Original Articles

Association between a Quantity of *Bifidobacterium longum* and *Fusobacterium nucleatum*, Clinical Symptoms, and Radiographic Findings in Infected Root canal of Primary Molars

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Abstract

To quantify *Bifidobacterium longum* and *Fusobacterium nucleatum* levels in the infected root canals of primary teeth and to analyse the association between these bacteria, clinical signs and symptoms, and radiographic findings. One hundred and twenty Thai children, aged 2 to 8 years old, were recruited from the Pediatric Dental Clinic, Maha Chakri Sirindhorn Dental Hospital, Mahidol University, Nakhon Pathom, Thailand. The treatment received was either pulpectomy or pulpotomy based on the diagnosis of the American Academy of Pediatric Dentistry (AAPD) guidelines. Clinical signs and symptoms and periapical radiographs of the infected primary teeth were recorded. A total of 120 samples were collected from primary molar teeth using aseptic techniques. DNA was extracted from samples and quantitative real-time PCR was performed using fluorescent dye (SYBR green). Participants included 62 boys (52%) and 58 girls (48%). Mean age±standard deviation was 5.62±1.22 years old. Eighty-three participants (69%) and 115 participants (96%) had clinical signs and symptoms and showed radiographic pathology of an infected root canal, respectively. There was a 100% (120/120) detection rate using the 16srRNA universal primers. *B. longum* and *F. nucleatum* were detected at 56% (67/120) and 57% (68/120), respectively. Range of total bacteria, *B. longum* and *F. nucleatum* detection were $1.63x10^{2}$-$1.64x10^{7}$, 0-$1.17x10^{6}$ and 0-$2.02x10^{6}$ cells/ml, respectively. Range of the ratio of *B. longum*/total bacteria and *F. nucleatum*/total bacteria were 0-$3.13x10^{-1}$ and 0-$7.13x10^{-1}$, respectively. The ratio of *B. longum*/total bacteria correlated with clinical signs and symptoms in only the sensitivity to percussion (p=0.043) while the ratio of *F. nucleatum*/total bacteria were correlated with three clinical signs and symptoms which are sensitivity to percussion (p=0.027), sensitivity to palpation (p=0.001), and the presence of gingival abscess (p=0.001). The ratio of *B. longum*/total bacteria were not correlated with any of radiographic findings while the ratio of *F. nucleatum*/total bacteria were associated with the widening periodontal ligament (PDL) space (p=0.004), periapical lesion (p=0.028), furcation involvement (p=0.002), and root resorption (p=0.027). In conclusion, *B. longum* and *F. nucleatum* were detected higher than 50% in the infected root canal of primary teeth. *F. nucleatum* levels showed positive correlation with many clinical symptoms and radiograph pathology, while *B. longum* levels showed positive correlation with sensitivity to percussion.

Keywords: *Bifidobacterium longum*, Early childhood caries, *Fusobacterium nucleatum*, Primary teeth, Real-time PCR, Root canal infection

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Introduction

Dental caries is one of the most prevalent chronic diseases in the world. More than 530 million children worldwide have dental caries in primary dentition which impacts their quality of life.[1] In Thailand, dental caries prevalence in three and five year olds were 47% and 72%, respectively.[2] Without proper treatment in time, bacteria will eventually invade into the dental pulp and root canal therapy is then needed to remove the infected pulp tissue.[3] The need for root canal treatment in three and five year-old Thai children were 10% and 19%, respectively.[4]

Dental biofilm on the occlusal surfaces of primary teeth is associated with active carious lesions.[5] Once the caries progresses deeper, bacteria which are located at the advanced frontline of the biofilms are directly involved in inducing damage and consequential inflammation of dental pulp tissue. Eventually, the microorganisms that initially occupy the pulp chamber and root canal lumen invade the entire root canal system. Root canal infection is a common consequence of dental caries. Much effort has been made to study and analyse the bacterial composition in caries lesions biofilm, especially in relation to advanced caries. Previous studies of the microbiology of advanced caries used a classical cultivation of the bacteria and mainly focused on mature plaque ecology.[5] Those studies have contributed to important information about the predominant composition of the biofilm in advanced carious surfaces, but do not include yet uncultured organisms.[6-8] Moreover, site-specific sampling of advanced caries lesions has already been performed and investigated by up-to-date molecular techniques, but the studies have been limited to only advanced stages of caries lesions involving the dentin.[9, 10] There is still a significant knowledge gap in bacterial composition within the root canals especially in primary teeth representing the complex ecology of root canal biofilm.

