Original

Characterizations of oral microbiota in elderly nursing home residents with diabetes

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Abstract: Over 700 bacterial species have been detected in the oral cavity. Several studies have suggested that periodontitis is associated with systemic disorders such as diabetes mellitus, indicating a key role for oral microbiota in human health. However, the relationship between oral microbiota and diabetes has not been well clarified. Therefore, we conducted microbiome analysis of saliva samples obtained from 15 elderly residents (3 with type 2 diabetes mellitus [DM] and 12 without diabetes [non-DM]) at three different nursing homes, as well as 9 young healthy controls (HC). Genomic DNA was extracted from each sample, and then the V4 region of the 16S rRNA gene was amplified and sequenced.

Alpha diversity, in terms of operational taxonomic unit richness, was significantly higher in samples from the non-DM group than in those from the HC group. Weighted UniFrac distance analysis showed that salivary microbial communities in the DM group were separately clustered. Furthermore, in the DM group, *Actinomyces* and *Selenomonas* showed significantly higher abundance, whereas *Alloprevotella* showed significantly lower abundance, relative to the non-DM group. Although our findings were limited by the small sample size, oral bacterial diversity in the DM group was clearly different from that in the non-DM group.

Keywords: salivary microbiota; diabetes; dysbiosis;
16S rRNA sequencing; nursing home.

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Introduction

Using 16S rRNA sequencing analysis, more than 700 bacterial species have been detected in the oral cavity. This degree of variety creates a unique community whose microbial composition is thought to be stable

within individuals (1). Aas and colleagues have found distinctively predominant bacterial flora in the oral cavities of healthy individuals that were highly diverse and showed site specificity (2). Recent studies have suggested that commensal bacterial species in the oral cavity may function as pathogens in a variety of systemic diseases, including aspiration pneumonia (3) and infective endocarditis (4), as well as having a relationship to pre-term low-weight births (5). Dysbiosis is defined as microbial imbalance, and the term oral dysbiosis has been applied to the association between oral bacterial composition and disorders such as periodontal disease (6) and obesity (7).

It has become widely recognized that the gut microbiota is closely associated with health and disease in humans. Alterations in the taxonomic and functional composition of gut microbiota have been observed in patients with chronic conditions such as diabetes (8,9), obesity (10,11), and Crohn's disease (12,13), and the composition is reportedly influenced by dietary nutrition (14,15). Similarly, the oral microbiota is also known to change in response to local and general conditions, and is considered to be an indicator of homeostatic deterioration (7,16,17). Although the association between oral and intestinal microbiota is unclear, recent studies have suggested that alterations in the microbial composition of dental plaque are associated with periodontal disease (6,18). In addition, a number of reports have suggested that periodontitis is linked to some systemic disorders, including diabetes mellitus (19,20). Although the oral microbiota seems to play a key role in human health, its relationship with diabetes has not been examined in detail. Considering the fact that microbial 16S rRNA analysis using next-generation sequencing is expensive, the present investigation using a small sample size was conducted as a pilot study to evaluate the feasibility, adverse events, and statistical validity of this approach, as well as the suitability of sample collection methods from individual subjects (general and oral).

In the present study, we performed microbiome analysis using salivary samples obtained from elderly residents of nursing homes, who all shared a similar living environment, in order to minimize the effects of controllable factors such as dietary intake, lifestyle, access to medical care, and oral hygiene consciousness. Our aim was to clarify the association between diabetes mellitus and the oral microbiota.

Materials and Methods

Study population

A total of 50 participants from three different nursing homes (Slowlife-Yao Long-term Care Health Facility,

Clover-no-Oka Long-term Care Health Facility, Suzunone Long-term Care Health Facility, Osaka, Japan) were recruited for this study. Among them, 15 elderly individuals (aged between 68 and 101 years) who provided sufficient quantities of unstimulated saliva for microbial analysis were selected: 3 with type 2 diabetes mellitus (DM) and 12 without evidence of diabetes (non-DM). In addition, 9 adults working at the Department of Prosthodontics, Gerodontology and Oral Rehabilitation, Osaka University Graduate School of Dentistry, Osaka, Japan (aged 25 to 53 years), were enrolled as healthy controls (HC). Individuals who had been treated with antimicrobial agents within the previous 3 months were excluded. The study protocol was approved by the Institutional Review Board of Osaka University Graduate School of Dentistry (approval number H22-E9), and the methods conformed to the approved guidelines. All participants provided written informed consent to participate.

