottom. This is so you can insert the re-sealable plastic bag through it and fold the edge of the bag over top of the collection bowl. The collection bowl is then lowered into centre of the toilet insert.

Plastic Toilet Insert
-Insert the collection bowl with bag into the toilet insert.
-Raise the toilet seat and place insert directly on porcelain rim of bowl.
-Lower seat and sit as usual.

Collection of Stool samples:

-Collect feces in a plastic bag folded over the top of a no-bottom collection bowl placed into the toilet insert
-Close the bag tightly then put it in the storage Ziploc container
-Keep the sample at 4C (Freezer) until the date of liver biopsy.
-For sample transportation to the Toronto General Hospital, maintain the sample between the 2 frozen ice bags in the cooler bag during transportation to the Toronto General Hospital (the stool should be collected as early as possible to the liver biopsy date, ideally within 24 hours)
APPENDIX 2

Activity Log Form

Please complete this activity log sheet for a period of the 7 days before your clinic visit.

According to the table provided below, please let us know how many units of exercise you have performed in a day.

Activities required for one unit of exercise:

<table>
<thead>
<tr>
<th>Intensity</th>
<th>Time (min)</th>
<th>Type of Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>30</td>
<td>Slow walking, Traveling by bus, Shopping, Housecleaning</td>
</tr>
<tr>
<td>Moderate</td>
<td>20</td>
<td>Faster walking or walking down stairs, Cycling, Doing heavy laundry, Ballroom dancing (slow)</td>
</tr>
<tr>
<td>Strenuous</td>
<td>10</td>
<td>Slow running, Climbing stairs, Disco dancing for the elderly, Playing volley ball or table tennis</td>
</tr>
<tr>
<td>Very strenuous</td>
<td>5</td>
<td>Jumping rope, Playing basketball, Swimming</td>
</tr>
</tbody>
</table>

NAME: ____________________ DAY: ___________ DATE: _____________

Activity Log Sheet

<table>
<thead>
<tr>
<th>Type of Activity</th>
<th>Start Time</th>
<th>Stop Time</th>
<th>Intensity Level</th>
<th>Duration of Activity</th>
<th>Unit(s) of Exercise</th>
</tr>
</thead>
</table>
12.0 REFERENCES


