The Role of Bacterial Products and Intestinal Microbiota in Non-Alcoholic Fatty Liver Disease

by

Hannah Elizabeth Da Silva

A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Nutritional Sciences
University of Toronto

© Copyright by Hannah E Da Silva, 2015
The Role of Bacterial Products and Intestinal Microbiota in Non-Alcoholic Fatty Liver Disease

Hannah Elizabeth Da Silva
Master of Science
Department of Nutritional Sciences
University of Toronto
2015

Abstract

Non-alcoholic fatty liver disease (NAFLD) includes simple steatosis (SS) and non-alcoholic steatohepatitis (NASH), which can progress to cirrhosis. NAFLD pathogenesis is complex. Recent research suggests a role for the intestinal microbiota (IM) with various potential mechanisms involving bacterial metabolism and products. This cross-sectional study compared bacterial products and metabolites in the blood and feces, and fecal levels of total bacteria, Bacteroidetes, C. coccoides, C. leptum, Bifidobacteria, Lactobacilli, E. coli and Archaea in biopsy confirmed SS, NASH, and healthy controls (HC). NAFLD patients (SS+NASH) had higher levels of bacterial fecal metabolites, including choline, trimethylamine, total short-chain fatty acids, propionate, isobutyric acid, and higher serum 2-hydroxybutyrate than HC. NASH patients had lower proportions of fecal C. leptum and higher amounts of E. coli than HC. Several bacterial metabolites correlated with histological findings, and with specific bacteria and diet. These findings suggest a potential role for bacteria and bacterial metabolism in NAFLD pathogenesis.
Acknowledgments

I would like to thank the many individuals who have contributed to this study and assisted me during my graduate studies.

First, I must thank our study participants who were willing to sacrifice their time to be part of our study during what was often a very stressful period in their lives.

Thank you to Dr. Allard who has been a constant source of motivation, support, and inspiration. You are and will continue to be a great role model for my career.

Thank you as well to the members of my advisory committee: Dr. Elena Comelli, Dr. Thomas Wolever, and Dr. Eberhard Renner. Your expertise and support has been influential throughout this process. It was an honour to work with each of you.

A very special thank you is owed to Dr. Bianca Arendt who was involved in planning and conducting this research long before me. Without your help in patient recruitment, grant writing, presentation edits, and guidance in the lab, this thesis would not have been possible.

I am very grateful for the expertise and support of our wonderful statistician, Anastasia Teterina. Thank you for your assistance with everything from course work to abstract writing to my final analyses and your veterinary expertise. Our research group is very lucky to have you.

Thank you to my other officemates past and present, especially Katherine Schwenger and Sultan Alenezi who have been with me since day one. A special thanks goes to Dr. Paulina Pettinelli who assisted with stool homogenization.

Thank you to Dr. Amel Taibi who completed our DNA extractions and the qPCR analyses. I am also very grateful for your willingness to answer all of my other laboratory associated questions.

Thank you to our many collaborators. Thank you to Dr. Scott Fung and Dr. David Wong who assisted with the recruitment of NAFLD patients. Thank you to Dr. Ian McGilvray and the entire Toronto General Hospital Multi-Organ Transplant Program team who assisted with recruiting
healthy controls. I am very appreciative of the excellent work of Dr. Sandra Fischer who assessed all liver biopsies.

Thank you to my colleagues and manager, Karen Smith, at Sunnybrook Health Science Centre for continuing to keep me on as a Registered Dietitian while also giving me the time to complete my graduate studies in a timely manner.

Lastly, but most importantly, I would like to thank my friends and family who have been a source of encouragement and support and who have accepted my absence in the last several months with understanding. Thank you to my brilliant husband Michael who believes that I can do amazing things. Your patience and encouragement have not gone unnoticed.
Table of Contents

Acknowledgments........................................................................................................... iii

Table of Contents.............................................................................................................. v

List of Tables ................................................................................................................. x

List of Figures................................................................................................................. xi

List of Appendices ........................................................................................................... xiii

List of Abbreviations ....................................................................................................... xiv

1 Introduction .................................................................................................................... 1

2 Review of the Literature................................................................................................. 3

  2.1 An Introduction to Non-Alcoholic Fatty Liver Disease Pathogenesis.......................... 3
    2.1.1 Insulin Resistance ............................................................................................... 3
    2.1.2 Lipid Peroxidation and Lipotoxicity .................................................................... 3
    2.1.3 Mitochondrial and Endoplasmic Reticulum Stress .............................................. 4
    2.1.4 Genetic Factors .................................................................................................... 4
    2.1.5 Diet and Physical Activity .................................................................................... 5
      2.1.5.1 Carbohydrates .............................................................................................. 5
      2.1.5.2 Fat .................................................................................................................. 6
      2.1.5.3 Vitamins ........................................................................................................ 7

  2.2 Role of intestinal microbiota in non-alcoholic fatty liver disease .............................. 7
    2.2.1 Increase energy uptake ....................................................................................... 8
    2.2.2 Increase Chronic Inflammation ......................................................................... 10
      2.2.2.1 Toll-Like Receptor 4 and Inflammatory Cytokines ....................................... 10
      2.2.2.2 Endotoxin ..................................................................................................... 10
      2.2.2.3 Intestinal Permeability .................................................................................. 11
2.2.3  The Role of Products of Bacterial Metabolism in NAFLD .............................. 12
  2.2.3.1  Short-Chain Fatty Acids ........................................................................ 12
  2.2.3.1.1  Butyrate .................................................................................................... 12
  2.2.3.1.2  Propionate ............................................................................................. 13
  2.2.3.1.3  Acetate .................................................................................................... 14
  2.2.3.2  Ethanol ........................................................................................................ 14
  2.2.3.3  Choline metabolism .................................................................................. 14
  2.2.4  NAFLD and the Intestinal Microbiota: Clinical Studies .............................. 15
  2.2.5  Probiotic and Prebiotic Trials ...................................................................... 18
  2.2.6  Proposed Mechanism .................................................................................. 20

3  Research Aim and Hypotheses ........................................................................... 21

4  Materials and Methods ....................................................................................... 22
  4.1  Study Design .................................................................................................... 22
  4.2  Clinical Data, Environmental Questionnaire, and Anthropometric Measurements .. 23
  4.3  Nutrition and Activity Assessment .................................................................. 23
  4.4  Clinical Biochemistry ...................................................................................... 25
  4.5  Liver Histology ............................................................................................... 26
  4.6  Plasma Endotoxin ........................................................................................... 28
  4.7  Serum Metabolites ......................................................................................... 29
  4.8  Stool Sample Collection and Analysis ............................................................ 31
    4.8.1  Stool Homogenization ................................................................................. 31
    4.8.2  Fecal Metabolite Analysis ............................................................................ 32
    4.8.3  DNA Extraction .......................................................................................... 32
    4.8.4  Quantitative Real-time PCR ........................................................................ 32
4.9 Statistical Analysis........................................................................................................................................... 33
  4.9.1 Data Analysis ........................................................................................................................................... 33
  4.9.2 Power Calculations............................................................................................................................... 34

5 Results ................................................................................................................................................................. 35
  5.1 Subjects ....................................................................................................................................................... 35
  5.2 Dietary Intake and Physical Activity ......................................................................................................... 40
  5.3 Bacterial Products ....................................................................................................................................... 45
    5.3.1 Plasma Endotoxin ............................................................................................................................... 45
    5.3.2 Short Chain Fatty Acids and Other Organic Acids ............................................................................ 46
    5.3.3 Metabolites Related to the Bacterial Metabolism of Choline ......................................................... 49
    5.3.4 Ethanol .................................................................................................................................................. 52
  5.4 Intestinal Microbiota .................................................................................................................................... 52
  5.5 Relationships between Bacterial Metabolites, Bacteria of Interest, Disease Status, and Diet ......... 55

6 Discussion .......................................................................................................................................................... 66
  6.1 Endotoxin....................................................................................................................................................... 66
  6.2 SCFA and Related Metabolites .................................................................................................................. 68
  6.3 Metabolites Related to the Bacterial Metabolism of Choline ............................................................... 71
  6.4 Ethanol ......................................................................................................................................................... 73
  6.5 Intestinal Microbiota .................................................................................................................................... 73
  6.6 Diet and Physical Activity .......................................................................................................................... 75
  6.7 Potential Confounders ............................................................................................................................... 76

7 Strengths and Limitations ............................................................................................................................... 79

8 Conclusions ....................................................................................................................................................... 81

9 Future Directions ............................................................................................................................................. 82

References .............................................................................................................................................................. 84
Appendices........................................................................................................................................104
## List of Tables

**Table 1:** Summary of human studies on intestinal microbiota and non-alcoholic fatty liver disease  
17

**Table 2:** Bacteria of interest in non-alcoholic fatty liver disease  
19

**Table 3:** Physical activity coefficients for estimated energy expenditure calculation  
25

**Table 4:** Assessment of liver histology: Brunt System  
27

**Table 5:** Metabolites measured in stool and serum samples  
31

**Table 6:** Standard curves used in the interpretation of the qPCR results  
33

**Table 7:** Subject demographics and health history  
37

**Table 8:** Biochemistry by disease group  
38

**Table 9:** Liver histology by disease group  
39

**Table 10:** Reported dietary intake and estimated requirements: Macronutrients  
41

**Table 11:** Reported dietary intake and estimated requirements: Micronutrients  
43

**Table 12:** Fecal and serum SCFA and organic acids in healthy controls and NAFLD patients  
49

**Table 13:** Bacterial taxa of interest from qPCR by disease group  
53

**Table 14:** Correlation between fecal and blood metabolites and NAFLD related parameters  
57

**Table 15:** Correlation between intestinal microbiota by qPCR and NAFLD related  
60
parameters

**Table 16:** Correlation between intestinal microbiota and metabolites in all subjects  

**Table 17:** Correlations between metabolites and diet in all subjects  

**Table 18:** Correlations between intestinal microbiota by qPCR and diet in all subjects
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Reported proportion of estimated energy requirement consumed</td>
<td>42</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Reported proportion of basal metabolic rate consumed</td>
<td>42</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Reported total daily physical activity completed</td>
<td>44</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Reported daily minutes of strenuous and very strenuous physical activity</td>
<td>44</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Comparison of plasma endotoxin concentration between groups</td>
<td>45</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Comparison of fecal butyric acid between groups</td>
<td>47</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Comparison of fecal propionate between groups</td>
<td>47</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Comparison of fecal acetic acid between groups</td>
<td>48</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Comparison of serum acetic acid between groups</td>
<td>48</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Fecal choline in healthy controls and NAFLD patients</td>
<td>50</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Fecal trimethylamine in healthy controls and NAFLD patients</td>
<td>50</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Serum choline in healthy controls and NAFLD patients</td>
<td>51</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Serum trimethylamine N-oxide in healthy controls and NAFLD patients</td>
<td>51</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Serum ethanol in healthy controls and NAFLD patients</td>
<td>52</td>
</tr>
<tr>
<td>Figure 15</td>
<td><em>C. leptum</em> relative abundance in stool samples from three study groups</td>
<td>54</td>
</tr>
<tr>
<td>Figure 16</td>
<td><em>E. coli</em> count in stool samples from three study groups</td>
<td>55</td>
</tr>
<tr>
<td>Figure 17</td>
<td>Relationship between fecal formic acid and total inflammation in NAFLD</td>
<td>58</td>
</tr>
</tbody>
</table>
patients

**Figure 18:** Relationship between fecal choline and body mass index in all subjects 58

**Figure 19:** Relationship between fecal trimethylamine and body mass index in all subjects 59

**Figure 20:** Relationship between serum acetic acid and hepatocyte ballooning intensity in NAFLD patients 59

**Figure 21:** Relationship between serum choline and liver steatosis in NAFLD patients 60

**Figure 22:** Relationship between Firmicutes count and liver steatosis in NAFLD patients 61

**Figure 23:** Relationship between *C. leptum* count and fecal butyric acid in all subjects 61

**Figure 24:** Relationship between relative abundance of *C. leptum* and reported dietary fibre intake in all subjects 65

**Figure 25:** Relationship between plasma endotoxin and reported carbohydrate intake in all subjects 65
## List of Appendices

<table>
<thead>
<tr>
<th>Appendix</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appendix 1:</td>
<td>Consent Forms</td>
<td>104</td>
</tr>
<tr>
<td>Appendix 2:</td>
<td>Instructions for stool sample collection and transportation</td>
<td>111</td>
</tr>
<tr>
<td>Appendix 3:</td>
<td>Environmental Questionnaire</td>
<td>112</td>
</tr>
<tr>
<td>Appendix 4:</td>
<td>7-day Food and Activity Log</td>
<td>115</td>
</tr>
</tbody>
</table>
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP</td>
<td>Alkaline Phosphatase</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine Transaminase</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate Transaminase</td>
</tr>
<tr>
<td>BMR</td>
<td>Basal Metabolic Rate</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of Variation</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>EER</td>
<td>Estimated Energy Requirement</td>
</tr>
<tr>
<td>ES</td>
<td>Effect Size</td>
</tr>
<tr>
<td>FFA</td>
<td>Free Fatty Acid</td>
</tr>
<tr>
<td>FMO3</td>
<td>Flavin Monooxygenase 3</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like Peptide</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose Transporter Type 4</td>
</tr>
<tr>
<td>GPR43</td>
<td>G-protein Coupled Receptor 43</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Hemoglobin A1c</td>
</tr>
<tr>
<td>HC</td>
<td>Healthy Controls</td>
</tr>
<tr>
<td>HDL</td>
<td>High Density Lipoprotein</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>Homeostasis Model Assessment Estimated Insulin Resistance</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IM</td>
<td>Intestinal Microbiota</td>
</tr>
<tr>
<td>kcal</td>
<td>Kilocalorie</td>
</tr>
<tr>
<td>LAL</td>
<td>Limulus Amebocyte Lysate</td>
</tr>
<tr>
<td>LDL</td>
<td>Low Density Lipoprotein</td>
</tr>
<tr>
<td>MM</td>
<td>Master Mix</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>n-3</td>
<td>Omega-3</td>
</tr>
<tr>
<td>NAFLD</td>
<td>Non-Alcoholic Fatty Liver Disease</td>
</tr>
<tr>
<td>NAS</td>
<td>NAFLD Activity Score</td>
</tr>
<tr>
<td>NASH</td>
<td>Non-Alcoholic Steatohepatitis</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NSAID</td>
<td>Nonsteroidal Anti-inflammatory Drug</td>
</tr>
<tr>
<td>PA</td>
<td>Physical Activity Coefficient</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen Activator Inhibiter</td>
</tr>
<tr>
<td>PEMT</td>
<td>Phosphatidylethanolamine N-methyltransferase</td>
</tr>
<tr>
<td>PNLPA3</td>
<td>Patatin-like Phospholipase 3</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated Fatty Acid</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reactions</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SCFA</td>
<td>Short-Chain Fatty Acid</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SS</td>
<td>Simple Steatosis</td>
</tr>
<tr>
<td>TG</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>TLR-4</td>
<td>Toll-like Receptor 4</td>
</tr>
<tr>
<td>TMAO</td>
<td>Trimethylamine N-oxide</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor Alpha</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very Low Density Lipoprotein</td>
</tr>
</tbody>
</table>
1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the Western World [1]. NAFLD describes a spectrum of disease severity from simple steatosis (SS) to non-alcoholic steatohepatitis (NASH) in the absence of significant alcohol consumption (<20g/day) [2]. SS is defined by the accumulation of fat (triglycerides) within the hepatocytes without hepatocyte injury and is considered a benign condition [2]. NASH involves steatosis and inflammation as well as hepatocyte injury which presents as ballooning and may also include spotty necrosis and/or fibrosis [2]. The gold standard for the diagnosis of NASH is liver biopsy [2] where histology is assessed using the Brunt scale to classify SS versus NASH and to grade disease severity using the NAFLD Activity Score (NAS) [3].

The World Gastroenterology Organisation estimates the prevalence of NALFD in the Western World to be approximately 27-34% in the general population, upwards of 75% in those with diabetes or obesity, and 75-92% in the morbidly obese [1]. The prevalence of NASH is significantly lower, estimated at 2-3% in the general population [4], but upwards of 18.5-30% in obese individuals [4, 5], and 55% in diabetics [6]. Approximately 3-15% of individuals with NASH will advance to cirrhosis, while 38-45% of those with cirrhosis will advance to liver failure within 7-10 years [1]. NASH cirrhosis is the third most common indication for liver transplant in the United States and has increased as the primary indication for liver transplant from 1.2% to 9.7% of all transplants performed from 2001 to 2009 [7].

Currently there are limited treatment options for NAFLD. The American College of Gastroenterology guidelines (2012) recommends weight loss via diet and exercise or bariatric surgery. Vitamin E supplementation is also recommended in non-diabetics [2]. However, due to difficulties achieving adequate weight loss, eligibility criteria limiting the number of candidates for, and the risk and invasiveness of bariatric procedures [8], few viable options exist for treating NAFLD and preventing its progression.

A significant barrier in developing these treatments is that the pathogenesis of NAFLD is complex and it is still not completely understood why some remain with only SS while others progress to the more severe form, NASH, with risks of developing cirrhosis and liver failure [9]. Lately, there has been emerging literature on the role of the intestinal microbiota in the
pathogenesis of NAFLD [10-15]. The aim of this research project was to characterize the intestinal microbiota of patients with NASH or SS versus healthy controls (HC) and determine if there were any differences in bacterial products or metabolites that could play a role in the pathogenesis of this disease.
2 Review of the Literature

2.1 An Introduction to Non-Alcoholic Fatty Liver Disease Pathogenesis

2.1.1 Insulin Resistance

Individuals with NAFLD are universally insulin resistant [16] and this is considered to be a driving factor behind much of NAFLD pathogenesis. Traditionally, the first stage of NAFLD pathogenesis is the accumulation of lipids within the hepatocytes, called steatosis. This occurs when the supply of free fatty acids (FFA) to the liver exceeds the needs for mitochondrial oxidation, phospholipid synthesis, and cholesterol ester synthesis [17-19]. Insulin resistance is associated with lipolysis from adipose tissue leading to increased circulating FFA and uptake by the liver [20]. FFA may also be supplied to the liver through dietary intake and absorption as chylomicrons. FFA uptake by the liver is unregulated [21] and therefore thought to be responsible for about two thirds of the lipid accumulation in the liver [22]. Elevated insulin levels resulting from insulin resistance also inhibit mitochondrial β-oxidation [23] and increase de novo lipogenesis in the liver via glycolysis [24]. High insulin levels can also inhibit the hepatic release of fatty acids as very low density lipoproteins (VLDL) by decreasing the production of apolipoprotein B100 [24].

The majority of lipids in steatosis are stored as triglycerides [25] which are benign [26] and have recently even been considered as protective against the oxidative stress and cellular damage that is seen in the progression from SS to NASH [27, 28]. It is therefore the FFA accumulation which causes the deleterious effects of steatohepatitis.

2.1.2 Lipid Peroxidation and Lipotoxicity

An increased presence of FFA in the liver leads to lipid peroxidation causing the release of inflammatory cytokines such as tumor-necrosis factor alpha (TNF-α) and interleukin six (IL-6) and reactive oxygen species (ROS) [29-31]. The resulting inflammation and oxidative stress from the release of these molecules leads to disease progression from the benign SS to the cellular damage and inflammation seen in NASH [32, 33]. Oxidative stress has also been shown to impair the proliferation of hepatocyte progenitors leading to impaired tissue recovery, fibrosis, and cirrhosis [34, 35].
The presence of FFA in hepatocytes may also have a direct toxic effect [36]. FFA is thought to be responsible for the significantly higher presence of apoptosis in NASH patients compared to healthy controls [37-39]. The type of fatty acid also seems to have an effect, with saturated fatty acids causing more apoptosis [40] than monounsaturated [36] or polyunsaturated fatty acids [41].

2.1.3 Mitochondrial and Endoplasmic Reticulum Stress

Another important component of NAFLD pathogenesis is the stress that the influx of fatty acids causes to the cellular organelles, particularly the mitochondria and endoplasmic reticulum. Mitochondrial stress and resulting dysfunction further promotes cellular damage through TNF-α and over-production of ROS [30, 42]. An accumulation of FFA can also lead to endoplasmic reticulum stress [43] which triggers the unfolded protein response. The unfolded protein response is an adaptive response initiated by the endoplasmic reticulum when its main function, protein folding, is disrupted by a variety of causes including the accumulation of FFA, a lack of energy from glucose, or an insufficient presence of calcium [44]. This response may start an inflammatory cascade which has been associated with apoptosis and exacerbation of metabolic diseases including obesity and insulin resistance, contributing to NAFLD pathogenesis [45].

2.1.4 Genetic Factors

Research has shown that genetic factors may play some role in NAFLD development and progression. The most consistent finding has been with polymorphisms in patatin-like phospholipase three (PNLPA3) [46-48]. Polymorphisms in PNLPA3 have been associated with an increased percentage of steatosis in NAFLD [49]. The homozygous 148M polymorphism for PNLPA3 has also been associated with NAFLD severity, including inflammation and fibrosis [50-52]. The Genetics of Obesity-Related Liver Disease Consortium found four additional gene polymorphisms that were associated with NAFLD. These were found in or near the genes: lysophospholipase like 1, protein phosphatase 1 regulatory subunit 3b, neurocan, and glucokinase regulatory protein [53]. Peterson et al. additionally found that the polymorphisms C-482T and T-455C in the gene for apolipoprotein 3C were associated with NAFLD and insulin resistance [54]. The role of genetics in NAFLD pathogenesis is still emerging.
2.1.5 Diet and Physical Activity

As obesity is a main risk factor for the development of SS and NASH it follows that energy balance, the difference between energy intake through diet and energy expenditure through the basal metabolic rate, thermic effect of food, and physical activity, could play an important role in NAFLD pathogenesis. Observational studies have consistently found that individuals with NAFLD have significantly lower fitness level and/or participation rates in physical activity [55-60]. The association between NAFLD and excessive energy intake, however, has only been shown in a few studies [61-63]. This relatively weak association in the literature may be due to underreporting by study participants, which is consistently encountered in studies using food frequency questionnaires and self-reported diet records, especially among obese individuals [64, 65]. This led to a more significant focus on the composition of the food consumed rather than the caloric content. A study from our research group, for instance, found that energy intake and macronutrient composition did not differ between healthy controls and biopsy confirmed SS and NASH patients, but that NASH patients were significantly more likely to have inadequate consumptions of micronutrients such as vitamin B6, folate, and zinc [60]. It is difficult at this point to think of a role for these micronutrients in the pathogenesis of NASH as other reports on low micronutrient intakes were primarily focused on antioxidants [66-68]. There is also additional literature on macronutrients, particularly fat and carbohydrates. Further details regarding dietary composition are discussed below.

