CLINICAL RESEARCH

Bacteriologic Conditions of the Apical Root Canal System of Teeth with and without Posttreatment Apical Periodontitis: A Correlative Multianalytical Approach

Sandra R. Hernández, MSc,[*†] *José F. Siqueira, Jr., PhD,*[*‡] *Danielle D. Voigt, PhD,*[*] *Giuliana Soimu, MSc,*[*] *Sabrina C. Brasil, PhD,*[*] *José C. Provenzano, PhD,*[*‡] *Ibrahimu Mdala, PhD,*[§] *Flávio R. F. Alves, PhD,*[*‡] *and Isabela N. Rôças, PhD*[*‡]

SIGNIFICANCE

Bacteria were found in the apical canal system in virtually all cases. However, factors other than bacterial levels, particularly bacterial diversity and host resistance, may have a more significant impact on the development and progression of apical periodontitis. 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.

From the *Postgraduate Program in Dentistry, University of Grande Rio, Rio de Janeiro, Rio de Janeiro, Brazil; †Department of Endodontics, Faculty of Dentistry, Francisco Marroquín University, Guatemala City, Guatemala; ‡Department of Dental Research, Faculty of Dentistry, Iguaçu University, Nova Iguaçu, Rio de Janeiro, Brazil; and §Department of General Practice, University of Oslo, Oslo, Norway

Address requests for reprints to Prof Flávio R.F. Alves, Programa de Pós-Graduação em Odontologia, Universidade do Grande Rio, Rua Professor José de Souza Herdy 1160, Duque de Caxias, RJ, Brazil 25071-202. E-mail address: flavioferreiraalves@gmail.com

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ABSTRACT

Introduction: This study used a correlative multianalytical approach to investigate the bacteriologic 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 lesions. **Methods:** Root apexes were obtained from recently extracted root canal–treated teeth with ($n = 23$) and without ($n = 22$) apical periodontitis lesions as demonstrated by cone-beam computed tomographic examination. The root apexes were sectioned and subjected to micro–computed tomographic (micro-CT) scanning. The specimens were cryopulverized, and DNA extracted from the powder was used as a template in real-time polymerase chain reaction assays to quantify total bacteria and members of the *Streptococcus* genus and Actinobacteria phylum. The bacteriologic findings were compared between the 2 groups and also evaluated for associations with cone-beam computed tomographic and micro–computed tomographic data. **Results:** Bacteria were detected in all apical canal samples except 1. The mean counts of total bacteria, streptococci, and actinobacteria did not differ significantly between teeth with or without apical periodontitis ($P > .05$). *Streptococcus* levels were significantly lower by 80% in the apical canals of teeth with small lesions compared with those without lesions ($P < .05$). The limit of filling >2 mm short was significantly associated with more total bacterial counts compared with canals filled 0–2 mm short ($P < .05$). An adequate coronal restoration was significantly associated with lesser counts of *Streptococcus* ($P < .05$). **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. *(J Endod 2024;50:154–163.)*

KEY WORDS

Cone-beam computed tomography; micro–computed tomography; posttreatment apical periodontitis; quantitative real-time polymerase chain reaction; root canal infection

Apical periodontitis is 1 of the most common inflammatory diseases that affect humans[1] and is caused by bacterial infection of the root canal system[2]. It is usually treated with a relatively high success rate by nonsurgical root canal therapy[3]. Although failure is much more common in cases in which treatment was inadequately performed[3,4], apical periodontitis can persist even in well-treated teeth in 5%–15% of cases[5]. The persistence of the lesion represents not only a health problem but also an economic burden because considerable financial amounts are spent trying to solve the problem via retreatment, surgery, or

even extraction. Although an extraradicular infection can occasionally be involved with posttreatment apical periodontitis, persistent/secondary intraradicular infections represent the most common causes of persistent periradicular inflammation[6].

