HEPATOLOGY
Official Journal of the American Association for the Study of Liver Diseases

Characterization of Gut Microbiomes in Nonalcoholic Steatohepatitis (NASH) Patients: A Connection Between Endogenous Alcohol and NASH

Lixin Zhu,[1] Susan S. Baker,[1] Chelsea Gill,[2] Wensheng Liu,[1] Razan Alkhouri,[1] Robert D. Baker,[1] and Steven R. Gill[2]

Nonalcoholic steatohepatitis (NASH) is a serious liver disease associated with obesity. Characterized by metabolic syndrome, hepatic steatosis, and liver inflammation, NASH is believed to be under the influence of the gut microflora. Here, the composition of gut bacterial communities of NASH, obese, and healthy children was determined by 16S ribosomal RNA pyrosequencing. In addition, peripheral blood ethanol was analyzed to monitor endogenous ethanol production of patients and healthy controls. UniFrac-based principle coordinates analysis indicated that most of the microbiome samples clustered by disease status. Each group was associated with a unique pattern of enterotypes. Differences were abundant at phylum, family, and genus levels between healthy subjects and obese patients (with or without NASH), and relatively fewer differences were observed between obese and the NASH microbiomes. Among those taxa with greater than 1% representation in any of the disease groups, Proteobacteria, Enterobacteriaceae, and *Escherichia* were the only phylum, family and genus types exhibiting significant difference between obese and NASH microbiomes. Similar blood-ethanol concentrations were observed between healthy subjects and obese non-NASH patients, but NASH patients exhibited significantly elevated blood ethanol levels. *Conclusions:* The increased abundance of alcohol-producing bacteria in NASH microbiomes, elevated blood-ethanol concentration in NASH patients, and the well-established role of alcohol metabolism in oxidative stress and, consequently, liver inflammation suggest a role for alcohol-producing microbiota in the pathogenesis of NASH. We postulate that the distinct composition of the gut microbiome among NASH, obese, and healthy controls could offer a target for intervention or a marker for disease.
(HEPATOLOGY 2013;57:601-609)

Nonalcoholic fatty liver disease (NAFLD), the hepatic manifestation of metabolic syndrome, is the most common cause of elevated liver enzymes in the United States.[1] NAFLD with inflammation and fibrosis is known as nonalcoholic steatohepatitis (NASH) because it resembles alcoholic liver disease (ALD) without a history of alcohol ingestion.[2] The incidence of NASH has been increasing over the past 20 years.[3] In the United States, the current prevalence of NAFLD and NASH could be as high as 46% and 12%, respectively.[4] Without an effective available treatment, the prognosis of NASH is not optimistic. NASH is responsible for approximately 10% of liver transplants in the United States and is projected to become the most common indication for liver transplantation in the near future.[5]

Approximately 25% of fatty livers progress to liver inflammation, fibrosis, and then cirrhosis, but the rest remain simple steatosis.[4] Therefore, it is likely that factor(s) other than, or in addition to, liver fat deposition are required for the development of NASH. Many studies have shown that an extra source of oxidative

Abbreviations: ADH, alcohol dehydrogenase; ALD, alcoholic liver disease; ANOVA, analysis of variance; BMI, body mass index; CDC, Centers for Disease Control; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; OS, oxidative stress; OTU, operational taxonomic unit; QIIME, Quantitative Insights into Microbial Ecology software; ROS, reactive oxygen species; rRNA, ribosomal RNA; TLR, toll-like receptor.

From the [1]Digestive Diseases and Nutrition Center, Department of Pediatrics, the State University of New York at Buffalo, Buffalo, NY; and [2]Department of Microbiology and Immunology, University of Rochester, Rochester, NY.

Received August 12, 2012; accepted September 24, 2012.

This work was supported by a grant from the Peter and Tommy Fund, Inc. (Buffalo, NY; to S.S.B.), a grant from the SKI4E Fund (to S.S.B.), and a departmental start-up fund (to L.Z.).

stress (OS) could be one such factor (e.g., as reviewed elsewhere[6]). These studies are the basis for the "two-hit hypothesis".[7] In addition to OS, Toll-like-receptors (TLRs)–mediated signaling,[8] adipose-tissue–derived signals,[9] endoplasmic reticulum stress,[10] and genetic factors[11] may be necessary for, or contribute to, the development of NASH.

Gut microbiota are thought to play a role in the pathogenesis of NASH for several reasons. First, gut microbiota are known to have a large effect on the digestion and absorption of nutrients.[12] Microbiota transplantation experiments in mice suggested that certain microbiota are capable of inducing obesity independent of other environmental factors.[13] Second, gut microbiota participate in the development and homeostasis of the overall immunity of the host.[14] Therefore, certain microbiota may influence the development of liver inflammation. The links between gut microbiota and the host immune system include TLRs and short-chain fatty acids.[15] For example, TLR5 knockout mice have a unique composition of gut microbiota, which induces hyperphagia, obesity, hyperglycemia, insulin resistance, and elevated levels of proinflammatory cytokines, when transplanted to wild-type germ-free mice.[16] Third, gut microbiota may influence the production of gut hormones, such as glucagon-like peptide 1, and, subsequently, have an effect on the overall metabolism of the host.[17]

Spencer et al. examined gut microbiomes of adult human subjects who had fatty livers induced by a choline-deficient diet.[18] They observed changes in gut microbiome composition upon liver fat induction, suggesting that gut microbiomes and liver health are closely related. In this report, we examined the gut microbiota of NASH, obese, and healthy children and adolescents. Composition of NASH microbiomes was found to be distinct from those of healthy and obese microbiomes. *Escherichia* stood out as the only abundant genus that differed between NASH and obese patients. Because *Escherichia* are ethanol producers, this finding is in concert with our previous report that alcohol-metabolizing enzymes are up-regulated in NASH livers.[19]

Patients and Methods

Human Subjects. This study was approved by the Children and Youth Institutional Review Board of the State University of New York at Buffalo. Three groups of children and adolescents were recruited in this study (NASH patients, obese patients, and healthy controls). All NASH patients included in this study (Table 1) underwent percutaneous liver biopsy and fulfilled Kleiner's criteria on hepatic fat infiltration, inflammation, and fibrosis.[20] Recruited in the obese group were patients whose body mass indices (BMIs) were higher than the 95th percentile, but whose liver function tests were normal. Patients with elevated transaminase, but without inflammation or fibrosis, were not included in the obese group because they may have had unidentified liver conditions that caused the elevated transaminases. Healthy controls were volunteers whose BMI was less than the 85th percentile. Enrolling only pediatric patients was an additional assurance that alcohol intake would not be a confounding factor in this study. Characteristics of human subjects are summarized in Table 1.

