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Original Article

Correlation analysis of the oral mucosal microbiome and diabetes mellitus using microbial DNA in elderly male subjects

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Oral microbiomes are associated with various systemic diseases, and the need for a deeper understanding of oral microbiomes has garnered substantial research interest. The differences between oral mucosal microbiomes of individuals with and without diabetes were analyzed in this study, to improve disease evaluation and therapeutic development. Mucosal samples were collected from 10 and 16 individuals with and without diabetes, respectively, during an implant-uncovering procedure. Subsequently, genomic DNA from the samples was used for 16S rRNA sequencing, and the compositions of the microbiomes were compared between the two groups. There were no significant differences in the alpha and beta diversities of the groups of individuals with and without diabetes. However, the group of individuals with/without diabetes presented a relatively higher abundance of the *Corynebacteriaceae* family (p=0.004) and the genus *Corynebacterium* (p=0.019). Our findings serve as a basis for future studies on the association between diabetes and bacterial species in the aforementioned taxonomic groups. The divergence between our results and past studies indicates the need for a unified study design for sample collection and the methods used for statistical analysis, when implementing a bioinformatics-based approach to assess microbiome sequencing data from oral mucosal samples.

Key Words: Diabetes mellitus; Microbiome; Oral mucosa; 16s rRNA

Introduction

Chronic infections caused by imbalances in oral bacterial communities lead to oral diseases, such as dental caries [1] and periodontal disease [2], which in turn are associated with cardiovascular disease [3,4], diabetes [5], and even cancer [6]. Furthermore, numerous studies have investigated the association between the oral microbiome and oral diseases, such as dental caries [7], periodontal disease [8], and systemic diseases [9].

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia, which is caused by the dysregula-

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tion of insulin, which regulates blood glucose levels in the body. Chronic hyperglycemia caused by diabetes results in microvascular complications, such as arteriosclerosis as well as cardiovascular and macrovascular diseases [10,11], leading to organ damage and dysfunction [12] and systemic inflammation [13]. In particular, diabetes aggravates periodontitis by maintaining the oral cavity in an inflammatory state [14]. Similarly, periodontitis worsens diabetes by increasing inflammatory precursor activity, thereby enhancing insulin resistance in the body [15]. In addition, one study has reported a correlation between dental caries and DM [16].

Based on this, it could be concluded that the oral environment and diabetes status are related, and indeed, this bilateral relationship has been demonstrated in several studies [15,17-19]. Reportedly, the delayed wound healing in the oral mucosa of patients with diabetes is caused by delayed vascularization, attenuated immunity, and decreased levels of growth factors, including insulin-like growth factor, transforming growth factor, platelet-derived growth factor, and nerve growth factor [20]. Furthermore, the healing of impaired oral wounds in mice with diabetes has been linked to increased fibroblast apoptosis leading to a decrease in fibroblast numbers [21]. These findings suggest that diabetic status and the oral environment are involved in a reciprocal relationship and are not independent of one another. Thus, comparing the oral microbiomes of individuals with and without diabetes may be helpful in assessing the risks as well as in diagnosing diabetes. Diabetes can affect gingival vasculature, inflammatory and immune responses, alterations in collagen synthesis, and genetic predisposition to diseases [22-27]; thus, we expected that the characteristic oral mucosal microbiome could be discovered through the analysis of oral mucosal tissues. Further, as seen in a study comparing vascular endothelial growth factor from gingival crevicular fluid (GCF) samples and gingival tissue samples from diabetic patients, gingival tissue yielded different results compared to the GCF samples [28]. Therefore, we conducted this study assuming that oral mucosal tissues would provide a unique characteristic microbiome in patients with diabetes. Currently, it is unclear as to whether the oral mucosal microbiome of patients with this disorder has characteristics different from those of individuals without diabetes. We hypothesized that we would find dominant or inferior strains in patients with diabetes or that there would be differences in the diversity of the microbiome, compared to that in subjects without diabetes. The aim of this study was to evaluate the effect of DM on the composition and diversity of the microbiomes in the oral mucosal tissue from patients with diabetes compared to patients without diabetes using 16S ribosomal RNA (rRNA) sequencing, a form of next-generation sequencing. We also investigated whether the oral mucosal tissue obtained during dental implant surgery can act as a representative sample of the microbiome more accurately than other sample types.

