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Evaluation of the microbiota of primary endodontic infections using checkerboard DNA–DNA hybridization

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Background/aims: The aim of this study was to evaluate the composition of the microbiota of primary endodontic infections in 111 selected cases of single-rooted teeth with necrotic pulp.
Methods: Samples were collected from the root canals using #15 Hedströen-type files and two sterile paper points, which were introduced 1 mm short of the apical foramen. The presence, levels, and proportions of 40 different bacterial species in each sample were determined using DNA probes and checkerboard DNA–DNA hybridization techniques.
Results: The mean number of species per sample was 22. *Enterococcus faecalis* (89.3%), *Campylobacter gracilis* (89.3%), *Leptotrichia buccalis* (89.3%), *Neisseria mucosa* (87.5%), *Prevotella melaninogenica* (86.6%), *Fusobacterium nucleatum* ssp. *vincentii* (85.7%), *Eubacterium saburreum* (75.9%), *Streptococcus anginosus* (75%), and *Veillonella parvula* (74.1%) were the most prevalent species. The species found in highest mean counts (over 10^5) were *F. nucleatum* ssp. *vincentii* (13.14×10^5), *E. saburreum* (5.67×10^5), *E. faecalis* (5.38×10^5), *N. mucosa* (4.19×10^5), *V. parvula* (3.63×10^5), *C. gracilis* (3.46×10^5), *Treponema socranskii* (3.34×10^5), *Porphyromonas endodontalis* (2.96×10^5), *Porphyromonas gingivalis* (2.85×10^5), *Micromonas micros* (2.81×10^5), *Prevotella nigrescens* (2.68×10^5) and *Fusobacterium nucleatum* ssp. *nucleatum* (2.64×10^5). Most of these species were also found in high proportions.
Conclusions: Our results suggest that several bacterial species considered to be oral pathogens seem to be implicated in the etiology of primary endodontic infections.

Key words: bacteria; DNA probes; endodontics; symptoms

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It is well established in the literature that bacteria and their by-products are critical factors in the development of pulp and periradicular pathosis. The harmful effects of these organisms on the pulp tissue have been the subjects of many studies since 1894 (13–15, 33).

Improvements in the culture techniques for anaerobic bacteria have allowed more detailed descriptions of the composition of the microbiota of endodontic infections (15, 33). However, isolating and maintaining viable cultures of some of these species in laboratory conditions are difficult tasks. Studies using molecular diagnostic methods, such as the checkerboard DNA–DNA hybridization (32) and polymerase chain reaction (PCR), have led to a better comprehension of the composition of the pulp and periapical microbiota (4, 6, 11, 27, 28).

The checkerboard DNA–DNA hybridization method allows the evaluation of a high number of samples and bacterial species and has the great advantage of not requiring bacterial viability, permitting the identification of species that are

difficult to grow in agar plates. This technique was described by Socransky et al. (32) and has been used to study the oral microbiota, particularly the composition of supragingival and subgingival plaque in periodontal health and disease (10, 31). This technique uses whole-genomic DNA probes and allows the simultaneous identification and quantification of a multitude of bacterial species in multiple clinical samples. Besides, it does not demand viable bacterial strains because only their preserved DNA is needed (32). Molecular techniques such as checkerboard DNA–DNA hybridization have the potential to enhance the understanding of the endodontic microbiota and, thereby, to provide more specific root canal therapies, based on the etiological agents of those infections.

Therefore, the aim of this study was to investigate the presence, levels, and proportions of 40 bacterial species in clinical samples from 111 cases of primary endodontic infections, using the checkerboard DNA–DNA hybridization technique.

Material and methods

Subject population

The samples used in this study were obtained from 111 subjects selected from the Endodontics Clinics of three different Brazilian Dental Schools: Rio de Janeiro State University (UERJ), Gama Filho University (UGF), and UNIGRANRIO University. All patients were informed about the study's nature and goals and they all signed an informed consent form before entering the study. This work was submitted to the UERJ Research Ethics Committee and received its approval (745-CEP/HUPE).

