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Signature of microbial dysbiosis in periodontitis.

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M.B.M. and F.B.H. contributed equally to this work.

Keywords: chronic periodontitis – health – microbiota – dysbiosis – ratio
Abstract

Periodontitis is driven by a disproportionate host inflammatory immune response induced by an imbalance in the composition of oral bacteria. It instigates microbial dysbiosis along with failed resolution of the chronic destructive inflammation.

The objective of this study is to identify microbial signatures for health and chronic periodontitis at the genus level and to propose a model of dysbiosis including the calculation of bacterial ratios.

Design and methods. Published sequencing data obtained from several different studies (196 sub-gingival samples from patients with chronic periodontitis and 422 sub-gingival samples from healthy subjects) were pooled and submitted to a new microbiota analysis using the same VAMPS (Visualization and Analysis of Microbial Population Structures) analysis pipeline to identify microbiota specific to health and disease. Microbiota were visualized using CoNet and Cytoscape. Dysbiosis ratio, defined as the percentage of genera associated with disease relative to the percentage of genera associated with health, were calculated to distinguish disease from health. Correlation between the proposed dysbiosis ratio and the periodontal pocket depth was tested on a different set of data obtained from a recent study to confirm the relevance of the ratio as a potential indicator of dysbiosis.

Results. Beta diversity showed significant clustering of periodontitis-associated microbiota according to clinical status at the genus level, independent of methods used. Specific genera were highly prevalent (>95%) in health (Veillonella, Neisseria, Rothia, Corynebacterium and Actinomyces) while other genera were associated with chronic periodontitis (Eubacterium, Campylobacter, Treponema and Tannerella). The calculation of dysbiosis ratio based on the relative abundance of these genera found in health versus periodontitis was tested. Non-periodontitis samples were significantly identifiable by low ratios as compared to chronic
periodontitis samples. When applied to a sub-gingival set with well-defined clinical data, the method showed a strong correlation between the dysbiosis ratio, as well as a simplified ratio (Porphyromonas, Treponema and Tannerella to Rothia and Corynebacterium), and pocket depth.

Conclusion. Microbial analysis of chronic periodontitis can be correlated with the pocket depth through specific signatures of microbial dysbiosis.

Importance

Defining microbiota typical of oral health or chronic periodontitis is a difficult task. Periodontal disease evaluation is currently based on probing of the periodontal pocket. However, the status of pockets “on the mend” or sulci at “risk of periodontitis” cannot solely be addressed through pocket depth measurements, nor by current microbiological tests available for practitioners. Thus, a more specific microbiological status of dysbiosis could help in future diagnoses of periodontitis. In this work, data from different studies were pooled to improve the accuracy of the results. However, analysis of multiple microbiota from different studies intensified the bacterial network and complicated the search for reproducible microbial signature. Despite different methods used in each study, the microbiota investigated at the genus level showed that some genera are prevalent (up to 95% of the samples/microbiota) in health or disease, allowing the calculation of bacterial ratios (i.e. dysbiosis ratios). The correlation between the proposed ratios and the periodontal pocket depth was tested which confirmed the link between dysbiosis ratios and the severity of the disease. The importance of this work is promising but longitudinal studies will be required to improve the ratios and define microbial signatures of the disease, which
will allow the monitoring of the periodontal pocket recovery and conceivably, the potential risk of periodontitis in healthy patients.
Introduction

Chronic periodontitis (CP) is a chronic inflammation characterized by alveolar bone loss with intermittent periods of remission and relapse. CP is currently considered as an infection mainly due to an increase of bacteria in the sulcus leading to the formation of a periodontal pocket (for review: 1, 2). The major pathogen linked to CP is *Porphyromonas gingivalis* with bacterial partners like *Treponema denticola* and *Tannerella forsythia*. These three bacteria have been considered as the major pathogenic “red complex” since 1998 (3). However, recent advances from metagenomics studies developed a new model of periodontal disease pathogenesis. CP does not result from individual pathogens but rather from polymicrobial synergy and dysbiosis (4) associated with a dysregulated immune response inducing inflammation-mediated tissue damage (5). Host genetic components have also been implicated in CP, where multiple genes contribute cumulatively to the hosts overall disease risk (or protection) through effects on the host immune response and the microbiome (6).