The root canal microbiome associated with posttreatment lesions differs significantly from that of primary lesions, usually showing different community profiles with lower diversity and different dominant taxa[7-10]. Species of the *Streptococcus* genus and the Actinobacteria phylum are among the most prevalent and abundant taxa found in teeth with posttreatment apical periodontitis[8,11], including samples taken exclusively from the apical part of the root canal system[12,13].

Bacteria present in intricate areas such as isthmuses, ramifications, recesses, and dentinal tubules may not be affected by the antimicrobial intracanal procedures[14,15] and are often the cause of persistent disease even in apparently well-treated teeth[16]. In addition, persistent disease is associated with bacteria located in the apical segment of the root canal[16]. The conventional bacteriologic sampling approach using paper points has important limitations in the sense that it usually fails to retrieve the bacterial cells located in those difficult-to-reach areas and cannot distinguish the region of the main canal sampled (apical, middle, or coronal)[17,18]. These limitations can be overcome by using the cryogenic grinding method[19]. There are many studies reporting on the apical microbiological conditions in teeth with posttreatment disease[12,13,20-23], but only 1 evaluated their association with other intracanal and periapical parameters[12]. Comparisons between treated teeth with and without apical periodontitis have already been conducted to evaluate the prevalence of *Enterococcus faecalis*[24,25] or the community profile using denaturing gradient gel electrophoresis[26]. However, none of these studies used samples taken exclusively from the apical root canal, which remain to be evaluated for differences in the bacterial load.

Data from periapical radiographs, including lesion size and the quality of the root canal fillings, have been widely used in microbiological studies for correlation purposes. However, radiographs have many limitations that can be circumvented by cone-beam computed tomographic (CBCT) imaging, which is more sensitive for lesion detection and can provide 3-dimensional (3D) information[27,28]. Micro–computed tomographic (micro-CT) imaging has a much higher resolution than CBCT imaging and has been widely used in endodontic studies, including for anatomic studies and evaluation of the technical quality of root canal fillings[29,30]. Because of the high radiation used, micro-CT imaging has only been used for the analysis of extracted teeth or in cadavers[31,32]. A correlative multianalytical study of teeth with posttreatment disease showed a significant association of the volume of an unfilled apical canal system, as determined by micro-CT imaging, with lesion size and bacterial counts[12]. Another study demonstrated that inadequate density of fillings was associated with the presence of symptoms and the size of posttreatment lesions[33].

This study used a correlative multianalytical approach to evaluate the bacteriologic conditions of the apical portion of teeth with posttreatment apical periodontitis in comparison with treated teeth with no disease. For this, the presence and counts of total bacteria and species of *Streptococcus* genus and Actinobacteria phylum were determined in cryopulverized root apical specimens using a sensitive quantitative real-time polymerase chain reaction (qPCR) assay. These data were analyzed for correlations with the technical quality of the previous root canal obturation (micro-CT imaging) and the presence and size/volume of apical periodontitis lesions (CBCT imaging).

MATERIALS AND METHODS

Sample Selection and Preparation

The sample consisted of 45 root canal–treated teeth from 25 patients with indication of extraction for reasons not related to this study, including the impossibility of being restored or not being included in the rehabilitation plan. All specimens were collected at the Clinic of Oral Surgery of the Faculty of Dentistry at Francisco Marroquín University. Immediately before tooth extraction, patients rinsed their mouth with 0.2% gluconate chlorhexidine solution for 1 minute. After extraction, blood, saliva, and tissue residues were removed from the tooth surfaces with sterile gauze soaked in saline solution, and the specimens were placed in sterile disposable plastic containers and stored at −20°C. The protocol of this research project was approved by the National Research Ethics Committee (resolution 36-2020). Patients were informed about the nature of the project and signed a consent form.

The inclusion criteria were root canal–treated teeth with and without apical periodontitis lesions as detected by periapical radiographs and further confirmed by CBCT imaging. The exclusion criteria were root fractures, periodontal disease, radicular resorptions, a root canal with separate instruments, a history of root end resection, and patients who received systemic antibiotic therapy during the last 12 weeks before extraction.

