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Original Article

Quantitative Analysis of *Bifidobacterium* and *Scardovia wiggsiae* in Dental Plaque from Children in Northern Thailand and Their Association with Caries Factors

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Abstract

Objectives The aim of this study were to measurably detect *Bifidobacterium* and *Scardovia wiggsiae* in plaque from severe early childhood caries (S-ECC) and caries-free children and to analyze the interrelation between these bacteria and clinical caries presentation and caries-related factors assessed by questionnaire.

Materials and Methods One-hundred forty supra gingiva plaque samples from children aged between 2 and 5 years were used in this study. There were 70 children in each group. Recorded plaque index, modified gingival index, and decay, missing, and filled tooth (dmft) scores. Parents' attitudes, child's oral hygiene, and diet were assessed by questionnaire. DNA was extracted from plaque samples and quantitative real-time polymerase chain reaction using fluorescent dye was performed.

Results Plaque ($p < 0.001$) and modified gingival indices ($p < 0.001$) in the S-ECC group were higher than in the caries-free group. Prevalence of *Bifidobacterium* ($p = 0.004$) and *S. wiggsiae* ($p < 0.001$) in the S-ECC group was higher than in the caries-free group. The numbers of total bacteria ($p = 0.003$), *Bifidobacterium* ($p < 0.001$), and proportion of *Bifidobacterium* to total bacteria ($p < 0.001$) were higher in the S-ECC group. Detections of both bacteria, *Bifidobacterium* + *S. wiggsiae* ($p < 0.001$), were higher in the S-ECC group than in the caries-free group. In the S-ECC group, dmft scores ($p < 0.001$; $p = 0.024$) and the modified gingiva index ($p = 0.004$; $p = 0.002$) were higher in the presence of *Bifidobacterium* and *S. wiggsiae,* respectively. In the S-ECC group, the dmft scores ($p = 0.005$) and modified gingiva index ($p = 0.004$) were higher in the presence of both *Bifidobacterium* + *S. wiggsiae.* There were positive correlations between the *Bifidobacterium* level ($p = 0.003$), the proportion of *Bifidobacterium* to total bacteria ($p = 0.017$), and S-ECC. The level of *Bifidobacterium* ($p < 0.001$) and ratio of *Bifidobacterium* to total bacteria ($p < 0.001$) were correlated with the dmft score and modified gingival index in the S-ECC group. From the questionnaire, S-ECC were associated with major caregiver ($p = 0.002$), parent education levels ($p = 0.02$), prolonged bottle-feeding (>18 months)

Keywords
- severe early childhood caries
- *S. wiggsiae*
- *Bifidobacterium*
- real-time PCR
- caries-related factors

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($p = 0.015$), night-time feeding ($p < 0.001$), eating cariogenic snacks ($p = 0.019$), and frequency of dental visits ($p = 0.003$).

Conclusions Levels of total bacteria, *Bifidobacterium*, *S. wiggsiae* and plaque, and the modified gingival indices were higher in the S-ECC group. Factors associated with S-ECC included the major caregiver, parent education levels, feeding patterns, cariogenic snacks consumption, and frequency of dental visits.

Introduction

Early childhood caries (ECC) is still one of common childhood diseases worldwide. Recent international survey showed that dental caries prevalence in 5 years old children ranged from 23 to 90%.[1] In Thailand, caries prevalence is higher than 50%.[2]

Severe ECC (S-ECC) is an aggressive form of ECC. Based on the definition of S-ECC by the American Academy of Pediatric Dentistry (AAPD), children aged between 3 and 5 years who have one or more cavitated lesions, caries-caused missing or filled smooth surface in primary teeth or decayed, missing or filled surfaces greater than or equal to four (age of 3), five (age of 4) or six (age of 5) are diagnosed as S-ECC patients. It occurs earlier in life, with more incidence, and affects children growth, even physical and psychological health of the subjects during their whole lifespan.[3] S-ECC is associated with decreased oral-health-related quality of life for the child, and high costs for families and society.[4] The etiology is complicated because it involves numerous factors, including biological, behavioral, psychological, cultural and lifestyle factors.[3]

