Original Article

Comparison of the gut microbial community between obese and lean peoples using 16S gene sequencing in a Japanese population

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Altered gut microbial ecology contributes to the development of metabolic diseases including obesity. In this study, we performed 16S rRNA sequence analysis of the gut microbiota profiles of obese and lean Japanese populations. The V3–V4 hypervariable regions of 16S rRNA of fecal samples from 10 obese and 10 lean volunteers were sequenced using the Illumina MiSeq™II system. The average body mass index of the obese and lean group were 38.1 and 16.6 kg/m^2, respectively (p<0.01). The Shannon diversity index was significantly higher in the lean group than in the obese group (p<0.01). The phyla Firmicutes and Fusobacteria were significantly more abundant in obese people than in lean people. The abundance of the phylum Bacteroidetes and the Bacteroidetes/Firmicutes ratio were not different between the obese and lean groups. The genera *Alistipes*, *Anaerococcus*, *Corpococcus*, *Fusobacterium* and *Parvimonas* increased significantly in obese people, and the genera *Bacteroides*, *Desulfovibrio*, *Faecalibacterium*, *Lachnoanaerobaculum* and *Olsenella* increased significantly in lean people. Bacteria species possessing anti-inflammatory properties, such as *Faecalibacterium prausnitzii*, increased significantly in lean people, but bacteria species possessing pro-inflammatory properties increased in obese people. Obesity-associated gut microbiota in the Japanese population was different from that in Western people.

***Key Words*: 16S sequence, datamining, Firmicutes, Bacteroides, SCFA**

Obesity is one of the most serious public health concerns worldwide.[1,2] More than 500 million people are obese, and its prevalence is dramatically increasing not only in developed countries but also in developing countries.[1,3] Obesity is associated with a higher risk for health problems such as heart disease, stroke, high blood pressure, diabetes mellitus and more.[1] A worldwide study in 2010 reported that obesity is associated with 3–4 million deaths, 4% of years of life lost, and 4% of disability-adjusted life-years lost.[4]

The effect of the gut microbiota on human health is recognized as a mutually beneficial interaction between human and indigenous microorganisms that contributes to normal physiology and immune homeostasis.[5] The gut microbiota regulates metabolic function and energy balance, and an altered microbial ecology contributes to the development of several metabolic diseases including obesity. For example, the gut microbiota profile of obese people is characterized by a reduced proportion of the phylum Bacteroidetes and an increased proportion of the phylum Firmicutes, compared to lean people.[6] These changes are considered to affect the metabolic potential of the gut microbiota and enhance the body's capacity to harvest energy from the diet.[7] Transfer of the gut microbiota from obese mice leads to a significantly greater accumulation of adipose tissue in recipient mice than a transfer of the gut microbiota from lean donors.[8] Thus, the gut microbiota is a critical environmental factor contributing to the development of obesity.

In previous studies, alteration of the gut microbiota in obese people has been studied mainly in Western countries.[3,6,7,9] De Filippo *et al.*[10] previously demonstrated a difference in the gut microbial structure between obese European children and lean African children, indicating that environmental factors such as diet, sanitation and hygiene are important for shaping the gut microbiota. This leads to the possibility that obesity-associated gut microbiota might be different between Western and Asian populations, since lifestyles including dietary habits are quite different. However, alteration of the gut microbiota profile has not been extensively investigated in Asian people in general or in the Japanese population in particular. Therefore, it is important to investigate the obesity-associated gut microbiota profile of Japanese people.

In the present study, we performed 16S rRNA sequence analysis of the gut microbiota profile in 10 obese and 10 lean Japanese people. Furthermore, bacterial species that contributed to the difference in the gut microbiota composition between obese and lean people were characterized.

