Received: 1 November 2023 | Accepted: 29 January 2024

DOI: 10.1111/iej.14046

INTERNATIONAL ENDODONTIC JOURNAL | WILEY

ORIGINAL ARTICLE

Identification of keystone taxa in root canals and periapical lesions of post-treatment endodontic infections: Next generation microbiome research

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Funding information
FEDER/Junta de Andalucía-Consejería de Economía y Conocimiento, Grant/Award Number: A1-CTS-174-UGR18; Ministry of Science and Innovation of Spain co-financed with European Regional Development Funds, Grant/Award Number: PID2020-120481RB-I00; University of Almería, Grant/Award Number: PPUENTE2021-006; Pérez-Carrasco V

Abstract

Aim: The aim of this study was to analyse and compare the microbiome present in root canals and periapical lesions of teeth with post-treatment infections, and to identify the presence of keystone taxa in both habitats using next-generation sequencing analysis.

Methodology: Apices and periapical lesions of patients with post-treatment apical periodontitis were surgically extracted. Specimens were cryo-pulverized, bacterial DNA was extracted, and the V3–V4 hypervariable regions of the 16S rRNA gene were sequenced using the Illumina Miseq platform. Bioinformatic analysis was carried out with Mothur software, whilst diversity indices were obtained using operational taxonomic units (OTUs). The diversity indices were compared with the Kruskal–Wallis test, and community composition differences were explored with Permutational Multivariate Analysis of Variance (PERMANOVA). A bacterial functional study was performed with the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis. Co-occurrence network analyses were performed using the Sparse Correlations for Compositional data (SparCC). Eigencentrality, clr-based abundance and ubiquitousness were applied to infer keystone taxa. *P* values <.05 were considered statistically significant.

Results: Thirty-two apices and thirty-nine periapical lesions were sequenced and analysed. A similar alpha-diversity ($p < .05$) and community composition ($p = .91$) was observed for apices and lesion samples. The most abundant OTUs identified amongst all samples included *Fusobacterium nucleatum*, *Prevotella loescheii, Streptococcus intermedius, Porphyromonas gingivalis, Parvimonas micra, Synergistetes bacterium, Tannerella forsythia* and *Peptostreptococcus stomatis*. The metabolic pathways with >0.81% abundances included membrane transport, genetic information processing and metabolic pathways. *F. nucleatum* was identified as a keystone taxon as it showed ubiquitousness, an eigenvector centrality value of 0.83 and a clr-based abundance >4.

Maria Teresa Arias-Moliz, Virginia Pérez-Carrasco, Jose Antonio García-Salcedo, Miguel Soriano contributed equally to this work.

was supported by a fellowship from the Ministry of Universities, Spain, Grant/Award Number: FPU20/03952

Conclusions: The microbiome in apices and periapical lesions of post-treatment endodontic infections showed a similar diversity and taxonomic composition. Co-occurrence network analyses at OTU level identified *F. nucleatum* as a keystone taxon candidate in these infections.

KEYWORDS

apices, keystone taxa, microbiome, next-generation sequencing, periapical lesions, persistent apical periodontitis

INTRODUCTION

Post-treatment apical periodontitis is an inflammatory response of the periapical tissues to the presence of microorganisms in the apical third of the root canal (Nair et al., 2005). These bacteria survived or were introduced during previous root canal treatment, or are the result of coronal leakage (Nair, 2006). Extraradicular microorganisms may also be detected in periapical tissues, contributing to the persistence of periapical disease (Bronzato et al., 2021; Noguchi et al., 2005; Noiri et al., 2002; Pérez-Carrasco et al., 2023; Signoretti et al., 2011; Sunde et al., 2003; Tronstad et al., 1990). However, some lesions appeared free of microorganisms (Noguchi et al., 2005; Saber et al., 2012; Sun et al., 2022; Sunde et al., 2002), most probably because of the clearance effect of immune cells.

Persistent endodontic infections are polymicrobial, and a number of different taxa have been identified, showing substantial inter-individual variability (Arias-Moliz et al., 2023; Ordinola-Zapata, Costalonga, Nixdorf, et al., 2023; Pérez-Carrasco et al., 2023). This finding demonstrates that different communities sharing similar functionality and virulence can lead to the same infectious disease, that is, the functional redundancy (Tian et al., 2020). Additionally, bacterial survival under the different environmental conditions of a treated root canal and the periapical tissues is known to depend on the virulence of the community and the nutrients available (Lamont et al., 2018; ter Steeg et al., 1987). Thus, bacteria with cell-invasion capacity and proteolytic activity —such as *Porphyromonas* and *Fusobacterium*— have been found in high relative abundance in persistent infections (Hou et al., 2022; Ordinola-Zapata, Costalonga, Nixdorf, et al., 2023; Pérez-Carrasco et al., 2023; Sun et al., 2022; Tzanetakis et al., 2015). Similarly, a high prevalence of species having a flagellum, which is a Toll-like receptor activator that induces inflammatory response, is related to symptomatic post-treatment infections (Pérez-Carrasco et al., 2023). Still, the factors that drive the virulence and function of the established community and lead to the progress of the infection are not well known.

