Microbiome composition comparison in oral and atherosclerotic plaque from patients with and without periodontitis

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Short title: Microbiome comparison of oral and atherosclerotic plaque

Abstract

There is no conclusive evidence regarding a causal relationship between periodontitis and atherosclerosis. In this study, we examined the microbiome in the oral cavity and atheromatous plaques from atherosclerosis patients with or without periodontitis to investigate the role of oral bacteria in the formation of atheromatous plaques. We chose four patients with and without periodontitis, who had undergone carotid endarterectomy. Bacterial samples were extracted from saliva on the tongue surface, from periodontal pocket (during the oral examination), and from the atheromatous plaques. We investigated the general and oral conditions from each patient and performed next-generation sequencing analysis for all bacterial samples. There were no significant differences between both groups concerning general conditions. However, the microbiome patterns of the gingival pocket showed differences depending on the absence or presence of periodontitis, while those of the saliva were relatively similar. The microbiome pattern of the atheromatous plaques was entirely different from that in saliva present on the tongue surface and gingival pocket, and oral bacteria were seldom detected. However, the microbiome pattern in atheromatous plaques was different in the presence

or absence of periodontitis. These results indicated that oral bacteria did not affect the formation of atheromatous plaques directly. However, the metabolic products of microbiome or the host inflammatory response might indirectly influence the composition of atheromatous plaques.

Introduction

More than 100 trillion microbes reside in niches within the human body. Collectively, these microbes constitute the microbiome [1]. The co-existence and interactions between eukaryotic and microbial cells is vital in regulating physiological functions. The microbiome balance has been linked with obesity, cancer, intestinal disorders, and mental disorders [2, 3], and periodontitis [4].

Periodontitis is a predominant oral infectious disease in which an excessive immune response directed at the microbiome on the tooth surface destroys the periodontal tissue, forming periodontal pockets. The microbiome in this pocket includes pathogenic anaerobic bacteria that can form biofilms, which are inherently a drug-resistant and challenge host immunity [5]. The mature biofilm causes further periodontitis progression because of the prolonged inflammation associated with the protracted immune response.

59 Therefore, periodontitis has two main features; it is an infectious disease caused by
60 microbiome imbalance and a chronic inflammatory disease caused by a dysregulated
61 immune response.

62 These two characteristic features of periodontitis are shared by various systematic
63 diseases, including diabetes, arteriosclerosis, cardiovascular diseases, brain diseases,
64 cancer, and non-alcoholic steatohepatitis and with preterm low birth weight [6-11]. We
65 also reported a case where the microbiome with pathogenic periodontal bacteria was
66 implicated as the cause of infective endocarditis [12]. Arteriosclerosis includes
67 atherosclerosis, in which an atherosclerotic plaque is formed on the blood vessel walls
68 through various mechanisms [13]. An association between atherosclerosis and
69 periodontitis has been suggested by some epidemiology reports. Moreover, bacterial
70 investigations aimed at detecting periodontal bacteria in the atherosclerotic plaque have
71 been conducted [14-16]. Although observational data support an association between
72 periodontitis and atherosclerotic vascular disease, the data do not yet justify a causative
73 relationship [17]. Multiple common factors, such as diabetes, high blood pressure,
74 dyslipidemia, and smoking, affect disease progression, and there is little data of a direct

75 involvement of periodontal bacteria in the development of atherosclerotic vascular
76 disease [18, 19].

77 An association between periodontitis and atherosclerotic vascular disease was
78 demonstrated *in vivo* using *Porphyromonas gingivalis* [20]. A clinical analysis sought to
79 detect the DNA of periodontal bacteria in atherosclerotic plaques [21]. The impact of the
80 disruption of the normal microbiome on various diseases is unclear and a comprehensive
81 analysis is necessary, since the microbiome could contribute to the formation of
82 microbiome atheroma.

83 Microbiome analysis has typically involved bacterial culture [22]. However, recent
84 comprehensive bacterial analyses using the gene for 16S ribosomal RNA (rRNA) have
85 been successful in detecting bacteria that are difficult to cultivate [23]. Next-generation
86 sequencing (NGS) has become a popular means of examining a large number and volume
87 of samples [24]. In this study, the association between periodontitis and atherosclerosis
88 in the context of the microbiome in the oral cavity and atherosclerotic plaque was
89 investigated by NGS.

90

Materials and Methods

Ethics statement

This study was approved by the ethics committee of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences and Okayama University Hospital (Authorization Number: 1603–059) and Brain Attack Center Ota Memorial Hospital (Authorization Number: 121). All enrolled patients provided written informed consent for the use of their resected tissue and oral samples.

Participants

The study focused on 12 patients who visited Brain Attack Center Ota Memorial Hospital between April 2016 and March 2018, and who were diagnosed with internal carotid artery stenosis. The patients were ≥ 40 years of age, underwent carotid endarterectomy, had more than ten teeth, and consented to participate.

Samples

107 Atheromatous plaques (AP) were extracted from an internal carotid artery. Bacteria in
108 the gingival pocket (GP) were collected using absorbent paper points (United Dental
109 Manufactures Inc., Johnson City, TN, USA). Bacteria in saliva from the tongue surface
110 (ST) were collected using forensic swabs (Sarstedt AG & Co. Nümbrecht, Germany).
111 Blood was collected from each patient and serum prepared as previously described [25].
112

113 ## Oral examination

114 Periodontal examinations were performed to evaluate the average pocket probing depth
115 (PPD) and rate of bleeding on probing (BOP) for each teeth of each patient. The patients
116 were then divided into three groups according to the Japanese Society for Periodontology
117 Clinical Practice Guideline for the Periodontal Treatment: periodontally healthy (control,
118 n = 4; H1-H4), mild periodontitis (n = 4, excluded from further analysis), and severe
119 periodontitis (periodontitis, n = 4; P1-P4).
120

121 ## DNA purification

APs were extensively minced using a scalpel and suspended in phosphate buffered saline (PBS). The collected material from paper points and swabs were resuspended using PBS. One milliliter of each resuspended bacterial sample was transferred to 2 ml Lysing Matrix B tubes (MP Biomedicals, Santa Ana, CA, USA) containing 0.1 mm silica beads and 500 µl ATL buffer (Qiagen, Hilden, Germany). The contents of each tube were homogenized using FastPrep 24 (MP Biomedicals) for 45 s at 6.5 m/s. Bacterial DNA was extracted using the QIAamp DNA Microbiome Kit (Qiagen) according to the manufacturer's instructions. The quality and quantity of the DNA were verified using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and the PicoGreen dsDNA assay kit (Life Technologies, Grand Island, NY, USA).

Polymerase chain reaction and NGS analysis

The first polymerase chain reaction (PCR) using 16S rRNA primers (forward: 5'-AGAGTTTGATCCTGGCTCAG-3', reverse: 5'-CGGTGTGTACAAGGCCCGGGAACG-3') and KAPA HiFi HotStart ReadyMix (Kapa Biosystems Inc., Wilmington, MA, USA). Thermal cycling conditions were as follows:

heating at 98°C for 3 min; 25 cycles of 98°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min. A second PCR was performed using the first PCR amplicons and V3-V4 primers (forward: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3', reverse: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') using the same reaction conditions. The quality and quantity of the DNA were verified using Qubit 4 Fluorometer (Invitrogen, Life Technologies, Grand Island, NY, USA) and the D1000 ScreenTape system (Agilent Technologies, Santa Clara, CA, USA).