Materials and Methods

Subjects and setting. Twenty volunteers (10 obese people and 10 lean people) were enrolled in this study. The body mass index (BMI) of the obese people was 38.1 ± 3.5 kg/m^2 (mean ± SD) (range 35.7–49.2 kg/m^2) and the BMI of the lean people was 16.6 ± 1.0 kg/m^2 (range 14.2–17.7 kg/m^2) (Table 1). No one was receiving medical treatment, drugs or supplements that could potentially modulate the gut microbiota. The Institutional Review Board of the Shiga University of Medical Science approved the study, and written informed consent was obtained from each person prior to enrolment.

DNA extraction. DNA was extracted from fecal samples according to a previously described method.[11] The fecal samples were suspended in a buffer containing 4 M guanidium thiocyanate, 100 mM Tris-HCl (pH 9.0) and 40 mM EDTA and beaten in the presence of zirconia beads using the FastPrep FP100A Instrument

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Table 1. Backgrounds of volunteers enrolled in this study

	Obese	Lean	*p* value
Gender (female/male)	5/5	5/5	
Age [mean (range)]	41 (35–55)	45 (31–58)	
Body weight (kg, mean ± SD)	101.1 ± 13.6	42.5 ± 4.1	<0.01
Height (cm, mean ± SD)	162.9 ± 9.0	159.8 ± 6.1	0.22
Body mass index (mean ± SD)	38.1 ± 3.5	16.6 ± 1.0	<0.01
Fasting blood sugar (mg/dl)	107.0 ± 34.7	85.8 ± 7.1	<0.05
Total chelesterol (mg/dl)	222.2 ± 26.6	216.6 ± 33.4	0.56
triglyceride (mg/dl)	136.5 ± 53.8	65.1 ± 35.7	<0.01

(MP Biomedicals, Irvine, CA). Thereafter, the DNA was extracted from the beads-treated suspension using a Magtration System 12GC and GC series Magtration–MagaZorb DNA Common Kit 200 N (Precision System Science, Chiba, Japan). The final concentration of DNA sample was adjusted to 10 ng/μl.

16S rRNA sequencing. 16S rRNA sequencing using the MiSeq™II system (Illumina, San Diego, CA) was performed according to a previously described method.[12] The V3–V4 hypervariable regions of 16S rRNA were PCR amplified from microbial genomic DNA using the following universal primers: 341F, 5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCCTACGGGAGGCAGCAGCCTACGGGAGGCAGCAG-3';[12] 806R, 5'-CAAGCAGAAGACGGCATAGAGATNNNNNNGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT-3'.[12] PCR products were purified through a MultiScreen PCR filter plate (Merck Millipore, Darmstadt, Germany). Barcoded V3 and V4 amplicons were sequenced using the paired-end, 2 × 250-bp cycle run on the MiSeq™II system with MiSeq Reagent Kit ver. 2 (500 Cycle) chemistry. The resulting sequences were then screened and filtered for quality and length. Paired-end sequencing with read lengths of 251 bp was joined together with the fastq-join program (http://code.google.com/p/ea-utils/). Only reads that had quality value (QV) scores of ≥20 for more than 99% of the sequence were extracted for further analysis. The nucleotide sequence dataset was deposited in the Sequence Read Archive of the DNA Data Bank of Japan (DDBJ) under the accession number DRA002295.

Principal component analysis and data mining. Principal component analysis (PCA) was performed using Metagenome@KIN (World Fusion, Tokyo, Japan). Data mining analysis was performed using SPSS Clementine14 software (IBM Japan, Tokyo, Japan). A dividing system using the Classification and Regression Tree (C&RT) approach, which is the most typical method for constructing decision trees, using the Gini coefficient[13] between obese (or lean) and operational taxonomic unit (OTU) data of the order level was applied. The records were divided into two subsets so that the records within each subset were more homogeneous than in the previous subset.