One important step towards a deeper understanding of endodontic infections is the characterization of microbial interactions that prove critical for the population dynamics and functional activity (Trosvik & de Muinck, 2015). Different microbial taxa do not influence ecosystem processes equally. Within each ecosystem, microorganisms interact with each other through various mechanisms, such as quorum sensing, cross-feeding and release of antimicrobial compounds (Tudela et al., 2021), leading to synergistic and antagonistic effects. Another mechanism entails the presence of keystone taxa. Microbial keystone species are highly connected taxa that exert a relevant influence on the structure and functioning of the microbiome regardless of their abundance over space and time (Banerjee et al., 2018). These microorganisms play a key role in the community pathogenicity, and their removal can cause a dramatic shift, for instance by disrupting the microbiome performance and changing the virulence of the consortium. In periodontitis, *Porphyromonas gingivalis* acts as a keystone taxon by causing tissue destruction and the release of nutrient-rich exudates, leading to dysbiosis and favouring the further growth of pathobionts (Lunar Silva & Cascales, 2021).

To date, there is no information in the literature about the presence of keystone species in post-treatment endodontic infections. Their identification and functional profiling would provide valuable insights about their impact on the overall biodiversity of the community aiding the development of novel treatment strategies by focusing on a limited number of bacterial targets that drive or control the disease (Hajishengallis et al., 2012). The aim of this study was therefore to analyse and compare the microbiome present in root canals and periapical lesions of teeth with post-treatment infections, and to identify the presence of keystone taxa in both habitats using next-generation sequencing analysis. The null hypotheses are that the microbiome present in root canals and lesions of teeth with secondary infections is similar, and that no keystone taxa can be found in the root canals and periapical lesions of teeth with post-treatment infections.

MATERIALS AND METHODS

The present work is an observational study reported under the guidelines of Strengthening the Reporting of Observational studies in Epidemiology (STROBE). The study was approved by the Ethics Committee of the University of Granada (ref: 354/CEIH/2017). Samples were collected by an endodontist from patients of a private practice from July 2019 to September 2022. All patients signed an informed consent form before their inclusion.

This partly secondary study includes sequencing data from samples pertaining to a previous study (Pérez-Carrasco et al., 2023) as well as new samples. Inclusion criteria consisted of patients showing radiographic evidence of persistent apical periodontitis. Excluded were patients with severe systemic diseases, pregnant or breastfeeding women, and patients under 18 years old. Teeth with periodontal pockets >4 mm, vertical root fracture, and history of trauma were also excluded.

Sample collection

Samples of root apices and lesions were taken under strictly aseptic conditions, as previously reported (Pérez-Carrasco et al., 2023). Briefly, patients rinsed their mouth with chlorhexidine solution for 30 s, and the intraoral surgical site was swabbed with a chlorhexidine soaked gauze. A mucoperiosteal flap was made with a 15C scalpel blade (Swann Morton, Sheffield, UK). The periapical lesion was exposed using an ultrasound SL2 F87512 insert (Acteon Satelec) and the apex was sectioned with an ultrasound tip NINJA F87545 (Acteon Satelec, Merignac, France). All clinical procedures were performed under magnification (Zeiss Pro Ergo; Carl Zeiss, Göttingen, Germany).

After extraction, the apical lesion was separated from the root with a sterile curette and tweezers. The external apex surfaces were cleaned as previously described (Alves et al., 2009). A sterile paper point was used to sample the outer surface of the apices in order to confirm their disinfection. The apex and the lesions were transferred into separate Eppendorf tubes with 200 mL of Tris-EDTA buffer (pH 8.0; Panreac Quimica, Barcelona, Spain) and they were stored at −20°C. Samples were then cryo-pulverized using a mortar and pestle containing liquid nitrogen, and stored at −20°C until further processing. A total of 98 samples (47 apices and 51 lesions) were included.

