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Abstract

Aims: This pilot study aimed to describe the majority and minority microbiota of saliva from individuals with advanced periodontitis, with or without type 2 diabetes mellitus (T2D), correlating the relative abundance of microorganisms with clinical parameters.

Material/Methods: Six individuals diagnosed with periodontitis were included and classified according to their diagnosis of T2D. Salivary pH, number of teeth with active caries lesions, number of remaining teeth, periodontal and glycemic parameters were evaluated. V4 region amplicons of the 16S rRNA from salivary DNA were sequenced at Ion PGM. Amplicon sequence variants (ASVs) were compared according to the clinical parameters.

Results: Correlation showed eight low-abundant bacteria significantly correlated with co-variables, either positively or negatively. The periodontitis-associated bacteria followed the increasing pH and number of remaining teeth.

Conclusions: This survey provided potential minority microbiota correlations with clinical parameters, such as number of remaining teeth, FBG, and salivary pH. The ubiquity of some low abundant microorganisms in individuals with advanced periodontitis, exposed or not to type 2 DM, can reveal microbial signatures not yet explored.

Keywords: periodontal disease; type 2 diabetes; low abundance microorganisms

1. INTRODUCTION

Changes in the salivary flow and composition are among the most critical factors for the oral microbiota shift. The balance of oral microorganisms can be disrupted, prompting oral diseases due to a dysbiosis (1). The salivary secretion is strongly affected by age, medications and hypoglycemia (2, 3), the last being particularly habitual in patients with diabetes mellitus (DM). This common condition has an established impact on periodontitis, but its relative importance in other oral diseases has been subject to considerable discussion (4, 5). Extensive research has shown that DM leads not only to salivary dysfunctions in terms of flow rates (hyposalivation) (3, 6-9), but also to significant changes in the composition of saliva that is secreted by the salivary glands, increasing the salivary glucose levels (10).

There is strong evidence demonstrating that glycemic control plays a crucial role in regulating the periodontal-associated subgingival biofilm (11-14). A potential impact of DM on the salivary microbiome has also been investigated (15-18). Correlations of the salivary microbiome with periodontitis and DM-related parameters were recently performed (19), and significant microbial composition changes were observed. However, those studies neither distinguish the severity of periodontitis nor correlate the microbiota to clinical oral conditions other than periodontal-related ones. Furthermore, research on the DM-associated salivary microbiome has mainly been restricted to the identification of most abundant microbiota associated with health or disease, overlooking the low-abundant microbiota. We believe that this strategy could cause a misunderstanding of whole environment as metabolic functions exerted by low-abundant microorganisms can be linked the dysbiotic microhabitats (20).

Since the salivary microbiome can reflect the entire oral microenvironment, saliva can be a more representative sample for searching the shifts to dysbiosis. By studying the salivary fluid of individuals with DM, oral manifestations of DM other than periodontitis affect the microbial composition can be better explored. The significant salivary dysfunctions in individuals with DM can be associated with variations in its microbiome. Goodson et al. evaluated changes in abundance of some bacterial species in the saliva of adolescents with high concentrations of salivary glucose, showing that the higher the salivary glucose the lower the pH of the saliva (21). As glucose is a well-known energy source for many oral bacteria, changes in its concentration may have a pivotal role in the salivary microbiome leading to reduced overall bacterial diversity and significant changes in the relative bacterial frequencies, favouring acidic bacterial species.

This pilot study aimed to describe the majority and minority microbiota of saliva from individuals with advanced periodontitis, with or without type 2 DM (T2D), correlating the relative abundance of organisms with the salivary pH, the number of teeth with active caries lesions, the number of remaining teeth and the periodontal and glycemic parameters.

2. MATERIAL AND METHODS

2.1. Ethics

This study was approved by the Research Ethics Committee of the School of Health Sciences of the University of Brasília (process number 87962818.4.0000.0030) in accordance with the declaration of Helsinki. All patients signed a formal consent form and received basic dental treatment.

2.2. Study Design, Setting and Participants

A pilot study enrolled six individuals diagnosed with periodontitis according to a New Classification of Periodontal Disease (22) at the "Diabetes dental clinic" at the University Hospital of Brasília. Patients were recruited and the data collected from August 2018 to March 2019.