2.1.5.1 Carbohydrates

Two observational studies have found an association between the percent of energy consumed from carbohydrates and the risk of advanced NAFLD [69, 70]. Solga et al. investigated dietary intake using a 24 hour recall in obese patients undergoing bariatric surgery. Liver biopsies taken at the time of surgery were evaluated for markers of inflammation and fibrosis. Higher percent of energy from carbohydrates was associated with higher odds of having inflammation on liver histology [69]. A similar study using a non-validated dietary questionnaire found that higher percent of energy from carbohydrates was associated with a greater risk of fibrosis [70].

Carbohydrate quality also appears to be important. The consumption of sugar sweetened beverages [71], soft drinks [57, 72], and fructose [73, 74] have been associated with a higher risk of NAFLD. Fructose has been of particular interest in NAFLD pathogenesis, upregulating de
**novo** lipogenesis, promoting lipogenesis, inhibiting β-oxidation, and contributing to the inflammatory cascade [75]. Fructose may also promote hepatic insulin resistance, further contributing to NAFLD development and progression [76, 77].

Diets low in beneficial carbohydrates such as fibre have also been observed in NAFLD patients [62, 78]. There are two main mechanisms through which fibre intake can influence NAFLD pathogenesis. Some fibres, such as cellulose and pectins can be fermented by the intestinal microbiota (IM) to produce short-chain fatty acids (SCFA) [79] which can exert many potentially beneficial roles in preventing NAFLD. There is more discussion on this in section 2.2.3.1 as this is relevant to this project. Fibre can also aid in increasing satiety [80-82] and improving glycemic control [83], including insulin sensitivity in NAFLD patients [84].

### 2.1.5.2 Fat

Several observational studies have found a higher dietary fat intake in individuals with NAFLD [62, 85-87] and NASH [88, 89] compared to healthy controls. Of these, saturated fats may increase hepatic inflammation, fibrosis and apoptosis [90, 91]. Several studies have found that there was a greater consumption of saturated fats in NAFLD patients [62, 73, 78, 85]. On the other hand, mono- and polyunsaturated fatty acids (PUFA), particularly omega-3 fatty acids, may be of benefit because of their anti-inflammatory effect [92]. Inadequate intake of these fatty acids in NAFLD patients was reported in several studies [62, 73, 78, 93], particularly for omega-3 (n-3) fatty acids [73, 89, 94].

As discussed in section 2.1.2, dietary fat is a main source of FFA in the liver and those with several double-bonds, like PUFA, are susceptible to lipid peroxidation, triggering oxidative stress and inflammation [29-31]. PUFA are fatty acids with multiple double bonds which can be classified at n-3, n-6, or n-9 depending on the placement of the first double bond. PUFA are particularly susceptible to lipid peroxidation due to their many double bonds. However, elongation of n-3 causes the production of anti-inflammatory eicosanoids but elongation of n-6 results in the production of pro-inflammatory prostaglandins and leukotrienes [92]. Therefore, the ratio of n-3:n-6 fatty acids can be important when considering disease progression through hepatic inflammation. Several studies found differences in fatty composition with reduction in n-3 or n-3:n-6 ratio in NASH [95-98]. Of additional note are findings that low levels of hepatic
PUFA, especially n-3 PUFA, are associated with a predisposition to lipogenesis over β-oxidation and export as VLDL [95, 99, 100].

2.1.5.3 Vitamins

Due to the driving force of oxidative stress in NAFLD pathogenesis it follows that diets deficient in antioxidant nutrients, including vitamin C and E, could promote NAFLD development and progression. Cross-sectional studies have found that dietary vitamin C [62, 78] and vitamin E [78] intake are lower in NAFLD patients compared to healthy controls. Vos et al. found that patients with lower vitamin E consumption had a higher grade of steatosis [66]. Plasma vitamin C and E levels have also been associated with disease status. Cankutaran et al. found that NAFLD patients with vitamin E deficiency had worse steatosis [67] while another study found that plasma vitamin E and C were lower in patients with NASH than with SS [68]. Given these findings and positive results from clinical trials [101] the current treatment guidelines suggest treating all non-diabetic, biopsy-proven NASH patients with 800 IU of vitamin E daily [2]. Choline is another nutrient found to play a role in NAFLD pathogenesis. It is discussed in the context of intestinal microbiota in section 2.2.3.3.

Therefore, there are several factors playing a role in the pathogenesis of NAFLD and lack of a healthy diet and sedentary lifestyle are important contributors. However, there is emerging literature suggesting that the intestinal microbiota may also play a role, possibly influenced by environmental factors such as diet and lifestyle. A role for intestinal microbiota is supported by a recent study from our group [13], showing a lower percentage of Bacteroidetes in NASH patients compared to healthy controls, independent of diet and body mass index. Therefore the present project was developed to further assess this relationship and determine if certain bacterial products or metabolites were associated with NAFLD.

2.2 Role of intestinal microbiota in non-alcoholic fatty liver disease

The human intestinal microbiota (IM) is composed of all microorganisms present in the gut. The IM is estimated to contain more than $10^{13}$ microorganisms [102] with between 15,000 and 35,000 species of bacteria [103]. The dominant phyla, Bacteroidetes and Firmicutes, make up approximately 90% of the bacteria in the human digestive system [104-107]. Until recently, very
little was known about the human IM composition because most IM bacteria are obligate anaerobes making them difficult to culture. Recent technological advances now allow researchers to identify IM species and groups and their relative abundance through the sequencing of the 16s rRNA gene in bacterial DNA [108]. Since the advent of this technology, researchers have discovered the many roles that the IM plays in human health and disease, including its potential role in NAFLD pathogenesis.

### 2.2.1 Increase energy uptake

One of the primary mechanisms through which the IM is thought to influence NAFLD development is by increasing energy absorption from the intestinal tract. In 2004, Bäckhed et al. colonized germ-free mice with the IM of conventionally raised mice [109]. Within 14 days, the colonized mice had a 60% increase in body fat and development of insulin resistance despite lower oral intake [109]. The proposed mechanism for this adiposity was an increased absorption of monosaccharides resulting from alterations in the IM which then lead to an increase in *de novo* lipogenesis [109]. The increased absorption was proposed to be due to an increase in the vascularization of the small intestinal villus epithelium [110]. An alternative mechanism was suggested by Turnbaugh et al. when metagenomic analyses from the microbiota of *ob/ob* and lean mice found that the *ob/ob* microbiome was enriched with genes coding for proteins involved in the breakdown of “indigestible” polysaccharides and import of fermentation products. The feces of these *ob/ob* mice also showed lower caloric content when measured by bomb calorimetry in comparison to lean mice [111]. A subsequent study from Bäckhed et al. found that germ-free mice were protected from diet induced obesity, gaining less weight and maintaining lower blood insulin, glucose, and FFA levels than conventionally raised mice when consuming a high-fat, high-sugar Western diet [112].

This relationship has also been studied by treating obese mice with antibiotics when exposed to an obesogenic (high fat, high sugar) diet. Changes in IM related to antibiotic treatment resulted in decreased body weight, fat mass, and glucose intolerance compared to controls [113] and a significant decrease in hepatic lipids after steatosis development on a fructose rich diet [114]. Le Roy et al. ran an interesting study that determined that NAFLD risk may be related to the IM, independent of obesity. Mice were fed a high fat diet leading to weight gain. Even though all mice gained weight, some mice also developed hyperglycemia and increased circulating
inflammatory cytokines [115]. These mice were called “responders”. Other mice remained normoglycemic with low cytokine levels and were called “nonresponders”. A donor mouse was selected from each group and IM was transplanted into recipient mice who were also placed on a high fat diet. All recipient mice became obese. Mice who received IM from a “responder” developed hepatic steatosis and insulin resistance with higher HOMA-IR, blood glucose, and insulin, and increased expression of genes involved in de novo lipogenesis than mice who received IM from the “non-responder”. Responder and non-responder recipients had distinct IM, suggesting that a certain IM composition lead to increased risk of developing NAFLD in response to high dietary fat intake [115]. Therefore, animal studies support the role of IM in obesity. Since obesity is associated with NAFLD, it is possible that IM will also contribute to NAFLD pathogenesis.

Human studies on obesity have also reported differences in the bacterial communities of lean versus obese adults [116-121] which are similar to animal studies. The first landmark study that suggested an association between obesity and the IM was published by Ley et al. in 2006 [116]. This simple study measured the IM of 12 obese patients for one year as they undertook a calorie-restricted diet that was either low-fat or low-carbohydrate. Before starting the diet obese patients had fewer Bacteroidetes and proportionally more Firmicutes. Through the course of their weight loss, Bacteroidetes increased and Firmicutes decreased with a significant positive correlation between proportion of Bacteroidetes and percentage of body weight lost. This was the start of a long debate on whether obesity in humans is associated with a lower abundance of Bacteroidetes and a proportionally increased abundance of Firmicutes. Most studies agree that there is a relationship [116, 117, 120, 121].

In studies that have also investigated bacterial function via metagenomics this difference in bacterial community was accompanied by an increase in genes associated with macronutrient metabolism [118, 119, 121]. The first study of this kind looked at the IM and microbiome (bacterial genes) of monozygotic and dizygotic twins and their mothers [119]. Obesity was associated with a lower overall bacterial diversity. At the metagenomics level they found that 383 genes were significantly different between obese and lean subjects and that 75% of the obesity enriched genes were from Actinobacteria (versus 0% of the lean enriched genes) and 42% of the lean enriched genes were from Bacteroidetes (versus 0% of the obese enriched genes). Functionally, these genes were associated with carbohydrate, lipid, and amino acid
metabolism [119]. This, again, suggests a role of bacterial energy harvest in obesity. Jumpertz et al. found that a 20% increase in Firmicutes was associated with a 150 kilocalorie (kcal) increase in energy harvest [121], a value that, if it occurred daily, would amount to about 1.3lb weight gain in one month or a nearly 16lb weight gain over the course of a year. It is therefore clear that alterations in the IM can be associated with increased energy absorption, leading to increased adiposity, which could in turn result in a greater risk of NAFLD.

### 2.2.2 Increase Chronic Inflammation

IM can also contribute to chronic systemic inflammation through several mechanisms. Considering that chronic systemic inflammation plays a role in insulin resistance and obesity [122], this certainly has relevance to NAFLD.

#### 2.2.2.1 Toll-Like Receptor 4 and Inflammatory Cytokines

There are several mechanisms leading to the increased production of inflammatory cytokines in NAFLD. One of them is the accumulation of hepatic triglycerides and particularly FFA promoting inflammation through the activation of toll-like receptor 4 (TLR-4). This mechanism is supported by animal studies. Cai et al. used a transgenic mouse model to show that hepatic triglyceride accumulation leads to the activation of NF-kB by TLR-4 which causes the release of inflammatory cytokines such as IL-6 and TNF-α [123]. TLR-4 activation has also been associated with insulin resistance [124], the activation of Kupffer cells (involved in fibrogenesis), and worsening liver histology [125]. The IM can also play a role in this as another mechanism activating TLR-4 is through exposure to lipopolysaccharide or endotoxin, which is a constituent of the outer membrane of Gram negative bacteria, which can cross the intestinal epithelium [126]. Several studies support a role for endotoxin, which is discussed in the next section.

#### 2.2.2.2 Endotoxin

Increased endotoxin levels have been associated with NAFLD pathogenesis in both animal [124, 127, 128] and human studies [129-132]. Cani et al. found that after four weeks on a high-fat diet mice developed a chronic increase in plasma endotoxin with an increased proportion of endotoxin producing IM [127]. To further examine the effect of endotoxemia on mice the investigators provided a constant infusion of endotoxin which resulted in increased fasting
glucose, insulin, and weight, including increased liver size and adipose tissue [127]. Brun et al. found that obese, leptin deficient mice had higher levels of endotoxin leading to inflammatory and fibrogenic hepatic responses [128]. Similar findings were published by Song et al. from mouse and tissue culture experiments [124] and Spruss et al. in studies on mice fed a fructose rich diet [133].

The first study that examined the role of endotoxin in NAFLD pathogenesis in humans was by Thuy et al. [129]. This cross-sectional study found that NAFLD patients had elevated hepatic expression of plasminogen activator inhibitor (PAI-1), which is used as a marker for hepatic damage, compared to healthy controls. PAI-1 was associated with an increased endotoxin concentration and hepatic expression of TLR-4. Harte et al. found that NAFLD patients had a significantly higher plasma endotoxin level than healthy controls and that endotoxin concentration was positively correlated with insulin resistance [130]. Another study measured IgG levels against endotoxin as a marker of chronic low level endotoxin exposure over time in severely obese NASH patients and severely obese healthy controls [131]. They found that despite both groups being obese, there was a significantly higher IgG level in NASH patients, which increased with NASH grade (severity) [131]. Volynets et al. similarly found that NAFLD patients had higher endotoxin and PAI-1, and additionally higher blood ethanol levels, and increased intestinal permeability than healthy controls [132].

2.2.2.3 Intestinal Permeability

Endotoxemia is present when there is an increase in intestinal permeability and this may also be influenced by IM. The finding of increased intestinal permeability is further supported by Miele et al. who found that NAFLD patients had higher gut permeability and prevalence of small intestinal bacterial overgrowth, and lower expression of tight junction protein ZO-1 than healthy controls, however, the barrier deficiency was not as severe as in untreated celiac disease patients [134]. A recent study by Jiang et al. found significant differences in the IM community between NAFLD patients and healthy controls. They also saw increased tight junction gaps, decreased numbers of vital immune factors (CD4+ and CD8+ T lymphocytes), and increased expression of inflammatory markers (TNF-α, IL-6) in the duodenal mucosa of NAFLD patients compared to healthy controls [15]. These studies suggest that NAFLD patients have increased blood endotoxin levels from an increase in endotoxin producing gram negative bacteria and/or
increased intestinal permeability which may lead to hepatic inflammation through the activation of TLR-4. However, these studies do not differentiate between patients with the generally benign SS and the more detrimental NASH. In addition, other bacterial products or metabolites may also play a role.

### 2.2.3 The Role of Products of Bacterial Metabolism in NAFLD

#### 2.2.3.1 Short-Chain Fatty Acids

One potential cause of the increased intestinal permeability observed in NAFLD patients is a decrease in the colonic production of butyrate. About 10-30% of consumed energy reaches the colon without being absorbed [135, 136]. These substrates, primarily starches and components of the plant cell-wall including cellulose, pectins, xylans, arabinogalactans, gums and mucilage, are fermented by the IM to form SCFA [79] and gases including CO$_2$, CH$_4$, and H$_2$ [137]. The main SCFA produced in the human colon are acetate, propionate, and butyrate in a molar ratio of about 60:20:20 which remains in the same proportions in the feces [138-140]. SCFA are a source of caloric intake and account for up to 10% of required daily energy intake in humans [135]. SCFA can also have other significant functions in NAFLD pathogenesis pathways. Lin et al. conducted a study on mice to look at the effects of SCFA on obesity and related measures [141]. They found that the administration of any of the three main SCFA protected against the development of obesity and insulin resistance on a high fat diet, but that only butyrate and propionate regulated food intake through the stimulation of gut hormones [141]. SCFA have also been shown to upregulate fatty acid oxidation [142]. Other important functions of butyrate, propionate, and acetate are discussed below.

#### 2.2.3.1.1 Butyrate

Butyrate’s main functional role is as an energy source for colonic mucosal cells, however it also strengthens this important immunological barrier through involvement in cell proliferation and differentiation [137, 143, 144]. Butyrate decreases intestinal permeability through effects on tight junction proteins [145-147] and may have anti-carcinogenic effects in the colon [148, 149]. Butyrate has also been demonstrated to have a significant anti-inflammatory role through the inhibition of NF-κB [150, 151] and may exhibit beneficial antioxidant functions, reducing the production of reactive oxygen species [152]. All of these functions could prove useful in preventing the progression of SS to NASH.
Butyrate producers are strict anaerobes and more than 90% belong to the *Eubacterium* or *Roseburia* genus from clostridium cluster XIVa or are *Faecalibacterium prausnitzii* from clostridium cluster IV [153]. A study by Wong et al. found that patients with NASH had a lower abundance of *Faecalibacterium* and therefore perhaps produced less butyrate, however, unfortunately SCFA were not measured in this study [14]. A probiotic supplementation study in rats on a NAFLD inducing choline deficient diet found that providing a probiotic that includes butyrate producing bacteria (*Clostridium butyricum*, MIYAIRI 588) led to lower blood endotoxin, insulin resistance, hepatic markers of inflammation, and hepatic lipid deposition [154]. The treated rats also had increased expression of tight junction proteins and decreased hepatic fibrosis and hepatocarcinogenesis [154]. A similar study in a mice model found that supplementation with the VSL#3 probiotic led to alterations in the IM, reduction in insulin resistance, weight, inflammatory markers (TNF-α, IL-6), FFA, and steatosis [155]. These beneficial effects were associated with increased levels of butyrate in the feces and plasma [155]. These findings suggest that butyrate and butyrate producing bacteria may have a beneficial effect in NAFLD by reducing intestinal permeability and translocation of bacterial products, thereby reducing chronic inflammation and insulin resistance. However, no human studies have assessed both butyrate and IM in stools.

### 2.2.3.1.2 Propionate

Propionate is primarily produced by colonic fermentation by *Bacteroides*, *Prevotella*, and *Clostridium* [156]. Thirty to fifty percent of propionate is taken up by the liver and the rest is taken up by the peripheral tissues [157, 158]. Propionate inhibits lipolysis in adipocytes, reducing plasma FFA, through the activation of free fatty acid receptor 2 [159, 160] and inhibits the production of new fatty acids in the liver [161, 162]. Propionate also has several anti-inflammatory properties, reducing the production of pro-inflammatory eicosanoids [163] and resistin, a pro-inflammatory cytokine [164]. Propionate has also been shown to inhibit the release of TNF-α by neutrophils in response to bacterial endotoxin, a proposed significant contributor to the inflammation seen in NASH [165]. As briefly mentioned above, propionate may also play a role in increasing satiety and has been shown to promote the release of leptin [159, 164, 166] and improve glucose uptake by peripheral tissues though increasing glucose transporter type four (GLUT4) [167]. Therefore, propionate may have beneficial effects in NAFLD.
2.2.3.1.3 Acetate

Acetate is produced by many bacteria, particularly Bacteroidetes and Bifidobacterium [156, 168]. It is primarily used as an energy source in the liver and for the production of cholesterol [158]. Acetate may also have an anti-inflammatory function through interaction with G-protein coupled receptor 43 (GPR43) [169]. Lastly, a study by Fukuda et al. suggested that the main source of Bifidobacterium’s prevention against intestinal infection by pathogens (E. coli) was its production of acetate [170]. The various functions of butyrate, propionate, and acetate are closely linked with various steps in NAFLD pathogenesis and warrant further investigation in NAFLD populations. Human studies in this area are lacking.

2.2.3.2 Ethanol

One of the bacterial products of particular interest to NAFLD pathogenesis is ethanol. Ethanol is formed during the fermentation of carbohydrates by the IM and absorbed into the portal vein for delivery to the liver where it is metabolized [171, 172]. Ethanol is a known hepatotoxic agent which has also been associated with increasing intestinal permeability [173]. Two studies have looked at blood ethanol levels in NAFLD patients. Volynets et al. found that NAFLD patients had a significantly higher blood ethanol concentration than healthy controls. Unfortunately this could not be tied back to the microbiota as IM characterization was not performed [132]. Zhu et al., however, measured both blood ethanol and IM composition in 22 children with NASH compared to 25 obese children and 16 healthy controls [11]. They found that blood ethanol concentrations were higher in NASH compared to healthy controls and obese subjects who did not differ significantly. When examining IM composition, NASH patients were found to have a higher abundance of Proteobacteria than the other groups, particularly Enterobacteriaceae which include Escherichia which are ethanol producers [11]. The role of bacterial ethanol production in NAFLD development warrants further investigation.

2.2.3.3 Choline metabolism

Several studies have used choline deficient diets to induce NAFLD in test subjects [10, 125, 154]. Choline deficiency is thought to induce NAFLD through the inhibition of VLDL formation and consequent accumulation of triglycerides within the liver [174], through mitochondrial dysfunction causing increased levels of reactive oxygen species [175-177] and through endoplasmic reticulum stress [178]. Choline is converted to trimethylamines by the IM
This could lead to reduced choline absorption from the colon despite adequate dietary intake. The resulting choline deficiency may promote NAFLD development via the mechanisms described above.

In a well-controlled inpatient study by Spencer et al. 15 women were placed on a choline deficient diet and were monitored for steatosis via magnetic resonance imaging (MRI) [10]. IM composition at baseline was associated with the development of steatosis during choline deficient diet. Specifically, Gammaproteobacteria abundance at baseline was negatively correlated with steatosis and Erysipelotrichi abundance at baseline was positively associated with steatosis development on MRI. This study also found that phosphatidylethanolamine N-methyltransferase (PEMT) genotype was an effective predictor of steatosis accumulation. The mechanism behind the relationship between choline, PEMT, and NAFLD pathogenesis is quite clear. The active form of choline, phosphatidylcholine, which is needed in the liver for VLDL formation, is lower in NAFLD patients [180]. The formation of this active form is catalyzed by PEMT; a polymorphism in PEMT has also been associated with NAFLD [181]. The role of the IM metabolism of choline in this process, however, remains unclear.

### 2.2.4 NAFLD and the Intestinal Microbiota: Clinical Studies

There have been six human studies that have identified significant differences between the IM in NAFLD patients and healthy controls. Table 1 summarizes the design and results of these studies. The first study, discussed above in section 2.2.3.3, found that a particular IM composition put participants at a greater risk of developing steatosis while on a choline deficient diet [10]. This study did not, however, look at liver histology and therefore no conclusion can be made regarding differences between NASH and SS. Additionally, this study was conducted using healthy women where steatosis was artificially induced therefore results cannot necessarily be extrapolated to the NAFLD population. A cross-sectional study from our research group investigated the intestinal microbiota of biopsy confirmed SS and NASH patients compared to healthy controls [13]. We found that patients with NASH had significantly lower fecal Bacteroidetes compared to both SS and healthy controls even when controlling for BMI and dietary intake [13]. This study, however, was conducted using quantitative polymerase chain reactions (qPCR) rather than pyrosequencing, therefore only selected bacterial groups were investigated and measurements of bacterial products were not conducted [13]. Raman et al.
compared the IM community and fecal volatile organic compounds (VOC) of obese patients with clinically suspected NAFLD diagnosis via ultrasound and liver enzymes with lean healthy controls recruited from a colon cancer screening program [12]. They found that patients with NAFLD had a significantly different IM community than lean controls, including a higher abundance of Lactobacilli and certain Firmicutes of the Lachnospiraceae family including *Dorea, Robinsoniella, and Roseburia* genus. NAFLD patients had a lower relative abundance of *Oscillibacter* and higher levels of VOC, which were primarily fatty acid esters [12]. Unfortunately, as liver biopsies were not conducted differences between SS and NASH could not be evaluated. In addition, dietary intake was not controlled prior to sample collection and not accounted for in data analysis.

