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ORIGINAL ARTICLE

Significant decrease in *Faecalibacterium* among gut microbiota in nonalcoholic fatty liver disease: a large BMI- and sex-matched population study

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Abstract

Background Compositional changes of the gut microbiota are known to occur in patients with nonalcoholic fatty liver disease (NAFLD); however, the changes did not corroborate between the studies. We evaluated the gut microbiota between NAFLD and non-NAFLD participants, excluding the influence of obesity and sex in this study involving a large number of participants.

Methods In total, 1148 adults participated in the health survey. NAFLD was defined as fatty liver by ultrasonography in the absence of other causes of steatosis. To exclude the influence of obesity and sex, NAFLD participants were matched to non-NAFLD participants based on BMI and sex. The relative abundance of each bacterial taxa in fecal samples was calculated using 16S ribosomal RNA amplification and was compared between NAFLD and non-NAFLD participants.

Results There were 205 (23.5%) participants defined as having NAFLD. Before matching, there were significant differences in the relative abundance of more than 1% in two classes, two orders, three families, and three genera including *Faecalibacterium* between NAFLD and non-NAFLD participants. After matching, 153 matched pairs were obtained. In terms of the relative abundance of more than 1%, the relative abundance of two taxa, including the family *Ruminococcaceae* and the genus *Faecalibacterium*, was significantly lower in NAFLD participants than in non-NAFLD participants ($p = 0.016$ and $p = 0.018$).

Conclusions The significant decrease in *Faecalibacterium* is a remarkable characteristic on BMI- and sex-matched analysis in NAFLD participants in a large study population. The decrease in *Faecalibacterium* is related to the pathogenesis of NAFLD.

Keywords Nonalcoholic fatty liver disease · Gut microbiota · *Faecalibacterium*

Abbreviation

AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
BMI	Body mass index
HbA1c	Hemoglobin A1c
HOMA-IR	Homeostasis model assessment of insulin resistance
LPS	Lipopolysaccharides
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis

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Introduction

Nonalcoholic fatty liver disease (NAFLD), the hepatic manifestation of metabolic syndrome, is currently the most prevalent chronic liver disease worldwide [1]. The spectrum of NAFLD ranges from simple steatosis to nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis. The gastrointestinal tract and the liver are closely related anatomically and physiologically. The "gut–liver axis," is considered a critical component for the onset and progression of NAFLD [2, 3]. The gut microbiota, in particular, plays an important role in the gut–liver axis [2, 3]. Many studies have shown a significant association between the gut microbiota

and NAFLD [4–11]. Although all of them demonstrated a compositional change of the gut microbiota in NAFLD patients, these findings did not agree among various studies [4–11]. This incompatibility could be because the sample size of the study was small and the participants included varied between each study, such as children, severely obese patients, or non-obese patients. The changes in composition in the human gut are observed to be different in obese individuals and between sexes [12–14]. The exclusion of these factors could help clarify the veridical characterization of gut microbiota in individuals with NAFLD.

The present observational study aimed to evaluate whether NAFLD is associated with gut microbiota, excluding the influence of obesity and sex, via a study involving a very large number of participants.

Methods

Study participants

A total of 1148 adults (age range 20–93 years) who participated in the Iwaki Health Promotion Projects Health Survey held in June 2016 in Hirosaki city located in north Japan (Fig. 1) were included. Of these, we excluded 44 participants with positive hepatitis B surface antigen or anti-hepatitis C virus tests; 196 participants with excess alcohol intake (male with an alcohol intake of more than 30 g/day and female with intake of more than 20 g/day); six participants administered agents associated with steatosis, such as amiodarone, methotrexate, prednisolone, and tamoxifen; and 28 participants whose stool samples were not collected. The data of the remaining 874 participants (323 male and 551 female) were collected and analyzed. We compared the relative abundance of 22 phylum, 42 class, 63 order, 139 family and 393 genus in the gut microbiota between NAFLD and non-NAFLD participants. Various intestinal bacteria in the gut microbiota were compared between NAFLD and non-NAFLD participants.