To be included, individuals should have age ≥30 years and at least two teeth with interproximal insertion loss ≥ 3mm. Exclusion criteria comprised DM complications and severe systemic comorbidities; transplanted patients; a positive history of epilepsy; a history of radiotherapy in the head and neck regions; and chemotherapy treatment prior to 3 months. DM status was established by a reported medical diagnosis of DM type 2 (T2D) and by the use of either insulin or other oral hypoglycemic medications. Six patients with periodontal disease were included in the present study and were divided according to the medical condition, as follows: three patients were not previously diagnosed with DM (P21, P10 e P2) and three had been previously diagnosed with DM (DMP14, DMP27 e DMP33).

2.3. Clinical examination

Trained examiners evaluated the periodontal status. The periodontal parameters such as probing depth, clinical attachment loss and bleeding on probing (BOP) were recorded using a Williams-type periodontal probe (Hu-Friedy MFG. Co. Inc., Chicago, IL, USA). The number of remaining teeth was counted by visual inspection. The number of teeth with either non-cavitated or cavitated caries lesions was also recorded, including the classification of lesion activity (23) by trained and calibrated examiners (weighted Kappa>0.7).

Participants were also tested regarding their fasting blood glucose (FBG) levels within three months before salivary collection (Sabin laboratories, Brasília, Brazil).

2.4. Salivary collection

Stimulated and passive salivary flow collects were performed in the morning (8-10 am) to minimize the effect of circadian rhythms. Individuals were asked to refrain from drinking, eating, and performing physical activities at least two hours before salivary collection (17). The collection time by passive drooling was 5 minutes. After collection, the unstimulated saliva was aliquoted (500μL) into sterile DNAse and RNAse free microtubes and stored at -20ºC until further DNA extraction and sequencing. The suspension was then centrifuged for 30 seconds at 10,000 rpm and the supernatant discarded.

2.5. Salivary flow, pH, and buffer capacity assays

The salivary pH and buffer capacity were tested in the stimulated saliva. After a minute of chewing a rubber device, the total volume of stimulated saliva was collected for 5 minutes by passive drool to a plastic container. The pH was checked using an indicator strip. Buffer capacity was assessed after adding 3mL of 0.005M hydrochloric acids for every 1mL of stimulated saliva. After 2 minutes, the pH was measured, considering a low buffering capacity when the pH values were less than 4.0.

2.6. Salivary DNA extraction and PCR

Extraction of the total DNA was performed following the protocol established by Smalla et al. (24). The protocol of the DNA amplification of the 16S rRNA gene using the universal primers F515/R806 was described elsewhere (25). Briefly, approximately 10ng of genomic DNA was added into a 50-μL PCR mixture, including a 1U Platinum Taq DNA polymerase (Tm 52 °C).

2.7. Amplicon sequencing and bioinformatics

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After the amplicons were purified using Agencount AMPure Beads (Beckman Coulter), a library construction was performed as described in the Ion Plus Fragment Library from an initial amount of 100ng of DNA. Sequencing was conducted on an Ion PGM System (Thermo Fisher) using an Ion 316 chip, following the manufacturer's instructions.

The 16S rRNA gene reads were submitted to the DADA2 (26) for a single read dataset of amplicon sequence variants (ASVs), a more stringent pipeline to identify low abundant microorganisms. Reads were filtered by quality and size, and error rates were estimated. Singletons were identified and removed during dereplication. Chimeras were removed, and taxonomy was assigned using the Silva v.132 database (27). ASVs assigned to Eukaryote, Chloroplast, or Mitochondria were removed for further analysis. The sequences were deposited at the National Biotechnology Information Center (NCBI) under the BioProject PRJNA558499.

2.8. Data analysis

Mann-Whitney test was applied to compare clinical parameters between groups after confirming the non-parametric condition of the data (SPSS software version 25.0 for Mac; SPSS Inc., Chicago, IL, USA). The values per ASVs were summed per phyla and then the average was calculated by the clinical characteristics. A metadata table was done with the clinical characteristics of the individuals, including salivary pH, number of active caries lesions, number of teeth, FBG, the proportion of sites with BOP, and proportion of insertion loss >5mm. Spearman correlation was used to compare ASVs relative abundance (RA) with these metadata. The saliva microbiome of individuals was also grouped according to clinical characteristics for a descriptive analysis. Mean and standard deviation were calculated for the clinical variables. The significance level considered was $p < 0.05$.

