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Comparison of the oral microbial composition between healthy individuals and periodontitis patients in different oral sampling sites using 16S metagenome profiling

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ABSTRACT

Purpose: The purpose of this study was to compare the microbial composition of 3 types of oral samples through 16S metagenomic sequencing to determine how to resolve some sampling issues that occur during the collection of sub-gingival plaque samples.
Methods: In total, 20 subjects were recruited. In both the healthy and periodontitis groups, samples of saliva and supra-gingival plaque were collected. Additionally, in the periodontitis group, sub-gingival plaque samples were collected from the deepest periodontal pocket. After DNA extraction from each sample, polymerase chain reaction amplification was performed on the V3-V4 hypervariable region on the 16S rRNA gene, followed by metagenomic sequencing and a bioinformatics analysis.
Results: When comparing the healthy and periodontitis groups in terms of alpha-diversity, the saliva samples demonstrated much more substantial differences in bacterial diversity than the supra-gingival plaque samples. Moreover, in a comparison between the samples in the case group, the diversity score of the saliva samples was higher than that of the supra-gingival plaque samples, and it was similar to that of the sub-gingival plaque samples. In the beta-diversity analysis, the sub-gingival plaque samples exhibited a clustering pattern similar to that of the periodontitis group. Bacterial relative abundance analysis at the species level indicated lower relative frequencies of bacteria in the healthy group than in the periodontitis group. A statistically significant difference in frequency was observed in the saliva samples for specific pathogenic species (*Porphyromonas gingivalis*, *Treponema denticola*, and *Prevotella intermedia*). The saliva samples exhibited a similar relative richness of bacterial communities to that of sub-gingival plaque samples.
Conclusions: In this 16S oral microbiome study, we confirmed that saliva samples had a microbial composition that was more similar to that of sub-gingival plaque samples than to that of supra-gingival plaque samples within the periodontitis group.

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INTRODUCTION

Periodontitis is a multifactorial chronic inflammatory disease that affects 20%–50% of the world population, and in those who are 65 years or older, it is associated with a 44% relative risk increase for cardiovascular disease [1]. Unless it receives appropriate treatment, periodontitis causes the formation of a periodontal pocket, followed by destruction of the alveolar bone, which eventually results in tooth loss. Various factors affect the onset and progress of periodontitis, of which the host immune response caused by the intraoral microbiota is a major factor. Multitudinous bacterial species reside in the mouth and maintain a symbiotic relationship and balance in the oral environment. In periodontitis, however, the numbers of anaerobic bacteria and Gram-negative bacteria increase in the periodontal pocket, leading to a change in their composition. In addition, microorganisms in the periodontal pocket can easily pass through inflammatory epithelial tissues and then move to other tissues in the body through blood vessels [2,3]. In other words, intraoral microorganisms can affect the entire body, which is supported by a recent study that suggests treatment of periodontitis could alleviate systemic diseases such as diabetes, hypertension, and cardiovascular disease [4]. Due to the clinical association between systemic diseases and periodontitis, periodontitis can cause the onset or exacerbation of systemic disease in patients with underlying diseases, as well as in those without diseases. Thus, it is essential to diagnose and treat periodontitis in the early stage to prevent periodontitis-induced systemic diseases. Currently, the diagnosis of periodontitis is performed by clinical examination and interpretation of radiographic images [5]. It is still difficult for clinicians to use histopathological or microbiological methods to diagnose periodontitis.

Microbiological studies related to periodontitis have been conducted. For example, the bacterial strains classified as "red complex," including *Porphyromonas gingivalis*, *Tannerella forsythia,* and *Prevotella intermedia* were frequently found in plaques of periodontitis patients, which were reported to be major causative bacterial strains of periodontitis [6]. In previous microbiological studies, genes were isolated from culturable bacteria and identified by Sanger sequencing (the first-generation sequencing method), which could be applied only to limited bacterial species, making it difficult to select candidate bacterial strains that could be used for microbial networks or biomarkers of certain phenomena [7]. After the appearance of next-generation sequencing (NGS) technology, it became possible to classify a broad range of bacteria, including unculturable bacteria, through metagenomic sequencing platforms with bacterial 16S ribosomal RNA (rRNA) gene sequencing or whole-metagenome shotgun sequencing [8]. The 16S rRNA gene sequencing platform is most widely applied for various microbiome studies. This platform is the most popular for researchers because it is less costly and analytically time-consuming than other metagenomic sequencing platforms, since it reads the sequence information of some or all variable regions (V4, V3-V4, or V1-V9) on the bacterial 16S rRNA gene [9-11]. Following recent advances in NGS and continuous human microbiome studies, it was found that the microbiome composition may be related to the onset of various human diseases, which led to attempts to apply metagenomic sequencing to the diagnosis of bacterial diseases [12,13].

The conventional method of identifying causative bacteria for periodontitis was to collect the bacterial biofilm of a plaque sample from a subgingival periodontal pocket. However,

clinicians with professional skills must be involved in collecting the required sub-gingival plaque, and this invasive procedure makes patients uncomfortable. Preceding studies reported that periodontitis-related bacteria were detected not only in the periodontal pocket, but also in other areas, including the oral mucosa [8,14]. Moreover, few studies have conducted comparative analyses between the microbiota of the supra-gingival plaque, sub-gingival plaque, and saliva.

In this study, to identify and compare the composition of the intraoral microbiomes between healthy individuals and periodontitis patients through a comprehensive microbiome analysis using 16S V3-V4 metagenomic sequencing, we collected intraoral saliva as well as supra-gingival and sub-gingival plaque. The diversity of microbiota from the paired samples in each group was comparatively analyzed to examine how the saliva and supra-gingival plaque samples differed from the plaque samples in the sub-gingival area and to explore the composition and frequency of various microorganisms related to oral diseases, including periodontitis, based on the complex oral microbiota. Finally, through a comparative microbiome analysis, we evaluated the question of which alternative clinical samples could solve some sampling issues (e.g., patient discomfort caused by invasive sampling) that can occur during the collection of sub-gingival plaque samples, which are considered the main basis for microbial diagnoses in patients with periodontal diseases.

MATERIALS AND METHODS

Subjects and clinical examination
All clinical experiments conducted for this study were approved by the Institutional Review Board of Wonkwang University Daejeon Dental Hospital (W2002/003-001). Of those 18 years or older who visited the Department of Periodontics, a total of 20 patients (10 in the control group and 10 in the periodontitis group) participated in this study. Before the clinical examination, patient information, including medical history, was collected through an interview and questionnaire. The exclusion criteria were as follows: patients who rejected participation in this study, those who had systemic diseases with potential periodontal effects, patients with a severe mental disorder, patients with substance abuse, patients who were pregnant, or patients who had active periodontal treatments (scaling, root planing, etc.) or took antibiotics within the past 6 months. All clinical examinations were performed by a dentist, who measured pocket depth (PD), clinical attachment loss (CAL), gingival index (GI), and plaque index (PI) through the full arch. The case group (periodontitis) included patients with severe periodontitis with at least 5 mm of CAL in more than one tooth (stage 3 or 4 periodontitis in the 2018 American Academy of Periodontology [AAP] classification). The control group had patients whose periodontal PD and CAL were 3 mm or less and had less than 20% bleeding on probing (corresponding to the conditions of periodontal health and gingival disease in the 2018 AAP classification).