Inclusion and exclusion criteria

To be included in the study subjects had to be in good systemic health and to present at least one single-rooted tooth with necrotic pulp and radiographic evidence of bone loss that had never received root canal treatment, characterized as primary endodontic infection. None of the sampled teeth presented either crowns or bridges. Sixty-eight teeth had permanent restorations (twenty-eight teeth were clinically and/or radiographically defective and 40 of them appeared to be clinically or radiographically sound). Cavities were evident in 60 teeth, 29 of them were open cavities. Thirty teeth presented chronic clinical symptoms as provoked pain (15 teeth presented pain to percussion, five teeth presented pain to palpation and 10 presented pain to both percussion and palpation) and 15 teeth had associated sinus tract. Twenty-nine cases presented with open cavities. Exclusion criteria were pregnancy, breastfeeding, and any systemic condition that could affect the progression of infectious disease or that required antibiotic coverage for routine dental therapy. In addition, subjects who had received antibiotic or anti-inflammatory therapy in the previous 6 months were excluded.

Sample collection

Samples were collected from 111 single-rooted teeth of adult subjects under strict aseptic conditions. Initially, the tooth was cleaned with pumice and isolated with a rubber dam. The tooth and the rubber dam were cleaned with a solution of 3% hydrogen peroxide and then disinfected with a solution of 2.5% NaOCl. The coronal access was performed using sterile burs, without water spray. The pulp chamber and the operating field were once again disinfected by swabbing with 2.5% NaOCl. This solution was inactivated with sterile 5% sodium thiosulfate. Samples were collected using a #15 H-type file (Dentsply/Maillefer®, Ballaigues, Switzerland) with the handle cut off. The file was introduced 1 mm short of the apical foramen with a discrete filing motion. Then, two sterile paper points were introduced to the same level as the file and were used to soak up the fluid found on the root canal for 1 min each. Both file and paper points were then transferred to Eppendorf tubes containing 150 µl of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.6). Then, 0.1 ml 0.5 M NaOH was added to each tube and the samples were frozen at –20°C until they were processed.

Microbiological assessment – checkerboard DNA–DNA hybridization

Preparation of DNA probes

All 40 bacterial strains used in the preparation of DNA probes are presented in Table 1. Bacterial stocks were rehydrated in *Mycoplasma* broth (Difco Laboratories, Detroit, MI) and grown for 3–7 days on trypticase soy agar with 5% defibrinated sheep blood (Baltimore Biological Laboratories, Cockeysville, MD) at 35°C under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2). Some bacterial strains were grown on supplemented or enriched media. *Tannerella forsythia* was grown on trypticase soy agar supplemented with 5% defibrinated sheep blood and 10 µg/ml *N*-acetylmuramic acid (Sigma Chemical Co., St Louis, MO). *Porphyromonas gingivalis* and *Porphyromonas endodontalis* were grown in a similar medium supplemented with 5% defibrinated sheep blood, 0.3 µg/ml menadione (Sigma) and 5 µg/ml hemin (Sigma). *Eubacterium* and *Neisseria* species were grown on fastidious anaerobic agar (Baltimore Biological Laboratories) with 5% defibrinated sheep blood. *Treponema denticola* and *Treponema socranskii* were grown in *Mycoplasma* broth supplemented with 1 mg/ml glucose, 400 µg/ml niacinamide, 150 µg/ml spermine tetrahydrochloride, 20 µg/ml sodium isobutyrate, 1 mg/ml L-cysteine, 5 µg/ml thiamine pyrophosphate and 0.5% bovine serum.

The bacterial growth was harvested and placed in 1.5-ml microcentrifuge tubes containing 1 ml TE buffer. Cells were resuspended and lyzed either with 10% sodium dodecyl sulfate and Proteinase K (20 mg/ml) (Sigma) for gram-negative strains or in 150 µl of an enzyme mixture containing 15 mg/ml lysozyme (Sigma) and 5 mg/ml achromopeptidase (Sigma) in TE buffer (pH 8.0) for gram-positive strains. The pelleted cells were resuspended by a 15-s sonication and incubated at 37°C for 1 h. DNA was isolated and purified as described by Smith et al. (30). The concentration of purified DNA was determined by spectrophotometric measurement of the absorbance at 260 nm. The purity of preparations was assessed by the ratio of DNA to protein (as measured by the ratio of absorbances at 260 and 280 nm). Whole-genomic DNA probes were prepared from each of the 40 test species by labeling 1 µg DNA with digoxigenin using the Random Primer Digoxigenin Labeling Kit (Boehringer Mannheim, Indianapolis, IN) (5).

Checkerboard DNA–DNA hybridization

The presence, levels, and proportions of 40 different bacterial species (Table 1) were determined by the checkerboard DNA–DNA hybridization method described by Socransky et al. (32).