Dental plaque, or biofilm, is composed of more than 1,000 species of microorganisms living together. Under normal circumstances, oral microbiota has a symbiotic relationship with all other microorganisms and live together in a non-disease environment. A shift of the microbiota in biofilm can cause disease such as dental caries, which initiates from the formation of biofilm on tooth surfaces, where the proportion of acidogenic and aciduric bacteria in biofilm are increasing and overbalance the healthy microbiota leading to a changing ecological equilibrium.[4] These bacteria produce acidic conditions by metabolizing fermentable carbohydrates. A frequent aciduric environment can disrupt microbial hemostasis in biofilm and promote demineralization of tooth structure when the pH is lower than 5.5.[4]

Streptococcus mutans is one of major microbial pathogens in S-ECC development.[5] However, not all children with S-ECC harbor *S. mutans*.[5] Microbiota associated with dental caries is highly complex and multiple members of the community can be implicated in caries development.[6,7], Besides *S. mutans*, studies in S-ECC have detected other pathogens involved in dental caries that are nonmutans bacteria, such as *Bifidobacterium* and *S. wiggsiae*.[6–9]

Bifidobacterium is a gram-positive, rod-shaped, anaerobic, nonfilamentous and nonmotile bacteria. It is acidogenic, aciduric and able to survive in an acidic environment.[9] When living in acidic conditions, it has several self-protection mechanisms even in the absence of an energy source in order to maintain their lives.[10] Besides its acidogenicity and aciduricity, it has an ability to produce an acidic environment to resist low pH and promote biofilm formation when coadhered with primary colonizers that were similar to *S. mutans*.[11,12] Recent studies found the link between *Bifidobacterium* and S-ECC including Thai children, and suggested that it might be a major pathogen in cavitated dentin caries because it has an ability to demineralize tooth structure at a pH below 4.2.[2,8,11–14] One of the studies in ECC and S-ECC children showed 80% detection of *Bifidobacterium* from plaque samples.[13] It was detected higher in children with caries (95%), when compared to caries-free children (9%).[12]

S. wiggsiae is anaerobic, gram-positive, bacilli-shaped bacteria. It is one of acid producer bacteria and able to tolerate in an acidic environment.[15] It can grow on agar at a low pH similar to *S. mutans*.[15] It has been detected from dental plaque, saliva, and infected dentin lesions in S-ECC in the presence of *S. mutans*.[15] However, *S. wiggsiae* has also been detected in children in the absence of *S. mutans*, which suggests that it might play an exclusive role in the caries process when *S. mutans* is not the main cariogenic specie.[15] Recent studies showed an interesting association between *S. wiggsiae* in the initial stages of caries and in children who are receiving orthodontic treatment.[6,15] Moreover, previous studies reported that the combination of *S. mutans* with *Bifidobacterium* and *S. wiggsiae* was associated with S-ECC and suggested that they might be microbiological markers in active caries lesions.[16,17]

The aims of this study were to detect *Bifidobacterium* and *S. wiggsiae* quantitatively in dental plaque samples from S-ECC and caries-free children in the Northern part of Thailand and analyze the association between these bacteria and clinical presentation as well as other factors assessed by questionnaire. The hypothesis is that the amounts of *Bifidobacterium* and *S. wiggsiae* in S-ECC and caries-free groups should be different.

Materials and Methods

Subject Selection

A statistician consultation was done based on previous studies, performed with $\alpha = 0.05$ and power of 80%, using the software package Primer of Biostatistics (McGraw-Hill, New York, United States).[7] A minimum of 61 children in each group was enough to achieve statistical difference.[8]

Total of 140 Thai children aged 2 to 5 years were recruited from four public childcare centers in Mae Lao district in the

Northern part of Thailand, Chiang Rai Province, Thailand. Consent forms were signed. Subjects were divided into two groups: Seventy children in an S-ECC group, and 70 children in a caries-free group. S-ECC was diagnosed based on the AAPD 2018 to 2019, which defines S-ECC. For children younger than 3 years of age, any sign of smooth-surface caries indicates S-ECC. For children aged between 3 and 5, one or more cavitated, missing (due to caries), or filled smooth surfaces in primary maxillary anterior teeth, or a decayed, missing, or filled score of less than or equal to 4 (age 3), less than or equal to 5 (age 4), or less than or equal to 6 (age 5) surfaces also indicate S-ECC.[17] For the caries-free group, subjects had no caries nor existing restorations (decay, missing, and filled tooth [dmft] = 0). Obtained bitewing radiograph only when interproximal caries was suspected. Subjects who had any systemic disease(s), taking any kind of antibiotics, had professional fluoride application or any dental treatment within 2 months prior to the sample collection period were excluded from the study.

