学位論文

DOCTORAL THESIS

Gut microbiota composition associated with hepatic fibrosis in non-obese patients with non-alcoholic fatty liver disease

（非肥満型非アルコール性脂肪性肝疾患における
腸内細菌叢と肝線維化との関連）

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HEPATOLOGY

Gut microbiota composition associated with hepatic fibrosis in non-obese patients with non-alcoholic fatty liver disease

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Key words
Eubacterium, Gut microbiota, Hepatic fibrosis, Non-alcoholic fatty liver disease, Non-obese NAFLD.

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Abstract

Background and Aim: Gut microbiota composition is associated with the pathogenesis of non-alcoholic fatty liver disease. However, the association between gut microbiota composition and non-alcoholic fatty liver disease in non-obese patients remains unclear. We compared clinical parameters and gut microbiota profiles of healthy controls and non-obese and obese patients with non-alcoholic fatty liver disease.

Methods: We examined the clinical parameters and gut microbiota profiles by 16S rRNA sequences and short-chain fatty acid levels in fecal samples from 51 non-obese patients with non-alcoholic fatty liver disease (body mass index <25 kg/m^2) and 51 obese patients with non-alcoholic fatty liver disease (body mass index ≥ 30 kg/m^2) who underwent pathological examination and 87 controls at five hospitals in Japan.

Results: Although no significant differences between the non-obese and other groups were observed in alpha diversity, a significant difference was found in beta diversity. We observed a significant decrease in serum alanine aminotransferase levels, *Eubacterium* population, and butyric acid levels in non-obese patients with non-alcoholic fatty liver disease compared with those in obese patients with non-alcoholic fatty liver disease. A significant negative correlation was found between the stage of hepatic fibrosis and *Eubacterium* abundance in non-obese patients with non-alcoholic fatty liver disease.

Conclusions: The decrease in the abundance of *Eubacterium* that produces butyric acid may play an important role in the development of non-alcoholic fatty liver disease in non-obese individuals. This study was registered at the University Hospital Medical Information Network clinical trial registration system (UMIN000020917).

Introduction

Non-alcoholic fatty liver disease (NAFLD) causes fat deposition in the liver in the absence of other known causes. NAFLD prevalence is increasing globally, with 20–30% of the population affected by NAFLD.[1,2] It is closely related to obesity and is a hepatic phenotype of the metabolic syndrome.[2]

However, NAFLD can progress in non-obese subjects, that is, those with "non-obese NAFLD" or "lean NAFLD."[3,4] Non-obese NAFLD patients make up 12–20% of all NAFLD patients.[5–8] In Asia, the commonly used criterion for non-obese NAFLD is NAFLD with a body mass index (BMI) <25 kg/m^2.[9] Our understanding of the risk factors, pathogenesis, and pathophysiological changes in non-obese NAFLD remains limited. Studies on Asian and European populations have reported no difference in clinical events or all-cause mortality. However, non-obese NAFLD is more strongly associated with a high risk of serious liver disease than overweight NAFLD.[10,11] Further data on the prognosis of non-obese NAFLD are required. According to studies focused on obese patients, altered composition of the gut microbiota is associated with NAFLD.[12–14] However, the role of the gut microbiome in non-obese subjects with NAFLD is unclear. Hence, we assessed pathological findings and the profiles of gut

microbiota in non-obese patients with NAFLD and compared them with those of obese patients with NAFLD and healthy control subjects. This study might lead to the identification of therapeutic options for non-obese patients with NAFLD.

Methods

Ethical approval. This clinical study was conducted at five sites in accordance with the principles of the Declaration of Helsinki and was approved by the local ethics committees: Yokohama City University Hospital, Kawasaki Medical Center, Kurume University Hospital, JA Hiroshima Kouseiren General Hospital, and Saga University Hospital. We obtained informed consent from all participants prior to enrolment. The study was registered as UMIN000020917 (the University Hospital Medical Information Network).

Study subjects. This was a multicenter, cross-sectional observational study. Between May 2016 and July 2019, we evaluated 74 healthy control subjects and 160 patients with NAFLD who underwent liver biopsy at Yokohama City University Hospital. In addition to the study at our facility, 57 patients with NAFLD and 13 control subjects in four other facilities were evaluated. We defined NAFLD with BMI < 25 kg/m^2 as non-obese NAFLD and NAFLD with BMI ≥ 30 as obese NAFLD. The subjects were categorized into three groups: non-obese NAFLD ($n = 51$); obese NAFLD ($n = 51$); and control ($n = 87$). The inclusion criteria are described in Appendix S1.

Patients and public involvement. Patients were involved in the conduct of the study. Especially, the development of the research question was based on patients' experiences. The results of this study will be disseminated by an international report to the patients and medical staff.

Clinical and laboratory evaluation. Blood samples were collected after 12 h of overnight fasting. Laboratory tests were performed using standard techniques. Blood endotoxin activity assays were performed as described previously.[15,16]

Pathological evaluation. Liver biopsy samples were collected from all patients with NAFLD. The procedure and the method of systematic evaluation are described in Appendix S2.

Fecal microbial community analysis. The interval between stool collection and pathological examination was <6 months. The method of intestinal microbial community analysis is described in Appendix S3.

Diversity analysis. We analyzed the alpha diversity at the genus level using the Chao 1 and Shannon index calculated with R (version 3.6.1). Beta diversity analysis was performed to evaluate the changes in genus complexity of the samples. Cluster analysis was performed using principal coordinate analysis based on the Bray–Curtis distance in R (version 3.6.1) to calculate the beta diversity values. We performed permutational multivariate analysis of variance using 5000× permutations to find the significant variation in beta diversity among the three groups.

