Advanced Caries Microbiota in Teeth with Irreversible Pulpitis

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Abstract

Introduction: Bacterial taxa in the forefront of caries biofilms are candidate pathogens for irreversible pulpitis and are possibly the first ones to invade the pulp and initiate endodontic infection. This study examined the microbiota of the most advanced layers of dentinal caries in teeth with irreversible pulpitis. **Methods:** DNA extracted from samples taken from deep dentinal caries associated with pulp exposures was analyzed for the presence and relative levels of 33 oral bacterial taxa by using reverse-capture checkerboard hybridization assay. Quantification of total bacteria, streptococci, and lactobacilli was also performed by using real-time quantitative polymerase chain reaction. Associations between the target bacterial taxa and clinical signs/symptoms were also evaluated. **Results:** The most frequently detected taxa in the checkerboard assay were *Atopobium* genomospecies C1 (53%), *Pseudoramibacter alactolyticus* (37%), *Streptococcus* species (33%), *Streptococcus mutans* (33%), *Parvimonas micra* (13%), *Fusobacterium nucleatum* (13%), and *Veillonella* species (13%). *Streptococcus* species, *Dialister invisus*, and *P. micra* were significantly associated with throbbing pain, *S. mutans* with pain to percussion, and *Lactobacillus* with continuous pain ($P < .05$). Quantitative polymerase chain reaction revealed a mean total bacterial load of 1×10^8 (range, 2.05×10^5 to 4.5×10^8) cell equivalents per milligram (wet weight) of dentin. Streptococci and lactobacilli were very prevalent but comprised only 0.09% and 2% of the whole bacterial population, respectively. **Conclusions:** Several bacterial taxa were found in advanced caries lesions in teeth with exposed pulps, and some of them were significantly associated with symptoms. A role for these taxa in the etiology of irreversible pulpitis is suspected. *(J Endod 2015;41:1450–1455)*

Key Words

Dentinal caries, irreversible pulpitis, *Lactobacillus*, microbiota, molecular biology, permanent teeth, *Streptococcus*

Pulpitis is the inflammation of the dental pulp and is commonly a sequel to caries (1). Bacteria located in the advanced frontline of the caries biofilm are directly involved in inducing damage and consequent inflammation in the pulp tissue (2–4). The bacterial effects on the pulp are caused either by bacterial virulence factors and antigens that diffuse through the dentinal fluid or by the bacterial cells themselves, which may reach the pulp via dentinal tubules, especially in very profound caries cavities (5, 6).

Pulpitis can be clinically classified as reversible or irreversible (7). In the former condition, removal of the causative agent usually permits the pulp to return to normality, whereas in the latter condition, direct intervention in the pulp tissue may be required for improved treatment outcome (8). Irreversible pulpitis usually develops when the pulp is frankly exposed to the caries biofilm (6).

Many studies evaluating the microbiota associated with deep dentinal caries revealed that the bacterial composition is substantially different from enamel caries (4, 9–11). This is highly likely to be a result of different ecological conditions associated with these lesions. In addition to lactobacilli, which are very prevalent in dentinal caries (4, 12–14), asaccharolytic and/or proteolytic anaerobic bacteria have been frequently detected (9–11, 13–15). Most of the species in carious dentin have also been detected in infected root canals (11, 14, 16–22), suggesting that in addition to being involved with pulpal damage, these dentinal lesions might well be the primary source of bacteria that initiate endodontic infections. However, there are not many studies evaluating the microbiota of advanced caries lesions in association with pulp conditions. A study identified bacteria isolated from carious lesion biofilms and vital carious exposures of pulps of deciduous teeth and observed that the microbiota of the cariously exposed pulps were similar in composition to those of carious lesion biofilms except that fewer species/taxa were identified from the pulps (23). *Actinomyces* and *Selenomonas* species were associated with carious lesions, whereas *Veillonella* species were associated with pulps. Other studies have reported a close association between pain and the presence of *Prevotella*, *Porphyromonas*, and *Fusobacterium* species in deep dentinal caries (3, 4). Black-pigmented anaerobic bacteria and *Streptococcus mutans* have been positively related to pulpal pain triggered by heat, whereas *Fusobacterium nucleatum* and *Actinomyces viscosus* have been associated with cold sensitivity (24). Positive associations between *Parvimonas micra* and *Porphyromonas endodontalis* detection in carious dentin and irreversible pulpitis have been found (2).