Previous studies have identified bacteria isolated from carious lesions and vital carious exposure of pulp tissue from primary teeth, results showed that the microbiota of the carious exposed pulp was similar to those of carious lesions.[11-16] The dominant bacteria detected in pulpitis were *S. mutans* and *Bifidobacterium*.[12] Another study found that the most frequently detected bacteria in deep dental caries and irreversible pulpitis were *S. mutans, Fusobacterium nucleatum, Veillonella, Lactobacillus* and *Enterococcus faecalis.*[17,18]

Bifidobacterium longum is a gram-positive, rod-shaped, non-filamentous, non-motile, non-spore forming, anaerobic bacteria.[19] It is frequently detected in the human oral cavity, although it may also be isolated from the human gastrointestinal tract or infections. Many *Bifidobacterium,* especially *B. longum,* have been isolated from root carious lesions and from occlusal carious lesions in children and adults.[9] Moreover, it is one of the bacteria that has the highest mean proportions in exposed vital primary pulp due to dental caries.[20] It is acidogenic and acid resistant, with the ability to survive prolonged exposure to low pH. In addition, it is able to proliferate in an acidic environment because of self-protection mechanisms in the absence of an energy source. This ability of *B. longum* in an acidic environment may account for the ability to proliferate caries lesions in the presence of *Streptococci* and *Lactobacilli.*[19] Recent studies have demonstrated an association between *Bifidobacterium* and early childhood caries (ECC).[21-23] A previous study in Thai children also reported that *Bifidobacterium* levels were significantly higher in the supra gingival plaque of ECC children when compared with caries-free children.[21] Also, our previous study showed that *Bifidobacterium* was detected significantly higher in the pulp necrosis group when compared with the irreversible pulpitis group.[23]

Fusobacterium nucleatum is gram-negative, rod-shaped, non-spore-forming, non-motile, obligate anaerobic bacteria that colonise in the oral cavity.[24] It has been isolated from primary endodontic infections in adults.[25] *F. nucleatum* is the most prevalent species found in root canal infections.[26-28] This microorganism has been found between 18%- 25% of the pulp infections in primary teeth.[29,30] Previous studies reported that *F. Nucleatum* is associated with the clinical condition and reflects the persistent instance of endodontic infection in primary teeth.[27,30] *Fusobacterium* species are predominant in teeth with apical abscesses and are also related to the degree of patient pain.[25] Even though many studies have investigated the great diversity of bacteria

involved in endodontic infections in primary teeth,[31-33] there are few studies among Thai children.[17,23] Our previous study found that *F. nucleatum* was significantly higher in the pulp necrosis group when compared with the irreversible pulpitis group. In addition, it was correlated with clinical swelling at the gingiva area.[23]

Quantitative real-time PCR shows a highly sensitive and specific assay for the identification and quantification of oral pathogens by using specific primers.[34] The purpose of this study is to quantify *B. longum* and *F. nucleatum* levels in the infected root canals of primary teeth and to analyse the association between these bacteria, clinical signs and symptoms, and radiographic pathology.

Materials and Methods

This study was approved by the Ethical Institutional Review Board, Faculty of Dentistry and the Faculty of Pharmacy, Mahidol University (MU-DT/PY-IRB 2020/DT049).

Participant selection

Based on a previous study with α = 0.05 and power of 80%, using the software package Primer of Biostatistics (McGraw-Hill, NY, USA). Sample size calculations determined that a minimum of 69 children in each group was enough to achieve statistical difference.[23] A total of 120 primary molar teeth from Thai children aged 2 to 8 years old were selected for the study. All participants were chosen from patients who came to the Pediatric Dental Clinic, Maha Chakri Sirindhorn Dental Hospital, Mahidol University, Nakhon Pathom, Thailand and needed pulpectomy or pulpotomy treatment. Consent forms were signed. One hundred and one samples were diagnosed with irreversible pulpitis and 16 samples with pulp necrosis.

Clinical examination, inclusion and exclusion criteria

All participants had normal physical growth and cooperated during dental treatment. Any who had any systemic disease(s), taking any kind of antibiotics, had professional fluoride application or any dental treatment within two months prior to the sample collection period were excluded.

