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CASE REPORT

In situ imaging and microbiome analysis of calculus-like deposits at the root apex: A case report of refractory apical periodontitis

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Abstract

Aim: This case report explored the application of next-generation sequencing (NGS) and fluorescence in situ hybridization (FISH) to visualize and characterize microbial populations in a refractory endodontic infection with apical calculus-like deposits, a rarely reported phenomenon.

Summary: Histopathologic analysis revealed bacterial biofilms and calcifications on the root surface, with Gram-positive bacteria predominant in both hard and soft tissues. Microbial sequencing showed *Pseudomonadota* dominated hard tissues, whereas *Bacillota* were prevalent in soft tissues, with distinct genera like *Lactibacterium* and *Streptococcus* identified. FISH imaging confirmed spatially distributed bacterial taxa, including *Actinomycetota* and *Chloroflexota*, within the biofilm, aligning with NGS findings. Notably, *Bacteroidota* was exclusive to soft tissues, whereas *Chloroflexota* was detected only in hard tissues. The presence of extensive calculus-like deposits on the root surface provided new insights into the microbial complexity of persistent endodontic infections and their management.

Key learning points:

1. The combination of NGS and FISH provided unprecedented insights into the microbial composition of refractory endodontic infections, revealing a diverse and spatially organized ecosystem.
2. Distinct microbial compositions in hard and soft tissues emphasize the importance of targeted therapeutic strategies for endodontic infections.
3. The presence of unique bacterial taxa and biofilms in calculus-like deposits offers new avenues for research into the pathogenesis and persistence of endodontic infections.

KEYWORDS

calculus-like deposits, endodontics, extraradicular infection, FISH, in situ biofilm, next-generation sequencing

INTRODUCTION

The oral cavity is a complex ecosystem of microorganisms that influence oral health and disease (Deo & Deshmukh, 2019). The dental pulp, normally sterile, becomes infected due to caries, trauma or periodontal disease, leading to pulp and periradicular inflammation (Siqueira & Rocas, 2009). Effective endodontic treatment aims to eliminate or reduce the microbial load and prevent reinfection through root canal filling (Nair et al., 2005). Although primary orthograde treatment often heals apical periodontitis (Sjogren et al., 1990), 10%–20% of cases fail because of persistent infections (Nair, 2004). These failures are linked to microorganisms in the root canal system, extraradicular regions and biofilms (Sun et al., 2022). Certain genera, such as *Actinomyces* and *Propionibacterium*, can colonize extraradicular spaces and contribute to treatment failure (Nair, 2004). Uncommon cases of extra-radicular infections with calculus-like deposits have been reported; however, comprehensive microbiome analyses are lacking (Harn et al., 1998; Ricucci et al., 2005).

Advancements in endodontic microbiology, from anaerobic cultures to high-throughput sequencing, have expanded knowledge of microbial diversity in persistent infections (Siqueira & Rocas, 2009). However, the spatial distribution of these microbes within biofilms remains poorly understood (Siqueira & Rocas, 2022). Fluorescence in situ hybridization (FISH), a noninvasive method using fluorescent probes, offers high-resolution visualization of bacterial species within clinical samples (Moter & Gobel, 2000). Despite its potential, few studies have applied FISH to endodontic infections, highlighting a promising area for future research (Schaudinn et al., 2009; Sunde et al., 2003).

This case report aimed to identify and visualize microorganisms in two periodontally healthy teeth with refractory sinus tracts and apical calculus-like deposits using next-generation sequencing (NGS) and FISH. By combining these techniques, the report sought to provide deeper insights into the microbial composition and spatial distribution within these lesions, enhancing the understanding of intraradicular and extraradicular infections.

CASE REPORT

Informed consent

The patient provided oral consent for the treatment and signed an informed consent form for the use of data and analysis in this report.

Clinical history

A 73-year-old care-dependent male with bipolar disorder, epilepsy and prostate pathology visited a general dental practitioner (GDP) in January 2019. He presented with multiple carious lesions, mild periodontal disease and persistent sinus tracts from periapical abscesses in teeth 22 and 23. Initial root canal treatment with BioRaCe files (FKG Dentaire Sàrl, Le Crêt-du-Locle, Switzerland) and calcium hydroxide dressing (Calasept, Directa AB, Upplands Väsby, Sweden) failed to resolve the sinus tracts, prompting referral to a specialist in May 2019.

At the specialist clinic, retreatment involved XP-Endo-Finisher (FKG Dentaire Sàrl, Le Crêt-du-Locle, Switzerland), extensive irrigation and intracanal medication over three sessions. Despite these measures, the sinus tract persisted, necessitating microsurgical endodontic treatment in November 2019. During surgery, interconnected lesions were observed, with the root surfaces coated in a grey, irregular, calculus-like deposit. Tooth 22 had extensive coverage from the apex to approximately 5–6 mm coronally, predominantly on the buccal surface, appearing as an amorphous mass. Tooth 23 showed less coverage, with dark patches exposing underlying dentin. The root apices were resected at the coronal limit of the deposits, and retrograde fillings of Total Fill BC RRM putty (FKG Dentaire Sàrl, Le Crêt-du-Locle, Switzerland) were placed.

At the 11-month follow-up, the patient was asymptomatic, with no clinical or radiological signs of pathology, though composite restorations had failed. The patient passed away in May 2023 before the scheduled 4-year follow-up. Representative images of the case are shown in Figure 1.

