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ORIGINAL ARTICLE

Microbiological analysis of root canals of primary teeth with pulp necrosis caused by caries or trauma

ABSTRACT

Aim: *This study aimed to investigate the microorganisms detected from root canals of primary teeth with pulp necrosis caused by dental caries or trauma.*
Methodology: *Microbial samples were taken from 44 cases in primary teeth with pulp necrosis either due to dental caries or trauma. DNA was extracted from the samples, which were analysed for the presence of fifteen endodontic pathogens by using PCR species-specific primers.*
Results: *The bacteria most detected in necrotic primary teeth due to caries (37/44) were P. micra (76.3%), P. nigrescens (76.3%), A. naeslundii (47.4%), and F. nucleatum (42.1%). On the other hand, A. naeslundii (83.3%), T. forsythia (83.3%), P. nigrescens (66.7%), and F. nucleatum (66.7%) were most frequently recovered from a root canal with pulp necrosis due to trauma (7/44). Significant associations were found between the presence of P. micra and the existence of caries (p=0.023) and sinus tract (p=0.044). The presence of T. forsythia was associated with the existence of trauma (p=0.035), and the presence of F. nucleatum was associated with pain on palpation (p=0.033).*
Conclusions: *The microbiota recovered from root canals of primary teeth with pulp necrosis caused by dental caries or trauma is similar, with the predominance of anaerobic microorganisms*

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Introduction

Primary teeth are subject to physical, chemical and/or biological injuries. Extensive dental caries and some traumatic injuries can result in pulp necrosis, which can lead to their premature loss, causing functional and aesthetic damage, as well as injuring the underlying permanent tooth germ, affecting its development (1).
Although pulp necrosis has been reported as a common sequel after dental caries and trauma in the primary dentition (2), its mechanisms vary according to the causal condition. After pulpal exposure by caries, pulp surface is colonized and covered by bacteria present in the caries biofilm, which will adhere to the dentinal walls colonizing these surfaces and forming root canal biofilms (1). This microbial load exerts a direct and by-product pulpal reaction characterized by severe inflammation (3). *Lactobacillus acidophilus, Streptococcus mutans*, and *Streptococcus sobrinus* have been related to the initial stages of dental caries and the pulpal surface colonization (4).
In some traumatic injuries, coagulation necrosis can happen as a result of a permanent break of the blood supply connected to the pulp tissues (5). The pulp and the periodontium communicate between themselves through the apical foramen and lateral canals, which is an acceptable pathway for bacterial entrance into the root canals (6). After this, the microenvironment of the pulp space becomes propitious to factors that influence the microbial colonization and multiplication (7). Anaerobic bacteria, such as black-pigmented rods (BPRs) and *Fusobacterium nucleatum*, have been reported as the most predominant microorganisms in primary teeth with endodontic infection after traumatic injury (8).
The success of root canal therapy depends directly on reducing or eliminating the endodontic microbiota. Several studies have evaluated the microbiota of permanent teeth (9-11), however few of them have investigated the primary dentition infection and also the effect of dental caries and trauma, on the microbial profile (8, 12, 13). Thus, this study aimed to investigate the microorganisms present in root canals of primary teeth with pulp necrosis caused by dental caries or trauma.

