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## Signature of Microbial Dysbiosis in Periodontitis

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- 1 **Signature of microbial dysbiosis in periodontitis.**
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- 5
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- 9
- 10 Running Head: Dysbiosis in Periodontitis
- 11
- 12
- 13 # Address correspondence to Vincent Meuric: [vincent.meuric@univ-rennes1.fr](mailto:vincent.meuric@univ-rennes1.fr)
- 14 M.B.M. and F.B.H. contributed equally to this work.
- 15
- 16 **Keywords:** chronic periodontitis – health – microbiota – dysbiosis – ratio

17 **Abstract**

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

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

24 Design and methods. Published sequencing data obtained from several different studies  
25 (196 sub-gingival samples from patients with chronic periodontitis and 422 sub-gingival  
26 samples from healthy subjects) were pooled and submitted to a new microbiota analysis  
27 using the same VAMPS (Visualization and Analysis of Microbial Population Structures)  
28 analysis pipeline to identify microbiota specific to health and disease. Microbiota were  
29 visualized using CoNet and Cytoscape. Dysbiosis ratio, defined as the percentage of genera  
30 associated with disease relative to the percentage of genera associated with health, were  
31 calculated to distinguish disease from health. Correlation between the proposed dysbiosis  
32 ratio and the periodontal pocket depth was tested on a different set of data obtained from a  
33 recent study to confirm the relevance of the ratio as a potential indicator of dysbiosis.

34 Results. Beta diversity showed significant clustering of periodontitis-associated microbiota  
35 according to clinical status at the genus level, independent of methods used. Specific genera  
36 were highly prevalent (>95%) in health (*Veillonella*, *Neisseria*, *Rothia*, *Corynebacterium* and  
37 *Actinomyces*) while other genera were associated with chronic periodontitis (*Eubacterium*,  
38 *Campylobacter*, *Treponema* and *Tannerella*). The calculation of dysbiosis ratio based on the  
39 relative abundance of these genera found in health *versus* periodontitis was tested. Non-  
40 periodontitis samples were significantly identifiable by low ratios as compared to chronic

41 periodontitis samples. When applied to a sub-gingival set with well-defined clinical data, the  
42 method showed a strong correlation between the dysbiosis ratio, as well as a simplified ratio  
43 (*Porphyromonas*, *Treponema* and *Tannerella* to *Rothia* and *Corynebacterium*), and pocket  
44 depth.

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

47

#### 48 **Importance**

49 Defining microbiota typical of oral health or chronic periodontitis is a difficult task.

50 Periodontal disease evaluation is currently based on probing of the periodontal pocket.

51 However, the status of pockets “on the mend” or sulci at “risk of periodontitis” cannot solely

52 be addressed through pocket depth measurements, nor by current microbiological tests

53 available for practitioners. Thus, a more specific microbiological status of dysbiosis could

54 help in future diagnoses of periodontitis. In this work, data from different studies were

55 pooled to improve the accuracy of the results. However, analysis of multiple microbiota from

56 different studies intensified the bacterial network and complicated the search for

57 reproducible microbial signature. Despite different methods used in each study, the

58 microbiota investigated at the genus level showed that some genera are prevalent (up to

59 95% of the samples/microbiota) in health or disease, allowing the calculation of bacterial

60 ratios (*i.e.* dysbiosis ratios). The correlation between the proposed ratios and the

61 periodontal pocket depth was tested which confirmed the link between dysbiosis ratios and

62 the severity of the disease. The importance of this work is promising but longitudinal studies

63 will be required to improve the ratios and define microbial signatures of the disease, which

- 64 will allow the monitoring of the periodontal pocket recovery and conceivably, the potential
- 65 risk of periodontitis in healthy patients.

66 **Introduction**

67 Chronic periodontitis (CP) is a chronic inflammation characterized by alveolar bone loss with  
68 intermittent periods of remission and relapse. CP is currently considered as an infection  
69 mainly due to an increase of bacteria in the sulcus leading to the formation of a periodontal  
70 pocket (for review: 1, 2). The major pathogen linked to CP is *Porphyromonas gingivalis* with  
71 bacterial partners like *Treponema denticola* and *Tannerella forsythia*. These three bacteria  
72 have been considered as the major pathogenic “red complex” since 1998 (3). However,  
73 recent advances from metagenomics studies developed a new model of periodontal disease  
74 pathogenesis. CP does not result from individual pathogens but rather from polymicrobial  
75 synergy and dysbiosis (4) associated with a dysregulated immune response inducing  
76 inflammation-mediated tissue damage (5). Host genetic components have also been  
77 implicated in CP, where multiple genes contribute cumulatively to the hosts overall disease  
78 risk (or protection) through effects on the host immune response and the microbiome (6).  
79 Since the Human Microbiome Project (HMP) (7), microbiota were analyzed based on partial  
80 sequencing of the 16S rRNA gene with different numbers of healthy and CP samples.  
81 However, comparison between studies is difficult because of the differences in methods  
82 used (*i.e.* clinical examination and diagnosis of periodontitis and oral health, sample  
83 collection protocols, DNA extraction protocols, hyper variable regions of the 16S rRNA gene  
84 analyzed). As there is a growing interest in the human microbiome, despite the difficulties  
85 mentioned earlier, the use of independent studies to look for “Signal in the Noise” should  
86 proceed as previously suggested (8), by reanalyzing all data with the same protocol. The  
87 difference between periodontal health- and disease-associated microbiota should be larger  
88 than the technical variations of the different studies and enable the identification of  
89 microbial signatures using NGS technologies. The first objective of this study was to explore