A clinical examination was performed by two pediatric dental residents. They were calibrated for clinical examination (kappa co-efficiency = 0.80). Oral examination was performed following the American Academy of Pediatric Dentistry (AAPD) guideline.[35] The diagnosis of a pulpal and periapical condition was based on the AAPD guidelines.[35] Clinical signs and symptoms of infected primary teeth included pain history, swelling and pathologic mobility (grade I, II). For clinical presentation or pulpal response, the presence of abscess or sinus tract, the presence of tenderness to percussion and tooth mobility were recorded. The roots should exhibit minimal or no resorption. For the diagnosis, tooth was diagnosed with irreversible pulpitis if it had 1) a history of pain; intense, lingering pain to temperature changes, spontaneous pain, diffuse or referred pain 2) Clinical examination; deep caries, response to thermal stimuli, hypersensitive to cold, excessive hemorrhage that is not controlled with a damp cotton pellet after being applied for several minutes 3) Radiographic examination; no evidence found of osseous changes.

Tooth was diagnosed with pulp necrosis if it has 1) A history of pain; a few months ago, or no history of pain 2) Clinical examination; deep caries that can be found on pulpal exposure, no response to thermal stimuli, pain on percussion if PDL (periodontal ligament) around apical region is inflamed 3) Radiographic examination; radiographic change and periapical lesions can be found. Pre-operative radiographs were taken before pulpectomy treatment in order to assess furcation involvement or periapical radiolucency, pathologic external root resorption and internal root resorption.

If the tooth was unrestorable, or root resorption was more than 2/3 of the root length, or the degree of tooth mobility was more than grade II, or showed a significant gingival recession or periodontal pockets deeper than 4 mm, they were excluded.

Sample collection

A sample collection was performed using an aseptic technique.[36] In each tooth, a single root canal was sampled in order to confine the microbial evaluation to a single ecologic environment. The criteria was to choose the root canal with periapical radiolucency or the largest canal: in the upper molars palatal canal, in the lower molars distal canal.[37,38] The teeth were cleaned with pumice and

isolated with a rubber dam. The operative field was sterilized with 20% iodine solution. An access cavity preparation was accomplished by sterile bur using sterile normal saline for the coolant. A sterile no. 15 K-file (Maillefer, Ballaigues, Switzerland) was introduced to a level approximately one mm short of the tooth apex, then a discrete filing motion was applied. Afterward, two sterile paper points were placed into the canal one by one, with each left for one minute for absorbing all the fluids. In case of a narrow root canal, filing was initiated respectively with files no. 10 and no. 15, without rinsing. These paper points were transferred to microcentrifuge tubes containing 1.0 ml TE buffer and then immediately frozen at -4°C. Samples were transferred to the Oral Biology Laboratory and frozen at -20°C until the DNA extraction process.

DNA extraction

DNA was extracted based on enzymatic lysis using a commercial kit (Flavogen, Pingtung, Taiwan) as previously described.[23] The extracted DNA concentration and purity was measured using a spectrophotometer at 260 nm/280 nm (Nanodrop 2000C Thermo Scientific, Delaware, USA).

Culture condition and standard strains

Two bacterial strains were used as standard strains. *B. longum* (subspecies 51139) was purchased from BIOTEC (National Center for Genetic Engineering and Biotechnology, Bangkok, Thailand) and cultured on BL agar. *F. nucleatum* (ATCC 25586) was cultured on Brain Heart Infusion agar. Both strains were incubated at 37°C for 24-48 hours in anaerobic conditions (5% CO_2). Genomic DNA was extracted from the overnight culture as described above. A ten-fold serial dilution, starting from 10^8 and diluted to 10^2 CFU/ml, was performed.

Conventional PCR

Conventional PCR was performed to check all extracted DNA samples with 16srRNA universal primers (Table 1). Conventional PCR was performed as previously described.[22] Thermocycle (GeneAmp PCR System 9600 PCR machine, PerkinElmer, CA, USA) was set at 45 cycles. The procedure started with preheating at 95°C for ten minutes. Each cycle consisted of a denaturing step at 95°C for 30 seconds, annealing at 55.9, extension at 72°C for 30 seconds, and incubation for an additional extension at 72°C for 10 minutes.

