



Determination of certain bacterial groups in gut microbiota and endotoxin levels in patients with nonalcoholic steatohepatitis

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ABSTRACT

Background/Aims: In recent years, the role of the gut microbiota has emerged in several diseases. Herein we aimed to determine the fecal microbiota, endotoxin levels, and inflammation markers in patients with nonalcoholic steatohepatitis (NASH) and healthy controls.

Materials and Methods: A total of 46 NASH patients and 38 healthy controls were included. NASH patients were diagnosed according to the steatosis, activity, and fibrosis score/fatty liver inhibition of progression algorithm. 16S rRNA gene-targeted specific primers were used for quantification of certain bacterial groups, and a plasmid library was constructed and sequenced in order to determine dominant *Lactobacillus* and *Bifidobacterium* members in patients and controls.

Results: Significantly decreased *Akkermansia muciniphila* and increased Enterobacteriaceae levels were determined in patients compared to healthy controls even after adjusting for the body mass index (BMI) and age. Patients with \geq F2 fibrosis had significantly higher Enterobacteriaceae levels compared to F0-F1 fibrosis. Serum endotoxin and high-sensitivity C-reactive protein levels were significantly higher in patients group. According to the sequencing results, *L. reuteri*, which was one of the dominant *Lactobacillus* species in the patient group, could not be detected in healthy controls. *Bifidobacterium infantis* was found in the patients' feces but not in the controls.

Conclusion: We demonstrated a BMI and age-independent association between the presence of NASH and levels of *A. muciniphila* and Enterobacteriaceae as well as increased endotoxin levels. *L. reuteri* was abundant in the patient group, suggesting that dominant *Lactobacillus* species should be considered before probiotic treatments.

Keywords: Nonalcoholic steatohepatitis, gut microbiota, *Akkermansia muciniphila*, endotoxin, C-reactive protein

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) includes a spectrum of hepatic pathology ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) and fibrosis (1). The exact mechanism of the disease is not well characterized, and no treatment strategy has proven effective to improve NAFLD prognosis (2). Studies on the identification of the mechanisms underlying the disease, particularly involving the microbiota, might shed light on both NAFLD pathogenesis and new therapeutic targets.

The effect of the gut microbiota on both health and the pathogenesis of multiple diseases is becoming an increasingly important issue. The data provided from The Human Microbiome Project and the MetaHit Consortium has enabled researchers to elucidate the composition of the gut microbiota and its role in health and pathological conditions (3). The gut microbiota has unique features, including energy harvest from the diet, balance of the immunological functions, change in gene expressions involved in de-novo lipogenesis, and protection of the gastrointestinal layer (4-6). Disruption

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of the balance among different gut bacteria may cause several metabolic disorders.

The gut and liver are closely related with each other. The liver receives 70% of its blood supply from the intestine through the portal vein (1). Thus, the liver is the first line of defense against gut-derived bacterial products. Bacterial overgrowth in the gut leads to an increase in intestinal permeability and the translocation of bacterial products, especially endotoxins (6-8). This is thought to be the major mechanism underlying disease pathogenesis. There is increasing evidence that NAFLD pathology is due to the acute phase response primarily caused by bacterial endotoxins (6,7,9). This endotoxin translocation across the intestinal barrier is likely to be caused by dysbiosis, which refers to microbial imbalance and subsequent disruption of gut barrier function due to alterations of the gut microbiota.

The aim of our study was to reveal the quantitative differences of certain bacterial species in fecal microbiota in patients with biopsy-proven NASH (a subgroup of NAFLD) and healthy controls, as well as to determine endotoxin levels and the inflammation status of these patients. We also aimed to determine the relative abundance of *Lactobacillus* and *Bifidobacterium* species in order to detect possible differences between patients and controls.

MATERIALS AND METHODS

Patients and Sample Collection

A total of 46 patients with biopsy-proven NASH according to the steatosis, activity, and fibrosis (SAF) score/fatty liver inhibition of progression algorithm who were admitted to the Gastroenterology Clinic were included from April 2014 to May 2015. The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in approval by the Ethics Committee. Patients who were diagnosed with NASH were enrolled in the study after obtaining their written informed consent. Exclusion criteria were as follows: men who consumed >20 g and women who consumed >10 g of alcohol per day, consumption of antibiotics, probiotics, prebiotics, or any other medications known to have an effect on steatohepatitis within the 6 months prior to sample collection, and patients with evidence of concomitant liver diseases, hemolysis, Gilbert's disease, human immunodeficiency virus infection, or who were on immunosuppressive therapy. Serum hepatitis B surface antigen, antibodies to hepatitis C virus, ceruloplasmin by copper oxidase method, anti-nuclear antibody, anti-smooth muscle antibody, anti-liver/kidney microsome antibody, serum protein electrophoresis, urinary copper, and Kayser-Fleischer ring on slit-lamp examination were used wherever indicated to exclude other causes of liver diseases. The stool samples and blood samples were collected on the same day. A health questionnaire was filled out and demographic information was recorded, including age, gender, alcohol use, smoking history, past medical and surgical history, waist circumference, body mass index (BMI), history of diabetes, and the presence of hypertension. Blood samples were drawn for rou-

