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Research Article

Quantitative Analysis of *Lactobacillus* and *Enterococcus faecalis* between Irreversible Pulpitis and Pulp Necrosis in Primary Teeth

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Abstract

Introduction: Root canal infection is a common consequence of dental caries which can cause premature loss in primary dentition. The exposure of pulp tissue to the oral environment allow oral microorganisms to access into the pulp chamber and lead to pulp necrosis without treatment. To identify bacteria associated with root canal infection would be beneficial and lead to providing more effective treatment.

Aims: To quantify *Lactobacilli* and *Enterococcus faecalis* levels in infectious root canal of primary teeth between irreversible pulpitis and pulp necrosis groups, and to analyze the association between these bacteria and clinical signs and symptoms.

Materials and methods: Total subjects were 170 Thai children aged 2-10 years old. All subjects were selected from patients who came to the pediatric dental clinic, Pediatric Department, Faculty of Dentistry, Mahidol University, Thailand and needed a pulpectomy treatment. DNA extraction and quantitative real-time PCR was performed.

Results: Mean age of the children was 5 ± 1.3 years old. One hundred and ten samples were diagnosed with irreversible pulpitis and 60 with pulp necrosis. One hundred and seven subjects (63%) had a history of pain. One hundred and twenty subjects (71%) had clinical signs and symptoms. One hundred and thirty-four radiographs (79%) showed radiographic pathology. The detection of total bacteria, *Lactobacillus* and *E. faecalis* was 100%,

100% and 84% (142/170), respectively. The ratio of *Lactobacillus* and *E. faecalis* to total bacteria levels was 20% and 9%, respectively. When compared between irreversible pulpitis and pulp necrosis groups, the quantities of total bacteria (p=0.000), *Lactobacillus* (p=0.000) and *E. faecalis* (p=0.001) in pulp necrosis group were higher than those of the irreversible pulpitis group. The ratio of *Lactobacillus* to total bacteria in the pulp necrosis group was higher than in the irreversible pulpitis group (*p*=0.004), whereas the ratio of *E. faecalis* to total bacteria was not different between the two groups. Only *Lactobacillus* was associated with a history of pain (*p*=0.013). Gingival swelling was correlated with *Lactobacillus* quantities (*p*=0.01). When analyzed within each group, *E. faecalis* quantity was correlated with clinical pain (*p*=0.01) in pulp necrosis group. In the irreversible pulpitis group, the data showed pathologic finding of lamina dura (*p*=0.000) and furcation involvement (*p*=0.016) which correlated with *E. faecalis* quantities.

Conclusion: The bacterial levels of total bacteria, *Lactobacillus* and *E. faecalis* in pulp necrosis group were higher than the irreversible pulpitis group. Levels of *Lactobacillus* and *E. faecalis* were associated with a history of pain, pathologic finding of lamina dura and furcation involvement. Gingival swelling was correlated with *Lactobacillus* quantities.

Keywords: Quantitative real-time PCR; Infected root canal; Primary teeth; Severe early childhood caries (S-ECC)

1. Introduction

Primary dentition is important as it guides the eruption of permanent dentition and contributes to jaw development. Premature loss of primary teeth leads to the disturbance of permanent teeth eruption [1]. Dental biofilm on occlusal surfaces of primary teeth is associated with active carious lesions [2]. Once the dental caries progresses deeper, bacteria which are located at the advanced frontline of the biofilms are directly involved in inducing damage and consequential inflam pulp tissue. Studies had identified bacteria isolated from carious lesion biofilms and vital carious exposure of pulp from primary teeth. The results show that the microbiota of the carious exposed pulp was similar to those of carious lesions [3-8].

Lactobacilli is a rod-shaped, gram-positive, none spore forming, highly acidogenic and acid-tolerant bacteria. It grows under anaerobic, facultative anaerobic or microaerophillic conditions and plays an important role as a pioneer in advanced dental caries, which rises significantly as caries progressed from the initial to deep lesions [9-12]. Previous study analyzed biofilm and pulp chamber bacteria samples using 16S rRNA sequencing. Results showed that the dominant species were *S. mutans, Actinomyces* and *Lactobacillus,* suggesting that these species might play direct or supporting roles in pulp pathology in children [3]. Another study evaluated bacteria associated with acute apical abscesses in children by analyzing purulence samples from infected primary teeth. The dominant bacteria were *Prevotella, Fusobacterium, Porphyromonas* and *Lactobacillus* [13].

