

Unveiling Subgingival Oral Microbiota Profile using Next Generation Sequencing Technology

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KEYWORDS

16s rRNA gene sequencing, periodontitis, subgingival oral microbiota

ABSTRACT

This study aimed to profile the subgingival microbiota of a selected Malaysian population. The subjects consisted of 21 periodontitis, 7 gingivitis and 13 healthy individuals. Subgingival plaque DNA were sequenced using next generation sequencing technology to study the oral microbial profile. In all the 3 groups, bacterial communities comprised 2 clusters whereby the first cluster was according to periodontitis status, while gingivitis and healthy groups clustered together. The phyla that dominated 80% of the bacteria community consisted of *Firmicutes*, *Fusobacteria*, *Bacteroidetes* and *Actinobacteria*. Among the healthy subjects, the bacterial composition was unique to respective individuals. It was obvious that periodontal condition influences the microenvironment and hence the microbial composition where periodontitis group presented with increased relative abundance in phylum *Spirochaetes*, *Synergistetes*, *Tenericutes* and *Chloroflexi*. At genus level, the periodontitis group was further represented by the increase in genus *Treponema* 2, *Filifactor*, *Fretibacterium* and *Peptostreptococcus*. This profiling shows the unique microbial features of a selected Malaysian population with mixed cultural background and habits. The results from this study demonstrates that changes in microbial composition are due to *in-situ* condition. These results served as a reference for future work related to the subgingival microbiota of Malaysian population.

INTRODUCTION

The oral microbiota is the second largest microbial community following gut microbiota in humans [1]. The composition of the oral microbial community has been shown to be predetermined by genetics and later influenced by factors such as oral health, oral hygiene and diet [2]. The profiling of bacterial community has evolved tremendously over the years, from culturing to massive parallel sequencing, allowing for a more comprehensive understanding of the microbial communities present in the oral cavity. To date, few authors have compared the subgingival microbiota of periodontal patients to those of healthy individuals using sequencing technique of 16S rRNA genes [3, 4, 5, 6]. However, all these results are mostly from

the European countries. Malaysia has a unique multi-ethnic society with different lifestyle and cultural background. Therefore, it is postulated that they have different oral bacteria profiles compared to their European counterparts.

Sequencing technology can provide a global view of the microbial community and detect all including unculturable members. Many studies using sequencing technology have been done in many European countries, as well as in the neighboring countries such as Thailand and Singapore. A study from India compared the abundance of phyla among periodontally healthy and gingivitis subjects. They observed that the concentration of *Firmicutes* and *Proteobacteria* was higher in healthy subjects. On the other hand, the abundance of *Candidatus saccharibacteria*, *Actinobacteria*, *Bacteroidetes*, and *Fusobacteria* was found to be higher in gingivitis. *Synergistetes* have only been observed in gingivitis samples [7].

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In another study from China, the researchers reanalyzed 47 studies to define the core salivary microbiome and factors such as geographic locations that influenced the human salivary microbiota [8]. They identified bacteria genera *Streptococcus*, *Neisseria*, and *Prevotella* as core salivary microbiome and reported that samples from China showed different microbial composition compared to samples from Western countries. A study involving 506 subjects from Belgium, Chile, Spain, and Peru also highlighted the importance of geographical factors in shaping subgingival bacterial communities [9].

The study on the oral microbial profile of the Malaysian population with its multiethnicity and multicultural uniqueness is scarce and have not been studied systematically. Online searches showed that a study published in 2017 on Malaysian population, studied the oral swab samples, using restriction fragment length polymorphism (RFLP) and targeted the V6 to V9 region of 16S rDNA [10]. However, the study did not provide the status of the oral condition (periodontal health, caries activity) which is the main determinant of the microbial profile [11]. In a more recent study, the salivary microbiome of the Malaysian aboriginal people were profiled but similarly, the researchers did not provide information on the oral conditions of the participants. Additionally, among the aboriginal people of Temiar tribe, 50% of them were pre-diabetic, which also increased the likelihood of co-occurrence of periodontitis, a factor that determines the profile of bacteria in the oral environment [11, 12]. Importantly, the profile of oral bacteria at baseline has been reported to influence the outcome following periodontal therapy [13]. Therefore, there is a clear need to systematically study the oral microbiota of the Malaysian population while considering their periodontal health status.

MATERIALS AND METHOD

This cross-sectional study was carried out from 2018-2019. Ethical approval for this study was obtained from the Medical Ethics Committee of the Dental Faculty [DF RD 1707/0029 (L)]. The study participants were conveniently recruited from the Outpatient clinic and the Periodontal clinic, Dental Faculty of University of Malaya.