Quantitative Real-time PCR

Using specific primers (Table 1), the reaction mixture (total volume of 20µl) contained 8.2µl of water, 10µl of 2X KAPA SYBR® FAST qPCR Master Mix, 0.4µl of 10 µM forward and reverse primer, and 1µl of standard bacteria DNA. The thermocycler (C1000™ Thermal cycler and CFX 96 Real-time System) was set for 40 cycles. Each cycle consisted of enzyme activation at 95°C for three minutes, denaturing at 95°C for three seconds, annealing at 55.9, 53°C and 56.3°C for 20 seconds for 16srRNA universal primers, *Bifidobacterium* and *F. nucleatum,* respectively. Melting curves were generated from 60°C to 95°C and read every 0.5°C for five seconds.[23]

Table 1 *Primers used in this study*

Primer name		Nucleotide sequence (5' to 3')	Expected amplicon (basepair)	Annealing Temp (°c)	References
UniversalBAC16S	F	5'-TGG AGC ATG TGG TTT AAT TCG A-3'	160	55.9	Sinsimer *et al,* 2005
	R	5'-TGC GGG ACT TAA CCC AAC A-3'			
B. longum	F	5'-CTC CTG GAA ACG GGT GG-3'	550	53	Matsuki *et al,* 2004
	R	5'-GGT GTT CTT CCC GAT ATC TAC A-3'			
F. nucleatum	F	5'-CGC CCG TCA CAC CAC GAG A-3'	75	56.3	Ammann *et al,* 2013
	R	5'-ACA CCC TCG GAA CAT CCC TCC TTA C-3'			

Agarose gel electrophoresis

Amplified PCR products were checked with 2% agarose gel (UltraPure Agarose, ThermoFisher Scientific, USA) which was stained with ethidium bromide and the gel images were captured with a digital imaging system (Molecular Imager® Gel docTM Systems, Bio-Rad Laboratories Inc., CA, USA).

Statistical Analysis

All data were recorded and analyzed using SPSS 23.0 software (Microsoft Corporation, USA). Data distribution was tested using Kolmogorov-Smirnov (p<0.001). The different amounts of two bacteria between two groups using a Mann Whitney U test for non-parametric data (p<0.05) were analyzed. Analysis for the correlation between the amount of each bacterium, clinical signs and symptoms and radiographic finding using Spearman's correlation test (p<0.05) was carried out.

Results

Total participants included 120 children, (62 boys (52%) and 58 girls (48%)) with mean age±standard deviation 5.62±1.22 years old. Ninety-five participants (80%) had a history of pain, 83 (69%) presented with clinical signs and symptoms, and 115 (96%) showed radiographic pathology (Table 2). One hundred and one participants (84%) were diagnosed with irreversible pulpitis, and 19 (16%) with pulp necrosis.

There was a 100% (120/120) detection rate using the 16srRNA universal primers. *B. longum* and *F. nucleatum* were detected at 56% (67/120) and 57% (68/120), respectively (Table 3). Range of total bacteria, *B. longum* and *F. nucleatum* detection were $1.63x10^2$-$1.64x10^7$, 0-$1.17x10^6$ and 0-$2.02x10^6$ cells/ml, respectively. Range of the ratio of *B. longum*/total bacteria and *F. nucleatum*/total bacteria were 0-$3.13x10^{-1}$ and 0-$7.13x10^{-1}$, respectively. Mean±standard deviation quantities of *B. longum* in irreversible pulpitis group and pulp necrosis group were $2.84x10^4$ and $1.08x10^5$ cells/ml, respectively. Mean±standard deviation quantities of *F. nucleatum* in irreversible pulpitis group and pulp necrosis group were $1.2x10^5$ and $3.07x10^5$ cells/ml, respectively. The ratio of *B. longum*/total bacteria was correlated with clinical signs and symptoms in only the sensitivity to percussion (p=0.043) while the ratio of *F. nucleatum*/total bacteria were correlated with three clinical signs and symptoms which are sensitivity to percussion (p=0.027), sensitivity to palpation (p=0.001), and the present of gingival abscess (p=0.001) (Table 4). When further analyzed the association between bacteria and type of radiographic pathology, results showed that the ratio of *B. longum*/total bacteria were not correlated with any of radiographic pathology. On the other hand, the ratio of *F. nucleatum*/total bacteria were associated with the widening periodontal ligament (PDL) space (p=0.004), periapical lesion (p=0.028), furcation involvement (p=0.002), and root resorption (p=0.027) (Table 5).