Clinical oral sample collection
We collected 3 types (supra-gingival plaque, sub-gingival plaque, and saliva) of oral samples from subjects corresponding to each comparison group. Sub-gingival plaque samples were only collected from the periodontitis group because it is hard to find dental calculus or plaque in the gingival sulcus of healthy individuals. Before collecting the saliva and plaque samples, all patients were instructed to fast for at least 3 hours and to refrain from oral hygiene. After rubbing the mouth wall 2–3 times, non-stimulating saliva samples were

collected using an NB gene saliva kit (Noble Biosciences, Hwaseong, Korea), followed by storage at −80°C. The supra-gingival plaque samples were collected from the mandibular anterior region and the buccolingual region of the maxillary and mandibular first and second molars using a curette after removing moisture from the regions using a cotton roll. The sub-gingival plaque was carefully collected from regions with at least 5 mm of PD using a curette.

Microbial genomic DNA isolation

All microbial genomic DNAs (mDNAs) were extracted from the clinical samples using the QIAamp PowerFecal DNA Kit (Qiagen, Hilden, Germany) following the protocol of the kit. An Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) checked the quality of the mDNA, followed by storage at 4°C until use.

Illumina sequencing library construction and 16S V3-V4 metagenomic sequencing

For sequencing each bacterial 16S rRNA gene, the case process for constructing the Illumina 16S metagenomic sequencing library was conducted using the Illumina official protocol (Illumina, San Diego, CA, USA). The present study targeted the V3-V4 hypervariable region on the bacterial 16S rRNA gene for metagenomic sequencing. The region was amplified by polymerase chain reaction (PCR) using KAPA Hot Start Ready Mix (2X) (Roche, Basel, Switzerland). A pair of amplicon primers targeting the 16S V3-V4 region was used for PCR amplification as recommended by Illumina. After PCR amplification, PCR products were purified using an AMPure XP bead (Beckman Coulter, Pasadena, CA, USA). To construct the 16S metagenomic sequencing library, additional PCR amplification was performed using Nextera XT Index Kit (Illumina), which contained the Illumina multiplexing dual index and sequencing adapter. Thereafter, PCR products were purified again by using an AMPure XP bead. Each sequencing library was subjected to metagenomic sequencing following the paired-end 2 ×300 bp Illumina MiSeq™ protocol.

16S metagenomic sequencing data analysis

The raw sequencing data generated from a MiSeq sequencer (Illumina) were applied to an analysis using the QIIME2™ microbiome bioinformatics analysis pipeline for the present oral microbiome study (**Supplementary Table 1**). All raw input data were converted to QIIME2 artifacts (.qza format) for compatibility with the QIIME2 pipeline. Next, amplicon sequence variant (ASV) data were created using the Divisive Amplicon Denoising Algorithm 2 (DADA2; **Supplementary Table 2**). Following denoising through the DADA2 to detect errors in amplicon sequencing data, unnecessary sequences were corrected, and potential chimeric sequences were filtered; the generated ASVs were utilized for bacterial classification, in which the sklearn-based Naïve Bayes classifier trained from the SILVA v138 99% 16S ribosomal RNA database was applied (**Supplementary Tables 3-5**). Alpha-diversity analysis representing the microbial richness and evenness of the microbiome present in the sample was conducted using the diversity analysis package included in the QIIME2 plugin and then visualized by box plots using the R bioinformatics package. The corresponding diversity score was estimated using 5 alpha-diversity indices (observed_OTUs, Chao 1, Shannon, Simpson, and Pielou_e). These diversity estimation indices were applied to analyze the differences in richness and evenness scores of microbial strains in the samples. The Kruskal-Wallis non-parametric variance and Mann-Whitney non-parametric tests were applied to test statistical significance. Beta-diversity for a comparative analysis of similarity among the microbiota of samples was estimated using the unweighted_UniFrac and the principal coordinate analysis of Bray-Curtis dissimilarity. Based on the classification confidence score in the generated microbiota

classification table, relative bacterial abundance was also analyzed using the average composition and frequency of the classified microorganisms in each group. Additionally, we performed linear discriminant analysis (LDA) coupled with the effect size to classify distinct bacterial taxa that showed a significant frequency difference between comparison groups. The threshold of the logarithmic LDA score for discriminative features was set to 2.0 (indicating a significant differential abundance).