Inferred metagenomic prediction of stool samples. We predicted functional profiles of the gut microbiota from each stool sample using Phylogenetic Investigation of Communities by Reconstruction of Unobserved State (Appendix S4).

Fecal short-chain fatty acids content. For the short-chain fatty acid (SCFA) analyses, we examined the stool samples obtained at Yokohama City University Graduate School of Medicine (31 non-obese patients, 37 obese patients, and 44 controls). The method is described in Appendix S5.

Statistical analysis. Data are expressed as the mean ± standard deviation, unless indicated otherwise. We analyzed the data using JMP 14.0 (SAS Institute Inc, Cary, North Carolina, USA) and BellCurve for Excel (Social Survey Research Information Co., Ltd., Tokyo, Japan). The Student's *t* test was used for univariate comparisons between the groups. All *t* tests were two-sided with a significance level of 5% ($P = 0.05$). In the gut microbiota analysis, false discovery rate (FDR) was derived using the Benjamini and Hochberg method for correction of multiple comparisons. We defined FDR < 0.1 as being statistically significant. The Jonckheere–Terpstra trend test was used to examine the association between pathological findings and characteristic microbiota in all patients with NAFLD. The correlation between clinical parameters and characteristic microbiota in all patients with NAFLD was examined using the Spearman's rank correlation coefficient. The non-obese NAFLD and obese NAFLD groups were compared using the linear discriminant analysis (LDA) effect size (LEfSe) method that could show both biological relationship and statistical significance. In the algorithm, we used the nonparametric factorial Kruskal–Wallis rank sum test and LDA to determine statistically significant features between the two groups and assess the effect size of the differences.[17] Differences were regarded as significant for adjusted *P* values <0.05 and a logarithmic LDA score cut-off ≥ 2. Important bacterial taxa in the LEfSe method were shown using the package "qgraph" in R (version 3.6.1).

Results

Characteristics of patients. A flow chart of patient selection is illustrated in Figure S1. In total, 217 patients with biopsy-proven NAFLD and 87 control subjects were enrolled for the study. Of the 217 patients with NAFLD, 51 (23.5%) were non-obese, and 51 (23.5%) were obese. The clinical laboratory and pathological characteristics of the study participants are presented in Table 1. Non-obese patients with NAFLD had lower alanine aminotransferase levels (50.8 *vs* 71.2 IU/L; $P = 0.008$) than obese patients with NAFLD. No significant differences in pathological findings were observed between obese and non-obese patients with NAFLD. Serum fibrosis markers indicated no significant difference, which was in line with the histological findings.

Table 1 Clinical, biochemical, and pathological characteristics of patients with non-obese non-alcoholic fatty liver disease (NAFLD) and obese NAFLD and control subjects

	Non-obese NAFLD (n = 51)	Obese NAFLD (n = 51)	Control (n = 87)
Clinical and laboratory evaluation			
Age (years) (mean ± SD)	61.9 ± 14.3	57.4 ± 13.3	55.6 ± 16.3
Male, n (%)	26 (51)	24 (47)	48 (55)
BMI (kg/m^2) (mean ± SD)	23.2 ± 1.2*#	32.8 ± 2.4	21.5 ± 3.8
AST (IU/L) (mean ± SD)	43.9 ± 20.1#	56.3 ± 38.7	24.0 ± 10.2
ALT (IU/L) (mean ± SD)	50.8 ± 29.3*#	71.2 ± 43.8	17.7 ± 12.7
GGT (IU/L) (mean ± SD)	63.3 ± 54.2#	90.7 ± 93.3	30.1 ± 33.3
Platelet count (/10^3 μL) (mean ± SD)	202 ± 74#	203 ± 67	250 ± 47
Albumin (g/dL) (mean ± SD)	4.3 ± 0.4	4.4 ± 0.4	4.4 ± 0.3
Ferritin (ng/mL) (mean ± SD)	217.9 ± 226.6	296.7 ± 265.2	153.2 ± 115.8
Insulin (μU/mL) (mean ± SD)	18.8 ± 27.0#	16.4 ± 8.5	11.2 ± 20.7
Type IV collagen 7s (ng/mL) (mean ± SD)	5.5 ± 1.7#	6.9 ± 6.1	4.4 ± 0.3
Hyaluronic acid (ng/mL) (mean ± SD)	69.2 ± 70.7#	85.1 ± 77.9	31.7 ± 12.7
Endotoxin activity (mean ± SD)	0.17 ± 0.09#	0.17 ± 0.07	0.07 ± 0.03
Pathological findings			
Steatosis (grade) (mean ± SD)	1.5 ± 0.8	1.7 ± 0.7	—
Steatosis, n (%)			
0	5 (9.8)	1 (1.9)	—
1	27 (52.9)	25 (49.0)	—
2	13 (25.5)	18 (35.3)	—
3	6 (11.8)	7 (13.7)	—
Lobular inflammation (grade) (mean ± SD)	1.2 ± 0.6	1.4 ± 0.5	—
Lobular inflammation, n (%)			
0	1 (2.0)	0 (0)	—
1	33 (64.7)	28 (54.9)	—
2	14 (27.5)	20 (39.2)	—
3	3 (5.9)	3 (5.9)	—
Ballooning (grade) (mean ± SD)	0.8 ± 0.6	0.7 ± 0.6	—
Ballooning, n (%)			
0	12 (23.5)	16 (31.4)	—
1	31 (60.8)	26 (51.0)	—
2	6 (11.8)	9 (17.6)	—
3	2 (3.9)	0 (0)	—
Fibrosis (stage) (mean ± SD)	1.8 ± 1.1	2.0 ± 1.0	—
Fibrosis, n (%)			
0	1 (1.9)	1 (1.9)	—
1	21 (41.2)	20 (39.2)	—
2	8 (15.7)	8 (15.7)	—
3	17 (33.3)	19 (37.3)	—
4	4 (7.8)	3 (5.9)	—
NAS (mean ± SD)	3.5 ± 1.4	3.8 ± 1.1	—
NAS, n			
0/1/2/3/4/5/6/7/8	0/3/4/17/13/8/3/2/1	0/0/7/12/12/13/3/1/2	—

*$P < 0.05$ *versus* obese NAFLD.

