Periodontal pathogens and clinical parameters in chronic periodontitis

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Summary	*Background*: The use of Next Generation Sequencing and bioinformatics have revealed the complexity and richness of the human oral microbiota. While some species are well known for their periodontal pathogenicity, the molecular-based approaches for bacterial identification have raised awareness about new putative periodontal pathogens. Although they are found increased in case of periodontitis, there is currently a lack of data on their interrelationship with the periodontal measures. *Methods*: We processed the sequencing data of the subgingival microbiota of 75 patients with haemochromatosis and chronic periodontitis in order to characterize well-described and newly identified subgingival periodontal pathogens. We used correlation tests and statistical models to assess the association between the periodontal pathogens and mean pocket depth, and to determine the most relevant bacterial biomarkers of periodontitis severity. *Results*: Based on correlation test results, nine taxa were selected and included in the statistical models. The multiple linear regression models adjusted for systemic and periodontal clinical variables showed that mean pocket depth was negatively associated with Aggregatibacter and Rothia, and positively associated with Porphyromonas. Furthermore, a bacterial ratio that was previously described as a signature of dysbiosis in periodontitis, (%Porphyromonas+%Treponema+%Tannerella)/(%Rothia+%Corynebacterium), was the most significant predictor. *Conclusion*: In this specific population, we found that the best model in

	predicting the mean pocket depth was microbial dybiosis using the dysbiosis ratio taxa formula. While further studies are needed to assess the validity of these results on the general population, such a dysbiosis ratio could be used in the future to monitor the subgingival microbiota.

Periodontal pathogens and clinical parameters in chronic periodontitis

Running Title: Dysbiosis in periodontitis

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Abstract

Background: The use of Next Generation Sequencing and bioinformatics have revealed the complexity and richness of the human oral microbiota. While some species are well known for their periodontal pathogenicity, the molecular-based approaches for bacterial identification have raised awareness about new putative periodontal pathogens. Although they are found increased in case of periodontitis, there is currently a lack of data on their interrelationship with the periodontal measures.

Methods: We processed the sequencing data of the subgingival microbiota of 75 patients with haemochromatosis and chronic periodontitis in order to characterize well-described and newly identified subgingival periodontal pathogens. We used correlation tests and statistical models to assess the association between the periodontal pathogens and mean pocket depth, and to determine the most relevant bacterial biomarkers of periodontitis severity.

Results: Based on correlation test results, nine taxa were selected and included in the statistical models. The multiple linear regression models adjusted for systemic and periodontal clinical variables showed that mean pocket depth was negatively associated with *Aggregatibacter* and *Rothia*, and positively associated with *Porphyromonas*. Furthermore, a bacterial ratio that was previously described as a signature of dysbiosis in periodontitis, (%*Porphyromonas*+%*Treponema*+%*Tannerella*)/(%*Rothia*+%*Corynebacterium*), was the most significant predictor.

Conclusion: In this specific population, we found that the best model in predicting the mean pocket depth was microbial dybiosis using the dysbiosis ratio taxa formula. While further studies are needed to assess the validity of these results on the general population, such a dysbiosis ratio could be used in the future to monitor the subgingival microbiota.

Keywords

Deep sequencing; potential periodontal pathogens; chronic periodontitis; dysbiosis.

1 Introduction

Periodontitis is a worldwide health problem and the 6th most common disease globally (Frencken et al., 2017). It is a chronic inflammation produced in response to a disease-associated multispecies bacterial community with intermittent episodes of remission and relapse, characterized by alveolar bone loss. The current model of periodontitis pathogenesis implies a synergistic polymicrobial community and a dysregulated host inflammatory response (G. Hajishengallis & Lamont, 2012). Indeed, a dysbiosis of the complex and diverse oral microbiota (Paster et al., 2001) that inhabits the sulcus is concomitant with the occurrence of a periodontal pocket. This pathognomonic sign of the periodontitis can be quantified as pocket depth (PD) and clinical attachment loss (CAL) (Lamont, Koo, & Hajishengallis, 2018).

Subgingival colonization by specific species, including the red complex bacterial species, i.e., *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*, has been reported to be related to PD and bleeding as determined by DNA-DNA checkerboard analysis (Socransky, Haffajee, Cugini, Smith, & Kent, 1998). *P. gingivalis* is also considered as the keystone pathogen of the disease, that is able to disrupt the immune system allowing for dysbiosis to arise (George Hajishengallis, Darveau, & Curtis, 2012; Jiao, Hasegawa, & Inohara, 2014). The development of contemporary molecular methods (Next Generation of Sequencing – NGS) has expanded the list of potential periodontal pathogens. The review by Pérez-Chaparro *et al*. identified in the literature 29 species and one genus putatively implicated in chronic periodontitis that have been cited by at least two different authors (Pérez-Chaparro et al., 2014). However, these results are usually based on health *versus* disease comparisons. The relationship between periodontitis measures and these taxa are lacking. Using NGS data from the literature, we have previously defined a bacterial ratio

as a signature of periodontitis that could score the dysbiosis of the subgingival microbiota (Meuric, Le Gall-David, et al., 2017). However, its relationship with the global periodontal status remains to be tested.

