The Oral Microbiome of Healthy Japanese People at the Age of 90

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Abstract: For a healthy oral cavity, maintaining a healthy microbiome is essential. However, data on healthy microbiomes are not sufficient. To determine the nature of the core microbiome, the oral-microbiome structure was analyzed using pyrosequencing data. Saliva samples were obtained from healthy 90-year-old participants who attended the 20-year follow-up Niigata cohort study. A total of 85 people participated in the health checkups. The study population consisted of 40 male and 45 female participants. Stimulated saliva samples were obtained by chewing paraffin wax for 5 min. The V3–V4 hypervariable regions of the 16S ribosomal RNA (rRNA) gene were amplified by PCR. Pyrosequencing was performed using MiSeq. Operational taxonomic units (OTUs) were assigned on the basis of a 97% identity search in the EzTaxon-e database. Using the threshold of 100% detection on the species level, 13 species were detected: *Streptococcus sinensis*, *Streptococcus pneumoniae*, *Streptococcus salivarius*, KV831974_s, *Streptococcus parasanguinis*, *Veillonella dispar*, *Granulicatella adiacens*, *Streptococcus*_uc, *Streptococcus peroris*, KE952139_s, *Veillonella parvula*, *Atopobium parvulum*, and AFQU_vs. These species represent potential candidates for the core make-up of the human microbiome.

Keywords: oral microbiome; pyrosequencing; core microbiome; elderly

1. Introduction

Microbiota inhabiting the human body have long been recognized as critical for a variety of human diseases and in maintaining human health [1–5]. The microbiome of humans has been extensively studied using accurate and efficient approaches involving high-throughput sequencing technologies and bioinformatics [6].

Among several microbiomes in the human body, the oral microbiome has been extensively studied. Specific bacterial taxa are responsible for oral infectious diseases, such as dental caries and periodontal diseases. Microbial diversity increases in parallel with the progression of periodontitis [7]. Several studies showed that there is a relationship between the human oral microbiome and certain systemic diseases, such as pancreatic cancer [8], Type 2 diabetes [9], pediatric Crohn's disease [10], heart disease [11], and low-weight preterm birth [12]. Certain oral bacterial species may contribute to

carcinogenesis [13]. The role of the oral microbiome involves the mediation of inflammation related to changes in systemic health and disease [14].

In addition to these intensive studies related to disease, health-associated species in the oral cavity have been identified. One of the primary goals of the Human Microbiome Project [15] launched by the National Institutes of Health was to characterize the core microbiome. The concept of the core human oral microbiome involves comprehensive, minimal bacteria that regularly inhabit the human body. The human core microbiome is hypothesized to be important for development, health, and functioning. Some diseases, including autoimmune disorders [16], diabetes, and obesity [17,18], are caused by perturbation of the core gut microbiome. Therefore, a beneficial oral microbiome and its associated ecosystem functions may ensure host health and wellbeing [19–21].

The aims of this study were to determine if a healthy core microbiome is meaningful in the context of disease prevention and to investigate whether the microbiome of healthy older people may be a suitable representation of a healthy microbiome. Data on the oral microbiome of healthy older people may be useful for a comparison with the microbiome of subjects with specific diseases in an effort to determine if their etiology is related to an imbalance of the microbiome or colonization by specific bacterial taxa. These data may be useful for the development of a healthy core microbiome from a young age. Several studies have investigated the oral microbiome of older people. These studies were focused on the etiology of diseases or disease conditions [22–31]. Few studies investigating healthy older people are available [32–34].

The aim of this study was to investigate the oral microbiome of a community of healthy people at the age of 90 in an attempt to identify their core oral microbiome.

2. Materials and Methods

2.1. Study Design, Setting, and Participants

The subjects that participated in this study were sourced from the Niigata study, a community-based cohort study investigating the relationship between oral health and systemic health in older subjects aged 70 in 2008. Sampling and data collection procedures were described in previous reports in detail [35,36]. A 20-year follow-up study was conducted in 2018 through mass examination. Dental and medical examinations were carried out. Briefly, all of our cohorts did not present with any comorbidities.

