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Abstract

Objective: The aim of this study was to use high-resolution whole metagenomic shotgun sequencing to characterize the subgingival microbiome of patients with/without type 2 Diabetes Mellitus and with/without periodontitis. Design: Twelve subjects, falling into one of the four study groups based on the presence/absence of poorly controlled type 2 Diabetes Mellitus and moderate-severe periodontitis, were selected. For each eligible subject, subgingival plaque samples were collected at 4 sites, all representative of the periodontal condition of the individual (i.e., non-bleeding sulci in subjects without a history of periodontitis, bleeding pockets in patients with moderate-severe periodontitis). The subgingival microbiome was evaluated using high-resolution whole metagenomic shotgun sequencing. Results: The results showed that: (i) the presence of type 2 Diabetes Mellitus and/or periodontitis were associated with a tendency of the subgingival microbiome to decrease in richness and diversity; (ii) the presence of type 2 Diabetes Mellitus was not associated with significant differences in the relative abundance of one or more species in patients either with or without periodontitis; (iii) the presence of periodontitis was associated with a significantly higher relative abundance of Anaerolineaceae bacterium oral taxon 439 in type 2 Diabetes Mellitus patients. Conclusions: Whole metagenomic shotgun sequencing of the subgingival microbiome was extremely effective in the detection of low-abundant taxon. Our results point out a significantly higher relative abundance of Anaerolineaceae bacterium oral taxon 439 in patients with moderate to severe periodontitis vs patients without history of periodontitis, which was maintained when the comparison was restricted to type 2 diabetics.

Keywords	metagenomics; microbiota; periodontitis; dental plaque; biofilms; periodontal pocket
Taxonomy	Periodontology, Periodontium, Microbiology

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UNIVERSITÀ DEGLI STUDI DI FERRARA
- EX LABORE FRUCTUS -

CENTRO INTER-DIPARTIMENTALE DI RICERCA PER LO STUDIO
DELLE MALATTIE PARODONTALI E PERI-IMPLANTARI
Direttore: Prof. Leonardo Trombelli

TO THE EDITOR IN CHIEF OF *ARCHIVES OF ORAL BIOLOGY*

Ferrara, April 13 2019

Dear Editor,

we would like to submit the amended version of our manuscript entitled "*Subgingival microbiota of diabetics and non-diabetics with different periodontal conditions: a pilot study using whole metagenomic shotgun sequencing*" for publication in *Archives of Oral Biology*.

We have carefully considered the points listed in your previous e-mail. Below please find a brief, structured explanation of the amendments that have been made. We hope that the manuscript is now suitable to undergo a peer review process, and we remain totally available if further amendments are needed.

Thank you in advance for your consideration.
Best regards,

Roberto Farina

NATURE OF THE STUDY

- Manuscript title: The title has been changed to "*Whole metagenomic shotgun sequencing of the subgingival microbiome of diabetics and non-diabetics with different periodontal conditions*";
- MAIN TEXT: the term "pilot" has been omitted from the entire manuscript text when referred to the nature of the study;
- STATISTICAL ANALYSIS: In the statistical analysis paragraph, we have added a section to explicitly declare the lack of previous studies on which an *a priori* sample size calculation could have been based: "*Due to the lack of previous studies with similar experimental design using*

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whole metagenomic shotgun sequencing and the absence of a true primary outcome to determine an expected inter-group difference, no sample size calculation could be performed a priori. Therefore, the size of each group arbitrarily determined.".

ABSTRACT

- The aim of the study has been rephrased as suggested: "*The aim of this study was to use high-resolution whole metagenomic shotgun sequencing to characterize the subgingival microbiome of patients with/without type 2 Diabetes Mellitus and with/without periodontitis*.".

ABBREVIATIONS

Abbreviations have been eliminated from the abstract and main manuscript text. The use of abbreviations should now be in accordance with the guidelines for Authors.

FIGURE 1 LEGEND

- The figure legend has been implemented with the following sentence: "*Data are represented as medians and minimum-maximum bars*.".

TABLES

When present, vertical lines have been eliminated from the Tables (Table 3 and 4).

DECLARATION OF AUTHORS' CONTRIBUTIONS

The declaration has been moved from the main manuscript text to the covering documents.

HIGHLIGHTS

The last highlight has been amended and now complies with the maximum length of 85 characters (including spaces).

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HIGHLIGHTS

- the subgingival microbiome tended to be less complex in presence of T2D and/or P
- the presence of T2D did not influence the relative abundance of subgingival species
- *Anaerolineaceae bacterium oral taxon 439* was more abundant in T2D+P+ vs T2D+P-

Whole metagenomic shotgun sequencing of the subgingival microbiome of diabetics and non-diabetics with different periodontal conditions

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RUNNING TITLE: Subgingival microbiome of diabetics and non-diabetics with/without periodontitis

KEYWORDS: metagenomics; microbiota; periodontitis; dental plaque; biofilms; periodontal pocket; gingival sulcus

NUMBER OF WORDS: 4523
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For *Archives of Oral Biology*,

Ferrara, April 15, 2019

ABSTRACT

Objective: The aim of this study was to use high-resolution whole metagenomic shotgun sequencing to characterize the subgingival microbiome of patients with/without type 2 Diabetes Mellitus and with/without periodontitis.

Design: Twelve subjects, falling into one of the four study groups based on the presence/absence of poorly controlled type 2 Diabetes Mellitus and moderate-severe periodontitis, were selected. For each eligible subject, subgingival plaque samples were collected at 4 sites, all representative of the periodontal condition of the individual (i.e., non-bleeding sulci in subjects without a history of periodontitis, bleeding pockets in patients with moderate-severe periodontitis). The subgingival microbiome was evaluated using high-resolution whole metagenomic shotgun sequencing.

Results: The results showed that: (i) the presence of type 2 Diabetes Mellitus and/or periodontitis were associated with a tendency of the subgingival microbiome to decrease in richness and diversity; (ii) the presence of type 2 Diabetes Mellitus was not associated with significant differences in the relative abundance of one or more species in patients either with or without periodontitis; (iii) the presence of periodontitis was associated with a significantly higher relative abundance of *Anaerolineaceae bacterium oral taxon* 439 in type 2 Diabetes Mellitus patients.

Conclusions: Whole metagenomic shotgun sequencing of the subgingival microbiome was extremely effective in the detection of low-abundant taxon. Our results point out a significantly higher relative abundance *of Anaerolineaceae bacterium oral taxon 439* in patients with moderate to severe periodontitis vs patients without history of periodontitis, which was maintained when the comparison was restricted to type 2 diabetics.

INTRODUCTION

Type 2 diabetes and periodontitis have a long established bi-directional relationship (Taylor, 2001; Sanz et al., 2018). Poorly controlled diabetes is a risk factor for periodontitis, contributing to more than doubled risk of periodontitis incidence (Lakschevitz, Aboodi, Tenenbaum & Glogauer, 2011; Taylor, Preshaw & Lalla, 2013) and negatively affecting its severity and progression (Miller et al., 1992; Taylor et al., 1996; Taylor & Borgnakke, 2008; Lalla & Papapanou, 2011). On the other hand, patients with severe periodontitis have an increased risk of developing type 2 diabetes (Saito et al., 2004, Demmer, Jacobs & Desvariuex, 2008, Morita et al., 2012). The mechanistic link between type 2 diabetes and periodontitis has been explored under a variety of aspects, including the composition of the periodontal microbiota. When evaluated though traditional methods, the periodontal microbiota did not reveal substantial differences between diabetics (affected by type 2 diabetes in the majority of studies) and non-diabetics (Polak & Shapira, 2017).

In the most recent years, high-throughput sequencing metagenomic techniques were applied to characterize the subgingival microbiome under health and disease conditions (Zhou et al., 2013; Casarin et al., 2013; Wang et al. 2013; Duran-Pinedo et al., 2014; Jorth, Turner, Gumus, Nizam, Buduneli, & Whiteley, 2014; Li et al., 2014; Shi et al., 2015; Babaev et al., 2017; Longo et al., 2018). In particular, three studies (Zhou et al., 2013; Casarin et al., 2013; Babev et al., 2017) evaluated the composition of the subgingival microbiota using 16S rDNA-based microbial profiling, considering type 2 diabetes and, in two of these (Zhou et al., 2013; Babev et al., 2017), periodontitis as comorbidities. In Brazilian subjects with severe, generalized chronic periodontitis (Casarin et al., 2013), poorly controlled type 2 diabetes was found to be associated with a higher prevalence of some species (including *F. nucleatum*, *V. parvula*, *V. dispar*, and *E. corrodens*) compared to systemic health. In a Chinese sample, Zhou et al. (Zhou et al., 2013) showed that the abundance of some periodontal pathogens (e.g., *T. forsythia*) and other species (e.g., *C. sputigena*) was positively influenced by the combined presence of type 2 diabetes and periodontitis, while the abundance of *Prevotellaceae* and *P. tannerae* was significantly lower in non-diabetics affected by periodontitis. In absence of periodontitis, different genera and species revealed significant differences in their abundance between diabetics and non-diabetics (Zhou et al.,

2013). Finally, Babaev et al. (Babev et al., 2017) found quantitative differences in the subgingival microbiome of patients with type 2 diabetes and periodontitis compared to periodontitis patients without type 2 diabetes or healthy subjects in a Russian sample of individuals. In particular, in periodontitis subjects the presence of type 2 diabetes was associated with significantly greater amounts of *P. gingivalis*, *T. denticola* and *F. nucleatum* (Babev et al., 2017). These studies, however, are affected by the limitations of 16S rDNA-based microbial profiling, where species identification is dependent on the extent of evolutionary diversification in those variable 16S regions where other genomic regions may be more informative for such speciation (Zhang et al., 2015).

More recently, whole metagenomic shotgun sequencing was used to profile viral families (Dimon, Wood, Rabbitts & Arron, 2013; Ma et al., 2014), for metagenomic studies (Hess et al., 2011; Nielsen et al., 2014), and for the Human Microbiome Project (Yang et al., 2011). Whole metagenomic shotgun sequencing allows for studying microbial communities in a cultivation-independent way, and provides enhanced information compared to the 16S rDNA-based microbial profiling. In particular, it provides with quantitative and qualitative information from taxa down to the level of species, regardless of the extent of evolutionary diversification in the variable 16S regions.

