

Intestinal Microbiota in Patients with Non-Alcoholic Fatty Liver Disease

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Abbreviations: ALT, alanine aminotransferase; BMI, body mass index; BMR, basal metabolic rate; HC, healthy control; IM, intestinal microbiota; IR, insulin resistance; LPS, lipopolysaccharide; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; SS, simple steatosis.

Abstract: Despite evidence that the intestinal microbiota (IM) is involved in the pathogenesis of obesity, the IM composition of patients with non-alcoholic fatty liver disease (NAFLD) has not been well characterized. This prospective, cross-sectional study was aimed at identifying differences in IM between adults with biopsy-proven NAFLD (simple steatosis [SS] or non-alcoholic steatohepatitis [NASH]) and living liver donors as healthy controls (HC). Fifty subjects were included: 11 SS, 22 NASH and 17 HC. One stool sample was collected from each participant. Quantitative real-time polymerase chain reaction was used to measure total bacterial counts, *Bacteroides/Prevotella* (here on referred to as Bacteroidetes), *C. leptum*, *C. coccoides*, bifidobacteria, *E. coli* and Archaea in stool. Clinical and laboratory data, food-records, and activity logs were collected. Patients with NASH had a lower percentage of Bacteroidetes (Bacteroidetes to total bacteria counts) compared to both SS and HC ($p=0.006$) and higher fecal *C. coccoides* compared to those with SS ($p=0.04$). There were no differences in the remaining microorganisms. As body mass index (BMI) and dietary fat intake differed between the groups ($p<0.05$), we performed linear regression adjusting for these variables. The difference in *C. coccoides* was no longer significant after adjusting for BMI and fat intake. However, there continued to be a significant association between the presence of NASH and lower percentage Bacteroidetes even after adjusting for these variables ($p= 0.002$; 95% CI= -0.06 to -0.02). Conclusion: There is an inverse and diet-/BMI-independent association between the presence of NASH and percentage Bacteroidetes in the stool, suggesting that the IM may play a role in the development of NAFLD.

Background:

Non-alcoholic fatty liver disease (NAFLD) is a term used to describe a spectrum of hepatic pathology that is closely linked to obesity and ranges from simple steatosis (SS), to steatosis with inflammation and/or fibrosis (non-alcoholic steatohepatitis; NASH) and even cirrhosis [1]. NAFLD is considered the hepatic manifestation of the metabolic syndrome. The pathophysiology of NAFLD is not entirely understood but it has been proposed to be the result of multiple 'hits' [2]. These include signals from the adipose tissue (e.g. fatty acids, cytokines), the diet (e.g. fructose, fatty acids), as well as the immune system [2]. Insulin resistance (IR) is frequently found in patients with NAFLD and is thought to contribute to its pathogenesis partly by enhancing lipolysis within the adipose tissue, subsequently increasing the flux of free fatty acids into the liver [2]. Research in the field of obesity has provided preliminary evidence that the intestinal microbiota (IM) may play a role in the development of obesity and the metabolic syndrome, suggesting a potential role in the pathogenesis of NAFLD [3].

The intestinal lumen is populated by trillions of microorganisms that carry 150 fold more genes compared to the host, collectively referred to as the microbiome [4, 5]. The IM is composed of bacteria, Archaea, yeasts and viruses [6, 7] Despite significant inter-individual variations in the IM of humans at lower taxonomical levels, the dominating phyla are Bacteroidetes and Firmicutes [8]. Several human studies in the field of obesity have suggested differences in the IM between obese and lean individuals. Ley et al. showed an increased fecal Firmicutes to Bacteroidetes ratio in obese subjects [9] but subsequent studies have shown inconsistent results, likely due to the uncontrolled effects of factors, such as diet and environment, as well as methodological issues that include variations in sample size and use of different techniques for the determination of the IM composition [10-12]. Very few studies have explored the role of IM in NAFLD and, to our knowledge, there are no studies directly assessing the IM composition of adults with non-experimental SS or NASH [13-15].

Animal studies have shown that the IM can contribute to all the histological components of NAFLD: hepatic steatosis, inflammation and fibrosis [3, 16-18]. The IM have the potential to increase intrahepatic fat through mechanisms, such as altered appetite signaling, increased energy extraction from the diet, altered expression of genes involved in *de novo* lipogenesis or β -oxidation, or via inflammation-driven steatosis [11, 16, 19-21]. Hepatocellular inflammation may be secondary to altered intestinal permeability and translocation of either intact bacteria or microbial cell components (such as lipopolysaccharide [LPS] derived from the cell wall of gram negative bacteria) to the circulation [22-26]. Bifidobacteria specifically, have been associated with enhanced integrity of the intestinal barrier of animals via increased expression of tight junction proteins [27]. Lastly, the IM may contribute to hepatic fibrosis via direct activation of hepatic stellate cells by LPS or via stimulation of pro-fibrotic pathways by TLR-9-dependent recognition of certain bacteria by Kupffer cells in the liver [28].

Despite the amount of evidence providing pathogenetic links between the IM and various components of NAFLD, there are no published studies focused at assessing IM composition of adults with this condition. Recently, Zhu et al. reported differences in the IM of children with NASH

compared to obese and normal-weight children [29]. In this study, NASH was associated with higher levels of ethanol-producing bacteria, as well as increased serum ethanol levels.

The aim of our study was to assess if there are any differences in the IM of adults with biopsy-proven SS, NASH and healthy controls (HC), taking into account potential confounders, such as dietary intake and BMI.

Methods:

General Protocol: This was a cross-sectional study performed at the University Health Network, Toronto, Canada. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the appropriate institutional review committee.