Oral examinations

Oral examinations were performed by a trained and registered dentist. The number of teeth and their status (decayed, missing and filled) were recorded. Usage of removable dentures was also noted.

Diagnosis of type 2 diabetes

Information regarding comorbidity was obtained from medical examinations conducted by physicians. We considered participants to have type 2 diabetes (ICD-10, code E11) if [1] they were receiving insulin or other oral hypoglycemic medications, [2] their fasting plasma glucose level was ≥ 100 mg/dL, and [3] their hemoglobin bA1c (HbA1c, NGSP) level was $\geq 6.5\%$.

Sample collection

During the period from November 2014 to January 2015, between 15:00 and 17:00 each day, saliva samples were collected in disposable cups after the subjects had refrained from all intake of food and drink, smoking, and use of toothpaste for at least 2 h. The samples were stored on ice during the collection procedure and immediately processed for genomic DNA extraction. We excluded participants who were unable to provide a sufficient volume of unstimulated saliva ($n = 29$, less than 1.0 mL within 5 min) or from whom an insufficient amount of genomic DNA was extracted ($n = 6$).

DNA extraction

Genomic DNA was extracted from each saliva sample using a previously described method (21) with minor modifications. Briefly, the samples were filtered using a

Flowmi Tip Strainer (40 μ m, Scienceware, Wayne, NJ, USA), then lysozyme at 15 mg/mL was added. After incubating the cell suspension for 1 h at 37°C, achromopeptidase (3 mg/mL) was added to the suspension, followed by incubation at 37°C for 30 min, and then proteinase K (2 mg/mL) was added, followed by further incubation at 55°C for 5 min. Subsequently, sodium dodecyl sulfate was added at a concentration of 1% and the incubation was continued for 1 h at 55°C. DNA purification was performed using both phenol-chloroform isoamyl alcohol (25:24:1, v/v) extraction and ethanol precipitation.

Preparation of a 16S rRNA library and DNA sequencing

A 16S ribosomal RNA library was constructed using a TruSeq DNA sample preparation kit (Illumina, San Diego, CA, USA). The V4 hypervariable region of bacterial 16S rRNA genes was amplified using custom barcode primers (forward: 5'-GTGCCAGCMGCCGCGGTAA-3', reverse: 5'-GGACTACHVGGGTWTCTAAT-3') and sequenced with paired-end 250-bp reads with an Illumina MiSeq.

Quality filtering

A FASTX-Toolkit (version 0.013 Hannon Lab, Howard Hughes Medical Institute, Chevy Chase, MD, USA) was employed to process the raw sequencing data, employing the following sequence quality criteria. [1] The minimum acceptable Phred quality score for the sequences was 20, with a score of ≥ 20 noted in more than 70% of the sequence bases. [2] After quality trimming from the sequence tail, sequences of over 100 bp were retained and these also had an acceptable Phred quality score of 20. [3] Both forward and reverse sequences (merged sequencing reads from both primers) that met the first and second requirements were retained for subsequent analysis.

Taxonomy assignment and sequence analyses

Bowtie 2 (22) was used to align the operational taxonomic units (OTUs) with the NCBI nucleotide database (fungal small subunit, internal transcribed spacer, large subunit region sequence) and the 16S rRNA database. A standard of 97% similarity with the database was applied, and sequencing reads that did not match the database were removed. Additional statistical analyses including those for alpha diversity and beta diversity were performed in an R language environment after normalization of all the samples to the same sample size (reads).

Table 1 Basic profiles of the participants

	DM	non-DM	HC
Number of participants	3	12	9
Male/Female	1/2	2/10	8/1
Age (years $\pm$ SD)	85.3 $\pm$ 4.5	83.9 $\pm$ 8.4	32.8 $\pm$ 7.8
BMI (kg/m ²)	21.4 $\pm$ 1.2	22.3 $\pm$ 3.4	22.4 $\pm$ 2.5
Denture wearers	2	10	0
Edentulous participants	1	2	0
Dentate participants	2	10	9
Number of teeth (range)	23-24	3-30	24-28
Decayed teeth (range)	0-0	0-4	0-0
Missing teeth (range)	4-5	2-25	0-4
Filled teeth (range)	19	1-14	0-7

