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BACTERIOLOGICAL CONDITIONS IN THE APICAL ROOT CANAL SYSTEM OF TEETH WITH AND WITHOUT POST-TREATMENT APICAL PERIODONTITIS: A MULTI-ANALYTICAL APPROACH

THESIS

SANDRA MILENA HERNÁNDEZ RINCÓN

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SANDRA MILENA HERNÁNDEZ RINCÓN

Thesis submitted to the Post-Graduation Program in Dentistry, of the Universidade do Grande Rio (UNIGRANRIO), as part of the requirements to obtain the degree of Doctor in Dentistry with emphasis in Endodontics.

Supervisors:

José Freitas Siqueira Jr. DDS, MSc, PhD
Isabela das Neves Rôças, DDS, MSc, PhD

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Approved on XXX XX, 2023

Committee Members

_____

Prof(a). Dr(a). Name of the Professor
Universidade do Grande Rio

_____

Prof(a). Dr(a). Name of the Professor
Universidade XXXX

_____

Prof(a). Dr(a). Name of the Professor
Universidade XXXX

_____

Prof(a). Dr(a). Name of the Professor
Universidade XXXX

This thesis is dedicated to my precious family,

Roy, Valeria, and Isabelle.

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ABSTRACT

Objectives: This study used a correlative multi-analytical approach to investigate the bacteriological conditions in the apical root canal system of treated teeth with or without apical periodontitis, and their correlation with the technical quality of the previous root canal obturation and the presence and volume of apical periodontitis lesion.

Materials and methods: Apical root fragments of extracted endodontically treated teeth with (n=23) and without (n=22) apical periodontitis were collected, as demonstrated by cone-beam computed tomography (CBCT). The root apexes were sectioned and subjected to micro-computed tomography (micro-CT) scanning. The specimens were cryopulverized and DNA extracted from the powder was used as template in real-time polymerase chain reaction (qPCR) assays to quantify total bacteria and members of the *Streptococcus* genus and Actinobacteria phylum. The bacteriological findings were compared between the two groups and also evaluated for associations with CBCT and micro-CT data. Additionally, parameters such as the apical limit, quality of the obturation, coronal restoration, and intraradicular post were examined in CBCT and micro-CT.

Results: Bacteria were detected in all apical canal samples, except one. The mean total counts of bacteria, Streptococci and Actinobacteria did not differ significantly between teeth with apical periodontitis (9.88×10^3) from teeth with no lesions (9.41×10^3). The lesion volume of the 23 root fragments with apical periodontitis was determined to be small for 16, and large for 7. Small apical periodontitis lesions presented higher counts of *Streptococcus* compared to apical fragments with no lesion and large lesion. Large lesions presented a total count of bacteria of 8.21×10^3; however, there were not significant differences compared to small lesions. The limit of filling >2 mm short was significantly associated with more total bacterial counts compared with canals filled 0 to 2 mm short ($p<0.05$). Adequate coronal restoration

and intraradicular post were significantly associated with 85% lesser counts of *Streptococcus.*

Conclusions: Comparable bacterial loads were observed in the apical canal system of treated teeth with and without apical periodontitis, suggesting that factors other than only the total bacterial levels may also influence the development and progression of apical periodontitis. Bacteria were found in the apical canal in virtually all cases, with a high prevalence of streptococci and actinobacteria. Streptococci counts were significantly higher in the apical canal of teeth with inadequate restorations and teeth with no lesions. Underfilled canals showed higher bacterial counts.

KEY WORDS: root canal infection, post-treatment apical periodontitis, residual intrarradicular bacteria.

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LIST OF ABBREVIATIONS

CBCT	Cone-Beam Computed Tomography
CBCTPAVI	Cone-beam Computed Tomography Periapical Volume Index
CFU	Colony-Forming Units
CHX	Chlorhexidine gluconate
CLSM	Confocal Laser Scanning Microscopy
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
FISH	Fluorescence *In Situ* Hybridization
HTS	High-Throughput Sequencing
Micro-CT	Computed microtomography
(MALDI-TOF)	Matrix-Assisted Laser Desorption Ionization-Time Off Light
MS	Mass spectrometry
NaOCl	Sodium Hypochlorite
NGS	Next Generation Sequencing
PAI	Periapical Index
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid
T-RFLP	Terminal - Restriction Fragment Length Polymorphism

1. INTRODUCTION AND LITERATURE REVIEW

1.1. Apical periodontitis. Generalities

Apical periodontitis is an inflammatory disease caused by opportunistic pathogens that colonize the root canal system, which manifests with a localized immune response, that can result in hard tissue resorption or destruction of periradicular tissues at different histopathological stages (SUNDQVIST, 1976; NAIR, 2004; SIQUEIRA *et al.*, 2011). It usually develops mainly as a sequel to dental caries; however, other factors such as trauma, periodontal infection, iatrogenesis, or dental development anomalies such as dens evaginatus or dens invaginatus can cause infection or damage to the pulp tissue (SIQUEIRA & RÔÇAS, 2009b).

Once inflammation of the pulp tissue begins due to the entry of microorganisms, necrosis occurs, and the infectious process is established. The host's immune system is unable to control the bacterial aggression expressed in number and virulence factors, establishing a chronic inflammation. This response can develop and expand without manifesting apparent symptoms. The slow progression of the infection could cause a widening of the periodontal ligament that may be detected radiographically as a consequence of initial bone resorption. The bone resorbs and is replaced by granulomatous tissue containing defense cells (lymphocytes, plasma cells, macrophages, neutrophils, and repair process components such as fibroblasts and newly formed nerves and blood vessels) (SIQUEIRA & RÔÇAS, 2022c). The bone resorption process provides space for a large number of defense cells in the area adjacent to the apical foramen, which prevents the infection from spreading to the periradicular tissues (TORABINEJAD *et al.*, 1985; NAIR, 1987; SIQUEIRA & RÔÇAS, 2022c). At the periphery of this granulomatous tissue, collagen fibers are deposited to encapsulate the lesion. This condition is histopathologically known as an apical

granuloma. As the lesion progresses, bone resorption increases until it manifests radiographically as a radiolucent area around the apical portion of the tooth. Malassez epithelial remnants can be activated by the chronic inflammatory response (bacteria and host factors) as a periodontal ligament response. The agglomeration of epithelial cells forms a cavity lined by stratified squamous epithelium known as an apical cyst (SIQUEIRA & RÔÇAS, 2022c).

Microorganisms such as fungi (PECIULIENE *et al.*, 2001a), archaea (SIQUEIRA *et al.*, 2005), and viruses (GLICK *et al.*, 1989) have been detected intraradicularly. However, a large number of studies confirm that the main etiological agent of apical periodontitis is the bacteria and their products, which favor the development of pulpal necrosis and, consequently, apical periodontitis (KAKEHASHI *et al.*, 1965; MÖLLER *et al.*, 1981)

Bacterial species are organized in biofilms and establish themselves intraradicularly in areas of the dentin walls or extraradicularly, in the apical portion, which enables them to obtain nutrients from the periradicular tissues affected by the infectious process (RICUCCI & SIQUEIRA, 2010).

Intraradicular endodontic infections can be either primary, secondary, or persistent. Primary infection is characterized by an initial invasion and microbial colonization of the pulp tissue. The secondary infection is caused by microorganisms not present during the primary infection and entered the root canal system after professional intervention. Finally, organisms from both primary and secondary infections that resist or are inaccessible to endodontic disinfection methods can survive periods of nutrient deprivation that cause persistent infections. Post-treatment apical periodontitis is caused by secondary and persistent infections that are difficult to distinguish clinically (SIQUEIRA & RÔÇAS, 2022a).

1.2. Post-treatment apical periodontitis

Post-treatment apical periodontitis is an inflammation of the periradicular tissues in a previously treated tooth that is caused by secondary and persistent endodontic infections that occur mainly intraradicularly or, in a smaller percentage (6%) of cases, extraradicularly (NAIR *et al.*, 1990; RICUCCI & SIQUEIRA, 2008; RICUCCI *et al.*, 2009; HAAPASALO *et al.*, 2011; SIQUEIRA *et al.*, 2020).

The diagnosis of post-treatment apical periodontitis is based on clinical examinations for resolution of signs/symptoms, radiographic evaluations (panoramic, periapical, and cone-beam computed tomography), and histopathology of specimens obtained during periapical surgery (CHUGAL *et al.*, 2003; TIBÚRCIO-MACHADO *et al.*, 2021).

Generally, it is asymptomatic, but when it manifests, the signs and symptoms are related to the persistence or recurrence of the infection in endodontically treated teeth with inadequate fillings, or poor disinfection of the root canals (SJÖGREN *et al.*, 1990). The clinical manifestations include persistent sinus tract, persistent symptoms (inflammation, and pain/discomfort upon percussion/palpation), interappointment flare-ups, and endodontic failure. Radiographically, it is revealed with a lateral, periapical, or periradicular radiolucency (MÖLLER *et al.*, 1981; HAAPASALO *et al.*, 2011; YU *et al.*, 2012; SIQUEIRA & RÔÇAS, 2022c).

The prevalence of post-treatment apical periodontitis has been reported in 5% to 10% of endodontically treated teeth with apical lesions present prior to treatment and, even in root canal treatments that were carried out with strict disinfection protocols (NAIR *et al.*, 1990; SIQUEIRA *et al.*, 2008). It has been demonstrated that post-treatment endodontic infections are more prevalent in teeth that presented apical periodontitis prior to the initial treatment (SJÖGREN *et al.*, 1990; SUNDQVIST *et al.*, 1998; RICUCCI *et al.*, 2011). On the other hand, cross-sectional studies conducted in

different countries revealed a relatively high prevalence (HÜLSMANN, 2016). Recently, a meta-analysis showed that endodontically treated teeth had a higher prevalence of post-treatment apical periodontitis (39%) than untreated teeth (3%) (TIBÚRCIO-MACHADO *et al.*, 2021)

Post-treatment apical periodontitis has been categorized as emergent, persistent, and recurrent. Emergent infections are those that develop after the endodontic treatment has been completed, persistent infections are those that resist the disinfection methods used during the endodontic treatment, and recurrent infections appear after a periapical or periradicular lesion has been repaired (SIQUEIRA *et al.*, 2014).

The treatment consists of a significant reduction of intraradicular and extraradicular microorganisms or prevention of reinfection in order to reestablish periradicular health and maintain the functional tooth in the oral cavity. In most cases, non-surgical endodontic retreatment is the first treatment option (MOLANDER *et al.*, 1998; HAAPASALO *et al.*, 2003). However, these cases are challenging since they need an optimized disinfection of the root canal system that allows a significant reduction of residual bacteria (SIQUEIRA *et al.*, 2014). Periapical surgery (HAAPASALO *et al.*, 2011; SIGNORETTI *et al.*, 2011) or intentional reimplantation (CORIA-VALDIOSERA *et al.*, 2020) are the treatment options indicated in those cases in which it was not possible to achieve the elimination of microorganisms via orthograde or in the presence of an extraradicular infection. Control of intraradicular or extraradicular infection is essential for the repair of periradicular tissues, but it also depends on the health and proper function of the host's immune system (LIN & ROSENBERG, 2011; SEGURA-EGEA *et al.*, 2022).

The initial condition of the dental pulp and periradicular tissues is directly related to the prognosis of post-treatment endodontic infection (SJÖGREN *et al.*, 1990; NG *et al.*, 2011; CURTIS *et al.*, 2018). Similarly, the prognosis is highly dependent on the

successful eradication of infection from the root canal system before obturation. On the other hand, the entry of microorganisms or their persistence during obturation indicates an elevated possibility of endodontic failure (SJÖGREN *et al.*, 1997).

In adequately treated teeth with post-treatment apical periodontitis, retreatment presents a success rate between 62%-88% (STRINDBERG, 1956; SJÖGREN *et al.*, 1990; ØRSTAVIK, 1996; SUNDQVIST *et al.*, 1998; FARZANEH *et al.*, 2004; IMURA *et al.*, 2007; DE CHEVIGNY *et al.*, 2008; KUNIN *et al.*, 2010; NG *et al.*, 2011; RICUCCI *et al.*, 2011).

A systematic review compared clinically and radiographically the success of endodontic retreatment and apical surgery. Apical surgery presented a better prognosis between 2 - 4 years (77.8%) compared to retreatment (70.9%). The results at 4 - 6 years showed that the retreatment presented a higher percentage of success (83%) compared in the same period of time with the apical surgeries (71.8%). However, the prognosis of the apical surgeries decreased at 6 years (62.9%). The authors concluded that apical surgery has a favorable initial prognosis that may decline over time. In contrast, retreatment offers a favorable long-term prognosis (TORABINEJAD *et al.*, 2009).

Tomographic evaluations (CBCT) of apical microsurgeries showed a greater reduction of apical periodontitis lesions (97.9%) compared to what was observed in retreatments (88.1%). None of the cases revealed full reparation throughout an approximate 22-months follow-up (CURTIS *et al.*, 2018).

The progression of persistent post-treatment lesions was evaluated clinically and radiographically in patients with at least 4 years of completion of root canal treatment. This study suggested the need to consider various criteria to determine a deteriorating progression of the lesion, including lesions >5 mm in diameter present for

more than 10 years, clinical indications such as pain when biting, sinuous tract, and/or a history of post-obturation flare-up (YU *et al.*, 2012).

The estimated time to achieve a complete repair of the periradicular tissues after retreatment is approximately 4 years (ØRSTAVIK, 1996; SIQUEIRA *et al.*, 2008; ZANDI *et al.*, 2019). In cases in which a complete repair of the periapical/periradicular lesions did not occur, the teeth remained asymptomatic and functional in 94% of them (DE CHEVIGNY *et al.*, 2008). A delayed repair can occur in 50% of the teeth between 10 and 17 years (FRISTAD *et al.*, 2004).

1.2.1. Post-treatment apical periodontitis: etiology and associated factors

1.2.1.1. Microbial factors

In endodontic infections, more than 500 bacterial species have been identified; however, only 20 to 30 are frequently detected (SIQUEIRA & RÔÇAS, 2022b). The main cause of post-treatment infections is bacterial persistence in the root canal system (NAIR *et al.*, 1990; LIN *et al.*, 1992; SIQUEIRA & RÔÇAS, 2008) or in some cases, it is caused by secondary endodontic infections (SIQUEIRA & RÔÇAS, 2008; SIQUEIRA *et al.*, 2014). This condition is also associated with extraradicular infections less often (6%) (RICUCCI & SIQUEIRA, 2010).

Microbiological culture studies (SUNDQVIST *et al.*, 1998; PINHEIRO *et al.*, 2003), microscopy studies (NAIR *et al.*, 1990; RICUCCI *et al.*, 2009), and numerous molecular techniques (RÔÇAS *et al.*, 2004a; SIQUEIRA & RÔÇAS, 2004) have shown that intraradicular infection is found in the majority of root canal treatments with poor fillings that did not present an adequate conformation and/or disinfection of the root canals, and even in adequately treated teeth (SJÖGREN *et al.*, 1990; SIQUEIRA,

2001). Similarly, extraradicular bacterial communities may grow as biofilm (TRONSTAD *et al.*, 1990) or as antimycotic colonies (HAPPONEN, 1986).

Bacterial penetration and persistence within the canal determine the secondary or persistent post-treatment infection type (SIQUEIRA & RÔÇAS, 2008; SIQUEIRA, 2011). In persistent post-treatment infections, bacteria are not effectively controlled or eradicated during the initial endodontic intervention. In contrast, bacterial introduction occurs during root canal therapy in secondary infections due to a break in the septic chain, minimally invasive accesses, missed canals, insufficient instrumentation, and/or inadequate post-endodontic treatment restoration (NAIR *et al.*, 1990; SIQUEIRA & RÔÇAS, 2008; VIEIRA *et al.*, 2020).

The microbiota of persistent endodontic infections is organized into complex entities known as biofilms (SIQUEIRA *et al.*, 2012). They are highly organized microbial communities that produce and agglomerate within an extracellular matrix of polymeric substances that adhere to a surface or substrate (FLEMMING *et al.*, 2016). The extracellular matrix enables the capture of nutrients and promotes metabolic cooperation between bacteria of the same or distinct species (SIQUEIRA *et al.*, 2012; NEELAKANTAN *et al.*, 2017).

The morphological structure of the biofilm differs between individuals. There is not a singular pattern for endodontic infections because its composition depends on the existing microorganisms and the available nutrients (RICUCCI & SIQUEIRA, 2010).

Microorganisms within the biofilm are more resistant than planktonic bacteria to antimicrobial agents and host defense response mechanisms (COSTERTON & STEWART, 2001). In addition, the biofilm provides an environment that allows microbial cells to undergo mutation, survive, and persist (NEELAKANTAN *et al.*, 2017).

The prevalence of biofilms in canals of treated and untreated teeth with apical periodontitis and their clinical, radiographic, and histopathological association was evaluated for the first time by RICUCCI & SIQUEIRA (2010). Biofilms were observed intraradicularly in 74% of treated and 80% of untreated canals. In small apical periodontitis lesions, they were detected in 62%, and in extensive lesions in 82%. The highest amount of biofilm was observed in cysts (95%), abscesses (83%), and granulomas (69.5%). These findings indicate biofilms are more likely to be associated with persistent pathologic processes, such as large lesions and cysts (RICUCCI & SIQUEIRA, 2010).

The persistence of intraradicular infection generally occurs due to the resistance of residual bacteria or due to the inaccessibility of instruments, irrigants, or medications in anatomical complexities such as lateral canals (RICUCCI *et al.*, 2013), apical ramifications and isthmuses (RICUCCI & SIQUEIRA, 2010) and dentinal tubules (VIEIRA *et al.*, 2012). Morphological studies have demonstrated that the apical segment of the root is compromised in most teeth with post-treatment endodontic infection, as it induces and maintains periradicular inflammation (RICUCCI *et al.*, 2009; ARNOLD *et al.*, 2013; RICUCCI *et al.*, 2013).

The proliferation and invasion of intracanal bacteria, mainly towards the periradicular tissues, occurs through the apical foramen. The host's immune system controls the spread of infection in alveolar bone; however, in some circumstances, bacteria overcome inflammatory barriers and establish themselves in periradicular tissues (SIQUEIRA *et al.*, 2014).