A pediatric study by Zhu et al., discussed in section 2.2.3.2, investigated the IM of children with biopsy confirmed NASH compared to obese and lean controls with normal liver enzymes [11]. Obese and NASH patients had increased Bacteroidetes and decreased Firmicutes which is contradictory to the majority of findings between obese and lean adults. Additionally, NASH patients had elevated blood ethanol levels and a higher proportion of ethanol producing Enterobacteriaceae compared to obese and lean controls [11]. Due to minimal diagnostic testing, however, we cannot confirm the liver status of obese controls who may have had SS. Wong et al. ran a small proof-of-concept trial of the probiotic and prebiotic formula, Lepicol, in adults with NASH [14]. At baseline NASH patients had a lower abundance of *Faecalibacterium* and *Anaerospirobacter* and increased *Parabacteroides* and *Allisonella* when compared to healthy controls. Probiotic supplementation led to improvements in steatosis which were associated with a decrease in the abundance of Firmicutes and an increase in Bacteroidetes [14]. Further probiotic and prebiotic trials are discussed in section 2.2.5. Finally, a recent study discussed in section 2.2.2.3, found that NAFLD patients (diagnosed by biopsy or imaging) had an increased abundance of *Escherichia, Anaerobacter, Lactobacilli, and Streptococcus* compared to healthy controls which was associated with impaired gut associated immunity and increased inflammation and permeability of the duodenal mucosa [15]. NASH and SS patients, however, were not distinguished. The body of literature on the role of the IM in NAFLD pathogenesis remains limited. Most studies did not use liver biopsies to differentiate NASH from SS which is important as NASH may progress to cirrhosis and hepatic failure while SS has a benign course. Additionally, the role of bacterial products in NAFLD has not been well described.
Table 1: Summary of human studies on intestinal microbiota and non-alcoholic fatty liver disease

<table>
<thead>
<tr>
<th>Reference</th>
<th>Study Design</th>
<th>Study Population</th>
<th>Liver Status Methodology</th>
<th>IM Methodology</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spencer et al. (2011)</td>
<td>Inpatient Intervention</td>
<td>Adult Women n=15</td>
<td>Magnetic Resonance Imaging</td>
<td>454-pyrosequencing</td>
<td>Women who started with a lower abundance of Gammaproteobacteria or a higher abundance of Erysipelotrichi were more vulnerable to the development of fatty liver during choline depletion</td>
</tr>
<tr>
<td>Mouzaki et al. (2013)</td>
<td>Cross-Sectional</td>
<td>Adults n=11 SS n=22 NASH n=17 Healthy Controls</td>
<td>Liver Biopsy for all groups</td>
<td>qPCR</td>
<td>NASH patients had lower fecal Bacteroidetes compared to both patients with SS and controls. Differences remained regardless of BMI and fat intake.</td>
</tr>
<tr>
<td>Raman et al. (2013)</td>
<td>Observational Case-Control</td>
<td>Adults n=30 Lean Controls n=30 NAFLD</td>
<td>Ultrasound and elevated liver enzymes (&gt;1.5 normal limit) for NAFLD Normal liver enzymes for six months prior to study for controls</td>
<td>Multitag pyrosequencing</td>
<td>NAFLD patients had a higher abundance of Lactobacilli and certain Firmicutes of the Lachnospiraceae family (Dorea, Robisoniella, Roseburia). NAFLD patients had a lower Oscillibacter and higher levels of fecal VOC, especially fatty acid esters.</td>
</tr>
<tr>
<td>Zhu et al. (2013)</td>
<td>Cross-Sectional</td>
<td>Children n=25 Obese n=22 NASH n=16 Lean Controls</td>
<td>Liver Biopsy for NASH Obese and lean controls had normal liver function tests</td>
<td>454-pyrosequencing</td>
<td>Obese and NASH patients had increased Bacteroidetes and decreased Firmicutes. Ethanol was higher in NASH vs Obese and Healthy Controls. Proteobacteria was higher in NASH versus Obese and Healthy Controls, primarily Enterobacteriaceae and Escherichia (an ethanol producer)</td>
</tr>
<tr>
<td>Wong et al. (2013)</td>
<td>Randomized Controlled Trial</td>
<td>Adults n=16 NASH (9 usual care, 7 probiotic) n=22 Healthy Controls</td>
<td>Liver Biopsy for NASH Normal liver function tests for healthy controls</td>
<td>454-pyrosequencing</td>
<td>NASH patients had lower Facecalibacterium and Anaerosporobacter and increased Parabacteroides and Allisonella abundance</td>
</tr>
<tr>
<td>Jiang et al. (2015)</td>
<td>Cross-Sectional</td>
<td>Adults n=53 NAFLD n=32 Healthy Controls</td>
<td>Liver Biopsy or imaging for NAFLD No diagnosis of liver disease for healthy controls</td>
<td>Illumina next-generation sequencing</td>
<td>NAFLD patients had higher Escherichia, Anaerobacter, Lactobacilli, Streptococcus, lower CD4+ and CD8+ T-lymphocytes and higher TNF-α, IL-6 in mucosa, microvilli abnormalities, and wider tight jxn gaps</td>
</tr>
</tbody>
</table>
2.2.5 Probiotic and Prebiotic Trials

Given the potential relationship between the IM and NAFLD pathogenesis, several studies have attempted to prevent or improve NAFLD with the provision of pre- or probiotic supplements. In animal studies, probiotic treatment led to improved steatosis, insulin resistance, and inflammation in mice on a high fat diet [182] and in rats on a choline deficient diet [154]. Probiotic supplementation in rats led to decreased hepatic expression of genes involved in lipogenesis and increased excretion of cholesterol transporters, reducing steatosis while on a high fat diet [183]. VSL#3, a probiotic used in many trials, reversed insulin resistance, inflammation, and NASH caused by increased intestinal permeability in mice [184]. Yadev et al. recently explored the function of VSL#3 in mice, showing that it prevented and treated genetic and diet induced obesity, reducing weight (fat mass), insulin resistance, steatosis, and inflammatory markers [155]. The beneficial effects of VSL#3 were associated with increased fecal and plasma butyrate which triggered increased expression of free fatty acid receptor 3, upregulating the release of glucagon-like peptide 1 (GLP-1) which is involved in appetite regulation, and therefore decreasing food intake [155]. VSL#3 also significantly altered the IM, decreasing Firmicutes and increasing Bacteriodetes and Bifidobacteria. Similarly, supplementary oligofructose (a prebiotic) in obese (fa//fa) rats led to slower weight gain and lower hepatic triglyceride concentrations when compared to controls [185].

Results have been equally promising in human studies; however, many studies are not able to sufficiently explain the mechanism behind the benefits of probiotic supplementation. Two studies have tested VSL#3 supplementation. Alisi et al. provided children with biopsy proven NAFLD either VSL#3 or a placebo for 4 months [186]. Children who received the probiotic had a significantly decreased probability of having severe NAFLD after four months than children receiving the placebo; 21% had no NAFLD after 4 months. VSL#3 supplementation was also associated with a higher plasma GLP-1 level and lower body mass index [186]. Loguercio et al. previously looked at VSL#3 supplementation in patients with NAFLD and other chronic liver disease, finding lower markers of lipid peroxidation and oxidative stress post-supplementation [187]. Aller et al. and Vajro et al. ran trials with other probiotic formulations, resulting in decreased liver enzymes, but no change in inflammatory measures, insulin resistance, or fatty liver [188, 189]. Trials with synbiotics (pre- and probiotics delivered together) appear more
promising, including the small trial discussed in section 2.2.4 [14]. Malaguarnera et al. found that synbiotic supplementation for 24 weeks resulted in a greater reduction in AST, insulin resistance, inflammatory markers, endotoxin levels, steatosis, and NASH activity score than diet and exercise alone [190]. Taken together these studies seem to suggest a beneficial role of probiotics, particularly VSL#3 or synbiotic combinations, in treating NAFLD. The mechanism of this improvement is unclear, but likely multifactorial. IM that may be of particular interest in NAFLD pathogenesis, considering the findings of previous studies, are included in Table 2.

**Table 2: Bacteria of interest in non-alcoholic fatty liver disease**

<table>
<thead>
<tr>
<th>Bacteria of Interest</th>
<th>Expected Finding</th>
<th>Notes on Mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroidetes</td>
<td>Lower in NASH vs SS &amp; HC</td>
<td>Increased energy absorption</td>
<td>[13]</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Higher in NAFLD</td>
<td></td>
<td>[11, 12]</td>
</tr>
<tr>
<td>Erysipelotrichi</td>
<td>Higher abundance increased susceptibility to NAFLD</td>
<td></td>
<td>[10]</td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td>Mixed results with lower or higher abundance in NAFLD</td>
<td></td>
<td>[10, 12]</td>
</tr>
<tr>
<td>Enterobacteriacea, especially <em>Escherichia</em></td>
<td>Higher in NAFLD/NASH vs HC</td>
<td>Ethanol producer</td>
<td>[11, 15]</td>
</tr>
<tr>
<td>Ruminococcaceae</td>
<td>Higher in HC</td>
<td>Butyrate producer</td>
<td>[15]</td>
</tr>
<tr>
<td><em>Faecalibacterium</em></td>
<td>Lower in NASH vs HC</td>
<td>Butyrate producer</td>
<td>[14]</td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>Higher in NAFLD (SS &amp; NASH)</td>
<td></td>
<td>[45, 78]</td>
</tr>
<tr>
<td><em>Prevotella</em></td>
<td>Higher in HC</td>
<td>Butyrate producer</td>
<td>[15]</td>
</tr>
<tr>
<td><em>Streptococcus</em></td>
<td>Higher in NAFLD vs HC</td>
<td></td>
<td>[15]</td>
</tr>
<tr>
<td><em>Clostridium</em> Clusters XIVa and IV</td>
<td>Lower in NASH vs HC &amp; SS</td>
<td>Butyrate producers</td>
<td>Not extensively studied in NAFLD population</td>
</tr>
<tr>
<td><strong>Table 2:</strong> Bacteria of interest in non-alcoholic fatty liver disease</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.2.6 Proposed Mechanism

Given the background information provided, the intestinal microbiota may contribute to NAFLD pathogenesis via the following mechanisms:

• Increased energy harvest by IM may increase total energy absorbed, contributing to obesity, a known risk factor for NAFLD

• Metabolism of choline by IM may promote steatosis by decreasing lipid excretion from the liver as VLDL

• Decreased production of butyrate by IM may impair the intestinal epithelium and increase intestinal permeability

• Bacterial endotoxin may cross the permeable intestinal mucosa causing the activation of TLR-4, triggering systemic inflammation and insulin resistance

• Alterations in IM composition may lead to increased production of ethanol while increased permeability may lead to increased absorption of ethanol entering the blood stream and causing increased metabolic stress and known risk to liver health.
3 Research Aim and Hypotheses

**Aim:** The aim of this research project is to compare bacterial products between biopsy confirmed SS, NASH and healthy controls (HC) as well as to further assess the IM between groups.

**Main Hypothesis:** Patients with NASH have higher plasma endotoxin compared to those with SS and HC.

**Secondary Hypothesis:** Patients with NASH have lower fecal butyrate compared to SS and HC.

**Additional Outcomes:** We will also measure whether there is a difference in serum choline, ethanol and other serum and fecal metabolites between the three groups and assess if there is any association with differences in IM and diet.
4 Materials and Methods

4.1 Study Design

This is a cross-sectional study comparing patients with liver biopsy proven simple steatosis (SS), non-alcoholic steatohepatitis (NASH) and healthy controls (HC).

Adult subjects seen by hepatologists of the University Health Network, Toronto, were approached for this study. Patients with a clinical suspicion of NAFLD, typically due to persistently elevated liver enzymes and obesity, were assessed by a hepatologist using standard medical practice. Patients were given general advice to lose weight and increase physical activity; no specific instructions for lifestyle change were provided. Patients who returned for their follow-up appointment approximately six months later without success in lifestyle improvement or weight loss were booked for a liver biopsy to assess the severity of NAFLD. During this visit (study visit 1) the study was explained in detail and informed consent was obtained from interested participants. See Appendix 1 for consent forms. Following consent, subjects were provided with detailed instructions for the completion of a 7-day food record, 7-day activity record, and environmental questionnaire (see below for further details). Patients were also provided with instructions for the collection and transport of their stool sample (Appendix 2), which they were asked to return along with their food/activity record on the day of their liver biopsy (study visit 2). On study visit 2, subjects underwent anthropometric measurements and provided a fasting blood sample for clinical (section 4.4) and study specific (section 4.6 and 4.7) bloodwork.

For healthy controls, individuals undergoing candidacy assessment as potential healthy living liver donors at Toronto General Hospital were approached during their first screening appointment. The study details were explained and informed consent was provided by interested participants. At this visit (study visit 1) the same instructions for the food/activity records, environmental questionnaire, and stool sample collection were provided. Approximately one week prior to liver donation, healthy controls returned for study visit 2 where they underwent anthropometric measurements, provided a fasting blood sample, and returned their stool sample and food/activity record. On study visit 3, during their donation surgery, a wedge liver biopsy was collected for histological analysis.
Inclusion criteria for this study were age greater than or equal to 18 years and confirmation of NAFLD or healthy liver status via liver biopsy. Exclusion criteria were diagnosis of liver disease other than NAFLD on liver biopsy, liver transplant expected to be required within one year, significant liver complications (e.g. variceal bleeding, jaundice, etc.) or any other contraindications for liver biopsy, alcohol intake greater than 20g per day, pregnancy or lactation, presence of gastrointestinal disease, use of medications known to cause steatohepatitis, insulin, NSAIDS (other than low dose acetylsalicylic acid), antibiotics, prebiotics, probiotics, or experimental drugs within the last three months.

4.2 Clinical Data, Environmental Questionnaire, and Anthropometric Measurements

Clinical data were collected on study visit one. Study participant’s smoking and alcohol consumption history and medication and supplement use were reviewed, including medications taken in the last three months. Study participants were also asked to answer a number of questions regarding their personal and family history of disease. Age, ethnicity, and menstrual history were also recorded.

An environmental questionnaire (Appendix 3) was completed and returned with the stool sample. This questionnaire collected information that may affect an individual’s IM composition and included questions such as country of origin, method of birth (vaginal versus caesarian section), breastfeeding, and pets at home.

Anthropometric measurements including height (ht), weight (wt), and waist circumference (WC) were measured by a trained research professional. Weight was measured using a calibrated hospital-grade chair scale; height was measured using a stadiometer. Waist circumference was measured at the umbilicus level. All measurements were taken in triplicate and the average value was used.

4.3 Nutrition and Activity Assessment

Each participant was given a food record and activity log (Appendix 4) to complete in the week prior to returning their stool sample. Detailed instructions were provided for the completion of both of these tools. The food log included all food and beverages consumed over each 24 hours for seven days. A three-day food record including one weekend day was also accepted.
Participants used the 2D Food Portion Visual Chart (Nutrition Consulting Enterprises, Framingham, MA) to estimate portion sizes. This is a validated tool which has been used in our previous studies [60, 191, 192]. Food records were reviewed by an experienced registered dietitian and were analyzed using Food Processor Diet and Nutrition Analysis Software (Version 7, ESHA Research, Salem, OR).

Physical activity logs were recorded for 7 days concurrent with the food records. Participants were asked to record any activity, including household chores, the duration of the activity, and the intensity level. Detailed instructions were provided including examples for each intensity level (mild, moderate, strenuous, and very strenuous). For details see Appendix 4. This information was used to calculate daily physical activity units: 1 unit = 30 minutes mild, 20 minutes moderate, 10 minutes strenuous, or 5 minutes very strenuous activity. This is a validated method for measuring physical activity level [193]. Basal metabolic rate (BMR) was calculated using the Harris-Benedict equation: BMR for men = 66.5 + [13.75 × wt(kg)] + [5.003 × ht(cm)] – [6.755 × age(y)], BMR for women = 655.1 [9.563 × wt(kg)] + [1.850 × ht(cm)] – [4.676 × age(y)]. Estimated energy requirement (EER) was calculated using Health Canada Guidelines: EER for men = 662 – [9.53 x age(y)] + PA x {[15.91 x wt(kg)] + [539.6 x ht(m)]}, and EER for women = 354 – [6.91 x age(y)] + PA x {[9.36 x wt(kg)] + (726 x ht(m))} where PA is the physical activity coefficients which are included in Table 3 [194].
Table 3: Physical activity coefficients for estimated energy expenditure calculation [194]

<table>
<thead>
<tr>
<th>Activity Level</th>
<th>Sedentary</th>
<th>Low Active</th>
<th>Active</th>
<th>Very Active</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description</td>
<td>Typical daily living activities (e.g., household tasks, walking to the bus)</td>
<td>Typical daily living activities PLUS 30 - 60 minutes of daily moderate activity (ex. walking at 5-7 km/h)</td>
<td>Typical daily living activities PLUS At least 60 minutes of daily moderate activity</td>
<td>Typical daily living activities PLUS At least 60 minutes of daily moderate activity PLUS An additional 60 minutes of vigorous activity or 120 minutes of moderate activity</td>
</tr>
<tr>
<td>Men</td>
<td>1.00</td>
<td>1.11</td>
<td>1.25</td>
<td>1.48</td>
</tr>
<tr>
<td>Women</td>
<td>1.00</td>
<td>1.12</td>
<td>1.27</td>
<td>1.45</td>
</tr>
</tbody>
</table>

4.4 Clinical Biochemistry

Blood was drawn after a 12 hour overnight fast on study visit 2. Liver markers were drawn as a routine clinical measure and included: aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and bilirubin. Measures of glucose metabolism included plasma glucose, insulin, hemoglobin A1c (HbA1c), and homeostasis model assessment estimated insulin resistance (HOMA-IR) which was calculated as fasting glucose (mmol/L) × fasting insulin (mU/L)/22.5 [195]. A lipid profile was also conducted, including total cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL), and triglycerides (TG). These analyses were conducted by the UHN Laboratory Medicine Program. Liver enzymes and lipid profile were measured using the Architect c8000 system (Abbott Laboratories). LDL was calculated from total cholesterol – HDL. Fasting plasma glucose and plasma insulin were measured by the enzymatic hexokinase method and radioimmunoassay, respectively.
4.5 Liver Histology

Liver biopsies were taken percutaneously (needle biopsy) for NAFLD patients and intraoperatively (wedge biopsy) for HC and preserved immediately in formalin. Liver biopsies were assessed by the same pathologist using standard stains for the diagnosis of NAFLD, morphologic evaluation, and to rule out any iron overload. The evaluation of NAFLD related measures of steatosis, inflammation, and fibrosis were conducted using the validated and reproducible Brunt system (Table 4) [196]. Disease severity was also evaluated using the NAS which accounts for degree of steatosis, lobular inflammation, and hepatocellular ballooning for a final score of 0-8 [197].
<table>
<thead>
<tr>
<th>Steatosis</th>
<th>% hepatocytes involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 0</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>Grade 1</td>
<td>5-33%</td>
</tr>
<tr>
<td>Grade 2</td>
<td>&gt;33-66%</td>
</tr>
<tr>
<td>Grade 3</td>
<td>&gt;66%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NASH (Inflammation + Steatosis)</th>
<th>Description</th>
</tr>
</thead>
</table>

**Grade 1: Mild**
- Steatosis: Predominantly macrovesicular, involves < 33% - 66% of the lobules
- Ballooning: Occasionally observed; zone 3 hepatocytes
- Lobular Inflammation: Scattered and mild acute (polymorphs) inflammation and occasional chronic inflammation (mononuclear cells)
- Portal Inflammation: None or mild

**Grade 2: Moderate**
- Steatosis: Any degree and usually mixed macrovesicular and microvesicular
- Ballooning: Obvious and present in zone 3
- Lobular Inflammation: Polymorphs may be noted associated with ballooned hepatocytes, pericellular fibrosis; mild chronic inflammation may be seen
- Portal Inflammation: Mild to moderate

**Grade 3: Severe**
- Steatosis: Typically > 66% (panacinar); commonly mixed steatosis
- Ballooning: Predominantly zone 3; marked
- Lobular Inflammation: Scattered acute and chronic inflammation; polymorphs may appear concentrated in zone 3 areas of ballooning and perisinusoidal fibrosis
- Portal Inflammation: Mild or moderate

**Fibrosis**
- Stage 1: Zone 3 perivenular perisinusoidal/pericellular fibrosis, focal or extensive
- Stage 2: As above with focal or extensive periportal fibrosis
- Stage 3: Bridging fibrosis, focal or extensive
- Stage 4: Cirrhosis
4.6 Plasma Endotoxin

Blood was drawn in a fasting state using an ethylenediaminetetraacetic acid (EDTA) coated vacutainer and separated by centrifugation at 2400 rpm for 10 minutes at 20°C. Plasma was then stored at -80°C until the time of measurement. Endotoxin quantification was conducted using the QCL-1000 chromogenic limulus amebocyte lysate (LAL) endpoint assay (Lonza, Walkersville, MD) in our own laboratory. This assay has been effectively used in the literature [130, 198-201]. The assay is based on the principle that endotoxin catalyzes the activation of a proenzyme that is found in LAL. The rate of activation is determined by the amount of endotoxin present. Once activated, the enzyme catalyzes the splitting of pNA from the colourless substrate. Once the reaction is stopped, the separated pNA can be measured via photometry at 405-410nm. The correlation between absorbance and endotoxin concentration is linear from 0.1 to 1.0 EU/mL. Endotoxin concentration is determined using a standard curve.

The LAL assay was optimized for use with our samples prior to study measurements. The ideal dilution and processing of samples was determined using a positive product control or “spiked” sample. Each sample dilution was spiked with 0.4 EU/mL. The spiked and unspiked sample dilution were then measured as per the study protocol (see below). Optimization occurred when spike recovery was in the 75-125% range, indicating that significant inhibition or enhancement has not occurred.