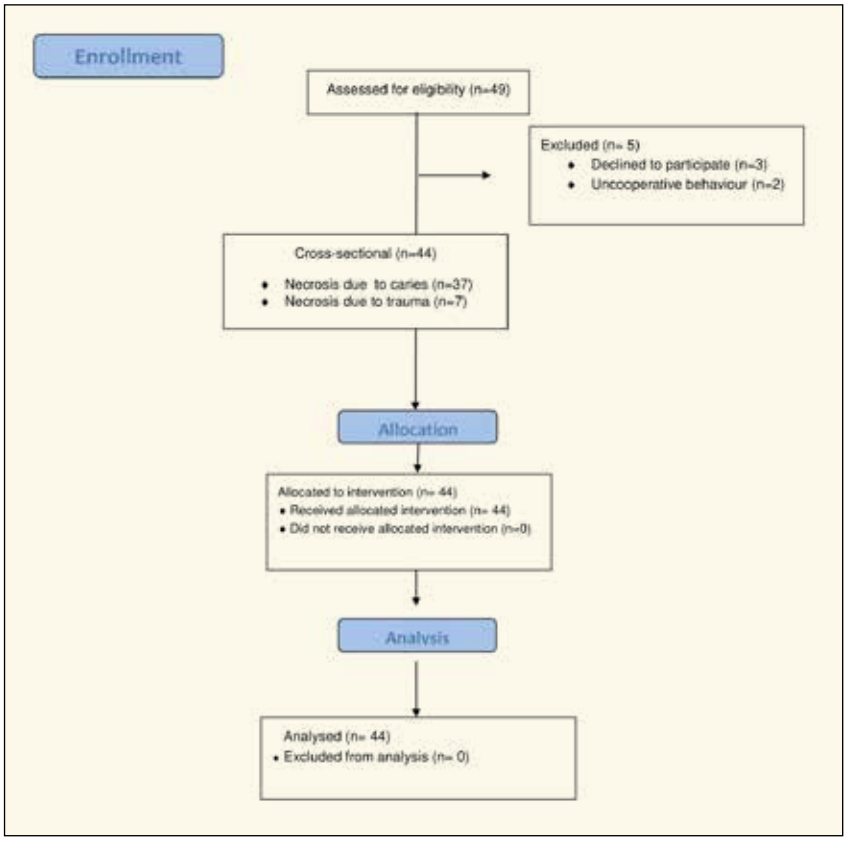

Figura 1
STROBE diagram of the patients' enrolment process.

Material and Methods

Patient selection
A cross-sectional study was conducted, following the *strengthening the reporting of observational studies in epidemiology* (STROBE) guidelines. Eligible 44 children, who had been referred for endodontic treatment and who presented at least 1 primary tooth with pulp necrosis due to carious lesions or dental trauma, were enrolled in this study (Figure 1).
The qualified participants had tooth with evidence of primary endodontic infection (necrotic pulp tissue, sinus tract, periapical lesion, no previous endodontic treatment), intact root or less than two thirds of root resorption, enough coronal structure to

Table 1
Clinical and radiographic features of the patients according to the groups studied

		Groups		
Variable	**Category**	**Total**	**Caries**	**Trauma**
Gender	Female	21	17	4
	Male	23	20	3
Age	≥6 years	30	30	0
	<6 years	14	7	7
Range of teeth	Single-rooted	7	0	7
	Bi-rooted	19	19	0
	Multi-rooted	18	18	0
Teeth localization	Upper	25	18	7
	Lower	19	19	0
Discoloration	Yes	7	0	7
	No	37	37	0
Pain on palpation	Yes	6	6	0
	No	38	31	7
Tenderness to percussion	Yes	6	6	0
	No	38	31	7
Sinus tract	Yes	21	17	4
	No	23	20	3
Mobility	Yes	1	1	0
	No	43	36	7
Periapical lesion ≤2mm	Yes	44	37	7
	No	0	0	0

allow a full isolation with a rubber dam, and absence of deep periodontal pockets in the involved tooth. Patients could not present systemic alterations or have used antibiotics or antifungals within the previous 3 months. Patients, who had used antibiotics or antimicrobial mouthwashes during the course of the trial and had an uncooperative behaviour, not allowing the microbial sample collection, were excluded from this study.

Clinical and radiographic features were observed and recorded for all patients, including presence or absence of teeth mobility, pain on palpation, tenderness to percussion, discoloration, sinus tract, and periapical lesion (Table 1).

This study was carried out in accordance with the International Code of Medical Ethics, and the research protocol was approved by the Institutional Research Ethics Committee (process n°. 224/10), including the description of the sample collection for this investigation. The purposes were fully explained to the guardians, who signed a written informed consent form authorizing their children's enrolment in the study, and the privacy rights of subjects were observed. An experienced operator performed the endodontic procedures and sample collection in all cases included in this investigation.

Clinical procedures

All clinical procedures have been previously described (10, 12). Firstly, an infiltrative local anaesthetic was applied followed by tooth isolation with a rubber dam. The rubber dam and the tooth were disinfected using 30% hydrogen peroxide and then 2.5% sodium hypochlorite (NaOCl). The later was neutralized with 5% sodium thiosulfate to avoid carry-over the antimicrobial effect of NaOCl during the bacteriological sampling.

The disinfection of the tooth surface was monitored by taking a swab sample from both external and internal surfaces of the crown and from its surrounding structure area. Next, a swab sample was streaked on a plate containing 5% defibrinated sheep blood and fastidious anaerobe agar (FAA – LAB M, Heywood, Lancashire, UK) before being incubated anaerobically and aerobically, respectively, for up to 14 days followed by DNA extraction from the swab and PCR run by using universal bacterial primers. If any positive culture or presence of bands on the agarose gel was detected, then the patient was excluded from the study.