Extraradicular infection is characterized by the formation of biofilm on the cementum surrounding the apical surface or around the apical foramina (PITT FORD, 1982; TRONSTAD *et al.*, 1990; RICUCCI *et al.*, 2013), that can present as an apical calculus (ANDERSON *et al.*, 2013; RICUCCI *et al.*, 2013) or as antimycotic colonies

within the body of the lesion (HAPPONEN, 1986). It is usually associated with chronic inflammatory processes related to endodontic failure and, rarely, in acute processes (RICUCCI *et al.*, 2009; SIQUEIRA *et al.*, 2014). Most cases are asymptomatic and associated with chronic apical abscesses (SIQUEIRA & RÔÇAS, 2022b). It is dependent on intracanal infection due to the continuous, direct interaction of microorganisms in the form of planktonic cells, flocs, or biofilms that extend to the periradicular tissues, which are a source of nutrients for the bacteria (RICUCCI *et al.*, 2013; SIQUEIRA *et al.*, 2014).

Histological evaluations of 24 teeth with post-treatment apical periodontitis (12 symptomatic and 12 asymptomatic) confirmed the dependence between intraradicular and extraradicular infection. Bacteria were observed intraradicularly in all cases, except in one case of vital pulp that later presented emergent infection, probably associated with a foreign body reaction due to extruded material. Extraradicularly, bacteria were observed in one asymptomatic case and 4 cases of symptomatic teeth (RICUCCI *et al.*, 2009). Some authors consider that adequate root canal treatments that fail are associated with extraradicular infection. It is probably due to biofilms adhering to the apical portion or periapical actinomycosis, in which disinfection techniques and antibiotics have no effect (SIQUEIRA, 2003; TRONSTAD & SUNDE, 2003).

1.2.1.2. Risk factors associated with the etiology of endodontic failure

Endogenous non-microbial factors (cholesterol crystals and true cysts) (NAIR, 1999; NAIR, 2004) and exogenous factors, such as the extrusion of filling materials, cellulose fibers from paper points, or remnants of cotton and vegetable-based food, have been proposed as possible causes for endodontic failure (SIMON *et al.*, 1982; KOPPANG *et al.*, 1989). Nevertheless, histobacteriological studies and advanced

molecular biology techniques have shown that post-treatment apical periodontitis is caused by intraradicular and extraradicular bacterial infections (SIQUEIRA & RÔÇAS, 2004).

In the past, overfillings were attributed to endodontic failure due to the apparent toxicity of filling materials (MURUZÁBAL & ERAUSQUIN, 1966). Nowadays, most of the materials used in the obturation of root canals are biocompatible (HAUMAN & LOVE, 2003; GIACOMINO *et al.*, 2019). The relationship that exists between overfillings and endodontic failure is due to the extrusion of infected smear layer as a result of overinstrumentation that precedes overfilling or inadequate apical sealing that favors the nutrient supply of residual bacteria (SIQUEIRA, 2011). Therefore, neither overinstrumentation and/or overfilling are considered a direct cause of endodontic treatment failure (BERGENHOLTZ *et al.*, 1979; LIN *et al.*, 1992; SJÖGREN *et al.*, 1997).

The unintentional extrusion of root canal sealer was evaluated in a retrospective study. Teeth with apical periodontitis revealed 79% of repair at a 4-year follow-up, and those without apical periodontitis showed 100% of repair. This study demonstrated that the sealer's extrusion does not compromise endodontic therapy's prognosis (RICUCCI *et al.*, 2016). The extrusion of filling materials into the periradicular tissues causes a localized inflammatory process and delays the repair (GREEN *et al.*, 1997; SELTZER, 1999).

Regarding the relationship between the true cyst and endodontic failure, one study evaluated the clinical, radiographic, histopathological, and histobacteriological characteristics of true and bay cysts. Biopsies (95) of apical lesions attached to the apex of endodontically treated and untreated teeth were examined. Bacteria were observed in the cavity of true (36%), and bay cysts (50%). Extraradicular biofilms were found in a few specimens. All the cysts evaluated presented an association with

intraradicular infection and, in some cases, with extraradicular infection. They only differ from the morphologic relationship of the cavity to the root canal. These results allow us to infer that true cysts are of infectious origin and are not self-sustaining (RICUCCI *et al.*, 2020).

The risk factors that can compromise endodontic therapy are preoperative, intraoperative, and/or postoperative. Preoperative risk factors are the anatomical complexity of the root canal system, obliteration of the canals due to hypermineralization and root resorptions (CHUGAL & LIN, 2017), the quality of the previous treatment and the size of the periapical lesion (STRINDBERG, 1956; NG *et al.*, 2011).

Among the intraoperative factors that can affect the prognosis of endodontic treatment are procedural errors (overfilling, underfilling, separation of instruments, and perforations) (RODRIGUES *et al.*, 2017), missed canals (COSTA *et al.*, 2019), non-instrumented canals (PÉREZ *et al.*, 2020), inadequate disinfection (SIQUEIRA *et al.*, 2002) and an inadequate quality of the obturation (GILLEN *et al.*, 2011; CRAVEIRO *et al.*, 2015).

The main postoperative factor that influences the success of endodontic treatment is the placement time and the coronal restoration quality. A permanent coronal restoration with adequate adaptation is necessary for the prevention of reinfection in a treated or retreated tooth (CHUGAL *et al.*, 2007; GILLEN *et al.*, 2011; RICUCCI & SIQUEIRA, 2011; PRATT *et al.*, 2016). An intraradicular post is sometimes required for the restoration of endodontically treated tooth that needs to retain coronal build-up or to reinforce the tooth with an extensive loss of tissue (TANG *et al.*, 2010). Abutments could be used for fixed and removable prostheses, which are teeth that are exposed to large horizontal and torquing forces during function (GOGA & PURTON, 2007). The post helps preserve tooth structure when endodontically treated teeth are

used as abutments for fixed partial dentures and subjected to loads (AKMAN *et al.*, 2012). The composition of the restorative materials does not influence on the condition of the periapical lesion (DAWSON *et al.*, 2016).

In secondary endodontic infections, coronal leakage is the main factor that leads to post-treatment apical periodontitis (SIQUEIRA & RÔÇAS, 2008). Nevertheless, several studies have demonstrated that instrumentation and adequate filling of the root canal can effectively prevent the entry of microorganisms even during extended periods of exposure to the filling material (RICUCCI & BERGENHOLTZ, 2003). Although most teeth with post-treatment endodontic infection rarely present bacteria in the coronal portion of the tooth, this is not a reason to underestimate the importance of coronal sealing (RICUCCI *et al.*, 2009). A study revealed that the main cause of extraction of endodontically treated teeth is prosthetic reasons due to loss of tooth structure (OLCAY *et al.*, 2018).

1.2.2. Microbiota associated with post-treatment endodontic infections

Post-treatment apical periodontitis exhibits a polymicrobial nature, as is attributed to the presence of several bacterial communities that differ in composition among individuals (SIQUEIRA *et al.*, 2004a; DE CASTRO KRULY *et al.*, 2022).

Bacteria in secondary or persistent infections have been identified by culture (ENGSTRÖM, 1964; MÖLLER, 1966; HASHIOKA *et al.*, 1992; MOLANDER *et al.*, 1998; SUNDQVIST *et al.*, 1998; PECIULIENE *et al.*, 2000; CHEUNG & HO, 2001; HANCOCK *et al.*, 2001; PECIULIENE *et al.*, 2001a; PINHEIRO *et al.*, 2003; GOMES *et al.*, 2004; SCHIRRMEISTER *et al.*, 2007; SCHIRRMEISTER *et al.*, 2009; GOMES *et al.*, 2021) or by molecular methods such as Polymerase Chain Reaction (PCR) (ROLPH *et al.*, 2001; RÔÇAS *et al.*, 2004a; RÔÇAS *et al.*, 2004; SIQUEIRA *et al.*,

2004; SIQUEIRA & RÔÇAS, 2004; FOUAD *et al.*, 2005; KAUFMAN *et al.*, 2005; SIQUEIRA & RÔÇAS, 2005b; SEDGLEY *et al.*, 2006; ZOLETTI *et al.*, 2006; SCHIRRMEISTER *et al.*, 2007; BLOME *et al.*, 2008; GOMES *et al.*, 2008; RÔÇAS *et al.*, 2008; SUBRAMANIAN & MICKEL, 2009; ANDERSON *et al.*, 2012; ENDO *et al.*, 2012; TENNERT *et al.*, 2014; ANTUNES *et al.*, 2015; BARBOSA-RIBEIRO *et al.*, 2020; BARBOSA-RIBEIRO *et al.*, 2021; GOMES *et al.*, 2021; SUN *et al.*, 2021), Denaturing Gradient Gel Electrophoresis (DGGE) (SIQUEIRA *et al.*, 2004a), checkerboard hybridization (GATTI *et al.*, 2000; SUNDE *et al.*, 2000; RÔÇAS & SIQUEIRA, 2012), Fluorescence *In Situ* Hybridization (FISH) with epifluorescence (SUNDE *et al.*, 2003) and 16S rRNA bacteria gene sequencing / pyrosequencing (SCHIRRMEISTER *et al.*, 2009; CHUGAL *et al.*, 2011; SABER *et al.*, 2012; ANDERSON *et al.*, 2013; HONG *et al.*, 2013; TZANETAKIS *et al.*, 2015; SIQUEIRA *et al.*, 2016; KESKIN *et al.*, 2017; BOUILLAGUET *et al.*, 2018; ZANDI *et al.*, 2019; DE CASTRO KRULY *et al.*, 2022; PÉREZ-CARRASCO *et al.*, 2023). The findings of several studies identifying the microbiota of post-treatment endodontic infections are presented in Table 1.

Table 1. Bacterial species found in endodontically treated teeth with post-treatment apical periodontitis.

Author(s)-Year	Title	N	Method of obtaining samples	Sample analysis method	Most prevalent bacterial phyla or species (%) and bacterial counts
Engström 1964,	The significance of enterococci in root canal treatment			Culture	*Enterococcus faecalis* (24)
Möller 1966	Microbial examination of root canals and periapical tissues of human teeth			Culture	*Enterococcus faecalis* (29) and *Candida albicans* (3)
Hashioka et al., 1992	The relationship between clinical symptoms and anaerobic bacteria from infected root canals.	28	Paper points (root canal)	Culture	**Percussion pain:** *Peptococcus* spp.: (*Peptococcus niger*, *Peptococcus magnus, Peptococcus saccharolyticus*), *Peptostreptococcus*, *Eubacteria*, *Porphyromonas* gingivalis, *Porhyromanas endodontalis* and *Bacteroides*. **Related to odor:** *Peptococcus*, *Peptostreptococcus*, *Eubacterium* e *Bacteroides*.
Sundqvist et al., 1998	Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment	54	Charcoaled paper points (root canal)	Culture (anaerobes)	Gram-positive anaerobes predominated. *Enterococcus faecalis* (38) and *Candida albicans* (8)
Molander et al., 1998	Microbiological status of root-filled teeth with apical periodontitis	100	Charcoaled paper points (root canal)	Culture	Gram-positive facultative anaerobes (69). *Enterococcus faecalis* (32)
Peciuliene et al., 2000	Isolation of *Enterococcus faecalis* in previously root-filled canals in a Lithuanian population	25	Paper points (root canal)	Culture	*Enterococcus faecalis* (70)
Gatti et al., 2000	Bacteria of asymptomatic periradicular endodontic lesions identified by DNA-DNA hybridization	36	Paper points (root canal)	DNA-DNA hybridization	*Bacteroides forsythus* (100) and *Actinomyces naeslundii* (100)
Sunde et al., 2000	Assessment of periradicular microbiota by DNA-DNA hybridization	34	Lesions obtained from periapical surgeries	DNA-DNA hybridization	*Actinobacillus actinomycetemcomitans* (>60%), *Bacteroides forsythus* (>60%). *Porphyromonas endodontalis* (94)
Peciuliene et al., 2001	Isolation of yeast and enteric bacteria in root-filled teeth with chronic apical periodontitis	33	Paper points (root canal)	Culture	*Enterococcus faecalis* (64) and *Candida albicans* (18)
Hancock et al., 2001	Bacteria isolated after unsuccessful endodontic treatment in a North American population	54	Paper points (root canal)	Culture	*Enterococcus faecalis* (30) and *Candida albicans* (3)
Cheung & Ho 2001	Microbial flora of root canal-treated teeth associated with asymptomatic periapical radiolucent lesions	18	Paper points (root canal)	Culture	*Pseudomonas aeruginosa, Pseudomonas* spp., *Streptococcus mutans, Streptococcus mitis, Streptococcus constellatus, Coagulase-negative staphylococci, Gemella morbillorum, Propionibacterium propionicus, Neisseria* spp., *Enterobacter cloacae, Acinetobacter calcoaceticus, Klebsiella oxytoca, Proteus mirabilis, Serratia* spp., *Peptostreptococcus prevotii, Peptostreptococcus asaccharolyticus, Peptostreptococcus* spp., *Eubacterium lentum*, *Veillonella* spp., *Campylobacter* spp., *Porphyromonas asaccharolytica* and *Candida albicans*
Rolph et al., 2001	Molecular identification of microorganisms from endodontic infections	26 Refractory cases	Paper points (root canal)	-Culture -PCR	*Capnocytophaga, Cytophaga, Dialister, Eubacterium, Fusobacterium, Gemella, Mogibacterium, Peptostreptococcus, Prevotella, Propionibacterium, Selenomonas, Solobacterium, Streptococcus, Veionella* and *Firmicutes*
Pinheiro et al., 2003	Microorganisms from canals of root-filled teeth with periapical lesion	60	Paper points (root canal)	Culture	*Enterococcus faecalis* (53), *Enterococcus faecium* (2), *Streptococcus costellatus* (8), *Streptococcus sanguis* (7), *Streptococcus mitis* (3), *Streptococcus anginosus* (3), *Streptococcus mutans*

					(2), *Streptococcus oralis* (2), *Streptococcus salivarius* (3), *Peptostreptococcus prevotii* (10), *Peptostreptococcus micros* (8), *Peptostreptococcus magnus* (2), *Peptostreptococcus saccharolyticus* (2), *Actynomices naeslundii* (7), *Actynomices odontolyticus* (5), *Actynomices viscosus* (5), *Prevotella buccae* (5), *Prevotella intermedia/nigrescens* (5), *Prevotella melaninogenica* (2), *Prevotella corporis* (3), *Prevotella loescheii* (2), *Propionibacterium acnes* (7), *Propionibacterium propionicum* (2), *Gemella morbillorum* (7), *Veillonella* spp.(7), *Fusobacterium necrophorum* (2), *Fusobacterium nucleatum (2), Fusobacterium spp. (2), Lactobacillus acidophilus* (3), *Staphylococcus lentus* (3), *Haemophilus aphrophilus* (2), *Eubacterium lentum* (2), *Bifidobacterium spp.* (2), *Clostridium subterminale* (2), *Lactococcus lactis cremoris* (2), *Capnocytophaga* spp. *(2)* and *Candida albicans* (3)
Sunde et al., 2003	Fluorescence in situ hybridization (FISH) for direct visualization of bacteria in periapical lesions of asymptomatic root-filled teeth	39	Lesions obtained from periapical surgeries	Fluorescence *In Situ* Hybridization (FISH) with epifluorescence and Confocal Laser Scanning Microscopy (CLSM)	*Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythensis* and *Treponemes* spp. *Streptococcus* spp.
Siqueira & Rôças, 2004	Polymerase chain reaction-based analysis of microorganisms associated with failed endodontic treatment	22	Paper points (root canal)	PCR	*Enterococcus faecalis* (77), *Pseudoramibacter alactolyticus* (52), *Propionibacterium propionicum* (52), *Dialister pneumosintes* (48), *Filifactor alocis* (48) and *Candida albicans* (9)
Rôças et al., 2004a	Association of *Enterococcus faecalis* with different forms of periradicular diseases	30 Cases of root-filled teeth	Paper points (root canal)	PCR	*Enterococcus faecalis* (67)
Rôças et al., 2004b	Polymerase chain reaction identification of microorganisms in previously root-filled teeth in South Korean Population	14	Paper points (root canal)	PCR	*Enterococcus faecalis* (64), *Streptoccus* spp. (21 and *Tannerella forsythensis* (14)
Gomes et al, 2004	Microbiological examination of infected dental root canals	19	Paper points (root canal)	Culture	*Peptostreptococcus spp.* (59), *Streptococcus* spp. (53), *Fusobacterium* (33), *Prevotella* spp. (32), *Enterococcus* spp. (13), *Gemella* spp. *(13), Staphylococcus* spp. (13), *Actinomyces* spp. (12), *Porphyromonas* spp. (12), *Lactobacillus* spp. (8), *Propionibacterium* spp. (7) e *Bacterorides* spp. (5)
Siqueira & Rôças, 2005	Uncultivated phylotypes and newly named species associated with primary and persistent endodontic infections	22 Root-filled teeth	Paper points (root canal)	16S rRNA gene-based nested or heminested PCR	*Dialister invisus* (14), *Synergistes oral clone BA*121 (4.5), *Olsenella uli* (4.5)
Fouad et al., 2005	Molecular detection of Enterococcus spp. in root canals of therapy-resistant endodontic infections	37	Paper points (root canal)	PCR	*Enterococcus* spp. (22)
Kaufman et al., 2005	Enterococcus spp. in endodontically treated teeth with and without perirradicular lesions	36	Paper points (root canal)	PCR	*Enterococcus* spp. (12)
Zoletti et al., 2006	Identification of Enterococcus faecalis in root-filled teeth with or without perirradicular lesions by culture dependent and independent approaches	27 Root-filled teeth with no periradicular lesion 23 Root-filled teeth with periradicular lesion	Paper points (root canal)	PCR	Root-filled teeth with no periradicular lesion: *Enterococcus faecalis* (81.5) Root-filled teeth with periradicular lesion: *Enterococcus faecalis* (78)
Sedgley et al., 2006	Real-time quantitative polymerase chain reaction and culture analyses of Enterococcus faecalis in root canals	48	Paper points (root canal)	- Culture - PCR	Culture: *Enterococcus faecalis* (8) PCR: *Enterococcus faecalis* (90)