Table 2 *General information of all subjects*

Variables	Total subjects N=120 (%)
Genders	Boys, 62 (52%)
	Girls, 58 (48%)
History of pain	95 (80%)
Clinical signs and symptoms	83 (69%)
Radiographic pathology	115 (96%)
Diagnosis	Irreversible pulpitis 101 (84%)
	Pulp necrosis 19 (16%)

Table 3 *Bacterial quantities detected by specific primers*

Bacteria	Prevalence	Median (cells/ml)	Range (cells/ml)
Total bacteria	**120/120 (100%)**	**$48.8x10^3$**	**$1.63x10^2$-$1.64x10^7$**
B. longum	67/120 (56%)	$0.358x10^3$	0-$1.17x10^6$
F. nucleatum	68/120 (57%)	$1.65x10^3$	0-$2.02x10^6$

Table 4 *The association of bacterial proportion with each type of clinical signs and symptoms*

Variables	*B. longum*/total bacteria		*F. nucleatum*/total bacteria	
	Correlation coefficient	*p*-value	Correlation coefficient	*p*-value
Sensitivity to percussion	0.185	0.043*	0.202	0.027*
Sensitivity to palpation	0.053	0.566	0.295	0.001*
Gingival abscess	0.053	0.566	0.304	0.001*
Tooth mobility	-0.025	0.786	0.113	0.218

** Correlation at the significant level of p<0.05*

Table 5 *The association of bacterial proportion with each type of radiographic pathology*

Variables	*B. longum*/total bacteria		*F. nucleatum*/total bacteria	
	Correlation coefficient	*p*-value	Correlation coefficient	*p*-value
Disrupt lamina dura	0.101	0.274	0.165	0.071
Widening PDL space	0.066	0.473	0.264	0.004*
Periapical lesion	0.082	0.372	0.201	0.028*
Furcation involvement	0.080	0.386	0.281	0.002*
Root resorption	-0.065	0.481	0.202	0.027*

** Correlation at the significant level of p<0.05*

Discussion

F. nucleatum is one of the predominant species that are isolated from acute periapical abscesses in both primary and permanent teeth by the cultured method.[39] Using a PCR technique, Siqueira and colleagues examined bacterial species in samples from pulp necrosis with periapical lesions, and reported that the prevalence of *F. nucleatum* was 14%.[31] Fabris and colleagues investigated 103 necrotic pulp samples and seven fistula samples from primary teeth. They found *F. nucleatum* at 25%.[27] In addition, a study by Yang and colleagues reported that one of the dominant taxa isolated from primary teeth with acute periapical abscesses was *F. nucleatum* at the prevalence of 18% using a polymerase chain reaction–denaturing gradient gel electrophoresis technique (PCR-DGGE).[26] In another study by Topcuoglu and colleagues, they investigated the microbial composition of endodontic infection from 30 root canals in primary teeth using microarray. They found that *F. nucleatum* was the most frequently isolated bacterium, as high as 97%.[40] From our previous study, *F. nucleatum* was found at 99%.[23] In this study, the prevalence of *F. nucleatum* was 57% which is lower than our previous study, which might be from the difference in sample demographic and the lower pulp necrosis group. Another previous study found the mean level of *F. nucleatum* in the irreversible pulpitis group and pulp necrosis with sinus tract group in permanent teeth were 15.38×10^5 and 5.59×10^5, respectively.[41] Their results showed the higher levels of *F. nucleatum* in the irreversible pulpitis group which is different from this study. A different detection rate among studies might be from the different guidelines of pulp diagnosis between permanent and primary teeth and the technique used to identify bacteria. Other reasons contributing to the different prevalence rate might depend on many factors, such as target sample, sample size, severity of infection, technical sensitivity, and microbial identification method.

Several studies previously revealed that *F. nucleatum* has been associated with clinical symptoms. In permanent teeth, *F. nucleatum* was reported to relate with a history of pain, tenderness to percussion, gingiva swelling, fistula, purulent exudate, and periapical radiolucency.[14,42] In addition, some studies in primary teeth