90 the disease-associated changes in the sub-gingival microbiota using a unique VAMPS  
91 (Visualization and analysis of microbial population structure) pipeline (9) at the genus level  
92 for beta-diversity (Bray-Curtis dissimilarity) on a large number of samples (from 6 different  
93 studies) and to confirm that the microbiota identified did not cluster according to the  
94 methods used (primer or study type). Sub-gingival microbiota from patients with diagnosed  
95 chronic periodontitis (196) and from healthy subjects (422) were included as well as external  
96 control samples (from dentine caries, supra-gingival plaque and the mid-vagina). The second  
97 objective was to determine a dysbiosis ratio of bacteria that could predict health or severity  
98 of the disease from the sub-gingival samples and finally to test it on an independent cohort  
99 of patients with well-described periodontal pocket measurements.

100 **MATERIAL and METHODS**

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

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

118

119 **Ecology diversity and taxonomy identifications.** Reads from the different datasets were  
120 analyzed using VAMPS using default parameters for taxonomy assignment to the genus level  
121 through the GAST process using the RDP classification to produce the best taxonomic  
122 assignment for each read. Reads identified as Archaea, Eukarya, Organelle and unknown  
123 reads were excluded for further analysis. The frequency of each taxonomic assignment

124 in the dataset was reported as a percentage (number of reads assigned to a taxonomy  
125 over total number of reads in the dataset). Alpha diversity as observed richness and  
126 Shannon-Weaver index were determined from the raw data sets. Differences between  
127 microbiota structures (beta-diversity) were assessed using principal coordinate analysis  
128 (PCoA) 2D tree on the Bray-Curtis distance through VAMPS. Samples were divided into five  
129 clusters (1 to 5), where visualizations were realized using Figtree software (v1.4.2) and 3D  
130 PCoA plots were generated using Emperor software. Relative abundances were studied  
131 when the average abundance was above 1% in at least one sample. Assessments of  
132 significant patterns of microbial co-occurrence or mutual exclusion at the genus level were  
133 performed using Cytoscape v3.2.1 (17) and the CoNet plugin (18). Only genera found in the  
134 high majority, in at least 95%, of the healthy sub-gingival samples or the CP samples (from  
135 the fifth cluster) are represented.

136

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

145

146 **Statistical analysis.** Normality tests for data distribution were realized. Data were studied by  
147 Spearman correlation test between biological origins, primers used, publication of sample

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

159 **RESULTS**

160 **Microbial community structure analysis.** Using a matrix correlation analysis, the possible  
161 clustering of microbiota according to nature of primers used, the site of sampling, or the  
162 study investigated was explored. Despite various studies, the analyzed data clustered into  
163 five groups according to the clinical status (healthy or CP) or sampling site as shown by the  
164 3D PCoA plots (Fig. 1). Healthy sub-gingival samples were, in majority, spread into two main  
165 clusters; control samples were clearly separated in two other clusters corresponding to  
166 saliva and dentine caries/vagina, while the majority of CP samples were found in a fifth  
167 cluster. Two-D beta-diversity analysis showed the precise distribution of the samples in the  
168 five clusters (Fig. 2). The search for an association between clusters and primers and/or  
169 study type showed that the fourth cluster was associated with V3V4 16S rRNA primers  
170 (correlation  $r=0.537$ ,  $p<0.001$ ) and with the Kianoush *et al.* study (15) that has used these  
171 specific primers (correlation  $r=0.608$ ,  $p<0.001$ ). No other correlation with primers was found.  
172 The 2 healthy clusters (1 and 2) were characterized by sub- and supra-gingival samples in  
173 similar proportions (Fig. 2, in blue). Focusing on healthy sub-gingival samples, the main  
174 difference between the two healthy clusters 1 and 2 was the distribution of samples from  
175 the HMP study and from the other studies in the clusters: 225/323 of the HMP study  
176 clustered into the healthy cluster 2 while the healthy cluster 1 was richer in samples deriving  
177 from the other studies (44/99). Cluster 3 was characterized by saliva as 91% of the saliva  
178 samples (258/284) are grouped within this cluster (Fig. 2, correlation  $r=0.892$ ,  $p<0.001$ ).  
179 Cluster 4 was characterized by samples from dentine caries 73% (80/110) and mid-vagina  
180 100% (60/60) (correlation  $r=0.603$  and  $r=0.638$  respectively,  $p<0.001$ ). Finally, the fifth  
181 cluster contained 90% of the CP samples (176/196, correlation  $r=0.708$ ,  $p<0.001$ ).