NGS was conducted using the MiSeq® system (Illumina Inc., San Diego, CA, USA). The obtained sequence was compared to the database using the CLC Genomics Workbench (CLC bio, Aarhus, Denmark). Principal component analysis (PCA) and clustering analysis were performed using R statistical software [26]. We also performed co-occurrence analysis for the 13 highly detected operational taxonomic units from control and periodontitis samples using the Quantitative Insights Into Microbial Ecology approach [27].

154

155 Plasma IgG antibody titer test against periodontal bacteria

156 Plasma IgG antibody titer against periodontal bacteria was determined as described
157 previously [28]. The selected periodontal pathogenic bacteria were *Aggregatibacter*
158 *actinomycetemcomitans* (Aa) Y4, Aa ATC29523, Aa SUNY67, *Eichenerra corrodens*
159 (Ec) FDC1073, *Fusobacterium nucleatum* (Fn) ATCC25586, *Prevotella intermedia* (Pi)
160 ATCC25611, Pi ATCC33563, *Capnocytophaga ochracea* (Co) S3, *Porphyromonas*
161 *gingivalis* (Pg) FDC381, Pg SU63, T*reponema denticola* (Td) ATCC35405, and
162 *Tannerella forsythia* (Tf)ATCC43037.

163

164 General condition evaluation

165 General conditions of patients were evaluated based on age, disease history, body mass
166 index, blood pressure, C-reactive protein, cholesterol, and HbA1c (Table 1).

Table 1 Characteristics of study participants			
Characteristic	healty (n = 4)	periodontitis (n = 4)	*p*
Man — no. (%)	4 (100)	4 (100)	
Age, years	74.8±4.3	75.0±3.2	0.884
Current smoker — no. (%)	0	1 (25)	
Known diabetes — no. (%)	2(50)	2(50)	
Known hypertension— no. (%)	2(50)	2(50)	
Family history of cardiovascular disease — no. (%)	0	1 (25)	
Body-mass index	25.7±3.2	25.4±1.7	0.885
Blood pressure — mm Hg			
· Systolic	128.5±24.2	134.5±16.3	0.772
· Diastolic	69.3±22.3	72.5±14.2	0.663
CRP—mg/dL	0.32±0.32	0.16±0.17	0.386
Leukocyte count—×10 ^ 4/μL	6535±754	6293±983	0.564
Cholesterol — mg/dL	163.3±31.7	187±40.3	0.309
· High-density lipoprotein	31.7±5.1	42.1±2.9	0.248
· Low-density lipoprotein	79.8±8.7	118.5±33.9	0.248
Triglycerides — mg/dL	234±224.4	164.5±107.3	0.773
HbA1c (NGSP) — %	6.1±0.6	6.8±0.9	0.248

Oral condition evaluation

We evaluated the periodontal condition for each patient group from oral examination and plasma IgG antibody titer test (Table 2).

Table 2 Periodontal Disease infection of study participants			
Variable	Controls (n = 4)	Patients (n = 4)	*p*
Total no. of teeth	24.8 ± 2.2	19.8 ± 7.4	0.561
periodontal pocket depth			
· 1～3mm —（ %）	97.9 ± 1.8	71.4 ± 4.5	0.021
· 4～6mm —（ %）	2.0 ± 1.8	27.2 ± 4.3	0.021
· over 7mm —（ %）	0.2 ± 0.3	2.9 ± 2.1	0.026
Sites with gingival bleeding (%)	7.8 ± 8.1	24.0 ± 12.8	0.081
Serume IgG Antibody Titer Test against Periodontal Bacteria			
· *Aggregatibacter actinomycetemcomitans* (*Y4*)	-0.06 ± 0.49	1.85 ± 1.33	0.021
· *Aggregatibacter actinomycetemcomitans* (*ATCC29523*	0.02 ± 0.38	1.96 ± 0.83	0.021
· *Aggregatibacter actinomycetemcomitans* (*SUNY67*)	0.04 ± 0.71	2.35 ± 1.21	0.021
· *Capnocytophaga ochracea* (*S3*)	-2.70 ± 2.53	1.94 ± 3.08	0.043
· *Eichenerra corrodens* (*FDC1073*)	-0.43 ± 0.91	0.43 ± 1.68	0.564
· *Fusobacterium nucleatum* (*ATCC25586*)	-0.11 ± 1.52	2.42 ± 3.82	0.248
· *Prevotella intermedia* (*ATCC33563*)	-0.95 ± 1.44	-0.63 ± 0.63	0.248
· *Prevotella intermedia* (*ATCC25611*)	-0.22 ± 0.98	0.50 ± 1.37	0.773
· *Porphyromonas gingivalis* （ *FDC 381*）	-0.98 ± 0.31	1.83 ± 1.63	0.021
· *Porphyromonas gingivalis* （ *SU 63*）	-0.70 ± 0.46	2.58 ± 4.25	0.083
· *Treponema denticola* (*ATCC35405*)	-0.03 ± 2.14	0.37 ± 1.94	0.564
· *Campylobacter rectus* (*ATCC33238*)	3.44± 6.40	17.35 ± 18.47	0.083
· *Bacteroides forsythus* (*ATCC43037*)	-0.90 ± 0.25	-0.41 ± 0.80	0.248

172

173 Statistical analysis

174 The statistical analysis was performed using the Mann-Whitney *U* Test. A *P*-value of
175 0.05 was considered significant and was determined using SPSS Ver. 23 (SPSS Inc.,
176 Chicago, IL, USA) for all the experimental results.

177

178 Results

179 The participants' characteristics are presented in Table 1. There were no significant
180 differences between both groups in terms of age, sex, other disease such as diabetes, and
181 markers of inflammation and cholesterol. The periodontal disease conditions of the
182 participants are presented in Table 2. In the control group, the ratio of PPD was < 3 mm,
183 while the ratio of PPD in the periodontitis group was significantly higher i.e., > 4 mm.
184 Serum IgG antibody titer was significantly higher in those with periodontitis that in
185 control group for Aa Y4, Aa ATCC29523, Aa SUNY67, Co S3, and Pg FDC381.

186

187 Characterization of microbiome in ST, GP, and AP

188 The microbiome pattern of ST was relatively similar between control samples and
189 periodontitis samples (Fig. 1A). Among them, the ratio of *Filifactor* sp., which was
190 reported to be virulent [29], was significantly higher in periodontitis patients than in the
191 respective controls (Fig. 1B).
192
193 **Fig. 1. Characterization of microbiome in ST**
194 The average ratio of the bacteria in ST from control and periodontitis patient samples
195 is presented. (A) Bacterial genera are indicated. (B) Thirteen bacterial species were highly
196 detected by NGS analysis. * indicates $P < 0.05$; Mann-Whitney *U* Test.
197
198 The microbiome pattern of GP was notably different between the control and
199 periodontitis samples (Fig. 2A). The ratio of *Rothia* sp. and *Neisseria* sp., which exist in
200 a healthy oral cavity, were lower in periodontitis than in control samples. Conversely, the
201 ratios of *Fusobacterium* sp. and *Filifactor* sp., which are present in the periodontitis oral
202 cavity, were higher in periodontitis than in control samples (Fig. 2A, B). The ratio of

203 *Desulfobulbus* sp., which was detected in the periodontal pocket in a recent report [30],

204 was significantly higher in periodontitis samples than in controls (Fig. 2B).