#$P < 0.05$ *versus* control.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGT, γ-glutamyl transpeptidase; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; SD, standard deviation.

Pathological findings are assessed by the Brunt classification.

Characteristics of gut microbiota in non-obese/obese non-alcoholic fatty liver disease and control. We determined the composition of the gut microbiota by 16S rRNA sequencing of fecal samples. First, we examined alpha diversity of intestinal bacteria using the Shannon index and Chao 1 (Figs. 1(a), and 1(b)). The Shannon index measures both richness and evenness, whereas the Chao 1 measures only richness. No significant differences were observed in richness among the three groups (Chao 1: non-obese NAFLD, 51.6; obese NAFLD, 49.2; control, 50.0; non-obese NAFLD *vs* obese NAFLD: $P = 0.28$; non-obese NAFLD *vs* control: $P = 0.47$; obese NAFLD *vs* control: $P = 0.69$). However, in obese patients with NAFLD, Shannon index was significantly lower than that in the control subjects (Shannon index: non-obese NAFLD, 2.21;

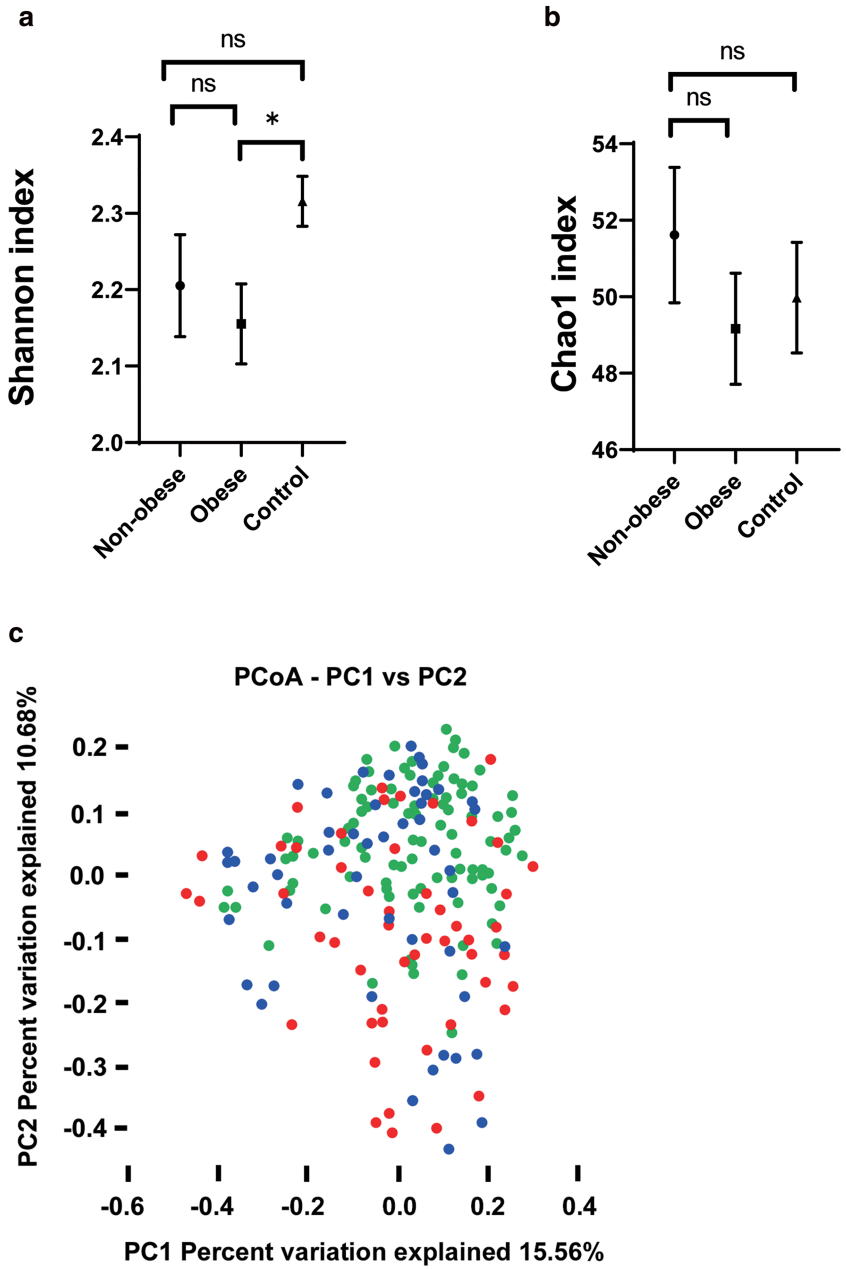

Figure 1 Diversity analysis of intestinal flora between obese non-alcoholic fatty liver disease (NAFLD) group and healthy group. Non-obese NAFLD group (n = 51); obese NAFLD group (n = 51); and control group (n = 87). Values are expressed as mean + standard error of the mean. (a) Shannon index: a significant difference in the diversity of intestinal flora between the two groups is seen; no difference is observed between the non-obese NAFLD group and the other groups. $^*P < 0.05$. (b) Chao 1: there is no significant difference between the non-obese NAFLD group and the other groups regarding the richness of the gut microbiota. $^*P < 0.05$. (c) Principal coordinate analysis: principal coordinates analysis was performed based on the Bray–Curtis distance to compare the bacterial communities among samples from non-obese NAFLD group, obese NAFLD group, and control subjects (non-obese NAFLD *vs* obese NAFLD, Pr (>F) = 0.006, non-obese NAFLD *vs* control, Pr (>F) = 0.0002, obese NAFLD *vs* control, Pr (>F) = 0.0004, permutational multivariate analysis of variance). (c) ●, Non-obese; ●, Obese; ●, Control.