16S rDNA-based taxonomic analysis and statistical analysis. Analyses of sequence reads were performed using the Ribosomal Database Project (RDP) Multiclassifier tool (http://rdp.cme.msu.edu/classifier/)[14] and BLAST search using the Metagenome@KIN analysis software (World Fusion) for Techno-Suruga Lab Microbial Identification Database DB-BA9.0 (Technosuruga laboratory, Shizuoka, Japan). Reads showing ≥97% homology were grouped in each taxonomic rank. To evaluate the strength of influence of bacterial species, the LogWorth statistic for partition models was calculated using JMP8 statistical software (SAS Institute, Cary, NC). Differences between different samples were checked for statistical significance ($p<0.05$) using the Student's t-tests. The data were analyzed using Statview 5.0 software (SAS).

Results

The baseline characteristics of the 20 subjects are shown in Table 1. Average body mass index (BMI) of the obese group (38.1 ± 3.5 kg/m^2) was significantly higher than that of the lean group (16.6 ± 1.0 kg/m^2) ($p<0.01$). Fasting blood sugar and triglyceride levels were also significantly higher in the obese group than in the lean group.

Initially, we performed 16S rRNA sequencing of the fecal samples from the obese and lean groups. The average of 20,486 reads per obese sample was significantly higher than the average of 17,358 reads per lean sample (Fig. 1A). In contrast, the Shannon diversity index (H') was significantly higher in the lean group than in the obese group (Fig. 1B). These results indicate that the fecal microbial structure of lean people is more complex as compared to that of obese people.

Based on the RDP database, all sequences were classified from the phylum to species. At the phylum level, Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria were dominant in both the obese and lean groups. The phyla Firmicutes and Fusobacteria were significantly more abundant in the obese group than in the lean group (Firmicutes: 42.6 ± 8.5% in obese vs 35.1 ± 5.2% in lean, $p = 0.018$) (Fig. 2). In particular, the phylum Fusobacterium was detected only in obese people (1.86 ± 4.20% in obese vs 0.00 ± 0.00% in lean, $p = 0.002$). Previous studies demonstrated an increase of the phylum Bacteroidetes in lean people compared to obese people,[6] but we could not detect such a difference in our samples (31.2 ± 14.1% in obese vs 32.9 ± 6.4% in lean, $p = 0.38$). Furthermore, there was no significant difference in the Bacteroidetes/Firmicutes ratio between obese (0.86 ± 0.63) and lean people (0.96 ± 0.27).

Principal component analysis (PCA) at the phylum level showed different distribution of obese and lean peoples (Fig. 3), suggesting a presence of potential difference between obese and lean microbial structure. Data mining was used to create a decision tree, which is a decision-supporting pathway (Fig. 4). Node 0 (the starting point for tree construction) was divided into Node 1 and Node 2 by the read number of the order *Clostridiales* with a cutoff value of 5617. Six of the 10 obese people were selectively allocated to Node 2 (*Clostridiales* read number >5,617), and 10 lean people and 4 obese people were allocated to Node 1 (*Clostridiales* read number ≤5,617). Node 1 was further subdivided into Nodes 3, 5 and 6. All lean people were allocated to Node 6, which was characterized by a read number for the order *Bacteroidales* >2,667. These results suggest that at the order level, the microbial community of obese people is characterized by higher *Clostridiales* and that of lean people is characterized by higher *Bacteroidales*.

At the genus level, the obtained sequences were assigned to 105 genera, and these were numerically dominated by *Bacteroides*, *Blautia*, *Bifidobacterium*, *Eubacterium*, *Faecalibacterium*, *Prevotella* and *Veillonella* (Table 2). The genera *Alistipes*, *Anaerococcus*, *Corpococcus*, *Fusobacterium* and *Parvimonas* significantly

