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Full Paper

A cross-sectional analysis from the Mykinso Cohort Study: establishing reference ranges for Japanese gut microbial indices

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The purpose of this study was to establish reference ranges for gut microbial indices by collecting real-world Japanese microbiome data from a Mykinso cohort. Although several large cohort studies have focused on the human gut microbiome, large cohort studies of the gut microbiome from Japanese populations are scarce, especially from healthy or non-diseased individuals. We collected stool samples and original survey lifestyle information from 5,843 Japanese individuals through the Mykinso gut microbiome testing service. From the obtained 16S rRNA sequence data derived from stool samples, the ratio and distribution of each taxon were analyzed. The relationship between different epidemiological attributes and gut microbial indicators were statistically analyzed. The qualitative and quantitative indicators of these common gut microbiota were confirmed to be strongly correlated with age, sex, constipation/diarrhea, and history of lifestyle-related diseases. Therefore, we set up a healthy sub-cohort that controlled for these attribute factors and defined reference ranges from the distribution of gut microbial index in that population. Taken together, these results show that the gut microbiota of Japanese people had high beta-diversity, with no single "typical" gut microbiota type. We believe that the reference ranges for the gut microbial indices obtained in this study can be new reference values for determining the balance and health of the gut microbiota of an individual. In the future, it is necessary to clarify the clinical validity of these reference values by comparing them with a clinical disease cohort.

Key words: gut microbiota, 16S rRNA, Japanese, reference range, microbial index

INTRODUCTION

Since the establishment of next-generation 16S rRNA sequencing analysis, multiple large cohort studies focusing on the human gut microbiome have been conducted, such as the US Human Microbiome Project [1] and MetaHIT in Europe [2]. An integrated catalog of human fecal microbial metagenomes from 1,200 people in the United States, China, and Europe has identified 9.9 million microbial genes across fecal microbiota [3]. However, studies on gut microbiome from Japanese populations are scarce, especially those reporting on healthy or non-diseased individuals. Further, in recent studies comparing gut microbiomes by race/nationality, clear impacts of dietary habits were demonstrated [4], suggesting that, owing to the unique Japanese food culture, the gut of Japanese individuals could harbor different flora from those of individuals in western countries. Therefore, to advance research on the gut microbiome and various diseases in Japan, it is critical to characterize the healthy gut microbiome in the Japanese population.

Host parameters such as age, gender, and body mass index (BMI) have been reported to be related to individual differences in gut microbiota composition [5–9]. Further, differences in dietary habits have been shown to affect the bacterial diversity and enterotype of human gut microbiota [10, 11], which may partially explain why differences in residential areas/countries are strongly associated with differences in gut microbiota composition [12, 13].

Recently, several studies have revealed gender differences in gut microbiota [14–17]. For instance, Min *et al.* [18] conducted an association study to identify bacterial compositions associated with men and women, and showed similar microbiota characteristics, including overall abundance and diversity,

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between men and women. However, they also showed gender differences at the species level between microbial taxa related to fat distribution, suggesting the existence of a gender-specific microbiome signature corresponding to gender-specific fat distribution, which may also contribute to the observed sex-specific immunity differences [19]. Thus, several immune pathophysiologies may be involved in gender differences in gut bacterial composition.

Age is also an important factor affecting the gut microbiota [8, 20–23]. Recent reports have described differences in gut microbiota between children and adults, and an adult-like composition of bacterial communities is established at around 3–4 years of age or older [7, 20, 24–26]. In addition, the intestinal microbiota has been shown to change with age, although the definition of old age has differed between reports and include individuals older than 60, 65, 70, or 100 years [14, 27–29]. The associated mechanism also remains unknown. Yatsunenko *et al.* [8] conducted a large study of subjects aged 0–83 years and showed continuous changes that occurred with age. They found that the period required to form an adult-like gut microbiota was the 3-year period following birth. Second, interpersonal variation was significantly greater between children than between adults. Third, the dominance of *Bifidobacterium* in the baby microbiota continued throughout the first year of life, although this dominance diminished with age. Nevertheless, owing to the limited number of subjects over 60 years of age, the specific continuous changes that occur in older people remain unknown. Recently, Odamaki *et al.* [7] reported age-related compositional differences from infants to centenarians in a Japanese cross-sectional study. They found that *Bifidobacterium* decreased and Enterobacteriaceae increased with age, as observed in some previous studies [8, 21–23].

Relationships have also been observed between gut microbiota and diarrhea/constipation. Vandeputte *et al.* [30] described an association between stool consistency and gut microbiota composition in 53 healthy female subjects. Tigchelaar *et al.* [31] also reported an association between stool consistency and the structure of gut microbiota. Hadizadeh *et al.* [32] demonstrated a correlation between the number of bowel movements and gut microbiota. In a Japanese cohort, Takagi *et al.* [33] reported significant differences in microbial structure between individuals with differences in stool consistency (Bristol stool scale type). Therefore, investigating the relationship between bowel habits and intestinal bacterial composition can provide important information on gastrointestinal motility function.

However, Japan has its own food culture and customs compared to western countries, and the intestinal flora of Japanese individuals contain more genes for polysaccharide-degrading enzymes derived from water-soluble dietary fiber than Americans [34]. This feature may be related to the longer life expectancy of Japanese people and their low BMIs [35, 36]. Nishijima *et al.* [37] clearly showed significant differences in the gut microbiota of the Japanese population compared to other countries, which cannot be explained by meals alone. Therefore, the structure of the intestinal flora may be highly dependent on an individual's country/region and lifestyle [38].

In this study, we investigated the relative abundance ranges of microbial taxa in stool samples from a large healthy human cohort. Further, we analyzed the relationship between the aforementioned genera or gut microbiota composition and Japanese demographic features, lifestyle, and bowel habits. Finally, we developed reference ranges using a large healthy Japanese cohort and considered the effects of age, gender, diarrhea, and constipation.