obese NAFLD, 2.19; control, 2.32; non-obese NAFLD *vs* obese NAFLD: P = 0.83; non-obese NAFLD *vs* control: P = 0.14; obese NAFLD *vs* control: P = 0.046). Non-obese NAFLD, obese NAFLD, and control groups could be distinguished in the principal coordinate analysis based on the Bray–Curtis distance, which represents the beta diversity values (Fig. 1c). Permutational multivariate analysis of variance indicated that there was a significant difference in beta diversity between each group (non-obese NAFLD *vs* obese NAFLD: Pr (>F) = 0.006; non-obese NAFLD *vs* control: Pr (>F) = 0.0002; obese NAFLD *vs* control: Pr (>F) = 0.0004).

To identify the differences in gut microbiota composition between non-obese and obese patients with NAFLD, LEfSe was conducted. The analysis was conducted to characterize the distinguishing phylogenetic types of the gut microbiota of the two NAFLD groups (Fig. 2). Verrucomicrobia (phylum), Verrucomicrobiae (class), Verrucomicrobiales (order), Verrucomicrobiaceae (family), *Akkermansia* (genus), *Butyricimonas* (genus), and *Pseudoramibacter* (genus) were enriched in the non-obese NAFLD group compared with the obese NAFLD group.

Characteristics of the gut microbiota at the genus level in non-obese patients with NAFLD are shown in Table 2. The genus most significantly associated with non-obese patients with NAFLD relative to obese patients with NAFLD and control subjects was *Eubacterium*, a genus belonging to Firmicutes. *Eubacterium* showed approximately 50% lower relative abundance in the non-obese NAFLD group than in the obese NAFLD

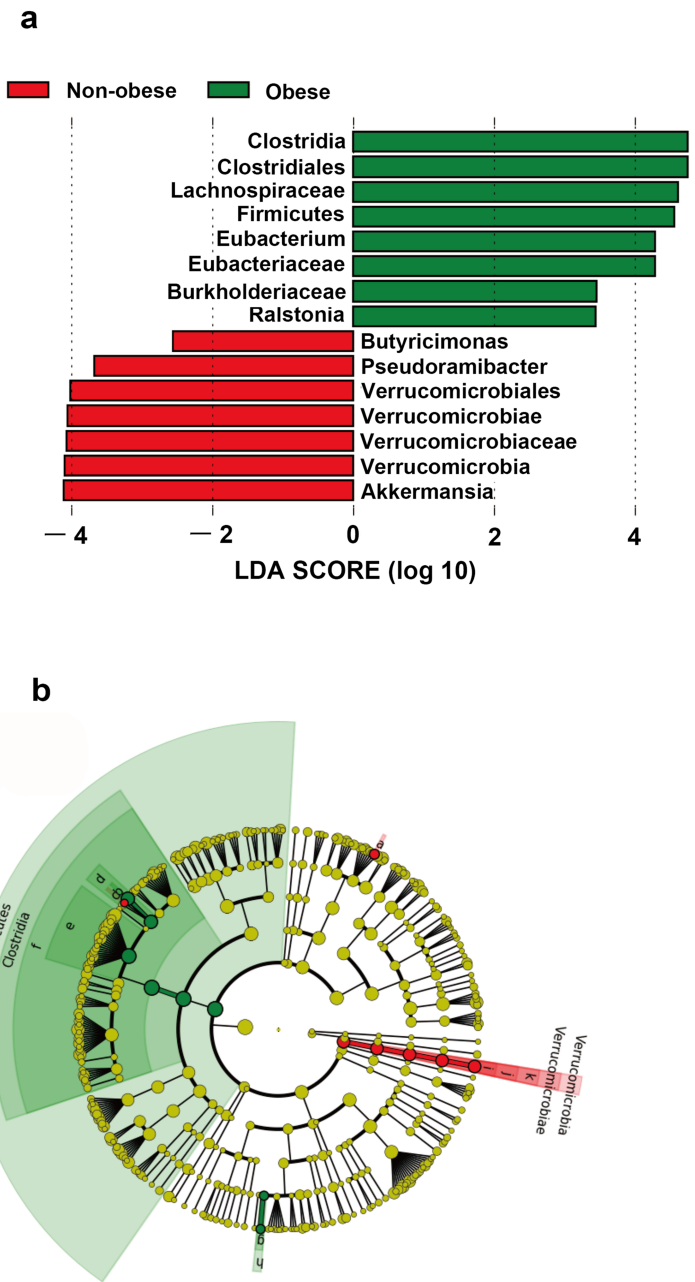

Figure 2 Linear discriminant analysis (LDA) effect size analysis in non-obese non-alcoholic fatty liver disease (NAFLD) group (n = 51); obese NAFLD group (n = 51); and control group (n = 87). (a) Operational taxonomic units (OTUs) with significant difference have LDA scores greater than 3.5 (threshold value). (b) OTUs that were significantly different between the non-obese and obese NAFLD groups are shown in the cladogram based on LDA effect size analysis. Taxonomic hierarchies were plotted from the inside (lower taxonomic level) to the outside (higher taxonomic level). Red and green nodes in the phylogenetic tree show differentially abundant OTUs in the non-obese and obese NAFLD groups, respectively. Yellow nodes indicate OTUs with no significant difference. (b) , a: *Butyricimonas*; , b: *Eubacterium*; , c: *Pseudoramibacter*; , d: Eubacteriaceae; , e: Lachnospiraceae; , f: Clostridiales; , g: *Ralstonia*; , h: Burkholderiaceae; , i: *Akkermansia*; , j: Verrucomicrobiaceae; , k: Verrucomicrobiales.