Table 1. Characteristics of the Study Groups

Characteristics	Control	Obese	NASH
Sex (F/M)	F6/M10	F12/M13	F10/M12
Age, years	14.4 ± 1.8*	12.7 ± 3.2	13.6 ± 3.5
BMI	20.4 ± 0.1	33.4 ± 0.3	34.0 ± 0.4
BMI *z*-score†	0.13 ± 0.04	2.36 ± 0.01	2.24 ± 0.02
AST	ND	28.4 ± 0.6	51.7 ± 1.3
ALT	ND	27.7 ± 0.6	66.9 ± 1.9
Ethnicity (black/white/other)	0/15/1	4/17/4	0/18/4

Abbreviations: F, female; M, male; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ND, not determined.

*Mean ± standard error.

†A *z*-score of 1.6449 is equivalent to the 95th percentile.

Dietary Assessment. Dietary intake for all individuals was assessed using three different methods. A 3-day diet history and a 24-hour dietary recall were used initially.[21] All patients were contacted by phone for detailed instructions on how to fill the 3-day diet history that was mailed to them. The 24-hour recall was obtained over the phone by a trained provider. Diet history and dietary recall data were analyzed with DINE Healthy version 7.0.1. The Centers for Disease Control (CDC) food frequency questionnaire was used[22] (CDC 2005 National health Interview Survey Questionnaire; available at: ftp://ftp.cdc.gov/pub/Health_Statistics/NCHS/Survey_Questionnaires/NHIS/2005/English/QCANCER.pdf) as an additional assessment tool for fiber intake.

Address reprint requests to: Lixin Zhu, Ph.D. (lixinzhu@buffalo.edu) or Susan S. Baker, M.D., Ph.D. (sbake@upa.chob.edu), Digestive Diseases and Nutrition Center, Department of Pediatrics, The State University of New York at Buffalo, 3435 Main Street, 422 BRB, Buffalo, NY14214. Fax: 716-829-3585.

View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep.26093

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

Genomic DNA Extraction, Sequencing, and Quantitative Analysis of the Microbiome Composition. Briefly, a single stool sample was collected from each patient and healthy control (details in Supporting Materials). Genomic DNA was isolated from stool samples using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) and mechanical lysis. 16S ribosomal RNA (rRNA) sequences were polymerase chain reaction amplified for pyrosequencing on a 454-FLX-Titanium Genome Sequencer (Roche 454 Life Sciences, Branford, CT). All raw 454 sequencing reads and the associated metadata are available at MG-rast (available at: http://metagenomics.anl.gov/linkin.cgi?project =1195). Pyrosequence reads were analyzed in the Quantitative Insights into Microbial Ecology (QIIME) software[23] version 1.4.0. For taxonomic assignment, sequence reads were grouped into operational taxonomic units (OTUs) at a sequence similarity level of 97%.

Enterotyping. Enterotypes were determined for all microbiome samples based on the criteria described by Arumugam et al.[24] Arumugam et al. detected three enterotypes: enterotype 1 is characterized by abundant *Bacteroides* (22%∼39%) and diminished *Prevotella* (0%∼1%); enterotype 2 is characterized by abundant *Prevotella* (6%∼36%) and less-abundant *Bacteroides* (2%∼17%); and enterotype 3 has diminished abundance in both genera (*Bacteroides*, 3%∼16%; *Prevotella*, 0%∼8%). Enterotype 3 is often also characterized by relatively more-abundant *Ruminococcus* (0.1%∼6.5%). Some of our microbiome samples were enriched in both *Bacteroides* and *Prevotella* and did not fit into the enterotypes described above. Therefore, we designated these samples as enterotype H, a hybrid between enterotypes 1 and 2.

Blast Search of the* Escherichia *OTU Sequence. From the QIIME-generated OTU table, all OTUs assigned to *Escherichia* were examined. The most abundant *Escherichia* OTU was identified to be OTU#20341. The DNA sequence of OTU#20341 was retrieved from a QIIME-generated FASTA file containing a representative sequence for each OTU. This sequence was used as the input for Blast search (available at: http://blast.ncbi.nlm.nih.gov), against the current 16S rRNA database (search performed on April 9, 2012).

Serum Alcohol Measurement. A single blood sample was collected from each patient at the time of liver biopsy (from July 26, 2010 through June 1, 2011). A single blood sample from each healthy and non-NASH obese subject was collected at the same period. Serum alcohol concentration was determined in subsets of obese and NASH patients in the microbiome study and a new cohort of healthy subjects. Serum samples were stored at −80 °C before ethanol concentrations were measured with an ethanol assay kit from BioVision (Milpitas, CA), following the manufacturer's instructions. Briefly, alcohol was oxidized by alcohol oxidase, and the product was subsequently measured by a colorimetric probe (at 570 nm). A linear standard curve between alcohol concentrations and OD570 was generated and used to calculate alcohol concentrations in serum samples.

Statistical Analysis. One-way analysis of variance (ANOVA) and post-hoc Tukey's honest significant difference tests for multiple comparisons were performed to evaluate differences in taxonomic abundance, alpha diversities, and serum ethanol concentrations among three groups. Fisher's exact test was performed to examine a possible association between enterotypes and health status. ANOVA, Tukey's, and Fisher's tests were performed with R version 2.14.0. A P value less than 0.05 was considered statistically significant.