MATERIALS AND METHODS

Study design and participants

From November 2015 through June 2019, a Mykinso cohort of 5,843 individuals who had submitted fecal samples (one sample per subject) was selected from data obtained through the Mykinso gut microbiome testing service. Informed consent was obtained from all participants in the study. All procedures complied with the principles of the Declaration of Helsinki and were approved by the Institutional Review Board (IRB) at our institution, and the study was registered as UMIN000028887 and UMIN000028888 in the UMIN Clinical Trials Registry System. The IRB-approved protocol specifically allows for a study involving a cross-sectional (one time per subject) analysis of the survey data and subsequent follow-up survey (multiple times per subject). In this study, we analyzed only cross-sectional data from the cohort study dataset.

Demographic features, bowel habits, and disease and medication data

Using an original survey (Supplementary Table 1), metadata were collected through the Mykinso gut microbiome testing service. The original survey included questions on demographic features, lifestyle, bowel habits, and disease. Individuals were scored positive for a disease if they replied yes to any original survey question, negative if they replied no, and unknown if data were unavailable across all original surveys.

Fecal sampling, DNA extraction, and sequencing

Fecal samples were collected using brush-type collection kits containing guanidine thiocyanate solution (Techno Suruga Laboratory, Shizuoka, Japan), transported at normal temperature, and stored at 4°C. DNA extraction from the fecal samples was performed using an automated DNA extraction system (GENE PREP STAR PI-480, Kurabo Industries Ltd, Osaka, Japan) according to the manufacturer's protocol. The V1–V2 region of the 16S rRNA gene was amplified using a forward primer (16S_27Fmod: TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG AGR GTT TGA TYM TGG CTC AG) and a reverse primer (16S_338R: GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GTG CTG CCT CCC GTA GGA GT) and KAPA HiFi HotStart ReadyMix (Roche). To sequence 16S amplicons by Illumina MiSeq platform, dual index adapters were attached using the Nextera XT Index kit. Each library was diluted to 5 ng/μL, and equal volumes of the libraries were mixed to 4 nM. The DNA concentration of the mixed libraries was quantified by qPCR with KAPA SYBR FAST qPCR Master Mix (KK4601, KAPA Biosystems) using primer 1 (AAT GAT ACG GCG ACC ACC) and primer 2 (CAA GCA GAA GAC GGC ATA CGA). The library preparations were carried out according to 16S library preparation protocol of Illumina (Illumina, San Diego, CA, USA). Libraries were sequenced using the MiSeq Reagent Kit v2 (500 Cycles), to produce 250 bp paired-end reads.

Taxonomy assignment based on 16S rRNA gene sequences

Paired-end reads of partial 16S rRNA gene sequences were clustered by 97% nucleotide identity and then assigned taxonomic information using the Greengenes database (v13.8) [39] through

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the QIIME pipeline (v1.8.0) [40]. The steps for data processing and assignment based on the QIIME pipeline were as follows: (i) joining paired-end reads; (ii) quality filtering with an accuracy of Q30 (>99.9%) and a read length > 300 bp; (iii) randomly extracting 10,000 reads per sample for subsequent analysis; (iv) clustering operational taxonomic units (OTUs) with 97% identity by UCLUST (v1.2.22q) [41]; and (v) assigning taxonomic information to each OTU using the RDP classifier [42] with the full-length 16S gene data of Greengenes (v13.8) to determine the identity and composition of bacterial genera.

Transformation of compositional microbiome data for hypothesis testing

Centered log-ratio (clr) transformed values were used as inputs for multivariate hypothesis testing [43] to manage 0 count values as both point estimates using the zCompositions R package [44] and as a probability distribution using the ALDEx2 package [45] available on Bioconductor.

Group differences in beta-diversity

Aitchison distance, the Euclidian distance between samples after clr transformation, and the distances between samples are the same as the phylogenetic ilr [43]. Replacement for β-diversity exploration of microbiome data is a variance-based compositional principal component (PCA) biplot [46], in which the relationship between inter-OTU variance and sample distance can be observed [47]. Compositional PCA biplots display the relationships between OTUs and distances between samples on a common plot to glean substantial and qualitative information regarding dataset quality and the relationships between groups [47].

Group differences in alpha-diversity

Microbiota diversity was assessed by the Shannon index based on 97% nucleotide sequence identity. These values were calculated using QIIME [40] with a depth of 10,000 reads. To test two-group differences between male and female groups, we calculated p values using the two-sided unpaired Welch's t-test. To test group differences among age groups in the diversity index, we calculated p values using one-way analysis of variance (ANOVA).

Group differences in taxonomic abundance

To compare the taxonomic abundance between the groups, we conducted the univariate statistical test using the ALDEx2 tool. The false discovery rate (FDR) control was performed based on the Benjamini-Hochberg procedure to correct for multiple testing, i.e., 'p.adjust' in R. Analysis was confined to taxa with a prevalence greater than 10% and a maximum proportion (relative abundance) greater than 0.005. An FDR-adjusted p-value less than 5% was considered to be significant.

RESULTS

Cohort characteristics

The participants primarily resided in Japan (n=5,843; Supplementary Table 2) and were characterized by a greater range in age, stool type, and lifestyle than the participants in other Japanese large-scale microbiome projects [7, 33, 37]. In the original survey, participants (n=4,479) reported demographic features, disease history, and lifestyle data (participants missing any of these data were excluded; Supplementary Table 3). In accordance with our IRB, all survey questions were optional (question response rate, 76.65%). Eligible subjects were male and female who were considered to not have disease history (ineligible subjects were those who self-reported any disease history; Supplementary Table 4). Eligible criteria included no self-reported history of any disease. Ultimately, 2,865 individuals were included in the subsequent analysis (Table 1, Supplementary Fig. 1).

Table 1. Distribution of primary eligible subjects

Demographic features	Female	Male
Number of samples	1,722	1,143
BMI	21.1 (3.0)	23.1 (3.5)
Age	40.16 (11.30)	41.77 (11.30)
Age group		
19 and under	20 (1.2%)	10 (0.9%)
20–29	322 (18.7%)	135 (11.8%)
30–39	559 (32.5%)	408 (35.7%)
40–49	485 (28.2%)	358 (31.3%)
50–59	249 (14.5%)	159 (13.9%)
60–69	73 (4.2%)	52 (4.5%)
70 and over	14 (0.8%)	21 (1.8%)

Mean (SD); n/N (%).
BMI: Body Mass Index.

