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# Microbial diversity of supra- and subgingival biofilms on freshly colonized titanium implant abutments in the human mouth

W. Heuer, M. Stiesch, W. R. Abraham

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1 **Microbial diversity of supra- and subgingival biofilms on freshly colonized titanium implant abutments in**  
2 **the human mouth**  
3

4 *W. Heuer<sup>1</sup>, M. Stiesch<sup>1</sup>, W. R. Abraham<sup>2</sup>*

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6 *<sup>1</sup>Department of Prosthetic Dentistry and Biomedical Materials Science, Hannover Medical School, Hannover,*  
7 *Germany*

8 *<sup>2</sup>Helmholtz Centre for Infection Research, Chemical Microbiology, Braunschweig, Germany*

9

10

11 **Correspondence to:**

12 Dr. Wolf-Rainer Abraham

13 Helmholtz Centre for Infection Research

14 Chemical Microbiology

15 Inhoffenstrasse 7

16 38124 Braunschweig, Germany

17 Tel.: +49-531-6181-4300/-4226

18 Fax: +49-531-6181-4699

19 E-mail: [Wolf-Rainer.Abraham@helmholtz-hzi.de](mailto:Wolf-Rainer.Abraham@helmholtz-hzi.de)

20

21

22 **Abstract:**

23 **Background:** Supra- and subgingival biofilm formation is considered to be mainly responsible for early implant  
24 failure caused by inflammations of periimplant tissues. Nevertheless, little is known about the complex microbial  
25 diversity and interindividual similarities around dental implants.

26 **Objectives:** An atraumatic assessment of the diversity of microbial communities around titanium implants by  
27 single strand conformation polymorphism (SSCP) analysis of the 16S rRNA gene amplicons and subsequent  
28 sequence analysis.

29 **Methods:** Samples of adherent supra- and subgingival peri-implant biofilms were collected from ten patients.  
30 Additionally, samples of sulcusfluid were taken at titanium implant abutments and remaining teeth. The bacteria  
31 in the samples were characterized by SSCP and sequence analysis.

32 **Results:** A high diversity of bacteria varying between patients and within one patient with location was found.  
33 Bacteria characteristic for sulcusfluid and supra- and subgingival biofilm communities were identified.  
34 Sulcusfluid of the abutments showed higher abundance of *Streptococcus* species than from residual teeth.  
35 *Prevotella* and *Rothia* species frequently reported from the oral cavity were not detected at the abutments  
36 suggesting a role as late colonizers.

37 **Conclusion:** Different niches in the human mouth are characterized by specific groups of bacteria. Implant  
38 abutments are a very valuable approach to study dental biofilm development *in vivo*.

39

40

41

42 **Keywords:**

43 Oral implants, single strand conformation polymorphism, microbial diversity, implant abutments, biofilm,  
44 microbial communities

45 **Introduction:**

46           The clinical success of osseointegrated oral implants led to extended application. Today many  
47 conventionally prosthetic treatments have been replaced by implant supported prosthetic supraconstructions.  
48 From a clinical viewpoint this fact is supported by multitudinous studies which analyzed structural and  
49 functional improvements of the implant surface. While the problem of primary osseointegration is solved in a  
50 high quality manner, inflammation of peri-implant tissues by the presence of bacterial biofilms is considered  
51 currently as one of the principle problems in oral implantation and, therefore, responsible for early implant  
52 failure. This early implant failures are clinically characterized by inflammation of the peri-implantary mucosa  
53 (Figure 1a) and a subsequent destruction of the peri-implantary bone (Figure 1b).