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Bacterial taxa present in the forefront of deep dentinal caries biofilms are candidate pathogens for irreversible pulpitis and are possibly the first ones to invade the pulp and initiate endodontic infection. The present study used reverse-capture checkerboard assay to evaluate the prevalence of several caries and endodontic bacterial pathogens in the most advanced layers of dentinal caries in teeth with the clinical diagnosis of irreversible pulpitis. Quantification of total bacteria, streptococci, and lactobacilli was also performed by using real-time quantitative polymerase chain reaction (qPCR). Associations between the presence and levels of the target bacterial taxa and clinical symptoms of irreversible pulpitis were also evaluated.

Materials and Methods

Subject Population

This study included 30 patients (23 female and 7 male) with deep occlusal caries in permanent maxillary or mandibular molars diagnosed with irreversible pulpitis. Patients ranged in age from 12 to 33 years. Each patient contributed 1 tooth. Medical history revealed no significant systemic condition or disease. Ethical approval for the study was granted by the Ethics Committee of the Federal University of Rio Grande do Norte, and informed consent was obtained from all subjects or their parents/guardians.

The diagnosis of irreversible pulpitis was based on clinical and radiographic findings and following the reports of the American Association of Endodontists Consensus Conference on diagnostic terminology (7). All cases had extensive caries lesions that led to pulp exposure. Intensity of pain was evaluated by using a visual analogue scale ranging from 0 (no pain) to 170 mm (severe pain). This scale permitted pain intensity to be ranked as mild, moderate, or severe. If present, pain was also recorded as provoked or spontaneous, intermittent or continuous, cold- or heat-evoked, localized or diffuse, and throbbing or after physical efforts. Pulp status was evaluated by thermal sensibility tests. Radiographic analysis involved extent of the caries lesion, presence of coronal restoration, stage of apical root formation, and conditions of the apical periodontal ligament space. Teeth with necrotic pulps or treated root canals and teeth with no evidence of pulp exposure after dentinal caries removal were excluded from the study.

Sample Taking and DNA Extraction

Selected teeth were cleaned with pumice, and the patient was anesthetized. Undermined enamel, superficial carious tissue, and debris were removed by using high-speed burs under water cooling. The target tooth was isolated with rubber dam, and the operative field, including the tooth, was cleaned with 6% hydrogen peroxide and disinfected with 2.5% sodium hypochlorite (NaOCl). The latter solution was inactivated with 10% sodium thiosulfate. The superficial layers of the caries lesion were removed by using a sterile spoon excavator and dismissed. Another sterile excavator was used to collect the deepest layer of dentinal caries in direct contact with the pulp, which was then transferred to cryotubes containing Tris-EDTA buffer. Transference of the material to the flasks in the clinical setting was always performed in the aseptic zone around a flame. Samples were immediately frozen at −20°C.

Caries dentin samples were weighed (wet weight), and DNA was extracted by using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA), following the protocol recommended by the manufacturer for tissues. DNA from a panel of several oral bacterial species was also prepared to serve as controls (25).

