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Original Article

Dentistry Section

Quantitative Analysis of *Streptococcus mutans*, *Streptococcus sobrinus* and *Streptococcus sanguinis* and their Association with Early Childhood Caries

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ABSTRACT

Introduction: Severe Early Childhood Caries (S-ECC) remains highly prevalent worldwide. *Streptococcus mutans* and *Streptococcus sobrinus* are causative pathogens of dental caries and strongly involved in plaque or oral biofilm formation. *Streptococcus sanguinis* has antagonistic relationship with *S. mutans* and it might delay the colonisation of *S. mutans* in the oral cavities.

Aim: To quantify *S. mutans, S. sobrinus* and *S. sanguinis* between 2 groups {S-ECC and Caries Free (CF)} of Thai children and to analyse the association between these bacteria and caries-related factors.

Materials and Methods: Supra-gingival overnight plaque samples were collected from 120 Thai children aged 2-5 years (S-ECC=CF=60) using sterile toothpicks and released in 1 mL of TrisBase and EDTA buffer from January 2015 to December 2017. They were asked to expectorate saliva into a cup, plaque and gingival indices and dmft scores were recorded, parent's demographic and children's oral hygiene care and diet were assessed using questionnaire. DNA extraction and quantitative real-time PCR was performed. Different amounts of each bacterium were analysed by Mann-Whitney U test ($p<0.05$). The correlation between amounts of each bacterium and other clinical factors were analysed by Spearman's correlation test ($p<0.05$).

Results: The guardian's demographic data, habit of milk bottle and breast feedings, oral hygiene care and consumption of cariogenic snacks were different between the two groups. *S.mutans* and *S.sobrinus* in plaque were higher in S-ECC. *S.sanguinis* in saliva and total bacteria were higher in the CF group. Plaque and gingival indices in S-ECC were higher than in the CF group. *S.mutans* levels in dental plaque and saliva was not significantly different.

Conclusion: *S.mutans* and *S.sobrinus* were associated with S-ECC while *S.sanguinis* was associated with caries-free. Socioeconomics and children's oral hygiene care and diet were important factors associated with S-ECC.

Keywords: Dental plaque, Oral biofilm, Real time-PCR

INTRODUCTION

The S-ECC is one of the most prevalent disease in children worldwide [1]. In Thailand, the prevalence of S-ECC in rural and capital areas is higher than 70% [2]. ECC causes not only local pain but also affects general growth and development, loss of self-esteem and might lead to psychological problems [1,3].

ECC results from an interaction between the acidogenic bacteria, sucrose, and host susceptibility [3,4]. Social and behaviour habits are contributing risk factors [3,4]. In the oral cavity, there are biofilm (dental plaque) which comprises of more than 800 species of microorganisms living in a complex community. It changes over time and the microorganisms population can shift between healthy and pathological environment when factor such as sugar is enhanced [4].

Streptococcus mutans and *Streptococcus sobrinus* are causative pathogen of dental caries [3-7]. *S.mutans* is commonly isolated microorganism from dental plaque [6-10]. Not only it is aciduric and acidogenic but also has the capability to adhere and deposit on the tooth surfaces. In the presence glucosyltransferases (Gtfs) (an enzyme of *S.mutans*), sucrose molecules are cleaved and the glucose component is polymerised into adherent glucans [10]. *S.mutans* is also able to generate the acid from carbohydrate and tolerate low pH environment [9-11].

S.sobrinus and *S.mutans* are different in many biochemical characteristics and virulence factors. *S.sobrinus* has greater acidogenic capacity than *S.mutans* [10,11]. The attachment of *S.sobrinus* happens when the pellicles are exposed to sucrose but this does not occur in *S.mutans.* The cell-associated Gtfs activity of *S.sobrinus* formed a higher percentage than that of *S.mutans* [11].

Previous studies showed that the prevalence of *S.mutans* in dental plaque of caries-active subjects was higher than *S.sobrinus* [12-14]. Numerous studies stated that children who were infected with both *S.mutans* and *S.sobrinus* have greater incidences of caries than those with *S.mutans* alone [13-15]. In contrast, study in Thai children found that when subjects were infected by both of them, the caries prevalence was the same as subjects infected by *S.sobrinus* alone [7].

Previous studies have reported the antagonistic relationship between *S. mutans* and *S. sanguinis.* They suggested that *S. sanguinis* delay the colonisation of *S. mutans* in the oral cavity and that caries-free children were colonised by high amounts of *S. sanguinis.* The interaction between *S. mutans* and *S. sanguinis* was also associated with caries outcome [16-19]. Previous studies have shown the relationship between *S. mutans* and *S. sobrinus* or *S.mutans* and *S.sanguinis* in dental plaque and saliva, none of them provided information on both sources in one study especially in Thai children [6,7].

Quantitative real-time PCR provides an accurate result and is a sensitive method for the detection and quantification of bacterial species [15]. This study aimed to detect *S.mutans, S.sobrinus* and *S.sanguinis* in dental plaque and saliva samples using real-time PCR from S-ECC and caries-free groups of Thai children, and to analyse the association between these bacteria and other caries-associated factors. The hypothesis is that the quantities of *S.mutans, S.sobrinus* and *S.sanguinis* from S-ECC and caries-free groups should be different.

MATERIALS AND METHODS

This cross-sectional study was conducted between January 2015 to December 2017 at Faculty of Dentistry, Mahidol University, Bangkok, Thailand. The approval from Human Institutional Review Board of the Faculty of Dentistry and the Faculty of Pharmacy, Mahidol University (MU-DT/PYIRB 2009/266.0910, 2013/027.2606) was obtained prior to initiation of the study. Sample size was calculated based on the previous study [6], with α=0.05 and power of 80%, using the software package Primer of Biostatistics (McGraw-Hill, NY, USA).

Subject Selection

Total subjects were 120 (caries-free=60, S-ECC=60) Thai children aged two to five-year-old. All subjects were randomly selected from public schools in Pathumthani province, Thailand. Consent forms were signed. A clinical examination was performed by 2 paediatric dental residents. They were calibrated for clinical examination (kappa co-efficiency=0.85). The diagnosis of S-ECC was based on the AAPD [20]. Children with any systemic disease(s), on any kind of antibiotics, had professional fluoride application or any dental treatment within 3 months prior to the sample collection period were excluded.

Clinical Examination, Plaque Index and Modified Gingival Index

Recorded dmft score and plaque index used a modified debris index of simplified oral hygiene index for deciduous dentition [21-23]. Gingival inflammation was recorded on a 0-4 scale following the modified gingival index [22,23].

The questionnaire: All participants' parents or caretakers were asked to complete the questionnaire by face-to-face interview. All questions were close ended. Besides the parents' general information, 3 categories were examined: 1) Child's general information; 2) Parental attitude towards child's diet: a) Is your child still bottle feeding?; b) Did your child ever have breast and/or bottle feeding ad lib?; c) Did your child breast and/or bottle feed ad lib and fall asleep?; d) Did you always give your child water after breast or bottle feeding?; e) What type of snacks does your child have per day?; f)Type and frequency of snacks; 3. Parent's attitude and behaviour in child's oral hygiene care: a) How many times per day do you brush your child's teeth?; b) When did you last take your child to the dentist. The Cronbach's alpha coefficient was 0.7, which is acceptable [6].

Plaque and Saliva Sample Collection

Supra-gingival overnight plaque samples were collected from bucco-gingival surfaces of all teeth using a sterile toothpick and released in 1 mL of TrisBase and EDTA buffer, then they were asked to expectorate saliva into a cup.

DNA Extraction

DNA was extracted based on enzymatic lysis using a commercial kit (Flavogen, Taiwan) as previously described [6]. Extracted DNA concentration and purity were measured by a spectrophotometer at 260 nm/280 nm (Nanodrop 2000C® Thermo Scientific, Delaware, USA).

Culture Condition and Standard Strains

S. mutans ATCC 25175, *S. sobrinus* ATCC 6715 and *S. sanguinis* OMZ 2176 strains were cultured in brain heart infusion agar and broth. Genomic DNA was extracted from the overnight culture as described above.

Conventional PCR

All extracted DNA samples were confirmed with 16srRNA universal primers [Table/Fig-1] [24-27]. Each reaction mixture (total volume of 25 µL) contained 2 µL of 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) using Thermocycle (GeneAmp PCR System 9600 PCR machine, PerkinElmer, CA, USA) as previously described [28].

Quantitative Real-time PCR

Using specific primers [Table/Fig-1], the reaction mixture (total volume of 20 µL) contained 8.2 µL of water, 10 µL of 2X KAPA SYBR® FAST qPCR Master Mix, 0.4 µL of 10 µM forward and reverse primer, and 1 µL of standard bacteria DNA. 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 [6].

Agarose Gel Electrophoresis

Amplified PCR products were checked with 1.5-2% agarose gel which stained with ethidium bromide and gel image were captured with a digital imaging system [Table/Fig-2] (Molecular Imager ®Gel docTM Systems, Bio-Rad Laboratories Inc., CA, USA) [6].

STATISTICAL ANALYSIS

SPSS 16.0 software (Microsoft Corporation, USA) was used to record and analyse the data. Kolmogorov-Smirnov and Shapiro-wink tests ($p<0.001$) were used to assess data distribution. The different amounts of each bacterium were analysed by Mann-Whitney U test ($p<0.05$). The correlation between amounts of each bacterium and other clinical factors were analysed by Spearman's correlation test ($p<0.05$). The association between caries status and demographic, socioeconomic, diet, and other factors were analysed by Pearson's Chi-Square test ($p<0.05$).

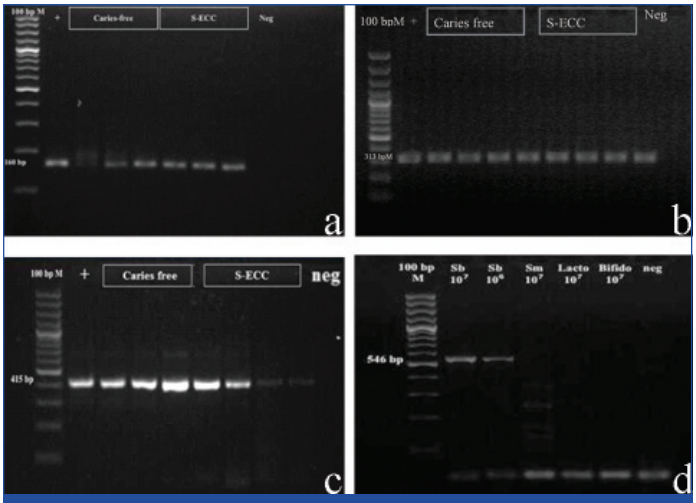
RESULTS

Participants

Total subjects were 120 (S-ECC=60, CF=60). Mean age of the children was 3.56±0.53 years. [Table/Fig-3-5] showed that the

Primer name		Nucleotide sequence (5' to 3')	Expected amplicon (bp)	Annealing Temp (°c)	Ref
Universal 16SBAC	F	5-TGG AGC ATG TGG TTT AAT TCG A-3′	160	56.7	Sinsimer D et al., [24]
	R	5-TGC GGG ACTTAACCC AAC A-3′			
S. mutans	F5	5'-AGCCATGCGCAATCA ACA GGT T-3'	415	59	Yano A et al., [25]
	R4	5'-CGCAACGCGAACATC TTG ATC AG-3'			
S. sobrinus	SobF	5'-CGCACTTGCTCCAGTGTTACTAA-3'	546	51	Sato T et al., [26]
	SobR	5'-GCC TTT AAC TTC AGA CTT AC-3'			
S. sanguinis	MKP-F	5'-GGATAGTGGCTCAGGGCAGCCAGTT-3	313	61.5	Hoshino T et al., [27]
	MKP-R	5'-GAACAGTTGCTGGACTTGCTTGTC-3'			

[Table/Fig-1]: Primers used in this study [24-27].



[Table/Fig-2]: Agarose gel electrophoresis of real-time PCR products (a) universal primers, (b) specific primers for *S. sanguinis* and (c) specific primers for *S. mutans* (d) specific primers for *S. sobrinus*.

Variables	Caries-free n (%)	S-ECC n (%)	p-value[1]
Children's gender			
Male	28 (46.7)	28 (46.7)	1.0
Female	32 (53.3)	32 (53.3)	
Guardian's education levels			
Primary school	4 (6.7)	16 (26.7)	**0.001***
High school or diploma	23 (38.3)	35 (58.3)	
≥Bachelor degree	33 (55)	9 (15)	
Guardian's occupation			
Worker for government or private company	32 (53.3)	14 (23.3)	**0.001***
Merchant	17 (28.4)	13 (21.7)	
Employee	5 (8.3)	23 (38.3)	
Housekeeper	6 (10)	10 (16.7)	
Relationship with the child			
Parents	50 (83.3)	37 (61.7)	**0.02***
Grandparents or senior relative	10 (16.7)	21 (35)	
Babysitters	0 (0)	2 (3.3)	
Monthly family income			
<10,000 baht	5 (8.3)	10 (16.7)	**0.001***
10,001-20,000 baht	17 (28.3)	37 (61.6)	
≥20,000 baht	38 (63.4)	13 (21.7)	
Family member's smoking			
Yes	11 (18.3)	38 (63.3)	**0.011***
No	49 (81.7)	22 (36.7)	

[Table/Fig-3]: Demographic characteristics of subjects in both groups.
[1]Pearson's Chi-square test. *p-value <0.05

guardian's demographic data, habit of milk bottle and breast feedings, oral hygiene care and consumption of cariogenic snacks were different between two groups.

Conventional PCR and Quantification Real-Time PCR of *S. mutans, S. sobrinus* and *S. sanguinis*

There was a 100% detection rate by the universal primers. [Table/Fig-6] shows the comparison of bacterial levels between the two groups. Both plaque and gingival indices were different between the two groups [Table/Fig-7]. [Table/Fig-8] shows the correlation of clinical parameters and microbial finding between two groups. When compared between plaque and saliva, *S. mutans* levels were not significantly different.

Variables	Caries-free n (%)	S-ECC n (%)	p-value[1]
Milk bottle			
Yes	18 (30)	45 (75)	**0.001***
No	42 (70)	15 (25)	
Frequency of milk bottle feeding			
1 time/day	7 (38.9)	13 (28.9)	0.555
2-3 times/day	8 (44.4)	19 (42.2)	
>3 times/day	3 (16.7)	13 (28.9)	
Duration of milk bottle feeding			
<10 minute/each feeding	11 (61.1)	14 (31.1)	**0.043***
10-30 minutes/each feeding	5 (27.8)	13 (28.9)	
>30 minute/each feeding	2 (11.1)	18 (40)	
Breast milk			
Yes	4 (6.7)	18 (30)	**0.001***
No	56 (93.3)	42 (70)	
Frequency of breast feeding			
1 time/day	3 (75)	13 (72.2)	0.751
2-3 times/day	1 (25)	3 (16.7)	
>3 times/day	0 (0)	2 (11.1)	
Duration of breast feeding			
<10 mins/time	4 (100)	12 (66.7)	0.400
10-30 mins/time	0 (0)	5 (27.8)	
>30 mins/time	0 (0)	1 (5.5)	
Sleep with bottle of milk			
Yes	13 (21.7)	41 (68.3)	**0.001***
No	47 (78.3)	19 (31.7)	
Brushing frequency			
>1 time/day	53 (88.3)	33 (55)	**0.001***
once a day	6 (10)	20 (33.3)	
once in 2 days	1 (1.7)	7 (11.7)	
Drinking water after milk feeding			
Always	39 (65)	20 (33.3)	**0.002***
Sometimes	18 (30)	31 (51.7)	
Never	3 (5)	9 (15)	
Dental treatment			
Regular	23 (38.3)	5 (8.3)	**0.001***
Irregular	6 (10)	18 (30)	
Never	31 (51.7)	37 (61.7)	

[Table/Fig-4]: Feeding pattern and oral hygiene care in 2 groups.
[1]Pearson's Chi-square test. *p-value <0.05

DISCUSSION

This is the first study in Thai children which determined the amount of *S. mutans, S. sobrinus* and *S. sanguinis* from both dental plaque and saliva. Previous studies reported that *S. mutans* and the ratio of *S. mutans* to total bacteria were higher in S-ECC [8,15]. Another research in Thai children found that the severity of ECC correlated with high levels of *S. mutans* and *S. sobrinus* [29]. Corresponding with this study, we found that S-ECC children had higher levels of *S. mutans*, *S. sobrinus*, *S. mutans*/total bacteria and *S. sobrinus*/total bacteria than caries-free children. From previous studies, *S. mutans* was detected in high level while *S. sobrinus* was detected in lower level in S-ECC than *S. mutans* [7-10]. Martinez-Martinez RE et al., compared the distribution of oral streptococci from saliva of caries-free and caries-affected Mexican children. They reported that *S. mutans* was identified in 80% of the caries-affected while *S. sobrinus* was detected 70% in the caries-affected children [16]. Contrast to the previous study in Thai Children, present study