Briefly, the samples were boiled for 10 min and neutralized using 0.8 ml 5 M ammonium acetate. The released DNA was then placed into the extended slots of a Minislot 30™ apparatus (Immunetics, Cambridge, MA), concentrated onto a 15 × 15 positively charged nylon membrane (Boehringer-Mannheim) and fixed to the membrane by baking it at 120°C for 20 min. A Miniblotter 45™ (Immunetics, Cambridge, MA) device was used to hybridize the 40

Table 1. Bacterial strains used for preparation of DNA probes used in this study

Species	Strains
Aggregatibacter actinomycetemcomitans	*
Actinomyces gerencseriae[1]	23860
Actinomyces israelii[1]	12102
Actinomyces naeslundii genospecies *I*[1]	12104
Actinomyces odontolyticus[1]	17929
Campylobacter gracilis[1]	33236
Campylobacter rectus[1]	33238
Campylobacter showae[1]	51146
Campylobacter gingivalis[1]	33624
Campylobacter ochracea[1]	33596
Campylobacter sputigena[1]	33612
Eikenella corrodens[1]	23834
Enterococcus faecalis[1]	29212
Eubacterium nodatum[1]	33099
Eubacterium saburreum[1]	33271
Fusobacterium nucleatum ssp. *nucleatum*[1]	25586
Fusobacterium nucleatum ssp. *polymorphum*[1]	10953
Fusobacterium nucleatum ssp. *vicentii*[1]	49256
Fusobacterium periodonticum[1]	33693
Gemella morbillorum[1]	27284
Leptotrichia buccalis	14201
Peptostreptococcus micros[1]	33270
Neisseria mucosa	19696
Porphyromonas endodontalis[1]	35406
Porphyromonas gingivalis[1]	33277
Prevotella intermedia[1]	25611
Prevotella melaninogenica[1]	25845
Prevotella nigrescens[1]	33563
Propionibacterium acnes	**
Selenomonas noxia[1]	43541
Streptococcus anginosus[1]	33397
Streptococcus constellatus[1]	27823
Streptococcus gordonii[1]	10558
Streptococcus intermedius[1]	27335
Streptococcus mitis[1]	49456
Streptococcus oralis[1]	35037
Tannerella forsythia[1]	43037
Treponema denticola[2]	B1
Treponema socranskii[2]	S1
Veillonella parvula[1]	10790

[1]ATCC (American Type Culture Collection, Rockville, MD) and [2]The Forsyth Institute.
*ATCC strains 43718 and 29523; **ATCC strains 11827 and 11828.

digoxigenin-labeled whole-genomic DNA probes at right angles to the lanes of the clinical samples. Bound probes were detected through the utilization of phosphatase-conjugated antibodies to digoxigenin and chemiluminescence (CDP-Star Detection Reagent™, Amersham Biosciences, Chicago, IL, USA). Signals were visually evaluated by comparison with the two standards. These standards consisted of a mixture at 10^5 and 10^6 cells of each bacterial species tested, placed in the last two lanes of each membrane. The signals were coded in six different classes in relation to the count levels observed: (0) not detected; (1) $<10^5$ cells; (2) nearly 10^5 cells; (3) between 10^5 and 10^6 cells; (4) nearly 10^6 cells; (5) $>10^6$ cells. Signals were also coded as (0) for presence and (1) for absence of each tested bacterial species. The sensitivity of this assay permitted the detection of 10^4 cells of a given bacterial species by adjusting the concentration of each DNA probe.

Statistical analysis

Microbiological data were available from all the root canals sampled at the baseline visit. Mean counts $\times 10^5$ of each species were computed for each root canal and then averaged across root canals. In a similar fashion, the percentage of the total DNA probe count was determined for each species in each root canal and averaged across root canals. Prevalence was computed by determining the proportions of the root canals colonized by each species at counts $\geq 10^4$ cells and then averaging these proportions across root canals.

Results

The population's age ranged from 18 to 70 years old (mean age 34 years) and 65% of the subjects were women. One hundred and five of the 111 samples evaluated had radiographic evidence of bone loss. Each of the 40 DNA probes tested hybridized with at least four different samples. The mean number of bacterial species detected in the samples was 22, ranging from 1 to 40 species per sample.

Figure 1 presents the prevalence (percentage of teeth colonized) and standard deviation (±SD) of the 40 bacterial species evaluated. The microorganisms found in more than 70% of the samples tested were *Enterococcus faecalis* (89.3% ± 0.31), *Campylobacter gracilis* (89.3% ± 0.31), *Leptotrichia buccalis* (89.3% ± 0.31), *Neisseria mucosa* (87.5% ± 0.31), *Prevotella melaninogenica* (86.6% ± 0.342), *Fusobacterium nucleatum* ssp. *vincentii* (85.7% ± 0.35), *Eubacterium saburreum* (75.9% ± 0.42), *Streptococcus anginosus* (75% ± 0.43), and *Veillonella parvula* (74.1% ± 0.44). Conversely, *Streptococcus intermedius* (24.1% ± 0.43), *Actinomyces naeslundii I* (17.0% ± 0.38), and *Actinomyces gerencseriae* (15.2% ± 0.36) were found in less than 25% of the teeth evaluated.