205

206 **Fig. 2. Characterization of microbiome in GP**

207 The average ratios of the bacteria in GP from control and periodontitis samples are shown.

208 (A) Bacterial genera are indicated. (B) Thirteen bacteria that were highly detected from

209 the NGS analysis. * indicates $P < 0.05$; Mann-Whitney *U* Test.

210

211 The majority of the bacteria found in the AP microbiome belonged to the soil bacterial

212 families *Burkholderiales*, *Bacillale*, and *Rhizobiales*. Their ratios were similar between

213 periodontitis and control patients (Fig. 3). The ratio of *Sphingomonadales,* which is a

214 normal constituent of the human bacteria flora, was higher in periodontitis samples than

215 in control samples.

216

217 **Fig. 3. Characterization of microbiome in AP**

The average ratio of the bacteria in GP from our patients (control and periodontitis samples) is depicted and the order of the bacteria is shown. Thirteen bacteria were every evident from the NGS analyses.

Comparison of PCA results between controls and periodontitis samples

Seventy five percent of the GP bacteria from periodontitis and control samples were positioned in the center right side of the PCA graph (separated by a red solid circle) and center of the plot (separated by a blue solid circle), respectively (Fig. 4). The two circular locations were sufficiently separated. ST bacteria from periodontitis samples were located in the center left side of the panel (separated by a red dotted circle). This position was slightly more toward to the right side than that of control samples (separated by a blue dotted circle). These two circular locations were comparatively closer. The bacteria in AP were located towards the lower middle region of the plot (separated by a green solid circle), and the control and periodontitis samples could not be clearly distinguished. The AP bacteria were located far from the oral samples (ST and GP).

Fig. 4. Comparison of PCA results between control and periodontitis samples

234 The PCA results from each sample are identified tagged as follows: □: ST; Saliva from
235 tongue surface, ○: GP; Gingival pocket, Δ: AP; Atheromatous plaques; controls: blue
236 color; periodontitis: red color; three GP bacteria from periodontitis and one GP from
237 controls, blue solid circle; three GP bacteria from controls and one GP from periodontitis,
238 red dotted circle; ST bacteria from periodontitis, blue dotted circle; ST bacteria from
239 controls, green solid circle; AP samples from both groups.

240

241 Comparison of clustering analysis results between control and
242 periodontitis samples

243 Similar to the PCA results, bacteria from oral samples (ST and GP) and AP were
244 completely different (Fig. 5). However, 75% of AP bacteria from periodontitis were
245 located at the lower part in the cluster and 75% of AP bacteria from controls were located
246 at the upper part of the periodontitis samples.

247

248 **Fig. 5. Comparison of clustering analysis results between control and periodontitis**
249 **samples**

The clustering analysis results from each sample were tagged as follows: ST; Saliva from tongue surface, GP; Gingival pocket, AP; Atheromatous plaques. Blue text represents control samples and red text specifies periodontitis samples.

Co-occurrence analysis of microbiome in AP

We evaluated the correlation of the microbiome in AP between the control and periodontitis group. In both groups, the major bacteria of the network were *Agrobacterium* sp., *Delftia* sp., and *Rhizobium* sp. This echoed a previous report [26]. However, the network around *Cutibacterium acnes* was different between control and periodontitis samples (Fig. 6).

Discussion

The human body is a complex habitat for about 1,000 species and 100-1000 trillion bacteria, wherein approximately 100 million bacteria specifically reside exclusively in the oral cavity [31]. Periodontitis is an infection caused by the members of the microbiome in the oral cavity, and is related to a number of systematic diseases. However, the detailed mechanism underlying this infection is still not completely understood [17].

As pathogenic factors for periodontitis, Red complex species (*P. gingivalis*, *T. denticola,* and *T. forsythia*) have been the focus of functional investigations [32]. Although there is no doubt regarding their relationship to periodontitis development, the microbiome is likely not comprised just of the pathogenic bacteria, but includes a mixture of various and diverse species of bacteria, with the total population ultimately affecting the development of this disease [33]. It has been suggested that 17 novel bacteria including *Filifactor alosis* probably induce periodontitis, even though these bacteria were not previously thought to be periodontitis-specific pathogenic bacteria [34].

The normal bacterial flora in the oral cavity, which was previously disregarded as insignificant, is actually very crucial for periodontitis development or progression. In general, pathogenic bacteria, such as *P. gingivalis*, configure the microbiome with the normal bacteria flora [29]. If the balance of pathogenic and normal bacteria in the microbiome is lost for some reason, the microbiome increases its pathogenicity and induces the disease. Therefore, a comprehensive microbiome analysis is necessary to investigate normal as well as pathogenic bacteria composition.

281 In this study, we performed a comprehensive microbiome analysis of the internal
282 carotid artery stenosis in patients affected with and without periodontitis. We harvested
283 bacteria samples from TS, GP, and AP from each patient and performed NGS analysis.
284 This analysis showed that the microbiome in the oral cavity was more numerous for
285 *Fusobacterium* sp. and *Filifactor* sp., which are periodontal bacterial pathogens, in the
286 periodontitis group compared to the control group. In particular, the ratio of *Filifactor* sp.
287 in TS was significantly higher in the periodontitis group in comparison to the control
288 group. Conversely, the ratio of *Rothia* sp and *Neisseria* sp in GP, which are constituents
289 of the normal bacterial flora in a healthy oral cavity, was lower in the periodontitis group
290 than in the control group [35, 36]. Thus, remarkably, the ratio of normal bacteria in GP
291 and TS decreased while that of pathogenic bacteria increased.

292 To investigate the possibility that periodontal bacteria might contribute to
293 atheromatous plaque formation directly on the vascular wall by hematogenous spread,
294 NGS analysis was done using the atheromatous plaque samples. Previous reports
295 established that *P. gingivalis* induces the expression of vascular cell adhesion molecule 1
296 from vascular endothelial cells, and promotes thrombus formation by macrophage

297 invasion into blood vessels, resulting in platelet aggregation [37, 38]. Another report
298 demonstrated that *P. gingivalis* infection accelerates the progression of atherosclerosis in
299 a heterozygous apolipoprotein E-deficient murine model [20]. Presently, oral bacteria
300 were barely detectable in AP, regardless of the presence or absence of periodontitis. The
301 patterns of the microbiome in AP were entirely different in TS and GP. A prior study
302 reported detection of some oral bacteria in the atheromatous plaque [39]. However, other
303 authors reported that *P. gingivalis* infection in an animal model induced atheromatous
304 plaque formation, although it was actually not detected in the atheromatous plaque [40].
305 Our data enables us to conclude that it is unlikely that the oral bacteria spread
306 hematogenously and directly induce the formation of atheromatous plaque on the aortic
307 wall.