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[34,35]. The size of apical periodontitis lesions was classified according to their volume on CBCT imaging as small (≤65 mm^3) or large (>65 mm^3)[35]. The lesion volume was determined using NemoScan 2017 software (Software Nemotech S. L., Madrid, Spain). The cutoff value of 65 mm^3 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 lesions on periapical radiographs[36]. The apical limit of filling was also determined on CBCT imaging and

TABLE 1 - Demographic Characteristics of the Study Participants and the Distribution of Clinical Parameters

Features	Results
Demographic characteristics (individual level) (n = 25)	
Sex	
Male	7 (28.0%)
Female	18 (72.0%)
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
Clinical variables (tooth level) (n = 45)	
Quality of filling	
Inadequate	7 (15.6%)
Adequate	38 (84.4%)
CBCT apical limit filling	
0–2 mm	29 (64.4%)
>2 mm	13 (28.9%)
Overfilled	3 (6.7%)
Micro-CT unfilled canal volume (mm^3), median (min, max)	0.2 (0.0, 1.75)
Micro-CT unfilled canal volume, median (min, max)	12.3 % (0.0%, 100.0%)

CBCT, cone-beam computed tomography; micro-CT, micro–computed tomography; SD, standard deviation.

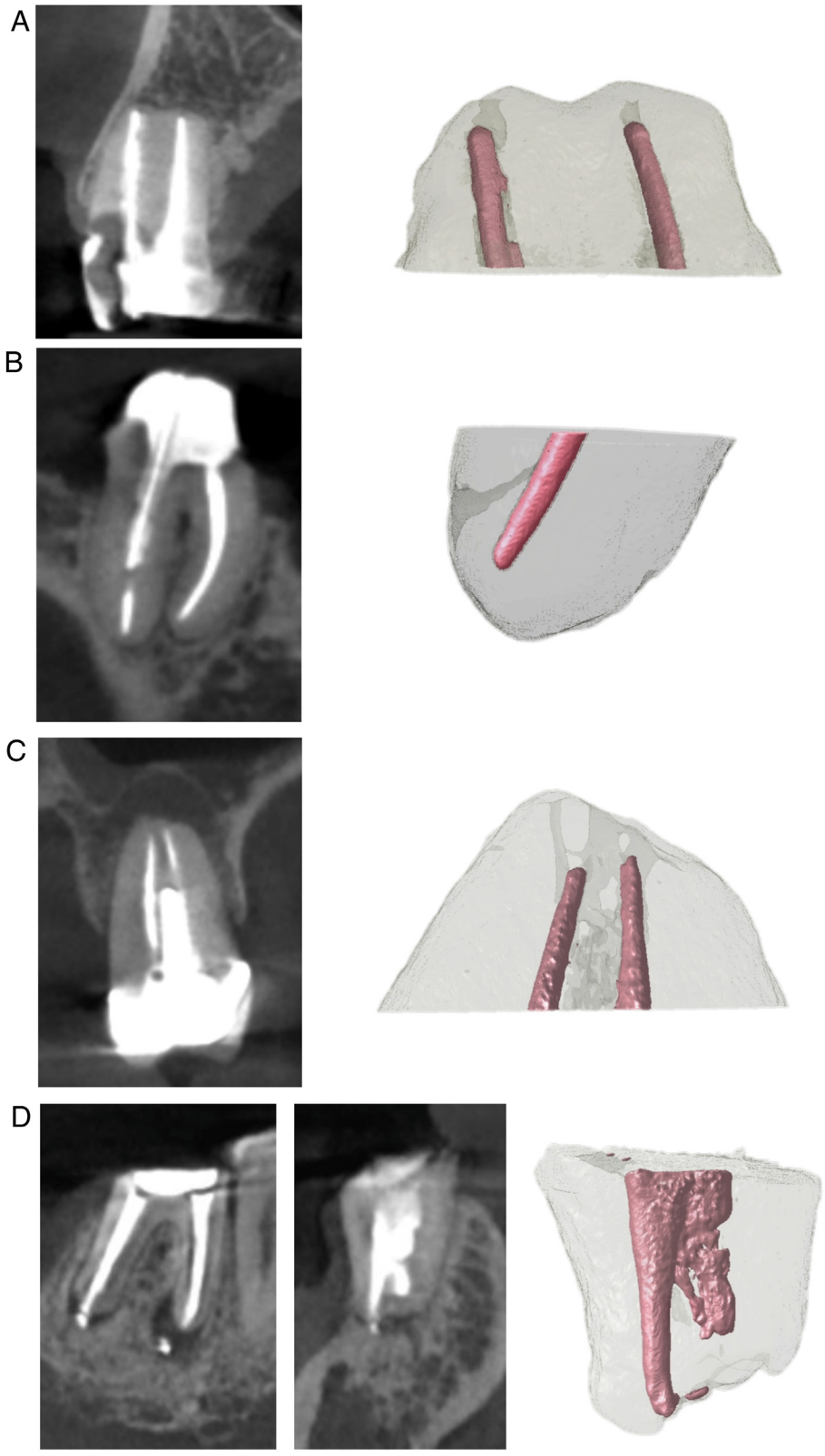

FIGURE 1 – Representative CBCT images of root canal–treated teeth and micro-CT scans of their respective apices. (*A*) A maxillary first premolar with no apical periodontitis lesion. (*B*) The mesial root of a mandibular second molar with no lesion. (*C*) A maxillary second molar with a large apical periodontitis lesion. (*D*) The distal root of a mandibular first molar with a small apical periodontitis lesion.

TABLE 2 - Mean Distribution of Counts of Bacteria by the Presence/Absence of Periapical Lesion and Lesion Size

Variable	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	.93	.65	.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
P value (reference: no lesion)	.84	.03	.56
P value (reference: no lesion)	.86	.48	.93

P values were obtained from unadjusted negative binomial regression models.