and controls (non-obese NAFLD, 2.8%; obese NAFLD, 6.1%; control, 5.8%; non-obese NAFLD *vs* obese NAFLD: FDR = 0.068; non-obese NAFLD *vs* control: FDR = 0.002; obese NAFLD *vs* healthy control: FDR = 0.82). Patients with a BMI of less than 23 were defined as lean NAFLD. The same analysis was performed in the lean NAFLD, obese NAFLD, and control groups. The characteristics of the gut microbiota at the genus level in lean NAFLD (BMI < 23) patients are shown in Table S1. *Eubacterium* was a significant microbiota in lean NAFLD.

The gut microbiota that differed between the non-obese and the control groups were *Faecalibacterium*, *Streptococcus*, and *Subdoligranulum*, which were the characteristic microbiota in all NAFLD patients, but not in non-obese NAFLD. *Faecalibacterium* abundance was significantly lower in non-obese and obese patients with NAFLD than that in the healthy control group (non-obese NAFLD, 4.6%; obese NAFLD, 4.1%; control, 7.7%; non-obese NAFLD *vs* obese NAFLD: FDR = 0.78; non-obese NAFLD *vs* healthy control: FDR = 0.016; obese NAFLD *vs* healthy control: FDR = 0.002). Non-obese patients with NAFLD had more than twice the abundance of *Streptococcus* than controls (non-obese NAFLD *vs* control: FDR = 0.01), whereas *Subdoligranulum* abundance was lower in non-obese patients with NAFLD (non-obese NAFLD *vs* control: FDR = 0.01). *Escherichia* tended to be higher in non-obese NAFLD than in the other two groups, although the difference was not significant after correction using the Benjamini and Hochberg method. In addition, *Akkermansia* was more

Table 2 Mean abundance of characteristic microbiomes at the genus level in patients with non-obese non-alcoholic fatty liver disease (NAFLD) and obese NAFLD and control subjects

Genus	Non-obese (%)	Obese (%)	Healthy control (%)
Alistipes	0.40	0.14[§]	0.25
Bacteroides	4.22	2.07[‡]	3.72
Clostridium	3.06	3.09	4.28
Escherichia	4.45	1.29	0.59
Eubacterium	2.81[†,‡]	6.14	5.83
Faecalibacterium	4.55[‡]	4.05[§]	7.74
Lactobacillus	2.52	2.18	0.59
Ruminiclostridium	0.22	0.19	0.41
Ruminococcus	2.39	3.02	3.82
Streptococcus	8.40[‡]	6.00	2.43
Subdoligranulum	1.55[‡]	2.04[§]	3.31

[†]FDR value (FDR < 0.1, non-obese NAFLD *vs* obese NAFLD).
[‡]FDR value (FDR < 0.1, non-obese NAFLD *vs* control).
[§]FDR value (FDR < 0.1, obese NAFLD *vs* control).
NAFLD, non-alcoholic fatty liver disease.
Non-obese NAFLD group (n = 51); obese NAFLD group (n = 51); and control group (n = 87). The occupation rates of genera for which significant P values (<0.05) were obtained are shown. The genera with an occupation rate of 0.1% in all groups were excluded. For correction of multiple comparisons, we computed the false discovery rate (FDR) by the Benjamini and Hochberg method.

Table 3 Mean abundance of characteristic microbiota at the phylum level in patients with non-obese non-alcoholic fatty liver disease (NAFLD) and obese NAFLD and control subjects

Phylum	Non-obese NAFLD (%)	Obese NAFLD (%)	Control (%)
Actinobacteria	9.03	8.69	6.69
Bacteroidetes	5.78[†]	2.60[§]	4.80
Firmicutes	74.2[†, ‡]	83.3	83.5
Fusobacteria	0.2	0.11	0.05
Proteobacteria	6.95[‡]	3.53	1.66
Verrucomicrobia	2.09	0.44	0.57

[†]FDR value (FDR < 0.1, *vs* obese NAFLD).
[‡]FDR value (FDR < 0.1, *vs* control).
[§]FDR value (FDR < 0.1, obese NAFLD *vs* control).
Lentisphaerae and Tenericutes are not listed because their abundance was 0% in all groups.
NAFLD, non-alcoholic fatty liver disease.
Non-obese NAFLD group (n = 51); obese NAFLD group (n = 51); and control group (n = 87). The occupation rates are shown. The genera with an occupation rate of 0.1% in all groups were excluded.

common in non-obese subjects, although this difference was not significant (non-obese NAFLD, 2.05%; obese NAFLD, 0.45%; control, 0.52%).