54           The early processes of biofilm formation, like generation of an acquired pellicle by salivary  
55 biopolymers or enzymes and adherence of initial microorganisms, is described as is the relationship between  
56 biofilm formation and periodontitis or periimplantitis. For example, *Streptococcus mitis*, *S. sanguinis* and *S.*  
57 *oralis* are known to create the pre-conditions for the accumulation of gram-negative anaerobic bacteria [1-5].  
58 Several studies were able to indicate, that during periimplant biofilm formation the microbial composition  
59 shifted towards a higher proportion of such gram-negative anaerobic microbiota [6-10]. However, these  
60 previously studies of microorganisms associated with diseases relied heavily upon cultivation or DNA  
61 checkerboard hybridization methods. The applied techniques are associated with the main disadvantages of a  
62 specific bacterial identification or time-consuming and fault-prone cultivation [11]. This led to the identification  
63 of several species as putative periodontopathogens based on frequency of their isolation in lesion sites. Among  
64 these are *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*,  
65 *Bacteroides forsythus*, *Treponema denticola*, *Fusobacterium nucleatum*, *Parvimonas micra*, *Campylobacter*  
66 *rectus*, *Eikenella corrodens* and *Selenomonas sputigena* [12-15]. However, concentrating only on those bacteria  
67 which can currently be cultivated and ignoring particularly those which cultivation fails to capture [16] will not  
68 give a complete picture of infections leading to periodontitis or periimplantitis [17]. Because of these limitations,  
69 it is also difficult to acquire a complete understanding of the dynamic changes of oral microbes as dental  
70 biofilms turn from harmless ones into detrimental microbial communities. Answers to this challenge are culture-  
71 independent methods to monitor microbial communities over space and time.

72           Therefore, the objective of the present study was to assess the diversity of microbial communities, by  
73 culture-independent methods, of initial periimplant biofilms in supra- and subgingival areas, to compare them  
74 with those of the sulcus fluid and to identify microbial communities posing potential problems for infections. In

75 addition, a sample of sulcusfluid was taken at one remaining tooth in the same jaw to evaluate cross-infections  
76 between implants and teeth in the initial phase of biofilm formation.

77

78

79 **Material and methods:**

80 *Subjects:*

81 The present study was approved by the ethics committee of Hannover Medical School (No. 3791). The  
82 examination was performed with the understanding and written consent of each patient.

83 Qualitative analysis was based on 10 healing abutments which were inserted for 14 days into 10  
84 patients, 6 women and 4 men, aged between 60 and 83 years (mean 70 years  $\pm$  6.7 years). Patients were selected  
85 to fulfil the following inclusion criteria: no systemic illness, no smoking, no pregnancy, no active periodontitis  
86 (probing pocket depth  $\leq$  4 mm), no pharmacological treatment or antibiotic therapy during or up to four months  
87 before the study. At the day of abutment insertion all patients received dental hygiene instructions but no  
88 professional tooth cleaning was performed during the observation period.

89 All patients were partially edentulous and had at least one oral two-piece implant made of titanium  
90 (Astra Tech, Mölndal, Sweden), which had been inserted three months before investigation. Two weeks after  
91 abutment surgery, the previously existing abutments were removed and new sterile healing abutments (Astra  
92 Tech, Mölndal, Sweden) for the analysis were inserted (Figure 2). Thereby no trimming of surrounding gingiva  
93 was performed.

94

95

96 *Sampling:*

97 Two weeks after insertion of the new sterile healing abutments, samples of adherent biofilm and  
98 crevicular fluid were taken. The periimplant saliva was removed by use of an air spray and cotton rolls. First,  
99 samples of crevicular fluid were taken with sterile paper points at abutments and remaining teeth. Afterwards,  
100 samples of adherent supragingival biofilm on the abutments were taken with sterile sponges. The samples of  
101 adherent subgingival biofilm could be collected after abutment removal. All samples were stored in sample tubes  
102 (Eppendorf AG) at -80 °C.

103

104 *DNA analysis:*

105 DNA was extracted using the Fast-DNA-Spin-Kit for soil (Bio 101, CA, USA) following the  
106 instructions of the manufacturer. The primers chosen for the amplification of bacterial 16S rRNA genes were  
107 forward primer Com1 (5'CAGCAGCCGCGGTAATAC3') and reverse primer Com2-Ph  
108 (5'CCGTCAATTCCTTTGAGTTT3' with 5'-terminal phosphate group) as published by Schwieger and Tebbe  
109 [18]. To obtain single-stranded DNA the phosphorylated strand of the PCR products was digested by lambda  
110 exonuclease (New England Biolabs, Schwalbach, Germany) following the protocol of Schwieger and Tebbe, and  
111 further purified using the Mini-elute-Kit (Qiagen, Hilden, Germany) as recommended by the manufacturer, and  
112 the remaining single stranded DNA was dried under vacuum. The DNA was then resuspended in denaturing  
113 SSCP loading buffer (47.5 % formamide, 5 mM sodium hydroxide, 0.12 % bromophenol blue and 0.12 % xylene  
114 cyanol), incubated at 95°C for 2 min and immediately cooled on ice. After 5 min, samples were loaded onto the  
115 gels and subjected to electrophoresis. The samples were electrophoresed in a MDE gel (9 ml 2x MDE (FMC  
116 Bioproducts, Rockland, Maine), 3 ml 10x TBE buffer, 18 ml bidest. water, 10% APS 120 µl and 12 µl TEMED).  
117 Gels (21 cm) were cast (spacers of 0.5 mm) and run with 1x TBE buffer. Gels were run at 400 V for 16 h at  
118 20°C in a Macrophor electrophoresis unit (LKB Bromma, Sweden) and subsequently silver stained [19].

