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Bacterial Community in Dental Biofilms Linked to Periodontal Disease: An Intra-subject Metagenomics Study

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Abstract

Objectives: To study the intra-subject subgingival plaque microbiota composition of gingivitis and periodontitis sites in periodontitis patients using 16s rRNA metagenomics.

Methods: Samples of subgingival plaque were collected from 16 periodontitis participants enrolled on the study. The extracted DNA from dental plaque was sent to the Illumina laboratory for 16s rRNA metagenomics. The V_3 and V_4 region of 16s RNA gene were amplified and sequenced using Illumina technology. After quality filtering, 313480 sequences were obtained and arranged in OTU based on the 97% threshold. The reads were assigned to species.

Results: The alpha diversity analyses revealed that periodontitis sites had more diverse and rich bacterial communities than gingivitis sites. However, the PCoA analysis (beta diversity) did not reveal any clustering of the bacterial community in gingivitis and periodontitis. Taxonomic analysis verified the presence of 56 known species. However, there was no apparent pattern in the bacterial community between gingivitis and periodontitis sites. Yet, a slight distinction was observed. Species like P. intermedia, R. dentocariosa and P. endodontalis were more abundant in gingivitis sites compared to periodontitis sites, while some other species like V. dispar, C. ochracea and A. segnis were more abundant in periodontitis sites.

Conclusion: This study supports the fundamental idea that individual bacterial species are not responsible for the advancement of gingivitis to periodontitis but rather the abundance of bacteria in the bacterial community.

Keywords: Intra-subject, Periodontitis, Gingivitis, Subgingival plaque, 16S RNA Metagenomics.

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INTRODUCTION

Gingivitis and periodontitis are the two most common forms of periodontal diseases linked to bacterial dental biofilm. Gingivitis, the reversible form, causes bleeding and inflamed gums. If gingivitis is left untreated, it can progress to periodontitis, an irreversible condition [1]. Approximately 775 bacterial species have been found in the oral cavity; however, only about 57% are culturable [2]. The possibility exists that periodontal tissue deterioration may be caused partly by bacteria that have never been cultivated or studied before. Thus, researches addressing the entire population of dental biofilm microorganisms are necessary.

Recent years have seen the introduction of high throughput sequencing technologies such as 16S rRNA metagenomics, which are time-efficient, cost-effective, and can also reveal information about non-culturable bacteria. This cutting-edge technology may aid in the study of how biofilm microorganisms contribute to oral disease, especially periodontal disease. Numerous studies have been conducted using 16s RNA metagenomics analysis to gain a better understanding of the oral microbiota [3,4].

It is well-known that the dental biofilm microbiota community varies between periodontally healthy and periodontitis-afflicted individuals [5], but how these bacterial populations induce periodontal disease is unknown. To further understand the role of microbiota in periodontal disease, numerous metagenomic analyses of the biofilm microbiota of healthy and periodontitis-affected individuals were designed [4,6]. However, the findings of these approaches could be questioned because the composition of the oral

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microbiota is influenced by a variety of individual factors, including lifestyle, diet, oral care habits, oral disease or systemic disease, host immune response, genetic susceptibility, and location in the oral cavity [2]. To eliminate all of these confounding factors, internal comparisons within subjects should be conducted. To date, only a few metagenomics studies have compared the biofilm microbial community within subjects, either among healthy sites [2] or between healthy and periodontitis-affected sites [4,7]. We didn't come across any studies that compared gingivitis and periodontitis-affected sites. Thus, the objective of this study was to evaluate the microbial communities of gingivitis and periodontitis sites within the same periodontitis subjects using 16s RNA metagenomics, where microbial community diversity, clustering, composition, and relative abundance were compared.

MATERIALS AND METHODS

Subject selection

This study recruited 16 periodontitis patients (9 males and 7 females) from Hospital Universiti Sains Malaysia. The ages of the subjects ranged from 42 to 62 years. Inclusion was determined based on the following criteria: (1) clinical probing depth greater than 5mm in 50% of teeth; (2) lost more than 30% of alveolar bone; (3) had more than 20 teeth in dentition; (4) had not received periodontal therapy in the previous 6 months; (5) had no systemic disease; (6) had not received antibiotic treatment in the previous 3 months; and (7) was neither pregnant nor nursing. Ethical approval was granted by Universiti Sains Malaysia (Ethical Ref Number: USM/JEPeM/15100370). The written informed consent was taken from all participants. The research had been carried out in accordance with the declaration of Helsinki.