Schirrmeister et al., 2007	Detection and eradication of microorganisms in root-filled teeth associated with perirradicular lesions: an in vivo study	20	Paper points (root canal)	- Culture - PCR	*Enterococcus faecalis* (31)
Gomes et al., 2008	Microbial analysis of canals of root-filled teeth with apical periodontitis using polymerase chain reaction before and after chemo-mechanical preparation	45	Paper points (root canal)	PCR	Gram-positives: *Enterococcus faecalis* (78), *Peptostreptococcus micros* (51), *Porphyromonas gingivalis* (36), *Porphyromonas endodontalis* (22), *Prevotella intermedia* (11), *Prevotella nigrescens* (11), *Filifactor alocis* (27), *Treponema denticola* (24), *Tanerella forsythia* (4)
Rôças et al., 2008	Microorganisms in root canal-treated teeth from a German population	17	Paper points (root canal)	Reverse -capture checkerboard hybridization assay	PCR: *Enterococcus faecalis* (47) *Candida albicans* (6) Checkerboard: *Enterococcus faecalis* (23.5). *Streptococcus* spp. (47), *Lactobacillus* spp. (35)., *Dialister invisus* (29), *Eubacterium infirmun* (29), *Prevotella intermedia* (29), *Selomonas sputigena* (29), *Synergistes* oral clone BA121 (29) and *Treponema denticola* (2)
Blome et al., 2008	Molecular identification and quantification of bacteria from endodontic infection using real-time polymerase chain reaction	20	Paper points (root canal)	PCR	*Peptostreptococcus micros* (45%), *Porphyromonas endodontalis* (20%), *Treponema denticola* (10%), *Tannerella forsythia* (20%), Fusobacterium *nucleatum* (20%), *Porphyromonas gingivales* (15%), *Prevotella intermedia* (5%) and *Enterococcus faecalis* (10%)
Subramanian, 2009	Molecular analysis of persistent periradicular lesions and root ends reveals a diverse microbial profile	34	Lesions obtained from periapical surgeries	PCR	*Enterococcus faecalis*, *Burkholderia cepacia*, *Campylobacter gracilis* *Streptococcus gordoniii*, *Atopobium rimae*, *Peptostreptococcus micros*, *Streptococcus genimospecies* C8. *Dialister* spp. E2_20 E1, *Eubacterium strain* A35MT
Schirrmeister et al., 2009	New bacterial composition in root-filled teeth with perirradicular lesions	18	Paper points (root canal)	- Culture - 16S rRNA gene sequencing	*Actinomyces* spp., *Atopobium rimae, Campylobacter* spp., *Enterobacter* spp., *Klebsiella* spp., *Megasphaera* spp., *Olsenella* spp., *Parvimonas* spp., *Porphyromonas* spp., *Propionibacterium* spp., *Staphylococcus* spp., *Streptococcus* spp., *Synergistes* spp., *Tannerella* spp., *Veillonella* spp., *Enterococcus* spp., *Vagococcus fluvialis*, *Solobacterium moorei*, *Fusobacterium nucleatum*, Parvimona *micra* and *Dialister invisus, Olsenella Uli,* and *Slackia exigua*
Chugal et al., 2011	Molecular characterization of the microbial flora residing at the apical portion of infected root canals of human teeth	26 18: Teeth with primary infection 8: Teeth with secondary infection	Lesions obtained from extracted teeth	PCR-DGGE	Primary and secondary infections: *Fusobacteria*, *Actynomices* spp., and *Anaeroglobus geminatus.* Secondary infections: *Burkholderiales* and *Pseudomonas* spp.
Rôças & Siqueira, 2012	Characterization of microbiota of root canal-treated teeth with posttreatment disease	42	Paper points (root canal)	-Capture-checkerboard DNA-DNA hybridization -Real time-PCR	*Propionibacterium acnes* spp. (52), *Fusobacterium nucleatum* (24), *Streptococcus* spp. (17), *Pseudoramibacter alactolyticus* (14), *Propionibacterium* acidifaciens (14) and *Enterococcus faecalis* (12) and Tannerella forsythia (12)
Saber et al., 2012	Bacterial flora of dental periradicular lesions analyzed by the 454-pyrosequencing technology	7	Periradicular lesions	Pyrosequencing of bacterial 16S rRNA	Bacterial phyla: *Proteobacteria* (33), *Firmicutes* (31), *Actinobacteria* (12), *Bacteroidetes* (11) Bacterial genera: *Fusobacterium* (21), *Streptococcus* (8), *Prevotella* (7.5), *Corynebacterium* (7), *Porphyromonas* (6), *Actinomyces* (6)
Endo et al., 2012	Quantification of cultivable bacteria and endotoxin in post-treatment apical periodontitis	15	Paper points (root canal)	PCR	Gram-negatives: *Prevotella nigrescens* (4/15), *Prevotella intermedia* (2/15), and *Tannerella forsythia* (2/15)
Anderson et al., 2012	Comprehensive analysis of secondary dental root canal infections: a combination of culture and culture-independent	21	Paper points (root canal)	- Culture - PCR amplification of 16S and 18S-rRNA	Bacterial phyla: Firmicutes, Actinobacteria Proteobacteria e Bacteroidetes

	approaches reveals new insights				Bacterial genera: *Enterococcus gallinarum/casseliflavus, Lactobacillus gasseri, Streptococcus* spp., *Streptococcus mutans, Selenomonas* spp., *Peptostreptococcus stomatis, Olsenella profusa, Proteus hauseri/vulgaris, Delftia* spp., *Exiguobacterium aurantiacum, Pantoea agglomerans, Neisseria* spp., *Phocaeicola abscessus, Candida parapsilosis*
Anderson et al., 2013	Comparison of the bacterial composition and structure in symptomatic and asymptomatic endodontic infections associated with root-filled using pyrosequencing	40	Paper points (root canal)	Pyrosequencing of 16S rRNA gene	Bacterial phyla: *Firmicutes* (30), *Proteobacteria* (26), *Actinobacteria* (23), *Bacteroidetes* (13), *Fusobacteria* (5). One sample was positive for fungi, and archaea could not be detected. Bacterial genera: *Streptococcus* (11), *Prevotella* (8), *Lactobacillus* (8), *Kocuria* (5), *Neisseria* (4). * *Streptococcus* spp. were prevalent in all symptomatic cases and in 21/23 asymptomatic cases
Hong et al., 2013	Microbial analysis in primary and persistent endodontic infections by using pyrosequencing	18 10: untreated teeth 8: root-filled teeth	Paper points (root canal)	Pyrosequencing of 16S rRNA	*Bacteriodetes* was the most abundant phylum in both primary and persistent infections
Tennert et al., 2014	New bacterial composition in primary and persistent/secondary endodontic infections with respect to clinical and radiographic findings	21 patients 6 cases: primary 6 cases: root-filled teeth	Paper points (root canal) after gutta-percha was removed.	16S rRNA	*Enterococcus faecalis* was most frequently isolated in secondary infections (33%). *Ikenella corrodens, Enterococcus faecalis, Eubacterium tedium, Filifactor alocis, Fusobacterium nucleatum, Lactobacillus casei, Lactobacillus rhamnosus, Mogibacterium pumilum, Prevotella buccae, Prevotella intermedia, Propionibacterium acidifaciens, Propionibacterium propionicus, Pseudoramibacter alactolyticus, Solobacterium moorei, Streptococcus mutans, Streptococcus constellatus, Streptococcus anginosus, Tannerella forsythia, Veillonella dispar,*and *Moraxella osloensis* The CFUs range from $1x10^3$ to 1.49 x 10^7 in primary and secondary endodontic infections.
Karygianni et al., 2014	Supplementary sampling of obturation materials enhances microbial analysis of endodontic treatment failures: a proof of principle study	5	- Filling root canal material and paper points	- Culture - 16S rDNA	*Phyla: Firmicutes, Actinobacteria and Bacteriodetes* in root canal filling material and in root canal. Quantification of microorganisms in root canal filling *3.30-7.50 x 10^3* CFU/ml. Quantification of microorganisms in root canal median *3.48-7.36 x 10^3* CFU/ml.
Antunes et al., 2015	Total and specific bacterial levels in the apical root canal system of teeth with post-treatment apical periodontitis	27	Apical root fragments of adequately treated teeth obtained during periradicular surgeries	- Cryogenic grinding - PCR	*Streptococcus* spp. (76), *Actinobacteria* phylum (52), *Pseudoramibacter alactolyticus* (19) and *Enterococcus faecalis* (14)
Tzanetakis et al., 2015	Comparison of bacterial community composition of primary and persistent endodontic infections using pyrosequencing	48 teeth 24: Teeth with persistent infections	Paper points (root canal)	Pyrosequencing of 16S rRNA	*Bacteroidetes* (36), *Proteobacteria* (6), *Tenericutes* (42), *Cyanobacteria* (0.018) and *Acidobacteria* (0.007)
Siqueira et al., 2016	Microbiome in the apical root canal system of teeth with post-treatment apical periodontitis	10	Apical portions obtained from surgeries	- Cryogenic grinding - 16rRNA gene sequencing	*Proteobacteria* (46), *Firmicutes* (18), *Fusobacteria* (15), *Actinobacteria* (8)
Keskin et al., 2017	Pyrosequencing analysis of cryogenically ground samples from primary and secondary/persistent endodontic infections	20	Extracted teeth	-Cryogenic grinding - Pyrosequencing of 16S rRNA	Phyla: *Proteobacteria* (36), *Cyanobacteria* (0.04), *Tenericure* (0.04), *Acidobacteria* (0.17). Genera: Fusobacterium (18)
Bouillaguet et al., 2018	Root microbiota in primary and secondary apical periodontitis	43	Apical portion (5 mm) of extracted teeth. The	16S rRNA gene amplicon sequencing	*Enterococcus faecalis* and *Fusobacterium nucleatum*

		21: primary apical periodontitis 22: secondary apical periodontitis	intraradicular content was collected with a file #15.		
Zandi et al., 2018	Microbial analysis of endodontic infections in root-filled teeth with apical periodontitis before and after irrigation using pyrosequencing.	10	Paper points (root canal)	Pyrosequencing of 16S rRNA	Phyla – initial (S1) and post-irrigation (S2): *Firmicutes, Fusobacteria, Bacteroidetes e Actinobacteria.* Genera - (S1 and S2): *Streptococcus* and *Fusobacterium* Especies - (S1 and S2): *Fusobacterium nucleatum ss. vincentii, Streptococcus oralis/mitos, Streptococcus intermedius and Streptococcus gordonii.*
Barbosa-Ribeiro et al., 2020	Microbiological investigation in teeth with persistent/secondary endodontic infection in different stages of root canal retreatment	20	Paper points (root canal)	Nested-PCR	*Enterococcus faecalis* and *Porphyromona gingivales* were frequently detected in all stages of root canal retreatment. Reduccion bacteriana: posterior a la preparacion quimico-mecanica (S2) (99.4%),despues de la medicacion intraconducto (99.5%). Los conteos no fueron reportados.
Siqueira et al., 2020	The apical root canal system of teeth with posttreatment apical periodontitis: correlating microbiologic, tomographic and histopathologic findings	36	Apical fragments obtained from periradicular surgery	- Cryogenic grinding - qPCR	All 36 cryopulverized apical root specimens were positive for Bacterial presence.Total Bacterial load was *1.82 x* 10^3 *Actinobacteria* (54) - 6.36 x 10^2 Streptococci (45) – 9.90 x 10^2 *E. faecalis* (19) – 1.88 x 10^3
Barbosa-Ribeiro et al., 2021	Microbiological analysis of endodontically treated teeth with apical periodontitis before and after endodontic treatment	20	Paper points (root canal) after root filling materials were removed (S1), after instrumentation (S2), after 30 days of intracanal medication (S3).	- 16S rRNA genetic sequencing - PCR	*Enterococcus faecalis* e *Porphiromona gingivalis* were the most prevalent species. *Parvimonas micra and p.gingivalis were associated with previous pain, P. gingivalis was associated with tenderness to percussion* and *E. Faecalis, Fusobacterium nucleatum* and *P. gingivalis were associated wit periapical* lesión $> 3\mathrm{mm}$.
Gomes et al., 2021	Identification of culturable and nonculturable microorganisms, Lipopolysaccharides, and Lipoteichoic acids from root canals of teeth with endodontic failure	50	Paper points (root canal). Sample collected after root filling material removal for retreatment	-Cuture -Nested-PCR	Culture: *Enterococcus faecalis* (28), *Gemella morbillorum* (28). Molecular analysis: *Porphyromonas gingivalis* (78), *Enterococcus faecalis* (76) *Fusobacterium nucleatum* (64), *Parvimonas micra* (50), *Gemella morbillorum* (44)
De Castro Kruly et al., 2022	Residual bacteriome after chemomechanical preparation of root canals in primary and secondary infections	19 10: Primary infections 9: Secondary infections	Paper points (root canal) after chemomechanical preparation	-Sequencing of 16S rRNA - Next generation sequencing (Illumina MiSeq instrument).	Fhyla (13): *Firmicutes* (55), *Bacteroidetes* (16), Actinobacteria (8.4%) in primary and secondary infections and *Proteobacteria* (15) in secondary infections. Predominant genera in primary and secondary infections: *Bacillus* (22.5), *Marinilactibacillus* (9).
Sun et al., 2022	Microbial communities in the extraradicular and intraradicular infections associated with persistent apical periodontitis	10 patients	Root canal fillings, root end surface (3 mm) and apical periodontitis lesions	16S rRNRA High-throughput sequencing	Phyla: *Proteobacteria* (31.5), *Firmicutes* (21), *Bacteriodetes* (13) and *Actinobacteria* (10.5). Genera: *Fusobacterium*, *Morganella*, *Porphyromonas*, *Streptoccocus* and *Bifidobacterium.*
Pérez-Carrasco et al., 2023	Microbiome in paired root apices and periapical lesions and its association with clinical signs in persistent apical periodontitis using next-generation sequencing	21 patients	Root apices and apical periodontitis lesions obtained from apical surgeries	- Cryogenic grinding - Sequencing of 16S rRNA - Next generation sequencing	Phyla: *Firmicutes* (38), *Bacteriodetes* (19), *Fusobacteria* (14), *Proteobacteria* (10), *Synergistetes* (7) and *Actinobacteria* (6) Genera: *Fusobacterium (13)*, *Porphyromonas (8)* and *Streptococcus (8), Pseudomonas (5), Fretibacterium (5) and Tannerella (3).*

The microbiota of previously treated teeth with persistent apical periodontitis differs widely from primary endodontic lesions (MOLANDER *et al.*, 1998; SUNDQVIST

et al., 1998; HANCOCK *et al.*, 2001; CHUGAL *et al.*, 2011). However, one study reported that the diversity of the microbiota in post-treatment infections is lower than that found in primary infections (SIQUEIRA & RÔÇAS, 2009b). In contrast, TZANETAKIS *et al.* (2015) suggested that the diversity is probably higher in persistent infections, while other studies found no significant differences between the bacterial communities of primary and secondary infections (CHUGAL *et al.*, 2011; HONG *et al.*, 2013; KESKIN *et al.*, 2017). Recently, a study reported that secondary or persistent infections are highly diverse compared to primary infections (DE CASTRO KRULY *et al.*, 2022). Differences between the results of the various studies can be attributed to the sample number, the method of obtaining the examined sample, the sequencing platforms, the assays used for identification, the quality of root canal treatments, and the geographic locations (SIQUEIRA *et al.*, 2016).

Phenotypic identification procedures have shown that the dominant microbiota in primary infections is gram-negative (GOMES *et al.*, 2004; SIQUEIRA & RÔÇAS, 2004). Facultative anaerobic gram-positive bacteria are predominant in secondary or persistent endodontic infections (MÖLLER *et al.*, 1981; MOLANDER *et al.*, 1998; GOMES *et al.*, 2004; SIQUEIRA & RÔÇAS, 2009b; BARBOSA-RIBEIRO *et al.*, 2021).

The microbiota of teeth with endodontic failure was identified using advanced microbiological techniques for anaerobic species. A total of 57.4% of the species identified in the study were classified as facultative anaerobes, while 83.3% were gram-positive bacteria, with *Enterococcus faecalis* being the most prevalent. Additionally, 42.6% of the species were categorized as obligate anaerobes (PINHEIRO *et al.*, 2003). Gram-negative species such as *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis*, have also been reported (BARBOSA-RIBEIRO *et al.*, 2020).

In endodontically treated teeth, it is typically observed that 1 to 2 anaerobic or facultative gram-positive organisms are predominantly identified by culture-based methods (PINHEIRO *et al.*, 2003). In cases of endodontic failure, PCR analyses have revealed the presence of an average of 3 distinct species in root canals that were filled 2 mm from the radiographic apex. On the contrary, canals filled beyond 2 mm from the apex revealed the presence of 5 species (SIQUEIRA & RÔÇAS, 2004) . Canals that are not treated adequately are more likely to harbor species, with an average of 3 species having been found in culture (PINHEIRO *et al.*, 2003) and 10 to 30 species in molecular studies (MUNSON *et al.*, 2002; SIQUEIRA *et al*., 2004a; SIQUEIRA & RÔÇAS, 2005a).The phyla commonly found in post-treatment endodontic infections have been Proteobacteria, Firmicutes, Actinobacteria, Fusobacteria, and Bacteroidetes (SABER *et al*., 2012; ANDERSON *et al*., 2013). The most predominant ones are Firmicutes, Proteobacteria and Actinobacteria (SIQUEIRA & RÔÇAS, 2007). Other phyla like Tenericutes, Synergistetes, Spirochaetes, GN02, TM7, Deinnococcus - Thermus, Cyanobacteria, Acidobacteria, Chloroflexi, SR1 y OD1 have been reported in very low percentages (ANDERSON *et al.*, 2013; TZANETAKIS *et al.*, 2015; SIQUEIRA *et al.*, 2016).

