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ORIGINAL ARTICLE

Microbiome in paired root apices and periapical lesions and its association with clinical signs in persistent apical periodontitis using next-generation sequencing

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Abstract

Aim: To assess and compare the microbiome of paired root apices and periapical lesions from cases with failed endodontic treatment and to associate the microbiome and bacterial metabolic pathways in both sites with asymptomatic apical periodontitis (AAP) and symptomatic apical periodontitis (SAP), using next-generation sequencing (NGS).

Methodology: Matched root apices and periapical lesions of patients with failed root canal treatments were surgically extracted. Specimens were cryopulverized, bacterial DNA was extracted and the V3–V4 hypervariable regions of the 16 S rRNA gene were amplified and sequenced using the Illumina Miseq platform. Diversity and community composition were studied in the paired samples, as well as in AAP and SAP cases. Diversity indices were compared in each case by means of the Wilcoxon matched-pairs signed rank and Mann–Whitney *U* tests. Differences in the community composition were explored with multivariate statistical analysis and Linear discriminant analysis Effect Size (LEfSe). Bacterial functional study was performed through the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis.

Results: Twenty-one paired apices and lesions were successfully sequenced and analysed, identifying a total of 21 phyla and 600 genera. A higher alpha-diversity was observed in the periapical lesions, although no global differences in the community composition between the two sites were found ($p = .87$), the most prevalent genera being *Fusobacterium*, *Porphyromonas* and *Streptococcus*. *Prevotella*, *Clostridiales_vadinBB60_group*, *Bosea*, *Phreatobacter*, *Afipia* and *Xanthobacteriaceae_unclassified* were enriched in SAP samples, while *Pseudopropionibacterium*, *Campylobacter* and *Peptoniphilus* were significantly more abundant in AAP cases ($p < .05$). Metabolic pathways involved in the amino acid metabolism or degradation and flagellum

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assembly were more abundant in SAP samples, whereas glucose metabolism-related pathways were associated with AAP.

Conclusions: The bacterial community composition was similar in the apices and periapical lesions. The microbiome was different in AAP and SAP samples, gram-negative bacteria showing higher relative abundances in SAP cases. An association was observed between amino acid degradation and flagellum assembly pathways, and the development of tenderness to percussion or palpation.

KEYWORDS

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

INTRODUCTION

One of the main factors associated with post-treatment apical periodontitis is the persistence of microbial biofilms in the apical portion of the root canal system (Nair et al., 2005) and in the periapical tissues (Noguchi et al., 2005; Signoretti et al., 2011). Extraradicular infection has long been a matter of controversy. While the periapical lesion is considered to be free of microorganisms except for exacerbating cases and periapical actinomycosis (Nair, 2006), biofilms can be observed attached to the outer root surface (Noguchi et al., 2005; Noiri et al., 2002; Signoretti et al., 2011; Tronstad et al., 1990) or immersed in the body of the lesion (Sunde et al., 2003; Tronstad et al., 1990), and bacterial DNA has been detected in periapical tissues analysed by molecular techniques (Bronzato et al., 2021; Handal et al., 2009; Lin et al., 2007; Noguchi et al., 2005; Pereira et al., 2017).

Because extraradicular microorganisms derive from inside the root canals, it is reasonable to surmise that intraradicular and extraradicular infections in post-treatment apical periodontitis share a similar composition (Montagner et al., 2010). However, once the microorganisms reach the periapical tissues, they must adapt to new ecological conditions, for example, a higher nutrient availability as compared to the treated root canal, less oxygen and the action of the host immune cells. To date, there is little evidence of the microbial correlation in paired samples – including apices and periapical lesions – or contradictory results (Pereira et al., 2017; Subramanian & Mickel, 2009; Sun et al., 2022). A deeper knowledge of the intraradicular and extraradicular infection in the sustained disease would shed light on the reasons behind failure of the root canal treatment.

The persistence of biofilms inside the root canal system and its surrounding environment is associated with several signs and symptoms (Siqueira & Rôças, 2013). Specifically, members of anaerobic genera, such as *Porphyromonas*, *Fusobacterium* and *Prevotella*, were shown to be associated with tenderness to percussion in studies employing culture methods and species-specific PCR (Gomes et al., 2004; Pinheiro et al., 2003). Results using next-generation sequencing (NGS) technology point to a similar diversity between symptomatic and asymptomatic cases of post-treatment apical periodontitis (Anderson et al., 2013; Hou et al., 2022), although several genera were demonstrated to be significantly more abundant in symptomatic cases (Hou et al., 2022). The high taxonomic variation of these infections makes mere characterization of the microbiome insufficient for understanding the disease evolution and the clinical signs. Bacterial functional pathways are considered intrinsic factors that contribute to microbial virulence and persistence in the host tissues. The widely studied virulence factors lipopolysaccharide and lipoteichoic acid have been associated with clinical signs (Gomes & Herrera, 2018; Jacinto et al., 2005) even though they represent a minor proportion of the microbial pathogenic mechanisms. Since the development of signs such as tenderness to percussion or palpation would be a consequence of the immune response to the presence of microorganisms (Martinho et al., 2021), the association between these clinical signs and the microbiome and bacterial functional genes merits further investigation. The aim of the present study was therefore two-fold: to assess and compare the microbiome from root apices and their associated periapical lesions retrieved surgically from cases with failed root canal treatments; and to associate the microbiome and bacterial metabolic pathways in both sites with clinical signs. The null hypotheses are that the microbiome in the matched root apices and periapical lesions would be similar, and there is no association between the microbiome and bacterial functional pathways of the two sites and the presence of clinical signs.

MATERIAL AND METHODS

The present work is an observational study reported under the Strengthening the Reporting of Observational studies

in Epidemiology (STROBE) guidelines. The study was approved by the Ethics Committee of the University of Granada (ref: 354/CEIH/2017). All patients signed an informed consent form before their inclusion in the study. Samples were collected from patients of a private practice by an endodontist with experience in endodontic microsurgery.

Patients included in the study were those that had a previously treated root canal with radiographic evidence of persistent apical periodontitis. Apical surgery was chosen instead of root canal retreatment because of persistent pain and apical lesion for more than 1 year after previous root canal treatment. Exclusion criteria included patients with factors that could interfere with the microbiota of the periapical lesion (such as severe systemic disease), use of antibiotics in the 3 months previous to surgery, teeth with periodontal pockets >4 mm, pulp cavity exposure to the oral environment, teeth with vertical root fracture, and history of trauma. Pregnant or breastfeeding women and patients under 18 years old were also excluded. A total of 38 patients were recruited in this study. Clinical and radiographic data recorded for each patient/tooth included age, gender, presence of symptomatic apical periodontitis (SAP; defined as tenderness to percussion or palpation) and asymptomatic apical periodontitis (AAP; no signs).