182 It is interesting to note that 10% of the CP samples were found in the 2 healthy clusters  
183 (19/196) and contained similar microbiota (analyzed by beta-diversity) as dentine caries  
184 and/or mid-vagina (1/196) at the genus level. Conversely, 16% of the healthy sub-gingival  
185 samples (69/422) and 17% of the dentine caries samples (19/110) were found in the fifth  
186 cluster.

187

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

197

198 **Patterns of microbial communities in sub-gingival samples (genus level):** Genera present in,  
199 at least, 95% of all healthy sub-gingival samples or 95% of the CP samples from the fifth  
200 cluster are presented in figures 4A and 4B respectively. Results showed that healthy sub-  
201 gingival samples are dominated by 8 major genera, *Fusobacterium*, *Actinomyces*,  
202 *Streptococcus*, *Neisseria*, *Capnocytophaga*, *Prevotella*, *Corynebacterium*, and *Rothia*, and by  
203 6 minor genera, *Leptotrichia*, *Veillonella*, *Porphyromonas*, *Granulicatella*, *Kingella* and  
204 *Gemella*. Associations were found between *Fusobacterium* and *Prevotella*, *Actinomyces* and  
205 *Rothia* and between *Leptotrichia* and *Porphyromonas*. Common genera found in CP were

206 less abundant with 4 major genera, *Treponema*, *Porphyromonas*, *Prevotella* and  
207 *Fusobacterium* followed by *Streptococcus*, *Eubacterium*, *Tannerella* and *Campylobacter*  
208 genera. Only one association was found between *Eubacterium* and *Treponema*, while  
209 *Fusobacterium* and *Treponema* presented a negative correlation.

210

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

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

222 The dysbiosis ratio found in CP samples from the fifth cluster (r=1.510) was significantly  
223 higher than the ratios of the majority of samples from cluster 1 and 2 (healthy sub-gingival  
224 r=0.015, shallow r=0.052 and CP samples r=0.088), and was also significantly higher than  
225 healthy sub-gingival samples (r=0.184) from the same fifth cluster (p<0.001). In clusters 1  
226 and 2, the dysbiosis ratio of CP samples was similar to the ratio of shallow sites. These two  
227 groups were significantly different from the healthy sub-gingival samples (p<0.05) in the  
228 same cluster.

229 Healthy sub-gingival samples (n=69) belonging to the fifth cluster exhibited a dysbiosis ratio  
230 ( $r=0.184$ ) significantly different from the other healthy sub-gingival samples ( $r=0.015$ ) but  
231 also from the majority of the CP samples (fifth cluster,  $r=1.510$ ). These results confirmed the  
232 possible difference of these healthy sub-gingival microbiota ( $p<0.001$ ) as compared with  
233 those of healthy clusters 1 and 2. Their ratio is also not significantly different from the CP  
234 sample ratio in cluster 1 and 2, which could be considered “on the mend”.

235

#### 236 **Validation of the dysbiosis ratio**

237 A different dataset from Bizzarro S. *et al.* (16), containing well-described samples (pocket  
238 depth from 2 to 8 millimeters), was used as an external control to confirm the relevance of  
239 the dysbiosis ratio of bacteria. The dysbiosis ratio at the genus level was correlated with the  
240 periodontal pocket depth ( $r=0.655$ ,  $p<0.001$ ) (Fig. 6A). These results, based on 37 patients  
241 (147 samples at different times and different procedures of the periodontal treatment),  
242 confirmed the link between the dysbiosis and the depth of periodontal pocket. The second  
243 simplified ratio of *Porphyromonas*, *Treponema* and *Tannerella* to *Rothia* and  
244 *Corynebacterium* showed a similar correlation ( $r=0.659$ ,  $p<0.001$ ) (Fig. 6B).

