RESEARCH ARTICLE

REVISED A pilot study of red complex and three genera subgingival microbiome in periodontitis subjects with and without diabetes, evaluated by MinION platform [version 3; peer review: 2 approved]

Boy M. Bachtiar¹, Citra F. Theodorea¹, Dicky L. Tahapary^{2,3}, Cindy Astrella^{3,4}, Natalina⁵, Endang W. Bachtiar¹

¹Department of Oral Biology and Oral Science Research Center, Faculty of Dentistry, Universitas Indonesia, Jakarta, 10430, Indonesia

²Division of Endocrinology, Department of Internal Medicine, Dr. Cipto Mangunkusumo National Referral Hospital, Faculty of Medicine, Dr. Cipto Mangunkusumo National Referral Hospital, Faculty of Medicine Universitas Indonesia, Jakarta, 10430, Indonesia

³Metabolic, Cardiovascular and Aging Cluster, The Indonesian Medical Education and Research Institute, Faculty of Medicine, Universitas Indonesia, Jakarta, 10430, Indonesia

⁴Department of Internal Medicine, Dr. Cipto Mangunkusumo National Referral Hospital, Faculty of Medicine, Universitas Indonesia, Jakarta, 10430, Indonesia

⁵Department of Periodontology, Faculty of Dentistry, Universitas Indonesia, Jakarta, 10430, Indonesia

v3 First published: 08 Feb 2021, 10:79
<https://doi.org/10.12688/f1000research.28216.1>
 Second version: 02 Jun 2021, 10:79
<https://doi.org/10.12688/f1000research.28216.2>
 Third version: 29 Jun 2021, 10:79
<https://doi.org/10.12688/f1000research.28216.3>
 Latest published: 12 Jul 2021, 10:79
<https://doi.org/10.12688/f1000research.28216.4>

Abstract

Background: Subgingival niche is one biofilm habitat containing rich microbiota, which plays an active role in maintaining the health of periodontal tissue and determining host response. As such, a study of changing subgingival biofilms is important for understanding the effect of a systemic condition. In this study, we compared the occurrence of six bacteria cohabiting in the subgingival area of periodontitis subjects, with (DP, n = 8) and without (NDP, n = 4) diabetes.

Methods: The six genus and species of targeted bacteria were confirmed by 16S rRNA amplicon sequencing on MinION nanopore platform. Descriptive statistic was used to describe the obtained data.

Results: We found that the six genus and species of targeted bacteria were detected but in different quantities in either group's periodontal pocket. Our data showed that *Tannerella forsythia* was the most abundant species in subgingival biofilms of the DP group of the red complex bacteria. In contrast, *Aggregatibacter* sp., which belongs to

Open Peer Review

Approval Status

	1	2
version 4		
(revision) 12 Jul 2021		
version 3		
(revision) 29 Jun 2021		
version 2		
(revision) 02 Jun 2021		
version 1		
08 Feb 2021		

1. Wan Izlina Binti Wan Ibrahim^{id}, Universiti Malaya, Kuala Lumpur, Malaysia

the phylum of proteobacteria, was present at a relatively lower level. In contrast, *Fusobacterium* sp., which belongs to orange complex bacteria, showed relative similarities in subgingival biofilms of both groups tested, while *Veillonella* sp., were abundant in the DP groups.

Conclusions: Our data show that the diversity of classic periodontopathogens increased in the subgingival niche of periodontitis subjects with diabetes. It is the first study in Indonesia to apply MinION-based, full-length 16S rRNA amplicon sequencing in periodontitis patients with and without diabetes.

Keywords

MinION, 16S rRNA, Red Complex bacteria, Diabetes, Periodontitis, Subgingival Microbiome

This article is included in the **Cell & Molecular Biology** gateway.

2. **Heni Susilowati** , Universitas Gadjah

Mada, Yogyakarta, Indonesia

Any reports and responses or comments on the article can be found at the end of the article.

Corresponding author: Endang W. Bachtiar (endang04@ui.ac.id)

Author roles: **Bachtiar BM:** Conceptualization, Formal Analysis, Methodology, Software, Writing – Original Draft Preparation; **Theodorea CF:** Investigation, Resources, Validation, Visualization; **Tahapary DL:** Data Curation, Investigation, Methodology, Supervision; **Astrella C:** Data Curation, Resources; **- N:** Resources, Validation; **Bachtiar EW:** Conceptualization, Formal Analysis, Funding Acquisition, Project Administration, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: This study was supported by The Grant from Ministry of Research and Technology/BRIN (UN2.RST/HKP.05.00/2020). *The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

Copyright: © 2021 Bachtiar BM *et al.* This is an open access article distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

How to cite this article: Bachtiar BM, Theodorea CF, Tahapary DL *et al.* **A pilot study of red complex and three genera subgingival microbiome in periodontitis subjects with and without diabetes, evaluated by MinION platform [version 3; peer review: 2 approved]** F1000Research 2021, 10:79 <https://doi.org/10.12688/f1000research.28216.3>

First published: 08 Feb 2021, 10:79 <https://doi.org/10.12688/f1000research.28216.1>

REVISED Amendments from Version 2

The reviewer's comment is regarding the explanation of sub-gingival plaque sampling and microbial samples. We agree, the explanation is rather confusing. Because they are the same work, we put both the paragraph 3 and 6, and rewritten them together as paragraph 1 under subheading "microbial sample."

Any further responses from the reviewers can be found at the end of the article

Introduction

Taking DNA straight from oral samples, without culture samples, is a fundamental principle of oral microbiome study. Currently used molecular methods generally rely on PCR, which can be used to target specific bacterial species. However, detection is only enabled for those that have primers. PCR can also detect all bacterial species using a broad range of 16S primers followed by sequencing, but problems can arise from contamination¹.

The advancement of sequencing technology has led to the mainstream in oral microbiology due to the increasing affordability and improvement in the speed of the sequencing process and quality of data obtained. In this context, MinION is the first sequencer available commercially that uses nanopore technology. Unlike other sequencing technology, the sequencing method provided by the MinION device does not rely on the synthesis of nucleotides. For this reason, we decide to use this long-read nanopore sequencing device to identify the dominant bacteria in diabetic condition-related periodontitis.

One of the systemic conditions that have been asserted to affect the host immune response to dental plaque is diabetes^{2,3}. However, the effect of diabetes on the composition of the subgingival microbiome is still inconclusive⁴. A previous study showed that an increased number of periodontal pathogens had been isolated from the subgingival plaque of diabetic patients⁵. However, another study showed a decrease of bacterial diversity associated with periodontitis⁶, indicating a putative role of specific oral bacteria species in the oral niche that might be correlated to the condition due to diabetes⁷.

Among the subgingival microbial community of periodontitis, the red complex bacteria (*Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*) have been considered as major periodontal pathogens^{8,9}. Their presence in the subgingival environment indicates that a selected bacteria species occurs due to the suitable anaerobic microenvironment that has been formed¹⁰. However, these red complex bacteria are usually preceded by members of other complex oral bacteria species, including those associated with a healthy periodontal pocket¹¹. This indicates that ecological stress has occurred, leading to the presence of periodontitis-related microorganisms leading to an imbalance of bacteria species in the dental plaque¹⁰. Consequently, in subgingival samples, the red complex bacteria could be measured as multiple oral bacteria species due to their role in progressive periodontitis.