Clinical Examination, Plaque and Modified Gingival Indices

Two examiners who are in a pediatric dentistry residency training program performed a clinical examination at schools based on World Health Organization criteria.[18] Decayed, missing, and filled tooth (dmft) scores, plaque and gingiva inflammation indices were recorded as mentioned in previous studies.[19–21]

The Questionnaire

The questionnaire was assessed by parents or caretakers in a face-to-face interview. All questions were close ended. Except the parents' general information, two categories were examined (►**Table 1**).

Plaque Sample Collection

Informed all parents/guardians to brush their child's teeth at 8.00 PM the night before plaque collection day. No food or drink before sample collection. Collected pooled overnight dental plaque using a sterile toothpick and released in 1 mL of Tris-EDTA buffer. Transported all samples on ice to the Oral Biology Laboratory and stored at −20°C until the DNA extraction process.

Table 1 Questionnaire used in this study

Categories	Questions
1. Demographic characteristics	1.Child's age 2. Child's gender 3.Guardian's age 4. Guardian's gender 5. Major caregiver 6. Parent's education levels
2. Child's diet and oral hygiene care	1. Prolonged bottle feeding 2. Night-time feeding 3. Eating cariogenic snacks 4. Night-time snack feeding 5. Frequency of dental visit

DNA Extraction

DNA was extracted based on enzymatic lysis using a commercial kit (Flavogen, Taiwan) as previously described.[8] In brief, we added 20 µL of Proteinase K, 400 µL of FABG buffer, and 20 µL of a lysozyme mixture (lysozyme 20 mg/mL and mutanolysin [Sigma Aldrich, United States] in 1:10 proteinase K) and mixed by means of rapid whirling. Incubated the mixture at 60°C for 1 hour; added 200 µL ethanol and centrifuged at 11,000 rpm for 30 seconds. Transferred the solution into a spin column and centrifuged for 1 minute. Discarded supernatant, then added 500 µL of W1 buffer and centrifuged for 1 minute. Again discarded supernatant and added 750 µL of wash buffer and centrifuged for 1 minute, then added 50 µL of elution buffer, left at room temperature for 3 minutes, before a final centrifuge for 2 minutes. Then measured the extracted DNA concentration and purity using a spectrophotometer at 260 nm/280 nm (Nanodrop 2000C Thermo Fisher Scientific, Delaware, United States).

Culture Condition and Standard Strains

Bacterial strains, *Bifidobacterium longum* (subspecies 51139) and *Streptococcus sobrinus* ATCC (6715), were used as standard strains. *Bifidobacterium longum* (subspecies 51139) was inoculated in Brain Heart Infusion broth and incubated at 37°C for 24 hours. *S. sobrinus* was grown anaerobically (5% CO_2) in Brain Heart Infusion broth at 37°C for 24 to 48 hours. After DNA extraction from the overnight culture as described above, performed a tenfold serial dilution, starting from 10^8 to 10^2 CFU/mL.

Quantitative Real-Time PCR

Using specific primers, the reaction mixture (total volume of 20 µL) contained water (2–9.1 µL), 10 µL of 2X KAPA SYBR FAST quantitative polymerase chain reaction (PCR) Master Mix, 0.4 µL of 10 µM forward and reverse primer, and 0.1 to 7.2 µL of 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 3 minutes, denaturing at 95°C for 3 seconds, annealing at 52°C, 53°C, and 53°C for 20 seconds for universal BAC16S (F 5′-TGG AGC ATG TGG TTT AAT TCG A-3′, R 5′-TGC GGG ACT TAA CCC AAC A-3′ amplicon 160 base pair),[22] *Bifidobacterium* (F 5′ CTC CTG GAA ACG GGT GG-3, R 5′ GGT GTT CTT CCC GAT ATC TAC A-3′ amplicon 550 base pair),[23,24] and *S. wiggsiae* (F 5′-GTGGACTTTATGAATAAGC-3′, R 5′-CTACCGTTAAGCAGTAAG-3′ amplicon 200 base pair),[11] respectively. Melting curves were generated from 60 to 95°C and read every 0.5°C for 5 seconds.[8]

