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Endodontic Microbial Communities in Apical Periodontitis

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1           **Analysis of oral microbiota in non-vital teeth and clinically intact external surface**  
2           **from patients with severe periodontitis using Nanopore sequencing: a case study**

3  
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16        **Abstract (189 words)**

17        Periodontal diseases include a wide range of pathological conditions, damaging the  
18        supporting structures of the teeth. Origin and propagation of periodontal disease is believed  
19        to be caused by dysbiosis of the commensal oral microbiota. The aim of this study was to  
20        evaluate the presence of bacteria in the pulp cavity of teeth with severe periodontal disease  
21        with clinically intact external surface. Periodontal (P) and endodontic (E) tissue samples of  
22        root canals from six intact teeth of 3 patients were sampled for analysis of microbial  
23        population using Nanopore technology. *Streptococcus* was the predominant genus in E  
24        samples. *Porphyromonas* (33.4%, p=0.047), *Tannerella* (41.7%, p=0.042) and *Treponema*  
25        (50.0%, p=0.0064) were significantly more present in P than in E samples. Some samples  
26        (E6 and E1) exhibited a remarkable difference in terms of microbial composition, whilst  
27        *Streptococcus* was a common signature in samples E2 to E5, all which were obtained from  
28        the same patient. In conclusion, bacteria were identified on both the root surface and the root  
29        canal system, thus demonstrating the possibility of bacteria to spread directly from the  
30        periodontal pocket to the root canal system even in the absence of crown's loss of integrity.

31

32        **Keywords:** microbiota, root canal, intact teeth, periodontitis, Nanopore

33 **Introduction (3,130 words).**

34 Although periodontal and endodontic tissues are prone to different pathologies, there are  
35 strict anatomical correlations through lateral and accessory canals and the apical foramen  
36 and dentinal tubules [1], favoring bacterial migration among contiguous tissues [1–3].

37 Bacterial biofilms associated to endodontic or periodontal infections are similar, confirming  
38 these pathways of migration [4] Endo-periodontal lesions (EPL) are bacterial infectious  
39 diseases that affect both periodontal and endodontic tissues of the same tooth, with  
40 periodontal tissue damage and pulp inflammation/necrosis, defined by a pathological  
41 communication between the pulpal and periodontal tissues [5].

42 The common pathologic communication between these tissues can occur by a carious or  
43 traumatic lesion that affects the pulp and, secondarily periodontium between apical foramen,  
44 accessory canals and dentinal tubules [6].

45 According to the classification of American Academy of Periodontology criteria, EPL occur  
46 in either an acute or a chronic form, and are classified according to signs and symptoms that  
47 have direct impact on their prognosis and treatment. The primary signs are deep periodontal  
48 pockets extending to the root apex and/or negative/altered response to pulp vitality tests.

49 Other signs/symptoms may include radiographic evidence of bone loss in the apical or  
50 furcation region, pain, suppuration, tooth mobility, sinus tract, and crown and/or gingival  
51 colour alterations. EPL can be associated to a root damage, generally determined by  
52 traumatic and/or iatrogenic factors that may include root perforation, fracture/cracking, or  
53 external root resorption. These conditions drastically impair the prognosis of the involved  
54 tooth. EPL without root damage are distinguished in two categories based on the presence of  
55 periodontitis. EPL are graded into 3 levels depending on morphology and width of the  
56 periodontal pocket. Grade 1 presents narrow deep periodontal pocket in 1 tooth surface,  
57 Grade 2 a wide deep periodontal pocket in the tooth surface and Grade 3 a deep periodontal  
58 pockets in > 1 tooth surface. EPL Grade 3 with root damage have the worst prognosis [5].

59 When it comes to pulpal pathology of periodontal origins, the matter is more controversial,  
60 especially in teeth that present only a periodontal pathology without root damages and/or  
61 coronal leakages [7,8] .

62 Massive periodontal destruction can secondarily affect the root canal system with  
63 dissemination of the inflammation, which can result in pulp necrosis [9]. It is still unclear if  
64 bacteria can reach the root canal system, or if inflammation is only due to their metabolic  
65 products. Overall, how bacteria can reach the root canal system from periodontal tissue is  
66 largely unclear. The aim of this study was to evaluate if in non-vital teeth with severe  
67 periodontal disease not reaching the apex root segment and with clinically intact external  
68 surface there is presence of bacteria in the endodontic space.

69

## 70 **Materials and methods**

### 71 *Case study*

72 Study participants were 3 patients (two female and one male patient; mean age 51 years)  
73 with chief complaint of severe lower anterior teeth mobility and severe periodontal  
74 compromise hindering any periodontal or conservative treatment. As control case, a  
75 patient (#1) referred with chief complaint due to lower third molar pericoronitis and  
76 indication for surgical extraction was recruited.