We analyzed the gut microbiota composition at the phylum level (Table 3). Firmicutes, Actinobacteria, Proteobacteria, and Bacteroidetes were the predominant phyla in all three groups of participants. In the phylum-level analysis, the abundance of Firmicutes in the non-obese NAFLD group was markedly different

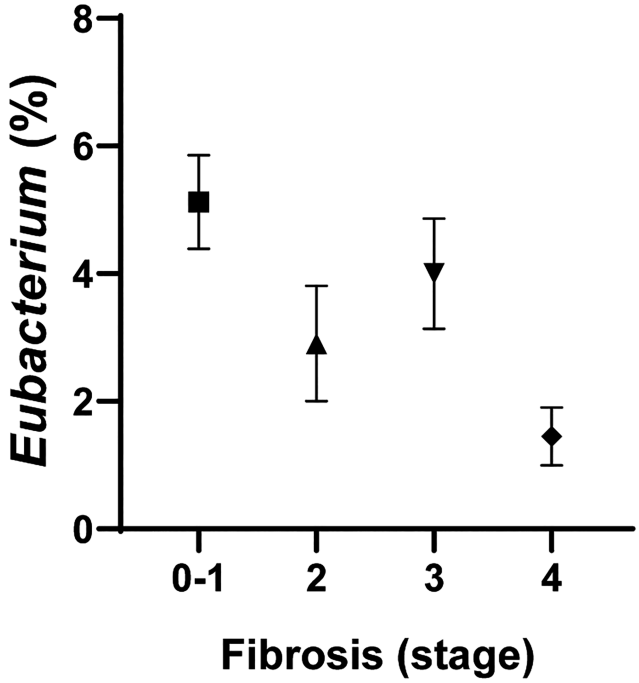

Figure 3 Correlation between microbiota and hepatic fibrosis in patients with non-alcoholic fatty liver disease. Values are expressed as mean + standard error of the mean. A steady stepwise decrease in abundance of *Eubacterium* was observed with an increase in severity of liver fibrosis in patients with non-obese and obese non-alcoholic fatty liver disease (Jonckheere–Terpstra trend test; $P < .0008$).

from that in the obese NAFLD and control groups. Firmicutes counts were significantly lower in the non-obese patients with NAFLD (non-obese NAFLD *vs* obese NAFLD: FDR = 0.008; non-obese NAFLD *vs* control: FDR = 0.002). The abundance of Bacteroidetes in non-obese patients with NAFLD was more than twice that in obese patients with NAFLD (non-obese NAFLD *vs* obese NAFLD: FDR = 0.063; non-obese NAFLD *vs* control: FDR = 0.48). Proteobacteria exhibited a significant increase in abundance in non-obese patients with NAFLD compared with that in controls (non-obese NAFLD *vs* obese NAFLD: FDR = 0.20; non-obese NAFLD *vs* control: FDR = 0.017).

Accurate analysis at the species level is difficult using the MiSeq platform due to regionally limited sequence analysis; however, we performed analysis at the species level (Table S2). At the species level, no characteristic microbiota were found in the non-obese NAFLD group compared with the other two groups. *Eubacterium ramulus* and *Eubacterium rectale* tended to be less abundant in non-obese NAFLD but were not significantly different compared with obese NAFLD. *Faecalibacterium prausnitzii* was significantly less in the non-obese NAFLD and obese NAFLD groups compared with the control group.

Table S3 shows the functions in the inferred metagenomic analysis that differed between the non-obese NAFLD group and the other two groups with $P < 0.05$. When the FDR was calculated using the Benjamini and Hochberg method, these functions were not found to be significantly different among the three groups of participants.

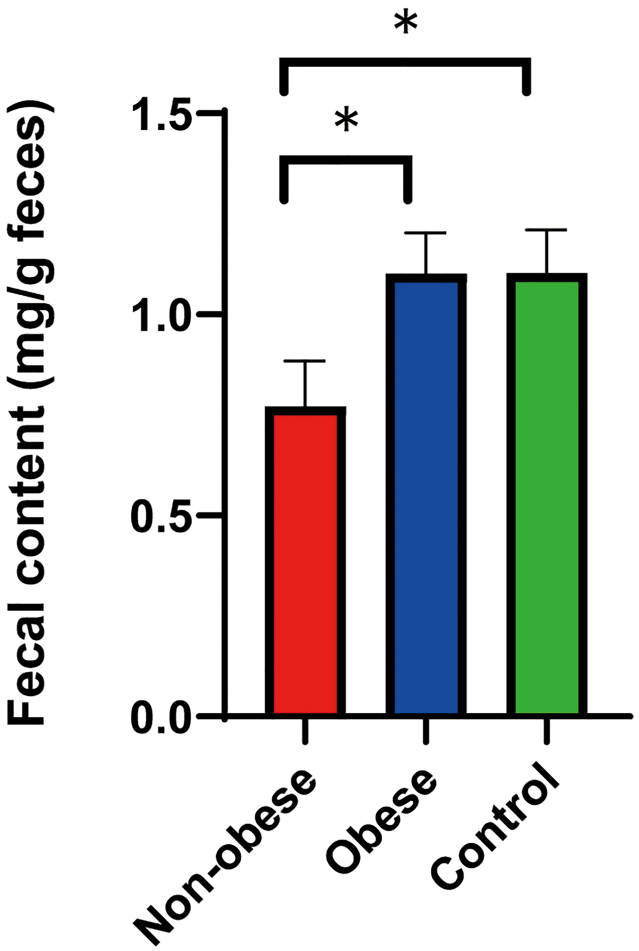

Figure 4 Analysis of short-chain fatty acids content in non-obese non-alcoholic fatty liver disease (NAFLD) group (n = 51); obese NAFLD group (n = 51); and control group (n = 87). Values are expressed as mean + standard error of the mean. Butyric acid levels were significantly lower in the non-obese NAFLD group than in other groups. *$P < 0.05$.

