ORIGINAL ARTICLE

Gut microbiota profile in patients with nonalcoholic fatty liver disease and presumed nonalcoholic steatohepatitis

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Background: The main composition of intestinal microbiota in nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) patients has not yet been elucidated. In this, case-control study, we identified differences of intestinal microbiota in male patients with NAFLD, presumed NASH, and healthy controls. **Materials and Methods:** We compared gut microbial composition of 25 patients with NAFLD, 13 patients with presumed NASH, and 12 healthy controls. Demographic information as well as clinical, nutritional, and physical activity data was gathered. Stool and blood samples were collected to perform the laboratory analysis. The taxonomic composition of gut microbiota was assessed using V4 regions of microbial small subunit ribosomal Ribonucleic acid genes sequencing of stool samples. **Results:** *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* were the most frequently phyla in all groups. Our results revealed that *Veillonella* was the only genus with significantly different amounts in presumed NASH patients compared with patients with NAFLD ($P = 2.76 \times 10^{-6}$, $q = 2.07 \times 10^{-4}$, logFC = 5.52). **Conclusion:** This pilot study was the first study to compare gut microbial composition in patients with NAFLD and presumed NASH in the Middle East. Given the potential effects of gut microbiota on the management and prevention of NAFLD, larger, prospective studies are recommended to confirm this study's findings.

Key words: Gut microbiota, nonalcoholic fatty liver disease, presumed nonalcoholic steatohepatitis

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) has been recently recognized as the most prevalent liver disease worldwide affecting over 25% of the population.[1] This disease is characterized by fat deposition in the liver cells and can progress to nonalcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis, and even hepatocellular carcinoma.[2]

The prevalence of NAFLD is rising parallel to other metabolic morbidities such as obesity, insulin resistance, metabolic syndrome, and dyslipidemia.[3] Various risk factors and pathophysiologies have been suggested for

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NAFLD, such as environmental, nutritional, genetic and immunological factors.[4,5]

The role of intestinal dysbiosis in NAFLD/NASH has also been debated by researchers.[6,7] The human intestinal tract houses over 10^{14} bacteria involved in food digestion and interactions with the immune system, and many studies have highlighted their role in the pathogenesis of NAFLD.[8,9] There are several mechanisms, which propose that microbiota dysregulation may affect NAFLD. Impaired intestinal permeability allows an increased flow of microbiota derivatives such as lipopolysaccharides (LPS) into the blood stream. Blood from the intestines is received by the liver through the portal vein, exposing it to the maximum concentration of these metabolites.[10] While liver Kupffer cells destroy most of these intruders,[11] the liver is still exposed to higher than normal levels of toxins, microbes and fatty compounds such as LPS, subsequently causing inflammation and damage to liver cells.[12,13]

Another mechanism by which intestinal microbiota can cause NAFLD is through ethanol-producing bacteria which produce ethanol by fermenting ingested sugar. Ethanol metabolism induces fatty acid synthesis which is deposited in the liver causing inflammation and damage.[14]

Some studies have shown dysbiosis to cause NAFLD by over-activation of *de novo* lipogenesis (DNL), a regular metabolic process in which excess carbohydrates consumed are converted to fatty acids and stored in adipose tissue as triglycerides to be used for energy production through beta-oxidation when needed. Greater fatty acid production caused by the over-activation of DNL leads to fat accumulation in the liver, causing inflammation through oxidative stress and ultimately the development of NAFLD.[15-17]

Finding the main variations in gut microbiota of patients with NAFLD, NASH, and healthy people may lead to novel strategies in the management of the disease.[6,7] Thus, we aimed to compare the profile of gut microbiota in NAFLD and presumed NASH patients as well as healthy controls in this case–control study.