Fig. 1 Characterization of microbial diversity in terms of alpha diversity values for the diabetic (DM), non-diabetic (non-DM), and healthy control groups. Six well-known parameters indicative of alpha diversity were calculated using 18,000 sequences from each dataset: (A) Richness ($q = 0$), (B) Chao index ($q = 0$ plus estimated unseen taxonomy), (C) Shannon index ($q = 1$), (D) True diversity ($q = 1$) for the DM (red bars) non-DM (yellow bars), and HC (green bars) groups, with standard deviation. * $P < 0.05$, two-tailed t -test. (E) Relative proportions of bacterial taxa at the phylum level in the DM, non-DM, and HC groups. (F) Scatter plots generated using principal coordinate analysis. Weighted UniFrac analysis was used for evaluation of the microbial community composition of the three groups.

Results

Microbial community structure

In saliva samples from the 24 subjects, a total of 217 operational taxonomic units (OTUs) were identified (a total of 1,525,260 reads matched to the quality filtering, and 1,190,320 taxonomy assignment reads), which belonged to 69 different genera in eight different phyla. The demographic characteristics and dental status of the participants as a whole are listed in Table 1.

We first compared microbial diversity (alpha diversity) among the three groups, whose basic profiles are shown in Table 1. OTU richness (observed OTUs and

Chao index) was significantly higher in samples from the non-DM group than in those from the HC group (Fig. 1A, B). A microbial diversity estimator (Shannon index) also indicated a significantly more diverse bacterial community in the non-DM group than in the HC group (Fig. 1C), true diversity also being higher in the non-DM group (Fig. 1D). In addition, the DM group showed a higher level of diversity, although the differences for each index were not significant.

Microbial diversity by UniFrac analysis

Next, we examined microbial composition. Firmicutes was the dominant phyla in the salivary microbiota. The DM group showed a significantly lower abundance of Bacteroidetes than the non-DM group (Fig. 1E). We then utilized UniFrac analysis to compare the degree of phylogenetic overlap in the microbial community composition among the three groups. The weighted UniFrac distance results showed that the salivary microbial communities in the DM group were clustered separately from those in the non-DM and HC groups (Fig. 1F). In addition, visualization of relative bacterial abundance using hierarchical clustering according to weighted UniFrac distance revealed a different composition in the DM group (Fig. 2).

Characterization of the salivary microbiota in subjects with diabetes

We compared the relative abundance of different genera. In the samples obtained from the DM group, *Actinomyces* and *Selenomonas* were significantly more abundant, whereas *Alloprevotella* showed lower abundance than in the non-DM group. In addition, *Actinomyces*, *Rothia*, *Fillifactor*, *Selenomonas*, and *Synergistes* showed significantly higher abundance, whereas *Virgibacillus*, *Abiotrophia*, *Veillonella*, and *Fusobacterium* showed significantly lower abundance in the non-DM group than in the HC group (Figs. 3, 4). Furthermore, 25 species, including major periodontal pathogens, such as *A. israelii*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *S. noxia*, were found to be more abundant in the samples from elderly subjects without diabetes than in samples from the HC group (Fig. 5).

Discussion

Type 2 diabetes is one of the most common chronic diseases worldwide, and its incidence is increasing. Its major complications include diabetic hyperglycemia, cardiovascular disease, stroke, kidney dysfunction, and eye damage. Its oral complications include periodontal disease (gingivitis, periodontitis), salivary dysfunction

Fig. 2 Salivary microbial proportions with hierarchical clustering. (Upper) Beta diversity clustering was utilized using the weighted UniFrac distance and is shown as a dendrogram. (Lower) Bacterial relative abundance of 24 samples from each group at the genus level, visualized as proportions. The major genera (top 20) are listed at bottom right.

(reduced saliva flow, changes in saliva composition), and taste dysfunction. Recently, it has been reported that the intestinal microbiota in humans with type 2 diabetes differs from that in non-diabetic individuals, the proportions of the phylum Firmicutes and the class Clostridia being significantly reduced in diabetics (23). On the other hand, an increase in the relative abundance of the phylum Firmicutes and a reduced level of Bacteroidetes have been observed in both obese mice (10) and humans (24). The present findings are in accord with those studies, indicating similar mechanisms of oral and intestinal dysbiosis.