We have worked on a cohort of patients with chronic periodontitis and hemochromatosis, that allowed us to show worsened periodontal measures in case of iron overload (Meuric, Lainé, et al., 2017). An early study (with 66 patients) of their subgingival microbiota showed a potential impact of iron through correlations between transferrin saturation and the abundance of *Porphyromonas* and *Treponema* (Boyer et al., 2018). Nevertheless, an extensive characterization of their microbiota (including dysbiosis) has not been performed yet, regarding occurrence, abundance and relationship of well-known and newly identified potential periodontal pathogens with periodontal measures.

The purpose of this study is three-fold. 1: to characterize all the well-described and newly identified periodontal pathogens (bacterial complexes described by Socransky *et al.* and reviewed by Pérez-Chaparro *et al.*) in the deepest pocket using the NGS in patients with chronic periodontitis (Pérez-Chaparro et al., 2014; Socransky et al., 1998). Two genera usually cited as implicated in health, *Corynebacterium* and *Rothia*, are also analyzed, as a part of the bacterial dysbiosis ratio. 2: to determine the association between the well-described, newly discovered periodontal pathogens and the periodontitis severity through continuous measures (mean PD, sites with PD≥4 mm). 3: to propose multiple regression linear models to refine the bacterial biomarkers of periodontitis severity adjusted for demographic and clinical data (including iron overload).

2 Materials and methods

Participants and collection of clinical data

The participants corresponded to a group of 75 patients with hemochromatosis and chronic periodontitis previously studied by Meuric *et al*. (Meuric, Lainé, et al., 2017). This case study series was approved by the local ethical committee (CPP Ouest V - 10/02-744). The informed consent for study participation has been obtained from all patients and all research was performed in accordance with relevant guidelines and regulations. These patients were recruited at the University Hospital of Rennes and benefited from a full-mouth periodontal examination, including PD, CAL, papillary bleeding index, gingival index, plaque score (Barnett, Ciancio, & Mather, 1980; Lobene, Weatherford, Ross, Lamm, & Menaker, 1986; Silness & Loe, 1964) and evaluation of periodontitis severity according to the case definitions from the Centers for Disease Control and Prevention and the American Association of Periodontology (CDC/AAP) (Eke, Page, Wei, Thornton-Evans, & Genco, 2012). For the 2018 classification, the algorithms considered the interdental CAL on two non-adjacent teeth and tooth loss (Graetz et al., 2019; Tonetti, Greenwell, & Kornman, 2018). The exclusion criteria were pregnancy, periodontal therapy within the last 12 months and treatment with either systemic antibiotics or drugs known to cause gingival hyperplasia within the last 3 months. Clinical data recorded at the time of dental examination included smoking status, alcohol consumption (non-drinkers, former drinkers, occasional drinkers and regular drinkers evaluated quantitatively in dose/day), number of dental visits per year and transferrin saturation. The assays of serum iron and transferrin were performed on an automated analyzer AU2700 Olympus (Beckman Coulter). Serum iron levels were measured by a standard colorimetric method that used TPTZ[2,4,6-Tri-(2-pyridyl)-5-triazine] as a chromogen and serum transferrin concentrations were quantified by immunoturbidimetry. Test procedures were conducted as described in the manufacturer's standard operating manual. Total iron binding capacity (TIBC) was calculated using the formula: (transferrin in g/l x 25) and transferrin saturation is calculated as follows: (iron in µmol/l / TIBC) × 100.

Sample collection

After supra-gingival plaque removal, sterile endodontic paper points (Henry Schein, France) were inserted into one of the deepest periodontal pockets for 30s. The material was transferred into a sterile tube with 100 µL of sterile distilled water and kept at 4°C overnight before DNA extraction.

DNA extraction and sequencing

DNA extraction from supernatants was performed using QIAamp DNA Mini Kit (Qiagen, France) according to the manufacturer's recommendations. DNA was then kept frozen at -80°C until amplification. The V3-V4 regions of the 16S rRNA gene were amplified with the primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') (Huse et al., 2008) and 802R (5'-TACNVGGGTATCTAATCC-3') (Claesson et al., 2009) using 25 amplification cycles with an annealing temperature of 45°C. PCR products were sequenced with the Illumina MiSeq of the Get-PlaGe platform (Toulouse, France). The sequence data were submitted to the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/Traces/ study/) under BioProject accession number PRJNA416501 and PRJNA517671.