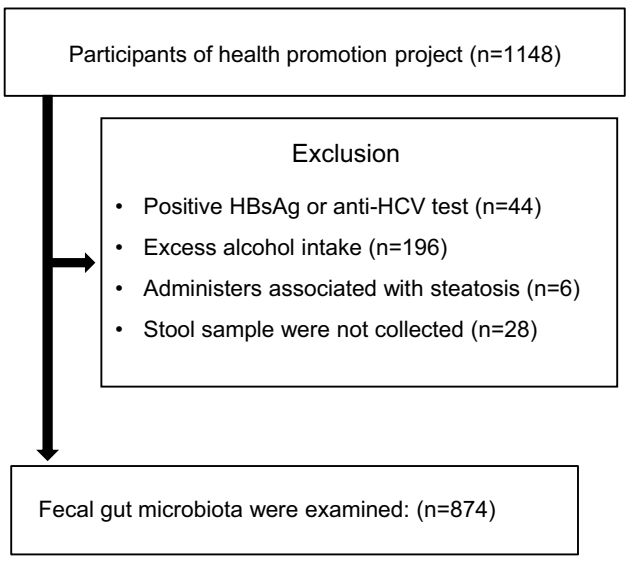

Fig. 1 Flowchart for participant selection. A total of 874 participants were enrolled from 1148 adults who participated in the Iwaki Health Promotion Projects in 2016

Diagnosis of NAFLD

Abdominal ultrasonography was performed using ProSound F37 (Hitachi Aloka Medical, Tokyo, Japan) by five well-trained hepatology specialists with more than 5 years of experience. Images were stored and reevaluated by a single hepatologist with more than 20 years of experience. A diagnosis of fatty liver on ultrasonography was defined as the presence of liver–renal echo contrast, bright liver, deep attenuation, and vessel blurring according to the protocol without referring to any of the subject's clinical data [15]. NAFLD was defined as fatty liver on ultrasonography in the absence of other causes of steatosis.

Biological parameters

Fasting blood samples were collected on the morning of the ultrasonography examination. The following clinical parameters were recorded: sex, age, height, body weight, waist circumference, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, glucose, insulin, hemoglobin A1c (HbA1c), uric acid, total cholesterol, triglycerides, platelets, hepatitis B surface antigen or anti-hepatitis C virus, and BMI (body mass index, calculated by dividing the weight in kilograms by the squared height in meters). The insulin resistance index was calculated using the homeostasis model assessment of insulin resistance (HOMA-IR) as follows: fasting glucose (mg/dL) × fasting insulin (μU/mL)/405 [16]. The FIB-4 index was calculated to predict the prevalence of participants with NASH: [age (years) × AST (U/L)]/[platelets (109) × square root ALT (U/L)] [17]. Liver fibrosis was defined as FIB-4 index ≥ 2.67, which had an 80% positive predictive value [18]. To exclude the influence of obesity, participants with NAFLD were matched to the participants without NAFLD using calipers of a width equal to 0.2 kg/m^2 of BMI by sex.

DNA extraction from fecal samples

On the morning of the survey after a course of overnight fasting, fecal samples were collected from each subject in commercial containers (TechnoSuruga Laboratory Co., Ltd., Shizuoka, Japan) and suspended in guanidine thiocyanate solution [100 mM Tris–HCl (pH 9.0), 40 mM