Following pulp exposure as a result of caries, the microorganisms that initially occupy the pulp chamber and root canal lumen invade the entire root canal system, which are dentinal tubules, lateral canals, accessory canals,

secondary canals, apical delta ramifications, apical foramen, apical root cementum surface and periapical tissues [13-15]. This infection leads to the development of apical periodontitis. Treatment of primary endodontic infections based on the elimination of the root canal infection [15]. In the beginning of the infection process, gram-positive facultative aerobes with cocci have been found in necrotic dental pulp tissues and *Enterococcus faecalis* has been reported in high prevalence in primary endodontic infections, especially secondary infection of faulty restorations [7, 16-21]. *E. faecalis* is a gram-positive facultative anaerobe, frequently found in re-infected root canals or chronic periodontitis [20, 21]. Even though the presence of different bacteria involved in endodontic infections of primary teeth, such as pulpitis, pulp necrosis and apical lesion has been reported, microbiological data are still scarce, especially in Thai children [22, 23]. Further study to quantitatively identify other root canal infected related bacteria would be beneficial and help to understand role of bacteria in infected root canals of primary teeth and lead to providing more effective treatment.

Quantitative real-time PCR provides an accurate result and is a sensitive method for detection and quantification of individual species in bacterial populations [23]. This study aimed to quantitatively identify *Lactobacillus* and *E. faecalis* in infected root canals of primary teeth between teeth diagnosed as irreversible pulpitis and pulp necrosis in Thai children using real-time PCR and to analyze the association between these bacteria and clinical signs and symptoms. The hypothesis was that quantities of *Lactobacillus* and *E. faecalis* in root canal samples of primary teeth diagnosed with pulp necrosis whould be higher than those of teeth diagnosed with irreversible pulpitis.

2. Materials and Methods

The study protocol was approved by the Human Institutional Review Board of the Faculty of Dentistry and the Faculty of Pharmacy, Mahidol University) MU-DT/PY-IRB 2016/014.0809, 2018/009.2301) A statistician consultation was done prior to the sample size calculation, which based on previous studies, used α=0.05 and power of 80%, with the software package Primer of Biostatistics (McGraw-Hill, NY, USA). Sample size calculations determined that a minimum of 69 children would be required to achieve the statistical difference [19, 22].

2.1 Subject selection and clinical examination

Total subjects were 170 Thai children aged 2-10 years old. One hundred and ten samples were diagnosed with irreversible pulpitis and 60 with pulp necrosis. All subjects were selected from patients who came to the pediatric dental clinic of the Pediatric Department, Faculty of Dentistry, Mahidol University and needed a pulpectomy treatment. Consent forms were signed. The participation was voluntary, and subjects were free to withdraw from the study at any time. A clinical examination was performed by 2 pediatric dental residents (PK and WW). They were calibrated for clinical examination (kappa co-efficiency=0.8). All patients had to have normal physical growth, no systemic disease and cooperative. Oral examination followed the AAPD guideline, assessing general health/growth, pain assessment, extra oral soft tissue, TMJ, intraoral examination, oral hygiene and periodontal health, developing occlusion, caries risk and child behavior. The presence or absence of tenderness to percussion and tooth mobility was recorded [12]. The infected primary molars indicated for pulpectomy treatment in this study had to exhibit

clinical or radiographic evidence of irreversible pulpitis or necrosed pulpal tissue due to dental caries. According to the Guideline on Pulp Therapy for Primary and Immature Permanent Teeth, pulpectomy treatment is provided to a primary tooth with irreversible pulpitis or necrosis or a tooth treatment planned for pulpotomy in which the radicular pulp exhibits clinical signs of irreversible pulpitis (eg. excessive hemorrhage that is not controlled with a damp cotton pellet applied for several minutes) or pulp necrosis (eg. suppuration, purulence) [12]. The roots should exhibit minimal or no resorption. If the tooth was unrestorable or root resorption was more than 2/3 of root length or degree of tooth mobility was more than grade II, it was excluded.