Patients referred to the hepatology clinics for persistently elevated liver enzymes and clinical suspicion of NAFLD were initially assessed as per standard of care to rule out other causes of liver disease. After 3-6 months of persistently elevated alanine aminotransferase (ALT) levels, patients underwent a liver biopsy to confirm the diagnosis of NASH and to assess its severity. During the initial visit, patients were invited to participate in this study. After providing written informed consent, they were instructed on how to collect and transport the stool sample, complete 7-day food records and 7-day activity logs. They were asked to return the stool sample and the food- and activity records the morning of their liver biopsy. On the day of but prior to liver biopsy, a blood sample was taken for metabolic, nutritional and hepatic parameters, as explained below.

Healthy subjects undergoing assessment for living donation by the Living Donor Liver Transplant Program at the University Health Network were invited to participate as controls. These subjects were rigorously assessed as per the protocol of the Transplant Program to ensure that they had no significant medical co-morbidities. After obtaining informed consent, subjects were given the same instructions for stool sample and food record/activity log collection as the NAFLD patients. Samples were returned one week prior to liver donation. Blood samples were also collected at that time. Histology was obtained either during a pre-donation biopsy (done to verify the healthy state of the liver) or during hepatectomy.

Inclusion criteria were age > 18 years and biopsy-confirmed NAFLD or healthy liver. Exclusion criteria were: liver disease other than NAFLD, anticipated need for liver transplantation within a year or complications of end-stage liver disease such as variceal bleeding or ascites; concurrent medical illnesses, contraindications for liver biopsy; use of medications known to cause or exacerbate steatohepatitis (such as corticosteroids) or antibiotics, pre- or probiotics in the preceding 6 months; consumption of more than 20 g of alcohol/day; use of vitamin E or fish oil supplements; chronic gastrointestinal diseases, previous gastrointestinal surgery modifying the anatomy; pregnancy or lactating state.

Clinical data: Patients provided information regarding medication use, alcohol consumption and smoking history. Past medical and surgical history was recorded and in addition, data on ethnicity were collected. Height and weight were measured and body mass index (BMI) was calculated.

Nutritional and Activity assessment: Subjects were asked to complete the 7-day food records the week prior to liver biopsy (or liver donation). The stool sample was collected at the end of this week and within the 24 hours preceding the biopsy. Portion sizes were estimated using the 2D Food Portion Visual chart (Nutrition Consulting Enterprises, Framingham, MA). Food-records were analyzed for macro- and micronutrient content using Diet Analysis Plus Version 7.0.1 (Thomson Wadsworth, Stamford, CT, USA).

The participants also recorded their physical activities for 7 days during the week preceding the biopsy. They listed the type of activity, duration and level of difficulty (mild, moderate, strenuous, very strenuous). Units of exercise were used to estimate physical activity as follows: 1 unit = 30min mild, 20 min moderate, 10 min strenuous, or 5 min very strenuous activity [30].

Basal Metabolic Rate (BMR) was calculated with the Harris-Benedict equation [men: $BMR = 66.5 + (13.75 \times \text{weight in kg}) + (5.003 \times \text{height in cm}) - (6.755 \times \text{age in years})$; women $BMR = 655.1 + (9.563 \times \text{weight in kg}) + (1.850 \times \text{height in cm}) - (4.676 \times \text{age in years})$] and the Estimated Energy Expenditure (EER) was calculated according to Health Canada Guidelines (<http://www.hc-sc.gc.ca/fn-an/nutrition/reference/index-eng.php>).

Biochemistry: Fasting plasma glucose was measured by the enzymatic hexokinase method on an Architect c8000 System (Abbot Laboratories, Abbot Park, IL, USA). Serum insulin was determined by radioimmunoassay (Immulite 2500, Siemens Diagnostics, Los Angeles, CA, USA). IR was calculated using the Homeostasis Model Assessment (HOMA)-IR. Hemoglobin A1c in plasma was measured by ion exchange HPLC (Variant II analyzer, Bio-Rad Laboratories Ltd., Montreal, QC, Canada). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) in plasma as well as serum triglycerides and total cholesterol were measured using the Architect c8000 system (Abbot Laboratories). Serum biochemistry was performed by the diagnostic testing laboratory at University Health Network, Toronto.

Histology: A pathologist blinded to clinical characteristics assessed liver histology for presence of steatosis, inflammation and fibrosis. Presence of NASH was determined using the Brunt scoring system, which is a validated and reproducible tool for the evaluation of NASH [31].

Stool samples and Quantification of IM: The stool collection kit included a plastic collection/storage container with a tightly closing lid, an insulated bag and cooling elements. Patients were asked to collect one sample within 24 hours of their next clinic appointment. The samples were immediately frozen in the patients' home freezer and transported to the hospital using the cooling elements and the insulated bag, similar to previously published methods [5]. Stools were then stored at -80°C until analysis.

The stool was thawed, immediately homogenized with a masticator blender, and 0.1g were used for DNA extraction using the E.Z.N.A.TM stool DNA Isolation Kit (Omega; Norcross, GA), as per the manufacturer's protocol. The extraction protocol was modified to include a lysozyme digestion step

(incubation at 37°C for 30 minutes). DNA concentration and purity were measured using ThermoScientific Nanodrop 1000 Spectrophotometer (ThermoScientific, Rockford, IL). DNA samples were subsequently stored at -20°C. Fifty nanograms of the extracted DNA were used for the quantification of fecal bifidobacteria, *Bacteroides/Prevotella*, *C. leptum*, *C. coccoides*, *E. coli*, as well as total bacteria and Archaea, by qPCR using a 7900HT thermocycler from Applied Biosystems (Foster City, CA) under default thermocycling conditions. Custom-made TaqMan primers for total bacteria [32], *C. coccoides* [32], *C. leptum* [32], *Bacteroides/Prevotella* [32, 33], bifidobacteria [32] and Archaea [34] were used. Real-time PCR for *E. coli* was done using SYBR Green Gene Expression master mix (Applied Biosystems) and the specific forward and reverse primer [32]. Number of cells of each microorganism in fecal samples was calculated by interpolation from standard curves and expressed as log cell counts/g feces. *Bacteroides/Prevotella* counts were considered representative of the Bacteroidetes phylum (as in [33]) and will herein be referred to as Bacteroidetes.