Sex-related gut microbiota

Taxonomic differences in microbial communities were evaluated at the genus level. The comparison of microbial composition between male and female subjects showed a significant richness in the abundances of 12 and 13 genera in male and female subjects, respectively (blue and red points, respectively, in Fig. 1). The results were characterized by a richness in the representative genera *Prevotella*, *Megamonas*, *Collinsella*, *Dorea*, *Megasphaera*, and *Fusobacterium* ($p<0.001$, FDR-adjusted p-value<0.005) in male subjects and an increase in representative genera *Oscillospira*, *Coprobacillus*, *Ruminococcus*, *Bacteroides*, *Eggerthella*, *Anaerotruncus*, *Trabulsiella*, and *Akkermansia* ($p<0.001$, FDR-adjusted p-value <0.005) in female subjects. Subsequently, we evaluated the alpha-diversity of gut microbiota using the Shannon index. The Shannon index showed no statistically significant differences between male (mean=6.013) and female (mean=6.008) subjects (Welch's two-sample t-test; t (2,352.5) =−0.198, p=0.843, 95% CI =−0.049 to 0.060). Next, the dissimilarity of the overall structure of the gut microbiome for male and female subjects, beta-diversity was calculated using Aitchison distance (Fig. 2). PCA revealed that there were structural differences between male and female subjects (PERMANOVA, R^2=0.060, p=0.001).

Age-related gut microbiota

Further, differences in the gut microbial structure in each age group were taxonomically evaluated at the phylum level (Fig. 3). In agreement with previous results, the microbiota composition included four predominant phyla (Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria). Of these, Actinobacteria showed a trend to decrease in the 60 years old

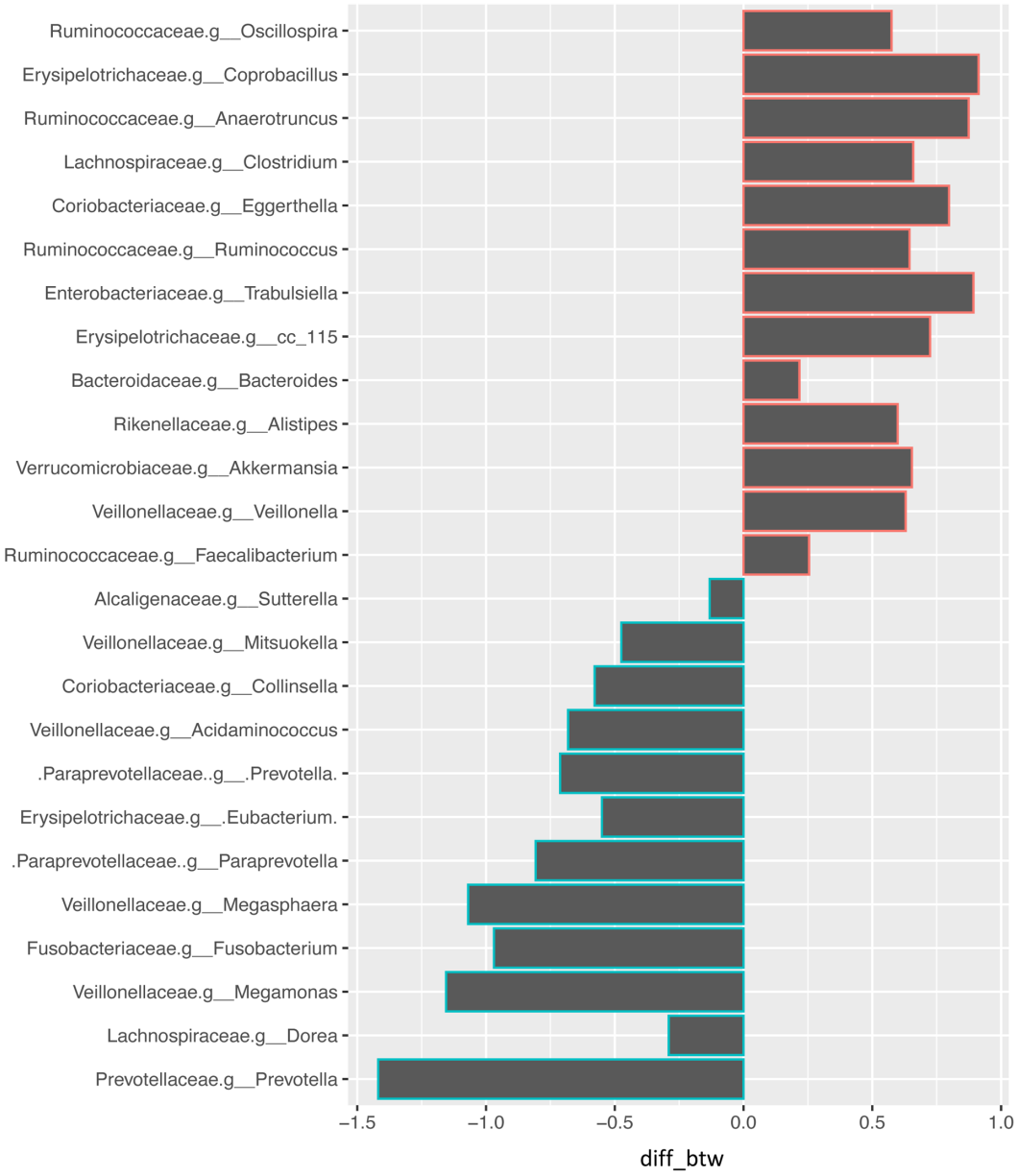

Fig. 1. Relative abundances of gut microbiota in male and female subjects. Genera were significantly different between male and female subjects. diff_btw = median difference in clr values between female and male groups. magenta: positive diff_btw in female group; cyan: negative diff_btw in female group.

group (p=0.040, FDR-adjusted p-value=0.640) and 70 years or older group (p=0.048, FDR-adjusted p-value=0.700), compared to the 19 years or under group. The alpha-diversity index showed significant differences across age groups in our cohort (ANOVA; $F(6,2851) = 5.045$, p<0.001; Fig. 4). We also tested the multiplicity correction of each pair difference with the Benjamini & Hochberg method, and statistically significant differences were found between 20s and 60s age groups (p<0.001), between 30s and 60s age groups (p=0.008), between 20s and 40s age group (p=0.006), and between 50s and 60s age groups (p=0.043; Table 2). Additionally, we created three age groups (young group, 0–19; adult group, 20–59; and elderly group, 60 years or older), and the overall structure of the gut microbiome using beta-diversity indices was calculated using Aitchison distance (Fig. 5). PCA revealed that there were microbial structural differences among the three age groups (PERMANOVA, R^2=0.034, p<0.001).