308 The co-occurrence analysis of the microbiome in AP revealed the most significant
309 bacteria were *Agrobacterium* sp.*, Delftia* sp.*,* and *Rhizobium* sp.*,* which constituted the
310 network in both groups. Although these are soil bacteria, they were also previously
311 detected in AP [26]. Another significant bacterium, *C. acnes,* configured the network in
312 the control group. The relationship with *C. acnes* was different among the control and

313 periodontitis groups. This bacterium is categorized as a normal bacterium present on the
314 skin and in the gut, although it was also detected in AP [41]. *C. acnes* reportedly can
315 cause sarcoidosis, sepsis, and infective endocarditis, and heat-killed *C. acnes* render mice
316 very susceptible to lipopolysaccharide (LPS) toxicity. *C. acnes* also promote the
317 production of cytokines, such as interleukin-12, interferon-gamma, and Toll-like receptor
318 4 [42]. Presently, it is conceivable that LPS produced by periodontal bacteria activated
319 *C. acnes* in the blood vessels, which then formed the atheromatous plaque. In this scenario,
320 the difference of the network in AP between the control and periodontitis samples might
321 be caused by LPS that is spread hematogenously, as well as by the chronic inflammatory
322 effect. Recently, it was reported that the production of trimethylamine-N-oxide, which
323 promotes atherosclerosis, depends upon the metabolism of the intestinal microbiome [43].
324 The prior and present data indicate that the loss of microbiome balance in the human body
325 affects the development of atherosclerosis. Periodontitis has a great effect on the
326 microbiome configuration in the oral cavity and promotes the formation of various
327 metabolic products. However, this detailed mechanism of atherosclerosis development

328 remains largely unknown. In a further study, we intend to investigate the relationship
329 between periodontitis and atherosclerosis.

330 Conclusion

331 The ratio of oral bacteria in AP was remarkably low, and the microbiome pattern was
332 entirely different from that found in the oral microbiome. In other words, oral bacteria
333 did not directly induce the atheromatous plaque configuration. However, the microbiome
334 pattern and the correlation of the microbiome in AP were different between the controls
335 and periodontitis samples. Thus, metabolic products of the microbiome, or the host's
336 inflammatory response, might indirectly affect the atheromatous plaque configuration.

337

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References

1. Grogan D. The microbes within. Nature. 2015;518(7540): S2. doi: 10.1038/518S2a.

2. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature. 2006;444(7122): 1027-1031. doi: 10.1038/nature05414.

3. Sharon G, Garg N, Debelius J, Knight R, Dorrestein PC, Mazmanian SK. Specialized metabolites from the microbiome in health and disease. Cell Metab. 2014;20(5): 719-730. Epub 2014/11/04. doi: 10.1016/j.cmet.2014.10.016.

4. Griffen AL, Beall CJ, Campbell JH, Firestone ND, Kumar PS, Yang ZK, et al. Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. ISME J. 2012;6(6): 1176-1185. Epub 2011/12/15. doi: 10.1038/ismej.2011.191.

5. Maddi A, Scannapieco FA. Oral biofilms, oral and periodontal infections, and

359 systemic disease. Am J Dent. 2013;26(5): 249-254. PubMed PMID: 24479275.

360 6. Lalla E, Papapanou PN. Diabetes mellitus and periodontitis: a tale of two common
361 interrelated diseases. Nat Rev Endocrinol. 2011;7(12): 738-748. Epub 2011/06/28.
362 doi: 10.1038/nrendo.2011.106.

363 7. Senba T, Kobayashi Y, Inoue K, Kaneto C, Inoue M, Toyokawa S, et al. The
364 association between self-reported periodontitis and coronary heart disease--from
365 MY Health Up Study--. J Occup Health. 2008;50(3): 283-287. Epub 2008/04/15.

366 8. Lafon A, Pereira B, Dufour T, Rigouby V, Giroud M, Béjot Y, et al. Periodontal
367 disease and stroke: a meta-analysis of cohort studies. Eur J Neurol. 2014;21(9):
368 1155-1161, e66-7. Epub 2014/04/08. doi: 10.1111/ene.12415.

369 9. Zeng XT, Deng AP, Li C, Xia LY, Niu YM, Leng WD. Periodontal disease and risk
370 of head and neck cancer: a meta-analysis of observational studies. PLoS One.
371 2013;8(10): e79017. Epub 2013/10/23. doi: 10.1371/journal.pone.0079017.

372 10. Yoneda M, Naka S, Nakano K, Wada K, Endo H, Mawatari H, et al. Involvement of
373 a periodontal pathogen, Porphyromonas gingivalis on the pathogenesis of non-
374 alcoholic fatty liver disease. BMC Gastroenterol. 2012;12: 16. Epub 2012/02/16.

375 doi: 10.1186/1471-230X-12-16. PubMed PMID: 22340817; PubMed Central

376 11. Sanz M, Kornman K; working group 3 of the joint EFP/AAP workshop.
377 Periodontitis and adverse pregnancy outcomes: consensus report of the Joint
378 EFP/AAP Workshop on Periodontitis and Systemic Diseases. J Periodontol.
379 2013;84(4 Suppl): S164-169. doi: 10.1902/jop.2013.1340016.

380 12. Isoshima D, Yamashiro K, Matsunaga K, Shinobe M, Nakanishi N, Nakanishi I, et
381 al. Assessment of pathogenesis of infective endocarditis by plasma IgG antibody
382 titer test against periodontal bacteria. Clin Case Rep. 2017;5(10): 1580-1586. Epub
383 2017/08/17. doi: 10.1002/ccr3.1066.

384 13. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med. 1999;340(2): 115-
385 126. doi: 10.1056/NEJM199901143400207.

386 14. Bahekar AA, Singh S, Saha S, Molnar J, Arora R. The prevalence and incidence of
387 coronary heart disease is significantly increased in periodontitis: a meta-analysis.
388 Am Heart J. 2007;154(5): 830-837. Epub 2007/08/20. doi:
389 10.1016/j.ahj.2007.06.037.

390 15. Ishihara K, Nabuchi A, Ito R, Miyachi K, Kuramitsu HK, Okuda K. Correlation

391 between detection rates of periodontopathic bacterial DNA in coronary stenotic
392 artery plaque [corrected] and in dental plaque samples. J Clin Microbiol. 2004;42(3):
393 1313-1315.
394 16. Okuda K, Ishihara K, Nakagawa T, Hirayama A, Inayama Y. Detection of
395 Treponema denticola in atherosclerotic lesions. J Clin Microbiol. 2001;39(3): 1114-
396 1117. doi: 10.1128/JCM.39.3.1114-1117.2001.
397 17. Lockhart PB, Bolger AF, Papapanou PN, Osinbowale O, Trevisan M, Levison ME,
398 et al. Periodontal disease and atherosclerotic vascular disease: does the evidence
399 support an independent association?: a scientific statement from the American Heart
400 Association. Circulation. 2012;125(20): 2520-2544. Epub 2012/04/18. doi:
401 10.1161/CIR.0b013e31825719f3.
402 18. Hujoel PP, Drangsholt M, Spiekerman C, DeRouen TA. Periodontal disease and
403 coronary heart disease risk. JAMA. 2000;284(11): 1406-1410.
404 19. Peacock ME, Carson RE. Frequency of self-reported medical conditions in
405 periodontal patients. J Periodontol. 1995;66(11): 1004-1007. doi:
406 10.1902/jop.1995.66.11.1004.

20. Li L, Messas E, Batista EL, Levine RA, Amar S. Porphyromonas gingivalis infection accelerates the progression of atherosclerosis in a heterozygous apolipoprotein E-deficient murine model. Circulation. 2002;105(7): 861-867.

21. Kurihara N, Inoue Y, Iwai T, Umeda M, Huang Y, Ishikawa I. Detection and localization of periodontopathic bacteria in abdominal aortic aneurysms. Eur J Vasc Endovasc Surg. 2004;28(5): 553-558. doi: 10.1016/j.ejvs.2004.08.010.

22. Papapanou PN, Sellén A, Wennstr JL, Dahlén G. An analysis of the subgingival microflora in randomly selected subjects. Oral Microbiol Immunol. 1993;8(1): 24-29.