119

#### 120 *Sequence analysis:*

121 Single bands were excised from the gels and eluted in extraction-buffer (10 mM Tris-buffer, 5 mM KCl,  
122 1.5 mM MgCl<sub>2</sub> x 6 H<sub>2</sub>O, 0.1 % Triton X 100, pH 9.0) at 95°C for 15 min. Extracts were centrifuged (12000 x g,  
123 1 min) and the DNA in the supernatant fluid was used for PCR with the primers described above. The PCR-  
124 product was cleaned (Mini-elute-Kit, Qiagen, Hilden, Germany) and sequenced with a sequencing kit  
125 (DYEnamic ET Terminator cycle sequencing kit by Amersham Biosciences, Freiburg, Germany) and both  
126 primers. The product was cleaned with the Dye Ex Spin Kit (Qiagen, Hilden, Germany) and the sequence  
127 analyzed on an ABI PRISMTM 337 DNA-Sequencer and 3100 Genetic Analyser. Comparisons of the sequences  
128 were performed using the BLAST program and the databases of EMBL [20] and GenBank [21]. The  
129 phylogenetic analysis was done using the MUSCLE version 3.6 software for sequence alignments [22]. Tree  
130 topologies were reconstructed with neighbour-joining algorithm (complete deletion of gaps/missing data,  
131 maximum composite likelihood model, bootstrap: 1000 replicates) (Figure 3) with the software MEGA 4 [23].  
132 All reference sequences used in the tree are from validated bacterial species: each has been cultured,  
133 characterized, deposited with at least two recognized culture collections, and named under the procedure  
134 described in the International Bacteriological Code of Nomenclature (ICNB), 1990 revision [24].The sequences

135 of the oral rDNA bands obtained from patients 1 to 10 (31 sequences, indicated in Figure 3) have been deposited  
136 in the GenBank database under accession numbers FN870024-FN870053 and FR666873.

137

138 *Statistics:*

139 Statistical analyses were done using the STATISTICA 6 software. For correlation analysis and  
140 discriminate analysis the presence/absence of a species was used. Discriminant analysis was performed applying  
141 the forward stepwise method setting the tolerance to minimum ( $10^{-10}$ ).

142

### 143 **Results**

144 All samples showed a large number of SSCP-bands and, hence, possess a high diversity of bacteria  
145 varying between patients and within one patient with location (Figure 4). Sequence analyses of 101 SSCP-bands  
146 led to the identification of 31 different bacterial taxa belonging to 5 different bacterial phyla (Firmicutes,  
147 Actinobacteria, Proteobacteria (Beta-, Gamma- and Epsilon-), Bacteroidetes and Fusobacteria). Remarkable  
148 were the diversity of *Veillonella*, *Streptococcus*, *Parvimonas*, *Fusobacterium* and *Neisseria* species detected.  
149 Species have only been identified down to the species level if they matched exactly with the sequence of the type  
150 strain (Figure 3). In the genus *Veillonella* gene sequences matching those of *Veillonella parvula* (*Veillonella*  
151 *parvula* 54-5) and *Veillonella atypica* (*Veillonella atypica* 133-5) could be identified. A third sequence  
152 (*Veillonella* sp. 112-3) was closer related to *Veillonella dispar*. As has been expected, many biofilm bacteria fell  
153 into the genus *Streptococcus*. Here *Streptococcus mitis* 42-1 and *Streptococcus intermedius* 63-1 could be  
154 identified together with two unknown *Streptococcus* species which were related to *Streptococcus intermedius*  
155 and *Streptococcus constellatus* (*Streptococcus* sp. 41-2 and 113-3). No sequence belonging to *Streptococcus*  
156 *mutans* was found. The genus *Fusobacterium* comprises strictly anaerobic bacteria and species of this genus are  
157 well known from the human mouth. The species *Fusobacterium naviforme* 72-3 and *Fusobacterium canifelinum*  
158 113-8 were identified from the corresponding SSCP amplicons together with a third *Fusobacterium* sequence  
159 (*Fusobacterium* sp. 63-3) which was related to *Fusobacterium naviforme*. As for the former two species of the  
160 genus *Neisseria* have been reported as common members of dental biofilms in humans and *Neisseria mucosa*  
161 133-2 was found together with the unknown *Neisseria* species 71-2 related to *Neisseria polysaccharea*.  
162 *Parvimonas micra*, widely distributed in human dental biofilms, was also detected in this study (*Parvimonas*  
163 *micra* 52-1). From one patient however, two distinct *Parvimonas* sequences have been identified (*Parvimonas*  
164 sp. 72-1 and 72-3) belonging to two undescribed *Parvimonas* species (Figure 3).