Statistical analyses: Results were expressed as median (range) as the data were not normally distributed. Kruskal-Wallis test was used to compare the three groups for demographic, dietary and laboratory data (Stata v.12, Stata®, College Station, TX). Non-parametric tests were used for statistical comparisons of the results of the fecal analyses as well (Kruskal-Wallis; Stata v.12 and GraphPad Prism v.4.0, GraphPad Software Inc., La Jolla, CA). For the microbiota, high and low outliers were defined as numbers higher than the third quartile plus 1.5 times the interquartile range (IQR) and lower than the first quartile minus 1.5 times the IQR, respectively [35]. Spearman correlation coefficients were used to assess bivariate relationships between variables. Analysis of covariance (ANCOVA) was used to investigate the associations between IM and liver disease state after controlling for variables that were found to be significant at the univariate level (these non-normally distributed variables had been normalized using their logarithmic value, prior to performing the ANCOVA). Statistically significant α was considered any value lower than 0.05. Considering the paucity of literature assessing the IM of patients with NAFLD, determination of the sample size was based on studies in the field of obesity, where differences at a phylum level have been detected between the groups with as few as 14 subjects in total [9].

Results:

A total of 50 patients were enrolled in this study: 17 HC, 11 SS and 22 NASH. The demographic and laboratory data are summarized in **Table 1**.

Demographics and anthropometrics: Patients with NASH and SS were older compared to HC. The gender distribution was not statistically different between groups. The majority of subjects in each group were Caucasians: 86% of the HC and 67% of both the SS and NASH patients. Patients with NASH had higher BMI when compared to HC (**Table 1**).

Laboratory data: Transaminases (ALT, AST) were higher in NASH compared to SS and HC. HOMA-IR was higher in patients with NASH compared to HC. No differences were found in ALP, glucose, hemoglobin A1c, cholesterol and triglyceride levels. All patients had normal liver synthetic function as determined by albumin and International Normalized Ratio levels (data not shown).

Histology: The median steatosis of the SS group was 12.5% (range: 5-35%) and 40% in the NASH group (range: 5-90%). Eighty percent of NASH patients had a variable degree of fibrosis (ranging from F1- F3). The median NAFLD activity score was 4 (range: 2-8).

Dietary data: The dietary data are summarized in **Table 2**. The total energy intake per day and the percentage of energy from carbohydrate and fat was not different among the groups. Adjusting the caloric intake for weight (total kcal/day divided by weight) revealed that HC were consuming more calories per kg compared to patients with NASH. The BMR was similar among subjects of all groups, as was the EER. The reported energy intake was lower than the EER in all 3 groups. When dividing the percentage fat intake by BMR, to adjust for factors such as age, the HC group was found to consume more energy from fat compared to patients with SS and NASH.

IM: Patients with NASH had higher fecal *C. coccoides* levels compared to those with SS as depicted in **Figure 1**. There were no differences between the groups for bifidobacteria, Bacteroidetes, *C. leptum*, *E. coli* and total bacteria ($p>0.05$). There were no differences in the Firmicutes to Bacteroidetes ratio between the groups ($p>0.05$; **Figure S1**). Archaea were only detectable in 5 HC, 2 SS and 2 NASH, which limited the statistical power for any comparisons (**Figure S2**).

The relative abundance of each microbe of interest (ratio of bacterial number to total bacteria) was also compared between the groups. The percentage of Bacteroidetes was significantly lower in patients with NASH, compared to both SS and HC (**Figure 2**). There were no differences between the groups in the percentages of the other microorganisms assessed.

Exploring for potential relationships between dietary intake and bacteria counts, we found no statistically significant correlations between total caloric intake, percentage fat or carbohydrate consumption and fecal Bacteroidetes, *C. leptum*, *C. coccoides*, bifidobacteria or *E. coli* ($p>0.05$). Performing the same correlations for the NAFLD cohort only (SS and NASH combined), there was a statistically significant negative association between total daily caloric intake and fecal Bacteroidetes counts (Spearman r : -0.43, $p=0.038$).

Taking into consideration that BMI and percentage of fat intake could be contributing to the association between the percentage of Bacteroidetes and NASH, ANCOVA was performed to control for these potential confounders. There was an independent association between the percentage of Bacteroidetes and the presence of NASH ($p=0.002$; 95% CI= -0.06 to -0.02). This was not the case with *C. coccoides*, which was no longer associated with NASH once BMI and percentage fat intake were taken into account ($p>0.05$).

We also assessed whether the percentage of Bacteroidetes was associated with IR, controlling for BMI. There was a trend ($r= -0.31$; $p=0.06$) towards a negative association between the percentage of Bacteroidetes and HOMA-IR.

Discussion:

To our knowledge, this is the first study assessing the IM of adults with non-experimental NAFLD and specifically comparing the IM composition of subjects classified as HC, SS or NASH based on histological data. We found a lower relative abundance of Bacteroidetes in NASH, which was independent of BMI and energy intake from fat in the diet.

The importance of classifying patients based on liver histology is significant, as one of the most challenging aspects in the pathophysiology of NAFLD is understanding the differences between mechanisms causing simple hepatic steatosis versus those that lead to steatohepatitis. Since bacteria are known to play a pathogenetic role in the development of inflammation, comparisons between all groups (HC, SS and NASH) allow for further elucidation of the effects of the IM on the liver.