MATERIALS AND METHODS

Characteristics

Fifty men 18–60 years of age referring to the Tehran Gastroenterology and Hepatology Clinic, were enrolled in the study. Given that previous studies have shown sex-related differences in gut microbiome, only men were included.[18] In this study, we had 25 NAFLD, 13 presumed NASH, and 12 healthy participants as control. NAFLD was diagnosed by transient elastography (FibroScan, Echosence, France) and defined as having a controlled attenuation parameter (CAP) score above 260 dB/m.[19] Presumed NASH was defined as having a CAP score above 260 dB/m along with a serum alanine transaminase (ALT) level >45 IU/L. Men with a CAP score below 260 dB/m who had normal ALT levels were chosen as controls.[20,21]

Exclusion criteria included having any of the following conditions: Hepatitis, autoimmune disorders, advanced liver disease, cancers, irritable bowel syndrome, inflammatory bowel disease, chronic diarrhea, liver enzymes 10 times above the normal values, any gastrointestinal surgeries, alcohol use >40 g per week, use of corticosteroids, probiotics, Vitamin E and fish oil supplements within 6 months, use of antibiotics within 6 weeks and dieting within 1 month.

The study protocol was approved by the Tehran University of Medical Sciences ethical committee and written consent was obtained from all participants.

Data collection

An interviewer-administered questionnaire was completed for each participant obtaining information on demographics, past medical and surgical history, medication history, alcohol use, and smoking. To assess dietary intake, a 90-item, nonquantitative food frequency questionnaire was completed. Physical activity was assessed using the 7-question, International Physical Activity Questionnaire,[22] through which the type, duration and difficulty level of different activities performed are questioned yielding a metabolic equivalent of task score for each individual. The Pittsburgh Sleep Quality Index was also measured using the standard validated questionnaire.[23] Height, weight and waist and hip circumferences were measured using the National Institute of Health protocols.

Sample collection

Fasting blood was collected and serum levels of blood sugar, aspartate and ALT, cholesterol, triglycerides, very low-density lipoprotein (VLDL) were measured by BT 3000 Auto Analyzer. Antinuclear antibody, insulin, C-reactive protein, and hemoglobin A1C were performed by Immunoturbidimetric.

Stool collection kits were given to all participants and participants were instructed to return their specimen within the 24 h. The stool was collected directly into a sterile 20 ml polypropylene fecal container with a spoon attached to the snap-on lid and stored in-80°C.

DNA was extracted from stool samples using FavorPrep TM Stool DNA Isolation Mini Kits (FAVORGEN, Taiwan). DNA concentration was evaluated by Nanodrop (IMPLEN, Germany). Genomic DNA was polymerase

chain reaction (PCR) amplified with primers (modified from the primer set employed by the Earth Microbiome Project (GTGYCAGCMGCCGCGGTAA and GGACTACNVGGGTWTCTAAT) targeting the V4 regions of microbial small subunit ribosomal Ribonucleic acid (RNA) genes. Amplicons were generated using a two-stage "targeted amplicon sequencing" protocol.[24] The primers contained 5′ common sequence tags (CS1 and CS2).[25] First and second stages PCR amplifications were performed using MyTaq HS 2X mastermix (Bioline). PCR conditions for first PCR amplification was 95°C for 5 min, followed by 28 cycles of 95°C for 30″, 55°C for 45″ and 72°C for 60″.

In second PCR amplification, each well received a separate primer pair with a unique 10-base barcode, obtained from the Access Array Barcode Library for Illumina (Fluidigm, South San Francisco, CA; Item# 100-4876). These primers contained the CS1 and CS2 linkers at the 3′ ends. Cycling conditions were: 95°C for 5 min, followed by 8 cycles of 95°C for 30″, 60°C for 30″ and 72°C for 30″.