The present study used high-resolution Whole metagenomic shotgun sequencing to characterize the subgingival microbiome of patients with/without type 2 diabetes and with/without periodontitis.

MATERIALS & METHODS

Experimental design and ethical aspects

The present case-control study was performed at the Research Centre for the Study of Periodontal and Peri-Implant Diseases, University of Ferrara, Italy. The study was approved by the Ethical Committee of Ferrara, Italy (protocol number: 150791). Each subject provided a written informed consent before participation. All

clinical procedures were performed in full accordance with the Declaration of Helsinki and the Good Clinical Practice Guidelines. All data were de-identified before database creation and data analysis.

Study population

Twelve adult (≥ 40 years) Caucasian individuals were recruited among current and permanent residents in the metropolitan area of Ferrara seeking care at the Research Centre for the Study of Periodontal and Peri-Implant Diseases, Ferrara, Italy. Based on the inclusion/exclusion criteria reported below, subjects were consecutively selected and assigned to one of the following groups (of 3 subjects each): patients affected by moderate to severe periodontitis and type 2 diabetes (T2D+P+ group); patients affected by moderate to severe periodontitis but not type 2 diabetes (T2D-P+ group); patients affected by type 2 diabetes but not periodontitis (T2D+P- group); and patients without either type 2 diabetes or periodontitis (T2D-P- group). To assess eligibility related to diabetic status, laboratory exams performed within 3 months prior to the study visit were considered and, if the subject was eligible, data were extracted for analysis. To assess eligibility related to periodontal conditions, data from a complete periodontal record chart performed within 1 month prior to the study visit were considered and, if the subject was eligible, extracted for analysis.

Inclusion criteria

T2D+P- group

- diagnosis of type 2 diabetes for at least two years according to the criteria of the American Diabetes Association (American Diabetes Association, 2014);
- insufficient metabolic control of diabetes (i.e., glycated haemoglobin serum level > 7%);
- currently receiving stable doses of oral hypoglycemic agents and / or insulin under supervision of a diabetologist;
- at least 20 teeth present;
- no history of periodontitis, either treated or not (i.e., no interproximal clinical attachment loss> 2 mm and no sites with probing depth> 4 mm).

T2D-P+ group

- no history of type 2 diabetes diagnosis;
- at least 20 teeth present;
- diagnosis of moderate to severe periodontitis, i.e., at least 30% of sites with clinical attachment loss≥ 3 mm (Armitage, 1999);
- at least 4 sites with probing depth≥ 5 mm.

T2D+P+ group

- diagnosis of type 2 diabetes for at least two years according to the criteria of the American Diabetes Association (American Diabetes Association, 2014);
- insufficient metabolic control of diabetes (i.e., glycated haemoglobin serum level > 7%);
- currently receiving stable doses of oral hypoglycemic agents and / or insulin under supervision of a diabetologist;
- at least 20 teeth present;
- diagnosis of moderate to severe periodontitis, i.e., at least 30% of sites with clinical attachment loss≥ 3 mm (Armitage, 1999);
- at least 4 sites with probing depth≥ 5 mm.

T2D-P- group

- no history of type 2 diabetes diagnosis;
- at least 20 teeth present;
- no history of periodontitis, either treated or not (i.e., no interproximal clinical attachment loss> 2 mm and no sites with probing depth> 4 mm).

Exclusion criteria (valid for all groups)

- current smoking or quit smoking less than 6 months prior to the screening visit;
- diseases or systemic conditions (in addition to type 2 diabetes) with a documented influence on periodontal status;
- use of drugs with a documented influence on periodontal status (e.g., bisphosphonates, cyclosporine, phenytoin, nifedipine, calcium channel blockers, corticosteroids and anti-inflammatory drugs);
- periodontal therapy within 12 months prior to the screening visit;
- local or systemic antibiotic therapy during the 3 months prior to the screening visit.

Collection and storage of subgingival plaque samples

For each eligible subject, 4 subgingival plaque samples were collected at 4 teeth. In T2D+P- and T2D-P- subjects, sampling was always performed at 4 sites randomly selected among those negative to bleeding on probing. In T2D+P+ and T2D-P+ subjects, sampling was performed at the 4 sites showing the deepest probing depth values among those positive to bleeding on probing.

At each site selected for plaque sampling, saliva was blotted with a gauze or cotton roll, and supragingival plaque was removed with a curette or scaler. Subgingival plaque was collected with a curette and wiped onto a sterile, coarse endodontic paper point. A commercially available kit (OMR-110; DNA Genotek, Ottawa, ON, Canada) was used for the storage of plaque samples. For each subject, the four samples were stored together in a sterile microcentrifuge tube containing a fixative solution containing sodium dodecyl sulphate, glycine, N,N'-trans-1,2-cyclohexanediylbis[N-(carboxymethyl)-, hydrate, and lithium chloride, at a temperature comprised between 15°C and 25°C, until lab processing for extraction of the oral microbiome DNA.

Sample Processing

Isolation of DNA

DNA was isolated from frozen samples by using the Maxwell RSC DNA Blood Kit (Promega) according to the manufacturer's protocol. The concentration of DNA was determined with the Qubit 2.0 Fluorometer (Life

Technologies) by using the Qubit dsDNA HS Assay Kit (Life Technologies). Prior to library preparation, DNA was fragmented using NEBNext® dsDNA Fragmentase for 30 minutes.

Library preparation and sequencing

Each library was generated from 20 ng of genomic DNA, in accordance with procedures described in the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina protocol (New England Biolabs). NEBNext® Multiplex Oligos for Illumina® (Dual Index Primers set 1) were used to produce the individual libraries and label them with specific molecular barcodes. Then, Agencourt AMPure XP beads (Beckman Coulter) were used for library purification. Finally, libraries were quantified by using the High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA) on the Bioanalyzer instrument (Agilent), diluted and pooled together in equimolar amounts. Samples were sequenced with an Illumina NextSeq 500 sequencer with 2 × 150-bp read, using NextSeq® 500/550 Mid Output Kit v2.

Bioinformatic analyses

The Illumina BaseSpace cloud platform (https://basespace.illumina.com/) generated FASTQ files that were used for the analysis of the shotgun fragments. Sequence quality was ensured by trimming the reads using Trimmomatic 0.36 (Bolger, Lohse, & Usadel, 2014), with the following parameters: CROP:147, HEADCROP:3, SLIDINGWINDOW:5:20, MINLEN:100.

All paired reads were aligned to the reference human genome (GRCh37-Hg19), using Bwa 0.7.15 (with option mem and parameters -M -t 20) (Li, 2013). Sequences displaying a concordant alignment (a mate pair that aligns with the expected relative mate orientation and with the expected range of distances between mates) against the human genomes and all orphan reads were then removed from all subsequent analyses using SAMtools (Li et al., 2009) and awk custom filter.

Then, BLASTN 2.6.0 (Li et al., 2009; Altschul, Gish, Miller, Myers & Lipman, 1990; Camacho, 2013) was applied for aligning the remaining non-human reads to the NCBI (NR/)NT reference database. The alignments

with an e-value lower than 1 × 10−6 were further filtered to exclude matches with percentage of identity lower or equal than 95% and length lower than 100 bp.

Finally, MEGAN6 (Huson, Auch, Qi & Schuster 2007) with default parameters was used to perform the taxonomical analysis of the data, ranked for genus and species.

Statistical analysis

Due to the lack of previous studies with similar experimental design using whole metagenomic shotgun sequencing and the absence of a true primary outcome to determine an expected inter-group difference, no sample size calculation could be performed *a priori*. Therefore, the size of each group arbitrarily determined. The patient was regarded as the statistical unit. Analyses were performed using STAMP v2.1.3 (Parks, Tyson, Hugenholtz & Beiko, 2014). Data were expressed as relative abundance of each taxonomic unit at either the bacterial genus or species level. Relative abundance was generated by normalizing the whole genome sequencing data of each individual over the subject with the lowest number of reads (IND5). To test the null hypothesis, White's non-parametric t-test (White, Nagarajan & Pop, 2009) and Kruskal-Wallis H-test plus post-hoc tests (e.g., Tukey–Kramer) were applied to compare profiles organized into two groups or multiple groups of profiles, respectively. To be more conservative, in the results section only comparisons (both for genera and species) with a nominal p-value lower than 0.01 will be discussed. The Benjamini-Hochberg procedure was applied to control for the false discovery rate. Alpha- and beta-diversity were used to describe the microbiome diversity within and between groups. Alpha-diversity (within-sample diversity) was measured with the Shannon H' diversity index, Pielou evenness J index and the Margalef d richness index. Beta-diversity within sample (intra-beta diversity), was evaluated with the D_β index (as a component of total community diversity, Dγ) while beta-diversity between individuals (inter-beta diversity) with the Bray-Curtis distance (Bray & Curtis, 1957) and visualized with principal coordinates analysis (PCoA). For a review about diversity indexes used, see Chiarucci et al., Lozupone and Knight and Tuomisto (Chiarucci, Bacaro & Scheiner, 2007; Lozupone & Knight, 2008; Tuomisto, 2010).

RESULTS

Study population

The characteristics of the study population are reported in Table 1. Diabetic subjects had received the diagnosis of type 2 diabetes at least 3 years before participation in the study, and their glycated haemoglobin level was comprised between 7.1% and 8.0%. Subjects with periodontitis had a number of sites with PD≥ 5 mm varying between 19 and 39 (T2D-P+ group) and between 18 and 29 (T2D+P+ group). The range of BoP score was 8%-25% in T2D-P- group, 17%-20% in T2D+P- group, 28%-49% in T2D-P+ group, and 30%-44% in T2D+P+ group (Table 1). The clinical characteristics of sites selected for subgingival plaque sampling are reported in Table 2.

Metagenomic analysis

Bioinformatic and Sequencing summary taxonomical profiling

The processing details of whole metagenomic shotgun sequencing for the twelve samples are shown in Table 3. The sequencing depth, obtained from the 12 samples, was similar among groups (Figure 1a) even after filtering out low-quality, chimeric, and non-bacterial sequences (Figure 1b).