Along with Firmicutes, Bacteroidetes comprise the majority of the human IM [36, 37]. In our cohort, the relative abundance of Bacteroidetes in the stool was lower in NASH compared to both SS and HC. This finding is in agreement with previously published literature in the field of obesity that has demonstrated lower Bacteroidetes in patients with higher BMI [9, 37]. The novelty of our study is the suggestion of a BMI-independent association between Bacteroidetes and liver disease state. Interestingly, our findings contrast those of Zhu et al., who showed increased Bacteroidetes in children with obesity or NASH, compared to those with a normal BMI [29]. These results may have been, in part, reflective of the imbalance in BMI between the groups in that study, as well as differences in environmental and dietary factors between the two study cohorts. In addition, the lack of classification of patients in each group based on liver histology may have also affected the results of the study by Zhu et al. Lastly, differences in age may have also played a role, as discussed below.

There are various theories to support an inverse correlation between Bacteroidetes and steatohepatitis. First, Bacteroidetes carry 45% of the lean metabolic potential in a study comparing the microbiome of lean and obese adults [21]. Lower percentage of Bacteroidetes could have affected energy balance by facilitating metabolic dominance of other bacteria that are more efficient in extracting energy from the diet. Jupertz et al., showed that a 20% increase in fecal Bacteroidetes is associated with a 150 kcal decrease in energy harvest from the diet [11]. A second theory is that an initial hit causes the cell death of Bacteroides leading to lipopolysaccharide (LPS) release from their cell wall and subsequent endotoxemia [19]. The latter leads to the development of NASH [19, 27]. It is not clear what would cause the death of these microorganisms. Changes in diet could play a role, as shown by studies in obese subjects, whose baseline lower fecal Bacteroidetes increase, when placed on a hypocaloric diet or after bariatric surgery [38, 39].

There is literature supporting an increase in intestinal permeability of subjects with IR, such as in obesity [40] and diabetes [41]. Recently, Zhu et al. reported higher serum ethanol levels in children with NASH, which was thought to be bacterially derived and, hence, potentially also contributing to increased intestinal permeability [29, 42]. In addition, there is scientific evidence linking endotoxemia with states of glucose intolerance, such as NAFLD [41, 43, 44]. Animals and humans exposed to low levels of endotoxin develop IR [19, 22]. Exploratory analysis from our study also

suggested a potential link between the intestinal microbiota and IR by showing a trend toward a negative association between Bacteroidetes and IR when controlling for BMI. This requires further studies.

We did not find lower bifidobacteria counts or higher Firmicutes to Bacteroidetes ratio in NASH compared to SS and HC. This is in contrast to some of the previously published literature in the field of obesity [9, 14, 45] and the recent study by Zhu et al. on children with NASH [29]. The inability to show differences in these bacteria may have been due to the sample size, however, the size of our cohort was similar to that of other cross-sectional studies on IM in obesity [9, 11, 12, 37] and NAFLD [29]. Our results on Firmicutes to Bacteroidetes ratio are in line with other smaller projects, which also failed to replicate the findings of Ley et al. [10-12]. Compared to previous reports, our study provides stronger evidence supporting these results, as it did not simply assess the differences in the IM composition between the groups, but it also carefully addressed the potential confounding effects of diet and BMI.

E. coli was not statistically different between the groups. Zhu et al. not only found *E. coli* to be higher in children with NASH compared to those who were obese without NASH, but also proposed that these bacteria may be contributing to the synthesis of ethanol with subsequent hepatotoxic effects [29]. In our cohort there was a low overall abundance of *E. coli* in the stool, which may have contributed to the difficulty in detecting potential differences between the groups. Ours is the first study addressing the presence of Archaea in the stool of adults with NAFLD. These organisms were only found in a small proportion of study subjects overall, limiting the power of statistical comparisons. Further studies are required to elucidate the role of *E. coli* and Archaea in the development of NASH in both children and adults.

We assessed the intestinal microbiota by using qPCR which is the gold-standard technique for bacterial enumeration [46]. It is currently employed for the compositional analysis of the gut microbiota in humans and animals and was therefore ideal to quantify, in this study, fecal microbes that are known to play a role in obesity. Because qPCR does not allow for the identification of novel species [46], future studies could include metagenomic approaches, such as those based on *16S rRNA* gene sequencing, potentially leading to the discovery of additional microbes associated with NAFLD. Moreover, a combination of these approaches with qPCR would provide an assessment of microbial diversity in healthy versus patients with NAFLD.

In our cohort, patients with NASH were older than HC. While the IM of infants and elderly patients appear to differ to that of adults, within the adult spectrum, it is unlikely that there are significant, age-dependent variations in the IM composition [33]. For that reason, age was not considered as a confounder and was not included in the ANCOVA. This factor, however, may in part explain the differences between the results of our study and those of Zhu et al., who assessed the IM of children with NASH [29].

The median BMI of HC was at the lower spectrum of the overweight range (**Table 1**). This is unlikely to have influenced the results of this study, as all subjects had had a biopsy proven

unaffected (non-steatotic, non-inflamed) liver. In addition, the higher BMI in the control group allowed for smaller differences in BMI between the groups overall, theoretically limiting the potential confounding effect of this factor.

As dietary intake contributes to the fecal microbial composition, all subjects provided a 7-day food record. The reported caloric intake was not different between the groups, similarly to the study by Zhu et al. [29]. In addition, there were no differences in calculated energy requirements, as expressed by BMR and EER. Considering the differences in BMI between the groups, dietary under-reporting may have influenced these results. A direct correlation between dietary under-reporting and BMI has been previously shown in the literature [47].

In summary, this is a novel study providing evidence for a link between percentage Bacteroidetes and the presence of NASH, which is independent of diet and BMI. Future research should address this topic, considering that the IM may serve as a potential therapeutic target in NASH, which is currently primarily managed by recommending weight loss and increased physical activity, which are notoriously difficult to sustain.