categorized as follows: overfilled, 0–2 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 the apical limit ending from 0–2 mm short of the apex. If present, coronal restoration was classified as adequate on both CBCT imaging 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 was also recorded.

Under aseptic conditions, the teeth were thawed at room temperature and the external root surfaces cleaned and disinfected using 3% hydrogen peroxide and then 2.5% sodium hypochlorite followed by inactivation with a 10% sodium thiosulfate solution. The entire root surfaces were scrubbed with these substances except for 1 mm around the apical foramen. The apical 5 mm of the root was measured with a digital caliper and transversally sectioned at this point with a diamond disc. The entire procedure was performed under magnification of an operating microscope. Specimens were placed in microcentrifuge tubes and again stored at −20°C.

Micro-CT Analysis

3D images of each apical root specimen were obtained using a micro-CT scanner (SkyScan1174v2; Bruker, Kontich, Belgium) to evaluate the volume of the apical root canal system that remained unfilled. Micro-CT scanning was performed with the specimens within microcentrifuge tubes to prevent contamination. The following parameters were used: 50 kV, 800 μA, isotropic resolution of 17 μm, 180° rotation around the vertical axis, rotation step of 1.0°, and a 0.5-mm thick aluminum filter. Next, the 3D images of the roots were reconstructed using the NRecon v.1.6.10.4 program (Bruker), and artifact reduction was performed using the following parameters: ring artifact correction = 6, beam hardening correction = 50%, and smoothing = 8. CTAn v. 1.12 software (Bruker) was used to evaluate the volume (mm^3) of the filling material and the unfilled apical root canal system. Representative 3D figures of the apical root specimens were created by CTvol software (CTvol 2.3.2, Bruker). After scanning, the specimens were stored frozen and subsequently subjected to cryogenic pulverization.