165 To detect interdependencies between bacteria species we correlated the occurrence of all species  
166 identified. Significant correlations were found between *Veillonella* species 112-3 and *Neisseria* species 71-2,  
167 *Haemophilus parainfluenzae* 112-5 and *Campylobacter* sp. 113-6, between *Veillonella parvula* 54-5 and  
168 *Oribacterium* species 54-3 and *Centipeda periodontii* 54-1 and between *Veillonella* sp. 133-5 and  
169 *Campylobacter gracilis* 54-7 and *Selenomonas noxia* 134-1. *Streptococcus mitis* 42-1 was significantly  
170 associated with *Lautropia mirabilis* 74-1, while the occurrence of *Streptococcus intermedius* 63-1 correlated  
171 with that of *Campylobacter* sp. 113-6. *Fusobacterium canifelinum* is closely related to *Fusobacterium nucleatum*  
172 which is known to have many interactions with other bacteria in dental plaques, but we found only a significant  
173 correlation between its occurrences with the two *Campylobacter* species.

174 We addressed the question whether the microbial communities are controlled by the individual host or  
175 the environment controls the composition of the community. To achieve this goal we used the bacteria detected  
176 in a sample to discern between the different types of samples. Discriminant analysis is used to find a  
177 combination of features (here bacteria species) which characterize or separate classes of objects (here sampling  
178 sites or patients). The resulting combination can be used to reduce the dimensionality. We applied discriminant  
179 analyses to identify such correlations and could not find microbial communities specific for the individual  
180 patients (data not shown). However, discriminant analyses identified occurrences of *Lactobacillus delbrueckii*  
181 92-2, *Parvimonas* sp. 72-1, *Haemophilus parainfluenzae* 112-5 and *Streptococcus mitis* 42-1 as discriminative  
182 between the subgingival bacteria and the other samples. The occurrences of *Parvimonas micra* 52-1 and  
183 *Neisseria mucosa* 133-2 were characteristic for the supragingival microbial communities, while those of the  
184 sulcusfluid from the abutments were characterized by the presence of *Burkholderia* sp. 113-1 (Figure 5a). In  
185 figure 5b the contribution of every discriminative strain to the plot shown in figure 5a was plotted for the first  
186 two coefficients factors. This presentation allows the fast identification of strains to a given sample set, e. g.  
187 *Parvimonas micra* 52-1 and *Neisseria mucosa* 133-2 contributed most to supragingival biofilms while  
188 *Parvimonas* sp. 72-1 was characteristic for subgingival biofilms. For the sulcusfluid from the residual teeth no  
189 discriminative bacteria could be identified as expected from the central position of this group in figure 5a.  
190 Comparing the sulcusfluid around the abutments and around the residual teeth only small differences in the  
191 microbial diversity was seen. One remarkable difference, however, was the higher diversity and the much higher  
192 frequency of *Streptococcus* sequences found in the sulcusfluid around the abutments compared to that from  
193 residual teeth.