3. RESULTS

3.1. Clinical characteristics

According to the extension of periodontal disease, all patients had generalized periodontitis, except the individual DMP33 that presented localized periodontitis. Two patients (DMP27 and P10) were diagnosed as periodontitis stage IV and four patients (DMP14, P2, P21, DMP33) as periodontitis stage III. Only one individual diagnosed with DM presented uncontrolled levels of blood glucose (369 ml/dl). Subjects without a diagnosis of DM had a borderline FBG level (~100 ml/dl).

Individuals with both periodontitis and DM (named as DMP group) were older than those presenting periodontitis and no DM (named as P group). The salivary pH in individuals with the diagnosis of DM ranged from pH=7-8, while in P it ranged from pH=6-7. The number of remaining teeth, the salivary pH, buffering capacity, and the number of teeth with active caries were similar between individuals from both groups (p>0.05). The proportion of sites with BOP and the proportion of teeth with clinical attachment loss >5mm were high and similar in both groups (Table 1).

3.2. Sequencing output and the relative abundance of the microbiota

A total of 252,964 reads were generated from saliva samples and 116,935 qualified reads were distributed in 442 ASVs. After screening and optimization, 332 ASVs remained for further analysis, and combined into 117 OTUs, of which two out of them were assigned to archaea. Figure 1 shows the relative abundance of the majority microbiota (ASVs ≥1%), representing more than 99% of the total sequences. At the phylum level (1A), the predominance of *Firmicutes* was observed in both groups; however, saliva of individuals with a diagnosis of DM (DMP group) were enriched with

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Bacteroidetes and *Proteobacteria*. In individuals with periodontitis but no DM, a tendency of enrichment of *Actinobacteria, Fusobacteria* and *Spirochaetes* was observed.

At the genus level (or respective ASV) (1B), the majority microbiota represented 93-94% of the whole microbiota in both groups. The salivary microbiome was dominated by the genus *Streptococcus,* representing ~30% of the total microbiota in both groups. The genera *Fusobacterium* (average DMP=6%; P=13%), *Porphyromonas* (average DMP=7.5%; P=7.8%)*,* and *Gemella* (average DMP=4.7%; P=5.1%) were also highly prevalent in both groups, presenting ≥1% of the total RA across all samples. The genus *Veillonella* presented a higher abundance in patients with a diagnosis of DM: >1.5% in all DMP samples (average=2.8%), while in all the P samples, it was <1% (average=0.6%). The same pattern was observed for genus *Prevotella*, which presented an average of 5.3% in DMP and only <0.3% across all P group samples. Conversely, *Treponema* and *Tannerella,* both periodontitis-associated genera, presented low abundance in the DMP group (average 0.03 and 0.1%, respectively), but higher in the P group (3% and 0.7%, respectively).

As we considered the ASV, it was possible to analyse the minority microbiota (<1%), representing 0.6 and 0.8% of the total relative abundance in the DMP and P, respectively, and dominated by phylum *Epsilonbacteraeota* (Figure 1C). Saliva of the P group was enriched with *Synergistes*. The most striking result was the identification of reads of two groups belonging to the domain *Archaea*, phyla *Thaumarchaeota* and *Euryarchaeota* (0.17% and 0.03%, respectively) in a single DMP sample (DMP14), which also presented the lowest salivary pH (pH=6) and the highest BOP (75%). Although in low abundance, ASVs affiliated to genera never or infrequently described in periodontitis were ubiquitous, found in all individuals: *Fermentimonas* (≥0.2%; same family as *Porphyromonas* and *Tannarela*), *Caldicoprobacter* (≥0.13%), *Rikenellaceae* (≥0.1%), *Lentimicrobiaceae* (≥0.05%), *Fastidiosipila* (≥0.08%), *Alishewanella* (≥0.07%), unnamed CK06-06-Mud-MAS4B-21 (≥0.05%). *Solobacterium* was found in all DMP samples, with at least 0.1% of relative abundance.

3.3 Correlation of the microbial composition and the clinical parameters

To better explore these findings from the descriptive analysis, a Spearman correlation was performed to compare the clinical parameters and the RA of microorganisms (Figure 2). Eight ASVs were significantly correlated to clinical variables, either positively or negatively. Interestingly, the pattern observed when the variables were grouped was not observed here. Neither the salivary pH nor the presence of lesions with caries activity presented significant correlations with the microbial composition.