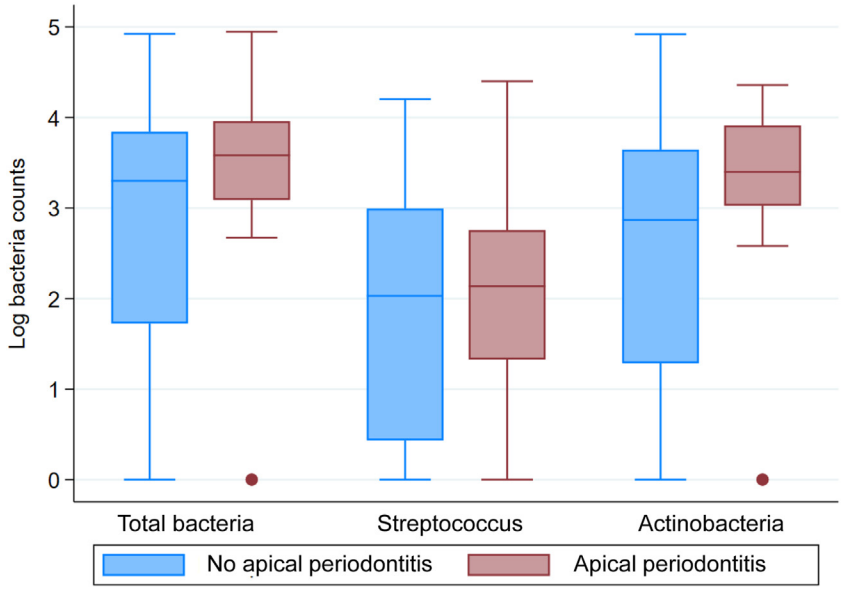

FIGURE 2 – Box plots showing the median distribution of counts of total bacteria, *Streptococcus*, and Actinobacteria (log transformed) by the presence or absence of apical periodontitis. The box plots are overlapping; thus, there are no differences between the 2 groups (apical periodontitis absent vs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

Cryopulverization and DNA Extraction

The specimens were thawed and had their external root surfaces once again cleaned and disinfected as described previously followed by inactivation with sodium thiosulfate, always with special care to not reach the apical foramen area. A sterility control sample was taken from the root surfaces using a 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 −20°C.

The apical specimens were pulverized in a cryogenic mill (6750 Freezer/Mill; Spex, Metuchen, NJ) under the temperature of liquid nitrogen. The obtained powder was conserved at −20°C until 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 root powder. DNA was extracted from the specimens using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. The final volume of the DNA extract solution for each sample was 200 μL, which was taken into account during bacterial quantification.

Bacterial Quantification with qPCR

Quantitative levels were obtained for total bacteria, *Streptococcus* species, and members of the Actinobacteria phylum. A qPCR assay based on the 16S ribosomal RNA gene was conducted with the Power SYBR Green Master Mix (Thermo Fisher Scientific,