2.2. Sampling and Microbial DNA Extraction

Saliva samples were collected by chewing paraffin wax for 5 min. Collected samples were kept on ice. Upon transporting them to the laboratory, samples were frozen at −20 °C until DNA extraction. Saliva samples were centrifuged at 3000 rpm for 10 min. The Maxwell 16 LEV Blood DNA Kit (Promega KK, Tokyo, Japan) was used for DNA extraction. The NanoDrop ND-2000 (Thermo Fisher Scientific KK, Tokyo, Japan) was used for the measurement of DNA concentration. DNA degradation was visually checked by electrophoresis on a 1% agarose gel using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific KK, Tokyo, Japan). The inclusion criteria for DNA samples subjected to further analysis were as follows: concentration > 20 ng/µL, volume ≥ 20 µL, $A_{260/280}$ ≥ 1.8, and $A_{260/230}$ >1.5 [32,37].

2.3. Microbial-Community Analysis

A polymerase chain reaction (PCR) was carried out using primers specific to the V3–V4 region involving pyrosequencing tags of the 16S ribosomal RNA (rRNA) gene. The taxonomic classification of each read was assigned on the basis of a search of the EzBioCloud 16S database [38,39]. By applying the data from this database, a hierarchical taxonomic classification was obtained [32,37]. These analyses were carried out by Chun Lab (Seoul, Korea).

2.4. Bioinformatics Analysis

The relative abundance of the 16S rRNA gene for each operational taxonomic unit (OTU) was used to determine the absolute abundance of each OTU by multiplying the respective relative abundance by

the total number of 16S rRNA gene copies. The Microbiome and Phyloseq packages in R software version 3.50 (Lucent Technologies, Murray Hill, NJ, USA) were used for analysis [40]. Heatmaps and core heatmaps [41] were used for visualization. Core line-plot and t-distributed stochastic neighbor embedding (t-SNE) [42] analyses were performed using the Microbiome Rtsne and Vegan packages.

2.5. Ethics Approval

All subjects who participated in this study were approved for the purpose of this study. Prior to saliva collection, they completed an informed-consent form. This study was approved by the Ethics Committee of the Tsurumi University School of Dental Medicine (Approval Number: 1332).

3. Results

3.1. Study Participants

The number of subjects participating in this study was 87. There were 41 men and 46 women who were all 90 years old. Adequate mount samples for pyrosequencing analysis were not obtained from one man and one woman.

3.2. Sequence Data

From the 85 subjects, 3,899,271 reads (minimum, 23,272; maximum, 108,597) passed quality control. From these reads, sequences were clustered into 24 phyla, 48 classes, 106 orders, 214 families, 529 genera, and 1216 species. The prevalence and abundances of all 1216 species are visualized using a heatmap in Figure S1 (Supplementary Materials).

The summary statistics of the alpha diversity indices are shown in Table S1 (Supplementary Materials). The rarefaction curve is presented in Figure S2 (Supplementary Materials).

3.3. Oral-Microbiome Structure

Figure 1 shows the relative abundance of detected bacteria. Data are presented separately on the (A) phylum and (B) genus levels. The Firmicutes phylum was most abundant, followed by Actinobacteria and Bacteroides. The abundance of these phyla constituted 92.6% of the sample. On the genus level, *Streptococcus* represented 44.5%, *Rothia* represented 15.2%, and *Veillonella* represented 9.0% of the sample. The composition bar plots for each sample on the phylum level are shown in Figure S3 (Supplementary Materials). The proportional ranges of these bacteria were 42.7% to 93.0% for Firmicutes, 4.7% to 39.8% for Actinobacteria, and <0.01% to 39.6% for Bacteroides. Taxon prevalence is shown in Figure 2. The prevalence of each species is plotted against their abundance. Highly prevalent phyla were Firmicutes and Actinobacteria. The core line plot is shown in Figure S4 (Supplementary Materials).
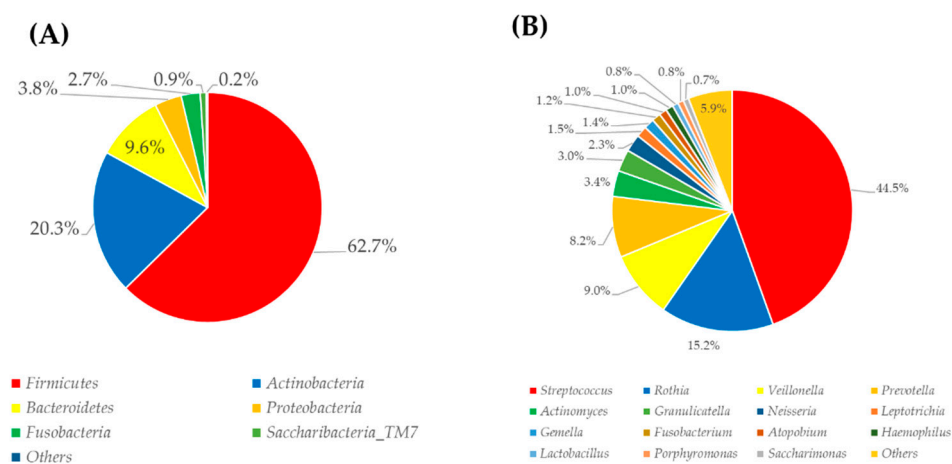