Sample collection

In order to avoid external contamination, before the microsurgery patients rinsed their mouth with 0.12% chlorhexidine solution for 30 s, and a sterile gauze soaked with the same solution was swabbed in the intraoral surgical site. Root apices and their respective lesions were taken under strictly aseptic conditions. After local anaesthesia, a full-thickness mucoperiosteal flap was made with a number 15C scalpel blade (Swann Morton). The apex was sectioned using an ultrasound tip NINJA F87545 (Acteon Satelec) and the periapical lesion was exposed with an ultrasound SL2 F87512 insert (Acteon Satelec). All clinical procedures were performed under magnification (Zeiss Pro Ergo; Carl Zeiss).

After the apex extraction, the external surface of each apex was rinsed with sterile saline solution, and the apical lesion from the root was removed with a curette and tweezers. The external apex surfaces were cleaned with a cotton pellet soaked in 3% hydrogen peroxide and disinfected the same way with 2.5% sodium hypochlorite (Alves et al., 2009). The latter was inactivated by sterile 5% sodium thiosulphate. After disinfection, a control sample was taken from the outer surface of the apices with a #60 sterile paper point in order to confirm its proper disinfection.

To avoid cross-contamination, each apex and the surrounding periapical lesion were transferred into separate Eppendorf tubes containing 200 mL of Tris-EDTA buffer (pH 8.0; Panreac Quimica) and they were stored at −20°C until cryopulverization. A sterile mortar and pestle containing 5 mL of liquid nitrogen were used to pulverize the samples. The powered samples were stored at −20°C until further processing.

DNA extraction and high-throughput sequencing

The bacterial DNA was extracted using a QIAmp DNA Mini Kit (Qiagen), following the protocol recommended by the manufacturer preceded by a 30-min preincubation step with lysozyme to optimize the DNA extraction from gram-positive bacteria. Controls were included in each extraction batch to check for any possible contamination. DNA quality and quantification were performed using a spectrophotometer (Nanodrop 2000 UV – Vis; ThermoFisher Scientific).

PCR amplification products of the V3-V4 hypervariable regions of 16 S rRNA gene were obtained using fusion universal primers 340F (Illumina adaptors +5′-CCTACGGG NGGCWGCAG-3′) and 800R (Illumina adaptors +5′-GACTACHVGGGTATCTAATCC-3′), as previously described (Soriano-Lerma et al., 2020). Similarly, negative amplification controls were included in each PCR batch. Amplicon multiplexing and sequencing was performed with a dual indexing tag-tailed design using 8 nt indexes from the Nextera XT Index Kit v2 (Illumina). Paired-end sequencing of 16 S rRNA amplicon libraries was performed by means of the Illumina MiSeq platform with v3 kit chemistry (300 + 300 bp). The raw sequencing data are available at the Sequence Read Archive (SRA) of the National Centre for Biotechnology Information (NCBI) under the Bioproject accession number PRJNA839210.

Bioinformatic analysis

Bioinformatic analysis and quality-filtering were performed using Mothur software (v 1.43.0; University of Michigan Medical School), following the standard MiSeq operating procedure. Chimeric reads were identified and excluded using Chimera UCHIME. Redundant, nonchimeric FASTA files were taxonomically classified using Silva v132 database. Abundance was expressed as a percentage with respect to the total number of sequences in each sample. Genera with a total abundance higher than 0.1% were considered for statistical analysis. Alpha-diversity in paired root apices and periapical lesions from the same patient, as well as in both sites according to the presence or absence of symptoms (SAP or AAP), was assessed by obtaining the species

richness (number of detected operational taxonomic units, OTUs) and the Inverse Simpson (InvSimpson), Shannon (H′) and Pielou (J′) diversity indexes. Bacterial functional analysis was performed by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis on high-throughput 16S rRNA gene sequencing data of the AAP and SAP cases (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., 2022).

Statistical analysis

After checking the absence of normality in the diversity indexes with the Shapiro–Wilk test, the nonparametric Wilcoxon matched-pairs signed rank test for paired root apices and lesions was performed, and the nonparametric Mann–Whitney *U* test was applied to SAP and AAP samples using GraphPad Prism version 8.0.0 (GraphPad Software). The similarity of the bacterial communities between samples was evaluated by Principal Component Analysis (PCA), based on Euclidean distances and performed using R software (R Foundation for Statistical Computing), and by Principal Coordinate Analysis (PCoA), based on Bray–Curtis distances and implemented in PRIMERe Permanova+ (PRIMER-E Ltd.). 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). For all statistical analyses, $p < .05$ was considered as significant. Identification of enriched bacteria taxa in the study groups was carried out by Linear discriminant analysis Effect Size (LEfSe) via Python 3.7.6, considering a *p* value of .05 and a linear discriminant analysis (LDA) value of 2 as significant (Segata et al., 2011). Statistical power achieved with the sample size was estimated with GPower 3.1 software (University of Kiel). A statistical power of 0.9002117 was obtained according to the detected effect size. Heatmap and SparCC correlation analysis were performed using *pheatmap* and *SpiecEasi* R packages respectively. Correlation and co-occurrence network diagrams were represented using Gephi v.0.9.2 with cut-off values of −0.2 and 0.2.

RESULTS

Microbial diversity and bacterial communities in paired root apices and periapical lesions

The sequencing of 16S rRNA gene amplicon libraries with Illumina Miseq platform gave a total of 805 800 sequences after bioinformatic processing (an average of 19 186 reads per sample). To minimize the sample size-induced bias amongst samples, all were rarefied by sub-sampling at 1250 sequence reads. This sequencing depth made it possible to obtain a coverage higher than 95% in all samples. To ensure the absence of external contaminants in the samples, disinfection, extraction and amplification controls were also amplified and sequenced, showing no significant amplification and a small number of sequences compared to the study samples. Seven patients were excluded from the study because their apex or lesion samples showed no significant PCR amplification. Of the remaining 31 participants, 10 patients were excluded because of an insufficient number of sequences (less than 1250 reads) in their matched apex and lesion, leading to a total of 21 paired apices and periapical lesions of 21 patients analysed (Figure 1). Ten patients presented AAP and 11 SAP. Table 1 offers the patient characteristics and the clinical data of the included samples.