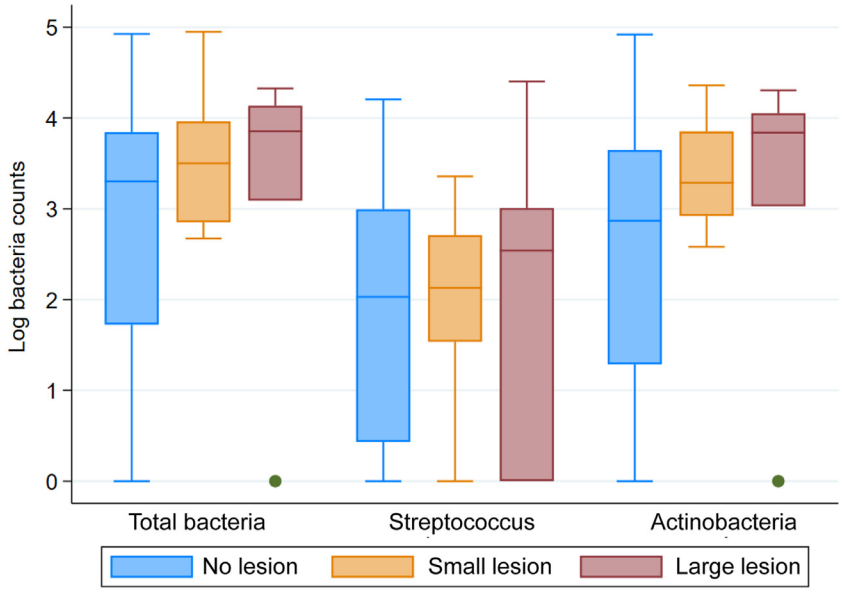

FIGURE 3 – Box plots showing the median distribution of counts of total bacteria, *Streptococcus*, and Actinobacteria (log transformed) by apical periodontitis lesion size. The box plots are overlapping; thus, there are no differences between groups (no lesion, small lesion, and large lesion). The *green-filled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

Foster City, CA) in an ABI 7500 real-time PCR device (Thermo Fisher Scientific). Universal and group-specific primers were as reported previously[37,38]. The reaction volume was 20 μL, containing a primer concentration of 0.5 μmol/L and 2 μL extracted DNA template. The temperature profile for the qPCR included initial denaturation at 95°C/10 minutes and 40 repeats of the following steps: 95°C/1 minute, annealing at 52°C/1 minute (for universal primers [total bacteria]) or 60°C/1 minute (for the *Streptococcus* and Actinobacteria primers), and extension at 72°C/1 minute. Controls and each sample were evaluated in triplicate. After amplification, melt curve analysis of the qPCR products was performed to determine the specificity of the reaction.

Data collection and analysis were performed using ABI 7500 v2.0.4 software (Thermo Fisher Scientific). The bacterial counts for each sample were based on standard curves constructed with known concentrations of DNA extracted from *Streptococcus mutans* strain ATCC 25175 (ATCC, Manassas, VA) (universal primers and specific primers for *Streptococcus* species) and a clinical isolate of *Actinomyces israelii* (primers for Actinobacteria). The isolated pure bacterial DNA was quantified, 10-fold diluted from 10^7 to 10^2 cells in Tris-EDTA buffer, and then used for preparation of the respective standard curves.

Statistical Methods

Frequencies and percentages were used to describe categoric variables. Numerical data were assessed for normality using the Shapiro-Wilk test, and normally distributed variables were summarized using means and standard deviations. Numerical variables that deviated from the normality assumption were summarized using medians and range (minimum and maximum). The negative binomial regression was used to model counts of bacteria, which were overdispersed with the variance of the counts far exceeding the distributional mean. The negative binomial models were used to examine whether the presence/absence of an apical periodontitis lesion and its size, as well as the other parameters, were associated with increased or decreased bacterial counts.

RESULTS

Among the patients included, there was a preponderance of females (18/25, 72%) (Table 1). The mean age for males was 60.7 years, on average being older than females. Almost 85% of the root canal fillings were categorized as of adequate quality based on CBCT imaging. The median volume of the unfilled canal system as determined by micro-CT imaging was 0.2 mm^3. The median proportion of the unfilled canal system was 12.3% (range, 0%–100%). Figure 1*A–D* shows representative correlative CBCT and micro-CT images of the specimens evaluated.

Table 2 shows the mean distribution of bacterial counts by the presence or absence of apical periodontitis and by lesion size. Findings showed that the mean counts of total bacteria, streptococci, and actinobacteria did not differ significantly between teeth with or without apical periodontitis ($P > .05$). The median distribution of the counts is shown in Figures 2 and 3.

Of the specimens evaluated, 23 were from teeth with posttreatment apical periodontitis (16 small and 7 large) and 22 from teeth with no detectable lesion on CBCT imaging. Bacteria were detected in all root apical specimens except for 1 from a tooth with a large lesion. Actinobacteria were found in 22 of 23 (96%) specimens with posttreatment lesions (16 small and 6 large) and 18 of 22 (82%) teeth with no lesions. Streptococci occurred in 20 of 23 (87%) specimens with lesions (15 small and 5 large) and 18 of 22 (82%) with no lesions.