Samples were then pooled in equal volume using an EpMotion5075 liquid handling robot (Eppendorf, Hamburg, Germany). The pooled library was purified using an AMPure XP cleanup protocol (0.6X, vol/vol; Agencourt, Beckmann-Coulter) to remove fragments smaller than 300 bp. The pooled libraries, with a 20% phiX spike-in, were loaded onto an Illumina MiniSeq mid-output flow cell. Based on the distribution of reads per barcode, the amplicons were re-pooled to generate a more balanced distribution of reads. The re-pooled library was purified using AMPure XP cleanup. The re-pooled libraries, with a 20% phiX spike-in, were loaded onto a Miniseq flow cell and sequenced. Fluidigm sequencing primers, targeting the CS1 and CS2 linker regions, were used to initiate sequencing. De-multiplexing of reads was performed on the instrument. Library preparation, pooling, and sequencing were performed at the University of Illinois at Chicago Genome Research Core within the Research Resources Center.

Forward and reverse reads were merged using PEAR.[26] Merged reads were trimmed to remove ambiguous nucleotides, primer sequences, and trimmed based on the quality threshold of $P = 0.01$. Reads that lacked either primer sequence or any sequences <225 bp were discarded. Chimeric sequences were identified and removed using the USEARCH algorithm with a comparison to Silva v132 reference sequence database.[27,28]

The standard QIIME pipeline was modified to generate taxonomic summaries using sub-OTU resolution of the sequence dataset.[29,30] Briefly, the resulting sequence files were then merged with sample information. All sequences were then dereplicated to produce a list of unique sequences. All sequences that had an abundance of at least 10 counts were designated seed sequences. USEARCH was then used to find the nearest seed sequence for any non-seed sequence with a minimum identity threshold of 97%. For any non-seed sequence that matched a seed sequence, its counts were merged with the seed sequence counts.[27] For any non-seed sequence that did not match a seed sequence, it would remain an independent sequence.

Taxonomic annotations for seed and unmatched non-seed sequences were assigned using the USEARCH and Silva v132 reference with a minimum similarity threshold of 90%.[27,28] In order to improve depth of annotation, the standard QIIME assignment algorithm was modified to only consider hits at each taxonomic level that had an assigned name. Furthermore, any hits in the reference database must have a minimum identity of 97% or 99% to be considered for genus or species level assignment, respectively. Taxonomic annotations and sequence abundance data were then merged into a single sequence table.

Statistical analysis

Analyses method for base-line characteristics

Quantile–quantile plot was used to assess normality of data. Mean ± standard deviation and median (range) were calculated for normal and skewed variables, respectively.

One-way analysis of variance (ANOVA) and Kruskal–Wallis tests were performed for normal and nonnormal variables, respectively. A $P < 0.05$ was considered to be statistically significant. Statistical analysis was performed using Stata version 12(StataCorp, Texas, USA).

Differential analysis of amplicon sequence data

Differential analyses of taxa as compared with experimental covariates were performed using the software package edge R on raw sequence counts. Prior to analysis, the data were filtered to remove any sequences that were annotated as chloroplast or mitochondria in origin as well as removing taxa that accounted for <0.1% of the total sequence counts. Data were normalized as counts per million. Normalized data were then fit using a negative binomial generalized linear model (GLM) using experimental covariates, and statistical tests were performed using a likelihood ratio test. Adjusted P values were calculated using the Benjamini–Hochberg false discovery rate (FDR) correction. Significant taxa were determined based on an FDR threshold of 5% (0.05).

Alpha and beta diversity analysis of amplicon sequence data

Shannon indices were calculated with default parameters in R using the vegan library. Prior to analysis, the data

were rarefied to a depth of 6500 counts per sample. The resulting Shannon indices were then modeled with the sample covariates using a GLM assuming a Gaussian distribution. Significance of the model ANOVA was tested using the *F* test. *Post hoc*, pair wise tests were performed using Mann–Whitney test. Plots were generated in R using the ggplot2 library.