A recent scoping review investigated the microbial profile in persistent endodontic infections. The most prevalent species were *Enterococcus faecalis*, *Parvimonas micra*, *Porphyromonas endodontalis*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Dialister invisus*, *Propionibacterium acnes*, *Tannerella forsythia*, and *Treponema denticola.* An increase in specific types of bacteria was observed in cases in which symptomatology or inadequate root canal filling was present. Likewise, teeth with deficient coronal restoration showed a more significant number of bacteria (PINTO *et al.*, 2023).

Proteobacteria are gram-negative opportunists that persist for prolonged periods in states of nutrient deprivation and grow in dense biofilm communities (BERGKESSEL & DELAVAINE, 2021). It has been the most abundant bacteria found by next-generation sequencing in post-treatment endodontic infections (SIQUEIRA *et al.*, 2016; DE CASTRO KRULY *et al.*, 2022). Another study, conducted with pyrosequencing, reported the presence of *Proteobacteria* (26.1%) in cases of endodontically treated teeth with asymptomatic apical periodontitis (ANDERSON *et al.*, 2013). In teeth with secondary and persistent infections with inadequate root canal treatment, *Proteobacteria* were detected by pyrosequencing in 6% (TZANETAKIS *et al.*, 2015). This phylum was also found in extracted teeth with primary (36.1%) and secondary infections (35.8%) that were processed with cryopulverization and pyrosequencing (KESKIN *et al.*, 2017).

Firmicutes include Gram-positive bacteria with a low DNA molecule. They are characterized by having a rigid cell wall that contains muramic acid (BATT & TORTORELLO, 2014). The microbiota of endodontically treated teeth with symptomatic and asymptomatic apical periodontitis was compared by pyrosequencing. *Firmicutes* were the most abundant phylum (29.9%) and the most prevalent in symptomatic patients. The genera commonly found were *Streptococcus*, *Prevotella,* and *Lactobacillus* (ANDERSON *et al.*, 2013).

The *Streptococcus* spp. and *Enterococcus* spp. are the most dominant species of the bacterial community in cases of post-treatment apical periodontitis (MOLANDER *et al.*, 1998; RÔÇAS & SIQUEIRA, 2012; RODRIGUES *et al.*, 2017; ZANDI *et al.*, 2018; ZANDI *et al.*, 2019).

Actinobacteria are a highly diversified gram-positive phylum characterized by their exceptional metabolic versatility (VAN BERGEIJK *et al.*, 2020). *Actinomyces* and *Propionibacterium propionicum* of the phylum *Actinobacteria* are capable of growing

and surviving for long periods in host tissues without causing symptoms (SIQUEIRA, 2003). They have been identified in post-treatment endodontic infections by molecular methods, mainly in asymptomatic cases (ANDERSON *et al.*, 2013; SIQUEIRA *et al.*, 2016). They are the main cause of extraradicular endodontic infections and periapical actinomycosis (DIOGUARDI *et al.*, 2020). Although the prevalence of periapical actinomycosis is low, it is considered part of post-treatment endodontic infections (SIQUEIRA, 2003).

Actynomyces is a normal commensal of the oral microbiota. Its penetration into the root canal system is believed to occur due to the lack of a coronal seal. They have also been found in apical periodontitis lesions where no root canal treatment has been performed (DIOGUARDI *et al.*, 2020).

Periapical actinomycosis is a chronic granulomatous infection characterized by suppuration, abscess formation, and a sinuous tract that may erupt through the mucosa or skin. Sulfur granules (small formations of yellow-colored bacteria) have been observed in the exudate and extraradicular mineralized biofilms, suggesting a diagnosis of actinomycosis; however, their presence is not sufficient to determine an infection because it is not yet clarified. In contrast, microscopic observation of characteristic fungal ray patterns of *Actinomyces* spp. is enough to establish the diagnosis (SUNDE *et al.*, 2002; SIQUEIRA, 2003; TRONSTAD & SUNDE, 2003). Actinomycotic colonies can live in equilibrium in the host tissues without inducing an acute response, but they can maintain chronic periapical inflammation. A high amount of *Actinomyces* spp. is necessary to cause a persistent infection. Their low pathogenicity and minimal host response could be the reason why they remain in a chronic periapical lesion (SIQUEIRA, 2003).

The microbiota of apical actinomycosis was evaluated immunocytochemically in 16 cases with a long-standing history of sinus tract and destruction of the cortical

bone plate. The predominant bacterial species found were *Actinomyces israelii, Arachnia propionica,* and *Actinomyces naeslundii* (HAPPONEN, 1986). A systematic review reported that the genera of *Actinomyces* frequently found in endodontic infections are *Actinomyces naeslundii, Actinomyces israelii, Actinomyces viscosus, Actinomyces odontolyticus and Actinomyces meyeri. Actinomyces gerencseriae, Actinomyces radicidentis, and Actinomyces halioti were* found less frequently (DIOGUARDI et al., 2020). In cases diagnosed with persistent intraradicular or extraradicular infections caused by *Actinomyces*, the recommended treatment is apical surgery or dental extraction to avoid future complications such as cervical facial actinomycosis or facial imperfections (DIOGUARDI *et al.*, 2020).

Fusobacteria is gram-negative obligate anaerobic non-spore forming bacilli (BENNETT & ELEY, 1993). It is one of the most abundant phyla in post-treatment endodontics infections (CHUGAL *et al.*, 2011; SIQUEIRA *et al.*, 2016).

Bacteroidetes are gram-negative anaerobes and have been the most abundant phylum reported in primary and secondary endodontic infections (HONG *et al.*, 2013; DE CASTRO KRULY *et al.*, 2022). It was the most prevalent phylum (59.4%) in primary and persistent endodontic infections identified by pyrosequencing (TZANETAKIS *et al.*, 2015).

Several species as *Streptococcus*, *Lactobacillus, Sphingomonas*, *Pseudoramibacter alactolyticus*, *Propionibacterium propionicum*, *Filifactor alocis*, *Dialister pneumosintes, Dialister invisus, Tanerella, Pseudomonas* and *Klebsiella, Stenotrophomonas* have been found in endodontic failures (CHEUNG & HO, 2001; TZANETAKIS *et al.*, 2015; SIQUEIRA *et al.*, 2016). Rare bacterial species have also been documented *Atopobium rimae, Megasphaera* spp., *Olsenella Uli, Slackia exígua, Vagococcus fluvialis* y *Solobacterium moorei* (SCHIRRMEISTER *et al.*, 2009).

Other microorganisms such as *Treponema denticola*, *Porphyromonas endodontalis*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Prevotella nigrescens* that have been detected in primary endodontic infections have also been identified in low percentages or absent in secondary and persistent infections (SIQUEIRA & RÔÇAS, 2004).

These microorganisms resist chemical-mechanical preparation due to their ability to form biofilms, synergism, expression of survival genes, and activation of the alternative metabolic pathway (PRADA *et al.*, 2019). The low occurrence and diversity of bacteria reported in secondary and persistent infections may be associated with the impossibility of complete eradication of microorganisms in primary infection due to the different aseptic conditions, instrumentation, and disinfection methods used by different operators during the process of root canal treatment or retreatment. The sample number, the method of obtaining the examined sample, the sequencing platforms, the identification assays used, the quality of root canal treatments, and the geographic locations may also influence the results (SIQUEIRA *et al.*, 2016).

The high prevalence of gram-positive bacteria in secondary and persistent infections was confirmed by culture, biochemical test, and molecular methods. A total of 154 gram-positive strains and 188 isolates were detected by culture. The biochemical test identified *Enterococcus faecalis* and *Gemella morbillorum*, while the molecular analysis detected a high frequency of *Porphyromonas gingivalis*, *Enterococcus faecalis* and *Fusobacterium nucleatum*. High levels of lipoteichoic acid revealed a significant association with post-treatment extensive apical periodontitis lesions (GOMES *et al.*, 2021).

In the apical portion of the root canal system with post-treatment apical periodontitis, *Streptococcus* (76%), followed by members of the Actinobacteria phylum

(52%) and *Pseudoramibacter alactolyticus* (19%), were the most dominant and prevalent bacterial population (ANTUNES *et al.*, 2015).

1.2.3. Relationship of pathogenicity threshold with intracanal disinfection strategies in post-treatment apical periodontitis

An infectious process results from the interaction between the virulence of microorganisms, their number, and the host's defenses. This concept proposed by SMITH (1934) has allowed us to understand the pathogenesis of apical periodontitis (SIQUEIRA, 2011).

The variability of the root canal system and the polymicrobial nature of endodontic infections make disinfection complex (NEELAKANTAN *et al.*, 2017). An infected root canal is a reservoir of bacteria and bacterial products; therefore, the primary goal of disinfection strategies in endodontic therapy is to reduce the number of bacteria to a level that is compatible with the host immune system that allows the periradicular tissue repair (HAAPASALO *et al.*, 2003; SIQUEIRA & RÔÇAS, 2008; RICUCCI & SIQUEIRA, 2010; HAAPASALO *et al.*, 2011).

Biofilm removal is accomplished through chemical-mechanical preparation using specific instruments, disinfectant solutions, and intracanal medications between appointments (SIQUEIRA, 2011; NEELAKANTAN *et al.*, 2017). The success of endodontic treatment may depend on various factors used during therapy, such as the type of irrigant, the irrigation technique, the size of the apical preparation, the use of intracanal medication, and the number of appointments (ORDINOLA-ZAPATA *et al.*, 2022).

The negative impact of bacterial presence in the root canal system during obturation has been demonstrated in several studies (SJÖGREN *et al.*, 1997; SUNDQVIST *et al.*, 1998; FABRICIUS *et al.*, 2006). The root canal of a tooth with post-

treatment infection may harbor 10^3 to 10^7 bacteria (PECIULIENE *et al.*, 2001b; SEDGLEY *et al.*, 2006; ANTUNES *et al.*, 2015; ZANDI *et al.*, 2019). This indicates that residual bacteria survive predominantly in the dentinal tubules or attached to the walls for prolonged times in endodontically treated root canals, preventing their dissemination to the periradicular tissues (SIQUEIRA *et al.*, 2014). In the apical portion, bacteria are more difficult to eliminate, and when they come into direct contact with periradicular tissues, they obtain nutritional sources that maintain inflammation and prevent repair (SIQUEIRA & RÔÇAS, 2008).

Other important factors include the virulence of the involved bacterial species and host resistance. The number of bacteria necessary to cause disease is inversely proportional to virulence, meaning that the greater the number of virulence factors, the smaller the number of bacteria (SIQUEIRA & RÔÇAS, 2008). Residual bacteria compromise the prognosis of endodontic therapy because they reach certain levels of pathogenicity, influenced by the diversity of bacterial communities and biofilm persistence, typically in the apical region (SUBRAMANIAN & MICKEL, 2009).

The parameters established for bacteria compatible with periapical/periradicular repair have been mostly obtained from cultures. Gram-positive bacterial cultures have shown a correlation between persistent endodontic infections and treatment prognosis (SJÖGREN *et al.*, 1997; SUNDQVIST *et al.*, 1998; WALTIMO *et al.*, 2005).

As reported in studies that identified the microbiota by cultures, the ideal result of endodontic therapy is the reduction of bacterial levels below 10^3 to 10^4. It has been demonstrated that there are no apparent clinical signs or symptoms at this level (SIQUEIRA, 2011). Radiographically, if a periapical radiolucency is not observed, it does not necessarily imply the absence of bacteria (BRYNOLF, 1967). Therefore, repair of periradicular tissues can occur in the presence of microorganisms that have survived in the root canal system (MOLANDER *et al.*, 1998). In the treatment of

endodontic infections, the bacterial population must reach a minimum level that either causes damage and initiates the disease or generates a host response mechanism (SIQUEIRA & RÔÇAS, 2007).

The development of disinfection techniques to remove biofilm or achieve "sterilization" in the root canal system has been the focus of numerous studies in endodontics (SIQUEIRA, 2011; NEELAKANTAN *et al.*, 2017). Although multiple disinfection strategies in endodontic therapy seek to reduce the bacterial load to a level compatible with the host's immune response that allows repair of the apical or periradicular tissues, to date, it has not been possible to demonstrate a complete removal of the microorganisms involved or to identify the relationship between the number of residual bacteria in the obturation compatible with periradicular health (SIQUEIRA, 2011; NEELAKANTAN *et al.*, 2017).

The repair of periapical tissues in the presence of residual bacteria was demonstrated in samples obtained from root canals prior to obturation and in post-treatment follow-ups (MOLANDER *et al.*, 1998; PINHEIRO *et al.*, 2003). The evaluation of bacterial levels in endodontically treated teeth with post-treatment apical periodontitis has been mainly based on samples obtained from root canals with sterile paper points, analyzed by molecular methods at different stages of retreatment (post-instrumentation and post-intracanal medication) (BLOME *et al.*, 2008; ZANDI *et al.*, 2019; BARBOSA-RIBEIRO *et al.*, 2020; BARBOSA-RIBEIRO *et al.*, 2021). The best methods for quantifying bacterial populations are polymerase chain reaction (PCR) or fluorescent in situ hybridization (MOTER & GÖBEL, 2000; MACKAY, 2004).

The influence of residual bacteria after root canal obturation was demonstrated radiographically and histologically in monkey teeth with apical periodontitis FABRICIUS *et al.* (2006). Non-repair of apical tissues was associated with 79% of residual bacteria found after endodontic treatment. In cases where no bacteria were

observed, repair occurred regardless of the quality of the root canal filling. On the contrary, in cases where residual bacteria predominated in poor root canal fillings, repair of the periapical tissues did not occur.

Another study quantified total bacterial loads in teeth with chronic apical periodontitis in primary and secondary infections with qPCR. Root canals with primary infection harbored significantly more bacteria than teeth with secondary infection. Teeth with post-treatment apical periodontitis showed high initial bacterial levels (2.1 x 10^6). These levels were significantly reduced after instrumentation (3.6 x 10^4) and intracanal medication (1.4 x 10^5). This study indicated that chemo-mechanical preparation reduced bacterial levels by 95% (BLOME *et al.*, 2008).

The total bacteria count and specific levels from apical fragments of endodontically teeth with post-treatment apical periodontitis were reported by ANTUNES et al. (2015). The mean total bacterial for 21 apical segments was 5.7 x 10^4.

A significant reduction in bacterial levels was found after chemical-mechanical preparation (99.4%) and intracanal medication placement (99.5%) in teeth previously treated with apical periodontitis. Intracanal samples were collected on paper points and analyzed with culture and nested-PCR (BARBOSA-RIBEIRO *et al.*, 2021).

A different study evaluated the influence of residual bacteria on teeth with persistent apical periodontitis using molecular techniques. Clinical and radiographic follow-ups of the retreatments were performed between 1 and 4 years. Samples were obtained before to instrumentation (S1), after instrumentation (S2) using 1% NaOCl (group 1) and 2% CHX (group 2), and after intracanal medication (S3). Periapical tissue repair was observed in 65% of group 1, and 64% of group 2 in the first year of follow-up. There were no clinically significant differences in outcomes between 1% NaOCl

and 2% CHX. However, the most significant repair was observed in S3, in which the bacterial loads were reduced to 3.12 x 10^3 (ZANDI *et al.*, 2019).

A recent study evaluated the composition and diversity of the microbiota in primary and secondary endodontic infections using high-throughput sequencing with MiSeq Illumina (DE CASTRO KRULY *et al.*, 2022). In addition, they examined the microorganisms that persist after non-surgical endodontic treatment. The samples were collected prior to the chemical-mechanical instrumentation (S1) and the obturation (S2). Individual differences in the bacterial composition of the intraradicular biofilm were observed. There were no significant variations in the bacterial composition. However, a high diversity of bacterial communities in low numbers was revealed after the chemical-mechanical preparation. This result suggests that certain microorganisms are able to survive disinfection procedures (DE CASTRO KRULY *et al.*, 2022).

The reduction of bacterial levels, adequate obturation and coronal restoration, and a favorable host immune response act on residual microorganisms to promote the repair of the periapical lesion (SIQUEIRA, 2011; SEGURA-EGEA *et al.*, 2022). More longitudinal studies are needed to expand knowledge about the effects of disinfection treatments and the associations of specific bacteria predominant in post-treatment endodontic infections (SIQUEIRA & RÔÇAS, 2022b).

1.3. Methods for the identification of the endodontic microbiota

1.3.1. Culture and molecular methods

Several studies have employed diverse methodologies to identify the microbiota associated with endodontic infections. These advancements have facilitated an understanding of the etiology and pathogenesis of primary, secondary, and

persistent infections. They are categorized into five generations (SIQUEIRA & RÔÇAS, 2009a; SIQUEIRA & RÔÇAS, 2013; SIQUEIRA & RÔÇAS, 2022a).

The first-generation studies were based on open-ended cultures and helped establish the role of anaerobic bacteria in apical periodontitis. In addition, they allowed the identification of various bacterial species, mainly obligate and facultative anaerobes. The first bacteria identified in root canals by culture were obligate anaerobes (e.g., *Fusobacterium* spp., *Peptostreptococcus* spp., *Treponema* spp., *Lactobacillus* spp. *Bacteroides* spp., *Actinomyces* spp.) (WITTGOW & SABISTON, 1975; MÖLLER *et al.*, 1981).

Microbial cultures provide valuable experimental models, facilitating the identification of pathogens, the study of antigens, the determination of antibiotic susceptibility, and the genetic exploration of microorganisms (GOMES *et al.*, 2021). Microorganisms are identified based on the phenotypic behaviors observed in the different strains with biochemical and physical properties for optimal growth (SIQUEIRA & RÔÇAS, 2013). However, no specific culture medium exists for all species in the root canal system (CROWLEY, 1963; GOMES *et al.*, 2021).

The culture technique may underestimate the prevalence of some pathogens since an error may occur during the isolation or growth of bacteria, especially the most fastidious ones, such as *Spirochaetes*, or due to the difficulty of maintaining their survival conditions in vitro, providing inconclusive or erroneous results (GOMES *et al.*, 2015).