Bowel habits-related gut microbiota

Considering the heterogeneity and varying generations of samples in this dataset, we excluded samples from the young (0–19) and elderly (60 years or older) groups, which might have caused bias in the subsequent analysis [7, 14, 27–29]. As a result, 2,675 samples were included in the resulting dataset (Table 3, Supplementary Fig. 1). The bowel habits (stool shape and defecation frequency) of all participants enrolled in this study were recorded and classified using the self-reported original survey. Additionally, perceived symptoms of diarrhea/constipation were recorded and classified. According to the

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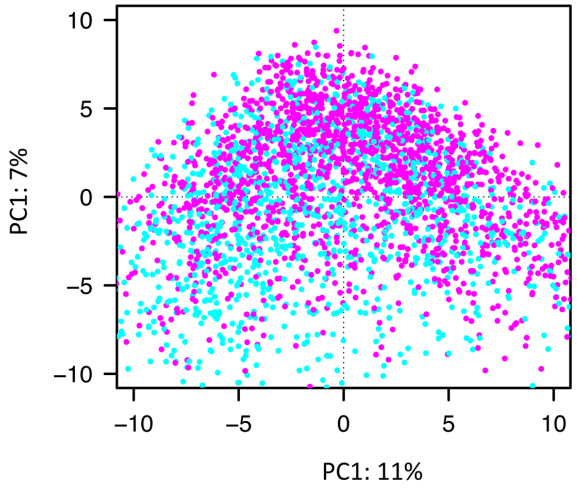

Fig. 2. Plot of individual samples from PCA output (magenta: female samples, cyan: male samples). The distance between points is proportional to the Euclidian distance of CLR vectors of the samples (Aitchison distance). The multivariate distance between samples was estimated using the Aitchison distance, which showed significantly different composition between female and male samples (ANOSIM, R^2=0.060, p=0.001).

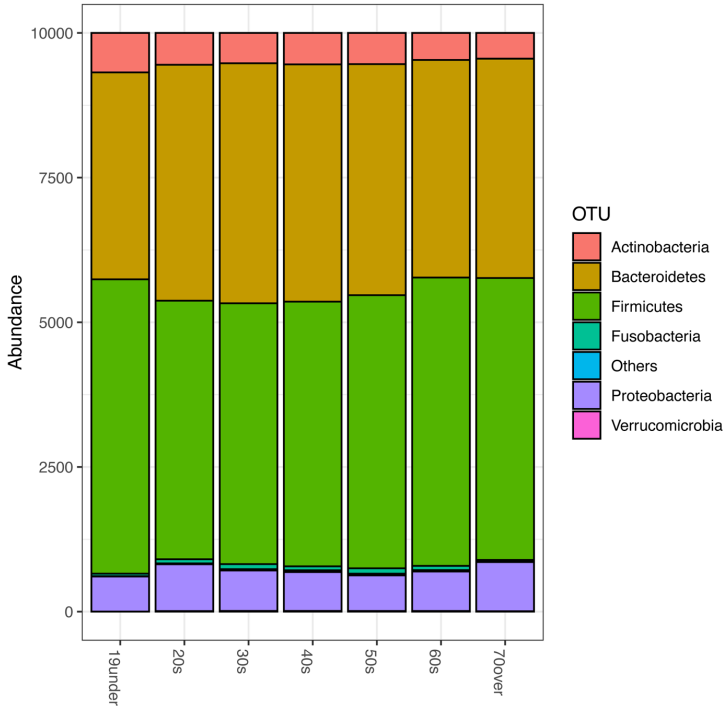

Fig. 3. Comparative analyses of the taxonomic composition of the microbial community at the phylum level for each age group. Each component of the cumulative bar chart indicates a phylum.

stool shape, bowel frequency, and perceived symptom scores, participants were classified as normal bowel habit type, diarrhea type, constipation type, or mixed type. Participants reporting stool type 1 (hard stool), defecation frequency type 4 (less than once per week), or frequent perception of constipation symptoms within 1 month were classified into the constipation type. Participants reporting stool type 7 (liquid stools), defecation frequency type 1 (more than three times per day), or frequent perception of diarrhea symptoms within 1 month were classified as the diarrhea type. Finally participants who fit into both the constipation and the diarrhea types were classified into the mixed type. The constipation group (female, n=337, 20.87%; male, n=37, 3.49%), diarrhea group (female, n=357, 22.11%; male, n=457, 43.11%), mixed group (female, n=139, 8.61%; male, n=29, 2.74%), and normal group (female, n=782, 48.42%; male, n=537, 50.66%) were observed (Table 3). Importantly, the Shannon index for each bowel habit group showed a significant difference among groups in our cohort (ANOVA; F(3,2667)=1.761, p<0.001; Fig. 6). We also tested each pair difference with the Benjamini & Hochberg method, which showed statistically significant differences between the normal and diarrhea groups (p<0.001), constipation and diarrhea groups (p<0.001), and mixed and diarrhea groups (p=0.001). These differences are illustrated in Fig. 6. Additionally, the beta-diversity indices among the four bowel habit groups using was calculated using Aitchison distance and visualized by PCA according to Aitchison distance (Fig. 7). An additional PERMANOVA analysis showed that the bowel habit type was a significant factor contributing to the variation of the structure of the gut microbiota (p<0.001). Approximately 0.7% of the variance in beta-diversity was explained by the bowel habit type (PERMANOVA; F(3,2667)=6.714, R^2=0.007, p<0.001), which was competitive with available measurements for clinical and environmental covariates. Subsequently, at the genus level, we identified several altered bacteria among the four bowel habit groups. Interestingly, a significantly higher relative abundances of *Fusobacterium* (p<0.001, FDR-adjusted p-value<0.005) and *Oscillospira* (p<0.001, FDR-adjusted p-value<0.005) were observed in the diarrhea and constipation groups, respectively. In addition, the relative abundances of *Ruminococcus*, *Anaerotruncus*, *Alistipes*, and *Akkermansia* (p<0.01, FDR-adjusted p-value<0.05) were significantly higher in the constipation group, whereas that of *Dorea* (p<0.01, FDR-adjusted p-value<0.05) was higher in the diarrhea group.