77 The exclusion criteria for this study were antibiotic therapy administered up to 3 months  
78 before tooth extraction, systemic diseases and pregnancy. Another exclusion criterion was  
79 evaluating that periodontal pockets could not reach the apical root segment. Moreover,  
80 inclusion criteria were teeth affected by EPL without clinically and radiographically  
81 identified caries lesions, cracks/fractures and/or restorations.

82 The patients involved in the research signed a formal written informed consent form.

83 EPL diagnosis was performed with periodontal probing that evaluate an average clinical  
84 attachment loss  $\geq 5$  mm on all root's surfaces and radiographic analyses on periapical

85 bidimensional radiographies using paralleling technique confirming bone loss extending to  
86 mid-third of root and beyond; moreover, periapical radiolucency was not observed. None of  
87 these teeth presented periodontal pockets reaching the apex. Teeth presented grade 2  
88 mobility with percussion and palpation sensitivity. In addition, thermal and electric pulp  
89 sensibility tests were performed returning non-responsivity and thus confirming pulpar  
90 necrosis. Thermal pulp test was performed with a #2 cotton pellet sprayed and fully  
91 saturated with 1,1,1,2-tetrafluoroethane and placed at the middle third of the buccal tooth's  
92 surface of the clinical crown for at least 20 seconds. Cold Pulp testing was selected as pulp  
93 sensibility test which is able to evaluate vital (specificity = 0.84) and nonvital (sensitivity =  
94 0.87) teeth [10–12]. No other EPL signs/symptoms (sinus tract, spontaneous pain, periapical  
95 radiolucency, suppuration) were observed.

96 Moreover, on intraoral inspection using loupes for magnification 4.0x and radiographic  
97 evaluation, the teeth did not present clinically identified caries lesions, cracks, fractures nor  
98 previous conservative restorations. The final diagnosis for all teeth was of EPL without root  
99 damage of Grade 3, according to the classification from the American Academy of  
100 Periodontology criteria. The sampled teeth clinically appeared without defects, decay or  
101 restorations and were affected by severe periodontitis (Stage IV) according to the  
102 classification of American Academy of Periodontology criteria [13]. Control sample  
103 consisted of an intact lower third molar without carious and periodontal pathologies,  
104 surgically extracted because affected by pericoronitis. All teeth affected by EPL were  
105 single-rooted (N=5 lower incisors and N=1 lower canine).

106 A total of 12 clinical samples of the study group were collected from periodontal (P) (n=6)  
107 and endodontic (E) (n=6) tissue samples of root canals from six intact teeth of 3 patients (P1  
108 and E1 from patient #1, P2 to P5 and E2 to E5 from patient #2 and P6 and E6 from #3).

109 All teeth were single-rooted (lower incisors and lower canine).

110 The study was performed in agreement with the ethical guidelines of the Declaration of  
111 Helsinki laid down in the 1964 and its later amendments or comparable ethical standards.  
112 The Ethics Committee of Azienda Unità Sanitaria Locale of Bologna approved this study  
113 with authorization nr. 844-2021-OSS-AUSLBO-21160-ID 3118-Parere CE-AVEC-ENDO-  
114 MICROBIOTA 09/2021.

115

### 116 *Root canal sampling*

117 Non-surgical periodontal treatment was applied using ultrasonic tips to remove  
118 supragingival dental biofilms and pre-operative mouthwash with chlorhexidine 0.20% for 60  
119 s to reduce bacterial load.

120 Subsequently, the teeth were anesthetized using articaine with adrenaline 1:100.000

121 (Septodont, Saint-Maur-des-Fossés, France). Sindesmotomy and luxation were performed

122 with a rounded periosteal elevator; extraction was gently performed with dental forceps and

123 tooth was positioned in a sterile tube (Eppendorf AG, Hamburg, Germany). An accurate

124 alveolar toilette was performed with mechanical debridement of granulation tissue and

125 subsequent intra-alveolar irrigation with sterile saline solution rinse. A resorbable collagen

126 sponge (Septodont, Saint-Maur-des-Fossés, France) was positioned in dental socket and a

127 criss-cross non-resorbable suture was performed to favor haemostasis. Only for the

128 extraction of the third molar, a mucoperiosteal flap was executed without ostectomy.

129 After extraction, all teeth were visually examined using loupes for magnification 4.0x to

130 exclude caries lesions, cracks, fractures nor previous conservative restorations.

131 Sampling procedures were carried out immediately after extraction using sterile gloves and

132 sterile materials/instruments. In detail, P samples were collected using a sterile swab to

133 scrub on root surface, chiefly where subgingival calculus was visible; subsequently, swabs

134 were inserted in sterile tubes (Aptaca Spa, Canelli AT, Italy) provided with Stuart transport

135 medium and stored at  $-80^{\circ}\text{C}$  until use.