245 **DISCUSSION**

246 Many studies have been published since the human microbiome project in 2009, increasing  
247 the number of microbiota data available for the research community. However, comparison  
248 between studies is challenging, at least at the species level, because of the use of different  
249 methods. This issue is a real limitation to understand disease as well as the low number of  
250 samples in each study. Additionally, it is more complicated for healthy sub-gingival status  
251 that usually represent less than a half of the samples when included in studies (10, 14). This  
252 work is a taxon-based analysis at the genus level of sequence reads from several studies.  
253 Studying a large number of samples minimized individual variations and overcame technical  
254 variations by increasing the effective sample size. Such analysis has already been proposed  
255 in a recent microbiota obesity study (8). Studies with described healthy (sulci  $\leq 3$ mm) and CP  
256 (pocket depth  $\geq 5$ mm) samples and available raw sequenced data in data banks were chosen.  
257 Data from the HMP resources (two different couples of primers used) were added to  
258 increase the number of sub-gingival healthy microbiota data available from 99 up to 422.  
259 The different microbiota clustered either by sampling site, such as out-groups used as  
260 controls for this study (saliva in cluster 3, dentine caries/vagina both rich in *Lactobacillus* in  
261 cluster 4), or by clinical status, such as sub-gingival samples (healthy samples in cluster 1 and  
262 2 and CP samples in the fifth cluster). CP sites can either show a greater microbial diversity  
263 and observed richness than healthy sub-gingival sites (12, 19), or present no significant  
264 difference in microbial diversity as it has been also reported between health and  
265 periodontitis (11). Thus, the high number of samples surpasses the technical variations, at  
266 least at the genus level with the primers used in these different studies, and the difference  
267 between periodontal health and disease is larger than the technical variations, as described  
268 by Kirst *et al.* (11). No difference was found between the healthy sub- and supra-gingival

269 samples when using beta-diversity analysis at the genus level, as previously described (20).  
270 Ninety per cent of CP samples were found in the fifth cluster. To define the fifth cluster as a  
271 “periodontitis cluster” by beta-diversity was appealing. However, the fifth cluster also  
272 contained healthy sub-gingival samples, indicating that further investigations were necessary  
273 to understand and develop prediction markers for chronic periodontitis.

274 A core community is usually identified in publications (genera present in at least 50% of the  
275 samples) and provides a basis for disease diagnosis, prevention and therapeutic targets (21,  
276 22). However, the genera variability expands as sample size increases, thus limiting its use  
277 to establish an easy microbiological marker for dysbiosis. In this work, genera present at a  
278 higher prevalence in at least in 95% of the samples were used to determine the genera  
279 implicated in health or in favor of the disease. The genera used to calculate the dysbiosis  
280 ratio in favor of periodontitis were *Treponema*, *Campylobacter*, *Eubacterium* and *Tannerella*.  
281 These genera were identified at high abundance and high prevalence in CP as compared to  
282 healthy samples. They include well-identified species (*T. forsythia* and *T. denticola*, *C. rectus*  
283 and *E. nodatum*) that are strongly associated with the disease (3, 23-25). It is noted that,  
284 while some species such as the newly-cultivated *Tannerella* clone BU063 (26, 27) that is  
285 supposed health-associated, is also found in active periodontal sites (28) and therefore still  
286 considered controversial. Despite a significant difference in abundance of the  
287 *Porphyromonas* genus (which includes *P. gingivalis* that is highly associated with  
288 periodontitis) between healthy samples (3.35%) and in CP samples (13%), it was  
289 excluded in the first dysbiosis ratio because of its similar prevalence. As the lowest  
290 abundance in genus accounted for the CP calculation was *Campylobacter*, 1.9%, this value  
291 was therefore chosen as a cut-off to minimize the number of genera used for the health  
292 calculation - *Rothia*, *Corynebacterium*, *Actinomyces*, *Veillonella* and *Neisseria*. Neither

293 *Capnocytophaga* nor *Leptotirichia* were included because of their high prevalence in  
294 periodontitis samples (more than 90%, data not shown). Species belonging to the genus  
295 *Rothia* have been repeatedly described as members of oral communities associated with  
296 periodontal health (29-34) or at least being more predominant in health (31). In the same  
297 way, *Corynebacterium* appeared to be more associated with “healthy” sub-gingival biofilm  
298 (35, 36). Moreover, *Rothia* and *Corynebacterium* were among the bacteria that showed the  
299 greatest increase after periodontal treatment (37), while a study suggested that  
300 *Corynebacterium* might be considered as a putative periodontal protector (38). *Veillonella*  
301 and *Actinomyces* have been negatively correlated with clinical markers in CP (39) and  
302 *Neisseria* was found in inactive sites (28). The calculated dysbiosis ratio distinguishes clearly  
303 healthy sub-gingival samples from CP samples.

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

311 Healthy sub-gingival samples are divided into 3 different groups. Two of them belonging to  
312 clusters 1 and 2 present the same low ratio and describe an absence of dysbiosis. The third  
313 group had a higher dysbiosis ratio, similar to shallow sites and CP samples from cluster 1 and  
314 2 but significantly inferior to CP or shallow samples from the fifth cluster. As “healthy  
315 patients” from HMP are defined as patients with pockets depths <4mm, some of them could  
316 have explained this high ratio group, however healthy patients from other studies (19/99)

317 were also included in this group. This result is similar to Zhou Y. *et al.* where few healthy  
318 subjects were detected with signals of disease such as an increase of *Treponema* (40).  
319 Therefore, patients who presented a relatively high ratio as such could be considered at “risk  
320 of periodontitis”.