Figure 1. Microbiome structure in saliva: (**A**) phylum level; (**B**) genus level.
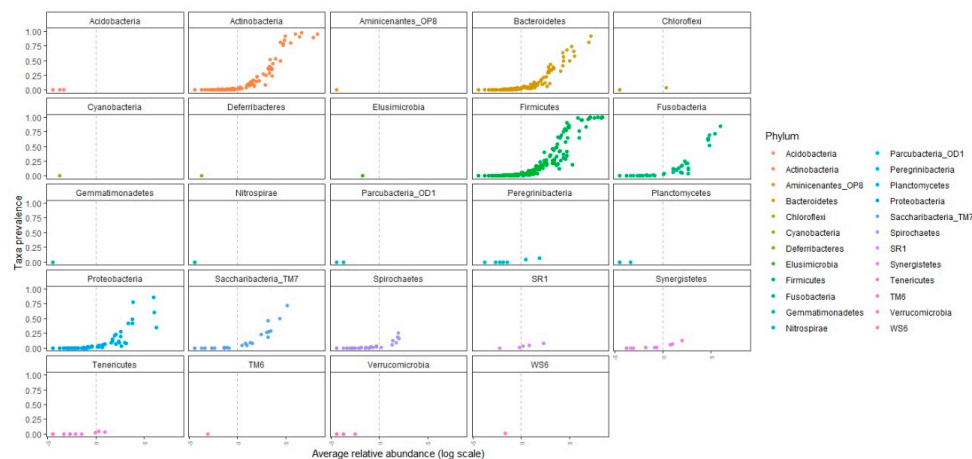
Figure 2. Overview of operational-taxonomic-unit (OTU) prevalence and taxonomic affiliations.

3.4. Candidates for Core Microbiome and Core-Microbiome Analysis

Table 1 shows the bacterial species detected in more than 90% of subjects. Thirteen species were detected in all subjects. Among these 13 species, eight species were from the *Streptococcus* genus. These species represent potential candidates for the core oral microbiome. These 13 species are visualized using a phylogenetic tree and core heatmap in Figure 3.

Table 1. Bacterial species detected in more than 90% of subjects.