Following this process the optimal LAL assay protocol was developed. Plasma samples were thawed at room temperature. An initial 1:5 dilution was heat treated at 70°C for 15 minutes to deactivate interference from plasma proteins. Samples were then cooled. Meanwhile a standard curve was produced, using the provided endotoxin standard and endotoxin free water. The standard curve including five points: 0.4 EU/mL, 0.3 EU/mL, 0.2 EU/mL, 0.1 EU/mL, and 0.05 EU/mL.

Once the 1:5 plasma dilution was cooled, a 1:60 dilution was produced by adding 0.1 mL of the 1:5 solution to 1.1 mL of 10mM MgCl₂. MgCl₂ was used to overcome the inhibitory effect of EDTA. An endotoxin-free 96 well plate was filled with 50 µL of each 1:60 sample dilution, the standard curve, and a water blank in triplicate. A sample blank was also plated in duplicate to control of any absorbance due to colour present in the sample prior to assay. The plate was then placed on a block heater and preheated to 37°C. Meanwhile the LAL reagent and substrate were
reconstituted with endotoxin-free water and the substrate was heated to 37°C in a water bath. All pipetting was done using endotoxin-free pipette tips and dilutions were made in endotoxin-free test tubes.

The timed test protocol then occurred as follows: at T=0, 50 µL of LAL reagent was added to each standard, blank, and test well using a repeater pipette. The plate was then tapped to mix and left to incubate for 10 minutes. At T=10 minutes, 100 µL of substrate (heated to 37°C) was added to each of these wells and the plate was tapped to mix. At T=16 minutes, 50 µL of a stop solution (10g/100mL sodium dodecylsulfate) was added to all wells, including the sample blanks and the plate was tapped to mix. Any bubbles present in the wells were then popped using a sterile syringe. Absorbance was measured at 405nm using the PHERAstar FS plate reader (BMG Labtech, Offenburg, Germany) and endotoxin concentrations were extrapolated from the standard curve using the MARS Data Analysis software (Version 3.01 R2, BMG Labtech, 2013).

4.7 Serum Metabolites

Serum metabolites, including choline, ethanol, and TMAO, were measured to evaluate potential implications of bacterial metabolism at a systemic level. For the list of metabolites measured see Table 5. Serum was drawn in a fasting state using a gel serum separation vacutainer and was immediately placed in an insulated container with cooling elements. Blood was separated by centrifuge at 4°C at 2800 x g for 20 minutes. Serum was then aliquoted and stored at -80°C until all study samples were collected. Serum samples were shipped to the Metabolomic Innovation Centre (Edmonton, AB) where metabolites were analyzed using nuclear magnetic resonance (NMR) spectrometry [202]. The following method was used by the centre [202]: serum samples were deproteinized using ultrafiltration. Filtrates were checked visually for a red tint, which indicates that the membrane was compromised. For those “membrane compromised” samples, the filtration process was repeated with a different filter and filtrates were inspected again. Then, each filtrate (300 µL) was combined with 35 µL of D2O and 15 µL of a standard buffer solution. The serum sample (350 µL) was then transferred to a standard Shigemi microcell NMR tube for subsequent spectral analysis.

All 1H-NMR spectra were collected on a 500 MHz Inova (Varian Inc., Palo Alto, CA) spectrometer. 1H-NMR spectra were acquired at 25°C using the first transient of the ttnoesy-presaturation pulse sequence, which was chosen for its high degree of quantitative accuracy
Spectra were collected with 128 transients and 8 steady-state scans using a 4 second acquisition time and a 1 second recycle delay.

All free induction decay NMR signals were zero-filled to 64k data points and subjected to line broadening of 0.5 Hz. The singlet produced by a known quantity of DSS methyl groups was used as an internal standard (set to 0 ppm). All $^1$H-NMR spectra were processed and analyzed using the Chenomx NMR Suite Professional software package version 6.0 (Chenomx Inc., Edmonton, AB), as previously described [204]. Each spectrum was processed and analyzed by at least two experienced NMR spectroscopists to minimize compound mis-identification and mis-quantification.

Serum trimethylamine N-oxide (TMAO) was not detectable using NMR therefore TMAO was measured using a targeted quantitative metabolomics approach by Liquid Chromatography Mass Spectrometry (LCMS). Isotopically-labeled internal standards were added to the serum to facilitate metabolite quantification. Sample extraction was performed on a 96 well plate with a 0.2 µm solvent filter. 10 µL of serum was spiked with the internal standard (TMAO D9) and then 150 µL of methanol with 10 mM ammonium acetate was added for extraction. Each sample was diluted with 150 µL of water. Seven calibrant solutions with known concentrations went through the same extraction steps. LCMS analysis was performed on AB SCIEX 4000 QTrap mass spectrometer with Agilent 1100 HPLC. 10 µL of the extracted samples were injected onto the Kinetex C18 (2.6 µm, 3.0x100mm, 100A) Column with guard column. Isobaric elution was performed with 90% A (10 mM ammonium formate in water, PH3) and 10% B (10 mM ammonium formate in 90:10 acetonitrile:water, PH3). Total LC method run time was 3 min with flow rate of 500 µl/min. A seven-point calibration curve was generated to quantify the concentration of TMAO in samples.
Table 5: Metabolites measured in stool and serum samples

<table>
<thead>
<tr>
<th>Stool Metabolites</th>
<th>Serum Metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic Acid*</td>
<td>Acetic Acid*</td>
</tr>
<tr>
<td>Butyric Acid</td>
<td>Choline*</td>
</tr>
<tr>
<td>Choline*</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Formic Acid*</td>
<td>Formic Acid*</td>
</tr>
<tr>
<td>Isobutyric Acid</td>
<td>2-Hydroxybutyrate</td>
</tr>
<tr>
<td>Isovalerate</td>
<td>L-Lactic Acid*</td>
</tr>
<tr>
<td>L-Lactic Acid*</td>
<td>Trimethylamine N-Oxide*</td>
</tr>
<tr>
<td>Propionate</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td></td>
</tr>
<tr>
<td>Trimethylamine*</td>
<td></td>
</tr>
</tbody>
</table>

* Metabolite measured in both stool and serum

4.8 Stool Sample Collection and Analysis

Stool collection kits included a plastic collection/storage container with a tightly closing lid, an insulated bag, and cooling elements. Within 24 hours of study visit 2, subjects collected one stool sample, which was frozen immediately after defecation in their home freezer (-20°C). Subjects brought the frozen sample in the insulated bag with cooling elements to the hospital, where it was stored at -80°C until homogenization. This method of stool collection was used in a landmark study to establish a human gut microbial gene catalogue [205].

4.8.1 Stool Homogenization

Stool samples were homogenized prior to DNA extraction and metabolite measurements. The entire sample was first transferred into a sterile masticator bag. The sample was allowed to thaw until a smooth consistency was reached, typically 2-3 hours depending on sample size. Once
thawed, excess air was released from the bag and the sample was homogenized for two minutes using a masticator blender (IUL, S.A., Barcelona, Spain). This was followed by one minute of hand mastication of any areas that were missed. The corner of the masticator bag was then cut and the sample was aliquoted: 1-2 g samples were stored for metabolite analysis and 0.1-0.2 g aliquots were stored for DNA extraction. Samples were immediately placed on dry ice and then transferred to -80°C for storage until analysis. Weight was recorded for each aliquot and pH was measured for each sample.

4.8.2 Fecal Metabolite Analysis

Homogenized fecal samples were transferred to the Metabolomic Innovation Centre on dry ice and were analyzed by NMR spectroscopy to identify 10 metabolites of interest (Table 5). Fecal samples were prepared by mixing 20 mg of frozen fecal material with 1 mL of saline phosphate buffer in deuterium oxide, followed by centrifugation (18 000 × g, 1 minute). Fecal supernatants were removed and filtered through 0.2 μm membrane filters as described by Le Gall and colleagues [206]. The measurement in fecal supernatant was then conducted as described above in section 4.7 for serum metabolites.

4.8.3 DNA Extraction

DNA was extracted using the E.Z.N.A. Stool DNA Kit (Omega Bio-Tek, Norcross, GA) and a modified manufacturer’s protocol with the addition of a lysozyme and proteinase digestion step (incubation at 37°C for 30 minutes) as used by Mouzaki et al. [13]. An elution volume of 100 μL was used. DNA was analyzed for purity and concentration using the Nanodrop 1000 Spectrophotometer (ThermoScientific, Rockford, IL). DNA samples were stored at -80°C.

4.8.4 Quantitative Real-time PCR

Total bacteria and selected microbial groups of interest: Bacteroidetes, Bifidobacteria, Clostridium leptum, Clostridium coccoides, Lactobacilli, E. coli, and Archaea were analyzed by qPCR. Assays were run in triplicate in a 384 well optical plate using a 7900HT thermocycler (Applied Biosystems, Foster City, CA) under default thermocycling conditions. Fifty nanograms of DNA with TaqMan Gene Expression Master Mix (MM) and specific TaqMan primers (Applied Biosystems) were used. Custom-made TaqMan primers for total bacteria, Lactobacilli, C. coccoides, C. leptum, Bacteroidetes, Bifidobacteria, and Archaea were used [207-209]. Real-
time PCR for *E. coli* was done using SYBR Green Gene Expression MM (Applied Biosystems) and the specific forward and reverse primer [207]. The ramping profile for SYBR Green amplifications was similar and a dissociation stage was added in the end. The number of cells of each microorganism in fecal samples was calculated by interpolation from pre-constructed standard curves (Table 6) and expressed as CFU/g of wet feces.

### Table 6: Standard curves used in the interpretation of the qPCR results

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Standard curve</th>
<th>Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bacteria</td>
<td>$y= -3.325x + 37.670$</td>
<td>99.87</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>$y= -3.289x + 33.635$</td>
<td>101.39</td>
</tr>
<tr>
<td><em>C. leptum</em></td>
<td>$y= -3.315x + 38.932$</td>
<td>100.29</td>
</tr>
<tr>
<td><em>C. coccoides</em></td>
<td>$y= -3.3236x + 37.013$</td>
<td>99.93</td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>$y= -3.371x + 37.660$</td>
<td>97.99</td>
</tr>
<tr>
<td>Bifidobacteria</td>
<td>$y= -3.357x + 37.826$</td>
<td>98.56</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>$y= -3.2928x + 34.939$</td>
<td>101.23</td>
</tr>
<tr>
<td>Archaea</td>
<td>$y= -3.351x + 39.680$</td>
<td>98.80</td>
</tr>
</tbody>
</table>

### 4.9 Statistical Analysis

#### 4.9.1 Data Analysis

Descriptive summaries of all measurements were calculated: means and standard deviations (SD) for normal variables, medians and first quartile and third quartile for skewed variables, and proportions for categorical variables. Plots were used as appropriate to examine the data. Bivariate relationships were assessed using Pearson’s or Spearman’s correlation coefficients, Chi-square and Fisher exact tests where appropriate. Comparisons across diagnosis groups were performed for normally distributed data using one-way ANOVA followed by the t-test (if necessary, data were log-transformed to normalize distribution), or for non-normally distributed data using the Kruskal-Wallis test followed by Wilcoxon rank-sums test. All tests were two-sided and performed at the 5% significance (alpha) level. Bonferroni’s correction method was
used to account for multiple comparisons. Outliers were identified by visual inspection and sensitivity analyses were run. If the exclusion of outliers altered the result of the statistical test this was noted in the results section. All statistical analyses were conducted using SAS version 9.4 (SAS Institute Inc., Cary, NC).

4.9.2 Power Calculations

Calculations were performed using standard statistical software packages, primarily SAS version 9.3 (SAS Institute Inc., Cary, NC). Calculations for the main variable (endotoxin) was based on a sample size of n=30 per group and estimates from Harte et al. obtained from a similar patient population of HC, SS, and NASH [130]. SD and effect size (ES), where ES=mean difference, were estimated from Harte et al. (SD=5.22, ES=3.84 EU/mL). The significance level was set at 5%. Power calculations were performed for one-way ANOVA. We estimated the power to detect differences between our three groups in ANOVA as >0.999.
5 Results

5.1 Subjects

A total of 30 HC, 15 SS, and 24 NASH patients were found eligible and provided consent to participate in this study. Demographics, health history, and anthropometric data for these subjects are described in Table 7. Both SS and NASH groups were significantly older than HC, but did not differ by sex. BMI in NASH patients was significantly higher than HC, but BMI did not differ between HC and SS or SS and NASH groups. Waist circumference also followed this pattern. There appeared to be a difference in diabetes prevalence between groups, but when Bonferroni correction was used for multiple comparisons this significance was lost. Prevalence of hypertension was greater in both patient groups than in HC and corresponded with a higher use of cardiovascular drugs which included antihypertensive medications. The use of lipid lowering drugs such as statins was higher in NASH patients and the use of mild non-steroidal anti-inflammatory drugs (low dose acetylsalicylic acid only) was higher in SS than in HC.

Biochemistry data is described in Table 8. ALT was significantly higher in SS patients versus HC and in NASH patients versus both SS and HC. AST was significantly higher in NASH patients than in both HC and SS patients. Fasting glucose appeared to be higher in NASH patients but this did not remain significant in post-hoc pairwise comparisons. Fasting insulin was significantly higher in NASH patients than in HC and SS patients, however, HOMA-IR and HbA1c where only significantly different between HC and NASH patients with NASH patients having higher levels. Lipids did not differ significantly between the groups with the exception of triglycerides which were significantly higher in NASH patients versus HC.

Liver biopsies were available for 17 HC, 15 SS, and 23 NASH patients. Healthy controls who did not have a liver biopsy had liver health confirmed via several diagnostic tests including blood measurements of liver enzymes (ALT, AST, ALP), computerized tomography and MRI scans which were reviewed by a panel of surgeons and hepatologists and confirmed to be healthy and without fat deposits. One NASH patient was diagnosed via liver biopsy at an outside facility and therefore histological analysis by our study pathologist was not possible.

Histological analysis is displayed in Table 9. Occasionally biopsies were not of sufficient size or quality for all analyses and the total inflammation score was not measured consistently for all
patients. The sample size available for each histological feature is therefore noted. NASH patients appear to have relatively mild disease with a median NAS of 5 out of a possible 8.

Environmental questionnaires were only received from 53% of study participants and were often incomplete therefore statistical analysis was limited. Patient’s country of birth was also collected as part of demography in 22 HC, 9 SS, and 16 NASH patients. There was a significant difference in the proportion of patients born in Canada between groups with more HC (86%) being born in Canada than SS (0%) and NASH (31%) patients (p<0.0001). Ethnicity, however, did not differ by group as shown in Table 7.
Table 7: Subject demographics and health history

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>HC</th>
<th>n</th>
<th>SS</th>
<th>n</th>
<th>NASH</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>30</td>
<td>37 (10)ab</td>
<td>15</td>
<td>47 (8)a</td>
<td>24</td>
<td>48 (9)b</td>
<td>0.0001</td>
</tr>
<tr>
<td>Sex (% female)</td>
<td>30</td>
<td>50% (15)</td>
<td>15</td>
<td>40% (6)</td>
<td>24</td>
<td>54 (13)</td>
<td>0.7</td>
</tr>
<tr>
<td>Ethnicity (% Caucasian)</td>
<td>19</td>
<td>84% (16)</td>
<td>9</td>
<td>44% (4)</td>
<td>12</td>
<td>58% (7)</td>
<td>0.09</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>30</td>
<td>26.2 (23.6, 28.8)a</td>
<td>15</td>
<td>27.4 (24.9, 32.5)</td>
<td>24</td>
<td>32.1 (29.7, 34.3)a</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Waist Circumference (cm)</td>
<td>28</td>
<td>89.9 (84.4, 94.8)a</td>
<td>13</td>
<td>97.1 (89.6, 106)</td>
<td>22</td>
<td>103 (97.8, 109)a</td>
<td>0.0002</td>
</tr>
<tr>
<td>Diabetes</td>
<td>30</td>
<td>0% (0)</td>
<td>15</td>
<td>7% (1)</td>
<td>24</td>
<td>17% (4)</td>
<td>0.049</td>
</tr>
<tr>
<td>Cardiovascular Disease</td>
<td>30</td>
<td>0% (0)</td>
<td>15</td>
<td>7% (1)</td>
<td>24</td>
<td>7% (1)</td>
<td>0.2</td>
</tr>
<tr>
<td>Hypertension</td>
<td>30</td>
<td>0% (0)ab</td>
<td>15</td>
<td>27% (4)a</td>
<td>24</td>
<td>38% (9)b</td>
<td>0.0003</td>
</tr>
<tr>
<td>Renal Disease</td>
<td>30</td>
<td>0% (0)</td>
<td>15</td>
<td>0% (0)</td>
<td>24</td>
<td>8% (2)</td>
<td>0.16</td>
</tr>
<tr>
<td>Cardiovascular Drugs</td>
<td>30</td>
<td>0% (0)ab</td>
<td>15</td>
<td>33% (5)a</td>
<td>24</td>
<td>25% (6)b</td>
<td>0.001</td>
</tr>
<tr>
<td>Diabetes Drugs</td>
<td>30</td>
<td>0% (0)</td>
<td>15</td>
<td>7% (1)</td>
<td>24</td>
<td>13% (4)</td>
<td>0.049</td>
</tr>
<tr>
<td>Lipid Lowering Drugs</td>
<td>30</td>
<td>3% (1)a</td>
<td>15</td>
<td>27% (4)</td>
<td>24</td>
<td>33% (8)b</td>
<td>0.008</td>
</tr>
<tr>
<td>Anti-Depressants</td>
<td>30</td>
<td>7% (2)</td>
<td>15</td>
<td>7% (1)</td>
<td>24</td>
<td>0% (0)</td>
<td>0.4</td>
</tr>
<tr>
<td>NSAID†</td>
<td>30</td>
<td>0% (0)a</td>
<td>15</td>
<td>27% (4)a</td>
<td>24</td>
<td>13% (3)</td>
<td>0.011</td>
</tr>
</tbody>
</table>

Continuous variables are expressed as mean (standard deviation) for normal variables and median (1\textsuperscript{st} quartile, 3\textsuperscript{rd} quartile) for non-normally distributed variables. Categorical variables are expressed as prevalence in percentage (count). Values with the same superscript are significantly different. † NSAID = nonsteroidal anti-inflammatory drug
**Table 8:** Biochemistry by disease group

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>HC</th>
<th>n</th>
<th>SS</th>
<th>n</th>
<th>NASH</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/L)</td>
<td>30</td>
<td>18 (14, 29)</td>
<td>15</td>
<td>45 (32, 54)</td>
<td>24</td>
<td>70.5 (45, 104)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ab</td>
<td></td>
<td>ac</td>
<td></td>
<td>bc</td>
<td></td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>30</td>
<td>20 (17, 25)</td>
<td>15</td>
<td>26 (21, 33)</td>
<td>24</td>
<td>45 (31, 67)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a</td>
<td></td>
<td>b</td>
<td></td>
<td>ab</td>
<td></td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>30</td>
<td>71 (21)</td>
<td>15</td>
<td>70 (18)</td>
<td>24</td>
<td>72 (19)</td>
<td>0.9</td>
</tr>
<tr>
<td>Bilirubin (µmol/L)</td>
<td>28</td>
<td>10 (7, 13)</td>
<td>14</td>
<td>8 (7, 13)</td>
<td>21</td>
<td>10 (8, 11)</td>
<td>0.9</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>29</td>
<td>4.9 (4.6, 5.1)</td>
<td>12</td>
<td>5.1 (4.7, 5.7)</td>
<td>23</td>
<td>5.7 (4.8, 6.2)</td>
<td>0.035†</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>24</td>
<td>36 (22, 56)</td>
<td>12</td>
<td>31 (23, 82)</td>
<td>22</td>
<td>102 (56, 191)</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a</td>
<td></td>
<td>b</td>
<td></td>
<td>ab</td>
<td></td>
</tr>
<tr>
<td>HOMA-IR‡</td>
<td>24</td>
<td>1.18 (0.80, 1.93)</td>
<td>11</td>
<td>1.33 (0.77, 4.65)</td>
<td>22</td>
<td>4.26 (2.03, 8.63)</td>
<td>0.0001</td>
</tr>
<tr>
<td>HbA1c</td>
<td>29</td>
<td>0.052 (0.049, 0.054)</td>
<td>12</td>
<td>0.056 (0.053, 0.059)</td>
<td>13</td>
<td>0.058 (0.053, 0.065)</td>
<td>0.001</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/L)</td>
<td>23</td>
<td>4.97 (1.00)</td>
<td>13</td>
<td>5.28 (1.07)</td>
<td>24</td>
<td>4.81 (1.55)</td>
<td>0.5</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>23</td>
<td>3.16 (2.67, 3.53)</td>
<td>12</td>
<td>3.37 (2.65, 4.27)</td>
<td>23</td>
<td>2.67 (2.09, 3.17)</td>
<td>0.06</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>23</td>
<td>1.30 (0.33)</td>
<td>12</td>
<td>1.26 (0.24)</td>
<td>24</td>
<td>1.10 (0.29)</td>
<td>0.07</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>23</td>
<td>0.94 (0.77, 1.23)</td>
<td>13</td>
<td>1.10 (0.86, 1.37)</td>
<td>24</td>
<td>1.63 (1.17, 2.62)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Values are expressed as mean (standard deviation) for normal variables and median (1st quartile, 3rd quartile) for non-normally distributed variables.

Values with the same superscript are significantly different.

† Despite significant comparison with Krusal-Wallis test, pairwise comparisons did not reach significance.

