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Periodontal pathogens and clinical parameters in chronic periodontitis

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Keywords:	Genetics < Oral Microbiology, Periodontal Disease, Ecology < Oral Microbiology, Dysbiosis
Summary	<p>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.</p> <p>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.</p> <p>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 <i>Aggregatibacter</i> and <i>Rothia</i>, and positively associated with <i>Porphyromonas</i>. 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.</p> <p>Conclusion: In this specific population, we found that the best model in</p>

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	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.

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3 1 **Periodontal pathogens and clinical parameters in chronic periodontitis**
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5 2 **Running Title:** Dysbiosis in periodontitis
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14 **Abstract**

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

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

26 Results: Based on correlation test results, nine taxa were selected and included in the statistical
27 models. The multiple linear regression models adjusted for systemic and periodontal clinical
28 variables showed that mean pocket depth was negatively associated with *Aggregatibacter* and
29 *Rothia*, and positively associated with *Porphyromonas*. Furthermore, a bacterial ratio that was
30 previously described as a signature of dysbiosis in periodontitis,
31 $(\%Porphyromonas + \%Treponema + \%Tannerella) / (\%Rothia + \%Corynebacterium)$, was the most
32 significant predictor.

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

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3 37 **Keywords**
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5 38 Deep sequencing; potential periodontal pathogens; chronic periodontitis; dysbiosis.
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For Review Only

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

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

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

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

83

84 2 Materials and methods

85 Participants and collection of clinical data

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3 86 The participants corresponded to a group of 75 patients with hemochromatosis and chronic
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5 87 periodontitis previously studied by Meuric *et al.* (Meuric, Lainé, et al., 2017). This case study
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7 88 series was approved by the local ethical committee (CPP Ouest V - 10/02-744). The informed
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10 89 consent for study participation has been obtained from all patients and all research was performed
11
12 90 in accordance with relevant guidelines and regulations. These patients were recruited at the
13
14 91 University Hospital of Rennes and benefited from a full-mouth periodontal examination,
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16 92 including PD, CAL, papillary bleeding index, gingival index, plaque score (Barnett, Ciancio, &
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18 93 Mather, 1980; Lobene, Weatherford, Ross, Lamm, & Menaker, 1986; Silness & Loe, 1964) and
19
20 94 evaluation of periodontitis severity according to the case definitions from the Centers for Disease
21
22 95 Control and Prevention and the American Association of Periodontology (CDC/AAP) (Eke,
23
24 96 Page, Wei, Thornton-Evans, & Genco, 2012). For the 2018 classification, the algorithms
25
26 97 considered the interdental CAL on two non-adjacent teeth and tooth loss (Graetz et al., 2019;
27
28 98 Tonetti, Greenwell, & Kornman, 2018). The exclusion criteria were pregnancy, periodontal
29
30 99 therapy within the last 12 months and treatment with either systemic antibiotics or drugs known
31
32 100 to cause gingival hyperplasia within the last 3 months. Clinical data recorded at the time of dental
33
34 101 examination included smoking status, alcohol consumption (non-drinkers, former drinkers,
35
36 102 occasional drinkers and regular drinkers evaluated quantitatively in dose/day), number of dental
37
38 103 visits per year and transferrin saturation. The assays of serum iron and transferrin were performed
39
40 104 on an automated analyzer AU2700 Olympus (Beckman Coulter). Serum iron levels were
41
42 105 measured by a standard colorimetric method that used TPTZ[2,4,6-Tri-(2-pyridyl)-5-triazine] as a
43
44 106 chromogen and serum transferrin concentrations were quantified by immunoturbidimetry. Test
45
46 107 procedures were conducted as described in the manufacturer's standard operating manual. Total
47
48 108 iron binding capacity (TIBC) was calculated using the formula: (transferrin in g/l x 25) and
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50 109 transferrin saturation is calculated as follows: (iron in $\mu\text{mol/l}$ / TIBC) \times 100.
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110 Sample collection