136 Subsequently the crown was disinfected with 2.5% sodium hypochlorite solution (NaOCl)  
137 (Niclor 2.5, Ognà, Maggiò, Italy) for 30 s [8,14]. The NaOCl solution was inactivated with  
138 5% sodium thiosulfate in order to avoid interference for diffusion of NaOCl in root canal  
139 system during cavity access preparation and bacteriological sampling.

140 To control the sterility of the operating field, two sterile cotton pellets were brushed on the  
141 disinfected tooth crown and transferred to a tube containing transport fluid. If bacterial  
142 growth was detected within 72 hours at 37 °C in laboratory incubator, the sample of the root  
143 canal was excluded from the study.

144

145 Preparation of the access cavity was performed using a sterile high-speed diamond bur  
146 (Maillefer, Ballaigues, Switzerland) under sterile saline solution flow. Before the pulp  
147 chamber was exposed, cleaning of the tooth was repeated as previously described. All the  
148 remaining pulpal tissues observed were evaluated clinically as non-bleeding, fibrotic and  
149 without chromatic aspects (red or pink coloring) traceable to vital pulp. Moreover, pulp  
150 space appeared more or less empty, to confirm clinical diagnosis of pulpal necrosis.

151 After gentle irrigation with sterile saline solution, a sterile #10 K-type stainless hand file  
152 (Maillefer, Ballaigues, Switzerland) was introduced into the canal at the tooth apex level. In  
153 the control case (lower molar) E samples were collected from the largest root canal (distal  
154 root). Working length was previously calibrated on clinical tooth's length to stop K-file and  
155 paper points at the level of the tooth apex level. These procedures were carried out by means  
156 of a visual inspection using magnification loupes to prevent the crossing of the apex by K-  
157 file and paper points.

158 Following gently scraping with instrumentation alongside the root canal walls with a sterile  
159 #10 K-type stainless hand file (Maillefer, Ballaigues, Switzerland) to disperse bacteria in the  
160 medium, sterile paper points #15 (Dentsply-Maillefer, Ballaigues, Switzerland) were  
161 positioned in the canals for 60 s, to collect "E" samples in sterile tubes (Eppendorf AG,

162 Hamburg, Germany), subsequently stored at  $-80^{\circ}\text{C}$  until use. Every procedure was  
163 executed using new sterile gloves.

164

#### 165 *DNA extraction*

166 Paper point samples were immersed in a 2 mL Eppendorf safe-lock tube containing  
167 Dulbecco Minimal Essential medium (DMEM). Subsequently, samples were homogenized  
168 by Tissue Lyser (Qiagen GmbH, Hilden, Germany) at 30 Hz for 5 min. Homogenized  
169 samples were centrifuged at  $10,000 \times g$  for 3 min. A total of 200  $\mu\text{L}$  of supernatants were  
170 subjected to DNA extraction using DNeasy PowerSoil PRO kit (Qiagen S.p.A., Milan, Italy)  
171 according to manufacturer's instructions. Negative controls of extraction (DMEM and  
172 reagents from extraction kits) were used at the same time as samples, to check for the  
173 presence of possible contamination during the extraction steps. To assess for bias in  
174 extraction and/or sequencing, commercially available mock community control composed of  
175 three Gram-negative and five Gram-positive bacteria with a range of GC content was used.  
176 Mock community DNA obtained by pooling DNA extracted from pure cultures  
177 (ZymoBIOMICS Catalog #D6306) was used as the input DNA.

178

#### 179 *PCR amplification of 16SrDNA gene and Nanopore sequencing*

180 A PCR protocol was performed on DNA extracts to amplify the full-length (1500bp)  
181 sequence of the 16SrRNA gene using universal primers [15] and TaKaRa LA Taq<sup>TM</sup> kit  
182 (Takara Bio Europe S.A.S., Saint-Germain-en-Laye, France). Afterwards, the 16S barcoding  
183 kit SQK-RAB204 (Oxford Nanopore Technologies, ONT, Oxford UK) was used to prepare  
184 libraries which were purified by Agencourt AMPure XP magnetic beads (Beckman  
185 Coulter<sup>TM</sup>), pooled and sequenced using MinION flongle Flow cell FLO-FLG001, version  
186 R9.4.1 adapted on the MinION- Mk1C device (ONT, UK) for 24h.

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### *Data analysis*

FastQ MinION files were uploaded on the online EPI2ME platform (<https://epi2me.nanoporetech.com/>) and analyzed by the Fastq 16S 2021.09.09 (Metricor Agent, ONT) workflow setting the following parameters: quality score 10, minimum length filter of 1500 bases and BLAST E-value of 0.01.

