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Microbiological examination of infected dental root canals

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Objectives: The aim of this study was to investigate the root canal microbiota of primary and secondary root-infected canals and the association of constituent species with specific endodontic signs and symptoms.
Methods: Microbial samples were taken from 60 root canals, 41 with necrotic pulp tissues (primary infection) and 19 with failed endodontic treatment (secondary infection). Strict anaerobic techniques were used for serial dilution, plating, incubation and identification.
Results: A total of 224 cultivable isolates were recovered belonging to 56 different bacterial species. Individual root canals yielded a maximum of 10 bacterial species. Of the bacterial isolates, 70% were either strict anaerobes or microphilic. The anaerobes most frequently isolated were: *Peptostreptococcus micros* (35%), *Fusobacterium necrophorum* (23.3%), *Fusobacterium nucleatum* (11.7%), *Prevotella intermedia/nigrescens* (16.7%), *Porphyromonas gingivalis* (6.7%) and *Porphyromonas endodontalis* (5%). The root canal microflora of untreated teeth with apical periodontitis was found to be mixed, comprising gram-negative and gram-positive and mostly anaerobic microorganisms and usually containing more than 3 species per canal. On the other hand, facultative anaerobic and gram-positive bacteria predominated in canals with failed endodontic treatment, which harbored 1–2 species per canal. Suggested relationships were found between anaerobes, especially gram-negatives, and the presence or history of pain, tenderness to percussion and swelling ($P < 0.05$). In particular, associations were found between: a) pain ($n = 29$) and *P. micros* ($P < 0.01$), *P. intermedia/nigrescens* and *Eubacterium* spp. (both $P < 0.05$); b) history of pain ($n = 31$) and *P. micros* ($P < 0.01$) *Porphyromonas* and *Fusobacterium* spp. ($P < 0.05$); c) tenderness to percussion ($n = 29$) and *Porphyromonas* spp. ($P < 0.01$), *Peptostreptococcus* and *Fusobacterium* spp. ($P < 0.001$); d) swelling ($n = 20$) and *Peptostreptococcus* spp. ($P < 0.01$), *Porphyromonas* and *Enterococcus* spp. ($P < 0.05$); e) wet canals ($n = 33$) and *Porphyromonas* and *Fusobacterium* spp. ($P < 0.05$); f) purulent exudate ($n = 20$) and *Porphyromonas, Peptostreptococcus* and *Fusobacterium* spp. ($P < 0.05$); previous endodontic treatment and *Enterococcus faecalis, Streptococcus* spp., *P. micros, F. necrophorum* ($P < 0.05$).
Conclusions: Our findings indicate potential complex interactions of species resulting in characteristic clinical pictures which cannot be achieved by individual species alone. They also indicate that the microbiota of primary infected canals with apical periodontitis differs in number and in species from the secondary infected canals by using the culture technique.

Key words: bacteria; endodontics; clinical signs

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Knowledge of the nature of the endodontic microbiota depends upon the recognition of those microorganisms present in the root canal system of teeth with necrotic pulp tissues and teeth with failed endodontic treatment. Even though infectious agents such as *Candida* (25, 36), human cytomegalovirus and Epstein-Barr virus (28) have been detected in infected dental root canals, bacteria and their by-products are the major cause of pulpal and periapical diseases (17, 21, 32). There are more than

500 bacterial taxa recognized today as normal inhabitants of the oral cavity (16). Comparatively, only a small group of microorganisms have been isolated and cultured from the root canals (about 150 reported microbial species). Individual root canals yield an average of 5–7 different species per canal (6, 11, 33). The most frequently bacterial genera isolated from necrotic pulps are *Peptostreptococcus, Prevotella, Porphyromonas, Fusobacterium, Eubacterium, Actinomyces* and facultative *Streptococcus* (5–11, 14–16, 21, 22, 32–34, 35, 38, 41). The selective pressures operating in the root canal environment suggest that certain bacteria are more capable of surviving and multiplying in the root canal than others, favoring the growth of obligate anaerobes in primary infected root canals (i.e. necrotic pulp tissues) (33) and the growth of facultatives in secondary endodontic infections (i.e. failed root-treated teeth) (20, 25–27, 36).