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

336 A major concern at the beginning of this work was the capacity to identify species with  
337 multiple datasets. However the V1V2 and V5V7 primers used in 3 studies were not suitable  
338 for species identification. At the genus level, as reported in Bizzarro S. *et al.* (16), the  
339 proposed dysbiosis ratio is a good microbial signature calculated using the online VAMPS  
340 software even by using a simplified dysbiosis ratio. Indeed, as *Rothia* and *Corynebacterium*

341 are the major healthy genera found, and even if *Porphyromonas* was found in both health  
342 and disease, its abundance increased significantly in disease (from 3.34% to 13%). The result  
343 was interesting, where it was found to be similar to the precedent ratio (correlation with  
344 pocket depth  $r=0.659$   $p<0.001$ ). However, more adjustments were needed as 43 out of 196  
345 CP samples presented none of the two healthy genera and a value of 0.1% was attributed for  
346 the calculation.

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

359  
360 In conclusion, this study aimed to define ratios of bacteria as microbial signature after the  
361 analysis of public raw data from different studies, independent of the technical methods  
362 used to generate the data. These ratios allow the differentiation of healthy and diseased  
363 microbiota in a majority of samples. Standardized protocols of sampling and complete  
364 metadata in the public bank are necessary to study dysbiosis in oral health and to improve

365 the proposed dysbiosis ratios. Adjunction of specific perio-protectors and potential specific  
366 pathogens to the calculation of the dysbiosis could also be promising. Longitudinal studies  
367 are necessary to predict exact pockets microbiologically “on the mend” or sulci with “risk of  
368 periodontitis”.

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370

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500

501 **Author Contributions** (names must be given as initials)

502 VM, FBH and MBM conceived and designed the research. VM realized the sampling. SLGD

503 realized the molecular biology. VM, SLGD, EB, LAA, BM, SBF, BM, performed the experiments

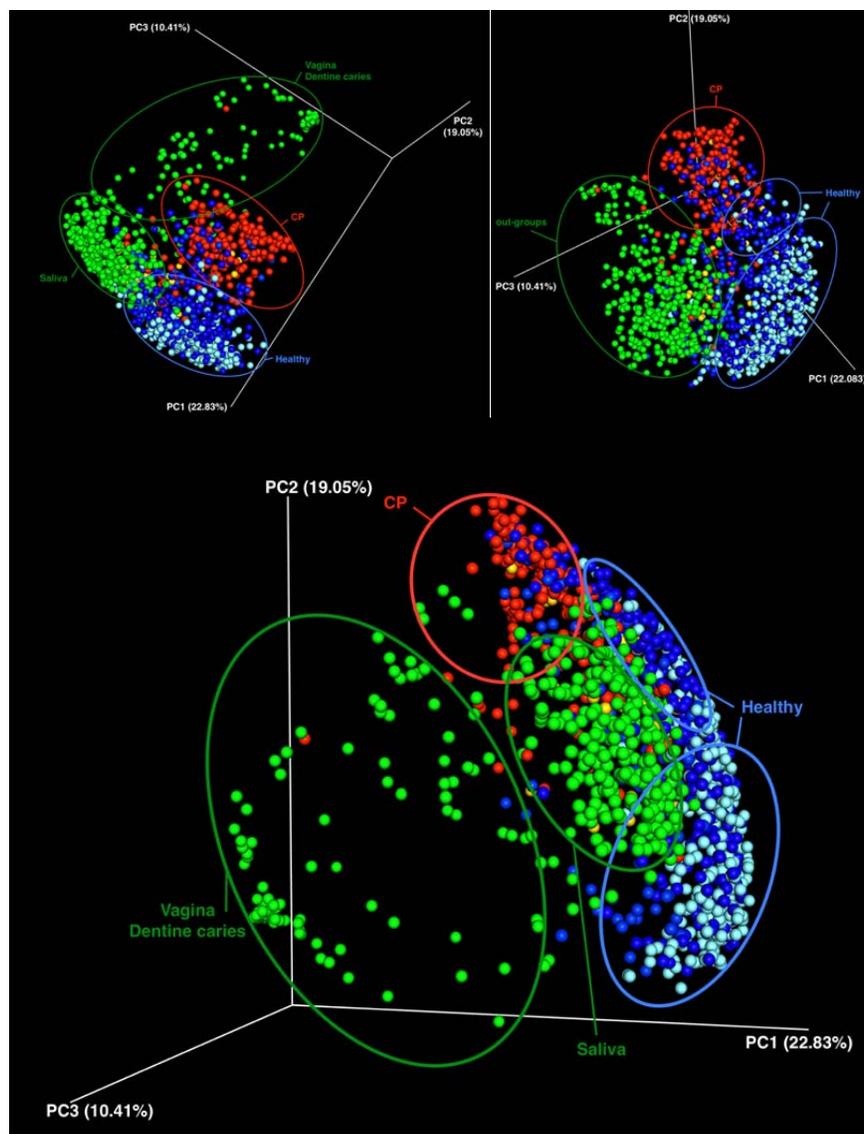
504 (bioinformatics analysis, statistics) and wrote the manuscript. MBM supervised the project.