Microbiological analysis

Taxonomy at the genus level was assigned using the "Visualization and Analysis of Microbial Population Structures" (VAMPS) analysis pipeline web tool (Huse et al., 2014). Default parameters were used for assignment to the genus level with the Ribosomal Database Project (RDP) classification (Cole et al., 2014). Through the Global Alignment for Sequence Taxonomy (GAST) process of VAMPS, the best taxonomic hit was assigned for each read. Reads identified as Archaea, Eukarya, Organelle and unknown were excluded from further analysis. Taxonomy at

the species level was assigned using the "Quantitative Insights Into Microbial Ecology" (QIIME 1.9.1) package software (Caporaso et al., 2010) with the default parameters (script: pick_open_reference_otus.py, uclust as the clustering method) and the Human Oral Microbiome Database (HOMD, version 15.1) as the reference database (Chen et al., 2010). The mean unassigned reads per sample was 6.4 % ± 6.2 %. These reads have been excluded from further species analyses. To challenge the assignment capacities used in this study, the V3V4 sequences of each taxon were extracted from the HOMD and entered in the previously described pipeline. Only three species (*Mogibacterium timidum*, *Anaeroglobus geminatus* and *Enterococcus faecalis*) could not be correctly classified by the pipeline, among all the putative periodontal pathogens described.

Statistics

Data were studied using R (version 3.3.3) (R. Core Team, 2015) with the RStudio software (RStudio Team, 2016). Statistical tests were considered significant for $p<0.05$. Results of the descriptive analysis are presented as mean ± standard error (SE) and with prevalence (%). Distribution of continuous variables was assessed using the Shapiro-Wilk test. The mean PD was analyzed according to the demographic and clinical data using the Mann-Whitney test. Correlations between taxa relative abundance and clinical parameters were calculated using Spearman correlation coefficient. Multiple linear regression models were used to study the association between periodontal parameters (i.e. mean PD and percentage of site with PD≥4mm) and bacterial taxa presenting a significant correlation ($r\geq0.20$, $p<0.05$) or the dysbiosis ratio (% *Porphyromonas* + % *Treponema* + % *Tannerella*) / (% *Rothia* + % *Corynebacterium*) described in Meuric *et al.* 2017 (Meuric, Le Gall-David, et al., 2017), adjusted for confounding factors including age, gender, smoking status, alcohol intake, plaque index, transferrin saturation,

gingival and bleeding index. The stepwise AIC algorithm was performed using the 'MASS' package for R (Venables & Ripley, 2002). Final regression coefficients, standard errors (SE) and statistics for each variable adjusted for confounding factors, with the characteristics of the models (adjusted R^2, *p*-value) are presented.

3 Results

- Clinical data

Based on the CDC/AAP case definition (Eke et al., 2012), all patients presented periodontitis; 44.3 % and 54.4 % had moderate and severe periodontitis, respectively. On average, 50. 0±1.96 % of sites per mouth had CAL ≥ 3 mm, 10.7±1.43 % of sites had CAL ≥ 5 mm, and 12.5±1.15 % of sites had PD ≥ 4 mm. The full description of the periodontal measures is available in Table S1. According to the 2018 classification, and using CAL and tooth loss as the main available criteria, 3/75 (4%) patients were classified as stage II, 59/75 (79%) as stage III and 13/75 (17%) as stage IV (Tonetti et al., 2018). A minority (9/75, 12%) was classified as localized. The average age was 50.6 ± 8 years. Males represented 56 %, and former or current smokers and drinkers were 62.6 % and 80 %, respectively. Most of the patients visited their dentist at least once a year (78.6 %). Table 1 presents the statistics exploring the potential impact of each demographic on the mean PD. The mean PD was increased in former or current drinkers, compared to patients who had never experienced alcohol abuse (p=0.028).

- Prevalence and proportion of bacteria

The average of reads per sample was 14 980 ± 740 (minimum = 5 298 reads). From the 74 genera and species related to periodontitis and described by Socransky *et al.*, Pérez-Chaparro *et al.* and

Meuric *et al.*, 47 taxa (64.4 %) have been identified in this study (Table 2). All the bacteria belonging to the orange and red complexes described by Socransky *et al.* were found. The lowest prevalence was *Fusobacterium periodonticum* (6.4 %), while *Fusobacterium nucleatum* and *T. forsythia* were found in all patients. For the 8 species which have been reported as highly implicated in periodontitis (reported by ≥ 4 studies according to the systematic review of the literature by Pérez-Chaparro *et al.*), five of them were found: i.e. *Porphyromonas endodontalis* (prevalence = 88.6 %), *Treponema lecithinolyticum* (62 %), *Treponema medium* (55.7 %), *Filifactor alocis* (40.5 %) and *Eubacterium saphenum* (11.4 %), while two were not detected (*Fretibacterium OT360* and *Selenomonas sputigena*). For the taxa cited 3 times, the following were not detected: *Bacteroidales* OT274, *Peptostreptococcus stomatis*, *Desulfobulbus* OT041 and phylum TM7. Species with lower evidence of implication with periodontitis (cited only by two studies) or belonging to the purple or green complex (*Actinomyces odontolyticus*, *Veillonella parvula* and *Eikenella corrodens* respectively) were not, or hardly detected (Table 2). *Mogibacterium timidum*, *Anaeroglobus geminatus* and *Enterococcus faecalis* were not found due to methodological limitations.