Reference ranges from the healthy Japanese cohort

Considering the heterogeneity and bowel habits of the sample dataset, we excluded samples from individuals with diarrhea or constipation (Supplementary Fig. 1), which might cause bias in reference ranges [30–33]. Ultimately, 1,319 samples were selected as a healthy reference dataset (Supplementary Fig. 1). We identified 453 genera and 20 phyla of Bacteria and Archaea in the gut microbiomes of the healthy reference dataset. The genera with an average relative abundance of ≥ 0.5% in the Japanese healthy reference dataset are listed in Supplementary Fig. 2. At the genus level, the Japanese healthy reference was characterized by the highest abundances of *Bacteroides*, *Faecalibacterium*, *Prevotella*, *Blautia*, *Bifidobacterium*, *Coprococcus*, and *Parabacteroides* (Supplementary Fig. 2).

In this study, health-related microbiome indices were selected based on peer-reviewed studies in academic journals and in-house data analyses (Table 4). To determine the reference ranges of 11 target microbiome indices, the dataset of 1,319 individuals

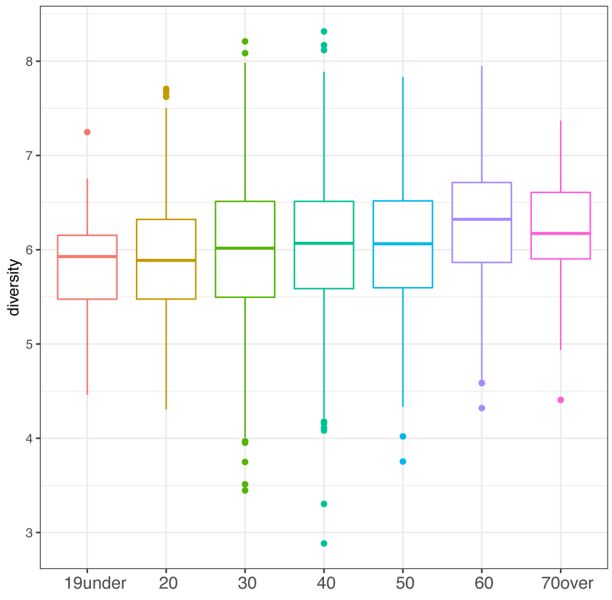

Fig. 4. Age-related differences in the Shannon index of gut microbiota.

Table 2. Pairwise comparisons of the Shannon index between age groups

Age-group pair	diff	lwr	upr	p.adj
20s–19under	0.001	−0.398	0.400	1.000
30s–19under	0.109	−0.284	0.502	0.983
30s–20s	0.108	−0.012	0.228	0.112
40s–19under	0.151	−0.242	0.545	0.917
40s–20s	0.151	0.027	0.274	0.006
40s–30s	0.043	−0.057	0.142	0.870
50s–19under	0.128	−0.272	0.529	0.965
50s–20s	0.127	−0.017	0.272	0.125
50s–30s	0.019	−0.106	0.145	0.999
50s–40s	−0.023	−0.151	0.105	0.998
60s–19under	0.349	−0.082	0.779	0.203
60s–20s	0.348	0.134	0.562	<0.001
60s–30s	0.240	0.039	0.441	0.008
60s–40s	0.197	−0.006	0.400	0.063
60s–50s	0.220	0.004	0.437	0.043
70over–19under	0.303	−0.224	0.830	0.618
70over–20s	0.302	−0.069	0.674	0.199
70over–30s	0.194	−0.170	0.559	0.701
70over–40s	0.152	−0.214	0.517	0.885
70over–50s	0.175	−0.198	0.548	0.812
70over–60s	−0.046	−0.451	0.359	1.000

diff: Differences in mean levels; lwr: 95% confidence lower level; upr: 95% confidence upper level
p.adj: p-value adjusted by Benjamin & Hochberg medhods.