Materials and Methods

Sampling procedure

Twenty-six subjects with and without type 2 diabetes (24 males and 2 females; mean age: 71.3±4.79), who were void of systemic diseases but had undergone an uncovering procedure following implant placement at the Department of Periodontology, Veterans Health Service Medical Center, were enrolled from July 2018 to December 2018. Patients who had received or required periodontal or dental caries treatment within 30 days, with uncontrolled systemic diseases, who had taken antibiotics within 30 days, who did not have either the will or the ability to make an informed consent, and female patients who were either pregnant or possibly pregnant were excluded. All patients were requested to avoid food intake after 11 pm the night before the uncovering procedure, and tooth-brushing on the day of sample collection. Sixteen subjects without and ten patients with diabetes were investigated. One subject was excluded because the sample size was not large enough for analysis, and two female subjects were also excluded because the presence of such a small number of female could cause statistical bias (Fig. 1). The demographics of the subjects is described in Table 1. Diagnoses of diabetes were made according to the recommendations of the American Diabetes Association [29]. This study was approved by the Institutional Review Board of VHS Medical Center (BOHUN IRB no. 2018-05-009), and all participants provided written

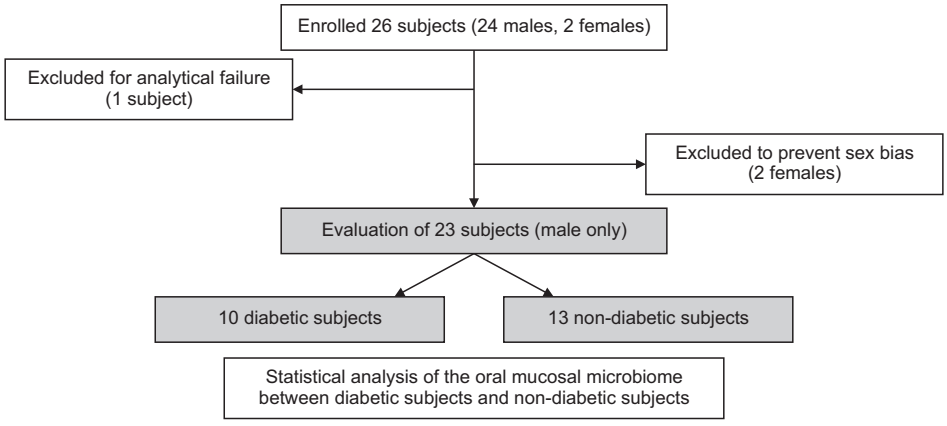

Fig. 1. Study flow.

Table 1. Patient demographics

Variable	Diabetes group (n=10)	Non-diabetes group (n=13)
Age (y)	73.9±4.392	71.5±2.098
BMI	25.00±1.392	24.61±2.241
HbA1c (%)	8.07±0.007	NA

Values are presented as the mean±standard deviation.
BMI, body mass index; HbA1c, haemoglobin A1c; NA, not available.

informed consent. Furthermore, this study was conducted in accordance with the tenets of the Helsinki Declaration of 1975 and its later revisions. Oral mucosal tissues removed during the second implant surgery were collected using a 15c blade. The samples were then placed in a sterile microtube and stored at -80°C.

DNA extraction and gene sequencing

DNA was extracted from samples using a MoBio DNeasy PowerSoil Kit (Cat. No. 12888-100; Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. Each sequenced sample was prepared according to Illumina 16S Metagenomic Sequencing Library protocols. Briefly, 16S amplicon PCR primers targeting 16S rRNA were used to produce PCR amplicon libraries. V4 hypervariable regions of 16S rRNA genes were PCR-amplified using specific 16S V3-V4 primers. The primer sequences are as follows:

16S Amplicon PCR Forward Primer
5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG- 3'

16S Amplicon PCR Reverse Primer
5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'

Subsequently, a limited-cycle amplification step was performed to add multiplexing indices and Illumina sequencing adapters. Each 25 μL PCR mixture contained 2.5 μL of template DNA, 5 μL of amplicon for PCR primer (5 μM), 12.5 μL KAPA's HiFi HotStart ReadyMix (2×), and 5 μL of distilled water. The amplicon PCR protocol used was as follows: 3 minutes at 95°C; 25 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C; and 5 minutes at 72°C. The index PCR protocol was as follows: 3 minutes at 95°C for denaturation; eight cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C; and 5 minutes at 72°C.