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References:

1. Brunt, E.M., *Pathology of nonalcoholic fatty liver disease*. Nat Rev Gastroenterol Hepatol, 2010. **7**(4): p. 195-203.
2. Tilg, H. and A.R. Moschen, *Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis*. Hepatology, 2010. **52**(5): p. 1836-46.
3. Abu-Shanab, A. and E.M. Quigley, *The role of the gut microbiota in nonalcoholic fatty liver disease*. Nat Rev Gastroenterol Hepatol, 2010. **7**(12): p. 691-701.
4. Clemente, J.C., et al., *The impact of the gut microbiota on human health: an integrative view*. Cell, 2012. **148**(6): p. 1258-70.
5. Qin, J., et al., *A human gut microbial gene catalogue established by metagenomic sequencing*. Nature, 2010. **464**(7285): p. 59-65.
6. Whitman, W.B., D.C. Coleman, and W.J. Wiebe, *Prokaryotes: the unseen majority*. Proc Natl Acad Sci U S A, 1998. **95**(12): p. 6578-83.
7. Arumugam, M., *Enterotypes of the human gut microbiome*. Nature, 2011. **12**(473): p. 6.
8. Backhed, F., et al., *Host-bacterial mutualism in the human intestine*. Science, 2005. **307**(5717): p. 1915-20.
9. Ley, R.E., et al., *Microbial ecology: human gut microbes associated with obesity*. Nature, 2006. **444**(7122): p. 1022-3.
10. Duncan, S.H., et al., *Human colonic microbiota associated with diet, obesity and weight loss*. Int J Obes (Lond), 2008. **32**(11): p. 1720-4.
11. Jumpertz, R., et al., *Energy-balance studies reveal associations between gut microbes, caloric load, and nutrient absorption in humans*. Am J Clin Nutr, 2011. **94**(1): p. 58-65.
12. Schwartz, A., et al., *Microbiota and SCFA in lean and overweight healthy subjects*. Obesity (Silver Spring), 2010. **18**(1): p. 190-5.
13. Loguercio, C., et al., *Beneficial effects of a probiotic VSL#3 on parameters of liver dysfunction in chronic liver diseases*. J Clin Gastroenterol, 2005. **39**(6): p. 540-3.
14. Malaguarnera, M., et al., *Bifidobacterium longum with fructo-oligosaccharides in patients with non alcoholic steatohepatitis*. Dig Dis Sci, 2012. **57**(2): p. 545-53.
15. Wigg, A.J., et al., *The role of small intestinal bacterial overgrowth, intestinal permeability, endotoxaemia, and tumour necrosis factor alpha in the pathogenesis of non-alcoholic steatohepatitis*. Gut, 2001. **48**(2): p. 206-11.
16. Backhed, F., et al., *The gut microbiota as an environmental factor that regulates fat storage*. Proc Natl Acad Sci U S A, 2004. **101**(44): p. 15718-23.
17. Maslowski, K.M., et al., *Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43*. Nature, 2009. **461**(7268): p. 1282-6.
18. Shi, H., et al., *TLR4 links innate immunity and fatty acid-induced insulin resistance*. J Clin Invest, 2006. **116**(11): p. 3015-25.
19. Cani, P.D., et al., *Metabolic endotoxemia initiates obesity and insulin resistance*. Diabetes, 2007. **56**(7): p. 1761-72.
20. Cani, P.D., C. Dewever, and N.M. Delzenne, *Inulin-type fructans modulate gastrointestinal peptides involved in appetite regulation (glucagon-like peptide-1 and ghrelin) in rats*. Br J Nutr, 2004. **92**(3): p. 521-6.

21. Turnbaugh, P.J., et al., *An obesity-associated gut microbiome with increased capacity for energy harvest*. *Nature*, 2006. **444**(7122): p. 1027-31.
22. Mehta, N.N., et al., *Experimental endotoxemia induces adipose inflammation and insulin resistance in humans*. *Diabetes*, 2010. **59**(1): p. 172-81.
23. Csak, T., et al., *Fatty acid and endotoxin activate inflammasomes in mouse hepatocytes that release danger signals to stimulate immune cells*. *Hepatology*, 2011. **54**(1): p. 133-44.
24. Ruiz, A.G., et al., *Lipopolysaccharide-binding protein plasma levels and liver TNF-alpha gene expression in obese patients: evidence for the potential role of endotoxin in the pathogenesis of non-alcoholic steatohepatitis*. *Obes Surg*, 2007. **17**(10): p. 1374-80.
25. Rivera, C.A., et al., *Toll-like receptor-4 signaling and Kupffer cells play pivotal roles in the pathogenesis of non-alcoholic steatohepatitis*. *J Hepatol*, 2007. **47**(4): p. 571-9.
26. Wagnerberger, S., et al., *Toll-like receptors 1-9 are elevated in livers with fructose-induced hepatic steatosis*. *Br J Nutr*, 2011: p. 1-12.
27. Cani, P.D., et al., *Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability*. *Gut*, 2009. **58**(8): p. 1091-103.
28. Miura, K., et al., *Toll-like receptor 9 promotes steatohepatitis by induction of interleukin-1beta in mice*. *Gastroenterology*, 2010. **139**(1): p. 323-34 e7.
29. Zhu, L., et al., *Characterization of the gut microbiome in non-alcoholic steatohepatitis (NASH) patients: A connection between endogenous alcohol and NASH*. *Hepatology*, 2012.
30. Pan, X.R., et al., *Effects of diet and exercise in preventing NIDDM in people with impaired glucose tolerance. The Da Qing IGT and Diabetes Study*. *Diabetes Care*, 1997. **20**(4): p. 537-44.
31. Brunt, E.M., et al., *Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions*. *Am J Gastroenterol*, 1999. **94**(9): p. 2467-74.
32. Furet, J.P., et al., *Comparative assessment of human and farm animal faecal microbiota using real-time quantitative PCR*. *FEMS Microbiol Ecol*, 2009. **68**(3): p. 351-62.
33. Mariat, D., et al., *The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age*. *BMC Microbiol*, 2009. **9**: p. 123.
34. Zhang, H., et al., *Human gut microbiota in obesity and after gastric bypass*. *Proc Natl Acad Sci U S A*, 2009. **106**(7): p. 2365-70.
35. Tukey, J., *Exploratory Data Analysis*. 1st ed. 1977: Wesley, A. 688.
36. DiBaise, J.K., et al., *Gut microbiota and its possible relationship with obesity*. *Mayo Clin Proc*, 2008. **83**(4): p. 460-9.
37. Turnbaugh, P.J., et al., *A core gut microbiome in obese and lean twins*. *Nature*, 2009. **457**(7228): p. 480-4.
38. Furet, J.P., et al., *Differential adaptation of human gut microbiota to bariatric surgery-induced weight loss: links with metabolic and low-grade inflammation markers*. *Diabetes*, 2010. **59**(12): p. 3049-57.
39. Santacruz, A., et al., *Interplay between weight loss and gut microbiota composition in overweight adolescents*. *Obesity (Silver Spring)*, 2009. **17**(10): p. 1906-15.