Reverse-capture Checkerboard Assay

The reverse-capture checkerboard assay used in this study was as described previously (26–28). DNA extracted from clinical samples was used as template in a 2-step 16S rRNA gene-based PCR protocol. In the first step, a practically full-length 16S rRNA gene fragment was amplified by using universal primers 8f and 1492r (26, 29, 30). In the second step, the resulting PCR product from each sample was used as template to run 2 sets of partial 16S rRNA gene amplification; one set used primers digoxigenin-8f and 519r, and the other set used primers digoxigenin-515f and 1492r (27). PCR amplifications were performed in 50 μL reaction mixture containing 1 μmol/L of each primer, 5 μL of 10× PCR buffer (Fermentas, Burlington, ON, Canada), 3 mmol/L $MgCl_2$, 2 U *Taq* DNA polymerase (Fermentas), and 0.2 mmol/L of each deoxyribonucleoside triphosphate (Invitrogen Life Technologies, Carlsbad, CA). Negative controls consisted of sterile ultrapure water instead of sample and were included with each batch of samples analyzed. Temperature profile for the first PCR reaction was 95°C/1 min, 26 cycles at 94°C/45 s, 50°C/45 s, 72°C/1.5 min, and 72°C/15 min, and for the second step it was 95°C/5 min, 28 cycles at 94°C/30 s, 55°C/1 min, 72°C/1.5 min, and 72°C/20 min. PCR products were separated by electrophoresis in agarose gels, which were then stained with GelRed (Biotium, Hayward, CA) and visualized under ultraviolet transillumination.

Labeled PCR products were mixed by using equal proportions of each (40 μL) and used in the checkerboard assay to determine the presence and levels of 33 bacterial taxa by using probes described and validated previously (26, 27, 31). In addition to the taxon-specific probes, 2 universal probes were included in each checkerboard membrane to serve as controls. Two lanes in the membrane contained extracted DNA standards at the concentration of 10^5 and 10^6 cells, which were treated the same way as the clinical samples. The reverse-capture checkerboard assay was performed by using the Minislot-30 and Miniblotter-45 system (Immunetics, Cambridge, MA). First, 100 pmol of probe in TE buffer (10 mmol/L Tris HCl, 1 mmol/L EDTA, pH 8.0) was introduced into the horizontal wells of the Minislot apparatus and cross-linked to the Hybond- N+ nylon membrane (AmershamPharmacia Biotech, Little Chalfont, Buckinghamshire, UK) by ultraviolet irradiation by using a Stratalinker 1800 (Stratagene, La Jolla, CA) on autocross-link position. Each probe has a polythymidine tail that is preferentially cross-linked to the nylon and leaves the specific probe available for hybridization. The membrane was then prehybridized at 55°C for 1 hour. Subsequently, 80 μL of the labeled PCR products mixed with 60 μL of 55°C preheated hybridization solution was denatured at 95°C for 5 minutes and loaded on the membrane by using the Miniblotter apparatus. Hybridization was carried out at 54°C for 2 hours.

After blocking in a buffer with casein, the membrane was incubated in antidigoxigenin antibody conjugated with alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany) and then in ultrasensitive chemiluminescent substrate CDP Star (Roche Molecular Biochemicals). Finally, a square of x-ray film was exposed to the membrane in a cassette to detect the hybrids.

Quantitative Real-time PCR

To quantify the total bacterial load and levels of streptococci and lactobacilli in caries samples, 16S rRNA gene-targeted qPCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on an ABI 7500 Real-time PCR instrument (Applied Biosystems) in a total reaction volume of 20 μL. The primers used were as described and validated elsewhere (32–36) (Table 1).

Primers in a concentration of 0.5 μmol/L each and DNA extract volume of 2 μL were added to the PCR master mix in MicroAmp Optical

TABLE 1. Primers Used for Bacterial Quantification in Samples from Advanced Caries Lesions in Teeth with Irreversible Pulpitis by Using Real-time PCR