It has been reported that the root canal microbiota recovered from asymptomatic teeth is different from that isolated from clinically symptomatic teeth (41). Sundqvist (32) was the first to associate pain with the presence of ''*Bacteroides melaninogenicus*''. Work by Griffee et al. (12), Haapasalo et al. (13), Yoshida et al. (41), Hashioka et al. (16) and Gomes et al. (7–11) also indicated that there may be significant associations between certain anaerobic species and some endodontic clinical features. On the other hand, recent work investigating the microbial findings of teeth with failure of endodontic treatment have reported a very limited assortment of microorganisms, with predominantly facultative anaerobic gram-positive species, especially *Enterococcus faecalis* (20, 25–27, 36).

The aim of this study was to investigate the microorganisms most commonly isolated from canals with primary and secondary endodontic infections and the association of constituent species with specific endodontic signs and symptoms in order to find new associations and validate the ones reported in previous studies.

Material and methods

Patient selection

Sixty patients were selected from those who attended the Dental School of Piracicaba, SP, Brazil, needing endodontic treatment/retreatment. Forty-one teeth presented necrotic pulp tissues and 19 teeth had previously been root-filled for more than 4 years and showed radiographic evidence of apical periodontitis. A detailed medical and dental history was obtained from each patient. Patients having received antibiotic treatment in the last 3 months or having a general disease were excluded from the study. The Human Volunteers Research and Ethics Committee of the Dental School of Piracicaba approved a protocol describing the specimen collection for this investigation, and all patients gave informed consent to participate in the study. The teeth involved in this study were mostly non-intact. Teeth which could not be fully isolated with a rubber dam were excluded from the study.

Clinical features

The following features were recorded for each patient so that they could be correlated with the microbial findings: age, gender, tooth type and pulp status. Clinical symptoms and signs included nature of pain, history of previous pain, tenderness to percussion, pain on palpation, mobility, presence of a sinus and its origin (endodontic or periodontal), presence of swelling of the periodontal tissues, probing depth of the periodontal pocket, history of previous and present antibiotic therapy and any other relevant medication, radiographic findings and the internal status of the canal such as dry canal or the presence of clear, hemorrhagic or purulent exudates, detected as a distinct dampening or stain on the sampling paper points. Each type of exudate was analyzed alone and also grouped with the other types under the denomination ''wet canal''.

Sampling procedure

The method followed for the microbiologic procedures has been previously described (6–11). After a two-stage access cavity preparation, which was made without the use of water spray, but under manual irrigation with sterile saline solution and employing sterile burs, the teeth involved were individually isolated from the oral cavity with a previously disinfected rubber dam. Disinfection of the rubber dam and teeth was carried out using firstly 30% hydrogen peroxide and then 2.5% sodium hypochlorite. The solution was inactivated with 5% sodium thiosulfate in order to avoid interference with the bacteriological sampling. Aseptic techniques were used throughout endodontic therapy and sample acquisition. After initial entry to the pulp space, the patency of the root canal was established with minimal instrumentation, where possible, and without the use of any chemically active irrigant. Preexisting root-filling was removed using Gates Glidden drills (Maillefer, Ballaigues, Switzerland) and endodontic files without the use of chemical solvents. Irrigation with sterile saline solution was performed to remove any remaining treatment materials prior to sample collection. In each case, even in the multirooted tooth, a single root canal was sampled in order to confine the microbial evaluation to a single ecologic environment. The criterion used to choose the canal to be microbiologically investigated in the multirooted teeth was the presence of exudation or, in its absence, the largest canal or the canal related with the periapical radiolucency.

For microbial sampling, a sterile paper point was introduced into the full length of the canal (as determined with a preoperative radiograph), and kept in place for 60 s (5–12). In cases where a dry canal was identified, a further sterile paper point, moistened in sterile saline, was used to ensure viable sample acquisition. In cases of wet canals (or those that had been previously irrigated with saline) as many paper points as necessary were used to absorb all the fluid inside the canal. The paper point sample from the root canal was immediately transferred to the VMGA III transport medium (4, 21) and forwarded within 15 min to an anaerobic workstation (Don Whitley Scientific, Bradford, UK) in the microbiology laboratory. The maximum time between sample collection and laboratory processing was 4 h.