The main goal of the current study was to use MinION of the full-length 16S rRNA gene to compare the profile of red complex bacteria and three other genera (*Aggregatibacter*, *Fusobacterium*, and *Veillonella*) in two different subgingival niches of periodontitis, subjects with (DP) and without (NDP) diabetes, in order to assess their microbiome.

Methods

Patients

This study was conducted between November 2018 and early June 2019. Twelve patients, 20 to 50 years of age, from consecutive participants were recruited from the Department of Internal Medicine, Faculty of Medicine, Universitas Indonesia, Cipto Mangunkusumo Hospital (FKUI-RSCM), Jakarta, Indonesia. The Ethics Committee of FKUI-RSCM has approved this study's protocol (No.1062/UN@.F1/ETIK/2018). The investigation procedure has been conducted according to the Declaration of Helsinki. Written informed consent was obtained from each subject to participate in this study.

Subjects excluded from this study were those who (i) had systemic disease other than diabetes mellitus; (ii) had received periodontal treatment or had taken antibiotic within the previous three months; (iii) and were smokers or pregnant. Participants in this study were those with periodontitis complicated by diabetes (DP, n = 8) and non-diabetes (NDP, n = 4), and diabetes criteria were determined from their medical record.

All patients consented to provide a subgingival dental plaque for this study. All subjects were diagnosed for periodontitis or healthy periodontal tissue according to criteria described by the American Academy of Periodontology¹². Subgingival dental plaque was collected by a registered dentist using sterile periodontal scalers and placed in individual microcentrifuge tube containing phosphate-buffered saline (PBS) buffer (pH 7.4). Samples were stored at -20°C until further processing. Extraction of DNA was performed using a DNA extraction kit Qiagen QIAamp DNA Mini Kit, as per the manufacturer's instruction. The obtained DNA's concentration and quality were determined using Qubit assay reagents (Invitrogen; Carlsbad, CA, USA). After dissolving in Tris-EDTA buffer, the DNA was cooled to -20°C for further analysis.

The effects and efficiency of pooling samples have been investigated in many studies^{13,14}. Therefore, in this study, we analysed pooled samples of subgingival biofilm of the two group patients with periodontitis for shifts in the subgingival community in response to a diabetic condition.

Microbial sample

Subgingival dental plaque was collected by a registered dentist using sterile periodontal scalers and placed in individual microcentrifuge tube containing phosphate-buffered saline (PBS) buffer (pH 7.4). The samples were taken from three sites in DP subjects and three periodontal pockets in NDP subjects. Bacterial subgingival biofilm was collected from one diseased site (when present) with probing depth ≥ 5 mm with bleeding

on probing. The collection area was isolated with cotton rolls, and a supragingival plaque was carefully removed with curettes. The collection was done with a sterile endodontic paper point by inserting the point to the depth of the sulcus and moving it laterally along the tooth's axis. Immediately following sampling, the paper point was placed in a microcentrifuge tube and stored at -70°C until processed.

In this study, the presence at genus and species level of the red complex bacteria (*Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*), and three other selected genera (*Aggregatibacter*, *Fusobacterium*, and *Veillonella*) were determined in subgingival biofilm samples. Therefore, we first extracted the DNA from the samples by using a DNA extraction kit Qiagen QIAamp DNA Mini Kit, as per the manufacturer's instruction. The obtained DNA's concentration and quality were further determined using Qubit assay reagents (Invitrogen; Carlsbad, CA, USA). After dissolving in Tris-EDTA buffer, the DNA was cooled to -20°C until further processing.

MinION sequencing and data analysis

The purified DNA were amplified by polymerase chain reaction (PCR) using specific primers (27F and 1492R) in a commercially available kit (16S Barcoding kit; SQK-RAB204; Oxford Nanopore Technologies/ONT, Oxford, UK). The procedure was conducted according to the protocol provided by 16S Barcoding Kit (SQK-RAB204; Oxford Nanopore Technologies/ONT, Oxford, UK). All PCR products for each subgingival biofilm sample obtained from their respective groups (DP and NDP) were pooled and purified. The concentration of each purified pool was measured using the Qubit dsDNA HS Kits by the Qubit Fluorometer (Invitrogen). For each purified pool, library preparation was prepared using the 16S Barcoding Kit mentioned above.

Two sequencing libraries were further prepared, one for a sample from DP and one for a sample from NDP. The amount of initial DNA used for both barcoding kits was 100 ng. Finally, each sequencing library was loaded into MinION flow cell of the MinION sequencing device (ONT) to be sequenced for 48 hours. Subsequently, the base calling of the generated data (fastq format) was analysed by using EPI2ME Desktop Agent (ONT). For microbiota profiling analysis, we followed the EPI2ME platform by selecting a workflow of 16S alignment for real time analysis. Alternatively, the obtained data can be analysed by a freely available software, NanoPipe (RRID:SCR_016852) (www.bioinformatics.uni-muenster.de/tools/nanopipe2). Since the base calling process is the central to improving the accuracy of nanopore sequencing technology¹⁵, in this study, only reads designated as 'pass' were included for further analyses. The analysis results were further generated in the form of a report in the EPI2ME platform.

Data analyses

Descriptive analyses were performed with GraphPad Prism 9.0 (GraphPad Software, Inc., San Diego, CA). If the six bacteria

were detected in the two sample groups, the group was regarded as positive for these bacteria.

Results and discussion

The ONT has been reported for its potential benefits to analyse microbial communities' composition and dynamics, including oral pathogen¹⁶. In this pilot study, we provide a new information on the state of ONTs MinION device for whole genome sequencing of some periodontal bacterial organisms. To describe the main finding of the results, we used the online EPI2ME platform, which contains a 16S workflow for analysing MinION reads.

Read analysis

We endeavoured to determine if the oral microbial community would reveal the different profiles of the six selected periopathogens in pooled samples collected from periodontitis subjects with (DP) and without (NDP) diabetes. We used plaque subgingival biofilm samples for practical and economic reasons, which have often been employed¹⁷.

The results of 16S rRNA amplicons on MiniION sequencing revealed a total of 113,654 sequence reads after base-calling, with more reads classified than unclassified in either group. However, the subgingival biofilms obtained from the DP group were found better classified and a greater number of species compared to those found in the subgingival microbiome of the NDP group. By comparing the read count, we found that the classified and unclassified sequence reads in the pooled sample of DP were 112,173 and 1988, respectively, while the classified and unclassified sequence reads in the pooled sample of NDP were 1478 and 172, respectively (Figure 1A). We found that the accuracy in pooled DP was 87% compared to those in pooled NDP that showed 85%. These results indicate that the long-read amplicons for sequencing on ONT covered nearly the full length of V1-V9 hypervariable regions of the 16S rRNA gene. In this study, all the reads were 1-directional base-calling, representing a sequence in the forward or reverse direction. Thus, the application of 16S rRNA-based using MinION platform has allowed tracking of bacterial cells' identity in subgingival niches, as shown in this study.