Taxa	Primer sequences	Annealing temperature (°C)	Fragment length (base pairs)	Reference
Universal 16S rRNA gene	5′ – GAT TAG ATA CCC TGG TAG TCC AC – 3′ 5′ – TAC CTT GTT ACG ACT T – 3′	52	733	(32, 33)
Streptococcus species	5′ – AGA TGG ACC TGC GTT GT – 3′ 5′ – GCT GCC TCC CGT AGG AGT CT – 3′	60	~120–130	(34, 35)
Lactobacillus species	5′ – TGG AAA CAG RTG CTA ATA CCG – 3′ 5′ – GTC CAT TGT GGA AGA TTC CC – 3′	62	223	(36)

(Life Technologies) 96-well reaction plates. Plates were sealed, centrifuged, and then subjected to amplification. Cycling conditions for universal bacteria and streptococci included 95°C/10 min and 40 repeats of the following steps: 95°C/1 min, annealing for 1 min (temperature shown in Table 1), and 72°C/1 min. The temperature profile for lactobacilli quantification was 50°C/2 min, 95°C/10 min, 40 cycles at 95°C/15 s, and 62°C/1 min. At each cycle, accumulation of PCR products was detected by monitoring the increase in fluorescence of the reporter dye (dsDNA-binding SYBR Green). All measurements were done in triplicate for samples and standards. In all experiments, triplicates of appropriate negative controls containing no template DNA were subjected to the same procedures. After amplification, melting curve analysis of PCR products was performed to determine the specificity of the amplified products. Melting curve was obtained from 60°C to 95°C, with continuous fluorescence measurements taken at every 1% increase in temperature. Data acquisition and analysis were performed by using the ABI 7500 software v2.0.6 (Applied Biosystems).

Standard curves for quantification of streptococci and lactobacilli were constructed with known concentrations of genomic DNA extracted from *S. mutans* ATCC 25175 and *Lactobacillus casei* ATCC 393, respectively. *S. mutans* DNA was also used for total bacteria quantification by using the pair of universal primers. Because the levels of total bacteria cannot be precisely calculated because of the differences in numbers of *rrn* operons among oral bacteria, *S. mutans*, which contains 5 copies of the 16S rRNA gene, was used, with 5 being regarded as the approximate average copy number in the range of most oral bacteria (http://www.cbs.dtu.dk/services/GenomeAtlas-3.0). DNA extracts were 10-fold diluted from 10^7 to 10^2 cells in ultrapure water and used for making the standard curves. Relative amounts were calculated as the percentage of streptococci or lactobacilli out of the total bacterial load.

Data Analysis

Data from the checkerboard assay were evaluated as the prevalence of the target taxa in the samples examined. The presence/absence of bacteria was analyzed in relation to clinical conditions by using the χ^2 or the Fisher exact test, and prevalence ratio and confidence interval were calculated. The chemiluminescent signals were also analyzed with ImageJ (http://rsb.info.nih.gov/ij/) and converted to counts by comparison with standards at known concentrations run on each membrane. Because of the recognized difficulties in inferring absolute counts for samples amplified by end-point PCR used in the checkerboard assay and because estimates had to be made for counting as-yet-uncultivated phylotypes, counts were transformed into semiquantitative data and categorized as follows: a level below detection (or absence), a level $<10^5$ bacteria, a level = 10^5 bacteria, a level $>10^5$ to $<10^6$ bacteria, a level = 10^6 bacteria, and a level $>10^6$ bacteria. Data from qPCR quantification of total bacteria, streptococci, and lactobacilli levels per milligram (wet weight) of dentin were transformed into log numbers for statistical analysis. The Mann-Whitney and Kruskall-Wallis tests were used to compare the semiquantitative data from checkerboard and the absolute bacterial counts from qPCR with the clinical parameters. For comparisons between groups after the Kruskal-Wallis test, the Mann-Whitney test with Bonferroni correction was used. Significance level was set at 5% ($P < .05$).

Results

Clinical conditions are shown in Table 2. All sample extracts were positive for the presence of bacterial DNA as demonstrated by pre-checkerboard PCR amplification and qPCR by using universal 16S rRNA gene primers. This also indicates that significant inhibitors of the PCR reaction were not present. Negative controls for PCR yielded no amplification.