FBG was positively correlated to *Absconditabacteriales* (SR1) and *Caldicoprobacter* and negatively correlated to *Leptotrichia, Parviromonas,* and *Balneolaceae*; unnamed CK06-06-Mud-MAS4B-21. *Abiotrophia* was negative, while *Johnsonella* was positively correlated to the number of remaining teeth. The clinical attachment loss >5mm was positively correlated to the relative abundance of the *Balneolaceae* CK06-06-Mud-MAS4B-21 and *Fastidiosipila.* Interestingly, none comprised the majority microbiota, but *Leptotrichia* (3 out of 6 samples with 1%)*, Absconditabacteriales* (SR1) (a single sample >1%) and *Parviromonas* (2 out of 6 samples with 1%).

The CCA triplot showed a contrary direction of the salivary pH and the FBG and the BOP (referred to as bleeding index in the figure) and the clinical attachment loss >5mm. The periodontitis-associated bacteria, *Tannarela, Treponema,* and *Porphyromonas*, followed the increasing pH and number of remaining teeth (Figure 3).

4. DISCUSSION

To gain insight into the correlation of the salivary microbiome and clinical parameters of patients with periodontitis with or without DM, this pilot study analyzing the abundance of microbial ASVs commonly found in the oral cavity sites using a next-generation sequencing approach. A core microbiome was similar to the ones related to dental biofilms, including *Streptococcus, Fusobacterium,* and *Gemella*, with some bacteria abundance strongly affected by the diagnosis of DM, as expected. However, the molecular survey used here provided a much broader picture of overall differences in the minority microbiota. *Fermentimonas*, *Caldicoprobacter*, *Rikenellaceae*, *Lentimicrobiaceae*, *Fastidiosipila*, *Alishewanella*, and unnamed CK06-06-Mud-MAS4B-21, were ubiquitous and in low abundance. *Solobacterium* was a low abundant organism associated with the presence of DM. The presence of an inflammatory process in the periodontal sites, the salivary pH fluctuation, the blood glucose level and the number of remaining teeth reflected intricate competition patterns among low abundance microbial populations, as significant correlations were observed between clinical parameters and ASVs representing the minority microbiota.

There has been a renewed interest in the influence of minority organisms in the microbiome accompanying dysbiosis, as primary functions can be assigned to minor members in the community. Studies of the minority microbiome may reveal unique signatures associated with both periodontal and diabetic status and may shed light on the mechanisms of microbial communities and how they prompt disease. However, few NGS-based studies has been describing the microbiota in low abundance (<1%), and the main reasons are the low detection power and a high chance of inaccuracy (20). This problem has been worked around here by using a more robust pipeline against detection errors in the data (single-read-based, instead of assembly-based classification), an issue that can be expected to be of much higher relevance in low-abundance species than in the high-abundance ones. We also observed the ubiquity in conjunction with a characteristic of abundance of taxa. The analysis of the low abundant organisms is not a search for a 'guilty', but a study on how some species metabolism can influence (or be impacted by) the transition from health to disease. Interestingly, none of the organisms correlated with clinical parameters comprised the majority microbiota, but *Leptotrichia* (3 out of 6 samples with 1%)*,* SR1 (a single sample >1%) and *Parviromonas* (2 out of 6 samples with 1%).

The abnormal blood sugar status can disrupt homeostasis, providing a more profound change on the microbiota profile particularly when combined with periodontitis (19). In a recent study, Matsha et al. detected nine phyla in plaque samples of individuals with DM and periodontitis, namely *Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria, and Actinobacteria* (13). Their findings agree with ours, except for *Proteobacteria* that is enriched in DM in the present study. Our results identified a positive correlation of blood glucose level with *Absconditabacteriales* [SR1, recently found in interproximal supragingival biofilms (28) and the anaerobic *Caldicoprobacter*. On the other hand, *Leptotrichia*, *Parviromonas*, and *Balneolaceae* significantly decreased with the increased blood glucose. The periodontal clinical parameters evaluated in the present study enable to find a positive correlation of clinical attachment level with *Balneolaceae* and *Fastidiosipila,* the last one detected in all samples. It is essential to point out that the most frequent genera strongly associated with periodontitis, such as *Tannerella*, *Treponema,* and *Porphyromonas* demonstrated a connection with increased pH and the number of remaining teeth (Figure 3). *Abiotrophia* was negative, while *Johnsonella* was positively correlated to the number of remaining teeth (Figure 2). Due to the lack of power to confirm those correlations, more studies are necessary focusing on the salivary minority microbiota. We project that it is necessary to recruit a minimum sample size of 14 to detect a correlation of 0.7, power of 80%, and alpha of 5% (Fisher's Z test).