Table 1 Characteristics before and after matching

Variables	Non-NAFLD $n=669$	NAFLD $n=205$	p value	Non-NAFLD $n=153$	NAFLD $n=153$	p value
Sex, male[a]	203 (30.3%)	120 (58.5%)	<0.001	59 (38.6%)	59 (38.6%)	1.000
Age (year)[b]	53.8 ± 17.1	55.1 ± 14.2	0.353	53.9 ± 16.8	56.0 ± 12.6	0.191
BMI (kg/m^2)[b]	22.0 ± 3.1	25.4 ± 4.8	<0.001	24.4 ± 2.4	24.4 ± 2.4	0.979
Waist circumference (cm)[b]	81.7 ± 8.7	92.5 ± 9.2	<0.001	88.2 ± 7.8	89.3 ± 6.2	0.069
AST (U/L)[b]	21.5 ± 7.3	26.2 ± 10.9	<0.001	21.5 ± 8.7	25.3 ± 9.3	<0.001
ALT (U/L)[b]	18.3 ± 10.9	32.1 ± 19.6	<0.001	20.2 ± 14.9	30.0 ± 18.2	<0.001
T-bil (mg/dL)[b]	0.80 ± 0.28	0.77 ± 0.29	0.170	0.76 ± 0.26	0.78 ± 0.29	0.625
Glucose (mg/dL)[b]	90 ± 21	98 ± 19	<0.001	93 ± 21	97 ± 16	0.090
Insulin (μU/mL)[b]	4.8 ± 2.5	8.2 ± 7.9	<0.001	5.8 ± 3.0	7.7 ± 8.5	0.013
HOMA-IR[b]	1.1 ± 0.7	2.0 ± 2.2	<0.001	1.3 ± 0.8	1.9 ± 2.4	<0.001
Hemoglobin A1c (%)[b]	5.8 ± 0.6	6.2 ± 0.8	<0.001	5.9 ± 0.7	6.1 ± 0.7	0.002
Uric acid (mg/dL)[b]	4.7 ± 1.4	5.6 ± 1.9	<0.001	4.9 ± 1.2	5.5 ± 1.4	<0.001
Total cholesterol (mg/dL)[b]	203 ± 36	212 ± 34	0.003	203 ± 32	213 ± 32	0.010
Triglycerides (mg/dL)[b]	82 ± 44	131 ± 88	<0.001	91 ± 47	125 ± 71	<0.001
Platelet (× 10^4/μL)[b]	24.1 ± 5.3	26.1 ± 6.3	<0.001	24.4 ± 5.9	26.2 ± 6.2	0.007
Fib-4 index[b]	1.27 ± 0.71	1.12 ± 0.56	0.005	1.25 ± 0.80	1.11 ± 0.51	0.083

ALT alanine aminotransferase, *AST* aspartate aminotransferase, *BMI* body mass index, *HOMA-IR* homeostasis model assessment of insulin resistance, *T-bil* total bilirubin

[a]Percentage

[b]Mean ± standard deviation

Tris–EDTA (pH 8.0), 4 M guanidine thiocyanate]. Until the DNA extraction procedure, these samples were kept at − 80 °C. Frozen fecal solids were agitated with zirconia beads at 5 m/s for 2 min by a FastPrep 24 Instrument (MP Biomedicals, Santana Ana, CA, USA). DNA was extracted from 200 μL of the suspension with a Magtration System 12 GC (Precision System Science, Japan) using MagdDEA DNA 200 (Precision System Science, Japan) as the reagent for automatic nucleic acid extraction.

Next-generation sequence analysis and 16S rDNA-based taxonomic analysis

According to the methods described in previous reports, samples were analyzed for a series of typical bacteria in the human gut microbiota using the primers for the V3–V4 hypervariable region of the 16S rDNA of prokaryotes [19, 20]. The sequencing was conducted by an Illumina MiSeq system (Illumina, San Diego, CA, USA). Quality filtering was carried out as follows: only reads that had quality value scores of ≥ 20 for more than 99% of the sequence were extracted for the analysis. Detection and identification of bacteria from the sequences were performed by Metagenome@KIN software (World Fusion Co., Tokyo, Japan) and the TechnoSuruga Lab Microbial Identification database DB-BA 10.0 (TechnoSuruga Laboratory) at 97% sequence similarity. We compared the relative abundance of various intestinal bacteria in the gut microbiota between NAFLD and non-NAFLD participants. The relative abundance is presented as the percentage composition of reads for each bacterium relative to the total number of reads. We investigated the gut microbiota with a relative abundance of more than 1%, which could influence the pathogenesis of NAFLD.

Statistical analysis

Statistical analyses of the clinical data were performed using the JMP ver. 12.1 (SAS Institute, Cary, NC) and the R software (R Foundation for Statistical Computing, version R-3.4.3). Categorical variables are shown as frequencies and percentages, and continuous variables are shown as the mean with standard deviation. Categorical variables were compared using the Chi square test, and continuous variables were compared using Student's t test. A p value less than 0.05 was considered significant. Alpha-diversity was evaluated using Shannon index and Chao1 index. Beta-diversity was evaluated by principal coordinate analysis and statistically analyzed using permutation multivariate analysis of variance.