Over all samples, the 87.16% of the high-quality sequences were classified into 18 phyla, 126 genera and 259 species. The most abundant phyla were *Bacteroidetes* (56.5%), followed by *Actinobacteria* (18.0%), *Firmicutes* (9.1%), *Proteobacteria* (6.6%), *Spirochaetes* (5.3%), *Synergistetes* (2.3%), and other phyla (2.2%). At the genus level, *Porphyromonas* clearly dominated (24.3%), followed by *Tannerella* (18.4%), *Prevotella* (13.4%), *Actinomyces* (7.3%), *Rothia* (6.9%), *Treponema* (5.3%), *Selenomonas* (2.7%), *Fretibacterium* (2.3%), *Olsenella* (2.2%), *Streptococcus* (2.1%). Other 58 genera, each showing relative abundance lower than 2%, accounted for the 15.1% overall. No obvious bias in the proportion of unclassifiable sequences among different sample groups was observed ($p = 0.339$).

All indices for diversity in microbial species (i.e. richness, evenness, alpha- and beta-diversity) did not differ significantly among groups (Figure 2). Microbial richness estimated by the Margalef d index was slightly higher in T2D-P- subjects. Moreover, the intra-subject diversity as expressed by other indices (evenness, alpha- and beta -diversity) showed a tendency to decrease in presence of periodontitis, and showed the lowest levels in T2D+P+ patients (Figure 2).

Taxonomical profiling

The bacterial compositions at the community level were compared between groups by PCoA of subgingival microbiota based on Bray-Curtis matrix distances (Figure 3, species level). Within subjects without type 2 diabetes, the microbiomes of periodontitis patients clustered together, whereas subjects without periodontitis showed a more sparse distribution based on their microbiome profile (Figure 3a). In type 2 diabets patients, distributions of individuals with and without periodontitis were both sparse, but were separated by the second component, indicating different taxonomic profiles according to the presence or absence of periodontitis (Figure 3b). In subjects without periodontitis, diabetics and non-diabetics did not show a clearly distinct microbiome (Figure 3c). Differently, periodontitis patients without diabetes clustered together and were separated (particularly for the third dimension) from periodontitis patients with type 2 diabetes, thus indicating different taxonomic profiles for these two groups (Figure 3d).

The forty most abundant genera and species in each of the four study groups are shown in Figure 4a and 4b, respectively. The results of White's non-parametric t-test applied to compare relative abundance between groups are shown in Table 4 where all genera and species with a nominal significance p-value lower than 0.01 before FDR correction are included.

Subgingival microbiome in individuals with and without periodontitis (irrespective of type 2 diabetes)

Figure 5a and 5b show the most abundant (> 0.5% in at least one group) genera and species, respectively, in individuals with periodontitis (n= 6) and without periodontitis (n= 6). The profiles of the oral microbiota showed

a trend (although not significant) towards more prevalent *P. gingivalis, T. forsythia, P. intermedia, Actinomyces sp. oral taxon* in P+ patients, and *R. dentocariosa* and *Veilonella parvula* in P- subjects.

At genus level, the main significant differences were observed for *Anaerolineaceae unclass., Parvimonas, Ottowia, Dialister, Microcella and Aggregatibacter.* At species level, *Anaerolineaceae bacterium sp. oral taxon 439, Parvimonas micra, Porphyromonas asaccharolytica, Ottowia sp. oral taxon* and *Dialister pneumosintes* were significantly more abundant in periodontitis patients, while*, Microcella alkaliphile* and 3 *Actinomyces sp*ecies were more abundant in subjects without periodontitis (Table 4). Only *Anaerolineaceae bacterium oral taxon 439* maintained the significance of the inter-group comparison after FDR correction (adjusted p= 0.001).

Subgingival microbiome in individuals with and without periodontitis in absence of type 2 diabetes (T2D-P+ vs T2D-P-)

T2D-P+ patients showed significant higher abundance of *Porphyromonas, Anaerolineaceae unclass.,* and *Aggregatibacter*, and significant lower abundance of *Microcella* compared to T2D-P- subjects (Table 4). At species level, T2D-P+ patients showed significant higher abundance of *Porphyromonas gingivalis, Anaerolineaceae bacterium oral taxon 439* and *Campylobacter rectus*, while *Microcella alkaliphila* was significantly more abundant in T2D-P- group (Table 4). None of the differences at either genus or species level, however, remained significant after FDR correction.

Subgingival microbiome individuals with and without periodontitis in presence of type 2 diabetes (T2D+P+ vs T2D+P-)

At genus level, T2D+P+ group showed significantly higher abundance of *Anaerolineaceae unclass.* and *Dialister,* and significantly lower abundance of *Campylobacter* when compared to T2D-P+ group (Table 4). After correction for FDR, only *Anaerolineaceae* and *Dialister* maintained their statistical significance (adjusted p: < 0.0001 and 0.052, respectively). Compared to T2D+P- group, T2D+P+ group showed a significantly higher abundance of *Anaerolineaceae bacterium oral taxon 439, Dialister pneumosintes, Leptotrichia sp. oral taxon* and *Treponema maltophilum* (with the last two being detected only in T2D+P+ subjects) and significantly

lower abundance of *Campylobacter gracilis* and *Rothia dentocariosa*. After FDR correction, only the relative abundance of *Anaerolineaceae bacterium oral taxon 439* remained significantly different between T2D+P+ group (1.5880%) and T2D+P- group (0.0087%) (adjusted p< 0.001).

Subgingival microbiome in individuals with and without type 2 diabetes (irrespective of the presence/absence of periodontitis)

Figure 5a and 5b show the most abundant (> 0.5% in at least one group) genera and species, respectively, in individuals with type 2 diabetes (n= 6) and individuals without type 2 diabetes (n= 6). *Filifactor* (and, within this genus, *Filifactor alocis*) and *Ornithobacterium* (and, within this genus, *Ornithobacterium rhinotracheale*) were significantly more abundant in subjects without type 2 diabetes compared to subjects with type 2 diabetes (Table 4), but none of these differences remained significant after FDR correction.

Subgingival microbiome in subjects with and without type 2 diabetes in absence of periodontitis (T2D-P- vs T2D+P-)

No significant inter-group differences in the relative abundance of one or more species were detected between T2D-P- vs T2D+P- groups (Table 4).

Subgingival microbiome in subjects with and without type 2 diabetes in presence of periodontitis (T2D-P+ vs T2D+P+)

At genus level, T2D+P+ group showed significantly higher abundance of *Dialister* and significantly lower abundance of *Filifactor* when compared to T2D-P+ group (Table 4). At species level, T2D+P+ group showed significantly higher abundance of *Dialister pneumosintes* and significantly lower abundance of *Filifactor alocis* and *Treponema sp. OMZ 838* when compared to T2D-P+ group (Table 4). None of the differences observed at either genus or species level maintained its significance after FDR correction.

DISCUSSION

In the present study, whole metagenomic shotgun sequencing was used to comparatively evaluate the subgingival microbiota of patients with and without type 2 diabetes with different periodontal conditions. Based on the presence/absence of type 2 diabetes and moderate-severe periodontitis, twelve subjects were selected, each falling into one of the four study groups. For each eligible subject, subgingival plaque samples were collected at 4 sites, all representative of the periodontal condition of the individual (i.e., non-bleeding sulci in subjects without a history of periodontitis, bleeding pockets in patients with moderate-severe periodontitis). The present study is the first using whole metagenomic analysis to provide with new insights on the potential impact of type 2 diabetes and periodontitis on the subgingival microbiome. Whole metagenomic shotgun sequencing has been increasingly recognized as the most comprehensive and robust method for metagenomics research (Ng & Kirkness, 2010; Noecker, McNally, Eng, & Borenstein, 2017).

Although all indices for diversity in microbial species did not show significant differences among study groups, a clear tendency of richness and diversity of the subgingival microbiome to decrease was observed in presence of type 2 diabetes and periodontitis, with the lowest levels being found in patients affected by both diseases. This finding is consistent with the existing literature, which indicates that the presence of either periodontitis (and also its severity, when present) or type 2 diabetes are negatively correlated with the diversity of the oral microbial community (Ai, Huang, Wen, Li, Zhu & Xia, 2017, Sabharwal et al. 2018). This inverse relationship is also common to other diseases, such as inflammatory bowel disease and obesity (Walker et al. 2011). Our results seem to confirm that periodontitis is due to a dysbiosis in the subgingival bacterial community resulting from the disruption of the host-microbial homeostasis by few bacterial species (Hajishengallis et al. 2011), rather than a true shift from some species to others.

The inclusion of type 2 diabetics in the study was subordinated to a poorly controlled diabetic status (as evaluated in terms of glycated haemoglobin). Several studies have pointed out the role of hyperglycaemia in determining an increased risk and severity of periodontitis (Cianciola, Park, Bruck, Mosovich, & Genco, 1982; Emrich, Shlossman & Genco, 1991; Morita et al., 2012) and, in particular, type 2 diabetes shows a strong association with periodontitis incidence and severity when it is poorly controlled. (Novaes, Gutierrez & Novaes,

1996; Tsai, Hayes & Taylor, 2002; Chen et al., 2010; Kowall et al., 2015; Khanuja, Narula, Rajput, Sharma & Tewari, 2017). Interestingly, recent studies using quantitative Polymerase Chain Reaction or 16S rDNA-based microbial profiling suggested that this relationship may reside in some alterations of the subgingival microbiota that are dependent on the level of metabolic control of diabetes. Miranda et al. (Miranda et al., 2017) observed that the frequencies and counts of some periodontal pathogens were increased in poorly controlled type 2 diabetes compared to well controlled type 2 diabetes. Also, Longo et al. (Longo et al., 2018) reported different levels of ecological diversity in the subgingival microbiome of type 2 diabetics with adequate or poor metabolic control, with poorly controlled type 2 diabetes showing increased levels of succinate/acetate producers and decreased levels of butyrate producers. Overall, these findings sustain the importance to characterize the subgingival microbiome of patients with poorly controlled type 2 diabetes with high-resolution techniques such as whole metagenomic shotgun sequencing.