Variables	Caries-free n (%)	S-ECC n (%)	p-value[1]
Frequency of protein			
None	11 (18.3)	5 (8.3)	0.092
Only in meal	33 (55)	30 (50)	
Between meal ≥1 time/day	16 (26.7)	25 (41.7)	
Frequency of fruit			
None	7 (11.7)	13 (21.7)	0.069
Only in meal	32 (53.3)	18 (30)	
Between meal ≥1 time/day	21 (35)	29 (48.3)	
Frequency of sugar coated snacks			
None	13 (21.7)	6 (10)	**0.001***
Only in meal	37 (61.6)	21 (35)	
Between meal ≥1 time/day	10 (16.7)	33 (55)	
Frequency of soft drink in one day			
None	27 (45)	3 (5)	**0.001***
Only in meal	14 (23.3)	15 (25)	
Between meal ≥1 time/day	19 (31.7)	42 (70)	
Frequency of hard candies in one day			
None	32 (53.3)	12 (20)	**0.001***
Only in meal	15 (25)	12 (20)	
Between meal ≥1 time/day	13 (21.7)	36 (60)	
Frequency of potato chip in one day			
None	10 (31.3)	2 (6.7)	**0.008***
Only in meal	13 (40.6)	9 (30)	
Between meal ≥1 time/day	9 (28.1)	19 (63.3)	
Frequency of Thai dessert in one day			
None	19 (59.3)	8 (26.7)	**0.034***
Only in meal	6 (18.8)	11 (36.7)	
Between meal ≥1 time/day	7 (21.9)	11 (36.6)	

[Table/Fig-5]: Type and frequency of snacks consumption in both groups.
[1]Pearson's Chi-square test. *p-value <0.05

Bacterial	Caries-free	S-ECC	p-value[1]
S. mutan			
Mature plaque	$2.87\times10^{4}\pm7.96\times10^{4}$	**$1.90\times10^{5}\pm5.75\times10^{5}$**	**0.005***
Total bacteria			
Mature plaque	**$5.33\times10^{7}\pm4.24\times10^{7}$**	$3.85\times10^{7}\pm3.51\times10^{7}$	**0.017***
***S. mutans*/total bacteria**			
Mature plaque	$6.91\times10^{-4}\pm2.23\times10^{-3}$	**$5.65\times10^{-3}\pm1.36\times10^{-2}$**	**0.003***
S. sobrinus			
Mature plaque	$59.34\pm1.58\times10^{2}$	**$7.45\times10^{2}\pm1.59\times10^{3}$**	**<0.001***
***S. sobrinus*/total bacteria**			
Mature plaque	$3.9\times10^{-6}\pm1.27\times10^{-5}$	**$4.67\times10^{-5}\pm1.24\times10^{-4}$**	**<0.001***
S. sanguinis			
Saliva	**$1.3\times10^{7}\pm5.3\times10^{6}$**	$2.4\times10^{6}\pm9.3\times10^{5}$	**0.004***
S. mutans			
Saliva	$4.8\times10^{4}\pm1.1\times10^{4}$	**$5\times10^{6}\pm1.5\times106$**	**0.001***
S. sanguinis*/*S. mutans			
Saliva	**$1.8\times10^{3}\pm9.9\times10^{2}$**	6±3.7	**0.001***
***S. sanguinis*/total bacteria**			
Saliva	**$6\times10^{-3}\pm2\times10^{-3}$**	$1.1\times10^{-3}\pm3.3\times10^{-4}$	**0.001***
***S. mutans*/total bacteria**			
Saliva	$1.1\times10^{-3}\pm1.1\times10^{-3}$	**$3.2\times10^{-3}\pm1\times10^{-3}$**	**0.001***

[Table/Fig-6]: Comparison of bacterial levels between caries free and S-ECC groups.
[1]Nonparametric Mann-Whitney U test. *p<0.05

Variable	CF Mean±sd	CF Median	S-ECC Mean±sd	S-ECC Median	p-value[1]
Plaque score	1.21±0.072	1.17	1.74±0.083	1.67	**0.001***
Gingival score	0.17±0.060	0.00	1.34±0.339	1.00	**0.001***

[Table/Fig-7]: Measurement of plaque and gingiva indices in both groups.
[1]Nonparametric Mann-Whitney U test. *p<0.05

Clinical characteristics	*S. sanguinis* CC	*S. sanguinis* p-value[1]	*S. mutans* (saliva) CC	*S. mutans* (saliva) p-value[1]	*S. Mutans* (Plaque) CC	*S. Mutans* (Plaque) p-value[1]	*S. sobrinus* (plaque) CC	*S. sobrinus* (plaque) p-value[1]
dmft	-0.34	0.001*	0.43	0.001*	0.337	<0.001*	0.732	<0.001*
Age	0.052	0.572	-0.208	0.023	-0.256	0.005*	0.037	0.691
PI	0.272	0.003*	0.62	0.001*	0.079	0.391	0.348	<0.001*
GI	-0.25	0.006*	0.323	0.001*	0.285	0.002*	-0.072	0.432

[Table/Fig-8]: Correlation of clinical parameters and microbial finding in both groups.
[1]Spearman correlation. *p<0.05
CC: Correlation coefficient

indicated that when subjects were infected by both *S.mutans* and *S.sobrinus*, the caries prevalence was the same as subjects infected by *S.sobrinus* alone. There might be a high possibility that *S.sobrinus* was not found without the presence of *S. mutans* [7].

In this study, it was found that in S-ECC dental plaque, mean level of *S.mutans* was higher than *S.sobrinus* which corresponds to other studies [7-10]. However, previous study reported that the acidogenicity of *S. sobrinus* was greater than *S. mutans* [30]. Several studies in children found that increasing *S.sobrinus* colonisation in dental plaque was correlated with aggravated caries activity as well as that in saliva [8,31,32]. From previous study, there was high baseline count of *S.sobrinus* in children with recurrent caries. This indicated that besides *S.mutans*, *S.sobrinus* can be used as a part of caries risk assessment, especially in high-risk group [33].

There are numerous studies about bacteria composition in dental plaque of caries-free and caries-active children. Li Y et al., analysed the diversity of microorganism in different caries status. They found that the diversity of microorganism in caries-free group was higher than that of caries active [34]. Other study reported that bacterial diversity was decreased when caries progress from healthy to active lesion [35]. Correspond to the present study, which found that total bacteria level in dental plaque of caries-free is higher than those in S-ECC group. From ecological plaque hypothesis, dental caries is the result from imbalance of oral micoorganisms. This results in shifting of bacterial community from healthy bacteria to acid tolerant bacteria. The new condition promotes the proliferation of cariogenic bacteria but decreases the diversity of the plaque community [34]. It can imply that the higher diversity of bacteria in caries-free condition can suppress the condition promoting dental caries [34,35].

Choi EJ and colleagues found that the level of *S.mutans* showed low correlation with dmfs scores while the ratio of *S.mutans* to total bacteria, the level of *S.sobrinus* and the ratio of *S.sobrinus* to total bacteria had positive correlation with dmfs score [8]. In this study, we found that the level of *S.mutans*, the ratio of *S.mutans* to total bacteria and the ratio of *S.sobrinus* to total bacteria in mature plaque were positively correlated with dmft scores. Various studies reported that the presence of dental plaque was associated with dental caries, but some studies did not [36,37]. We found that the level of *S. sobrinus* and the ratio of *S. sobrinus* to total bacteria in mature plaque was positively correlated with plaque index. Law V and Seow WK reported that *S.mutans* and *S.sobrinus* infection was correlated with dental plaque amount [37]. Prevalence of *S.mutans* was higher in children with visible plaque than that of plaque-free group [38-40]. This study showed that the level of *S.mutans* and the proportion of *S.mutans* to total bacteria in dental plaque correlated with gingival index. This is similar to previous study which reported that the prevalence of *S.mutans* was positive correlated with visible dental plaque, gingival inflammation and bleeding [38]. Beighton D

et al., also reported that level of *S.mutans* and *S.sobrinus* in saliva showed correlation with gingival scores [41].

For *S.sanguinis*, in this study, its level in saliva was different and higher in caries-free group. Previous study from Thai children also demonstrated that *S.sanguinis* level was higher in caries-free group when compared with those of S-ECC [6]. Moreover, *S.sanguinis* level was inversely correlated with dmft scores. This study corresponded with previous studies [6,42,43].