What stands out in the patients' clinical parameters is the slightly lower salivary pH in the presence of DM. The superior amount of glucose in the saliva and the crevicular gingival fluid increases the acidogenicity of bacteria (21). It can be accountable for reducing the pH, which can contribute to the proliferation of fermenting organisms and metabolizing acids. Yang et al., for

example, observed an enrichment of *Lactobacillus fermentum* in the saliva of patients with DM that decreased after DM treatment (18). Furthermore, important pH regulating mechanisms are lactate metabolism that facilitates the pH neutralization, producing propionate, acetate, CO_2 and H_2; the nitrogen utilization by some species; and the release of basic substances to lower pH (29). Those characteristics reflected the enrichment of some bacteria in the DM group, such as *Veillonella* and *Prevotella,* both observed in the CCA triplot following the decreased pH and increased FBG. *Veillonella* is considered an "acid sink" organism linked to the classical Socransky's purple-complex together with *A. odontolyticus* (30). The differences in the abundance of *Veillonella* in saliva compared to the subgingival plaque can be expected, as in periodontal pockets the O_2 is reduced favouring this species growing. Nevertheless, its enrichment in the presence of DM can reflect the increased salivary glucose levels and should be better investigated. Strikingly, a previous study has confirmed the gene activity of *Veillonella* at low pH, identifying metabolic activities that are possibly responsible for a pH of recovery linked to *Veillonella*, recognizing this species as a pH-neutralizing organism (31). Furthermore, these health-related organisms in periodontal sites (32) supplies through its metabolism the menadione (vitamin K) indispensable for the development of *Porphyromonas gingivalis* and *Prevotella intermedia* (33).

The abundance of bacterial genera found in the saliva could be impacted by the abundance and composition of bacteria attached to the dental surfaces since all patients included in the presented study were diagnosed with periodontitis regardless of their systemic condition. These results reinforce that the saliva reflects the microbiota of the subgingival environment, although differences in abundance are expected, and it is a simple and representative means for analysing the microbiome of individuals with periodontitis. We believe that saliva should be the sample choice to analyse complex oral conditions, particularly when affected by a systemic condition. Besides, the gut microbiota of individuals with DM showed a higher proliferation of some phyla such as *Proteobacteria, Bacteroidetes* and *Firmicutes* in comparison with healthy individuals. In this pilot study, *Bacterioidetes* and *Proteobacacteria* were also enriched in the salivary microbiome of DMP, somehow reflecting the pattern the gut microbiome (34), and this can suggest a correlation of the gut and oral microbiome that should be investigated.

Here, all participants presented high degrees of periodontitis. The clinical inflammatory parameter BOP increasing was related to the enrichment of *Actinobacteria* and a lower BOP to *Spirochaetes.* Sun et al. also demonstrated that periodontal parameters were positively correlated with several well-known periodontitis microbiotas, including *P. gingivalis*, *T. forsythia, P. capri*, *Alloprevotella rava, P. denticola, Catenibacterium mitsuokai, Prevotella stercorea* and *Treponema medium* (19). Furthermore, *Porphyromonas gingivalis* described as a keystone in periodontitis, was highly prevalent in the salivary microbiome across all samples. That corroborates with the theory that *P. gingivalis* can orchestrate the inflammophilic microbiota promoting the imbalance of the oral environment. Following the concepts of the 'polymicrobial synergy and dysbiosis' (PSD) model (35), what means that those microorganisms do not always lead to converting a symbiotic microbiota to a dysbiotic one but are benefiting from it and help maintain it.

Based on the implicating evidence from a small sample size, caution must be applied. Besides, further studies are needed to confirm the impact of controlled/uncontrolled DM. Lastly, it is always important to emphasize the choice of primer in the conception of sample diversity, although the V4 hypervariable region is traditionally selected due to the lower error rate and adequate information for taxonomic classification of microbial communities.

In conclusion, these preliminary results possibly suggest that clinical parameters can be reflected in the minority salivary microbiome of patients with T2D and periodontitis. Results suggested putative signatures of the number of the low-abundance microbiota correlated with the number of remaining teeth, FBG, and salivary pH. It is indispensable to perform more studies with

larger sample sizes to understand these patterns of oral manifestations of DM for effective prevention and treatment strategies. This pilot study can be helpful for further research planning on salivary microbiome considering the low abundant species, as well as supporting research for further manipulate oral microbiota in clinic for medical benefit. Once confirmed, low abundant microorganisms correlated to clinical parameters can be a potential strategic therapeutic target.