‡ HOMA-IR = fasting glucose (mmol/L) × fasting insulin (mU/L)/22.5.
Table 9: Liver histology by disease group

<table>
<thead>
<tr>
<th></th>
<th>HC (n=17)</th>
<th>SS (n=15)</th>
<th>NASH (n=23)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Steatosis (% hepatocytes)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Median (min, max)</em></td>
<td>0 (0, 2.5)</td>
<td>15 (5, 70)</td>
<td>40 (10, 90)</td>
</tr>
<tr>
<td><strong>Fibrosis Stage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Proportion of subjects (count)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>79% (11)</td>
<td>67% (10)</td>
<td>13% (3)</td>
</tr>
<tr>
<td>1</td>
<td>21% (3)</td>
<td>20% (3)</td>
<td>26% (6)</td>
</tr>
<tr>
<td>2</td>
<td>0% (0)</td>
<td>13% (2)</td>
<td>35% (8)</td>
</tr>
<tr>
<td>3</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>13% (3)</td>
</tr>
<tr>
<td>4</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>13% (3)</td>
</tr>
<tr>
<td><strong>Lobular Inflammation Intensity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Proportion of subjects (count)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100% (15)</td>
<td>87% (13)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>1</td>
<td>0% (0)</td>
<td>13% (2)</td>
<td>69.5% (16)</td>
</tr>
<tr>
<td>2</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>22% (5)</td>
</tr>
<tr>
<td>3</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>8.5% (2)</td>
</tr>
<tr>
<td><strong>Total Inflammation†</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Proportion of subjects (count)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>100% (11)</td>
<td>60% (3)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>Mild</td>
<td>0% (0)</td>
<td>40% (2)</td>
<td>27% (3)</td>
</tr>
<tr>
<td>Moderate</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>73% (8)</td>
</tr>
<tr>
<td>Severe</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>0% (0)</td>
</tr>
<tr>
<td><strong>Ballooning Intensity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Proportion of subjects (count)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>100% (16)</td>
<td>100% (15)</td>
<td>4% (1)</td>
</tr>
<tr>
<td>Mild</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>61% (14)</td>
</tr>
<tr>
<td>Obvious</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>35% (8)</td>
</tr>
<tr>
<td><strong>NAFLD Activity Score</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Median (min, max)</em></td>
<td>0 (0, 0)</td>
<td>1 (1, 4)</td>
<td>5 (3, 8)</td>
</tr>
</tbody>
</table>

† This measure was not consistently recorded in all patients.
5.2 Dietary Intake and Physical Activity

Food records were completed for at least three days by 25 HC, 10 SS, and 20 NASH patients. Analysis of energy and macronutrients is detailed in Table 10. Reported energy intake differed by group with NASH patients reporting significantly fewer kilocalories than HC. To investigate for possible underreporting, reported energy intake was compared with EER and BMR. Reported proportion of EER consumed did not differ significantly between groups (p=0.14) (Figure 1). Reported proportion of BMR consumed, however, was significantly lower in NASH patients than HC (p=0.011) with the median reported proportion consumed at less than 100% for NASH patients (Figure 2). As BMR is the estimated energy required for basic physiological functions and none of these patients was losing weight we suspected that underreporting of energy intake may have occurred, particularly in the NASH patients. For this reason macro and micronutrient intake was expressed as a proportion of energy consumed to describe dietary composition. Protein derived energy did differ slightly between groups but did not remain significant in pairwise comparisons after Bonferroni correction.

Reported micronutrient intake is displayed in Table 11. Micronutrient intake did not differ between groups. Data regarding omega 6:3 ratio and choline intake may not be reliable due to incomplete data in the Food Processor database, but it is important to note that relative intake did not differ between the three groups. The proportion of all subjects with acceptable intakes for each nutrient (where requirements are available) are also noted in Table 10 and Table 11. These proportions were also calculated and compared by disease group but did not differ significantly (Fisher p>0.05). Notably, reported dietary intake was quite poor for many nutrients with less than 50% of subjects having acceptable intakes for saturated fat, fibre, calcium, and sodium.

Physical activity records were completed by 24 HC, 11 SS, and 19 NASH patients. Total daily activity (physical activity units per day) did not differ between groups (p=0.8) (Figure 3). As strenuous physical activity may have a more significant effect on health as well as the IM, the combined time spent participating in strenuous and very strenuous activity was also compared between groups (Figure 4). Although there is a trend for higher strenuous activity in HC compared to other subjects this did not reach significance (p=0.1).
Table 10: Reported dietary intake and estimated requirements: Macronutrients

<table>
<thead>
<tr>
<th>Variable</th>
<th>HC (n=25)</th>
<th>SS (n=10)</th>
<th>NASH (n=20)</th>
<th>p-value</th>
<th>Requirement (%) total subjects with acceptable intake*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy Intake (kcal/kg/day)</td>
<td>27 (23, 33)</td>
<td>24 (20, 30)</td>
<td>18 (13, 21)</td>
<td>0.001</td>
<td>25-35 kcal/kg (22%)</td>
</tr>
<tr>
<td>Energy Intake (kcal/day)</td>
<td>2122 (569)</td>
<td>1798 (525)</td>
<td>1639 (612)</td>
<td>0.024</td>
<td></td>
</tr>
<tr>
<td>EER (kcal/day)</td>
<td>2582 (2141, 3316)</td>
<td>2399 (2185, 2639)</td>
<td>2565 (2117, 3052)</td>
<td>0.6</td>
<td>N/A</td>
</tr>
<tr>
<td>BMR (kcal/day)</td>
<td>1544 (1436, 1760)</td>
<td>1655 (1316, 1834)</td>
<td>1705 (1469, 1953)</td>
<td>0.4</td>
<td>N/A</td>
</tr>
<tr>
<td>Fat-derived Energy (%)</td>
<td>35 (7)</td>
<td>29 (7)</td>
<td>32 (10)</td>
<td>0.17</td>
<td>20-35% kcal (53%)</td>
</tr>
<tr>
<td>Protein-derived Energy (%)</td>
<td>17 (15, 19)</td>
<td>21 (18, 22)</td>
<td>19 (17, 21)</td>
<td>0.031†</td>
<td>10-35% kcal (98%)</td>
</tr>
<tr>
<td>Carbohydrate-derived Energy (%)</td>
<td>48 (44, 54)</td>
<td>51 (44, 56)</td>
<td>48 (39, 51)</td>
<td>0.5</td>
<td>45-35% kcal (58%)</td>
</tr>
<tr>
<td>Saturated Fat-derived Energy (%)</td>
<td>11 (10, 13)</td>
<td>8 (7, 11)</td>
<td>11 (10, 12)</td>
<td>0.15</td>
<td>&lt;10% kcal (40%)</td>
</tr>
<tr>
<td>Cholesterol (mg/day)</td>
<td>270 (174, 336)</td>
<td>239 (119, 354)</td>
<td>207 (146, 356)</td>
<td>&lt;300 mg</td>
<td>(64%)</td>
</tr>
<tr>
<td>Cholesterol (mg/1000 kcal)</td>
<td>129 (88, 156)</td>
<td>134 (99, 155)</td>
<td>143 (99, 208)</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Fibre (g/day)</td>
<td>24 (19, 27)</td>
<td>18 (14, 27)</td>
<td>17 (13, 21)</td>
<td></td>
<td>21-38 g (22%)</td>
</tr>
<tr>
<td>Fibre (g/1000 kcal)</td>
<td>11 (9, 13)</td>
<td>10 (8, 15)</td>
<td>10 (8, 12)</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Sugar-derived Energy (%)</td>
<td>19 (8)</td>
<td>15 (6)</td>
<td>16 (8)</td>
<td>0.17</td>
<td>N/A</td>
</tr>
<tr>
<td>Omega 6:3</td>
<td>6 (5, 8)</td>
<td>6 (4, 8)</td>
<td>7 (5, 8)</td>
<td>0.9</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Values are expressed as mean (standard deviation) for normal variables and median (1st quartile, 3rd quartile) for non-normally distributed variables.

Values with the same superscript are significantly different.

* Intake was compared to the Dietary Reference Intakes [210] and American Heart Association guidelines [211].

† Despite significant comparison with Krusal-Wallis test, pairwise comparisons did not reach significance.
Figure 1: Reported proportion of estimated energy requirement consumed

There was no statistically significant difference in proportion of estimated energy requirement (EER) consumed between healthy control (HC), simple steatosis (SS), and non-alcoholic steatohepatis (NASH) groups (HC: n=25; SS: n=10; NASH: n=20).

Figure 2: Reported proportion of basal metabolic rate consumed

There was no statistically significant difference in proportion of basal metabolic rate (BMR) consumed between healthy control (HC), simple steatosis (SS), and non-alcoholic steatohepatis (NASH) groups (HC: n=25; SS: n=10; NASH: n=20).
Table 11: Reported dietary intake and estimated requirements: Micronutrients

<table>
<thead>
<tr>
<th>Variable</th>
<th>HC (n=25)</th>
<th>SS (n=10)</th>
<th>NASH (n=20)</th>
<th>p-value</th>
<th>Requirement (% total subjects with acceptable intake)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A (IU/day)</td>
<td>11117 (5478, 16688)</td>
<td>6785 (5039, 12014)</td>
<td>8514 (6711, 13635)</td>
<td></td>
<td>2333-3000 IU (89%)</td>
</tr>
<tr>
<td>Vitamin A (IU/1000 kcal)</td>
<td>4892 (2687, 8683)</td>
<td>4935 (2921, 7409)</td>
<td>6465 (2418, 12198)</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Vitamin C (mg/day)</td>
<td>161 (91, 227)</td>
<td>108 (61, 132)</td>
<td>128 (69, 218)</td>
<td></td>
<td>75-90mg (71%)</td>
</tr>
<tr>
<td>Vitamin C (mg/1000 kcal)</td>
<td>79 (52, 112)</td>
<td>62 (42, 82)</td>
<td>76 (41, 177)</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>Choline (mg/day)</td>
<td>234 (183, 284)</td>
<td>212 (165, 303)</td>
<td>189 (119, 258)</td>
<td></td>
<td>425-550 mg (2%)</td>
</tr>
<tr>
<td>Choline (mg/1000 kcal)</td>
<td>117 (86, 147)</td>
<td>122 (95, 155)</td>
<td>120 (102, 155)</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Calcium (mg/day)</td>
<td>752 (666, 1269)</td>
<td>657 (437, 871)</td>
<td>509 (354, 1031)</td>
<td></td>
<td>1000-1200 mg (35%)</td>
</tr>
<tr>
<td>Calcium (mg/1000 kcal)</td>
<td>443 (313, 624)</td>
<td>325 (271, 604)</td>
<td>322 (205, 704)</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Iron (mg/day)</td>
<td>15 (11, 21)</td>
<td>15 (13, 20)</td>
<td>11 (9, 16)</td>
<td></td>
<td>8-18 mg (74.5%)</td>
</tr>
<tr>
<td>Iron (mg/1000 kcal)</td>
<td>7 (6, 9)</td>
<td>9 (8, 13)</td>
<td>7 (6, 10)</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Sodium (mg/day)</td>
<td>2619 (2233, 3400)</td>
<td>1971 (1620, 3290)</td>
<td>2199 (1529, 2709)</td>
<td></td>
<td>&lt;2300 mg (45%)</td>
</tr>
<tr>
<td>Sodium (mg/1000 kcal)</td>
<td>1340 (1047, 1791)</td>
<td>1381 (917, 1722)</td>
<td>1365 (1095, 1658)</td>
<td>0.9</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean (standard deviation) for normal variables and median (1st quartile, 3rd quartile) for non-normally distributed variables.
Values with the same superscript are significantly different.
* Intake was compared to the Dietary Reference Intakes [212-216].
Figure 3: Reported total daily physical activity completed

There was no statistically significant difference in average daily physical activity units completed between healthy control (HC), simple steatosis (SS), and non-alcoholic steatohepatis (NASH) groups (HC: n=24; SS: n=11; NASH: n=19).

Figure 4: Reported daily minutes of strenuous and very strenuous physical activity

There was no statistically significant difference in average daily physical activity units completed between healthy control (HC), simple steatosis (SS), and non-alcoholic steatohepatis (NASH) groups (HC: n=24; SS: n=11; NASH: n=19).
5.3 Bacterial Products

5.3.1 Plasma Endotoxin

Plasma endotoxin was not significantly different between groups (p=0.9) with a slightly narrower range of values for NASH compared to HC and SS (Figure 5). Intra-assay coefficient of variation (CV) was consistently below 10% and inter-assay CV was acceptable at 13.5%.

Figure 5: Comparison of plasma endotoxin concentration between groups

There was no statistically significant difference in plasma endotoxin concentration (EU/mL) between healthy control (HC), simple steatosis (SS), and non-alcoholic steatohepatitis (NASH) groups (HC: n=25; SS: n=14; NASH: n=25).
5.3.2 Short Chain Fatty Acids and Other Organic Acids

Our secondary outcome, fecal butyric acid, was not significantly different between groups (p=0.4) (Figure 6). Along with butyric acid the other main SCFA, propionate and acetate (measured as acetic acid), were also examined. Fecal propionate was significantly higher in SS patients than in HC (Figure 7). Fecal acetic acid and serum acetic acid did not differ between groups, p=0.19 and p=0.09 respectively (Figure 8 and 9).

The small number of subjects per group, particularly for SS, and the absence of significant differences between the three disease groups despite a tendency for SS and NASH values to be similar and HC to be different from SS and NASH led us to suspect that there might have not been sufficient power for 3-way comparisons. We therefore chose to combine the SS and NASH group into one NAFLD group for further metabolite analyses. Comparisons between HC and NAFLD for the above SCFA and other related organic acids are displayed below in Table 12.

When comparing these organic acids between HC and NAFLD subjects, total fecal SCFA, fecal propionate and isobutyric acid and serum 2-hydroxybutyrate were all significantly higher in NAFLD patients versus HC. The ratio of median acetate:propionate:butyrate in fecal samples for HC was 62.4:22.6:15.0 and was similar for NAFLD patients with a ratio of 61.5:22.6:15.9.
**Figure 6:** Comparison of fecal butyric acid between groups

There was no statistically significant difference in fecal butyric acid concentration (µM) between healthy control (HC), simple steatosis (SS), and non-alcoholic steatohepatitis (NASH) groups (HC: n=28; SS: n=14; NASH: n=24).

**Figure 7:** Comparison of fecal propionate between groups

Patients with simple steatosis (SS) had a significantly higher fecal propionate concentration (µM) than healthy controls (HC). HC and SS patients did not have a significantly different fecal propionate concentration than non-alcoholic steatohepatitis (NASH) patients (HC: n=28; SS: n=14; NASH: n=24).
Figure 8: Comparison of fecal acetic acid between groups

There was no statistically significant difference in fecal acetic acid concentration (µM) between healthy control (HC), simple steatosis (SS), and non-alcoholic steatohepatis (NASH) groups (HC: n=28; SS: n=14; NASH: n=24).

Figure 9: Comparison of serum acetic acid between groups

There was no statistically significant difference in serum acetic acid concentration (µM) between healthy control (HC), simple steatosis (SS), and non-alcoholic steatohepatis (NASH) groups (HC: n=28; SS: n=15; NASH: n=18).
Table 12: Fecal and serum SCFA and organic acids in healthy controls and NAFLD patients

<table>
<thead>
<tr>
<th>Fecal Metabolite (μM)</th>
<th>HC (n=28)</th>
<th>NAFLD (n=38)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butyric Acid</td>
<td>1366 (993, 2072)</td>
<td>1753 (1130, 2266)</td>
<td>0.2</td>
</tr>
<tr>
<td>Propionate</td>
<td>2052 (1598, 2419)</td>
<td>2485 (1985, 3649)</td>
<td>0.019</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>5665 (4025, 7061)</td>
<td>6781 (4619, 8384)</td>
<td>0.09</td>
</tr>
<tr>
<td>Formic Acid</td>
<td>60 (50, 73)</td>
<td>56 (42, 68)</td>
<td>0.2</td>
</tr>
<tr>
<td>Total SCFA (sum of above)</td>
<td>9251 (3286)</td>
<td>11633 (4832)</td>
<td>0.020</td>
</tr>
<tr>
<td>Isobutyric Acid</td>
<td>249 (199, 285)</td>
<td>293 (239, 372)</td>
<td>0.017</td>
</tr>
<tr>
<td>Isovalerate</td>
<td>181 (115, 223)</td>
<td>172 (136, 208)</td>
<td>0.9</td>
</tr>
<tr>
<td>Succinate</td>
<td>121 (67, 175)</td>
<td>94 (58, 187)</td>
<td>0.5</td>
</tr>
<tr>
<td>Serum Metabolite (μM)</td>
<td>HC (n=28)</td>
<td>NAFLD (n=33)</td>
<td>p-value</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>51 (37, 69)</td>
<td>37 (33, 59)</td>
<td>0.06</td>
</tr>
<tr>
<td>Formic Acid</td>
<td>55 (48, 64)</td>
<td>58 (51, 61)</td>
<td>0.6</td>
</tr>
<tr>
<td>2-Hydroxybutyrate</td>
<td>51 (40, 66)</td>
<td>69 (48, 78)</td>
<td>0.011</td>
</tr>
</tbody>
</table>

Values are expressed as mean (standard deviation) for normal variables and median (1st quartile, 3rd quartile) for non-normally distributed variables.

5.3.3 Metabolites Related to the Bacterial Metabolism of Choline

Fecal choline and trimethylamine as well as serum choline and TMAO were measured. Both fecal choline (Figure 10) and fecal trimethylamine (Figure 11) were significantly higher in NAFLD patients than in HC. Despite this difference in fecal values, serum choline (Figure 12) and TMAO (Figure 13) did not differ between HC and NAFLD patients, p=0.9 and p=0.5 respectively.
Figure 10: Fecal choline in healthy controls and NAFLD patients

There was a significantly higher level of fecal choline (µM) in the non-alcoholic fatty liver disease group (NAFLD) than in healthy control (HC) group (HC: n=28; NAFLD: n=38).

Figure 11: Fecal trimethylamine in healthy controls and NAFLD patients

There was a significantly higher level of fecal trimethylamine (µM) in the non-alcoholic fatty liver disease group (NAFLD) than in healthy control (HC) group (HC: n=28; NAFLD: n=38).
**Figure 12:** Serum choline in healthy controls and NAFLD patients

There was no statistically significant difference in serum choline concentration (µM) between the healthy control (HC) and non-alcoholic fatty liver disease (NAFLD) groups (HC: n=28; NAFLD: n=33).

**Figure 13:** Serum trimethylamine N-oxide in healthy controls and NAFLD patients

There was no statistically significant difference in serum trimethylamine N-oxide (TMAO) concentration (µM) between the healthy control (HC) and non-alcoholic fatty liver disease (NAFLD) groups (HC: n=28; NAFLD: n=33).
5.3.4 Ethanol

Serum ethanol was compared in the three disease groups, but levels did not differ significantly (p=0.5). There was also no significance when comparing HC with the merged NAFLD group (p=0.8) (Figure 14). Average daily reported alcohol intake was similar in HC vs SS vs NASH: 0 (0, 1.9) g vs 0.39 (0, 3.0) g vs 0 (0, 0.36) g (p=0.4).

Figure 14: Serum ethanol in healthy controls and NAFLD patients

There was no statistically significant difference in serum ethanol concentration (µM) between the healthy control (HC) and non-alcoholic fatty liver disease (NAFLD) groups (HC: n=28; NAFLD: n=33).

5.4 Intestinal Microbiota

Intestinal microbiota was compared between the three disease groups for total bacteria, Archaea, and presence of bacterial taxa of interest as a percentage of total bacteria and as bacterial counts (log cells/g wet feces). Firmicutes was defined as the sum of Lactobacilli, C. coccoides, and C. leptum. Full statistical analyses for all taxa are included in Table 13. There was no statistically significant difference in total bacteria or any other taxa of interest with the exception of C. leptum as a percent of total bacteria which was significantly lower in NASH versus HC (Figure 15) and E. coli count which was significantly higher in NASH versus HC (Figure 16).
Proportion of individuals with Archaea present was not significantly different between HC, SS, and NASH groups: 36%, 40%, and 50% respectively (p=0.6).

Table 13: Bacterial taxa of interest from qPCR by disease group

<table>
<thead>
<tr>
<th>Microbiota (log cells/g wet feces)</th>
<th>HC (n=28)</th>
<th>SS (n=15) †</th>
<th>NASH (n=24)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Bacteria</td>
<td>24.4 (0.62)</td>
<td>24.1 (0.43)</td>
<td>24.3 (0.75)</td>
<td>0.4</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>8.8 (0.66)</td>
<td>8.6 (0.83)</td>
<td>8.3 (1.2)</td>
<td>0.14</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>22.8 (0.97)</td>
<td>22.4 (0.93)</td>
<td>22.6 (0.97)</td>
<td>0.5</td>
</tr>
<tr>
<td>Firmicutes:Bacteroidetes</td>
<td>1.1 (0.65)</td>
<td>1.1 (0.73)</td>
<td>1.5 (1.2)</td>
<td>0.2</td>
</tr>
<tr>
<td>Bifidobacteria</td>
<td>9.4 (8.4, 9.7)</td>
<td>9.6 (9.2, 10.2)</td>
<td>9.2 (7.5, 9.8)</td>
<td>0.4</td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>6.4 (0.92)</td>
<td>6.4 (0.90)</td>
<td>6.5 (0.83)</td>
<td>0.8</td>
</tr>
<tr>
<td>C. coccoides</td>
<td>9.4 (0.42)</td>
<td>9.4 (0.37)</td>
<td>9.5 (0.32)</td>
<td>0.7</td>
</tr>
<tr>
<td>C. leptum</td>
<td>9.8 (9.5, 10.0)</td>
<td>9.5 (9.2, 9.8)</td>
<td>9.4 (8.5, 9.9)</td>
<td>0.10</td>
</tr>
<tr>
<td>E. coli</td>
<td>6.9 (1.2)</td>
<td>7.8 (0.99)</td>
<td>7.8 (1.4)</td>
<td>0.016</td>
</tr>
<tr>
<td>Archaea ‡</td>
<td>6.9 (2.3)</td>
<td>5.8 (2.1)</td>
<td>6.9 (2.3)</td>
<td>0.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Microbiota (% total bacteria)</th>
<th>HC (n=28)</th>
<th>SS (n=15) †</th>
<th>NASH (n=24)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroidetes</td>
<td>2.4 (0.92, 3.8)</td>
<td>3.5 (0.29, 7.4)</td>
<td>0.78 (0.30, 2.8)</td>
<td>0.19</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>23 (9.0)</td>
<td>21.4 (10.5)</td>
<td>21.7 (10.1)</td>
<td>0.9</td>
</tr>
<tr>
<td>Bifidobacteria</td>
<td>5.1 (0.73, 12.3)</td>
<td>17.2 (4.5, 27.6)</td>
<td>2.7 (0.14, 14.6)</td>
<td></td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>0.005 (0.002, 0.020)</td>
<td>0.008 (0.002, 0.064)</td>
<td>0.011 (0.003, 0.023)</td>
<td>0.7</td>
</tr>
<tr>
<td>C. coccoides</td>
<td>6.5 (4.3, 9.5)</td>
<td>8.6 (4.9, 11.3)</td>
<td>8.7 (5.5, 14.6)</td>
<td>0.3</td>
</tr>
<tr>
<td>C. leptum</td>
<td>15.9 (8.8, 17.9)</td>
<td>10.2 (5.5, 14.2)</td>
<td>7.4 (1.5, 14.8)</td>
<td>0.030</td>
</tr>
<tr>
<td>E. coli</td>
<td>0.14 (0.02, 0.63)</td>
<td>0.08 (0.01, 0.43)</td>
<td>0.06 (0.003, 0.86)</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Values are expressed as mean (standard deviation) for normal variables and median (1st quartile, 3rd quartile) for non-normally distributed variables. Values with the same superscript are significantly different.