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

115 DNA extraction and sequencing

116 DNA extraction from supernatants was performed using QIAamp DNA Mini Kit (Qiagen,
117 France) according to the manufacturer's recommendations. DNA was then kept frozen at -80°C
118 until amplification. The V3-V4 regions of the 16S rRNA gene were amplified with the primers
119 338F (5'-ACTCCTACGGGAGGCAGCAG-3') (Huse et al., 2008) and 802R (5'-
120 TACNVGGGTATCTAATCC-3') (Claesson et al., 2009) using 25 amplification cycles with an
121 annealing temperature of 45°C. PCR products were sequenced with the Illumina MiSeq of the
122 Get-PlaGe platform (Toulouse, France). The sequence data were submitted to the NCBI
123 Sequence Read Archive ([https://www.ncbi.nlm.nih.gov/Traces/](https://www.ncbi.nlm.nih.gov/Traces/study/) study/) under BioProject
124 accession number PRJNA416501 and PRJNA517671.

125 Microbiological analysis

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

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3 132 the species level was assigned using the "Quantitative Insights Into Microbial Ecology" (QIIME
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5 133 1.9.1) package software (Caporaso et al., 2010) with the default parameters (script:
6
7 134 pick_open_reference_otus.py, uclust as the clustering method) and the Human Oral Microbiome
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10 135 Database (HOMD, version 15.1) as the reference database (Chen et al., 2010). The mean
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12 136 unassigned reads per sample was $6.4 \% \pm 6.2 \%$. These reads have been excluded from further
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15 137 species analyses. To challenge the assignment capacities used in this study, the V3V4 sequences
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17 138 of each taxon were extracted from the HOMD and entered in the previously described pipeline.
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19 139 Only three species (*Mogibacterium timidum*, *Anaeroglobus geminatus* and *Enterococcus*
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21 140 *faecalis*) could not be correctly classified by the pipeline, among all the putative periodontal
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24 141 pathogens described.

27 142 Statistics

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30 143 Data were studied using R (version 3.3.3) (R. Core Team, 2015) with the RStudio software
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32 144 (RStudio Team, 2016). Statistical tests were considered significant for $p < 0.05$. Results of the
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35 145 descriptive analysis are presented as mean \pm standard error (SE) and with prevalence (%).
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37 146 Distribution of continuous variables was assessed using the Shapiro-Wilk test. The mean PD was
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40 147 analyzed according to the demographic and clinical data using the Mann-Whitney test.
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42 148 Correlations between taxa relative abundance and clinical parameters were calculated using
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44 149 Spearman correlation coefficient. Multiple linear regression models were used to study the
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46 150 association between periodontal parameters (i.e. mean PD and percentage of site with $PD \geq 4$ mm)
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48 151 and bacterial taxa presenting a significant correlation ($r \geq 0.20$, $p < 0.05$) or the dysbiosis ratio
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50 152 ($\% \textit{Porphyromonas} + \% \textit{Treponema} + \% \textit{Tannerella} / (\% \textit{Rothia} + \% \textit{Corynebacterium})$)
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53 153 described in Meuric *et al.* 2017 (Meuric, Le Gall-David, et al., 2017), adjusted for confounding
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56 154 factors including age, gender, smoking status, alcohol intake, plaque index, transferrin saturation,
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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.

159

160 **3 Results**

161 - Clinical data

162 Based on the CDC/AAP case definition (Eke et al., 2012), all patients presented periodontitis; 163 44.3 % and 54.4 % had moderate and severe periodontitis, respectively. On average, 50. 164 0 ± 1.96 % of sites per mouth had $CAL \geq 3$ mm, 10.7 ± 1.43 % of sites had $CAL \geq 5$ mm, and 165 12.5 ± 1.15 % of sites had $PD \geq 4$ mm. The full description of the periodontal measures is 166 available in Table S1. According to the 2018 classification, and using CAL and tooth loss as the 167 main available criteria, 3/75 (4%) patients were classified as stage II, 59/75 (79%) as stage III 168 and 13/75 (17%) as stage IV (Tonetti et al., 2018). A minority (9/75, 12%) was classified as 169 localized. The average age was 50.6 ± 8 years. Males represented 56 %, and former or current 170 smokers and drinkers were 62.6 % and 80 %, respectively. Most of the patients visited their 171 dentist at least once a year (78.6 %). Table 1 presents the statistics exploring the potential impact 172 of each demographic on the mean PD. The mean PD was increased in former or current drinkers, 173 compared to patients who had never experienced alcohol abuse ($p=0.028$).