Bray-Curtis indices were calculated with default parameters in R using the vegan library. Prior to analysis the normalized data were square root transformed. The resulting dissimilarity indices were modeled and tested for significance with the sample covariates using the ADONIS test. Additional comparisons of the individual covariates were also performed using ANOSIM. Plots were generated in R using the ggplot2 library.

RESULTS

Clinical characteristics

Overall, 50 men (41.3 ± 8.9 years) entered this study, 12, 25, and 13 of whom were in the control, NAFLD, and presumed NASH groups, respectively [Table 1]. The number of individuals overweight or obese (body mass index [BMI] >25) was significantly greater in the presumed NASH group compared to NAFLD and in the presumed NASH and NAFLD groups, compared to controls. Serum triglycerides, VLDL and aspartate transaminase were also significantly greater in the presumed NASH and NAFLD groups ($P < 0.05$).

Alpha and beta diversity

Beta diversity was not significantly different at the genus level using ADONIS ($P = 0.466$, $R2 = 0.041$) and ANOSIM ($P = 0.573$, $R = -0.0121$) methods [Figure 1]. Alpha diversity assessed at the genus level via the Simpson (A), Evenness (B) and Richness (C) methods did not yield significant differences either, $P = 0.482$, $P = 0.573$ and $P = 0.464$, respectively [Figure 2].

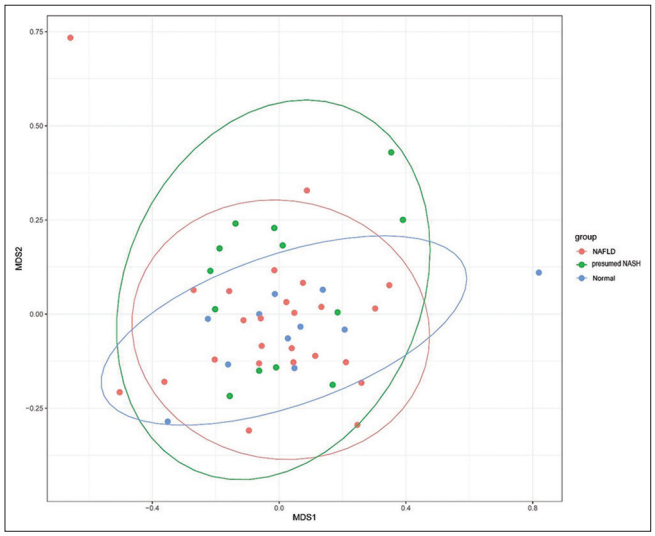

Figure 1: Nonmetric multidimensional scaling plot of Bray-Curtis dissimilarity indices computed using the taxonomic summary data at the genus level. Points are colored based on sample group

Microbiome comparison

The relative average phyla distribution of gut microbiomes in the NAFLD, presumed NASH, and control groups are reported in Figure 3. *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* were dominant in all study groups; differences in these phyla were insignificant. At the phyla level, there were no significant differences. At the Genus level, only *Veillonella* were found to be significantly different among the study groups [Figure 4]. *Veillonella* was more abundant in the presumed NASH group than the NAFLD group ($P = 2.76 \times 10^{-6}$, $q = 2.07 \times 10^{-4}$, logFC = 5.52).

DISCUSSION

Intestinal microbial composition affects host metabolism. Accumulating evidence suggests a relationship between microbial composition and fatty liver pathogenesis.[6,7] In this study, we compared gut microbiota in presumed NASH, NAFLD and healthy individuals at phyla, class, order, family and genus levels, and accounted for factors (diet, sleep quality and physical activity) that were shown to be associated with microbial composition as well as NAFLD in the prior studies.