Statistical Analysis

Data was analyzed using SPSS 18.0 software (Microsoft Corporation. California, United States). Normality of the data was tested using a Kolmogorov–Smirnov and Shapiro–Wilk test ($p < 0.05$). The different levels of bacteria between the S-ECC and caries free groups were analyzed using a Mann–Whitney U test ($p < 0.05$). The association between *Bifidobacterium* and *S. wiggsiae* was analyzed using McNemar's test ($p < 0.05$). Pearson chi-squared test was used for

Table 2 General information of subjects between caries-free and S-ECC groups

	Caries-free	S-ECC	*p*-Value
Age (months)	Mean ± SD = 35.54 ± 6.90 Median = 35	Mean ± SD = 35.07 ± 6.88 Median = 34	0.664[a]
Gender			
- Male	44(62.9%)	33(47.1%)	0.062[b]
- Female	26(37.1%)	37(52.9%)	

Abbreviations: SD, standard deviation; S-ECC, severe early childhood caries.
[a]Mann–Whitney U test.
[b]Pearson chi-squared test.

analysis of the detection of each bacteria and association of factors in the questionnaire for caries status.

Ethical Considerations

This study was approved by the Ethics Approval Review Board, Faculty of Dentistry and the Faculty of Pharmacy, Mahidol University (MU-DT/PY-IRB 2020/DT039).

Results

Children's age and gender between caries-free and S-ECC groups were not different (►**Table 2**). Mean ± standard deviation (SD) of the plaque index in caries-free and S-ECC groups was 0.87 ± 0.56 and 1.40 ± 0.64, respectively. Mean ± SD of the modified gingival index in caries-free and S-ECC groups was 0.29 ± 0.44 and 0.61 ± 0.46, respectively. The plaque ($p < 0.001$) and modified gingival indices ($p < 0.001$) in the S-ECC group was higher than in the caries-free group (►**Table 3**).

Prevalence of *Bifidobacterium* in the caries-free and S-ECC groups was 6 (9%) and 19 (27%), respectively. Prevalence of *S. wiggsiae* in the caries-free and S-ECC groups was 1 (2%) and 27 (39%), respectively. The prevalence of *Bifidobacterium* ($p = 0.004$) and *S. wiggsiae* ($p < 0.001$) in the S-ECC group was significantly higher than in the caries-free group (►**Fig. 1**).

The median numbers (minimum, maximum) of total bacteria, *Bifidobacterium*, and proportion of *Bifidobacterium* to total bacteria in the S-ECC group were 6.63×1025 (9.37×10^5, 1.92×10^7), 0 (0, 7.61×10^5), and 0 (0, 8.5×10^{-2}), respectively. The median numbers (minimum, maximum) of total bacteria, *Bifidobacterium*, and proportion of *Bifidobacterium* to total bacteria in the caries-free group were 5.27×1025 (1.14×10^6, 3.06×10^7), 0 (0, 3.43×10^4), and 0 (0, 3.51×10^{-03}), respectively.

The median numbers of total bacteria ($p = 0.003$), *Bifidobacterium* ($p < 0.001$), and proportion of *Bifidobacterium* to total bacteria ($p < 0.001$) were higher significantly in the S-ECC group in compared to the caries-free group (►**Table 4**). Detections of both bacteria in combination, *Bifidobacterium* + *S. wiggsiae* ($p < 0.001$), were higher in the S-ECC group than in the caries-free group (►**Fig. 1**).

►**Table 5** shows clinical parameter comparisons between the presence and absence of bacteria in the S-ECC group. In the S-ECC group, the mean ± SD of dmft scores in the presence and absence of *Bifidobacterium* were 8.47 ± 3.58 and 5.06 ± 3.52, respectively. Mean dmft scores were significantly higher in the presence group than in the absence group ($p < 0.001$). Mean ± SD of the modified gingiva index in the presence and absence of *Bifidobacterium* was 0.87 ± 0.4 and 0.52 ± 0.41, respectively. Mean dmft scores ($p < 0.001$) and modified gingiva index ($p = 0.004$) were significantly higher in the presence group than in the absence group. Same as *Bifidobacterium,* in the S-ECC group, mean ± SD dmft scores in the presence and absence of *S. wiggsiae* were 7.04 ± 3.67 and 5.33 ± 3.82, respectively. Mean ± SD of the modified gingiva index in the presence and absence of *S. wiggsiae* was 0.82 ± 0.48 and 0.48 ± 0.39, respectively. The mean dmft scores ($p = 0.024$) and modified gingiva indexes ($p = 0.002$) were significantly higher in the presence group than in the absence group.