Differences in the subgingival microbiome between subjects with and without type 2 diabetes were observed for a limited number of genera and species, only in presence of periodontitis and not in subjects without history of periodontitis. In particular, in presence of moderate-severe periodontitis, poorly controlled type 2 diabetes was associated with higher abundance of *Dialister pneumosintes* and lower abundance of *Filifactor alocis* and *Treponema sp. OMZ 838* compared to systemically healthy subjects. While *Dialister pneumosintes* and *Filifactor alocis* are two species with a well documented history of association with several forms of periodontal, peri-implant and endodontic infections, including periodontitis (Contreras et al., 2000; Ferraro, Gornic, Barbosa, Peixoto & Colombo, 2007; Colombo et al., 2009; Silva-Boghossian, Neves, Resende & Colombo, 2013; Aruni, Chioma, & Fletcher, 2014, Aruni et al. 2015; Gogeneni et al., 2015; Collins et al., 2016; Gonçalves et al., 2016; Perez-Chaparro et al., 2018), *Treponema sp. OMZ 838* has been only recently associated with necrotizing ulcerative gingivitis lesions in an anecdotal report (Chan et al., 2014). These few differences, however, were not maintained after FDR correction, thus indicating that type 2 diabetes is not robustly associated with substantial alterations of the subgingival microbiota, but rather determines mild changes in the abundance of few species (which include keystone pathogens, i.e. such species that initiate the

cascade leading to dysbiosis and clinical manifestation of disease). Although this general finding is shared with previous studies, the bacterial species that showed differences between patients with and without type 2 diabetes differed from those identified in other studies (Zhou et al., 2013; Casarin et al., 2013; Babaev et al., 2017). Differences can be partly explained by differences in patient selection criteria (including factors such as smoking, see Gomes et al., 2006), case definitions and severity of type 2 diabetes and periodontitis, clinical characteristics of the sampled sites, and technique used for the analysis of the subgingival microbiome.

In the present material, subgingival plaque samples were collected at non-bleeding sulci in subjects without a history of periodontitis, while at bleeding pockets in patients with moderate to severe periodontitis. This methodological approach was used due to the well-known impact of the periodontal condition (particularly in terms of probing depth) of the sampled site on the subgingival microbiome (Ge, Rodriguez, Trinh, Gunsolley & Xu, 2013; Pérez-Chaparro et al., 2018).

When comparing the subgingival microbiome of individuals with and without periodontitis, red complex species (specifically, *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*) showed a tendency to be present in higher abundance in periodontitis patients. The difference in *Porphyromonas gingivalis* reached the statistical significance (p= 0.004) in T2D-P+ vs T2D-P-. After statistical correction, only *Anaerolineaceae bacterium oral taxon 439* maintained its statistical significance, resulting in higher relative abundance in periodontitis patients vs individuals without periodontitis, both in the overall population and in the type 2 diabetes sub-sample. *Anaerolineaceae bacterium oral taxon* 439 is an oral anaerobe bacterium isolated from pure cultures of human oral samples, but little is known about its relationship with destructive periodontal disease (Dewhirst et al., 2010). The *Anaerolineaceae bacterium oral taxon* 439 was firstly described only recently (Vartoukian et al. 2016). The difficulties in the isolation of *Anaerolineae bacterium HOT-439* in plaque cultures were proven to be dependent on the need for “helper” bacteria (e.g., *Fusobacterium nucleatum*) for its growth *in vitro* (Vartoukian et al. 2016). Inferences from the available genomic data for this *Anaerolineaceae* sp. suggest a niche-specific adaptation for survival in host environments, with regard to the ability to evade host defence system and the ability to scavenge material from lysed cells/tissue (Campbell et al., 2014).

Anaerolineae bacterium HOT-439 represents the first oral taxon from the *Chloroflexi* phylum that has been cultivated from plaque samples obtained from deep periodontal pockets. While this strain was frequently isolated (three over four samples) in the study by Vartoukian et al. (2016), other studies based on molecular analyses suggested that the *Chloroflexi* taxon is a low-abundance member of the human oral cavity (Dewhirst et al., 2010; Campbell et al., 2014), with a prevalence of 0.003% (HOMD). A possible explanation for this inconsistency could come from the presence of base mismatches between the 'universal' bacterial primers, commonly used in 16S rRNA sequencing, and the *Anaerolineae bacterium oral taxon 439* specific gene sequence, that might result in a molecular detection bias against this taxon (Camanocha & Dewhirst, 2014) with the 16S approach.

Based on whole metagenomic shotgun sequencing, our analyses overcome some technical bias observed in 16S rDNA-based microbial profiling being more effective in the detection of low-abundant taxon, such as *HOT-439*. Thus, our results consolidate the existing literature (Abusleme et al., 2013; Szafranski et al., 2015) that assign this *Chloroflexi* taxon to the 'core' periodontitis-associated microbiome (Abusleme et al., 2013) and suggests it as a potential biomarker for periodontitis (Szafranski et al., 2015).

In conclusion, the results of the present study showed that: (i) the presence of type 2 diabetes and/or periodontitis were associated with a tendency of the subgingival microbiome to decrease in richness and diversity; (ii) the presence of type 2 diabetes was not associated with significant differences in the relative abundance of one or more species in patients either with or without periodontitis; (iii) a significantly higher relative abundance *of Anaerolineaceae bacterium oral taxon 439* in periodontitis patients vs individuals without periodontitis, which was maintained when the comparison was restricted to patients with type 2 diabetes.

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CONFLICT OF INTERESTS

The authors declare they have no conflict of interests related to the present study.

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TABLES

Table 1. Characteristics of the study population.

Table 2. Clinical characteristics of sites selected for subgingival plaque sampling.

Table 3. Statistics of whole metagenome shotgun sequencing of plaque samples.

Table 4. Bacteria genera/species having a nominal significantly difference ≤ 0.01 level at White's non-parametric t-test applied to compare relative abundance between groups.

FIGURE LEGENDS

Figure 1. Number of reads in each group. a. Total reads sequenced (p= 0.546). **b.** Total reads after filtering (p= 0.281). Data are represented as medians and minimum-maximum bars.

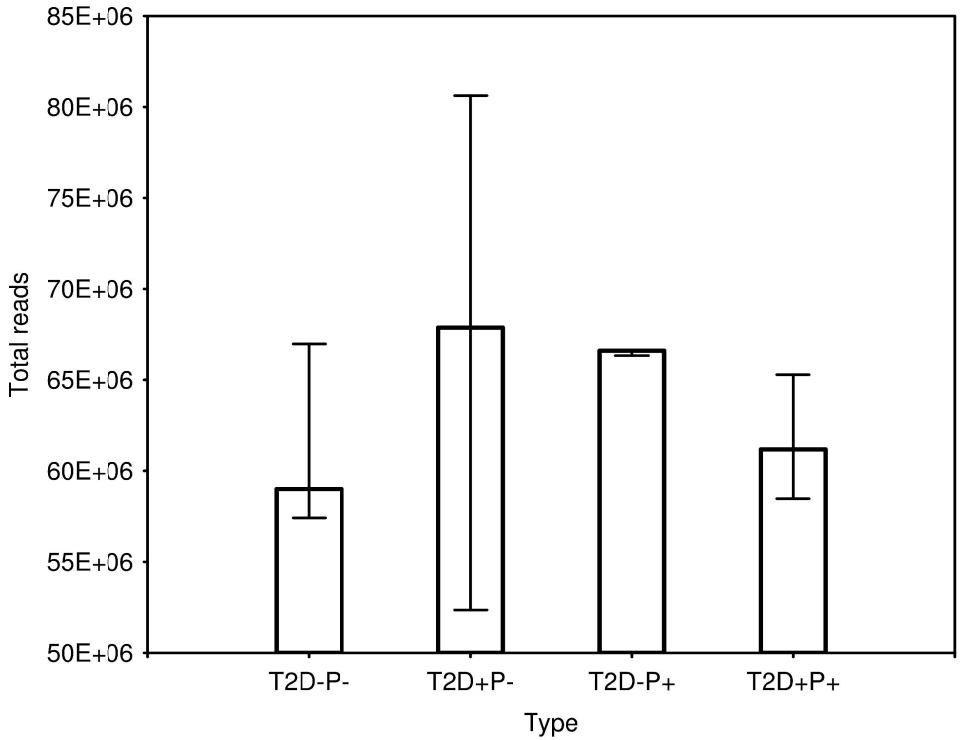
Figure 2. Intrapersonal diversity of the subgingival microbiome. a. Richness (p= 0.218). **b.** Evenness Index (J) (p= 0.838). **c.** alpha-diversity index (H') (p= 0.789). **d.** Beta-diversity Index (p= 0.789). Data are represented as medians and minimum-maximum bars.

Figure 3. Plots of principal coordinates analysis (PCoA) of subgingival microbiota based on Bray-Curtis matrix distances (species level). Each point represents the microbiota composition of one participant. **a.** T2D-P- (n= 3) and T2D-P+ (n= 3) groups; **b.** T2D+P- (n= 3) and T2D+P+ (n= 3) groups; **c.** T2D-P- (n= 3) and T2D+P- (n= 3) groups; **d.** T2D+P+ (n= 3) and T2D-P+ (n= 3) groups.