Mean counts ($\times 10^5$; ±SD) of the 40 microorganisms studied were presented in Fig. 2. The species found at the highest levels were *F. nucleatum* ssp. *vincentii* (13.14 ± 3.03), *E. saburreum* (5.67 ± 1.85), *E. faecalis* (5.38 ± 1.61), *N. mucosa* (4.19 ± 1.61), *V. parvula* (3.63 ± 1.61), *C. gracilis* (3.46 ± 1.33), *T. socranskii* (3.34 ± 1.65), *P. endodontalis* (2.96 ± 1.62), *P. gingivalis* (2.85 ± 1.33), *Peptostreptococcus micros* (2.81 ± 1.34), *Prevotella nigrescens* (2.68 ± 1.33), and *F. n.* ssp. *nucleatum* (2.64 ± 1.33). The species found in the lowest numbers were *Actinomyces israelii* (0.18 ± 1.05), *Capnocytophaga gingivalis* (0.15 ± 0.51), *Aggregatibacter actinomycetemcomitans* (0.12 ± 0.50), *Streptococcus oralis* (0.09 ± 0.25), *Campylobacter rectus* (0.08 ± 0.20), and *A. gerencseriae* (0.07 ± 0.48).

The mean percentage of DNA probe counts (proportions) of each species evaluated is shown in Fig. 3. The species found in the lowest proportions were *A. actinomycetemcomitans* (0.5 ± 0.11; $\times 10^5$; ±SD), *C. rectus* (0.47 ± 0.10), *S. oralis* (0.47 ± 0.13), *A. israelii* (0.45 ± 0.13), and *A. gerencseriae* (0.2 ± 0.09). Each of these species represented less than 0.5% of the total microbiota evaluated. Conversely, *F. n.* ssp. *vincentii* (12.9 ± 3.9), *E. faecalis* (9.05 ± 2.65), *E. saburreum* (7.45 ± 3.45), *V. parvula* (6.2 ± 3.63), *N. mucosa* (6.12 ± 4.22), and *T. forsythia* (4.8 ± 4.09) were present in the highest proportions. Each of them represented more than 4% of the total microbiota studied.