Since the Human Microbiome Project (HMP) (7), microbiota were analyzed based on partial sequencing of the 16S rRNA gene with different numbers of healthy and CP samples. However, comparison between studies is difficult because of the differences in methods used (i.e. clinical examination and diagnosis of periodontitis and oral health, sample collection protocols, DNA extraction protocols, hyper variable regions of the 16S rRNA gene analyzed). As there is a growing interest in the human microbiome, despite the difficulties mentioned earlier, the use of independent studies to look for “Signal in the Noise” should proceed as previously suggested (8), by reanalyzing all data with the same protocol. The difference between periodontal health- and disease-associated microbiota should be larger than the technical variations of the different studies and enable the identification of microbial signatures using NGS technologies. The first objective of this study was to explore...
the disease-associated changes in the sub-gingival microbiota using a unique VAMPS (Visualization and analysis of microbial population structure) pipeline (9) at the genus level for beta-diversity (Bray-Curtis dissimilarity) on a large number of samples (from 6 different studies) and to confirm that the microbiota identified did not cluster according to the methods used (primer or study type). Sub-gingival microbiota from patients with diagnosed chronic periodontitis (196) and from healthy subjects (422) were included as well as external control samples (from dentine caries, supra-gingival plaque and the mid-vagina). The second objective was to determine a dysbiosis ratio of bacteria that could predict health or severity of the disease from the sub-gingival samples and finally to test it on an independent cohort of patients with well-described periodontal pocket measurements.
MATERIAL and METHODS

Microbiome datasets for comparison. Read sequences from healthy and chronic periodontitis sub-gingival samples of five different studies i.e. Abusleme et al. (10), Kirst et al. (11), Griffen et al. (12) (shallow site samples also included), Zhou et al. (13), and Camelo-Castillo et al. (14) were retrieved from either the NCBI Sequence Read Archive (SRA), or the MG-RAST server (Table 1). Twenty-four samples analyzed using V3V4 primer from patients with chronic periodontitis recruited between June 2010 and September 2011 at the University Hospital (Rennes, France) were added (publication in progress). Each data set was manually imported into VAMPS, while numerous healthy sub-gingival samples were added from the HMP (2 different sub-gingival datasets using V1V3 and V3V5 primers available in VAMPS (9), https://vamps.mbl.edu/). Three mouth control microbiota datasets were used from the HMP (saliva and supra-gingiva, both V1V3 and V3V5 regions, available in VAMPS) and dentine caries from Kianoush et al. (V3V4 regions, PRJEB5178) (15). One mid vagina microbiome dataset from the HMP (V1V3 region, available in VAMPS) was used as an external mouth control.

Finally, the dataset from Bizzarro S. et al. (16), containing well-described sample pocket depth (from 2 to 8 mm) was used to independently challenge the relevance of the dysbiosis ratio of bacteria involved in periodontitis.

Ecology diversity and taxonomy identifications. Reads from the different datasets were analyzed using VAMPS using default parameters for taxonomy assignment to the genus level through the GAST process using the RDP classification to produce the best taxonomic assignment for each read. Reads identified as Archaea, Eukarya, Organelle and unknown reads were excluded for further analysis. The frequency of each taxonomic assignment
in the dataset was reported as a percentage (number of reads assigned to a taxonomy over total number of reads in the dataset). Alpha diversity as observed richness and Shannon-Weaver index were determined from the raw data sets. Differences between microbiota structures (beta-diversity) were assessed using principal coordinate analysis (PCoA) 2D tree on the Bray-Curtis distance through VAMPS. Samples were divided into five clusters (1 to 5), where visualizations were realized using Figtree software (v1.4.2) and 3D PCoA plots were generated using Emperor software. Relative abundances were studied when the average abundance was above 1% in at least one sample. Assessments of significant patterns of microbial co-occurrence or mutual exclusion at the genus level were performed using Cytoscape v3.2.1 (17) and the CoNet plugin (18). Only genera found in the high majority, in at least 95%, of the healthy sub-gingival samples or the CP samples (from the fifth cluster) are represented.