505

506 **Competing interests**

507 The authors declare no competing financial interests.

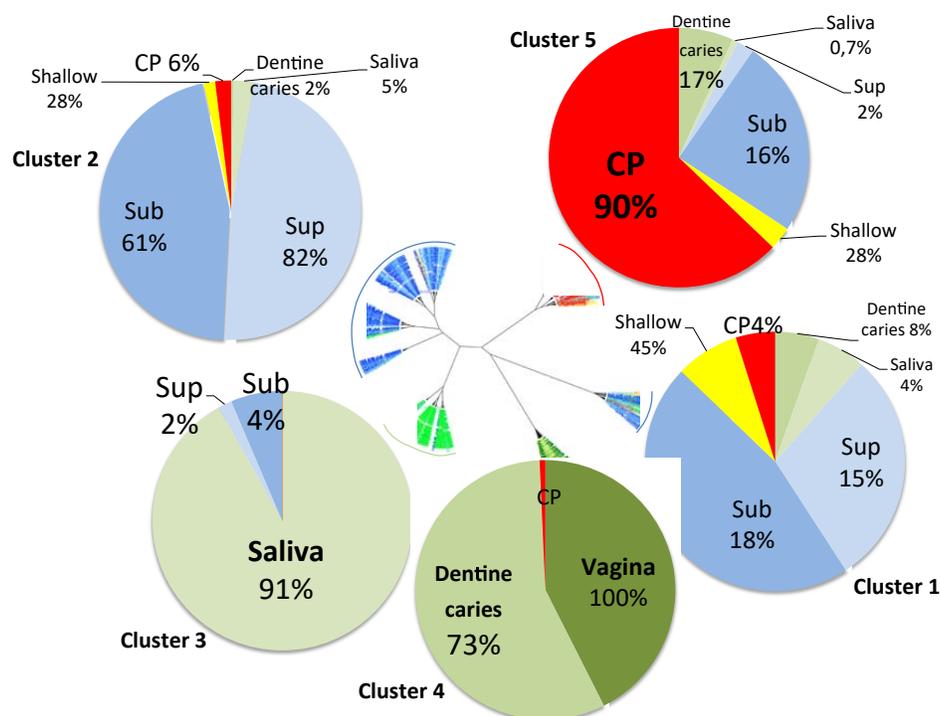
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510 **Fig. 1: Different views of 3D PCoA plots illustrating the beta-diversity of bacterial**511 **populations as a function of sampling site and diagnosis. Light blue: supra-gingival samples;**512 **dark blue: healthy sub-gingival samples; green: out-groups as saliva, mid-vagina and dentine**513 **caries; red: chronic periodontitis - CP).**

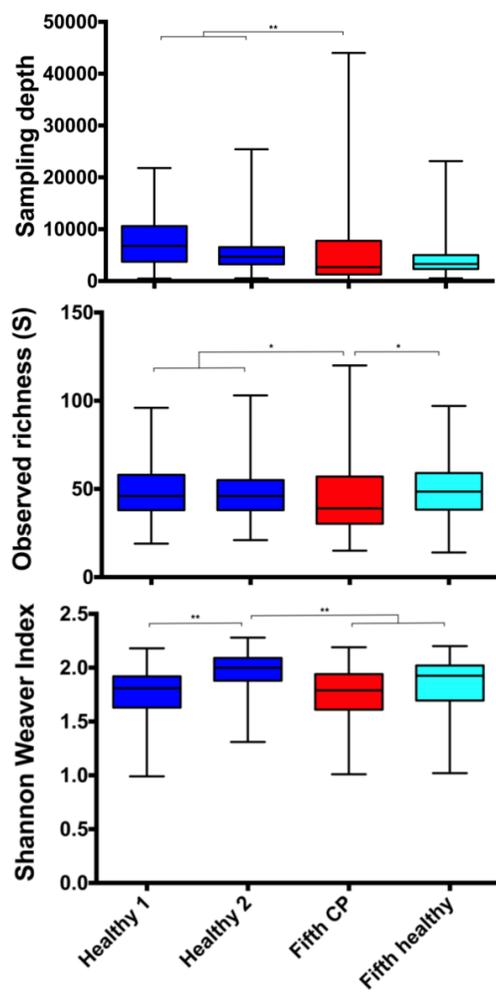
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516 **Fig. 2: Unrooted tree displaying genus Bray Curtis beta-diversity clustering microbiota and**517 **pie charts related to sample origin within each cluster. The tree was realized using Figtree**518 **software v1.4.2. Distribution of microbiota in each cluster is represented by pie charts with**519 **different colors according to sampling sites (supra-gingival: Sup in light blue, saliva: light**520 **green, dentine caries: green and mid vagina: dark green) and diagnosis for sub-gingival**521 **samples (healthy: Sub in dark blue, shallow in yellow and chronic periodontitis in red).**522 **Written percentages correspond to the number of samples from a given specific sampling**523 **site in a given cluster to the total number of samples from the same specific sampling site.**