Taxonomy was obtained through interrogation of the NCBI database non redundant using BLAST with a minimum horizontal coverage of 30% and a minimum accuracy of 77% as default parameters. Reads data obtained were organized in Microsoft Office Excel. Only Taxa scoring a  $\geq 0.1\%$  relative abundance in samples were considered and thus analyzed using the “Plotly.py” open-source library for Python 3.7.9 [16]. The computed data were then represented as interrogable BarPlot charts.

In addition, “Krona” visualization tool was employed to organize and display the communities at a species level in multi-layered pie [17]. Multiple comparisons of the bacterial sequence reads obtained in the P and E tissue samples was compared using Kruskal-Wallis test with Dunn test as post hoc test. Moreover, categorical dichotomous data (P and E tissue samples and presence/absence of bacteria in samples) were described as counts and percentages and evaluated by Exact Fisher test. Statistical analyses were performed using the freely available online tool EZR [18] for personal computers. A p-value  $< 0.05$  was considered for statistical significance.

### *Diversity indexes*

Statistical analyses were performed with R v.4.1.3 using the library “vegan” (<https://vegandevs.github.io/vegan/>). Alpha diversity for sample was assessed using Shannon index and measure of biodiversity was evaluated using Richness Menhinick’s index. Shapiro–Wilk test was performed to evaluate the normality of distribution of data. Two-sided Student’s t test for independent samples or Mann–Whitney U test were

214 performed on the calculated alpha diversity and biodiversity values on the basis of “P” and  
215 “E” categories. To identify possible sample stratification, beta diversity was assessed using  
216 Bray–Curtis index and Principal Coordinate Analysis (PCoA) was performed for each pair  
217 of categories. ANOVA test and Tukey Honestly Significant Difference as post hoc test were  
218 carried out on the calculated beta diversity values. The statistical significance was set at  
219 0.05.

220

## 221 **Results**

222 All the crown samples tested negative in the sterility test. Bacterial DNA was identified in  
223 all the 12 samples analyzed from the study group and the control sample. Negative  
224 extraction controls (DMEM and reagents from the DNA extraction kit) did not result in  
225 library prep due to low DNA concentration and were not sequenced. Mock community  
226 control DNA included in the sequencing runs most closely approximated to the theoretical  
227 composition of the mock community.

228

229 After quality control of Nanopore sequence data, a total of 445,215 bacterial 16SrRNA gene  
230 sequence reads (mean 37,101, median 12,870, range 3,255-23,0280) were obtained in the 12  
231 analyzed samples but only 36 sequence reads in the control sample. A total of 123  
232 Operational Taxonomic Units (OTUs) were identified in the P and E samples and assigned  
233 to 8 phyla, 29 genera and 86 species using Fastq 16S 2021.09.09 workflow. Overall, the  
234 most abundant phylum detected was *Bacillota* (80%) followed by *Actinomycetota* (6.1%)  
235 and *Bacteroidetes* (5.6%). The prominent genus was *Streptococcus* (72.9%) followed by  
236 *Veillonella* (3.6%), *Actinomyces* (3.6%), *Parvimonas* (3.5%) and *Prevotella* (3.1%) (Table  
237 1, Figure 1) whilst *Streptococcus mutans* (50.7%) was the predominant species followed by  
238 *Streptococcus anginosus* (6.3%) and *Parvimonas micra* (3.5%) (Figure 2).

239 Although the six collected teeth samples did not present neither clinically evident coronal  
240 leakages nor root damages, *Streptococcus* (54.7%) was predominant in E samples (Table 1),  
241 and it was also identified in P samples (18.2%) (Table 1, Figure 1).

242 The bacterial genera identified in the analyzed samples is reported in Table 2 whilst Table 3  
243 shows the distribution of bacterial genera, expressed as percentage, in P and E tissue  
244 samples from the root canals. Overall, the most prevalent bacterial genera in the twelve  
245 collected samples were *Streptococcus* (100.0%, 12/12), *Actinomyces* (83.4%, 10/12),  
246 *Fusobacterium* (66.7%, 8/12), *Parvimonas* (66.7%, 8/12), *Prevotella* (66.7%, 8/12),  
247 *Tannerella* (66.7%, 8/12), *Treponema* (66.7%, 8/12) and *Veillonella* (66.7%, 8/12) (Table 2  
248 and 3).

249  
250 Alpha diversity among the samples, calculated using Shannon index, ranged between 0.053-  
251 0.909 (mean = 0.564; median = 0.611) whilst the biodiversity value using Richness  
252 Menhinick's index ranged between 0.010 and 0.239 (mean = 0.091; median= 0.076).  
253 Comparisons of alpha diversity and biodiversity values did not reach the thresholds of  
254 statistical significance ( $p>0.05$ ) for the considered categories.