The most abundant genus was *Fusobacterium* (mean = 26.1±1.7 %) followed by *Prevotella* (12±1 %), *Porphyromonas* (7±0.8 %), *Streptococcus* (5.8±0.7 %), *Treponema* (5.6±0.6 %) and *Tannerella* (5.4±0.6 %). The most abundant species were *F. nucleatum* (15.3±1.4 %) followed by *T. forsythia* (4.5±0.5 %), *P. endodontalis* (3.1±0.4 %), and *P. gingivalis* (2.8±0.6 %).

- Microbiological correlation with periodontal data

Analysis showed that the species exhibiting significant correlations with periodontal clinical data were *P. gingivalis* (r=0.33 and 0.41, with mean PD and percentage of sites with PD≥4 mm, respectively), *T. forsythia* (r=0.25 and 0.30, respectively), and *E. nodatum* (r=0.35 and 0.34,

respectively). *Desulfobulbus*, *Treponema* and *Porphyromonas* genera were also found significantly correlated with these periodontal data. Significant inverse correlations were found for the taxa of *Campylobacter*, *Aggregatibacter* and *Rothia*. No correlation was found between the sampled pocket depth and any of the bacterial taxa. Finally, the calculated dysbiosis ratio corresponding to (%*Porphyromonas* + %*Treponema* + %*Tannerella*) / (%*Rothia* + %*Corynebacterium*) showed the highest correlation with mean PD (r=0.37, p=0.001) and with the percentage of sites with PD ≥ 4 mm (r=0.35, p=0.0017), as compared with individual taxa. Table 3 presents all the significant results of the correlation between bacterial/clinical data and mean PD, which were further used in the following statistical experiments. As shown in Table 3, the periodontal indices rating the plaque, gingival inflammation and bleeding on probing, were found significantly correlated with the mean PD. Therefore, they were also used in the statistical models as confounding variables.

- Multiple linear regression analyses

The multiple linear regression was performed with the stepwise method which selects the most significant variables affecting the mean pocket depth. After adjustment for age, sex, smoking status, alcohol status, plaque index, transferrin saturation, bleeding index and gingival index, the mean PD was found independently associated with *Aggregatibacter, Rothia* and *Porphyromonas* genera (β=-0.053, -0.063 and 0.051, respectively, p<0.05) in the first model (adjusted R^2 = 0.3798, p<0.001). The most significant data were obtained in the second model using the dysbiosis ratio and *Aggregatibacter* as independent variables with β=0.062 (p<0.001) and β=-0.053 (p<0.01) respectively and an adjusted R^2 = 0.4868 (p<0.00001) (Table 4). By using another

periodontal measure as the outcome variable – the percentage of sites with PD ≥ 4 mm –, the dysbiosis ratio ($p < 0.001$ – Table S2) remained as the most significant predictor.

4 Discussion

Owing to decades of oral microbiology studies, some species are well known for their periodontal pathogenicity. Culture-independent and molecular-based approaches to bacterial identification has highlighted new putative periodontal pathogens, that were found increased in periodontitis patients. However, few studies assess the correlation between the severity of periodontal disease and the abundance of these taxa. The identification of taxa that are significantly associated with the periodontal status is the first step in the search for biomarkers that could predict the onset of the disease.

Most of the periodontal pathogens were identified in the analyses due to the sequencing depth. However, six species were not detected. *Bacteroidales OT274*, *Peptostreptococcus stomatis*, *Desulfobulbus OT041*, *Fretibacterium sp.*, TM7 and *Selenomonas Sputigena* could not be found while they are highly cited (≥3 times) in the literature as being implicated in periodontitis. Only three (*Mogibacterium timidum*, *Anaeroglobus geminatus* and *Enterococcus faecalis*) could not be identified through the pipeline of assignation, due to methodological limitations. The lack of detection of *Fretibacterium* is surprising since it was proposed as a diagnostic bacterial biomarker for periodontitis screening in a Japanese population (Khemwong et al., 2019). Indeed, *Fretibacterium* was not detected in our Caucasian population. As for the other five remaining taxa, we have confirmed that they were not detected in this population. Thus, different population could carry different taxon associated with periodontitis. However, previous works on periodontitis microbiota analysis through NGS have used a binary approach (healthy or disease) to determine in which condition taxon are increased (Abusleme et al., 2013;