Results

Ecological Diversities of Microbiomes in Healthy Subjects, Obese Patients, and NASH Patients. Gut microbiomes of healthy, obese, and NASH children and adolescents (Table 1) were analyzed by 16S rRNA pyrosequencing. A total of 835,591 sequencing reads were obtained from a total of 63 samples. Ecological diversity within each sample was evaluated by a phylogenetic distance metric (Supporting Fig. 1A). No significant difference was observed among three groups. However, as a measurement of species richness, Chao1 metric revealed significant differences among three groups (Supporting Fig. 1B). P(ANOVA) values were <0.05 at sampling sizes 1,268, 2,526, 3,784, and 5,042 OTUs. Post-hoc Tukey's tests indicated that differences were significant between obese and healthy controls (Tukey's $P < 0.05$ at sampling sizes 1,268, 2,526, 3,784, and 5,042 OTUs). Species abundance in the NASH group was marginally lower than that of the healthy group, with Tukey's P values of approximately 0.1.

Ecological diversities within each group were accessed by UniFrac analysis. The majority of the samples clustered by health status (Fig. 1), but not by age, gender, or ethnicity (Supporting Fig. 2), indicating a specific connection between health status and gut microbiomes. Exceptions from all three groups were observed, reflecting the effect from other genetic and environmental factors on these microbiomes.

To investigate possible effect of dietary habits on gut-microbiome composition of patients, dietary

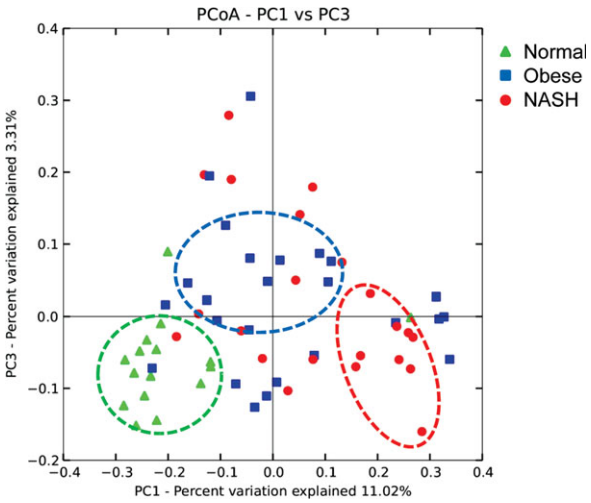

Fig. 1. UniFrac-based principle coordinates analysis: gut microbiomes clustered by health status with exceptions. The beta diversity of the study cohort was evaluated by UniFrac-based analysis. The majority of the samples clustered by health status at the PC1 versus PC3 plot, indicating that health status was a major effect factor for the phylogenetic composition of these samples. Exceptions from all three study groups were observed, reflecting the effect from other genetic and environmental factors on these microbiomes.

assessments were conducted to analyze dietary intake at the time of fecal-sample collection (Supporting Table 1). No significant difference in percent energy from protein, fat, or carbohydrate was found among healthy, obese, and NASH subjects. Dietary fructose, fiber, and aspartame (a potential source of methanol) were also similar among the three study groups. No significant dietary source of alcohol was identified for any of the patients or healthy controls.

Obesity and NASH Are Associated With Specific Enterotypes of Gut Microbiomes. Fifty-three of sixty-three microbiome samples fit into the enterotypes 1 (enriched in *Bacteroides*), 2 (enriched in *Prevotella*), and 3 (diminished in both *Bacteroides* and *Prevotella*), as described by Arumugam et al.,[24] but the remaining 10 samples did not fall into a previously defined enterotype (Supporting Table 2). These 10 samples were characterized by abundant representation in both *Bacteroides* and *Prevotella*, therefore termed enterotype H (hybrid between enterotypes 1 and 2). The majority of the healthy gut microbiomes were classified into