255  
256 Beta diversity for P and E categories were assessed by using Bray–Curtis index and PCoA  
257 plot graphs were produced (Figure 3). Comparisons of beta diversity of samples did not  
258 reveal statistical significance ( $p>0.05$ ) for the categories.

259  
260 The genera most frequently identified in P samples (n=6) were *Fusobacterium* (50%),  
261 *Prevotella* (50.0%), *Streptococcus* (50.0%), *Treponema* (50.0%), *Actinomyces* (41.7%),  
262 *Olsenella* (41.7%), *Parvimonas* (41.7%) and *Tannerella* (41.7%). *Streptococcus* (50.0%)  
263 and *Actinomyces* (41.7%) were the most frequently detected genera in E samples (n=6)  
264 (Table 2 and 3). *Actinomyces* and *Streptococcus* were detected with equal prevalence (41.7-

265 50.0%) in both P and E samples whilst *Fusobacterium*, *Prevotella* and *Treponema* were  
266 detected with higher prevalence in P (50.0%) than in E samples (16.7%) (Table 3).  
267 *Atopobium* (41.7%, 5/12), *Selenomonas* (41.7%, 5/12), *Lactobacillus* (33.4%, 4/12),  
268 *Capnocytophaga* (25.0%, 3/12), *Leptotrichia* (25.0%, 3/12), *Paludibacter* (25.0%, 3/12) and  
269 *Schaalia* (25.0%, 3/12) were detected with equal or comparable prevalence in both P (16.7-  
270 25%) and E samples (8.3-16.7%). Conversely, *Campylobacter* (41.7%, 5/12),  
271 *Porphyromonas* (41.7%, 5/12) and *Bifidobacterium* (25.0%, 3/12) were identified more  
272 frequently in P samples (25.0-33.4%) than in E samples (0.0-8.3%) (Table 3). Multiple  
273 comparison between the obtained sequence reads in P and E samples revealed that the  
274 genera *Porphyromonas* (33.4% vs 8.3%,  $p=0.047$ ), *Tannerella* (41.7% vs 25%,  $p=0.042$ )  
275 and *Treponema* (50.0%,  $p=0.0064$ ) were significantly more present in P than in E samples.

276  
277 Samples P1 and P6 were collected from patient #1 and #3, respectively and samples P2 to  
278 P5 were collected from patient #2. In the P samples collected from the three patients, yet in  
279 the context of an expected microbiome diversity, we observed the presence of the genera  
280 *Actinomyces*, *Fusobacterium*, *Olsenella*, *Parvimonas*, *Prevotella*, *Streptococcus*, *Tannerella*  
281 and *Treponema*. Samples E6 and E1 exhibited a remarkable difference in terms of microbial  
282 composition, whilst the genus *Streptococcus*, was a common signature in samples E2 to E5  
283 obtained from the same patient (#2) (Table 2).

284

## 285 **Discussion**

286 In this study teeth affected by EPL Grade 3 with clinically intact crown' surface and bone  
287 loss not reaching the apex were used. Therefore, a primary endodontic involvement was  
288 ruled out. The rationale for the study was based on the hypothesis that periodontal bacteria  
289 may reaches the root canal system even before the periodontal disease reaches the apical  
290 root segment.

291 In our study, all the teeth but the control case presented bacteria on the root surface and in  
292 the root canal system, demonstrating the possibility of bacteria to spread directly from the  
293 periodontal pocket to the root canal system, despite the apical foramen was not reached by  
294 periodontal lesion. It is well known that periodontal diseases may determine the exposure of  
295 other anatomical communications, over the apical segment, between periodontal tissues and  
296 the root canal system, with possible invasion of the root canal system from periodontal  
297 bacteria and/or their toxic metabolic products [19]. “Retrograde” pulpitis is an  
298 inflammatory pulpal condition caused by response to bacterial invasion and toxic products  
299 entering through anatomical communications that became exposed to the oral fluids. Total  
300 pulp necrosis is determined when the blood supply to all root canals is interrupted by  
301 periodontal disease that involves apical root segment determining a vascular damage and  
302 subsequent hypoxia [20]

303 Alveolar bone resorption causes exposure of cementum that can be subsequently eroded  
304 mechanically, removed, and abraded with various processes. Loss of cementum exposes the  
305 dentinal tubules and allow bacterial entrance into the tooth. Moreover, the cementum-  
306 enamel junction (CEJ) presents a great morphological diversity, with frequent gaps between  
307 enamel and cementum with exposure of dentin, also among different surfaces of the same  
308 tooth [21]. Although in healthy teeth CEJ is normally protected and covered by gingival  
309 tissues (epithelial attachment and connectival attachment) [22], it can be exposed for  
310 gingival recession and/or periodontal disease, with the possibility of an immediate exposure  
311 of dentinal tubules.

312 Lateral and accessory canals may distribute bacteria and toxins from the periodontal  
313 apparatus into the dental pulp [23,24].

314 Since they are located more at the coronal level than at the apical foramen, deep periodontal  
315 pockets can expose these communications without reaching the apex of the root. The control  
316 tooth in our study was also positive for bacterial DNA, revealing a low number of bacterial

317 reads in the root canal system. This can suggest that pulpal tissue is not completely sterile  
318 also in the absence of deep periodontal pockets.

319 There are many controversial opinions regarding implications of severe periodontitis on  
320 inflammatory and degenerative alterations in the dental pulp. Some researchers suggest that  
321 periodontal disease can cause pulpal changes [25–29], whereas others do not [30,31].

322 Ricucci et al. evaluated teeth affected by periodontal disease with no clinically identified  
323 caries lesions, reporting histological and bacteriologic results consistent with bacterial  
324 colonization of the outer end of dentinal tubules when loss of integrity of the radicular  
325 cementum occurred. In some cases the authors described histological aspects of pulpal  
326 degeneration due to bacterial colonization of the orifice of a lateral canal, with subsequent  
327 vascular damage and bacterial invasion of the pulpal bloodstream even before pulpal tissue  
328 necrosis [32]. These data support the results of this study, suggesting the possibility for  
329 periodontal bacteria to migrate in the root canal system also in absence of evident root  
330 damages, loss of crown integrity or massive periodontal disease reaching the apex.

331  
332 Moreover, some bacteria seem to have a greater ability of migration between the two spaces.  
333 In fact, *Actinomyces*, *Streptococcus* [14], *Parvimonas* and *Veillonella* were present with  
334 similar frequencies both in the periodontal pocket and in the root canal system.

335 Although their high frequency on root surfaces, some bacteria such as *Porphiromonas* and  
336 *Prevotella* were not identified in the root canal system. Several factors, including bacterial  
337 size, adhesive properties, motility or micro-environmental selectivity may affect the degree  
338 of permeability to the dentinal tubules and virulence [21]

339 Overall, our study presents some limitations. The total sample size is relatively small  
340 and likely a larger sample size could be more useful to identify trends in the oral  
341 microbiome in these pathologies. Unfortunately, teeth affected by advanced periodontal  
342 disease without no clinically loss of external integrity and/or coronal leakages, which are



343 ideal for similar studies, are not commonly observed in the clinical practice. In presence of  
344 teeth affected by EPL with loss of crown's integrity there is an objective difficulty to  
345 establish if the primary bacterial involvement took place from the periodontal space or  
346 endodontic space.

347 Moreover, histologic investigations with bacterial staining were not carried out in our study  
348 to investigate the patterns of tissue invasion by bacteria in the endoperiodontal  
349 environments.

350 The study was based on teeth with poor prognosis, hindering any periodontal or endodontic  
351 treatment and with indication of extraction. It is unclear if endodontic contamination from  
352 periodontal space may occur also in teeth affected by less severe periodontitis.

353  
354 The present study confirms the complexity of oral microbiome, organized in multispecies  
355 communities that may present important limits in microbiological evaluation using classic  
356 microbiological tests such as in vitro cultivation, typing with primers and probes and direct  
357 sequencing [33–35]. Massive sequencing techniques are a novel molecular method that may  
358 be applied to unveil the convoluted pictures of polymicrobial communities including low-  
359 abundance taxa or non-cultivable species of oral microbiota [8,15,34,36–38]. In this study  
360 we relied on a Nanopore 16S rDNA protocol to generate sequence data at a population level  
361 on the microbiological community. 16S rDNA gene is a universal target for bacterial  
362 characterization with nine variable regions intermingled with conserved regions. Unlike  
363 other NGS techniques, Nanopore technology allows to generate sequence data on the full  
364 length 16S rDNA gene increasing the accuracy of characterization. Also, PCR-based  
365 enrichment with consensus primers allows to obtain data from biological matrices with low  
366 density bacterial communities, for which otherwise, other sequencing approaches would not  
367 be feasible.

368

369 Considering the capacity of migration among periodontal and endodontic tissues,  
370 in case of surgical/non-surgical periodontal therapies and/or conservative rehabilitations of  
371 teeth affected by deep periodontal pockets, particular attention should be always paid to  
372 pulpal sensibility tests and pulpal symptoms to evaluate necrotic pulp or hyperresponsive  
373 vital pulp. In these cases, the root canal system should be considered as potentially  
374 contaminated by bacteria, and potentially acts as bacterial reservoir that may serve as  
375 recontamination source of residual pockets and/or periodontal tissues after surgical/non-  
376 surgical therapies [39], although not all bacterial species seem to possess the same capacity  
377 of migration. At the same time, an untreated deep periodontal pocket may serve as a source  
378 of periodontal bacteria to contaminate/re-contaminate the root canal system, determining  
379 pulpal/periapical pathology. Also, therapies that may remove cementum, such as root  
380 planning, should be carefully pondered for the potential exposure of dentinal tubules  
381 creating breaches for bacterial entry. [31].

382 In conclusion, the results of the present study demonstrated the possibility of bacteria to  
383 spread directly from the periodontal pocket to the root canal system even in the absence of  
384 crown's loss of integrity.

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467

## 468 **Declarations**

## 469 **Ethics approval and consent to participate**

470 The study was performed in agreement with the ethical guidelines of the Declaration of  
471 Helsinki laid down in the 1964 and its later amendments or comparable ethical standards.

472 The Ethics Committee of Azienda Unità Sanitaria Locale of Bologna approved this study

473 with authorization nr. 844-2021-OSS-AUSLBO-21160-ID 3118-Parere CE-AVEC-ENDO-  
474 MICROBIOTA 09/2021. Informed consent was obtained from all subjects and/or their legal  
475 guardian(s) for participation and publication.

476

477 **Consent for publication**

478 Not applicable

479

480 **Availability of data and materials**

481 All data generated or analyzed in this study are included in this published article.

482

483 **Competing Interests**

484 The authors declare no competing interests.

485

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489

490 **Authors' contributions**

491 A.B. contributed to conception and design of the study, wrote the first draft of the  
492 manuscript; F.P. performed the experiments, organized the database, wrote the first draft of  
493 the manuscript; G.L. performed the statistical analysis and wrote the first draft of the  
494 manuscript; G.D. performed the experiments; M.S.L. performed the experiments; F.Z. wrote  
495 sections of the manuscript; M.C. reviewed and edited the manuscript; M.G.G. wrote sections  
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508

509 **Figure legends**

510 **Figure 1** Sequence read distribution of bacterial genera detected in the periodontal (P), endodontic  
511 (E) and control (M) tissue samples of root canals from teeth of patients expressed as BarPlot charts

512

513 **Figure 2** Sequence read distribution of bacterial species detected in the periodontal (P), endodontic  
514 (E) and control (M) tissue samples of root canals from teeth of patients expressed as multi-layered  
515 pie charts (Krona visualization tool).

516

517 **Figure 3** Bray–Curtis index (a) and Principal Coordinate Analysis (PCoA) (b) for periodontal (P)  
518 and endodontic (E) samples expressed as plots.

519 **Table 1:** Distribution of sequence data, expressed as percentage, per bacterial genera in samples  
520 collected from periodontal (P) and endodontic (E) tissue samples from root canals of patients  
521 affected by severe periodontitis (Stage IV) according to the American Academy of Periodontology  
522 criteria.

Bacterial genera	Total reads (%)	P (%)	E (%)
<i>Actinomyces</i>	3.6	2.7	0.9
<i>Atopobium</i>	0.2	0.1	0.1
<i>Bifidobacterium</i>	0.7	0.7	0.0
<i>Campylobacter</i>	0.4	0.4	0.0
<i>Capnocytophaga</i>	0.1	0.1	0.0
<i>Corynebacterium</i>	0.1	0.1	0.0
<i>Dialister</i>	0.1	0.1	0.0
<i>Enterococcus</i>	0.1	0.1	0.0
<i>Fusobacterium</i>	1.6	1.4	0.2
<i>Lactobacillus</i>	1.8	1.7	0.1
<i>Leptotrichia</i>	0.2	0.2	0.0
<i>Mycoplasma</i>	0.6	0.6	0.0
<i>Neisseria</i>	0.1	0.1	0.0
<i>Olsenella</i>	0.4	0.4	0.0
<i>Oribacterium</i>	0.1	0.1	0.0
<i>Paludibacter</i>	0.2	0.1	0.1
<i>Parvimonas</i>	3.5	2.2	1.3
<i>PeptoStreptococcus</i>	0.4	0.4	0.0
<i>Porphyromonas</i>	1.3	1.3	0.0
<i>Prevotella</i>	3.1	3.1	0.0
<i>Pseudomonas</i>	0.1	0.1	0.0
<i>Rothia</i>	0.3	0.3	0.0
<i>Scardovia</i>	0.2	0.2	0.0
<i>Schaalia</i>	1.3	1.3	0.0
<i>Selenomonas</i>	0.5	0.5	0.0
<i>Streptococcus</i>	72.9	18.2	54.7
<i>Tannerella</i>	0.7	0.6	0.1
<i>Treponema</i>	1.9	1.8	0.1
<i>Veillonella</i>	3.6	2.1	1.4
Total	100.0	41.0	59.0

523



524 **Table 2:** Presence of bacterial genera in periodontal (P) and endodontic (E) tissue samples of root canals from patients affected by severe  
 525 periodontitis