Dysbiosis calculations – ratios of bacteria: To measure the dysbiosis, a first ratio based on the relative abundance of genera highly prevalent (>95%) in CP samples (Eubacterium, Campylobacter, Treponema and Tannerella) to genera highly prevalent (>95%) in healthy microbiota (Veillonella, Neisseria, Rothia, Corynebacterium and Actinomyces) was calculated. The ratios were normalized between samples using GraphPad Prism V6 software before comparison. A second simplified ratio of Porphyromonas, Treponema and Tannerella to Rothia and Corynebacterium was also tested. When no specific genus was detected and as "no detection" does not mean "absence", a value of 0.1% was attributed.

Statistical analysis. Normality tests for data distribution were realized. Data were studied by Spearman correlation test between biological origins, primers used, publication of sample...
origins and microbiota clusters. Observed richness ($S$, number of taxa per sample), Shannon-Weaver index and dysbiosis ratio of the genera found in disease to the genera found in health were analyzed with a non-parametric Anova Kruskal-Wallis test. Tests were carried out using GraphPad Prism V6 software and were considered significant when $p < 0.05$. The significant patterns of microbial co-occurrence and mutual exclusion were analyzed as described by Faust (18): a compilation of statistical analysis (Spearman, Pearson correlations and Bray Curtis, Kullback-Leibler dissimilarity measures) was used with a threshold set at 0.5. The data matrix was randomized by 100 row-wise permutations. The $P$ values were adjusted by Benjamini-Hochberg false discovery rate (FDR) correction for the number of tests, retaining only $p < 0.05$. Finally, the ratios of genera and pocket depth were controlled for normality followed by Spearman correlation test.
RESULTS

Microbial community structure analysis. Using a matrix correlation analysis, the possible clustering of microbiota according to nature of primers used, the site of sampling, or the study investigated was explored. Despite various studies, the analyzed data clustered into five groups according to the clinical status (healthy or CP) or sampling site as shown by the 3D PCoA plots (Fig. 1). Healthy sub-gingival samples were, in majority, spread into two main clusters; control samples were clearly separated in two other clusters corresponding to saliva and dentine caries/vagina, while the majority of CP samples were found in a fifth cluster. Two-D beta-diversity analysis showed the precise distribution of the samples in the five clusters (Fig. 2). The search for an association between clusters and primers and/or study type showed that the fourth cluster was associated with V3V4 16S rRNA primers (correlation r=0.537, p<0.001) and with the Kianoush et al. study (15) that has used these specific primers (correlation r=0.608, p<0.001). No other correlation with primers was found. The 2 healthy clusters (1 and 2) were characterized by sub- and supra-gingival samples in similar proportions (Fig. 2, in blue). Focusing on healthy sub-gingival samples, the main difference between the two healthy clusters 1 and 2 was the distribution of samples from the HMP study and from the other studies in the clusters: 225/323 of the HMP study clustered into the healthy cluster 2 while the healthy cluster 1 was richer in samples deriving from the other studies (44/99). Cluster 3 was characterized by saliva as 91% of the saliva samples (258/284) are grouped within this cluster (Fig. 2, correlation r=0.892, p<0.001). Cluster 4 was characterized by samples from dentine caries 73% (80/110) and mid-vagina 100% (60/60) (correlation r=0.603 and r=0.638 respectively, p<0.001). Finally, the fifth cluster contained 90% of the CP samples (176/196, correlation r=0.708, p<0.001).
It is interesting to note that 10% of the CP samples were found in the 2 healthy clusters (19/196) and contained similar microbiota (analyzed by beta-diversity) as dentine caries and/or mid-vagina (1/196) at the genus level. Conversely, 16% of the healthy sub-gingival samples (69/422) and 17% of the dentine caries samples (19/110) were found in the fifth cluster.