Clinical signs and symptoms of infected primary teeth, including pain history, swelling, pathologic mobility (grade I, II), presence of abscess or sinus tract, were recorded in documents by the examiners. Recent preoperative radiographs were taken from all patients before treatment. All samples were categorized to the irreversible pulpitis group or pulp necrosis group. The characteristics of pain and pain history were categorized as spontaneous pain, provoked pain or no history of pain. Clinical signs, including presence of clinical abscess or fistula and tooth mobility were recorded for analysis. Subjects who had any systemic disease(s), were taking any kind of antibiotic therapy in the 3 months prior to the examination, showed significant gingival recession or periodontal pockets deeper than 4 mm were excluded from the study.

For radiographic assessment, appropriate radiographs by periapical film using a paralleling technique were taken before the dental procedure to assess periradicular lesion, periodontal status, presence of underneath succedaneous tooth, and to measure initial working length. The roots should exhibit minimal or no resorption [12]. For pulpal diagnosis of irreversible pulpitis, a clinical diagnosis based on subjective and objective findings indicates incapable healing and vital inflamed pulp. In addition, irreversible pulpitis can be divided into symptomatic and asymptomatic groups. Pulp necrosis is a clinical diagnostic category indicating degeneration of the dental pulp. The tooth diagnosed with pulp necrosis should present no pulp tissue remaining when the pulp chamber is accessed or indicate the presence of purulent discharge from the canal root [12].

2.2 Sample collection

Samples were collected by using strict asepsis with slight modification [10]. Access to the root canal was made using a sterile bur without water spray, but using sterile normal saline for the coolant. In each tooth, multiple root canals were sampled in order to confine the microbial evaluation to a single ecologic environment. The tooth was cleansed with pumice and isolated with a rubber dam and the surrounding field was sterilized with Iodine solution. Complete access preparations were made by using sterile burs with sterile normal saline solution. Samples were initially collected by means of a #15 K-file (Maillefer, Ballaigues, Switzerland). The files were introduced to a level approximately 1 mm short of the tooth apex, and a discrete filing motion was applied. Afterward, two sequential paper points were placed at the same level and used to soak up the fluid in the canal. The paper points were left in the wet canal for 60 seconds and then transferred to tubes containing 1.0 ml of TE buffer. Samples were immediately frozen at -20°C.

2.3 DNA extraction

DNA was extracted based on enzymatic lysis using a commercial kit (Flavogen, Taiwan) as previously described [12]. In brief, 20 µl of Proteinase K was added, 400 µl of FABG buffer and 20 µl of a lysozyme mixture (lysozyme 20 mg/ml and mutanolysin (Sigma Aldrich, USA) in 1:10 proteinase K) and vortex. This was incubated at 60°C. for 1 h.; 200 µl ethanol was added and centrifuged at 11,000 9 rpm for 30 s. The solution was transferred into a spin column and centrifuged for 1 min. The supernatant was discarded, 500 µl of W1 buffer was added and centrifuged for 1 min. The supernatant was discarded. Then 750 µl of wash buffer was added and centrifuged for 1 min. The next step was adding 50 µl of elution buffer, left at room temperature for 3 min, before a final centrifuge for 2 min. The extracted DNA concentration and purity was measured using a spectrophotometer at 260 nm/280 nm (Nanodrop 2000C Thermo Scientific, Delaware, USA).

2.4 Culture condition and standard strains

Lactobacillus fermentum (ATCC 14931) and *Enterococcus faecalis* (ATCC 29212) were cultured on Rogosa SL agar (Difco, Sparks, MD) and selective agar and broth, respectively. Both of them were grown at 37°C. anaerobically (5% CO_2) for 24-48 hrs. Genomic DNA was extracted from the overnight culture as described above. A ten-fold serial dilution, starting from 10^8-10^2 CFU/ml, was performed.