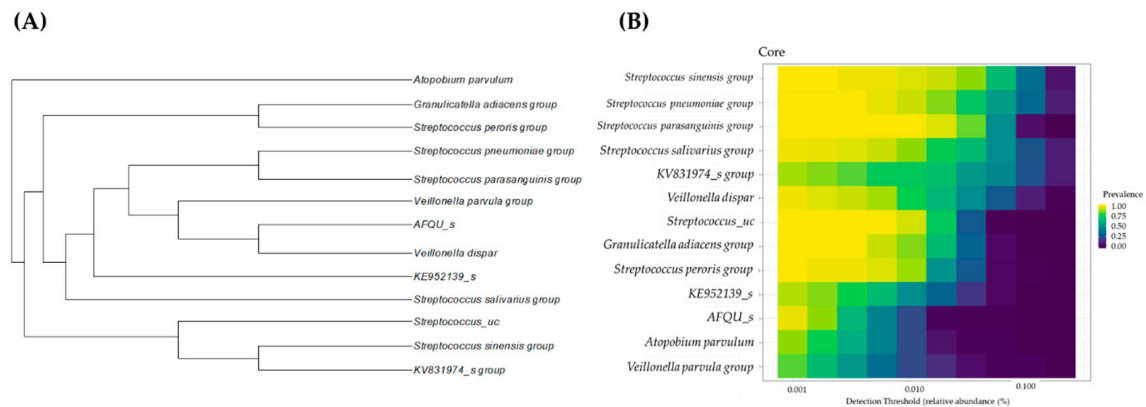|	Taxonomy			Prevalence (*n*%)		Abundance (Average)
	Phylum	Genus	Species			
100%	Firmicutes	*Streptococcus*	*Streptococcus sinensis*	85/85	100%	10.14%
	Firmicutes	*Streptococcus*	*Streptococcus pneumoniae*	85/85	100%	9.61%
	Firmicutes	*Streptococcus*	*Streptococcus salivarius*	85/85	100%	8.75%
	Actinobacteria	*Rothia*	KV831974_s	85/85	100%	8.30%
	Firmicutes	*Streptococcus*	*Streptococcus parasanguinis*	85/85	100%	6.13%
	Firmicutes	*Veillonella*	*Veillonella dispar*	85/85	100%	4.31%
	Firmicutes	*Granulicatella*	*Granulicatella adiacens*	85/85	100%	2.90%
	Firmicutes	*Streptococcus*	*Streptococcus*_uc	85/85	100%	2.80%
	Firmicutes	*Streptococcus*	*Streptococcus peroris*	85/85	100%	2.67%
	Actinobacteria	*Actinomyces*	KE952139_s	85/85	100%	1.72%
	Firmicutes	*Veillonella*	*Veillonella parvula*	85/85	100%	1.15%
	Actinobacteria	*Atopobium*	*Atopobium parvulum*	85/85	100%	0.89%
	Firmicutes	*Streptococcus*	AFQU_s	85/85	100%	0.77%
99%–95%	Firmicutes	*Veillonella*	*Veillonella atypica*	84/85	98.8%	2.22%
	Actinobacteria	*Actinomyces*	*Actinomyces*_uc	84/85	98.8%	0.30%
	Firmicutes	*Veillonella*	*Veillonella*_uc	84/85	98.8%	0.25%
	Actinobacteria	*Rothia*	*Rothia mucilaginosa*	83/85	97.6%	5.43%
	Firmicutes	*Gemella*	*Gemella haemolysans*	83/85	97.6%	1.23%
	Actinobacteria	*Rothia*	*Rothia*_uc	83/85	97.6%	0.28%
	Proteobacteria	*Campylobacter*	*Campylobacter concisus*	83/85	97.6%	0.10%
	Bacteroides	*Prevotella*	*Prevotella melaninogenica*	82/85	96.5%	2.92%
	Firmicutes	*Streptococcus*	*Streptococcus gordonii*	82/85	96.5%	1.78%
	Firmicutes	*Moryella*	*Stomatobaculum longum*	82/85	96.5%	0.31%
	Actinobacteria	*Actinomyces*	JVLH_s	82/85	96.5%	0.18%
	Actinobacteria	*Rothia*	*Rothia dentocariosa*	81/85	95.3%	1.36%
	Firmicutes	*Bulleidia*	*Solobacterium moorei*	81/85	95.3%	0.20%
>90%	Fusobacteria	*Fusobacterium*	*Fusobacterium nucleatum*	80/85	94.1%	0.87%
	Firmicutes	*Megasphaera*	*Megasphaera micronuciformis*	80/85	94.1%	0.30%
	Actinobacteria	*Actinomyces*	*Actinomyces odontolyticus*	80/85	94.1%	0.24%
	Firmicutes	*Lachnoanaerobaculum*	*Lachnoanaerobaculum saburreum*	80/85	94.1%	0.17%
	Bacteroides	*Prevotella*	*Prevotella histicola*	79/85	92.9%	2.39%
	Proteobacteria	*Haemophilus*	*Haemophilus parainfluenzae*	79/85	92.9%	0.88%
	Bacteroides	*Prevotella*	*Prevotella*_uc	79/85	92.9%	0.42%
	Firmicutes	*Streptococcus*	*Streptococcus sanguinis*	78/85	91.8%	0.92%
	Actinobacteria	*Actinomyces*	*Actinomyces graevenitzii*	78/85	91.8%	0.52%
	Bacteroides	*Prevotella*	*Prevotella salivae*	77/85	90.6%	0.25%
	Firmicutes	*Oribacterium*	*Oribacterium asaccharolyticum*	77/85	90.6%	0.24%

(A)

(B)

Figure 3. Core visualization: (**A**) phylogenetic tree and (**B**) core heatmap of 13 species detected in all subjects.