Diagnosis of periodontitis was according to the Centers for Disease Control and Prevention-American Academy of Periodontology case definition classification in periodontal medicine

research. Subjects with periodontal inflamed surface area (PISA) >100mm², clinical attachment loss (CAL) >1mm were grouped into periodontitis (P); PISA > 100mm² with CAL <1mm were grouped as gingivitis (G), while the rest as healthy (H). The exclusion criteria were (i) subjects who were pregnant, (ii) had concurrent systemic or debilitating conditions such as diabetes mellitus and (iii) smoking.

All patients were given Patient Information Sheet (PIS) which explained the nature of the study, its purpose, possible benefits and drawbacks. Informed consent, which was available in both English and Malay languages were obtained. Demographic data, dental visits and brushing frequency were obtained using a questionnaire. Full mouth periodontal examination excluding the 3rd molars was performed for all subjects.

The collection of subgingival plaque samples were done using curettes. The details has been described in our previous paper [11]. All plaque samples obtained were stored in a -80°C freezer. MasterPure™ DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA) was used to perform DNA extraction from subgingival plaque samples as published previously [11]. The extracted DNA was subjected to 16s rRNA library preparation and sequencing, spanning the variable region V3-V4 using Illumina MiSeq technology.

The raw data obtained were processed using CLC genomic workbench and annotated against SILVA ribosomal RNA database. Only taxa that were present in at least 85% of the samples were included for analysis. The raw abundance of each operational taxonomy unit (OTU) was normalised with the total number of reads to yield its relative abundance. Principal Coordinate (PCo) Analysis was performed to estimate beta diversity, which is similarity between one sample from the other. The results presented below were parts of a bigger study.

RESULTS

Table 1 shows the demographic data of the subjects. There were 13 healthy, 7 gingivitis and 21 periodontitis subjects who contributed their plaque samples. Subjects presented with gingivitis and periodontitis had higher PISA scores. Samples were noted to cluster according to periodontal condition, where healthy and gingivitis (red and blue dots) were more similar and clustered on one side, while periodontitis (black dots) clustered on the other side (Figure 1). This is explained by PCo1 (11%).

Table 1 Demographic background of subjects

	Healthy (n=13)	Gingivitis (n=7)	Periodontitis (n=21)
Ethnicity (n (%))			
Malay	8 (61.5)	4 (57.1)	9 (42.9)
Chinese	5 (38.5)	2 (28.6)	9 (42.9)
Indian	-	1 (14.3)	3 (14.3)
Age			
(years±SD)	38.3 (±14.1)	36.3 (±10.8)	42.5 (±12.5)
Gender (n)			
Male	2	2	9
Female	11	5	12
Periodontal status (mm±SD)			
PISA	32.2 (±30.8)	386.5 (±180.8)	847.7 (±638.0)
PD	1.8 (±0.3)	2.1 (±0.3)	3.3 (±0.7)
CAL	0.5 (±0.2)	0.6 (±0.2)	4.5 (±2.7)

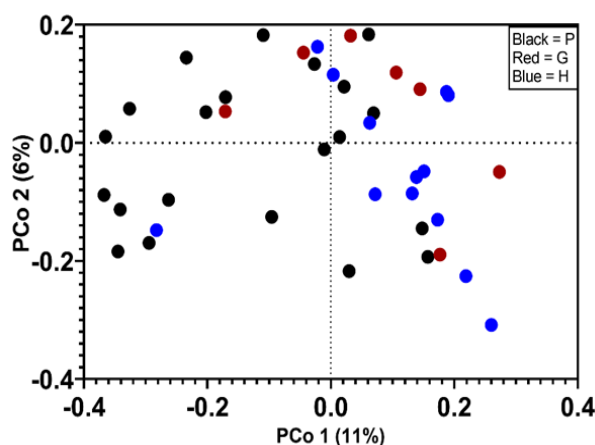
**Figure 1** Beta diversity based on Principal Coordinate (PCo) Analysis showed clustering of samples according to periodontal condition

Table 2 showed the relative abundance of oral microbe according to phyla. When comparing gingivitis to healthy, phyla *Firmicutes*, *Fusobacteria*, *Proteobacteria*, *Patesibacteria* and *Spirochaetes* showed increased relative abundances, and a reduction in relative abundance of *Actinobacteria*. When comparing periodontitis to healthy, similarly there is reduction in relative abundance of *Actinobacteria*, while increase in relative abundance of *Spirochaetes* and *Synergistetes*.