DNA extraction and high-throughput sequencing

These steps were done as previously described (Pérez-Carrasco et al., 2023). The QIAmp DNA Mini Kit (Qiagen, Valencia, CA, USA), preceded by a 30-min preincubation with lysozyme, was used for bacterial DNA extraction. In each extraction batch controls were included to check for any possible contamination. DNA quality and quantification were performed using a spectrophotometer (Nanodrop 2000 UV – Vis; ThermoFisher Scientific, Waltham, MA, USA).

PCR amplification products of the V3–V4 hypervariable regions of 16S rRNA gene were obtained using fusion universal primers 340F (Illumina adaptors + 5′-CCTACGGGNGGCWGCAG-3′) and 800R (Illumina adaptors + 5′-GACTACHVGGGTATCTAATCC-3′) (Soriano-Lerma et al., 2020). Negative amplification controls were included in each PCR batch. Amplicon multiplexing and sequencing were performed with a dual indexing tag-tailed design using 8 nt indexes from the Nextera XT Index Kit v2 (Illumina, San Diego, CA, USA). Paired-end sequencing of 16S rRNA amplicon libraries was performed with the Illumina MiSeq platform. The raw sequencing data are available at the Sequence Read Archive (SRA) of the National Centre for Biotechnology Information (NCBI) under the Bioproject accession numbers PRJNA839210 (Pérez-Carrasco et al., 2023) and PRJNA1033443.

Bioinformatic analysis

Bioinformatic analysis and quality-filtering were performed with Mothur software (v 1.43.0, University of Michigan Medical School, Ann Arbor, MI, USA). Chimeric reads were excluded using Chimera UCHIME and redundant, non-chimeric FASTA files were taxonomically classified using the Silva v132 database. Alpha-diversity was examined by means of operational taxonomic units (OTUs) at 3% dissimilarity and the distance-based greedy clustering (DGC) algorithm, obtaining the coverage and species richness (number of detected operational taxonomic units, OTUs), as well as the Chao1, Inverse Simpson (InvSimpson), Shannon (H′) and Pielou (J′) diversity indexes. Abundance was expressed as a percentage with respect to the total number of sequences in each sample. OTUs with a total abundance higher than 0.01% were considered for statistical analysis. Bacterial functional analysis relied on Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis of 16S rRNA gene sequencing data (Douglas et al., 2018). Kyoto Encyclopedia of Genes and Genomes (KEGG) microbial pathways classified at level 3 were analysed as previously described (Díaz-Faes et al., 2021; Soriano-Lerma et al., 2020).

Statistical analysis

After checking for the absence of normality in the diversity indexes with the Shapiro–Wilk test, the non-parametric Mann–Whitney *U*-test was applied using GraphPad Prism version 8.0.0 (GraphPad Software, San Diego, California, USA). The similarity of bacterial communities between samples was evaluated by principal coordinate analysis (PCoA), based on Bray-Curtis distances and implemented in PRIMERe Permanova+ (PRIMER-E Ltd, Plymouth, UK). A multivariant PERMANOVA test was also carried out using PRIMERe Permanova+ with square root transformation and permutation of residuals under a reduced model (9999 permutations). SparCC (Sparse Correlations for Compositional data) correlation analysis was performed by means of the *SpiecEasi* package in R. Correlation and co-occurrence network diagrams were represented using Gephi v.0.9.2 with cut-off values of −0.2 and 0.2. Network statistics such as eigencentrality, betweenness centrality or modularity class, were obtained using Gephi v.0.9.2. To identify keystone taxa, we adopted the combination of three criteria as previously described (Wu-Chuang et al., 2022): (1) eigencentrality values higher than 0.75, (2) clr-based abundance higher than the mean clr value (i.e. 4), and (3) ubiquitousness (presence of the bacteria in all samples). In all cases, p values <.05 were considered statistically significant.

RESULTS

The sequencing of 16S rRNA gene amplicon libraries gave a total of 1.164.873 reads, and an average of 21 562 reads in samples from apices and 10 797 reads in samples from periapical lesions. Samples were rarefied by sub-sampling at 1200 sequences to obtain a coverage higher than 95% in all samples. Disinfection, extraction and amplification controls were also amplified and sequenced, showing no significant amplification and a small number of sequences. Of the 98 samples taken (47 apices and 51 lesions), 15 apices and 12 periapical lesions were excluded because they showed no significant PCR amplification or an insufficient number of sequences, giving a total of 32 apices and 39 periapical lesions analysed (Figure 1).