Processing of tissue specimens

The apices of the teeth (hard tissue) and a portion of surrounding granulation-like tissue (soft tissue) were fixed immediately in 4% paraformaldehyde solution at room temperature for at least 24 h; hard tissue was demineralized. The hard and soft tissues were then washed in distilled water (2×20 min), dehydrated in ascending concentrations of ethanol (2×30 min 70%, 30 min 90%, 2×30 min 96%, 30 min 100%, 45 min 100%) and cleared in xylene (2×60 min). Dehydrated hard and soft tissues were immersed in molten paraffin (56°C) overnight and embedded in the same material. Embedded samples were sectioned at 5 μm thickness along the longitudinal axis. Morphological analysis was performed using haematoxylin–eosin (HE), Giemsa and Gram staining at the Clinical Pathology Department of Umeå University Hospital. Stained histological sections were scanned using

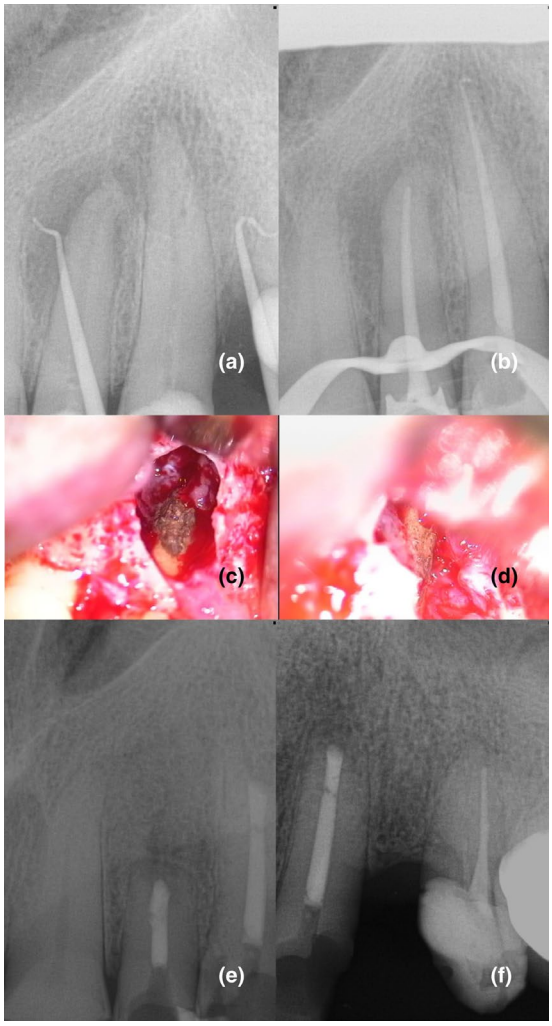

FIGURE 1 (a) Diagnostic radiographs taken at the endodontics specialist clinic, showing apical radiolucencies in teeth 22 and 23 with visible sinus tracts; (b) completed root canal treatment; (c, d) clinical photographs of tooth 22 (c) and tooth 23 (d) during apical surgery, revealing calculus-like structures; and (e, f) radiographs taken at the 11-month postoperative follow-up, demonstrating healing in teeth 22 and 23. All radiographs were captured using SIRONA imaging equipment.

a Panoramic III scanner (3D Histech, Hungary), and digital images were obtained. The digital image analysis (DIA) was conducted using the 3D Histech QuantCenter software (3D Histech, Hungary).

Next-sequencing microbiome identification

DNA extraction for microbial analysis

Bacterial deoxyribonucleic acid (DNA) was extracted from formalin-fixed, paraffin-embedded (FFPE) hard and soft tissue samples. Initial scrolls from the FFPE blocks were discarded, followed by deparaffinization of eight 10-μm sections using xylene and subsequent rehydration with a graded ethanol series (100% → 70% → 50%). DNA extraction was conducted using the QIAamp DNA Micro Kit (QIAGEN) with the following modifications. Chemical lysis was performed using lysozyme (20 mg/mL) and mutanolysin (200 U/mL), whereas mechanical lysis was achieved using NucleoSpin Bead Tubes Type B (Macherey-Nagel) for 3 min at 50 Hz. The microtome was decontaminated with DNA AWAY (Thermo Scientific) between samples, and sterile swabs were routinely tested and sequenced as controls.

Illumina MiSeq sequencing

The extracted DNA samples were shipped to the Forsyth Research Institute (Cambridge, MA, USA) for microbial analysis using NGS. The V3–V4 hypervariable regions of the 16S rDNA were amplified using the forward primer 341F and reverse primer 806R, as described by Monoharan et al. (2020). Following amplification, amplicons were purified using AMPure beads (Beckman Coulter Genomics). Each library, totaling 100 ng, was pooled, gel purified, quantified and supplemented with 20% PhiX before being run on the MiSeq system. The MiSeq produced paired-end reads (2 × 250 bp), which were fused. The data processing included the removal of barcodes, primers, and any ambiguous or chimeric sequences, followed by amplicon sequence variant (ASV) predictions and abundance calculations. ASVs with total abundances below 10% of samples with at least five counts were filtered out. This was performed using QIIME2 in combination with DADA2. Taxonomy of the ASVs was assigned using HOMD reference sequences (Manoharan et al., 2020).

Fluorescence in situ hybridization

Mono-labelled oligonucleotide probes used for hybridization in FISH

Tooth sections (hard tissues) were analysed using FISH with mono-labelled 16S rRNA oligonucleotide probes (Appendix A: Table A1). Three probe sets were applied to target different bacterial groups: the first set detected bacteria (EUB 338_I), Gammaproteobacteria (Gam 42a with a competitor) and Actinomycetota (HGC 69a with a competitor); the second set targeted Gram-positive bacteria with low GC content (LGC354a, LGC354b, and LGC354c); and the third set identified Chloroflexota (GNSB 941 and CFX1223).

Detection of bacteria by FISH

Mono-labelled oligonucleotide probes were utilized to visualize the location of selected members within the bacterial community, using the protocol described by Lee (2019). FISH was performed on formalin-fixed, paraffin-embedded (FFPE) sections. Positive and negative control samples were included, with *Herpetosiphon aurantiacus* CCUG 48726T serving as the positive control and *Fusobacterium nucleatum* subsp. *polymorphum* CCUG 9126T as the negative control. Additionally, FISH was performed on samples without fluorescent probes to evaluate the extent of autofluorescence. Before hybridization, the FFPE sections were deparaffinized using xylene (10 min, twice) and rehydrated through a graded ethanol series: absolute ethanol (10 min), 95% ethanol (10 min), 90% ethanol (10 min), 70% ethanol (10 min), followed by distilled water (10 min). Each probe at a final concentration of 2 nM was applied to samples in hybridization solution (900 mM NaCl, 20 mM Tris, pH 7.5, 0.01% SDS) with adequate concentrations of formamide and incubated at 46°C for 12 h in a humidified condition with corresponding concentrations of formamide. Slides were then washed in saline Tris-EDTA buffer (215 mM NaCl, 20 mM Tris, pH 7.5, 5 mM EDTA) at 48°C for 2 h, dipped in cold water, air-dried, and mounted on microscope slides in VectraShield medium with DAPI (Dao, Agilent Tech. #S3023) at a final concentration of 1 μg/mL.