Interestingly, in the S-ECC group, there was a detection of the combination of two bacteria that was not found in the caries-free group. In the S-ECC group, mean ± SD dmft scores in the presence and absence of a two bacteria presence,

Table 3 Plaque index and modified gingival index in caries-free and S-ECC groups

Clinical parameters	Caries-free		S-ECC		*p*-Value
	Median (min, max)	Mean ± SD	Median (min, max)	Mean ± SD	
Plaque index	0.75 (0, 2.50)	0.87 ± 0.56	1.33 (0.33, 3.00)	1.40 ± 0.64	<0.001[a]
Modified gingival index	0 (0, 1.80)	0.29 ± 0.44	0.67 (0, 2.00)	0.61 ± 0.46	<0.001[a]

Abbreviations: SD, standard deviation; S-ECC, severe early childhood caries.
[a]Mann–Whitney U test at the significant level of $p < 0.05$.

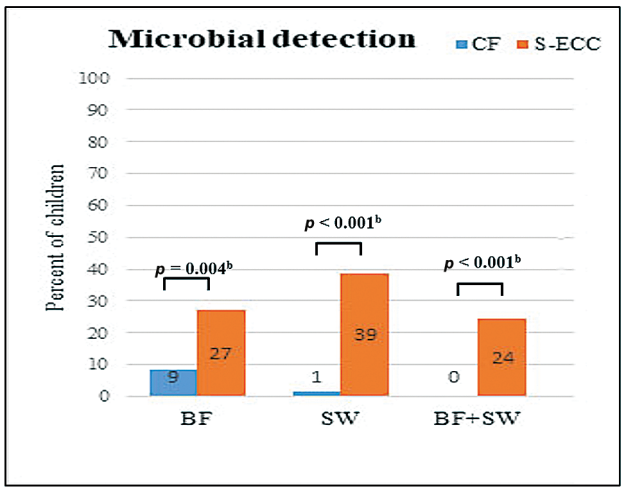

Fig. 1 The prevalence of bacteria between caries-free and severe early childhood caries (S-ECC) groups. BF, *Bifidobacterium*; CF, caries-free; SW, Scardovia *wiggsiae*. [b]Pearson chi-squared test at the significant level of $p < 0.05$.

Bifidobacterium + *S. wiggsiae*, were 8.12 ± 4.04 and 5.3 ± 3.53, respectively. In the S-ECC group, mean ± SD of the modified gingiva index in the presence and absence of two bacteria, *Bifidobacterium* + *S. wiggsiae*, was 0.91 ± 0.48 and 0.52 ± 0.41, respectively. The mean dmft scores ($p = 0.005$) and modified gingiva index ($p = 0.004$) were significantly higher in the presence group than in the absence group (►**Table 5**). There was a positive correlation between the levels of *Bifidobacterium* ($p = 0.003$) and the proportion of *Bifidobacterium* to total bacteria ($p = 0.017$) in the S-ECC group.

In the S-ECC group, the correlation coefficiency between *Bifidobacterium* and dmft, plaque index and modified gingiva index was 0.489, 0.165, and 0.378, respectively. The correlation coefficiency between the ratio of *Bifidobacterium* to total bacteria and dmft, plaque index, and modified gingiva index was 0.449, 0.168, and 0.381, respectively. The level of *Bifidobacterium* ($p < 0.001$) and the ratio of *Bifidobacterium* to total bacteria ($p < 0.001$) was significantly correlated with the dmft score. Also, the level of *Bifidobacterium* ($p = 0.001$)

Table 4 Number of bacteria between caries-free and S-ECC groups

Bacteria	Caries-free Median (min, max)	S-ECC Median (min, max)	*p*-Value
Total bacteria	5.27E + 06 (1.14E + 06,3.06E + 07)	6.63E + 06 (9.37E + 05, 1.92E + 07)	0.003[a]
Bifidobacterium	0 (0, 3.43E + 04)	0 (0, 7.61E + 05)	0.001[a]
Bifidobacterium/total bacteria	0 (0, 3.51E–03)	0 (0, 8.50E–02)	0.001[a]

Abbreviation: S-ECC, severe early childhood caries.
[a]Mann–Whitney U test at the significant level of $p < 0.05$.