According to Figure 3, eight species of *Streptococcus* were not adjacent. *Streptococcus sinensis* and *Streptococcus pneumoniae* were highly prevalent and abundant species.

3.5. Ordination Analysis

The t-SNE plot allows embedding high-dimensional data into a two-dimensional map on the basis of each data point. Each species was categorized according to its prevalence (Figure 4A) or genus (Figure 4B). Through this graphical observation, highly prevalent species were conglomerated, and low-prevalence species were separated into distinct groups. Species were not separated on the basis of their genus. These results indicate that, on the species level, oral bacteria formed groups, and their coexistence was not regulated by their genus.
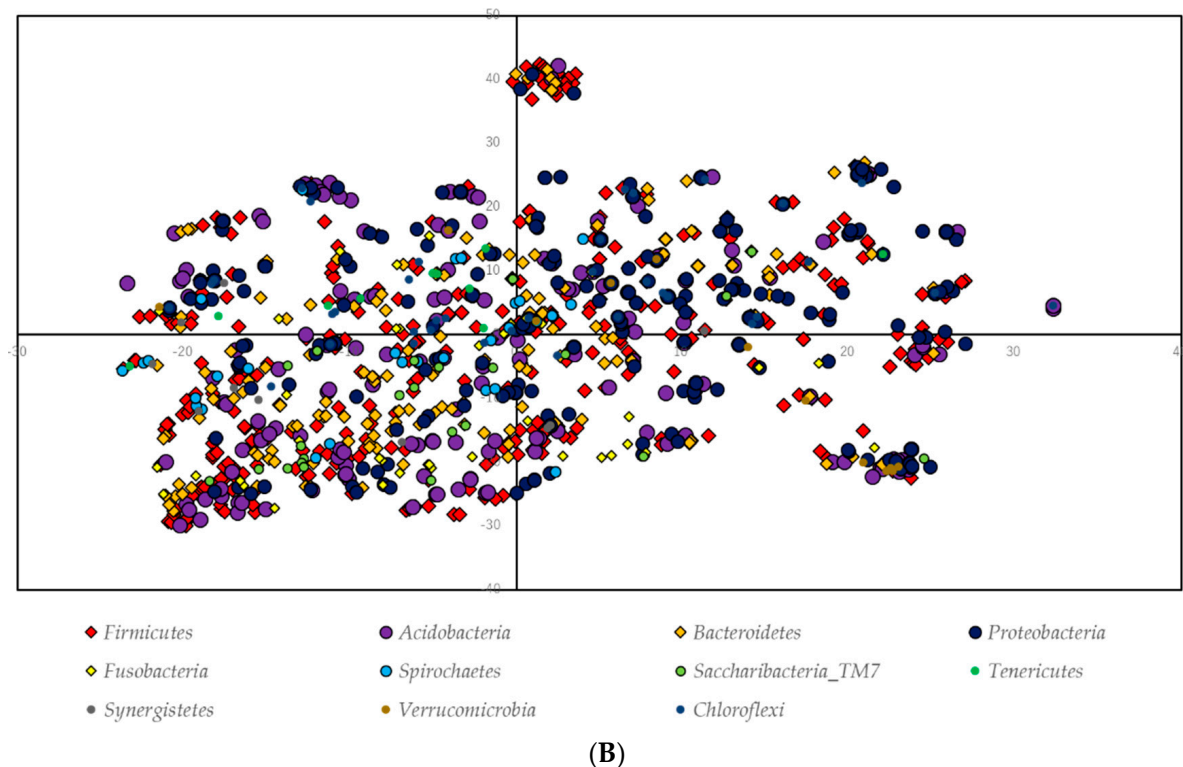

(A)

Figure 4. *Cont.*

(B)
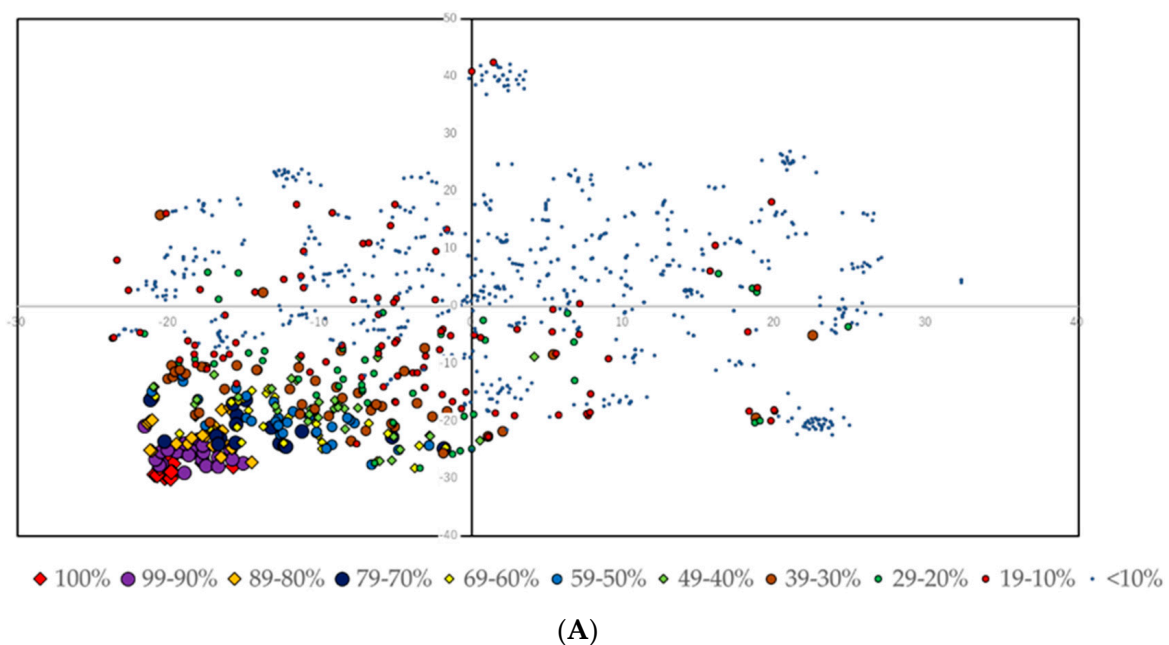
Figure 4. Ordination analysis as a function of t-distributed stochastic neighbor embedding (t-SNE) plot categorized by (**A**) prevalence and (**B**) genus.

4. Discussion

In this study, we investigated 85 healthy people at the age of 90, focusing on analyzing their core oral microbiome. On the basis of the pyrosequencing analysis, 389,927 valid reads were obtained, and 1216 species were detected. Thirteen species were detected in all 85 subjects. Among them, seven belonged to the *Streptococcus* genus. These species represent potential candidates for the core human oral microbiome.

In this study, the most abundant phylum was Firmicutes, whereas *Streptococcus* constituted 45% of the genera present. The microbiome structure on the phylum level was categorized as follows: *Firmicutes* (62.7%), *Bacteroides* (9.6%), *Proteobacteria* (3.8%), *Fusobacteria* (2.7%), and *Actinobacteria* (20.3%). A previous study investigating subjects aged 60 years and older in China showed that the abundant phyla were *Firmicutes* (29.6%), *Bacteroides* (22.4%), Proteobacteria (20.4%), Fusobacteria (16.2%), and Actinobacteria (7.6%) [43]. Another study showed that the core oral microbiome predominantly comprises Firmicutes, followed by Proteobacteria and Bacteroides [44]. *Firmicutes*, *Proteobacteria*, *Bacteroides*, *Fusobacteria*, and *Actinobacteria* constitute more than 98% of the oral microbiome [45]. The oral-microbiome structure may change as a function of growth [46], food [47,48], oral diseases [49–51], or infection [51,52]. These results are consistent with the results of this study.