ACKNOWLEDGMENTS

AUTHORS CONTRIBUTIONS

DAC and NDT contributed to conception, design, data acquisition and analysis, drafted and critically revised the manuscript; JAC, DCG, MCMG, LAO and LPS contributed to data acquisition and analysis, and critically revised the manuscript; LM and LGAB contributed to data acquisition, data analysis, figure creation, and critically revised the manuscript. All authors gave final approval and agreed to be accountable for all aspects of the work.

DATA STATEMENT

https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA558499

DECLARATIONS OF INTEREST

The authors have no conflicts of interest to declare.

FUNDING

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6. TABLES AND FIGURES

Table 1. Clinical characteristics of the individuals with periodontitis.

	Diagnosis of Diabetes mellitus	
Individual	**Yes**	**No**
Gender (Female/Male)	1/2	2/1
Age (mean±SD)**	60±9.8	48±5.6
Fasting blood glucose (mg/dl)**	236±188*	106±17
Number of remaining teeth	21.3±6.3	20.33±10.9
Salivary pH (ml/min)	6.67±0.58	7.67±0.58
Salivary buffer capacity	3.67±1.15	3.50±0.71
Number of teeth with active caries	1.33±1.52	1.0±1.0
Insertion loss >5mm (average %)	77.0±34.7	34.2±28.9
Bleeding on probing (average %)	39.6±31.4	51.1±11.5

*1 missing data (individual DMP27)