A single examiner performed a full-mouth periodontal examination on six tooth surfaces mesiobuccal, buccal, distobuccal, distolingual, lingual, and mesiolingual. The periodontal pocket depth (PPD) and bleeding on probing (BOP) were measured using the University of North Carolina probe (UNC-15, Hu-Friedy, Chicago, IL, USA).

The PPD was determined by measuring the distance between the free gingival margin and the periodontal pocket base. During probing, sites were examined for the presence or absence of bleeding to quantify Bleeding On Probing (BOP). For each subject, three teeth with pocket depth (PD) on gingivitis sites (PD ≤ 3 mm) and three with periodontitis sites (PD ≥ 5 mm) were selected for subgingival plaque sample collection.

Subgingival plaque sample collection

Teeth and gingiva were air-dried before being separated with cotton rolls. A Gracey curette was initially used to remove a supragingival plaque (Hu-Friedy, USA). Subgingival plaque was then collected using a new sterile curette by inserting it parallel to the long axis of the tooth into the deepest part of the pocket and then moving coronally, scraping along the root surface. Subgingival plaque was subsequently dispersed in a 1.5 ml microcentrifuge tube containing 0.5 ml Tris-EDTA buffer solution (Sigma Aldrich, Germany). For the gingivitis and periodontitis site of each subject, dental plaque from three teeth was pooled and stored in a single centrifuge at -80° C until needed.

DNA extraction

The DNA was extracted from dental plaque samples using the Epicentre Masterpure DNA Purification Kit (Cambio, Irvine, UK), following the manufacturer's instructions. The concentration of extracted DNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific-GE, USA), and only DNA with a 260/280 ratio between 1.8 and 2.0 was included in the study. Gel electrophoresis was used to further verify the DNA's integrity. Sixteen gingivitis and fourteen periodontitis samples yielded DNA in sufficient quantity and quality were selected for 16s metagenomic analysis using Illumina technology. DNA plaque samples were kept at -80° C before being sent to the Illumina MiSeq lab (Malaysian Genomic Institute, Kajang Selangor, Malaysia) for Illumina sequencing.

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PCR Amplification and Sequencing Analysis

We used the following primers to amplify the V3 and V4 region of 16S rRNA gene [8]:

Forward primer:

5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

Reverse primer:

5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

PCR reaction was performed using ReadyMix PCR Kit, KAPA HiFi HotStart DNA Polymerase (Roche, Switzerland). Briefly, a 25µl PCR reaction was prepared by adding 2.5µl of DNA sample, 5µl of each forward and reverse primers, and 12.5µl 2x KAPA ready mix (Roche, Switzerland). Thermal cycling was set to consist of an initial denaturation at 95 °C for 3 min, followed by 25 cycles of denaturation at 95° C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 30 sec. The reaction was completed after a 5-minute extension at 72 ºC. The PCR amplicons were then purified using AMPure XP beads (Beckman Coulter, USA). Nextera XT Index Kit (Illumina, San Diego, USA) was used to add specific oligonucleotides to the amplicons, and then AMPure XP beads were used to purify the amplicons (Beckman Coulter, USA). The quality and size of the library were assessed using Agilent 2100 bioanalyzer (Agilent Technologies, USA). The library sequencing was performed on a sequencing platform (MiSeq System, Illumina, USA) using a Miseq v3 600 cycles kit (Illumina, USA). Following sequencing, image analysis and base calling were performed using MiSeq Reporter software (Illumina, USA), with raw sequencing data recorded in FASTQ format.

Bioinformatic analysis

The processing for 16S sequence data was performed on the bioinformatics pipeline, Quantitative Insights into Microbial Ecology (QIIME) software v1.9.1[9].