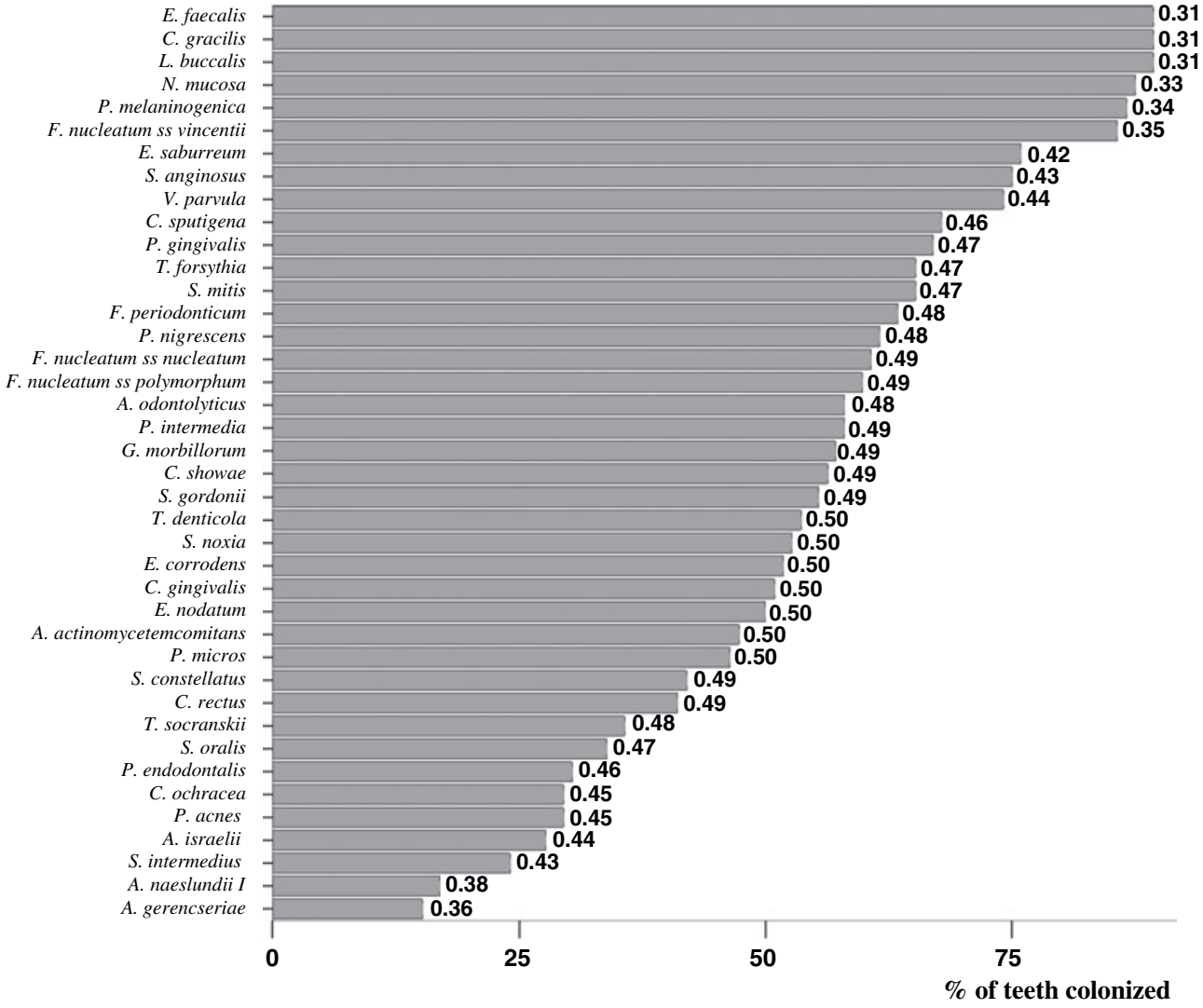

Fig. 1. Prevalence (% of teeth colonized; ±SD) of the 40 bacterial species in the 111 evaluated samples. The species are represented in decreasing order according to prevalence.

Discussion

From a theoretical standpoint, any bacterial species found in the oral cavity could colonize the root canal system. However, only a fraction of the total number of species is observed in that particular habitat (34–36). Unfortunately, most of the investigations that aimed to evaluate the composition of the endodontic microbiota have studied either only a few samples or only a few bacterial species. Therefore, to comprehensively study the microbiota of primary endodontic infections, the present study evaluated a large number of samples and microorganisms. The presence, levels, and proportions of 40 bacterial species were investigated in 111 infected root canals.

In general, the microbiota of primary endodontic infections is mixed, with predominance of anaerobic bacteria, such as *Porphyromonas*, *Prevotella*, *Fusobacterium*, *Peptostreptococcus*, and *Campylobacter* species and facultative organisms, such as *Streptococcus* species (4, 7, 8, 21, 25, 26). Our findings agree to a large extent with those results, with more than 70% of the root canal samples comprising *E. faecalis*, *C. gracilis*, *L. buccalis*, *N. mucosa, P. melaninogenica*, *F. n.* ssp. *vincentii*, *E. saburreum, V. parvula*, and *S. anginosus*. Other studies using different diagnostic techniques also showed a high prevalence of *F. nucleatum* in these infectious processes (4, 33, 34, 37, 38). Among the black-pigmented bacteria tested in the present study, *P. melaninogenica* was the most prevalent species (86.6% ± 0.34), followed by *P. gingivalis* (67% ± 0.47), *P. nigrescens* (64.6% ± 0.48), *Prevotella intermedia* (58% ± 0.496) and *P. endodontalis* (30.4% ± 0.46). Using the checkerboard DNA–DNA hybridization technique, de Souza et al. (4) also indicated that *P. melaninogenica* and *P. gingivalis* (75%) were the black-pigmented bacteria species detected in highest prevalences. Molecular diagnostic methods usually detect higher prevalence of black-pigmented bacteria than the traditional culture methods (4, 6, 16, 37). The technical difficulties involved in cultivating these species could have underestimated their prevalence and counts in some of the studies.