All probes were commercially synthesized at the biopolymer factory BIOMERS (www.biomers.net, Ulm, Germany).

Slides were stored in the dark overnight at 4°C before microscopy and analysed using a Zeiss LSM 710 confocal microscope equipped with a Zeiss AXIO imager M2 under a 63× oil objective. Detectors and filters were set for simultaneous monitoring of red fluorescence (Exmax 647 nm/Emmax 665 nm), green fluorescence (Exmax 495 nm/Emmax 519 nm) and blue fluorescence (Exmax 350 nm/Emmax 470 nm), and scanned sequentially to eliminate spectral overlap between probes. Photomicrographs were obtained and visualized with ZEN software.

Quantitative analysis of the fluorescence intensity

Fluorescence intensity was analysed using ImageJ v.1.47 (NIH, USA). Each fluorescent channel was assessed separately, with intensity expressed as a mean ± standard deviation. A threshold was set to distinguish bacterial signals from the background, and the fraction of the area occupied by each probe-detected taxon was calculated as the percentage of all detected bacteria.

Bioinformatic analysis

The Wilcoxon signed-rank test was used to compare median taxa occurrence with the most frequently detected taxon. Statistical analysis was performed using GraphPad Prism 10.1.2 (GraphPad Software Inc.).

Histopathologic and histobacteriologic observations

HE and Giemsa staining revealed an amorphous material on the root surface, compatible with a bacterial biofilm, alongside areas of calcification (Figure 2a,b). Interestingly, Giemsa staining highlighted similar bacterial structures within the dentinal tubules (Figure 2b). Gram staining revealed Gram-positive bacteria within the biofilm on the tooth surface (Figure 2c).

HE staining of the soft tissue revealed a predominantly lymphoplasmacytic inflammatory infiltrate with Russell bodies, occasional polymorphonuclear cells and bacterial colonies (Figure 3a). Gram staining further confirmed infection by Gram-positive bacteria within the soft tissues, though the bacterial load was notably lower than in the adjacent hard tissues (Figure 3b).

Microbial spectrum assessment and quantification

The microbial diversity of hard and soft tissues at the phylum level, analysed through Illumina MiSeq sequencing, is presented in Figure 4. Five dominant bacterial taxa at the phylum level were observed in both hard and soft tissues; however, their prevalence differed significantly between them. In hard tissues, *Pseudomonadota* was the most prevalent phylum, whereas *Bacillota* predominated in soft tissues. Notably, *Bacteroidota* was exclusively detected in soft tissues, whereas *Chloroflexota* was found solely in hard tissues (Figure 4a).

Moreover, the heat map (Figure 4b) illustrates the relative abundance of bacterial taxa across the samples. In hard tissues, significant abundances were observed for *Lactobacterium aquatile* (10.7%), *Gammaproteobacteria* (9.1%), *Fusobacterium nucleatum* (8.8%) and *Pseudomonas* spp. (6.9%). In contrast, the soft tissue samples were dominated by *Streptococcus* spp. (19.9%), members of the