A similar alpha-diversity (species richness, Chao1, Inverse Simpson, Shannon and Pielou indexes) ($p > .05$) was observed for apices and lesion samples (Figure 2a). No differences were found in the community composition according to principal coordinate analysis (PCoA) between the two groups at OTU level (Figure 2b), which was quantitatively confirmed with a multivariant PERMANOVA test ($p = .91$).

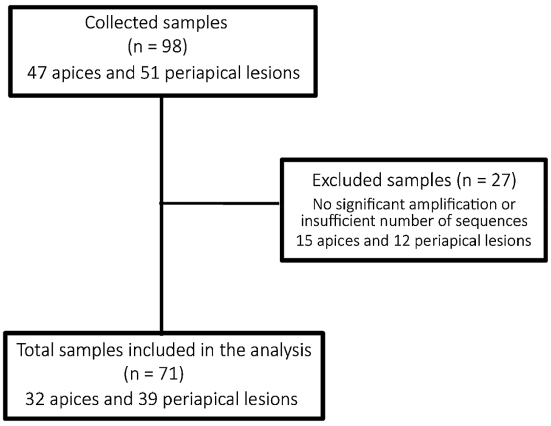

FIGURE 1 Flow chart of the samples included in the study.

Taxonomic classification at OTU level gave a total of 11 963 OTUs, of which 222 showed a relative abundance higher than 0.01% and were therefore included in the subsequent analysis. The most dominant OTUs observed amongst all samples (>2.2%) included *Fusobacterium nucleatum* (19.8%), *Prevotella loescheii* (4.18%), *Streptococcus intermedius* (4.17%), *P. gingivalis* (4.06%), *Parvimonas micra* (3.9%), *Synergistetes bacterium* (3.37%), *Tannerella forsythia* (3.32%), *Peptostreptococcus stomatis* (3.29%), *Pseudomonas gessardii* (2.72%) and *Pseudoramibacter alactolyticus* (2.2%) (Figure 3).

The metabolic pathways that showed abundances >0.81% included membrane transport pathways (transporters, abc transporters, secretion system), genetic information processing pathways (translation, transcription and replication and repair systems), and metabolic pathways (amino acid, carbohydrate, energy and nucleotide metabolisms, and metabolism of cofactors and vitamins) (Figure 4).

SparCC correlation and co-occurrence network analysis at OTU level were undertaken to identify essential bacterial species in the microbial community and possible candidates for keystone taxa (Figure 5). Only one bacterial species, *F. nucleatum*, fulfilled the three requirements to be considered keystone: ubiquitousness, an eigenvector centrality value of 0.83, and a clr-based abundance higher than the mean clr value (i.e. 4).

DISCUSSION

In this study, the microbiome and the presence of keystone taxa in apices and periapical lesions of secondary root canal infections were investigated. The detection of keystone species is relevant for the understanding of microbial interactions within a community, how they remain stable over long periods of time, and how they adapt to environmental changes of the habitat (Tudela