Bacterial diversity and structure of the subgingival samples of periodontitis subjects, with and without diabetes

In the current study, the long length sequences were taxonomically assigned using a workflow from the cloud-based EPI2ME, which supported the NCBI 16S database, allowing classification down to the phylum, genus and species level, respectively. Therefore, we searched which taxa were responsible for overall community differences between DP and NDP groups. Our data showed that in general, subgingival microbiome patterns were similar between DP and NDP groups, a phenomenon that have been reported by^{18,19}. Other studies²⁰ showed differences between glycaemic status and the proportion of several phyla. This study did not separate the tested sample according to the

Figure 1. Read classification across pooled subgingival microbiota samples collected from diabetic (DP) and non-diabetic (NDP) patients. (A) Taxa-level; (B) phylum; (C and D) root taxa in DP (C) and NDP (D) groups.

glycaemic level in diabetic subjects. We aimed to examine whether diabetes mellitus might affect composition of six targeted bacterial communities in the subgingival niche. Thus, we observed these subgingival bacteria by phylum to species level.

The obtained sequences were first analysed on taxonomic basis at the phylum level. Eleven phyla (Firmicutes, Proteobacteria, Fusobacteria, Bacteroides, Actinobacteria, Spirochaetes, Synergistetes, Tenericutes, Acidobacteria, Planctomycetes, and Verrucomicrobia) were detected in DP group, while the last five phyla were not detected in NDP group. This finding indicates that the ONT technology allowed sequencing of the entire 16S rRNA gene region of the bacteria belonging to these phyla associated with the disease processes in our diabetic patients. This result agrees with previous findings in the subgingival bacterial microbiome diversity study in diabetic patients²¹. Interestingly, the long sequence of Synergistetes, which were only found in the DP group, has been identified in the area of periodontitis²². The colonization are located in the outermost region of subgingival biofilm, indicating they are opposed to inflammatory cells²³. However, no genus belonging to Synergistic phylum was detected in this study. We assumed this

is because of the low-read accuracy by MinION platform²⁴, which complicates our complex samples' analysis.

Distribution of the subgingival microbiota at the phylum and family level

In this study, the subgingival microorganisms' samples were collected from the periodontal pocket at the same depth (≥ 5 mm). Firstly, we found that the seven major phyla detected in subgingival microbiota of DP group were Firmicutes, Proteobacteria, Fusobacteria, Bacteroidetes, Actinobacteria, Spirochaetes, and Synergistetes, contain 99% of the taxa. The remaining phyla, Tenericutes, Acidobacteria, Planctomycetes and Verrucomicrobia containing the remaining 1% of the Taxa. Compared to the subgingival microbiota in the NDP group, containing four major phyla (99%): Firmicutes, Proteobacteria, Fusobacteria, and Bacteroides, while the Actinobacteria was the remaining bacteria containing 1% in NDP group. At the phyla level, the phyla occurrences belong to the six targeted bacteria in both groups are the same, but the proportions differed. The Firmicutes phylum dominated the microbial community with relative abundance higher than 80% in both pooled samples (Figure 1B). This result demonstrated that using MinION, it is possible to associate the single cell level for nearly all subgingival plaque

bacteria, from each group tested, to one of the major taxonomic units.

Furthermore, as shown in **Figure 1C and D**, the subgingival microbiota profiles observed at the families level were relatively similar between DP and NDP groups, indicating that the predominant cooperative network microbiome is still conserved. However, when the six targeted bacteria were analysed at family level, the DP and DP groups' bacterial profiles were predominantly by *Pasteurellaceae*, followed by *Veillonellaceae*, *Porphyromonadaceae*, *Tannerellaceae*, and *Spirochaetaceae*. All bacteria belonging to these family were increased in the DP group compared with the NDP group (**Figure 2A**). Therefore, the obtained length-sequences were further analysed to determine if diabetes, which alters the nature of inflammatory response²⁵, also influenced the relative abundance of six genera of periodontal pathogens selected in this study.

Distribution of six subgingival microbial at genus and species level

In this study, we focused to on identifying the red complex (*P. gingivalis*, *T. denticola*, and *T. forsythia*), which a lot of studies have described as the most important pathogens in adult periodontal disease^{26,27}. We analysed the red complex at

genus and species levels of the subgingival niche, as species identification is important because it provides information regarding periodontal disease's pathogenicity and a detailed description of the subgingival microbiome in a diabetic subject. We found that among the three genera belong to these species, the topmost prevalent genera were *Porphyromonas*, followed by *Tannerella*, and *Treponema* (**Figure 2B**). As shown in **Figure 2B**, the cumulative reads belong to these genera were significantly increased in DP subjects compared with the NDP group, and more specifically, *Treponema* was only found in the DP group. This result might indicate that the different quantities of red complex bacteria are more likely due to host diabetic-related responses. However, as with other systemic factors, there are very diverse clinical and medical parameters that might affect the composition of the oral microbiome in systemic disorder patients²⁸. Hence, it is more likely that the red complex bacteria in subgingival plaque microbiome observed in periodontitis subject was affected by several concurrent factors, which we did not include in this study.

In addition to the red complex bacteria differences observed between DP and NDP subjects, we studied the differences in the individual microbial genera belong to *Fusobacteria*, *Veillonella*, and *Aggregatibacter*. In general, we found that the

Figure 2. Subgingival microbiota of diabetic (DP) and non-diabetic (NDP) patients. Relative abundance of (A) six bacterial families; (B) genera of the red complex bacteria; (C) genera of selected non-red complex bacteria.

most abundant bacteria in both subject groups (DP and NDP) belonged to *Fusobacterium* sp. (Figure 2C). This species has a role in the progression of periodontal disease due to its ability to build a physical relationship (co-aggregation) with other oral bacterial species, notably with *P. gingivalis* and *T. denticola*, in the formation of biofilm²⁹.

When analysis was focused to the genus *Porphyromonas*, our data indicated that these genera, where its sequences were abundant in the DP group, comprise mainly species *P. catoniae*, followed by *P. pasteri*, *P. gingivalis*, and *P. endodontalis*, while only *P. catoniae* and *P. endodontalis* sequences were detected in NDP group (Figure 3A–C). For *Treponema*s, which are typically restricted to the subgingival crevice³⁰, the full length of 16S rRNA gene belonging to this genus were found in six species and one subspecies in DP group, i.e. *T. medium*, *T. denticola*, *T. lecithinolyticum*, *T. maltophilum*, *T. amylovorum*, and *T. socranskii* as well as its subspecies (Figure 4A and B). Lastly, sequences identified as *Tannerella* was *Tannerella forsythia*, which was more abundant in the DP than in the NDP group (Figure 4C). However, by comparing to *P. gingivalis* and *T. denticola*, the cumulative reads of *T. forsythia* were found to be higher (not shown), suggesting that individuals with diabetes may have an increase in the subgingival abundance of the *T. forsythia*,

When species analysis was focused on *P. gingivalis*, literature shows that this species has been proposed as an important

keystone pathogen-induced dysbiosis in periodontitis conditions³¹. It has the ability to modify the oral microbiota composition³². In this study, the bacterium was only found in a sample collected from DP patients, while we obtained all the samples from chronic periodontitis patients. Additionally, in the EPI2ME 16S workflow, nanopore sequence reads are blasted against the NCBI database for 16S DNA. Although it is possible that certain species are not represented in the database, this was not the case for *P. gingivalis*, as its 16S rRNA gene sequence can be retrieved from NCBI refSeq database. Thus, our study contradicts the previous report showing that *P. gingivalis* was associated with periodontitis in patients without diabetes³³.