2.5 Conventional PCR

All extracted DNA samples were confirmed with 16srRNA universal primers (Table 1). Each reaction mixture (total volume of 26 µl) contained 2 µl of extracted DNA sample, 16.5 µl of nuclease-free water, 1 µl of 10 mM deoxynucleoside triphosphate (dNTP), 1 µl of each primer, 1.5 µl of 50 mM $MgCl_2$, 2.5 µl of 10X PCR buffer minus Mg, and 0.5 µl of *Taq* DNA polymerase (KAPA Biosystems, USA). Thermocycle (GeneAmp PCR System 9600 PCR machine, PerkinElmer, CA, USA) was set at 45 cycles. The procedure started with preheating at 95°C for 10 minutes. Each cycle consisted of a denaturing step at 95°C for 30 seconds, annealing at 52°C for 30 seconds, extension at 72°C for 30 seconds, and incubated for an additional extension at 72°C for 10 minutes [23].

2.6 Quantitative real-time PCR

A standard curve was generated with 10-fold serial dilutions of each bacteria using specific primers (Table 1). The reaction mixture (total volume was 20 µl) contained 9.2 µl of nuclease-free water, 10 µl of 2X KAPA SYBR® FAST qPCR Master Mix (KAPA Biosystems, USA), 0.4 µl of 10 µM forward and reverse primer, and 1µl of standard bacteria DNA. The reaction for DNA plaque samples from plaque samples was similar to the standard strains. We set the thermocycler (C1000™ Thermal cycler and CFX 96 Real-time System) for 40 cycles. Each cycle consisted of enzyme activation at 95°C for 3 minutes, denaturing at 95°C for 3 seconds, annealing for 20 seconds and extension for 30 seconds. Melting curves were generated from 60°C to 95°C and read every 0.5°C for 5 seconds.

Primers		Nucleotide sequence 5' to 3'	Expected amplicon (bp)	Annealing Temp (°C)	Ref.
Universal BAC16S	F	5'-TGG AGC ATG TGG TTT AAT TCG A-3′	160	52	Sinsimer et al. [24]
	R	5'-TGC GGG ACT TAA CCC AAC A-3′			
Lactobacillus	F	5'-TGGAAACAGGTGCT AATACCG-3'	247	53.6	Mcorist et al. [25]
	R	5'-CCATTGTGGAAGA TTCCC-3'			
Enterococcus faecalis	F	5'-GTTTATGCCGCATGGCATAAGAG-3′	310	66.9	Siqueira et al. [26]
	R	5'-CCGTCAGGGGACGTTCAG-3′			

Table 1: Primers used in this study.

2.7 Agarose gel electrophoresis

Amplified PCR products from conventional PCR and quantitative real-time PCR were checked with 2% agarose gel (Broad Separation Range for DNA/RNA agarose, Fisher Scientific, UK), respectively, using 1xTris–borate EDTA buffer (100 mM Tris, 90 mM borate; 1 mM EDTA, pH 8.4). The gels were stained with ethidium bromide and image results were captured with a digital imaging system (Molecular Imager ®Gel docTM Systems, Bio-Rad Laboratories Inc., CA, USA).

3. Statistical Analysis

All data were recorded and analyzed using SPSS 16.0 software (Microsoft Corporation, USA). Data distribution was tested with Kolmogorov-Smirnov. The different bacteria quantities between irreversible pulpitis and pulp necrosis were analyzed using a Mann-Whitney U test ($p \leq 0.05$). The correlation between clinical signs and symptoms were analyzed using Spearman's correlation test ($p \leq 0.05$).

4. Results

4.1 Participants

Total subjects were 170. Mean age of the children was 5 ± 1.3 years (age range between 2.1-8.2 years). All subjects were examined for pulpal status at baseline before sample collection, 110 samples were diagnosed with irreversible pulpitis and 60 with pulp necrosis. One hundred and seven subjects (63%) had a history of pain. One hundred and twenty subjects (71%) had clinical signs and symptoms. One hundred and thirty-four radiographs (79%) showed radiographic pathology, and 4 subjects had no permanent tooth bud (Table 2).