23. Goodrich JK, Waters JL, Poole AC, Sutter JL, Koren O, Blekhman R, et al. Human genetics shape the gut microbiome. Cell. 2014;159(4): 789-799. doi: 10.1016/j.cell.2014.09.053.

24. Buffie CG, Bucci V, Stein RR, McKenney PT, Ling L, Gobourne A, et al. Precision microbiome reconstitution restores bile acid mediated resistance to Clostridium difficile. Nature. 2015;517(7533): 205-208. Epub 2014/10/22. doi: 10.1038/nature13828.

423 25. Kudo C, Naruishi K, Maeda H, Abiko Y, Hino T, Iwata M, et al. Assessment of the
424 plasma/serum IgG test to screen for periodontitis. J Dent Res. 2012;91(12): 1190-
425 1195. Epub 2012/09/26. doi: 10.1177/0022034512461796.
426 26. Ziganshina EE, Sharifullina DM, Lozhkin AP, Khayrullin RN, Ignatyev IM,
427 Ziganshin AM. Bacterial Communities Associated with Atherosclerotic Plaques
428 from Russian Individuals with Atherosclerosis. PLoS One. 2016;11(10): e0164836.
429 Epub 2016/10/13. doi: 10.1371/journal.pone.0164836.
430 27. Shi W, Qin M, Chen F, Xia B. Supragingival microbial profiles of permanent and
431 deciduous teeth in children with mixed dentition. PLoS One. 2016;11(1): e0146938.
432 Epub 2016/01/11. doi: 10.1371/journal.pone.0146938.
433 28. Murayama Y, Nagai A, Okamura K, Kurihara H, Nomura Y, Kokeguchi S, et al.
434 Serum immunoglobulin G antibody to periodontal bacteria. Adv Dent Res.
435 1988;2(2): 339-345. doi: 10.1177/08959374880020022401.
436 29. Pérez-Chaparro PJ, Gonçalves C, Figueiredo LC, Faveri M, Lobão E, Tamashiro N,
437 et al. Newly identified pathogens associated with periodontitis: a systematic review.
438 J Dent Res. 2014;93(9): 846-858. Epub 2014/07/29. doi:

439 10.1177/0022034514542468.

440 30. Oliveira RR, Fermiano D, Feres M, Figueiredo LC, Teles FR, Soares GM, et al.
441 Levels of candidate periodontal pathogens in subgingival biofilm. J Dent Res.
442 2016;95(6): 711-718. Epub 2016/03/02. doi: 10.1177/0022034516634619.

443 31. Sender R, Fuchs S, Milo R. Revised estimates for the number of human and bacteria
444 cells in the body. PLoS Biol. 2016;14(8): e1002533. Epub 2016/08/19. doi:
445 10.1371/journal.pbio.1002533.

446 32. Socransky SS, Smith C, Haffajee AD. Subgingival microbial profiles in refractory
447 periodontal disease. J Clin Periodontol. 2002;29(3): 260-268.

448 33. Page RC, Kornman KS. The pathogenesis of human periodontitis: an introduction.
449 Periodontol 2000. 1997;14: 9-11.

450 34. Takeshita T, Nakano Y, Kumagai T, Yasui M, Kamio N, Shibata Y, et al. The
451 ecological proportion of indigenous bacterial populations in saliva is correlated with
452 oral health status. ISME J. 2009;3(1): 65-78. Epub 2008/10/02. doi:
453 10.1038/ismej.2008.91.

454 35. Wang J, Qi J, Zhao H, He S, Zhang Y, Wei S, et al. Metagenomic sequencing reveals

455 microbiota and its functional potential associated with periodontal disease. Sci Rep.
456 2013;3: 1843. doi: 10.1038/srep01843.
457 36. Takeshita T, Matsuo K, Furuta M, Shibata Y, Fukami K, Shimazaki Y, et al. Distinct
458 composition of the oral indigenous microbiota in South Korean and Japanese adults.
459 Sci Rep. 2014;4: 6990. Epub 2014/11/11. doi: 10.1038/srep06990.
460 37. Khlgatian M, Nassar H, Chou HH, Gibson FC, Genco CA. Fimbria-dependent
461 activation of cell adhesion molecule expression in Porphyromonas gingivalis-
462 infected endothelial cells. Infect Immun. 2002;70(1): 257-267.
463 38. Imamura T, Travis J, Potempa J. The biphasic virulence activities of gingipains:
464 activation and inactivation of host proteins. Curr Protein Pept Sci. 2003;4(6): 443-
465 450.
466 39. Toyofuku T, Inoue Y, Kurihara N, Kudo T, Jibiki M, Sugano N, et al. Differential
467 detection rate of periodontopathic bacteria in atherosclerosis. Surg Today.
468 2011;41(10): 1395-1400. Epub 2011/09/16. doi: 10.1007/s00595-010-4496-5.
469 40. Jain A, Batista EL, Serhan C, Stahl GL, Van Dyke TE. Role for periodontitis in the
470 progression of lipid deposition in an animal model. Infect Immun. 2003;71(10):

6012-6018. PubMed PMID: 14500522; PubMed Central PMCID: PMCPMC201045.

41. Koren O, Spor A, Felin J, Fåk, F, Stombaugh J, Tremaroli V, et al. Human oral, gut, and plaque microbiota in patients with atherosclerosis. Proc Natl Acad Sci U S A. 2011;108 Suppl 1: 4592-4598. Epub 2010/10/11. doi: 10.1073/pnas.1011383107.

42. Tsutsui H, Imamura M, Fujimoto J, Nakanishi K. The TLR4/TRIF-mediated activation of NLRP3 inflammasome underlies endotoxin-induced liver injury in mice. Gastroenterol Res Pract. 2010;2010: 641865. Epub 2010/06/16. doi: 10.1155/2010/641865.

43. Tang WH, Wang Z, Levison BS, Koeth RA, Britt EB, Fu X, et al. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. N Engl J Med. 2013;368(17): 1575-1584. doi: 10.1056/NEJMoa1109400.