The spirochetes have been associated with pulp infections since 1894 (14) and

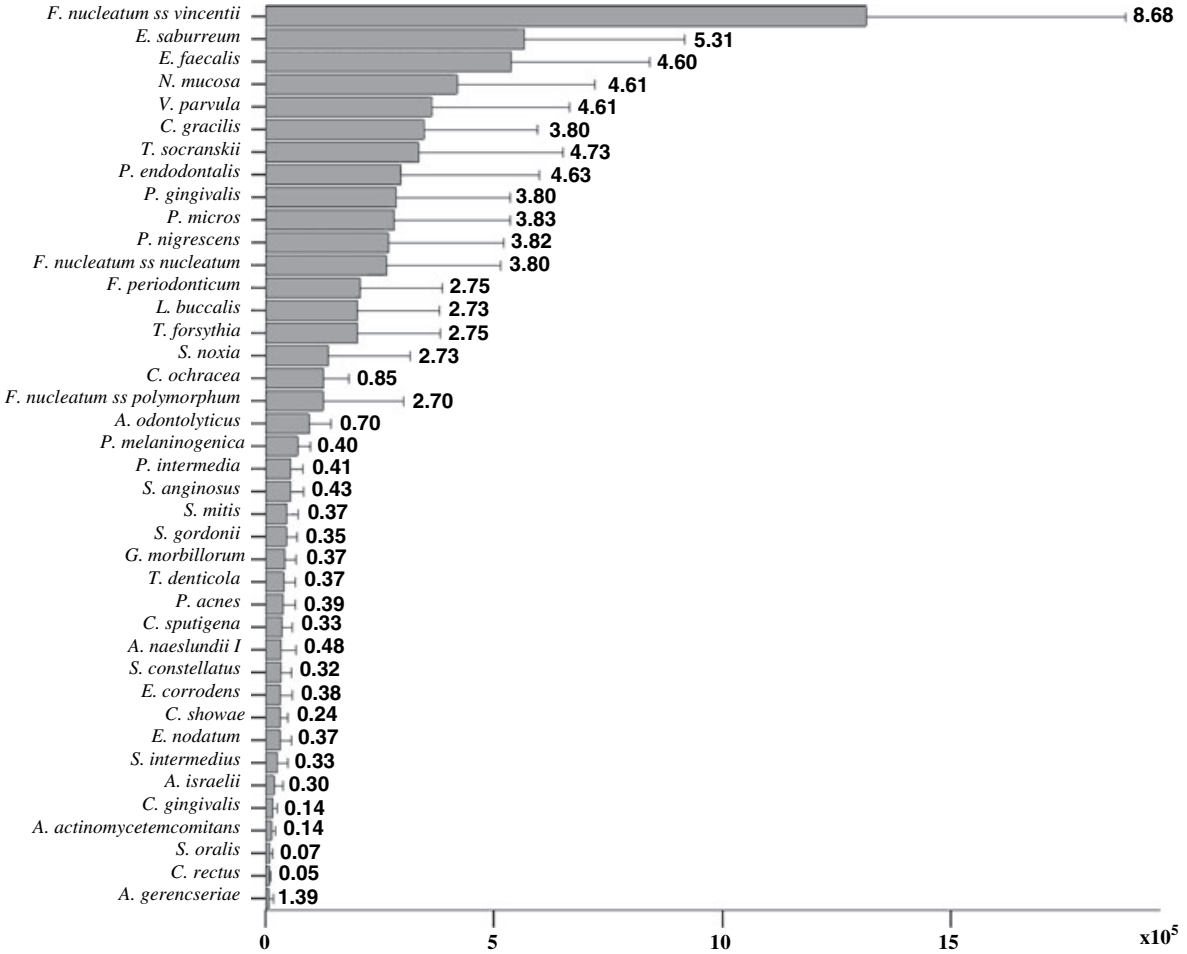

Fig. 2. Mean counts ($\times 10^5$ bacterial cells; ±SD) of the 40 bacterial species tested in the 111 samples evaluated. The species are represented in decreasing order according to mean count levels.

they are also known to be periodontal pathogens (10, 31). Their presence in infectious endodontic processes was underestimated for a long time because they are strictly anaerobic and fastidious bacteria that do not grow on solid media without special techniques. Only after the emergence of the molecular detection methods in the 1990s, more than a century after their first association with tooth infections, were *Treponema* species again pointed out as possible endodontic pathogens (24). In this paper, *T. denticola* was detected in 53.6% (±0.50) of the samples, while *T. socranskii* was found in 35.7% (±0.48). Several studies using PCR (2, 17, 19, 22) also found a high prevalence of spirochetes in primary endodontic infections. However, the results from other studies employing the checkerboard technique (4, 28) and those from the present study appear to be somewhat conflicting. Some points that could explain these divergent results are the differences among the clinical scenarios of samples (acute abscess vs. chronic infections), sampling technique (aspiration or K-type file vs. H-type one), storage of samples (TSB-DMSO vs. TE buffer and NaOH) and number of samples (no more than 27 vs. 111 in the present study). More studies with these factors standardized should be conducted to discover the actual relevance of spirochetes in each clinical scenario of endodontic infection.