Kirst et al., 2015). In this study, we have performed correlation analyses between the abundance of species considered as periodontal pathogens (from previous studies) and continuous periodontal measures, in haemochromatosis patients with periodontitis to assess the current weight of their implication in the disease. Adjusted regression models were then used to determine the best bacterial biomarkers of periodontitis severity. Taxa already well described as implicated in the disease were found correlated with the mean PD or the percentage of sites with PD ≥ 4 mm: *P. gingivalis* and *T. forsythia* (red complex) and *E. nodatum* (orange complex) (Socransky *et al.*, 1998). From the newly identified putative periodontal pathogen, only *Desulfobulbus* genus (Pérez-Chaparro *et al*., 2014) was found correlated with the mean pocket depth. Correlation coefficients between periodontal data and bacterial taxa remained inferior to 0.4, similarly to other microbiota analyses in chronic periodontitis (Khemwong *et al.*, 2019; Lourenço *et al.*, 2014; Silva-Boghossian, Cesário, Leão, & Colombo, 2018). Finding associations is especially more difficult when looking at the species level. However, it is not surprising as this disease is a multifactorial pathology, as each taxon might be partially implicated in the disease. For instance, *T. denticola* which belongs to the red complex (Socransky *et al.,* 1998), was not correlated with the mean PD. Nevertheless, the *Treponema* genus, which contains *T. denticola* and other species highly suspected to be periodontal pathogens (i.e. *T. vincentii*, *T. lecithinolyticum* and *T. medium*, as reported in the systematic review by Pérez-Chaparro *et al.*), was found correlated with the PD measures.

Surprisingly, *Campylobacter* was negatively associated with the mean PD, while *Campylobacter* species are mainly classified as periodontal pathogens (i.e.: *C. showae*, *C. gracilis*, and *C. rectus* belonging to the orange complex and *C. concisus* to the green complex (Socransky *et al.*, 1998)). However, this repartition of species is controversial: *C. gracilis* belongs to the orange complex, though it was found increased in healthy subjects (Henne, Fuchs, Kruth,

Horz, & Conrads, 2014) and decreased in severe periodontitis (Macuch & Tanner, 2000). Finally, the *Campylobacter* genus was still negatively associated with percentage of PD ≥ 4mm in the regression model.

This analytical approach is also interesting when focused on the prevalence or occurrence of periodontal pathogens in patients. Taxa are not found in all patients. Even the prevalence of *P. gingivalis,* which is well described as one of the keystone pathogens in the disease (George Hajishengallis *et al.*, 2012), was only found at 62% in this study. Moreover, *P. gingivalis* has been detected in 25% of healthy Caucasian (Griffen, Becker, Lyons, Moeschberger, & Leys, 1998), up to 40% in healthy Japanese (Kato, Imai, Ochiai, & Ogata, 2013) and not higher than 79% in chronic periodontitis (Griffen et al., 1998). The chronic nature and clinical course of the disease, with alternating episodes of exacerbation and remission (Goodson, Tanner, Haffajee, Sornberger, & Socransky, 1982), could explain that the bacteria are more difficult to detect. The single sampling could also be a potential modifier of the microbiota and a limitation, as different periodontal pockets in the same patient could show various microbiota composition. Long-term follow-up with regular and multi-site sampling is certainly needed, which could address these issues. As such studies are difficult to implement, a ratio of taxa was developed (Meuric, Le Gall-David, et al., 2017) to explore the periodontal dysbiosis responsible for chronic periodontitis (Kilian et al., 2016). This dysbiosis ratio showed the highest and the most significant correlation with mean PD in this study (p=0.001) and could be calculated for each patient, whereas *P. gingivalis* could only be detected in 62% of the patients. Thereafter, in the first multiple regression model, each taxon found significantly correlated with mean PD was entered, while *Porphyromonas, Treponema*, *P. gingivalis*, *T. forsythia* and *Rothia* were replaced in the second model by the dysbiosis ratio which is computed with their relative abundance. The models were

refined with the stepwise method (Venables & Ripley, 2002) adjusted with the clinical and demographic data as for example, alcohol intake, which is considered as a risk factor for periodontitis (Wang, Lv, Wang, & Jiang, 2016) and a worsened mean PD in our population. Since the patients come from a sub-cohort of a previous study where high transferrin saturation (specifically for the hemochromatosis disease) was implicated in explaining clinical severity of periodontitis, we have added the transferrin saturation into the multiple linear regression models as a confounding factor. As described previously, periodontitis through mean PD was associated with transferrin saturation in the two models (Meuric, Lainé, et al., 2017). For each dependent variable (mean PD or percentage of sites with PD ≥ 4 mm), the most significant result, appeared in the second model with the dysbiosis ratio (p<0.001) which is compiled with majority of the periopathogens (i.e.: *Porphyromonas, Treponema* and *Tannerella* genera) and the genera usually found in health (Meuric, Le Gall-David, et al., 2017). This result is independent from the transferrin saturation (reflecting the iron status of the hemochromatosis disease). Thus, the dysbiosis ratio confirmed its utility in exploring the microbial dysbiosis to explain periodontal disease (Meuric, Le Gall-David, et al., 2017). Similar microbial dysbiosis ratios have also been calculated in Crohn's disease (Gevers et al., 2014) and in peri-implantitis (Kröger et al., 2018).