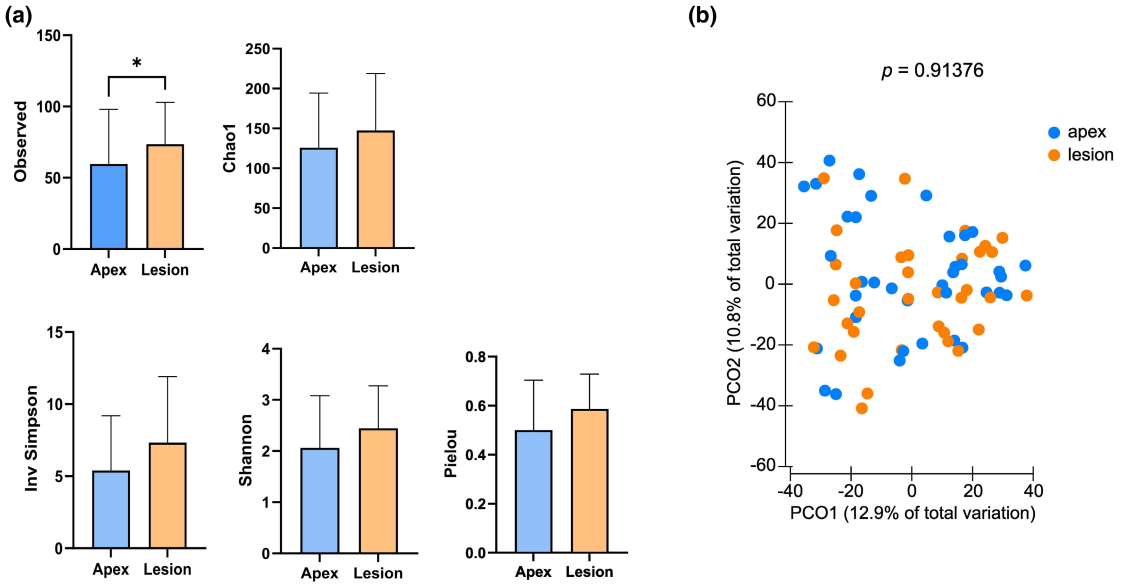

FIGURE 2 (a) Alpha-diversity indices (observed species, Chao1, Inverse Simpson, Shannon and Pielou) of the bacterial community in apices (blue) and periapical lesions (orange). Statistical analysis between both sites was performed by Mann–Whitney *U*-test (*.01 < *p* < .05). (b) Principal coordinate analysis (PCoA) of apices and periapical lesions samples at OTU level. Samples are represented by dots.

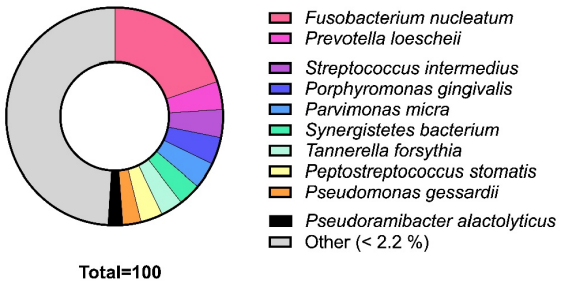

FIGURE 3 Relative abundances of the predominant OTUs. A representative sequence of each OTU was extracted and used as input in a BLAST analysis for the identification of the bacterial species.

et al., 2021), for example, the treated root canal. To our knowledge, this is the first study to statistically identify a candidate of these highly connected taxa in endodontic infections.

A total of 15/47 (31.9%) apices and 12/51 (23.5%) lesions were excluded because they showed no significant amplification or insufficient number of sequences. The exclusion of the samples can be attributed to various factors such as low DNA concentration, unsuccessful amplification as a consequence of the presence of inhibitory substances in the PCR, variability in the microbial community composition and errors in the sampling procedure. Some samples could also be free of microorganisms. Actually, according to the literature it is possible that some lesions are free of bacteria (Noguchi et al., 2005; Saber et al., 2012; Sun et al., 2022; Sunde et al., 2002). A similar bacterial community was observed in the apices and periapical lesions, a finding in agreement with previous studies (Pérez-Carrasco et al., 2023; Sun et al., 2022). The first null hypothesis was therefore accepted. The most predominant OTUs identified were: *F. nucleatum*, *P. loescheii, S. intermedius, P. gingivalis, P. micra, S. bacterium, T. forsythia* and *P. stomatis* (Hong et al., 2013; Hou et al., 2022; Keskin et al., 2017; Ordinola-Zapata, Costalonga, Dietz, et al., 2023; Ordinola-Zapata, Costalonga, Nixdorf, et al., 2023; Pérez-Carrasco et al., 2023; Sun et al., 2022; Tzanetakis et al., 2015). Despite variations in the ecological conditions at each site —such as adhesion surfaces or the presence of host cells— the absence of differences in the microbiome of apices and lesions may be due to the same microorganisms passing through the apical foramen and sharing a similar nutrient source. In fact, this anaerobic microbial community closely resembles that seen in periodontal infections, where nutrients appear to be a main ecological determinant (ter Steeg et al., 1987; ter Steeg & Van Der Hoeven, 1989). In our samples, a mixture of fast and slow-growing microorganisms, primarily feeding on proteins and glycoproteins from inflammatory periapical serum, yielded to a likely serum degrading consortium. Thus, the availability of glycoproteins may have favoured the presence of saccharolytic microorganisms such as *S. intermedius* and *P. loescheii* (Fei et al., 2016; Könönen et al., 2022), which is supported by the enrichment of carbohydrate metabolic pathways (Figure 4). On the other hand, the proteins and the protein core left from the glycoproteins in the serum would explain the high relative abundance of proteolytic microorganisms such as *F. nucleatum*,