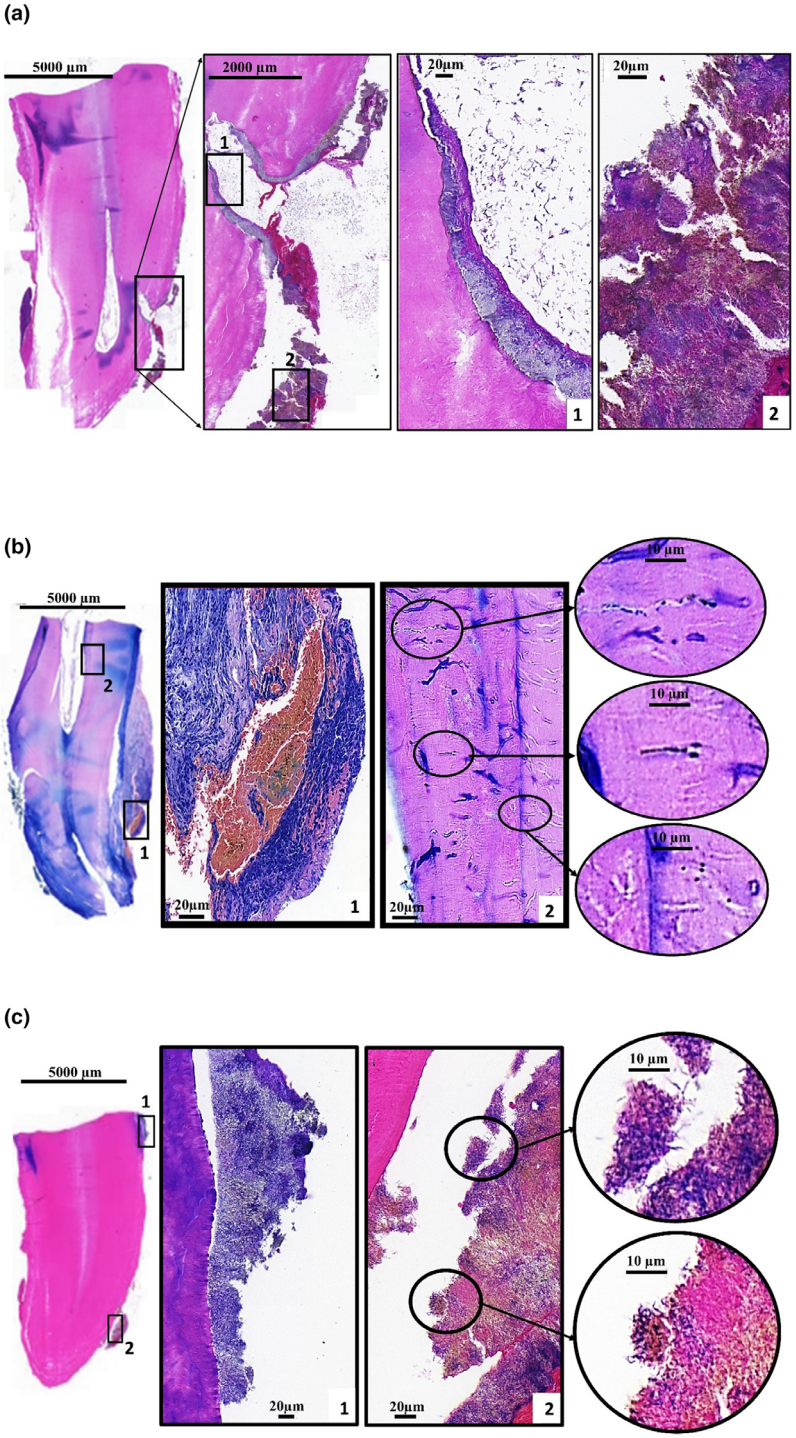

FIGURE 2 Morphological staining of hard tissues (teeth apices) sectioned along the longitudinal axis. Bacterial identification was primarily based on morphology, as eukaryotic tissues also stain. (a) Haematoxylin and eosin (H&E); (b) Giemsa; and (c) Gram.

Propionibacteriaceae family (12.3%) and *Porphyromonas pasteri* (12.1%).

Bacterial biofilms and visualization of selected bacterial taxa location in the tooth sample using FISH

FISH analysis revealed that the root surface was covered by a dense biofilm composed of various bacterial taxa (Table 1). Negative controls confirmed that background fluorescence and nonspecific interactions with the fluorescent dyes and the sample were negligible or lower than the positive FISH signals. Representative images from at least three independent experiments are shown in Figure 5. The detected bacterial taxa displayed distinct spatial distributions within the biofilm (Figure 5). Representatives of the *Actinomycetota* phylum were mainly located within the biofilm, mostly on one side of the root fragment (Figure 5a), and were the most

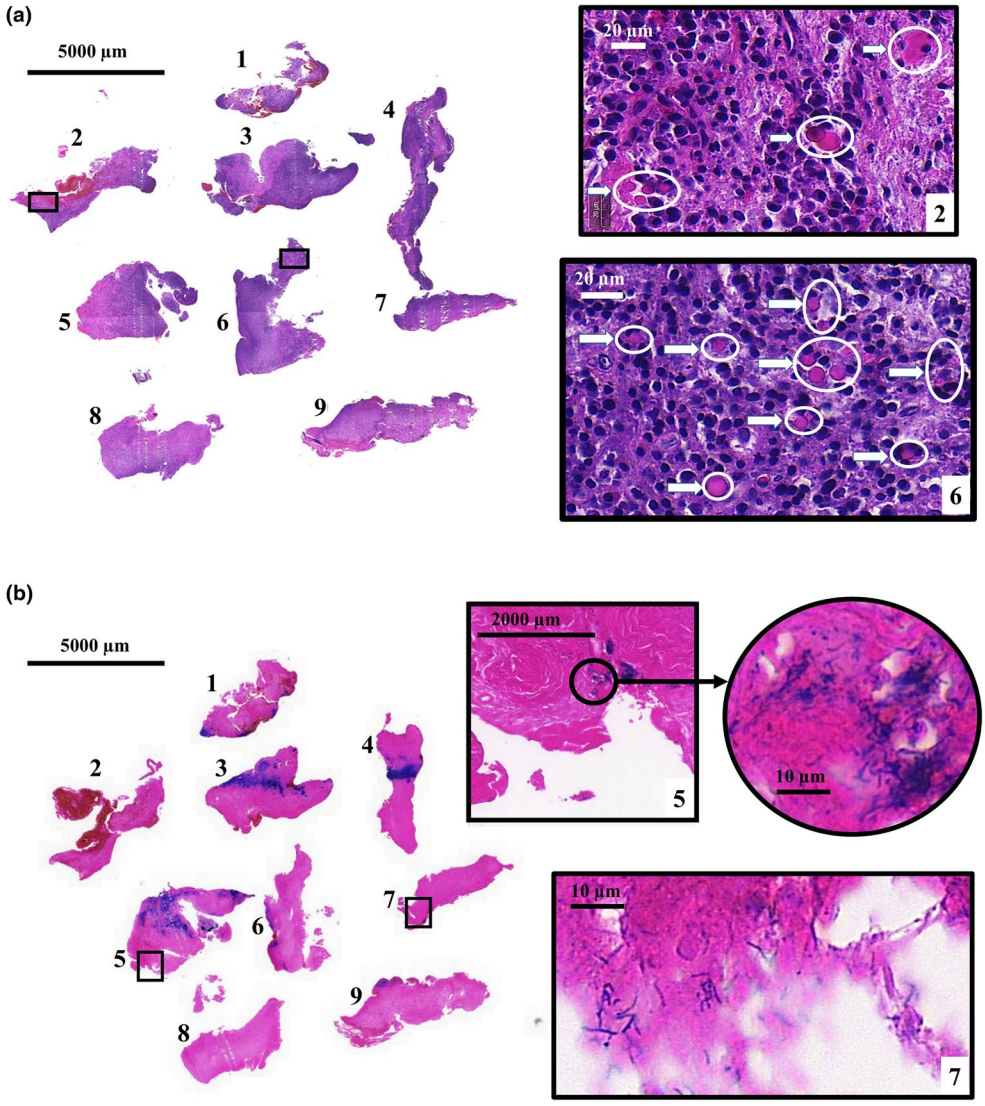

FIGURE 3 Morphological staining of soft tissues. (a) Haematoxylin and eosin (H&E) and (b) Gram staining. Representative areas show Russel bodies (H&E staining, arrows within circles) and bacteria (Gram staining).

abundant taxa detected in the first probe set (Figure 5b). Representatives of the *Gamma Proteobacteria* class were mainly observed on the outer layer of the biofilm (Figure 5a).