Another periodontal pathogen, *T. forsythia*, was first identified in infected necrotic pulps by Conrads et al. (3), using PCR. This species was found in 65.2% (±0.47) of the samples in the present study and in 55% of the samples examined by de Souza et al. (4). Other studies also using the checkerboard DNA–DNA hybridization technique found lower prevalences of *T. forsythia*, varying from 29.6% (28) to 40% (27). A possible explanation for these differences is the different methods used in the studies, such as number of samples evaluated (4, 27, 28), a different clinical scenario or type of sample (28), and the storage of these samples (27, 28). For example, while some investigations (4), including the present study, stored the samples in TE buffer and NaOH, other researchers used TSB-DMSO (27, 28). Also, the authors of the present study opted for collecting samples by means of an H-type file instead of a K-type one (4, 19, 27) because the design of the H-type file allows for a larger amount of sample material to be taken.

In this study, *E. faecalis* was the most prevalent bacterial species (89.3% ± 0.31) and it was also present in root canals at high levels and proportions (Figs 1, 2, and 3). This finding was unanticipated to some extent because this species is usually associated with persistent infections (8, 18, 29) and has been found with lower prevalence in samples of primary endodontic infections evaluated by microbial culture (1, 29) and molecular meth-

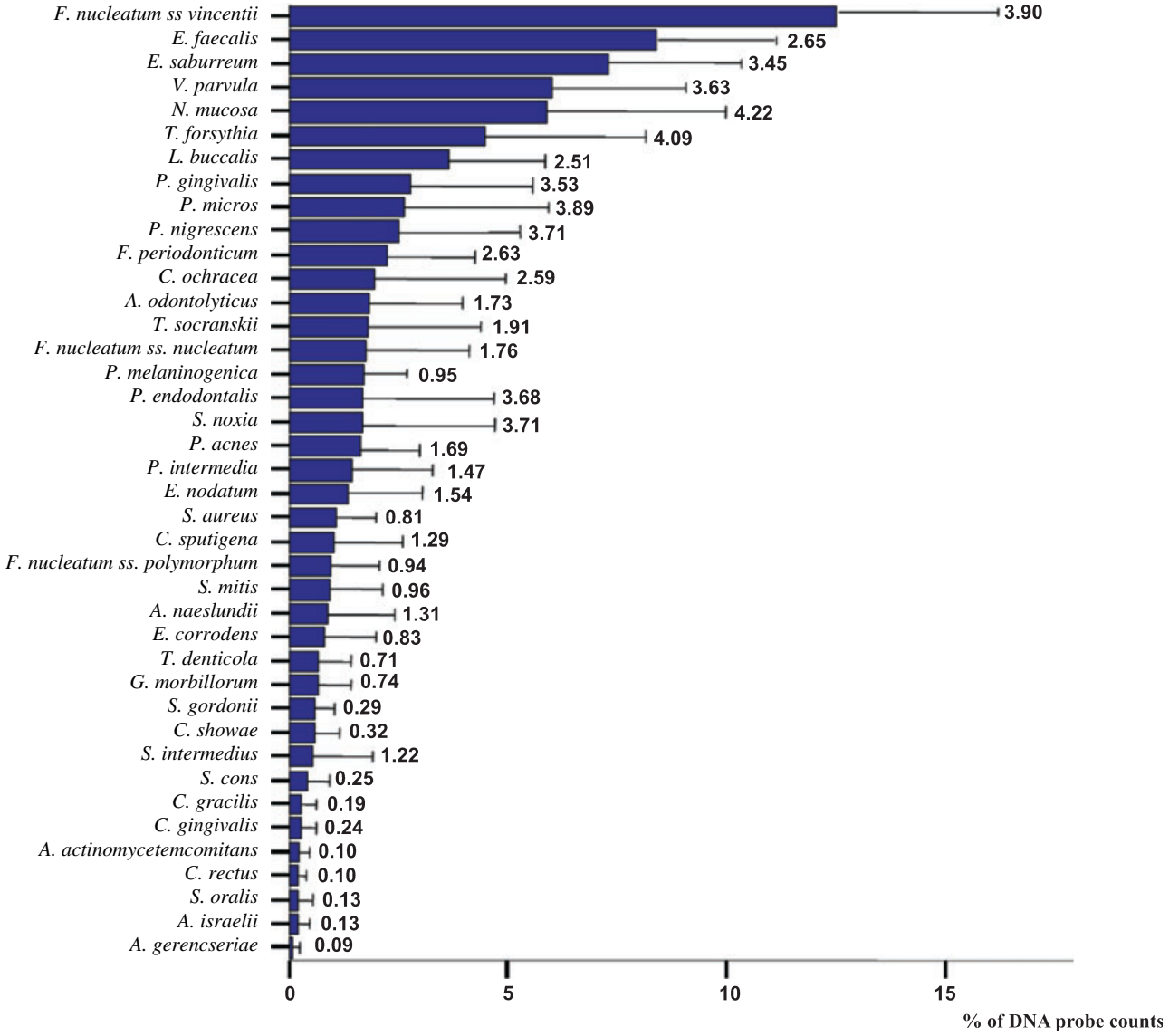

Fig. 3. Proportion of the total DNA probe counts (mean percentage of DNA probe counts; ±SD) of the 40 bacterial species evaluated. The species are represented in decreasing order according to mean percentage of DNA probe counts.

ods (4, 27, 28). However, two recent studies presented data similar to ours. Sedgley et al. (20), using real-time PCR detected *E. faecalis* in 67.5% of the 40 samples of the primary endodontic infections investigated. The authors found the species in even higher levels (16.8×10^5) than in the present study (5.38×10^5). Gomes et al. (9), using PCR, found *E. faecalis* in 82% of the cases of primary endodontic infection that they studied.

The results of the bacterial counts obtained in this study indicated that most of the species observed at high prevalence were also found in high levels and proportions in the tested samples (Figs 1, 2 and 3). This was the case for *F. n.* ssp. *vincentii*, *E. saburreum*, *E. faecalis*, *N. mucosa, V. parvula*, *C. gracilis*, and *P. gingivalis*. Similarly, the species found at low prevalence also presented with low levels and proportions, such as *A. naeslundii I*, *S. intermedius*, *A. israelii*, *S. oralis*, and *A. gerencseriae*. It is noteworthy to point out that several species found at low prevalence and levels, such as *Streptococcus* and *Actinomyces* species, have a less virulent profile (31). Conversely, two important pathogens, *T. socranskii* and *P. endodontalis*, were found at high levels and were detected in roughly 30% of the tested samples. Other known pathogens exhibiting relatively elevated levels and prevalences were *P. micros*, *P. nigrescens*, *F. n.* ssp. *nucleatum*, *Fusobacterium periodonticum* and *T. forsythia*.