Variables	Irreversible pulpitis N=110	Pulp necrosis N=60
Age (Years)	4.1 ± 1.3	5.3 ± 1.5
Gender, n (%)	Boys, 47 (42.72%) Girls, 63 (57.28%)	Boys, 36 (60%) Girls, 24 (40%)

History of pain, n (%)	64 (58.18%)	43 (71.67%)
Clinical signs and symptoms, n (%)	75 (44.12%)	45 (75%)
Radiographic pathology, n (%)	83 (48.82%)	51 (85%)

Table 2: Subjects information in this study.

4.2 Conventional PCR and quantification real-time PCR

The detection of total bacteria, *Lactobacillus* and *E. faecalis* was 100%, 100% and 83.53% (142/170), respectively. The ratio of *Lactobacillus* and *E. Faecalis* to total bacteria levels was 20.41% and 8.83%, respectively. When compared between the irreversible pulpitis and pulp necrosis groups, the quantities of total bacteria (p=0.000), *Lactobacillus* (p=0.000) and *E. faecalis* (p=0.001) in pulp necrosis group were higher than those of irreversible pulpitis group (Table 3). The ratio of *Lactobacillus* to total bacteria in pulp necrosis group was higher than in the irreversible pulpitis group *(p*=0.004) whereas the ratio of *E. faecalis* to total bacteria was not different between two groups (Table 4). Only *Lactobacillus* was associated with a history of pain (*p*=0.013) (Table 5).

Bacteria	**Pulpal status**	**N**	**Mean quantity ± SD *(*cells/ml)**	**Median *(*cells/ml)**	***p*-value**
Total bacteria	Irreversible pulpitis	110	$9.44 \times 10^5 \pm 2.55 \times 10^6$	5.20×10^4	0.000*
	Pulp necrosis	60	$2.86 \times 10^6 \pm 4.13 \times 10^6$	1.04×10^6	
Lactobacillus	Irreversible pulpitis	110	$5.93 \times 10^4 \pm 1.84 \times 10^5$	4.36×10^3	0.000*
	Pulp necrosis	60	$6.24 \times 10^5 \pm 1.28 \times 10^6$	1.22×10^5	
E. faecalis	Irreversible pulpitis	110	$1.44 \times 10^4 \pm 4.08 \times 10^4$	1.21×10^3	0.001*
	Pulp necrosis	60	$1.32 \times 10^5 \pm 2.77 \times 10^5$	6.81×10^3	

*Median was analyzed by Mann-Whitney U-test at p-value <0.05.

Table 3: Comparison of bacterial quantities between irreversible pulpitis and pulp necrosis.

The ratio of bacteria	**Pulpal status**	**N**	**percentage**	***p*-value**
Lactobacillus to total bacteria	Irreversible pulpitis	110	18.89%	0.004*
	Pulp necrosis	60	23.19%	
E. faecalis to total bacteria	Irreversible pulpitis	110	9.67%	0.738
	Pulp necrosis	60	7.28%	

*p-value <0.05, Mann-Whitney U-test

Table 4: Comparison of specific bacteria to total bacteria between irreversible pulpitis and pulp necrosis groups.

Variables	Total bacteria		*Lactobacillus*		*E. faecalis*	
	Correlation coefficient	*p*-value	Correlation coefficient	*p*-value	Correlation coefficient	*p*-value
History of pain	0.122	0.112	0.191*	0.013*	0.043	0.574
Clinical signs and symptoms	0.084	0.277	-0.024	0.756	-0.045	0.557
Radiographic pathology	0.04	0.995	0.018	0.818	-0.144	0.061

*p-value <0.05, Spearman's rank correlation coefficient

Table 5: The correlations between bacterial quantities and the history of pain.