Bacteria from the *Bacillota* phylum with low GC content were widely present within the biofilm (Figure 5c) and were confined to one side of the root fragment. Additionally, *Bacillota* representatives were observed in the root canal space. The most abundant FISH signals were observed with the LGC-B probe (Figure 5d), which targeted genera such as *Staphylococcus, Bacillus, Aneurnibacillus, Paenibacillus* and *Alisyclobacillus*.

The third probe set targeted the *Chloroflexota* phylum, combining two probes to expand the target spectrum and FISH signal intensity. The results are shown in Figure 5e.

Chloroflexota members were identified exclusively on the external surface of the tooth sections, with no detection within the dentinal tubules. This finding aligned qualitatively and quantitatively with the NGS results (Figure 4a).

This case report was written in accordance with the Preferred Reporting Items for Case Reports in Endodontics (PRICE) 2020 guidelines (Nagendrababu et al., 2020).

DISCUSSION

This study examined the microbiome of rare apical calculus-like deposits in a 73-year-old male, providing significant insights into the complex microbiome composition and spatial distribution using NGS, FISH and histological analysis. Distinct microbial compositions

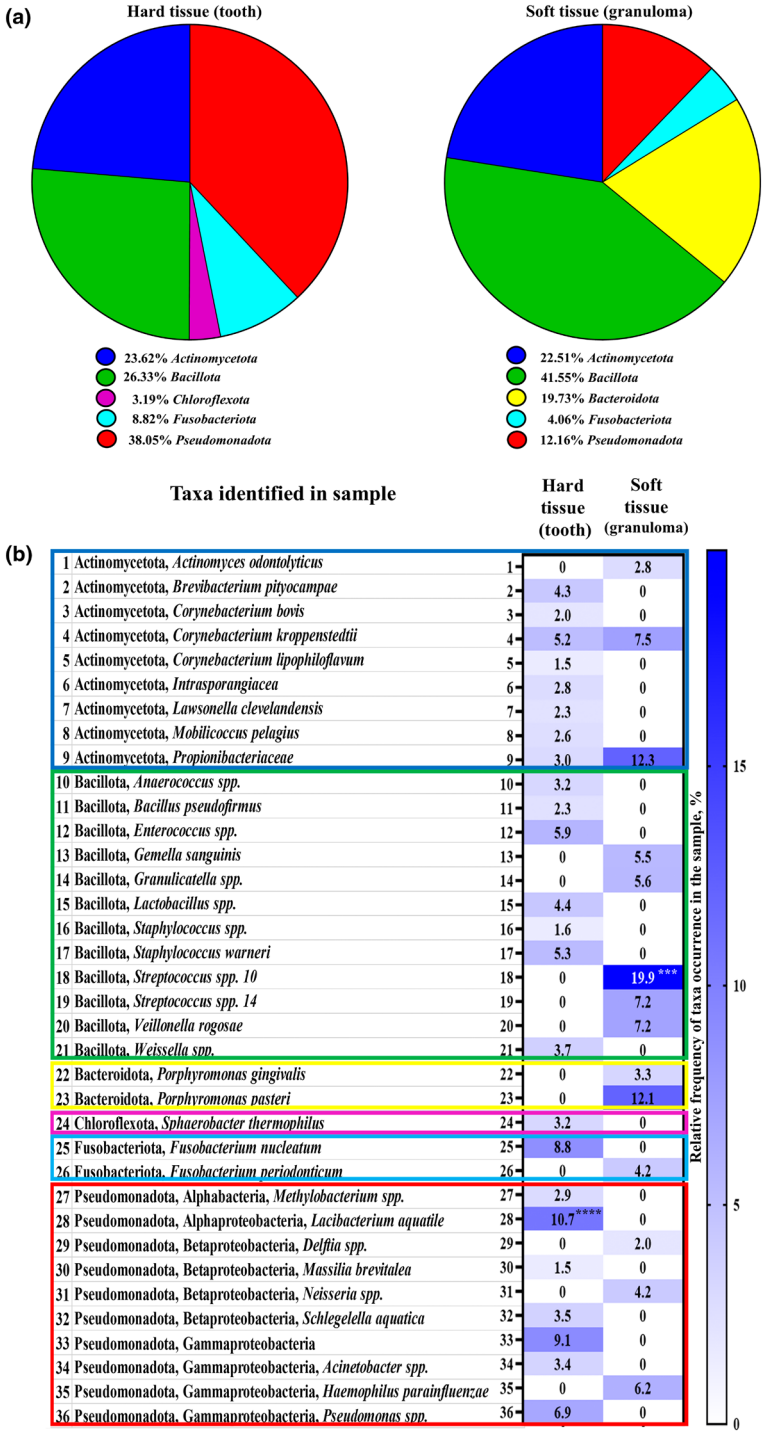

FIGURE 4 (a) Relative abundance of taxa detected within each phylum in hard tissues (tooth) and soft tissues (granuloma) microbial communities and (b) heatmap showing the frequency of taxa occurrence in the samples. A one-sample Wilcoxon signed-rank test was performed to assess the statistical difference between the taxon with the highest occurrence frequency and the median occurrence frequency of all taxa detected in the tooth or tissue microbial communities. Statistical significance was set at $p = .05$: ***$p = .0005$; ****$p < .0001$.

were identified on hard and soft tissues, as well as on different root surfaces, with taxa spanning six phyla: *Actinomycetota, Bacillota, Bacteroidota, Fusobacteriota, Pseudomonadota* and *Chloroflexota*.

Methods

Periradicular surgery remains a rigorous method for examining apical and extraradicular biofilms, making it a preferred approach for such studies (Ricucci et al., 2023). Technical limitations prevented FISH analysis of soft tissue. Although contamination from adjacent periodontal tissues is unlikely, it cannot be entirely ruled out.