RESULTS

Demographic characteristics and clinical parameters of subjects

We performed a comprehensive microbiome analysis for comparing the microbial composition within the human oral cavity in healthy (n=10) and periodontitis patient (n=10) groups using the 16S V3-V4 metagenomic sequencing method (**Figure 1**). The average age of subjects was 31.1 years for the control group and 43.8 years for the case group (i.e., the case group was older on average), and 2 patients in the control group and 4 patients in the case group were smokers. First, we collected 3 types (supra-gingival plaque, sub-gingival plaque, and saliva) of oral samples from subjects corresponding to each comparison group. Sub-gingival plaque samples were collected only from the case group because it is difficult to find dental calculus or plaque in the periodontal pocket in healthy individuals (without periodontitis). Based on these considerations, we collected a total of 50 clinical oral samples, including 20 saliva, 20 supra-gingival, and 10 sub-gingival plaque samples. For the PD, CAL, GI, and PI values, the difference between 2 groups was assessed using the Mann-Whitney U test. In the case group, the PD and CAL values were calculated only in the spots where the
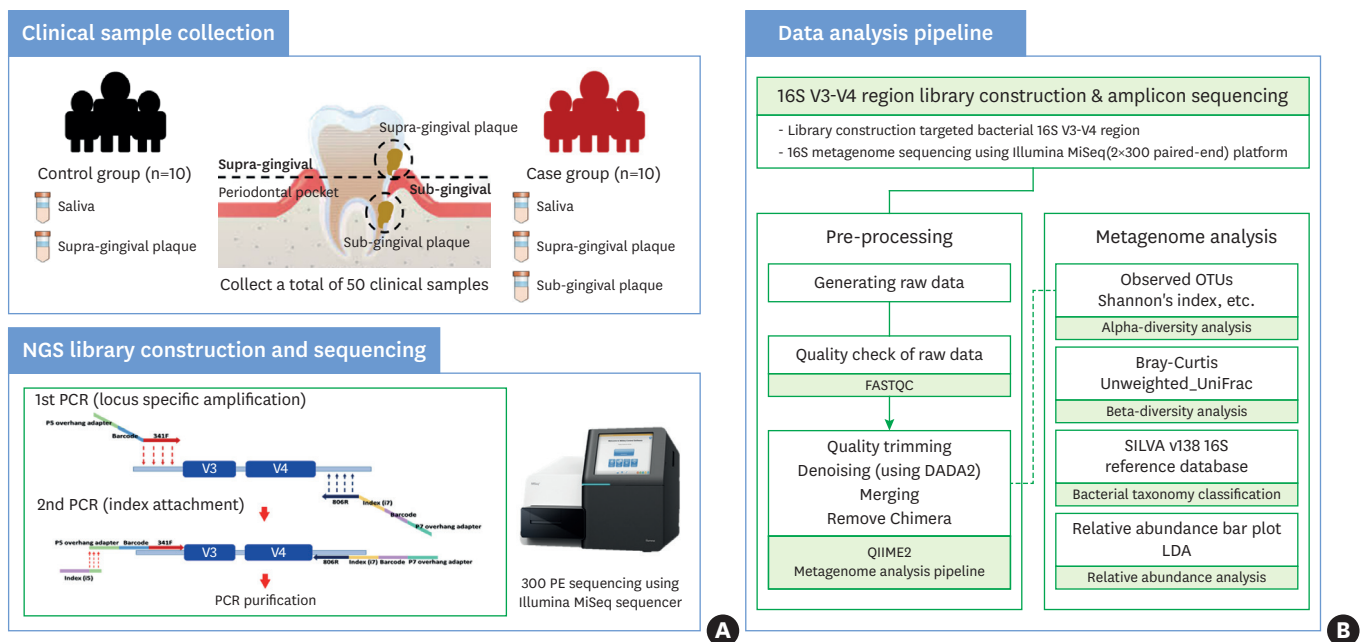
Figure 1. Overall schematic workflow showing the experimental and metagenomic data processing in this study. We conducted a comprehensive 16S microbiome analysis using oral specimens (supra-gingival plaque, sub-gingival plaque, and saliva) collected from each of 10 subjects in the healthy group and periodontitis group to compare oral microbial compositions in periodontitis-related sampling sites. (A) Overall experimental process of the oral specimen collection and 16S V3-V4 sequencing library preparation for metagenomic sequencing. (B) Schematic diagram showing pre-processing for sequencing data and the bioinformatic process for 16S microbiome analysis.
LDA: linear discriminant analysis, OUT: operational taxonomic unit, PCR: polymerase chain reaction.

Table 1. Demographic characteristics and clinical parameters of periodontal disease group and healthy group

Characteristic	Control group (healthy)	Test group (periodontitis)	Statistical significance
Sex			
Male	6	7	
Female	4	3	
Age (yr)	31.1[a]±10.64	43.8±13.40	$P<0.01$
Examination			
PD (mm)	2.19±0.61	5.86±2.17	$P<0.01$
CAL (mm)	2.21±0.62	6.11±2.04	$P<0.01$
GI	0.18±0.45	1.49±0.95	$P<0.01$
PI	0.36±0.61	1.36±0.93	$P<0.01$
Smoking	2	4	

Values are presented as mean±standard deviation.
PD: probing depth, CAL: clinical attachment loss, GI: gingival index, PI: plaque index.

pocket depth was more than 5 mm. As a result of sampling, we confirmed that the average PD, CAL, GI, and PI values calculated in the clinical examinations were significantly higher (*P*<0.01) in the case group than in the control group (**Table 1**).

16S V3-V4 sequencing data processing for microbiome analysis

To conduct the NGS-based 16S V3-V4 metagenomic sequencing, we extracted mDNA from all previously collected clinical samples. To minimize the potential for misclassification of the microbial composition within each sample, we thoroughly checked the quality of the mDNA (e.g., DNA degradation, concentration, and purity). Next, we successfully constructed sequencing libraries targeting the bacterial 16S V3-V4 hypervariable region using all mDNA samples and produced sequencing data. As a result of sequencing, the average demultiplexed reads count output of all samples was 43,479, of which 20,854 denoised reads were filtered through low-quality data using the DADA2 pipeline (e.g., removing chimeric sequences; **Supplementary Table 1**). Finally, the average pre-processed read count (e.g., non-chimeric reads or base calling quality score more than the Q30) applied for 16S microbiome analysis was 13,848. Based on the data on pre-processed reads, the bacterial ASV taxonomy number, classified using the SILVA v138 16S rRNA gene reference database, was 1362 (sequence alignment more than the 70% confidence threshold value; **Supplementary Table 2**).

Microbial diversity analysis (alpha and beta)