Gingival swelling was correlated with *Lactobacillus* quantities (p=0.01, Table 6). When analyzed within each group, *E. faecalis* quantity was correlated with clinical pain (p=0.01) in the pulp necrosis group while there was no association of each bacteria and clinical signs and symptoms in the irreversible pulpitis group (Table 7). In the irreversible pulpitis group, pathologic finding of lamina dura (p=0.000) and furcation involvement (p=0.016) were correlated with *E. faecalis* quantities (Table 8). There was no association between radiographic pathology and quantities of bacteria in the pulp necrosis group.

Variables	Total bacteria		*Lactobacillus*		*E. faecalis*	
	Correlation coefficient	*p*-value	Correlation coefficient	*p*-value	Correlation coefficient	*p*-value
Clinical pain	-0.082	0.286	0.005	0.948	-0.150	0.051
Sensitivity to air-blow	0.045	0.051	0.563	0.505	-0.110	0.153
Gingival swelling	0.114	0.138	0.197*	0.01*	0.100	0.193
Tooth mobility	0.051	0.511	0.730	0.091	-0.131	0.089

*p-value <0.05, Spearman's rank correlation coefficient

Table 6: The association between bacterial quantities and clinical signs and symptoms.

Variables	Pulp necrosis					
	Total bacteria		*Lactobacillus*		*E. faecalis*	
	Correlation coefficient	*p*-value	Correlation coefficient	*p*-value	Correlation coefficient	*p*-value
Clinical pain	-0.003	0.980	0.132	0.314	**0.305***	**0.018**
Sensitivity to air-blow	0.094	0.475	0.151	0.248	-0.155	0.237
Gingival swelling	0.075	0.569	0.122	0.354	0.045	0.731

Tooth mobility	-0.03	0.818	0.032	0.811	-0.223	0.086

*p-value <0.05, Spearman's rank correlation coefficient

Table 7: The association between bacterial quantities and clinical signs and symptoms in pulp necrosis group.

Variables	**Irreversible pulpitis**					
	Total bacteria		***Lactobacillus***		***E. faecalis***	
	Correlation coefficient	***p*-value**	**Correlation coefficient**	***p*-value**	**Correlation coefficient**	***p*-value**
Lamina dura	-0.051	0.600	-0.055	0.567	**-0.199***	**0.00***
Periodontal space	-0.077	0.424	-0.134	0.163	-0.181	0.059
Periapical lesion	-0.159	0.980	-0.082	0.395	-0.070	0.465
Furcation invlovement	-0.119	0.214	-0.135	0.159	-0.230*	0.016*
Root resorption	0.019	0.846	-0.012	0.900	0.175	0.068

*p-value <0.05, Spearman's rank correlation coefficient

Table 8: The association between bacterial quantities and radiographic pathology in irreversible pulpitis group.

5. Discussion

Due to a limited number of studies reporting bacteria involving infected root canals in primary teeth, there was no previous study that had reported quantitative data of *Lactobacillus* and *E. faecalis* in root canal infection of primary teeth before. However, in this study total bacteria quantity from the pulp necrosis group was higher than in the irreversible pulpitis group, which similar to a previous study using checkerboard DNA hybridization that showed results taken from 55 infected root canal samples. Their results showed that the total bacteria were higher in the pulp necrosis group compared with the irreversible pulpitis group [19]. Because the bacterial community had changed after the bacterial invasion to the pulp, in the coronal part the major composition of microorganisms was gram-positive facultative anaerobes. When the lesion progressed to the root canal, anaerobic bacteria represented over 70% in the root canals of primary molars that had not been treated. As expected, the bacterial community in pulp necrosis should be found to be higher than in irreversible pulpitis.

Few studies which were done in primary teeth have reported the prevalence of *Lactobacillus* in advanced carious lesions with pulp exposure. A previous study using real-time PCR showed high level of *Lactobacillus* in dental plaque of deep carious lesions with chronic pulpitis and they suggested that *Lactobacillus* was important in caries development, especially in dentin [17]. In this study, *Lactobacillus* was found in all samples (100%), whereas *E. faecalis* was found in 83.53%. The prevalence of *Lactobacillus* in this study was similar to a previous study using

quantitative real-time PCR. That study showed 97% of *Lactobacillus* prevalence in advanced caries with irreversible pulpitis [21]. Another study presented 44% of *Lactobacillus* prevalence in 9 carious exposed pulp lesions using a culture technique [3]. Other studies were done in Chinese children aged 5.4-7.6 years old showed the prevalence of *Lactobacillus* in 11 purulence samples (27.3%) from acute apical abscess using the PCR-DGGE method [21]. A study by Lana and colleagues found 19.35% of *Lactobacillus* in necrotic root canals of permanent teeth [13].