Table 5 The association of clinical parameters and bacteria in S-ECC group

	Presence		Absence		*p*-Value
	Median (min, max)	Mean ± SD	Median (min, max)	Mean ± SD	
Bifidobacterium					
- dmft	8 (4,16)	8.47 ± 3.58	4 (1,19)	5.06 ± 3.52	<0.001[a]
- Plaque index	1.50 (0.67, 3.00)	1.63 ± 0.74	1.33 (0.33, 3.00)	1.31 ± 0.58	0.141
- Modified gingiva index	1.00 (0.04, 2)	0.87 ± 0.49	0.54 (0, 1.17)	0.52 ± 0.41	0.004[a]
Scardovia wiggsiae					
- dmft	6 (2,16)	7.04 ± 3.67	4 (1, 19)	5.33 ± 3.82	0.024[a]
- Plaque index	1.60 (0.33, 3.00)	1.55 ± 0.74	1.17 (0.33, 3.00)	1.30 ± 0.55	0.178
- Modified gingiva index	1.00 (0, 2.00)	0.82 ± 0.48	0.50 (0, 1.00)	0.48 ± 0.39	0.002[a]
Bifidobacterium+ *S. wiggsiae*					
- dmft	8 (2, 16)	8.12 ± 4.04	4 (1, 19)	5.30 ± 3.53	0.005[a]
- Plaque index	1.67 (0.50, 3.00)	1.62 ± 0.80	1.33 (0.33, 3.00)	1.31 ± 0.56	0.242
- Modified gingiva index	1.00 (0, 2.00)	0.91 ± 0.48	0.50 (0, 1.17)	0.52 ± 0.41	0.004[a]

Abbreviations: dmft, decay, missing, and filled tooth; SD, standard deviation; S-ECC, severe early childhood caries.
[a]Mann–Whitney U test at the significant level of $p < 0.05$.

and ratio of *Bifidobacterium* to total bacteria ($p = 0.001$) were significantly correlated with the modified gingival index.

Caries-related factors assessed by the questionnaire showed that major caregiver ($p = 0.002$), parent education levels ($p = 0.02$), prolonged bottle-feeding (>18 months of age) ($p = 0.015$), night-time feeding ($p < 0.001$), eating cariogenic snacks ($p = 0.019$), night-time snack feeding ($p = 0.023$), and frequency of dental visits ($p = 0.003$) were associated with S-ECC (►**Table 6**).

Discussion

In this study, in S-ECC group, plaque, and gingival indices were significantly higher than in the caries-free group, which are similar to previous studies.[3,8] Dental plaque or oral biofilm on tooth surfaces actively supports the metabolism of cariogenic microbes, which brings about an acidic environment, growth, and proliferation of aciduric and acidogenic bacteria, and tooth demineralization.[9] This study

Table 6 The association between related factor and caries status

Variable	Caries-free *n* (%)	S-ECC *n* (%)	*p*-Value
Demographic characteristics			
Child's age (months)	35.54 ± 6.90	35.07 ± 6.88	0.664[a]
Child's gender			
Male	44 (62.9)	33(47.1)	0.062[b]
Female	26 (37.1)	37(52.9)	
Guardian's age (years)	33.40 ± 8.73	33.87 ± 8.42	0.727[a]
Guardian's gender			
Male	13 (18.6)	12(17.2)	0.825[b]
Female	57 (81.4)	58(82.8)	
Major caregiver			
Father or mother	60 (85.7)	44 (62.9)	0.002[b]
Grandparents or others	10 (14.3)	26 (37.1)	
Parent's education levels			
<Bachelor's degree	46 (65.7)	58 (82.9)	0.02[b]
≥Bachelor's degree	24 (34.3)	12 (17.1)	
Child's diet and oral hygiene care			
Prolonged bottle feeding			
Yes	20 (28.6)	34 (48.6)	0.015[b]
No	50 (71.4)	36 (51.4)	
Night-time feeding			
Yes	10 (14.3)	37 (52.9)	<0.001[b]
No	60 (85.7)	33 (47.1)	
Eating cariogenic snack			
Never or in meal	14 (20.0)	15 (21.4)	0.019[b]
Between meal ≤2 times/day	47 (67.1)	33 (47.1)	
Between meal >2 times/day	9 (12.9)	22 (31.4)	
Night-time snack feeding			
Yes	20 (28.6)	33 (47.1)	0.023[b]
No	50 (71.4)	37 (52.9)	
Frequency of dental visits			
Regularly	23 (32.9)	26 (37.1)	0.003[b]
When child had symptom	8 (11.4)	22 (31.4)	
Never	39 (55.7)	22 (31.4)	