Microbial isolation

Inside the anaerobic chamber the transport media were shaken thoroughly in a mixer for 60 s (Vortex, Marconi, São Paulo, SP, Brazil). The transport media contained glass beads 3 mm in diameter to facilitate mixing and homogenization of the sample prior to cultivation. Serial 10-fold dilutions were made up to 1 : 104 in tubes containing Fastidious Anaerobe Broth (FAB, Lab M, Bury, UK). Fifty μl of the serial dilutions $1:10^2$, $1:10^3$ and $1:10^4$ were plated, using sterile plastic spreaders, into 5% defibrinated sheep blood Fastidious Anaerobe Agar (FAA, Lab M), in which 1 ml/l of hemin and 1 ml/l of vitamin K1 were added, so as to culture non-selectively obligate anaerobes and facultative anaerobes. The following selective culture media were also used:

- 5% sheep blood FAA + NAL (0.001% w/v nalidixic acid) + VAN (0.5 mg/l vancomycin) to select gram-negative anaerobic bacteria;
- 5% sheep blood FAA + KAN (kanamycin) + VAN to select ''black-pigmented bacteria'';

- 5% sheep blood FAA + NEO (0.0075% w/v neomycin) for clostridia and other anaerobes;.
- 5% sheep blood FAA + NAL (0.001% w/v nalidixic acid) for gram-positive anaerobes and *Actinomyces*.

The plates were incubated at 37°C in an anaerobic atmosphere for up to 14 days to permit detection of very slow-growing strains.

The same dilutions were plated in 5% sheep blood Brain Heart Infusion (BHI) agar (Oxoid, Basingstoke, UK) to allow aerobic or facultative microorganism growth, and in Agar Dextrose Sabouraud (Oxoid) supplemented with 100 μg/ml of chloramphenicol (Medley, Campinas, SP, Brazil) for yeasts, both incubated aerobically at 37°C during 2 days.

Microbial identification

Preliminary characterization of microbial species was based on colony features (i.e. size, color, shape, high, lip, surface, texture, consistency, brightness and hemolysis) visualized under a stereoscopic lens (Lambda Let 2, Atto instruments Co., Hong Kong). Isolates were then purified by subculture, gram-stained, tested for catalase-production, and their gaseous requirements were established by incubation for 2 days aerobically and anaerobically. Based on this information it was possible to select appropriate procedures for identification of the species as follows:

- Rapid ID 32A (BioMérieux SA, Marcy-l'Etoile, France) for strict anaerobic, gram-negative rods.
- RapID ANA II System (Innovative Diagnostic Systems Inc., Atlanta, GA) for anaerobic gram-positive cocci.
- API Staph (BioMérieux SA) for staphylococci and micrococci (gram-positive cocci, catalase-positive).
- Rapid ID 32 Strep (BioMérieux SA) for streptococci (gram-positive cocci, catalase-negative).
- RapID NH System (Innovative Diagnostic Systems Inc.) for *Eikenella, Haemophilus, Neisseria* and *Actinobacillus*.
- API C Aux (BioMérieux SA) for yeasts.

Three complementary tests were also used to ensure the identification of bacteria from the genus *Prevotella* and *Porphyromonas:*

1. fluorescence under long-wave (366 nm) UV light – Black-Ray lamp, model UVL-56 (UVP Inc, San Gabriel CA);
2. lactose fermentation – by the application of the fluorogenic substrate 4-methylumbelliferyl-β-galactoside (Sigma Chemical Co., St Louis, MO – M-1633), according to Alcoforado et al. (1); and
3. trypsin-like activity by the application of the synthetic fluorogenic peptide L-arginin-7-amino-4-methylocoumarin amido-HCl (C9396) (23, 31).