**Microbiota richness and alpha-diversity in sub-gingival samples:** Cluster comparison showed that sampling depth (number of reads sequenced) was higher in healthy sub-gingival clusters 1 and 2 than in the fifth cluster. Nevertheless, no significant difference between healthy sub-gingival and CP samples of the fifth cluster was found (Fig. 3). The observed richness (S) was lower in the CP samples from fifth cluster than samples of both healthy clusters 1 and 2 and of healthy sub-tingival samples from the fifth cluster (Fig. 3). However, the Shannon Weaver diversity index showed that the diversity of healthy cluster 2 was significantly higher than the diversity index that is similar in healthy cluster 1 and of all samples from the fifth cluster.

**Patterns of microbial communities in sub-gingival samples (genus level):** Genera present in, at least, 95% of all healthy sub-gingival samples or 95% of the CP samples from the fifth cluster are presented in figures 4A and 4B respectively. Results showed that healthy sub-gingival samples are dominated by 8 major genera, *Fusobacterium, Actinomyces, Streptococcus, Neisseria, Capnocytophaga, Prevotella, Corynebacterium, and Rothia*, and by 6 minor genera, *Leptotrichia, Veillonella, Porphyromonas, Granulicatella, Kingella* and *Gemella*. Associations were found between *Fusobacterium* and *Prevotella*, *Actinomyces* and *Rothia* and between *Leptotrichia* and *Porphyromonas*. Common genera found in CP were...
less abundant with 4 major genera, *Treponema, Porphyromonas, Prevotella* and *Fusobacterium* followed by *Streptococcus, Eubacterium, Tannerella* and *Campylobacter* genera. Only one association was found between *Eubacterium* and *Treponema*, while *Fusobacterium* and *Treponema* presented a negative correlation.

**Dysbiosis calculation – ratio of bacteria:** The dysbiosis ratio of genera found mainly in chronic periodontitis (*Eubacteria-Campylobacter-Treponema-Tannerella*) to the genera found mainly in health samples (*Veillonella-Neisseria-Rothia-Corynebacterium-Actinomyces*) was significantly different between samples according to their diagnosis. The dysbiosis ratio of healthy sub-gingival samples (from HMP, n=323, r=0.016 and from the other studies, n=99, r= 0.021) obtained a median r=0.018, shallow sites r=0.071 and samples from chronic periodontitis r=1.229 (p<0.001) (figure 5A).

Despite achieving a different clustering through beta-diversity, no significant difference was found between the ratios of cluster 1 and 2 according to the clinical status (healthy, shallow and CP). Pooling of samples according to clinical status was done and the resulting ratios were compared to ratios of the fifth cluster as shown in figure 5B.

The dysbiosis ratio found in CP samples from the fifth cluster (r=1.510) was significantly higher than the ratios of the majority of samples from cluster 1 and 2 (healthy sub-gingival r=0.015, shallow r=0.052 and CP samples r=0.088), and was also significantly higher than healthy sub-gingival samples (r=0.184) from the same fifth cluster (p<0.001). In clusters 1 and 2, the dysbiosis ratio of CP samples was similar to the ratio of shallow sites. These two groups were significantly different from the healthy sub-gingival samples (p<0.05) in the same cluster.
Healthy sub-gingival samples (n=69) belonging to the fifth cluster exhibited a dysbiosis ratio (r=0.184) significantly different from the other healthy sub-gingival samples (r=0.015) but also from the majority of the CP samples (fifth cluster, r=1.510). These results confirmed the possible difference of these healthy sub-gingival microbiota (p<0.001) as compared with those of healthy clusters 1 and 2. Their ratio is also not significantly different from the CP sample ratio in cluster 1 and 2, which could be considered “on the mend”.