Another study by Sundqvist and colleagues showed the prevalence of *Lactobacillus* to total bacteria was 32% from bacteria that had been isolated from the infected root canal without intact pulp chamber walls [27]. Taken together, the presenting of a high prevalence of *Lactobacillus* in root canal infections in previous studies, including this study, supports the ability of *Lactobacillus* to invade into infected pulpal tissue and might be one of the pulpal pathogens in primary teeth. For the proportion of *Lactobacillus* to total bacteria in pulp necrosis and irreversible pulpitis, they were 23.19% and 19.4%, respectively. A previous study presented the role of *Lactobacillus* in the initial stages of polymicrobial infection of dental pulp and found that after pulpal exposure, *Lactobacillus* were displayed in the early stage of pulpal infection [28].

Others findings also showed that *Lactobacillus* was detected close to the pulp due to acidic environment changes at deeper levels [28, 29]. Afterwards, *Lactobacillus* levels rose again to initiate root canal biofilms [29]. In the early stage of pulpal infection or irreversible pulpitis, *Lactobacillus* plays an important role as one of the pulpal pathogens and rises significantly as caries progresses to initiate endodontic infection by playing as a pioneer for root canal biofilm formation [30]. It could support the findings of this study that *Lactobacillus* can be found in necrotic root canals higher than in irreversible pulpitis root canal samples. For the prevalence of *E. faecalis* in this study, it was found to be higher than 80%, which is in line with previous studies. Those studies reported that the prevalence of *E. faecalis* in the infected root canals of primary teeth was between 14-83% [19, 31, 32]. A study by Cogulu and colleagues using cultural and PCR methods investigated *E. faecalis* in necrotic primary and permanent teeth, their results showed that the prevalence of this bacteria were 26% and 32% in primary teeth and permanent teeth, respectively [33].

In another study using checker board DNA hybridization, results showed that 83% *of E. faecalis* was detected in primary teeth necrotic pulp samples [31]. Interestingly, results from previous studies, including this study, showed that the prevalence of *E. faecalis* in root canal infection of primary teeth was higher than permanent teeth (4-40%) [33]. In addition, the previous study in permanent teeth presented the ratio of *E. faecalis* to total bacterial level as less than 1% of the total bacterial level in samples of oral rinse, saliva, dental plaque and subgingival biofilm samples [34, 35]. The reason might be due to the morphology of primary teeth roots which have more accessory canals and large dentinal tubules compared to permanent teeth. *E. faecalis* has the capacity to invade and live within the dentinal tubule, which might relate to the invasion of *E. faecalis* in primary root canal infections [36, 37].

Furthermore, *E. faecalis* had been reported in high prevalence in primary and secondary endodontic infections and was found to be over 50% in necrotic pulp with fistula samples [38]. Sedgley and colleagues reported that *E.*

faecalis was detected in 1.1×10^3-1.7×10^6 cells/ml. from 88 root canal samples (48 failed root canal treatments, 40 primary infection root canals) of permanent teeth using real-time PCR [39]. Another study using conventional PCR showed that *E faecalis* was cultured from 2 of 50 necrotic canals (4%) and from 21 of 50 root-treated canals (42%) [27]. There were no previous studies presenting quantitative levels of *E. faecalis* in infectious root canals of primary teeth before, most previous studies presented the prevalence of *E. faecalis* between primary infections compared with secondary infections. From previous study, they compared *E. faecalis* prevalence between primary infection and secondary infection and found that the prevalence of *E. faecalis* rose significantly after secondary infection due to their capacity to invade and adapt their cell surface to live within the dentinal tubule, this microorganism showed high resistance to calcium hydroxide and irrigating solutions used in root canal treatment [38, 40].