The reference strains included were: *Porphyromonas gingivalis* ATCC 33277; *Porphyromonas endodontalis* ATCC 35406; *Porphyromonas asaccharolytica* ATCC 25260; *Prevotella intermedia* ATCC 25611; *Prevotella nigrescens* ATCC 33536; *Prevotella melaninogenica* ATCC 25845; *Prevotella loescheii* ATCC 15930; *Prevotella corporis* ATCC 33457 and *Prevotella denticola* ATCC 33185.

Statistical analysis

The data collected for each case (clinical features, and the bacteria isolated) were typed onto a spreadsheet and statistically analyzed using SPSS for Windows (SPSS Inc., Chicago, IL). The Pearson chi-square test or the one-sided Fisher's Exact test, as appropriate, was chosen to test the null hypothesis that there was no relationship between endodontic clinical symptoms and signs and the presence of a specific group of bacteria in the root canals sampled.

Results

A total of 224 cultivable isolates, belonging to 56 different microbial species and 21 different genera, were recovered from the 60 root canals sampled (Table 1). Necrotic canals (41/60) yielded 188 isolates, 124 of them being gram-positives and 137 strict anaerobic bacteria. Root-treated canals (19/60) held 36 different bacterial species, 27 of them being gram-positives and 16 facultative microorganisms.

All different colony types isolated from the primary cultures were subcultured and identified. Individual root canals yielded a maximum of 10 bacterial species each. Six root canals, all of them with previous root canal treatment, had no cultivable bacteria. A single microorganism was found in eight cases (two of them with primary infection). Four cases presented two species (*Streptococcus sanguis* and *Staphylococcus aureus; Actinomyces odontolyticus* and *S. sanguis* from necrotic canals; *Peptostreptococcus micros* and *Peptostreptococcus prevotii; Propionibacterium acnes* and *E. faecalis* from root-treated canals) and 42 cases were polymicrobial infections consisting of three or more species per canal.

Of the bacterial isolates, 70% were either strict anaerobes or microphilic. The anaerobes most frequently isolated were: *P. micros* (35%), *Fusobacterium necrophorum* (23.3%), *Fusobacterium nucleatum* (11.7%), *P. intermedia/nigrescens* (16.7%), *P. gingivalis* (6.7%) and *P. endodontalis* (5%) (Table 1).

Gram-negative bacteria accounted for 32.6% of the total species isolated from the 60 canals. They accounted for 34% of isolates in primary endodontic infected canals (41/60) and for 25% of isolates in secondary root canals infected (19/60). Gram-positive bacteria accounted for 67.4% of the total isolates, 66% of isolates in canals with necrotic pulp tissue and 75% of isolates in canals with failed root canal treatments.

The bacterial genera most frequently recovered from the root canals were *Peptostreptococcus* (58.3%), *Streptococcus* (53.3%), *Fusobacterium* (33.3%), *Prevotella* (31.7%), *Enterococcus* (13.3%), *Gemella* (13.3%), *Staphylococcus* (13.3%), *Actinomyces* (11.7%), *Porphyromonas* (11.7%), *Clostridium* (10%), *Eubacterium* (10%), *Veillonella* (10%), *Lactobacillus* (8.3%), *Propionibacterium* (6.7%), *Bacteroides* (5%), *Cardiobacterium* (3.3%) and *Tissierella* (3.3%). Genera isolated once included *Bifidobacterium, Capnocytophaga, Haemophilus* and *Neisseria*.

Root canals with primary endodontic infection yielded *P. micros, P. prevotii, F. necrophorum, P. intermedia/nigrescens, Streptococcus constellatus, Streptococcus anginosus, S. sanguis, F. nucleatum, Gemella morbillorum, Streptococcus mitis, P. gingivalis, P. loescheii* and *Lactobacillus acidophilus* (Table 1).

Peptostreptococcus saccharolyticus, Peptostreptococcus tetradius, Staphylococcus lentus, Streptococcus salivarius and *Actinomyces naeslundii* (all of them gram-positive bacteria) were isolated only from root canal treated teeth. *Prevotella buccae* and especially *E. faecalis* predominated in treated canals (Table 1).

P. acnes, Peptostreptococcus magnus and *P. denticola* were present in equal proportions in both primary and secondary infected root canals.