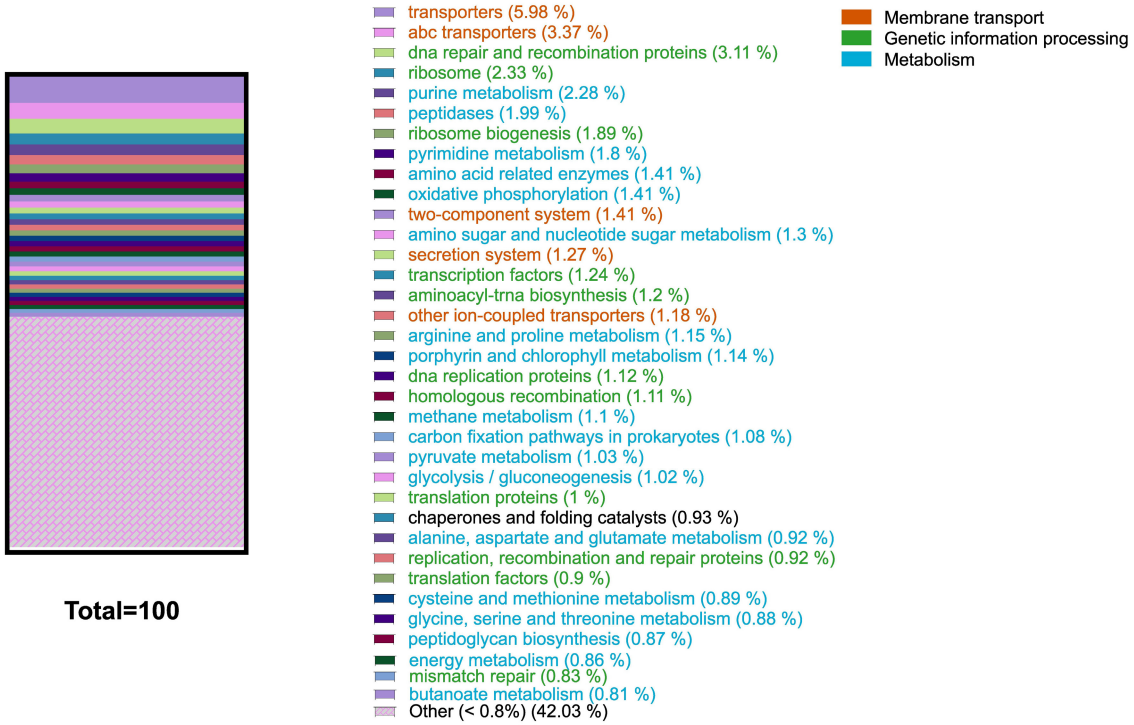

FIGURE 4 Relative abundances of the predominant KEGG microbial pathways. Bacterial pathways related to membrane transport are noted in orange, those related to genetic information processing in green, and those related to metabolism in blue.

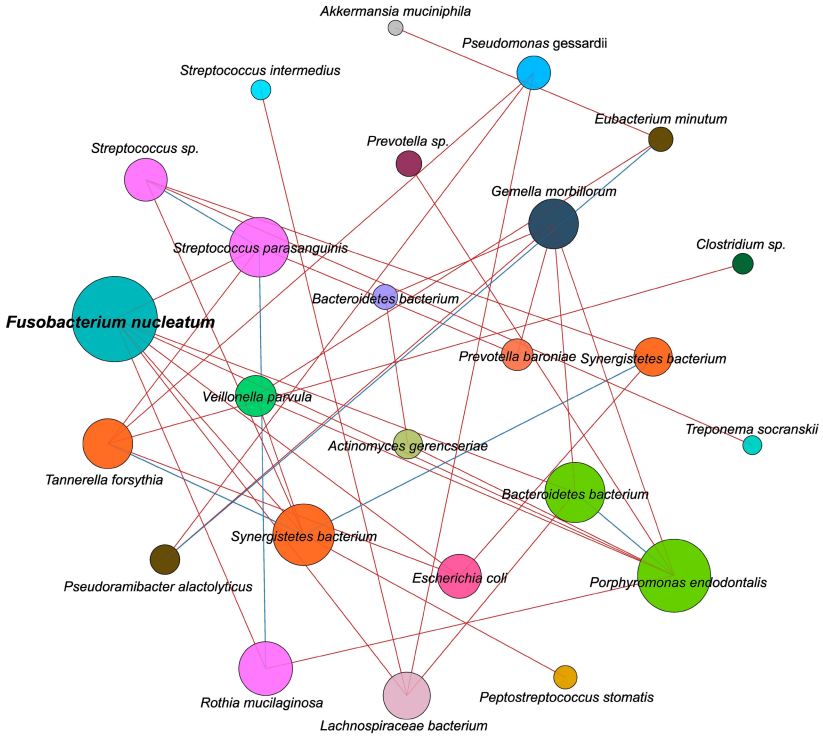

FIGURE 5 SparCC correlation and co-occurrence analysis of bacterial OTUs in all samples. A representative sequence of each OTU was extracted and used as input in a BLAST analysis for the identification of the bacterial species. Nodes represent bacterial species, edges represent correlations. Only edges with correlation values less than −0.2 and higher than 0.2 are included. The colour of the nodes is based on the modularity class to represent different bacterial co-occurrence clusters. The node size depends on the betweenness centrality, representing the influence of each node in the network. Correlations are drawn using a red–blue scale; red marks negative correlations and blue marks positive correlations.