tine biochemical tests and also for circulating inflammatory cytokines, endotoxin levels, and high-sensitivity C-reactive protein (hs-cRP) levels. Blood samples were centrifuged at 3000 rpm for 10 min to separate serum. Serum samples were kept at -80°C until use. Patients were instructed to collect stool samples. Stool samples were immediately stored at -80°C after a 200 mg aliquot was taken into a 1.5 mL collection tube for DNA isolation. The DNA isolation from stool samples was performed with a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Input/output DNA amounts were calculated to convert the quantitative polymerase chain reaction (qPCR) results into log₁₀ copy/g feces.

Liver Histology

Tissue obtained at biopsy was fixed in formalin and subjected to routine paraffin embedding and hematoxylin and eosin staining, ensuring adequate sections to examine the entire biopsy. Sections were also stained with reticulin and Masson's trichrome stain.

Histological confirmation of NAFLD was made using the SAF score. The steatosis score (S) assessed the quantities of large or medium-sized lipid droplets, but not foamy microvesicles, on a scale from 0 to 3 (S0: <5%; S1: 5%-33%, mild; S2: 34%-66%, moderate; S3: >67%, marked) (10,11). Specimens were evaluated for necro-inflammation and fibrosis by an experienced pathologist who was blinded for patient's data. Significant fibrosis was defined as fibrosis ≥2 and minimal fibrosis as F0 or F1.

Quantitative Polymerase Chain Reaction Analysis

As an internal control for qPCR experiments, the following bacterial ATCC (American type culture collection) standard strains were used: *Akkermansia muciniphila* ATCC BAA-835, *Faecalibacterium prausnitzii* ATCC 27766, *Bifidobacterium breve* ATCC 15700, *Lactobacillus acidophilus* ATCC 4356, *Bacteroides fragilis* ATCC 25285, *Escherichia coli* ATCC 25922. *Lactobacillus acidophilus* and *Bifidobacterium breve* were cultured on Man Rogosa Sharpe agar, *A. muciniphila* was grown on Brain Heart Infusion Agar supplemented with 5 µg/mL hemin and 0.1 µg/mL vitamin K1, *F. prausnitzii*, which is a strictly anaerobic bacteria, was cultured on M2GSC medium with 30% rumen fluid, and *E. coli* was cultured on MacConkey Agar plates. All of the bacterial cultures except *E. coli* were grown under anaerobic conditions and incubated anaerobically (anaerobic jars with Anaerocult A gas packs; Oxoid). Sufficient numbers of bacterial colonies from pure cultures were suspended in phosphate buffer saline, and DNA was extracted with High-Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany). The amount of DNA was measured using a Nano Drop ND-1000 (Thermo Scientific, Wilmington, DE). The copy number/µL was calculated according to the concentration and molecular mass (number of base pairs×660) depending on the genome size of the target bacteria.

For the construction of the standard curves, a 10-fold dilution series, including 10², 10³, 10⁴, 10⁵, 10⁶, 10⁷ copy number/µL was prepared. The standard curve was generated by using at least

Table 1. Specific primer pairs used in this study

Bacterium	Standard strain	Primer Seq (5'-3')	Target (bp)	Primer	Annealing (oC)	Tm*
<i>Bifidobacterium</i> spp.	<i>B. breve</i> ATCC 15700	CTCCTGGAACGGGTGG GGTGTCTTCCCAGATCTACA	550	g-Bifid-F g-Bifid-R	56	90
<i>B. fragilis</i> group	<i>B. fragilis</i> ATCC 25285	ATAGCCTTTCGAAAGRAAGAT CCAGTATCAACTGCAATTTTA	495	g-Bfra-F g-Bfra-R	50	86
<i>Lactobacillus</i> spp.	<i>L. acidophilus</i> ATCC 4356	AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG	341	Lact-F Lact-R	55	86
<i>A.muciniphila</i>	<i>A.muciniphila</i> ATCC BAA-835	CAGCACGTGAAGGTGGGGAC CCTTGC GGTTGGCTTCAGAT	327	AM-1 AM-2	60	90
<i>F. prausnitzii</i>	<i>F. prausnitzii</i> ATCC 27766	GATGGCCTCGCTCCGATTAG CCGAAGACCTTCTCTCC	199	Fprau223F Fprau420R	60	88
Enterobacteriaceae	<i>E. coli</i> ATCC 25922	CATTGACGTACCCGCAGAAGAAGC CTCTACGAGACTCAAGCTTGC	195	Eco1457F Eco1652R	63	87