As also shown in literature, the presence of red complex bacteria in subgingival niche are usually found with consortia, which include various species belong to the “orange, green, and purple complex”^{34,35} as well as non-pathogenic microorganisms³⁶. Since this polymicrobial consortium comprising the mix species induced significant increased alveolar bone resorption than the mono species³⁷, our result may suggest the difference in host response between the DP and NDP groups, and we did not explore this in this study. Furthermore, our results are in line with a previous study that recovered several periodontal pathogens, including *A. actinomycetemcomitans*, *Campylobacter rectus*, *F. nucleatum*, and *P. intermedia*, which was similar in both diabetic and non-diabetic subjects, but *P. gingivalis* was more frequently detected in individuals with diabetes³⁸. Our finding

Figure 3. *Porphyromonas* sp. in pooled subgingival microbiota samples obtained from diabetic (DP) and non-diabetic (NDP) patients. (A) Abundance of *Porphyromonas* sp.; (B and C) dendrograms showing the variability of *Porphyromonas* sp. in DP (B) and NDP (C) groups.

Figure 4. *Treponema* and *Tannerella* sp. in pooled subgingival microbiota samples obtained from diabetic (DP) and non-diabetic (NDP) patients. Relative abundance of (A) six *Treponema* sp; and (B) Dendrogram showing the variability of *Treponema* sp., across pooled samples. (C) *Tannerella forsythia*.

is also consistent with previous reports, in which *P. gingivalis* is a quantitatively minor constituent of biofilms associated human periodontitis^{39–41}, in addition to its association with progressive bone loss in periodontitis patients⁴², particularly those with diabetes⁴¹.

Other studies showed that the red complex species can be detected in higher numbers when the disease reaches the advanced state⁸. However, this study showed that only the read counts of *T. forsythia* were found higher in patients with diabetes than the other red complex bacteria species. Our result supports the idea of polymicrobial synergy and dysbiosis for periodontitis, which highlights the importance of other bacterial species in keystone pathogenesis⁴³. Thus, species other than the red complex species may have similar keystone role in periodontitis³¹, as shown in this study. Another interesting finding was that we observed *T. forsythia* to be associated with periodontitis, and it did not relate to diabetes as its DNA was detected in all samples obtained from DP and NDP, and to lesser extent was the DNA of *P. endodontalis*. Similar findings have been reported previously^{44,45}. Similarly, *P. catoniae*, which has been found in the mouth of infants before eruption of their teeth⁴⁶, was detected in both DP and NDP groups in our study.

In this study, we observed that although the presence of the red complex species in DP group had similar trends as was seen in NDP group, two of them (*T. denticola* and *T. forsythia*) showed differences in abundance. This result might indicate that the different quantity is more likely due to host diabetic-related response. However, there are very diverse clinical and medical parameters that might affect the composition of the oral microbiome in systemic disorder patients²⁸. Hence, it is more likely that the subgingival plaque microbiome observed in this study was affected by several concurrent factors.

Analysis of *Aggregatibacter*, *Fusobacterium*, and *Veillonella*

In addition to the red complex bacteria differences observed between DP and NDP subjects, we studied the differences in the individual microbial species belong to *Aggregatibacter*, *Fusobacterium*, and *Veillonella*.

Regarding *Aggregatibacter* sp., *A. actinomycetemcomitans* have been officially designated as aetiology agents of periodontitis, together with *P. gingivalis*, and *T. forsythia*^{47,48}. Hence, our aim was to find out the presence of *A. actinomycetemcomitans* in a sample obtained from DP and NDP groups. While the DNA

sequence of *A. actinomycetemcomitans* was not present at any samples tested, we did find *A. aphrophilus* and *A. segnis*. These two species have been known to belong to the genus of *Aggregatibacter*, in addition to *A. actinomycetemcomitans*⁴⁹. Therefore, this finding is the first step towards understanding the potential contribution and a partnership between *A. aphrophilus* and *A. segnis* with *P. gingivalis*, and *T. forsythia* in periodontitis patients with and without diabetes. Comparison of the cumulative reads of the two species (*A. aphrophilus* and *A. segnis*), between DP and NDP groups is shown in **Figure 5A and B**. Additionally, despite the presence of *A. aphrophilus* and *A. segnis*, our data are consistent with a previous report that species belonging to genus *Aggregatibacter* were present at a relatively low level compared to other periodontal pathogenic species⁵⁰. Another study also showed that both *A. actinomycetemcomitans* and *Prevotella intermedia* are of only minor importance in periodontal disease progression⁵¹.

In terms of *Fusobacteria*, within oral cavity *F. nucleatum* is the most abundant species, in both diseased and healthy individuals^{52,53}. This species has a role in the progression of periodontal disease due to its ability to build a physical relationship (co-aggregation) with other oral bacterial species, notably with *P. gingivalis* and *T. denticola* formation of biofilm²⁹. Also, in the subgingival model, the count of *P. gingivalis* and some tested bacteria significantly decreased in the presence of *Fusobacterium sp./spp.*⁵⁴. Our data showed that, although the species variability of *Fusobacterium sp.* was relatively similar between the two groups tested, the cumulative reads of *F. nucleatum* was found more abundant in the DP group (**Figure 6A–C**). In contrast, the reverse was found for *P. gingivalis* (**Figure 3A**). Hence, it is important to carry out studies that evaluate the possibility of host response-associated diabetes regulating the interaction between *F. nucleatum* and *P. gingivalis*.

In this study, Firmicutes bacteria at genus level to be analysed was *Veillonella sp.* We found that the annotation accuracy for *Veillonella* at the genus level was 88%. The cumulative reads of sequences belonging to *Veillonella sp.* consisted of eleven and five species in DP and NDP, respectively (**Figure 7A–C**). We found that *V. parvula* was the predominant Firmicutes bacteria in subgingival microbiota of both groups, with more abundance in the DP group. Additionally, the results of this study was similar with a previous report elsewhere, in which *V. rogosae* was detected at a low number in DP patients⁵⁵, and was not detected in NDP individuals. Although it had been proposed to be used as an index for the state of chronic periodontitis⁵⁵, there is no clear explanation at present regarding the increased number of *V. parvula* in subgingival biofilms of diabetic patients. Our result, however, may indicate different environment conditions due to diabetes that may lead to increased number of *V. parvula* in subgingival niche. Interestingly, *Veillonella sp.* have been reported to have the ability to inhibit the host-cell effect of *P. gingivalis*⁵⁶, the red complex species that we found in lower abundance in the subgingival niche of DP patients in the current study. Thus, the difference in the amount and other bacterial species is not sufficient to explain the difference in periodontitis severity in a patient with diabetes. Although the host's immunological response may be influenced by diabetes²⁵, in the case of our subjects, other risk factors, including genetic background⁵⁷ may also affect inflammation and periodontal disease expression⁵⁸, which we did not include in this study. Considering these facts, we suggest that in periodontitis patient with diabetic, the subgingival microbiota formed by a low level of red complex and other representative bacteria may indicate that the red complex bacteria are necessary but insufficient to be linked to diabetes.

There are some limitations of this study. First, we compared the subgingival microbiome profile based on pooled PCR

Figure 5. *Aggregatibacter sp.* in pooled subgingival microbiota samples obtained from diabetic (DP) and non-diabetic (NDP) patients. (A) Abundance of *Aggregatibacter sp.*; (B) dendrograms showing the same *Aggregatibacter sp.* across pooled samples.