Overall, our results indicate no significant difference in alpha and beta diversity, among the study groups. Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria have been recognized as the four dominant bacterial phyla.[31] Some studies have reported Firmicutes and Bacteroidetes to be the dominant phyla,[32-34] while our study, found Firmicutes, followed by Actinobacteria and Bacteroidetes to be dominant. These variations can be explained by ethnicity, genetics, diet, and lifestyles.[35]

Recent systematic reviews in NAFLD/NASH patients have also shown heterogeneity in biodiversity at both phyla and genus levels.[6,7] While some have reported significant differences at the phyla level between NASH patients and controls,[32-34] others observed no significant differences.[35,36] Among those reporting significant differences, the composition in the cases and controls did not follow a similar pattern, consistent with our findings; in some studies, Bacteroidetes increased in NASH patients and Firmicutes decreased,[32-34] while in others it was the opposite.[37] In this study, while Firmicutes increased in presumed NASH patients compared to the NAFLD and controls, no significant differences were observed at the phyla level.

Table 1: Demographic and paraclinic characteristics of study participants

Variables	Control (*n*=12)	NAFLD (*n*=25)	presumed NASH (*n*=13)	Total (*n*=50)	*P*
Age (years), mean±SD	41.8±9.7	41.8±10.0	39.8±6.1	41.3±8.9	0.8
BMI (kg/m^2), mean±SD	25.0±1.9	28.9±8.0	28.7±4.6	27.9±6.4	0.098
BMI>25 (kg/m^2), *n* (%)	7 (58.3)	15 (60.0)	11 (84.6)	33 (66.0)	0.026
Tobacco, *n* (%)	6 (50.0)	12 (48.0)	9 (69.2)	27 (54.0)	0.437
Quality of sleep, mean±SD	11.6±1.2	12.1±2.1	13.1±2.1	12.2±2.0	0.138
FBS (mg/dl), median (minimum-maximum)	92.0 (89-93.8)	92.0 (88-98)	91.0 (88-103.5)	91.5 (72.0-224.0)	0.8
Cholesterol (mg/dl), median (minimum-maximum)	162.5 (144.3-171.0)	168.0 (153.5-189.5)	162.0 (155.5-199)	165.0 (104.0-246.0)	0.7
TG (mg/dl), median (minimum-maximum)	86.5 (62.0-115.0)	115.0 (97.0-160.0)	141.0 (99.5-214.0)	114.0 (45.0-386.0)	0.03
HDL (mg/dl), median (minimum-maximum)	35.0 (32.3-37.8)	32.0 (30.0-38.5)	33.0 (28.5-36.5)	33.0 (24.0-59.0)	0.4
Cho/HDL (ratio), median (minimum-maximum)	4.7 (3.8-5.7)	5.1 (4.3-5.9)	5.4 (4.4-6.5)	5.0 (3.0-7.0)	0.4
VLDL (IU/L), median (minimum-maximum)	17.3 (12.4-23.0)	23.0 (19.4-32)	32.0 (19.9-47.1)	22.5 (9-244)	0.02
LDL-Cho (mg/dl), median (minimum-maximum)	105.5 (86.8-122.5)	99.0 (91.5-125.5)	97.0 (85.0-150.0)	100.0 (11.0-197.0)	0.9
ALT (U/L), median (minimum-maximum)	19.5 (10.0-44.0)	28.0 (13.0-43.0)	58.0 (46.0-132.0)	29.5 (10.0-132.0)	0.0001
AST (U/L), median (minimum-maximum)	20.5 (17.3-24.3)	19.0 (16.5-21.0)	30.0 (29.0-39.5)	21.0 (11-44.0)	0.0001
CRP (mg/L), median (minimum-maximum)	4.5 (2.5-5.8)	3.0 (1.5-6.0)	3.0 (1.0-6.0)	4.0 (1.0-25.0)	0.6
HbA1C (%), median (minimum-maximum)	5.2 (5.0-5.5)	5.3 (5.0-5.6)	5.6 (5.0-5.9)	5.3 (4.5-9.7)	0.5
ANA (U/ml), median (minimum-maximum)	2.8 (2.0-3.4)	2.3 (1.7-3.7)	2.7 (2.0-4.1)	2.0 (0.0-9.0)	0.7
MET, median (minimum-maximum)	2086.5 (1073.3-4807.0)	1278.0 (495.0-2206.5)	1173.0 (367.5-4410.0)	1279.5 (66.0-15588.0)	0.1
Calorie intake, median (minimum-maximum)	1628.7 (1259.0-2369.9)	2196.6 (1955.0-3009.0)	2427.3 (1861.7-3089.1)	2186.5 (1048.7-3969.1)	0.09
Systolic blood pressure, median (minimum-maximum)	110.0 (100.0-120.0)	110.0 (105.0-125.0)	120.0 (100.0-130.0)	110.0 (100.0-160.0)	0.7
Diastolic blood pressure, median (minimum-maximum)	80.0 (70.0-80.0)	80.0 (70.0-80.0)	80.0 (75.0-85.0)	80.0 (50.0-110.0)	0.5