40. Moreno-Navarrete, J.M., et al., *Circulating zonulin, a marker of intestinal permeability, is increased in association with obesity-associated insulin resistance*. PLoS One, 2012. **7**(5): p. e37160.
41. Creely, S.J., et al., *Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes*. Am J Physiol Endocrinol Metab, 2007. **292**(3): p. E740-7.
42. Miele, L., et al., *Increased intestinal permeability and tight junction alterations in nonalcoholic fatty liver disease*. Hepatology, 2009. **49**(6): p. 1877-87.
43. Miele, L., et al., *Increased intestinal permeability and tight junction alterations in nonalcoholic fatty liver disease*. Hepatology, 2009. **49**(6): p. 1877-87.
44. Thuy, S., et al., *Nonalcoholic fatty liver disease in humans is associated with increased plasma endotoxin and plasminogen activator inhibitor 1 concentrations and with fructose intake*. J Nutr, 2008. **138**(8): p. 1452-5.
45. Fanning, S., et al., *Bifidobacterial surface-exopolysaccharide facilitates commensal-host interaction through immune modulation and pathogen protection*. Proc Natl Acad Sci U S A, 2012. **109**(6): p. 2108-13.
46. Fraher, M.H., P.W. O'Toole, and E.M. Quigley, *Techniques used to characterize the gut microbiota: a guide for the clinician*. Nat Rev Gastroenterol Hepatol, 2012. **9**(6): p. 312-22.
47. Freisling, H., et al., *Dietary reporting errors on 24 h recalls and dietary questionnaires are associated with BMI across six European countries as evaluated with recovery biomarkers for protein and potassium intake*. Br J Nutr, 2012. **107**(6): p. 910-20.

Table 1: Demographic and laboratory results

Variables	HC (n=17)	SS (n=11)	NASH (n=22)
Age (years) ⁺	36 (23-58) ^{a,b}	48 (22-55) ^b	47 (29-68) ^a
Gender (% male)	59	36	45
BMI (kg/m ²) [*]	26.0 (18.8-30.5) ^a	28.6 (23.5-44.2)	32.4 (24.2-49.5) ^a
ALT (U/L) ^{**}	17 (6-41) ^{a,b}	32 (14-54) ^b	69 (22-168) ^a
AST (U/L) [*]	22 (12-31) ^a	21 (16-40)	44 (18-114) ^a
ALP (U/L)	65 (49-98)	64 (42-105)	77 (37-114)
Glucose (mmol/L)	5.0 (4.1-6.5)	5.1 (4.6-6.5)	5.9 (4.1-7.6)
Insulin (pmol/L)	21 (15-61)	33 (15-465)	97 (29-437)
HOMA-IR ^{**}	0.60 (0.5-2.0) ^a	2.15 (0.5-13.7)	4.15 (1.0-34.6) ^a
Hemoglobin A1c	0.054 (0.046-0.057)	0.055 (0.050-0.072)	0.061 (0.050-0.074)
Triglycerides (mmol/L)	0.94 (0.58-3.32)	1.10 (0.62-3.97)	1.58 (0.28-3.29)
Total cholesterol (mmol/L)	4.4 (2.78-7.46)	5.05 (3.75-6.88)	4.56 (2.65-6.94)

⁺p=0.003; ^{*}p=0.001; ^{**}p<0.001

BMI: Body Mass Index; ALT: Alanine transaminase; AST: Aspartate transaminase; ALP: Alkaline phosphatase; HOMA-IR: Homeostasis Model of Assessment for Insulin Resistance

The values are expressed as medians (range). For each comparison, identical letters indicate the groups between which the statistical difference was significant.