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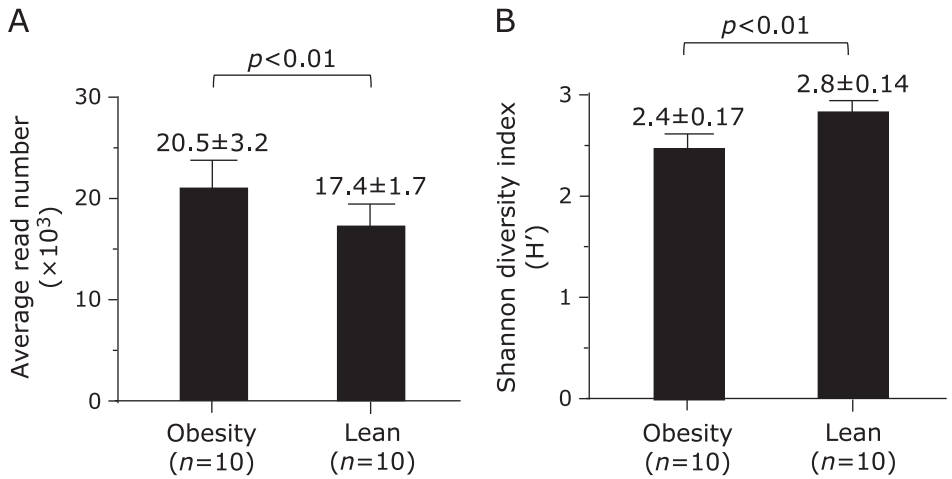

Fig. 1. Results of 16S rRNA sequencing of fecal samples from obese (n = 10) and lean people (n = 10). (A) The average read number. The average number of reads per obese person was significantly higher than the average number of reads per lean person. (**p<0.01). (B) Shannon diversity index. The average of the Shannon diversity index was significantly higher in lean people than in obese people (**p<0.01). Data is expressed as mean ± SD (n = 10).

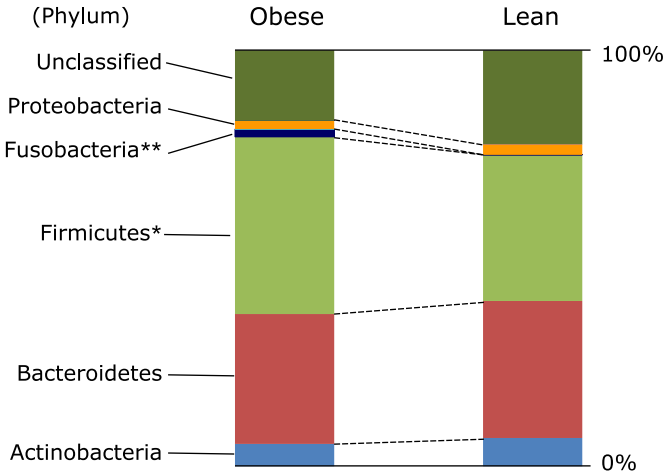

Fig. 2. The gut microbial composition of obese and lean people (phylum level). *p<0.05, **p<0.01.

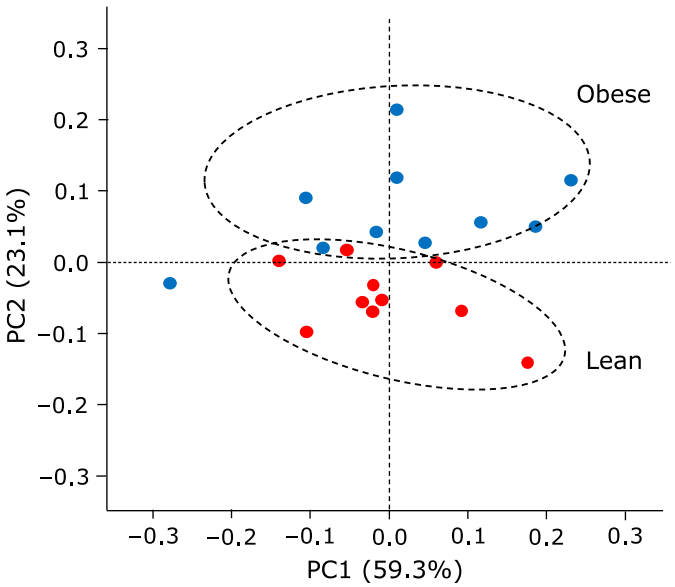

Fig. 3. Principal component analysis (PCA) at the phylum level between obese and lean peoples. PCA based on PC1 (proportion of contribution 59.3%) and of PC2 (proportion of contribution 23.1%) showed different distribution of obese and lean peoples.

increased in obese people as compared to lean people. In contrast, the genera *Bacteroides*, *Desulfovibrio*, *Faecalibacterium*, *Lachnoanaerobaculum* and *Olsenella* significantly increased in lean people compared to obese people.