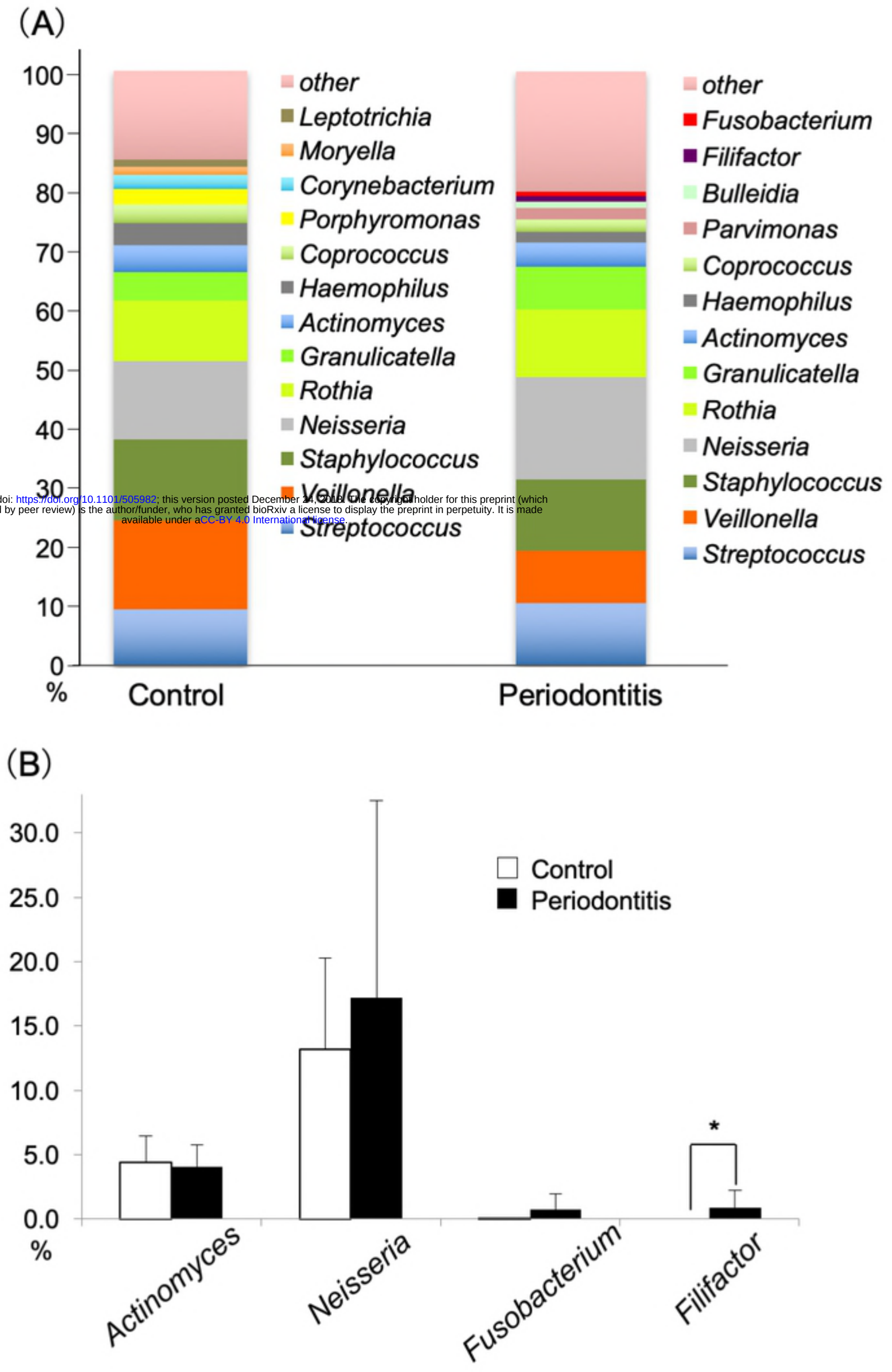


Figure 1

Figure 1

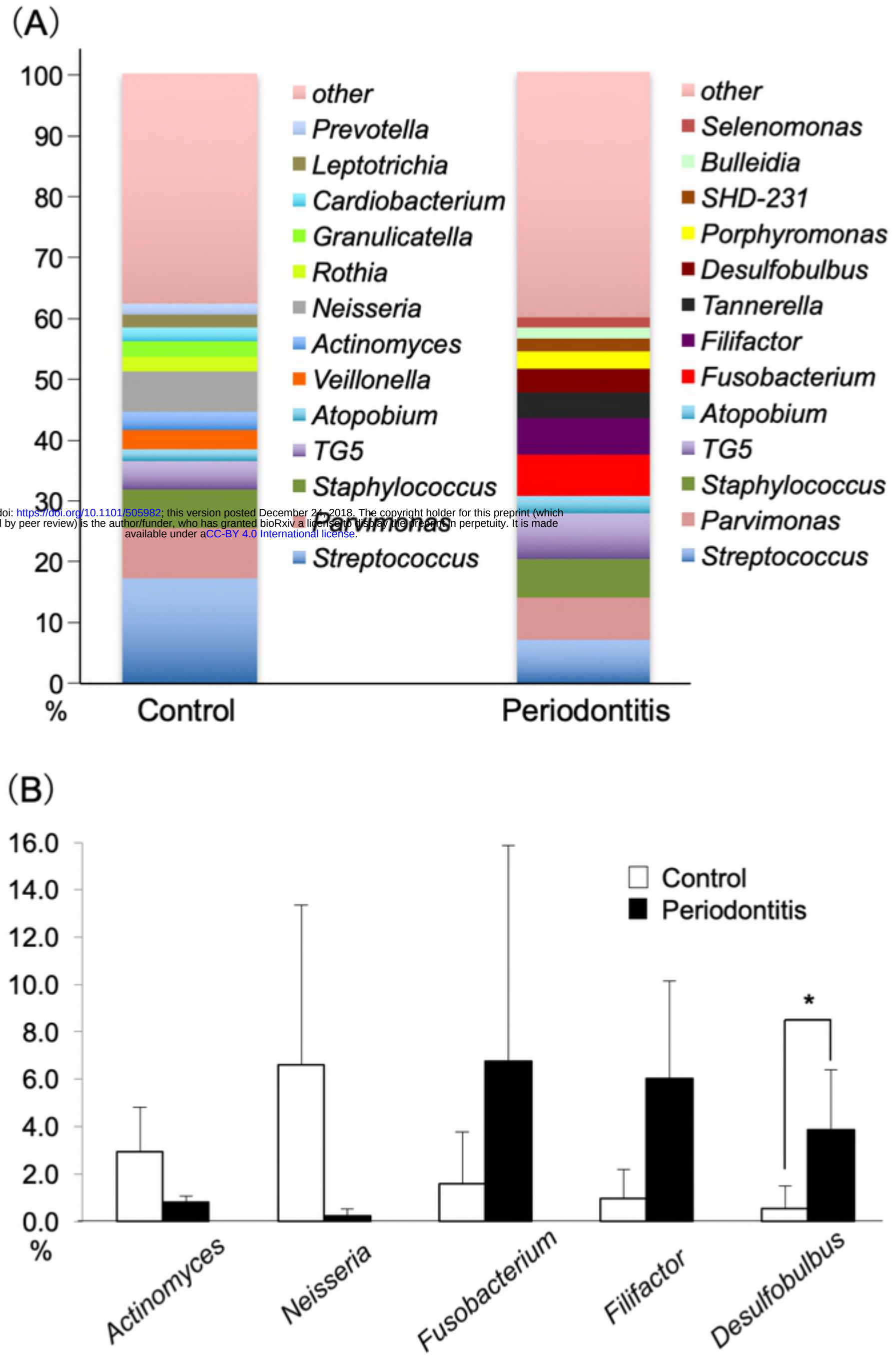


Figure 2

Figure 2

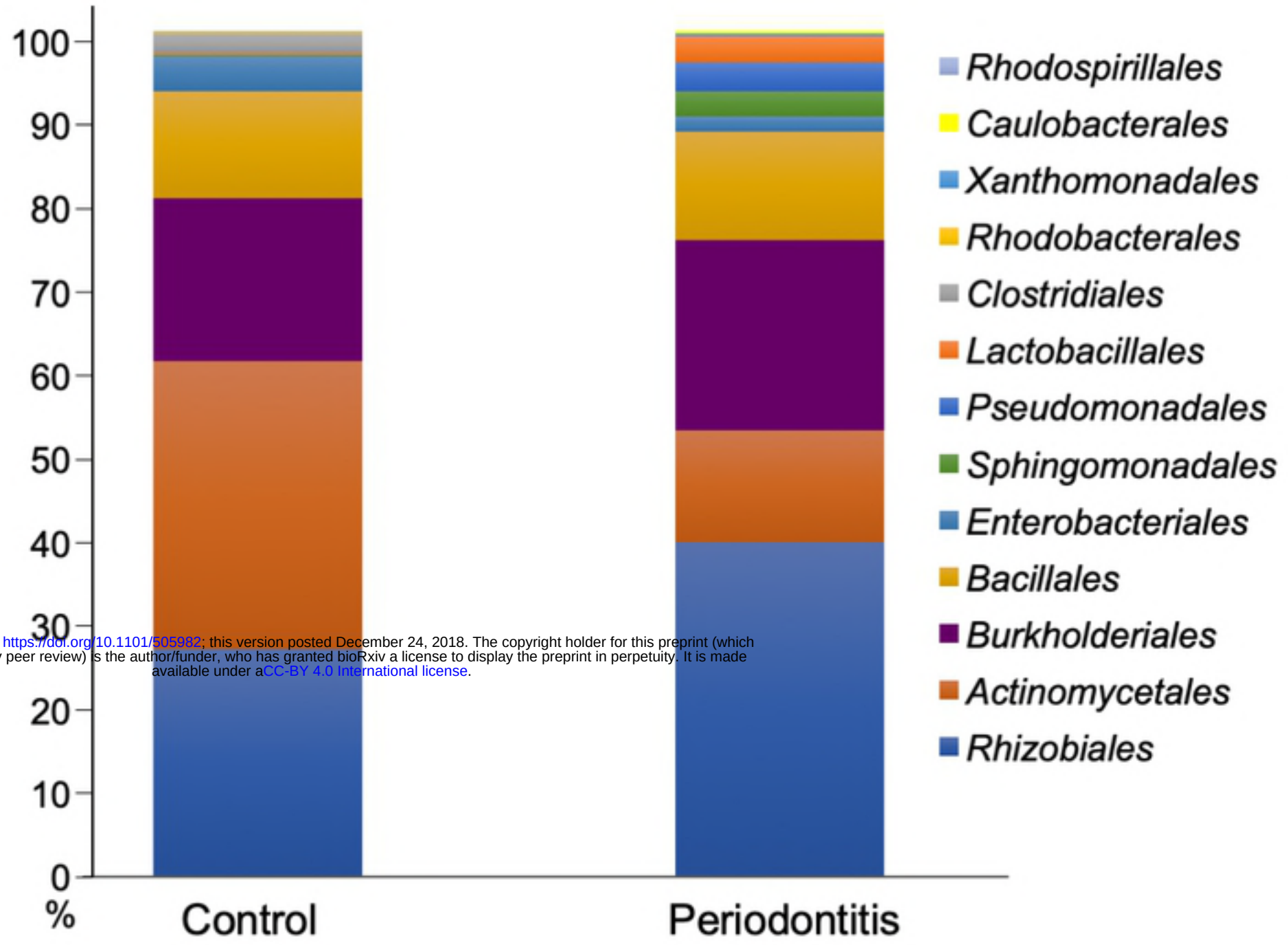


Figure 3

Figure 3