FISH was applied alongside next-generation sequencing to leverage the complementary strengths of both methods, using a hierarchical probe strategy; however, due to the short, conserved sequences obtained from NGS, we could only use phylum- or subphylum-level probes and more specific probes require longer gene sequences from

TABLE 1 Taxa detected in hard tissue and soft tissues.

Hard tissue			Soft tissue		
Phylum	**NGS**	**FISH**	**Phylum**	**NGS**	**FISH**
Pseudomonadota	(Alpha PB) *Lacibacterium*	#	Pseudomonadota	(Beta PB) *Delftia*	#
	(Alpha PB) *Methylobacterium*	#		(Beta PB) *Neisseria*	#
	(Beta PB) *Massilia*	#		(Gamma PB) *Haemophilus*	#
	(Beta PB) *Schlegelella*	#			
	Gammaproteobacteria	Gammaproteobacteria			
	(Gamma PB) *Acinetobacter*	Gammaproteobacteria			
	(Gamma PB) *Pseudomonas*	Gammaproteobacteria			
Bacillota	*Anaerococcus*		Bacillota	*Gemella*	#
	Bacillus	*Bacillota* subgroup B		*Granulicatella*	#
	Enterococcus	*Bacillota* subgroup C			
	Lactobacillus	*Bacillota* subgroup A			
	Staphylococcus	*Bacillota* subgroup B			
	Weissella	#			
Bacteroidota		#	Bacteroidota	*Porphyromonas*	#
Actinomycetota	*Brevibacterium*	Actinomycetota	Actinomycetota	*Actinomyces*	#
	Corynebacterium	Actinomycetota		*Corynebacterium*	#
	Intrasporangiaceae	Actinomycetota		*Propionibacteriaceae*	#
	Lawsonella	Actinomycetota			
	Mobilicoccus	Actinomycetota			
	Propionibacteriaceae	Actinomycetota			
Fusobacteriota	*Fusobacterium*	#	Fusobacteriota	*Fusobacterium*	#
Chloroflexota	*Sphaerobacter*	Chloroflexota	Chloroflexota		#

Note: # – was not applied.

Abbreviations: FISH, fluorescence in situ hybridization; NGS, next-generation sequencing.

alternative techniques. The EUB probe, in the FISH analysis, was used to detect and estimate the total bacterial load (Eubacteria domain). The selection of additional probes was based on prior knowledge of key bacterial taxa associated with refractory apical periodontitis and the findings from DNA (NGS) analysis in this study.

The FISH method successfully revealed the spatial distribution of different bacterial taxa across the samples. However, weak or absent FISH signals were observed for certain taxa, which may indicate their low abundance, poor viability or absence. However, the lack of signals could also result from the probes being suboptimal for detecting specific taxa (Lee, 2019). FISH was not performed on soft tissue samples in this case, which limits the findings. This method applied to fixed tissue was not used to assess cell viability.

As a complement to Illumina sequencing, which targets only conserved regions (V3–V4), the probes used in the FISH analysis were designed over two decades ago and targeted regions outside the V3–V4 area. Future research should focus on optimizing FISH techniques for oral microbiological studies, including the development of updated, region-specific probes and the exploration of advanced FISH methodologies to improve signal detection (Lee, 2019).

Results

Apical calculus-like deposits in apical periodontitis, often dark-coloured and unrelated to periodontal disease, are rarely documented as a cause of endodontic failure. A literature review identified nine case reports involving 12 patients and 12 teeth, along with case-series studies reporting deposits in 2%–4% of over 500 apical surgery cases (Harn et al., 1998; Petitjean et al., 2018; Ricucci et al., 2005, 2016, 2018, 2023; Ricucci & Siqueira, 2010; Rud & Andreasen, 1972; Song et al., 2011; Tan et al., 2014; Toubes et al., 2019; Yang et al., 2010). Some authors have speculated on how the calculus could have formed, noting that whilst a bacterial biofilm is necessary for its development, the salts required for mineralization could originate either internally—such as from inflammatory exudate—or from saliva (Petitjean et al., 2018; Tan et al., 2014). Nevertheless, it is noteworthy that all identified cases exhibited a communication with the oral cavity