P. gingivalis, *P. micra*, *T. forsythia* and *P. stomatis*, and the predominance of pathways such as peptidases, and arginine and proline metabolism (Takahashi, 2015).

Additionally, these putative periodontal pathogens show important virulence factors that might be involved in the disease (Ordinola-Zapata, Costalonga, Dietz, et al., 2023). For instance, their strong proteolytic activity —depending on proteolytic enzymes such as gingipains of *P. gingivalis* and proteasases of *P. loescheii*— is responsible for tissue destruction (Costalonga & Herzberg, 2014; Siqueira & Roças, 2007). Some of these bacteria have an intracellular invasion capacity (*P. gingivalis*, *P. intermedia, F. nucleatum* and *T. forsythia*) that helps them survive and pervade tissues (Colombo et al., 2007; Lamont et al., 1995). These bacteria can also trigger immune responses through the activation of host immune cells and the release of proinflammatory cytokines. Finally, the capsule formation of *S. intermedius* can provide protection from phagocytosis clearance and immune system recognition (Skov Sørensen et al., 2016).

Interestingly, the OTU *Pseudomonas lactis* was detected in high relative abundance in our samples. This genus, previously reported in secondary root canal infections (Anderson et al., 2013; Arias-Moliz et al., 2023; Ordinola-Zapata, Costalonga, Nixdorf, et al., 2023; Pérez-Carrasco et al., 2023; Sánchez-Sanhueza et al., 2018; Siqueira Jr. et al., 2016), includes microorganisms not commonly found in the human body and oral ecosystems. Their presence in post-treatment infections could be a consequence of external contamination during the previous root canal treatment (Siqueira Jr. et al., 2016), which highlights the importance of keeping an aseptic environment during the treatment performance.

Keystone species of the human microbiome, such as *Bacteroides fragilis* and *Helicobacter pylori*, have been often identified based on empirical evidence (Banerjee et al., 2018). Yet bioinformatics is increasingly being used to detect keystone taxa, with co-occurrence or co-abundance networks applied to 16S rRNA gene sequencing being the most widely developed procedure (Tudela et al., 2021). In the present study, the influence of the node on the co-occurrence network, the abundance and the presence of the OTU across all the samples were used to infer the identification of keystone taxa (Wu-Chuang et al., 2022).

The results of this study point to the OTU *F. nucleatum* as a keystone taxa candidate in secondary infections. Thus, the second null hypothesis was rejected. Several mechanisms could explain the relevant participation of *F. nucleatum* in the community. The first one is that this species shows an important coaggregation capacity that relies on the presence of several adhesins, including Fap2, RadD and aid1 (Han, 2015; Kaplan et al., 2010, 2014). Adhesins allow *F. nucleatum* to act as a supportive bridge between primary colonizers (e.g. *Streptococcus* spp.) and anaerobic secondary colonizers (such as *Tannerella* spp. and *Porphyromonas* spp.) (Kolenbrander, 2000; Kolenbrander et al., 2010; Wang & Fang, 2023). Multispecies bridging also facilitates multispecies community co-existence, hence bacterial communication, and metabolic and cross-feeding interactions (Alon-Maimon et al., 2022). In the presence of early colonizers such as *Streptococci*, *F. nucleatum* would therefore favour the availability of amino acids, promoting the production of polyamides like butyrate that stimulate the outgrowth of *P. gingivalis* to disrupt host homeostasis and induce host damage (Sakanaka et al., 2022). Additionally, when in coinfection with other oral species, for example, *T. forsythia*, *P. gingivalis* and *Streptococci*, a synergistic virulence behaviour is detected as a consequence of increased bone loss, abscess or cell death (Hajishengallis & Lamont, 2016; Lamont et al., 2018). These interspecies interactions, widely described for *F. nucleatum* in periodontal infections, could similarly occur in root canal infections. In fact, the overall similar taxonomic composition of both infections (Ordinola-Zapata, Costalonga, Nixdorf, et al., 2023), along with the relatively comparable microenvironment (in terms of oxygen and nutrients), could support this hypothesis.