Few reports presented the core microbiome on the genus or species level. On the genus level, *Neisseria* (12.5%), *Leptotrichia* (11.1%), *Streptococcus* (10.7%), *Prevotella* (7.0%), *Veillonella* (6.9%), *Fusobacterium* (5.4%), *Capnocytophaga* (4.2%), *Prevotella* (4.1%), *Corynebacterium* (2.6%), *Saccharibacteria* (2.6%), *Actinomyces* (2.6%), *Haemophilus* (2.3%), and *Porphyromonas* (2.2%) were most prevalent [43]. Abundant genera according to another study were *Streptococcus* (26.1%), *Veillonella* (21.9%), *Neisseria* (16.9%), *Haemophilus* (10.7%), *Actinomycetes* (2.6%), *Rothia* (3.1%), and *Oribacterium* (1.7%) [53]. On the species level, a study carried out in Japan identified *Streptococcus salivarius* (9.5%), *Prevotella melaninogenica* (9.2%), *Rothia mucilaginosa* (8.8%), *Veillonella atypica* (6.0%), and *Neisseria flavescens* (5.8%) as species exhibiting > 5% abundance on the tongue surface [34]. When compared with our results, shown in Table 1, four of these species were detected in all our subjects, except for

Neisseria flavescens. *Neisseria* sp. is often detected in samples obtained from the dorsal surface of the tongue [54]. On the phylum level, the core microbiome observed in our study coincided with that in the literature. However, on the genus or species level, predominant bacteria varied across studies.

There are environmental and cultural differences, such as food consumption, that affect microbiome structure [49,55–57]; in our study, the proportions of Firmicutes and *Streptococcus* were higher than those found in other studies.

Streptococcus spp. are abundant in human milk, and they play an important role in the establishment of the oral-microbiome structure for breastfed infants [58,59]. Some *Streptococcus* spp. act as probiotic bacteria [60,61]. In contrast to these beneficial effects of *Streptococcus* for the human body, pathogenic *Streptococcus sinensis*, which is responsible for bacteremia [62] and endocarditis [63], and *Streptococcus pneumoniae* [64] were detected in all samples.

Two species of *Veillonella*, which are also classified as Firmicutes, were detected in all samples. *Veillonella* is known to be prevalently detected at various sites in the oral cavity, such as dental plaque, saliva [65], and oral mucosa [66]. *Veillonella parvula* is associated with the development of dental caries [67], endodontic infections [68], and periodontitis [69]. The most abundant species in our study was *Streptococcus sinensis*, followed by *Streptococcus pneumoniae*.

In this study, *Fusobacterium nucleatum* was detected in 94.1% of subjects. A previous study showed that Fusobacteria represent a predominant taxon in the oral microbiome [69]. Fusobacteria mediate the coaggregation of nonaggregating microbiota, and they are a structural element of plaque in both healthy and disease conditions [70]. They may contribute to the diversity of the oral microbiome.

A pioneering study of the oral microbiome using pyrosequencing suggested that the concept of a healthy core microbiome was supported by abundant oral taxa found in the oral cavity of healthy individuals [66,71]. Phylogenetic trees of 118 of the most predominant taxa identified at several sampling sites in the oral cavity were presented [71]. Among the 13 species detected in all subjects, five species were not included in this phylogenetic tree. These 13 species included pathogenic bacteria leading to human diseases. These bacteria were also detected in healthy older persons; however, they are not proposed as candidates for a healthy core oral microbiome.

It has been suggested that periodontal disease can be a major risk factor for some systemic diseases [72–77]. Recent advances in research on oral and general health have shown that there are protective host factors for periodontal-related systemic diseases [78–80]. The limitation of this study is its cross-sectional study. Further study is necessary to investigate the oral microbiome that can be the risk for mortality in combination with these host factors for older people.

In this study, we aimed to identify the core oral microbiome in healthy older people. However, on the basis of prevalence and abundance, pathogenic bacteria were also included. The human oral microbiome plays a crucial role in diseases and human health. Simple descriptive analysis as a function of prevalence and abundance may not be enough to define a healthy core microbiome. The effect of bacteria should be considered when defining a healthy human oral microbiome.

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