** Mann-Whitney test <0.05
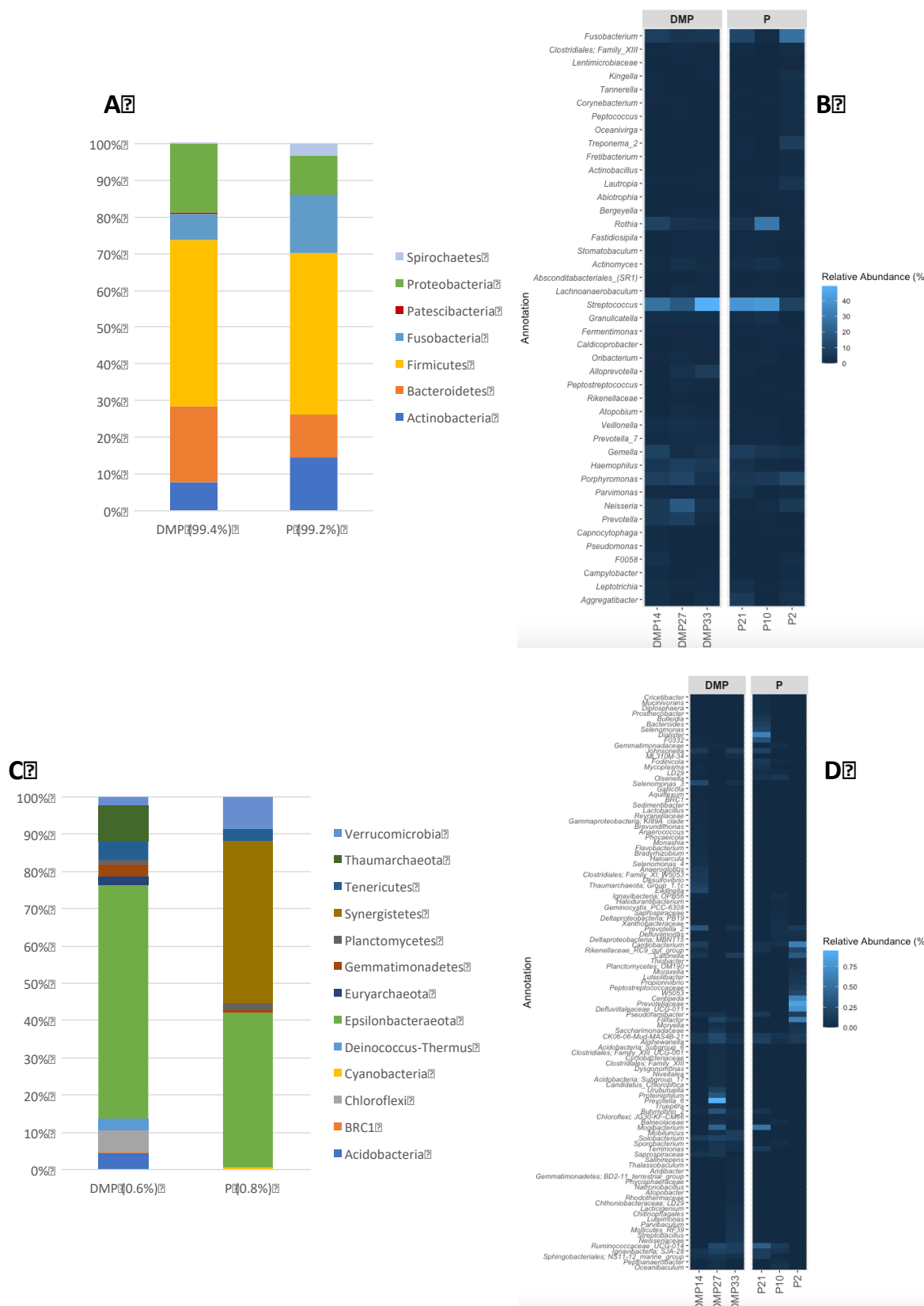
Figure 1. The relative abundance of the salivary microbiome of individuals with periodontitis diagnosed with DM (DMP) and without DM (P). (A) The mean of relative abundance at phylum level of the majority microbiota (≥1%). (B) Heatmap of the relative abundance at genus level of the majority microbiota (≥1%). (C) The mean of relative abundance at phylum level of the minority microbiota (<1%). (D) Heatmap of the relative abundance at genus level of the minority microbiota (<1%).
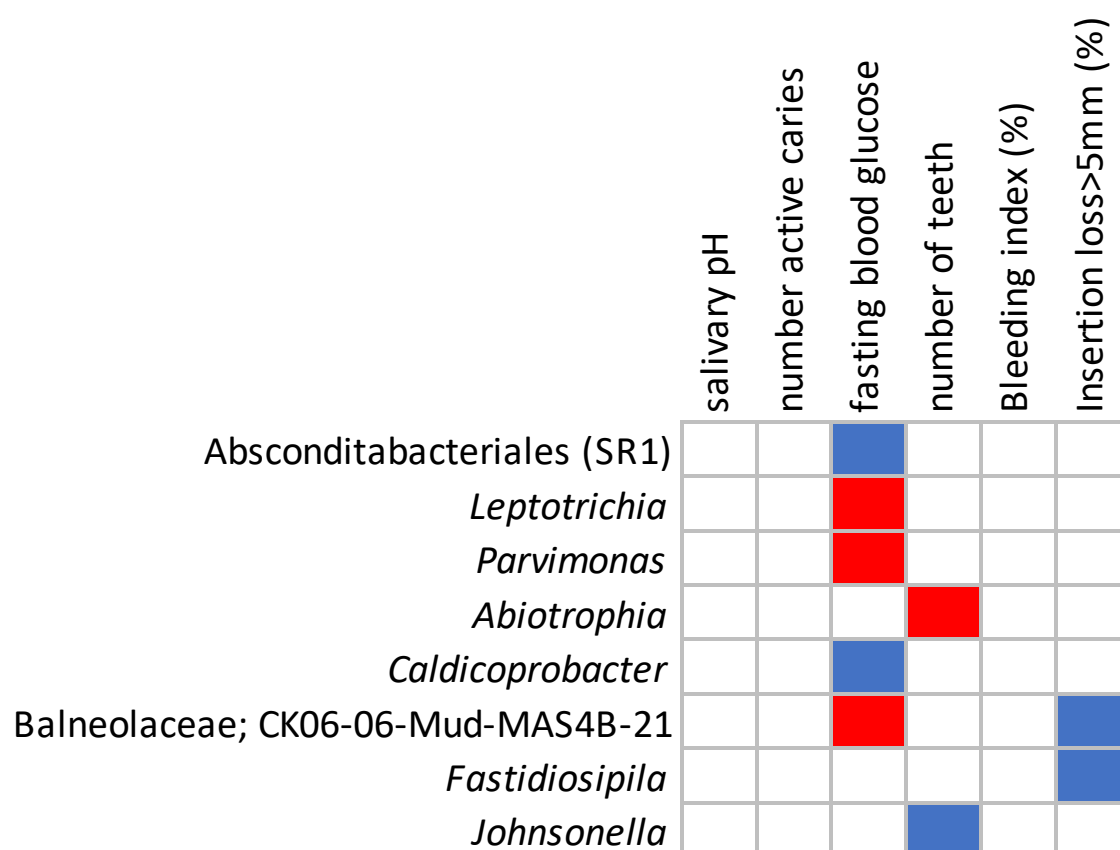
Figure 2. Correlation between clinical variables and the relative abundance of OTUs in the salivary microbiome of patients with periodontitis (Spearman correlation; p<0.05). Blue color means positive correlation; Red color means negative correlation.