A greater alpha-diversity was observed in the lesions as compared with their matched apex, as higher values of species richness, InvSimpson, Shannon and Pielou indexes ($p < .05$) were obtained (Figure 2a). No clear distinction was observed in the community composition in the Principal Component Analysis (PCA) and Principal Coordinate Analysis (PCoA) between the two groups at genus level (Figure 2b), which was quantitatively confirmed with a multivariant PERMANOVA test ($p = .87$). However, the genera *Clostridium_sensu_stricto_1*, *Blautia* and *Veillonella* were significantly enriched ($p < .05$) in the periapical lesions, whereas only *Granulicatella* genus was enriched in the apices according to LEfSe analysis (Figure 2c and Table S1).

Taxonomic classification found a total of 21 phyla and 600 genera, of which 14 phyla and 82 genera showed

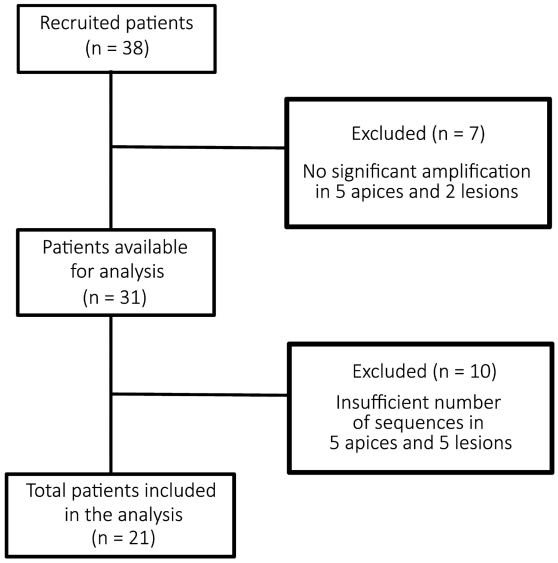

FIGURE 1 STROBE flowchart of the study participants.

TABLE 1 Patient characteristics and clinical data of the 21 matched paired samples analysed in the study.

Patient	Tooth	Sex	Age (y)	AAP/SAP	Reason for root canal retreatment
P1	Mandibular molar	F	35	SAP	Separated file
P2	Mandibular molar	M	37	AAP	Persistent apical lesion
P3	Mandibular incisive	F	42	SAP	Crown and post
P4	Maxillary molar	M	27	SAP	Persistent pain and apical lesion
P5	Maxillary premolar	M	43	SAP	Persistent pain and apical lesion
P6	Maxillary premolar	F	45	AAP	Crown and post
P7	Maxillary premolar	F	54	SAP	Apical canal deviation
P8	Maxillary premolar	F	56	SAP	Extruded gutta-percha
P9	Maxillary molar	F	48	SAP	Persistent pain and apical lesion
P10	Maxillary premolar	F	76	AAP	Crown and post
P11	Maxillary molar	M	63	SAP	Persistent pain and apical lesion
P12	Maxillary incisive	F	37	AAP	Persistent apical lesion
P13	Maxillary incisive	F	34	SAP	Persistent pain and apical lesion
P14	Maxillary molar	F	51	SAP	Persistent pain and apical lesion
P15	Maxillary molar	M	35	AAP	Missed canal
P16	Maxillary incisive	M	39	AAP	Persistent apical lesion
P17	Maxillary incisive	F	27	AAP	Persistent apical lesion
P18	Maxillary molar	F	69	AAP	Crown and post
P19	Maxillary molar	M	41	SAP	Crown and post
P20	Maxillary incisive	F	47	AAP	Persistent apical lesion
P21	Maxillary incisive	M	58	AAP	Persistent apical lesion

Abbreviations: AAP, asymptomatic apical periodontitis; SAP, symptomatic apical periodontitis.

a relative abundance >0.1%. *Firmicutes* (37.89%) and *Bacteroidetes* (18.84%) were the dominant phyla, followed by *Fusobacteria* (14.42%), *Proteobacteria* (9.76%), *Synergistetes* (7.13%) and *Actinobacteria* (6.47%), both in apices and periapical lesions (Figure 3). At genus level, *Fusobacterium* (12.93%), *Porphyromonas* (8.05%), *Streptococcus* (7.65%), *Pseudomonas* (4.95%), *Fretibacterium* (4.77%) and *Tannerella* (3.22%) were the most prevalent genera identified. In the apices, *Fusobacterium* was the most abundant, followed by *Streptococcus*, *Porphyromonas*, *Enterococcus*, *Pseudomonas* and *Tannerella*, whereas *Porphyromonas* followed by *Fusobacterium*, *Streptococcus*, *Fretibacterium* and *Pseudomonas* were dominant in the periapical lesions (Table 2).

Microbial diversity and bacterial communities in AAP and SAP cases

Figure 4a shows the alpha-diversity in apices and periapical lesions in AAP and SAP samples. A greater diversity was observed in the periapical lesions than in the apices within the AAP samples, while no significant differences were observed amongst SAP samples. In addition, a greater diversity was obtained in the apices of SAP samples when compared with the apices of AAP samples, although no significant differences were observed in the lesions according to the presence or absence of symptomatology.

The PCA showed that the bacterial communities in AAP and SAP groups were separated along the Y axis, explaining 7.9% of bacterial variation (Figure 4b). Differences between the two groups were quantitatively confirmed by PERMANOVA ($p = .03$). In the apices, the most abundant genera in AAP were *Fusobacterium* (19.68%), *Streptococcus* (10.78%) and *Parvimonas* (7.40%); in SAP they were *Fusobacterium* (12.78%), *Pseudomonas* (9.06%) and *Enterococcus* (8.10%). Within the periapical lesions, *Streptococcus* (9.81%), *Fretibacterium* (9.01%) and *Fusobacterium* (7.45%) were the most prevalent genera in AAP, and *Porphyromonas* (13.07%), *Fusobacterium* (11.93%) and *Pseudomonas* (9.78%) in SAP samples (Table S2).