Figure 6. *Fusobacterium* sp. in pooled subgingival microbiota samples obtained from diabetic (DP) and non-diabetic (NDP) patients. (A) Abundance of *Fusobacterium* sp.; (B and C) dendrograms showing the variability of *Fusobacterium* sp. in DP (B) and NDP (C) groups.

amplicons separated by the DP and NDP groups, respectively, not with health and disease sites as controls. It is also well known that detailed site-specific information might be lost when using pooled samples for microbial analysis⁵⁹. Although being inferior to the non-pooling sample, this study suggests the pooling approach for sequencing studies, particularly if there are budgetary constraints that do not permit individual sample runs' analytical execution. Lastly, the descriptive analysis prevented us from testing the directional relationship between diabetes and periodontitis.

Conclusion

This is the first study in Indonesia to show that using the Nanopore MinION sequencing technology, we can investigate the presence of a consortium of red complex bacteria (*P. gingivalis*,

T. forsythia, and *T. denticola*) that includes three genera (*Aggregatibacter*, *Fusobacterium*, and *Veillonella*) in periodontitis subjects with and without diabetes. The present study revealed that the abundance of the sequence reads of six selected bacteria in subgingival microbiome were strongly affected by diabetic condition. All sequences observed in a large number were derived from the DP group. However, the six selected periodontal pathogens profile was relatively similar between DP and NDP pooled DNA samples. Therefore, we reject the hypothesis that the composition of subgingival biofilm in DP patients is more variable than in periodontitis subjects without diabetes. Additionally, one species belonging to the red complex bacteria (*P. gingivalis*) was only found in the subgingival microbiome of DP. Lastly, the capability of differentiating bacterial species, and even subspecies, as

Figure 7. *Veillonella* sp. in pooled subgingival microbiota samples obtained from diabetic (DP) and non-diabetic (NDP) patients. (A) Abundance of *Veillonella* sp.; (B and C) dendrograms showing the variability of *Veillonella* sp. in DP (B) and NDP (C) groups.

shown in this study, makes the MinION sequencer useful for pathogen detection in periodontitis subjects since it enables full-length 16S rRNA amplicon sequencing, while the reads can be analysed in real-time. However, we suggest, when investigating the subgingival microbiome of periodontitis patient with diabetes, there should be evidence in the presence of the targeted bacteria before the detection of attachment loss or bone loss

Data availability

Underlying data

Open Science Framework: A pilot study of red complex and three genera subgingival microbiome in periodontitis subjects with and without diabetes, evaluated by MinION, <https://doi.org/10.17605/OSF.IO/DQE6F60>.

This project contains the following underlying data

- Subject data
- Fastq files

Data are available under the terms of the [Creative Commons Zero “No rights reserved” data waiver](#) (CC0 1.0 Public domain dedication).

Acknowledgements

The authors would like to acknowledge to Astri Deviana for MinION Nanopore work at Department of Oral Biology, Faculty of Dentistry Universitas Indonesia.

References

1. Böttger EC: **Frequent contamination of Taq polymerase with DNA.** *Clin Chem.* 1990; **36**(6): 1258-9. [PubMed Abstract](#) | [Publisher Full Text](#)
2. Zhou M, Rong R, Munro D, et al.: **Investigation of the effect of type 2 diabetes mellitus on subgingival plaque microbiota by high-throughput 16S rDNA pyrosequencing.** *PLoS One.* 2013; **8**(4): e61516. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
3. Taylor GW, Borgnakke WS: **Periodontal disease: associations with diabetes,**