Tm: melting temperature; *B. breve*: *Bifidobacterium breve*; *B. fragilis*: *Bacteroides fragilis*; *L. acidophilus*: *Lactobacillus acidophilus*; *A. muciniphila*: *Akkermansia muciniphila*; *F. prausnitzii*: *Faecalibacterium prausnitzii*; *E. coli*: *Escherichia coli*

four of the standard dilutions with the appropriate efficiency number and was used to determine copy numbers in the samples. The species, genus, and group-specific oligonucleotide primers used in the present study were already designed and validated in other studies (12-14) (Table 1).

The amplification reaction was carried out in a total volume of 20 µL and consisted of 4 mM MgCl₂, 0.25 µM of each primer, 2 µL of LightCycler FastStart DNA Master SYBR Green I (Roche), and 2 µL of DNA template. Amplification involved an initial denaturation at 95°C for 10 min following by 45 cycles of denaturation at 95°C for 10 s, annealing at the specific annealing temperature (Table 1) for 5 s, and extension at 72°C for 10 s. Melting curve analysis was also performed in order to determine the specificity of the PCR reactions. Melting temperatures for the primers determined in the study are shown on Table 1.

Plasmid Gene Library Construction and Sequencing

In order to determine dominant *Lactobacillus* and *Bifidobacterium* species in patients and controls, a plasmid gene library was constructed from pooled samples and sequenced. Isolated DNA samples from 46 NASH patients and 35 control subjects were pooled within each group by using the same DNA concentration for each sample (50 ng/µL). The DNA pools were amplified with genus-specific primers for *Lactobacillus* and *Bifidobacterium* species. Amplicon size and purity were examined by agarose gel electrophoresis. After purification with a Pure-Link PCR Purification Kit (Invitrogen), purified amplicons were ligated into the PCR 2.1 TA Cloning plasmid vector (Invitrogen) and cloned into *E. coli* JM 109 competent cells. The *E. coli* clones were cultured in Luria-Bertani agar containing 100 mg/L ampicillin. Approximately 50 colonies from each group were randomly selected and sequenced with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) after the colony PCR and purification step with a plasmid DNA Purification Kit (Qiagen). The 16S rRNA gene sequence generated from transformed *E. coli* colonies was subjected to a BLAST search against known

sequences in GenBank. All sequences obtained showed more than 98% sequence identity to their nearest database entries.

Inflammation Markers

Serum samples were kept at -80°C until use. To determine the IL-6 and TNF-α serum concentrations, Human TNF-alpha Instant Enzyme-Linked ImmunoSorbent Assay (ELISA) and Human IL-6 Instant ELISA kits (EBioscience; CA, USA) were used according to the manufacturer's instructions. The absorbance was read at 450 nm using a plate reader. The standard curve was made from the standards to calculate the serum concentrations of the samples (EU/mL). hs-CRP levels were determined by the nephelometric method using CardioPhase hs-CRP (Siemens Healthcare Diagnostics, Marburg, Germany).

Endotoxin Analysis

A commercially available limulus amoebocyte lysate (LAL) chromogenic quantification assay (Pierce LAL Chromogenic Endotoxin Quantitation Kit, Pierce Biotechnology, Rockford, IL) was used to determine the bacterial endotoxin concentration in the serum samples. Following dilution of the serum samples 1/50, the samples were kept at 70°C for 15 min in order to eliminate inhibitory substances in human serum. Briefly, diluted serum samples were mixed with the LAL reagent and incubated at 37°C. After adding stop solution (25% acetic acid) the absorbance of the samples was measured at 405 nm. The linear relation between the absorbance of endotoxin standard concentrations and the samples was calculated by constructing a standard curve.

Statistical Analysis

Statistical Package for Social Sciences version 15.0 (SPSS Inc.; Chicago, IL, USA) statistical software was used to analyze the data. Statistical review of the study was performed by a biomedical statistician. Standard statistical methods were used for the calculation of means and standard deviations. Independent t-test was used for intergroup comparison of continuous data.