† Analysis of Bifidobacteria was done in n=14 patients.
‡ Archaea was only present in 10 HC, 6 SS, 12 NASH patients.
**Figure 15:** *C. leptum* relative abundance in stool samples from three study groups

*C. leptum* bacteria as a percentage of total bacteria was significantly lower in the non-alcoholic steatohepatitis (NASH) group versus the healthy control (HC) group. There was no difference in *C. leptum* relative abundance between the HC and simple steatosis (SS) groups or between the NASH and SS groups (HC: n=28; SS: n=15; NASH: n=24).
5.5 Relationships between Bacterial Metabolites, Bacteria of Interest, Disease Status, and Diet

Several correlations were studied to investigate potential mechanisms for bacteria and their metabolites on disease status as well as the possible influence of diet. Correlations between metabolites and NAFLD related parameters were investigated first (Table 14). These correlations were run only in NAFLD patients with the exception of BMI which was examined in all patients. Correlations that were considered moderately strong and relevant to potential disease mechanisms are featured in Figures 17 to 21. In fecal metabolites there was a significant positive correlation between fecal formic acid and total inflammation on liver histology, and between
both fecal choline and fecal trimethylamine and BMI. In serum metabolites there was a significant negative correlation between serum acetic acid and ballooning intensity and between serum choline and liver steatosis. A negative correlation also appeared to be significant between serum choline and ballooning intensity ($r_s=-0.40, p=0.022$) but is believed to be superfluous following visual inspection.

Relationships between bacterial taxa of interest and NAFLD related parameters were also examined in the same method as mentioned above. Results are summarized in Table 15. Bacterial count and relative abundance were examined in all correlations, however, only measures where there was some significant relationships are shown. The measure displayed is noted in a footnote below the table. A significant positive correlation was seen between liver steatosis and Firmicutes count (Figure 22).

Relationships between bacterial taxa of interest and metabolites were then investigated using Spearman’s correlations in all patients (Table 16). There were several positive correlations between bacterial taxa and metabolites, particularly between fermenters such as Firmicutes, *C. leptum*, and *C. coccoides*, and fermentation products including butyric acid, formic acid, and acetic acid. One of the strongest correlations was between *C. leptum* and fecal butyric acid (Figure 23). *E. coli* count and relative abundance were not correlated to serum ethanol levels ($p<0.05$).

Dietary composition was also compared with metabolites (Table 17) and bacterial taxa (Table 18) in all patients. Dietary variables examined included macronutrient composition (protein, fat, and carbohydrates as a proportion of total energy), fibre (g/1000 kcal), and micronutrients (vitamin C, E, A, and calcium in units per 1000 kcal). Some products of protein metabolism by bacteria including succinate and isovalerate were positively correlated with protein intake while some products of carbohydrate fermentation including butyric and acetic acid were correlated with fibre and vitamin consumption. Although relatively weak, a relationship of particular interest was seen between *C. leptum* and fibre intake (Figure 24). A positive correlation was also observed between carbohydrate intake and plasma endotoxin (Figure 25).

Correlations between those metabolites that were measured in both stool and serum, for example fecal choline and serum choline, were not significant ($p>0.05$). Correlations discussed above
were also done for HC and NAFLD groups separately in an attempt to investigate differences in bacterial metabolism. Relationships were generally similar between disease groups.

Table 14: Correlation between fecal and blood metabolites and NAFLD related parameters

<table>
<thead>
<tr>
<th>Fecal Metabolite</th>
<th>Steatosis (%)</th>
<th>Fibrosis</th>
<th>Total Inflammation</th>
<th>Lobular Inflammation</th>
<th>Ballooning Intensity</th>
<th>NAS</th>
<th>HOMA-IR</th>
<th>BMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butyric Acid</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Propionate</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Formic Acid</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Isobutyric Acid</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Isovalerate</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>r_s=0.36 p=0.040 n=32</td>
<td>NS</td>
</tr>
<tr>
<td>Succinate</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Choline</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>r_s=0.43 p=0.0003 N=66</td>
</tr>
<tr>
<td>Trimethylamine</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>r_s=0.32 p=0.010 N=66</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Blood Metabolite</th>
<th>Steatosis (%)</th>
<th>Fibrosis</th>
<th>Total Inflammation</th>
<th>Lobular Inflammation</th>
<th>Ballooning Intensity</th>
<th>NAS</th>
<th>HOMA-IR</th>
<th>BMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic Acid</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>r_s=-0.44 p=0.012 n=32</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Formic Acid</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>2-Hydroxybutyrate</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>r_s=-0.40 p=0.022 n=32</td>
<td>NS</td>
<td>NS</td>
<td>r_s=-0.37 p=0.003 n=61</td>
</tr>
<tr>
<td>Ethanol</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Choline</td>
<td>r_s=-0.40</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>r_s=-0.40 p=0.022 n=32</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>r_s=-0.48</td>
<td>p=0.005 n=32</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMAO</td>
<td>r_s=0.38</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Correlations are for NAFLD patients only for all parameters except for BMI which was compared in all patients.
Figure 17: Relationship between fecal formic acid and total inflammation in NAFLD patients

![Graph showing the relationship between fecal formic acid and total inflammation score.]

**NAFLD patients only (n=15)**  
$r_s=0.61$  \( p\text{-value}=0.015 \)

Figure 18: Relationship between fecal choline and body mass index in all subjects

![Graph showing the relationship between fecal choline and BMI.]

**All Subjects (n=66)**  
$r_s=0.43$  \( p\text{-value}=0.0003 \)
**Figure 19:** Relationship between fecal trimethylamine and body mass index in all subjects

![Graph showing the relationship between BMI and fecal trimethylamine](image)

**All Subjects (n=66)**  
$r_s=0.32$  
*p-value=0.010*

**Figure 20:** Relationship between serum acetic acid and hepatocyte ballooning intensity in NAFLD patients

![Graph showing the relationship between serum acetic acid and ballooning intensity](image)

**NAFLD patients only (n=32)**  
$r_s=-0.44$  
*p-value=0.012*
Figure 21: Relationship between serum choline and liver steatosis in NAFLD patients

![Graph showing the relationship between serum choline and liver steatosis in NAFLD patients.](image)

NAFLD patients only (n=32) $r_s=-0.40$ p-value=0.022

Table 15: Correlation between intestinal microbiota by qPCR and NAFLD related parameters

<table>
<thead>
<tr>
<th></th>
<th>Steatosis (%)</th>
<th>Fibrosis</th>
<th>Total Inflammation</th>
<th>Lobular Inflammation</th>
<th>Ballooning Intensity</th>
<th>NAS</th>
<th>HOMA-IR</th>
<th>BMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroidetes</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>C. coccoides</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>C. leptum</td>
<td>$r_s=0.41$</td>
<td>p=0.012</td>
<td>$r_s=0.25$</td>
<td>p=0.038</td>
<td>$r_s=0.48$</td>
<td>p=0.005</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Bifidobacteria</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>E. coli</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>$r_s=0.48$</td>
<td>p=0.005</td>
</tr>
<tr>
<td>Archaea</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>$r_s=0.48$</td>
<td>p=0.005</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>$r_s=0.47$</td>
<td>p=0.003</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Correlations are for % of total bacteria except for E. coli and Archaea where count/g wet feces was used. Correlations are for NAFLD patients only for all parameters except for BMI which was compared in all patients.
Figure 22: Relationship between Firmicutes count and liver steatosis in NAFLD patients

![Graph showing the relationship between Firmicutes count and liver steatosis in NAFLD patients. The x-axis represents Steatosis (% of hepatocytes) ranging from 0 to 100, and the y-axis represents Log Cells Firmicute per g Feces ranging from 8 to 11. The data points are shown for NAFLD patients only (n=38), with \( r_s = 0.47 \) and p-value = 0.003.]

Figure 23: Relationship between \( C. \) leptum count and fecal butyric acid in all subjects

![Graph showing the relationship between \( C. \) leptum count and fecal butyric acid in all subjects. The x-axis represents Fecal Butyric Acid (\( \mu \)M) ranging from 330 to 8320, and the y-axis represents Log Cells \( C. \) leptum per g Feces ranging from 5 to 11. The data points are shown for all subjects (n=66), with \( r_s = 0.41 \) and p-value = 0.0006.]
Table 16: Correlations between intestinal microbiota and metabolites in all subjects

<table>
<thead>
<tr>
<th>METABOLITE</th>
<th>Fecal (n=66)</th>
<th>Blood (n=59)</th>
<th>BACTERIAL TAXA OF INTEREST</th>
<th>Correlations with IM presented are for %bacteria except for Firmicutes, Archaea, C. leptum, E. coli, and C. coccoides where cell count/g wet feces was used.</th>
<th>Bacteroidetes</th>
<th>Firmicutes</th>
<th>Lactobacilli</th>
<th>C. coccoides</th>
<th>C. leptum</th>
<th>Bifidobacteria</th>
<th>E. coli</th>
<th>Archaea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butyric Acid</td>
<td>NS</td>
<td>r_s=-0.38</td>
<td>NS</td>
<td>r_s=-0.41</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Propionate</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>r_s=-0.41</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>r_s=-0.27, p=0.031</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Formic Acid</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>r_s=-0.27, p=0.031</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Isovalerate</td>
<td>r_s=-0.31</td>
<td>p=0.010</td>
<td>NS</td>
<td>r_s=-0.31, p=0.010</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Succinate</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>r_s=0.27, p=0.026</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Choline</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>r_s=0.27, p=0.026</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Trimethylamine</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>r_s=0.27, p=0.026</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>2-Hydroxybutyrate</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>r_s=0.28, p=0.029</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Ethanol</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>r_s=0.28, p=0.029</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Choline</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>r_s=-0.34, p=0.009</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>TMAO</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>r_s=-0.34, p=0.009</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>r_s=-0.34, p=0.009</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>
Table 17: Correlations between metabolites and diet in all subjects

<table>
<thead>
<tr>
<th>METABOLITE</th>
<th>REPORTED DIETARY INTAKE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Fecal (n=52)</strong></td>
</tr>
<tr>
<td></td>
<td>Fat (%kcal/BMR)</td>
</tr>
<tr>
<td>Butyric Acid</td>
<td>NS</td>
</tr>
<tr>
<td>Propionate</td>
<td>r_s =-0.31, p=0.028</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>NS</td>
</tr>
<tr>
<td>Isobutyric Acid</td>
<td>r_s =-0.29, p=0.037</td>
</tr>
<tr>
<td>Isovalerate</td>
<td>NS</td>
</tr>
<tr>
<td>Succinate</td>
<td>NS</td>
</tr>
<tr>
<td>Choline</td>
<td>NS</td>
</tr>
<tr>
<td>TMA</td>
<td>NS</td>
</tr>
<tr>
<td>Blood (n=48)†</td>
<td></td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>NS</td>
</tr>
<tr>
<td>2-Hydroxybutyrate</td>
<td>NS</td>
</tr>
<tr>
<td>L-Lactic Acid</td>
<td>NS</td>
</tr>
<tr>
<td>Ethanol</td>
<td>NS</td>
</tr>
<tr>
<td>Choline</td>
<td>NS</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>r_s =-0.33, p=0.019</td>
</tr>
</tbody>
</table>

† n=50 for plasma endotoxin correlations only
**Table 18:** Correlations between intestinal microbiota by qPCR and diet in all subjects

<table>
<thead>
<tr>
<th>Bacterial Taxa of Interest</th>
<th>Fat (%kcal/BMR)</th>
<th>Carbohydrate (%kcal)</th>
<th>Protein (%kcal)</th>
<th>Fibre (g/1000kcal)</th>
<th>Vitamin A (IU/1000kcal)</th>
<th>Vitamin E (mg/1000kcal)</th>
<th>Vitamin C (mg/1000kcal)</th>
<th>Calcium (mg/1000kcal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroidetes</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td><em>C. coccoides</em></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td><em>C. leptum</em></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>r_s = 0.29</td>
<td>r_s = 0.33</td>
<td>r_s = 0.38</td>
<td>r_s = 0.38</td>
<td>r_s = 0.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p = 0.032</td>
<td>p = 0.015</td>
<td>p = 0.005</td>
<td>p = 0.015</td>
<td>n = 53</td>
</tr>
<tr>
<td>Bifidobacteria</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Archaea</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>r_s = 0.38</td>
<td>r_s = 0.38</td>
<td>r_s = 0.38</td>
<td>r_s = 0.38</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p = 0.005</td>
<td>p = 0.016</td>
<td>p = 0.005</td>
<td>p = 0.015</td>
<td>n = 53</td>
</tr>
</tbody>
</table>

Correlations with IM displayed are for bacterial taxa as a percent of total bacteria except for Firmicutes, *E. coli* and Archea where count/g wet feces is shown.
**Figure 24:** Relationship between relative abundance of *C. leptum* and reported dietary fibre intake in all subjects

![Scatter plot showing the relationship between relative abundance of *C. leptum* and dietary fibre intake.](image)

All Subjects n=53  \( r_s = 0.29 \) p-value=0.032

**Figure 25:** Relationship between plasma endotoxin and reported carbohydrate intake in all subjects

![Scatter plot showing the relationship between plasma endotoxin and carbohydrate intake.](image)

All Subjects (n=50)  \( r_s = 0.39 \) p-value=0.005
6 Discussion

To our knowledge, this is the first human study that measures both the IM and bacterial metabolites in adults with biopsy confirmed SS, NASH, and HC. The primary and secondary study outcome, plasma endotoxin and fecal butyrate, did not differ between these groups. However, other SCFA and metabolites showed differences between NAFLD patients and HC. NAFLD patients had higher fecal total SCFA, propionate and isobutyric acid and higher serum 2-hydroxybutyrate than HC. Significant differences were also found between NAFLD and HC for fecal compounds related to the bacterial metabolism of dietary choline. Fecal choline and trimethylamine were higher in NAFLD patients but serum choline and TMAO did not differ from HC. HC, SS, and NASH patients had similar total bacteria counts in stool samples as measured by qPCR and the proportion of subjects who had detectable Archaea did not differ between groups. NASH patients were found to have lower proportions of Clostridium leptum and higher counts of Escherichia coli in their feces. Some bacterial metabolites and IM taxa were correlated with histological findings and dietary intake, suggesting potential contribution to NAFLD pathogenesis.

6.1 Endotoxin

Based on the findings of four human studies in NAFLD patients [129-132], three of which measured blood endotoxin levels using a similar method to ours [129, 130, 132], we predicted that NASH patients would have a significantly higher plasma endotoxin level than SS or HC. We found no significant differences between the three disease groups, and no perceivable trend. This was also found in a study released last month which found no difference in endotoxin measured via LAL assay between HC and NAFLD patients [217]. Endotoxin levels in the entire study population ranged from 4.54 EU/mL to 7.14 EU/mL with a slightly narrower range for NASH patients (5.14-7.00 EU/mL). This was similar to the levels found by Harte et al. (~3.2-14.8 EU/mL) who used the same assay [130], but higher than the levels seen by Thuy et al. and Volynets et al. who used the same assay type from a different company and whose values ranged from about 0.001-0.28 EU/mL [129, 132]. Studies in other disease populations also appear to be shifted up or down as an entire study population, but one would still expect to see a difference between groups if it was present. There are two main factors which we believe may have played
a role in the lack of significant differences in endotoxin level between our groups: diet and
disease severity.

Dietary intake was measured in both Thuy et al. and Volynets et al. and in both of these studies
NAFLD patients were found to have a higher dietary intake of carbohydrates, particularly
fructose in Thuy’s study [129, 132]. Volynets also found a positive correlation between
carbohydrate intake and endotoxin level. Although the mechanism for this correlation is unclear
it is possible that in both of these studies the higher endotoxin level in the NAFLD group was
due to higher carbohydrate intake levels rather than disease state. Interestingly, we also found a
positive correlation between plasma endotoxin and dietary carbohydrate intake, but our disease
groups did not differ in levels of carbohydrate intake. It is conceivable therefore that we did not
see a difference in endotoxin between groups because their carbohydrate intake was equal.

Notably a negative correlation was detected in our patients between reported fat intake and
endotoxin level. This is in contradiction to previous animal studies that have found a positive
correlation between these two variables [127, 218-220]. This is thought to be due to a fat
mediated uptake of endotoxin whereby fats are incorporated into chylomicrons, which also have
a high affinity for endotoxin, and therefore endotoxin enters circulation along with chylomicrons
[221, 222]. It is important to note that the blood used for endotoxin analysis in our patients was
drawn after a 12 hour fast therefore recent fat intake would not have had a significant effect on
endotoxin intake. Fat absorption occurs in the small intestine rather than the colon so colonic
endotoxin as a result of microbiota should not be influenced by fat uptake. It is therefore
plausible that this is a superfluous correlation that reflects a dietary composition with high
carbohydrate intake rather than low fat intake being related to higher endotoxin levels.

The third study that measured plasma endotoxin in a NAFLD population did not account for
dietary intake, but endotoxin levels were positively correlated with HOMA-IR [130]. We did see
a difference in HOMA-IR between our HC and NASH group, but the magnitude of this
difference was far lower than that seen in Harte’s study [130]. In their study NASH patients were
much more insulin resistant with a median HOMA-IR of 8.6 whereas our NASH patients only
had a HOMA-IR of 4.26. This is likely because the diabetic individuals in our study were taking
medication and were well controlled. If our patients had poorer control and greater insulin
resistance we may have seen a greater level of endotoxin in this group. A study by Verdam et al.
that looked at IgG levels against endotoxin rather than blood endotoxin concentration found that IgG levels were positively correlated with NASH severity [131]. In our study the NASH patients had relatively mild disease with a median NAS of only 5 out of a possible 8. It is conceivable that the severity of the disease was not great enough to cause endotoxin differences between disease groups.

6.2 SCFA and Related Metabolites

This is the first human study to measure fecal butyric acid in biopsy confirmed NAFLD patients versus HC. A study by Wong et al. found that NASH patients had a lower prevalence of butyrate producing *Faecalibacterium*, but they did not measure butyrate levels [14]. Animal studies showed beneficial effects of butyrate producing probiotic supplementation including improvements in weight, steatosis, FFA, inflammation, and reduced intestinal permeability [154, 155]. One of these studies was also able to correlate these beneficial improvements with fecal and plasma butyrate concentrations [155]. In our study there was no difference in fecal butyrate between the three groups or between the combined NAFLD group and HC. There was, however, a significant difference in the prevalence of the butyrate producing *C. lepum* bacteria, with a significantly lower relative abundance in NASH patients. This will be discussed separately below.

It is perhaps naïve to presume that we would see a clear connection between bacterial prevalence of butyrate producers, fecal butyrate, and disease state. The concentration of fecal SCFA is frequently used as a proxy for measuring the bacterial production of these compounds, but it does not account for differences in colonic absorption. We assume that a higher concentration of fecal butyrate means that more butyrate was produced and more butyrate will therefore be absorbed in the colon. A study by Vogt and Wolever, however, found otherwise [223]. Vogt and Wolever conducted a study where subjects infused a SCFA solution into their colon, retained it for several minutes, and then voided. Absorption of the three main SCFA as well as combinations thereof were measured and compared with SCFA composition in the subject’s normal feces. They found that the acetate absorption from the colonic infusion was negatively correlated with acetate concentration in normal stools and that other SCFA did not have significant correlation between these two measures [223]. This could mean that a lower fecal concentration of a certain SCFA indicates a greater absorption rather than a lower production
from the IM. The amount of SCFA produced and absorbed can also be influenced by fecal transit time, which was not measured in this study [224]. Further research is needed to better understand the production and uptake of butyrate in different disease states. It is also important to note that the proposed mechanism for the beneficial role of butyrate uptake in healthy patients was in preventing excessive intestinal permeability. Jiang et al. and Volynets et al. did find increased intestinal permeability in NAFLD patients, but did not look at relationships between permeability, IM, and SCFA production [15, 132].

The ratio of SCFA in our NAFLD patients and HC (approximately 62:23:15 acetate:propionate:butyrate) was fairly similar to the commonly quoted ratio of 60:20:20 and 60:25:15 noted in the literature [224]. When looking at the other main SCFA and related carboxylic acids in fecal and serum samples we found a significantly greater level of total fecal SCFA, fecal propionate, fecal isobutyric acid, and serum 2-hydroxybutyrate in NAFLD patients compared to HC. A greater amount of total fecal SCFA, particularly propionate, was previously found in overweight and obese adults compared to lean controls [118, 225]. As discussed in section 2.2.1 it has been suggested that the human obese IM is enriched with genes that code for the bacterial metabolism of macronutrients leading to an increased energy absorption of up to 150 kcal per day [119, 121]. The increased energy harvest from SCFA is one potential mechanism through which these metabolites may affect NAFLD pathogenesis. However, in order to confirm this we would have needed to conduct bomb calorimetry on stool samples, which was not included in our study protocol.

The primary role of propionate, once absorbed and taken up by the liver, is to function as a substrate for gluconeogenesis [226]. As an additional energy substrate propionate could increase adiposity and potentially contribute to steatosis. Further studies on the mechanistic role of propionate on steatosis and energy balance, including gene expression would be helpful.