174

175 - Prevalence and proportion of bacteria

176 The average of reads per sample was $14\,980 \pm 740$ (minimum = 5 298 reads). From the 74 genera 177 and species related to periodontitis and described by Socransky *et al.*, Pérez-Chaparro *et al.* and

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3 178 Meuric *et al.*, 47 taxa (64.4 %) have been identified in this study (Table 2). All the bacteria
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5 179 belonging to the orange and red complexes described by Socransky *et al.* were found. The lowest
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7 180 prevalence was *Fusobacterium periodonticum* (6.4 %), while *Fusobacterium nucleatum* and *T.*
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9 *forsythia* were found in all patients. For the 8 species which have been reported as highly
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11 181 implicated in periodontitis (reported by ≥ 4 studies according to the systematic review of the
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13 182 literature by Pérez-Chaparro *et al.*), five of them were found: i.e. *Porphyromonas endodontalis*
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15 183 (prevalence = 88.6 %), *Treponema lecithinolyticum* (62 %), *Treponema medium* (55.7 %),
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17 184 *Filifactor alocis* (40.5 %) and *Eubacterium saphenum* (11.4 %), while two were not detected
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19 185 (*Fretibacterium OT360* and *Selenomonas sputigena*). For the taxa cited 3 times, the following
20
21 186 were not detected: *Bacteroidales OT274*, *Peptostreptococcus stomatis*, *Desulfobulbus OT041*
22
23 187 and phylum TM7. Species with lower evidence of implication with periodontitis (cited only by
24
25 188 two studies) or belonging to the purple or green complex (*Actinomyces odontolyticus*, *Veillonella*
26
27 189 *parvula* and *Eikenella corrodens* respectively) were not, or hardly detected (Table 2).
28
29 190 *Mogibacterium timidum*, *Anaeroglobus geminatus* and *Enterococcus faecalis* were not found due
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31 191 to methodological limitations.
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35 193 The most abundant genus was *Fusobacterium* (mean = 26.1 ± 1.7 %) followed by *Prevotella*
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37 194 (12 ± 1 %), *Porphyromonas* (7 ± 0.8 %), *Streptococcus* (5.8 ± 0.7 %), *Treponema* (5.6 ± 0.6 %) and
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39 195 *Tannerella* (5.4 ± 0.6 %). The most abundant species were *F. nucleatum* (15.3 ± 1.4 %) followed by
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41 196 *T. forsythia* (4.5 ± 0.5 %), *P. endodontalis* (3.1 ± 0.4 %), and *P. gingivalis* (2.8 ± 0.6 %).
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49 198 - Microbiological correlation with periodontal data

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51 199 Analysis showed that the species exhibiting significant correlations with periodontal clinical data
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53 200 were *P. gingivalis* ($r=0.33$ and 0.41 , with mean PD and percentage of sites with $PD \geq 4$ mm,
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55 201 respectively), *T. forsythia* ($r=0.25$ and 0.30 , respectively), and *E. nodatum* ($r=0.35$ and 0.34 ,
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3 202 respectively). *Desulfobulbus*, *Treponema* and *Porphyromonas* genera were also found
4
5 203 significantly correlated with these periodontal data. Significant inverse correlations were found
6
7 204 for the taxa of *Campylobacter*, *Aggregatibacter* and *Rothia*. No correlation was found between
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9 205 the sampled pocket depth and any of the bacterial taxa. Finally, the calculated dysbiosis ratio
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11 206 corresponding to $(\%Porphyromonas + \%Treponema + \%Tannerella) / (\%Rothia +$
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13 207 $\%Corynebacterium)$ showed the highest correlation with mean PD ($r=0.37, p=0.001$) and with
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15 208 the percentage of sites with $PD \geq 4$ mm ($r=0.35, p=0.0017$), as compared with individual taxa.
16
17 209 Table 3 presents all the significant results of the correlation between bacterial/clinical data and
18
19 210 mean PD, which were further used in the following statistical experiments. As shown in Table 3,
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21 211 the periodontal indices rating the plaque, gingival inflammation and bleeding on probing, were
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23 212 found significantly correlated with the mean PD. Therefore, they were also used in the statistical
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25 213 models as confounding variables.
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33 215 - Multiple linear regression analyses