194

195 **Discussion**



196           The use of healing abutments to study the microbial diversity of initial biofilms on oral implant  
197 abutments using an unspecific molecular biological detection method allows an *in-vivo* analysis of biofilms in  
198 supra and subgingival areas along oxic-anoxic gradients. The anaerobic environment enables realistic formation  
199 of the same biofilm as during development of mucositis or periimplantitis. The advantage to remove healing  
200 abutments atraumatically for sampling adherent bacteria in subgingival areas without contaminating the sample  
201 with supragingival microbiota was an important precondition for a realistic comparison of supra- and  
202 subgingival biofilm compositions. In the present study a short term investigation of biofilm formation on healing  
203 abutments was performed over a period of 14 days. The analysis was performed over this specific period to  
204 display the realistic and typical microorganisms in the initial phase of biofilm formation. Furthermore previous  
205 investigations approved microbiota around implants to remain unchanged for six months after a comparable  
206 period [25]. In recent studies, a similar method directed to the typical periodontal pathogens *Aggregatibacter*  
207 *actinomycetemcomitans* or *Porphyromonas gingivalis* in the periimplant crevicular fluid was used [26, 27].  
208 However, structural differences between healing abutments and implant retained prostheses have to be  
209 considered while interpreting the results. Implant anchored prostheses like crowns or bridges exhibit an anatomic  
210 form with typical plaque retentive sites. Furthermore, a gap between the definitive abutments and the dental  
211 prostheses can boost the biofilm formation because of their modular configuration. However, an analysis of  
212 initial adherent biofilms along oxic-anoxic gradients is not possible with implant retained dental prostheses  
213 because sampling of adherent bacteria in subgingival areas without removal of the construction is leading to a  
214 contamination with supragingival microbiota in the majority of cases.

215           In the present study several species of the genera *Veillonella*, *Streptococcus*, *Parvimonas*, *Fusobacterium*  
216 and *Neisseria* were detected all of them well known from dental plaques. Not all of them fitted to the 16S rRNA  
217 gene sequences reported from the type strains of accepted species. However, the identification of the *Veillonella*  
218 species is complicated as it has been reported that as least some *Veillonella* species have a rather high intra-  
219 chromosomal heterogeneity between their four rRNA gene copies [28]. No *Streptococcus mutans* sequences  
220 were found, however, the essential role of *Streptococcus mutans* in the caries process has been questioned [29]  
221 as some strains of *Streptococcus oralis* exhibited levels of caries induction in rats that were close to those of  
222 *Streptococcus mutans* [30]. *Streptococci* are the principal group of the early colonizing bacteria showing  
223 interactions with a number of other bacteria. One example is a metabolic interaction with *Veillonella* species,  
224 which coaggregate with *Streptococci* [31]. In this food chain *Veillonella* uses lactic acid produced by the  
225 *Streptococci* as an end product of carbohydrate fermentation [32]. *Streptococcus* species were detected in 75%

226 and *Veillonella* species in 50% of the patients. Only in one patient no *Streptococcus* but *Veillonella parvula*  
227 could be found.

228 Species of the genus *Fusobacterium* are obligate anaerobes and implicated in the pathogenesis of  
229 periodontal disease. However, this organism has also been frequently identified in high numbers in healthy  
230 adults. Consequently it was found in six out ten patients. The diversity of *Fusobacterium* species between the  
231 different patients and also between the different locations was high. Such diversity has also been observed at the  
232 clonal level of *F. nucleatum* isolates [33]. Interestingly, in this study also a large dynamic between the clones  
233 could be shown. Furthermore, it is known from *F. nucleatum* that is a typically middle colonizer of dental  
234 surfaces showing many interactions with the surrounding bacteria cells [34]. Both observations can explain the  
235 diversity observed in our study but not the absence of *Fusobacterium* strains in some patients. This finding  
236 points to alternative routes to anaerobic communities and plaque formation not involving *Fusobacterium*.

237 Well known primary colonizers are species of the genus *Neisseria* and found in the majority of the  
238 patients but in two out of ten patients they could not be detected. Again it is reasonably to assume that *Neisseria*  
239 is not essential for the primary colonization of dental implants and can be substituted by other species. In  
240 addition to *Neisseria* many of the bacteria identified in this study including the *Burkholderia* species were also  
241 reported from the initial colonization of enamel [35], childhood caries [36] and in periodontal pockets [37].  
242 Interestingly, no *Prevotella* or *Rothia* species was detected which were reported to be rather abundant in a recent  
243 study on the oral microbiota of 10 individuals with healthy oral tissues [38]. It is tempting to speculate that these  
244 bacteria appear late in the oral biofilm formation and were, therefore, not observed in our two-weeks-study. Such  
245 an information can only obtained with methods which are at least “semiquantitative” but are hard to deduce from  
246 a metagenomic approach [39]. A further advantage of the culture-independent approach applied here is the  
247 detection of novel species, e. g. *Oribacterium* sp. 54-3. According to the gene databases such strain has never  
248 been detected before and judging from its remote relatedness to *Oribacterium sinus* it may well belong to a still  
249 undescribed genus.