[a]Mann–Whitney U test or [b]Pearson chi-squared test at the significant level of $p < 0.05$.

showed that the *Bifidobacterium* prevalence in the S-ECC group was higher than in the caries-free group significantly. Moreover, the amount of *Bifidobacterium* and proportion of *Bifidobacterium* to total bacteria in the S-ECC group were higher than in the caries-free group, which were in the same direction to previous studies.[16,17,26] Our findings showed no detection of *Bifidobacterium* in the caries-free group, which is similar to the previous study by Zhai et al.[27] Their study performed PCR from saliva and plaque samples from 30 caries-free and 40 S-ECC subjects, aged between 3 and 5 years. Their results showed that *Bifidobacterium* was detected 47.5% and 0 in the S-ECC and caries-free groups, respectively. There was significant difference between the two groups ($p < 0.05$).[27] It was previously reported that a higher level of *Bifidobacterium* was detected in mature plaque from subjects with poor oral hygiene when compared with good oral hygiene subjects, which was in the same direction as our results.[8,10] This study also found the correlation between levels of *Bifidobacterium* and the proportion of *Bifidobacterium* to total bacteria with the gingival index in the S-ECC group, which is similar to previous studies.[2,28] One of our previous studies showed a higher level of *Bifidobacterium* significantly in the S-ECC group than in the caries-free group and also found that the level of *Bifidobacterium* and the proportion of *Bifidobacterium* to total bacteria were positively related to the dmft scores in S-ECC group, which were similar to this study.[8] Conversely, our recent study analyzed the association between *Bifidobacterium* in plaque from S-ECC children recruited from the Southern part of Thailand showed the detection and quantities of *Bifidobacterium* from the S-ECC group and caries-free group were not different significantly.[2] The reason could be from the level of caries severity and demographic. Although both previous studies and this study were done on Thai children, subjects in each study were recruited from different regions. We noticed that the mean dmft scores in this study and the study in 2017 were higher than the study in 2022. It is possible that the dental caries severity might be related to the *Bifidobacterium* quantity, thus resulting in different outcome. Previous study showed the positive correlation between *Bifidobacterium* levels and dentin active lesions ($p = 0.001$) in children aged between 2 and 5 years. This suggested that the higher detection levels of *Bifidobacterium* may be linked to lesion activity.[16,17] The current literature mentions special properties of *Bifidobacterium* that increase the violence in caries development because of its abilities to store polysaccharides inside their cells and degrade them into acids under limited conditions of carbohydrate. Moreover, it can tolerate to fluoride because of the availability of unique metabolic pathway.[17] Taken together, *Bifidobacterium* seems to play an important role in caries progression. The association between *Bifidobacterium* and caries severity should be assessed and confirmed in the future.