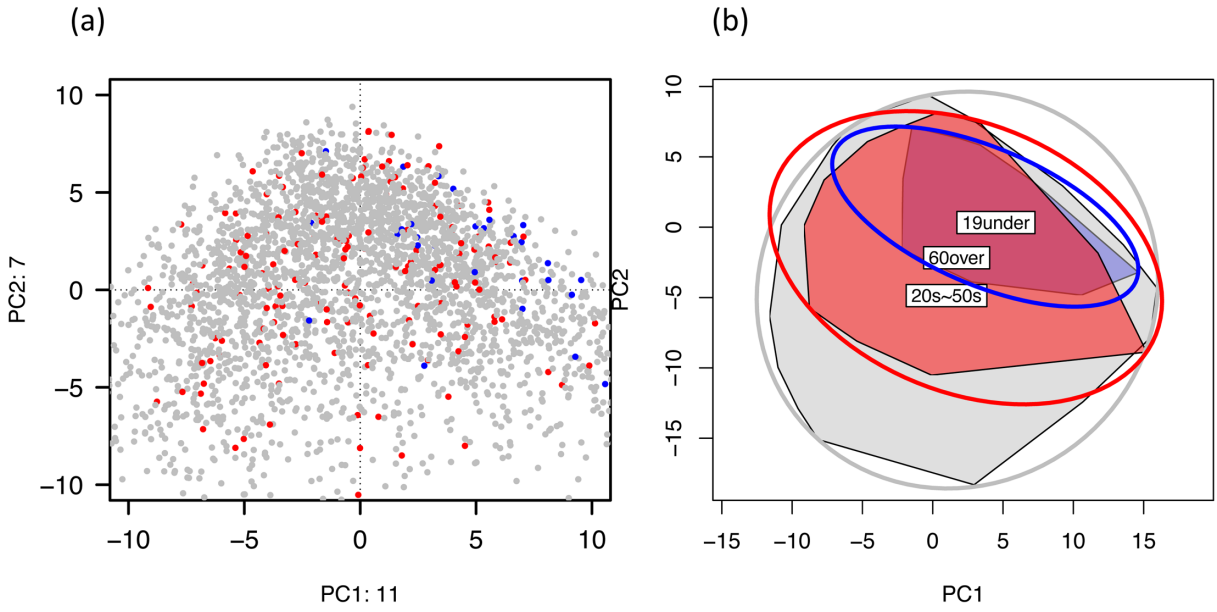

Fig. 5. (a) Plot of individual samples from PCA output (red: elderly samples, blue: young samples, and gray: adult samples). The distance between points is proportional to the Euclidian distance of CLR vectors of the samples (Aitchison distance). (b) The multivariate distance between samples was estimated using the Aitchison distance, which showed significantly different compositions in the junior, adult and senior samples (red: elderly r, blue: young, and gray: adult) (PERMANOVA, R^2=0.034, p<0.001).

selected from the Mykinso cohort as described above was established. Microbiome data from this dataset were analyzed to determine the empirical reference ranges for two indices of overall community structure, two complex genus indices, one class, and six genera. For each of the 1,319 samples, we determined the relative abundance of each target within the microbial population, revealing the distribution of the relative abundance of each target in the cohort (Table 4). These data were used to define a central 80% healthy range with confidence intervals for each target. Many of the targets show significant spread, highlighting the importance of defining reference ranges for health-related indices.

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Table 3. Distribution of bowel habits by sex and age group (20–50)

Age-group: gender	Normal stool	Diarrhea	Constipation	Mixed	Number of samples
20–29: male	59	69	2	5	135
20–29: female	149	76	57	40	322
30–39: male	196	185	14	13	408
30–39: female	243	134	128	54	559
40–49: male	185	150	15	8	358
40–49: female	233	112	111	29	485
50–59: male	97	53	6	3	159
50–59: female	157	35	41	16	249
Sum	1,319	814	374	168	2,675

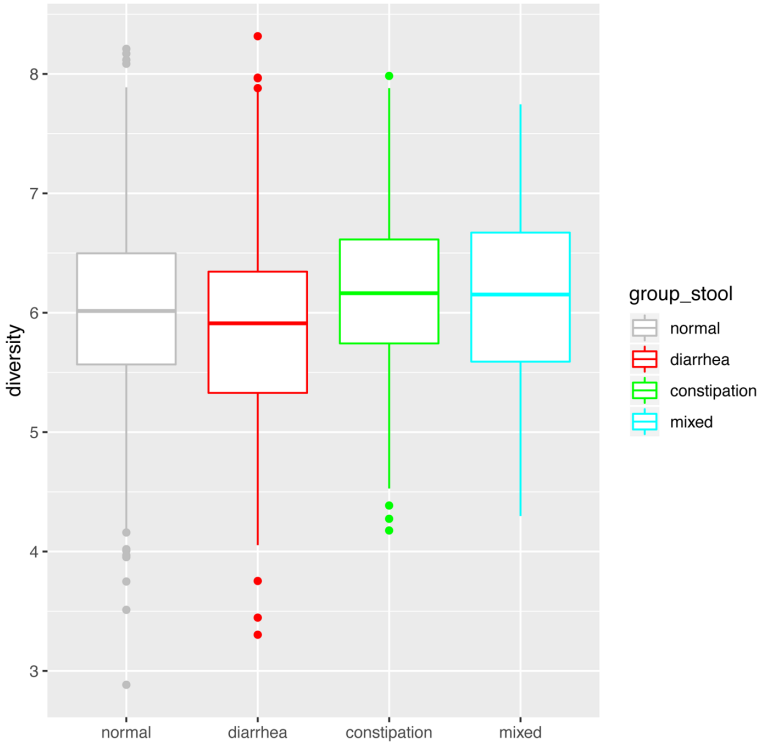

Fig. 6. Alteration of the Shannon index of gut microbiota associated with stool consistency. Comparison of α-diversity indices: Shannon index (OTU evenness estimation). Bowel habit was categorized into four groups: normal, diarrhea, constipation, and mixed.

DISCUSSION

We developed reference ranges using a large healthy Japanese cohort. The reference ranges consider the effect of age, gender, diarrhea, and constipation to aid physicians with accurate diagnosis of the intestinal bacterial composition ratio using a standard value derived from a healthy population. Eighteen intestinal bacterial indicators suggested to be associated with health status were selected. Using intestinal bacterial composition test panels, the detection of intestinal bacterial indicators outside of their healthy ranges can be useful evidence to support a medical plan.

Gut microbiota and sex/gender

Some characteristics of gender-specific immune differences are induced by gut microbiota. Fransen *et al.* [48] investigated significant gender differences in bacterial groups at the family or genus level. Females had higher abundances of *Desulfovibrionaceae*, *Lactobacillaceae* (*Lactobacillus* at the genus level), and *Verrucomicrobiaceae* (*Akkermansia* at the genus level), whereas males had higher abundances of *Ruminococcaceae* and *Rikenellaceae* (*Alistipes* at the genus level). In this study, several characteristic differences were observed between male and female subjects regarding the abundances of gut microbiota at the genus level. The genera *Prevotella*, *Megamonas*, *Fusobacterium*, and *Megasphaera* were significantly abundant in male subjects, whereas *Bifidobacterium*, *Ruminococcus*, and *Akkermansia* were significantly abundant in female subjects. These results are consistent with the results of previous Japanese studies [7, 33] and may be considered as the characteristic gender differences in the composition of intestinal microbiota in the Japanese population.