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35 216 The multiple linear regression was performed with the stepwise method which selects the most
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37 217 significant variables affecting the mean pocket depth. After adjustment for age, sex, smoking
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39 218 status, alcohol status, plaque index, transferrin saturation, bleeding index and gingival index, the
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41 219 mean PD was found independently associated with *Aggregatibacter*, *Rothia* and *Porphyromonas*
42
43 220 genera ($\beta=-0.053, -0.063$ and 0.051 , respectively, $p<0.05$) in the first model (adjusted $R^2 =$
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45 221 $0.3798, p<0.001$). The most significant data were obtained in the second model using the
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47 222 dysbiosis ratio and *Aggregatibacter* as independent variables with $\beta=0.062 (p<0.001)$ and $\beta=-$
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49 223 $0.053 (p<0.01)$ respectively and an adjusted $R^2 = 0.4868 (p<0.00001)$ (Table 4). By using another
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224 periodontal measure as the outcome variable – the percentage of sites with $PD \geq 4$ mm –, the
225 dysbiosis ratio ($p < 0.001$ – Table S2) remained as the most significant predictor.

226

227 **4 Discussion**

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

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

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3 248 Kirst et al., 2015). In this study, we have performed correlation analyses between the abundance
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5 249 of species considered as periodontal pathogens (from previous studies) and continuous
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7 250 periodontal measures, in haemochromatosis patients with periodontitis to assess the current
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9 251 weight of their implication in the disease. Adjusted regression models were then used to
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11 252 determine the best bacterial biomarkers of periodontitis severity. Taxa already well described as
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13 253 implicated in the disease were found correlated with the mean PD or the percentage of sites with
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15 254 PD \geq 4 mm: *P. gingivalis* and *T. forsythia* (red complex) and *E. nodatum* (orange complex)
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17 255 (Socransky *et al.*, 1998). From the newly identified putative periodontal pathogen, only
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19 256 *Desulfobulbus* genus (Pérez-Chaparro *et al.*, 2014) was found correlated with the mean pocket
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21 257 depth. Correlation coefficients between periodontal data and bacterial taxa remained inferior to
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23 258 0.4, similarly to other microbiota analyses in chronic periodontitis (Khemwong *et al.*, 2019;
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25 259 Lourenço *et al.*, 2014; Silva-Boghossian, Cesário, Leão, & Colombo, 2018). Finding associations
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27 260 is especially more difficult when looking at the species level. However, it is not surprising as this
28
29 261 disease is a multifactorial pathology, as each taxon might be partially implicated in the disease.
30
31 262 For instance, *T. denticola* which belongs to the red complex (Socransky *et al.*, 1998), was not
32
33 263 correlated with the mean PD. Nevertheless, the *Treponema* genus, which contains *T. denticola*
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35 264 and other species highly suspected to be periodontal pathogens (i.e. *T. vincentii*,
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37 265 *T. lecithinolyticum* and *T. medium*, as reported in the systematic review by Pérez-Chaparro *et al.*),
38
39 266 was found correlated with the PD measures.