The raw metagenomic sequence read adapters were removed using paired-end trimmer PEAT v1.2.[10]. The forward and reverse reads were merged with paired read merger BBMerge v7.3 [11]. The sequence shorter than 100 bp, with Phred score lower than 20 were removed by FASTX-Toolkit v 0.0.13. The remaining sequences were used for de novo binning of operational taxonomic units (OTUs) based on sequence similarity followed by selection of representative OTUs per bin. The tags were clustered into OTUs using QIIME at 97% sequence similarity. The 16S Metagenomics workflow classified organisms based on V3 and V4 amplicons using 16S rRNA data database. The taxonomic classification was assigned using the greengene database version 13.8 [12]. Alpha and beta diversity were computed on the basis of a previously constructed OTU table by QIIME. Bacterial species with a prevalence of less than 0.1% were excluded [13]. Bacterial species were ranked in groups to obtain relative abundance. The percentage of total abundance in each group was estimated by combining the abundance of individual components in each Taxa. Only genera and species were analyzed in this paper.

Statistical analysis

The Shapiro-Wilk test was used to analyse the normality of the data. The independent sample t-test or Mann-Whitney U test was used to identify the statistically significant differences between the patient's clinical variables, age, α-diversity, and taxonomic ranks. All these statistical tests were performed using IBM SPSS version 25.

An OTU table was used for alpha diversity indices calculation, where Chao1 was used as the richness estimator, and Shannon was used to representing evenness and diversity. Beta diversity was determined by computing distance matrices, which were represented by weighted Unifrac distances that consider both abundance and phylogenetic distances among taxa [14]. It was used to construct the hierarchal clustering, which was calculated on QIIME and constructed on PAST software. The principal coordinates analysis (PCoA) was performed on the OTU level to evaluate the similarity of microbial community structure on

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EMPEROR software. The non-parametric permutational multivariate analysis of variance (PERMANOVA) was used to measure the multivariate community-level difference between the gingivitis and periodontitis groups [15]. This analysis was performed using QIIME software v1.9.1.

RESULTS

Demographic and periodontal parameters of study subjects

The study included sixteen periodontitis patients, nine males and seven females. The average age of the subjects was 46.3 years, ranging from 31 to 62 years. The median and interquartile range for subjects' PPD (mm) and BOP (%) are 5.6 (0.9mm) and 85 (10%), respectively. The periodontal parameters of the teeth involved in the collection of dental plaque samples are shown in Table 1.

Table 1. Periodontal parameters of teeth selected for dental plaque sample collection

		Gingivitis (n = 96)	Periodontitis (n = 96)	*p* - value*
Periodontal Parameters				
	PPD (mm)	2.5 (0.7)	5.6 (0.8)	<0.001
	BOP (%)	21 (5)	80 (19)	<0.001

The parameters are presented as median and interquartile range (IQR). The significance level was set at $p = 0.05$, and n = 96.
* = Mann-Whitney U test.

16S rRNA metagenomics

This study used 16s RNA metagenomics to analyze the microbiome within the gingivitis and periodontitis sites of the subjects. Each sample from gingivitis and periodontitis sites yielded 50K sequences in total. After merging, filtering, and deleting chimaeras, a total of 313480 sequences were retrieved.

Alpha diversity

Rarefaction curves were generated for the Chao1 index (species richness) and the Shannon index (species evenness), with their values plotting to a plateau, indicating that sufficient sequencing was performed to reveal an excellent representation of microbial community diversity and richness (Figure 1). The Chao1 and Shannon index values were higher in the periodontitis group than in the gingivitis group, indicating a more diverse and balanced microbial community in the periodontitis group. However, these differences were not statistically significant.

Beta diversity

The principal coordinates analysis (PCoA) revealed that the microbial compositions of gingivitis and periodontitis sites do not cluster distinctively (Figure 2). However, a closer examination of the gingivitis and periodontitis samples using a Hierarchical clustering plot indicated a taxonomic cluster link (Figure 3). Not only is the microbial composition of gingivitis and periodontitis samples clustered, but some gingivitis and periodontitis samples have notable similarities. However, in hierarchical clustering, the majority of gingivitis and periodontitis samples from single subjects exhibit a distance in similarity.

The statistical analysis PERMANOVA, using weighted Unifrac distances, revealed that the difference between the abundance of OTU in the gingivitis and periodontitis group was not statistically significant (permutations = 999, p-value = 0.219).

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Figure 1. Rarefaction curve of Chao1 (A) and Shannon (B) of gingivitis and periodontitis samples.