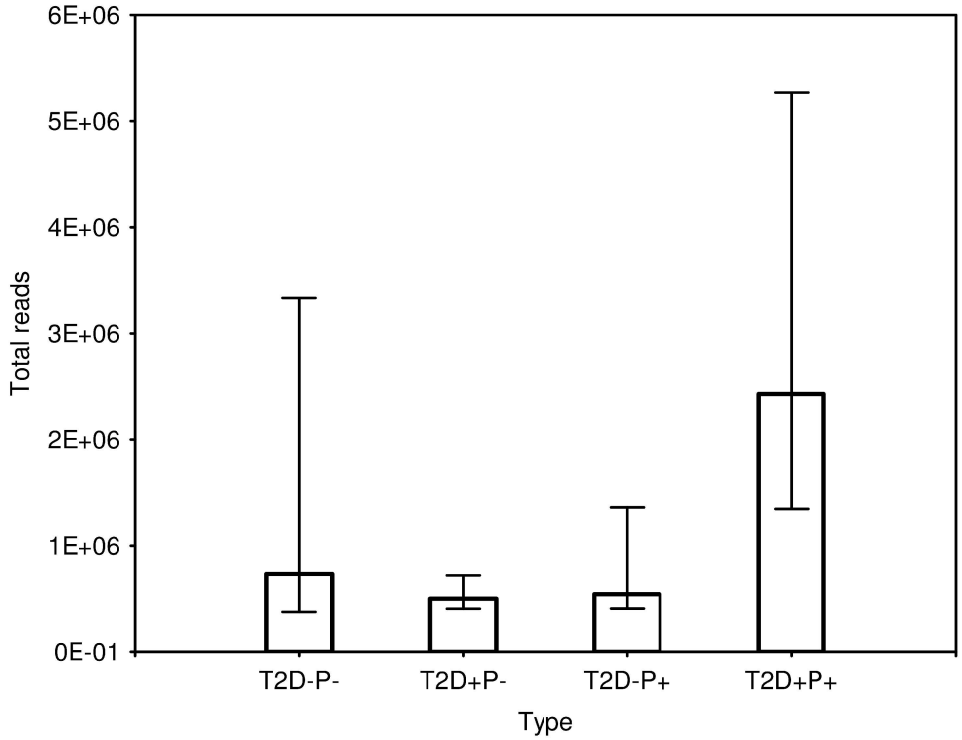
Figure 4. Forty most abundant genera (a) and species (b) in the four study groups. Bar heights correspond to relative abundance percentage, and are log-scaled. The error bar indicates one unit of standard error.

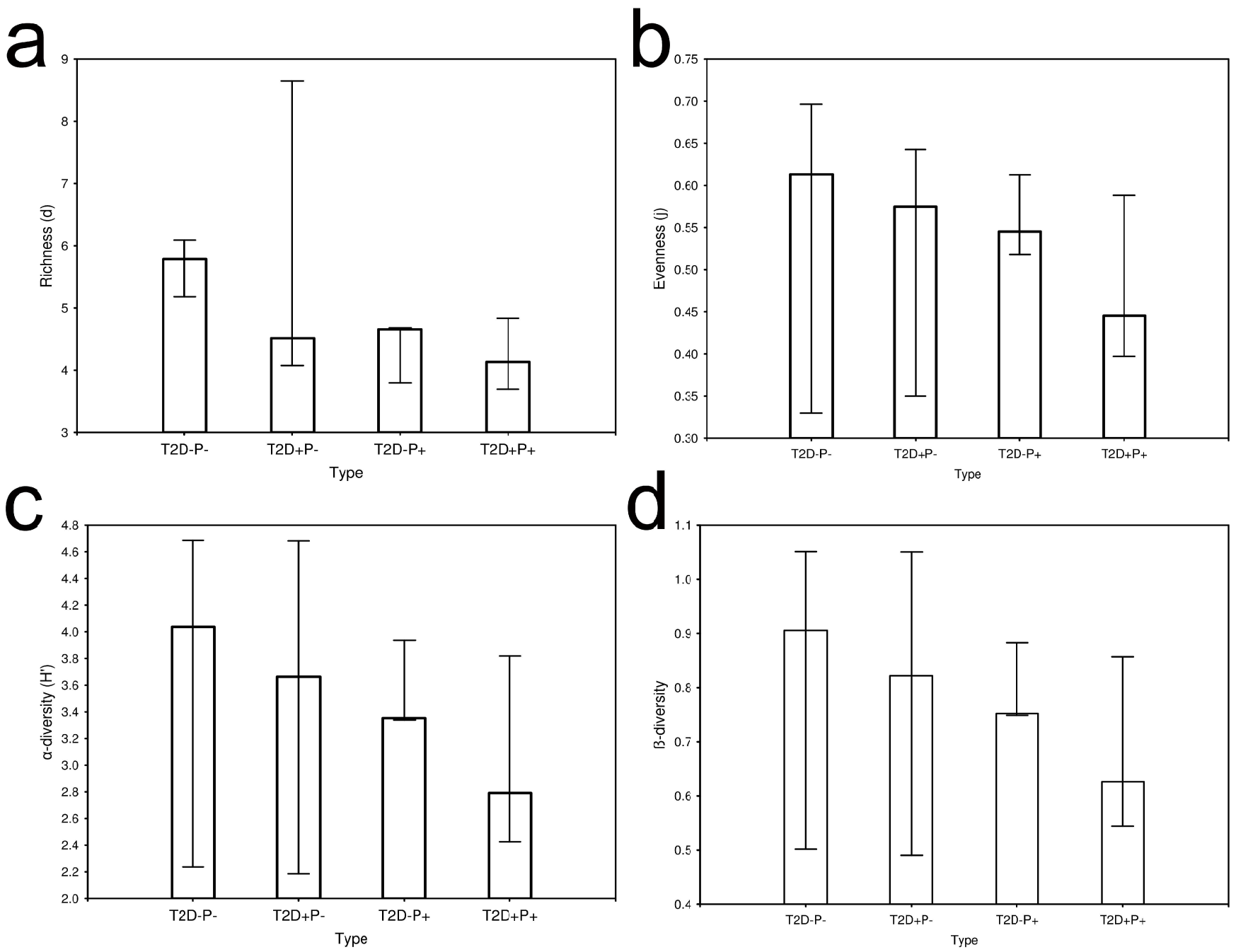
Figure 5. Bacterial genera and species with relative abundance > 0.5% in: a. Bacterial genera in patients with periodontitis (n= 6) and individuals without periodontitis (n= 6); **b.** Bacterial species in patients with periodontitis (n= 6) and individuals without periodontitis (n= 6); **c.** Bacterial genera in patients with type 2 diabetes (n= 6) and individuals without type 2 diabetes (n= 6); **d.** Bacterial species in patients with type 2 diabetes (n= 6) and individuals without type 2 diabetes (n= 6).

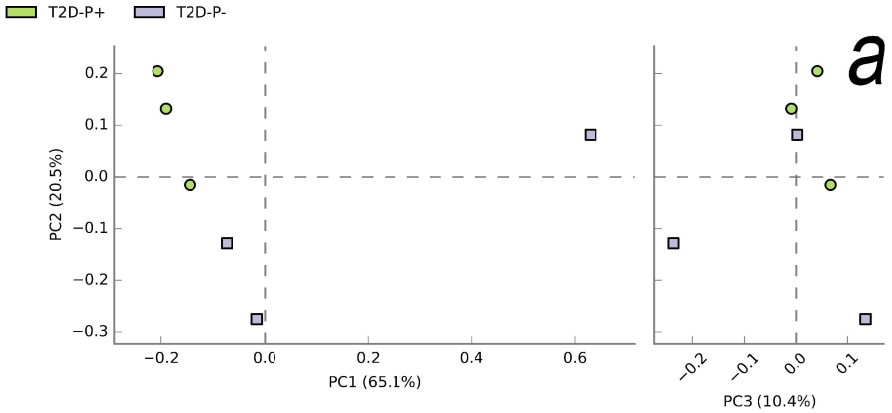
a

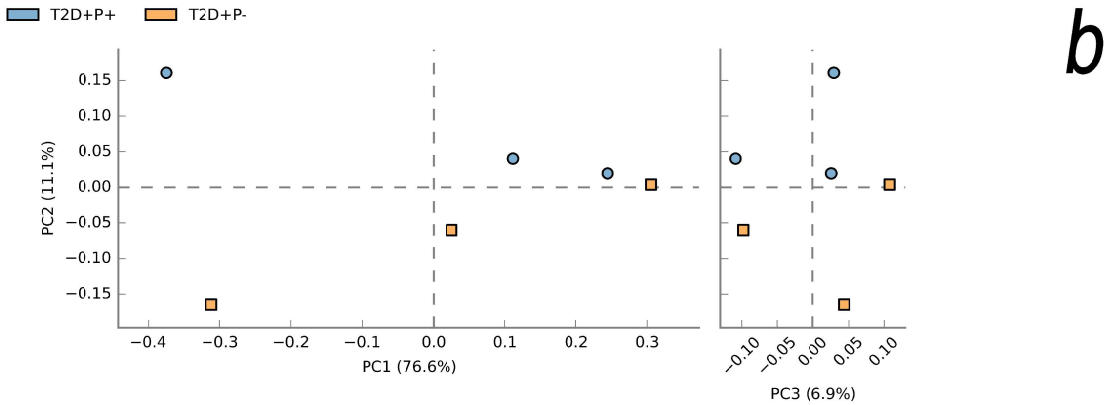

85E+06
80E+06
75E+06
70E+06
65E+06
60E+06
55E+06
50E+06
Total reads
T2D-P-
T2D+P-
T2D-P+
T2D+P+
Type

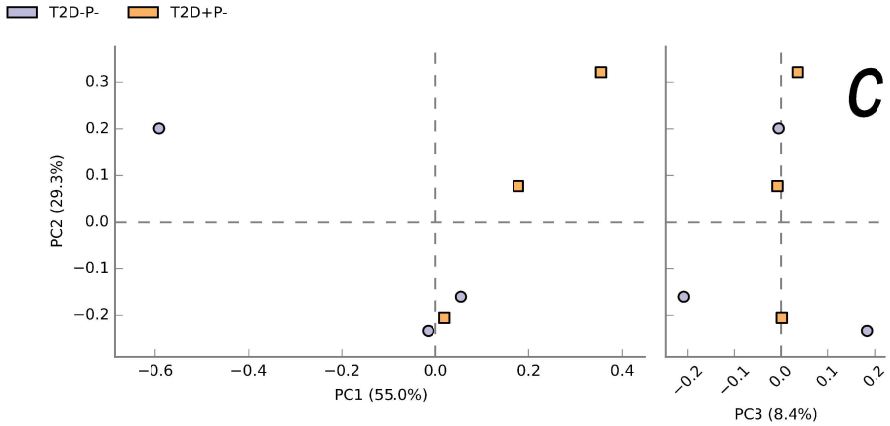
b

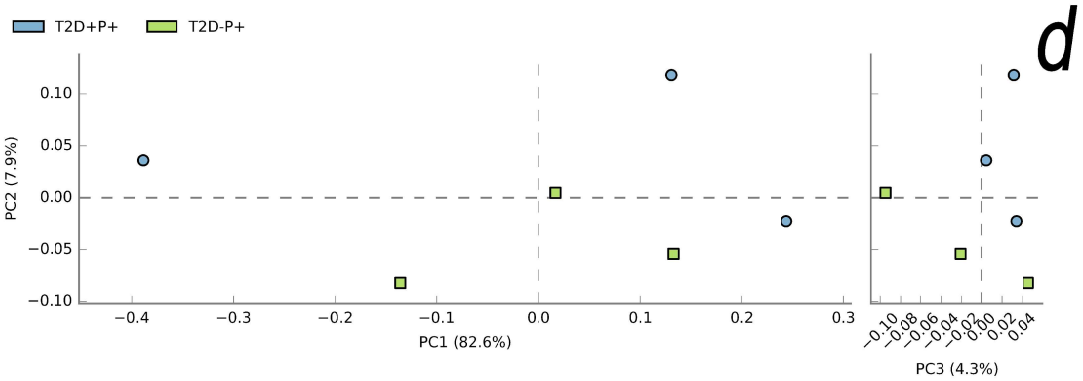

6E+06
5E+06
4E+06
3E+06
2E+06
1E+06
0E-01
Total reads
T2D-P-
T2D+P-
T2D-P+
T2D+P+
Type