250 Having assessed the microbial diversity of young biofilms in the human mouth it is interesting to  
251 compare the findings with detrimental biofilms reported in the literature [40]. Socransky and coworkers assessed  
252 the microbial diversity of a large number of plaque samples and correlated it with the clinical assessment [15].  
253 Multivariate analyses revealed five groups of bacteria increasingly related to periodontitis. We compared this  
254 finding with our results and found that none of the species in the group strikingly related to periodontal disease  
255 was detected in our study. This indicates no forms of gingivitis or periodontitis. Beside pathogens related to  
256 dental diseases other pathogens were found in a few samples, e. g. *Neisseria mucosa*, *Actinomyces israelii* or

257 *Haemophilus parainfluenzae*. The detection of *Haemophilus parainfluenzae* fits to the suggestion that typical  
258 respiratory pathogens may colonize the teeth of hospitalized intensive care and nursing home patients [41].

259           The occurrences of some bacteria were found to be discriminative for some sites. *Streptococcus mitis*  
260 and *Lactobacillus delbrueckii* contributed together with *Parvimonas* sp. 72-1 to the discrimination of subgingival  
261 biofilm communities while *Neisseria mucosa* and *Parvimonas micra* were mainly contributing to the  
262 discrimination of supragingival biofilms. This also confirms their role as primary colonizers. Our results  
263 confirmed earlier findings [42] but extended the number of site specific bacteria considerably. We also showed  
264 that *Prevotella* and *Rothia* species, abundant in the oral cavity of healthy individuals, are not members of the  
265 young biofilm communities. Concerning the experimental set-up used our results show as well that healing  
266 abutments can be used as a convenient model to study initial colonization of implants under natural conditions in  
267 humans.

268

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370 **Figure legends:**

371

372 **Figure 1:** Clinical signs of periimplantitis with an inflammation of the peri-implantary mucosa (arrows) (a) and a  
373 subsequent destruction of the peri-implantary bone with the typical signs of radiographic bone loss around the  
374 dental implant (arrows) (b).

375

376 **Figure 2:** New sterile Healing abutment (regio 12) *in situ*.

377

378 **Figure 3:** Unrooted phylogentic tree based on 16S rRNA gene sequences obtained by sequencing SSCP bands  
379 and the nearest type strain using neighbour joining. Bootstraps values are shown at the branching points; only  
380 values above 50% are shown. Only sequences with more than 350 unambiguously determined base pairs were  
381 included. Numbers in bold stand for sequences of SSCP bands deposited at GenBank. Scale bar represents 5  
382 nucleotide substitutions per 100 bases.

383

384 **Figure 4:** SSCP samples from 5 patients; sp = supragingival, sb = subgingival, ab = sulcusfluid from abutment,  
385 rt = sulcusfluid from residual teeth. Numbered bands are 1 = *Neisseria mucosa*, 2 = *Veillonella parvula*, 3 =  
386 *Burkholderia* sp. 113-1, 4 = *Marinobacter santoriniensis*, 5 = *Parvimonas micra*, 6 = *Corynebacterium* sp. 51-2,  
387 7 = *Leptotrichia* sp. 51-3, 8 = *Klingella* sp. 112-2, 9 = *Campylobacter gracilis*, 10 = *Centipeda periodontii*, 11 =  
388 *Oribacterium* sp. 54-3, 12 = *Fusobacterium nucleatum*, 13 = *Fusobacterium* sp. 63-3, 14 = *Parvimonas* sp. 72-1,  
389 15 = *Parvimonas* sp. 72-2, 16 = *Fusobacterium naviforme*, 17 = *Lautropia mirabilis*, 18 = *Neisseria* sp. 71-2, 19  
390 = *Streptococcus mitis*, 20 = *Lactobacillus delbrueckii*, 21 = *Streptococcus intermedius*, 22 = *Capnocytophaga*  
391 *sputigena*.