Figure 3. CCA triplot showing the correlation of every OTU of the saliva microbial composition with the clinical variables of patients with periodontitis. Plot A represents all OTUs. The plot B represents OTUs with relative abundance >1% (for OTUs <1%, no response was observed in the graph).
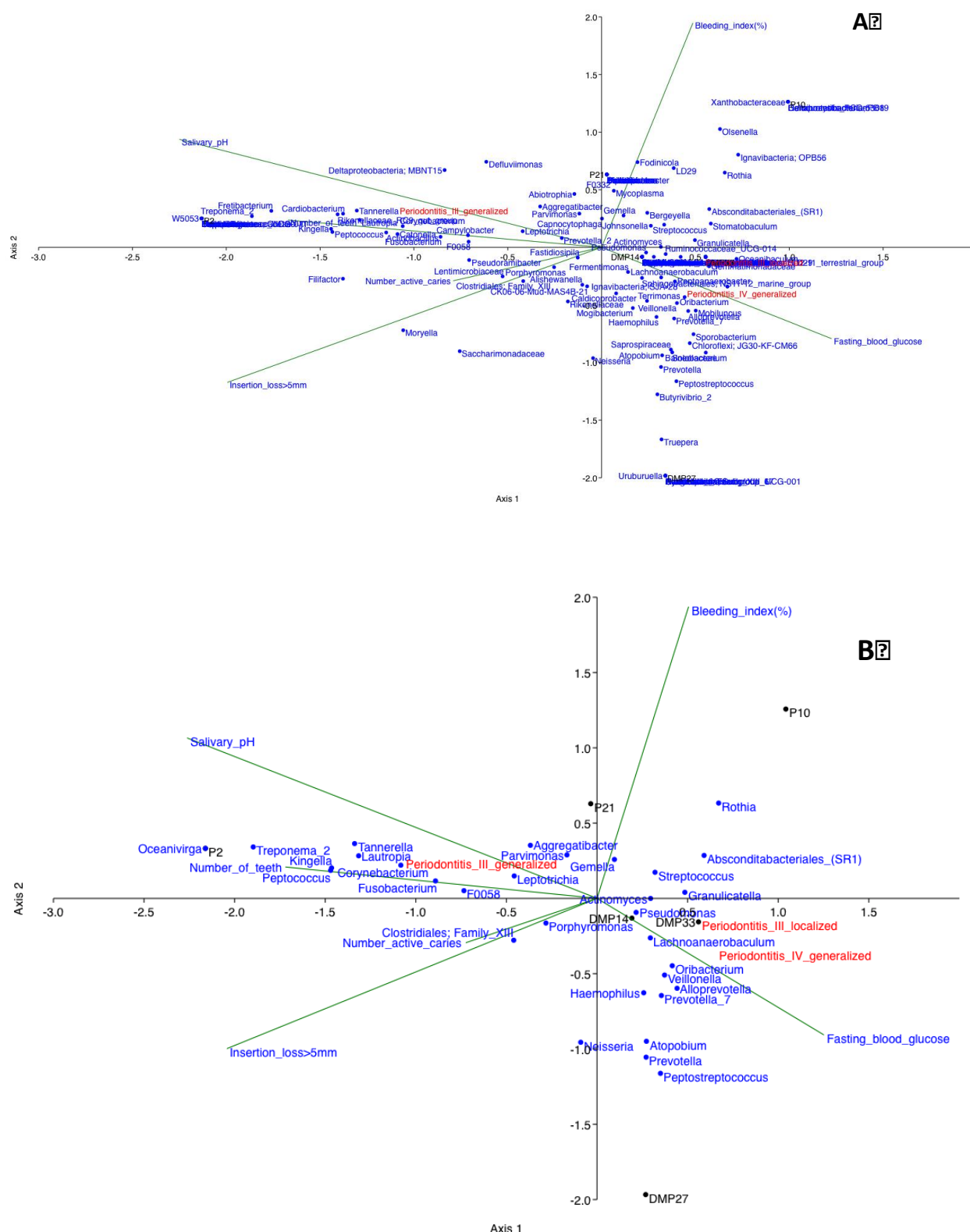
This preprint was submitted under the following conditions: