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# Residual bacteriome after chemomechanical preparation of root canals in primary and secondary infections.


## Abstract

Secondary infections may be linked to the presence of residual microorganisms within dental root canals. The purpose of this study was to investigate the bacterial composition of primary and secondary root canal infections, before and after chemomechanical treatment. Samples were collected before chemomechanical preparation (S1) and prior obturation (S2) from 19 subjects (10 primary and 9 secondary infections). DNA was extracted and the V3/V4 region of the 16SrRNA gene was amplified using the 347F/803R primers and paired-end sequenced using the Illumina MiSeq instrument. Sequencing analysis yielded partial 16S rRNA gene sequences that were taxonomically classified into 10 phyla and 143 genera. The most prevalent phyla in S1 and S2 samples were found to be Firmicutes and Bacteroides, however, when comparing between sample groups, Proteobacteria seem to have been enriched in secondary infections. The dominant genera in the primary S1 samples were *Bacillus, Streptococcus and Prevotella while Bacillus, Streptococcus* and *Selenomonas* dominated the secondary infection S1 samples. *Bacillus* and *Marinilactibacillus* were the most dominant genera in primary and secondary S2. The mean number of OTUs per sample was 32,656 (±12,124 SD) and 37,113 (± 16,994 SD) in S1 and S2 samples, respectively. Alpha and Beta diversities presented the same pattern within samples from both groups. Great inter-individual variations in the bacterial composition of the root canal biofilms were observed. There was no difference in the bacterial composition before and after treatment, although some genera survived and seems to be part of a residual microbiome. Our findings revealed a high diversity of the bacterial communities present in root canal infections after chemomechanical treatment, although the majority of the taxa detected were in low abundance.



**Keywords:** Microbiota, Pulpitis, Endodontic inflammation, Next generation sequencing, 16S rRNA sequencing.

## Introduction

Apical periodontitis is an inflammatory oral disease characterised by contaminated dental pulp and apical tissues, and necrotic root canals. Infection is triggered by oral opportunistic pathogens invading and colonising the root canals due to carious lesions, trauma, tooth fracture or disruptions by dental procedures exposing dental pulp (1-3). Endodontic treatment is the recommended clinical approach which typically consists of the removal of infected pulp tissues from the root canal system, chemomechanical disinfection, filling procedures and tooth obturation to prevent re-infection (4). A primary infection refers to the first ever infection of a root canal. Studies have reported variable rates of treatment success, from 70% (5) to as high as 95% (6). A review based on 26 clinical studies reported a success rate around 80% (7).

However, endodontic treatment failures lead to re-infection, and are considered secondary infections or persistent infections, which may be caused by persistent inflammation linked to the presence of residual microorganisms within the root canals after chemomechanical treatment (8-10). Also, secondary infections can be associated with the re-entry of isolated microorganisms or biofilms into the root canal, which may occur due to the delay in placing a definitive coronal restoration with adequate sealing (11-14). Other reported causes of treatment failure include missed canals during treatment, insufficient enlargement of root canals, perforation, residual caries and root fractures (15). Indeed, dental root canal systems include an isthmus, lateral canals, and apical ramifications, which can be difficult to access during endodontic therapy, particularly when microorganisms have already colonised these areas and can remain viable following treatment procedures (12, 16).

Residual bacteria surviving chemomechanical procedures have been investigated using cultural and molecular approaches (17, 18). It has been reported that apico-coronal seals may become ineffective and allow host glycoproteins to percolate into the root canal environment, therefore providing an endogenous nutrient source to residual bacteria allowing them to proliferate and cause periradicular lesions (13, 19). Bacterial genera isolated from necrotic root canals have been found to be mostly strict and facultative anaerobes such as *Peptostreptococcus, Prevotella, Porphyromonas, Fusobacterium, Eubacterium*, *Actinomyces*, as well as streptococci (11). Interestingly, secondary root canal infections have been reported to have distinct microbial populations compared to non-treated ones. Some of these bacteria have been found to be resistant to conventional antimicrobials used in endodontic treatment and are able to remain viable in root-filled teeth (11, 20). Studies found in the literature vary in methodologies and types of periapical diseases included. To the best

of our knowledge, only two studies assessed the microbiome of primary and secondary endodontic infections including different periapical diseases using next-generation sequencing, but both only collected samples after chemomechanical treatment (21, 22). Although the presence of a residual bacterial community during the root filling procedure represents a poor prognosis, no specific species has been linked to it (23). Irrespective of the individual distinctions in species composition, sophisticated molecular methods to detail the community's composition may help establish strategies for more effective and tailored antimicrobial treatments improving the success rates of endodontic treatments.

The understanding of the microbial diversity and ecology related to endodontic infections is important to help guide clinicians towards the ideal therapeutic approach (12, 24). The aim of this longitudinal study was to investigate the bacterial diversity of primary and secondary root canal infections using high-throughput sequencing to answer the following questions: What is the microbial composition and abundance of infected root canals? Does the microbiological diversity differ between primary and secondary infections? And which bacterial species may persist after standard root canal treatment?

## Materials and Methods

### Subjects

The sample size of this study was determined following statistical advice by a qualified biostatistician at the Centre of Epidemiology and Biostatistics, University of Leeds. There are significant differences in the anatomy, ecosystem, infection nature and disease pathogenesis when comparing the root canal system to other body sites. Hence a decision was made to conduct a pilot study. Three studies that used a similar NGS approach in the form of pyrosequencing (25-27) reported recruitment of 7,10 and 17 participants respectively. The usual pilot study with a sample size of 30 does not apply here because our observed outcome was not expected to be normally distributed data. Therefore, based on this and previous literature as well as the time available for patient recruitment and sampling which was limited, we had proposed to recruit 20 participants with an expected dropout rate of <15 %.

The study population included subjects who had non-vital infected teeth with evidence of chronic apical pathology confirmed by clinical signs and symptoms, such as tenderness to percussion, soft tissue palpation and/or presence of sinus tract, negative response to (thermal and/or electrical) pulp testing, apical radiographic changes that indicated an apical pathology in line with clinical signs and symptoms.

This clinical study included subjects with both primary and secondary infections. The demographic and clinical data of teeth included in the study are described in supplementary Table S1. Only one tooth per subject was included in this study. The research team included two experienced and trained dental nurses who were involved in the participants' recruitment and clinical care. Only one trained dentist performed clinical diagnosis and endodontic treatments. Ethical approvals were obtained from the National Research Ethics Service (NRES) Committee of Leeds East (REC reference number: 13/YH/0035) and the Leeds Research and Development Directorate (R&D) approval was obtained from Leeds Teaching Hospitals (LTHT R&D number DT 13/ 10723).

*Eligibility criteria*

The inclusion criteria were individuals with a teeth with primary (previously untreated) or secondary (previously root filled) root canal infections; Restorable teeth; Stable periodontal condition and absence of periodontal pockets > 4mm. Exclusion criteria were individuals under 18 years old; any immune deficiency such as HIV or leukemia; pregnant; who had antibiotics in the last month; teeth with severe anomalies; cases where microbiological sampling may not be optimum or compromised by an ineffective coronal seal, for instance: teeth with post(s), teeth with root curvature of >15◦ and teeth which fail to show radiographic evidence of patent canals. Clinical characteristics were balanced between groups (pain: 6 primary, 5 secondary; sinus: 2 patients per group; swelling: 3 primary, 2 secondary; apical periodontitis with radiolucency >10mm 1 primary, 2 secondary) (supplementary table S1).

**Endodontic treatment and sample collection**

The research was conducted at the Leeds Dental Translational and Clinical Research Unit (DenTCRU), Leeds Teaching Hospitals. The root canal (re)treatment was performed over three clinical visits in all cases according to the agreed protocol (Supplementary Figure S1).

Root canal biofilm samples were collected following the protocol described by Moller (28). In total, two types of biofilm samples were collected from each subject: S1 was collected during the first visit, prior chemomechanical treatment (using 2.5% NaOCl, CaOH dressing and manual instruments). S2 was collected during the third visit, immediately prior obturation of the root canal. See Figure S3 for further details.

The root canal treatment procedures of this study were tailored to achieve this aim as well as to optimize the quality of the study in accordance with the ethics and regulations of the UK. Although the selected cases were of a chronic nature, the definitive diagnoses varied and,

hence, some details of the treatment needed to be personalized for each given case. In addition, other factors such as tooth morphology, the restorative status or those related to the patient were vital when judging the most appropriate treatment choice. Despite all of this, the clinical protocol was designed to be as similar as possible for all patients. This, in addition to collection of samples at exact time intervals, was aimed to obtain a more comparable, reflective picture of the microbiological status of the infected root canals.

Sample collection procedure were as follows: the canal was filled with about 0.5-2 ml of sterile saline. A new sterile surgical glove was worn before sampling and a sterile file (Dentsply, UK) of at least size 20 was introduced into the canal and moved with gentle filing motion to disrupt the biofilm. The file was then placed in the sample collection tube (Bijou) which contained 1.5 ml of reduced transfer fluid (RTF). A sterile paper point was then inserted in the canal to the full working length to absorb the canal contents and then transferred to the collection tube. This was repeated until all fluid and biofilm were absorbed. In multi rooted teeth, the sample was collected from the canal with the apical pathology. Upon collection, the sample was immediately placed in a jar with anaerobic sachet and immediately transferred to the oral microbiology laboratory. Upon arrival, the collection tube was vortexed for 30 seconds and then placed in the anaerobic workstation for further laboratory analyses.

**16S rRNA sequencing**

DNA was extracted using the QIAamp DNA Mini Kit (Qiagen), following the manufacturer's protocol. The regions V3-V4 from the 16S rRNA gene were amplified using the Q5 High Fidelity DNA polymerase kit (New England BioLabs Inc.,Life Technologies Inc.US) with the 347F and 803R primers (Eurogentec, Belgium). The master mixture (supplementary table S2) was distributed as aliquots of 23 µl, plus 2 µl of the templates DNAs. The PCR tubes were then loaded onto the thermal cycler (Techne, Bibby Scientific, UK) (thermal cycling conditions are described in supplementary Table S3). The presence of PCR products was checked using agarose gel electrophoresis, with 1 µl of GelRed DNA stain (Biotium, UK). The microCLEAN (Microzone ltd,UK) was used to purify the PCR product samples. The DNA pellet was resuspended in 55.5 µl of nuclease-free water (Ambiol). The cleaned DNA was added to 3.0 µl End Prep Enzyme mix and 6.5 µl of End Repair Reaction Buffer (10X) to yield a total volume of 65 µl. The mixture tube was then placed in the thermal cycler (Techne, Bibby Scientific, UK) (thermal cycling conditions are described in supplementary table S4). Next, 15 µl Blunt/TA ligase master mix, 2.5 µl NEBNext Adaptor for Illumina and 1 µl ligation enhancer were directly added to the end Prep reaction mixture.

AMPure XP beads (Beckerman Coulter, Inc) was used for the cleanup, and the product was then eluted into 28 µl of 0.1X TE buffer. Finally, 23 µl of the solution was mixed with 25 µl of

NEBNext High Fidelity 2XPCR master mix, 1 µl of universal PCR primer, and 1 µl of Primer Index 1-38 (one unique index for each sample). After mixing by pipetting and a brief centrifuge, the mixture tubes were then placed in the thermal cycler for PCR amplification (see supplementary Table S5 for setting details). AMPure XP beads (Beckerman Coulter, Inc) was used again for another clean-up. Amplicon sizes were assessed with 2200 Tapestation System using 1 µl from each DNA sample, without dilution. The Qubit Kit Assays (Invitrogen, Life Technologies) was utilized to quantify the libraries. The final multiplexed indexed library was pooled by adding equimolar concentration of the libraries into a 2.0 ml collection tube and then sequenced on the Illumina MiSeq platform.

**Data analysis**

The demultiplexed paired-end reads were denoised with DADA2 using the Quantitative Insights into Microbial Ecology (QIIME2) bioinformatics pipeline (29) and clustering was performed at 99% identity to create OTUs. The taxonomy was using the Greengenes_13_8 database (30). Alpha diversity was evaluated with Chao1 richness estimator and Shannon diversity index was calculated. Beta diversity was also determined, and microbial structures compared using Unifrac. Unweighted Unifrac distances were used to perform a principal coordinate analysis in R software.

**Results**

The sample consisted of 19 participants, 14 females and 5 males, with an average age of 42.89±13.05. The number of primary and secondary infected root canals were n=10 and n=9, respectively. Discomfort/pain was related from 11 subjects and n=3 had radiolucency higher than 10mm (Supplementary table S1).

The number of OTUs detected in each sample is shown in supplementary Table S6. Overall, the average number of OTUs detected in S1 samples was 32,656 (± 144.8 SD) OTUs/sample compared to 37,113 (± 140 SD) in S2 samples. A total of thirteen bacterial phyla were assigned (Figure 1). At lower classification levels, 27 different bacterial classes, 49 orders and 86 families were identified in the root canal samples. On average, the four most abundant phyla were *Firmicutes (55.1%)*, *Bacteroidetes (15.7%), Proteobacteria (15.0%)* and *Actinobacteria* (8.4%) (Figure 1). *Firmicutes*, *Bacteroides*, *Proteobacteria* and *Actinobacteria* were in the same order of abundance and had similar percentages in primary and S1 samples (Figure 2). Secondary infection and S2 samples showed a similar phyla profile. *SR1* and

*Chloroflexi* were found only in primary samples, and *Synergistetes* only in one secondary sample. A detailed examination of S2 samples revealed a notable increase in Actinobacteria and decrease in Fusobacteria (Figure 2).

The most abundant classes were *Bacilli* (36.7%) and *Clostridia* (18.2%), both with similar abundance in primary and secondary infection samples, and *Clostridia* was more abundant in S1 than in S2 samples, while *Bacilli* showed similar percentage of abundance in both S1 and S2 samples. *Clostridiales* was the most abundant order (18.6%), followed by *Bacillales* (16.5%) and *Lactobacillales* (17.6%). The most abundant family was Bacillaceae (22.6%).

With regards to genera, 135 different genera were found in the samples. Of these, only 20 were found at an abundance of >1% in the overall abundance (data not shown). 70% of the top ten genera belonged to the *Firmicutes* phylum.

On average, the most abundant genera (all 33 samples included) were *Bacillus* (22.5%), *Marinilactibacillus* (9.2%), *Streptococcus* (7.3%), *Defluviitelea* (6.5%) and *Pseudomonas* (6.2%). The abundance of the main genera present in primary and secondary infection samples is shown in Figure 3. *Bacillus, Marinilactibacillus* and *Pseudomonas* were more abundant in S2 samples than in S1 samples. The 10 most abundant genera also included *Clostridium, Selenomonas, Nonlabens, Anaerosinus and Rothia*.

The most dominant genera in primary S1 samples were *Streptococcus* (4.4%), *Bacillus* (4.1%) and *Prevotella* (2.9%) whereas those in secondary S1 samples were *Bacillus* (6.2%), *Marinilactibacillus* (2%) and *Selenomonas* (1.9%). Interestingly, *Bacillus* and *Marinilactibacillus* were also the most dominant genera in primary S2 and secondary S2 samples. This may indicate survival and resilience properties of these genera.

The 10 most abundant bacterial species were different between sample groups. Those from primary S1 samples are shown in supplementary Figure S2. *Streptococcus agalactiae* was the most abundant bacterium in primary S1 samples, whilst *Marinilactibacillus psychrotolerans* was most abundant in secondary S1 samples. *Streptococcus agalactiae*, *Defluviitalea saccharophila*, *Anaerosinus glycerini*, *Bacillus alkalinitrilicus*, *Marinilactibacillus psychrotolerans* and *Rheinheimera perlucida* were among the top 10 most abundant bacterial species in all four groups (Supplementary Figures S2-S5).

The abundance of most represented taxonomies in the samples is also presented in a heatmap (Figure 4).

## Alpha diversity

To assess the diversity within the samples, alpha rarefaction diversity was calculated and displayed in supplementary figures S6 and S7. The diversity analyses of observed species, according to infection type (primary or secondary), and sample type (S1 or S2), resulted in a similar pattern. The boxplots showing Chao1 and Shannon estimators for primary and secondary infections, and S1 and S2 samples are presented in Figure 5.

## Beta diversity

To assess bacterial diversity between samples, beta diversity analysis was carried out. A principal coordinate analysis (PCoA) based on unweighted UniFrac plot is shown in Figure 6. No distinct clustering between sample groups was observed, indicating that the samples had relatively similar microbial diversities.

## Discussion

High throughput sequencing using Illumina's MiSeq was used to explore the diverse composition of endodontic infection samples, before and after chemomechanical preparation of root canals in primary and secondary infections. In contrast with our study, some studies in the literature that assessed the microbiome in endodontic infections included only apical periodontitis samples (11, 31), and one did not make it clear if they included other periapical diseases (20). Besides, this particular study used a checkerboard DNA-DNA hybridization to identify the microbiota present in root canal samples (20). Two studies collected samples from extracted teeth (11, 32), and in one of them the teeth were pulverized with a cryogenic grinder (32).

Next generation sequencing (NGS) data analysis indicated no significant difference in OTUs abundance before and after root canal treatment (supplementary Table S6). Our study showed no difference in the bacterial composition before and after root canal treatment, although it was expected to observe a reduction in the bacterial load due to the chemomechanical treatment (33-36). However, when comparing primary and secondary infections, a clear difference in the phylum composition was observed, with an enrichment of *Proteobacteria* in secondary infections. This result can indicate resistance of taxa belonging to this phylum.

Our finding emphasizes the current knowledge that existing root canal preparation procedures usually fail to disinfect and clean large parts of the root canal system. It might

reduce, but not eliminate bacteria from the canal (33). The presence of slightly higher proportions of OTUs in S2 samples when compared to S1 may hold clinical implications. These OTUs may have been present in low abundance in the primary samples. The change in the environment and the reduction in selection pressure following treatment might have enriched these species. These OTUs may also come from viable and non-viable microorganisms that remained in the root canal because either they survived the treatment protocol or they were located and persisted in lateral canals that were not accessible to instrumentation and intracanal medication (12). Some components from non-viable species remaining in the canal may serve as nutrient source for the remaining microorganisms, leading to persisting or recurring infections. Moreover, other remnants of bacterial cells such as endotoxins may be involved in inflammatory reactions, as they stimulate the release of cytokine and MMPs, which contribute to the inflammatory process (37, 38).

Previous NGS studies support our findings related to the number of phyla and genera detected. One study using pyrosequencing detected 15 bacterial phyla and 160 genera in twenty teeth (32), while in the apical root canal infections another study detected 84 genera and 10 phyla (27). Other researchers (39) studied 23 extracted teeth and compared apical and coronal segments in which they detected 24 phyla. Other NGS studies detected between 9 and 18 phyla (11, 22, 31, 40, 41).

*Firmicutes* were the dominant phylum in primary and secondary infections, similar to the findings from a study using the Illumina HiSeq2000 instrument (41). The results of other studies comparing primary and secondary infections may vary. *Firmicutes* were found to dominate secondary infections in one study (40), while *Bacteroidetes* were the most abundant phylum in primary infections in another study (33).

The enrichment of *Proteobacteria* in secondary infections has previously been described. A metagenomics study described similar findings to ours (24). Furthermore, Keskin et al. showed a high abundance of this phylum in both, primary and secondary infections. These reinforce the need of further studies on *Proteobacteria*'s persistence into root canals.

Further evidence from Vengerfeldt et al. (2014) supports our findings of high abundance of *Firmicutes* and *Bacteroidetes* in S1 samples (41). In a study from 2018, *Firmicutes* were also detected as being among the five most abundant phyla (11).

*Streptococcus* was the most dominant genus in primary S1 samples, and the same result was found in a number of culture, molecular and pyrosequencing studies (40, 42-44). In secondary infection samples, genera such as *Fusobacterium*, *Streptococcus* and

297 *Actinomyces* identified in this study also appeared in other studies as was described in a
298 recent review (13).

299 In the primary infection samples, dominant genera remained mostly the same before and
300 after chemomechanical treatment of root canal (*Streptococcus* and *Bacillus* in S1*, Bacillus*
301 *and Marinilactibacillus* in S2 samples) (Supplementary Figure S8). Similarly, in secondary
302 infection samples, the dominant genera detected in both S1 and S2 samples were *Bacillus*
303 and *Marinilactibacillus,* which gives evidence of their resilience.

304 Primary and secondary S1 samples had a similar OTU count. However, dominant genera
305 were found to be different: *Streptococcus*, *Bacillus* and *Prevotella* were dominant in primary
306 samples whereas *Bacillus*, *Marinilactibacillus* and *Selenomonas* were dominant in secondary
307 infection samples. Nevertheless, most of the assigned bacteria were found in both primary
308 and secondary infection samples, although at different abundance. Some studies have also
309 found varied bacterial communities in primary and secondary endodontic infections (11), but
310 other studies found no difference between the two types of infection (32). One consensus
311 between these studies is that endodontic infections are polymicrobial, complex, with some
312 predominant genera but may still remain variable between individuals (10, 11). The
313 differences in the dominant phyla and/or genera observed in these studies might be due to
314 several aspects. These include variations in clinical conditions and anatomical locations,
315 sampling methods, NGS platforms and read lengths used for analysis. In addition, site-
316 specific endodontic bacterial communities can also contribute to variations (24).

317 Due to the change in environmental conditions after endodontic treatment, bacteria persisting
318 in root canal and identified in secondary infections are usually the ones that can survive harsh
319 conditions such as wide pH range and low nutrient availability (10). Some studies have
320 detected in secondary infections, species such as *Enterococcus faecalis (23),* which was not
321 detected in this study. A recent systematic review showed that studies found *E. faecalis*
322 mostly in secondary infection samples, sometimes in high abundance (45). However, other
323 studies have, like ours, also not found *E. faecalis* in secondary infections (46) or detected it
324 in low abundance (20, 36). Reasons for its absence or low detection include sample selection,
325 patient condition and the detection method used (46). It might also be due to the fact that in
326 this study, the samples were taken from inside the entire root canal, and not from the root
327 surface or different thirds of the root canal (apical or cervical). Elucidating the ecology and
328 pathogenicity of microbial communities requires the thorough identification of site-specific
329 microbial species present in low abundance (47). We might especially recognise that the
330 dental pulp was initially a sterile environment (26). This aspect might be important in

331 treatment strategies aiming for better rates of long-term success and reducing the need for
332 expensive, unnecessary additional interventions.

333 *Streptococcus agalactiae*, the most abundant species in primary S1 samples, is a Gram
334 positive, facultative anaerobic bacterium. It can be commensal but is usually considered an
335 opportunistic pathogen, as it has been associated with systemic infections (48).

336 *Marinilactibacillus* is a relatively new Gram positive, facultative anaerobic genus with only 2
337 species described so far (49, 50), therefore there is currently sparse information in the
338 literature about the species *Marinilactibacillus psychrotolerans* and its association with
339 endodontic infections. More research is necessary to confirm our findings and better
340 understand this microorganism.

341 One of the secondary objectives of this study was to investigate the prokaryote
342 microorganisms that can resist after chemomechanical preparation. Opting for a multiple root
343 canal treatment visit approach allowed for this investigation as well as for comparison with
344 pre-preparation samples. Although a Cochrane Review (51) detected no significant
345 differences in the effectiveness of root canal treatment between single and multiple visits, it
346 concluded that the former is associated with higher frequency of symptoms. In addition, for
347 teeth with necrotic pulps and apical disease, as in this study, multiple visit root canal treatment
348 is the traditional treatment option as it allows the use of inter-appointment medication which
349 may be beneficial for the cases with more established infections.

350 Limitations of this study include the lack of discrimination from dead and live microorganisms
351 and hence all genetic materials were assessed, which may have overestimated bacterial load
352 (52). However, it is argued that an assessment of both live and dead microorganisms is
353 important because these bacteria may have been predominant in the early phases of disease
354 or played a part in biofilm formation (53). Besides, targeting fragments of 16S rRNA variable
355 regions using short-read sequences (up to ~300 bp) instead of the full gene or a shotgun
356 sequencing approach, does not provide the same level of accuracy for identification to the
357 species level (54). Such profiling also lacks the necessary details required for a full
358 understanding of the microbiota including non-bacterial micro-organisms. About 37% of the
359 reads could not be assigned to any taxa at the phylum level, and this may be due to PCR
360 artefact, sequencing errors, or possibly unknown bacterial phyla (45). The results should be
361 interpreted with caution due to the heterogeneous samples regarding clinical variables such
362 as pain and swelling, teeth with sinus tract, and chronic apical periodontitis, although this was
363 minimized by balancing these characteristics within groups. Further investigations are
364 needed to complete a thorough profiling of endodontic biofilms.

## Conclusions

Secondary infections have shown to have similar diversity to primary infections, however, with different bacterial abundance. Similar diversity was also found before and after chemomechanical preparation of the root canal, although some bacteria such as *Bacillus* and *Marinilactibacillus* were the most dominant genera in primary and secondary S2 and seem to be part of a residual microbiome. This is an indication that specific bacteria are able to survive the standard root canal disinfection procedure, therefore strict aseptic procedures, more specific, targeted disinfection technique, irrigation and washing time may be recommended.

Further studies are essential to further explore the understanding of the ecology within the infected root canal and apical regions and guide strategies for treatment improvement.

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## Data availability

Sequences are deposited in the public repository NCBI Sequence Read Archive (SRA) accession number (PRJNA750799).

## Disclosure of interest

The authors report no conflict of interest, and no financial affiliation.

**Figure 1**: Relative abundance of phyla in all samples. Eleven of the 13 identified phyla are displayed and listed in the legend (SR1 and Synergistetes' abundance ranged between 0-0.2 and 0-0.1% respectively and were removed for ease of visualisation).

**Figure 2:** Relative abundance of phyla in S1, S2, primary and secondary infection samples. Eleven of the 13 identified phyla are displayed and listed in the legend (SR1 and

Synergistetes' abundance ranged between 0-0.2 and 0-0.1% respectively and were removed for ease of visualisation).

**Figure 3**: Genera abundance in S1, S2, Primary and Secondary infection samples.

**Figure 4**: Abundance of taxonomies described in a heatmap. The percentage abundance is represented as log 10 values and shown as a colour gradient ranging from yellow to blue, with yellow being the most abundant features.

**Figure 5:** Alpha diversity indices (Chao1 and Shannon) for primary and secondary infections. The boxplots display indices grouped by S1 (before canal treatment) and S2 (prior obturation) samples.

**Figure 6:** Principal coordinate analysis (PCoA) of Bray–Curtis similarity of bacterial communities. The analysis was based on square root-transformed proportions of OTUs and included all samples.

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