Figure 2 shows relative abundance according to phyla for all groups. There were 4 phyla that make up 80% of the subgingival oral microbiota namely *Firmicutes*, *Fusobacteria*, *Bacteroidetes* and *Actinobacteria*. Gingivitis and healthy groups have almost similar proportion of the bacteria profile. In the periodontitis group, there was an increase in the relative abundance of *Bacteroidetes* while a reduction in *Actinobacteria* and *Proteobacteria* was noted. At the same time, the other phyla with

increased relative abundance in the periodontitis group include *Synergistetes*, *Tenericutes*, and *Chloroflexi*.

Table 2 Relative abundance (%) of oral microbe according to phyla

	Healthy (%)	Gingivitis (%)	Periodontitis (%)
<i>Firmicutes</i>	25.8	27.1	28.0
<i>Fusobacteria</i>	24.0	26.6	23.5
<i>Bacteroidetes</i>	21.1	17.7	25.1
<i>Actinobacteria</i>	11.5	8.8	5.2
<i>Proteobacteria</i>	9.0	10.2	4.1
<i>Patesibacteria</i>	4.1	6.8	5.0
<i>Epsilonbacteraeota</i>	3.9	2.0	2.4
<i>Spirochaetes</i>	0.4	0.7	4.6
<i>Synergistetes</i>	0.2	0.1	1.9
<i>Tenericutes</i>	0	0	0.1
<i>Chloroflexi</i>	0	0	0.1

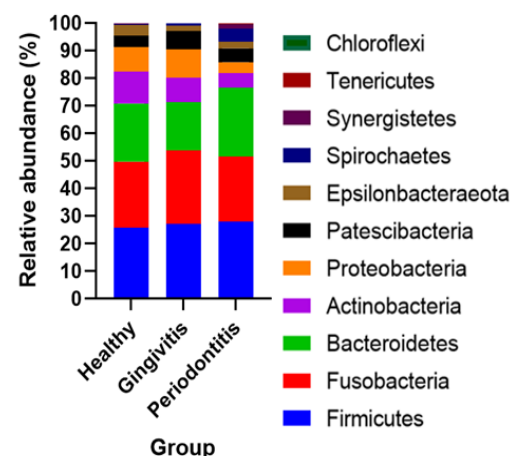
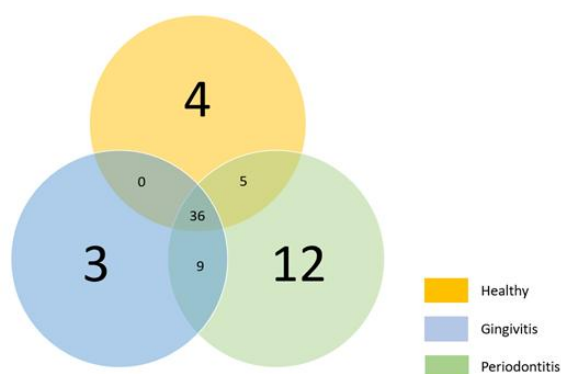
**Figure 2** Relative abundance according to phyla for all the groups

Table 3 shows the top 20 bacteria genera specific to each group. At genus level, healthy and gingivitis groups presented with similar bacteria profile, represented by *Leptotrichia*, *Fusobacterium*, *Corynebacteria*, *Campylobacter*; while periodontitis group was dominated by disease-associated genera *Porphyromonas*, *Streptococcus*, *Treponema*. In the periodontitis group, the genus associated with diseases were noted to have higher relative abundance, namely *Treponema*, *Filifactor*, *Fretibacterium* and *Peptostreptococcus*.

Figure 3 shows the Venn diagram of number of bacteria species unique to individual groups and shared among the groups. When looking at all bacteria species that are successfully annotated, there are 36 species shared by all 3 groups, while those unique to individual groups were 4 in healthy, 3 in gingivitis and 12 in periodontitis.