showed that *F. nucleatum* was detected more often in teeth that were tender to percussion and where mobility was present.[14,42] Another study obtained samples from 30 teeth in children with both primary and permanent dentitions found a relationship between *F. nucleatum* and hemorrhagic exudate, purulent exudate, and periapical radiolucency.[39] This study added more correlation between *F. nucleatum* and each type of clinical signs and symptoms and types of radiographic pathology as shown in the results. Correlation analysis between bacterial proportion and clinical signs and symptoms in this study show that sensitivity to percussion, sensitivity to palpation, and gingival abscess were correlated with the proportion of *F. nucleatum,* which is similar to a previous study by Yun and colleagues. They found that *F. nucleatum* was associated with the clinical condition and reflected the progression of endodontic infection in primary teeth.[29] Another study revealed that acute symptoms of pain, history of previous pain, tenderness to percussion and swelling in permanent teeth were associated with *F. nucleatum.*[40] Data from this study help to confirm the role of *F. nucleatum* in the root canal infection in the primary teeth. Previous studies had reported that gram-negative bacteria cell wall containing endotoxin can stimulate the release of bradykinin which is a pain mediator that is associated with acute symptoms such as pain.[43] The apical part of a root canal has low oxygen tension and large availability of proteins and glycoproteins which contributes to anaerobic bacteria establishment. Most of them are strictly anaerobic species, such as *F. nucleatum, Porphyromonas endodontalis, Tannerella forsythia* and *Treponema denticola.*[43]

This was the first quantitative analysis of *B. longum* in infected root canals in primary teeth in Thai children. The difference between this study and our previous study was that in this study, *B. longum* was specifically detected while in our previous study *Bifidobacterium* genus was identified and results in this study showed that the detection was higher.[23] Our previous study has led us to further evaluate *B. longum* species specifically. Moreover, we would like to confirm the role of these bacteria by selecting study participants from different demographics. Another difference between this study and our previous study was that in this study, the correlation between these bacteria and radiographic findings was further analyzed which was lacking in our previous study. Most previous studies were done to analyze the association between this bacteria and advanced dental caries. Previous studies reported that *Bifidobacterium* was detected in human saliva, the levels of *Bifidobacterium* in dental plaque were significantly higher in children who had carious lesions compared with caries-free children.[22-24] A previous study found that, in active cavitated enamel lesion, *Bifidobacterium* were notably abundant and present in the outer layers of the biofilm at the cavity entrance.[44] Several previous studies found *Bifidobacterium* mostly in deep dentinal caries.[9,45,46] Previous studies have suggested that bacteria located in advanced dental caries are directly involved in inducing damage and consequent inflammation in the pulp tissue, and *Bifidobacterium* is one of those bacteria that are involved in pulpal inflammation and initiate endodontic infection.[13,47] In addition, it was found in the primary teeth with necrotic pulps in children aged 4-7 years old together with *Streptococcus intermedius*.[37] In this study, it was found that *B. longum* was significantly associated with the sensitive to percussion clinically. *Bifidobacterium* were shown to have similar acidogenicity and aciduricity to *S. mutans* and the ability to produce an acidic environment, to resist low pH and to promote biofilm formation when co-adhered with primary colonizers.[47] The acidogenic and aciduric ability of *B. longum* might play a role in helping it to survive in deep dental caries and invade the pulp in the early stage of pulpitis. Haukioja and colleagues reported that *Bifidobacterium* bound well to *F. nucleatum* coated surfaces, indicating the importance of other oral bacteria in modulating the colonization potential of the strains.[44] This might be one of the reasons that the detection of *Bifidobacterium* was in the same direction as *F. nucleatum.* Taken together, *Bifidobacterium* was detected in the advanced dentinal caries and was found in the early stage of reversible pulpitis. When the reversible pulpitis progressed to irreversible pulpitis or necrotic pulp, *F. nucleatum* was detected more often and correlated with more clinical signs and symptoms and radiographic pathology as mentioned

above. More studies from different populations would be recommended to confirm the relationship between *B. longum* and endodontic infection. This study might not be immediately usable in the clinical application. However, bacterial infection is the cause of caries and pulp infection. This finding could be part of precision preventive dentistry, e.g. to develop a caries risk assessment tool kit from saliva. Or when technology is more accessible, the bacterial biomarker of an individual could be useful to precisely select root canal treatment materials. Furthermore, this study added up the important role of these bacteria and their association with the clinical symptoms and radiographic findings which only a small number of studies were published.

In conclusion, *B. longum* and *F. nucleatum* were detected in more than 50% of the infected root canals of primary teeth. The ratios of *B. longum* and *F. nucleatum* to total bacteria were associated with clinical signs and symptoms of pulp infection in primary teeth. The ratio of *F. nucleatum* to total bacteria was positively correlated with sensitivity to percussion, sensitivity to palpation, gingival abscess, and radiographic pathology including the widening periodontal ligament (PDL) space, periapical lesion, furcation involvement, and root resorption while the ratio of *B. longum* to total bacteria was positively correlated with sensitivity to percussion.

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