For the correlation of history of pain, clinical signs and symptoms and bacterial levels, this study found that *Lactobacillus* was associated with a history of pain and clinical signs and symptoms, which is similar to a study by Rocas and colleagues which found that the quantity of *Lactobacillus* increased when continuous pain occurred [28]. Due to the capacity to produce acid and initiate an inflammation process, *Lactobacillus* may affect pain from pulpal pathology [34, 39, 41-43]. When lesions progress nearly to the pulp horn, dental nerves are activated by mediators such as IL-10, histamine or organic acid from bacteria. Mediator accumulation affects pulpal pressure and low compliance of pulpal blood flow, which can cause pulpal inflammation [43]. *E. faecalis* significantly associated with only a history of pain, it can be related to pulpal pathology of the periapical area. There was some evidence showing that *E. faecalis* was most frequently found involving chronic periodontitis, and another study found *E. faecalis* was related with tenderness on percussion and periapical radiolucency [10, 28]. In addition, one study found that *E. faecalis* had a strong correlation with tenderness on percussion in permanent teeth [43].

However, the findings from the present study did not find an association between *E. faecalis* quantities and clinical signs and symptoms similar to previous study, which found no correlation of *E. faecalis* and pain in primary infection [24]. According to characteristic features of these bacteria, which are frequently found in secondary infections or failed root canal treatments and persistent of antibiotics, it would be better if we studied the prevalence of *E. faecalis* in secondary infections which can be represented as a pathogen. Although the relationship between *E. faecalis* levels and history of spontaneous pain was not found in this study, the study of Cogulu and colleagues presented an association between *E. faecalis* and previous pain in both primary and permanent teeth [6]. Moreover, Vineet and colleagues found that *E. faecalis* was strongly correlated with tenderness on percussion in permanent teeth [43]. Although the success of endodontic treatment depends on many factors, the reduction of bacteria is the important key. According to this finding, clinicians should consider taking more histories of pain in children because it might influence the treatment outcome in terms of bacteria elimination.

The results showed no significant difference between *E. faecalis* and *Lactobacillus* levels in the presence of clinical abscess, both for acute and chronic abscess. In this study, *E. faecalis* and *Lactobacillus* in root canal samples without the clinical abscess presence showed higher levels than samples with abscess presence. The previous studies by Yang and colleagues showed that acute apical abscess samples in primary teeth with root canal infection

presented the predominance of *Prevotella* (24%), *Fusobacterium* (18%) and *Porphyromonas* (14%) [41]. Moreover, *Actinomyces, Streptococcus mitis,* and *Streptococcus oralis* were found in high prevalence in abscesses of primary teeth infection. Studies from permanent teeth showed that the predominate microorganism of extra-radicular infection, which presented as gingival or periapical abscess was similar to the microflora in periodontal pockets and the pioneer bacteria that can develop scaffold building, which was *Actinomyces* [40].

History taking and clinical examination were examined before doing the dental procedure. All data were informed by children and guardians about history of pain, including spontaneous pain, provoked pain or pain on mastication, for the clinical examination, it included pain on percussion, tooth mobility, gingival swelling or gingival abscess. The weak point for collecting history of pain was reliability of information from children or guardians who did not recognize or were uncertain of the occurrence, which might be one drawback in this study.

Although, endodontic treatment of primary teeth is routine in dental practice to control endodontic infections and preserve the teeth by elimination of microorganisms, participating in the pathogenesis and success of treatment depends on many factors [21]. From the findings of this study, history taking and clinical examination together with radiographic finding affects the proper treatment and success of outcomes. In conclusion, the bacterial levels of total bacteria, *Lactobacillus* and *E. faecalis* in necrotic pulp necrosis group were higher than those of irreversible pulpitis group. The proportion of *Lactobacillus* to total bacteria in pulp necrosis group was significantly different from irreversible pulpitis group. *Lactobacillus* and *E. faecalis* levels were significantly associated with a history of pain. Only *Lactobacillus* levels were associated with a history of pain in irreversible pulpitis group.

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