Fecal isobutyric acid is a branched chain fatty acid that is formed by the bacterial catabolism of branched chain amino acids [224]. Isobutyric acid is an isomer of butyric acid and is also closely related to propionate (with the addition of a methyl branch) [227]. The functional role of isobutyric acid is not well described, but as it is an intermediate in protein breakdown it is possible that elevated levels in the NAFLD group are secondary to the increased protein intake that was observed in these individuals.
2-hydroxybutyrate (alpha-hydroxybutyrate) is a metabolite formed during amino acid catabolism and glutathione anabolism [228]. Although this compound has been mentioned as an intermediary in the bacterial metabolism of amino acids, it is not clear how much is actually produced and absorbed in the human colon and incorporated into the blood [229]. It is therefore more likely that the serum levels measured in our study, which were found to be significantly higher in NAFLD patients, are due to the presence of NAFLD and its related metabolic syndrome. Oxidative stress can increase the production of glutathione in the liver and may therefore increase the amount of 2–hydroxybutyrate produced [228]. Research has recently suggested that the oxidative stress triggered by insulin resistance, a primary mechanism in NAFLD pathogenesis, can increase circulating levels of 2–hydroxybutyrate [230, 231]. Li et al. conducted metabolic profiling in 48 diabetic patients and 31 healthy controls [230]. They found 5 biomarkers that accurately distinguished diabetic patients from healthy controls, including 2–hydroxybutyrate [230]. Similarly, a study by Gall et al. conducted non-targeted biochemical profiling on 399 subjects who also underwent the gold-standard hyperinsulinemic euglycemic clamp testing and oral glucose tolerance testing for identification and grading of insulin resistance [231]. After statistical analysis, 2–hydroxybutyrate was identified as the best candidate biochemical to separate insulin resistant from insulin sensitive individuals independent of age, sex, or BMI [231]. It is therefore likely that the higher 2–hydroxybutyrate levels that we see in NAFLD patients are due to insulin resistance and resulting oxidative stress rather than alterations in IM metabolism. Indeed, 2-hydroxybutyrate was the only serum metabolite correlated with BMI, pointing to its potential relationship to the metabolic state of NAFLD patients.

Two SCFA were also correlated with markers of disease severity in NAFLD patients. Fecal formic acid, the smallest of the SCFA, was positively correlated with total inflammation. This is a novel finding in NAFLD and little research exists on the physiological effects of formic acid. One study found an increased level of serum formic acid in patients with inflammatory bowel disease in comparison to healthy controls, which may also support a pro-inflammatory role, but the mechanism remains unknown [232]. Serum acetic acid seemed to have a more beneficial effect in NAFLD patients with a negative correlation with hepatocyte ballooning intensity. Recently it was suggested that acetate may have an anti-inflammatory function through interaction with GPR43, but the exact mechanism through which acetic acid may reduce ballooning intensity is not known [169].
In relation to dietary intake, some of the fecal and serum metabolites measured were positively correlated with potential substrates for their production. Fecal isovalerate and succinate, products of the catabolism of protein by IM, were positively correlated with protein intake [224]. Fecal butyric acid was correlated with fibre and vitamin A intake. Although not all fibres are fermentable, many of the substrates used for butyrate production would be categorized as a fibre by the data analysis software. Vitamin A may also be a marker of these substrates as they are both primarily found in plant-based foods. Serum acetic acid was positively correlated with Vitamin E, which is again a primarily plant-derived vitamin which may be an indicator of a relationship between this SCFA and plant-based substrates available for fermentation.

### 6.3 Metabolites Related to the Bacterial Metabolism of Choline

Another proposed mechanism for IM in NAFLD pathogenesis is through the bacterial metabolism of dietary choline to trimethylamine. We hypothesized that NAFLD patients would have a lower circulating choline level due to an increased bacterial breakdown of choline which would also result in increased fecal trimethylamine and serum TMAO. We did observe a higher fecal trimethylamine level as well as a higher fecal choline level. Unfortunately we cannot link fecal choline with dietary choline intake as this dietary information was incomplete in our database. We did, however, see a slightly higher intake of proteins in NAFLD patients which may include choline rich foods such as eggs, meat, and fish. This may have led to a higher amount of choline reaching the colon. This is supported by the positive correlation that we found between both fecal choline and fecal trimethylamine and reported dietary protein intake. We could therefore suggest that a higher fecal choline (substrate) level resulted in a higher fecal trimethylamine (product) level. Serum choline and TMAO, however, did not differ between groups.

Previous human studies looking at the role of choline in NAFLD pathogenesis have not consistently measured serum choline. The only study that did so was by Nehra et al. They were looking at a potential role for choline deficiency as a result of celiac sprue in NAFLD pathogenesis [233]. They found no correlations between disease severity and serum choline levels. They did not compare choline levels in NAFLD patients to HC. It is important to note that the proposed role for choline deficiency in NAFLD pathogenesis is through a decreased production and export of VLDL from the liver as a result of lower phosphatidylcholine
availability. A previous study from our research group did find that SS and NASH patients had lower hepatic phosphatidylcholine than HC [180]. It is plausible therefore that although serum levels are not different between our groups, phosphatidylcholine levels are reduced as a result of reduced choline bioavailability. The issue with this theory is that fecal choline levels were higher in NAFLD patients. Like SCFA, however, we are unsure of whether a high fecal level reflects a high colonic absorption or a low colonic absorption.

Our study is the first in humans to measure both serum choline and NAFLD characteristics on liver histology. Interestingly, serum choline was negatively correlated with liver steatosis and hepatic ballooning intensity in NAFLD patients. The role of dietary choline deficiency in steatosis is well known [10, 119, 154] and a potential role of low choline availability in disease progression through oxidative and endoplasmic reticulum stress has also been discussed [175-178]. Despite no significant difference in levels between disease groups our findings still support the role of reduced choline availability in NAFLD pathogenesis.

As mentioned previously, trimethylamine is absorbed in the colon and processed in the liver to form TMAO. This conversion is catalyzed by flavin monooxygenase 3 (FMO3) [234]. The expression of FMO, however, can vary from person to person and result in varying levels of serum TMAO [235]. It is therefore plausible that a significantly greater amount of fecal TMA is absorbed in NAFLD patients but may not have resulted in a significantly higher level of TMAO. The effect of high TMA levels in the liver and circulation appear to be benign. Individuals who lack FMO3 due to a genetic mutation develop a condition called trimethylaminuria where high levels of TMA results in a fishy odour in the patient’s sweat, urine, and saliva, however no other physiological consequences are mentioned [235]. Serum TMAO levels, however, has been associated with several chronic diseases including cardiovascular disease [236-238] and it is known that NAFLD patients are at increased risk of cardiovascular disease [239]. However, our current findings do not show higher levels of TMAO in NALFD patients or associations with NAFLD pathogenesis.

Lastly, it is important to note that both fecal choline and fecal trimethylamine were correlated with BMI. It is possible that as individuals with NAFLD are heavier, greater BMI but not disease state is associated with higher levels of these two metabolites. Unfortunately as our groups differ significantly by BMI it is not possible to decipher the effect of disease versus BMI. Further
studies with a sufficient sample size of obese healthy controls would better distinguish the difference between BMI and NAFLD.

6.4 Ethanol

The last metabolite of particular interest in our study was serum ethanol. We predicted, in line with the findings of Zhu et al., that patients with NASH would have a significantly higher ethanol level than HC and SS patients and that this would be related to a higher prevalence of ethanol producing bacteria such as *E. coli* [11]. We did not find a significant difference in serum ethanol levels between disease groups, but our mean levels were quite similar to the mean level for the HC and obese patients in the Zhu et al. study which was approximately 25 µM compared to their NASH group which was approximately 35 µM [11]. It is important to consider, however, that the Zhu study was conducted on children who were assumed to have zero alcohol intake. It is known that increased exposure to ethanol leads to increased metabolic tolerance, meaning that ethanol is more readily metabolized and blood ethanol levels decrease more quickly after repeated exposures [240]. It is therefore possible that as our NAFLD patients were adults they were better able to tolerate ethanol exposure and therefore their serum levels quickly returned to the normal levels seen in the HC, whereas in the children a slight increase in ethanol exposure had a more lasting effect and continued to be elevated in serum. This does not mean that ethanol exposure from bacterial metabolism in adult NAFLD patients is benign as the ethanol will still travel directly from the colon to the liver, potentially causing an increased production of toxic metabolic intermediates such as acetaldehyde or increased intestinal permeability [173, 241].

6.5 Intestinal Microbiota

Disease groups did not differ significantly in the relative abundance or counts of Bacteroidetes, Firmicutes, Archaea, Bifidobacteria, Lactobacilli, or *C. coccoides*. Two main differences, however, were found in the bacterial composition of fecal samples in NASH patients compared to healthy controls. NASH patients had a significantly lower proportion of the *C. leptum* bacterial group as a percent of total bacteria and a significantly higher count of *E. coli* compared to healthy controls.

The *C. leptum* bacterial group was lower in NASH patients. It is also known as Clostridial Cluster IV which compose approximately 16-25% of the intestinal bacteria [242, 243]. The
bacteria contained in this group ferment carbohydrates in the colon to produce SCFA, particularly the most abundant bacteria in this group, *Faecalibacterium prausnitzii* which is a butyrate producer [153]. Indeed, *C. leptum* was positively correlated with dietary fibre intake which would include potential substrates for this fermentation. One of the few human studies on NAFLD and microbiota by Wong et al. similarly found that patients with NASH had a significantly lower abundance of *Faecalibacterium* [14]. They suggested that *F. prausnitzii* may have protective anti-inflammatory properties, preventing NASH [244]. Another possible mechanism could be that lower *Faecalibacterium* in that study’s NASH group could reduce the production of butyrate which is known to have anti-inflammatory action [150, 151] and strengthening effects on the intestinal epithelium [145-147]. Although differences in fecal butyric acid between disease groups was not significant in our study, we did see a positive correlation between *C. leptum* abundance and fecal butyric acid levels.

The significantly higher abundance of *E. coli* in NASH patients in comparison to healthy controls is also supported by the literature. Zhu et al. found that children with NASH had a significantly higher abundance of *Escherichia* than both HC and obese children [11]. This was associated with higher serum ethanol in NASH patients [11]. As discussed above our study did not find a significant difference in ethanol levels between groups and additionally serum ethanol was not correlated with *E. coli* abundance. This again may be due to adults’ capacity to clear ethanol efficiently. Future metagenomic studies may be able to look at the genetic capacity of the entire IM to better understand if the bacterial production of ethanol is associated with NASH.

A previous study by our research group, using some of the same subjects, found a significantly lower relative abundance of Bacteroidetes in NASH patients compared to HC [13]. Although we saw a trend towards this relationship, it did not reach significance. It is possible that this is simply because the relationship was lost with a larger sample size, particularly of healthy controls, or this may be influenced by length of stool sample storage. Research has shown that with extended storage of stool samples at -80 °C the ratio of Firmicutes:Bacteroidetes increases [245]. It is therefore possible that the relationship that was previously seen is no longer significant due to changes that occurred in storage. A sub-analysis comparing results from the subjects with qPCR from DNA extracted in 2012 and DNA extracted in 2014 is planned to further investigate differences in IM that may occur during storage.
Lastly, although Firmicutes did not differ between groups, Firmicutes count was positively correlated with hepatic steatosis. The Firmicutes phylum contains many fermenting bacteria and is therefore responsible for much of the SCFA production [111]. It is therefore possible that the difference in the amount of total fecal SCFA in the feces of the NAFLD patients compared to HC is due, in part, to a higher count of Firmicutes which produce an influx of energy to the liver, contributing to fat accumulation.

6.6 Diet and Physical Activity

The dietary data used in this study was collected from a 7-day food record with the assistance of validated 2D visual models for portion estimates and clear instructions provided by a nutrition expert. All food records were reviewed and follow-up questions were asked of the subject to clarify any missing or unclear details regarding method of preparation or ingredients consumed. The use of a 7-day food record in this study is superior to the food frequency questionnaires used in many studies because it accounts for recent dietary intake which is of greatest importance for the IM and metabolites and is a better estimate of individual micro and macronutrient intake [246]. In addition, 7-day food records are completed at the time of consumption and therefore reduce the risk of memory-related errors.

Dietary intake between the three disease groups was similar with the exception of energy intake. A trend was seen for higher protein intake in SS patients but was not significant after Bonferroni correction for multiple comparisons. Reported kilocalorie intake was significantly lower in NASH patients compared to HC, however, there was no difference between HC and SS patients or NASH and SS patients. The EER (energy requirements including needs for reported physical activity) and BMR (energy required for basic physiological functioning) were then compared with reported energy intake, a method used to estimate underreporting [247]. The proportion of EER reported was not different between groups, but the proportion of BMR reported was. The NASH patients reported consuming a significantly smaller proportion of their BMR than the HC with a median that was below 100%. Physiologically someone who is consuming less than their BMR should be losing weight, however, none of our subjects reported weight loss. There are two possible explanations for this discrepancy. One is that the BMR did not accurately estimate these patients’ metabolic rate, which may be depressed secondary to metabolic disturbances, reduced lean body mass or a sedentary lifestyle. The second is that NASH patients may be underreporting
their dietary intake. Underreporting is particularly suspected as this has been reported disproportionately in overweight and obese individuals [65, 248, 249].

To address the possibility of underreporting in study subjects we chose to focus on dietary composition rather than energy intake. We have therefore expressed nutrients as a proportion of energy intake and in the case of fat have also adjusted for age and weight by dividing the percent of kilocalories from fat by BMR as was done in one of our previous publications [13]. These energy-controlled values were used for all statistical analyses and dietary intake remained similar between groups. Notably dietary quality was quite poor in all patient groups, reflecting a typical Western-style diet with low dietary fibre intake and high intakes of saturated fat and sodium. This was also seen in a previous study from our lab [60].

Physical activity was recorded in a self-reported 7-day activity record. Average daily physical activity units completed and average daily minutes of strenuous and very strenuous physical activity did not differ between disease groups, however, a trend was observed for a lower level of high intensity physical activity in SS and NASH patients. Previous work from our team saw a significantly greater proportion of NASH patients than HC who were classified as sedentary [60]. This is significant as physical activity can reduce excess body weight and improve insulin resistance and other metabolic abnormalities that contribute to NAFLD [56, 250-252]. However, only vigorous activity was reported to be associated with decreased risk of developing NASH and advanced fibrosis [58]. Like dietary intake, it is possible that physical activity was overestimated in our obese subjects who may have perceived some physical activities as more strenuous than HC [253, 254]. Literature in the field of IM only saw differences in bacteria based on physical activity between sedentary individuals and athletes therefore it is unlikely that any small, statistically insignificant, differences between physical activity in our groups affected IM and metabolites [255].

6.7 Potential Confounders

Aside from the variables already discussed above there were a few other demographic and clinical variables that differed between disease groups: age, country of origin, BMI, insulin resistance, chronic disease diagnosis and medication use. Many of these differences were expected as they are risk factors for or part of the pathogenesis of NAFLD. The potentially confounding effect of each is discussed below.
Patients in both the SS and NASH group were significantly older than HC. This was expected because age is a risk factor for the development of NAFLD [1]. Age may affect the production of bacterial metabolites secondary to an increase in colonic transit time [224, 256, 257], however, the research regarding the effect of age on transit time is mixed [258, 259]. It is also unclear at what age transit time slows with the majority of studies focusing on seniors who were older than our patient population. IM composition has also been shown to change with age, but remains relatively stable in adults until they are elderly meaning that age is unlikely to affect the IM composition of our subjects [260].

Although the ethnicity of our patients does not differ significantly between groups, we did see a significantly higher proportion of Canadian-born individuals in the HC group than in the SS and NASH groups. The intestinal microbiota of children and adults has been shown to differ significantly between countries [261-264]. The differences between these populations, however, have been largely attributed to dietary differences such as an increased amount of fermentable fibre in the diet of African children versus those in Europe [261]. What is unknown is whether individuals who immigrate to Canada develop a ‘Westernized IM’ or retain their original IM composition. Further studies are needed to understand this transition.

BMI was significantly higher in the NASH group than HC and trended higher for SS versus HC but this difference did not reach statistical significance. This was expected as BMI is a primary risk factor for NAFLD [1]. This was also expected because although the HC group did have a median BMI in the overweight range, the BMI of these patients was somewhat limited secondary to the nature of their recruitment. HC were recruited from a group of healthy living liver donors. Due to the high prevalence of NAFLD in obese individuals, typically obese people are not considered as potential donors. Due to the fact that BMI and disease status were closely related in our patient samples, the representation of diagnostic groups within each BMI category was very unbalanced, which prevented the possibility of stratified analysis or ANCOVA to control for BMI in order to distinguish the effect of BMI from that of disease diagnosis. Interestingly only fecal choline, fecal trimethylamine, and serum 2-hydroxybutyrate were significantly correlated with BMI.

Biochemical differences between disease groups were also secondary to disease state including higher AST and ALT in SS and NASH patients than in HC and higher insulin, HOMA-IR, and
blood triglyceride level in NASH patients. NASH patients also had an increased prevalence of hypertension, which is unsurprising as obesity is a known risk factor for both diseases [1, 265]. Medication use differed in accordance with clinical conditions with a significantly higher proportion of NASH and SS patients taking cardiovascular medications, primarily antihypertensives, than HC and with significantly more NASH patients taking lipid lowering drugs than HC. Interestingly, SS patients took significantly more NSAIDS than HC. Only patients taking low dose NSAIDS were included in this study, but it is possible that without these anti-inflammatory medications some of these patients may have progressed on to NASH. No studies to date have investigated this potential preventative treatment.
7 Strengths and Limitations

This was the first study in humans to measure both intestinal microbiota and bacterial products in biopsy confirmed SS and NASH patients in comparison to healthy controls. This is one step forward in our understanding of the role of IM in NAFLD pathogenesis through various bacterial products. Given the high prevalence of NAFLD and limited treatment options it is worthwhile to consider the role of the IM and its metabolites for further research on potential therapies or preventative strategies.

With the exception of one previous study from our group [13] no other human study has compared the IM of NAFLD patients with HC where all patient groups had liver biopsies available for histological analysis. Liver biopsies are currently the gold standard for NASH diagnosis and disease grading [2] but previous studies have been limited by the ethical issue for obtaining a liver biopsy from a healthy patient. Our healthy living liver donor group allows us to obtain this vital data without a separate invasive procedure. Even in HC who were unable to undergo surgery and therefore did not provide a liver biopsy, analysis of liver health was significantly more detailed than previous studies which relied primarily on normal liver enzymes.

Although dietary and physical activity data may have been limited by self-reporting, it is a strength of this study that we were able to compare these lifestyle factors between groups and examine the relationship between diet, IM, and metabolite composition. This data therefore allowed us to make connections that were limited in other studies.

There were some limitations in this study as well. As this was a cross-sectional study, causal relationships cannot be drawn and further work is therefore necessary to understand the physiological mechanisms behind the relationships that we observed. A significant limitation was the small sample size in our SS group which was half of what was determined to be required a priori. This was due to the fact that SS is largely a benign and asymptomatic disease that is usually detected by ultrasound and does not require a liver biopsy. Although our SS patients had elevated liver enzymes that necessitated a referral to a hepatologist and subsequent liver biopsy, those patients are now generally followed with new surrogate markers such as Fibrotest [266] or Fibroscan [267] as per the American Gastroenterological Association guidelines [2], so liver biopsies are no longer performed unless patients demonstrate features suggestive of NASH or...
fibrosis. Despite this limitation ours is one of few studies on NAFLD and IM that diagnosed SS via liver biopsy.

In this study IM composition was limited to the taxa selected for qPCR, meaning that an understanding of the full IM community is not possible. The value of having bacterial counts, however, is not to be underestimated as the actual amount of bacteria present is of particular interest when comparing bacteria and metabolites. Future analyses that compare the full IM community using Illumina 16s rRNA sequencing and metagenomics are in progress.
8 Conclusions

The findings of this study provide further evidence for a relationship between the IM, bacterial products, and disease severity in NAFLD. More specifically we have observed in NAFLD patients: a higher presence of some fermentation products, the production of potentially harmful metabolites, the altered metabolism of an essential nutrient (choline), and differences in IM composition. In relation to the study aims, hypotheses, and proposed mechanisms, the study results were as follows:

- The study did not find a significant difference in plasma endotoxin or fecal butyric acid levels between HC, SS, and NASH patients.
- NAFLD patients had higher fecal total SCFA, propionate, isobutyric acid, choline, and trimethylamine, and serum 2-hydroxybutyrate than HC. Fecal choline and trimethylamine and serum 2-hydroxybutyrate were positively correlated with BMI.
- Metabolites including fecal formic acid, serum choline, and serum acetic acid were correlated with histological features of NAFLD.
- NASH patients had lower prevalence of *C. leptum* and higher amounts of *E. coli* in their stool. No other differences existed for IM composition.
- Reported energy intake was significantly lower in NASH patients than in HC and tended to be higher in protein derived energy for SS and NASH patients than for HC.
- Positive correlations were observed between substrates and products of bacterial metabolism: protein with succinate and isovalerate, and sources of fermentable carbohydrates with butyric and acetic acid.
- Bacterial taxa were also correlated with substrates and in some cases, products of their metabolic processes, including: *C. leptum* with sources of fermentable carbohydrates and serum acetic acid, and Firmicutes with sources of fermentable carbohydrates, fecal butyric acid, and serum acetic acid.

This study contributes to growing evidence suggesting the potential benefit of therapeutic alteration of the IM in NAFLD prevention and treatment. Future research is necessary to further evaluate the role of the IM and bacterial products in NAFLD pathogenesis and to investigate the potential exploitation of this relationship for disease prevention and treatment.
9 Future Directions

There are several future research projects that could expand on the findings presented in this thesis. Of primary interest would be the metagenomics analysis of the bacterial IM of these patients. This data would allow us to fill in some of the gaps in the mechanistic information coming from the current study. We would, for instance, be able to look at the gene expression for carbohydrate fermentation and butyrate production to better understand if the higher relative abundance of butyrate producers that we observed actually results in a higher butyrate production. Current methods are limited by our incomplete understanding of the relevance of fecal SCFA content in relation to SCFA production and absorption. Metagenomic studies would also be able to determine if a higher *E. coli* count results in a greater production of ethanol or if the overall metagenome results in differences in ethanol production between NASH patients and HC. Planning for this type of study is underway.

Another important step to undertake in order to better understand the potential role of butyrate would be to measure the abundance of butyrate producers, or ideally the bacterial gene expression for butyrate production, while at the same time measuring fecal butyrate and intestinal permeability/integrity. Similar studies, although not as comprehensive, have been able to look at the role of butyrate producing probiotics with fecal butyrate and intestinal permeability in mice, but this has not been examined in humans [154, 155]. Intestinal permeability can be measured in a variety of ways in adults with NAFLD. Previous studies have used transmission electron microscopy to observe microvilli and tight junctions or immunohistochemical analysis of zona occludins-1 expression in intestinal biopsies [15, 134] or less invasive techniques including the measurement of lactulose/mannitol or (51)Cr-ethylene diamine tetraacetate in urine [132, 134].