Table 3 Relative abundance of the top 20 bacteria genera in all the groups

Relative abundance, %		
Healthy	Gingivitis	Periodontitis
<i>Leptotrichia</i> , 12.8	<i>Leptotrichia</i> , 15.8	<i>Leptotrichia</i> , 12.7
<i>Fusobacterium</i> , 11.2	<i>Fusobacterium</i> , 10.8	<i>Fusobacterium</i> , 10.2
<i>Corynebacterium</i> , 8.3	<i>Streptococcus</i> , 9.1	<i>Porphyromonas</i> , 9
<i>Capnocytophaga</i> , 7.5	<i>Corynebacterium</i> , 7	<i>Streptococcus</i> , 8.8
<i>Selenomonas</i> 3, 7.2	<i>Capnocytophaga</i> , 5.6	<i>Treponema</i> 2, 4.5
<i>Streptococcus</i> , 7.2	<i>Veillonella</i> , 3.7	<i>Prevotella</i> 7, 3.8
<i>Porphyromonas</i> , 5.1	<i>Neisseria</i> , 3.3	<i>Selenomonas</i> 3, 3.7
<i>Veillonella</i> , 5	<i>Selenomonas</i> 3, 3	<i>Prevotella</i> , 3.6
<i>Campylobacter</i> , 3.9	<i>Prevotella</i> , 2.6	<i>Capnocytophaga</i> , 3
<i>Neisseria</i> , 3.4	<i>Haemophilus</i> , 2.6	<i>Filifactor</i> , 2.9
<i>Cardiobacterium</i> , 2.6	<i>Porphyromonas</i> , 2.4	<i>Corynebacterium</i> , 2.6
<i>Prevotella</i> 7, 2.6	<i>Prevotella</i> 7, 2.3	<i>Neisseria</i> , 2.4
<i>Prevotella</i> , 1.8	<i>Tannerella</i> , 2	<i>Campylobacter</i> , 2.3
Uncultured Candidatus	<i>Campylobacter</i> , 2	<i>Tannerella</i> , 2
<i>Saccharibacteria bacterium</i> , 1.5	<i>Prevotella</i> 2, 1.7	<i>Fretibacterium</i> , 1.9
<i>Lachnoanaerobaculum</i> , 1.4	<i>Lachnoanaerobaculum</i> , 1.6	<i>Selenomonas</i> , 1.6
<i>Tannerella</i> , 1.4	<i>Aggregatibacter</i> , 1.4	<i>Veillonella</i> , 1.4
<i>Actinomyces</i> , 1.2	<i>Selenomonas</i> , 1.3	<i>Lachnoanaerobaculum</i> , 1.2
F0058, 1.1	D_4__Saccharimonadaceae,	D_4__Saccharimonadaceae,
<i>Aggregatibacter</i> , 1	D_5__uncultured Candidatus	D_5__uncultured Candidatus
<i>Prevotella</i> 2, 1	<i>Saccharibacteria bacterium</i> , 1.1	<i>Saccharibacteria bacterium</i> , 1.2
	<i>Lautropia</i> , 1	<i>Peptostreptococcus</i> , 1

**Figure 3** Venn diagram showing the number of bacteria species shared by 3 groups, and unique to individual groups

Bacteria species with higher abundance in the periodontitis group were *Flexistipes-like* sp. oral clone BB062 (Phyla Synergistetes), *Porphyromonas-like* sp. oral clone DA064 (phyla Bacteroidetes), *Veillonellaceae bacterium* oral taxon 14 (phyla Firmicutes), *Prevotella baroniae* (Bacteroidetes), *Capnocytophaga* sp. JCM 8568 (phyla Bacteroidetes), *Selenomonas* sp. oral clone AJ036 (phyla Firmicutes), *Catonella* sp. oral clone FL037 (phyla Firmicutes), *[Eubacterium] yurii subsp. yurii* (phyla Firmicutes), *Prevotella denticola*

(Bacteroidetes), *Selenomonas* sp. oral clone JS031 (phyla Firmicutes), *Prevotella* sp. oral clone GI030 (Bacteroidetes) and *Treponema* sp. oral taxon 230 (phyla Spirochaetes).

DISCUSSION

This study investigated the subgingival bacteria profile of a selected Malaysian population using 16S rDNA amplicon sequencing techniques, which investigates the composition of the microbial community in an open-ended manner. The research identified core bacterial species in relation to periodontal health.

The core bacteria species are indicated by phyla Firmicutes, *Fusobacteria*, *Bacteroidetes* and *Actinobacteria* which represent 80% of the community across all groups. This is quite similar to the results of published Malaysian data in 2017 [10], where the authors identified Firmicutes, Proteobacteria, *Fusobacteria*, *Bacteroidetes* and *Actinobacteria* at the phylum level; and also similar to the results from Singapore [14], China, Korea and Japan, where the vast majority of the sequences were assigned to five bacterial phyla, Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria and

Fusobacteria [15]. Singapore has a similar multi-ethnic population of Malays, Chinese and Indians as Malaysia, where Chinese is the majority (76%), followed by Malay (15%) and Indian (7.5%) [16]. However, the current study did not analyse the effect of different ethnicity due to the low number of participants.

From the Venn diagram, we can observe that there are 9 bacteria species detected in higher relative abundance and co-occurred in both gingivitis and periodontitis groups. This might be the bacteria signature associated with dysbiosis. However, this requires further investigation such as using metaproteomics which measures protein intensities to capture active protein functions and taxonomic units within the microbiome [17].