To investigate the microbial community diversity of the oral samples, the Kruskal-Wallis non-parametric analysis of variance test was used to compare richness and evenness based on alpha-diversity indices, including observed_OTUs, Chao 1, Shannon, Simpson, and Pielou_e (**Figure 2**). When the observed_OTUs and Chao 1 diversity indices were applied, focusing on the microbial richness of the collected supra-gingival samples, there were no significant differences between the case group and the control group (**Figure 2A**). Similarly, there were also no significant differences in the alpha-diversity indices measured based on Shannon, Simpson, and Pielou_e to test both richness and evenness (**Table 2**). While the supra-gingival samples collected from the control group and the case group had a similar richness of microbial community, the case group had less evenness and lower overall mean values than the control group. Likewise, there were no statistically significant differences in alpha-diversity for each of the collected saliva samples between the case group and the control group in terms of richness and evenness (**Figure 2B**, **Table 2**). However, the case group showed higher estimated values than the control group. Lastly, alpha-diversity in the supra-gingival, sub-gingival, and saliva samples collected from the case group was compared by the Mann-Whitney test (**Figure 2C**, **Table 3**). The saliva samples of the case group had higher mean alpha-diversity values of the microbial community than the supra-gingival samples.
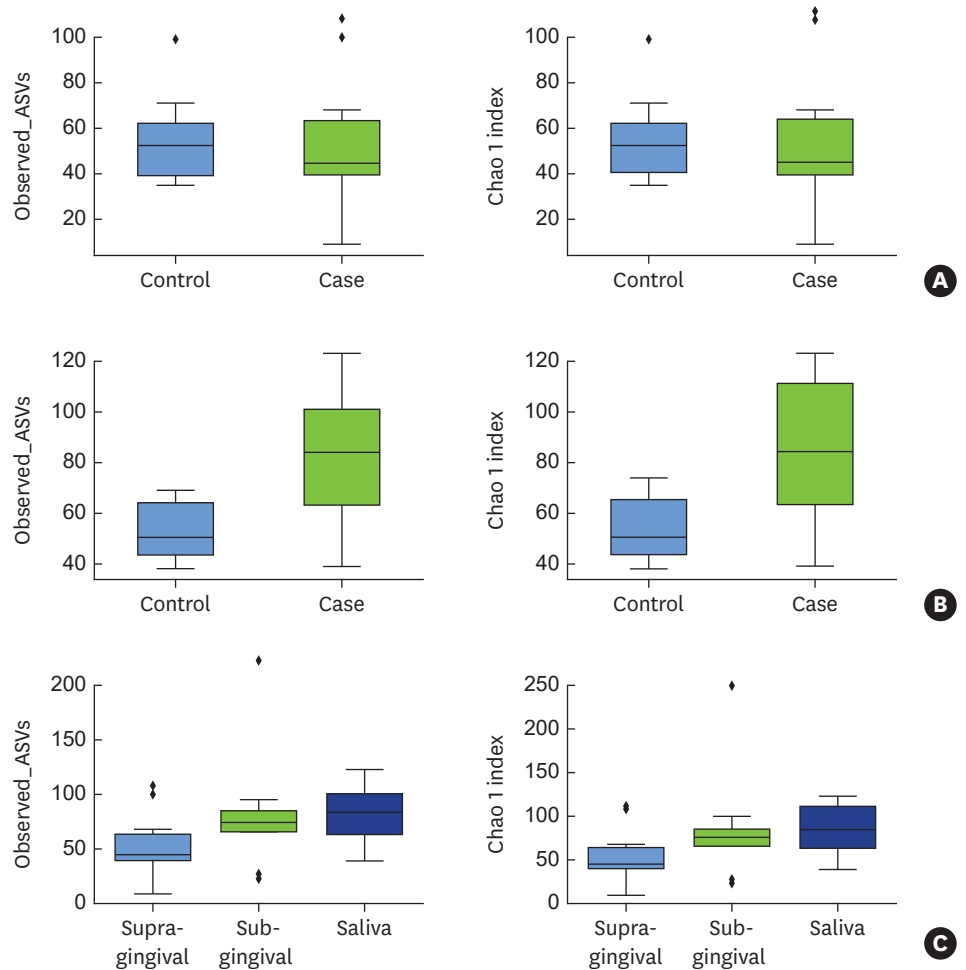
Figure 2. Box plots showing the alpha-diversity estimation scores between 2 comparison groups and each sampling site within the case group, which were calculated using the Observed_ASVs and Chao 1 alpha-diversity indices. (A) In supra-gingival plaque samples: the control group versus the case group. (B) In saliva samples: the control group versus the case group. (C) Within the case group: supra-gingival plaque versus sub-gingival plaque versus saliva samples.

Table 2. Alpha-diversity analysis of bacterial communities between the 2 comparison groups

Alpha-diversity index	Control	Case	P value
Supra-gingival plaque samples			
Observed_OTUs	42.9±16.35	41.8±27.62	n.s.
Chao1	43.2±16.23	43.48±30.55	n.s.
Pielou_e	0.79±0.08	0.63±0.21	n.s.
Simpson	0.9±0.08	0.75±0.22	n.s.
Shannon	4.25±0.8	3.29±1.43	n.s.
Saliva samples			
Observed_OTUs[a]	42.9±11.07	62.5±24.16	n.s.
Chao1[a]	43.34±11.45	63.79±25.62	n.s.
Pielou_e[b]	0.78±0.04	0.76±0.15	n.s.
Simpson[b]	0.9±0.03	0.88±0.13	n.s.
Shannon[b]	4.2±0.39	4.49±1.17	n.s.

Values are presented as mean±standard deviation. The Mann-Whitney test was used to assess the statistical significance of differences according to disease status.
n.s.: not significant.
[a]Richness: Observed_OTUs, Chao1 diversity index; [b]Richness & Evenness: Pielou_e, Simpson, and Shannon diversity index.

Table 3. Alpha-diversities of bacterial communities between sampling sites within the case group

Alpha-diversity index	Sample type					
	Supra-gingival	Sub-gingival	Saliva	Supra-gingival vs. Sub-gingival	Supra-gingival vs. Saliva	Sub-gingival vs. Saliva
Observed_OTUs[a]	41.8±27.62	62.5±24.16	64.5±47.77	n.s.	$P<0.05$	n.s.
Chao1[a]	43.48±30.55	63.79±25.62	69.04±60.03	n.s.	$P<0.05$	n.s.
Pielou_e[b]	0.63±0.21	0.73±0.17	0.76±0.15	n.s.	n.s.	n.s.
Simpson[b]	0.75±0.22	0.85±0.16	0.88±0.13	n.s.	$P<0.05$	n.s.
Shannon[b]	3.29±1.43	4.26±1.38	4.49±1.17	n.s.	$P<0.05$	n.s.

Values are presented as mean±standard deviation. The Mann-Whitney test was used to assess the statistical difference between sampling sites.
n.s.: not significant.
[a]Richness: Observed_OTUs, Chao1 diversity index; [b]Richness & Evenness: Pielou_e, Simpson, and Shannon diversity index.

However, there were no significant differences in alpha-diversity of the microbial community between the sub-gingival and the saliva samples.

Beta-diversity between the control group and case group, as well as for each collected sample type was measured focusing on the unweighted_UniFrac and the PCA method of Bray-Curtis dissimilarity (**Figure 3**, **Table 4**). First, we compared the dissimilarity distance of microbial species estimated in the supra-gingival and saliva samples between 2 different comparison groups (**Figure 3A and B**). In the case of the sub-gingival sample, this comparison was limited because it was collected only in the case group (as mentioned above in the first part of the RESULTS section). We confirmed through this comparison that microbial clustering was clearly distinguished between the comparison groups within the 2 different sample types (Bray-Curtis: $P<0.05$; unweighted_UniFrac: $P<0.05$). Next, we compared the microbial clustering for each sample type in the case group (**Figure 3C**). A comparison within the case group confirmed that the other sample types had little differences in microbial clustering from the sub-gingival plaque samples.