Root canals were accessed through the crown with high-speed round diamond burs (KG Sorensen Industria e Comercio, São Paulo, SP, Brazil), which was made without the use of water spray but under manual irrigation with sterile saline solution. The pulp chamber was irrigated with sterile saline to remove the contents from the pulp space. The microbial sample was obtained from a single root canal. If the

Table 2

PCR primers sequences, with expected amplicon size and thermocycling parameters

Target bacteria	Primers pairs (5' to 3')	Amplicon size (bp)	Cycles
Actinomyces naeslundii	Forward: GCG CCT TTT TTG GTG TTT TTG G Reverse: CAC CCA CAA ACG AGG CAG GCC TG	274	Initial denaturation at 94 °C for 1 min and 35 cycles of: 94 °C for 1 min, 60 °C for 1 min, 72 °C for 90s and final step continued at 72 °C for 10 min
Dialister pneumosintes	Foward: TTC TAA GCA TCG CAT GGT GC Reverse: GAT TTC GCT TCT CTT TGT TG	1105	Initial denaturation at 95 °C for 2min and 36 cycles of: 94 °C for 30s, 55 °C for 1min, 72 °C for 2 min and a final step 72 °C for 2 min
Enterococcus faecalis	Forward: CCG AGT GCT TGC ACT CAA TTG G Reverse: CTC TTA TGC CAT GCG GCA TAA AC	138	Initial denaturation at 95 °C for 2 min and 36 cycles of: 95 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min and a final step 72 °C for 7 min
Filifactor alocis	Forward: CAG GTG GTT TAA CAA GTT AGT GG Reverse: CTA AGT TGT CCT TAG CTG TCT CG	594	Initial denaturation at 95 °C for 2 min and 26 cycles of: 95 °C for 30 s, 58 °C for 1 min, 72 °C for 1 min and a final step 72 °C for 2 min
Fusobacterium nucleatum	Foward: AGT AGC ACA AGG GAG ATG TAT G Reverse: CAA GAA CTA CAA TAG AAC CTG A	1000	Initial denaturation at 95 °C for 5min and 30 cycles of: 94 °C for 30 s, 40 °C for 1 min, 72 °C for 2 min and a final step 72 °C for 10 min
Parvimonas micra	Forward: AGA GTT TGA TCC TGG CTC AG Reverse: ATA TCA TGC GAT TCT GTG GTC TC	207	Initial denaturation at 95 °C for 2 min and 36 cycles of: 94 °C for 30s , 60 °C for 1 min, 72 °C for 1 min and a final step 72 °C for 10 min
Porphyromonas endodontalis	Forward: GCT GCA GCT CAA CTG TAG TC Reverse: CCG CTT CAT GTC ACC ATG TC	672	Initial denaturation at 95 °C for 2 min and 36 cycles of: 94 °C for 30 s, 58 °C for 1 min, 72 °C for 2 min and a final step 72 °C for 10 min
Porphyromonas gingivalis	Forward: AGG CAG CTT GCC ATA CTG CG Reverse: ACT GTT AGC AAC TAC CGA TGT	404	Initial denaturation at 95 °C for 2 min and 36 cycles of: 94 °C for 30 s, 60 °C for 1 min, 72 °C for 2 min and a final step 72 °C for 2 min
Prevotella intermedia	Forward: TTT GTT GGG GAG TAA AGC GGG Reverse: TCA ACA TCT CTG TAT CCT GCG T	575	Initial denaturation at 95 °C for 2 min and 36 cycles of: 94 °C for 30 s, 58 °C for 1 min, 72 °C for 2 min and a final step at 72 °C for 10 min
Prevotella nigrescens	Forward: ATG AAA CAA AGG TTT TCC GGT AAG Reverse: CCC ACG TCT CTG TGG GCT GCG A	804	Initial denaturation at 95 °C for 2 min and 36 cycles of: 94 °C for 30 s, 58 °C for 1 min, 72 °C for 2 min and a final step at 72 °C for 10 min
Streptococcus mitis	Forward: GTC GAA GGT GAT GAT ATG AC Reverse: GAC AGT ACG CAG TCT TAC GTC	372	Initial denaturation at 94 °C for 2 min and 30 cycles of: 94 °C for 1 min, 54 °C for 1 min, 72 °C for 1min and a final step 72 °C for 10 min
Streptococcus mutans	Forward: ATT GAA GGC GAG CCT TTA GAA AG Reverse: CTA GGA CAA TAG CAA C	351	Initial denaturation at 94 °C for 2 min and 30 cycles of: 94 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min and a final step 72 °C for 10 min
Streptococcus sanguis	Forward: GTC GAT GGC GAG GAT CTA GAG C Reverse: TGC CGA GCG CTC TAA CTC CA	208	Initial denaturation at 94 °C for 2 min and 30 cycles of: 94 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min and a final step 72 °C for 10 min
Tannerella forsythia	Forward: GCG TAT GTA ACC TGC CCG CA Reverse: TGC TTC AGT GTC AGT TAT ACC T	641	Initial denaturation at 95°C for 1 min and 36 cycles of: 95 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min and a final step at 72 °C for 2 min
Treponema denticola	Forward: TAA TAC CGA ATG TGC TCA TTT ACA T Reverse: TCA AAG AAG CAT TCC CTC TTC TTC TTA	316	Initial denaturation at 95 °C for 2 min and 36 cycles of: 94 °C for 30 s, 60 °C for 1 min, 72 °C for 2 min, and a final step at 72 °C for10 min
Universal 16S rDNA	Forward: TCC TAC GGG AGG CAG CAG T Reverse: GGA CTA CCA GGG TAT CTA ATC CTG TT	466	Initial denaturation at 95 °C for 10 min and 40 cycles of: 95 °C for 10 s, 60 °C for 10 s and a final extension step at 72 °C for 25 s