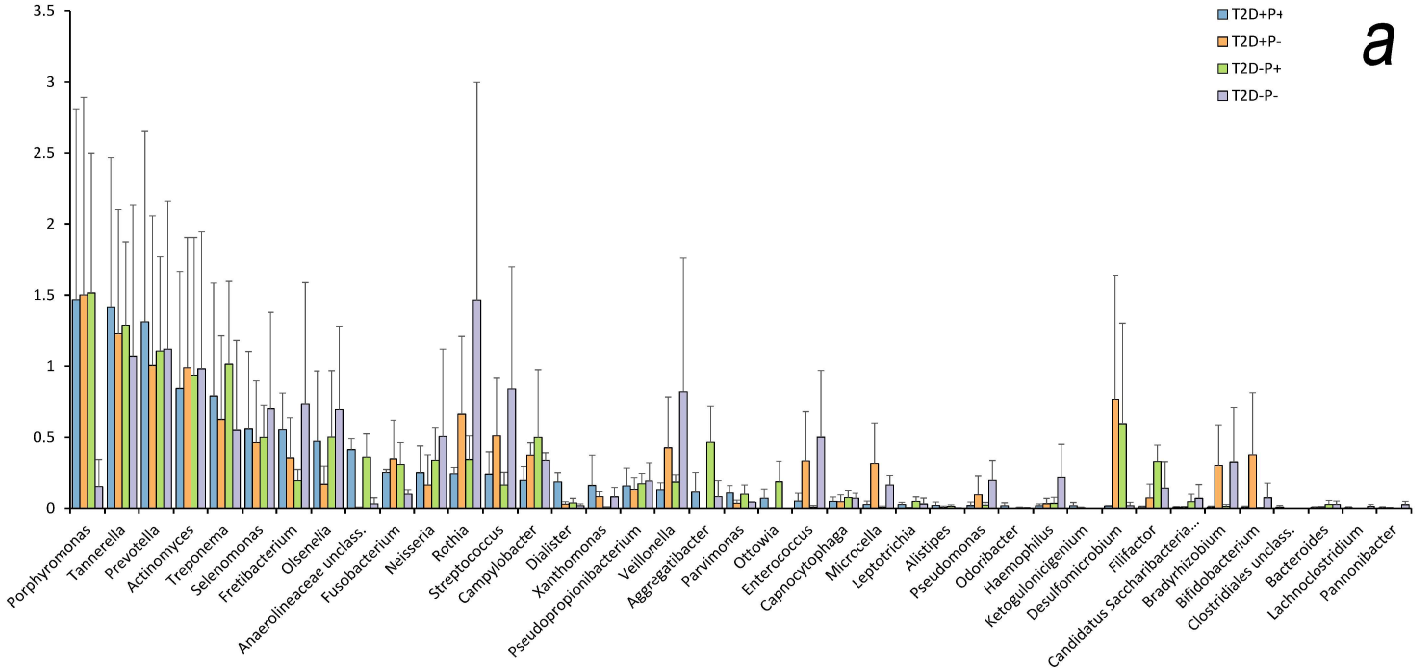

a
Richness (d)
9
8
7
6
5
4
3
T2D-P-
T2D+P-
T2D-P+
T2D+P+
Type
b
Evenness (j)
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
T2D-P-
T2D+P-
T2D-P+
T2D+P+
Type
c
α-diversity (H')
4.8
4.6
4.4
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
T2D-P-
T2D+P-
T2D-P+
T2D+P+
Type
d
β-diversity
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
T2D-P-
T2D+P-
T2D-P+
T2D+P+
Type

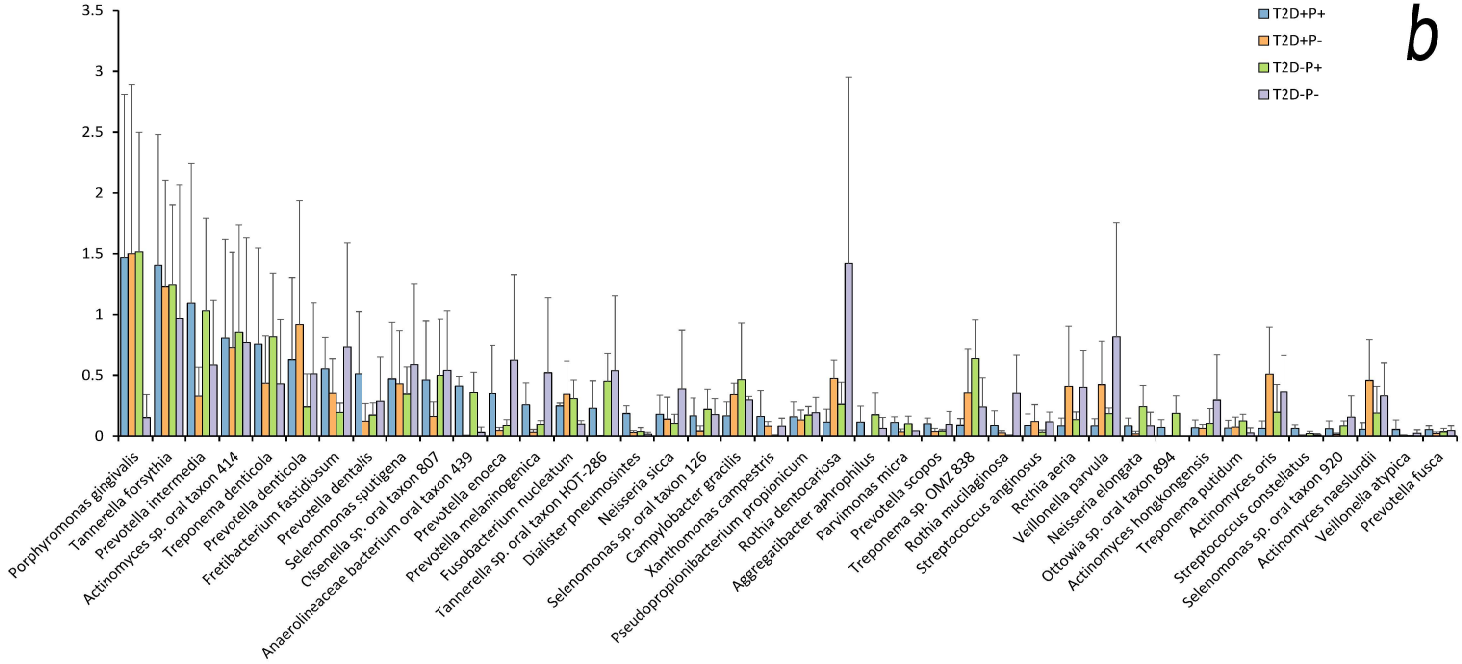

T2D-P+
T2D-P-
a
PC2 (20.5%)
PC1 (65.1%)
PC3 (10.4%)


T2D+P+
T2D+P-
b
PC2 (11.1%)
PC1 (76.6%)
PC3 (6.9%)


T2D-P-
T2D+P-
c
PC2 (29.3%)
PC1 (55.0%)
PC3 (8.4%)


T2D+P+
T2D-P+
d
PC2 (7.9%)
PC1 (82.6%)
PC3 (4.3%)


a
T2D+P+
T2D+P-
T2D-P+
T2D-P-
3.5
3
2.5
2
1.5
1
0.5
0
Porphyromonas
Tannerella
Prevotella
Actinomyces
Treponema
Selenomonas
Fretibacterium
Olsenella
Anaerolineaceae unclass.
Fusobacterium
Neisseria
Rothia
Streptococcus
Campylobacter
Dialister
Xanthomonas
Pseudopropionibacterium
Veillonella
Aggregatibacter
Parvimonas
Ottowia
Enterococcus
Capnocytophaga
Micrrcella
Leptotrichia
Alistipes
Pseudomonas
Odoribacter
Haemophilus
Ketogulonicigenium
Desulfomicrobium
Filifactor
Candidatus Saccharibacteria...
Bradyrhizobium
Bifidobacterium
Clostridiales unclass.
Bacteroides
Lachnoclostridium
Pannonibacter


b
T2D+P+
T2D+P-
T2D-P+
T2D-P-
3.5
3
2.5
2
1.5
1
0.5
0
Porphyromonas gingivalis
Tannerella forsythia
Prevotella intermedia
Actinomyces sp. oral taxon 414
Treponema denticola
Prevotella denticola
Fretibacterium fastidiosum
Prevotella dentalis
Selenomonas sputigena
Olsenella sp. oral taxon 807
Anaerolineaceae bacterium oral taxon 439
Prevotella enoeca
Prevotella melaninogenica
Fusobacterium nucleatum
Tannerella sp. oral taxon HOT-286
Dialister pneumosintes
Neisseria sicca
Selenomonas sp. oral taxon 126
Campylobacter gracilis
Xanthomonas campestris
Pseudopropionibacterium propionicum
Rothia dentocariosa
Aggregatibacter aphrophilus
Parvimonas micra
Prevotella scopos
Treponema sp. OMZ 838
Rothia mucilaginosa
Streptococcus anginosus
Rothia aeria
Veillonella parvula
Neisseria elongata
Ottowia sp. oral taxon 894
Actinomyces hongkongensis
Treponema putidum
Actinomyces oris
Streptococcus constellatus
Selenomonas sp. oral taxon 920
Actinomyces naeslundii
Veillonella atypica
Prevotella fusca