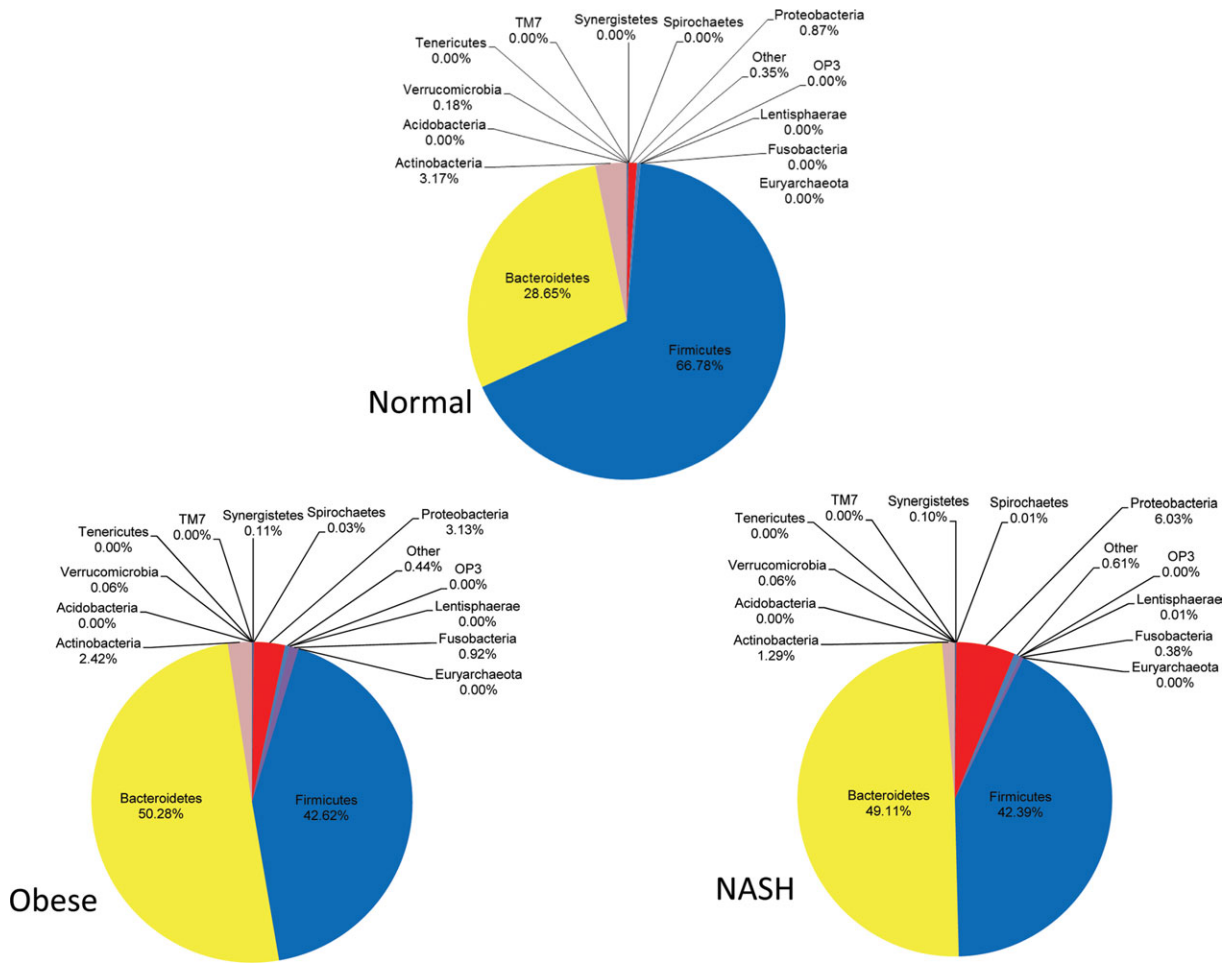

Fig. 2. Average phylum distribution of gut microbiomes of healthy subjects, obese patients, and NASH patients.

Table 2. Abundant Taxa in Gut Microbiomes of Healthy Subjects, Obese Patients, and NASH Patients

	Healthy*		Obese†		NASH‡		*P*(ANOVA)
Actinobacteria§	**3.17**		**2.42**		**1.29**		**0.0262**
Bifidobacteriaceae‖	2.19		1.54		0.64		0.0446
Bifidobacterium¶		2.16		1.47		0.63	0.0454
Bacteroidetes	**28.65**		**50.28**		**49.11**		**0.0092**
Bacteroidaceae	21.02		24.11		23.31		0.8960
Bacteroides		21.02		24.11		23.31	0.8960
Porphyromonadaceae	1.69		5.01		3.79		0.1920
Parabacteroides		1.40		1.69		1.34	0.8780
Porphyromonas		0.12		3.17		2.25	0.2210
Prevotellaceae	3.32		19.92		20.65		0.0295
Prevotella		3.05		19.71		20.62	0.0282
Rikenellaceae	1.97		0.74		0.41		0.0046
Alistipes		1.92		0.70		0.40	0.0048
Firmicutes	**66.78**		**42.62**		**42.39**		**0.0022**
Clostridiales family XI	4.21		12.87		13.59		0.2110
Anaerococcus		0.35		1.82		2.21	0.1240
Finegoldia		0.03		2.00		1.96	0.0882
Peptoniphilus		0.36		2.85		4.10	0.0372
Unknown#		3.39		5.94		4.82	0.8140
Lachnospiraceae	32.23		12.84		14.42		0.0008
Blautia		12.76		3.17		4.94	0.0058
Clostridium		1.13		1.22		1.11	0.9280
Coprococcus		3.69		1.32		1.03	0.0006
Eubacterium		1.18		0.36		0.29	0.0000
Roseburia		4.48		1.68		2.13	0.0155
Ruminococcus		1.74		1.21		0.77	0.0815
Ruminococcaceae	18.82		10.42		7.01		0.0007
Faecalibacterium		8.15		4.73		4.27	0.1040
Oscillospira		3.31		1.31		0.71	0.0254
Ruminococcus		1.66		1.39		0.33	0.0473
Unknown#		1.71		1.20		0.46	0.0270
Veillonellaceae	7.55		3.70		4.55		0.3460
Acidaminococcus		0.24		0.04		1.16	0.1520
Dialister		1.02		1.46		1.84	0.3770
Megamonas		2.72		0.03		0.27	0.2710
Proteobacteria	**0.87**		**3.13**		**6.03**		**0.0007**
Alcaligenaceae	0.21		0.97		1.11		0.0084
Campylobacteraceae	0.04		1.53		2.06		0.1470
Campylobacter		0.04		1.53		2.06	0.1470
Enterobacteriaceae	0.37		0.43		2.61		0.0075
Escherichia		0.30		0.37		2.36	0.0124

Numbers listed under study groups are percentages.
*Average abundance in gut microflora of healthy subjects (n = 16).
†Average abundance in gut microflora of obese patients (n = 25).
‡Average abundance in gut microflora of NASH patients (n = 22).
§Phyla with average abundance greater than 1% in any of the disease groups are listed.
‖Families with average abundance greater than 1% in any of the disease groups are listed.
¶Genera with average abundance greater than 1% in any of the disease groups are listed.
#16S rRNA sequence distinct from any known genera in this family.

enterotypes 1 and 3, reflecting the fact that healthy microbiomes are scarcely represented by *Prevotella*, whereas obese and NASH microbiomes are more frequently classified into enterotype 2 (*Prevotella* type). NASH samples further differentiated from obese samples in that only one NASH sample was classified as enterotype 3 and seven NASH samples were classified as enterotype H. Fisher's exact test suggested that each of the three groups was associated with a specific enterotyping pattern ($P < 0.01$).

Comparison of Microbiomes at the Phylum Level. Fourteen bacteria phyla were detected in gut microbiomes in this study (Fig. 2). *Bacteroides* and Firmicutes were the dominant phyla in these samples. Although exhibiting a broad distribution (Supporting Fig. 3), a statistically significant and drastic increase in *Bacteroides* and decrease in Firmicutes was apparent in the obese and NASH groups, compared to the healthy group (Figs. 2 and 3A). The abundance of *Bacteroides* and Firmicutes were similar between the obese and

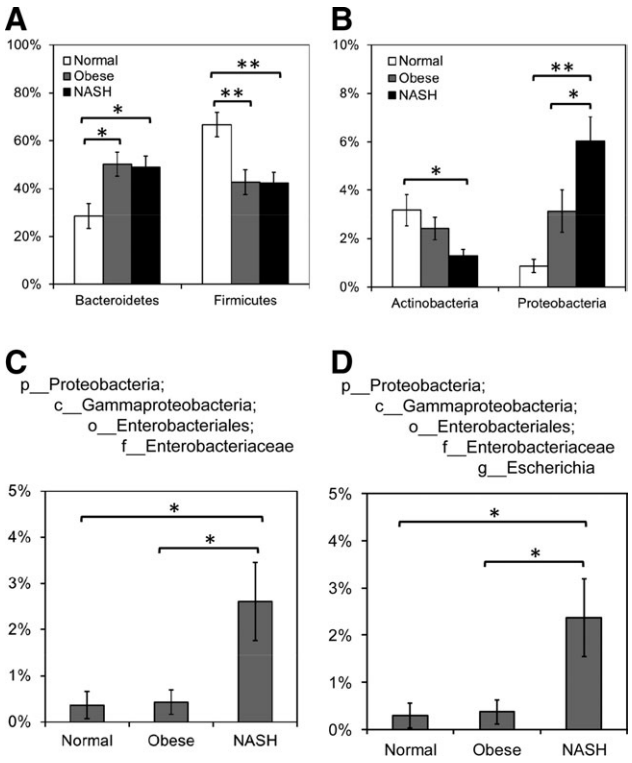

Fig. 3. Comparison of the taxonomic abundance among the healthy, obese and NASH groups. ANOVA tests indicated that these three groups were significantly different in all four abundant phyla in gut microbiomes: *Bacteroides*, Firmicutes (A), Actinobacteria, and Proteobacteria (B). Proteobacteria is the only phylum exhibiting significant difference between the obese and NASH groups. Within Proteobacteria, Enterobacteriaceae (C) and *Escherichia* (D) exhibited a significant difference between the obese and NASH groups. $*P < 0.05$ in Tukey's honest significance test; $**P < 0.01$ in Tukey's honest significance test.

NASH groups. Another two phyla, Actinobacteria and Proteobacteria, exhibited >1% abundance in at least one of the groups. ANOVA analysis indicated that these two phyla were also significantly different among the three groups (Fig. 3B). Tukey's tests showed that Actinobacteria was significantly lower in the NASH group, compared to the healthy group. A gradually increased abundance of Proteobacteria was observed from the healthy group to the obese group and then to the NASH group. Proteobacteria was the only abundant phylum exhibiting a significant difference between the obese and NASH groups.

Comparison of Microbiomes at Family and Genus Levels. To gain a clear image of the taxonomic changes in obese and NASH gut microbiomes, abundant families and genera with >1% average abundance in any of the groups were examined (Table 2).

Within phylum Actinobacteria (Table 2), the only abundant family Bifidobacteriaceae and the only abundant genus *Bifidobacterium* were differently represented in the study groups. A progressive decreased abundance was observed from the healthy group to the obese group and then to the NASH group.

Within phylum Bacteroidetes (Table 2), family Prevotellaceae exhibited a >6-fold increase in the obese group and NASH group, compared to the healthy group, accounting for most of the increased abundance in Bacteroidetes phylum in the obese and NASH groups. Most of the Prevotellaceae sequences belonged to a single-genus *Prevotella*. Another noteworthy fact in this phylum was that there was a ~20 fold increase of abundance in the genus *Porphyromonas* (family Porphyromonadaceae), but statistical significance was not achieved because of the large intragroup variances with the obese group and the NASH group. In contrast, a small, but significant, decrease was observed with Rikenellaceae, in which most of the sequences belonged to a single-genus *Alistipes*.

The decreased representation of Firmicutes in the obese group and the NASH group were mostly explained by the decreased abundance in two families: Lachanospiraceae and Ruminococcaceae (Table 2). Although most of the genera in these two families exhibited a similar trend (i.e., decreased abundance in the obese group and the NASH group, compared to the healthy group), it is noteworthy that the often pathogenic genus, *Clostridium*, exhibited similar representation among all groups. Also worth noting is that the most abundant genera in the Firmicutes phylum, *Blautia* and *Faecalibacterium*, showed the greatest reduction in abundance in the obese group and the NASH group.

Increased abundance of Proteobacteria in the obese and NASH groups was mainly explained by the increased abundance of Enterobacteriaceae (Table 2). Importantly, Enterobacteriaceae was the only abundant family (within the whole bacteria domain) exhibiting a significant difference between the obese group and the NASH group (Table 2; Fig. 3C). Most of the Enterobacteriaceae sequences belonged to *Escherichia* (Table 2; Fig. 3D), which is the only abundant genus within the whole bacteria domain exhibiting a significant difference between the obese group and the NASH group.