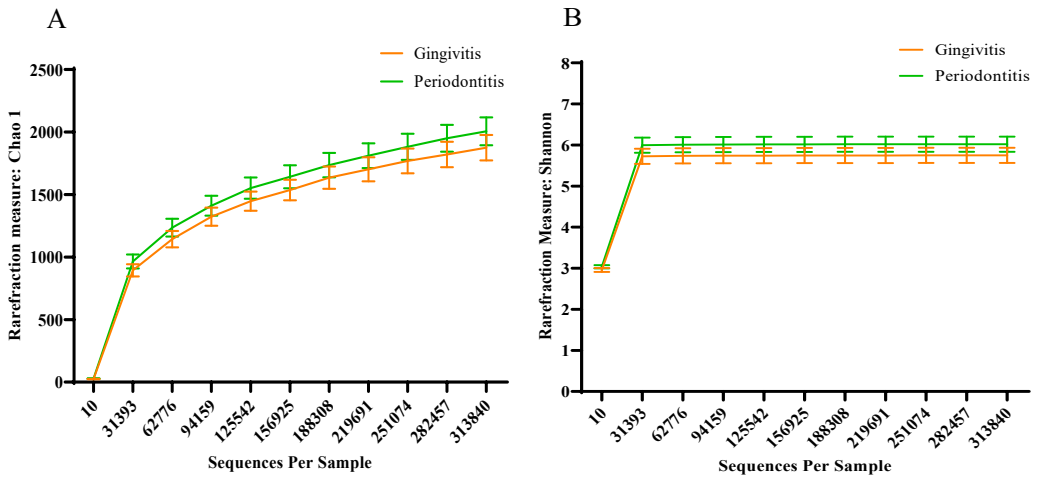
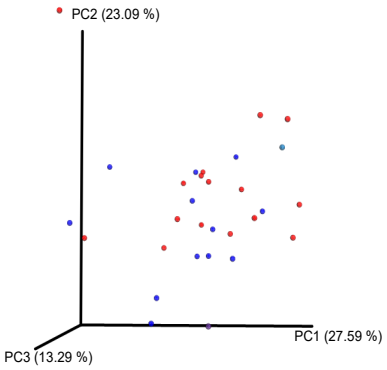


Figure 2. A 3D PCoA plot based on the weighted Unifrac distance in gingivitis and periodontitis samples. The percentage of variance along each axis is indicated in brackets to illustrate beta diversity. Periodontitis samples are indicated by blue dots, while gingivitis samples are indicated by red dots.



Taxa Abundance Analysis

The taxonomic community analysis revealed the presence of 287 known species in gingivitis and periodontitis sites. Among them, 56 species were taxonomically recognised. After excluding those with less than 0.1 % relative abundances, only 26 species were included in the analysis.

Top abundant species

Prevotella intermedia (P. intermedia), Veillonella dispar (V. dispar), Rothia dentocariosa (R. dentocariosa), and *Porphyromonas endodontalis (P. endodontalis)* were the most abundant species in gingivitis and periodontitis sites. Among these, *V. dispar* were present in the greatest abundance (28.4%) in periodontitis sites, followed by *P. melaninogenica* (13.95) and *P. intermedia* (12.9%). *P. intermedia* were the most prevalent (22.2%) in gingivitis sites, followed by *V. dispar* (16.2%) and *R. dentocariosa* (15.3%). Figure 4 compares the abundance of 26 bacterial species in gingivitis and periodontitis sites. Comparing gingivitis and periodontitis sites reveals a change in bacterial species abundance, either an increase or a decrease. There was, however, no statistically significant difference.