The identification of bacteria with statistical differences for both sites (Figure 5a), root apices (Figure 5b) or periapical lesions (Figure 5c), between the AAP and SAP groups, was assessed by means of LEfSe. Seven genera including *Prevotella*, *Clostridiales_vadinBB60_group*, *Bosea*,

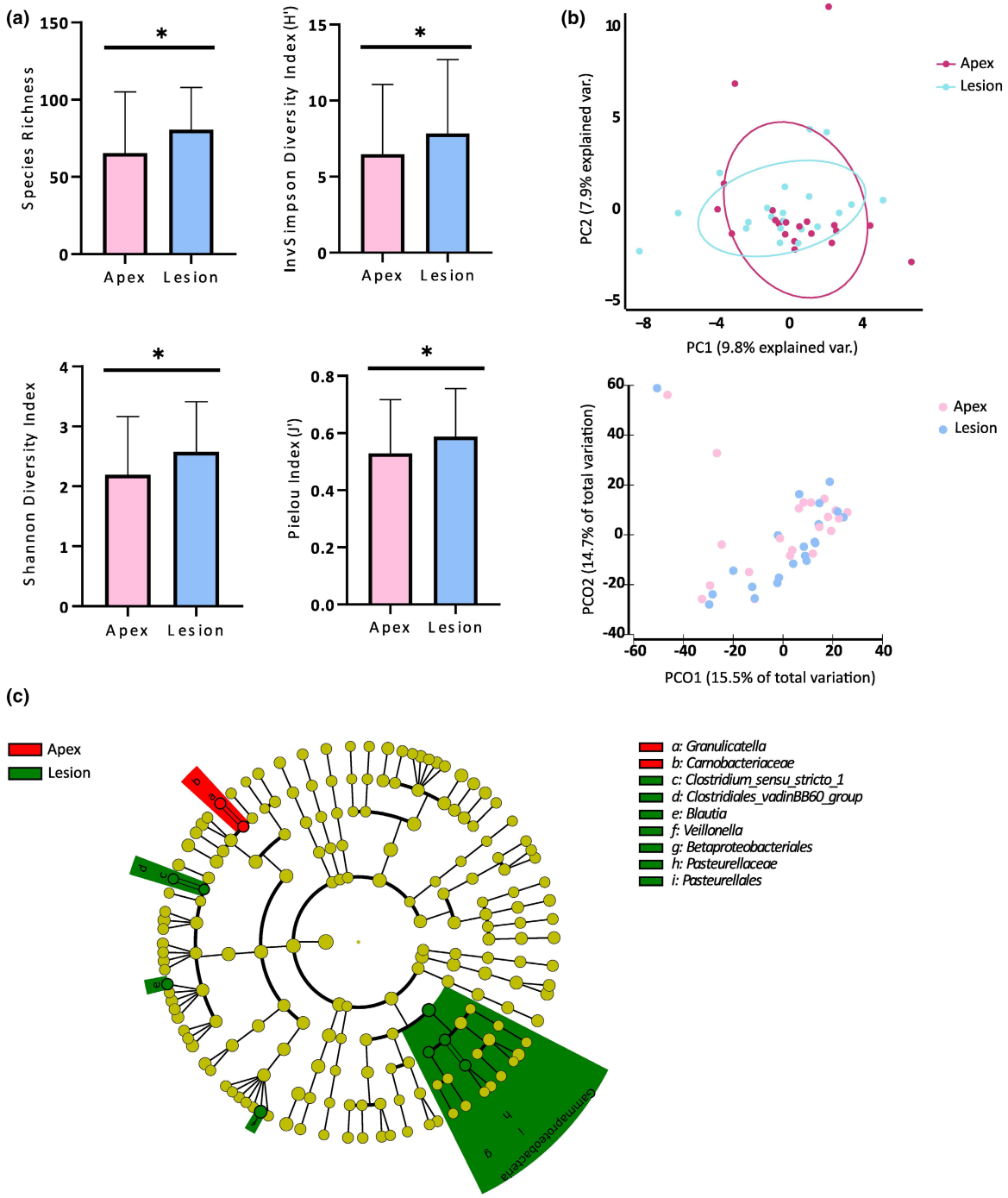

FIGURE 2 (a) Alpha-diversity indices (species richness, InvSimpson, Shannon and Pielou) of the microbiome in paired apices and periapical lesions. Differences between the two sites were assessed by Wilcoxon matched-pairs signed rank test (*$.01 < p < .05$). (b) Principal Component (PC) and Principal Coordinate (PCO) analyses of the apices (pink) and lesion (blue) samples at genus level. Samples are represented by dots. (c) Linear discriminant analysis Effect size (LEfSe). Cladogram shows differentially distributed bacterial taxa ($p < .05$, LDA > 2.0) between the two sites. Taxonomic features are represented in a hierarchical structure, with higher taxonomic levels oriented towards the inner part of the plot. Taxa with significant differences are coloured according to whether they are enriched in apices (red) or in lesions (green); yellowish green for non-significant.

Phreatobacter, *Afipia*, *Erysipelotrichaceae_UGC_006* and *Xanthobacteriaceae_unclassified* were enriched in SAP samples ($p < .05$), while *Pseudopropionibacterium*, *Campylobacter* and *Peptoniphilus* were significantly more abundant in AAP patients ($p < .05$), considering both root apices and periapical lesions (Figure 5a and Table S1). When considering the apices, enriched bacteria were detected only in the SAP group (Figure 5b); whereas three