Furthermore, the OTUs within *Escherichia* were examined. A single OTU was found to dominate the sequences in *Escherichia*: OTU #20341 was found to account for 83%, 88%, and 90% of the *Escherichia* sequences in the healthy, obese, and NASH groups, respectively. The representative sequence of this OTU was then used to BLAST against the 16S rRNA sequences (Bacteria and Archaea) on the National Center for Biotechnology Information website (available at: http://blast.ncbi.nlm.nih.gov). The top four matches were

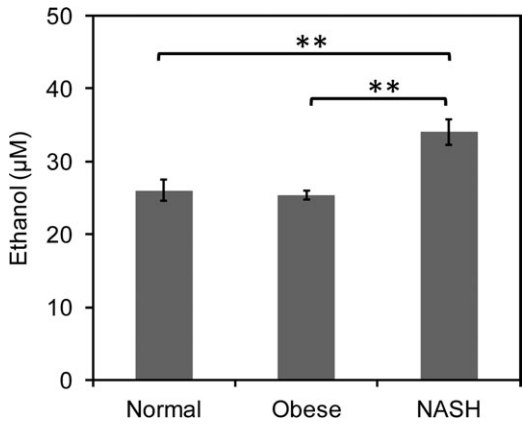

Fig. 4. Elevated serum ethanol concentration in NASH patients. Serum ethanol of healthy subjects (healthy; n = 10), obese patients (n = 7), and NASH patients (n = 13) were measured using an ethanol assay kit from BioVision (Milpitas, CA). Data represent mean ± standard error of the mean. Significant difference was detected among three groups ($P < 0.001$; ANOVA). $^{**}P < 0.01$ in Tukey's honest significance test.

sequences of *Escherichia* species, followed by two sequences of *Cronobacter*, another Enterobacteriaceae genus (Supporting Table 3; Supporting Fig. 4). Note that *Shigella* species have been reclassified as *Escherichia* coli strains based on genetic evidence.[25] Similar results were obtained when the OTU #20341 sequence was searched against the Ribosomal Database Project database with the SeqMatch tool (Supporting Table 4).

Serum Alcohol Concentration in Healthy Subjects and Obese and NASH Patients. Elevated alcohol-producing bacteria in the NASH microbiota prompted us to examine the endogenous ethanol production in patients and healthy controls. Because it is not feasible to obtain portal blood where highest ethanol concentration is expected, peripheral blood was used to determine serum ethanol concentrations of healthy subjects and obese and NASH patients (Fig. 4). A significantly elevated serum ethanol concentration was observed with NASH patients, when compared to healthy subjects and obese patients. Serum ethanol concentration was not different between healthy subjects and obese patients.

Discussion

Here, we characterized gut microbiomes of NASH, obese, and healthy children and adolescents. Ecological diversities (alpha and beta) were different among three groups, indicating a strong connection between gut microbiomes and liver health. Each health status is associated with a unique pattern of enterotyping. Abundant differences among three groups were observed at phylum, family, and genus levels (Table 2). However, fewer differences were observed between obese and NASH microbiomes. Among taxa with greater than 1% representation, Proteobacteria, Enterobacteriaceae, and *Escherichia* were the only phylum, family, and genus exhibiting significant difference between obese and NASH microbiome. Proteobacteria/Enterobacteriaceae/*Escherichia* was similarly represented between healthy and obese microbiomes, but was significantly elevated in NASH. A strikingly similar pattern was observed with blood alcohol concentrations of healthy, obese, and NASH patients.

Liver ultrasound indicated that some obese patients had fatty liver and others did not. No significant difference was observed between these two subgroups in gut microbiomes at all taxonomic levels, possibly the result of the small sample sizes of both subgroups. Future studies with larger sample sizes may reveal differences in gut microbiomes between these two subgroups of obese patients.

Under normal conditions, alcohol is constantly produced in the human body.[26] Intestinal microbiota is the major source of endogenous alcohol, as suggested by the increased blood alcohol level after intake of alcohol-free food.[26-28] This endogenously produced alcohol is immediately and almost completely removed from portal blood by liver alcohol dehydrogenases (ADHs), catalases, and microsomal ethanol-oxidizing system. When ADH is inhibited, blood alcohol levels increase.[26] Production of ethanol in the gut is also reflected by the fact that the liver and gastrointestinal tract have the highest activities of ADHs.[29]

Elevated breath alcohol levels are observed in obese mice, in which abnormal intestinal microbiota is the source for increased alcohol production and neomycin treatment decreases alcohol concentration.[30] Because NASH patients are generally obese and liver histology is the same as that observed in ALD, it was hypothesized that NASH patients may also have elevated blood alcohol.[30] The alcohol hypothesis of NASH could also explain the observation of increased gut permeability[31] and, possibly, elevated serum lipopolysaccharide in NASH patients,[32] because alcohol is known to increase gut permeability.[33] The first evidence in support of this hypothesis was that the gene expression of all three major pathways for ethanol catabolism in NASH liver are highly elevated.[19] Recently, elevated blood ethanol concentration was observed in NAFLD patients.[34] The observation of Volynets et al.[34] provides a link between blood alcohol and NAFLD. Our data further clarified that the blood ethanol concentration of obese patients without NASH is not elevated; however, obese patients with NASH had significantly elevated blood ethanol.

In anaerobic conditions, Enterobacteriaceae, including *Escherichia*, takes the mixed-acid fermentation pathway, a major product of which is ethanol.[35-39] Because the pediatric patients had no access to frequent alcoholic beverages and no dietary source for alcohol was found, the colon microbiota is likely the major source for the elevated blood alcohol concentration in our NASH patients. Our findings of increased abundance of *Escherichia* in NASH microbiomes, and of the elevated blood alcohol in NASH patients, together with the well-established role for alcohol metabolism in the generation of reactive oxygen species (ROS) and, consequently, liver inflammation,[40] suggest a novel mechanism for the pathogenesis of NASH is: Gut microbiota enriched in alcohol-producing bacteria (e.g., *E. coli*) constantly produce more alcohol than healthy microbiota and therefore supply a constant source of ROS to the liver, which, in turn, causes liver inflammation.