Molecular microbiology-based tools and methods were introduced in endodontic research in the late 1990s to overcome the limitations of cultures. These have been widely used to discover the diversity of endodontic bacterial species. Although knowledge of the taxonomy of microorganisms has increased and been defined with advances in molecular methods, identification provides limited information

on the physiology and pathogenicity within bacterial communities (MARON *et al.*, 2007). This information can be obtained by evaluating the bacterial products secreted in the environment or determining the gene expression pattern (SIQUEIRA & RÔÇAS, 2022b).

Molecular methods are classified in the second to fifth generation (SIQUEIRA & RÔÇAS, 2013; SIQUEIRA & RÔÇAS, 2022a). These are used for the detection of specific species (closed-ended analysis), of all or the most dominant species (broad-range or open-ended analysis), or to identify profiles of microbial communities (community analysis) (SIBLEY *et al.*, 2012).

The second generation consists of closed-ended molecular detection studies of culturable bacteria, such as species-specific PCR and checkerboard hybridization assays using whole genomic probes. These methods are more sensitive than culture and were used to detect difficult-to-grow bacterial species (SIQUEIRA *et al.*, 2001; SIQUEIRA *et al.*, 2001a; BAUMGARTNER *et al.*, 2003; GOMES *et al.*, 2006).

The third generation involves open-ended DNA molecular studies such as broad-range PCR followed by cloning and Sanger sequencing, terminal restriction fragment length polymorphism (T-RFLP) or denaturing gradient gel electrophoresis (DGGE) to detect bacterial species in a sample. These methods include culturable and non-cultivable bacterial species. They have disadvantages such as the long time needed to process the samples and their high cost, which can lead to the analysis of few samples or only of dominant bacteria (SIQUEIRA & RÔÇAS, 2022a). An advantage of these methods is that they allow the identification of the profile of the structure of bacterial communities (SIQUEIRA & RÔÇAS, 2009a).

Data from the third generation gave rise to the fourth generation, based on closed-ended molecular methods such as PCR, reverse-capture checkerboard hybridization, and microarrays. Finally, the fifth generation comprises high-generation

open-ended analysis deep-coverage technologies known as High-Throughput Sequencing (HTS) or Next Generation Sequencing (NGS) (SIQUEIRA & RÔÇAS, 2022b).

1.3.2. Molecular methods used in endodontics

PCR-based and sequencing technologies have been commonly employed molecular methods. Various techniques, including 16S rRNA sequencing, matrix-assisted laser desorption ionization-time of flight (MALDI-TOF), and mass spectrometry (MS), have been employed for bacterial isolation and identification (SONG et al., 2003; DINGLE & BUTLER-WU, 2013). Additionally, this method enables the identification of different primary colonies that do not require subcultures (Siqueira & Rôças, 2022a).

The PCR technique was introduced by Kary Mullis, for which he received the Nobel Prize in 1993 (MULLIS & FALOONA, 1987). This technique involves the use of a DNA polymerase enzyme to amplify a substantial quantity of DNA or a specific gene. It enables the exponential replication of a small segment of DNA, usually less than 3000 base pairs, resulting in a substantial increase in the number of copies, often reaching around one million-fold. These specific replicas facilitate the utilization of additional molecular biology applications, including the assessment of base size and nucleotide sequencing. The target sequence of DNA to be amplified is identified by a specific pair of DNA primers, usually oligonucleotides of about 20 nucleotides in length, that designate the boundaries of the product to be amplified (SPRATT, 2004). This method requires the design of 16S rRNA gene sequences public databases specific primers for the species to be evaluated (SIQUEIRA & RÔÇAS, 2022a).

The species-specific single PCR is one of the simplest methods to detect bacterial species. Nested-PCR is based on two rounds of amplification that can be

applied to species-specific assays with higher sensitivity and specificity (SIQUEIRA & RÔÇAS, 2022a). In addition, due to its difficulty in growth, it allows the detection of non-culturable or fastidious microorganisms. Its sensitivity is superior to cultures or a single-PCR (SPRATT, 2004; GOMES *et al.*, 2008). It is an important tool to monitor the effects of endodontic procedures on some microbial species (DUQUE *et al.*, 2019).

` The multiplex PCR can detect more than one species simultaneously. Real-time PCR has been the most widely used quantification method for total bacteria based on universal primers of the 16S rRNA gene. Its higher sensitivity and specificity provide quantitative results (qPCR) in species-specific assays (RAOULT *et al.*, 2004). There are two types of real-time PCR, SYBR green (HIGUCHI *et al.*, 1992) and Taqman (HEID *et al.*, 1996). SYBR Green is a simple and accessible method (MAEDA *et al.*, 2003). Although this assay is very sensitive, it has decreased specificity since the dye binds to all the double strands of DNA present in the reaction, which can cause the dimers of the primers to show false readings (BUSTIN, 2000). The Taqman method is more specific compared to the SYBR Green assay (HEID *et al.*, 1996); however, no significant differences have been found with SYBR Green regarding the specificity, quantification, and sensitivity in the identification of oral bacteria (MAEDA *et al.*, 2003).

Unlike the previously described PCRs, the reverse-transcriptase PCR allows detection and identification in mRNA or rRNA and is commonly used to show cell viability. mRNA provides information on function and physiology, while rRNA allows species to be identified (SIQUEIRA & RÔÇAS, 2022a).

The use of 16S (small subunit) nucleotide sequence data and the rRNA gene is the method of choice because it is present in all organisms and always performs the same function. Its sequence and regions remain conserved with an adequate size (ca 1500 bases), that allows easy sequencing with sufficient information for identification and phylogenetic analysis (SPRATT, 2004). The 16S rRNA is the commonly used

method for bacterial quantification; however, it is important to note that the results of such quantifications correspond to estimates. This occurs because most species contain more than one rRNA operon, and some reach up to 15 copies (ESPEJO & PLAZA, 2018). Therefore, the results of investigations of bacterial loads in endodontic infections are an approximate representation since it is not possible to obtain a precise quantification when using this gene. The number of rRNA operons is currently unknown for most oral bacterial species. To establish standard bacterial total quantification curves, studies generally use bacterial strains with 4-5 copies of the 16S rRNA gene, as the approximate median copy number in the range of best-known oral bacteria (RÔÇAS & SIQUEIRA, 2012; ANTUNES *et al.*, 2015).

Broad-range PCR is an open-ended method that has been widely used to investigate bacteria in various environments. It is more effective than cultures because it allows amplification of long gene fragments which, can result in reduced sensitivity, yet provides a more variable sequence that provides information for accurate identification and can be more specific. In addition, it reduces the risk of amplifying DNA contaminated by the reagents, which are usually fragmented (MAIWALD, 2004).

Denaturing Gradient Gel Electrophoresis (DGGE) or Terminal-Restriction Fragment Length Polymorphism (T-RFLP) are valuable methods for fingerprinting and identification of bacterial communities. In the investigation of the microbiota of endodontic infections, the DGGE was introduced in 2004 (SIQUEIRA *et al.,* 2004a). This technique is highly sensitive and allows simultaneous and reliable identification of microbial species or strains that are difficult or impossible to grow by culture. Another advantage is the possibility of identifying members of the bacterial community by sequencing their bands (MUYZER & SMALLA, 1998).

Technologies such as High-Throughput Sequencing (HTS) or Next Generation Sequencing (NGS) have allowed a greater understanding of microbial communities in

humans or the environment (KUNIN *et al.*, 2010; ANDERSON *et al.*, 2013). This technology performs numerous readings in a single cycle that allows for in-depth identification of the most dominant and least abundant taxa (KUNIN *et al.*, 2010). The Illumina MiSeq platform is currently the most widely used HTS platform in oral microbiota research, because it manages to combine high quality readings with low cost. A new generation of sequencing platforms are available, Pacific Biosciences (PacBio) and Oxford Nanopore (MinION), which require improving the precision in the sequencing and the application of specific correction tools (SIQUEIRA & RÔÇAS, 2022a).

DNA-DNA hybridization methods have also been used to identify the microbiota in endodontics. The most used methods have been DNA-DNA hybridization (macroarray) and DNA microarray. Their great advantage is the simultaneous detection of hundreds of samples of bacterial taxa (SOCRANSKY *et al.*, 1994; SIQUEIRA & RÔÇAS, 2022a)

1.3.2.1. Advantages of the use of molecular methods in the evaluation of the endodontic microbiota

For many years, cultures have been used to identify the endodontic microbiota and have contributed significantly to the understanding of the etiology and pathophysiology of apical periodontitis (SIQUEIRA & RÔÇAS, 2009b). Similarly, these methods have been widely used for evaluating the response of bacteria to intracanal disinfection techniques and systemic and local antibiotics. Nevertheless, these techniques have disadvantages, such as the meticulous attention the samples require to keep the cells viable from their collection to their culture in the laboratory. The transport medium must have special conditions that preserve cell viability and prevent bacterial growth until it is processed in the laboratory (SIQUEIRA & RÔÇAS, 2022a).

The laboratory process requires dispersion, dilution, cultivation, quantification, and isolation in a culture medium containing the essential nutrients/growth factors and identification (SIQUEIRA & RÔÇAS, 2022a). In contrast to traditional cultural techniques, second to fifth-generation molecular approaches exhibit a less complicated procedure due to the absence of cell viability maintenance and eliminating the need for a specific transport method.

One of the significant limitations of cultures is reproducing species' physiological and nutritional conditions from different environments to obtain adequate growth in the laboratory. Another limitation they present is a characterization based on phenotypic traits that take strains as references, which can lead to difficulties in identification and even obtaining false positive results. Therefore, the low sensitivity of cultures can lead to erroneous results, even in samples with abundant numbers of cells (SIQUEIRA & RÔÇAS, 2022a). On the contrary, molecular methods present a high sensitivity to detect cultivable and non-cultivable species that allow confirming the microbial profiles and obtaining a more precise and complete quantification of the analyzed samples. In addition, they facilitate the endodontic microbiota expansion and provide more reliable results on the efficacy of antimicrobial methods used in endodontic therapy (SIQUEIRA & RÔÇAS, 2005a; SIQUEIRA & RÔÇAS, 2009b; RÔÇAS *et al.*, 2014).

Traditionally, most cultures have only assessed the prevalence of bacterial species and have not been identified and quantified by culture. In contrast, molecular methods allow bacterial quantification and rarely interfere with the results of function and physiology. However, neither method fails to provide precise information regarding the specific location of the bacteria in the root canal system (SIQUEIRA & RÔÇAS, 2022a).

A controversial issue regarding molecular techniques, especially PCR, is the possibility of acquiring extremely low cell numbers and DNA from non-viable cells. For

these reasons, it is important to acknowledge that the enhanced sensitivity of PCR might be perceived as either advantageous or disadvantageous. For example, obtaining negative results indicates that the quantities of bacteria are exceedingly low or non-existent. In a complex bacterial community, a low number of species can play an important role in ecology, physiology, and even pathogenicity (SOGIN *et al.*, 2006; HAJISHENGALLIS *et al.*, 2011; HAJISHENGALLIS *et al.*, 2012). Therefore, a high sensitivity is ideal in this method to obtain reliable results. Nevertheless, the results of the sensitivity of the molecular methods do not correspond exactly to what is found in the clinic because there are inhibitory factors in the samples and the dilution as small aliquots (SIQUEIRA *et al.*, 2017).

The presence of non-viable cells can be considered advantageous or limiting in the context of molecular analysis. One potential advantage is the ability to detect fastidious bacteria that are non-cultivable during sampling, transport or isolation (WANG *et al.*, 1996; RANTAKOKKO-JALAVA *et al.*, 2000). Identifying ADN from non-viable cells at infection sites can result in inaccurate findings on the disease's pathophysiology (JOSEPHSON *et al.*, 1993; KEER & BIRCH, 2003). Experimental designs in endodontics are usually cross-sectional, so it is not possible to assume that the samples obtained contain bacteria that were not involved in the etiology of the infection (SIQUEIRA & RÔÇAS, 2022a). When cell death occurs, especially in areas of infection, the DNases released by viable bacteria are responsible for degrading non-viable cells to levels so low that they are not detectable by molecular methods (LEDUC *et al.*, 1995; BRUNDIN *et al.*, 2010). Detection of non-viable cells may be of concern in studies evaluating intracanal antimicrobial effectiveness because recent DNA release from dead cells can still be detected (SIQUEIRA & RÔÇAS, 2022a). This situation can be minimized with RNA-based methods (e.g., RT-PCR), primers that generate extensive amplicons (MCCARTY & ATLAS, 1993), viable/non-viable cell

stains that inhibit DNA amplification from dead cells (NOCKER & CAMPER, 2006; NOCKER *et al.*, 2006; NKUIPOU-KENFACK *et al.*, 2013) or DNase treatments during sample preparation (İRIBOZ *et al.*, 2018).

1.3.3. Cryogenic grinding

The cryogenic grinding technique was first introduced in the field of forensic medicine as an option for molecular analysis and identification of DNA extracted from teeth and bones (SWEET & HILDEBRAND, 1998; LIJNEN & WILLEMS, 2001).

This method consists of grinding mineralized tissue in a freezer mill that works with liquid nitrogen. The samples are placed in metallic tubes that contain magnets that facilitate crushing and act by the action of oscillating movements and the cooling of liquid nitrogen, which also protects the bacterial DNA from degradation by heat (PRETTY & SWEET, 2001). In endodontics, samples of specimens obtained by cryopulverization are more representative since they can include bacteria from the root canal, lateral/accessory canals, isthmus, dentinal tubules and apical ramifications (ALVES *et al.*, 2009). One study evaluated the microbiota of the apical, middle, and coronal thirds of extracted teeth with primary endodontic infections. This study demonstrated that the profiles of bacterial communities colonizing the apical third of teeth with apical periodontitis differ from the middle and coronal thirds. A high variation was observed interindividual (samples taken from different patients from the same region) and intra-individual (samples taken from different regions of the same tooth) (ALVES *et al.*, 2009).

Some studies have identified the microbiota of post-treatment endodontic infections using the cryogenic grinding technique (ANTUNES *et al.*, 2015; SIQUEIRA *et al.*, 2016; KESKIN *et al.*, 2017).

1.3.4. Limitations of traditional sampling and advantages of the use of cryogenic grinding

Traditionally, most studies that have evaluated the microbiota of the root canal system have used the method of sampling with paper points or endodontic files. This method consists of taking a microbiological sample with a file or several paper points covering the root canal's entire length. Obtaining a representative sample is not a simple procedure due to the narrowness of the root canals. This task becomes even more difficult in canals that require retreatment because, during the removal of the obturation material, the microorganisms present may blend, leading to a possible underestimation of the number of bacteria (SIQUEIRA & RÔÇAS, 2004).

The limitation of this technique is the inaccessibility of the file or the paper points to areas of difficult access, such as dentinal tubules, isthmus, ramifications, apical deltas, or lateral canals, in which most of the bacteria involved in endodontic treatment failure are found. Therefore, the results of such studies may present low bacterial loads that do not necessarily indicate bacteria were not present, suggesting the diversity of the endodontic microbiota could be considerably higher than established (SUNDQVIST *et al.*, 1998; ZOLETTI *et al.*, 2006). Determining the exact location is another limitation of this method (SIQUEIRA *et al.*, 2016).

Cryopulverization is a reliable technique for detecting the microbiota in endodontic infections since it allows obtaining intracanal representative samples (TRAN *et al.*, 2013; SIQUEIRA *et al.*, 2016). One of the limitations of this method is that it only allows obtaining samples of specimens from apical surgeries or extracted teeth. An additional significant limitation comes from the damage suffered by the samples, making them unsuitable for use in longitudinal studies with the objective to assess the efficacy of antimicrobial treatments (SIQUEIRA & RÔÇAS, 2022a).

During the cryopulverization process, there is the possibility of external contamination of the samples due to the presence of saliva or crevicular fluid during their collection through apical surgery or extraction. Therefore, a disinfection process is carried out to remove any microorganism from the external surface of the sample to be evaluated. Consequently, if there is extraradicular biofilm attached to the apical section, its removal limits the assessment of the extraradicular biofilm, which is crucial for understanding post-treatment endodontic infections (SIQUEIRA *et al.*, 2016; SIQUEIRA & RÔÇAS, 2022a). Sterility controls of the root surface are necessary to ensure that the detected bacteria were found intraradicularly (SIQUEIRA & RÔÇAS, 2022a).

In bacterial count studies, it is recommended to calculate the powder weight obtained from cryopulverization to permit analysis of data as the number of bacterial cells per 100 mg. The reason for this is because the samples vary in sizes (ANTUNES *et al.*, 2015).

1.4. Micro-computed tomography in endodontics

Micro-computed tomography (micro-CT) is a non-destructive, exact, and reproducible technique that allows the generation of two-dimensional cross-sectional images of the external and internal part of specimens through the use of X-rays of microfocal origin and high-resolution detectors that construct an image three-dimensional with the help of software (BARBOSA-RIBEIRO *et al.*, 2020; ORHAN, 2020). This equipment has significant limitations, including a high price and an extensive scanning time and rebuilding volume process (VERSIANI & KELES, 2020).

The use of this tool in endodontics was suggested in 1995 for evaluations of the morphology of the root canal system and to assess the changes that occur on

intraradicular surfaces after instrumentation or obturation in extracted teeth (NIELSEN *et al.*, 1995).

A wide variety of micro-CT studies have evaluated the anatomy of the root canal system, and the presence of anatomical complexities such as C-shaped canals, lateral/accessories canals, and ramifications (LEE *et al.*, 2006; AMOROSO-SILVA *et al.*, 2015; MARCELIANO-ALVES *et al.*, 2016; PIASECKI *et al.*, 2016).

The micro-CT allows reliable results on the effects and efficacy of the chemical-mechanical preparation and the obturation. The chemical-mechanical preparation has been evaluated in images obtained from micro-CT and identified with a color code that correlates the chemical-mechanical preparations pre-treatment and post-treatment. The analysis is carried out in a software, and results are obtained by subtracting percentages of the total number of voxels in treated channels from those untreated surfaces. The evaluation of untreated surface areas is relevant because they contain biofilm remnants. This equipment is also helpful for evaluating instrumentation faults in canals (apical transportations), untreated canals, and the quantity and quality of the obturation (VERSIANI & KELES, 2020).