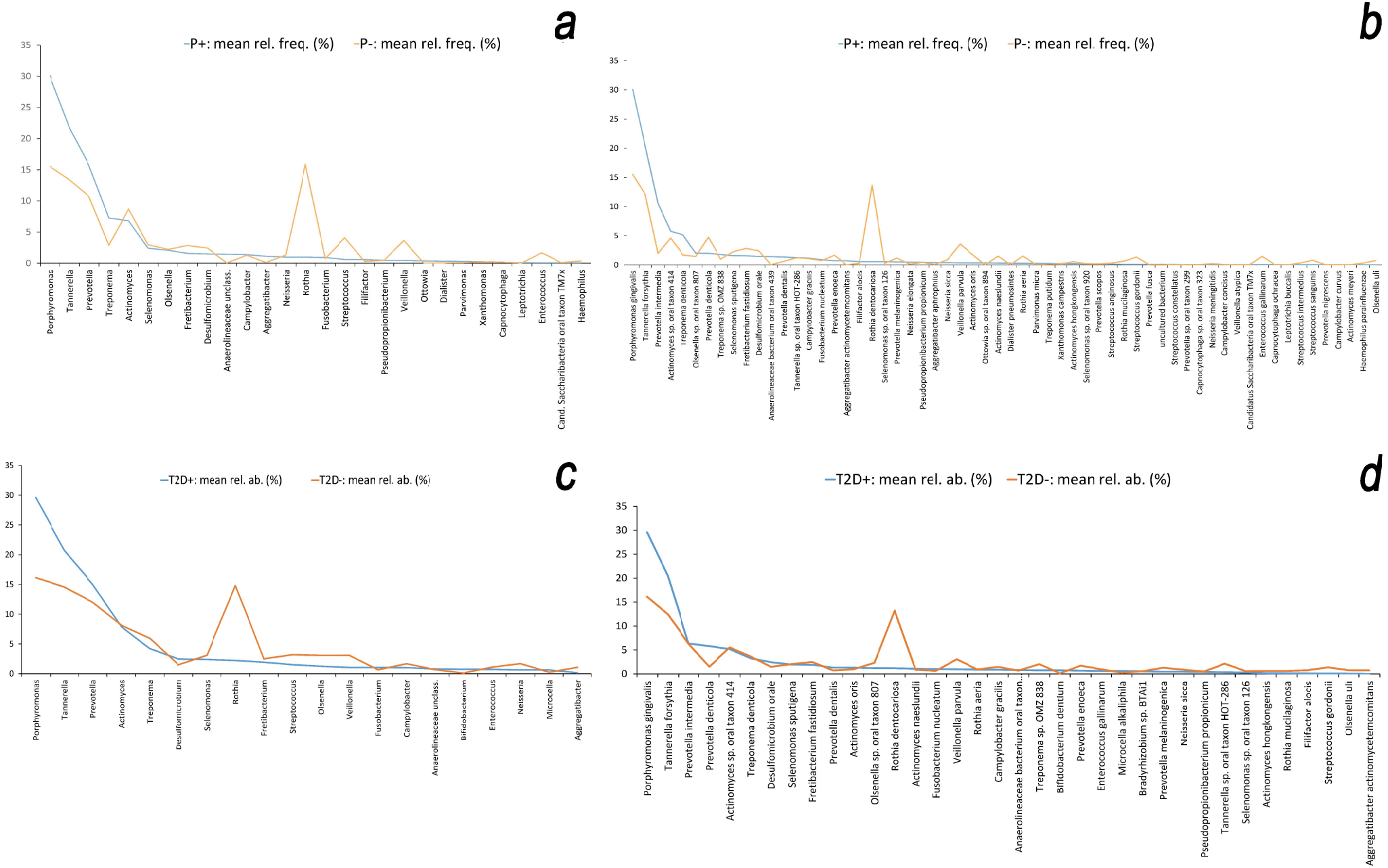

a
P+: mean rel. freq. (%)
P-: mean rel. freq. (%)
Porphyromonas
Tannerella
Prevotella
Treponema
Actinomyces
Selenomonas
Olsenella
Fretibacterium
Desulfomicrobium
Anaerolineaceae unclass.
Campylobacter
Aggregatibacter
Neisseria
Rothia
Fusobacterium
Streptococcus
Filifactor
Pseudopropionibacterium
Veillonella
Ottowia
Dialister
Parvimonas
Xanthomonas
Capnocytophaga
Leptotrichia
Enterococcus
Cand. Saccharibacteria oral taxon TM7x
Haemophilus
b
P+: mean rel. freq. (%)
P-: mean rel. freq. (%)
Porphyromonas gingivalis
Tannerella forsythia
Prevotella intermedia
Actinomyces sp. oral taxon 414
Treponema denticola
Olsenella sp. oral taxon 807
Prevotella denticola
Treponema sp. OMZ 838
Selenomonas sputigena
Fretibacterium fastidiosum
Desulfomicrobium orale
Anaerolineaceae bacterium oral taxon 439
Prevotella dentalis
Tannerella sp. oral taxon HOT-286
Campylobacter gracilis
Fusobacterium nucleatum
Prevotella enoeca
Aggregatibacter actinomycetemcomitans
Filifactor alocis
Rothia dentocariosa
Selenomonas sp. oral taxon 126
Prevotella melaninogenica
Neisseria elongata
Pseudopropionibacterium propionicum
Aggregatibacter aphrophilus
Neisseria sicca
Veillonella parvula
Actinomyces oris
Ottowia sp. oral taxon 894
Actinomyces naeslundii
Dialister pneumosintes
Rothia aeria
Parvimonas micra
Treponema putidum
Xanthomonas campestris
Actinomyces hongkongensis
Selenomonas sp. oral taxon 920
Prevotella scopos
Streptococcus anginosus
Rothia mucilaginosa
Streptococcus gordonii
Prevotella fusca
uncultured bacterium
Streptococcus constellatus
Prevotella sp. oral taxon 299
Capnocytophaga sp. oral taxon 323
Neisseria meningitidis
Campylobacter concisus
Veillonella atypica
Candidatus Saccharibacteria oral taxon TM7x
Enterococcus gallinarum
Capnocytophaga ochracea
Leptotrichia buccalis
Streptococcus intermedius
Streptococcus sanguinis
Prevotella nigrescens
Campylobacter curvus
Actinomyces meyeri
Haemophilus parainfluenzae
Olsenella uli
c
T2D+: mean rel. ab. (%)
T2D-: mean rel. ab. (%)
Porphyromonas
Tannerella
Prevotella
Actinomyces
Treponema
Desulfomicrobium
Selenomonas
Rothia
Fretibacterium
Streptococcus
Olsenella
Veillonella
Fusobacterium
Campylobacter
Anaerolineaceae unclass.
Bifidobacterium
Enterococcus
Neisseria
Microcella
Aggregatibacter
d
T2D+: mean rel. ab. (%)
T2D-: mean rel. ab. (%)
Porphyromonas gingivalis
Tannerella forsythia
Prevotella intermedia
Prevotella denticola
Actinomyces sp. oral taxon 414
Treponema denticola
Desulfomicrobium orale
Selenomonas sputigena
Fretibacterium fastidiosum
Prevotella dentalis
Actinomyces oris
Olsenella sp. oral taxon 807
Rothia dentocariosa
Actinomyces naeslundii
Fusobacterium nucleatum
Veillonella parvula
Rothia aeria
Campylobacter gracilis
Anaerolineaceae bacterium oral taxon...
Treponema sp. OMZ 838
Bifidobacterium dentium
Prevotella enoeca
Enterococcus gallinarum
Microcella alkaliphila
Bradyrhizobium sp. BTAi1
Prevotella melaninogenica
Neisseria sicca
Pseudopropionibacterium propionicum
Tannerella sp. oral taxon HOT-286
Selenomonas sp. oral taxon 126
Actinomyces hongkongensis
Rothia mucilaginosa
Filifactor alocis
Streptococcus gordonii
Olsenella uli
Aggregatibacter actinomycetemcomitans

Table 1. Characteristics of the study population.

Group	Patient	Gender	Age (years)	Year of type 2 diabetes diagnosis	Glycated haemoglobin (%)	Teeth present (n)	Sites with probing depth≥ 5 mm (n)	Bleeding on probing score (%)
T2D+P+	#1	male	67	1990	7.3	24	18	30
	#2	female	60	2000	8.0	24	29	36
	#3	male	70	2003	7.4	20	26	44
T2D+P-	#1	male	47	2012	8.0	26	0	18
	#2	male	54	1995	7.8	25	0	17
	#3	male	46	2009	7.1	28	0	20
T2D-P+	#1	female	62	-	-	24	19	49
	#2	female	45	-	-	24	39	49
	#3	female	51	-	-	22	23	28
T2D-P-	#1	female	54	-	-	28	0	8
	#2	male	47	-	-	26	0	25
	#3	female	59	-	-	32	0	12

Table 2. Clinical characteristics of sites selected for subgingival plaque sampling.

Group	Patient	Tooth	Tooth aspect	Probing depth (mm)	Bleeding on probing (+/-)
T2D+P+	#1	1.6	distolingual	7	+
		1.4	mesiolingual	6	+
		4.1	mesiobuccal	6	+
		3.2	distobuccal	6	+
	#2	2.5	distobuccal	7	+
		2.6	distobuccal	8	+
		3.5	distobuccal	6	+
		4.5	distolingual	6	+
	#3	3.1	mesiobuccal	9	+
		4.3	distobuccal	8	+
		3.2	distobuccal	5	+
		2.3	distobuccal	6	+
T2D+P-	#1	1.3	mesiobuccal	2	-
		4.4	mesiobuccal	3	-
		2.3	mesiobuccal	2	-
		3.4	mesiobuccal	3	-
	#2	4.4	mesiobuccal	3	-
		1.6	mesiobuccal	3	-
		2.6	mesiobuccal	3	-
		3.5	mesiobuccal	3	-
	#3	1.1	distobuccal	3	-
		1.3	distolingual	3	-
		4.2	mesiobuccal	3	-
		3.4	mesiobuccal	3	-
T2D-P+	#1	1.4	distobuccal	6	+
		2.6	distobuccal	7	+
		3.3	distobuccal	7	+
		4.4	distolingual	6	+
	#2	2.2	mesiobuccal	5	+
		2.1	distolingual	6	+
		3.2	mesiobuccal	6	+
		4.2	mesiobuccal	6	+
	#3	4.7	mesiobuccal	5	+
		1.7	mesiolingual	6	+
		2.5	distolingual	6	+
		2.6	distolingual	8	+
T2D-P-	#1	4.3	mesiobuccal	2	-
		2.6	mesiolingual	3	-
		1.6	mesiobuccal	3	-
		3.4	distobuccal	3	-
	#2	4.3	mesiobuccal	2	-
		3.4	mesiobuccal	3	-
		2.4	mesiobuccal	2	-
		1.2	mesiobuccal	2	-
	#3	3.2	mesiobuccal	3	-
		4.2	distobuccal	3	-
		2.4	mesiobuccal	3	-
		1.3	distobuccal	3	-

Table 3. Statistics of whole metagenome shotgun sequencing of plaque samples.