There were many researchers who studied the interaction between oral streptococci in saliva and dental plaque, few studies reported the antagonism action between *S.sanguinis* and *S.mutans*. In this study, *S. mutans* level was higher in S-ECC groups than those of caries-free group which corresponded to previous studies that *S. mutans* is the main cariogenic microorganism and they might inhibit the growth of *S. sanguinis* [44,45]. Kreth J et al., investigated the molecule mechanism of the antagonism interaction between *S. mutans* and *S. sanguinis,* they reported that *S. sanguinis* could inhibit the growth of *S. mutans* by hydrogen peroxide (H_2O_2) whereas mutacins produced by *S. mutans* are involved in *S. sanguinis* growth inhibition and the mutacins mutants had reduced ability to inhibit the growth of *S. sanguinis* [45]. In this study, we found positive correlation between level of *S. sanguinis* and dmft score. *S. sanguinis* might also be used as an indicator with *S. mutans* for predicting dental caries. From previous studies, they also suggested that the ratio of *S. mutans/S. sanguinis* can indicate the risk of caries [46,47]. Loesche WJ and Syed SA reported that the percentages of *S. sanguinis* decreased as the plaque score increased [23]. The proportions of *S. sanguinis* steadily declined as the gingivitis developed [44]. Corresponded with this study where plaque and gingival scores were higher in S-ECC group. Furthermore, *S. sanguinis* was inversed correlated with plaque and gingival indices [44].

In the present study, we found the relationships between caries status and related factors from questionnaire. Several studies reported that ECC are commonly found in children with low economic status [42,43,46]. Sarumathi T et al., found that low socioeconomic children had higher risk of developing dental caries than those in middle or high socioeconomic status [43]. The result of this study found that caries-free children had greater proportion of high household income than S-ECC group. Correlation between caries status and parent's education level has been reported. Various studies suggested that ECC was commonly found in children whose guardians have low literacy level [42,46]. Sarumathi T et al., study found that the children whose parents had high educational level had lower caries prevalence similar to this study [43].

The results of this study found that S-ECC group had more smokers in the house than caries-free group. Household tobacco smoke increased risk of ECC [44,45]. Infection is caused by chemical toxin in tobacco which suppresses or modulates immune system [44]. *In-vitro* study mentioned that phagocytes acitivities of neutrophils and monocytes were inhibited by nicotine [44]. Vitamin C in blood levels of smokers and children with second-hand smoking were decreased [47-49]. This condition prefers the growth of *S. mutans* [49]. Second-hand smoked children received the same toxins as the active smoker but in lower doses, so they might receive the same oral health affect as those in active smoking. Previous study reported that the levels of *S. mutans* were increased in active smoking [50]. Same as the study from Sakki T et al., which mentioned that nicotine in tobacco increased the growth of *S. mutans* and it was transmitted from guardians to their children [47]. Another possible factor is that active smoking parent might have poor dietary behaviour, unhealthy lifestyle and lack of oral health awareness. This condition might affect their children's oral health.

In this study, we found that S-ECC group had frequent consumption of food in every types of dietary except fruit and protein. Various studies mentioned about the correlation between high frequency of food and beverage consumption and dental caries [51,52]. The frequency and amount of beverage consuming in S-ECC children were greater than that of caries-free children [53].

Numerous studies found the association between bottle feeding and dental caries [3-5]. In this study, we found that S-ECC had more duration of bottle feeding than caries-free children and S-ECC was associated with history of sleeping with bottle or breast feeding. Furthermore, S-ECC children had history of sleeping with bottle or breast feeding more than caries-free children. The relation between bottle sleeping and caries was found [3]. Du M et al., study stated that bottle-fed children were five times higher risks in developing early childhood caries than breast-fed children [54]. There were various advantages from breast-feeding. Infant received nutrition and immunological protection through human milk. In contrast, the association between breastfeeding and dental caries was inconclusive. From systematic review of epidemiological evidence mentioned that children who breast-fed longer than 1 year or at night might had higher prevalence of dental caries [55]. Dissimilarly, other systematic review stated that there was no scientific evidence about the cariogenicity of human milk [56].

Limitation(s)

The cross-sectional design of the study limit in finding out the predicting factor in questionnaires. In addition, this study used the subjects from the district that have similar socioeconomics and demographic information. The result need to be interpreted carefully so as to generalise the representatives of Thai children.

CONCLUSION(S)

S.mutans and *S.sobrinus* were associated with S-ECC while *S.sanguinis* was associated with caries-free group. Socioeconomics and children's oral hygiene care and diet were important factors which were found associated with S-ECC.

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