Relative abundance in the human oral microbiota

To confirm the relative microbial composition classified from each oral sample, we compared the bacterial classification at the phylum and species level between the 2 comparison groups (except for the sub-gingival plaque sample) and within the case group (all sample types; **Figures 4** and **5**, **Tables 5** and **6**, **Supplementary Tables 3-5**). At the phylum level, *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Fusobacteria* phyla generally exhibited high relative frequencies; in particular, the sub-gingival and saliva samples within the case group showed more similar microbial compositions to each other than to the supra-gingival sample. At the species level, we identified obvious differences in the relative microbial proportion associated with periodontitis through comparisons of both supra-gingival plaque and saliva samples between the 2 comparison groups. In the supra-gingival plaque samples, *Streptococcus sanguinis* and *Corynebacterium durum* showed lower relative frequencies in the case

Table 4. Statistical results of beta-diversity analysis for each comparison group

Beta-diversity matrix	R[2]	P value
Supra-gingival plaque samples		
Bray-Curtis	0.110022561	<0.01
Unweighted_UniFrac	0.094794984	<0.05
Saliva samples		
Bray-Curtis	0.09732198	<0.01
Unweighted_UniFrac	0.177864779	<0.01
Within the case group		
Bray-Curtis	0.081274976	n.s.
Unweighted_UniFrac	0.103469133	<0.05

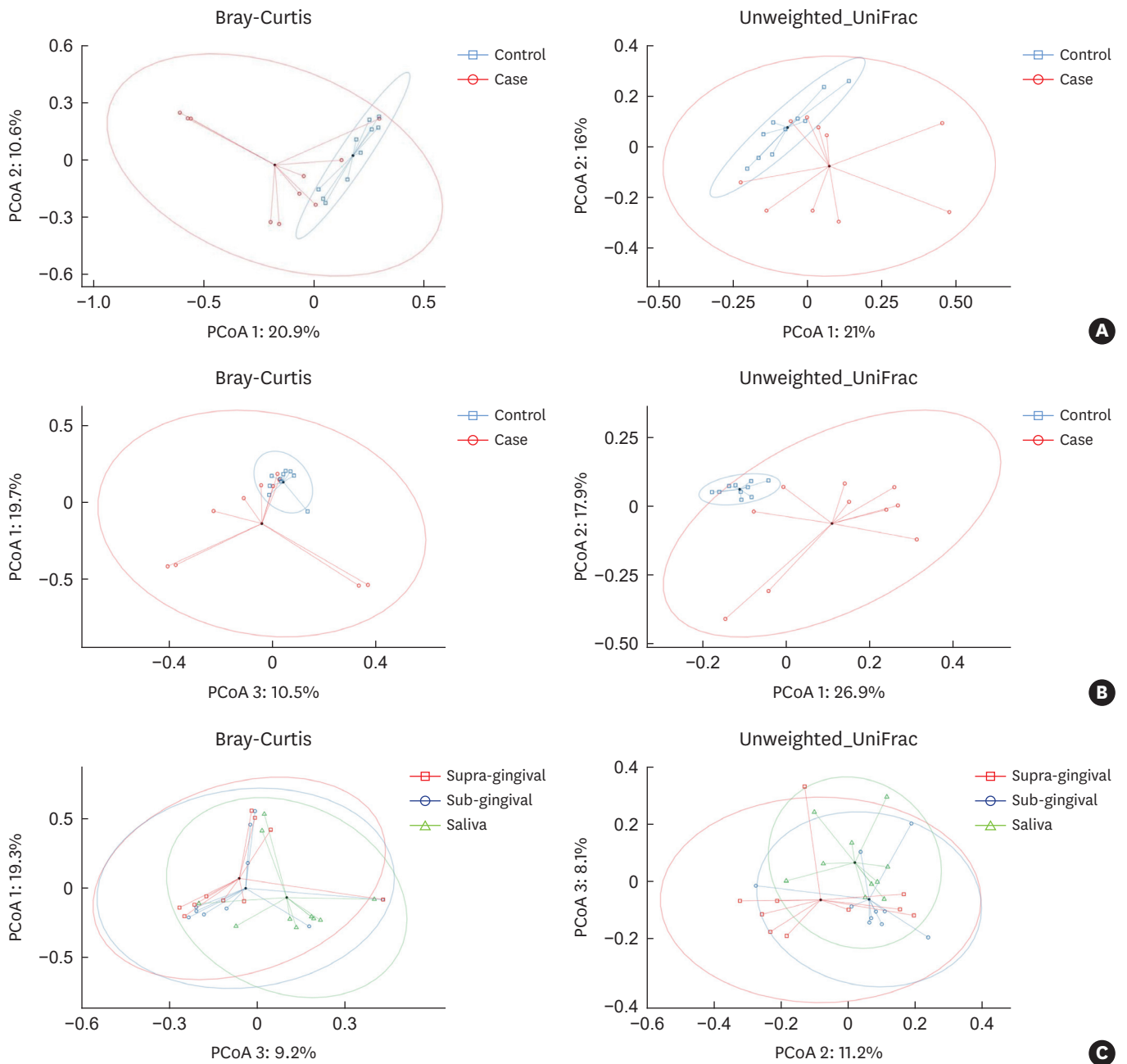R^2: correlation rho factor, n.s.: not significant.

Figure 3. Bacterial beta-diversity calculated using principal coordinate analysis based on the Bray-Curtis and unweighted_UniFrac distance matrices. (A) In supra-gingival plaque samples. (B) In the saliva samples. (C) In each sampling site within the case group.

group than in the control group. The relative frequencies of *P. intermedia*, *Selenomonas noxia*, and *Actinomyces massiliensis*, which are associated with periodontal disease occurrence were higher in the case group. In the saliva sample, the relative microbial frequencies of *T. forsythia*, *Treponema denticola*, and *P. intermedia* were higher in the case group than in the control group. In particular, we confirmed that the relative frequencies of *P. gingivalis, T. denticola,* and *T. forsythia* were statistically significantly higher (*P*=0.002) in the case group than in the control group. Next, the relative frequency of *Haemophilus parainfluenzae* showed the statistically most significant difference (*P*=0.001) between the 2 different comparison groups. However, the
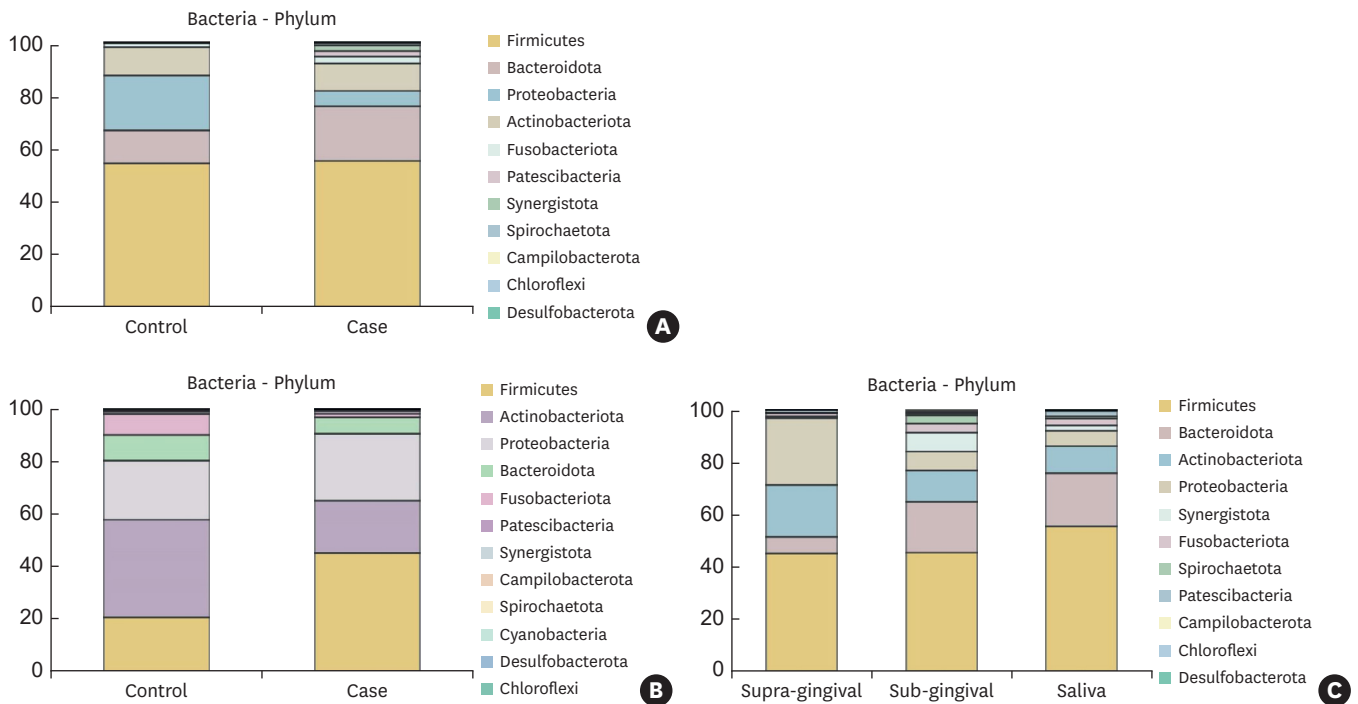
Figure 4. A relative abundance bar plot showing the relative bacterial proportions at the phylum level between 2 comparison groups and each sampling site within the case group. (A) In supra-gingival plaque samples: the control group versus the case group. (B) In saliva samples: the control group versus the case group. (C) Within the case group: supra-gingival plaque vs. sub-gingival plaque vs. saliva samples.