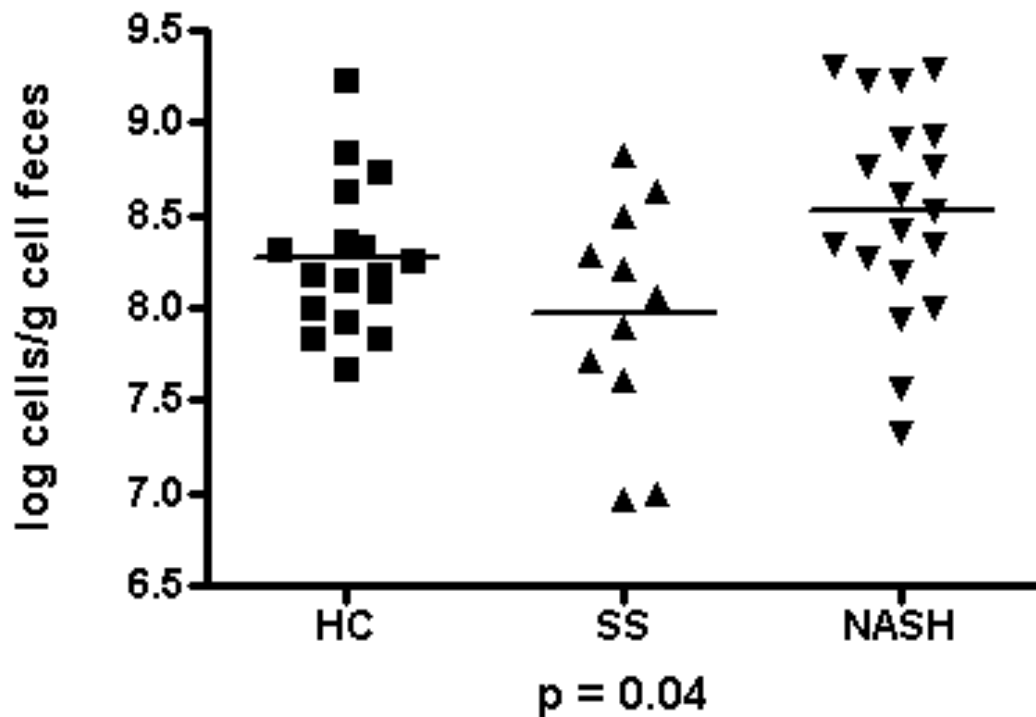
Table 2: Dietary data and calculations

Variables	HC (n=17)	SS (n=11)	NASH (n=22)	p value
Energy intake (kcal/day)	2094 (1103-2796)	1715 (1248-2348)	1617 (1025-2653)	0.23
Fat-derived energy %	37 (29-45)	33 (22-39)	34 (18-43)	0.06
Carbohydrate-derived energy %	49 (4-57)	53 (39-74)	47 (4-65)	0.42
Weight-adjusted energy intake (kcal/kg/day)	26.9 (13.8-64.1) ^a	21.7 (15.3-27.9)	17.4 (10.7-31.6) ^a	<0.01
BMR (kcal/day)	1541 (1223-2145)	1655 (1252-2224)	1635 (1329-2052)	0.99
EER (kcal/day)	2683 (1675-4115)	2639 (2042-3681)	2418 (1768-3899)	0.62
Energy intake/BMR	1.18 (0.6-2.3)	1.15 (0.7-1.2)	0.88 (0.6-1.6)	0.40
% fat intake/BMR	0.023 (0.016-0.035) ^{a, b}	0.019 (0.012-0.470) ^b	0.020 (0.013-0,031) ^a	0.04

BMR: Basal Metabolic Rate; EER: Estimated Energy Requirement

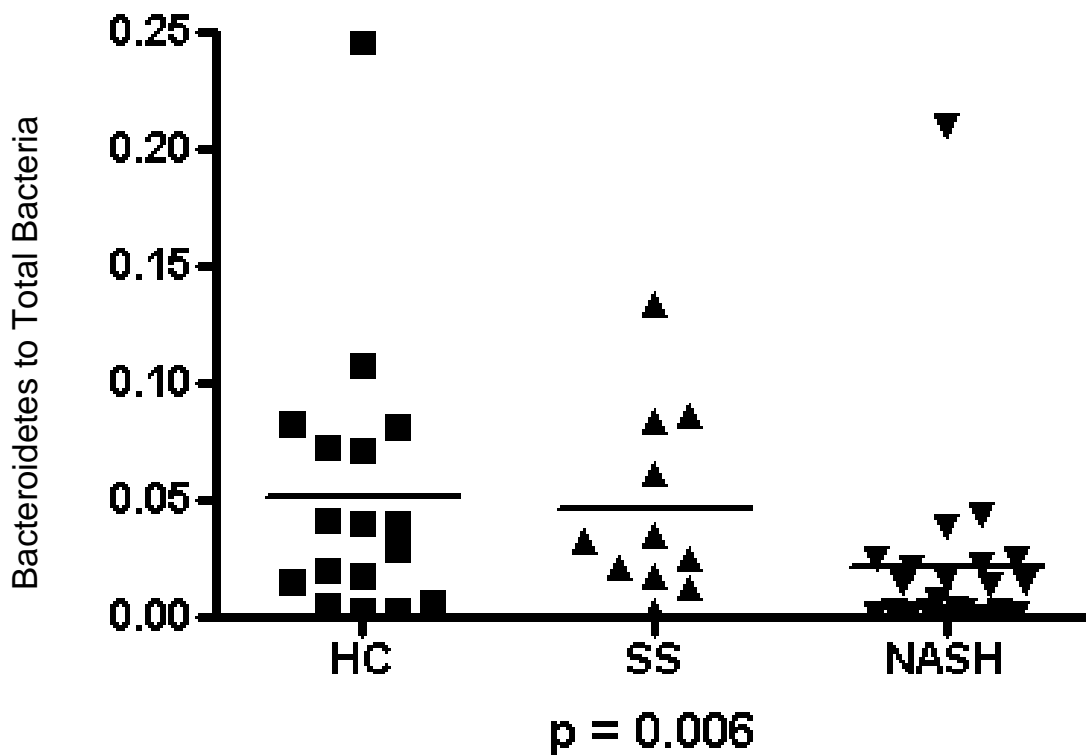
The values are expressed as medians (range). For each comparison, identical letters indicate the groups between which the statistical difference was significant.

Figure 1: Comparisons for fecal *C. coccooides* between the groups.