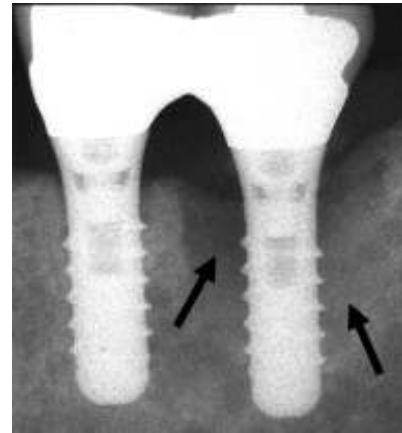
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393 **Figure 5:** Discriminant analysis between the four sampling sites and the presence/absence of their bacteria. Only  
394 roots 1 and 2 are shown. **5a** (left): Plot of the four different sample types; circle: supragingival biofilms (solid  
395 ellipse), square: subgingival biofilms (dashed ellipse), rhomb: sulcusfluids of the abutments (mixed dashed  
396 ellipse), triangle: sulcusfluids of the residual teeth (solid ellipse). **5b** (right): factor loadings of the bacteria  
397 discriminating the four groups.

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399 **Figures:**

400 Figure 1 a/b:



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409 **Figure 2:**



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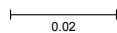
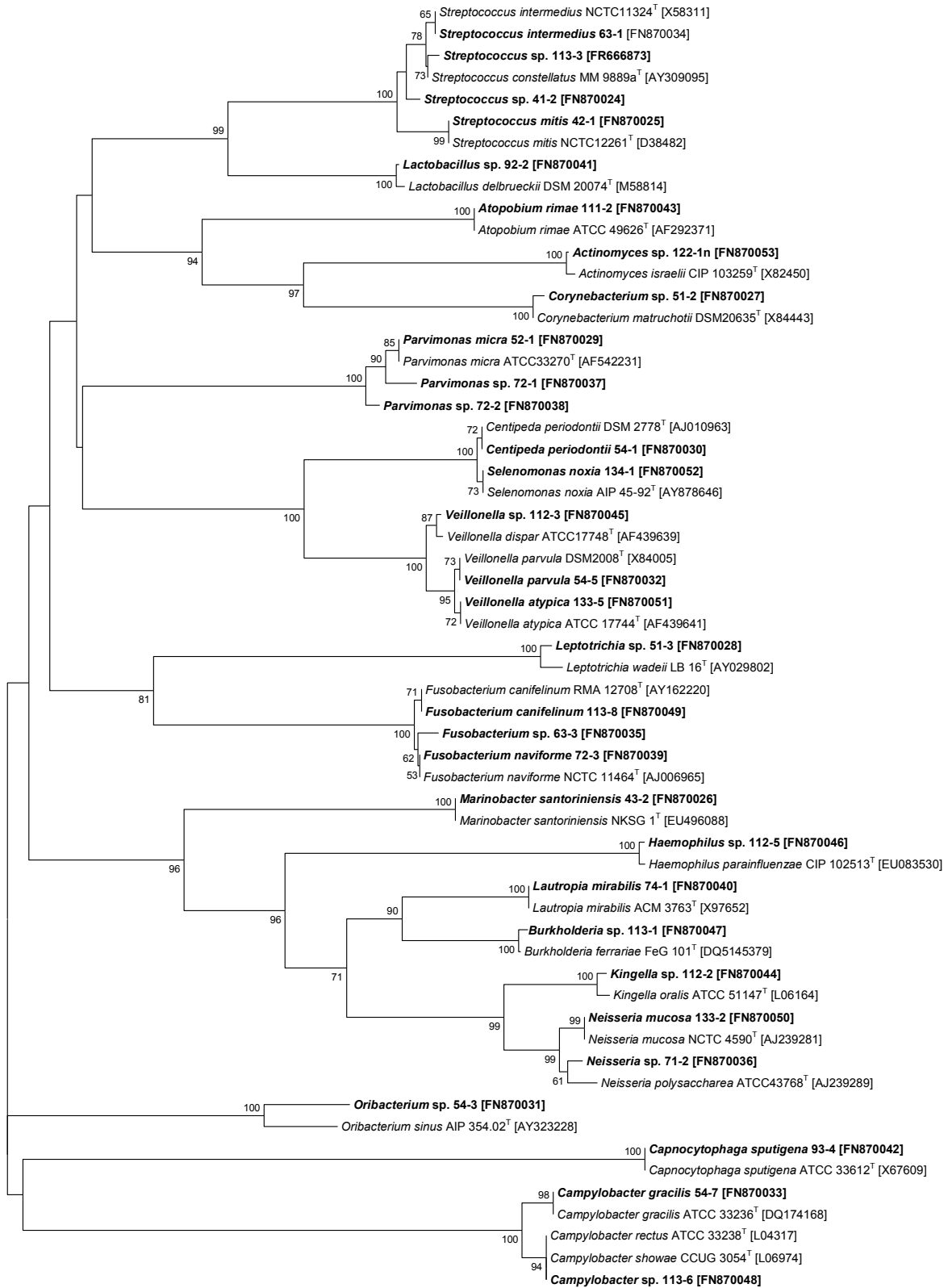
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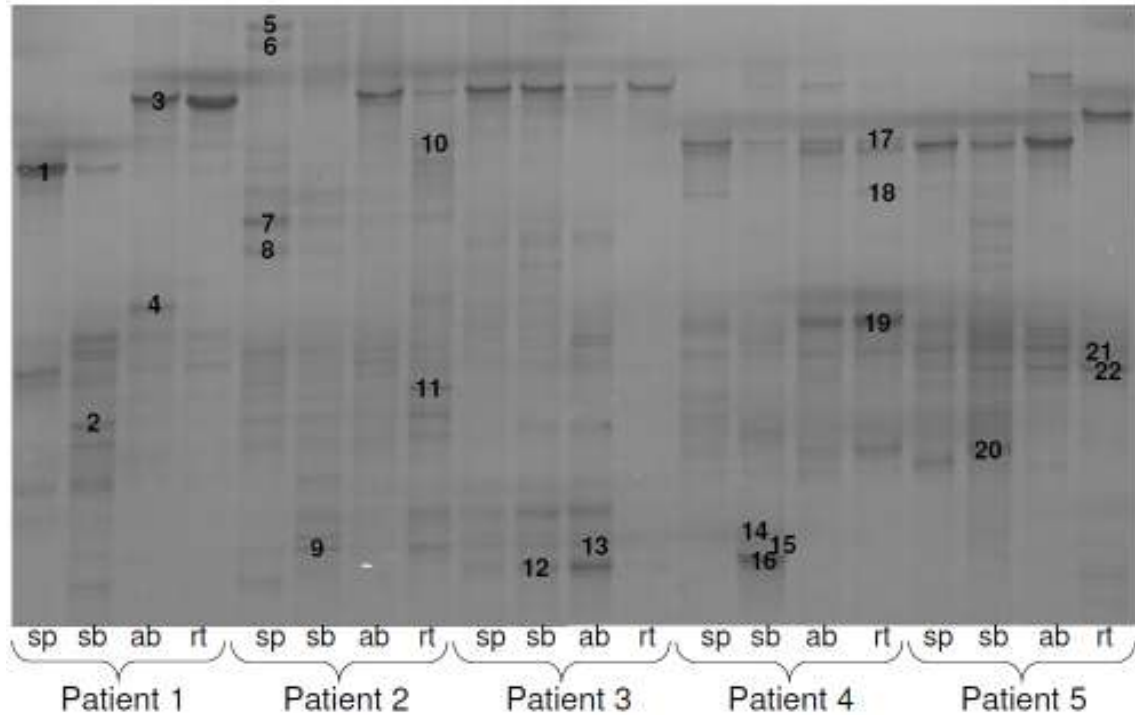




418 Figure 4:

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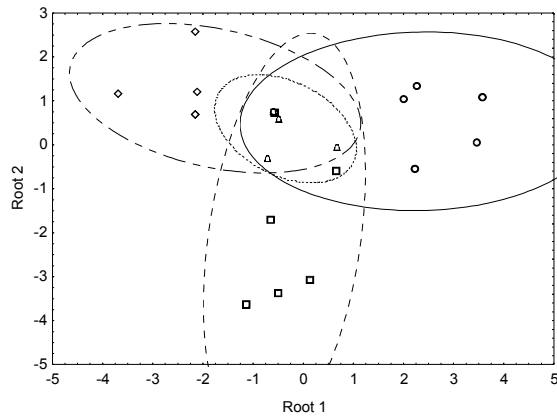


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422 Figure 5:

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