Gut microbiota and age/generation

Recent reports have shown a clear difference in the composition of the intestinal microbiota of infants, adults, and the elderly [7,

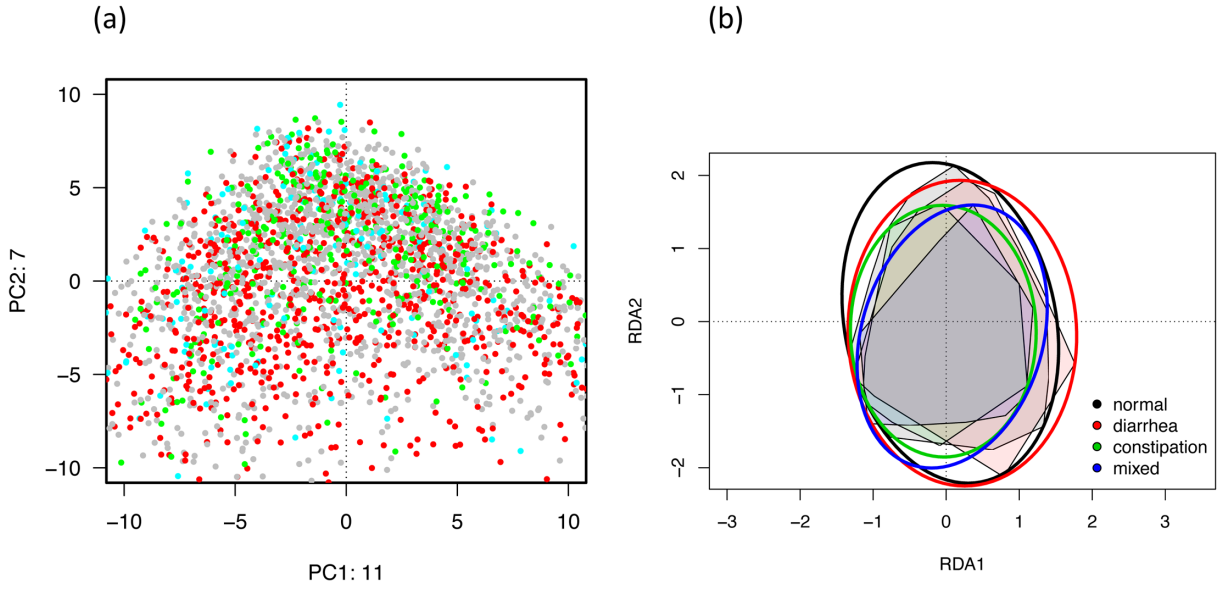

Fig. 7. (a) Plot of individual samples from PCA output (gray: normal samples, red: diarrhea samples, green: constipation samples, and blue: mixed type samples). The distance between points is proportional to the Euclidian distance of CLR vectors of the samples (Aitchison distance). The multivariate distance between samples was estimated using the Aitchison distance, which showed significantly different compositions between the bowel habit type (PERMANOVA, R^2=0.007, p<0.001). (b) RDA triplot of CLR vectors of the samples constrained by bowel habit group.

8, 33]. The microbiota composition initially shifts after birth, followed by significant shifts during childhood and in later years [20]. In this study, we segmented the population by age group (young, adult, and elderly group). Our results are in agreement with studies indicating clear differences in gut microbiota composition among infants, adults, and the elderly [7, 49, 50]. It was found that the Actinobacteria abundance and alpha-diversity index were gut microbiome indices related to aging. The most dramatic changes in gut microbiota diversity occur in early childhood [20], but recent large cross-sectional cohort studies have also reported increases in adulthood [7, 51]. In our cross-sectional cohort, the alpha-diversity index showed slight increasing trend from 20 to 69 years old (Fig. 4) that was consistent with recent previous reports [7, 51]. On the other hand, other recent studies have suggested that both *Bacteroides* abundance and species diversity decline in the feces of elderly subjects and that the abundance of *Bifidobacterium* is reduced [27]. The gut microbiota composition of elderly subjects is expected to be in a state of flux [20]. In our cross-sectional cohort, most elderly individuals were community dwellers not in long-term residential care; this state of healthy aging may maintain a high diversity of gut microbiota.

Gut microbiota and bowel habits (diarrhea/constipation)

Similarly to Vandeputte *et al.* [30], we found a significant association between bowel habits (stool shape and defecation frequency) and gut microbiota diversity. Furthermore, deviations of the gut microbiota composition in several genera, including *Oscillospira*, *Ruminococcus*, *Anaerotruncus*, *Alistipes*, and *Akkermansia*, in constipation subjects and *Fusobacterium* and *Dorea* in diarrhea subjects were confirmed, which is consistent with a previous report [33]. Although the role of these genera in stool consistency remains unclear, the results illustrate the effect of gut microbiota on stool consistency in healthy Japanese subjects.

Gut microbiota and racial/regional differences

Our results showed that Japanese adults (20–59 years old) had a greater abundance of the genera *Bacteroides* and *Faecalibacterium,* interquartile ranges (IQRs) of 27.43% (19.03–35.26) and 6.83% (3.39–10.06), respectively, and a relatively lower abundance of the genera *Clostridium* (IQR 0.20%, 0.04–0.44), compared with previous studies in other Japanese cohorts [37]. However, the estimated abundances of *Bifidobacterium* and *Blautia* were greater (IQRs of 2.47% (0.86–5.78), and 5.31% (2.94–7.85), respectively) than those of a previous study in other nations (<0.5% and >5%, in the US and China, respectively) [37]. These bacterial compositions may be characteristic of the intestinal microbiota of the Japanese population but may also reflect differences in DNA extraction methods [52, 53] and the amplified region of the 16S rRNA [54].

A high abundance of *Bifidobacterium* has also been observed in the gut microbiome of Japanese children by 16S rRNA gene analysis [12], indicating its high prevalence throughout the Japanese population. *Bifidobacterium* is thought to be a beneficial microbe that contains more glycoside hydrolases for degrading starch than other intestinal microbes [55, 56]. Therefore, the high abundance of *Bifidobacterium* may be a consequence of the intake of various saccharides in traditional and unique Japanese foods. However, it is unknown which foods or nutrients contribute to the high abundance of *Bifidobacterium*. As future prospects, it is essential to create a reference microbiota for the Japanese population by age group and to increase the number of subjects in the young and the elderly age groups. Additionally, investigations of geographical differences within Japan are of interest.