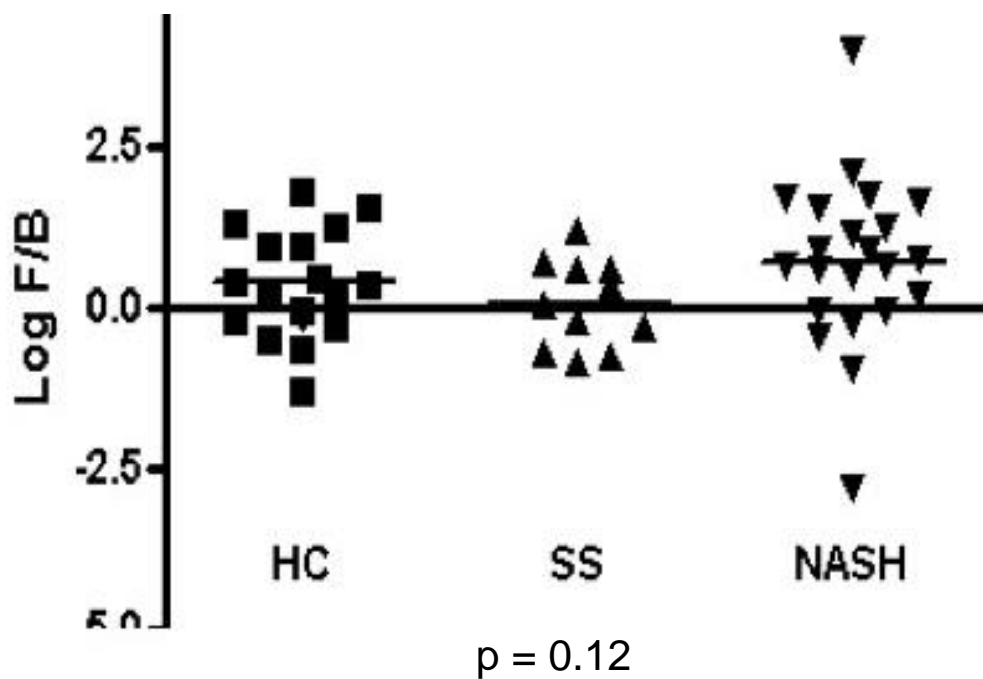
Patients with non-alcoholic steatohepatitis (NASH) have higher fecal *C. coccooides* compared to patients with simple hepatic steatosis (SS). N= 17 HC, 11 SS, 19 NASH.

Figure 2: Comparisons for percentage Bacteroidetes (Bacteroidetes to total bacteria ratio) in stool between the groups.



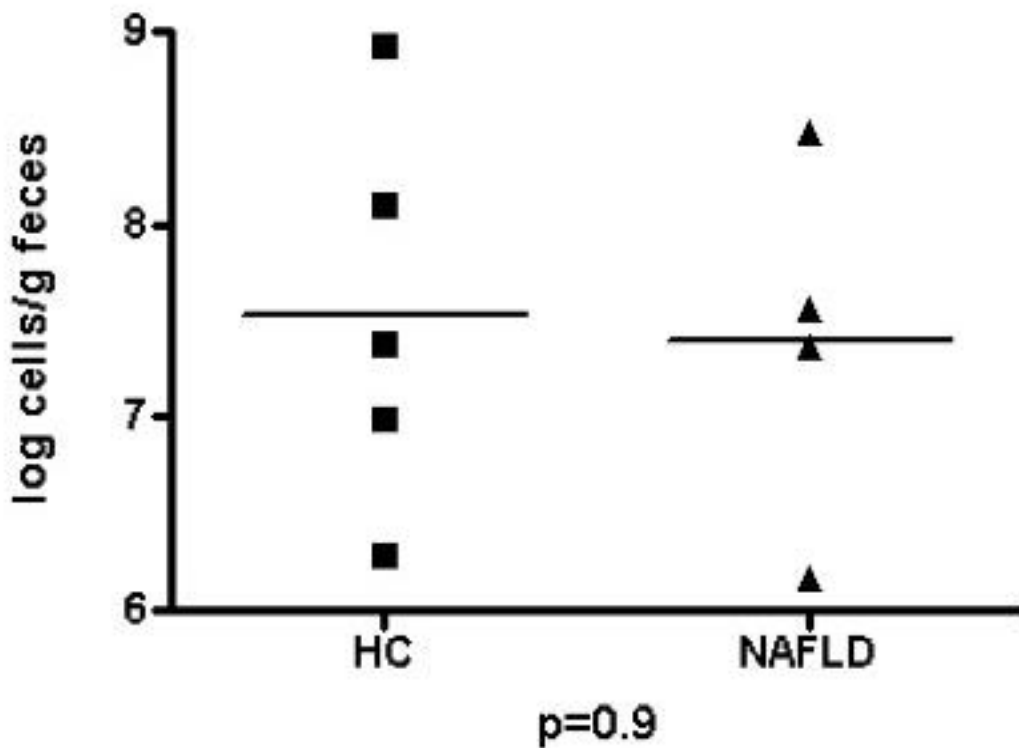
Patients with non-alcoholic steatohepatitis (NASH) have lower fecal percentage Bacteroidetes compared to both patients with simple hepatic steatosis (SS) and healthy controls (HC). N= 17 HC, 11 SS, 22 NASH

Figure S1: Comparisons for Firmicutes to Bacteroidetes ratio between the groups.



There are no differences in Firmicutes to Bacteroidetes (F/B) between the groups (n=17 HC, 11 SS, 22 NASH).

Figure S2: Comparisons for Archaea between healthy controls (HC) and patients with non-alcoholic fatty liver disease (NAFLD).



There are no differences in Archaea between the groups (n=5 HC, 4 NAFLD). The rest of the cohort did not have detectable Archaea in their stool.