Correlation between microbiota and other findings. We assessed the correlation between gut microbiota and pathological findings or clinical parameters to investigate the potential impact of the gut microbiota on NAFLD development. Figure 3 shows stepwise increases in the occupation rate of *Eubacterium* with an increase in the stage of hepatic fibrosis ($P = 0.008$ by the Jonckheere–Terpstra trend test). When the non-obese group (BMI < 25) was changed to the lean group (BMI < 23), similar results were obtained (Fig. S2; $P = 0.032$). Regarding steatosis, lobular inflammation, and ballooning, there was no correlation between the grades and the abundance of *Eubacterium* (Fig. S3). Table S4 summarizes the Spearman's rank correlations between clinical parameters related to *Eubacterium*. There were no clinical parameters correlated with *Eubacterium*.

Short-chain fatty acids in the non-obese non-alcoholic fatty liver disease group. Short-chain fatty acids are the main metabolites of fermentation of non-digestible carbohydrates by the gut microbiota. They are considered to be involved in the pathogenesis of NAFLD because they potentially contribute to the maintenance of body weight, intestinal homeostasis, and improved metabolism of glucose and lipids.[18–20] Butyric acid levels were significantly lower in the non-obese NAFLD group than in the obese NAFLD and control groups. No significant differences were observed in the other SCFAs among the three groups (Fig. 4).

Discussion

Our results suggest that the gut microbiota of non-obese patients with NAFLD are different from those of obese NAFLD and control subjects. The gut microbiota of patients with NAFLD exhibit less diversity than those of healthy persons.[21,22] Lower diversity in the gut microbiota has been linked to obesity, higher insulin resistance, higher visceral fat, and numerous inflammatory conditions.[23–25] Thus, gut microbiota diversity could be linked not only to NAFLD but also to body weight. Here, alpha diversity did not differ between the non-obese NAFLD group and the other two groups, whereas beta diversity differed among the three groups. This indicated that the richness and evenness of the gut microbiota in non-obese individuals with NAFLD were similar to those of the other two groups; however, inter-individual and intergroup variability in the bacterial community structure were observed among the three groups. We showed that Firmicutes was the predominant phylum in patients with NAFLD with a notable decrease in non-obese patients with NAFLD. The abundance of Bacteroidetes was higher in the non-obese NAFLD group than in the obese NAFLD group. Obese people have fewer Bacteroidetes and more Firmicutes than leaner ones.[26] With the progression from mild and moderate to advanced fibrosis in NAFLD, Proteobacteria abundance increases significantly, whereas that of Firmicutes decreases.[27] Bacteroidetes abundance has been found to be significantly lower in patients with non-alcoholic steatohepatitis than in those with simple steatosis and control individuals.[28] The gut microbiota composition also changes with the progression of fatty liver disease. We showed that Firmicutes and Bacteroidetes abundance in patients with NAFLD changed not only with the progression of NAFLD but also with BMI changes.

Proteobacteria at the phylum level and *Escherichia* and *Streptococcus* at the genus level are abundant in NAFLD.[29,30] *Escherichia* in the Proteobacteria is an ethanol-producing bacterium; elevated blood alcohol levels are observed in NASH patients, suggesting that the increased abundance of the ethanol-producing *Escherichia* may be a risk factor for the accelerated progression of NASH.[30] In this study, Proteobacteria and *Escherichia* tended to be more abundant in the non-obese NAFLD group and may be involved in the pathogenesis of the disease. *Akkermansia muciniphila* was reported to have an anti-fat effect.[31] In this study, in line with the previous report, *Akkermansia* was higher in non-obese NAFLD than in the other two groups, although not statistically significant.

Although previous studies have assessed the relationship between non-obese NAFLD and gut microbiota, their findings differ from ours. Erysipelotrichaceae UCG-003, *Ruminococcus*, *Romboutsia*, *Clostridium sensu stricto 1*, and Ruminococcaceae UCG-008 were enriched in lean patients with NAFLD.[32] In another study, a significant decrease in Desulfovibrionaceae was

observed in lean patients with NAFLD.[33] These studies have not shown a consistent composition of gut microbiota, possibly because of environmental factors and genetic factors such as diet, region, country, and race.[14,27,28,30,34,35] At the genus level, our study was the first to show that *Eubacterium* abundance was significantly lower in non-obese patients with NAFLD than in obese patients with NAFLD and controls. The prevalence of *Eubacterium ventriosum* and *Eubacterium hasrum* is higher, while that of *Eubacterium fissicatena* is lower in obese subjects.[36,37] The variation in the abundance of *Eubacterium* with BMI should be taken into account. However, the abundance of *Eubacterium* was significantly lower in the non-obese NAFLD group, compared with the control and obese NAFLD groups. This cannot be explained in relation to BMI alone, but is likely to be related to the pathogenesis of non-obese NAFLD.