The clinical characteristics of the 60 canals studied were as follows: pain (29/60), previous pain (31/60), tenderness to percussion (29/60), swelling (20/60), sinus (7/60), tooth mobility (6/60), periapical radiolucency (53/60), wet canal (33/60), purulent exudate (20/60), hemorrhagic exudate (9/60), and clear exudate (4/60). Necrotic pulp tissue was observed in 41 canals and the remaining 19 canals had previously been root filled with gutta-percha.

Suggested relationships were found between anaerobes, especially gram-negatives, and the presence or history of previous pain, tenderness to percussion and

Table 1. Prevalence of bacterial species in 60 root canals

Species	Number of root canals with necrotic pulp	Number of root canals with endodontic failure
1. Peptostreptococcus micros	**18**	**3**
2. Peptostreptococcus prevotii	**17**	**1**
3. Fusobacterium necrophorum	**12**	**2**
4. Prevotella intermedia/nigrescens	**9**	**1**
5. Streptococcus constellatus	**9**	**1**
6. Enterococcus faecalis	**2**	**6**
7. Streptococcus anginosus	**6**	**2**
8. Streptococcus sanguis	**6**	**2**
9. Fusobacterium nucleatum	7	0
10. Gemella morbillorum	**6**	**1**
11. Streptococcus mitis	**5**	**2**
12. Prevotella corporis	6	0
13. Veillonella spp.	6	0
14. Actinomyces odontolyticus	5	0
15. Staphylococcus epidermidis	5	0
16. Porphyromonas gingivalis	**3**	**1**
17. Prevotella loescheii	**3**	**1**
18. Propionibacterium acnes	**2**	**2**
19. Streptococcus parasanguis	4	0
20. Actinomyces meyeri	3	0
21. Bacteroides gracilis	3	0
22. Eubacterium aerofaciens	3	0
23. Eubacterium lentum	3	0
24. Lactobacillus acidophilus	**2**	**1**
25. Porphyromonas endodontalis	3	0
26. Prevotella buccae	**1**	**2**
27. Actinomyces naeslundii	0	**2**
28. Cardiobacterium hominis	2	0
29. Clostridium subterminale	2	0
30. Gemella haemophylus	2	0
31. Peptostreptococcus magnus	**1**	**1**
32. Prevotella denticola	**1**	**1**
33. Prevotella oralis	2	0
34. Staphylococcus sacharolyticus	2	0
35. Streptococcus oralis	2	0
36. Tissierella praecuta	2	0
37. Bifidobacterium breve	1	0
38. Capnocytophaga spp.	1	0
39. Clostridium hastiforme	1	0
40. Clostridium butyricum	1	0
41. Lactobacillus plantarum	1	0
42. Neisseria spp.	1	0
43. Peptostreptococcus anaerobius	1	0
44. Peptostreptococcus asacharolyticus	1	0
45. Peptostreptococcus sacharolyticus	0	**1**
46. Peptostreptococcus tetradius	1	0
47. Prevotella melaninogenica	0	**1**
48. Prevotella oris	1	0
49. Staphylococcus aureus	1	0
50. Staphylococcus lentus	0	**1**
51. Streptococcus intermedius	1	0
52. Streptococcus mutans	1	0
53. Streptococcus salivarius	0	**1**

Boldface: species recovered from primary and secondary endodontic infections.
Dark gray: microorganisms recovered in greater numbers from primary endodontic infection.
Light gray: microorganisms recovered in equal numbers from both types of canals.
Hatched boxes: microorganisms recovered in greater numbers from endodontic treatment failures.

swelling ($P < 0.05$). In particular, associations were found between:

- pain and *P. micros* ($P < 0.01$), *P. intermedia*/*nigrescens* and *Eubacterium* spp. (both $P < 0.05$);
- history of pain and *P. micros* ($P < 0.01$), *Porphyromonas* and *Fusobacterium* spp. ($P < 0.05$);
- tenderness to percussion and *Porphyromonas* spp. ($P < 0.01$), *Peptostreptococcus* and *Fusobacterium* spp. ($P < 0.001$);
- swelling and *Peptostreptococcus* spp. ($P < 0.01$), *Porphyromonas* and *Enterococcus* spp. ($P < 0.05$);
- wet canals and *Porphyromonas* and *Fusobacterium* spp. ($P < 0.05$);
- purulent exudate and *Porphyromonas, Peptostreptococcus* and *Fusobacterium* spp. ($P < 0.05$);
- periapical radiolucency and *P. micros* ($P < 0.05$);
- previous endodontic treatment and *E. faecalis, Streptococcus* spp., *P. micros, F. necrophorum* ($P < 0.05$).