The effectiveness of two endodontic instrumentation systems was assessed using micro-CT images, which identify the unprepared areas of the canal. The presence of pulp tissue remnants, residual bacteria, or biofilms in the uninstrumented areas was assessed by histobacteriological analyses since micro-CT cannot obtain soft tissue nuances. Abundant amounts of dentin debris and bacterial biofilms were detected (PÉREZ *et al.*, 2020).

The possible defects caused in the dentin (micro-cracks) by various mechanized instruments during the preparation and after the obturation of the root canals have been evaluated with micro-CT (DE-DEUS *et al.*, 2016; DE-DEUS *et al.*, 2017;

MANDAVA *et al.*, 2018). Micro-CT has also been useful in demonstrating the efficacy of different obturation techniques (CHELLAPILLA *et al.*, 2021).

A study in teeth with persistent bacterial infection performed with micro-CT found a significant association between unfilled areas and increased bacterial counts in extensive apical periodontitis lesions (SIQUEIRA *et al.*, 2020). Another study showed that an inadequate filling density influences the symptoms and size of apical lesions in teeth with post-treatment infections (VILLA-MACHADO *et al.*, 2020).

1.5. Determination of the size of apical periodontitis lesion

Bone defects associated with apical periodontitis can only be observed radiographically in the presence of a radiolucent area. These defects may correspond to a perforation of the osseous cortex, erosion of the internal surface of the osseous cortex, or extensive erosion or defects on the external surface (BENDER & SELTZER, 2003). As part of the defects, the cancellous bone may also be affected. SHOHA *et al.* (1974) were the first to demonstrate that cancellous bone of apical periodontitis could be observed using conventional radiographs. An *in-vivo* study has recently shown 97.6% of cortical bone lesions and 91.2% of cancellous bone lesions are detectable on periapical radiographs. Lesions with a diameter of 6 mm are more likely to involve the cortical bone, whereas lesions with a diameter of 4 mm were associated with the cancellous bone. This study suggested that the size of the lesion is a reliable predictor for identifying the periapical lesion, regardless of the osseous defect (CHANG *et al.*, 2020).

The cone-beam computed tomography (CBCT) was developed at the end of the 90s and introduced as a diagnostic tool that provides valuable three-dimensional images of dental and maxillofacial structures (MOZZO *et al.*, 1998; ARAI *et al.*, 1999). Currently, CBCT is the most sensitive, specific, and accurate method for detecting

apical periodontitis than periapical radiographs (LOFTHAG-HANSEN *et al.*, 2007; ESTRELA *et al.*, 2008).

CBCT was used to identify and classify types of bone defects in endodontically treated teeth with persistent apical periodontitis. The classification consists of five types: I. cancellous bone defect, II. buccal/labial bone defect, III. palatal/lingual bone defect, IV. through-and-through bone defect, and V. apical root protrusion through bone plate (YOSHIOKA *et al.*, 2011).

The traditional standard method for determining the periapical status of teeth is the Periapical Index (PAI). PAI is a 5-point scale system that ranges from healthy periapex (scores 1 and 2) and increases according to the severity of the apical periodontitis (scores 3, 4, and 5) (ORSTAVIK *et al.*, 1986). This visual reference scale allows observers to be calibrated in research studies, helps reduce the risk of personal bias associated with subjective radiographic assessments, and permits follow-up evaluations of apical periodontitis in prospective studies (ERIKSEN & BJERTNESS, 1991). However, this classification was performed on two-dimensional images in which spatial information of three-dimensional structures is lost (HARIDAS *et al.*, 2016).

In 2008, Estrela et al. introduced the CBCT-PAI by interpreting CBCT scans of periapical lesions. Six categories were proposed: 0. Intact periapical bone structures; 1. Diameter of periapical radiolucency 0.5 - 1.0 mm; 2. Diameter of periapical radiolucency 1 - 2 mm; 3. Diameter of periapical radiolucency 2 - 4 mm; 4. Diameter of periapical radiolucency 4 - 8 mm; 5. Diameter of periapical radiolucency 8 mm. Also, the classification added variables such as the expansion of cortical bone (E) and destruction of cortical bone (D) (ESTRELA *et al.*, 2008). This classification has limitations since lesion measurements were obtained on orthogonal planes, which prevent a standard lesion measurement (ESPOSITO *et al.*, 2011) and periapical lesion volume was not included (BOUBARIS *et al.*, 2021). Therefore, this classification has

been suggested to evaluate healing of post-treatment apical periodontitis (SĂLCEANU *et al.*, 2016).

A new classification volume-based Cone-beam Computed Tomography Periapical Index (CBCTPAVI) using semiautomatic segmentation technique was suggested by BOUBARIS *et al.* (2021). The accuracy rate of this new classification was 98.169%. CBCTPAVI was classified from small to large apical periodontitis lesions based on volume (mm^3) in 5 rules: Rule 1. □ 0.07; Rule 2. 0.07-0.68; Rule 3. 0.69-8.18; Rule 4. 8.19-67.73; Rule 5. ≥ 67.74. The results of this study indicated there is a significant variability between the sizes of the volumes obtained from apical periodontitis lesions, for which a 7-score index (0-6) was suggested.

Using a three-dimensional analysis, the sphericity of the apical periodontitis lesions and their relationship with CBCTPAVI were demonstrated. The results indicated that the apical periodontitis have a semispherical shape in their spread. As the size of the lesion increases, it expands and becomes less uniform in the cancellous bone. Consequently, larger apical periodontitis are less uniform than smaller lesions (BOUBARIS *et al.*, 2022).

2. JUSTIFICATION

This study provides new insights into the bacteriological conditions of root canals that were endodontically treated with and without apical periodontitis. Streptococci and Actinobacteria loads were determined using cryogenic grinding and quantitative polymerase chain reaction (qPCR). In addition, CBCT and micro-CT evaluations confirmed that an adequate root canal filling and restoration are essential to improve the prognosis of the endodontic therapy.

Obtaining the volumes of the apical periodontitis lesions of the endodontically treated teeth to determine if a correlation between size and intracanal bacterial loads was an additional significant contribution of this study.

The knowledge of the residual bacteria loads associated with periapical health or disease are crucial for improving or developing effective and predictable antimicrobial protocols to prevent post-treatment periapical infection.

3. OBJECTIVES

3.1. General objective

Quantify Streptococci and Actinobacteria levels in apical root fragments of endodontically treated teeth without and with post-treatment apical periodontitis.

3.2. Specific objectives

- Establish the association between the bacterial loads of *Streptoccocus* spp. and Actinobacteria of endodontically treated root canals without and with post-treatment apical periodontitis and the quality of the filling, limit obturation and restoration evaluated by CBCT and micro-CT

- Determine the relationship between bacterial counts in endodontically treated apical fragments of teeth without and with post-treatment endodontic infection and the volume of the periapical lesion size

4. MATERIALS AND METHODS

4.1. Ethical considerations

The protocol and informed consent for this research project was approved by the National Ethics Committee of the Ministry of Public Health and Social Assistance in Guatemala City, Guatemala (resolution 36-2020). Prior to the extraction of the teeth, each patient signed an informed consent form. Radiographic analyses of the extracted teeth were performed for diagnostic purposes.

4.2. Study type and design

This study was observational and cross-sectional in nature.

4.3. Methodology

4.3.1. Sample collection

Forty-five endodontically treated teeth from 25 patients with indication of extraction due to unrestorability, need of placement of an immediate dental implant, or any other reason that prevented the tooth from remaining functional in the oral cavity were collected.

Extracted teeth were obtained between October 2020 and November 2021 from patients referred to the Oral Surgery Clinic of the Dental School at Francisco Marroquín University, Guatemala City, Guatemala. Data such as the clinical history, age, sex, and smoking status, were recorded. As part of the diagnosis, a CBCT was taken of every tooth indicated to be extracted.

Immediately before tooth extraction, patients rinsed their mouth with 0.2% gluconate chlorhexidine solution for 1 minute. All tooth extractions were performed under aseptic conditions by undergraduate or graduate students with the supervision of an expert oral surgeon.

After extraction, blood, saliva and tissue residues were removed using a sterile gauze soaked in saline solution. The specimens were placed in 60 mL universal sterile collectors (NIPRO, Osaka, Japan) and stored at -20°C.

4.3.2. Selection criteria

The samples were selected according to the inclusion criteria. Periapical radiographs and CBCT determined the presence or absence of apical periodontitis lesion in each specimen.

4.3.2.1. Inclusion criteria

All extracted teeth used in this study had to meet the following inclusion criteria:

- Single or multirooted endodontically treated permanent teeth with and without apical periodontitis lesion from patients older than 18 years.
- Endodontically treated teeth that had a periapical radiograph and CBCT prior to extraction.
- Endodontically treated teeth with or without coronal restoration/intraradicular post.

4.3.2.2. Exclusion criteria

This study did not include endodontically treated teeth of patients exhibiting the following characteristics:

- Radicular fractures involving the canal
- Periodontal disease
- Surgically treated teeth (apicoectomy)
- Radicular resorption
- Root canals with separate instruments

- Patients who received systemic antibiotic therapy during the last 12 weeks prior to dental extraction.

4.3.3. Cone-beam computer tomography evaluations

The periapical status of each endodontically treated tooth was assessed in axial, sagittal, and coronal CBCT scans. An apical periodontitis lesion was determined as present when the lamina dura was disrupted and the radiolucency associated with the root apex was at least twice the width of the periodontal ligament space (PATEL *et al.*, 2012; ZHANG *et al.*, 2021). Additionally, parameters such as the apical limit, quality of the obturation, coronal restoration, and intraradicular post were evaluated. The apical limit of filling was categorized as: overfilled, 0 to 2 mm short of the root apex, and > 2 mm short of the root apex. The quality of the root canal filling was classified as adequate when all canals were obturated, with no detectable voids and apical limit ending from 0 to 2 mm short of the apex. If present, coronal restoration was classified as adequate on both CBCT and radiographs when it was apparently intact, with no detectable radiographic signs of overhangs, open margins, recurrent caries, or presence of temporary restoration. The presence of posts and the abutment tooth was also recorded.

4.3.4. Determination of the apical periodontitis lesion volume

The volume of the apical periodontitis lesion of each root was obtained from tomographic images taken with the CS 8100 Carestream 2018 radiographic equipment with an FOV of 5 x 5 cm, 150 µm voxel and with an exposure of 90 KV 3.2 mA 15s.

A centered image of each tooth with periapical lesion was obtained with CS 3D Imaging Carestream software (V3.10.22, Carestream Dental LLC, Atlanta, USA). To achieve an alignment of each root to be evaluated, in the oblique slicing the frontal

plane was identified in which the axial plane was oriented at the level of the cemento-enamel junction. Each lesion was centered in its axial, sagittal, and coronal planes. Next, the plane was rotated until an orthoradial view of the tooth was obtained. The sagittal and coronal planes of each root were aligned parallel to its long axis.

DICOM (Digital Imaging and Communications in Medicine) files were imported into the NemoScan 3D 2021 3D software (Nemotec S. L., Madrid, Spain). Axial, coronal and sagittal planes were obtained for each tooth with NemoScan. The volume of the apical periodontitis lesion was determined in mm^3 with manual adjustments. Using the sinus segmentation tool, the lesion was delimited in the coronal, axial, and sagittal planes to determine the seed point by navigating through it in search of an intermediate point between the highest and lowest grayscale readings of 0 HU and a tolerance limit of 250 HU. The volume was then calculated in mm^3. The areas of the volume that did not correspond to the lesion were removed, and then the geometry tool was used to determine the lesion volume. The apical periodontitis lesion size volume was classified as small and large using 65 mm^3 as the cut-off value. It was based on the calculated volume of a spherical lesion with a diameter of 5 mm, which is the parameter commonly used to categorize the size of apical periodontitis lesion on periapical radiographs (NGMANN et al., GULABIVALA, 2008).

4.3.5. Sample preparation

Under aseptic conditions, each tooth was removed from the universal collector with dressing pliers. The apical 5 mm of each root was then measured with a digital caliper and sectioned with a small diamond disc at high speed. The entire process was carried out under 2.5X magnification by a single operator. Then, each root fragment

was placed in a sterile 1.5 mL Eppendorf tube and labeled for identification before being stored at -20ºC.

4.3.6. Micro-CT analysis

All apical root specimens were scanned in a SkyScan 1174 v2 micro-CT (Bruker, Kontich, Belgium), with a 50-kV X-ray source at 800 µA. Scanning parameters included a 17 µm isotropic resolution, a rotation step of 1º, 180˚ rotation around the vertical axis and a 0.5-mm-thick aluminum filter, generating at the end of the scan 188 images each sample. The datasets images were reconstructed with the NRecon v. 1.6.10.4 software (Bruker micro-CT, Kontich, Belgium), using a ring artifact reduction of 6, beam hardening correction of 50% and smoothing of 8.

The differentiation between dentine and the filling material, as well as the volume quantification of the remaining material, was performed with the CTAn v. 1.12 software (Bruker micro-CT). The analyses were performed in all apical fragments.

Measurements of sample length (mm), filling material volume (mm^3), total canal volume (mm^3), unfilled canal volume (mm^3), and the distance from filling material to the foramen (mm) were performed using ImageJ software v.1.51n (Fiji implementation; National Institutes of Health, Bethesda, MD) (NEVES *et al.*, 2015). After dentin segmentation, root canal volume was obtained through task list, in the custom processing preview option and then the binary value set on the histogram (255/255) that provided the filling material representation in the CTAn software was selected in morphometry preview (3D analysis). CTVol software (v.2.3.2; Bruker micro-CT) was used for qualitative 3D visualization. After scanning, the specimens were stored frozen and subsequently subjected to cryogenic pulverization.

4.3.7. Cryogenic grinding

Root fragments were thawed and then subjected to a cleaning and disinfection procedure using sterile cotton applicators in the external portion, always with special care to not reach the apical foramen area. In a laminar flow chamber, each sample was cleaned with 3% hydrogen peroxide, disinfected with 5% NaOCl, and then inactivated with 10% sodium thiosulfate. A sterility control sample was taken from the root surfaces using a #20 paper point moistened in Tris-EDTA buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 7.6) and stored in the same buffer at -20ºC.

Each root fragment was transferred to an individual polycarbonate grinding vial containing a magnetic steel bar that oscillated back and forth against two stationary stainless steel end plugs, closing the vial (volume = 25 mL) until pulverization was achieved. Three grinding vials were placed in a metal tube that was hermetically sealed and then inserted into a 6750–freezer mill (Spex, Metuchen, NJ) operated with liquid nitrogen at -200°C. The parameters used in the freezer mill were the following: 1. precooling for 15 minutes (before grinding, when the vial was immersed in liquid nitrogen); 2. grinding for 1 minute; 3. recooling for 0.1 minute (a period between two working cycles when the oscillation of the impact bar was interrupted to avoid heating resulting from attrition). Two grinding cycles were performed per specimen. The impact bar frequency was set at 10, which is 20 actual impacts per second (one to each side). After samples were ground, the obtained powder was placed in sterile Eppendorf tubes under the laminar flow chamber and conserved at -20°C until DNA extraction.

4.3.8. DNA extraction

For greater accuracy of bacterial counts and comparison with other studies, the apical root powder was weighed, and the bacterial counts obtained were converted to the equivalent per 100 mg of root powder. The apical root powder was suspended in

Tris-EDTA buffer, and the DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. For bacterial quantification, the final volume of the extracted DNA solution for each sample was approximately 200 □L. The samples were frozen at -20ºC until qPCR analysis.

4.3.9. Bacterial quantification by qPCR

The levels of total bacteria, *Streptococcus* spp. and members of Actinobacteria phylum were determined for each root fragment. A qPCR assay based on the 16S ribosomal RNA gene was conducted with the Power SYBR Green Master Mix (Thermo Fisher Scientific, Foster City, CA, USA) in an ABI 7500 real-time PCR device (Thermo Fisher Scientific).

A template containing 20 □L of total reaction volume, 0.5 □mol/L of primer concentration, and 2 □L of extracted DNA was used for each sample. Reactions were processed in MicroAmp Optical 96-well plates (Applied Biosystems, Foster City, CA, USA) that were sealed, centrifuged, and subsequently subjected to amplification.

The temperature profile for the qPCR included cycles of 95□C/10min and 40 repetitions with the following steps: 95□C/1min, annealing at 52□C/1 min (for universal primers - total bacteria) or 60□C/1 min (for the *Streptococcus* and Actinobacteria primers), and 72□C/1 min. At each cycle, the accumulation of PCR products was detected by monitoring the increase in fluorescence of the dye that indicated double-stranded DNA binding. Each sample and controls were obtained in triplicate. After amplification, melt curve analysis of the PCR products was performed to determine the specificity of the amplified products. The melt curve was obtained from 60□C a 95□C, with continuous fluorescence measurements taken at every 1% increase in temperature.

Data collection and analysis were performed using ABI 7500v2.0.4 software (Applied Biosystems). Bacterial counts for each sample were based on standard curves constructed by dilutions of known concentrations of DNA extracted from the following strains: *Streptococcus mutans* ATCC 25175 (American type Culture Collection, Manassas, VA, USA) (for universal primers and specific primers for *Streptococcus* spp.) and a clinical isolate of *Actinomyces israelii* (for Actinobacteria primers).

The isolated pure bacterial DNA was quantified, diluted 10-fold from 10^7-10^2 cells in Tris-EDTA buffer, and then used for the construction of the standard curves. The prevalence of the species was calculated according to the positive results obtained in the qPCR. The relative abundance was calculated as the proportion of each bacterial taxon in relation to the total bacterial load in the same sample. Consequently, each target taxon was categorized as low abundant (<10% of the entire community), dominant (10%-90% of the entire community), and highly dominant (>90% of the entire community).

4.3.10. Statistical methods

Frequencies and percentages were used to describe categorical variables. Numerical data were assessed for normality using the Shapiro-Wilk test, and normally distributed variables were summarized using the means and standard deviations. Numerical variables that deviated from the normality assumption were summarized using the medians and range (minimum, maximum).