S. wiggsiae belongs to the Bifidobacteriaceae family.[29] It was first detected in S-ECC in 2002.[29] This study found a significantly higher prevalence of *S. wiggsiae* in the S-ECC group than in the caries-free group, which is similar to previous studies.[2,29–33] The prevalence of *S. wiggsiae* in the S-ECC group was 39% in this study, while in our previous study it was 49%.[2] This study showed that dmft scores and the gingival index of children presented with *S. wiggsiae* were higher significantly than in children who were absent of *S. wiggsiae*. Previous studies showed a positive correlation between dmft scores and levels of *S. wiggsiae* in saliva, which was similar to our study.[2,34–36] This is the second study to show the association between gingival index and *S. wiggsiae* in Thai children, which supported the association between *S. wiggsiae* and mature plaque, which is a highly complex oral community, consistent with numerous microorganisms associated with gingivitis and dental caries.[35,37] A previous study reported that when *S. wiggsiae* metabolized sugar, the environmental pH immediately decreased into 3.5.[35] Due to its characteristics, *S. wiggsiae* is an acidogenic and aciduric bacteria, similar to *S. mutans*. It can grow in acid agars (pH 5) and produce high acid that induces demineralization and increased caries progression.[35] *S. wiggsiae* might be one of bacteria involved in caries development. Previous studies have shown that in S-ECC groups, a combination of *S. mutans* and *S. wiggsiae* was detected, and this combination was not found in the caries-free groups.[2] This study also showed that the combination of *Bifidobacterium* and *S. wiggsiae* was detected in the S-ECC group, whereas they were not found in the caries-free group. Moreover, the dmft scores and gingival index of the S-ECC group, with the presence of these bacteria groups, were significantly higher than the absence of the bacteria group. As for the results in the amount of *S. wiggsiae,* which was detected in higher amounts in the S-ECC group and associated with the dmft scores and the gingival index. Our previous study was done in Thai children also, but from the Southern part of Thailand, and showed similar results. This is the second study in Thai children to confirm this result.[2]

According to the questionnaire, the education level of guardians and major caregivers was significantly associated with S-ECC. The higher the education (bachelor's degree or higher) of guardians, the more caries-free children, compared to those whose guardians had a lower education, below a bachelor degree. Similar to previous studies, major caregivers with university levels had better knowledge and attitudes, hence a better practice to improve the oral health care of their children.[2,36,37] In this study, S-ECC were significant associated with prolonged bottle-feeding, night-time, eating cariogenic snacks, and night-time snack feeding. As found in previous studies, on-demand bottle-feeding habits and nocturnal bottle-feeding in children aged over 12 months, with irregular brushing, could increase the risk of developing caries because of extended contact time with sugary liquids on teeth and declined salivary flow at night.[38] More frequent activities of bottle-feeding and sugary foods consumption at age 12 months increased the risk of dental caries when the children were 3 years old.[39] Consumption of cariogenic snacks, which contain low-nutrients but high-energy, such as candies, sugar-coated starchy foods, cookies, Thai desserts, sticky sweets, juice, sweetened milk, sweetened beverages and other sweets, was associated with caries status.[2,37,39] Frequent sugary food intake is especially related

to a higher chance of developing S-ECC. Night-time consumption of sweetened drinks is also associated with caries production.[38,39] Moreover, this study showed a significant difference of frequency of dental visits between the S-ECC group and the caries-free group. Generally, children who had never seen a dentist associated with increased risk of caries development.[39] However, our study found that children in the S-ECC group had more dental visits than those of the caries-free group. Previous studies found that most parents and caregivers did not focus on the children's oral health status, due to many reasons, until their children unavoidably visited the dentist due to severe toothache.[1] This may assume that children in the S-ECC group had made more dental visits in the past due to several oral health problems, while children in the caries-free group had visited dentists less. Limitations of this study are that it is a cross-sectional study that is able to show only a short period of time. The oral biofilm community is dynamic and might need longer time to observe. Second, only two bacteria were investigated and analyzed. Analysis of multiple bacteria is strongly recommended in the future to more accurately represent the oral environment and the relationship between pathological bacteria and caries initiation. Third, larger sample size from multiple areas of Thailand is strongly recommended in the future study.

In conclusion, the prevalence of *Bifidobacterium* and *S. wiggsiae* in an S-ECC group was higher than in a caries-free group significantly. Levels of *Bifidobacterium* and the proportion of *Bifidobacterium* to total bacteria were higher in the S-ECC group significantly compared with the caries-free group. There was positive correlation between levels of *Bifidobacterium* and the proportion of *Bifidobacterium* to total bacteria and the dmft scores and gingival index. The detection of *Bifidobacterium*, *S. wiggsiae,* and combinations of these bacteria in the S-ECC group presented significantly higher modified gingival index and dmft scores. The factors from the questionnaire that were significantly associated with S-ECC were the major caregiver, parent's education levels, prolonged bottle-feeding ($>$ 18 months of age), night-time feeding, eating cariogenic snacks, night-time snack feeding, and frequency of dental visits.

Conflict of Interest

None declared.

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