- glycemic control and complications. *Oral Dis.* 2008; **14**(3): 191–203.
[PubMed Abstract](#) | [Publisher Full Text](#)
4. Ohlrich EJ, Cullinan MP, Leichter JW: **Diabetes, periodontitis, and the subgingival microbiota.** *J Oral Microbiol.* 2010; **2**.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 5. Aemaimanan P, Amimanan P, Taweekaisupapong S: **Quantification of key periodontal pathogens in insulin-dependent type 2 diabetic and non-diabetic patients with generalized chronic periodontitis.** *Anaerobe.* 2013; **22**: 64–8.
[PubMed Abstract](#) | [Publisher Full Text](#)
 6. Ai D, Huang R, Wen J, et al.: **Integrated metagenomic data analysis demonstrates that a loss of diversity in oral microbiota is associated with periodontitis.** *BMC Genomics.* 2017; **18**(Suppl 1): 1041.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 7. Taylor JJ, Preshaw PM, Lalla E: **A review of the evidence for pathogenic mechanisms that may link periodontitis and diabetes.** *J Clin Periodontol.* 2013; **40** Suppl 14: S113–34.
[PubMed Abstract](#) | [Publisher Full Text](#)
 8. Socransky SS, Haffajee AD, Cugini MA, et al.: **Microbial complexes in subgingival plaque.** *J Clin Periodontol.* 1998; **25**(2): 134–44.
[PubMed Abstract](#) | [Publisher Full Text](#)
 9. Holt SC, Ebersole JL: ***Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*: the “red complex”, a prototype polybacterial pathogenic consortium in periodontitis.** *Periodontol 2000.* 2005; **38**: 72–122.
[PubMed Abstract](#) | [Publisher Full Text](#)
 10. Marsh PD: **Are dental diseases examples of ecological catastrophes?** *Microbiology (Reading).* 2003; **149**(Pt 2): 279–294.
[PubMed Abstract](#) | [Publisher Full Text](#)
 11. Zhang Y, Shan TL, Li F, et al.: **A novel phage from periodontal pockets associated with chronic periodontitis.** *Virus Genes.* 2019; **55**(3): 381–393.
[PubMed Abstract](#) | [Publisher Full Text](#)
 12. Caton JG, Armitage G, Berglundh T, et al.: **A new classification scheme for periodontal and peri-implant diseases and conditions - Introduction and key changes from the 1999 classification.** *J Periodontol.* 2018; **89** Suppl 1: S1–S8.
[PubMed Abstract](#) | [Publisher Full Text](#)
 13. Kusunmano K, Netzer M, Baumgartner C, et al.: **Effects of Pooling Samples on the Performance of Classification Algorithms: A Comparative Study.** *ScientificWorldJournal.* 2012; **2012**: 278352.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 14. Anand S, Mangano E, Barizzone N, et al.: **Next Generation Sequencing of Pooled Samples: Guideline for Variants’ Filtering.** *Sci Rep.* 2016; **6**: 33735.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 15. Shabardina V, Kischka T, Manske F, et al.: **NanoPipe-a web server for nanopore MinION sequencing data analysis.** *Gigascience.* 2019; **8**(2): gij169.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 16. Calus ST, Ijaz UZ, Pinto AJ: **NanoAmpli-Seq: a workflow for amplicon sequencing for mixed microbial communities on the nanopore sequencing platform.** *Gigascience.* 2018; **7**(12): gij140.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 17. Belstrøm D, Sembler-Møller ML, Grande MA, et al.: **Microbial profile comparisons of saliva, pooled and site-specific subgingival samples in periodontitis patients.** *PLoS One.* 2017; **12**(8): e0182992.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 18. Lalla E, Kaplan S, Chang SMJ, et al.: **Periodontal infection profiles in type 1 diabetes.** *J Clin Periodontol.* 2006; **33**(12): 855–62.
[PubMed Abstract](#) | [Publisher Full Text](#)
 19. Hintao J, Teanpaisan R, Chongsuvivatwong V, et al.: **The microbiological profiles of saliva, supragingival and subgingival plaque and dental caries in adults with and without type 2 diabetes mellitus.** *Oral Microbiol Immunol.* 2007; **22**(3): 175–81.
[PubMed Abstract](#) | [Publisher Full Text](#)
 20. Saeb ATM, Al-Rubeaan KA, Aldosary K, et al.: **Relative reduction of biological and phylogenetic diversity of the oral microbiota of diabetes and pre-diabetes patients.** *Microb Pathog.* 2019; **128**: 215–229.
[PubMed Abstract](#) | [Publisher Full Text](#)
 21. Benítez-Páez A, Portune KJ, Sanz Y: **Species-level resolution of 16S rRNA gene amplicons sequenced through the MinION™ portable nanopore sequencer.** *Gigascience.* 2016; **5**: 4.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 22. Kumar PS, Griffen AL, Moeschberger ML, et al.: **Identification of candidate periodontal pathogens and beneficial species by quantitative 16S clonal analysis.** *J Clin Microbiol.* 2005; **43**(8): 3944–55.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 23. Zijne V, Ammann T, Thurnheer T, et al.: **Subgingival biofilm structure.** *Front Oral Biol.* 2012; **15**: 1–16.
[PubMed Abstract](#) | [Publisher Full Text](#)
 24. Laver T, Harrison J, O'Neill PA, et al.: **Assessing the performance of the Oxford Nanopore Technologies MinION.** *Biomol Detect Quantif.* 2015; **3**: 1–8.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 25. Grossmann V, Schmitt VH, Zeller T, et al.: **Profile of the Immune and Inflammatory Response in Individuals With Prediabetes and Type 2 Diabetes.** *Diabetes Care.* 2015; **38**(7): 1356–64.
[PubMed Abstract](#) | [Publisher Full Text](#)
 26. Suzuki N, Yoneda M, Hirofuji T: **Mixed red-complex bacterial infection in periodontitis.** *Int J Dent.* 2013; **2013**: 587279.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 27. Wade WG: **Has the use of molecular methods for the characterization of the human oral microbiome changed our understanding of the role of bacteria in the pathogenesis of periodontal disease?** *J Clin Periodontol.* 2011; **38** Suppl 11: 7–16.
[PubMed Abstract](#) | [Publisher Full Text](#)
 28. Belstrøm D: **The salivary microbiota in health and disease.** *J Oral Microbiol.* 2020; **12**(1): 1723975.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 29. Kigure T, Saito A, Seida K, et al.: **Distribution of *Porphyromonas gingivalis* and *Treponema denticola* in human subgingival plaque at different periodontal pocket depths examined by immunohistochemical methods.** *J Periodontol Res.* 1995; **30**(5): 332–41.
[PubMed Abstract](#) | [Publisher Full Text](#)
 30. Segata N, Haake SK, Mannon P, et al.: **Composition of the adult digestive tract bacterial microbiome based on seven mouth surfaces, tonsils, throat and stool samples.** *Genome Biol.* 2012; **13**(6): R42.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 31. Hajishengallis G, Lamont RJ: **Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology.** *Mol Oral Microbiol.* 2012; **27**(6): 409–19.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 32. Honda K: ***Porphyromonas gingivalis* sinks teeth into the oral microbiota and periodontal disease.** *Cell Host Microbe.* 2011; **10**(5): 423–5.
[PubMed Abstract](#) | [Publisher Full Text](#)
 33. Castrillon CA, Hincapié JP, Yepes FL, et al.: **Occurrence of red complex microorganisms and *Aggregatibacter actinomycetemcomitans* in patients with diabetes.** *J Investig Clin Dent.* 2015; **6**(1): 25–31.
[PubMed Abstract](#) | [Publisher Full Text](#)
 34. Carrouel F, Viennot S, Santamaria J, et al.: **Quantitative Molecular Detection of 19 Major Pathogens in the Interdental Biofilm of Periodontally Healthy Young Adults.** *Front Microbiol.* 2016; **7**: 840.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 35. Darveau RP, Tanner A, Page RC: **The microbial challenge in periodontitis.** *Periodontol 2000.* 1997; **14**: 12–32.
[PubMed Abstract](#) | [Publisher Full Text](#)
 36. Kuramitsu HK, He X, Lux R, et al.: **Interspecies interactions within oral microbial communities.** *Microbiol Mol Biol Rev.* 2007; **71**(4): 653–70.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 37. Kesavalu L, Sathishkumar S, Bakthavatchalu V, et al.: **Rat model of polymicrobial infection, immunity, and alveolar bone resorption in periodontal disease.** *Infect Immun.* 2007; **75**(4): 1704–12.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 38. Thorstensson H, Dahlen G, Hugoson A: **Some suspected periodontopathogens and serum antibody response in adult long-duration insulin-dependent diabetics.** *J Clin Periodontol.* 1995; **22**(6): 449–58.
[PubMed Abstract](#) | [Publisher Full Text](#)
 39. Kumar PS, Leys EJ, Bryk JM, et al.: **Changes in periodontal health status are associated with bacterial community shifts as assessed by quantitative 16S cloning and sequencing.** *J Clin Microbiol.* 2006; **44**(10): 3665–73.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 40. Abusleme L, Dupuy AK, Dutzan N, et al.: **The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation.** *ISME J.* 2013; **7**(5): 1016–25.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 41. Preshaw PM, Alba AL, Herrera D, et al.: **Periodontitis and diabetes: a two-way relationship.** *Diabetologia.* 2012; **55**(1): 21–31.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 42. Chaves ES, Jeffcoat MK, Ryerson CC, et al.: **Persistent bacterial colonization of *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Actinobacillus actinomycetemcomitans* in periodontitis and its association with alveolar bone loss after 6 months of therapy.** *J Clin Periodontol.* 2000; **27**(12): 897–903.
[PubMed Abstract](#) | [Publisher Full Text](#)
 43. Rosier BT, De Jager M, Zaura E, et al.: **Historical and contemporary hypotheses on the development of oral diseases: are we there yet?** *Front Cell Infect Microbiol.* 2014; **4**: 92.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 44. Kumar PS, Griffen AL, Barton JA, et al.: **New bacterial species associated with chronic periodontitis.** *J Dent Res.* 2003; **82**(5): 338–44.
[PubMed Abstract](#) | [Publisher Full Text](#)
 45. Leys EJ, Lyons SR, Moeschberger ML, et al.: **Association of *Bacteroides forsythus* and a novel *Bacteroides* phylotype with periodontitis.** *J Clin Microbiol.* 2002; **40**(3): 821–5.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 46. Nononen E, Kanervo A, Takala A, et al.: **Establishment of oral anaerobes during the first year of life.** *J Dent Res.* 1999; **78**(10): 1634–9.
[PubMed Abstract](#) | [Publisher Full Text](#)
 47. Haffajee AD, Socransky SS: **Microbial etiological agents of destructive periodontal diseases.** *Periodontol 2000.* 1994; **5**: 78–111.
[PubMed Abstract](#) | [Publisher Full Text](#)