At the species level, the obtained sequences were assigned to 345 species. Table 3 shows bacteria species that significantly increased in obese people compared to lean people. These included *Acidaminococcus intestini*, *Actinomyces meyeri*, *Atopobium parvulum*, *Bacteroides vulgatus*, *Eubacterium hadrum*, *Klebsiella pneumoniae* and *Roseburia faecis*. The genus *Fusobacterium (F.)* including *Faecalibacterium (F.) mortiferum*, *F. nucleatum*, and *F. varium* increased in obese people, but the difference was not significant.

Table 4 shows the bacteria species that increased in lean people as compared to obese people. *Clostridium ramosum*, *Clostridium citroniae*, *Faecalibacterium prausnitzii*, *Eubacterium desmolans*, *Eubacterium fissicatena*, and *Holdemania filiformis* significantly increased in lean people compared to obese people (p<0.05). *Bilophila wadsworthia* tended to increase in lean people, but the difference was not significant.

Discussion

In this study, we analyzed the fecal microbial community of obese and lean people in the Japanese population. Japan is an island nation and Japanese people are a single ethnic group. In addition, a distinctive food culture developed and has been maintained in this country. Therefore, a unique gut microbial community in the Japanese population is expected. Indeed, Nakayama *et al.*[15] recently reported a specific fecal microbial community in Japanese children that consists of more *Bifidobacterium* and fewer potentially pathogenic bacteria compared to the microbial communities of children in other Asian countries. Previous studies of obesity-associated gut microbiota mainly targeted people living in Western countries, but extensive studies have not