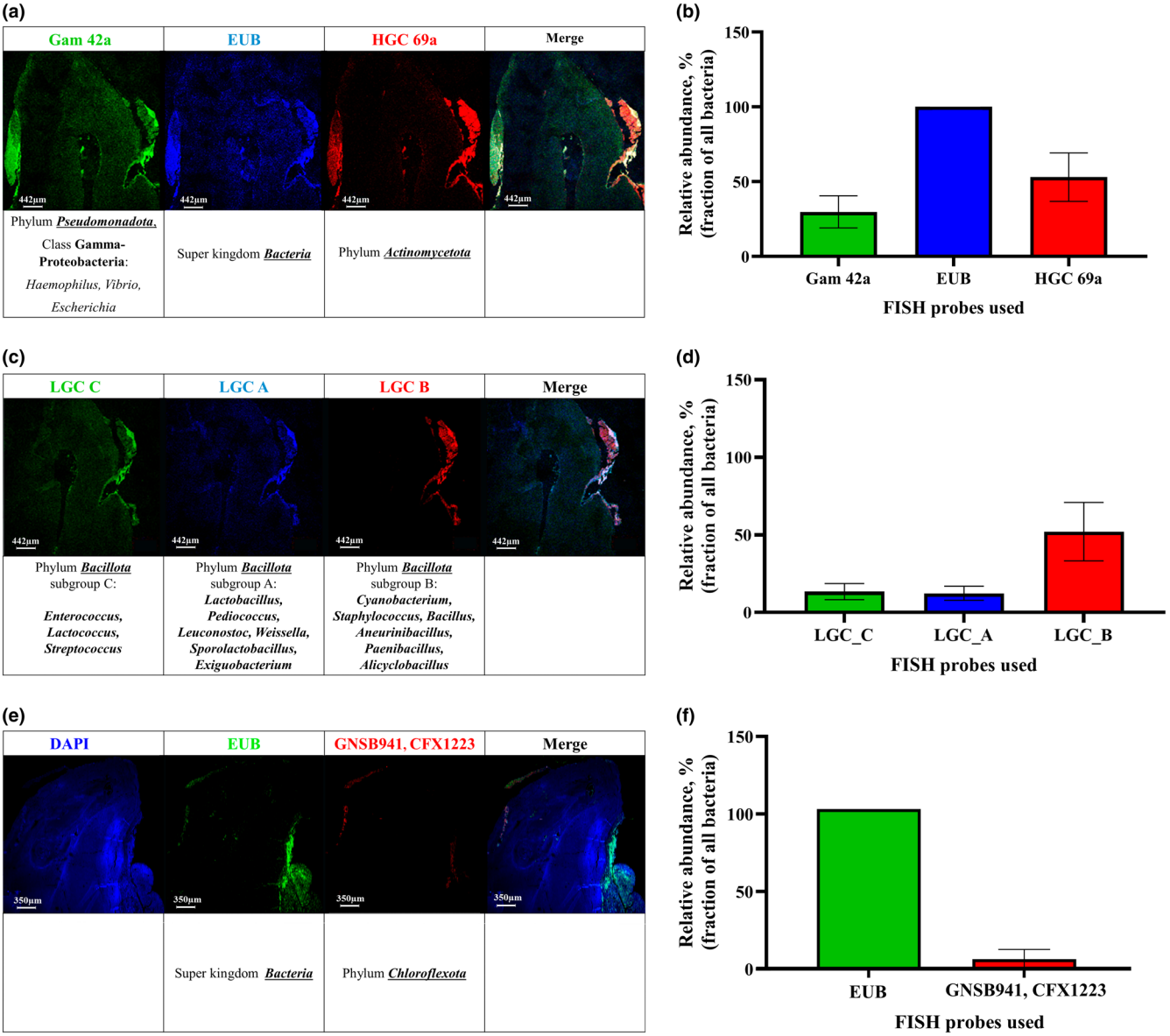

FIGURE 5 FISH detection of bacteria in tooth sections using three sets of probe combinations targeting different taxa. (a) The first set includes probes targeting *Gammaproteobacteria*, the *Eubacteria* domain, and *Actinomycota* (GAM42a, EUB, HGC69a); (b) relative abundance of bacteria detected by the probes in a; (c) the second set includes probes targeting Gram-positive bacteria with low GC content (LGC-C, LGC-A, LGC-B); (d) relative abundance of bacteria detected by the probes in c; (e) the third set includes probes targeting the *Eubacteria* domain (green) and *Chloroflexota* (red), combined with DAPI (blue); and (f) relative abundance of bacteria detected by the probes in e.

through a sinus tract. Apical surgery seemed effective for promoting healing, although case reports primarily focused on successful outcomes. These reports lacked advanced microbiological analyses (Ricucci et al., 2005, 2016, 2018; Toubes et al., 2019; Yang et al., 2010).

Histological examination in the present report revealed inflamed tissue with extraradicular bacteria and biofilm, confirmed by FISH. Illumina sequencing revealed significant differences in microbial composition between hard and soft tissues, with substantial abundances of *Actinomycetota*, *Bacillota* and *Pseudomonadota*.

Nearly a quarter of the microbiota in the analysed tooth and tissue samples belonged to the Actinomycetota phylum, which consists predominantly of anaerobic bacteria. The Propionibacteriaceae family was overrepresented in soft tissue samples, where *Actinomyces odontolyticus* was also found. Given their established role in extraradicular infections, the presence of *Propionibacterium* and *Actinomyces* was expected (Pinheiro et al., 2003).

The isolated *Bacillus pityocampae* has never been reported as pathogenic. Although species of the *Bacillus* genus are well known for their environmental prevalence in soil and plant material, *B. pityocampae* is not typically associated with infections in the root canal or periapical regions (Mahapatra et al., 2022). Additionally, an undefined species from the family *Intrasporangiaceae* was detected. To date, only two species from this family have been documented in the oral microbiome: *Arsenicococcus bolidensis* and *Janibacter indicus* (Chen et al., 2010). Another notable genus identified was *Lawsonella*, specifically *L. clevelandensis*, which has been described in monomicrobial abscesses, indicating its pathogenic potential (Bell et al., 2016).

The phylum *Bacillota*, formerly known as Firmicutes, includes *Enterococci* and *Streptococci*, two Gram-positive bacterial genera commonly found in the oral environment. *Streptococci* are often found in primary infected root canals, and *Enterococci* in failed endodontic treatments (Rocas & Siqueira, 2012). Phylum representatives were identified in the tooth biofilm; however, they were confined to one side of the tooth. In the soft tissue, *Streptococcus* spp. were dominant. They may have gained access via the fistula rather than solely through sample contamination, although the latter cannot be completely ruled out. In the present case, *enterococci* were only found in the tooth sample, confirming that *Enterococcus* does not primarily sustain extraradicular infections (Komiyama et al., 2016; Najafi et al., 2020).

Findings from the NGS and FISH analyses (Figures 4b and 5c) align with the previous studies demonstrating that *Lactobacilli* and members of the *Staphylococcus* genus are commonly detected in infected root canals (Campos et al., 2023; Siqueira & Lima, 2002). *Weissella* species, which have been isolated from the human oral cavity, are recognized as opportunistic pathogens (Li et al., 2015).

The extracted tooth had a distinct black coloration, making taxa associated with pigmentation phenotypes, such as *Porphyromonas* and *Prevotella*, particularly interesting. *Actinomyces* species may be involved in black stain formation as well through the production of hydrogen sulphide that may react with metal ions such as iron—likely present in apical tissues (Basic et al., 2015; Washio et al., 2005). If bacterial activity was not responsible for the discoloration, an alternative hypothesis is that pigments from the oral cavity (such as those from food) enter through the sinus tract. Additionally, calculus or biofilm may exhibit greater absorbency than dentin. However, the patchy distribution of discoloration on tooth 23 does not support this origin. *Porphyromonas* was found in the tissue samples. Interestingly, representatives of the *Bacteroidota* phylum were absent from the tooth sample despite their common presence in infected root canals. This absence may be attributed to the effectiveness of orthograde endodontic treatment, which is known to successfully eliminate gram-negative bacteria (Siqueira, 2002). However, the presence of other gram-negative bacteria suggests the possibility of additional sources or factors influencing bacterial colonization in the tooth.