Quantification and quality evaluation of DNA was performed using PicoGreen (Invitrogen, Carlsbad, CA, USA) and a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). The final products were normalized and pooled using PicoGreen, and the library sizes were verified using a TapeStation DNA screentape D1000 (Agilent, Santa Clara, CA, USA); the microbiomes of the specimens were analyzed using 16S rRNA sequencing on an Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA; Fig. 2). Negative extraction blank controls were included for each batch of sample extraction to detect contaminants [30].

Data collection and statistical analysis

After sequencing, the MiSeq raw data were used to ex-

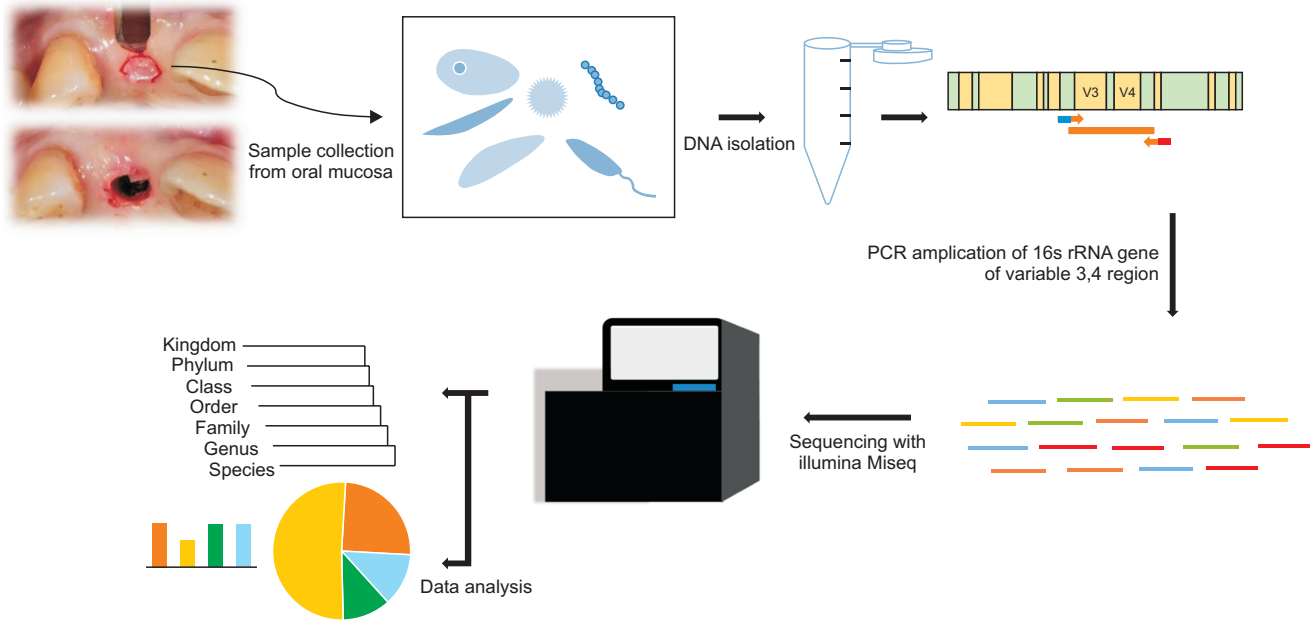

Fig. 2. Flow diagram showing the study design.
Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA).

tract high-quality sequences of approximately 440–465 bp in length with a 120–160-bp overlap using FLASH [31]. The sequences obtained were clustered with sequences showing more than 97% sequence similarity using CD-HIT-out [32], a CD-HIT-EST-based operational taxonomic unit (OTU) analysis program that removes low quality sequences, ambiguous sequences, and chimeric sequences, considered to be sequencing errors, to form species-level OTUs. The representative sequence of each OTU was taxonomically assigned by selecting subjects showing highest similarities in terms of organism information via BLASTN [33], in accordance with the National Center for Biotechnology Information (NCBI) 16S Microbial reference database (DB). If the query coverage of a best hit that matched the DB was less than 85%, and the identity of the matched area was less than 85%, its taxonomy was considered as undefined. Using the OTU-related data, a comparative analysis of various microbial communities was performed using QIIME [34].