The second mechanism is implied by the *F. nucleatum* FadA adhesion, known to bind and invade endothelial cells, which are considered relevant mechanisms for colonization, dissemination and evasion of host defence (Han, 2015). Specifically, FadA binds to the cell-junction molecules known as cadherins, increasing the permeability of the endothelium (Fardini et al., 2011), and allowing other bacteria in the area to penetrate through the tissues (Han, 2015).

Finally, a variety of host responses are triggered by *F. nucleatum*. It activates lymphocyte apoptosis by Fap2 and RadD (Kaplan et al., 2010), and is a strong stimulator of inflammatory cytokines, interleukin-6 (IL-6), IL-8, and tumour necrosis factor-α (TNF-α), with harmful consequences for the host tissues (Han et al., 2000; Park et al., 2014). It induces necrosis and apoptosis of neutrophils which promotes the coaggregation of late colonizers such as *P. gingivalis* (Chen et al., 2022). *F. nucleatum* furthermore stimulates the toll-like receptor 4 (TLR4) mediated responses, inducing exacerbated inflammation (Park et al., 2014). Such impairment of host immunity could allow bacterial outgrowth, whilst the resulting inflammatory environment could provide nutrients for the community (Hajishengallis & Lamont, 2021).

The results of the present study underline a predominant serum like degrading bacterial consortia in apices

and periapical lesions which may well benefit from the inflammatory conditions in apical periodontitis. They also suggest that *F. nucleatum* is a keystone taxon candidate in post-treatment endodontic infections. These results provide a starting point for achieving a deeper understanding of how the endodontic microbiome functions. However, the present study shows some limitations. The sample size for the keystone taxa analysis is limited and only samples from one geographical location were included. Network scores and co-occurrence patterns are based on correlations, yet correlations do not mean causation (Banerjee et al., 2018). In order to corroborate the present findings and to demonstrate the impact of the keystone species on the microbiome structure and performance, it is therefore necessary to increase the sample size and complement statistical evidence with empirical evidence and omics technology, such as metatranscriptomics and metaproteomics. Finally, knowledge on the response of keystone taxa to environmental changes (keystone resilience), for example, antimicrobial agents, is further needed.

CONCLUSIONS

The microbiome in root canal apices and periapical lesions of post-treatment endodontic infections showed a similar diversity and taxonomic composition. Co-occurrence network analyses at OTU level identified *F. nucleatum* as a keystone taxon candidate in these infections.

AUTHOR CONTRIBUTIONS

Maria Teresa Arias-Moliz took part in the conceptualization of this study, performed laboratory work and contributed to the writing, reviewing and editing. Virginia Perez-Carrasco processed the samples, performed the bioinformatic analysis and contributed to writing. David Uroz-Torres collected the samples. Jose Santana Ramos processed the samples and performed the bioinformatic analysis. Miguel Soriano and Jose Antonio García-Salcedo took part in the conceptualization of this study and revised the article for important content related to microbiome analysis.

ACKNOWLEDGEMENTS

The authors acknowledge Francisca Castillo Pérez for her technical assistance.

FUNDING INFORMATION

The study was partly funded by the FEDER/Junta de Andalucía-Consejería de Economía y Conocimiento (Project ref: A1-CTS-174-UGR18) and the Ministry of Science and Innovation of Spain co-financed with European Regional Development Funds (Project ref: PID2020-120481RB-I00) and University of Almería (Project Ref: PPUENTE2021-006). Pérez-Carrasco V was supported by a fellowship from the Ministry of Universities, Spain (FPU20/03952). Funding for open access charge: Universidad de Granada / CBUA.

CONFLICT OF INTEREST STATEMENT

The authors deny any conflicts of interest with this article.

DATA AVAILABILITY STATEMENT

All datasets supporting the conclusions of this article are available in the Sequence Read Archive (SRA) of the National Centre for Biotechnology Information (NCBI) under the Bioproject numbers PRJNA839210 and PRJNA1033443. Authors can confirm that all relevant data are included in the article.

ETHICAL APPROVAL

This research involved human samples. The study was approved by the Ethics Committee of the University of Granada (ref: 354/CEIH/2017).

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How to cite this article: Arias-Moliz, M.T., Pérez-Carrasco, V., Uroz-Torres, D., Santana Ramos, J.D., García-Salcedo, J.A. & Soriano, M. (2024) Identification of keystone taxa in root canals and periapical lesions of post-treatment endodontic infections: Next generation microbiome research. *International Endodontic Journal*, 00, 1–10. Available from: https://doi.org/10.1111/iej.14046