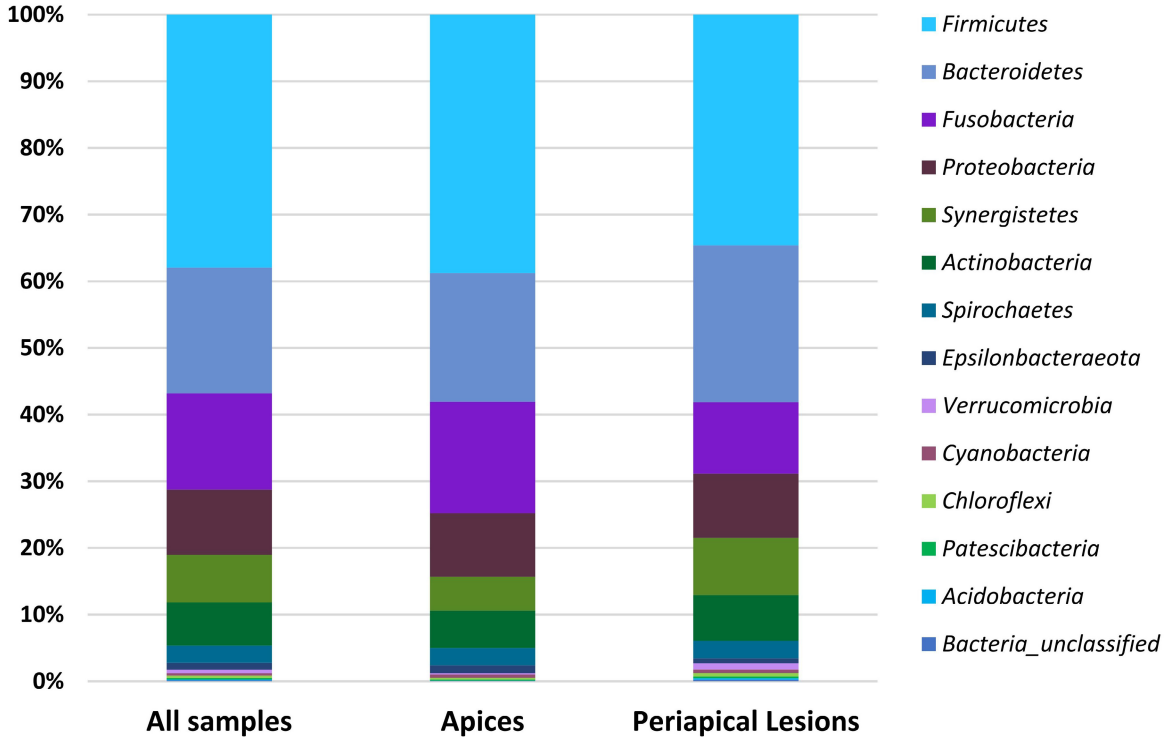

FIGURE 3 Relative abundance of bacterial phyla considering apices, periapical lesions and all samples together.

genera in the SAP group and two genera in group AAP were enriched in periapical lesions (Figure 5c).

SparCC correlation and co-occurrence network analysis at genus level was performed to analyse the interaction between bacteria in AAP and SAP groups. The network characteristics varied for each group. Several clusters or communities of bacterial genera were observed in both groups: 10 clusters in AAP patients and seven in SAP patients (Figure S1A,C). Bacterial genera showed positive correlation with other members of the same cluster, as well as with other genera from different clusters (Figure S1B,D). Different genera showed negative correlation values with genera of other clusters such as *Streptococcus–Tannerella*, *Treponema–Actinomyces*, *Treponema–Rothia* and *Fusobacterium–F0058* in AAP patients (Figure S1B), and *Prevotella–Campylobacter*, *Porphyromonas–Gemella*, *Moryella–Prevotella_2*, *Porphyromonas–Veillonella* in the SAP group (Figure S1D).

Bacterial functional analysis in AAP and SAP groups

The evaluation of the bacterial functional data obtained by PICRUSt analysis showed that several KEGG pathways differed between the AAP and SAP when both the apices and periapical lesions were considered. Eleven KEGG pathways (level 3) were enriched amongst SAP samples, including several pathways involved in amino acid metabolism or degradation and flagellum assembly. Gene families involved in glucose metabolism were significantly associated with AAP (Table 3 and Figure S2).

DISCUSSION

Apical periodontitis is an inflammatory oral disease that is caused by bacterial biofilms colonizing the root canal walls, the radicular cementum and in some cases the apical tissues (Nair et al., 2005). To the best of the authors' knowledge this is the first study to predict the bacterial functional pathways in biofilms from root apices and periapical lesions.

Samples were initially cryopulverized because it allows for a more accurate retrieval of the microorganisms present in the apical portion of the root canal system, including the filling material, and in the periapical lesion (Alves et al., 2009; Karygianni et al., 2015). NGS techniques make it possible to quantitatively characterize the microbiome, providing information on microbial communities and identifying uncultured microbes (Perez-Carrasco et al., 2021). In this study, the V3–V4 hypervariable region of 16S rRNA gene was amplified and analysed. The short length of the V3–V4 amplicon as compared to V1–V2 has the advantage of improved overlapping, thus reducing sequencing errors (Soriano-Lerma et al., 2020). The amplification step was performed using fusion universal primers and a total of 25 cycles to reduce PCR artefacts (Soriano-Lerma et al., 2020).

TABLE 2 Relative abundance of the main genera in paired apices and periapical lesions at abundances >0.5%.

Genera	All samples	Apices	Periapical lesions
Fusobacterium	12.93	16.06	9.79
Porphyromonas	8.05	5.89	10.2
Streptococcus	7.65	7.65	7.64
Pseudomonas	4.95	4.75	5.14
Fretibacterium	4.77	2.75	6.78
Tannerella	3.22	3.11	3.34
Peptostreptococcus	2.96	2.46	3.47
Parvimonas	2.88	3.84	1.92
Veillonella	2.63	0.49*	4.76*
Enterococcus	2.62	4.8	0.44
Treponema_2	2.56	2.53	2.59
Phocaeicola	2.54	2.74	2.34
Oribacterium	2.36	2.96	1.75
Actinomyces	2.29	2.03	2.57
Pyramidobacter	2.03	2.31	1.76
Paludibacteraceae_F0058	1.96	2.58	1.35
Pseudoramibacter	1.82	3.14	0.51
Prevotella_7	1.63	1.55	1.72
Rothia	1.52	1.81	1.24
Candidatus_Arthromitus	1.32	1.81	0.83
Clostridiales_FamilyXIII_ge	1.12	1.11	1.13
Prevotella	1.11	0.81	1.36
Lactobacillus	1.09	0.76	1.43
Lachnospiraceae_ge	1.05	0.62	1.47
Campylobacter	0.97	1.19	0.75
Gemella	0.85	0.75	0.94
Moryella	0.76	0.74	0.78
Leptotrichia	0.73	0.59	0.86
Alloprevotella	0.65	0.39	0.9
Rikenellaceae_RC9_gut_group	0.54	0.39	0.69
Filifactor	0.52	0.48	0.55
Afipia	0.52	0.72	0.31
Akkermansia	0.51	0.15	0.85

*Significant differences between apices and periapical lesions ($p < .05$).

Two out of 38 lesions (5.3%) were discarded because of the low amount of DNA in the samples, meaning that some periapical lesions would be free of microorganisms, as previously reported (Noguchi et al., 2005; Saber et al., 2012; Sun et al., 2022; Sunde et al., 2002). Paired-end sequencing of 16S rRNA gave an average of 19186 reads per sample and a coverage higher than 97% in all samples, confirming an adequate sequencing depth.