Of particular interest in the role of bacterial metabolism of choline would be to look at the expression of flavin monooxygenases in the liver, which is responsible for the conversion of trimethylamine to TMAO. This may help us to better understand whether trimethylamine metabolism after absorption is related to NAFLD disease status. Comparisons of fecal and serum choline to phosphatidylcholine and expression of genes involved in the conversion of choline to this active form, e.g. *PEMT*, would also be of interest since we did not see the expected lower serum choline level in NAFLD patients, despite a higher production of trimethylamine.
A useful method for future studies would be to match NAFLD and HC by BMI. As BMI is so closely tied to the NAFLD disease pathogenesis this will be a difficult task, but larger centers, or multicenter trials may be able to accomplish this matching which would allow for a better understanding of factors involved in NAFLD pathogenesis irrespective of weight.

The use of chemostat models, recently adapted to allow for the growth and manipulation of human IM communities may be of particular use in future research [268]. Chemostats may be particularly interesting when trialing the effects of potential pre or probiotic supplements or dietary modifications as researchers can observe how these interventions alter IM and bacterial metabolites directly. These basic science studies may then provide the data needed to plan for larger human intervention trials with supplementation or potentially fecal transplantation. Fecal transplantation has been successfully used for the treatment of Clostridium difficile infection [269, 270] and was recently shown to improve insulin sensitivity [271].


Appendices

APPENDIX 1

Patient Information Sheet & Consent Form

**Study Title:** Non-alcoholic Steato-hepatitis (NASH) Versus Simple Hepatic Steatosis: Is There a Difference in the Nutritional Factors Influencing Lipid Peroxidation and Inflammation?

**PRINCIPAL INVESTIGATOR:** Dr. Johane P. Allard  **Tel:** (416) 340-5159

**SPONSOR:** Canadian Liver Foundation & Canadian Institutes of Health Research

**Introduction**

You were referred to a liver specialist because of elevated liver enzymes and/or fat infiltration in your liver detected by blood work or abdominal ultrasound. As part of standard medical care, a hepatologist (liver specialist) ordered a liver biopsy to make a diagnosis. A biopsy consists of a very small sample of your liver taken with a needle. Prior to having the liver biopsy, a research study coordinator will contact you to ask you if you would be interested in participating in a research study. The only way to determine if you are eligible for the study is to examine the sample of tissue taken from your liver biopsy, but, we first need your permission to be able to look at the tissue.

If you agree to allowing us to look at a portion of the liver biopsy, and if the tissue confirms the diagnosis of liver steatosis (fat in the liver) or non-alcoholic steatohepatitis (fat with inflammation) and no other causes are found or you have a completely normal liver biopsy result, the research coordinator will contact you to see if you agree to have further assessments. It is important that you understand the purpose, procedures, benefits, discomfort, risks and precautions associated with the study before you agree to participate, so that you can make an informed decision. This is known as the informed consent process. If there is anything in this form that you do not understand, please consult the study coordinator. Make sure all your questions have been answered to your satisfaction before signing this document. Of course, you have the right to refuse to participate and also the right to withdraw from the study at any time.

**Purpose of the Study**

Fatty infiltration of the liver can be associated with alcohol, obesity, diabetes and abnormal fat and sugar handling by your body. When alcohol intake is excluded, this is called a “Non-Alcoholic Fatty Liver” or “NAFLD”. If it is associated with inflammation (cell damage), it is called “Non-Alcoholic Steato-Hepatitis” or “NASH”. No one knows why some patients with excess fat in their liver remain stable while others progress to a more advanced disease like cirrhosis.

Therefore, the purpose of this study is to determine if nutritional factors measured in your blood and diet or the kind of bacteria that are found in the intestinal tract can have an effect on liver tissue damage, the type of fat deposited in the liver and also the genes involved in fat and sugar metabolism. We are also measuring bacterial products in stool and blood samples.
About 190 patients with liver-biopsy proven simple steatosis (fat infiltration), NASH (fat with inflammation) or people with minimal findings on liver biopsy will participate in this study at The Toronto General Hospital and The Toronto Western Hospital. For the purpose of this study, nutritional assessment and blood work will be done only in one occasion, and we will ask the participants for one single stool sample. Fifty patients with healthy liver will serve as a control group.

**Study Procedures**

If you consent to participate in this study, the following study procedures are to be performed at each study visit.

**First Visit:** (Approximately 30 minutes)
This visit is the present clinical appointment with your liver specialist where the decision is made to do the liver biopsy. You are now presented with the consent form for the study and meet with the study coordinator who explains the study and ask you to sign the consent. You will be instructed to record your food intake and physical activity for a period of 7 days. You will also receive instructions and the material to collect a stool sample.

**Second Visit**

**According to standard care,** you need a liver biopsy. This is an in-hospital procedure performed by a liver specialist using local anesthesia. Local anesthesia means that a small area of your skin over the liver will be frozen with a very small needle and then a specially designed needle will be inserted between the ribs into the liver. The biopsy takes only a moment and there is slight discomfort during the procedure. In some cases, the liver specialist cannot perform the liver biopsy due to anatomical reasons. In this case, you will be referred to the ultrasound department for your liver biopsy. The procedure is the same, but ultrasound is used to locate the best place to obtain the liver sample, and the person performing the procedure will be an interventional radiologist. During the procedure, usually 1 – 2 small standard samples (3.0 cm long x 1 cm thickness (liver specialist) or 2.5 cm long x 1 mm thickness (radiology)) will be taken from your liver to assess for the presence of fat and inflammation (you will be provided with the standard consent form routinely used for performing liver biopsy procedure). The study coordinator is present during the liver biopsy and takes a portion of the sample for future tests. After the biopsy you will be kept under observation for 2 to 4 hours.

We will ask you to collect a stool sample the day before your liver biopsy and bring the sample with you to the hospital, where you give it to the study coordinator. We will ask you to fill out an environmental questionnaire that inquires about factors that could influence the bacteria composition in your stool.

The saved portion of the liver biopsy and the stool sample will be identified by a code and if you decide not to participate in the study, they will be destroyed.

*This entire visit will last 4-5 hours.*

**Third Visit: Follow-up with liver specialist for liver biopsy results (1-2 months after)**

After the liver biopsy, you will need a follow-up appointment with the liver specialist to discuss the results of the liver biopsy and possible treatments. You will be contacted by the study
coordinator about 10 days before the follow-up visit if the diagnosis of simple steatosis or NASH is made and you qualify for the rest of the study. You will be instructed to record your food intake for a period of 7 days (forms will be given to you in the first visit). Before your visit, you will need to be in a fasting state for 12 hours (nothing to eat or drink overnight) for blood work and nutritional assessment.

During this visit, you will return the 7-days food and activity record. The study coordinator will record your medical history and review your medical chart for your drug regimen and previous blood results. Your body fat will be measured (by taking the circumferences of your waist and hip). Using a device, your skinfold thickness will be measured at several sites on your body (mainly at your arms and at the back of your shoulders). You will have blood taken (5 tablespoons) for different measurements.

This visit will take 30 minutes with the study coordinator.

**Participation and Withdrawal**
Your participation in this study is voluntary. You can choose not to participate and you may withdraw at any time without affecting your medical care. During the course of the study, you will be kept informed of any new treatments or finding that may influence your participation in the study. It is your responsibility to follow the directions and rules of the study. The data gathered from this study is being monitored on an on-going basis by the study coordinator. You will be notified of the results of the study once the study is completed.

**Risks**
There is always a small risk involved in invasive procedures such as the liver biopsy. These risks are not related to research procedures and they are associated with your routine clinical care. To minimize this risk, the biopsies will be done in the hospital premises by a very experienced liver specialist or interventional radiologist. There may be some pain (in about 10% of cases), which is usually mild and does not last long. Occasionally the pain is more severe and requires painkillers. However, this also usually passes within a few hours. Bleeding may occur in about 1 in 5000 to 1 in 10,000 cases. Rarely the needle will penetrate another organ, such as the gall bladder. All such complications are usually managed without requiring surgery. There may also be a risk associated with drawing blood with a needle which include discomfort, bruising, or infection where the needle goes in.

**Benefits**
You may not receive any medical benefit from your participation in this study. However, depending on the results of your tests, you may be asked in the near future (2-3 months) to participate in another study where an intervention will be proposed to improve your liver condition.

**Remuneration**
There will be no costs to you for participating in the study and you will not be charged for any research procedure. You will receive $50.00 for participating in the study to compensate for your time and traveling expenses once you complete the study. If you leave the study early, you will be reimbursed for the time you have spent in the study.
**Compensation**
If you become ill or are physically injured as a result of participation in this study, medical treatment will be provided. The reasonable costs of such treatment will be covered by your health insurance for any injury or illness that is directly a result of participation in this trial. In no way does signing this consent form waive your legal rights nor does it relieve the investigators, sponsors or involved institutions from their legal and professional responsibilities”.

**Confidentiality**
All information obtained during the study will be held in strict confidence. All the samples will be identified by a code. No names or identifying information will be used in any publication or presentations. For the purpose of verification of clinical trial procedures and/or data, members of specific agencies in the presence of the investigator or his/her staff may look at sections of your medical records that are related to the study without participant’s names attached. These agencies include: members of the Research Ethic Board and a representative from the Canadian Liver Foundation and the Canadian Institutes of Health Research. By signing this form, you are agreeing to make your study records available to the above-mentioned individuals. The above-mentioned individuals will not see information that is not related to the study. No information identifying you will be transferred outside the investigators in this study.

**Contact Person**
If you have any questions about the study, please contact Bianca Arendt (Study Coordinator) at (416) 340-4104 or Dr. Johane Allard at (416) 340-5159.
If you have any questions about your rights as a research subjects, please call the chair of the University Health Network Research Ethics Board at (416) 340-4557.

**Consent**
I have had the opportunity to discuss this study and my questions have been answered to my satisfaction. I consent to take part in the study with the understanding that I may withdraw at any time without affecting my medical care. I have received a signed copy of this consent form. I voluntarily consent to participate in this study. I also understand that there is no guarantee that this study will provide any benefit to me.

_______________________
Patient’s Name (Please Print)

_______________________
Patient’ Signature

______________
Date

The potential benefits, risks and procedures associated with this study have been fully explained to the volunteer and he/she has had ample time and opportunity to ask questions and to decide whether or not to participate in the study. A copy of the signed consent form has been given to the participant.

_______________________
Name of Person Obtaining Consent

_______________________
Signature

______________
Date
Patient Information Sheet & Consent Form
Healthy Liver Donors

**Study Title:** Non-alcoholic Steato-hepatitis (NASH) Versus Simple Hepatic Steatosis: Is There a Difference in the Nutritional Factors Influencing Lipid Peroxidation and Inflammation?

**PRINCIPAL INVESTIGATOR:** Dr. Johane P. Allard  **Tel:** (416) 340-5159

**SPONSOR:** Canadian Liver Foundation & Canadian Institutes of Health Research

**Introduction**
You were seen in the Transplant Clinic of the University Health Network and you have consented to operative and treatment procedures to donate part of your liver for liver transplantation. In addition, you have signed consent to store a sample of your liver tissue for future research. We are seeking your permission to use a portion of your liver, and samples of your subcutaneous (fat under your skin) and mesenteric fat (fat around the internal organs) tissue (0.5-1 g) as a control for a nutritional study.

If you agree to allow us to look at portions of your liver and fat tissue, the research coordinator will contact you to see if you agree to have further nutritional assessments.

It is important that you understand the purpose, procedures, benefits, discomfort, risks and precautions associated with the study before you agree to participate, so that you can make an informed decision. This is known as the informed consent process. If there is anything in this form that you do not understand, please consult the study coordinator. Make sure all your questions have been answered to your satisfaction before signing this document.

Of course, you have the right to refuse to participate and also the right to withdraw from the study at any time.

**Purpose of the Study**
Fatty infiltration of the liver can be associated with alcohol, obesity, diabetes and abnormal fat and sugar handling by your body. When alcohol intake is excluded, this is called a “Non-Alcoholic Fatty Liver” or “NAFLD”. If it is associated with inflammation (cell damage), it is called “Non-Alcoholic Steato-Hepatitis” or “NASH”. No one knows why some patients with excess fat in their liver remain stable while others progress to a more advanced disease like cirrhosis and liver failure requiring liver transplantation. The purpose of this study is to determine if nutritional factors measured in the blood and diet or the kind of bacteria that are found in the intestinal tract can have an effect on liver tissue damage, the type of fat deposited in the liver and also the genes involved in fat and sugar metabolism. We are also measuring bacterial products in stool and blood samples.

You are asked to serve as a healthy control subject for this study. We would like to have similar measurements from people with a healthy liver for comparison.

About 190 patients with liver-biopsy proven simple steatosis (fat infiltration), NASH (fat with inflammation) or people with minimal findings on liver biopsy will participate in this study at The Toronto General Hospital and The Toronto Western Hospital. For the purpose of this study, nutritional assessment and blood work will be done only in one occasion, and we will ask the
participants for one single stool sample. Fifty patients with healthy liver will serve as a control group.

**Study Procedures**
If you consent to participate in this study, the following study procedures are to be performed.

**First Visit: (Approximately 30 minutes)**
This visit is the present clinical appointment in the Transplant Clinic. You are now presented with the consent form for the study and meet with the study coordinator who explains the study and ask you to sign the consent. The study coordinator will review your medical chart and may ask you some questions regarding your medical history and medications that you are taking. You will then be instructed to record your food intake and physical activity for a period of 7 days. Your body fat will be measured (by taking the circumferences of your waist and hip). Using a device, your skinfold thickness will be measured at several sites on your body (mainly at your arms and at the back of your shoulders).

You will need to be in a fasting state for 12 hours (nothing to eat or drink overnight) for blood work. If you are fasting for this visit, you will have blood taken (5 table spoons) for different measurements. If you are not in a fasting state, this will be done in the next visit. You will also receive instructions and the material to collect a stool sample and an environmental questionnaire that inquires about factors that could influence the bacteria composition in your stool. This form should be filled out on the same day you are collecting the stool.

**Second Visit (The day of the surgery)**
During this visit, you will return the 7-days food, the activity record, the environmental questionnaire and the stool sample. The study coordinator is present during the surgery and takes the samples for future tests. The stool sample and the saved tissue portions will be identified by a code and if you decide not to participate in the study, they will be destroyed.

**Participation and Withdrawal**
Your participation in this study is voluntary. You can choose not to participate and you may withdraw at any time without affecting your medical care. It is your responsibility to follow the directions and rules of the study. The data gathered from this study is being monitored on an on-going basis by the study coordinator.

**Risks**
There may be a small risk associated with drawing blood with a needle which include discomfort, bruising, or infection where the needle goes in.

**Benefits**
You may not receive any medical benefit personally from your participation in this study. However, your participation will help understand the mechanism by which fatty liver disease can develop and progress.

**Remuneration**
There will be no costs to you for participating in the study and you will not be charged for any research procedure. You will receive $50.00 for participating in the study to compensate for your time once you complete the study. If you leave the study early, you will be reimbursed for the time you have spent in the study.

**Compensation**
If you become ill or are physically injured as a result of participation in this study, medical treatment will be provided. The reasonable costs of such treatment will be covered by your
health insurance for any injury or illness that is directly a result of participation in this trial. In no way does signing this consent form waive your legal rights nor does it relieve the investigators, sponsors or involved institutions from their legal and professional responsibilities”.

Confidentiality
All information obtained during the study will be held in strict confidence. All the samples will be identified by a code. No names or identifying information will be used in any publication or presentations. For the purpose of verification of clinical trial procedures and/or data, members of specific agencies in the presence of the investigator or his/her staff may look at sections of your medical records that are related to the study without participant’s names attached. These agencies include: members of the Research Ethic Board and a representative from the Canadian Liver Foundation and the Canadian Institutes of Health Research. By signing this form, you are agreeing to make your study records available to the above-mentioned individuals. The above-mentioned individuals will not see information that is not related to the study. No information identifying you will be transferred outside the investigators in this study.

Contact Person
If you have any questions about the study, please contact Bianca Arendt (Study Coordinator) at (416) 340-4104 or Dr. Johane Allard at (416) 340-5159.
If you have any questions about your rights as a research subject, please call the chair of the University Health Network Research Ethics Board at (416) 340-4557.

Consent
I have had the opportunity to discuss this study and my questions have been answered to my satisfaction. I consent to take part in the study with the understanding that I may withdraw at any time without affecting my medical care. I have received a signed copy of this consent form. I voluntarily consent to participate in this study. I also understand that there is no guarantee that this study will provide any benefit to me.

__________________________
Patient’s Name (Please Print)  Patient’ Signature  Date

The potential benefits, risks and procedures associated with this study have been fully explained to the volunteer and he/she has had ample time and opportunity to ask questions and to decide whether or not to participate in the study. A copy of the signed consent form has been given to the participant.

__________________________
Name of Person Obtaining Consent  Signature  Date
APPENDIX 2

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 3

Environmental Questionnaire

Patient ID ____________  Visit Date ____________

1. What country were you born in? _____________________________

2. If you were not born in Canada, when did you immigrate? ___________

3. What is your ethnic background? (Check all that apply)
   □ African-Canadian  □ Asian  □ Caucasian
   □ First Nation  □ Hispanic  □ Hispanic-Black
   □ Middle Eastern  □ Other ________________________ (specify)

4. How were you born?
   □ Caesarian section (C-section)  □ Natural birth  □ I don’t know

5a. As an infant, were you ever breastfed?
   □ Yes  □ Never  □ I don’t know

5b. If “Yes”, how long were you breastfed?
   □ Less than 6 month  □ More than 6 months  □ I don’t know

6a. Do you have any allergies or food intolerances?
   □ Yes  □ No  □ I don’t know

6b. If “Yes”, please specify: ________________________________

7a. Do you have any history of gastro-intestinal diseases (e.g. inflammatory bowel disease, celiac disease, gastric ulcer)?
   □ Yes  □ No  □ I don’t know

7b. If “Yes”, please specify: ________________________________

8a. Did you ever have any major abdominal surgery?
   □ Yes  □ No  □ I don’t know
8b. If “Yes”, please specify: _______________________
    Date of surgery: _____________________   (mm/yyyy)

9a. During the last 6 months, did you use any antibiotics?
    □ Yes    □ No    □ I don’t know

9b. If “Yes”, when was the last time you used antibiotics?
    _____________________   (dd/mm/yy)

10a. During the last 6 months, did you use any laxatives?
    □ Yes    □ No    □ I don’t know

10b. If “Yes”, how often do you use a laxative?   _________________

10c. If “Yes”, when was the last time you used a laxative?
    _____________________   (dd/mm/yy)

11. Are you using any pre- or probiotic or synbiotic products on a regular basis (as a food product or as a supplement such as tablets, powder, etc.)?
    □ Yes    □ No    □ I don’t know
    Name(s) of the product(s): ________________________

12. Did you travel outside of North-America during the last 6 months?
    □ Yes    Country: ________________    □ No

13a. Did you have any gastrointestinal infections during the last 6 months (e.g. traveller’s diarrhea)
    □ Yes    □ No    □ I don’t know

13b. If “Yes”, when did you have the last infection?
    _____________________   (dd/mm/yy)

14. Do you have any pets at home?
    □ Yes    What animal(s)? ________________    □ No

15a. Female Participants ONLY: Are you using any contraceptives?
    □ Yes    Specify: ________________    □ No
15b. When was the first day of your last period?
____________________ (dd/mm/yy) □ I am past my menopause

15c. Usual length of your menstrual cycle? ___ days □ I don’t know
APPENDIX 4

Diet Intake Records

General Information:

Please read instructions before beginning to record your food intake.

Please keep an accurate record of everything you eat and drink for 3 days – 1 day should be on the week-end. Include all snacks, alcoholic beverages, cigarette smoking, and condiments.

- List each food item in as much detail as possible.
- Try to describe the amount of food eaten.
- Include the portion size model for each food item consumed.
- For each food, briefly describe how it was prepared.
- Include brand names where possible.

<table>
<thead>
<tr>
<th>Recommendations of 2D Models Used To Describe Foods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume Models (A Side)</td>
</tr>
<tr>
<td>Beverages</td>
</tr>
<tr>
<td>Cakes, quiches, pies</td>
</tr>
<tr>
<td>Canned goods, vegetables, fruits, fish, etc.</td>
</tr>
<tr>
<td>Casseroles, stews, potted meats, cottage cheese</td>
</tr>
<tr>
<td>Cereals, beans, nuts, chips</td>
</tr>
<tr>
<td>Fresh cooked vegetables, fruits</td>
</tr>
<tr>
<td>Frozen cooked vegetables, fruits</td>
</tr>
<tr>
<td>Lettuce and other salad items</td>
</tr>
<tr>
<td>Noodles, macaroni, spaghetti, rice, etc.</td>
</tr>
<tr>
<td>Sauces, soups, gravy, salad dressing, etc.</td>
</tr>
<tr>
<td>Sugar and other condiments</td>
</tr>
<tr>
<td>Weight Model (B Side)</td>
</tr>
<tr>
<td>Beef, lamb, pork, fish</td>
</tr>
<tr>
<td>Luncheon meats</td>
</tr>
<tr>
<td>Solid cheese</td>
</tr>
<tr>
<td>Sliced chicken, turkey</td>
</tr>
<tr>
<td>Biscuits, muffins, cakes, some cookies</td>
</tr>
</tbody>
</table>

E.g. Beef and Broccoli Stir Fry – should be recorded as:

<table>
<thead>
<tr>
<th>Food Item</th>
<th>Description</th>
<th>No.</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef strips</td>
<td>Lean, stir fry</td>
<td>1.0</td>
<td>A15</td>
</tr>
<tr>
<td>Broccoli</td>
<td>Fresh, stir fry</td>
<td>0.5</td>
<td>A15</td>
</tr>
<tr>
<td>Pea Pods</td>
<td>Fresh, stir fry</td>
<td>0.25</td>
<td>A15</td>
</tr>
<tr>
<td>Beef Sauce</td>
<td>Thick salty</td>
<td>0.25</td>
<td>A15</td>
</tr>
</tbody>
</table>

Checklist: Have you included the amount and kind of:

- Spread on bread, toast, vegetables, rice, etc.....
- Milk and/or LAct-aid, LActaeze, soy milk in cereal and beverages
- Salad dressing; fats, oils in cooling, frying, etc
- Sugar, jams, jellies, syrups
- Candy, chocolate
- Snack foods such as potato chips, pretzels, etc.
- All fluids/beverages
# DAILY FOOD INTAKE

ID: _________________________   Day of Week: ____________   Date: ________________

<table>
<thead>
<tr>
<th>Time</th>
<th>Food Item</th>
<th>Description (frozen, fried, broiled; salted; brand name; etc.)</th>
<th>No. of serving</th>
<th>Amount/ Model</th>
<th>Multiple/ B side Thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**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>

ID: ________________________  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 of Exercise</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>