Validation of the dysbiosis ratio

A different dataset from Bizzarro S. et al. (16), containing well-described samples (pocket depth from 2 to 8 millimeters), was used as an external control to confirm the relevance of the dysbiosis ratio of bacteria. The dysbiosis ratio at the genus level was correlated with the periodontal pocket depth (r=0.655, p<0.001) (Fig. 6A). These results, based on 37 patients (147 samples at different times and different procedures of the periodontal treatment), confirmed the link between the dysbiosis and the depth of periodontal pocket. The second simplified ratio of Porphyromonas, Treponema and Tannerella to Rothia and Corynebacterium showed a similar correlation (r=0.659, p<0.001) (Fig. 6B).
DISCUSSION

Many studies have been published since the human microbiome project in 2009, increasing the number of microbiota data available for the research community. However, comparison between studies is challenging, at least at the species level, because of the use of different methods. This issue is a real limitation to understand disease as well as the low number of samples in each study. Additionally, it is more complicated for healthy sub-gingival status that usually represent less than a half of the samples when included in studies (10, 14). This work is a taxon-based analysis at the genus level of sequence reads from several studies. Studying a large number of samples minimized individual variations and overcame technical variations by increasing the effective sample size. Such analysis has already been proposed in a recent microbiota obesity study (8). Studies with described healthy (sulci ≤3mm) and CP (pocket depth ≥5mm) samples and available raw sequenced data in data banks were chosen. Data from the HMP resources (two different couples of primers used) were added to increase the number of sub-gingival healthy microbiota data available from 99 up to 422. The different microbiota clustered either by sampling site, such as out-groups used as controls for this study (saliva in cluster 3, dentine caries/vagina both rich in Lactobacillus in cluster 4), or by clinical status, such as sub-gingival samples (healthy samples in cluster 1 and 2 and CP samples in the fifth cluster). CP sites can either show a greater microbial diversity and observed richness than healthy sub-gingival sites (12, 19), or present no significant difference in microbial diversity as it has been also reported between health and periodontitis (11). Thus, the high number of samples surpasses the technical variations, at least at the genus level with the primers used in these different studies, and the difference between periodontal health and disease is larger than the technical variations, as described by Kirst et al. (11). No difference was found between the healthy sub- and supra-gingival
samples when using beta-diversity analysis at the genus level, as previously described (20). Ninety per cent of CP samples were found in the fifth cluster. To define the fifth cluster as a “periodontitis cluster” by beta-diversity was appealing. However, the fifth cluster also contained healthy sub-gingival samples, indicating that further investigations were necessary to understand and develop prediction markers for chronic periodontitis. A core community is usually identified in publications (genera present in at least 50% of the samples) and provides a basis for disease diagnosis, prevention and therapeutic targets (21, 22). However, the genera variability expands as sample size increases, thus limiting its use to establish an easy microbiological marker for dysbiosis. In this work, genera present at a higher prevalence in at least in 95% of the samples were used to determine the genera implicated in health or in favor of the disease. The genera used to calculate the dysbiosis ratio in favor of periodontitis were Treponema, Campylobacter, Eubacterium and Tannerella. These genera were identified at high abundance and high prevalence in CP as compared to healthy samples. They include well-identified species (T. forsythia and T. denticola, C. rectus and E. nodatum) that are strongly associated with the disease (3, 23-25). It is noted that, while some species such as the newly-cultivated Tannerella clone BU063 (26, 27) that is supposed health-associated, is also found in active periodontal sites (28) and therefore still considered controversial. Despite a significant difference in abundance of the Porphyromonas genus (which includes P. gingivalis that is highly associated with periodontitis) between healthy samples (3.35%) and in CP samples (13%), it was excluded in the first dysbiosis ratio because of its similar prevalence. As the lowest abundance in genus accounted for the CP calculation was Campylobacter, 1.9%, this value was therefore chosen as a cut-off to minimize the number of genera used for the health calculation - Rothia, Corynebacterium, Actinomyces, Veillonella and Neisseria. Neither...
Capnocytophaga nor Leptotirichia were included because of their high prevalence in periodontitis samples (more than 90%, data not shown). Species belonging to the genus Rothia have been repeatedly described as members of oral communities associated with periodontal health (29-34) or at least being more predominant in health (31). In the same way, Corynebacterium appeared to be more associated with “healthy” sub-gingival biofilm (35, 36). Moreover, Rothia and Corynebacterium were among the bacteria that showed the greatest increase after periodontal treatment (37), while a study suggested that Corynebacterium might be considered as a putative periodontal protector (38). Veillonella and Actinomyces have been negatively correlated with clinical markers in CP (39) and Neisseria was found in inactive sites (28). The calculated dysbiosis ratio distinguishes clearly healthy sub-gingival samples from CP samples.