40
41 267 Surprisingly, *Campylobacter* was negatively associated with the mean PD, while
42
43 268 *Campylobacter* species are mainly classified as periodontal pathogens (i.e.: *C. showae*,
44
45 269 *C. gracilis*, and *C. rectus* belonging to the orange complex and *C. concisus* to the green complex
46
47 270 (Socransky *et al.*, 1998)). However, this repartition of species is controversial: *C. gracilis* belongs
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49 271 to the orange complex, though it was found increased in healthy subjects (Henne, Fuchs, Kruth,
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3 272 Horz, & Conrads, 2014) and decreased in severe periodontitis (Macuch & Tanner, 2000). Finally,
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5 273 the *Campylobacter* genus was still negatively associated with percentage of PD \geq 4mm in the
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8 274 regression model.
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11 275 This analytical approach is also interesting when focused on the prevalence or occurrence
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13 276 of periodontal pathogens in patients. Taxa are not found in all patients. Even the prevalence of
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15 277 *P. gingivalis*, which is well described as one of the keystone pathogens in the disease (George
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17 278 Hajishengallis *et al.*, 2012), was only found at 62% in this study. Moreover, *P. gingivalis* has
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19 279 been detected in 25% of healthy Caucasian (Griffen, Becker, Lyons, Moeschberger, & Leys,
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21 280 1998), up to 40% in healthy Japanese (Kato, Imai, Ochiai, & Ogata, 2013) and not higher than
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23 281 79% in chronic periodontitis (Griffen *et al.*, 1998). The chronic nature and clinical course of the
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25 282 disease, with alternating episodes of exacerbation and remission (Goodson, Tanner, Haffajee,
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27 283 Sornberger, & Socransky, 1982), could explain that the bacteria are more difficult to detect. The
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29 284 single sampling could also be a potential modifier of the microbiota and a limitation, as different
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31 285 periodontal pockets in the same patient could show various microbiota composition. Long-term
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33 286 follow-up with regular and multi-site sampling is certainly needed, which could address these
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35 287 issues. As such studies are difficult to implement, a ratio of taxa was developed (Meuric, Le Gall-
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37 288 David, *et al.*, 2017) to explore the periodontal dysbiosis responsible for chronic periodontitis
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39 289 (Kilian *et al.*, 2016). This dysbiosis ratio showed the highest and the most significant correlation
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41 290 with mean PD in this study ($p=0.001$) and could be calculated for each patient, whereas
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43 291 *P. gingivalis* could only be detected in 62% of the patients. Thereafter, in the first multiple
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45 292 regression model, each taxon found significantly correlated with mean PD was entered, while
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47 293 *Porphyromonas*, *Treponema*, *P. gingivalis*, *T. forsythia* and *Rothia* were replaced in the second
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49 294 model by the dysbiosis ratio which is computed with their relative abundance. The models were
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3 295 refined with the stepwise method (Venables & Ripley, 2002) adjusted with the clinical and
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5 296 demographic data as for example, alcohol intake, which is considered as a risk factor for
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7 297 periodontitis (Wang, Lv, Wang, & Jiang, 2016) and a worsened mean PD in our population.
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10 298 Since the patients come from a sub-cohort of a previous study where high transferrin saturation
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12 299 (specifically for the hemochromatosis disease) was implicated in explaining clinical severity of
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14 300 periodontitis, we have added the transferrin saturation into the multiple linear regression models
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16 301 as a confounding factor. As described previously, periodontitis through mean PD was associated
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18 302 with transferrin saturation in the two models (Meuric, Lainé, et al., 2017). For each dependent
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20 303 variable (mean PD or percentage of sites with $PD \geq 4$ mm), the most significant result, appeared
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22 304 in the second model with the dysbiosis ratio ($p < 0.001$) which is compiled with majority of the
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24 305 periopathogens (i.e.: *Porphyromonas*, *Treponema* and *Tannerella* genera) and the genera usually
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26 306 found in health (Meuric, Le Gall-David, et al., 2017). This result is independent from the
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28 307 transferrin saturation (reflecting the iron status of the hemochromatosis disease). Thus, the
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30 308 dysbiosis ratio confirmed its utility in exploring the microbial dysbiosis to explain periodontal
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32 309 disease (Meuric, Le Gall-David, et al., 2017). Similar microbial dysbiosis ratios have also been
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34 310 calculated in Crohn's disease (Gevers et al., 2014) and in peri-implantitis (Kröger et al., 2018).

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40 311 These results are based on a specific population suffering from haemochromatosis.
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42 312 Extrapolations outside of this population need to be validated, though the iron status of the
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44 313 patients has been included as a confounding variable in statistical models. Nonetheless, these
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46 314 results are in accordance with the existing view that periodontitis or peri-implantitis are
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48 315 orchestrated by a bacterial consortium rather than a single pathogen (Darveau, 2010).