ANOVA was used for normal variables and Kruskal–Wallis was used for nonnormal variables. NAFLD=NonAlcoholic fatty liver disease; presumed; NASH=Presumed nonalcoholic steatohepatitis; BMI=Body mass index; FBS=Fasting blood sugar; TG=Triglycerides; HDL=High-density lipoprotein; VLDL=Very low density lipoprotein; ALT=Alanine transaminase; AST=Aspartate transaminase; CRP=C-reactive protein; HbA1c=Hemoglobin A1C; ANA=Antinuclear antibody; MET=Metabolic equivalent of task; ANOVA=Analysis of variance

Inconsistent results may be explained by technical heterogeneity such as stool sampling and storage, as well as DNA extraction methods. Ethnicity and genetics have also been proposed to influence gut microbial composition. We used 16s rRNA analysis as the standard method to study taxonomic and phylogenetic composition of microbiota.[38,39] This study is the first evaluation of gut microbiota in NAFLD/presumed NASH patients in Middle East Region; hence, different results compared to studies in other countries were expected. Interestingly, some studies have shown that even within a single nation with similar lifestyles and dietary habits, microbial composition can vary significantly.[40]

BMI >25 was found to be significantly different among NAFLD/presumed NASH patients and controls. Prior to adjusting, microbial composition varied significantly, so the association of BMI with NAFLD/NASH may not be independent from microbial composition.

Veillonella was the only genus exhibiting a significant difference between the NAFLD and presumed NASH groups, with greater abundance among those with presumed NASH. Given that some studies have shown Veillonella to be greater in cirrhotic patients,[41,42] it is possible that this genus increases as liver disease progresses.

Veillonella, highly recognized for its involvement in lactate fermentation, causing the release of acetate and propionate.[43] Buildup of high amounts of these compounds has been previously shown to trigger gluconeogenesis and