proportions of the pathogenic bacterial strains within the supra-gingival plaque and saliva samples were lower than in the sub-gingival sample. However, the core bacterial strains (including *P. gingivalis*) for the microbial diagnosis of periodontitis were more frequently identified within the saliva sample than in the supra-gingival plaque sample.

Comparison of differences in bacterial strains at the species level

To compare the differences in the classified bacterial taxonomy at the species level between comparison groups (in supra-gingival plaque, saliva, and within the case group), we investigated bacterial abundance using LDA (**Figure 6**). In the supra-gingival sample, only 7 of the classified 169 bacterial species showed significant differences between the case and the control group (**Figure 6A**). However, there were significant differences in 20 bacterial species in the saliva sample, with a relative frequency difference between the 2 different comparison groups among the 139 classified bacterial species (**Figure 6B**). Within the case group, we cross-validated through LDA that the relative frequency of infectious bacterial strains associated with periodontal disease was higher than that of other samples in the sub-gingival plaque of each sample type (**Figure 6C**). Among the 242 classified bacterial species, 19 bacterial species showed significant differences within the case group.

DISCUSSION

Periodontal diseases are chronic inflammatory diseases that tend to induce the repeated formation of periodontal pockets and destruction of the alveolar bone due to bacterial colonies. It is widely known that periodontal diseases deteriorate with changes in the microbial

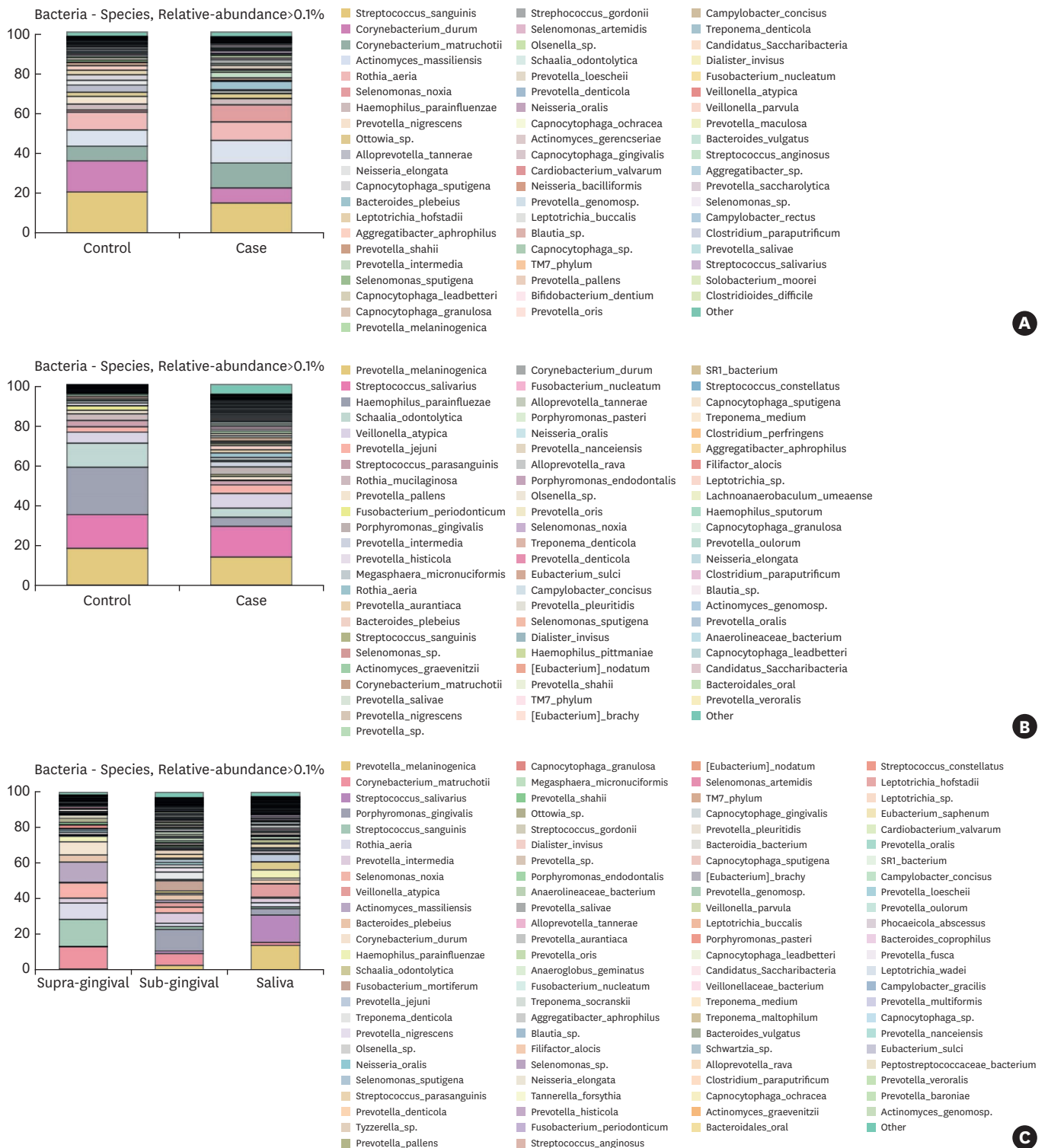**Figure 5.** The relative abundance bar plot shows the relative bacterial proportion at the genus level between two comparison groups and each sampling site within the case group. (A) In supra-gingival plaque samples: the control group versus the case group. (B) In saliva samples: the control group versus the case group. (C) Within case group: supra-gingival plaque versus sub-gingival plaque versus saliva samples.