P/E	Samples											
	P1	P2	P3	P4	P5	P6	E1	E2	E3	E4	E5	E6
Patient	#1	#2	#2	#2	#2	#3	#1	#2	#2	#2	#2	#3
<b>Bacterial Genera</b>												
<i>Actinomyces</i>	+	+	+	+	+	-	+	+	+	+	+	-
<i>Atopobium</i>	-	+	-	+	+	-	-	+	+	-	-	-
<i>Bifidobacterium</i>	-	+	-	+	+	-	-	-	-	-	-	-
<i>Campylobacter</i>	+	+	+	-	+	-	+	-	-	-	-	-
<i>Capnocytophaga</i>	+	+	-	-	-	-	+	-	-	-	-	-
<i>Corynebacterium</i>	-	-	-	-	-	-	+	-	+	-	-	-
<i>Dialister</i>	+	-	-	-	-	-	-	-	-	-	-	-
<i>Enterococcus</i>	-	-	-	-	-	-	-	-	-	+	-	-
<i>Fusobacterium</i>	+	+	+	+	+	+	+	-	-	-	-	+
<i>Lactobacillus</i>	-	-	-	+	+	-	+	-	-	+	-	-
<i>Leptotrichia</i>	+	+	-	-	-	-	-	+	-	-	-	-
<i>Mycoplasma</i>	-	-	-	-	-	+	-	-	-	-	-	-
<i>Neisseria</i>	+	-	-	-	-	-	-	-	-	-	-	-
<i>Olsenella</i>	+	+	+	+	+	-	-	-	-	+	-	-
<i>Oribacterium</i>	-	-	-	-	+	-	-	-	-	-	-	-
<i>Paludibacter</i>	+	-	-	-	-	+	+	-	-	-	-	-
<i>Parvimonas</i>	+	+	+	+	+	-	+	-	+	-	+	-
<i>PeptoStreptococcus</i>	-	-	+	+	-	-	-	-	-	-	-	-
<i>Porphyromonas</i>	-	+	+	+	+	-	-	-	-	+	-	-
<i>Prevotella</i>	+	+	+	+	+	+	+	-	-	-	+	-
<i>Pseudomonas</i>	-	-	-	-	-	-	+	-	-	-	-	-
<i>Rothia</i>	-	-	-	+	-	-	-	-	-	-	-	-
<i>Scardovia</i>	-	-	-	-	+	-	-	-	-	-	-	-
<i>Schaalia</i>	-	+	-	-	+	-	-	-	-	+	-	-

<i>Selenomonas</i>	-	-	+	+	+	-	+	+	-	-	-	-
<i>Streptococcus</i>	+	+	+	+	+	+	+	+	+	+	+	+
<i>Tannerella</i>	+	+	+	+	+	-	+	-	+	-	+	-
<i>Treponema</i>	+	+	+	+	+	+	+	-	-	+	-	-
<i>Veillonella</i>	+	+	-	+	+	-	-	+	+	+	+	-

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+: Presence; -: Absence; •: same patient

526  
527

528 **Table 3.** Distribution of bacterial genera, expressed as percentage, in periodontal (P) and  
 529 endodontic (E) tissue samples of root canals from patients affected by severe periodontitis.

Bacterial genera	P (%)	E (%)
<i>Actinomyces</i>	41.7	41.7
<i>Atopobium</i>	25.0	16.7
<i>Bifidobacterium</i>	25.0	0.0
<i>Campylobacter</i>	33.4	8.3
<i>Capnocytophaga</i>	16.7	8.3
<i>Corynebacterium</i>	0.0	8.3
<i>Dialister</i>	8.3	0.0
<i>Enterococcus</i>	0.0	8.3
<i>Fusobacterium</i>	50.0	16.7
<i>Lactobacillus</i>	16.7	16.7
<i>Leptotrichia</i>	16.7	8.3
<i>Mycoplasma</i>	8.3	0.0
<i>Neisseria</i>	8.3	0.0
<i>Olsenella</i>	41.7	8.3
<i>Oribacterium</i>	8.3	0.0
<i>Paludibacter</i>	16.7	8.3
<i>Parvimonas</i>	41.7	25.0
<i>PeptoStreptococcus</i>	16.7	0.0
<i>Porphyromonas</i>	33.4	8.3
<i>Prevotella</i>	50.0	16.7
<i>Pseudomonas</i>	0.0	8.3
<i>Rothia</i>	8.3	0.0
<i>Scardovia</i>	8.3	0.0
<i>Schaalia</i>	16.7	8.3
<i>Selenomonas</i>	25.0	16.7
<i>Streptococcus</i>	50.0	50.0
<i>Tannerella</i>	41.7	25.0
<i>Treponema</i>	50.0	16.7
<i>Veillonella</i>	33.4	33.3

530