Alpha diversity was assessed using the following indices: Chao1, which represents the richness estimate for a defined OUT; Shannon, which considers the number and evenness of species; and inverse Simpson, which represents the probability that two randomly selected individuals in a habitat belong to the same species. Beta diversity between samples was assessed using the weighted UniFrac distance, and flexible relationships between samples were visualized via a principal coordinate analysis (PCoA).

Data analyses of the relative abundance of OTUs were performed using the R Statistical Package, Version 4.0.1 (R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was set at $p<0.05$. Student's t-tests were performed to test for differences in alpha diversity between patients with and without diabetes. OTUs with low counts across all patients were excluded prior to commencing differential expression analyses. OTUs involving counts per million of 100, or greater, in at least two samples were used for differential expression analyses and retained for alpha and beta diversity analyses. Differentially abundant OTUs that were significantly associated with disease states were identified via the Robinson and Smyth approach [35], and later incorporated into a generalized linear model framework (GLM). A negative binomial GLM was fitted to the count data following which likelihood ratio tests were performed to compare abundance levels between groups. For multiple comparison tests, the false discovery rate (FDR) was controlled using the Benjamini–Hochberg step-up procedure.

Results

Sequencing results and diversity indices

We compared the oral microbiota of 10 patients with and 16 individuals without diabetes. The average number of analyzed sequences was 149,114 (max: 173,275, min: 112,220) for the 10 specimens from patients with diabetes and 139,598 (max: 191,755, min: 1,038,963) for the 16 specimens from individuals without diabetes. The *p*-values for OTU, as well as Chao1, inverse Simpson, and Shannon indices were 0.717, 0.728, 0.904, and 0.802 respectively (Table 2). There was no significant difference in alpha-diversities between the two groups (Student's t-test; Fig. 3).

Table 2. Comparison of richness and diversity estimates between diabetes and non-diabetes groups

Variable	Diabetes group	Non-diabetes group	*p*-value
OTUs	153.70±77.40	165.54±82.51	0.730
Chao1	154.67±77.82	166.20±82.63	0.737
Inverse Simpson	0.93±0.06	0.91±0.10	0.749
Shannon	5.35±1.13	5.40±1.21	0.919

Values are presented as the mean±standard deviation.
OTU, operational taxonomic unit.

Comparison of beta diversity between the groups

To compare community structure differences, a PCoA based on a weighted UniFrac matrix was used (Fig. 4). Evident grouping of subjects with diabetes and those without diabetes was not observed.

Taxonomy-based comparisons of oral microbiota between the groups

We compared the relative abundance of taxa to investigate possible differences between specific bacterial taxa in the oral microbiota of subjects with and without diabetes.

A likelihood ratio test was performed for each OUT detected depending on the diabetes status variable. When the FDR value was maintained at 0.05, a significant difference between the diabetes and non-diabetes groups was observed for only one family (*Corynebacteriaceae*) and one genus (*Corynebacterium*) (Table 3). These two showed a relatively higher abundance in the non-diabetes group. No significant differences were found at any other phylum, class, order, or species level.

Bar plots showing taxonomic profiles of diabetes patients and non-diabetes subjects at the phylum (Fig. 5A) and genus (Fig. 5B) levels are presented. Only those with a relative abundance >1% are listed.