Reverse-capture Checkerboard Assay

The results of the reverse-capture checkerboard analysis revealed that 15 of the 33 oligonucleotide probes tested were reactive with 1 or more dentinal samples. All samples but 2 were positive for at least 1 taxon-specific probe. The number of target taxa per sample ranged from 1 to 8.

The most frequently detected taxa were *Atopobium* genomospecies C1 (16 samples, 53%), *Pseudoramibacter alactolyticus* (11 samples, 37%), *Streptococcus* species (10 samples, 33%), *S. mutans*

TABLE 2. Description of Independent Clinical Variables

Variables	Categories	*n*	%
Coronal restoration	Yes	12	40
	No	18	60
Provoked pain	Present	14	47
	Absent	16	53
Spontaneous pain	Present	16	53
	Absent	14	47
Intermittent pain	Present	7	23
	Absent	23	77
Continuous pain	Present	9	30
	Absent	21	70
Localized pain	Present	21	70
	Absent	9	30
Diffuse pain	Present	2	7
	Absent	28	93
Cold-evoked pain	Present	25	83
	Absent	5	17
Heat-evoked pain	Present	7	23
	Absent	23	77
Pain to percussion	Present	5	17
	Absent	25	83
Throbbing pain	Present	12	40
	Absent	18	60
Pain after physical efforts	Present	0	0
	Absent	30	100
Visual analogue scale	Absent to mild	6	20
	Moderate	12	40
	Severe	12	40

(10 samples, 33%), *P. micra* (4 samples, 13%), *F. nucleatum* (4 samples, 13%), and *Veillonella* species (4 samples, 13%) (Fig. 1). Associations were found between some target taxa and clinical conditions. As for the presence/absence data, associations were found for *S. mutans* with pain to percussion and *P. micra* with throbbing pain. As for the semiquantitative data, the significant associations observed are depicted in Table 3.

Quantitative Real-time PCR

Analysis by using universal primers revealed a mean total bacterial load of 1×10^8, ranging from 2.05×10^5 to 4.5×10^8 per mg (wet weight) of dentin. Streptococci were detected in 28 of the 30 cases, whereas lactobacilli occurred in 29. The average numbers of cells of streptococci and lactobacilli were 3.6×10^5 and 2.32×10^6, respectively. As for proportion, streptococci comprised from 0.0004% to 91.5% of the total bacterial counts (mean, 4.3%; median, 0.12%), whereas lactobacilli comprised from 0.01% to 98.1% of the total bacteria (mean, 8.2%; median, 2%). The only significant association observed for the qPCR data was between lactobacilli and continuous pain ($P = .04$). Quantitative data are summarized in Table 4.