The negative binomial (NB) regression was used to model counts of bacteria, which were overdispersed with the variance of the counts far exceeding the distributional mean. The NB models were used to examine whether the presence or

absence of apical periodontitis lesion and its size, as well as the other parameters, were associated with increased or decreased bacterial counts. The level of significance was .05.

5. RESULTS

5.1. Demographic characteristics of the study participants

A total of 25 patients were included in this study; there was a preponderance of female sex (18/25; 72%) (Table 2). The overall mean age was 57.7 □ 11.5 years with males, on average being older than females. Most of the participants (76%) were non-smokers.

Table 2. Demographic characteristics of the study participants

Demographic characteristics (Individual level): (n = 25)	**n (%)**
Gender	
Male	7 (28.0)
Female	18 (72.0)
Smoking status	
Non-smoker	19 (76.0)
Current smoker	6 (24.0)
Overall mean age in years (Mean ± SD)	57.7 ± 11.5
Males (Mean ± SD)	60.7 ± 11.2
Females (Mean ± SD)	55.7± 11.4

5.2. Clinical parameters of the evaluated root canals

Evaluations of the quality of filling and limit obturation were performed for each tooth in CBCT and micro-CT. The quality of obturation was classified as inadequate or adequate using CBCT, and the percentage of unfilled canals was determined with micro-CT (Table 3).

Out of the 22 roots assessed without apical periodontitis lesions, only 2 exhibited inadequate root canal filling. Additionally, 8 roots belonged to teeth without coronal

restorations; 4 had poor coronal restorations, and 10 had satisfactory coronal restorations. In contrast, among the roots assessed with root canal treatment and apical periodontitis, 18 out of 23 exhibited an acceptable root canal filling. Regarding coronal restoration, it was absent in 9 teeth, inadequate in 10, and only appropriate in 4. The distribution of clinical variables showed that almost 85% of the root canal fillings can be described as adequate quality. Micro-CT findings revealed that the median percentage of unfilled teeth was 0.2% mm^3.

The limit of obturation was determined in CBCT as 2mm, between 0.5 and 2 mm, and overfilled. The micro-CT allowed for more precise measurements of the obturation limit. The limit at 0 (anatomical apex of the tooth) was the largest percentage (66.7%), whereas the CBCT images revealed that most root canals were located between 0 and 2 mm (64.4%). Figure 1 shows representative correlative CBCT and micro-CT images of the specimens evaluated.

Table 3. Distribution of clinical parameters of the study participants

Clinical variables (tooth level): (n = 45)	**n (%)**
CBCT - Quality of filling	
Inadequate	7 (15.6)
Adequate	38 (84.4)
Micro CTU unfilled canal: Median (min, max)	0.2 (0.0, 1.75)
Unfilled (%): Median (min, max)	12.3 (0.0, 100.0)
Micro CT limit obturation	
0	30 (66.7)
0.5 - 2	11 (24.4)
> 2	4 (8.9)
CBCT limit obturation	
0 - 2	29 (64.4)
> 2	13 (28.9)
Overfilled	3 (6.7)

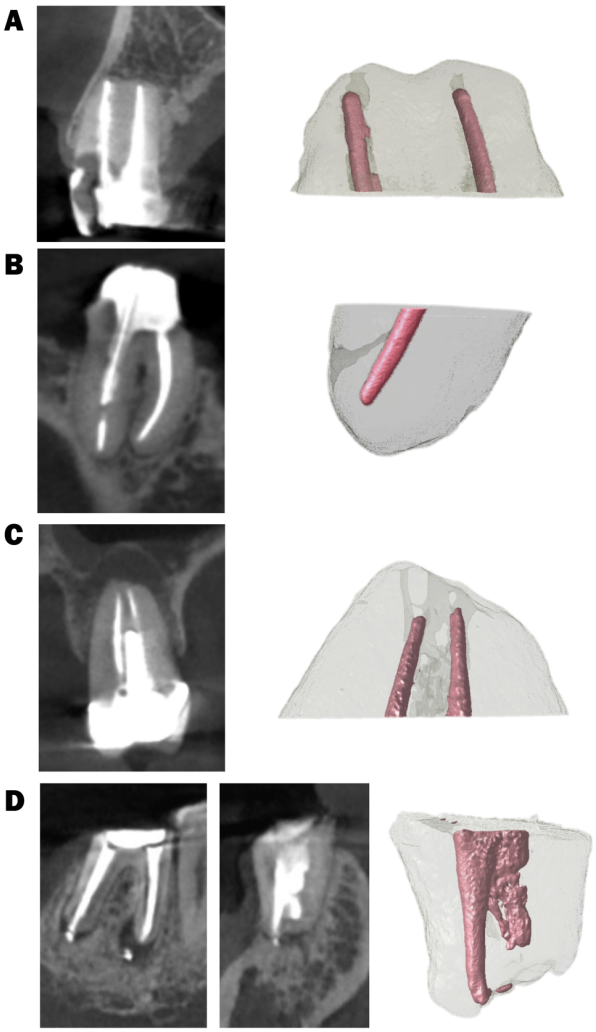

Figure 1. Representative cone-beam-computed tomographic images of root canal-treated teeth and micro-computed tomographic scans of their respective apices. **A.** Maxillary first premolar with no apical periodontitis lesion. **B.** Mesial root of a mandibular second molar with no lesion. **C.** Maxillary second molar with a large apical periodontitis lesion. **D.** Distal root of a mandibular first molar with a small apical periodontitis lesion.

5.3 Distribution of bacterial counts by the presence or absence of apical periodontitis and by lesion size

This study evaluated 45 apical fragments, 23 with apical periodontitis lesion and 22 from teeth with no detectable lesion on CBCT. Using the same universal qPCR assay used to assess total bacteria, sterility control samples obtained prior to cryogenically grinding demonstrated negative results for bacterial DNA. All root fragments with apical periodontitis (23) were positive for Streptococci and

Actinobacteria, whereas only 4 of the 22 fragments without apical periodontitis were negative for bacteria. Streptococci occurred in 20/23 (87%) specimens with lesion (15 small and 5 large), and 18/22 (82%) with no lesion. Streptococci were detected in specimens with apical periodontitis in 1.45 x 10^3 and without lesions in 1.98 x 10^3. Actinobacteria were found in 22/23 (96%) specimens with posttreatment lesion (16 small and 6 large), and 18/22 (82%) teeth with no lesion. Actinobacteria loads in apical fragments with lesion were 6.08 x 10^3 cells, and in fragments without apical lesions were 7.93 x 10^3 cells, showing an amount higher than *Streptococcus* spp. Findings showed that the mean total counts of bacteria, Streptococci and Actinobacteria did not differ significantly between sites with apical periodontitis from sites that did not have lesions. Root fragments without apical periodontitis contained a mean of 9.41 x 10^3 bacterial cells while those with apical periodontitis presented 9.88 x 10^3 (Table 4).

Table 4. Mean distribution of counts of bacteria by the presence/ absence of periapical lesion and lesion size

	Total bacteria	***Streptococcus***	**Actinobacteria**
Apical periodontitis			
No	9.41×10^3	1.98×10^3	7.93×10^3
Yes	9.88×10^3	1.45×10^3	6.08×10^3
Overall mean counts	9.65×10^3	1.71×10^3	6.98×10^3
P-value	0.93	0.65	0.64
Apical periodontitis lesion size			
No lesion	9.41×10^3	1.98×10^3	7.93×10^3
Small lesion	1.06×10^4	4.03×10^2	5.50×10^3
Large lesion	8.21×10^3	3.86×10^3	7.39×10^3
Overall mean counts	9.65×10^3	1.71×10^3	6.98×10^3
[1]*P*-value (ref: No lesion)	0.84	**0.03**	0.56
[2]*P*-value (ref: No lesion)	0.86	0.48	0.93

P-values were obtained from unadjusted negative binomial regression models
[1]Intragroup observations
[2]Intergroup observations

The lesion volume of the 23 root fragments with apical periodontitis lesion was determined to be small for 16 and large for 7 specimens. Small lesions presented

higher counts of *Streptococcus* spp. compared to apical fragments with no lesion and large lesion with significantly differences p=.03. Large lesions presented a total count of 8.21 x 10^3 bacterial cells. However, there was no significant differences in bacterial counts comparing the lesion size. Table 3 shows the mean distribution of bacterial counts by the presence or absence of apical periodontitis and by lesion size.

The median distribution of total bacteria counts by the presence or absence of apical periodontitis lesion is shown in Figure 1. The median distribution of the total bacterial counts by lesion size is presented in Figure 2.

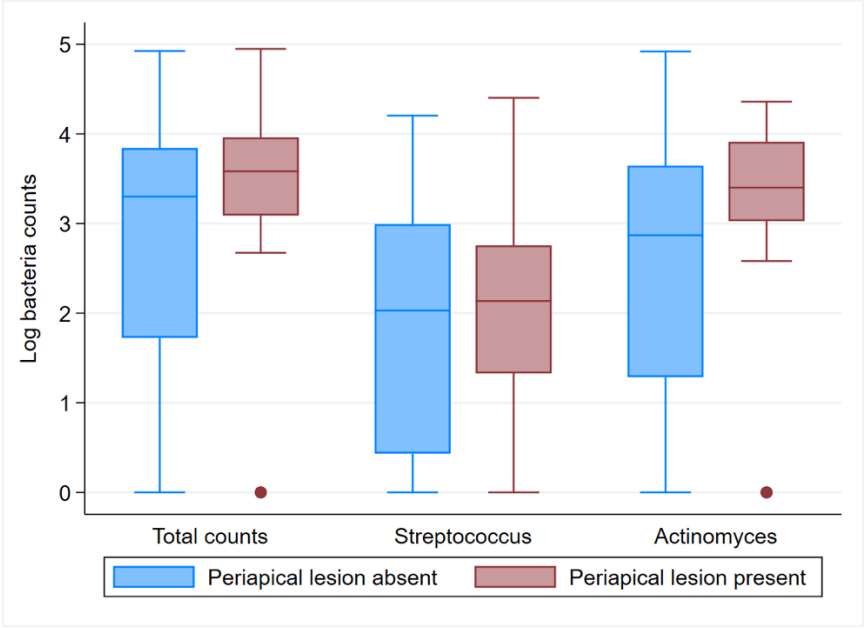

Figure 2. Box plots showing the median distribution of total bacteria, *Streptococcus* and Actinobacteria (log-transformed) by the presence or absence of apical periodontitis. The box plots are overlapping, thus there are no differences between the two groups (apical periodontitis absent vs. present). The red filled circles in the plot indicate outliers.

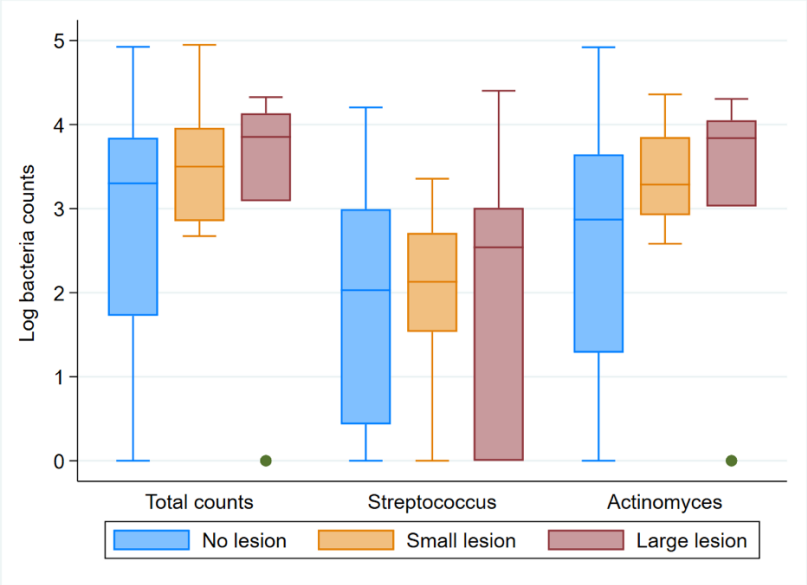

Figure 3. Box plots showing the median distribution of total bacteria, *Streptococcus* and Actinobacteria (log-transformed) by apical periodontitis lesion size. The box plots are overlapping, thus there are no differences between the three groups (no lesion, small lesion, and large lesion). The green filled circles in the plot indicate outliers.

5.4. Factors associated with bacterial counts

Table 5 shows unadjusted estimates of the Incidence Rate Ratio (IRR) with their 95% confidence intervals (CIs). Our findings showed that counts of *Streptococcus* spp. were significantly lower by 80% in small lesions than in no lesions. Female patients presented a significant higher risk of total bacterial counts 3.39 (1.19, 9.63) and *Streptococcus* spp. counts were 9.07 (2.54, 32.39) times significantly higher than Actinobacteria among female patients compared to male.

CBCT limit of obturation > 2 mm short was significantly associated with more total bacterial counts than CBCT obturation limit at 0-2 mm short increasing the risk 3.30 (1.05, 10.33) times. Adequate coronal restoration and intraradicular post were significantly associated with 85% lesser counts of *Streptococcus spp.* The presence of an adequate intraradicular post decreased the risk of developing high counts of

Streptococcus spp. by 88% when compared to canals that lacked a post. The results from the adjusted analyses were not statistically significant and are not presented.

Table 5. Estimates of IRR and their 95% CIs showing factors that were associated with bacterial counts

	Total counts		Streptococcus		Actinobacteria	
	IRR (95% CI)	***P*-value**	**IRR (95% CI)**	***P*-value**	**IRR (95% CI)**	***P*-value**
Apical periodontitis presence (ref: absent)						
Present	1.05 (0.36, 3.03)	0.93	0.73 (0.19, 2.77)	0.65	0.77 (0.25, 2.31)	0.64
Apical periodontitis lesion size (ref: No lesion)						
Small	1.13 (0.35, 3.62)	0.84	**0.20 (0.05, 0.83)**	**0.03**	0.69 (0.21, 2.33)	0.56
Large	0.87 (0.19, 4.08)	0.86	1.94 (0.31, 12.39)	0.48	0.93 (0.19, 4.63)	0.93
Demographics:						
Age in years	0.97 (0.93, 1.01)	0.19	0.98 (0.92, 1.04)	0.47	0.98 (0.94, 1.02)	0.30
Gender (ref: Male)						
Female	**3.39 (1.19, 9.63)**	**0.02**	**9.07 (2.54, 32,39)**	**< 0.01**	2.83 (0.94, 8.50)	0.06
Smoking (ref: No)						
Yes	0.95 (0.28, 3.25)	0.93	0.29 (0.06, 1.34)	0.11	1.42 (0.39, 5.11)	0.59
Clinical factors:						
Quality of filling (ref: Adequate)						
Inadequate	2.03 (0.48, 8.67)	0.34	0.81 (0.13, 5.08)	0.82	2.92 (0.66, 12.98)	0.16
Micro CT unfilled canal	0.47 (0.09, 2.42)	0.37	1.08 (0.13, 9.07)	0.94	0.49 (0.07, 3.36)	0.47
Unfilled	1.01 (0.99, 1.03)	0.29	1.03 (0.98, 1.08)	0.31	1.02 (1.00, 1.04)	0.11
Micro CT limit obturation (ref: 0)						
0.5 - 2	0.58 (0.17, 1.99)	0.39	1.82 (0.38, 8.75)	0.45	0.78 (0.22, 2.78)	0.71
> 2	2.36 (0.37, 15.22)	0.37	1.52 (0.14, 16.16)	0.73	3.68 (0.54, 25.17)	0.19
CBCT limit obturation (ref: 0 – 2)						
> 2	**3.30 (1.05, 10.33)**	**0.04**	3.36 (0.79, 14.18)	0.10	2.91 (0.88, 9.58)	0.08
Overfilled	3.24 (0.41, 25,82)	0.27	6.35 (0.46, 87.18)	0.17	4.17 (0.48, 36.52)	0.20
Coronal restoration (ref: absent)						
Inadequate	0.66 (0.19, 2.33)	0.52	0.51 (0.11, 2.43)	0.40	0.94 (0.25, 3.51)	0.93
Adequate	0.33 (0.09, 1.17)	0.09	**0.15 (0.03, 0.71)**	**0.02**	0.42 (0.11, 1.55)	0.19
Abutment tooth (ref: No)						
Yes	0.75 (0.16, 3.56)	0.72	0.18 (0.03, 1.23)	0.08	1.00 (0.20, 5.06)	1.00
Intraradicular post (ref: No)						
Yes	0.74 (0.22, 2.54)	0.63	0.91 (0.19, 4.27)	0.90	1.01 (0.28, 3.66)	0.98
Intraradicular post quality (ref: absent)						
Inadequate	1.29 (0.20, 8.31)	0.79	2.28 (0.23, 22.42)	0.48	1.82 (0.26, 12.66)	0.55
Adequate	0.43 (0.10, 1.84)	0.25	**0.12 (0.02, 0.73)**	**0.02**	0.55 (0.12, 2.53)	0.45

6. DISCUSSION

This multi-analytical study evaluated the bacteriological conditions in the apical region of the root canal system of teeth with and without post-treatment apical periodontitis. Bacterial counts in the cryopulverized root apexes were evaluated for their correlation with the presence/absence of apical periodontitis lesion, as well as its volume as determined by CBCT, and the technical quality of the obturation as determined by both CBCT and micro-CT.

Post-treatment endodontic infections usually result from persistent infections caused by microorganisms that were involved in either primary or secondary infections. The infections persist mainly intraradicularly, or less frequently, extraradicularly after root canal treatment (SIQUEIRA, 2001; RICUCCI & SIQUEIRA, 2008; RICUCCI *et al.*, 2009; NEELAKANTAN *et al.*, 2017).