The bacterial community in paired apices and lesions was similar (Sun et al., 2022). Therefore, the first null hypothesis was accepted. *Firmicutes* was the most abundant phylum identified, representing 37.89% of the total reads, and it was followed by *Bacteroidetes*, found in 18.84% of all the sequences (Siqueira et al., 2011; Tzanetakis et al., 2015; Vengerfeldt et al., 2014). The most predominant genera were *Fusobacterium*, *Porphyromonas*, *Streptococcus*, *Pseudomonas*, *Fretibacterium*, *Tannerella*, *Parvimonas* and *Peptostreptococcus*, all frequent pathogens in post-treatment infections in these sites (Hong et al., 2013; Hou et al., 2022; Keskin et al., 2017; Saber et al., 2012; Sun et al., 2022; Tzanetakis et al., 2015). *Porphyromonas* and *Treponema* were demonstrated in relative abundance similar to that of periodontal infections (Costalonga & Herzberg, 2014). *Porphyromonas* has the ability to internalize into epithelial cells, replicate and survive inside them, and inhibit the apoptosis of the infected cells (Houalet-Jeanne et al., 2001; Tribble & Lamont, 2010). The invasion capacity, likewise observed for *Treponema* (Inagaki et al., 2016), helps the microorganisms evade the extracellular clearance debilitating the innate immune system (Lamont & Jenkinson, 2000). This mechanism is characteristic of periodontal disease and could justify the presence of these bacteria in the periapical tissues.

Enterococcus was amongst the most abundant genera identified in the apices, where it represented 4.8% of total reads, in contrast to just 0.44% for the lesions. This high prevalence can be attributed to a patient for whom 84.47% of the sequences corresponded to *Enterococcus* (data not shown), meaning the apex hosted high loads of this bacterium. *Enterococcus* was present in 38.10% (8/21) of the apices and in 47.62% (10/21) of the lesions, in line with studies using the NGS technique (Bouillaguet et al., 2018; Zandi et al., 2018) and contrasting with results obtained by culture and close-ended molecular methods (Manoil et al., 2020; Tennert et al., 2014). These findings suggest some previous overestimation of its role in secondary and persistent endodontic infections.

Regarding the clinical signs, a higher diversity was observed in the apices of symptomatic cases. Indeed, no differences were obtained in the periapical lesions. Previous studies have reported no differences in the diversity in root canals of failed treated cases according to the symptomatology (Anderson et al., 2013). Diverse reasons, for example, root canal ecological factors during the progress of the infection, could be responsible for the differences in diversity obtained in the present study. One interesting finding is that the microbial community differed according to the symptomatology regardless of the site, so that our second null hypothesis was partly rejected. There is no clear consensus in the literature regarding a possible

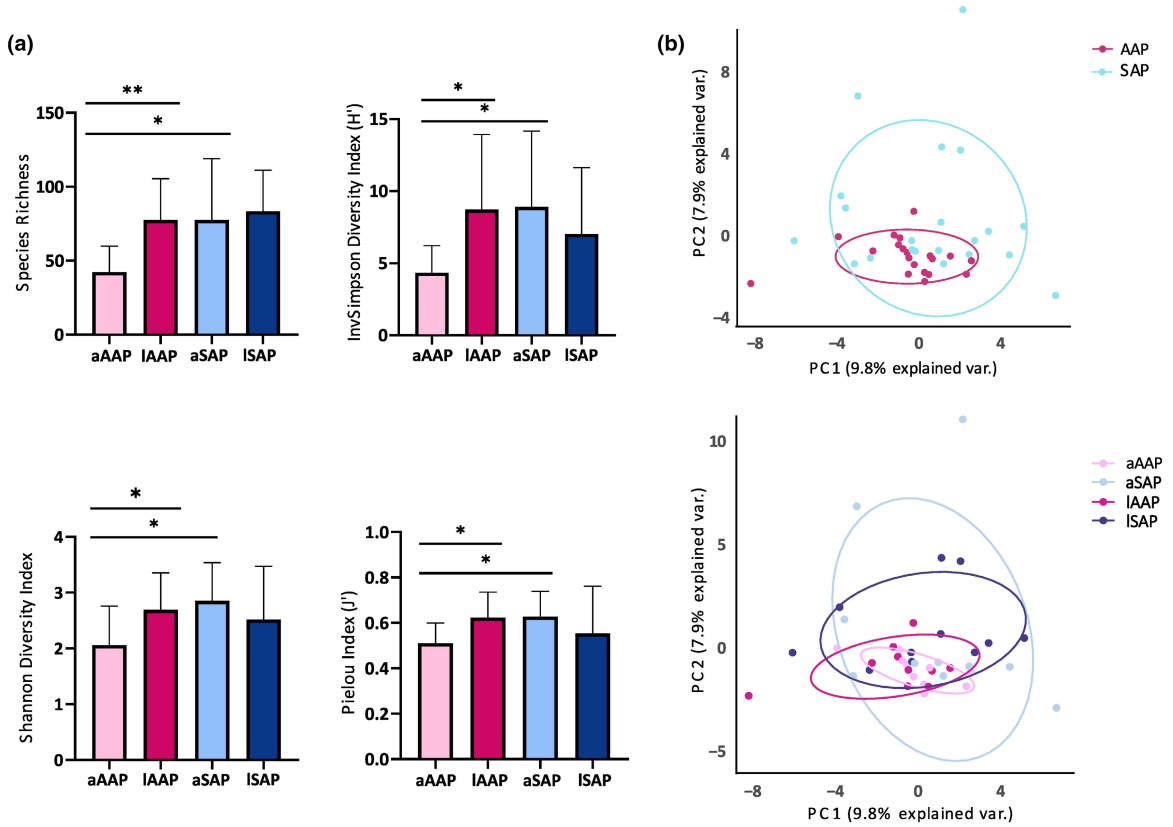

FIGURE 4 (a) Alpha-diversity indices (species richness, InvSimpson, Shannon and Pielou) of the microbiome in apices of asymptomatic apical periodontitis patients (aAAP), in apices of symptomatic apical periodontitis patients (aSAP), in periapical lesions of asymptomatic apical periodontitis patients (lAAP) or in periapical lesions of symptomatic apical periodontitis patients (lSAP). Differences between groups were assessed by Mann–Whitney *U* test (*$.01 < p < .05$; **$.001 < p < .01$). (b) Principal Component (PC) analysis of the AAP (pink) and SAP (blue) samples with and without taking into account the sample site (apex or lesion) at genus level.

association between specific microorganisms and the development of clinical signs. Shifts in the microbial composition according to the signs have been previously reported by certain authors (Sánchez-Sanhueza et al., 2018), yet others demonstrated no significant changes (Anderson et al., 2013; Hou et al., 2022).