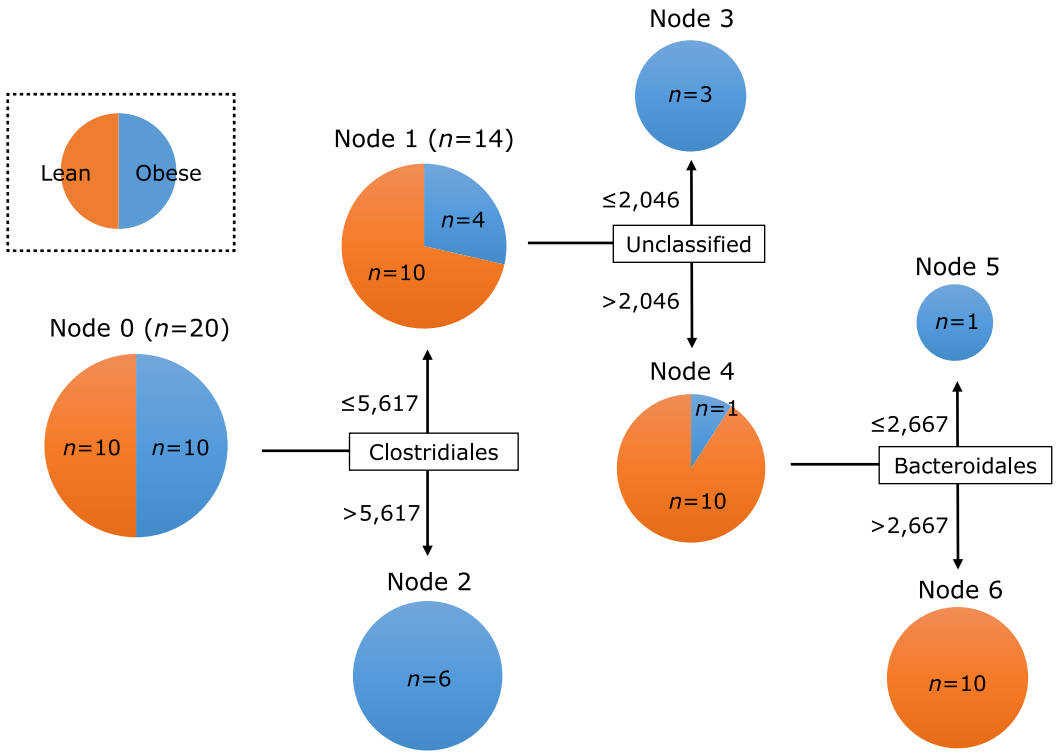

Fig. 4. Decision tree constructed using the Classification and Regression Tree (C&RT) approach. The cutoff value to create each node was calculated from the read number of sequence data at the order level, using the Gini coefficient and the C&RT method. Similar steps were repeated to construct the decision tree. Node 0 is the starting point for tree construction, and the terminal node is one that cannot be further divided. The details related to the pathway leading to the terminal node clearly indicate the orders involved and their relative quantities, which contribute to creating the subject groups.

been performed on the Japanese population. To understand the influence of certain dietary habits and ethnicity on the gut microbial composition, it would be valuable to characterize the obesity- and lean-associated gut microbial communities of the Japanese population.

In this study, we demonstrated that the microbial community of obese Japanese people was characterized by a reduced diversity and an increased abundance of the phyla Firmicutes and Fusobacteria as compared to that of lean people. A reduced diversity and an increased abundance of the phylum Firmicutes in obese people were consistent with previous studies in Western populations.[6,16,17] On the other hand, significance of the phylum Bacteroidetes in the obesity-associated gut microbiota remains to be discussed. Several studies confirmed a decrease of the phylum Bacteroidetes in obese individuals and animal models,[7,18–21] but others did not report any difference between obese and lean subjects or even found the opposite relationship.[22,23] In this study, we could not detect a significant difference in the phylum Bacteroidetes. So, we further investigated the microbial structure at the order level using data mining analysis. Data mining was used to create a decision tree, as shown in Fig. 3, which clearly categorized the obese and lean people. Six of the 10 obese people were allocated to Node 2, which is characterized by a higher abundance of the order *Clostridiales*. In contrast, all of the lean people were allocated to Node 6, which is characterized by a higher abundance of the order *Bacteroidales*. Thus, the dominance of the order *Bacteroidales* in lean Japanese people became clear, although there were no definitive findings at the phylum level.

Short-chain fatty acids (SCFAs: acetate, propionate and butyrate) are generated through the fermentation of dietary fiber by the gut microbiota.[24] The bacterial SCFAs provide 10% of the total dietary energy supply in humans,[24] and fecal SCFA levels increase significantly in obese people compared to lean people.[22] Both the phyla Firmicutes and Bacteroidetes contribute to SCFA generation. It is known that alteration of the gut microbial composition affects changes in SCFA concentration,[25] and a higher Firmicutes/Bacteroidetes ratio is associated with obesity via increased generation of SCFAs.[7,18,19] In this study, however, we did not detect a significant difference in the Firmicutes/Bacteroides ratio between obese and lean people, although a higher proportion of the phylum Firmicutes was confirmed in obese people. Similar negative results have been reported previously,[22,23] and Murphy *et al.*[26] reported that changes in the proportions of the major phyla were unrelated to SCFA concentrations and energy harvest. These results suggest that the linkage between gut microbial composition, SCFA-related energy harvest and obesity may be complex and require further extensive investigations in the future.