Table 3 depicts unadjusted estimates of the incidence rate ratio with their 95% confidence intervals. Findings showed that counts of *Streptococcus* species were significantly lower by 80% in the apical root canals of teeth with small lesions than in teeth with no lesions ($P < .05$). Total bacterial counts and counts of *Streptococcus* were 3.39 and 9.07 times significantly higher among female patients compared with males, respectively ($P < .05$).

The limit of root canal filling >2 mm short as determined by CBCT imaging was significantly associated with more total bacterial counts in the apical canal system than canals filled up to 0–2 mm short ($P < .05$). The presence of an adequate coronal restoration was significantly associated with lesser counts of *Streptococcus* species ($P < .05$). The results from the adjusted analyses were not statistically significant and are not presented.

DISCUSSION

This multianalytical study evaluated the bacteriologic conditions in the apical region of the root canal system of teeth with and without posttreatment apical periodontitis. Bacterial counts in the cryopulverized root apexes were evaluated for their correlation with the presence/absence of apical periodontitis lesions as well as lesion volume as determined by CBCT imaging and the technical quality of the obturation as determined by both CBCT and micro-CT imaging.

Bacteria were detected virtually in all root apical specimens. The prevalence and mean counts of total bacteria, actinobacteria, and streptococci in the apical segment of the root canal system did not differ significantly when comparing root canal–treated teeth with or without apical periodontitis. No significant differences in counts were observed when comparing teeth with small and large lesions either. Overall, the mean bacterial counts were low, in the order of 10^3, which have been

TABLE 3 - Estimates of Incidence Rate Ratio (IRR) and Their 95% Confidence Intervals (CIs) Showing Factors That Were Associated with Bacterial Counts

	Total counts		*Streptococcus*		Actinobacteria	
Variable	**IRR (95% CI)**	***P* value**	**IRR (95% CI)**	***P* value**	**IRR (95% CI)**	***P* value**
Apical periodontitis presence (reference: absent)						
Present	1.05 (0.36–3.03)	.93	0.73 (0.19–2.77)	.65	0.77 (0.25–2.31)	.64
Apical periodontitis lesion size (reference: no lesion)						
Small	1.13 (0.35–3.62)	.84	**0.20 (0.05–0.83)**	**.03**	0.69 (0.21–2.33)	.56
Large	0.87 (0.19–4.08)	.86	1.94 (0.31–12.39)	.48	0.93 (0.19–4.63)	.93
Demographics						
Age in years	0.97 (0.93–1.01)	.19	0.98 (0.92–1.04)	.47	0.98 (0.94–1.02)	.30
Sex (reference: male)						
Female	**3.39 (1.19–9.63)**	**.02**	**9.07 (2.54–32.39)**	**.01**	2.83 (0.94–8.50)	.06
Smoking (reference: no)						
Yes	0.95 (0.28–3.25)	.93	0.29 (0.06–1.34)	.11	1.42 (0.39–5.11)	.59
Clinical factors						
Quality of filling (reference: adequate)						
Inadequate	2.03 (0.48–8.67)	.34	0.81 (0.13–5.08)	.82	2.92 (0.66–12.98)	.16
Micro-CT unfilled canal (mm^3)	0.47 (0.09–2.42)	.37	1.08 (0.13–9.07)	.94	0.49 (0.07–3.36)	.47
Micro-CT unfilled canal (%)	1.01 (0.99–1.03)	.29	1.03 (0.98–1.08)	.31	1.02 (1.00–1.04)	.11
CBCT limit obturation (reference: 0–2 mm)						
>2 mm	**3.30 (1.05–10.33)**	**.04**	3.36 (0.79–14.18)	.10	2.91 (0.88–9.58)	.08
Overfilled	3.24 (0.41–25.82)	.27	6.35 (0.46–87.18)	.17	4.17 (0.48–36.52)	.20
Coronal restoration (reference: absent)						
Inadequate	0.66 (0.19–2.33)	.52	0.51 (0.11–2.43)	.40	0.94 (0.25–3.51)	.93
Adequate	0.33 (0.09–1.17)	.09	**0.15 (0.03–0.71)**	**.02**	0.42 (0.11–1.55)	.19
Abutment tooth (reference: no)						
Yes	0.75 (0.16–3.56)	.72	0.18 (0.03–1.23)	.08	1.00 (0.20–5.06)	1.00
Intraradicular post (reference: no)						
Yes	0.74 (0.22–2.54)	.63	0.91 (0.19–4.27)	.90	1.01 (0.28–3.66)	.98