tooth was multi-rooted, the largest canal or the canal with periapical lesion was chosen. Three sterile absorbent paper points of a diameter compatible with the root canals were successively introduced and maintained into the canals for 60 seconds each, up to 1 mm shorter of the root apex, established by the initial radiograph. In the case of a dry canal, 0.85% sterile saline was used to moisten the root

canals to allow better sample collection. In case of a wet canal (or those that have been previously irrigated with saline) numerous paper points were used to absorb all the fluid inside the canal.
The paper points were then transferred to sterile Eppendorf tubes containing VMGA III transport medium and were frozen immediately at -80 ºC and stored until assayed by Polymerase Chain Reaction (PCR).

DNA extraction and PCR assay
DNA from clinical samples was extracted and purified using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The DNA concentration was recorded by a DNA quantification machine (NanoDrop 2000; Thermo Scientific, Wilmington, DE, USA).
The PCR reactions were performed as previously described (14) (MyCycler; Bio-Rad, Hercules, CA, USA) with modification to consist of a final volume of 25 µL of reaction mixture. The primer sequences and PCR cycling parameters are listed in Table 2 (27, 28). Once the PCR reactions were concluded they were loaded into 1% agarose gel electrophoresis, stained with ethidium bromide, and analysed under ultraviolet transillumination. The presence of determined bacteria was confirmed by a positive band of positive control in the expected molecular weight and a negative band of negative control.

Data analysis
Data were tabulated in Excel spread sheet (Microsoft, Redmond, WA, USA) and statistically analysed by using SPSS 21 (IBM, Chicago, IL, USA). A descriptive analysis was performed on all data. Fisher´s exact or Pearson Chi-square tests, when appropriate, were used to test the null hypothesis that there is no relation between clinical and radiographic features and presence of specific bacteria. Significance level was set at 5% ($P<0.05$).
A Venn's diagram was drawn to easily visualize both common and different species found in necrosis after caries and trauma (15).

Results

Clinical and radiographic features
Forty-four patients were enrolled in this study, 37 were due to dental caries, while 7 were due to trauma. Eligible children were selected from 2 to 9-year-old, mean of 6 years old (±1), being 21 (47.72%) females and 23 (52.28%) males.
Traumatised teeth corresponded to the anterior single-rooted, while necrotic teeth due to caries were posterior and bi-rooted or multi-rooted. All teeth presented periapical lesion equal or lower than 2 mm.
Regarding the patients' clinical features, none of them reported acute pain (acute abscess). However, 21 patients presented sinus tract (chronic abscess). A detailed explanation of the clinical features according to each group (necrosis caused by dental caries or trauma) is shown in Table 1.