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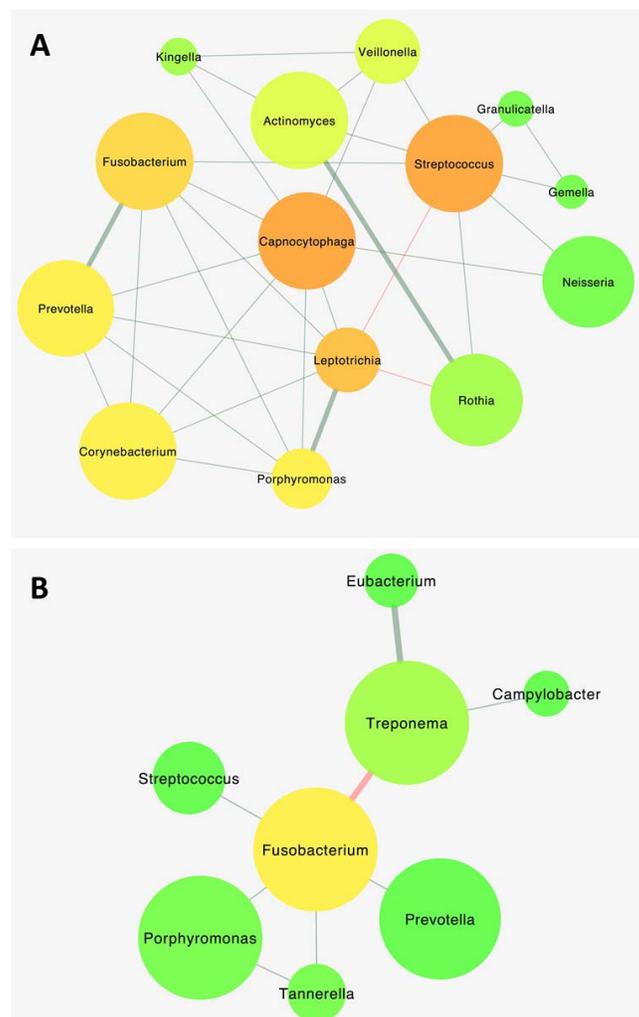


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526 **Fig. 3: Alpha diversity index.** Microbiota sampling depth, observed richness (S, number of  
527 different taxa per sample) and diversity (Shannon Weaver index) comparisons in sub-gingival  
528 samples between samples of healthy clusters 1 and 2 (blue) and samples of cluster 5, either  
529 with chronic periodontitis (red) or sub-gingival healthy samples (light blue). \* $p < 0.05$ ,

530 \*\* $p < 0.01$ .

531



532

533 **Fig. 4: Patterns of sub-gingival microbial communities.** A: Patterns of genera present at

534 least in 95% of all healthy sub-gingival samples. B: Patterns of genera present at least in 95%

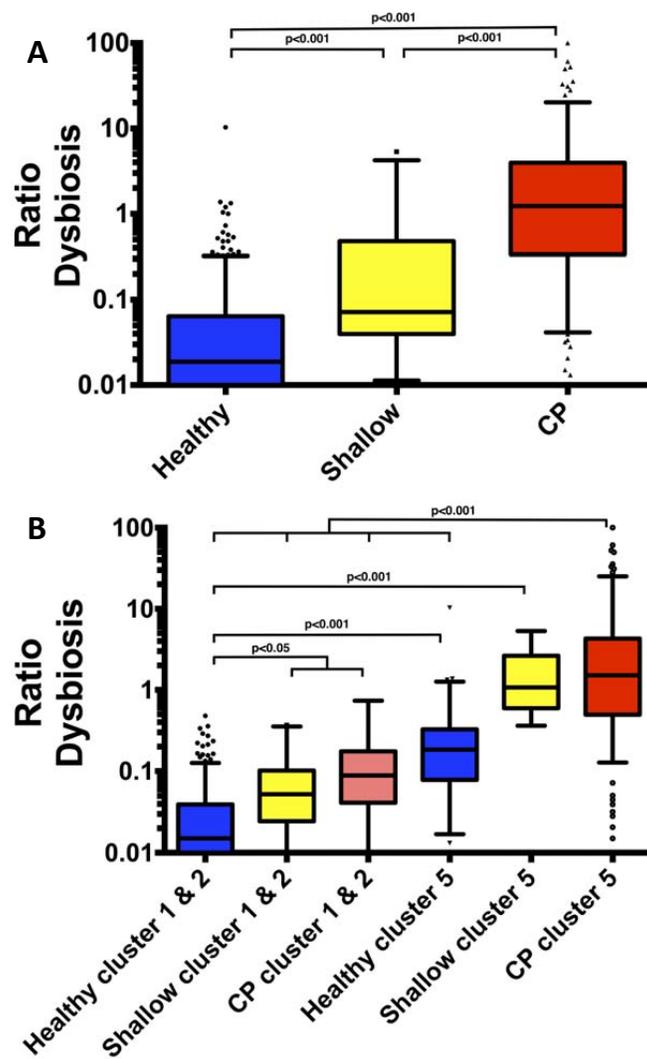
535 of all chronic periodontitis (CP) samples from the cluster 5. Edges represent one (thin line) or