CBCT, cone-beam computed tomography; micro-CT, micro–computed tomography.
Data with significant *P* values are in boldface.

suggested to be compatible with a normal radiographic status of periradicular tissues in some cases[39] and helps explain the counts found in teeth with no lesion in this study. However, these findings are somewhat unexpected because 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 bacterial diversity in the root canal[40-43], 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[40,42,44]. A possible reason for the lack of significant differences in bacterial counts may be related to the fact that the cryopulverization sampling approach, despite its numerous advantages (see below), cannot reveal the specific location of the detected bacteria in the apical canal system. Thus, the possibility exists that most bacteria detected in teeth with no lesion might be sequestered into areas of the system with no frank contact with the periradicular tissues to cause disease. However, it is also 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[45]. 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. A study using samples taken from the full main canal found higher bacterial richness in teeth with posttreatment apical periodontitis compared with teeth with no apical disease[26]. Further community profiling studies focusing exclusively on the apical microbiome are required. The host susceptibility is certainly another factor of great impact on this equation[2].

Streptococcus species are among the most commonly found taxa in teeth with posttreatment apical periodontitis, as demonstrated by studies that evaluated the full canal length[8,11,46,47] or only the apical portion[12,13]. The present findings confirm the high prevalence of streptococci in the apical canal system of root canal–treated teeth. These bacteria were found in higher counts in samples from female patients. Curiously, the streptococci counts were substantially lower in

the apical canal of teeth with small lesions compared with no lesions. This may represent a temporal shift in the composition of the apical microbiome. 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 among the most abundant taxa in saliva and plaque[48,49].

The quality of root canal filling may affect the treatment outcome[50] 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[12,33]. The present findings of unfilled canal space as determined by micro-CT imaging 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 imaging. This is in consonance with the reports of a significantly lower success rate for teeth with underfillings[51]. It is likely that in these underfilled cases, the length of the unfilled canal may have also been unprepared, allowing the bacterial infection to remain unaffected.

This study has some 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 using qPCR to evaluate lesions obtained during extraction.

One of the strengths of this study was the use of a multianalytical approach devised to evaluate exclusively the apical portion of the root canal system, an area regarded as of critical relevance for disease pathogenesis and treatment[52]. CBCT imaging, which has higher sensitivity to detect bone lesions in comparison with periapical radiographs[27,28], was used to determine the presence of apical periodontitis lesions. In addition, most studies that evaluated the size of the lesion were based on its largest bidimensional diameter; this study used CBCT imaging 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 a 5-mm diameter, a cutoff used in previous studies to determine the lesion size by diameter[36]. Another strength was the use of micro-CT imaging to evaluate with higher resolution and 3-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[19]. Finally, qPCR is a highly used, sensitive, and accurate approach to detect and quantify bacteria, including difficult-to-grow and uncultivated taxa[18]. This method was used here to target total bacteria and some taxonomic groups commonly associated with posttreatment apical periodontitis.

In conclusion, 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.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Sandra R. Hernández: Formal analysis, Investigation, Writing – original draft. **José F. Siqueira:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision. **Danielle D. Voigt:** Investigation. **Giuliana Soimu:** Formal analysis, Data curation. **Sabrina C. Brasil:** Investigation. **José C. Provenzano:** Investigation. **Ibrahimu Mdala:** Formal analysis. **Flávio R.F. Alves:** Formal analysis, Writing – review & editing. **Isabela N. Rôças:** Conceptualization, Investigation, Writing – review & editing, Supervision.

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The authors deny any conflicts of interest related to this study.

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