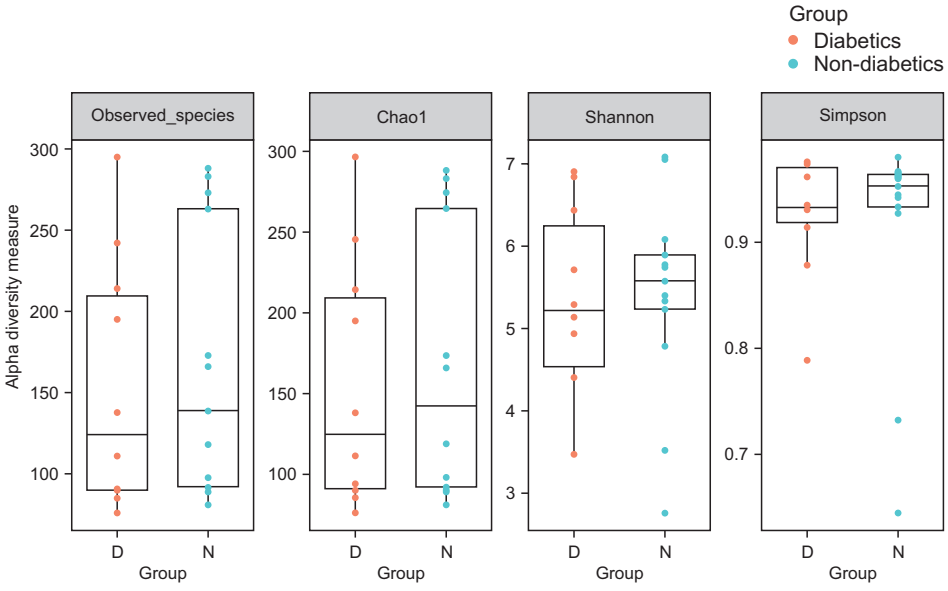

Fig. 3. Comparison of alpha diversity (Observed, Chao1, Shannon and Inverse Simpson indices) between the diabetes group (diabetics) and the control group (non-diabetics). The Student's t-test was used.

Discussion

Microbial dysbiosis reportedly affects inflammatory as well as chronic metabolic disease states [36,37], as demonstrated by the correlation between gut microbiomes and inflammatory bowel diseases [38]. Similarly, microbial changes in the oral cavity might increase the severity of secondary chronic diseases, such as diabetes. This may be due to the oral microbiota playing a regulatory role in the initiation of systemic inflammatory conditions [39]. Accordingly, several studies have investigated the association between diabetes and oral cavity microbiomes.

This study only showed a significant difference in the *Corynebacteriaceae* family, and the genus *Corynebacterium*. These results are in contrast to those of other studies. A previous study analyzed the microbiomes of 29 morbidly obese individuals, including 13 patients with diabetes, and reported that the genus *Bifidobacterium* had a significantly lower abundance in patients with diabetes [40]. Another study, which investigated 20 patients with diabetes and 11 control subjects, indicated that streptococci and lactobacilli were more abundant in the former than in the latter [41]. These studies and ours had a relatively small sample

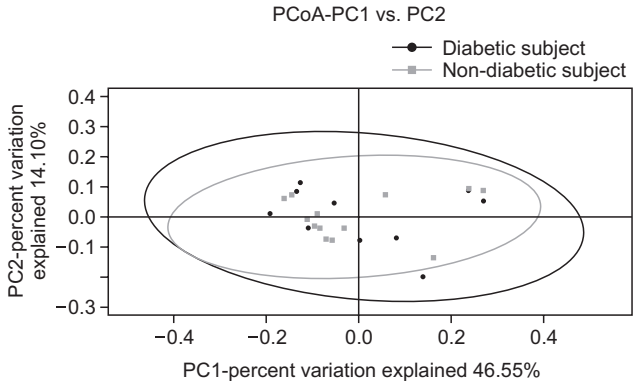

Fig. 4. Beta diversity. There was no significant difference between groups (p=0.753). Principal Coordinate Analysis (PCoA) plot using weighted UniFrac of diabetic patients (black circles) and non-diabetic subjects (gray squares). The ellipses represent 95% confidence intervals for each group.