The most direct support for this hypothesis would come from the measurement of portal blood ethanol, which is not feasible with human subjects. Because no adequate NASH animal model bearing human microbiomes is available, we planned to examine the alcohol production by cultured endogenous *Escherichia*, but found that the experiment was performed many times 60 years ago. A typical result is that 1 g (wet weight) of *E. coli* produces 0.8 g of ethanol in 1 hour.[35] Because the adolescent colon is 1-2 L,[41] one would estimate that gut microbiomes of NASH patients could produce a significant ethanol insult for the liver, considering the fact that chronic intake of ~30 g of alcohol per day will cause liver damage.[42,43] Moreover, because fat deposition sensitizes the liver to alcohol insult, even a relatively lower level of alcohol is sufficient to cause inflammation and fibrosis.[44]

A recent study using inflammasome-deficient mice supports that modulation of gut microbiomes exerts a critical influence on liver health, in particular, the progression of NAFLD.[45] Nevertheless, before direct evidence is available, we cannot exclude the opposite scenario: the possibility that change in gut microbiomes is a consequence of liver disease. More specifically, could fatty liver cause increased abundance of *Escherichia* in the gut? This seems unlikely, because Spencer et al. demonstrated that induction of fatty liver diseases caused a decreased abundance of Proteobacteria (to which *Escherichia* belongs) in gut microbiomes.[18]

Besides *Escherichia*, other gut microbial genera, including *Bacteroides*,[46] *Bifidobacterium*,[47] and *Clostridium*,[48] are capable of producing alcohol, and, collectively, these genera may generate a significant burden for liver-alcohol–scavenging mechanisms. These bacteria and, perhaps, yeast not assessed in this study may explain why some NASH patients had blood ethanol concentrations higher than healthy subjects, even though their microbiomes did not exhibit an increased abundance in *Escherichia*.

In conclusion, our study revealed unique characters in the composition, ecological diversity, and enterotyping patterns of gut microbiomes of NASH patients, in comparison to those of healthy and obese patients. The most outstanding character was the elevated representation of alcohol-producing bacteria in NASH microbiomes. Increased blood alcohol concentration was also observed with NASH patients. Our data suggest that microbiomes rich in ethanol-producing *Escherichia* may be a risk factor in driving the disease progression from obesity to NASH. An immediate future task is to characterize and quantitate the activities of the alcohol-producing genes in microbiomes. Another important future direction is to identify the cause(s) for the increased level of *Escherichia* in NASH gut microbiomes. Possible causes for elevated *Escherichia* include special dietary components, as suggested by a recent report that subtherapeutic doses of antibiotics leads to increased *Escherichia* in swine gut microbiomes.[49] Microbiomes in patients with NASH may offer a unique opportunity for interrupting disease progression.

Acknowledgments: The authors thank Dr. Pearay Ogra (UB) and Dr. W. Allan Walker (Harvard) for their critical comments and invaluable suggestions and Jonathan E. Bard (UB Next-Generation Sequencing and Expression Analysis Core) for his expert assistance with the QIIME.

References

1. Vernon G, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. Aliment Pharmacol Ther 2011;34: 274-285.
2. Ludwig J, Viggiano TR, McGill DB, Oh BJ. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. Mayo Clin Proc 1980;55:434-438.
3. Younossi ZM, Stepanova M, Afendy M, Fang Y, Younossi Y, Mir H, Srishord M. Changes in the prevalence of the most common causes of chronic liver diseases in the United States from 1988 to 2008. Clin Gastroenterol Hepatol 2011;9:524-530.e521.
4. Williams CD, Stengel J, Asike MI, Torres DM, Shaw J, Contreras M, Landt CL, et al. Prevalence of nonalcoholic fatty liver disease and non-alcoholic steatohepatitis among a largely middle-aged population utilizing ultrasound and liver biopsy: a prospective study. Gastroenterology 2011;140:124-131.
5. Charlton MR, Burns JM, Pedersen RA, Watt KD, Heimbach JK, Dierkhising RA. Frequency and outcomes of liver transplantation for nonalcoholic steatohepatitis in the United States. Gastroenterology 2011;141: 1249-1253.
6. Berson A, De Beco V, Letteron P, Robin MA, Moreau C, El Kahwaji J, et al. Steatohepatitis-inducing drugs cause mitochondrial dysfunction