These results are based on a specific population suffering from haemochromatosis. Extrapolations outside of this population need to be validated, though the iron status of the patients has been included as a confounding variable in statistical models. Nonetheless, these results are in accordance with the existing view that periodontitis or peri-implantitis are orchestrated by a bacterial consortium rather than a single pathogen (Darveau, 2010).

5 Conclusion

In this study, we have confirmed that most of the well-described and newly identified periodontal pathogens can be found in the deepest periodontal pocket of patients suffering from haemochromatosis and periodontitis. Yet, only few taxa were correlated to mean pocket depth. The relative abundance of these taxa was therefore used as a predictor, with confounding variables, in multiple linear regression models that allowed for the prediction of mean pocket depth. While *Porphyromonas*, *Aggregatibacter* and *Rothia* returned significant results, the previously described dysbiosis ratio – (% *Porphyromonas* + % *Treponema* + % *Tannerella*) / (% *Rothia* + % *Corynebacterium*) – was the most reliable biomarker to predict periodontitis severity in our patients. Longitudinal studies in patients with no systemic disease, as well as several sampling sites and time points during the episodes of disease-active periodontitis should be considered to further validate and generalize the findings of this study.

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Data availability: The sequence data were deposited to the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/Traces/study/) under BioProject accession numbers PRJNA416501 and PRJNA517671. The detailed periodontal and demographic data for every patient can be accessed in Table S3.

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Demographics	Mean PD (mm)	*p*-value
Gender		
Male (*n* = 42)	2.32 ± 0.06	
Female (*n* = 33)	2.29 ± 0.06	0.863
Smoking status		
Never (*n* = 28)	2.29 ± 0.04	
Former (*n* = 30) and Current (*n* = 17)	2.31 ± 0.06	0.81
Alcohol status		
Never (*n* = 15)	2.12 ± 0.07	
Former (*n* = 2) and Current (*n* = 58)	2.35 ± 0.05	**0.028**
Frequency of dentist visits		
Once or twice a year (*n* = 59)	2.32 ± 0.06	
Less than once a year (*n* = 16)	2.27 ± 0.09	0.535

Table 1: Mean probing depth (PD) according to demographic data. Data are presented as mean ± standard error (SE). Bold *p*-values indicate significant Mann-Whitney test.

Phylum	Genera	Species	HOT	Author	Year	Identidied in	Mean % genera	Mean % species	+/-SE	Occurence (%)
Actinobacteria	*Corynebacteria*			Meuric	2017	Health	0.143		0.028	83.5
Actinobacteria	*Rothia*			Meuric	2017	Health	0.215		0.050	73.4
Firmicutes	*Streptococcus*			Socransky	1998	yellow complex	5.782		0.728	100
Firmicutes	*Veillonella*	*parvula*	*161*	Socransky	1998	purple complex	ND			
Actinobacteria	*Actinomyces*						0.237		0.054	77.2
Actinobacteria	*Actinomyces*	*odontolyticus*	*701*	Socransky	1998	purple complex	ND			
Proteobacteria	*Eikenella*	*corrodens*	*577*	Socransky	1998	green complex	ND			
Bacteroidetes	*Capnocytophaga*						1.667		0.262	98.7
Bacteroidetes	*Capnocytophaga*	*gingivalis*	*337*	Socransky	1998	green complex		0.002	0.001	11.4
Bacteroidetes	*Capnocytophaga*	*sputigena*	*775*	Socransky	1998	green complex		0.509	0.087	83.5
Bacteroidetes	*Capnocytophaga*	*ochracea*	*700*	Socransky	1998	green complex		0.002	0.001	19.0
Bacteroidetes	*Campylobacter*						4.279		0.365	100.0
Bacteroidetes	*Campylobacter*	*concisus*	*575*	Socransky	1998	green complex		0.190	0.041	77.2
Bacteroidetes	*Porphyromonas*						7.054		0.833	98.7
Bacteroidetes	*Porphyromonas*	*gingivalis*	*619*	Socransky	1998	*red complex*		2.769	0.626	62.0
Spirochaetes	*Treponema*						5.591		0.584	100
Spirochaetes	*Treponema*	*denticola*	*584*	Socransky	1998	*red complex*		1.061	0.170	87.3
Bacteroidetes	*Tannerella*						5.368		0.584	100
Bacteroidetes	*Tannerella*	*forsythia*	*613*	Socransky	1998	*red complex*		4.522	0.531	100
Firmicutes	*Eubacterium*						1.415		0.200	97.6
Firmicutes	*Eubacterium*	*nodatum*	*694*	Socransky	1998	*orange complex*		0.177	0.043	62.0
Fusobacteria	*Fusobacterium*						26.140		1.733	100
Fusobacteria	*Fusobacterium*	*nucleatum*	*200. 202. 420. 698*	Socransky	1998	orange complex		15.292	1.458	100
Fusobacteria	*Fusobacterium*	*periodonticum*	*201*	Socransky	1998	orange complex		0.0003	0.0002	6.3
Firmicutes	*Parvimonas*						1.019		0.186	92.4
Firmicutes	*Parvimonas*	*micra*	*111*	Socransky	1998	orange complex		1.015	0.176	91.1
Bacteroidetes	*Prevotella*						12.006		1.043	100
Bacteroidetes	*Prevotella*	*intermedia*	*643*	Socransky	1998	orange complex		0.774	0.228	58.2
Bacteroidetes	*Prevotella*	*nigrescens*	*693*	Socransky	1998	orange complex		1.558	0.290	82.3
Firmicutes	*Streptococcus*	*constellatus*	*576*	Socransky	1998	orange complex		0.789	0.177	82.3
Proteobacteria	*Campylobacter*	*gracilis*	*623*	Socransky	1998	orange complex		1.363	0.202	92.4
Proteobacteria	*Campylobacter*	*rectus*	*748*	Socransky	1998	orange complex		1.740	0.240	94.9
Proteobacteria	*Campylobacter*	*showae*	*763*	Socransky	1998	orange complex		0.012	0.002	53.2
Proteobacteria	*Aggregatibacter*						0.851		0.167	86.1
Proteobacteria	*Aggregatibacter*	*actinomycetemcomitans*	*531*	Socransky	1998			0.027	0.012	17.7
Bacteroidetes	*Prevotella*	*denticola*	*291*	Perez-Chapparo	2014	2 studies		0.868	0.226	75.9
Bacteroidetes	*Alloprevotella*	*tannerae*	*466*	Perez-Chapparo	2014	2 studies	ND			