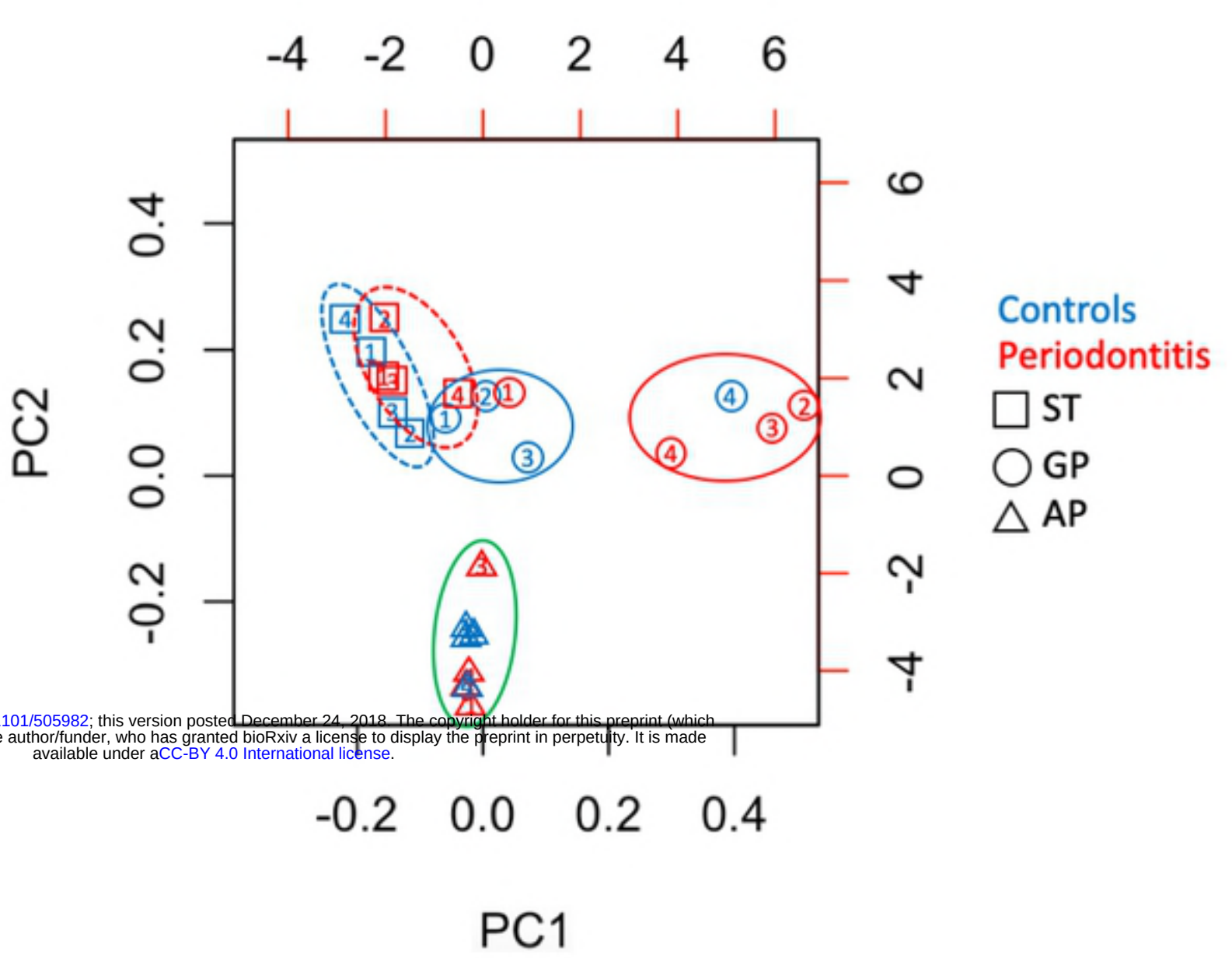


Figure 4

Figure 4

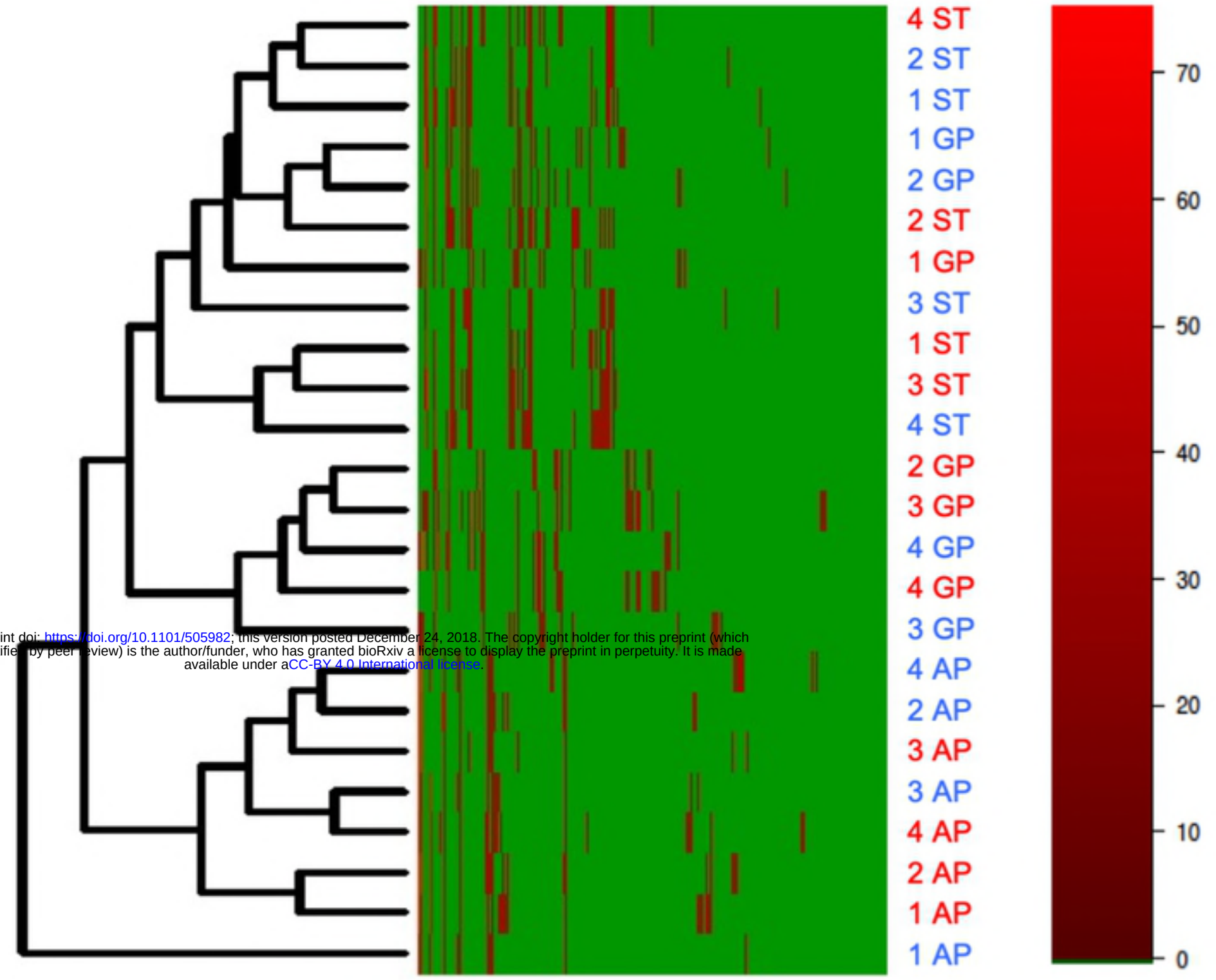
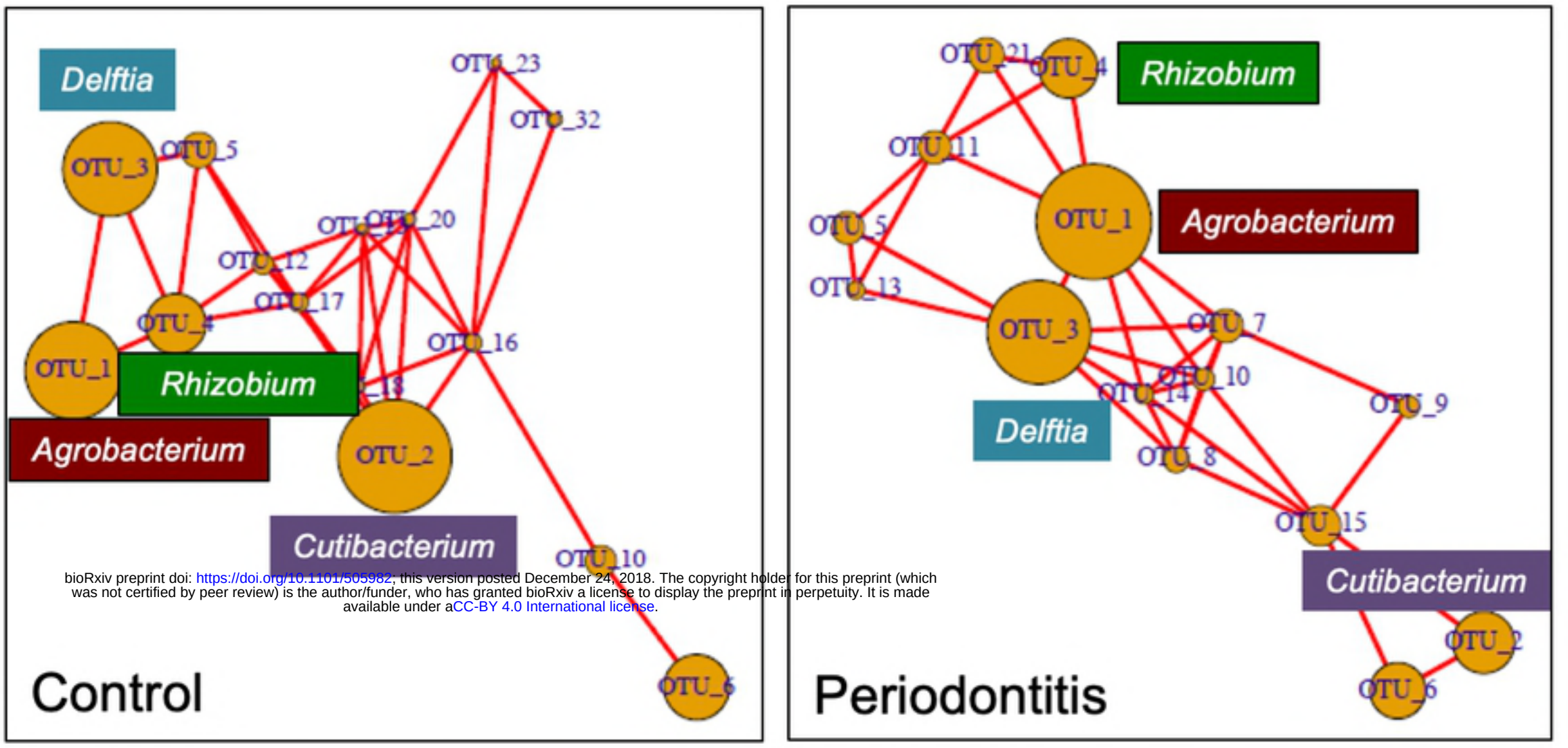


Figure 5

Figure 5



OUT_1:o__*RhizobialeST*. g__*Agrobacterium*
OTU_2:o__*ActinomycetaleST*. g__*GProGPionibacterium*
OUT_3:o__*BurkholderialeST*. g__*Delftia*
OUT_4:o__*RhizobialeST*. g__*Rhizobium*

Figure 6

Figure 6