ID	Type	N° Tot Reads	N° Human Reads	N° Non-Human Reads (after quality filter)	% Human reads	% Non-Human (after quality filter)	N° of Blast hits	N° of Blast hits (after quality filter)	N° Reads in Megan	N° Species Identified by Megan	N° Genus Identified by Megan	Dominanat genus
1	T2D+P+	65,284,068	60,968,338	4,310,210	93.39	6.60	37,344,300	9,166,227	1,346,372	91	44	Prevotella
3	T2D+P+	61,186,636	50,173,223	11,006,180	82.00	17.99	126,597,122	47,494,569	5,268,893	76	30	Tannerella
7	T2D+P+	58,469,398	53,133,961	5,323,208	90.87	9.10	79,159,464	30,735,681	2,429,845	69	34	Porphyromonas
2	T2D+P-	80,622,922	76,292,571	4,324,334	94.63	5.36	66,603,563	21,019,944	500,936	143	78	Actinomyces
6	T2D+P-	67,873,856	66,190,415	1,681,216	97.52	2.48	19,792,224	8,184,688	720,295	76	36	Porphyromonas
12	T2D+P-	52,357,072	51,299,375	1,054,246	97.98	2.01	14,136,306	4,410,286	405,213	83	35	Porphyromonas
4	T2D-P+	66,338,258	64,936,977	1,397,736	97.89	2.11	11,182,043	4,127,100	542,826	71	33	Porphyromonas
9	T2D-P+	66,614,552	65,194,288	1,416,982	97.87	2.13	13,477,533	3,936,184	408,515	86	34	Actinomyces
11	T2D-P+	66,644,814	62,977,577	3,662,942	94.50	5.50	46,010,795	16,768,321	1,360,609	86	35	Porphyromonas
5	T2D-P-	66,979,592	65,292,747	1,684,614	97.48	2.52	10,635,227	2,624,044	376,877	93	55	Prevotella
8	T2D-P-	57,425,098	41,002,074	16,416,896	71.40	28.59	276,171,550	76,039,977	3,334,364	105	42	Actinomyces
10	T2D-P-	59,001,928	55,316,534	3,678,986	93.75	6.24	132,078,475	18,357,675	733,856	104	69	Rothia

Periodontitis patients *vs* individuals without periodontitis (Genera)	Periodontitis Mean ± s.d.	No periodontitis Mean ± s.d.	Nominal p-val	FDR p-val	Periodontitis patients *vs* individuals without periodontitis (Species)	Periodontitis Mean ± s.d.	No periodontitis Mean ± s.d.	Nominal p-val	FDR p-val
Anaerolineaceae unclass.	**1.439 ± 0.387**	**0.042 ± 0.083**	**0.000**	**0.001**	***Anaerolineaceae bacterium oral taxon 439***	**1.439 ± 0.387**	**0.042 ± 0.083**	**0.000**	**0.001**
Parvimonas	0.276 ± 0.141	0.096 ± 0.038	0.003	0.213	*Parvimonas micra*	0.276 ± 0.141	0.096 ± 0.038	0.003	0.410
Ottowia	0.362 ± 0.349	0.002 ± 0.005	0.008	0.342	*Actinomyces sp. Chiba101*	0.006 ± 0.009	0.037 ± 0.024	0.003	0.295
Dialister	0.315 ± 0.256	0.056 ± 0.040	0.009	0.289	*Actinomyces sp. pika_114*	-	0.014 ± 0.012	0.004	0.231
Microcella	0.040 ± 0.051	0.764 ± 0.731	0.010	0.246	*Porphyromonas asaccharolytica*	0.017 ± 0.016	-	0.007	0.343
Aggregatibacter	1.116 ± 1.015	0.105 ± 0.234	0.010	0.215	*Ottowia sp. oral taxon 894*	0.362 ± 0.349	0.002 ± 0.005	0.008	0.337
					Dialister pneumosintes	0.315 ± 0.256	0.056 ± 0.040	0.009	0.336
					Microcella alkaliphila	0.04 ± 0.051	0.764 ± 0.731	0.010	0.313
					Actinomyces oris	0.369 ± 0.539	1.774 ± 1.319	0.010	0.283

T2D-P+ *vs* T2D-P- (Genera)	T2D-P+ Mean ± s.d.	T2D-P- Mean ± s.d.	Nominal p-val	FDR p-val	T2D-P+ *vs* T2D-P- (Species)	T2D-P+ Mean ± s.d.	T2D-P- Mean ± s.d.	Nominal p-val	FDR p-val
Porphyromonas	31.819 ± 8.595	0.420 ± 0.552	0.002	0.265	*Porphyromonas gingivalis*	31.779 ± 8.595	0.42 ± 0.552	0.004	0.998
Microcella	0.014 ± 0.020	0.463 ± 0.167	0.005	0.321	*Campylobacter rectus*	0.065 ± 0.013	0.008 ± 0.011	0.005	0.596
Anaerolineaceae unclass.	1.291 ± 0.466	0.075 ± 0.106	0.007	0.299	*Microcella alkaliphila*	0.014 ± 0.020	0.463 ± 0.167	0.008	0.700
Aggregatibacter	1.924 ± 0.788	0.209 ± 0.296	0.010	0.342	*Anaerolineaceae bacterium oral taxon 439*	1.291 ± 0.466	0.075 ± 0.106	0.010	0.653

T2D+P+ *vs* T2D+P- (Genera)	T2D+P+ Mean ± s.d.	T2D+P- Mean ± s.d.	Nominal p-val	FDR p-val	T2D+P+ *vs* T2D+P- (Species)	T2D+P+ Mean ± s.d.	T2D+P- Mean ± s.d.	Nominal p-val	FDR p-val
Anaerolineaceae unclass.	**1.588 ± 0.195**	**0.009 ± 0.012**	**0.000**	**0.001**	***Anaerolineaceae bacterium oral taxon 439***	**1.588 ± 0.195**	**0.009 ± 0.012**	**0.000**	**0.001**
Dialister	0.538 ± 0.160	0.072 ± 0.038	0.001	0.059	*Leptotrichia sp. oral taxon 212*	0.016 ± 0.004	-	0.001	0.103
Campylobacter	0.581 ± 0.249	1.365 ± 0.228	0.003	0.114	*Rothia dentocariosa*	0.301 ± 0.282	1.984 ± 0.418	0.002	0.161
					Treponema maltophilum	0.024 ± 0.008	-	0.002	0.148
					Dialister pneumosintes	0.538 ± 0.16	0.072 ± 0.038	0.003	0.141
					Campylobacter gracilis	0.467 ± 0.308	1.201 ± 0.239	0.010	0.412

Patients with type 2 diabetes *vs* Individuals without type 2 diabetes (Genera)	Type 2 diabetes Mean ± s.d.	No type 2 diabetes Mean ± s.d.	Nominal p-val	FDR p-val	Patients with type 2 diabetes *vs* Individuals without type 2 diabetes (Species)	Type 2 diabetes Mean ± s.d.	No type 2 diabetes Mean ± s.d.	Nominal p-val	FDR p-val
Filifactor	0.106 ± 0.195	0.762 ± 0.574	0.005	0.628	*Filifactor alocis*	0.106 ± 0.195	0.762 ± 0.574	0.006	0.999
Ornithobacterium	-	0.020 ± 0.019	0.006	0.377	*Ornithobacterium rhinotracheale*	-	0.020 ± 0.019	0.007	0.888

T2D+P- *vs* T2D-P-	T2D+P-	T2D-P-	Nominal	FDR	T2D+P- *vs* T2D-P-	T2D+P-	T2D-P-	Nominal	FDR

(Genera)	Mean ± s.d.	Mean ± s.d.	p-val	p-val	(Species)	Mean ± s.d.	Mean ± s.d.	p-val	p-val
-	-	-	-	-	-	-	-	-	-
T2D+P+ *vs* T2D-P+ (Genera)	**T2D+P+ Mean ± s.d.**	**T2D-P+ Mean ± s.d.**	**Nominal p-val**	**FDR p-val**	**T2D+P+ *vs* T2D-P+ (Species)**	**T2D+P+ Mean ± s.d.**	**T2D-P+ Mean ± s.d.**	**Nominal p-val**	**FDR p-val**
Filifactor	0.026 ± 0.011	1.137 ± 0.305	0.006	0.717	*Filifactor alocis*	0.026 ± 0.011	1.137 ± 0.305	0.004	0.967
Dialister	0.538 ± 0.160	0.092 ± 0.078	0.009	0.598	*Treponema sp. OMZ 838*	0.228 ± 0.136	3.360 ± 1.083	0.008	0.999
					Dialister pneumosintes	0.538 ± 0.160	0.092 ± 0.078	0.009	0.805

AUTHOR DECLARATION

Ferrara, April 9 2019

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.
We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.
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R.F., L.T. and C.S. contributed to the conception and design of the study; M.S. performed clinical assessments and collected plaque samples; S.S. and E.M. performed DNA sequencing and data analysis; A.C. performed bioinformatic and statistical analysis. R.F., L.T. and C.S. wrote the initial draft of the manuscript. All authors contributed to manuscript revision and gave final approval before submission.