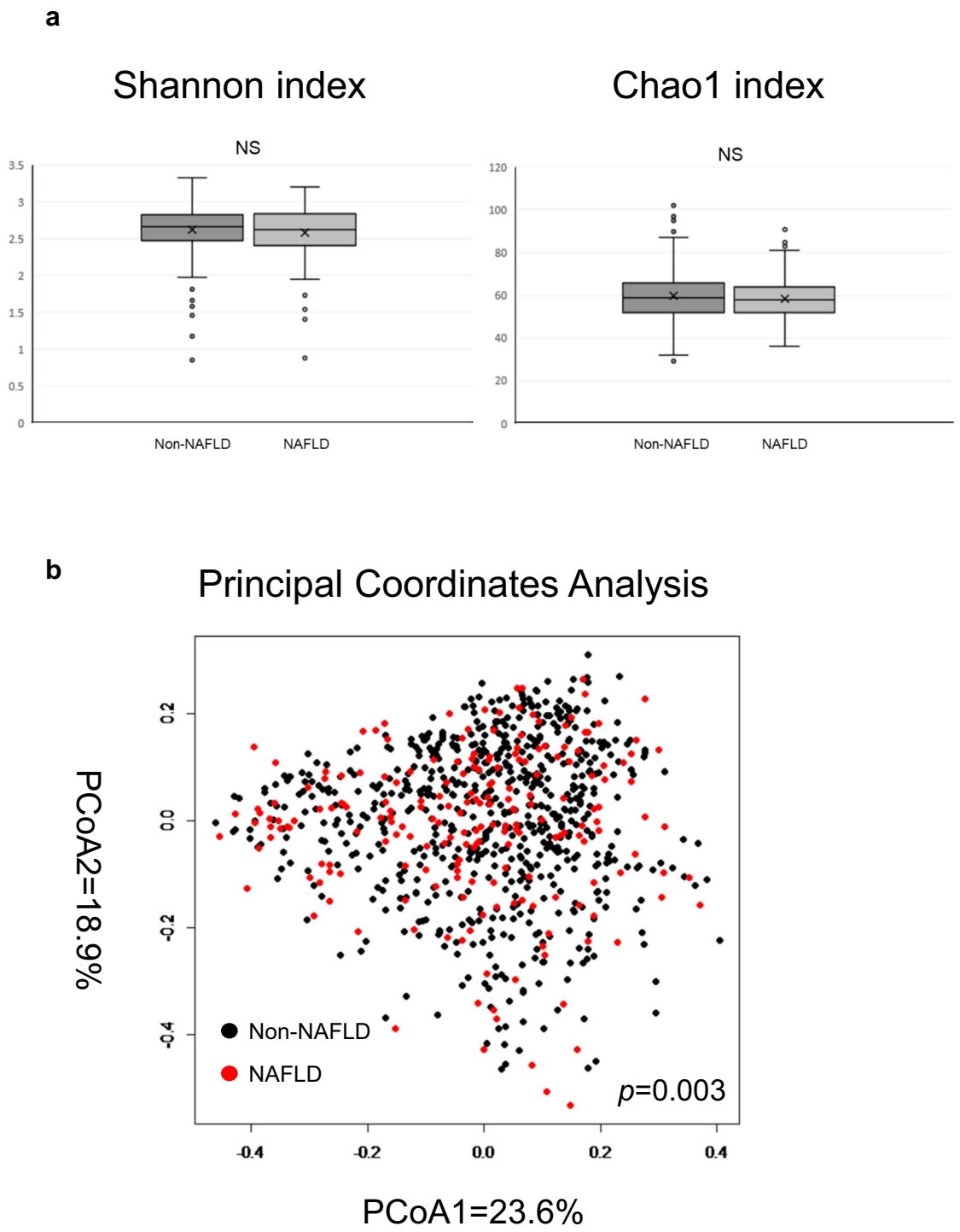

Fig. 2 **a** The Shannon index and Chao1 index of gut microbiota in the NAFLD and non-NAFLD participants. **b** Principal coordinate analysis between NAFLD and non-NAFLD participants

Results

There were 205 (23.5%) participants [male 120 (58.5%), female 85 (41.5%)] who were defined as having NAFLD. There were significant differences in the values of BMI, AST, ALT, HOMA-IR, HbA1c, and Fib4-index between NAFLD participants and non-NAFLD participants (Table 1). The prevalence of NASH according to the Fib-4 index was 1.5% in NAFLD participants.