The phyla associated with periodontitis were *Synergistetes* and *Spirochaetes*; while phyla *Actinobacteria* and *Proteobacteria* were noted to decrease. The results were similar as reported in a study from India [7]. It is noted that health-associated bacteria are equally present in the disease stage which signifies their cooperation with the disease-associated bacteria, indicating the symbiotic relationship. This has been shown in transcriptomic studies [18] which reported that some of the commensals expressed virulence factors under pathogenic conditions. *Streptococcus* has been shown to provide a protective mechanism for the survival of *Porphyromonas gingivalis* whereby the adherence to *Streptococci* can modulate the pathogenic potential of *P. gingivalis* [19]. Hence, it is not surprising to observe the high relative abundance of both *Porphyromonas* and *Streptococcus* in the periodontitis group in this study.

The salivary microbiome of the indigenous people in Malaysia has been reported in a recent publication [20]. In that study, the core bacteria genera were *Streptococcus*, *Veillonella*, *Haemophilus*, *Prevotella*, *Gemella*, *Neisseria*, *Lactobacillales*, *Actinomyces* and *Fusobacterium*. However, the information of their oral status (periodontal health, caries activity) was not available. Additionally, the study populations were the aborigines with high prevalence of diabetes which also increased the likelihood of co-occurrence of periodontitis, a factor that determines the profile of oral bacteria. These factors might explain the differences noted from the findings in this current study.

Reports from Thailand showed that the bacteria species identified from periodontitis group are *P*

gingivalis, *P. intermedia*, *F. alocis*, *P. endodontalis*, *T. denticola*, *T. forsythia* and *T. medium* [21]. This is different from the findings in this study. The results from Japan also showed different results, *Prevotella* and *Veillonella* were associated with periodontitis, whereas larger proportions of *Neisseria*, *Haemophilus* and *Porphyromonas* were associated with periodontal health [22]. Although Malaysia and Thailand are neighbours, Malaysian population have different eating choices and habits which might be the main contributing factor in the different findings [23]. However, this will need to be included and investigated in the future study design.

The degree of severity for periodontal health (gingivitis vs periodontitis) determines the oral microbial profile. In the current study microbial profile in gingivitis was still somewhat similar to health, however, periodontitis showed distinct differences with healthy. This was due to the fact that gingivitis is the intermediate state between the healthy and periodontitis statuses, as reported previously [24]. In that study, similarity of co-occurrence networks were noted in healthy and gingivitis. This may explain why gingivitis is considered a reversible condition to health if treated and maintained properly.

The presence of specific subgingival bacteria may influence the response of individuals towards treatment. It has been reported that subjects who presented with higher abundance of specific bacteria such as *Actinobacteria* might indicate a favorable response, while others like *Synergistetes* may predict non-responsiveness following periodontal therapy [13]. The researchers suggested that the baseline bacteria profiles will determine the outcome of treatment. In the future it will be possible to design microbiome modulation strategies to improve host health [25].

The current study presents with some limitations as the population was from an urban area with a mixed ethnic population. The dietary habits of these subjects were not captured which may potentially influence the bacterial composition. Therefore, future studies should include larger samples that encompasses the major ethnicity group and record the dietary practice of the participants. Information from 16S profiling of bacteria did not portray a true picture of the interactions within the bacteria communities. Metaproteomic studies will be able to provide information on the function of bacteria especially in influencing disease regression or progression. It also provides molecular level details on systems

consisting of mixed microbial membership, interpret the protein-based function of microbial communities and to determine potential useful biomarkers [17].

CONCLUSION

The subgingival microbial profile of this selected Malaysian population provides a global picture of the bacterial community. The main factor that differentiates the bacteria profile is the health status of the periodontal condition. The oral microbiota's composition and its influencing factors is crucial for advancing oral health research. Therefore, these results serve as a reference for future work related to the subgingival microbiota of

the Malaysian population. It also highlights the way forward for a customized/ personalized approach when outlining treatment plans for populations with different demographic backgrounds to ensure more predictable treatment outcomes.

ACKNOWLEDGEMENT

This study was supported by Fundamental Research Grant Scheme, grant number (FP017-2017A) of Malaysia Ministry of Education.

DECLARATION OF INTEREST

Authors declare no conflict of interest.

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Editorial History

Date of Submission: 7 Apr 2025

Review & Revision: 29 May 2025 – 10 Mar 2026

Accepted: 23 Apr 2026

Published: 8 May 2026

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