Firmicutes	*Selenomonas*	*genus*	*124. 125. 126. 130. 133. 134. 136. 137. 138. 139. 146. 149. 388. 442. 478. 479. 481. 501. 639. 892. 919. 920. 936. 937*	Perez-Chapparo	2014	2 studies	ND			
Firmicutes	*Johnsonella*	*oral taxon 166*	*166*	Perez-Chapparo	2014	2 studies	ND			
Firmicutes	*Eubacterium [X1] [G-3]*	*brachy*	*557*	Perez-Chapparo	2014	2 studies		0.005	0.001	30.4
Firmicutes	*Peptostreptococcaceae [XIII] [G-1]*	*oral taxon 113*	*113*	Perez-Chapparo	2014	2 studies	ND			
Firmicutes	*Lachnospiraceae [G-8]*	*oral taxon 500*	*500*	Perez-Chapparo	2014	2 studies	ND			
Firmicutes	*Dialister*						1.518		0.197	94.9
Firmicutes	*Dialister*	*pneumosintes*	*736*	Perez-Chapparo	2014	2 studies	ND			
Proteobacteria	*Acinetobacter*						0.081		0.030	45.6
Proteobacteria	*Acinetobacter*	*baumannii*	*554*	Perez-Chapparo	2014	2 studies	ND			
Proteobacteria	*Escherichia*						0.065		0.016	55.7
Proteobacteria	*Escherichia*	*coli*	*574*	Perez-Chapparo	2014	2 studies	ND			
Spirochaetes	*Treponema*	*phylogroup II*	*584. 743*	Perez-Chapparo	2014	2 studies		1.106	0.194	86.7
Synergistetes	*Fretibacterium*	*oral taxon 359*	*359*	Perez-Chapparo	2014	2 studies	ND			
TM7	*TM7 [G-1]*	*oral taxon 346. 349*	*346. 349*	Perez-Chapparo	2014	2 studies	ND			
SR1	*SR1 [G-1]*	*oral taxon 345*	*345*	Perez-Chapparo	2014	2 studies	ND			
Bacteroidetes	*Bacteroidales [G-2]*	*oral taxon 274*	*274*	Perez-Chapparo	2014	3 studies	ND			
Firmicutes	*Mogibacterium*						0.293		0.037	93.7
Firmicutes	*Mogibacterium*	*timidum*	*0 42*	Perez-Chapparo	2014	3 studies	ND			
Firmicutes	*Peptostreptococcus*						0.192		0.040	77.2
Firmicutes	*Peptostreptococcus*	*stomatis*	*112*	Perez-Chapparo	2014	3 studies	ND			
Firmicutes	*Anaeroglobus*	*geminatus*	*121*	Perez-Chapparo	2014	3 studies	ND			
Proteobacteria	*Desulfobulbus*						1.515		0.285	81.0
Proteobacteria	*Desulfobulbus*	*sp. oral taxon 041*	*0 41*	Perez-Chapparo	2014	3 studies	ND			
Spirochaetes	*Treponema*	*vincentii*	*0 29*	Perez-Chapparo	2014	3 studies		0.034	0.009	41.8
Synergistetes	*Fretibacterium*	*oral taxon 362*	*362*	Perez-Chapparo	2014	3 studies	ND			
Synergistetes	*Fretibacterium*	*fastidiuosum*	*363*	Perez-Chapparo	2014	3 studies	ND			
TM7	*TM7 [G-5]*	*oral taxon 356*	*356*	Perez-Chapparo	2014	3 studies	ND			
Bacteroidetes	*Porphyromonas*	*endodontalis*	*273*	Perez-Chapparo	2014	4 studies		3.105	0.421	88.6
Firmicutes	*Enterococcus*	*faecalis*	*604*	Perez-Chapparo	2014	4 studies	ND			
Spirochaetes	*Treponema*	*lecithinolyticum*	*653*	Perez-Chapparo	2014	4 studies		0.696	0.158	62
Synergistetes	*Fretibacterium*	*oral taxon 360*	*360*	Perez-Chapparo	2014	4 studies	ND			
Firmicutes	*Eubacterium [XI] [G-5]*	*saphenum*	*759*	Perez-Chapparo	2014	5 studies		0.002	0.001	11.4
Firmicutes	*Filifactor*						2.202		0.305	97.5
Firmicutes	*Filifactor*	*alocis*	*539*	Perez-Chapparo	2014	5 studies		0.041	0.011	40.5
Firmicutes	*Selenomonas*	*sputigena*	*151*	Perez-Chapparo	2014	5 studies	ND			
Spirochaetes	*Treponema*	*medium*	*667*	Perez-Chapparo	2014	5 studies		0.044	0.010	55.7
Dysbiosis Ratio	(% *Porphyromonas* + % *Treponema* + % *Tannerella*) / (% *Corynebacterium* + % *Rothia*)			Meuric	2017	**Mean %** 61 816		**+/-SE** +/- 19 257		**Occurence** 100