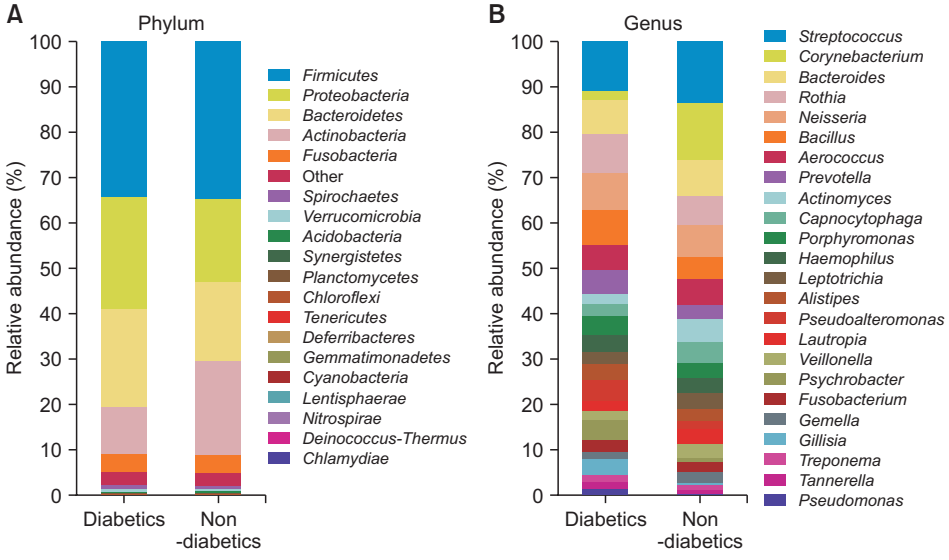

Fig. 5. Oral microbial community. (A) Bar plots showing the taxonomic profiles of diabetes patients and subjects without diabetes at the phylum level. (B) Bar plots showing taxonomic profiles of diabetes patients and subjects without diabetes at the genus level.

Table 3. Relative abundances[a] of OTUs in the diabetes group compared to the non-diabetes group

OTU	*p*-value	FDR adjusted *p*-value	Log_2 fold-change (logFC)	Average log_2 counts per million (logCPM)
Corynebacteriaceae (family level)	<0.001	0.008	−4.298	17.01
Corynebacterium (genus level)	<0.001	0.008	−4.545	17.06

FDR, false discovery rate.

[a]Only significant results are shown. The degree of differential abundance is represented by a logFC, which indicates a positive or negative interaction (logFC >0 or <0) of the specified operational taxonomic unit (OTU). LogFC was calculated for the non-diabetes group as the denominator and the diabetes group as the numerator.

size compared to others; however, unlike in this study, only certain types of bacteria were assessed. A study that evaluated the association between oral microbiomes and diabetes risk without the above limitations reported that the relative abundance of phylum Actinobacteria was associated with a reduced risk for diabetes, while the relative abundance of genus *Actinomyces* was strongly associated with an increased risk [42]. In contrast, a study comparing a diabetes group with a high-risk group (fasting glucose levels >7 mmol/L) and a low-risk group (fasting glucose levels <7 mmol/L) reported that certain genera such as *Leptotrichia*, *Staphylococcus*, *Catonella*, and *Bulleidia* were relatively enriched in the high-risk group [43]. These differences highlight the variation in oral microbiome depending on the study design and the parameters of the cohort.

Here, we have analyzed microbial diversity pertaining to diabetes status within each group (alpha diversity) as well as between groups (beta diversity). In a previous study that analyzed the relationship between diabetes and the saliva microbiome, patients with diabetes showed significant decreases in alpha diversity (Observed, Chao1, ACE, and Shannon indices) and beta diversity compared to individuals without diabetes. However, the differences reported were prominent at the genera level, but not at the species level [44]. In contrast, in an uncontrolled diabetic status study, some OTUs were either significantly more or significantly less abundant in samples from individuals with diabetes than in those without diabetes [45]. Interestingly, the results of the present study did not show a significant difference between alpha or beta diversities of either group, with the groups diverging only in the relative abundance of the *Corynebacteriaceae* family and the genus *Corynebacterium* in individuals without diabetes. A previous study, wherein more families and genes were correlated between the oral microbiome and diabetes, as determined using a mouth rinse sample, reported similar results with respect to the relative abundance of the *Corynebacteriaceae* family and the genus *Corynebacterium* among subjects without diabetes [34]. This may serve as a basis for the association with diabetes for these two bacterial taxa in the future.

In a study comparing chronic wounds from diabetic ulcers with intact skin, more *Corynebacterium* were detected in the wound group [46,47]. This suggests that *Corynebacterium* is a significant opportunistic contributor in chronic skin infections. However, in this study, *Corynebacterium* were detected at higher levels in the mucosal samples from subjects without diabetes than in diabetes patients, which is contradictory to the results of previous studies. This difference is presumed to be due to the difference between the role of *Corynebacterium* as an opportunistic infection in the skin of diabetes patients and the role of healed mucosa in the oral cavity of diabetes patients. This suggests that elucidating the oral role of *Corynebacterium* is important to characterize the oral microbiome of diabetic patients. Therefore, additional research is needed to explore the role of these pathogens in the oral mucosal tissues of diabetes. *Corynebacterium* attenuates the virulence of *Staphylococcus aureus* by regulating a specific pathway which decreases the transcription of virulence genes [48]. The risk of *S. aureus* bacteremia is substantially increased with diabetes, thus potentially aggravating disease risk and mortality [49,50]. Therefore, a decrease in *Corynebacterium* in patients with diabetes might lead to an increase in the pathogenesis of *S. aureus*, which might affect the diabetic status of the patients and present complications such as diabetic foot ulcers. A future study is required to uncover such a relationship.