Shallow samples were divided into two groups that can be easily explained based on the origin of the sampling (healthy sub-gingival sites in mouth presenting chronic periodontitis). Two thirds of the samples had a low ratio (cluster 1 and 2) and can be considered “microbiologically healthy”. The remaining one third of the samples (cluster 5) presented a high ratio certainly due to contamination of the sampling sites by bacteria from surrounding CP sites and could be considered as “at risk of periodontitis”. Thus, shallow samples may represent an intermediate stage in disease development as proposed by Griffen et al. (12).

Healthy sub-gingival samples are divided into 3 different groups. Two of them belonging to clusters 1 and 2 present the same low ratio and describe an absence of dysbiosis. The third group had a higher dysbiosis ratio, similar to shallow sites and CP samples from cluster 1 and 2 but significantly inferior to CP or shallow samples from the fifth cluster. As “healthy patients” from HMP are defined as patients with pockets depths <4mm, some of them could have explained this high ratio group, however healthy patients from other studies (19/99)
were also included in this group. This result is similar to Zhou Y. et al. where few healthy subjects were detected with signals of disease such as an increase of Treponema (40). Therefore, patients who presented a relatively high ratio as such could be considered at “risk of periodontitis”.

Conversely, a few CP samples with deep periodontal pockets (i.e. ≥ 5mm) had a low dysbiosis ratio. A hypothesis of appropriate host response (such as a stronger immune response and/or better hygiene) could explain this discrepancy between dysbiosis ratio and diagnosis: these patients might be microbiologically “on the mend”, as revealed by both clustering and dysbiosis ratio. Another hypothesis is a sampling concern between the top and base of the periodontal pocket (to be discussed later). To study the microbiota “on the mend” hypothesis and the dysbiosis calculated by the ratio, a recent study presenting a follow-up after treatment with well-defined depth of periodontal pocket metadata was used (16). This study was conducted with a different set of primers (V5V7) and allowed to test the dysbiosis ratio at the genus level on a new set of primers that have not been used to determine the ratio. Consequently, this comparative analysis can be considered as a validation experiment of the ratios. A strong correlation was obtained between the dysbiosis ratio and the pocket depths, thus highlighting the value of calculating the dysbiosis ratio (using the selected genera of our study) as a microbial signature to evaluate the microbiota of chronic periodontitis.

A major concern at the beginning of this work was the capacity to identify species with multiple datasets. However the V1V2 and V5V7 primers used in 3 studies were not suitable for species identification. At the genus level, as reported in Bizzarro S. et al. (16), the proposed dysbiosis ratio is a good microbial signature calculated using the online VAMPS software even by using a simplified dysbiosis ratio. Indeed, as Rothia and Corynebacterium
are the major healthy genera found, and even if Porphyromonas was found in both health and disease, its abundance increased significantly in disease (from 3.34% to 13%). The result was interesting, where it was found to be similar to the precedent ratio (correlation with pocket depth \( r=0.659 \ p<0.001 \)). However, more adjustments were needed as 43 out of 196 CP samples presented none of the two healthy genera and a value of 0.1% was attributed for the calculation.

Finally, using ratios, some data points still showed discrepancies in predicting the periodontal status. The variability in microbial composition and spatial distribution could explain these results. Deep periodontal pockets in CP patients may present gradients of oxygen tension, pH and nutrients as well as host defense factors from the base of the pocket to the top (opening). This may explain why some genera are typically found at the base of the pocket (Porphyromonas, Treponema) (41, 42). However, the sampling could induce bias even after careful removal of the supra-gingival plaque. Healthy genera may be found predominantly at the top (opening) of the pocket as compared with the genera more closely associated with CP being located at the base of the pocket. Indeed, with the use of NGS analysis, while the architecture of the periodontal pocket has not been clearly studied yet, the importance of the biogeography of microbiome at the micron scale has clearly been shown recently (43).