Eubacterium is an obligate anaerobic bacterium that ferments dietary fiber to produce SCFAs including butyric acid.[38] SCFAs play important roles in NAFLD pathogenesis because of their potential effects on the maintenance of body weight, intestinal homeostasis, and improvement in glucose and lipid metabolism.[18,19] Fructose intake was inversely correlated to the presence of *Eubacterium* in obese patients.[39] Feeding a carbohydrate-rich diet to mice reduces *Eubacterium rectale* levels, a species linked to butyrate production. There is a negative association between *Eubacterium* abundance and fructose intake.[40] In this study, analysis at the species level indicated that *Eubacterium rectale* were the gut microbiota that tended to be reduced in the non-obese NAFLD group, compared with the other two groups. We hypothesized that dietary content and intestinal bacteria such as *Eubacterium* are involved in non-obese NAFLD development. Gut microbiota play key roles in the progression and homeostasis of the host immune system.[41] SCFAs such as propionate and butyrate suppress the expression of lipopolysaccharide (LPS)-induced cytokines, interleukin-6 (IL-6), and IL-12p40 in human mature dendritic cells.[42,43] Butyrate suppresses IL-6, tumor necrosis factor-alpha (TNF-α), and myeloperoxidase activity by inhibiting nuclear factor-kappa B activation in Kupffer cells.[44] Accumulation of triglycerides and decrease in fatty acid oxidation and insulin responsiveness in the liver have been observed in a murine model of Kupffer cell depletion, largely mediated by TNF-α.[45] Therefore, SCFAs, including butyrate, may influence the development of liver inflammation. The decreased butyric acid levels we observed could be involved in non-obese NAFLD development. According to the results of our inferred metagenomic analysis, LPS biosynthesis, which represents the ability of a cell to produce endotoxin, was higher in non-obese NAFLD, although the difference was found to not be significant upon FDR correction. Higher LPS biosynthesis in non-obese NAFLD may be related to the disease pathogenesis. However, blood endotoxin (endotoxin activity assay values) levels were not elevated in the non-obese NAFLD group compared with those in the obese NAFLD group. We showed a negative correlation between *Eubacterium* abundance and hepatic fibrosis. However, the mechanism that links *Eubacterium* abundance and hepatic fibrosis is unclear. We hypothesized that a decrease in *Eubacterium* levels lowers SCFA production, which causes abnormalities in fat metabolism and induction of hepatic fibrosis. Thus, the decrease in *Eubacterium* caused by high carbohydrate intake may be one of the causes of progression of non-obese NAFLD.

This study has several advantages over previous studies that have investigated the gut microbiota in non-obese NAFLD. First, our subjects were pathologically well-characterized as patients with NAFLD who underwent liver biopsies. Second, this study had a larger number of subjects (51 non-obese patients with NAFLD) than previous studies (<10 subjects).[44,46] Finally, the present study was a multicenter study with more generalizable findings than those of single-center studies. Our study also has several limitations. First, the presented associations between the gut microbiota and non-obese NAFLD were based on statistical associations from cross-sectional observations. Therefore, it is unclear whether the decrease in *Eubacterium* levels is responsible for non-obese NAFLD, or if the progression of NAFLD causes a shift in the gut microbiota. Future studies should include the administration of *Eubacterium* to non-obese NAFLD model mice. Second, the subject registration process may have led to a selection bias. The patients were enrolled from tertiary care centers in Japan, where liver biopsies were conducted for patients with NAFLD with more severe liver conditions. Therefore, studies in patients recruited from general hospitals are needed to confirm the results. Third, although patients using antibiotics or fermented foods were excluded, insufficient data on diet and medications limit the interpretation of the results. The influence of medications such as proton pump inhibitors, and conditions such as diabetes, which modulate intestinal bacteria, should have been considered.[47,48] Lastly, the composition of the gut microbiota varies depending on the database used in the analysis. In this study, the Ribosomal Database Project was used to analyze the taxonomy, but the gut microbiota composition also changed when compared to other databases (e.g. Greengenes and SILVA),[49] which may also account for the low abundance of Bacteroidetes we observed.

In conclusion, we determined the relationship between the gut microbiota composition and the progression of non-obese NAFLD. This is the first report to show that *Eubacterium* abundance was significantly decreased in non-obese patients with NAFLD compared with that in obese patients with NAFLD and healthy subjects. Furthermore, our results demonstrated a negative correlation between *Eubacterium* and hepatic fibrosis. The decrease in the abundance of *Eubacterium* that produces butyric acid may play an important role in the development of non-obese NAFLD.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Mean abundance of characteristic microbiomes at the genus level in patients with lean (BMI < 23) non-alcoholic fatty liver disease (NAFLD), obese NAFLD, and control subjects.
Supplementary Table 2. Mean abundance of characteristic microbiota at the species level in patients with non-obese non-alcoholic fatty liver disease (NAFLD), obese NAFLD, and control subjects.
Supplementary Table 3. Inferred metagenomic analysis.
Supplementary Table 4. Correlation between microbiota and clinical parameters.
Figure S1. Flow diagram of patients with non-alcoholic fatty liver disease (NAFLD) and controls.
Non-obese NAFLD group (n = 51); obese NAFLD group (n = 51); and control group (n = 87).
Figure S2. Correlation between microbiota and hepatic fibrosis in patients with non-alcoholic fatty liver disease (NAFLD) when the non-obese group (BMI < 25) was changed to the lean group (BMI < 23).
Values are expressed as mean + standard error of the mean (SEM). A steady stepwise decrease in abundance of Eubacterium was observed with an increase in severity of liver fibrosis in patients with lean (BMI < 23) and obese NAFLD (Jonckheere-Terpstra trend test; $P < 0.032$).
Figure S3 a, 3b, and 3c. Correlation between *Eubacterium* abundance and pathological evaluation.
There was no correlation between the abundance of *Eubacterium* and the pathological findings in steatosis, lobular inflammation, and ballooning (Kruskal-Wallis test; steatosis $P = 0.72$, lobular inflammation $P = 0.42$, ballooning $P = 0.83$).
In ballooning, there were only 2 cases of grade 3, and grades 2 and 3 were unified.

論　文　目　録

Ⅰ　主　論　文

Gut microbiota composition associated with hepatic fibrosis in non-obese patients with non-alcoholic fatty liver disease. Iwaki M, Kessoku T, Ozaki A, Kasai Y, Kobayashi T, Nogami A, Honda Y, Ogawa Y, Imajo K, Yoneda M, Maeda A, Tanaka Y, Nakajima S, Ohno H, Usuda H, Kawanaka M, Kawaguchi T, Torimura T, Kage M, Hyogo H, Takahashi H, Eguchi Y, Aishima S, Wada K, Kobayashi N, Sumida Y, Saito S, Nakajima A: Journal of gastroenterology and hepatology. Vol.36, No.8, Page 2275-2284, 2021.