and lipid peroxidation in rat hepatocytes. Gastroenterology 1998;114: 764-774.
7. Day CP, James OF. Steatohepatitis: a tale of two "hits"? Gastroenterology 1998;114:842-845.
8. Rivera CA, Adegboyega P, van Rooijen N, Tagalicud A, Allman M, Wallace M. Toll-like receptor-4 signaling and Kupffer cells play pivotal roles in the pathogenesis of non-alcoholic steatohepatitis. J Hepatol 2007;47:571-579.
9. Moschen AR, Molnar C, Geiger S, Graziadei I, Ebenbichler CF, Weiss H, et al. Anti-inflammatory effects of excessive weight loss: potent suppression of adipose interleukin 6 and tumour necrosis factor alpha expression. Gut 2010;59:1259-1264.
10. Greene MW, Burrington CM, Ruhoff MS, Johnson AK, Chongkrairatanakul T, Kangwanpornsiri A. PKC{delta> is activated in a dietary model of steatohepatitis and regulates endoplasmic reticulum stress and cell death. J Biol Chem 2010;285:42115-42129.
11. Romeo S, Kozlitina J, Xing C, Pertsemlidis A, Cox D, Pennacchio LA, et al. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nat Genet 2008;40:1461-1465.
12. Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, et al. Metagenomic analysis of the human distal gut microbiome. Science 2006;312:1355-1359.
13. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 2006;444:1027-1031.
14. Noverr MC, Huffnagle GB. Does the microbiota regulate immune responses outside the gut? Trends Microbiol 2004;12:562-568.
15. Bohmig GA, Krieger PM, Saemann MD, Wenhardt C, Pohanka E, Zlabinger GJ. n-butyrate downregulates the stimulatory function of peripheral blood-derived antigen-presenting cells: a potential mechanism for modulating T-cell responses by short-chain fatty acids. Immunology 1997;92:234-243.
16. Vijay-Kumar M, Aitken JD, Carvalho FA, Cullender TC, Mwangi S, Srinivasan S, et al. Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. Science 2010;328:228-231.
17. Vrieze A, Holleman F, Zoetendal EG, de Vos WM, Hoekstra JB, Nieuwdorp M. The environment within: how gut microbiota may influence metabolism and body composition. Diabetologia 2010;53: 606-613.
18. Spencer MD, Hamp TJ, Reid RW, Fischer LM, Zeisel SH, Fodor AA. Association between composition of the human gastrointestinal microbiome and development of fatty liver with choline deficiency. Gastroenterology 2011;140:976-986.
19. Baker SS, Baker RD, Liu W, Nowak NJ, Zhu L. Role of alcohol metabolism in non-alcoholic steatohepatitis. PLoS One 2010;5:e9570.
20. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. HEPATOLOGY 2005;41: 1313-1321.
21. Thompson F, Subar A. Dietary assessment metholology. In:Coulston A,Boushey C, eds. Nutrition in the Prevention and Treatment of Disease, 2nd ed. San Diego, CA: Academic Press; 2008:3-39.
22. Buzzard IM, Stanton CA, Figueiredo M, Fries EA, Nicholson R, Hogan CJ, Danish SJ. Development and reproducibility of a brief food frequency questionnaire for assessing the fat, fiber, and fruit and vegetable intakes of rural adolescents. J Am Diet Assoc 2001;101:1438-1446.
23. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 2010;7:335-336.
24. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, et al. Enterotypes of the human gut microbiome. Nature 2011; 473:174-180.
25. Lan R, Reeves PR. *Escherichia coli* in disguise: molecular origins of Shigella. Microbes Infect 2002;4:1125-1132.
26. Sarkola T, Eriksson CJ. Effect of 4-methylpyrazole on endogenous plasma ethanol and methanol levels in humans. Alcohol Clin Exp Res 2001;25:513-516.
27. Blomstrand R. Observations of the formation of ethanol in the intestinal tract in man. Life Sci II 1971;10:575-582.
28. Watanabe-Suzuki K, Seno H, Ishii A, Kumazawa T, Suzuki O. Ultrasensitive method for determination of ethanol in whole blood by headspace capillary gas chromatography with cryogenic oven trapping. J Chromatogr B Biomed Sci Appl 1999;727:89-94.
29. Engeland K, Maret W. Extrahepatic, differential expression of four classes of human alcohol dehydrogenase. Biochem Biophys Res Commun 1993;193:47-53.
30. Cope K, Risby T, Diehl AM. Increased gastrointestinal ethanol production in obese mice: implications for fatty liver disease pathogenesis. Gastroenterology 2000;119:1340-1347.
31. Miele L, Valenza V, La Torre G, Montalto M, Cammarota G, Ricci R, et al. Increased intestinal permeability and tight junction alterations in nonalcoholic fatty liver disease. HEPATOLOGY 2009;49:1877-1887.
32. Ruiz AG, Casafont F, Crespo J, Cayon A, Mayorga M, Estebanez A, 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;17:1374-1380.
33. Rao RK, Seth A, Sheth P. Recent advances in alcoholic liver disease I. Role of intestinal permeability and endotoxemia in alcoholic liver disease. Am J Physiol Gastrointest Liver Physiol 2004;286:G881-G884.
34. Volynets V, Kuper MA, Strahl S, Maier IB, Spruss A, Wagnerberger S, et al. Nutrition, intestinal permeability, and blood ethanol levels are altered in patients with nonalcoholic fatty liver disease (NAFLD). Dig Dis Sci 2012;57:1932-1941.
35. Dawes EA, Foster SM. The formation of ethanol in *Escherichia coli*. Biochim Biophys Acta 1956;22:253-265.
36. Paege LM, Gibbs M. Anaerobic dissimilation of glucose-C14 by *Escherichia coli*. J Bacteriol 1961;81:107-110.
37. Stokes JL. Fermentation of glucose by suspensions of *Escherichia coli*. J Bacteriol 1949;57:147-158.
38. Clark DP. The fermentation pathways of *Escherichia coli*. FEMS Microbiol Rev 1989;5:223-234.
39. Brooks JB, Basta MT, el Kholy AM. Studies of metabolites in diarrheal stool specimens containing *Shigella* species by frequency-pulsed electron capture gas-liquid chromatography. J Clin Microbiol 1985;21:599-606.
40. Lieber CS. Microsomal ethanol-oxidizing system (MEOS): the first 30 years (1968-1998)—a review. Alcohol Clin Exp Res 1999;23:991-1007.
41. Hernandez RJ, Gutowski D, Guire KE. Capacity of the colon in children. AJR Am J Roentgenol 1979;133:683-684.
42. Anderson P, Cremona A, Paton A, Turner C, Wallace P. The risk of alcohol. Addiction 1993;88:1493-1508.
43. Bellentani S, Saccoccio G, Costa G, Tiribelli C, Manenti F, Sodde M, et al. Drinking habits as cofactors of risk for alcohol induced liver damage. The Dionysos Study Group. Gut 1997;41:845-850.
44. Xu J, Lai KK, Verlinsky A, Lugea A, French SW, Cooper MP, et al. Synergistic steatohepatitis by moderate obesity and alcohol in mice despite increased adiponectin and p-AMPK. J Hepatol 2011;55:673-682.
45. Henao-Mejia J, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T, et al. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature 2012;482:179-185.
46. Frantz JC, McCallum RE. Growth yields and fermentation balance of *Bacteroides fragilis* cultured in glucose-enriched medium. J Bacteriol 1979;137:1263-1270.
47. Amaretti A, Bernardi T, Tamburini E, Zanoni S, Lomma M, Matteuzzi D, Rossi M. Kinetics and metabolism of *Bifidobacterium adolescentis* MB 239 growing on glucose, galactose, lactose, and galactooligosaccharides. Appl Environ Microbiol 2007;73:3637-3644.
48. Weimer PJ, Zeikus JG. Fermentation of cellulose and cellobiose by *Clostridium thermocellum* in the absence of *Methanobacterium thermoautotrophicum*. Appl Environ Microbiol 1977;33:289-297.
49. Looft T, Johnson TA, Allen HK, Bayles DO, Alt DP, Stedtfeld RD, et al. In-feed antibiotic effects on the swine intestinal microbiome. Proc Natl Acad Sci U S A 2012;109:1691-1696.