Clinical relevance and reference ranges

All 11 microbiome indices successfully identified using 16S rRNA gene sequencing were associated with specific health conditions. Alpha-diversity, including the Shannon index, and

Table 4. Reference ranges from healthy Japanese subjects for 11 clinically relevant indices

Microbiome Index	unit	group	lower limit [10%]	median [50%]	upper limit [90%]	Reference
Shannon	value	all	5.08	6.01	6.88	[58, 59]
		male	5.15	6.03	6.93	
		female	5.05	6	6.85	
Observed genera	genus	all	54	65	80	[58, 59]
		male	54	65	81	
		female	54	66	79	
Bifidobacterium	%	all	0.18	2.47	9.6	[60, 61]
		male	0.12	2.18	8.45	
		female	0.21	2.79	10.41	
Faecalibacterium	%	all	0.55	6.83	12.87	[62]
		male	0.37	6.36	12.09	
		female	0.57	7.23	13.27	
Butyric acid-producing genera group *1	%	all	4.25	12.16	20.47	[59]
		male	3.64	11.53	20.03	
		female	4.66	12.88	20.89	
Clostridium	%	all	0	0.19	0.79	[59]
		male	0	0.19	0.7	
		female	0	0.2	0.89	
Lactobacillales genera group verified lactic acid producers *2	%	all	0	0.01	0.22	[59, 63]
		male	0	0.01	0.24	
		female	0	0.01	0.21	
Streptococcus	%	all	0.05	0.38	2.58	[63, 65]
		male	0.03	0.34	2.46	
		female	0.05	0.41	2.65	
Genera group popular in oral cavity *3	%	all	0.18	1.4	9.74	[63, 65]
		male	0.15	1.31	9.71	
		female	0.2	1.48	9.9	
Fusobacterium	%	all	0	0	1.37	[59, 64]
		male	0	0	2.92	
		female	0	0	0.83	
Observed genera within class *Gammaproteobacteria*	class	all	2	5	8	[59, 65]
		male	2	5	8	
		female	2	5	8	

*1: This index included *Coprococcus*, *Roseburia*, *Butyricicoccus*, *Faecalibacterium*, *Anaerostipes*, and *Butyricimonas*.
*2: This index included *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Lactococcus*, and *Weissella*.
*3: This index included *Streptococcus*, *Fusobacterium*, and *Enterobacter* genera.

observed genera number appeared to be associated with better health [57]. A recent meta-analysis proposed reduced alpha-diversity as a reliable indicator of diarrhea-associated dysbiosis [58]. These microbiota diversity indices (Shannon index and observed OTUs) revealed significant differences in the healthy aging group, indicating that a healthy, diverse diet promotes greater diversity in the gut microbiota [57].

Previous studies have proposed that *Bifidobacterium* is inversely associated with inflammatory bowel disease (IBD) and diarrhea-associated dysbiosis [59] and the consumption of probiotics, inulin, and oligofructoses promotes an increase in *Bifidobacterium* abundance [60]. Additionally, *Faecalibacterium* has been proposed to be a dominant member of the human intestinal microbiota in healthy adults and especially to be a health sensor for active Crohn's disease patients [61]. A recent meta-analysis showed a reduction in butyrate-producing *Clostridiales*, including *Coprococcus*, *Roseburia*, *Butyricicoccus*, *Faecalibacterium*, *Anaerostipes*, and *Butyricimonas*, which are associated with a healthy gut [59].

Although not all genera within the order *Lactobacillales* are verified lactic acid producers, the dominant genera within this order (including *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Lactococcus*, and *Weissella*) are known to harbor genes for lactic acid production and are often enriched in the case of patients across multiple diseases [58]. *Lactobacillales* genera have been shown to adapt to the lower pH of the upper gastrointestinal tract [62]. Thus, the shared disease-associated taxa may be indicators of shorter stool transit times and disruptions in the redox state and/or pH of the lower intestine, rather than specific pathogens. Genera within *Lactobacillaceae* and *Streptococcaceae* families are dominant in the upper gastrointestinal tract and are present in the stool of many individuals at low frequency [58]. These taxa likely become enriched with faster stool transit time (i.e., a diarrhea signature) [58].

Previous studies have proposed Fusobacterium to be associated with various human diseases [63]. Dysbiosis associated with

colorectal cancer is generally characterized by increased prevalence of known pathogenic or pathogen-associated *Fusobacterium* and *Enterobacter* genera, which were shown to be higher in colorectal cancer patients in two or more studies [58]. Furthermore, other oral community genera, such as members of *Porphyromonas*, *Peptostreptococcus*, and *Parvimonas*, were found with *Fusobacterium* on colonic tumors [64].

Limitations

We concede that this study has several limitations. First, this research was a participatory observational study. This kind of study may be self-selecting and have a tendency for illness behaviors, which may create a biased cohort rather than a true representation of the Japanese population [65]. As shown in Supplementary Table 1, the study cohort contained mostly females in their 30s and 40s, followed by males in their 30s and 40s. In addition, as shown in Table 3, in the cohort of the adult (20–59) age group, 50% or more of individuals in the group answered that they had diarrhea or constipation, a higher prevalence than in the general Japanese population. On the other hand, we believe that it was possible to adjust for these biases by screening the analysis dataset as shown in Supplementary Fig. 1. We excluded individuals with a medical history, the elderly, the young, and those with diarrhea/constipation symptoms to extract the reference value population. We did not exclude obese individuals from our reference dataset because Japan has one of the lowest rates of prevalence of obesity (about 4–4.5% of the adult population) in the world [66], and the obese population (BMI >30) in our healthy reference dataset was even lower (3.8% for men, 3.5% for women). Therefore, we believe they did not make a strong impact on the reference range values. However, it might be better to reconsider adding the BMI criterion for a more rigorous definition of "healthy" in future studies. Second, the scope of the present study did not extend to analysis of the influence of medication such as antibiotics on the gut microbiota profile, thus representing a qualitative limitation.

CONCLUSION

Regardless of health status, there are many microorganisms that are clinically related to health and disease in the intestines of all people, and the exquisite balance of these microorganisms varies greatly from person to person, making the definition of "good flora" difficult. However, to understand and monitor the health and balance of an individual's gut microbiota, it is essential to first know the reference ranges available from a large, healthy population such as the one presented here. By further expanding the cohort of healthy subjects and accumulating a cohort of various health conditions and attributes, more valuable indicators can be identified, leading to the realization of personalized precision medicine using microbiome information in the future.

DATA AVAILABILITY

Requests for materials and/or data should be addressed to the corresponding author, SW.

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