One remarkable finding is that 68.32% of the reads in SAP samples corresponded to gram-negative bacteria, as compared to the 46.08% for AAP samples ($p < .05$), in genera with more than 0.1% of sequences. The outer membrane of gram-negatives holds lipopolysaccharide, which can stimulate immune cells to release inflammatory mediators, thus resulting in clinical signs and explaining their higher abundance in symptomatic cases (Bronzato et al., 2021; Hou et al., 2022; Prada et al., 2019). Moreover, *Prevotella*, *Clostridiales_vadinBB60_group*, *Bosea*, *Phreatobacter*, *Afipia* and *Xanthobacteriaceae_unclassified* were significantly enriched and sometimes exclusively detected in symptomatic cases, all of them being gram-negative. The correlation and co-occurrence analysis showed the presence of different bacterial clusters or communities in AAP and SAP patients. Interestingly, all these genera enriched in patients with clinical signs were detected as members of the same bacterial cluster.

The heterogeneity seen within the samples denotes that there is no specific pathogen responsible for the development of the disease; rather, the community and its potential function as a whole would play a role in the pathogenicity and possibly the clinical signs and symptoms. In order to understand the functional role of the microbial community, a prediction of the bacterial metabolic pathways was performed by PICRUSt. It is noteworthy that SAP samples displayed a higher abundance of predicted pathways related to amino acid degradation and flagellum assembly, which are relevant for bacterial virulence and pathogenicity. For instance, leucine, serine and lysine degradation up-regulate pro-inflammatory cytokines (Cheng et al., 2020; Han et al., 2018; Zhang et al., 2017) and similarly, serine inhibits macrophage- and neutrophil-mediated inflammatory responses (He et al., 2019). This increase in the amino acid degradation is accompanied by a higher abundance, although not significant, of proteolytic bacteria in symptomatic cases like *Porphyromonas* and *Prevotella*.

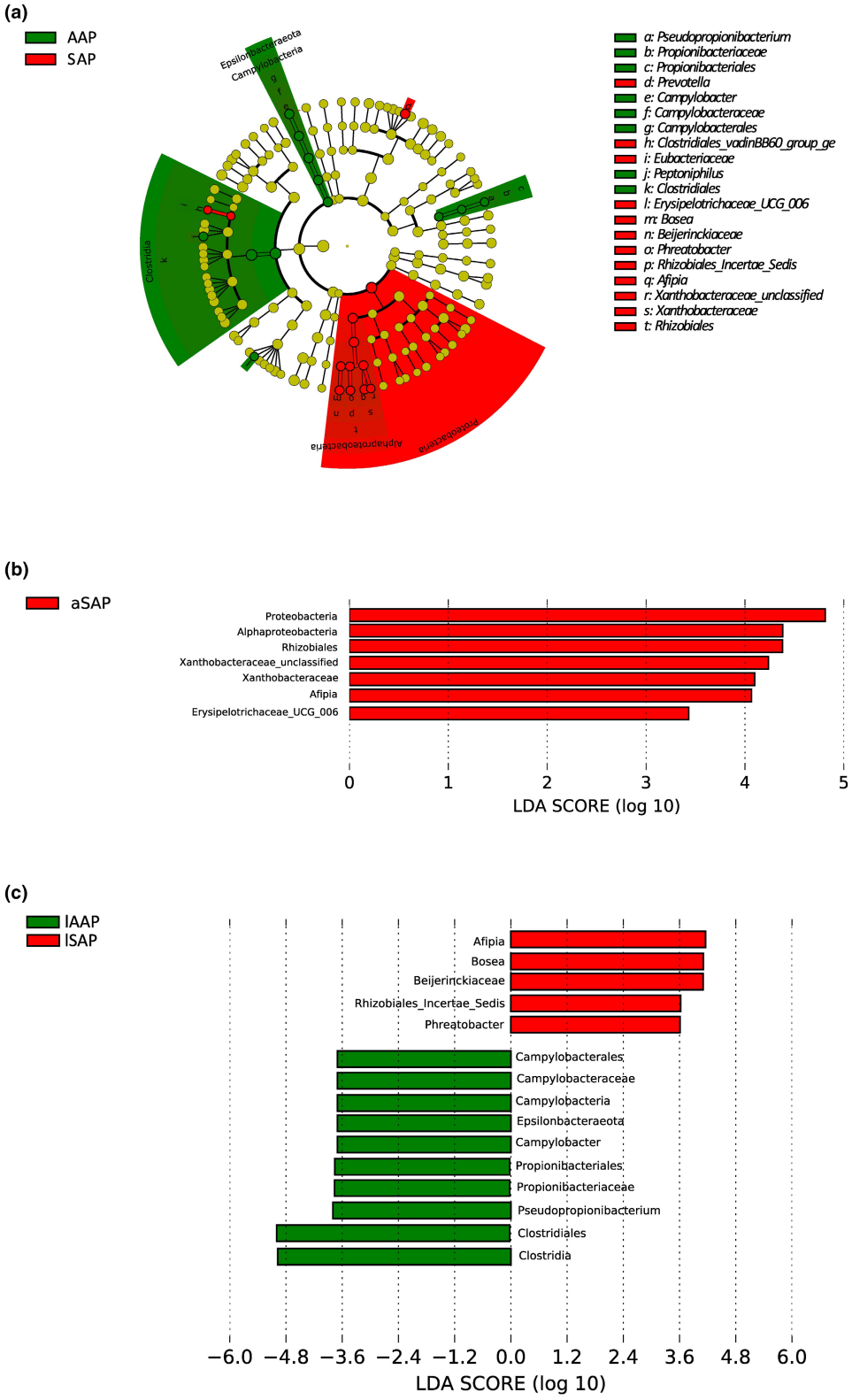

FIGURE 5 Enriched bacterial taxa in asymptomatic apical periodontitis (AAP) or symptomatic apical periodontitis (SAP) patients identified by Linear discriminant analysis Effect Size (LEfSe) ($p < .05$, LDA > 2.0). (a) Cladogram generated by LEfSe using the apices and lesions of AAP or SAP cases. Taxa with significant differences are coloured according to whether they are enriched in AAP (green) or in SAP (red); yellowish green for non-significant. (b) Bar plot generated by LEfSe including only apices of AAP and SAP (aSAP) cases. No enriched bacterial taxa were identified in the apices of AAP cases. (c) Bar plot generated by LEfSe including only periapical lesions of AAP (lAAP) and SAP (lSAP) patients. Taxa with significant differences are coloured according to whether they are enriched in lesions of AAP (green) or of SAP (red) cases.

TABLE 3 Kyoto Encyclopedia of Genes and Genomes (KEGG) microbial pathways enriched in asymptomatic apical periodontitis (AAP) or symptomatic apical periodontitis (SAP) cases.