The presence of *F. nucleatum* in hard tissues was confirmed using both Illumina sequencing and FISH, corroborating previous reports of its high prevalence in primary root canal infections (Manoharan et al., 2020). *F. nucleatum* is a Gram-negative anaerobe that contributes to endodontic infections by promoting biofilm formation and cytokine release (Hasse, 1978; Rakhimova et al., 2021; Sun et al., 2024).

Interestingly, *Fusobacterium periodonticum* was identified through Illumina sequencing in the granulomatous tissue sample. This obligate, anaerobic, nonspore-forming, nonmotile, Gram-negative rod has previously been isolated from periodontitis lesions (Park et al., 2019). However, its specific role in apical granulomas remains poorly understood, as no prior studies have documented its presence or potential pathogenicity in these lesions. The identification of *F. periodonticum* in this context raises questions about its involvement in periapical pathology. Due to technical limitations, the FISH technique could not be applied to soft tissue samples in this study, preventing further investigation of this species. Although careful specimen management minimized the risk of contamination, the possibility of contamination from adjacent periodontal tissues cannot be entirely excluded.

Gammaproteobacteria are microaerophilic and belong to the phylum *Pseudomonadota*. They were detected in the external parts of the biofilm on the tooth.

Gammaproteobacteria is a large and diverse group of Gram-negative bacteria, including many different genera. Their presence in the root canal likely contributes to the polymicrobial environment characteristic of root canal infections (Kesim et al., 2023).

The phylum *Chloroflexota* is a rare member of the oral microbiome (Campbell et al., 2014). In environmental settings, this ecologically and physiologically diverse group of bacteria is highly abundant in anaerobic habitats, including sediments, hot springs and methanogenic anaerobic sludge digesters, where they are abundant (Petriglieri et al., 2023).

In this report, members of the *Chloroflexota* phylum were identified in hard tissues using both Illumina sequencing and FISH, with FISH signals detected exclusively on the external surface of the tooth sections. Although *Chloroflexota* has been associated with periodontal

disease, its specific role in endodontic infections remains unclear. Further investigation is warranted to clarify its potential involvement in disease progression (Campbell et al., 2014).

CONCLUSION

In conclusion, this study successfully identified and visualized microorganisms within two adjacent periodontally healthy teeth with a refractory endodontic infection with apical calculus-like deposits using NGS and FISH. The findings revealed a diverse microbial ecosystem in both hard and soft tissues. The detection methods highlighted the robust presence of certain genera, with some detected using both NGS and FISH. Variations in microbial community composition between tissue types highlight the need for tissue-specific analyses to better understand microbial ecology and its implications for endodontic infections. This detailed characterization provides deeper insights into the microbial populations involved and their potential roles in disease progression; however, broader conclusions require analysis of larger and more diverse samples.

AUTHOR CONTRIBUTIONS

Fernando J. Mota de Almeida: conceptualization (equal), investigation (equal), methodology (equal), writing – original draft preparation (lead), writing – review and editing (equal). **Olena Rakhimova:** data curation (lead), formal analysis (lead), investigation (lead), resources (lead), validation (lead), visualization (lead), writing – original draft preparation (equal), writing – review and editing (equal). **Nelly Romani Vestman:** funding acquisition (equal), methodology (lead), writing – review and editing (equal). **Natuschka M. Lee:** supervision (equal), writing – review and editing (equal). **Malin Brundin:** conceptualization (lead); funding acquisition (lead), project administration (lead), supervision (lead), writing – original draft preparation (equal); writing – review an editing (lead).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data other than those presented in this report are not shared.

ETHICS STATEMENT

Case reports do not fall under the general requirements of Swedish law for ethical review; therefore, approval from the Swedish Ethical Review Authority was not sought.

PATIENT CONSENT STATEMENT

The patient provided oral consent for the treatment and signed an informed consent form for the use of data and analysis in this report.

RELEVANT REPORTING GUIDELINES

This report followed the Preferred Reporting Items for Case Reports in Endodontics (PRICE) 2020 guidelines, as described in the manuscript.

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APPENDIX A

TABLE A1 Oligonucleotide probes used for hybridization in FISH.

Target taxa	Probe	References	Gene	Target taxa probe sequence (5′-3′)	Fluorochrome
Bacteria: all bacteria	EUB338	(Amann et al., 1990)	16S rRNA	GCTGCCTCCCGTAGGAGT	Alexa Fluor 350 (or Cy3)
Gamma-Proteobacteria, *Pseudomonadota* phylum: *Haemophilus*, *Vibrio*, *Escherichia*	GAM42a	(Manz et al., 1992)		GCCTTCCCACATCGTTT	Cy3
Actinomycetota phylum	HGC69a	(Roller et al., 1994)	23S rRNA	TATAGTTACCACCGCCGT	Cy5
Firmicutes *Bacillota* phylum subgroup A: *Lactobacillus*, *Pediococcus*, *Leuconostoc, Weissella*, *Sporolactobacillus*, *Exiguobacterium*	LGC354a	(Meier et al., 1999)	16S rRNA	TGGAAGATTCCCTACTGC	Alexa Fluor 350
Firmicutes *Bacillota* phylum subgroup B: *Staphylococcus, Bacillus*, *Aneurinibacillus, Paenibacillus*, *Alicyclobacillus*	LGC354b	(Meier et al., 1999)	16S rRNA	CGGAAGATTCCCTACTGC	Cy5
Firmicutes *Bacillota* phylum subgroup C: *Enterococcus*, *Lactococcus, Streptococcus*	LGC354c	(Meier et al., 1999)	16S rRNA	CCGAAGATTCCCTACTGC	Cy3
Chloroflexota phylum	CFX1223	(Bjornsson et al., 2002)	16S rRNA	CCATTGTAGCGTGTGTGTMG	Cy5
	GNSB941	(Gich et al., 2001)	16S rRNA	AAA CCA CAC GCT CCG CT	Cy5