No correlation was found between sinus tracts and tooth mobility and the presence of any specific bacterial species. No significant association was found between patient tooth type, gender or age group and the presence of clinical features.

Discussion and conclusion

The present study investigated the microbiota of primary and secondary infected root canals. Primary infected root canals are untreated canals where microorganisms are able to access and colonize the pulpal tissue and impair its function. The most common pathways to the pulp are direct extension from carious lesions and via cavity preparation, which frequently opens up extensive dentinal tubular access. Other pathways include exposure of accessory canals and apical foramina in periodontal disease, exposure of dentin following caries, erosion or cracking the enamel, restorative procedures, anachoresis, and direct exposure of pulp (41). All 41 teeth with primary endodontic infection presented necrotic pulp tissues and periapical lesions, which are induced by bacteria present in infected root canals.

Secondary infected root canals indicate a failure of the endodontic treatment, especially due to the persistence of microbial infection in the root canal system. These microorganisms may have survived the biochemical procedures or invaded the canal via coronal leakage of the root filling (27). The 19 teeth investigated in the present study had previously been root-filled for more than 4 years and showed radiographic evidence of apical periodontitis.

In this study the only exclusion criteria were the impracticality of isolation with rubber dam and prior antibiotic therapy. Therefore, this selection of source material is more representative of the range of clinical situations encountered daily in patients referred for endodontic therapy.

Our findings indicate potential complex interactions of species resulting in characteristic clinical pictures which cannot be achieved by individual species alone. However, the range of microorganisms clinically involved may be even wider, because some microorganisms will not grow reliably on available culture media despite the use of anaerobic techniques (10).

To optimize the recovery of anaerobic species, the present study employed pre-

reduced nutritious media, which were promptly incubated under anaerobic conditions and prolonged incubation period to ensure the recovery of slow-growing species. We did not detect cultivable bacteria in 10% (6/60) of root canals, all of them with previous root canal treatment (6/19). Cultural studies by Yoshida et al. (41), Wasfy et al. (38), Gomes et al. (6–11), Sundqvist et al. (36) and Molander et al. (20) also did not isolate detectable microorganisms in several endodontic samples. It is possible that the sampling with paper point in retreatment cases may result in the detection of a lower number of bacteria, because some microorganisms may be removed together with the debris produced when the canal is re-accessed and opened up (7).

In the present study, acute symptoms of pain, history of previous pain, tenderness to percussion and swelling were associated with gram-negative species, especially *Prevotella, Porphyromonas* spp. and fusobacteria, agreeing with the findings of other authors (5–11, 14–16, 32–36, 41). Gram-negative bacteria contain endotoxin, which can stimulate production of bradykinin, a potent pain mediator. It is likely that it is associated with painful infectious exacerbations during endodontic therapy (29).

P. micros, a gram-positive coccus, was statistically associated with pain, previous pain, tenderness to percussion and wet canals. Such findings corroborate work by Yoshida et al. (41), Gomes et al. (7, 10) and Drucker et al. (5). *P. micros* has also been associated with both endodontic (3, 39, 40) and periodontal abscesses (24).