However, since there are only a few reports on the correlation between *Corynebacterium* and oral conditions in diabetes patients, it was difficult to make assumptions regarding this relationship. Therefore, further studies are needed to determine how *Corynebacterium* affect the complex oral system in terms of oral inflammation, pathology, or immune system and its correlation to diabetes.

Notably, previous investigations have predominantly used supra- or subgingival plaque, or saliva samples, and only a few microbiome studies have used oral mucosa samples. Therefore, it is difficult to make an accurate comparison between our results and those of the other studies. Further discrepancies could be explained by differences between the sample collection methods used by our study and those used by the other studies. Saliva has previously proven to be a useful specimen which reflects oral health [51] and has been used in several microbiome studies due to the ease and non-invasiveness of its collection. Plaque and biofilms might also be similarly useful as specimens for bacterial

analysis [52]. However, to our knowledge, few studies have used the oral mucosa samples for the purpose of microbiome analysis as we elected to do here. Since our results did not agree with those of previous studies using different sampling methods, we presume that oral mucosal tissue from the edentulous region might not be as representative of the intraoral microbiome as other samples.

Furthermore, the previous all had different study designs. The diabetic status of subjects at the time of sample collection was slightly different for each study, and hence the statistical methods used to compare the relative abundance of OTUs were different. Some studies have overlooked the high probability of statistical error that arises when multiple OTUs are compared, by neglecting the concept that statistical error increases as the number of comparisons is increased. This may have led to results that have compromised the reproducibility and reliability of microbiome experiments [53,54]. Therefore, further studies with improved coordination between sampling methods and statistical analyses might be needed.

Since dental caries or periodontitis, which exist independently of diabetes, can affect the composition of the oral microbiome [55,56], which in turn, can also affect the mucosal supernatant, and since systemic diseases other than diabetes can also affect the oral microbiome [57-59], the above inclusion criteria were established to exclude these effects. However, as a limitation, since diabetes can be correlated with oral disease [60,61] as well as other systemic diseases, future studies also need to consider subjects with oral and systemic diseases related to diabetes.

Some studies have suggested that gender has an effect on microbiome composition [62], while others have not [63,64]. Due to the nature of this hospital, only a limited number of female were enrolled initially (two), and therefore, the gender distribution was not even. In order to eliminate the slight bias that may occur due to this gender imbalance, only data that were derived from male patients were used for the statistical analysis. However, it should be noted that although the inclusion of the data from the two females did not affect the statistical results, the effect of differences in subject gender distribution should be evaluated in future studies.

In addition, this study was conducted on crestal mucosa obtained during implant-uncovering surgery. However, a previous study has reported that the bacterial composition can be affected by the position of the tooth or the tooth surface from which the sample was taken [65]. Unlike the case for previous studies, our study is different because it targeted the oral mucosa, but the limitation of this study is that the microbiome composition may be affected depending on the sampling site.

Although the amplicon sequence of the 16S rRNA gene could capture widespread changes in community diversity, it has low sensitivity and limited resolution. Therefore, future studies seems necessary to use a device of whole-community shotgun metagenome with several advantages [66,67].

Moreover, this study attempted to analyze the oral mucosal microbiome, but the existence of saliva on the upper surface of the collected tissue was not considered. Since saliva present on the oral mucosal tissue surface can affect the microbiome composition of the sample, a step that includes its removal from the sample before analysis should be conducted in future studies.

To the best of our knowledge, this is the first preliminary study to analyze the correlation between the oral mucosal microbiome and DM using 16S rRNA gene amplicon sequencing. Previous studies that have explored the interaction between the oral environment and the microbiome have been limited to only a few species [66]. Our result can serve as a basis for future studies investigating the relationship between systemic diseases and the oral cavity. Additionally, to achieve optimum results, microbiome-related studies should be compared and analyzed with studies selecting for dental plaque and saliva samples.

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Conflicts of Interest

The authors declare that they have no competing interests.

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