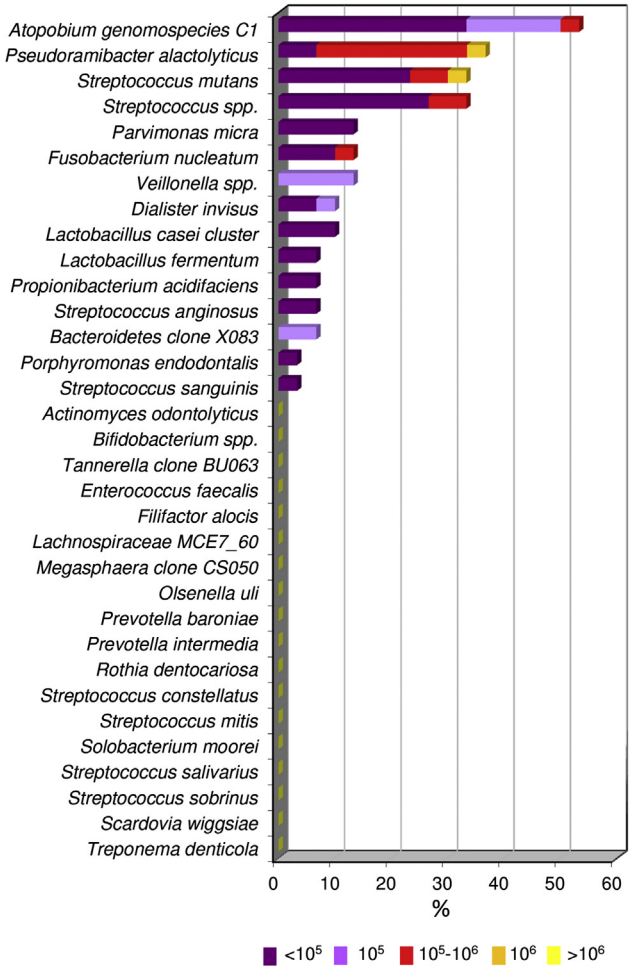

Figure 1. Stacked bar chart of frequency of detection and levels of bacterial species/phylotypes in deep dentinal caries lesions associated with pulp exposure and irreversible pulpitis. Total length of each bar stack indicates percentage of positive samples (ie, prevalence of bacterial species/phylotypes). Different colors within each bar indicate percentage of samples containing different levels of the species.

TABLE 3. Bacterial Taxa Significantly Associated with Demographic or Clinical Conditions Based on Semiquantitative Data from Reverse-capture Checkerboard Hybridization Assay

Bacterial taxa	*P* value	Variable
Streptococcus species	.03	Provoked pain
	.03	Intermittent pain
	.04	Throbbing pain
Dialister invisus	.03	Throbbing pain
	.03	Presence of restoration
Parvimonas micra	.01	Throbbing pain

Discussion

Bacteria present in the advanced front of carious dentin can be considered as etiologically significant in the development of pulpitis. In the present study, the checkerboard DNA hybridization method was used to evaluate the prevalence of several bacterial taxa commonly found in both deep dentinal caries lesions and in endodontic infections. There are numerous studies evaluating the microbiota associated with these 2 conditions separately (11, 14, 16–22). Because endodontic infections are usually a sequel to caries, studies evaluating the process in between (ie, initial pulp invasion by caries bacteria) may provide valuable information about the bacteria that cause irreversible pulpitis and participate in the early phases of pulp colonization preceding endodontic infections. There are not many studies in this regard, and the present one was intended to help bridge this gap in knowledge.

Of the taxa targeted in checkerboard, the most prevalent were *Atopobium* genomospecies C1, *P. alactolyticus*, *Streptococcus* species, *S. mutans*, *P. micra*, *F. nucleatum*, and *Veillonella* species. *Atopobium* genomospecies C1, *S. mutans* and other streptococci, and *Veillonella* species have been very frequently associated with advanced caries, whereas *P. alactolyticus*, *P. micra, F. nucleatum*, and *Streptococcus* species are commonly associated with endodontic infections. Therefore, it seems that there is a coexistence of caries and endodontic pathogens in very advanced caries lesions. This may indicate a shift in the microbiota as the caries process advances to the pulp. Shifts are expected to be governed by ecological changes in the affected tissues, including presence of inflammation and the dominant species in the consortium. For instance, inflammation may change the ecology by making some nutrients more abundant, especially glycoproteins from the inflammatory exudate, and/or may exert a selective pressure as a result of the host defense attack. Moreover, the most dominant species may set the stage for establishment of appropriate partners for metabolic interactions (37). Longitudinal studies in animals evaluating the microbiota shifts from initial caries to endodontic infection are required to confirm these assumptions and reveal the most significant species occurring in different phases of the pathologic process.