Table 5. Average relative frequency of the core microbiome associated with periodontitis between different comparison groups

Bacterial taxon	Control	Case	P value
Supra-gingival plaque samples			
Streptococcus sanguinis	20.515911	15.083355	n.s.
Corynebacterium durum	15.466772	0.042655	n.s.
Actinomyces massiliensis	8.06943	11.234816	n.s.
Selenomonas noxia	0.984091	8.505496	n.s.
Prevotella intermedia	0	0.006506	n.s.
Saliva samples			
Porphyromonas gingivalis	0	3.71924	<0.05
Tannerella forsythia	0	0.173792	n.s.
Treponema denticola	0	0.589467	<0.05
Prevotella intermedia	0.013797	2.55814	<0.05
Haemophilus parainfluenzae	23.487303	4.557439	<0.05

The Mann-Whitney test was used to assess the statistical difference between disease status.
n.s.: not significant.

Table 6. Relative abundance of the core microbiome associated with periodontitis of each sample site within the case group

Bacterial taxon	Supra-gingival	Sub-gingival	Saliva
Porphyromonas gingivalis	0.047558	12.091163	3.71924
Tannerella forsythia	0.086586	0.984971	0.173792
Treponema denticola	0.193873	4.074156	0.589467
Prevotella intermedia	2.790426	5.794574	2.55814
Fusobacterium nucleatum	0	0.34107	1.09473
Streptococcus mutans	0.011734	0.009383	0.013309

composition [15]. In addition, the sub-gingival environment resulting from the formation of periodontal pockets is different from the oral environment, so its microbial composition is also different. Unlike previously used bacterial culture tests, NGS is not dependent on bacterial culture; therefore, previously unidentified bacterial strains could be detected by 16S rRNA gene sequencing, enabling more in-depth research. This study collected saliva, supra-gingival plaque, and sub-gingival plaque samples from the healthy and periodontitis groups, then characterized and analyzed them through 16S rRNA gene sequencing.

In an alpha-diversity analysis to examine microbial community diversity in the groups, there was no significant difference in saliva samples between the control and case groups; however, the periodontitis group had higher diversity. Preceding studies collected and compared oral mucosa and sub-gingival plaque from control and periodontitis groups, and found the periodontitis group had higher microbial community diversity than the control group [8]. Although the collection sites in our study were different, they showed the same pattern as those in the present study. Periodontal diseases were accompanied by higher microbial community diversity, as shown above. The formation of deeper periodontal pockets due to the destruction of periodontal tissues provides larger spaces for the accumulation of plaque, which in turn serves as an ideal environment for more stable plaque accumulation compared to other collection sites such as supra-gingival plaque or saliva [8]. In addition, saliva samples showed significantly higher alpha-diversity than supra-gingival samples, while there was no significant difference in diversity between sub-gingival and saliva samples. This indicates that the microbial community diversity of the sub-gingival area, which is the main cause of periodontitis, might be similarly estimated in saliva samples, but not in the supra-gingival samples. Beta-diversity was also analyzed to examine the similarity among the microbial communities in the samples. Beta-diversity distance calculations confirmed the presence of identifiable differences in the clustering of estimated microbial composition between the
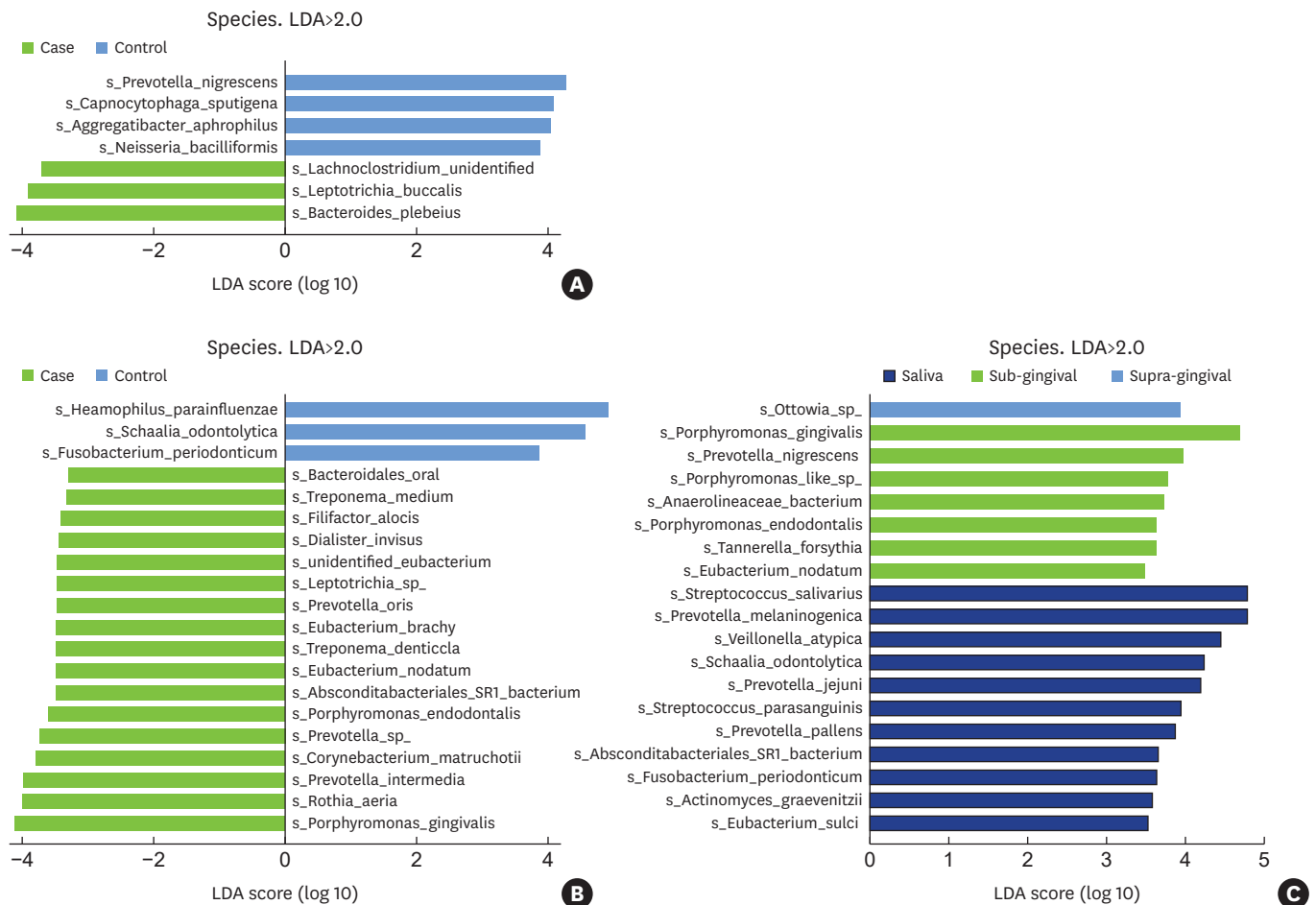
Figure 6. LDA effect size (LEfSe) analysis of supra-gingiva samples. The threshold on the logarithmic LDA score for discriminative features was set to 2.0. This bar graph reflects bacterial species with a significant difference in relative frequency between each group. (A) In supra-gingival plaque samples: the control group versus the case group. (B) In saliva samples: the control group versus the case group (C) Within case group: supra-gingival plaque versus sub-gingival plaque versus saliva samples.
LDA: linear discriminant analysis.