Table 2. Demographic and laboratory results of the participants

Characteristic	NASH (n=46) Mean±SD	Control (n=38) Mean±SD	p*
Male (%)	47.8	32.4	0.100
Age (years)	48±12	36±10	<0.001
BMI (kg/m ²)	29±4	22±2	<0.001
Waist circumference	94±9	83±6	0.001
ALT (U/L)	50±41	20±11	<0.001
AST (U/L)	36±18	21±4	<0.001
ALP (U/L)	105±38	62±18	<0.001
Total cholesterol (mg/dL)	202±43	207±43	0.613
HDL (mg/dL)	45±9	57±10	<0.001
LDL (mg/dL)	127±39	140±38	0.065
Triglyceride (mg/dL)	184±80	96±46	<0.001
Glucose (fasting; mg/dL)	102±22	87±12	<0.001

NASH: nonalcoholic steatohepatitis; BMI: body mass index; ALT: alanine transaminase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; HDL: high-density lipoprotein; LDL: low-density lipoprotein

*p-values were determined by independent t-test except male %, which was determined by chi-square test.

Table 3. Steatosis, ballooning, and lobular inflammation parameters of NASH patients (n=46)

Parameter	Score	n (%)
Steatosis	0	0 (0)
	1	9 (19.6)
	2	30 (65.2)
	3	7 (15.2)
Lobular inflammation	0	0 (0)
	1	31 (67.4)
	2	15 (32.6)
Ballooning	0	0 (0)
	1	33 (71.7)
	2	13 (28.3)

For categorical variables such as gender, the chi-square test was used. Multiple linear regression analysis was performed in order to adjust for the variables that are significant between the groups. Possible correlations between BMI and bacterial counts were assessed using Pearson's correlation coefficient. Relative abundances of *Lactobacillus* and *Bifidobacterium* species according to sequencing results are shown as percentages. A p value of <0.05 was used to establish significance.

RESULTS

Demographics and Laboratory Data

A total of 46 patients with biopsy-proven NASH and 38 healthy controls were enrolled in the study. The demographic and lab-

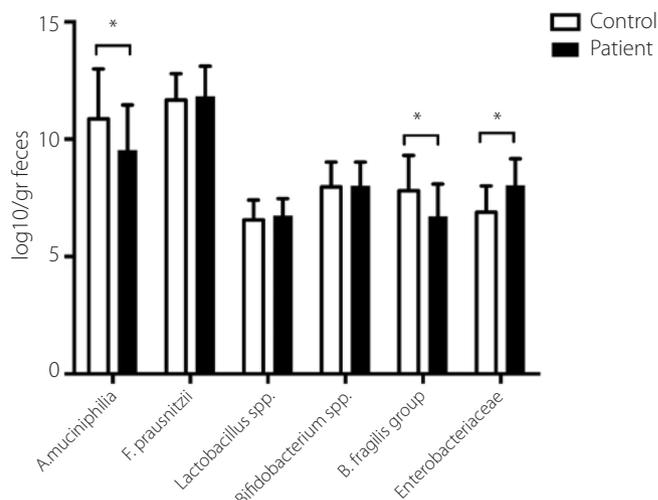


Figure 1. Comparison of log₁₀/g feces levels of bacterial groups between patients with NAFLD and healthy controls
NAFLD: nonalcoholic fatty liver disease

oratory data are shown in Table 2. The histopathological features of NASH (steatosis, ballooning, and lobular inflammation) are presented in Table 3. NASH patients had higher BMI and waist circumference when compared to the healthy controls, as was expected. Within the patient group, 41.3% were obese and 50.0% were overweight, whereas 8.7% of the patients were normal weight. In terms of laboratory data, significantly elevated alanine transaminase, aspartate aminotransferase, and alkaline phosphatase levels were observed in the patient group. Moreover, high-density lipoprotein, triglyceride, and glucose levels were also higher compared to healthy controls. A total of 23 patients had a fibrosis degree of ≥2 (significant fibrosis), while 23 of the patients had a fibrosis score of either F0 or F1.

qPCR Analysis

Specificity of the primers was assessed by conventional PCR and gel electrophoresis in both pure cultures and stool samples. Expected sizes were obtained, and no cross-reactions were observed in mixed communities (data not shown). The detection limits for the SYBR Green-I qPCR assay were determined using 10-fold serial dilutions of extracted DNA from pure cultures of the standard bacteria. PCR efficiency values were calculated for each standard curve made by the 10-fold dilutions of the internal controls.

According to the qPCR results, *A. muciniphila* and *B. fragilis* were significantly lower in patients with NASH (*A. muciniphila*, 10.86±2.12 vs. 9.10±1.90 log₁₀/g feces, p=0.003 and *B. fragilis*, 7.81±1.50 vs. 6.80±1.39 log₁₀/g feces, p=0.001, respectively). The Enterobacteriaceae family members were found to be significantly higher in the NASH group (6.89±1.12 vs. 8.10±1.21 log₁₀/g feces, p<0.001). No significant differences in terms of *F. prausnitzii*, *Lactobacillus* spp., or *Bifidobacterium* spp. levels were observed between the patients and controls (Figure 1). Because BMI and age distribution were significantly differ-

Table 4. Prevalence of Lactobacillus and Bifidobacterium species in patients and controls detected by sequencing genus-specific PCR amplicon libraries

	Control (%)	NASH (%)
<i>Lactobacillus</i> spp.		
<i>L. delbrueckii</i>	8.7	3.6
<i>L. mucosae</i>	2.2	10.7
<i>L. gastricus</i>	2.2	7.1
<i>L. sakei</i>	4.3	-
<i>L. ruminis</i>	50.0	44.6
<i>L. acidophilus</i>	4.3	12.5
<i>L. salivarius</i>	4.3	3.6
<i>L. helveticus</i>	6.5	-
<i>L. reuteri</i>	-	21.4
<i>L. johnsonii</i>	-	5.4
Uncultured <i>Lactobacillus</i> spp. clone	17.4	5.4
<i>Bifidobacterium</i> spp.		
<i>B. adolescentis</i>	54.9	42.3
<i>B. longum</i>	15.7	36.5
<i>B. bifidum</i>	5.9	1.9
<i>B. kashiwanohense</i>	9.8	1.9
<i>B. catenulatum</i>	3.9	7.7
<i>B. ruminantium</i>	2.0	-
<i>B. infantis</i>	-	7.7
Uncultured <i>Bifidobacterium</i> spp. clone	7.8	9.6

NASH: nonalcoholic steatohepatitis

ent between patients and controls, we performed a multiple linear regression analysis in order to adjust the data for those variables. After adjusting for BMI and age, *A. muciniphila* and Enterobacteriaceae levels in the NASH group compared to controls were significantly different ($p=0.0152$ and $p=0.0186$). However, the difference in *B. fragilis* was no longer significant. The Enterobacteriaceae levels were significantly higher in NASH patients with a significant fibrosis degree of $\geq F2$ when compared to the fibrosis degree of F0 and F1 (7.80 ± 1.11 vs. 9.01 ± 0.89 log₁₀/g feces, $p < 0.001$). There was a significant positive correlation between BMI and Enterobacteriaceae levels (Pearson's $r=0.282$, $p=0.021$). *A. muciniphila* and *B. fragilis* levels were negatively correlated with BMI, but the association was not significant.

Our study cohort had 19 obese patients (BMI > 30) within the NASH group. When we only considered the obese subgroup with NASH, *A. muciniphila* levels were significantly lower ($p=0.037$) and Enterobacteriaceae levels and *F. prausnitzii* levels were significantly higher ($p=0.0019$ and $p=0.002$, respectively) when compared to the normal-weight healthy controls.

Fecal *F. prausnitzii* was found at higher levels as compared to other bacterial groups studied in both patients and controls (11.75 log₁₀/g feces).

Dominant Lactobacillus and Bifidobacterium Species

The most abundant *Lactobacillus* species was *L. ruminis* in both patients (44.6%) and healthy controls (50.0%). However, *L. reuteri*, which was the second-most dominant *Lactobacillus* member in the patient group (21.4%), was not observed in healthy controls (Table 4). Healthy microbiota contains *L. sakei* and *L. helveticus*, which were not seen in patients with NASH. Fecal microbiota in terms of *Bifidobacterium* species was found to be similar for both patients and controls, and both groups had *B. adolescentis* and *B. bifidum* as the dominant species (Table 4). The main difference was that the patient group had 7.7% *B. infantis*, which could not be detected in healthy controls. The *Bifidobacterium* microbiota of the healthy control group also contained *B. catenulatum* and *B. ruminantium*, which were not seen in the NASH group.

Serum Levels of Inflammatory Markers and Endotoxin

There was no significance difference regarding TNF- α and IL-6 between patients and controls ($p=0.193$ and $p=0.348$, respectively). However, the serum hs-CRP level, which is a well-known inflammatory marker, was significantly higher in patients with NASH (0.37 ± 0.44 vs. 0.12 ± 0.12 mg/dL, $p < 0.001$) (Figure 2). Serum endotoxin levels were also significantly higher in NASH patients when compared to controls (9.05 ± 11.73 EU/mL vs. 2.75 ± 1.31 EU/mL, $p < 0.001$) (Figure 2).

All of the samples were studied in duplicate, and the intra-assay CV was less than 10% for all assays (TNF- α , 5.3% and 7.8%; IL-6, 4.6% and 3.8%; hs-CRP, 9.8% and 3.4%; and endotoxin, 2.6% and 9.6% for patients and controls, respectively).

DISCUSSION

In the present study, we showed that the NASH patients had significantly lower *A. muciniphila* and higher Enterobacteriaceae as well as elevated serum endotoxin and hs-CRP levels compared to healthy controls. Many studies have demonstrated the association between microbiota disruption (dysbiosis) and obesity (4,15). However, the exact mechanism and the direct relationship between microbiota composition and specific bacterial load in NAFLD remains unclear. To our knowledge, there are only a few studies in the literature regarding intestinal microbiota composition in human subjects with biopsy-proven NASH. Our study provides quantitative intestinal microbiota data from patients with biopsy-proven NASH. Because minor pathological differences may have an effect on the gut microbiota composition, the liver histology, which is the gold standard, is of importance for definitive diagnosis of NASH (1,16,17).

In our patient cohort, 41.3 % of the patients were obese, and BMI and waist circumference values were significantly higher in the patient group compared to the healthy controls, as was

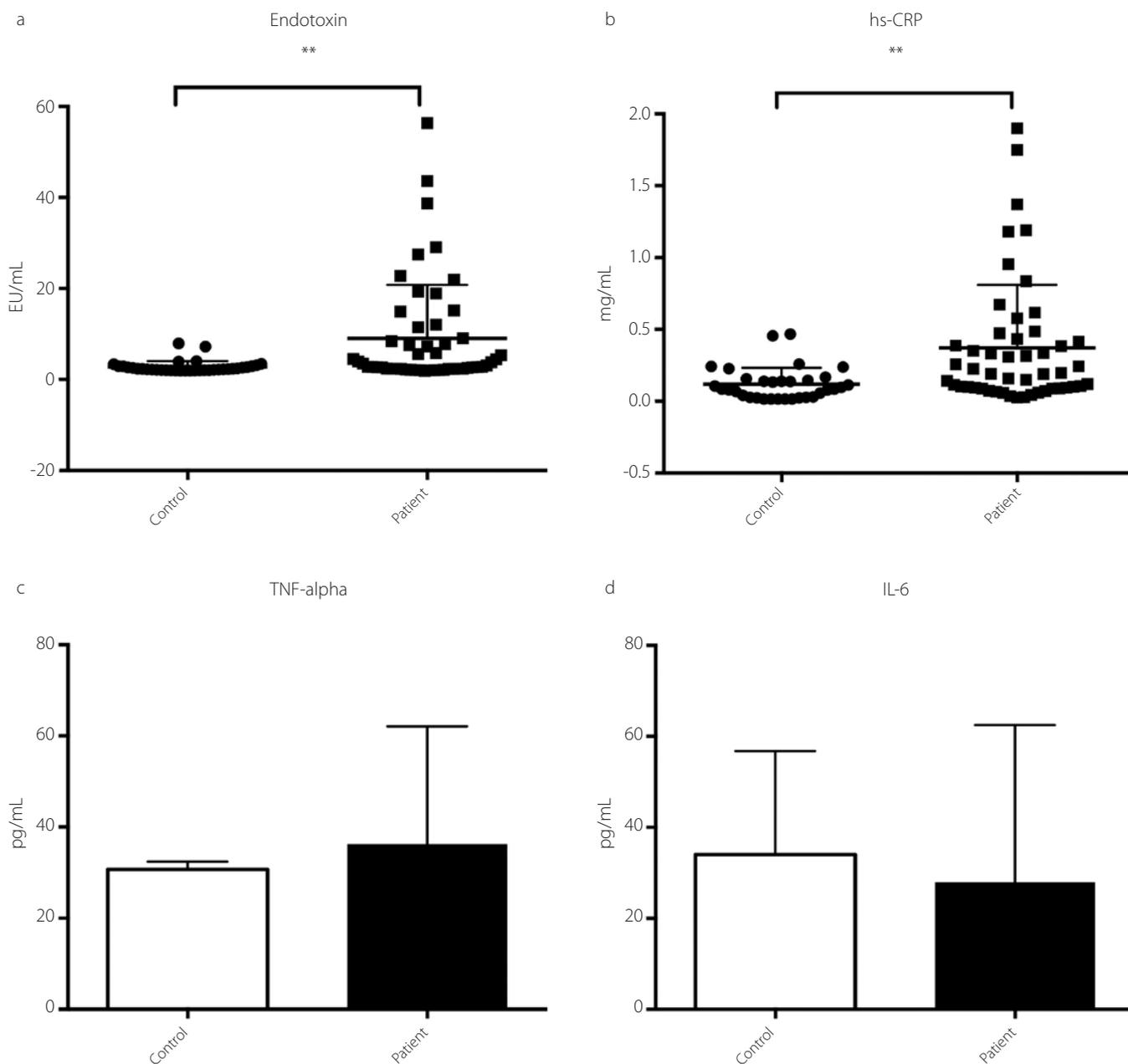


Figure 2. a-d. Serum levels of endotoxin (a), hs-CRP (b), TNF- α (c), and IL-6 (d) between patients and controls

expected. For this reason, we performed linear regression analysis to adjust for significantly different variables. After adjusting for BMI and age, the difference in *B. fragilis* group was no longer significant between the groups, while *A. muciniphila* and Enterobacteriaceae remained significantly different. There are controversial results regarding Bacteroidetes levels in NAFLD (9,18). However, the majority of the reports have indicated that Bacteroidetes is closely related with the obesity-associated microbiota along with Firmicutes (4,15,19). The results of the present study and the previous data in the literature suggest that *B. fragilis* levels are more likely to be related with the BMI status than with hepatic pathology (15,20,21). Thus, our study suggests a BMI-independent association of *A. muciniphila* and Enterobacteriaceae with NASH phenotype.

The degree of fibrosis in NAFLD was recently suggested to be considered in histopathological evaluation of NASH (22). Enterobacteriaceae was significantly higher in patients with F2 fibrosis compared to F0-F1, suggesting a strong relationship between Enterobacteriaceae levels and liver pathology.

F. prausnitzii belongs to phylum Firmicutes and is known to be a dominant member of healthy microbiota with its anti-inflammatory and butyrate production (23). We also found higher levels of *F. prausnitzii* in the whole study cohort indicating that *F. prausnitzii* is a dominant member of the fecal microbiota. It has been shown to be reduced in the intestinal microbiota of patients with inflammatory bowel disease and irritable bowel syndrome (23-25). Wong et al. (25) found lower levels of fecal *F. prausnit-*

F. prausnitzii in 16 NASH patients compared to 22 healthy controls by 16S rRNA sequencing. There was no significant difference in terms of *F. prausnitzii* levels between patients and the control group in our study cohort. However, the results from Wong et al. (25) were obtained from a small subpopulation, which might not reflect the overall NASH phenotype features. Interestingly, when we only considered the obese NASH patient subgroup, *Akkermansia* and *Enterobacteriaceae* remained significantly different, and *F. prausnitzii* become significantly higher in that group. The obese microbiome is known to have an increased capacity of energy harvest from food, which leads to fat accumulation (4). It has been shown that the Firmicutes to *Bacteroidetes* ratio is significantly higher in obese patients (4,15). Increased *F. prausnitzii* levels have been shown to be significantly related with obesity in children (26). Despite its beneficial health features, *F. prausnitzii* is also responsible for increased energy intake like the other members of the Firmicutes group (4,26).

We found lower levels of *A. muciniphila* in patients with NASH. *A. muciniphila* has been recently identified as an abundant mucin-degrading bacterium residing in the mucus layer (27). It has been shown to have beneficial effects on gut barrier function via an unknown mechanism, and its abundance is inversely correlated with body weight (12,21). Everard et al. (12) have recently demonstrated dramatically decreased levels of *A. muciniphila* in both diet-induced and genetically obese mice. They also suggest that obesity is associated with decreased mucus thickness, which may be the key feature for increased gut permeability and metabolic endotoxemia. Moreover, further *A. muciniphila* treatment restored the mucus layer. In our study population, we also demonstrated higher circulating endotoxin levels in the patient group along with lower *A. muciniphila* levels suggesting the role of this bacterium in maintaining the gut barrier function. *A. muciniphila* was also significantly different in the obese patient subgroup compared to controls. Because *A. muciniphila* has been shown to protect gut barrier function, these results were expected when we consider that both obesity and NASH are associated with increased gut permeability and metabolic endotoxemia.

We also found higher Enterobacteriaceae levels in the NASH group independent of BMI and age. Consistent with the increased Enterobacteriaceae levels in NASH patients, higher serum endotoxin levels were also shown in those patients as expected. This finding supports the possible underlying mechanism of NASH with respect to the increased gut permeability, which accelerates the bacterial translocation that is likely to be caused by reduced *A. muciniphila* levels and increased Enterobacteriaceae levels. Enterobacteriaceae members are known to produce alcohol as a fermentation product, which promotes gut permeability and leads to metabolic endotoxemia (18).

Two cross-sectional studies in the literature regarding the intestinal microbiota in NAFLD patients have conflicting results (9,18). Using a qPCR approach, Mouzaki et al. (9) showed decreased

Bacteroidetes and increased *Clostridium coccooides* levels in 22 NASH patients compared to 17 healthy controls. They did not show a difference in *E. coli* levels. Zhu et al. (18) screened gut microbiota in a pediatric cohort of 16 healthy controls, 25 obese subjects, and 22 patients with biopsy-proven NASH using 16S ribosomal RNA pyrosequencing. They showed that the Enterobacteriaceae family and *Escherichia* genus were similar in obese and healthy microbiomes; however, they showed a significant increase in NASH patients regarding this family and genus. Our findings on Enterobacteriaceae are similar to those of Zhu et al. (18). However, their study was different in terms of study population. Both studies were limited by a small sample size, which might not reflect the overall NASH phenotype microbiota.

There was no significant difference in either *Bifidobacterium* or *Lactobacillus* levels between NASH patients and the control group. We could not find any study regarding the fecal *Lactobacillus* and *Bifidobacterium* levels in NASH patients. There are a few reports of clinical studies with limited results regarding outcomes of probiotic intervention in NASH patients (28,29). The outcome of VSL#3 therapy in NASH patients is not clear in those studies, which all had limited sample sizes and used diagnoses based on liver enzyme levels and ultrasonography. Identification of relative abundances of *Lactobacillus* and *Bifidobacterium* species, which have well-known probiotic characteristics, is of critical importance because there is an increasing number of controversial results regarding the benefits of probiotic treatment. We hypothesized that patients with NASH and healthy microbiota might have some distinct characteristics in terms of dominant species. To evaluate the general population differences in patients and healthy controls, we constructed a plasmid library from PCR amplicons of the *Lactobacillus* and *Bifidobacterium* genus. According to our results, the dominant *Lactobacillus* and *Bifidobacterium* species seem to be different between healthy controls and NASH patients, which supports the idea that certain species may be unique for the pathological condition. Especially *L. reuteri*, which is one of the most-studied probiotic species for its effects on certain disease conditions (30,31), was found to be the second-most dominant *Lactobacillus* member in our NASH patient cohort (30). It was also found to be associated with obesity in a previous study (32). According to our present results and the literature, we suggest that *L. reuteri* administration in NASH patients might not be beneficial because it might shift the balance in the gut microbiota when it is already abundant. According to our present results and the literature, we suggest that the relative abundances of *Lactobacillus* and *Bifidobacterium* species in the gut microbiota should be considered before using probiotic treatments.

To demonstrate the inflammation levels in patients with NASH, we also determined the serum IL-6 and TNF- α levels, which show conflicting results in the literature as inflammation markers in patients with liver injury (33). In our study cohort, we found higher levels of TNF- α in serum samples of the patients, however the difference compared to healthy controls was not

significant. There was also no difference in terms of circulating IL-6 levels. However, we found significantly elevated hs-CRP levels in patients, even after adjustment for age and BMI. According to our results from a large cohort of NASH patients, we conclude that hs-CRP is an independent clinical feature in NASH patients in accordance with the results from previous studies (34,35).

Our study included 46 NASH patients who were diagnosed with liver biopsy and classified into two groups according to the degree of fibrosis. Liver histology is the gold standard for diagnosis of NASH (17). Our results suggest a direct relationship between NASH and fecal *A. muciniphila* and Enterobacteriaceae levels independent of BMI and age in a large cohort of histologically diagnosed patients. Because there are only a few studies addressing NASH microbiota, and those are all with relatively small sample sizes, our results provide important data on NASH-specific microbiota. Moreover, we also showed elevated circulating endotoxin levels in the patient group and higher hs-CRP levels indicating inflammation, which supports the possibly increased gut bacterial translocation in NASH patients.

Further studies with metagenomic approaches on NASH patients might shed light on the exact effect of the microbiota. Studies addressing novel probiotic replacements focusing on *A. muciniphila* might also be beneficial.

Akkermansia muciniphila was decreased and Enterobacteriaceae and serum hs-CRP were increased in patients with NASH. *L. reuteri* was abundant among other Lactobacillus species in the patient group. Further metagenomics approaches are needed to better characterize the differences in gut microbiota in this population.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Gazi University (Decision No: 23.12.2013/250).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

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