536 2 to 3 (thick line) significant correlation between genera (green: positive; red: negative).

537 Node colors represent the number of partners ranging from one (green) to 7 (dark orange).

538 Node sizes represent the abundance of each taxon.

539



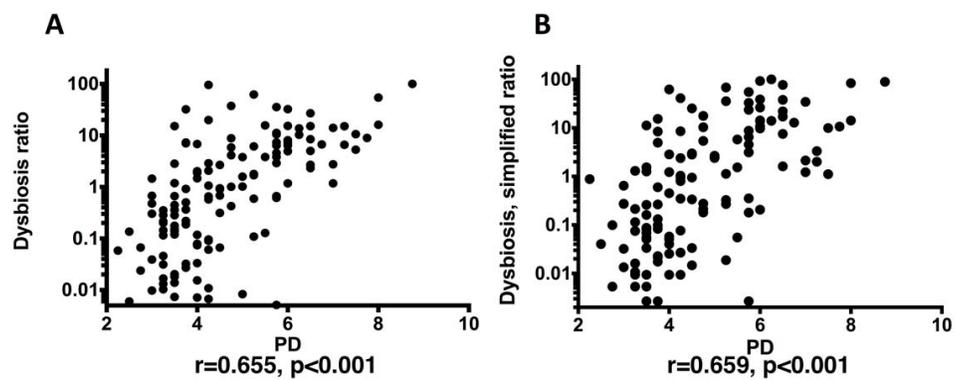
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541 **Figure 5: Sub-gingival dysbiosis ratio.** Ratio = *Eubacterium*, *Campylobacter*, *Treponema* and542 *Tannerella* to *Veillonella*, *Neisseria*, *Rothia*, *Corynebacterium* and *Actinomyces*. A. Between

543 healthy, shallow and chronic periodontitis samples (CP) from all clusters. B. Between clusters

544 1&amp;2 and Cluster 5 for healthy, shallow and CP samples.

545



546

547

548 **Figure 6: Correlation between pocket depth and dysbiosis.** Samples from Bizzarro S. *et al.*,

549 2016 (16) were analyzed by VAMPS followed by the calculation of the dysbiosis ratio. A.

550 Ratio = *Eubacterium*, *Campylobacter*, *Treponema* and *Tannerella* to *Veillonella*, *Neisseria*,551 *Rothia*, *Corynebacterium* and *Actinomyces*). B. Simplified ratio = *Porphyromonas*, *Treponema*552 and *Tannerella* to *Rothia* and *Corynebacterium*.

553

554

555

556 Table 1: Sub-gingival microbiota used in this study

Authors	Accession Number	Number of sub-gingival microbiota				16S rRNA gene regions
		Health	Shallow #	CP		
				Diagnosis	Follow up after treatment	
Abusleme <i>et al.</i> (10)	SRA051864	10		44		V1V2
Kirst <i>et al.</i> (11)	PRJNA269205	25		25		V1V3
Griffen <i>et al.</i> (12)	SRP009299	29	29	29		V1V2 and V4
Camelo-Castillo <i>et al.</i> (14)	MG-RAST: ID 12161	22		60		V1V3
Zhou <i>et al.</i> (40)	SRA062091	13		18		V1V3
Meuric <i>et al.</i>	In progress <sup>§</sup>			24		V3V4
HMP (7)		119				V1V3
HMP (7)		204				V3V5
Bizzarro <i>et al.</i> (16)	PRJNA289294			37*	110*	V5V7

557 \* CP microbiota from patients with a follow up treatment used to confirmed the dysbiosis

558 ratios hypothesis (16).

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

560 <sup>§</sup> Data available on VAMPS for the reviewers, dataset: "Y\_Hemoparo"

561