Baumgartner et al. (2) microbiologically investigated 40 canals of teeth with necrotic pulp and periodontitis. Seventeen canals were associated with purulent exudate, and black-pigmented bacteria were found in 9/17 cases (*P. nigrescens* in 6 cases and *P. intermedia* in 3/9 cases). Sundqvist et al. (35) found black-pigmented bacteria in 16 out of 22 canals associated with purulent exudation. In our study, purulent exudate was statistically associated with *P. gingivalis, P. endodontalis, P. micros, F. nucleatum and S. sanguis* (all $P < 0.05$). Microorganisms not statistically associated with the clinical characteristics studied included *P. intermedia/nigrescens, P. prevotii, F. necrophorum, Eubacterium aerofaciens, Actinomyces meyeri, A. odontolyticus, Veillonella* spp. and *Streptococcus constellatus*. Brook et al. (3), Sundqvist et al. (35), van Winkelhoff et al. (37), Gomes et al. (7, 10) and Baumgartner et al. (2) also detected those microorganisms in endodontic infections.

Teeth with endodontic failure were statistically associated with facultative microorganisms such as *Enterococcus* and *Streptococcus* ($P < 0.05$), in accordance with the findings of Molander et al. (20), Sundqvist et al. (36), and Pinheiro et al. (26, 27). The present study also found failed endodontic treatment to be associated with strict anaerobic bacteria including *P. micros* and *F. necrophorum* ($P < 0.05$).

Gram-positive bacteria accounted for 67.4% of the total species isolated per root canal. A similar percentage of gram-positive bacteria has been reported (7, 10). The high proportion of gram-positive bacteria may reflect the nature of the root canal samples in the present study, which involved teeth with primary (necrotic pulp tissues) and also secondary (cases of retreatment) endodontic infections.

A clear difference was found in the microbial composition of primary and secondary infected root canals. The root canal microflora of untreated teeth with apical periodontitis was found to be mixed, comprising gram-negative and gram-positive and mostly anaerobic microorganisms and usually containing more than 3 species per canal. On the other hand, facultative anaerobic and gram-positive bacteria predominated in canals with failed endodontic treatment; these canals generally harbored 1–2 species. The increased occurrence of gram-positive bacteria may be due to increased resistance to instrumentation and to antiseptic agents (9, 20). According to Molander et al. (20) facultative anaerobes, especially the gram-positives, can survive in a quiescent phase with low metabolic activity for a period of time, and factors like coronal leakage during or after root canal treatment can change the nutritional conditions and contribute to bacterial growth. *E. faecalis* was isolated as a single species from 6/19 canals with failed endodontic therapy. This organism has demonstrated the capacity to survive in an environment in which there are scant nutrients and in which commensality with other bacteria is minimal (36). It has been postulated that a virulence factor of *E. faecalis* in failed endodontically treated teeth may be related to the ability of *E. faecalis* cells to maintain the capability to invade dentinal tubules and adhere to collagen in the presence of human serum (18).

The cell walls of gram-positive bacteria such as *Peptostreptococcus* and *Eubacterium* spp. include peptidoglycans and lipoteichoic acids, which can influence inflammatory reactions and enhance the pathogenicity of ''black-pigmented *Bacteroides*'' (5, 7, 34), and are also related to acute symptoms and destruction of periapical tissues (7, 10, 12, 15, 16, 41). Studies have shown that the combination of *P. micros* and *Prevotella* spp. is associated with pain and swelling (5, 7–9, 10, 34). One explanation for the synergy between these species is the known enhancement of the endotoxin effect by gram-positive superantigens (5).

The use of modern molecular assays has identified species from infected root canals that are difficult to culture, such as *Tannerella forsythensis* (*Bacteroides forsythus)* and *Treponema denticola* (16, 30). Although no correlation was found between clinical endodontic features and these specific bacteria, it should be cautioned that the methodologies used did not enumerate the total number of bacteria collected in a sample. Lyons et al. (19) found higher levels of *P. gingivalis* in samples from subjects with periodontitis than in sites without evidence of disease using Real-Time PCR, a method that offers the ability to determine the absolute and relative amounts of an specific microorganism in a mixed sample without the need for culturing. This points out the importance of considering, when using molecular assays, not only the presence of specific microorganisms but also the number of cells in a sample, in order to associate it with the signs and symptoms. To date there is no single method capable of recovering and quantifying every microorganism found in the pulp space and related structures. Ongoing research using molecular techniques aims to analyze, in more details, endodontic samples in order to obtain a more comprehensive description of the endodontopathic microorganisms.

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