A novel finding in this study was an increased abundance of the phylum Fusobacteria in obese people. This finding was also confirmed at the genus and species levels. The levels of *F. infirmum*, *F. nucleatum* and *F. varium* were higher in obese people than in lean people, although the difference was not significant. The phylum Fusobacteria induces host inflammatory response and possesses virulence characteristics that promote their adhesiveness to host epithelial cells and their ability to invade into epithelial cells.[27] Recent studies focused on the association of the phylum Fusobacteria with colorectal cancer,[27,28] and *F. nucleatum* has been shown to potentiate intestinal tumorigenesis by modulating β-catenin signaling.[28] *F. varium* has also been reported to be associated with the pathophysiology of ulcerative colitis.[29] However, the association between the phylum Fusobacteria and obesity has not been described previously. Our results do not imply a causal relationship between the phylum Fusobacteria and obesity,

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Table 2. Difference in bacterial composition between obese and lean peoples (Genus level)

Genus	Obese (%)	Lean (%)	*p* value
Increased in obese peoples			
Alistipes	**2.20**	**0.50**	**0.04**
Anaerococcus	**0.01**	**0.00**	**0.01**
Barnesiella	10.24	7.81	0.10
Butyricimonas	0.01	0.00	0.10
Campylobacter	0.05	0.00	0.08
Coprococcus	**0.03**	**0.28**	**0.03**
Delftia	1.34	0.74	0.10
Eubacterium	1.86	0.00	0.10
Fusobacterium	**0.01**	**0.00**	**0.03**
Holdemania	0.03	0.00	0.10
Parvimonas	**0.01**	**0.00**	**0.01**
Raoultella	0.01	0.00	0.09
Shigella	0.00	0.00	0.06
Solobacterium	0.54	1.26	0.06
Turicibacter	2.54	0.50	0.09
Increased in lean peoples			
Allisonella	0.00	0.02	0.08
Bacteroides	**0.08**	**0.20**	**0.05**
Bifidobacterium	0.00	0.01	0.11
Collinsella	0.00	0.00	0.09
Coprobacillus	0.00	0.00	0.08
Corynebacterium	0.03	0.34	0.06
Desulfovibrio	**0.02**	**0.10**	**0.05**
Enterococcus	3.93	5.94	0.10
Faecalibacterium	**0.00**	**0.02**	**0.04**
Finegoldia	**0.00**	**0.10**	**0.03**
Howardella	0.00	0.00	0.10
Lachnoanaerobaculum	**0.00**	**0.02**	**0.05**
Olsenella	**0.34**	**1.15**	**0.03**
Subdoligranulum	**0.00**	**0.00**	**0.04**
Sutterella	16.90	22.50	0.10
Veillonella	0.03	0.00	0.09

Table 3. Increased bacteria species in obese peoples

Species	Obese (%)	Lean (%)	*p* value
Acidaminococcus intestini	**1.851**	**0.102**	**0.013**
Actinomyces meyeri	**0.018**	**0.009**	**0.039**
Atopobium parvulum	**0.006**	**0.001**	**0.022**
Bacteroides coprophilus	0.137	0.002	0.099
Bacteroides finegoldii	0.282	0.044	0.076
Bacteroides ovatus	1.886	0.546	0.097
Bacteroides vulgatus	**10.586**	**2.456**	**0.023**
Blautia wexlerae	5.589	3.798	0.086
Clostridium butyricum	0.005	0.000	0.172
Clostridium difficile	0.007	0.000	0.130
Clostridium nexile	0.171	0.006	0.081
Coprococcus comes	0.635	0.225	0.065
Eubacterium hadrum	**2.157**	**0.438**	**0.050**
Eubacterium infirmum	0.001	0.000	0.086
Fusobacterium mortiferum	1.376	0.002	0.107
Fusobacterium nucleatum	0.008	0.000	0.093
Fusobacterium varium	0.047	0.001	0.157
Gemella haemolysans	**0.008**	**0.001**	**0.020**
Granulicatella adiacens	**0.012**	**0.005**	**0.023**
Klebsiella pneumoniae subsp.	**0.076**	**0.003**	**0.042**
Lactococcus garvieae	0.000	0.004	0.088
Peptostreptococcus stomatis	**0.007**	**0.000**	**0.007**
Pseudomonas koreensis	0.001	0.000	0.084
Roseburia faecis	**0.881**	**0.324**	**0.026**
Rothia mucilaginosa	**0.007**	**0.001**	**0.049**
Ruminococcus gnavus	1.747	0.544	0.083