Table 2: Taxonomy and dysbiosis ratio (PTT/RC) implicated in chronic periodontitis, with the mean, standard error (SE) and occurrence of the bacterial taxa and of the dysbiosis ratio in the present study. ND: not detected

Correlation with mean PD	Rho	*p*-value	Mean ± SE	Occurrence (%)
Bacterial data				
Dysbiosis ratio	0.37	0.001	3 573 ± 202	100
E. nodatum	0.35	0.01	0.18 ± 0.04	62
P. gingivalis	0.33	0.01	2.77 ± 0.63	62
Desulfobulbus	0.28	0.05	1.52 ± 0.29	81
Treponema	0.26	0.05	5.59 ± 0.58	100
T. forsythia	0.25	0.05	4.52 ± 0.53	100
Porphyromonas	0.23	0.05	7.05 ± 0.83	98.7
Aggregatibacter	-0.31	0.01	0.85 ± 0.17	86.1
Campylobacter	-0.27	0.05	4.28 ± 0.36	100
Rothia	-0.26	0.05	0.22 ± 0.05	73.4
Clinical data				
Plaque index	0.25	0.03	0.19 ± 0.03	
Gingival index	0.35	0.002	0.41 ± 0.03	
Papillary bleeding index	0.36	0.002	0.4 ± 0.03	

Table 3: Correlation between mean probing depth (PD) and bacterial or clinical data. Only significant correlations are presented.
Dysbiosis ratio: (%*Porphyromonas*+%*Treponema*+%*Tannerella*)/(%*Rothia*+%*Corynebacterium*); SE: standard error. Plaque index from Silness & Loe. 1964, Gingival index from Lobene *et al.* 1986 and Papillary bleeding index from Barnett *et al.* 1980.

Coefficients:	Estimate	Std. Error	t value	*p-value*
Model 1				
(Intercept)	1.484	0.221	6.730	3.54e-08
Transferrin saturation (binary)	0.198	0.076	2.612	0.05
Plaque index	-0.049	0.195	-0.252	NS
Papillary bleeding index	0.301	0.161	1.872	NS
log(*Aggregatibacter*)	-0.053	0.021	-2.492	0.05
log(*Rothia*)	-0.063	0.025	-2.482	0.05
log(*Porphyromnas*)	0.051	0.025	2.035	0.05
Model 2				
(Intercept)	1,280	0.147	8.686	4.39e-12
Log(Dysbiosis ratio)	0.062	0,016	3.791	0.001
Log(*Aggregatibacter*)	-0.053	0.018	-2.895	0.01
Papillary bleeding index	0.349	0.128	2.732	0.01
Transferrin saturation (binary)	0.159	0.069	2.309	0.05
Male	0.171	0.069	2.457	0.05
Alcohol	0.121	0.088	1.373	NS

Table 4: Multiple linear adjusted regression models with bacteria positively associated before or dysbiosis ratio and *Aggregatibacter* as predictors of **mean periodontal pocket depth**, adjusted for age, sex, smoking status, alcohol intake, transferrin saturation, plaque index, gingival index and bleeding index.
Characteristics of the model 1: multiple $R^2 = 0.4573$, adjusted $R^2 = 0.3798$, F-statistic = 5.899, *P*-value = 0.0001569. (42 degrees of freedom)
Characteristics of the model 2: multiple $R^2 = 0.5349$, adjusted $R^2 = 0.4868$, F-statistic = 11.12, *P*-value = 3.219e-08. (58 degrees of freedom)