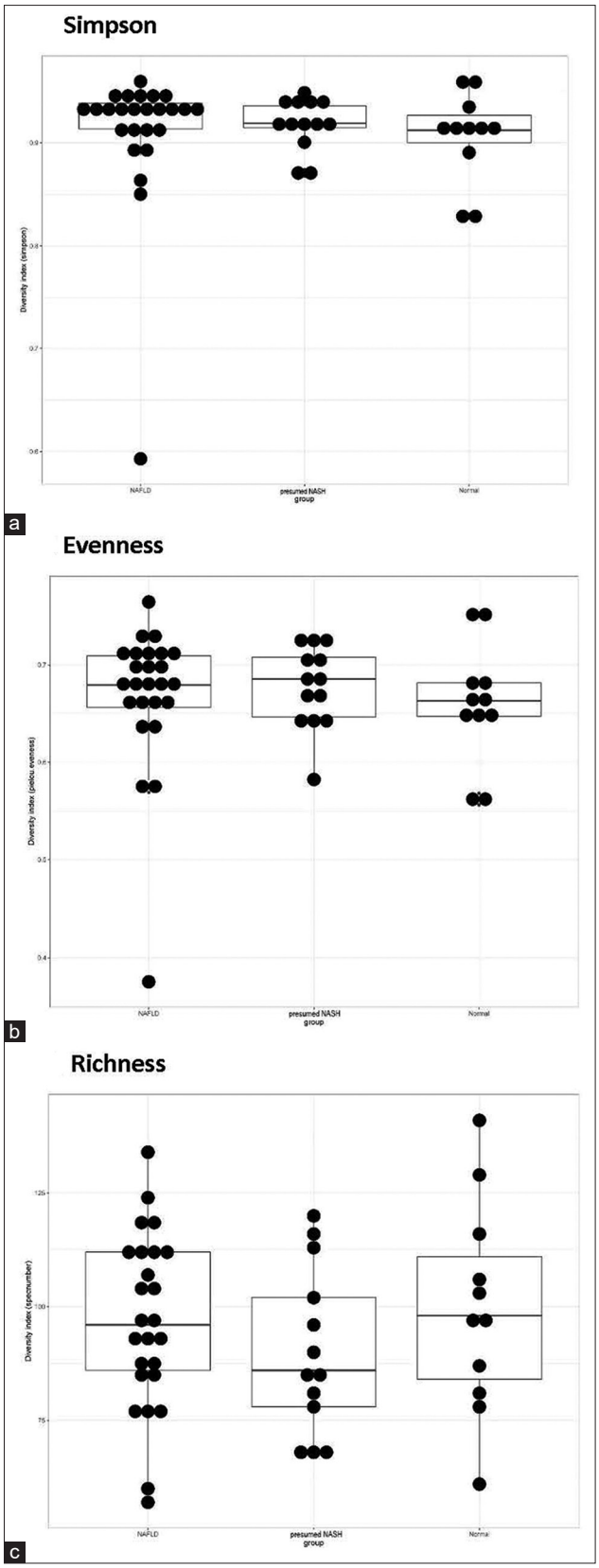

Figure 2: Dot and box plots of (a) Shannon, (b) Pielou's eveness and (c) richness diversity indices as compared with sample group