Table 2 Median relative abundance of gut microbiota with 1% occurrence in the whole population (percentage of the total bacterial reads) between NAFLD and non-NAFLD subjects

	Before matching			After matching		
	Non-NAFLD	NAFLD	*p* value	Non-NAFLD	NAFLD	*p* value
Phylum						
Firmicutes	58.64	58.82	0.375	58.88	58.04	0.403
Bacteroidetes	24.88	25.69	0.269	24.88	26.02	0.675
Actinobacteria	8.03	8.92	0.991	9.26	9.41	0.851
Proteobacteria	1.59	1.55	0.278	1.77	1.59	0.292
Class						
Clostridia	48.23	46.40	**0.015**	49.14	46.40	0.067
Bacteroidia	24.86	25.66	0.261	24.88	26.02	0.671
Actinobacteria	8.02	8.92	0.991	9.26	9.41	0.851
Negativicutes	2.46	2.99	**0.004**	2.41	2.81	0.171
Bacilli	1.85	1.52	0.289	1.93	1.65	0.601
Erysipelotrichia	1.03	1.01	0.841	1.03	0.95	0.943
Order						
Clostridiales	48.20	46.40	**0.015**	49.13	46.40	0.067
Bacteroidales	24.86	25.66	0.261	24.88	26.02	0.671
Bifidobacteriales	5.87	5.15	0.465	6.17	5.73	0.593
Selenomonadales	2.46	2.99	**0.004**	2.41	2.81	0.171
Coriobacteriales	1.70	2.08	0.166	1.79	2.07	0.895
Lactobacillales	1.49	1.23	0.662	1.55	1.43	0.582
Erysipelotrichales	1.03	1.01	0.841	1.03	0.95	0.942
Family						
Lachnospiraceae	26.07	25.55	0.586	26.37	26.61	0.965
Ruminococcaceae	16.29	14.94	**0.007**	15.96	14.66	**0.016**
Bacteroidaceae	16.41	13.98	**0.032**	17.99	12.79	0.096
Bifidobacteriaceae	5.87	5.15	0.465	6.17	5.72	0.593
Coriobacteriaceae	1.70	2.08	0.166	1.79	2.08	0.895
Veillonellaceae	1.46	1.85	**0.024**	1.69	1.82	0.482
Porphyromonadaceae	1.78	1.75	0.828	1.71	1.70	0.837
Erysipelotrichaceae	1.03	1.01	0.841	1.03	0.95	0.942
Streptococcaceae	1.17	1.00	0.489	1.25	1.12	0.709
Genus						
Bacteroides	9.93	7.75	**0.024**	9.84	7.70	0.097
Faecalibacterium	7.97	6.86	**0.025**	7.92	6.55	**0.018**
Blautia	6.13	6.29	0.644	6.14	6.69	0.432
Bifidobacterium	5.90	5.18	0.376	6.30	5.54	0.562
Anaerostipes	3.89	3.54	0.629	3.77	3.86	0.655
Roseburia	3.01	3.41	0.752	4.48	3.44	0.246
Ruminococcus2	3.61	3.26	0.759	3.88	3.18	0.381
Collinsella	2.51	3.15	0.085	2.96	3.07	0.824
Gemmiger	2.31	1.87	0.234	2.34	1.67	0.445
Lachnospiracea_incertae_sedis	1.86	1.63	**0.031**	1.81	1.74	0.407
Fusicatenibacter	1.48	1.58	0.457	1.49	1.62	0.239
Ruminococcus	1.55	1.31	0.402	1.02	1.15	0.595
Clostridium IV	1.01	0.85	0.288	0.89	0.86	0.704

Bold indicates significance

There were no significant differences in the Shannon index and the Chao1 index between NAFLD and non-NAFLD participants (Fig. 2a). However, the principal coordinate analysis revealed that there were microbial structural differences between NAFLD and non-NAFLD participants ($p = 0.003$), (Fig. 2b). In this study, 22 phyla,

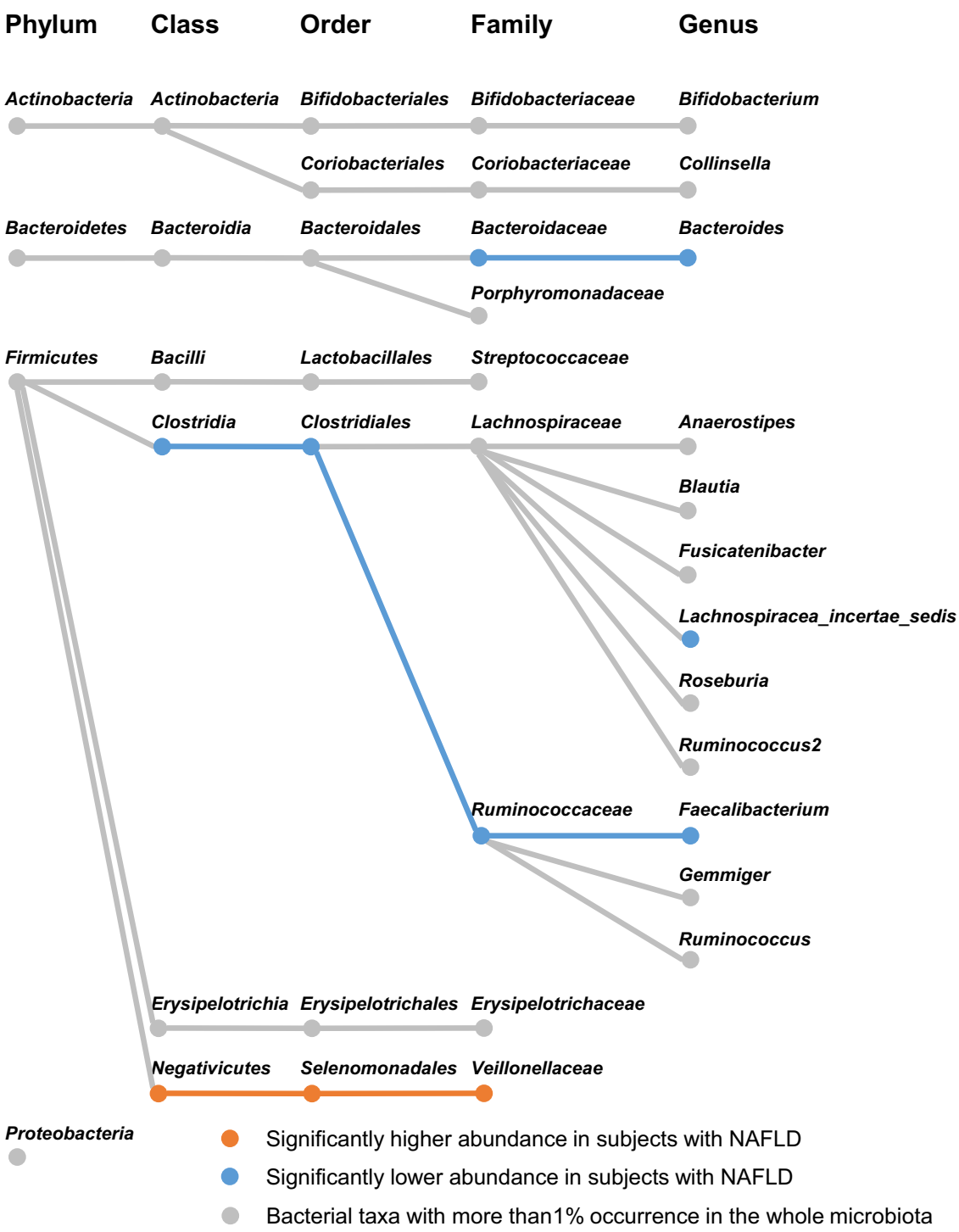

Fig. 3 Summary of the association between NAFLD status and bacterial taxa with 1% occurrence in the whole microbiota prior to matching

42 classes, 63 orders, 139 families, and 393 genera were identified in the gut microbiota. In terms of the relative abundance of more than 1%, there were significant differences in the relative abundance of two classes, two orders, three families, and three genera between NAFLD and non-NAFLD participants (Table 2). The relative abundance of three taxa including the genus *Faecalibacterium*, *Bacteroides*, and *Lachnospiracea_incertae_sedis* was significantly lower in NAFLD participants than in non-NAFLD participants (Fig. 3).

Fig. 4 Summary of the association between NAFLD status and bacterial taxa with 1% occurrence in the whole microbiota after matching

After matching for BMI and sex, 153 matched pairs were obtained, and no significant differences in the characteristics in terms of the BMI, sex, and waist circumference were observed between two groups (Table 2). In the relative abundance of more than 1%, the relative abundance of only two taxa, namely the family *Ruminococcaceae* and the genus *Faecalibacterium* was significantly lower in NAFLD participants than in non-NAFLD participants ($p = 0.016$ and $p = 0.018$) (Fig. 4).