Overall microbial findings
Samples collected from the operatory field, including external and internal surfaces of the crown and its surrounding structures using sterile swabs presented no positive cultures and no bacterial DNA after performing culture and molecular methods, respectively.
The mean number of species detected was 4.16 (±1.88), ranging from 1 to 9 species per sample. In samples from pulp necrosis caused by caries, the mean number of species detected was 3.97 (±1.64), ranging from 1 to 7, while in samples from pulp necrosis caused by trauma the mean was 5.33 (±2.94), ranging from 2 to 9 species. No statistically significant differences were observed among specific bacteria from pulp necrosis caused by caries or trauma ($p>0.05$).
Prevotella nigrescens (75%), *Parvimonas micra* (70.5%), *Actinomyces naeslundii* (52.3%), and *Fusobacterium nucleatum* (45.5%) were the most prevalent species in primary teeth with primary endodontic infection (n=44).
The bacteria most detected in necrotic primary teeth due to caries (37/44) were *P. micra* (76.3%), *P. nigrescens* (76.3%), *A. naeslundii* (47.4%), and *F. nucleatum* (42.1%). On the other hand, *A. naeslundii* (83.3%), *Tannerella forsythia* (83.3%), *P.*

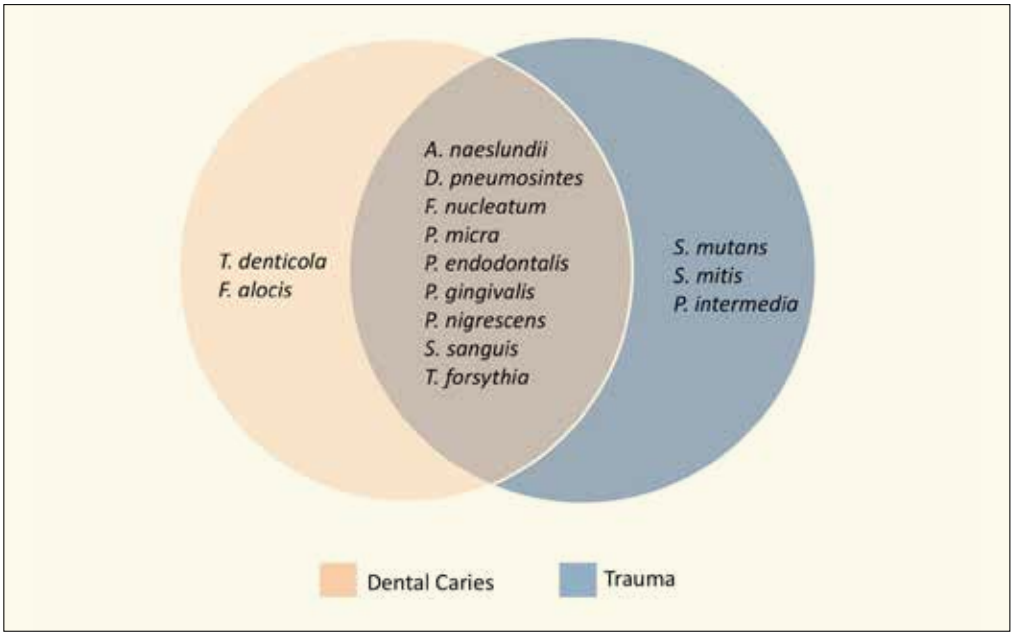

Figura 2
Venn's diagram showing the overlap of bacterial species detected by PCR in pulp necrosis due to trauma or caries.

nigrescens (66.7%), and *F. nucleatum* (66.7%) were most frequently recovered from a root canal with pulp necrosis due to trauma (7/44).
Treponema denticola and *Filifactor alocis* were detected only from a root canal with pulp necrosis due to caries. *S. mutans*, *S. mitis* and *P. intermedia* were detected only from a root canal with pulp necrosis due to trauma. *Enterococcus faecalis* was not observed in any of the root canals (Figure 2).
Significant associations were found between the presence of *P. micra* and the existence of caries (p=0.023) and sinus tract (p=0.044). The presence of *T. forsythia* was associated with the existence of trauma (p=0.035), and the presence of *F.nucleatum* was associated with positive pain on palpation (p=0.033).