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318 **5 Conclusion**

319 In this study, we have confirmed that most of the well-described and newly identified
320 periodontal pathogens can be found in the deepest periodontal pocket of patients suffering from
321 haemochromatosis and periodontitis. Yet, only few taxa were correlated to mean pocket depth.
322 The relative abundance of these taxa was therefore used as a predictor, with confounding
323 variables, in multiple linear regression models that allowed for the prediction of mean pocket
324 depth. While *Porphyromonas*, *Aggregatibacter* and *Rothia* returned significant results, the
325 previously described dysbiosis ratio – ($\% \textit{Porphyromonas} + \% \textit{Treponema} + \% \textit{Tannerella}$) / ($\% \textit{Rothia} + \% \textit{Corynebacterium}$) – was the most reliable biomarker to predict periodontitis severity
326 in our patients. Longitudinal studies in patients with no systemic disease, as well as several
327 sampling sites and time points during the episodes of disease-active periodontitis should be
328 considered to further validate and generalize the findings of this study.
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15 336 report no conflicts of interest related to this study.
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20 337 **Data availability:** The sequence data were deposited to the NCBI Sequence Read Archive
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22 338 (<https://www.ncbi.nlm.nih.gov/Traces/study/>) under BioProject accession numbers
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24 339 PRJNA416501 and PRJNA517671. The detailed periodontal and demographic data for every
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26 340 patient can be accessed in Table S3.
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Demographics	Mean PD (mm)	<i>p</i> -value
<i>Gender</i>		
Male (<i>n</i> = 42)	2.32 ± 0.06	
Female (<i>n</i> = 33)	2.29 ± 0.06	0.863
<i>Smoking status</i>		
Never (<i>n</i> = 28)	2.29 ± 0.04	
Former (<i>n</i> = 30) and Current (<i>n</i> = 17)	2.31 ± 0.06	0.81
<i>Alcohol status</i>		
Never (<i>n</i> = 15)	2.12 ± 0.07	
Former (<i>n</i> = 2) and Current (<i>n</i> = 58)	2.35 ± 0.05	0.028
<i>Frequency of dentist visits</i>		
Once or twice a year (<i>n</i> = 59)	2.32 ± 0.06	
Less than once a year (<i>n</i> = 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.