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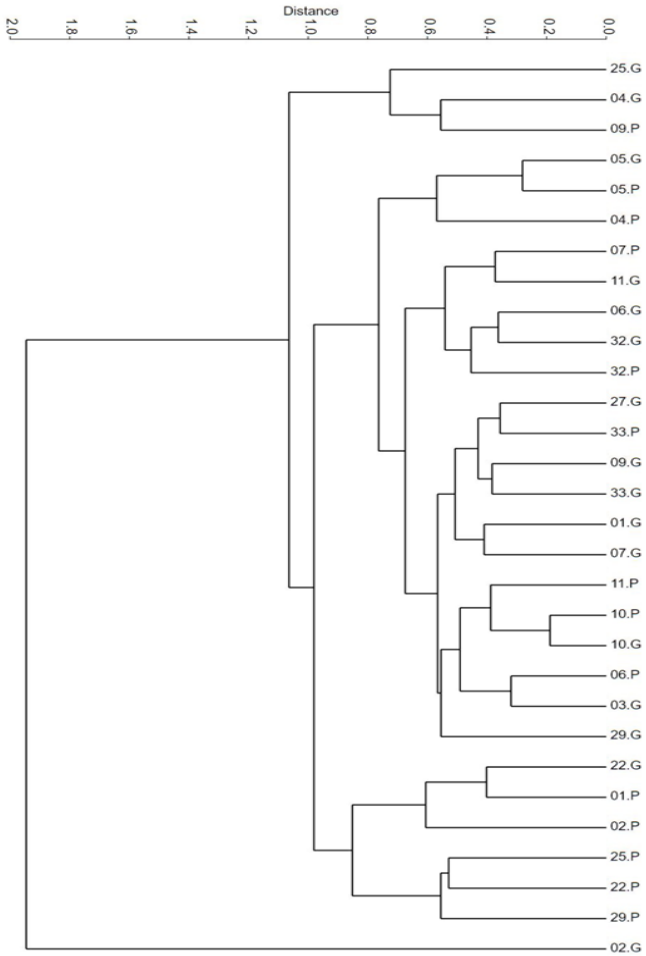
Figure 3. Weighed Unifrac-based hierarchical clustering of Gingivitis (G) (n = 16) and periodontitis (P) (n = 14) samples. Distance = Weighted Unifrac value.



DISCUSSION

Alpha diversity

The values of alpha diversity (richness estimator chao1 and Shannon) revealed a trend of increase in periodontitis samples compared to gingivitis samples, although the difference was not statistically significant. This is consistent with a previous study which found no statistically significant difference in Shannon values between healthy, gingivitis, and periodontitis sites in the same individual [4]. Even in the inter-subject study, the species Evenness between healthy and diseased periodontal sites was reported to be not significantly different [16–18]. However, previous studies also revealed contradictory results, showing that sites with periodontitis had statistically greater microbial diversity and evenness than healthy sites [3]. Another study reported significantly higher evenness in the periodontitis group than in the healthy group. However bacterial community diversity was not significant [19]. High bacterial community diversity in periodontal disease sites may result from a nutritionally richer environment or a diminished immunological competence [3]. Although Alpha diversity can be a good predictor of disease-associated dysbiosis, which could be the cause of periodontal disease [20], our findings do not appear to support the role of bacterial

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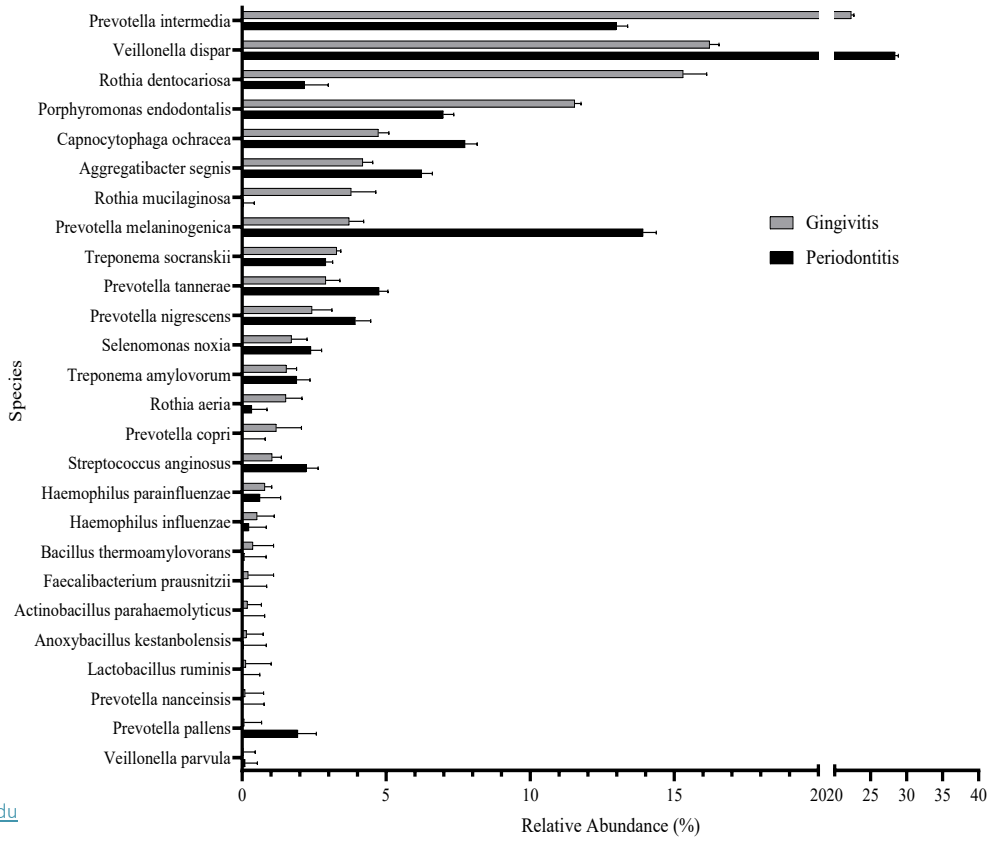
community diversity in the progression of gingivitis to periodontitis.