Within the oral cavity of any given individual, the environment can differ according to tissue location, the bacterial composition of the buccal area, vestibule, tongue, palate, tonsil, tooth surface and gingiva being site-specific (2). The major phylum present in saliva samples is low-GC gram-positive bacteria (Firmicutes), and this includes both healthy subjects and patients with periodontal disease (17). Although the methods we used for sequence analyses were not the same as those used in previous studies, our present results were concordant. So far, there has been no evidence to indicate a relationship between gut and oral microbiota, as the oral cavity and

Fig. 3 Heat maps showing microbial abundance at the genus level. (Upper) Clustering of each sample based on correlation distance. (Left) Phylogenetic correlation of all genera detected in 24 samples from each group.

gastrointestinal tract provide quite different environments in terms of variables such as gas environment, nutritive factors, and temperature. According to a study by David et al., the dynamics of saliva microbiota remain more stable over time than those of gut microbiota in response to environmental alterations (25). This suggests that the oral and gut microbiomes have different origins, as reflected in the difference evident in their microbial phyla. Therefore, saliva may be regarded as a better sampling source than fecal specimens for assessment of diversity at the individual level.

Selenomonas noxia and related members of the genus are anaerobic, crescent-shaped, gram-negative bacteria present at sites of periodontal disease (26,27). Using DNA probe analysis, Goodson et al. demonstrated that *S. noxia* was present more frequently in saliva samples

from overweight individuals (7). *S. noxia* is known to actively ferment glucose and produce propionic acid, which seems concordant with our present findings in diabetic elderly individuals. Goodson et al. also noted an abundance of *Actinomyces* and *A. israelii*, as was the case in our subjects. *Actinomyces* is a widely recognized pathogen causing actinomycosis, and several case reports of actinomycosis complicating diabetes have been published (28,29). However, details of the relationship between actinomycosis severity and diabetes remain unknown. Further studies are required to explore the link between diabetes mellitus, as the phenotype of a pathological condition, and causative pathogens, while it is also important to determine whether such pathogens act independently or as members of the overall flora. Recently, Sakanaka et al. have reported that *Strepto-*

Fig. 4 Relative abundance of microbiota at the genus level. Microbial compositions at the genus level with significant differences are shown in a graph for comparisons of (A) diabetic (red bars) vs. non-diabetic (yellow bars) elderly subjects, and (B) elderly (yellow bars) vs. young (green bars) subjects without diabetes, with standard deviation. * $P < 0.05$, two-tailed t -test.

coccus gordonii ArcD plays crucial roles in mediating arginine uptake and promoting bacterial growth, particularly under low-arginine conditions (30). It has also been suggested that metabolic cross-feeding occurs in complex biofilms, enabling the bacterial community to function cooperatively and resist the effects of antimicrobial agents to a degree up to 1,000 times greater than is possible for individual planktonic cells (31).

In saliva samples from the present diabetic group, the phylum Bacteroidetes and genus *Alloprevotella* were decreased, while the genera *Actinomyces* and *Selenomonas* were increased. Although our findings are limited by the very small sample size, they are considered to be of interest, as no previously reported studies have addressed the relationship between oral microbiota and diabetes. Additional studies are needed to better clarify the association of the oral microbiome with health status.

In this study, we used the NCBI database for nucleotide matching. However, Korenori et al. have reported that the NCBI database is unreliable for bacterial classification, as it includes too much sequence data, including fungal small subunits, internally transcribed spacers, large subunit regions as well as the 16S rRNA sequence (Korenori et al. Jpn J Lactic Acid Bact 23, 24-34, 2012).

Fig. 5 Relative abundance of microbiota at the species level. Microbial composition at the species level with significant differences for comparisons of diabetic elderly subjects (red bars), non-diabetic elderly subjects (yellow bars), and young subjects without diabetes (green bars), with standard deviation. * $P < 0.05$, two-tailed t -test.

This would also have been a limitation of our study design. Future research will need to focus on the association between oral microbiota and general condition, systemic disease and dietary nutrition. It will also be necessary to clarify the link between oral microbiota and the effects of general medical and dental interventions, such as medication, use of oral hygiene and professional care, using a larger sample.

In summary, through microbiome analyses of saliva samples from 15 elderly individuals, we have shown that alpha diversity, in terms of operational taxonomic unit richness, was significantly higher in samples from non-DM individuals than in those from HC individuals. Weighted UniFrac distance analysis showed that salivary microbial communities in the DM group were separately clustered. Although the present findings are limited by the small sample size, our data suggest that saliva may be a preferable material for assessment of oral bacterial diversity in elderly individuals.

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Conflict of interest

None declared.

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