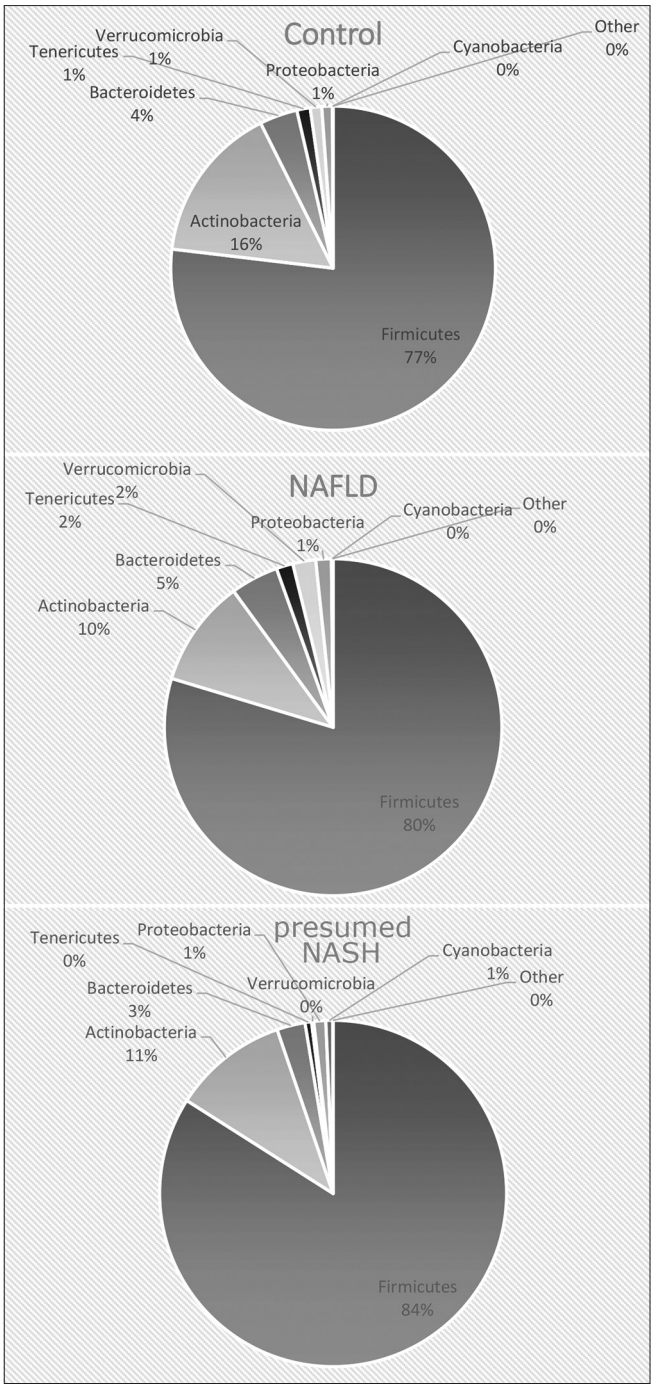

Figure 3: Relative sequence abundance of main bacterial phyla as compared with sample group

lipogenesis, increasing lipid storage in the liver and body tissues.[44] Veillonella has been shown to play a role in small intestinal bacterial overgrowth (SIBO),[45] which occurs more frequently in overweight/obese individuals.[46] In addition, SIBO is correlated with increased TLR4 expression and Interleukin 8 secretions, both of which affect inflammatory pathways involved in NAFLD pathogenesis.[47]

In this study, many factors, known to affect microbial composition in NAFLD/NASH patients were measured and controlled. For example, given that even small alterations in diet can affect the microbial composition, only individuals

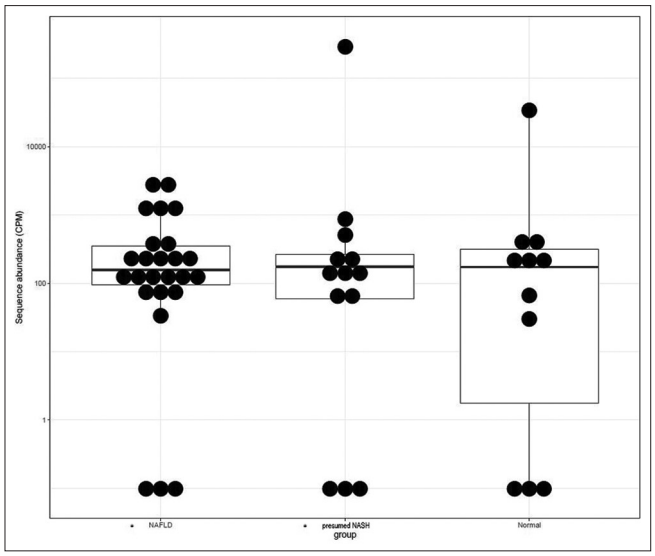

Figure 4: Relative sequence abundance of genus Veillonella as compared with sample group. *Veillonella was significantly different between presumed nonalcoholic steatohepatitis and nonalcoholic fatty liver disease group

who had stable, routine diets in the month prior to study recruitment were eligible to enter the study and dietary information was obtained for all individuals to control for variations. As expected, individuals with NAFLD and NASH consumed higher overall calories as well as calories from fat compared to healthy controls.

CONCLUSION

The dominant phyla in this study population were different from those of many other populations; however, these results were not significantly different among the healthy individuals and those with fatty liver. Larger, longitudinal cohort studies are needed to better control for all factors affecting NAFLD/presumed NASH development and capture long-term changes in microbial composition of this patient population.

Acknowledgments

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Conflicts of interest

There are no conflicts of interest.

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