	LDA (log 10)	*p* Value
KEGG microbial pathways enriched in AAP		
RNA transport	2.2392	.0495
Pentose phosphate pathway	2.4921	.0095
Glycerophospholipid metabolism	2.5203	.0032
Cysteine and methionine metabolism	2.5383	.0284
Pyruvate metabolism	2.7032	.0035
Glycolysis–Gluconeogenesis	2.7574	.0041
Aminoacyl-tRNA biosynthesis	2.9527	.0109
KEGG microbial pathways enriched in SAP		
Valine, leucine and isoleucine degradation	2.9075	.0065
Membrane and intracellular structural molecules	2.8737	.0035
Flagellar assembly	2.7887	.0219
Pores ion channels	2.7391	.0126
Glycine, serine and threonine metabolism	2.6716	.0413
Glyoxylate and dicarboxylate metabolism	2.6583	.0065
Lysine degradation	2.6371	.0156
Geraniol degradation	2.6209	.0179
Beta-alanine metabolism	2.5914	.0179
Toluene degradation	2.4239	.0439
Naphthalene degradation	2.3359	.0249

Abbreviation: LDA, linear discriminant analysis.

The enriched flagellum assembly pathway is a consequence of the higher presence of motile bacteria in symptomatic cases such as *Treponema* and *Pseudomonas*. The flagellum is a virulence factor involved in bacterial chemotaxis, tissue colonization and maintenance at the infection site (Chaban et al., 2015). In particular, *Pseudomonas* flagellum could be involved in the development of the clinical signs evaluated, as flagellin can be recognized by the Toll-like receptor 5 (TLR5) that induces inflammatory response as well as mechanical allodynia (Yang & Yan, 2017). The levels of *Pseudomonas* were high, especially in the periapical lesions. It has epithelial cell invasion capacity, which seems to be enhanced when in coinfection with *Porphyromonas* and *Fusobacterium* (Pan et al., 2009), and may provide a mechanistic basis for the association between this microorganism and apical periodontitis. *Pseudomonas* is not a resident microorganism of oral ecosystems; however, it has been previously detected in post-treatment infections (Anderson et al., 2013; Sánchez-Sanhueza et al., 2018; Siqueira et al., 2016). Its presence in the samples suggests the occurrence of secondary infections, probably as a consequence of a breach in the aseptic workflow during the previous root canal treatment (Siqueira et al., 2016). Further investigations into the role of this bacterium as an external contaminant in post-treatment infections are needed.

Glucose metabolism was over-represented in AAP samples – including the pentose phosphate pathway, pyruvate metabolism and the glycolysis gluconeogenesis routes – possibly due to the increase of saccharolytic microorganisms such as *Streptococcus*, *Peptostreptococcus* and *Parvimonas*. Interestingly, *Pseudopropionibacterium* was significantly enriched in the lesions of asymptomatic samples as well as *Actinomyces*, with no significant differences in the latter. Both microorganisms have been associated with the development of asymptomatic extraradicular actinomycosis (Siqueira, 2003). In this study, *Pseudopropionibacterium* was found in 50% of the AAP samples (5/10) in abundances ranging from 0.02% to 4.34%, and it was always in coinfection with *Actinomyces*. In turn, *Actinomyces* was detected in 90% (9/10) of the asymptomatic cases and the abundances ranged from 0.02% to 32%. Although in different frequencies, these microorganisms have been detected previously in periapical lesions (Siqueira & Rôças, 2003; Sunde et al., 2002). Their ability to form clusters held together by an extracellular matrix can provide them with protection against immune cells and establish an equilibrium with the host, which would explain their presence in refractory apical periodontitis (Siqueira, 2003).

Despite the aseptic sampling procedures undertaken in this study, a main problem of working with surgical samples is the risk of contamination, especially with saliva. However, the proportion of microorganisms present in saliva is different from the one discovered in this study, meaning the probability of contamination would be low. Saliva presents a high abundance of *Veillonella*, *Prevotella* and *Neisseria*, which were less abundant in our samples. Instead, our samples were rich in *Fusobacterium* and *Porphyromonas*, genera demonstrated in small proportions in saliva (Costalonga & Herzberg, 2014). Periapical lesion samples might also be contaminated with microorganisms residing in the apical foramen that are dislodged during surgery and sampling procedures (Nair, 2004). Such contamination is difficult to control, and its significance requires further investigation. Notwithstanding, the higher alpha diversity observed in the lesions as compared with the root apices leads one to surmise that some periapical lesions could contain a proper microbiome (Sunde et al., 2003).

The results of the present study stress the importance of investigating the bacterial genes involved in disease to understand the microbial role in the progress of root

canal infection. Efforts to continue classifying the taxonomy of root canal bacteria may be limited if the functional profile of the microorganisms is not characterized. Still, some limitations need to be considered. First, NGS can detect free extracellular DNA and DNA from dead cells (Brundin et al., 2014, 2015; Klein et al., 2012; Siqueira & Rôças, 2005a, 2005b, 2022). Previous research showed that the half-life of free DNA in infected root canals is very short (Siqueira, 2008), although other authors report that this DNA can be preserved for months (Boutsioukis et al., 2022; Brundin et al., 2014, 2015). To overcome this problem, a metatranscriptomics approach would be necessary. Secondly, the symptomatology can be influenced by factors such as previous analgesic consumption and time of the infection (Read et al., 2014). In addition, SAP is established by percussion tenderness, and results can be recorded as present or absent. A more objective method, such as a bite force transducer, could provide for a better scale to grade the severity of SAP (Khan et al., 2007; Read et al., 2014). Another important limitation is the observational nature of the study, which precludes any strong conclusion about the degree of involvement of certain microorganisms and bacterial genes present in causation of the infection and the clinical signs (Siqueira & Rôças, 2013). Consequently, only an association with disease and signs can be inferred. Finally, 16 S rRNA gene sequencing gives no information about the bacterial viability and location of microorganisms in the samples, and does not consider the gene expression. Future research combining microscopy with molecular techniques, such as fluorescence *in situ* hybridization (FISH), shotgun metagenomic sequencing and metatranscriptomics, is required to determine the location of the microorganisms and their viability, and to validate the specific functional pathways involved in the microbial virulence of these infections.

CONCLUSIONS

The bacterial community studied was similar in the apices and periapical lesions. The microbiome was different in symptomatic and asymptomatic cases; gram-negative bacteria being present in higher relative abundances in symptomatic cases. An association was observed between amino acid degradation and flagellum assembly pathways, and the development of tenderness to percussion or palpation.

AUTHOR CONTRIBUTIONS

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

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CONFLICT OF INTEREST

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 number PRJNA839210. Authors can confirm that all relevant data are included in the article and/or its supplementary information files.

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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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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