Beta diversity

Based on Weighted Unifrac distances, the PCoA graph presented with no distinctive cluster of gingivitis and periodontitis samples. The hierarchical clustering graph showed that certain samples from a single subject exhibited similarity, while most samples exhibited dissimilarity. There was no study that investigated the beta diversity in gingivitis and periodontitis for us to compare. However, a study showed no significant difference in the weighted unifrac distance between healthy and periodontitis salivary samples, and there was no apparent clustering between the two groups [21]. Another study also found no significant difference in beta diversity but did find apparent clustering in healthy and periodontitis samples [22]. There was also no significant difference in beta diversity or apparent clustering between gingivitis subjects with different oral hygiene statuses [23]. In contrast, another researcher reported apparent clustering of healthy and periodontitis samples in PCOA plots from the same individual [17]. Studies also found a significant difference in the weighted unifrac distance of bacterial species when healthy and periodontitis subjects were compared [16,24]. A well-controlled study is required to answer the question of how bacterial plaque distribution is associated with periodontal health conditions.

Figure 4. Mean relative abundance of bacteria species with standard error of the mean (SEM) in gingivitis and periodontitis samples. Only recognised species with a relative abundance greater than 0.1% were included. Mann-Whitney test was used for statistical analysis, and the significance level was set at $p = 0.05$. No statistically significant were found between the two groups.



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Composition of bacterial species in the dental plaque of gingivitis and periodontitis sites

In partial agreement with our findings, others reported a higher abundance of *V. dispar* in plaque samples of diseased sites compared to healthy sites in periodontitis patients [7]. In contrast, a higher abundance of *V. dispar* was discovered in healthy implant sites compared to sites with periodontitis and periimplantitis [25], and *V. dispar* was shown to be more prevalent in shallow pocket sites than in deeper places [26]. In agreement with our finding, *P. melaninogenica* was detected in great abundance in the saliva of periodontitis patients compared to healthy ones [27]. However, *P. melaninogenica* was also found to be more prevalent in plaque samples from healthy sites than in periodontitis sites [28]. Additionally, similar to our observation, *P. intermedia* was found abundant in gingivitis plaque samples compared to periodontitis [29].

On the other hand, it was shown to be more prevalent in the subgingival plaque sample of the periodontitis than in gingivitis sites [30]. As for *R. dentocariosa*, it was shown that its abundance decreased as the severity of periodontal disease increased, which is consistent with our observation that gingivitis sites had a higher abundance [19,31]. However, compared to healthy sites, the species were more abundant in sites with periodontitis [32].

CONCLUSION

Within the limitations of this study, it may be concluded that there is no apparent divergence of the dental biofilm communities in gingivitis and periodontitis. Nevertheless, certain species displayed a trend of increased or decreased abundance in gingivitis or periodontitis sites. These patterns of species abundance in gingivitis and periodontitis have also been reported in contradictory ways in other studies, rendering the conclusions inconclusive. If this fundamental understanding is to be confirmed, bigger and well-controlled studies are required.

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