Some differences in the composition of the caries microbiota can be observed when comparing the present reverse-capture checkerboard findings with a previous study that used the very same method to evaluate the microbiota of the deepest layers of dentinal caries lesion with no pulp exposure (15). For instance, fewer species were found in the present study, and there were some subtle differences in prevalence of some taxa. Although several of the most prevalent taxa occurred in both studies, *P. micra* and *P. alactolyticus* were more commonly found in deep dentin layers associated with pulp exposures than in cases with no exposure. *P. micra* has been previously shown to be associated with irreversible pulpitis (2). Whether the high prevalence of these species in irreversible pulpitis cases represents a random finding or a shift in the biofilm composition remains to be determined. The

TABLE 4. Prevalence and Quantitative Data for Total Bacteria, Streptococci, and Lactobacilli in Advanced Caries Lesions Associated with Irreversible Pulpitis as Determined by Quantitative Real-time PCR

Target	Prevalence (%)	Absolute counts per mg dentin			% Total bacteria		
		Mean	Median	Range	Mean	Median	Range
Total bacteria	30/30 (100)	1.00×10^8	3.07×10^7	2.05×10^5 to 4.50×10^8	100	100	100
Streptococcus species	28/30 (93)	3.60×10^5	2.89×10^4	1.32×10^2 to 2.63×10^6	4.3	0.12	0.0004–91.5
Lactobacillus species	29/30 (97)	2.32×10^6	5.66×10^5	1.85×10^2 to 1.26×10^7	8.2	2	0.01–98.1

reduced number of species and the appearance of new species may be a result of the ecological changes in the environment. Species more frequently found in advanced caries lesions in association with pulp exposures are candidate pathogens for irreversible pulpitis.

Some species were significantly associated with signs/symptoms of pulpitis. For instance, *Streptococcus* species, *Dialister invisus*, and *P. micra* were more frequent in cases with throbbing pain, whereas *S. mutans* was associated with pain to percussion. *Lactobacillus* species occurred in significantly higher levels in cases with continuous pain. Previous studies have described some species associated with pulpal symptoms, including black-pigmented anaerobic bacteria, *Fusobacterium* species, *S. mutans*, and *A. viscosus* (3, 4, 24). Some bacterial metabolic products have been demonstrated to be involved in the development of symptoms. Ammonia and indole, which are produced by many anaerobic bacteria found in deep caries, can make intradental sensory nerves more susceptible to stimuli that evoke pain (38, 39). The amount of lipopolysaccharide in caries was positively associated with pulpal pain (40). Lipopolysaccharide-mediated pain may be related to the proinflammatory effects of this bacterial component (41) or its direct effects on sensory nerve fibers (42).

Quantitative PCR analysis was also carried out to evaluate the presence and levels of streptococci and lactobacilli, which are bacterial groups very frequently associated with caries (13–15, 31, 36, 43). Both *Streptococcus* species and *Lactobacillus* species were much more prevalent in the qPCR analysis as compared with the checkerboard approach, and this can be explained by the higher sensitivity of the former. The high prevalence of these bacterial groups, especially lactobacilli, in deep caries lesions is in agreement with several previous reports in the literature (4, 12–15, 36). Counts of lactobacilli were significantly increased in cases of continuous pain. Although very frequent, both streptococci and lactobacilli were rarely found in high relative abundance. These bacterial groups corresponded to more than 10% of the total population in only 2 cases each. Further quantitative studies should reveal the bacterial taxa that dominate the microbiota in advanced caries lesions.

Knowledge of the microbiota involved in irreversible pulpitis may be invaluable for the development of therapeutic approaches to improve the predictability of vital pulp therapy. For instance, the use of antimicrobial agents that selectively target the species associated with irreversible pulpitis may open new perspectives on the conservative treatment of teeth with this condition.

In conclusion, this study identified some bacterial taxa associated with advanced caries lesions in teeth with irreversible pulpitis. Some species were more frequently detected in the presence of symptoms. Bacteria found in high prevalence in the forefront of caries lesions that resulted in pulp exposure may be important pathogens in evoking pulp inflammation. Also, they may be pioneer species in the pulp colonization process to initiate endodontic infections.

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