Table 4. Increased bacteria species in lean peoples

Species	Obese (%)	Lean (%)	*p* value
Clostridium ramosum	**0.002**	**0.008**	**0.042**
Ruminococcus torques	0.230	0.685	0.058
Acetivibrio ethanolgignens	0.000	0.014	0.061
Anaerotruncus colihominis	0.000	0.022	0.089
Bacteroides dorei	1.987	5.673	0.086
Bacteroides stercorirosoris	0.000	0.018	0.089
Bilophila wadsworthia	0.078	0.202	0.051
Christensenella minuta	0.000	0.003	0.070
Clostridium citroniae	**0.002**	**0.008**	**0.019**
Clostridium xylanolyticum	0.046	0.208	0.054
Desulfovibrio piger	0.026	0.276	0.037
Dialister succinatiphilus	0.000	0.276	0.084
Eggerthella lenta	0.016	0.102	0.057
Eubacterium callanderi	0.000	0.066	0.094
Eubacterium coprostanoligenes	0.004	0.157	0.101
Eubacterium desmolans	**0.002**	**0.103**	**0.029**
Eubacterium fissicatena	**0.004**	**0.016**	**0.038**
Eubacterium ruminantium	0.002	0.189	0.061
Faecalibacterium prausnitzii	**3.932**	**5.943**	**0.050**
Flavonifractor plautii	0.069	0.152	0.067
Gordonibacter pamelaeae	0.001	0.011	0.041
Holdemania filiformis	**0.000**	**0.003**	**0.034**
Lactococcus lactis	0.004	0.020	0.088
Lactococcus garvieae	0.000	0.004	0.088
Parabacteroides distasonis	0.001	0.007	0.054
Roseburia hominis	0.006	0.099	0.061
Streptococcus intermedius	0.001	0.004	0.086
Subdoligranulum variabile	0.544	1.261	0.056

but suggest that the obesity-associated gut microbial community of the Japanese population may have a different composition compared to that of Western people. The exact role of the phylum Fusobacteria in obesity should be investigated in the future.

One of the mechanisms by which the intestinal microbiota affects obesity is the induction of systemic low-grade inflammation.[30] The gut microbiota increases mucosal permeability in obese mice, thereby promoting translocation of bacterial products (e.g., lipopolysaccharide) and stimulating the low-grade inflammation that is characteristic for obesity.[30] So, bacterial species abundantly present in the lean microbiota with anti-inflammatory properties may have a protective effect on obesity. One of such bacteria species, *Faecalibacterium (F.) prausnitzii* has strong anti-inflammatory activities via butyrate production and the induction of regulatory T cells,[31–33] and has been reported to negatively correlate with inflammatory markers in obese subjects.[34,35] In this study, we actually observed a significant increase in the abundance of *F. prausnitzii* in lean peoples as compared to obese peoples. These results suggest that *F. prausnitzii* may play a protective role against obesity via its anti-inflammatory actions. In contrast, we observed that the abundance of the phylum Fusobacteria and *Bacteroides (B.) vulgatus* increased significantly in obese peoples as compare to lean peoples. The phylum Fusobacteria and *B. vulgatus* might play a causative role for obesity, since previous studies have reported the proinflammatory properties of these bacteria[27,36] and a positive correlation between the abundance of *B. vulgatus* and BMI.[37] Thus, it is likely that the

balance of inflammatory and anti-inflammatory bacteria may be one of factors affecting the development of obesity.

In conclusion, we found that obesity-associated gut microbiota of Japanese people is somewhat different from that of Western people. In Japanese people, a decrease of the phylum Bacteroidetes and a decrease in the Firmicutes/Bacterodetes ratio were not detected. We found an increase of the phylum Fusobacteria in obese people for the first time. The differences between the Japanese population and Western populations could be caused by a variety of factors (e.g., dietary habits, hygiene and genetics).

Acknowledgments

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Conflict of Interests

No potential conflicts of interest were disclosed.

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