In conclusion, this study aimed to define ratios of bacteria as microbial signature after the analysis of public raw data from different studies, independent of the technical methods used to generate the data. These ratios allow the differentiation of healthy and diseased microbiota in a majority of samples. Standardized protocols of sampling and complete metadata in the public bank are necessary to study dysbiosis in oral health and to improve
the proposed dysbiosis ratios. Adjunction of specific perio-protectors and potential specific pathogens to the calculation of the dysbiosis could also be promising. Longitudinal studies are necessary to predict exact pockets microbiologically “on the mend” or sulci with “risk of periodontitis”.
References


Author Contributions (names must be given as initials)

VM, FBH and MBM conceived and designed the research. VM realized the sampling. SLGD realized the molecular biology. VM, SLGD, EB, LAA, BM, SBF, BM, performed the experiments (bioinformatics analysis, statistics) and wrote the manuscript. MBM supervised the project.

Competing interests

The authors declare no competing financial interests.
Fig. 1: Different views of 3D PCoA plots illustrating the beta-diversity of bacterial populations as a function of sampling site and diagnosis. Light blue: supra-gingival samples; dark blue: healthy sub-gingival samples; green: out-groups as saliva, mid-vagina and dentine caries; red: chronic periodontitis - CP).
Fig. 2: Unrooted tree displaying genus Bray Curtis beta-diversity clustering microbiota and pie charts related to sample origin within each cluster. The tree was realized using Figtree software v1.4.2. Distribution of microbiota in each cluster is represented by pie charts with different colors according to sampling sites (supra-gingival: Sup in light blue, saliva: light green, dentine caries: green and mid vagina: dark green) and diagnosis for sub-gingival samples (healthy: Sub in dark blue, shallow in yellow and chronic periodontitis in red). Written percentages correspond to the number of samples from a given specific sampling site in a given cluster to the total number of samples from the same specific sampling site.
Fig. 3: Alpha diversity index. Microbiota sampling depth, observed richness (S, number of different taxa per sample) and diversity (Shannon Weaver index) comparisons in sub-gingival samples between samples of healthy clusters 1 and 2 (blue) and samples of cluster 5, either with chronic periodontitis (red) or sub-gingival healthy samples (light blue). *p<0.05, **p<0.01.
Fig. 4: Patterns of sub-gingival microbial communities. A: Patterns of genera present at least in 95% of all healthy sub-gingival samples. B: Patterns of genera present at least in 95% of all chronic periodontitis (CP) samples from the cluster 5. Edges represent one (thin line) or 2 to 3 (thick line) significant correlation between genera (green: positive; red: negative). Node colors represent the number of partners ranging from one (green) to 7 (dark orange). Node sizes represent the abundance of each taxon.
Figure 5: Sub-gingival dysbiosis ratio. Ratio = Eubacterium, Campylobacter, Treponema and Tannerella to Veillonella, Neisseria, Rothia, Corynebacterium and Actinomyces. A. Between healthy, shallow and chronic periodontitis samples (CP) from all clusters. B. Between clusters 1&2 and Cluster 5 for healthy, shallow and CP samples.
Figure 6: Correlation between pocket depth and dysbiosis. Samples from Bizzarro S. et al., 2016 (16) were analyzed by VAMPS followed by the calculation of the dysbiosis ratio. A. Ratio = Eubacterium, Campylobacter, Treponema and Tannerella to Veillonella, Neisseria, Rothia, Corynebacterium and Actinomyces. B. Simplified ratio = Porphyromonas, Treponema and Tannerella to Rothia and Corynebacterium.
Table 1: Sub-gingival microbiota used in this study

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<td>V3V5</td>
</tr>
<tr>
<td>Bizzarro et al. (16)</td>
<td>PRJNA289294</td>
<td>37*</td>
<td>110*</td>
<td>V5V7</td>
<td></td>
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</tr>
</tbody>
</table>

* CP microbiota from patients with a follow up treatment used to confirmed the dysbiosis hypothesis (16).

# Site defined as healthy in patients with periodontitis (12).

$ Data available on VAMPS for the reviewers, dataset: “Y_Hemoparo”