Discussion

In this large population study, we observed that there were significant differences in beta-diversity of gut microbial communities between NAFLD and non-NAFLD participants, and after BMI- and sex-matched, the relative abundances of the family *Ruminococcaceae* and the genus *Faecalibacterium* were significantly lower in NAFLD participants than in non-NAFLD participants. The decrease in the genus *Faecalibacterium* in gut microbiota could be closely related to the pathogenesis of NAFLD.

Several studies have reported that the gut microbiota may have an important role in the pathogenesis of NAFLD [4–11]. In this study, the difference in relative abundance of *Faecalibacterium* between NAFLD and non-NAFLD participants was small but significant. Similar to our study, three studies reported a decrease in *Faecalibacterium* in NASH or NAFLD patients [5, 9, 10]. Others studies did not have results that were in accordance with the findings of our study [4, 6–8, 11]. The main reason could be the adjustment for BMI and sex in this study, which influenced the gut microbiota. Other reasons could be because other studies included only a small number of subjects, fewer than several dozen subjects, and due to the discrepancy between subjects with NAFLD, NASH, obesity, and healthy controls in each study.

The genus *Faecalibacterium* is composed of only a sole species, *Faecalibacterium prausnitzii*. *F. prausnitzii* is an oxygen-sensitive, butyrate-producing bacterium of the gut microbiota and plays an important role in a healthy gut as a biomarker of intestinal health [21]. Indeed, decreased levels of *F. prausnitzii* have been found in patients with intestinal and metabolic disorders such as inflammatory bowel diseases, irritable bowel syndrome, and celiac disease [22, 23].

The mechanism of the decrease in *Faecalibacterium* in gut microbiota in NAFLD participants has not been clearly elucidated. However, in those studies that have reported their results, it was observed that *Faecalibacterium* negatively correlated with serum lipopolysaccharide (LPS) levels, known as endotoxins [24, 25]. Exposing the liver to LPS plays a role in the pathogenesis of NAFLD. In patients with NAFLD, increased serum level of LPS was observed in previous studies [26, 27]. LPS binding to TLR4 in hepatic immune cells causes activation of the NF-κB pathway and the associated inflammatory pathway ultimately leads to hepatic injury and fibrosis [27, 28].

Before matching for BMI and sex, the relative abundance of *Bacteroides* and *Lachnospiracea_incertae_sedis* was also significantly lower in NAFLD participants than in non-NAFLD participants. A few studies on NAFLD patients revealed the increase in these two family taxa [8, 10]. However, after matching for BMI and sex, these significant differences disappeared. The influence of BMI and sex was related to these two genus taxa. Meanwhile, the rate of decrease of *Faecalibacterium* in NAFLD participants increased after the matching. Therefore, *Faecalibacterium* may have a high association with NAFLD.

The prevalence of NAFLD has been increasing. A large Japanese multicenter study that involved ultrasonographic examination reported that the prevalence of NAFLD was 29% [29], which was higher than that in our study. The proportion of male was 52% in a previous study [29], whereas it was 38% in our study. Generally, the prevalence of NAFLD is higher in male than in female [30]. Between the two studies, the difference in the prevalence of NAFLD could be due to the proportion of male. In this study, the prevalence of NASH according to the Fib-4 index was less than 2%, which was similar to a previous Japanese study [29]. However, this study population would be at a low risk for NASH compared to general population. Therefore, this study could not evaluate the gut microbiota between participants with NAFLD and participants with NASH; hence, further research is required.

There are some limitations to this study. First, although sex and BMI were matched, we could not preclude the influence of other factors, such as age or diet, that could affect the gut microbiota. Second, the diagnosis of fatty liver was made using ultrasonography examination but not using liver biopsy, which is an invasive procedure. In the current study based on a mass survey, it was not possible to perform a liver biopsy. Therefore, the diagnostic accuracy could be low. However, a common ultrasonographic definition of fatty liver was established and used [29]. Third, we revealed the significant association between the gut microbiota and NAFLD. However, because this was an observational study, it could not prove a cause-and-effect relationship between the change in gut microbiota and NAFLD.

In conclusion, a significant decrease in the relative abundance of the family *Ruminococcaceae* and the genus *Faecalibacterium* in the gut microbiota was observed in NAFLD participants on BMI- and sex-matched analysis of a large number of participants. Among NAFLD participants, the decreased abundance of *F. prausnitzii* is a remarkable characteristic.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was performed in accordance with the ethical standards of the Declaration of Helsinki and was approved by the Hirosaki University Medical Ethics Committee.

Informed consent Written informed consent was obtained from all participants.

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