The microbiota of previously treated teeth with persistent apical periodontitis differs widely from primary endodontic lesions (MOLANDER *et al.*, 1998; SUNDQVIST *et al.*, 1998; HANCOCK *et al.*, 2001; CHUGAL *et al.*, 2011). The most predominant phyla found in post-treatment endodontic infections are Firmicutes, Proteobacteria and Actinobacteria (SIQUEIRA & RÔÇAS, 2007). Phenotypic identification procedures have shown that facultative anaerobic gram-positive bacteria are predominant in secondary/persistent endodontic infections (MÖLLER *et al.*, 1981; MOLANDER *et al.*, 1998; GOMES *et al.*, 2004; SIQUEIRA & RÔÇAS, 2009b; BARBOSA-RIBEIRO *et al.*, 2021). This study quantified two groups of gram-positive species that have been well-documented in treated teeth with post-treatment infection *Streptococcus* spp. and *Actinobacteria* (PINHEIRO *et al.*, 2003; SCHIRRMEISTER *et al.*, 2009; CHUGAL *et al.*, 2011; RÔÇAS & SIQUEIRA, 2012; ANTUNES *et al.*, 2015; SIQUEIRA *et al.*, 2020; DE CASTRO KRULY *et al.*, 2022).

Streptococcus spp. were detected in specimens with apical periodontitis in a mean cell number of 1.45 x 10^3 and without lesions in a mean of 1.98 x 10^3. Actinobacteria loads were higher than *Streptococcus* spp. in apical fragments with periapical lesion (6.08 x 10^3), and in fragments without lesions (7.93 x 10^3). Overall, the mean bacterial counts were low, in the order of 10^3, which have been suggested to be compatible with a normal radiographic status of the periradicular tissues in some cases (ZANDI *et al.*, 2019), and helps explain the counts found in teeth with no lesion in this study. Our results support the findings from ANTUNES *et al.* (2015), in which total bacterial counts of endodontically treated teeth with apical periodontitis were lower for *Streptococcus* spp. (4 x 10^1) and higher for Actinobacteria (6.4 x 10^3).

The high prevalence and dominance of *Streptococcus* spp. suggest that they are relevant in post-treatment endodontic disease (ANTUNES *et al.*, 2015; SIQUEIRA *et al.*, 2020). The interactions of *Streptococcus* spp. with other species are multifaceted because these include intra and interspecies as well as interactions with the host (KRETH *et al.*, 2009). The pathogenicity and virulence of *Streptococci* are dependent upon their metabolic mechanisms, which are influenced by their ecological interactions with commensal bacteria in each particular biofilm environment (SHELBURNE *et al.*, 2008; SHELBURNE *et al.*, 2008).

Member of the Actinobacteria phylum are normal commensals of the oral microbiota that can live in equilibrium in the host tissues without inducing an acute response, but they can maintain chronic periapical inflammation. Their relatively low virulence and ability to form cohesive colonies could be the reason why they remain in a chronic periapical lesion, being one of the predominant microorganisms in extraradicular infections (SIQUEIRA, 2003; DIOGUARDI *et al.*, 2020).

The development of disinfection techniques to remove biofilm or achieve "sterilization" in the root canal system has been the focus of numerous studies

(SIQUEIRA, 2011; NEELAKANTAN *et al.*, 2017). Although multiple disinfection strategies in endodontic therapy seek to reduce the bacterial load to levels compatible with the host immune response, that allow repair of the apical or periradicular tissues, to date, it has not been possible to demonstrate a complete removal of the microorganisms involved or to identify the relationship between the number of residual bacteria in the obturation compatible with periradicular health (HAAPASALO *et al.*, 2003; SIQUEIRA & RÔÇAS, 2008; RICUCCI & SIQUEIRA, 2010; HAAPASALO *et al.*, 2011; NEELAKANTAN *et al.*, 2017).

Various factors used during endodontic therapy, such as the type of irrigant, the irrigation technique, the size of the apical preparation, the use of intracanal medication, and the number of visits may determine the success of the treatment (ORDINOLA-ZAPATA *et al.*, 2022). However, the variability of the root canal system and the polymicrobial nature of endodontic infections make the disinfection process complex (NEELAKANTAN *et al.*, 2017). The persistence of intraradicular bacteria generally occurs due to their resistance or inaccessibility to instruments, irrigants, or medications in anatomical complexities such as lateral canals (RICUCCI *et al.*, 2013), apical ramifications, isthmuses (RICUCCI & SIQUEIRA, 2010) and dentinal tubules (VIEIRA *et al.*, 2012).

Several studies have provided evidence for the existence of residual bacteria in the root canal system during obturation, which indicates an elevated possibility of endodontic failure (SJÖGREN *et al.*, 1997; SUNDQVIST *et al.*, 1998; FABRICIUS *et al.*, 2006). This study found by cryogenic grinding and qPCR that apical fragments of endodontically treated teeth without apical periodontitis contained a total of 9.41 x 10^3 bacterial cells, while those with apical periodontitis held 9.88 x 10^3 cell equivalents. The results indicated that the mean total counts of *Streptococcus* spp. and Actinobacteria did not differ significantly, suggesting that post-treatment apical

periodontitis harbors residual bacteria after root canal disinfection regardless of the presence or absence of apical periodontitis. To our knowledge, the present study is the first to identify total bacteria counts by cryogenic grinding and qPCR in endodontically treated teeth without apical periodontitis.

Most studies that have used molecular techniques to evaluate the microbiota in post-treatment apical periodontitis have focused on identifying the composition and diversity of the microbiota (GOMES *et al.*, 2021; DE CASTRO KRULY *et al.*, 2022; PÉREZ-CARRASCO *et al.*, 2023). Some studies, that have evaluated bacterial counts using culture and molecular techniques, agree that an endodontically treated tooth with post-treatment harbor bacterial loads between 10^3 and 10^7 (PECIULIENE *et al.*, 2001b; SEDGLEY *et al.*, 2006; ANTUNES *et al.*, 2015; ZANDI *et al.*, 2019). The evaluation of bacterial levels in endodontically treated teeth with post-treatment apical periodontitis has been mainly based on samples obtained from root canals with sterile paper points, and analyzed by molecular methods at different stages of retreatment (post-instrumentation and post-intracanal medication) (BLOME *et al.*, 2008; ZANDI *et al.*, 2019; BARBOSA-RIBEIRO *et al.*, 2020; BARBOSA-RIBEIRO *et al.*, 2021). All molecular studies agree on a gradual reduction in bacterial loads, depending on the stage of endodontic treatment, obtaining the greatest reduction (10^3 to 10^5) after intracanal medication (BLOME *et al.*, 2008; ANTUNES *et al.*, 2015; ZANDI *et al.*, 2018).

In persistent post-treatment infections, bacteria are not effectively controlled or eradicated during the initial endodontic intervention. Bacteria introduction occurs in secondary infections during root canal therapy due to a break in the aseptic chain, minimally invasive accesses, missed canals, insufficient instrumentation, and/or inadequate post-endodontic treatment restoration (NAIR *et al.*, 1990; SIQUEIRA & RÔÇAS, 2008; VIEIRA *et al.*, 2020). Bacteria have the ability to survive in normal or

reduced presence of nutrients in situations where nutrient levels decrease, and also to develop strategies for adapting to new environmental conditions (SIQUEIRA & RÔÇAS, 2008).

After chemomechanical instrumentation large amounts of bacteria may remain adhered in irregular canals or in areas of difficult access such as recesses, ramifications and isthmuses (PÉREZ *et al.*, 2020). Also, the possibility exists that the endodontic sealer entombs microorganisms in anatomical complexities after filling, preventing their access to periradicular tissues or even causing their death. Even when entombed, some bacterial species can survive prolonged periods of nutrient deprivation or acquire nutrients from remaining pulp tissue, dead cells, or even from the metabolic products of other species. Microleakage or filtration of substrates through inadequate restorations, periradicular tissue fluids, or inflammatory exudates from lateral canals or through the apical foramen/foramina may promote intraradicular bacterial growth. As the number of bacteria colonizing the root canal increases, they are able to migrate to the periradicular tissues and establish themselves to initiate or sustain periradicular infection (SIQUEIRA, 2001).

Another aspect to consider is the bacterial interactions that determine the degree of virulence of the community, which is considered the unit of pathogenicity of apical periodontitis (SIQUEIRA & RÔÇAS, 2022b). Virulence factors are structural components, and secreted products of bacterial cells. They are involved in cell adhesion, invasion, and proliferation, causing direct or indirect damage to host tissues. Also, they participate in bacterial survival strategies and the host's immune response (SIQUEIRA, 2011).

Most studies, that have evaluated the microbiota in post-treatment endodontic infections, have used samples obtained from the root canal (ZANDI *et al.*, 2018; BARBOSA-RIBEIRO *et al.*, 2021; DE CASTRO KRULY *et al.*, 2022). However,

morphological studies have demonstrated that the apical segment of the root has the greatest number of anatomical complexities, which may favor bacterial persistence to maintain periradicular inflammation (SJÖGREN *et al.*, 1990; ABOU-RASS & BOGEN, 1998; RICUCCI *et al.*, 2009; ARNOLD *et al.*, 2013; RICUCCI *et al.*, 2013).

Few studies have evaluated the microbiota and bacterial counts of post-treatment apical periodontitis using apical fragment samples obtained from periradicular surgeries or extracted teeth (ANTUNES *et al.*, 2015; SIQUEIRA *et al.*, 2016; KESKIN *et al.*, 2017; BOUILLAGUET *et al.*, 2018; SIQUEIRA *et al.*, 2020). The present study used standardized apical fragments with and without apical periodontitis from extracted teeth that were cryopulverized and analyzed with qPCR.

These results indicate that most of the teeth used in this investigation were adequately filled with an acceptable apical seal. Likewise, adequate coronal restoration and intraradicular post were significantly associated with lesser counts of *Streptococcus* spp. The endodontically treated teeth without apical periodontitis used in this study showed that, although residual bacteria were present, an adequate coronal and apical seal is compatible with the health of periapical tissues. In addition, streptococci were also in lower numbers in teeth with an adequate coronal restoration, suggesting that a defective coronal sealing may favor the entrance and colonization of the root canals by these bacteria, which are amongst the most abundant taxa in saliva and plaque (AAS *et al.*, 2005; BELSTRØM *et al.*, 2014). The quality of root canal filling may affect the treatment outcome (NGMANNRAHBARAN *et al.*, 2008) and may be used as a surrogate for the other steps involved in infection control, particularly root canal preparation. Previous studies have shown that the amount of unfilled space may influence some features of posttreatment apical periodontitis, including lesion size and the presence of symptoms (SIQUEIRA *et al.*, 2020; VILLA-MACHADO *et al.*, 2020). The present findings of unfilled canal space as determined by micro-CT have not

confirmed these previous reports. However, a significantly higher bacterial load was observed in the apical canal system of teeth filled more than 2 mm short of the apical foramen as determined by CBCT. This is in consonance with the reports of a significantly lower success rate for teeth with underfillings (SJÖGREN *et al.*, 1990). It is likely that in these underfilled cases, the length of unfilled canal may have also been unprepared, allowing bacterial infection to remain unaffected. These results can be attributed to the fact that a favorable immune response from the host is an additional factor that influences the action of residual microorganisms, favoring the repair of the apical periodontitis lesion (SIQUEIRA, 2011; SEGURA-EGEA *et al.*, 2022).

The radiographic evaluation of the size of the apical periodontitis in treated teeth has been an important indicator in the success rate of endodontic treatment, being the repair associated with success and health of the periradicular tissues, and its persistence or the increase of the lesion size related to a post-treatment apical periodontitis (CHUGAL *et al.*, 2017). CBCT has demonstrated its efficacy and accuracy to identify the volume (mm^3) in small and large apical periodontitis lesions (BOUBARIS *et al.*, 2021). In the present study, the volume of the 23 root fragments with apical lesion was determined to be small for 16 and large for 7. Small lesions presented higher count of *Streptococcus* spp. compared to apical fragments with no lesion and large lesion. These findings agree with those reported by SIQUEIRA *et al.* (2020) in apical fragments of teeth endodontically treated with apical periodontitis. The authors suggest that this may due to the fact that the *Streptococcus* spp. could be present in the initial phase of the formation of the apical lesion. More research is necessary to confirm this finding.

Large lesions presented a total count of 8.21×10^3 bacterial cells; however, this was not significantly different from teeth with small lesions. CHEUNG & HO (2001) showed the non-correlation between the microbiota and the size of the lesion in 18

endodontically treated teeth associated with asymptomatic apical periodontitis. Facultative gram-positive cocci were present in all the canals evaluated. Most of the samples contained less than 2.5 x 10^4 CFU/ml. The size of the periapical radiolucency did not show any relationship with the quantity of microorganisms recovered by culture. These findings are somewhat unexpected, as teeth with apical periodontitis, particularly with larger ones, are expected to harbor a higher infectious load. Although the size of the apical periodontitis lesion has been shown to be directly proportional to the bacterial diversity in the root canal (SUNDQVIST, 1976; SIQUEIRA *et al.*, 2007; RÔÇAS & SIQUEIRA, 2008; AMARAL *et al.*, 2020), data on the bacterial load (cell numbers) are rare in endodontic microbiological studies, based on nonsensitive culture methods, and inconsistent in terms of looking for associations with lesion size (SUNDQVIST, 1976; BYSTRÖM *et al.*, 1987; SIQUEIRA *et al.*, 2007) . However, it is important to point out that the pathogenicity of a given bacterial community depends not only on the number of cells (load), but also on the diversity represented by the richness (number of different species) and abundance (proportion of the species in the community) as well as the interactions among the different community members and between them and the surrounding environment (SIQUEIRA & RÔÇAS, 2022c). These factors were not evaluated in this study and based on the present findings on counts, they are likely to be the most relevant in influencing the development and progress of posttreatment apical periodontitis. The host susceptibility is certainly another factor of great impact on this equation (SIQUEIRA, 2002).

It is interesting to note that in this study female patients presented a significant higher risk of total bacterial counts 3.39 (1.19, 9.63) and *Streptococcus* spp. counts were 9.07 (2.54, 32,39) times significantly higher than Actinobacteria spp. among female patients compared to male. Although women were more prevalent (72%) than men (28%) in this study, the reasons for differences in bacterial counts are not

apparent. Retrospective studies have shown periapical granulomas have a predilection for women (BECCONSALL-RYAN *et al.*, 2010; TAVARES *et al.*, 2017; COUTO *et al.*, 2021). An epidemiological study that evaluated the prevalence of periapical granuloma, radicular cyst or periapical abscess with histopathological results in 10,381 cases of Brazilian subjects found these chronic infections were more frequent in women (56.1%) compared to men (41.6%) (COUTO *et al.*, 2021). Similarly, another retrospective study evaluated 647 inflammatory apical periodontitis lesions, including 244 periapical granulomas and 403 periapical cysts, in which females (56%) were more prevalent than males in the presence of periapical granulomas (61%) and periapical cysts (53%) (TAVARES *et al.*, 2017). Generally, the frequency in women in the results of epidemiological studies of chronic endodontic infections has been attributed to their concern for oral health care (GEORGOPOULOU *et al.*, 2005). However, this female gender factor should be considered in future research on post-treatment endodontic infections in order to determine whether or not there is an association.

This study has many strengths. The multi-analytical approach was devised to evaluate exclusively the apical portion of the root canal system, an area regarded as of critical relevance for disease pathogenesis and treatment (SIMON, 1994) . CBCT, which has higher sensitivity to detect bone lesions in comparison with periapical radiographs (DE PAULA-SILVA *et al.*, 2009; PATEL *et al.*, 2015), was used to determine the presence of the apical periodontitis lesion. In addition, most studies that evaluated the size of the lesion were based on its largest bidimensional diameter; this study used CBCT for a more accurate and 3D evaluation of the lesion size by determining its volume. The parameter for classifying the lesion as small or large was considering the volume of a sphere with 5 mm in diameter, a cut-off used in previous studies to determine the lesion size by the diameter (NG et al., 2008). Another strength

was the use of micro-CT to evaluate with higher resolution and three-dimensionally the volume and proportion of the unfilled areas in the apical canal. Moreover, cryopulverization was used to process samples for microbiological examination. Sample taking is undoubtedly a key factor in obtaining consistent and reliable microbiological results. The cryopulverization approach used herein can circumvent many of the limitations associated with the conventional paper point approach, including the ability to incorporate the whole root canal system, including the main canal, dentinal tubules, isthmus, ramifications and recesses, in the final sample (ALVES *et al.*, 2009). Finally, qPCR is a highly used, sensitive and accurate approach to detect and quantify bacteria, including difficult-to-grow and uncultivated taxa (SIQUEIRA & RÔÇAS, 2022a). This method was used here to target total bacteria and some taxonomic groups commonly associated with posttreatment apical periodontitis.

This study also has limitations. Because of the difficulties in obtaining this type of clinical samples based on extracted teeth, the sample size may have been relatively small, especially when comparing subgroups, such as lesion size. Another limitation was that the quality of coronal restorations was evaluated on the basis of CBCT findings, which may have overlooked areas of leakage that might have interfered with the microbiological results. In addition, for most cases, the information about the time elapsed since root canal treatment was not available. The possibility exists that some lesions might have been in a healing process, which justifies the low amount of bacteria in some root apical specimens. Finally, this study has not evaluated the possibility of an extraradicular infection being present, either as a bacterial biofilm attached to the outer root surface or within the body of the inflammatory lesion. This was because any external bacteria were eliminated by the root disinfection procedure, to make sure that detected bacteria were indeed within the canal system and not externally as a contaminant during extraction. The lesion was not available for microbiological

evaluation because it was submitted to histopathology and it would be very difficult to distinguish infection from contamination by using qPCR to evaluate lesions obtained during extraction. Despite these limitations, the results of bacterial counts of endodontically treated teeth with post-treatment apical periodontitis are consistent with those previously reported. Further longitudinal studies are needed to broaden the knowledge about the interactions of specific residual bacteria predominant in post-treatment endodontic infections and the effects of current disinfection methods.

CONCLUSIONS

This study found no significant differences in the bacterial load located specifically in the apical canal system of treated teeth with or without apical periodontitis. This suggests that factors other than only bacterial levels, particularly bacterial diversity and host resistance, may have a more significant impact on the development and progression of apical periodontitis. Bacteria were found in the apical canal in virtually all cases, with a high prevalence of streptococci and actinobacteria. Streptococci counts were significantly higher in the apical canal of teeth with inadequate restorations and those with no lesions. Underfilled canals showed higher bacterial counts.

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