FOR REVIEW ONLY

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

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3				124. 125. 126. 130. 133. 134.							
4	Firmicutes	<i>Selenomonas</i>	genus	136. 137. 138. 139. 146. 149.	Perez-Chapparo	2014	2 studies	ND			
5				388. 442. 478. 479. 481. 501.							
6				639. 892. 919. 920. 936. 937							
7	Firmicutes	<i>Johnsonella</i>	oral taxon 166	166	Perez-Chapparo	2014	2 studies	ND			
8	Firmicutes	<i>Eubacterium [XI] [G-3]</i>	brachy	557	Perez-Chapparo	2014	2 studies		0.005	0.001	30.4
9	Firmicutes	<i>Peptostreptococcaceae [XIII] [G-1]</i>	oral taxon 113	113	Perez-Chapparo	2014	2 studies	ND			
10	Firmicutes	<i>Lachnospiraceae [G-8]</i>	oral taxon 500	500	Perez-Chapparo	2014	2 studies	ND			
11	Firmicutes	<i>Dialister</i>						1.518		0.197	94.9
12	Firmicutes	<i>Dialister</i>	pneumosintes	736	Perez-Chapparo	2014	2 studies	ND			
13	Proteobacteria	<i>Acinetobacter</i>						0.081		0.030	45.6
14	Proteobacteria	<i>Acinetobacter</i>	baumannii	554	Perez-Chapparo	2014	2 studies	ND			
15	Proteobacteria	<i>Escherichia</i>	coli	574	Perez-Chapparo	2014	2 studies	ND			
16	Spirochaetes	<i>Treponema</i>	phylogroup II	584. 743	Perez-Chapparo	2014	2 studies		1.106	0.194	86.7
17	Synergistetes	<i>Fretibacterium</i>	oral taxon 359	359	Perez-Chapparo	2014	2 studies	ND			
18	TM7	TM7 [G-1]	oral taxon 346. 349	346. 349	Perez-Chapparo	2014	2 studies	ND			
19	SR1	SR1 [G-1]	oral taxon 345	345	Perez-Chapparo	2014	2 studies	ND			
20	Bacteroidetes	<i>Bacteroidales [G-2]</i>	oral taxon 274	274	Perez-Chapparo	2014	3 studies	ND			
21	Firmicutes	<i>Mogibacterium</i>						0.293		0.037	93.7
22	Firmicutes	<i>Mogibacterium</i>	timidum	0 42	Perez-Chapparo	2014	3 studies	ND			
23	Firmicutes	<i>Peptostreptococcus</i>						0.192		0.040	77.2
24	Firmicutes	<i>Peptostreptococcus</i>	stomatis	112	Perez-Chapparo	2014	3 studies	ND			
25	Firmicutes	<i>Anaeroglobus</i>	geminatus	121	Perez-Chapparo	2014	3 studies	ND			
26	Proteobacteria	<i>Desulfobulbus</i>						1.515		0.285	81.0
27	Proteobacteria	<i>Desulfobulbus</i>	sp. oral taxon 041	0 41	Perez-Chapparo	2014	3 studies	ND			
28	Spirochaetes	<i>Treponema</i>	vincentii	0 29	Perez-Chapparo	2014	3 studies		0.034	0.009	41.8
29	Synergistetes	<i>Fretibacterium</i>	oral taxon 362	362	Perez-Chapparo	2014	3 studies	ND			
30	Synergistetes	<i>Fretibacterium</i>	fastidiosum	363	Perez-Chapparo	2014	3 studies	ND			
31	TM7	TM7 [G-5]	oral taxon 356	356	Perez-Chapparo	2014	3 studies	ND			
32	Bacteroidetes	<i>Porphyromonas</i>	endodontalis	273	Perez-Chapparo	2014	4 studies		3.105	0.421	88.6
33	Firmicutes	<i>Enterococcus</i>	faecalis	604	Perez-Chapparo	2014	4 studies	ND			
34	Spirochaetes	<i>Treponema</i>	lecithinolyticum	653	Perez-Chapparo	2014	4 studies		0.696	0.158	62
35	Synergistetes	<i>Fretibacterium</i>	oral taxon 360	360	Perez-Chapparo	2014	4 studies	ND			
36	Firmicutes	<i>Eubacterium [XI] [G-5]</i>	saphenum	759	Perez-Chapparo	2014	5 studies		0.002	0.001	11.4
37	Firmicutes	<i>Filifactor</i>						2.202		0.305	97.5
38	Firmicutes	<i>Filifactor</i>	alocis	539	Perez-Chapparo	2014	5 studies		0.041	0.011	40.5
39	Firmicutes	<i>Selenomonas</i>	sputigena	151	Perez-Chapparo	2014	5 studies	ND			
40	Spirochaetes	<i>Treponema</i>	medium	667	Perez-Chapparo	2014	5 studies		0.044	0.010	55.7
41	Dysbiosis							Mean %		+/-SE	Occurrence
42	Ratio	(% <i>Porphyromonas</i> + % <i>Treponema</i> + % <i>Tannerella</i>) / (% <i>Corynebacterium</i> + % <i>Rothia</i>)			Meuric	2017		61 816		+/- 19 257	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	<i>p</i> -value	Mean ± SE	Occurrence (%)
<i>Bacterial data</i>				
Dysbiosis ratio	0.37	0.001	3.573 ± 202	100
<i>E. nodatum</i>	0.35	0.01	0.18 ± 0.04	62
<i>P. gingivalis</i>	0.33	0.01	2.77 ± 0.63	62
<i>Desulfobulbus</i>	0.28	0.05	1.52 ± 0.29	81
<i>Treponema</i>	0.26	0.05	5.59 ± 0.58	100
<i>T. forsythia</i>	0.25	0.05	4.52 ± 0.53	100
<i>Porphyromonas</i>	0.23	0.05	7.05 ± 0.83	98.7
<i>Aggregatibacter</i>	-0.31	0.01	0.85 ± 0.17	86.1
<i>Campylobacter</i>	-0.27	0.05	4.28 ± 0.36	100
<i>Rothia</i>	-0.26	0.05	0.22 ± 0.05	73.4
<i>Clinical data</i>				
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.

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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(<i>Aggregatibacter</i>)	-0.053	0.021	-2.492	0.05
log(<i>Rothia</i>)	-0.063	0.025	-2.482	0.05
log(<i>Porphyromnas</i>)	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(<i>Aggregatibacter</i>)	-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 R² = 0.4573, adjusted R² = 0.3798, F-statistic = 5.899, P-value = 0.0001569. (42 degrees of freedom)

Characteristics of the model 2: multiple R² = 0.5349, adjusted R² = 0.4868, F-statistic = 11.12, P-value = 3.219e-08. (58 degrees of freedom)