control and periodontitis groups in the supra-gingival plaque and saliva samples. Between each oral sample type in the periodontitis group, the distance dissimilarity calculated using the Bray-Curtis matrix (considering microbial abundance) between the 3 types for estimated microbial composition showed no significance. However, in the unweighted_UniFrac matrix (considering phylogeny), the dissimilarity in estimated microbial composition between each sample type was significant ($P<0.05$). Based on these microbial diversity analyses, we performed a relative abundance analysis to classify the microbial taxonomy within each oral sample type to identify differences in the microbial composition between each comparison group.

Preceding studies reported that the buccal mucosa and sub-gingival area were occupied mainly by 5 significant phyla (*Firmicutes, Proteobacteria, Bacteroidetes, Actionobacteria,* and *Fusobacteria*) [16]. When the relative bacterial abundance at the phylum level was examined between the normal group and the periodontitis group in the present study, the above 5 phyla also accounted for high proportions. *Firmicutes* showed the highest proportion in all groups, except the supra-gingival plaque samples from the control group. In the periodontitis group, *Bacteroidetes* and *Synergistota* were observed more often in the sub-gingival and saliva samples than in the supra-gingival samples. On the contrary, the supra-gingival samples had higher proportions

of *Proteobacteria* and *Actinobacteria*. While the above 5 phyla were commonly detected, their compositions differed across studies, which seemed to be attributable to differences in the sample collection site, collection method, and patient group. The collection site and collection method should be standardized to apply the phylum information clinically. We confirmed that the early oral microbial colonies and beneficial groups dominated the control group at the species level. *S. sanguinis* and *C. durum* are known to inhibit the proliferation of infectious bacterial strains related to periodontitis and positively change the environment in the oral cavity through the metabolites they produce [17,18]. In contrast, opportunistic infection bacteria that can induce various periodontitis, including *P. gingivalis*, dominated within the case group. Several studies have reported that bacteria known as periodontal pathogens, such as *P. gingivalis*, were more abundant in patients with periodontal diseases than in healthy people [19-21], and Chen et al. [22] reported a high correlation of *P. gingivalis*, *T. denticola*, and *T. forsythia* with GI, PD, and CAL. In the present study, the periodontitis group, which had higher GI, PD, and CAL values than the control group, contained significantly more pathogenic bacterial strains, including *P. gingivalis,* and *T. denticola*, suggesting that these bacterial strains play important roles in periodontitis.

Particularly, *P. gingivalis, T. denticola,* and *P. intermedia* were detected significantly more frequently in the saliva samples of the periodontitis group than in the control group, unlike the supra-gingival samples. Among them, *P. gingivalis* showed the most considerable difference. Although the saliva samples had lower measurement values than the sub-gingival samples, they displayed a similar pattern, which is consistent with the findings of a previous study that showed significant differences in the abundance of *P. gingivalis*, *Treponema forsythia*, and *T. denticola* between the periodontitis group and the control group [23].

In LDA, saliva samples showed a difference in the frequency of "red complex" strains associated with periodontal diseases such as *P. gingivalis* and *T. denticola*, mirroring the results of the relative abundance analysis, which showed an evident difference between the 2 comparison groups. Within the case group, some normal flora (such as *Streptococcus parasanguinis*) in the oral cavity was distinctly classified within the saliva sample, and various bacterial species were identified more often than in the supra-gingival plaque sample, including opportunistic pathogens (such as *Prevotella melaninogenica*, *Veillonella atypica*, *Prevotella pallens*, and *Fusobacterium periodonticum*) associated with periodontal disease [24-27]. In addition, in previous studies, *Fretibacterium* in the *Synergistetes* phylum was reported to be relatively abundant in the sub-gingival area, and *P. gingivalis* and *P. intermedia*, which are known periodontal pathogens, were detected more in saliva than in the supra-gingival samples [28,29].

In conclusion, the present study demonstrated that when affected by periodontal diseases, the alpha-diversity of bacteria was higher, and this pattern was more prominent in the saliva and sub-gingival area than in the supra-gingival area. Regarding the relative frequency and composition changes in bacterial communities classified based on the 16S rRNA gene database, the saliva and the sub-gingival area were similar. In this respect, it is possible that collecting saliva for the microbial diagnosis of periodontal disease could replace collecting oral plaque samples, alleviating the burdens of sub-gingival sampling for patients. There were some differences in its composition depending on the sample collection site and whether it was affected by periodontal disease. However, the present study should be interpreted in light of the following limitations: it had an insufficient number of subjects; since the control group had no subgingival plaque sample, there were sample differences due to the study design; and there was no investigation of other variables such as smoking, which

is well known as a risk factor for periodontal diseases. Hence, the interaction between risk factors for periodontal diseases and bacteria should be further studied using more samples.

SUPPLEMENTARY MATERIALS

Supplementary Table 1
Statistical results of 16S V3-V4 metagenome sequencing data pre-processing

Click here to view

Supplementary Table 2
Bacterial taxonomy classification results with more 70% confidence threshold

Click here to view

Supplementary Table 3
Relative bacterial frequency data in supra-gingival plaque sample of two different comparison group

Click here to view

Supplementary Table 4
Relative bacterial frequency data in saliva sample of two different comparison group

Click here to view

Supplementary Table 5
Relative bacterial frequency data within case group

Click here to view

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