Discussion

There is a well-established consensus that microorganisms are the main cause of endodontic infections. Of all microorganisms inhabiting the oral cavity, only a few of them can invade the pulp and compromise its function. Studies have well-documented these species in endodontic infection in permanent teeth either by culture method (10) or molecular techniques (9, 12). However, few studies have been carried out to identify microorganisms in root canals of primary teeth (8, 11-13, 16-18). The present study sought to investigate 15 bacterial species frequently detected from root canals of primary teeth with pulp necrosis after caries or trauma using PCR.
The microbiota of primary teeth with pulp necrosis, identified in this study, was similar to permanent teeth regarding the bacteria respiration metabolism, being the anaerobes frequently detected (10, 12). This result suggests that, despite the different causes of pulp necrosis and different mechanisms of microbial invasion, the conditions inside the root canal probably favours the colonization and multiplication of a restricted group of species with a predominance of anaerobic species.
Actinomyces naeslundii was found commonly in necrotic root canals either after caries (47.4%) or after trauma (83.3%). *Actinomyces naeslundii*, a facultative Gram-positive bacterium, has been associated with secondary endodontic infections. A low frequency of this microorganism has been reported previously in primary infections of permanent teeth using culture techniques (10).
Prevotella nigrescens was found often in necrotic root canals either after caries (76.3%) or after trauma (66.7%). *Fusobacterium nucleatum* was also regularly detect-

ed in necrotic root canals either after caries (42.1%) or trauma (66.7%), and it was associated with positive pain on palpation (p=0.033). *Prevotella* and *Fusobacterium*, which are Gram-negative anaerobic bacilli, have been previously associated with the presence of acute symptoms of pain, history of previous pain, tenderness to percussion and swelling in permanent teeth (10). In our study, *Fusobacterium* was associated with pain, and this may be explained by the presence of lipopolysaccharide, an outer membrane component of Gram-negative bacteria that induces, among other issues, up-regulation of bradykinin, a potent pain mediator (10).

Parvimonas micra were the species most commonly detected in root canals of primary teeth with pulp necrosis after caries (76.3%). This species was not generally detected in pulp necrosis after trauma and had an association with the existence of caries (p=0.023) and sinus tract (p=0.044). *P. micra,* Gram-positive anaerobic cocci, have been previously associated with a history of pain, tenderness to percussion and wet canals in permanent teeth (10).

Treponema denticola (Gram-negative anaerobic bacilli) and *Filifactor alocis* (Gram-positive anaerobic bacilli) were detected only from samples of root canals with pulp necrosis after caries. These bacteria have been previously observed in great prevalence in primary endodontic infections in permanent teeth, implying they may be implicated in the pathogenesis of periapical diseases (19, 20).

Tannerella forsythia was the species most frequently detected in root canals of primary teeth with pulp necrosis after trauma (83.3%). This bacterium had an association with the presence of trauma (p=0.035), and it was not frequently detected in pulp necrosis after caries.

Our results have similarities with other findings (18), which showed a low frequency of *T. forsythia* in primary teeth with pulp necrosis caused after caries. *T. forsythia,* a Gram-negative anaerobic bacillus, has been previously associated with tenderness to percussion, mobility, wet canals, and purulent exudate in permanent teeth (21). *S. mutans*, *S. mitis* (both facultative Gram-positive cocci) and *P. intermedia* (black-pigmented anaerobic Gram-negative bacilli) were detected only from a root canal with pulp necrosis after trauma. According to the literature, *P. intermedia* have been most frequently detected in permanent teeth with necrotic pulp, and are related to the appearance of signs and symptoms of periapical disease in permanent dentition (22).

Enterococcus faecalis was not detected in any root canal in this study. This result is in disagreement with a previous study (18) which observed a high frequency of *Enterococcus* spp. (50%) in the necrotic pulp of children using PCR. *E. faecalis* was also previously detected in 63% in primary teeth using culture technique (23). *E. faecalis* was frequently detected in cases of primary and secondary infections in permanent teeth using PCR (19), and in the root canals and their combined periodontal pockets in cases of endodontic-periodontal lesions (24). Our results are consistent with other studies, which say the microbial composition of unexposed and exposed pulp tissues do not present impressive distinction (25, 26). This evidence suggests pulpal exposure may not play an important role in establishing the selection of bacteria present in infected root canals (26).

Conclusions

The microbiota recovered from root canals of primary teeth with pulp necrosis caused by dental caries or trauma is very similar, with the predominance of anaerobic microorganisms.

Clinical Relevance

Despite the different causes of pulp necrosis and different mechanisms of microbial invasion, the conditions inside the root canal probably favors the colonization and multiplication of a restricted group of species with a predominance of strict anaerobic species.

Conflicts of Interest

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

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