48. Armitage GC: **Periodontal diseases: diagnosis.** *Ann Periodontol.* 1996; **1**(1): 37–215.
[PubMed Abstract](#) | [Publisher Full Text](#)
49. Murra M, Lützen L, Barut A, *et al.*: **Whole-Genome Sequencing of *Aggregatibacter* Species Isolated from Human Clinical Specimens and Description of *Aggregatibacter kilianii* sp. nov.** *J Clin Microbiol.* 2018; **56**(7): e00053-18.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
50. He J, Huang W, Pan Z, *et al.*: **Quantitative analysis of microbiota in saliva, supragingival, and subgingival plaque of Chinese adults with chronic periodontitis.** *Clin Oral Investig.* 2012; **16**(6): 1579–88.
[PubMed Abstract](#) | [Publisher Full Text](#)
51. Cullinan MP, Hamlet SM, Westerman B, *et al.*: **Acquisition and loss of *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* and *Prevotella intermedia* over a 5-year period: effect of a triclosan/copolymer dentifrice.** *J Clin Periodontol.* 2003; **30**(6): 532–41.
[PubMed Abstract](#) | [Publisher Full Text](#)
52. Field CA, Gidley MD, Preshaw PM, *et al.*: **Investigation and quantification of key periodontal pathogens in patients with type 2 diabetes.** *J Periodontal Res.* 2012; **47**(4): 470–8.
[PubMed Abstract](#) | [Publisher Full Text](#)
53. Miranda TS, Feres M, Retamal-Valdés B, *et al.*: **Influence of glycemic control on the levels of subgingival periodontal pathogens in patients with generalized chronic periodontitis and type 2 diabetes.** *J Appl Oral Sci.* 2017; **25**(1): 82–89.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
54. Thurnheer T, Karygianni L, Flury M, *et al.*: ***Fusobacterium* Species and Subspecies Differentially Affect the Composition and Architecture of Supra- and Subgingival Biofilms Models.** *Front Microbiol.* 2019; **10**: 1716.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
55. Mashima I, Nakazawa F: **The interaction between *Streptococcus* spp. and *Veillonella tobetsuensis* in the early stages of oral biofilm formation.** *J Bacteriol.* 2015; **197**(3): 2104–2111.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
56. Tenorio EL, Klein BA, Cheung WS, *et al.*: **Identification of interspecies interactions affecting *Porphyromonas gingivalis* virulence phenotypes.** *J Oral Microbiol.* 2011; **3**.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
57. Michalowicz BS, Diehl SR, Gunsolley JC, *et al.*: **Evidence of a substantial genetic basis for risk of adult periodontitis.** *J Periodontol.* 2000; **71**(11): 1699–707.
[PubMed Abstract](#) | [Publisher Full Text](#)
58. Repeke CE, Trombone APF, Ferreira SB Jr, *et al.*: **Strong and persistent microbial and inflammatory stimuli overcome the genetic predisposition to higher matrix metalloproteinase-1 (MMP-1) expression: a mechanistic explanation for the lack of association of *MMP1-1607* single-nucleotide polymorphism genotypes with MMP-1 expression in chronic periodontitis lesions.** *J Clin Periodontol.* 2009; **36**(9): 726–38.
[PubMed Abstract](#) | [Publisher Full Text](#)
59. Socransky SS, Haffajee AD: **Periodontal microbial ecology.** *Periodontol.* 2000; **38**: 135–87.
[PubMed Abstract](#) | [Publisher Full Text](#)
60. Bachtar EW: **A pilot study of red complex and three genera subgingival microbiome in periodontitis subjects with and without diabetes, evaluated by MinION.** 2021.
<http://www.doi.org/10.17605/OSF.IO/DQE6F>

Open Peer Review

Current Peer Review Status:

Version 3

Reviewer Report 06 July 2021

<https://doi.org/10.5256/f1000research.58058.r88545>

© 2021 Susilowati H. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Heni Susilowati

Oral Biology Department, Universitas Gadjah Mada, Yogyakarta, Indonesia

Thank you for the response from the author to the previous suggestions. However, there are still similarities between the work described in paragraph 3 of the Methods with the beginning of paragraph 5.

In addition, I suggest a description of the DNA isolation procedure in the Microbial Sampling section, following paragraph 5.

Thank you.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Oral Microbiology and Immunology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 06 Jul 2021

Boy Muchlis Bachtiar

Dear Editor,

I have read the Reviewer's suggestion, and I revised the paragraph in the method section as suggested by the Reviewer.

Thank you

Competing Interests: No competing interests were disclosed.

Version 2

Reviewer Report 21 June 2021

<https://doi.org/10.5256/f1000research.55175.r87054>

© 2021 Susilowati H. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Heni Susilowati ¹ Oral Biology Department, Universitas Gadjah Mada, Yogyakarta, Indonesia² Oral Biology Department, Universitas Gadjah Mada, Yogyakarta, Indonesia

This research is well organized and benefits the development of microbiology and periodontology. The research background is well explained. Results are well presented and easy to understand. The analysis is comprehensive. The conclusions represent the answers to the research problems.

For the Methods section: The sequence of the study in the form of sampling of subgingival plaque isolates and microbial samples, respectively, is written in paragraphs 3 and 6 of the Methods section, whether they are two stages of the same or different work. The order of explanation of these stages is a bit confusing. Paragraph 6 may be more appropriate to be written in paragraph 3.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Oral Microbiology and Immunology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 22 Jun 2021

Boy Muchlis Bachtiar

We appreciate for the reviewer (two) comments and suggestion. Therefore, we have revised this manuscript accordingly. Thank you.

The reviewer's comment is regarding the explanation of subgingival plaque sampling and microbial samples. We agree, the explanation is rather confusing. Because they are the same work, we put both the paragraph 3 and 6, and rewritten them together as paragraph 1 under subheading "microbial sample."

Competing Interests: No competing interests were disclosed.

Reviewer Report 16 June 2021

<https://doi.org/10.5256/f1000research.55175.r86513>

© 2021 Wan Ibrahim W. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Wan Izlina Binti Wan Ibrahim

¹ Department of Oral and Craniofacial Sciences, Faculty of Dentistry, Universiti Malaya, Kuala Lumpur, Malaysia

² Department of Oral and Craniofacial Sciences, Faculty of Dentistry, Universiti Malaya, Kuala Lumpur, Malaysia

I have no further comments to make.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Oral Biology (Oral Biochemistry, Oral Microbiology, Oral Histology, Oral Physiology, Oral Environment); Traditional Medicine and Natural Products (Free radical biochemistry & DNA damage); Biotechnology, Medical Biotechnology (Gel- & non-gel-based proteomics, liquid chromatography & mass spectrometry); Proteins (Proteomics & glycoproteomics - cancer, disease biomarkers, human and mammalian milk)

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 02 March 2021

<https://doi.org/10.5256/f1000research.31209.r79850>

© 2021 Wan Ibrahim W. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Wan Izlina Binti Wan Ibrahim

¹ Department of Oral and Craniofacial Sciences, Faculty of Dentistry, Universiti Malaya, Kuala Lumpur, Malaysia

² Department of Oral and Craniofacial Sciences, Faculty of Dentistry, Universiti Malaya, Kuala Lumpur, Malaysia

³ Department of Oral and Craniofacial Sciences, Faculty of Dentistry, Universiti Malaya, Kuala Lumpur, Malaysia

General comments:

- The manuscript presents interesting findings regarding the presence of red complex and three genera subgingival microbiome in periodontitis subjects with and without diabetes, using ONT MinION platform, which is one of the most recent sequencing technologies.
- While detailing the advantages of the platform, it would be great to also describe about the limitations or drawbacks of this technology, because the authors made quite a number of postulations, some can be confounding, based on literatures that used other technologies or techniques. This platform is known to be error prone, particularly in studying small or

rare sequence differences.