The group of the 20 microorganisms found at the highest levels (from *F. n.* ssp. *vincentii* to *P. melaninogenica*) in Fig. 2 was mostly composed of presumed virulent species. In contrast, most of the 20 species found at the lowest levels (from *P. intermedia* to *A. gerencseriae*) are considered to be more host-compatible when present in plaque samples (31). This finding suggests that the levels of bacterial species found in samples may be an important indicator of the pathogenicity of the infectious process (21). Jung et al. (12) suggested that the degree of severity

of an endodontic infection is related not merely to the presence of pathogens but to the numbers of those organisms in the infected site. In this regard, the checkerboard DNA–DNA hybridization method used in this study has the clear-cut advantage of allowing the quantification of the bacterial species found in the infected tooth (31, 32). Although the PCR technique is identified as one of the most sensitive of the molecular diagnostic methods (39), it does not allow the quantification of bacterial species. Only real-time PCR allows this kind of quantification, but the high cost of this technique limits its utilization to few samples and bacterial species (23).

Although the infectious nature of endodontic pathosis has been established for many decades, only recently, with the development of the modern microbiological diagnostic techniques, has the composition of these infections been more comprehensively studied. From that standpoint, the current study presents additional information because of the large number of samples and bacterial species evaluated. Studies of this nature could guide the establishment of more specific therapies, for different kinds of endodontic infections.

Conclusions

The microbiota found in primary endodontic infections is mixed and presents a large variety of bacterial species. Several microorganisms considered to be oral pathogens, such as *F. n.* ssp. *vincentii*, *C. gracilis*, *T. socranskii*, *P. endodontalis*, *P. gingivalis*, *P. micros*, *P. nigrescens*, and *F. n.* ssp. *nucleatum* and also *E. faecalis*, *N. mucosa*, and *V. parvula* were found at high prevalence, levels and proportions in the infected root canals. These data suggest a possible role for these species in the etiology of primary endodontic infections.

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