- It would be also be really great if the authors consider to include a method for bacterial identification and quantification that is able to validate these results, perhaps a preliminary study, if there is any?
- Suggest proofread again to recheck incorrect sentence structure, missing preposition, as well as minor spelling and grammar mistakes. E.g. phylum are sometimes capitalized when it is not actually a proper noun.

Specific comments:

Method

Microbial assessment:

- Eppendorf is a brand. Should be written as 'a microcentrifuge tube' instead of "an Eppendorf tube".
- I suggest changing the subheading "Microbial assessment", to 'Microbial sample collection'.
- It is unclear what the authors meant by "*The presence at genus and species level of the red complex bacteria (Phorphyromonas gingivalis, Treponema denticola, and Tannerella forsythia), and three other selected genera (Aggregatibacter, Fusobacterium, and Veillonella) were determined in subgingival biofilm samples.*" - How did the authors determine "the presence" of these bacteria? Or the authors are actually already referring to the MinION sequencing already? If yes, kindly consider revising the sentence.
- Third paragraph "MinION™" - The authors should be consistent, whether to use MinION with TM (superscript) or without, throughout the text.

MinION sequencing and data analysis:

- "Polymerase Chain Reaction" is not a proper noun, so there is no need to capitalize each word.

Results and discussion

Read analysis:

- Second paragraph, third sentence "*By comparing the read count, we found that the classified sequence reads in the pooled sample of DP were 112.173 and 1988, respectively, while in the pooled sample of NDP were 1478 and 172, respectively (Figure 1A).*" Consider revising this sentence.
- Perhaps the authors intended to write 'we found that the classified and unclassified sequence reads in the pooled sample of DP were 112.173 and 1988, respectively, while the classified and unclassified sequence reads in the pooled sample of NDP were 1478 and 172, respectively.'

Distribution of the subgingival microbiota at the phylum and family level

- Second paragraph, "*All bacteria belonging to these phyla were increased in the DP group compared with the NDP group (Figure 2A).*". I believe 'phyla' should be replaced with 'families'.

Analysis of Aggregatibacter, Fusobacterium, and Veillonella

- Figure 5 - In the text, the authors described Figure 5A and 5B as showing the cumulative

reads of *A. aphrophilus* and *A. segnis* between DP and NDP groups. However, the legend says (A) Abundance of *Aggregatibacter* sp.; (B) Dendrogram showing the variability of *Treponema* sp., across pooled samples. (C) *Tannerella forsythia*. Kindly revise the legend.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Oral Biology (Oral Biochemistry, Oral Microbiology, Oral Histology, Oral Physiology, Oral Environment); Traditional Medicine and Natural Products (Free radical biochemistry & DNA damage); Biotechnology, Medical Biotechnology (Gel- & non-gel-based proteomics, liquid chromatography & mass spectrometry); Proteins (Proteomics & glycoproteomics - cancer, disease biomarkers, human and mammalian milk)

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 03 Mar 2021

Boy Muchlis Bachtiar

General comments:

We highly appreciate the reviewers' insightful and helpful comments on our manuscript.

- According to the reviewer suggestions, we will carefully rewrite the incorrect sentence structure, missing preposition, as well as minor spelling and grammar mistakes. For example, as suggested, the typo of "Phylum" will be changed to 'phylum', in the new version of this manuscript.
- We also would like to correct some mistake we made in the original manuscript.

- In the new version of this manuscript, we will explain the limitations of the MinION platform, as well as adding the needed reference, and will put it in the last sentence of the second paragraph when explain the Synergistic phylum, as follows:
"However, no genus belonging to Synergistic phylum was detected in this study. We assumed this is because of the low-read accuracy by MinION platform (Laver 2015), which complicates our complex samples' analysis."
- Regarding the other identification and quantification method to validate this sequencing platform, our response is we did not include bacterial identification and quantification method, such as real time-PCR to validate the sequencing result, as our main purposed was to estimate microbial community diversity using MinION platform.

Specific comments:

Method

Microbial assessment:

- As suggested, the word "Eppendorf tube" has been changed to "microcentrifuge tube". Thank you.
- The sub heading "Microbial assessment" has been changed to "Microbial sample". Thank you.
- As suggested, the sentence, "The presence at genus and species level of the red complex bacteria (Phorphyromonas gingivalis, Treponema denticola, and Tannerella forsythia), and three other selected genera (Aggregatibacter, Fusobacterium, and Veillonella) were determined in subgingival biofilm samples, will be changed in the new version. Thus, the new sentence become," *in order to detect the The presence at genus and species level of the red complex bacteria (Phorphyromonas gingivalis, Treponema denticola, and Tannerella forsythia), and three other selected genera (Aggregatibacter, Fusobacterium, and Veillonella) were determined in subgingival biofilm samples.*
- According to the reviewer suggestion, we have changed the word MinION™, and only write it as MinION throughout the text. Thank you.
- Yes, we agree, we will rewrite the "Polymerase Chain Reaction" without the capital word. Thank you.

C. Results and discussion

Read analysis:

- "By comparing the read count, we found that the classified sequence reads in the pooled sample of DP were 112.173 and 1988, respectively, while in the pooled sample of NDP were 1478 and 172, respectively (Figure 1A)". Consider revising this sentence.

Author response: Yes, thank you for this important correction. As suggested, we will change the wrong sentence, In the new version, the corrected sentence will become "By comparing the read count, we found that the classified *and unclassified* sequence reads in the pooled sample of DP were 112.173 and 1988, respectively, while the classified and unclassified sequence reads in the pooled sample of NDP were 1478 and 172, respectively".

- Under the subtitle; within the second paragraph, "Distribution of the subgingival

microbiota at the phylum and family level”.

Author response: We agree with the suggestion. Hence the word phyla have been changed to be families. Thank you.

Analysis of Aggregatibacter, Fusobacterium, and Veillonella:

- Figure 5 - In the text, the authors described Figure 5A and 5B as showing the cumulative reads of *A. aphrophilus* and *A. segnis* between DP and NDP groups. However, the legend says (A) Abundance of *Aggregatibacter* sp.; (B) Dendrogram showing the variability of *Treponema* sp., across pooled samples. (C) *Tannerella forsythia*. Kindly revise the legend.

Author response: Thank you very much for this very important correction. Accordingly, in the new version, the legend will be revised.

Competing Interests: No competing interests

Comments on this article

Version 2

Author Response 22 Jun 2021

Boy Muchlis Bachtiar

We appreciate for the reviewer (two) comments and suggestion. Therefore, we have revised this manuscript accordingly. Thank you.

The reviewer's comment is regarding the explanation of subgingival plaque sampling and microbial samples. We agree, the explanation is rather confusing. Because they are the same work, we put both the paragraph 3 and 6, and rewritten them together as paragraph 1 under subheading "microbial sample".

Competing Interests: No competing interests were disclosed.

The benefits of publishing with F1000Research:

- Your article is published within days, with no editorial bias
- You can publish traditional articles, null/negative results, case reports, data notes and more
- The peer review process is transparent and collaborative
- Your article is indexed in PubMed after passing peer review
- Dedicated customer support at every stage

For pre-submission enquiries, contact research@f1000.com

F1000Research