Association between *S. mutans* and *S. sanguinis* in Severe Early childhood Caries and Caries-Free Children A Quantitative Real-Time PCR Analysis

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Objectives: *To identify S. mutans and S. sanguinis in initial and overnight plaque between 2 groups and to analyze the association between them and caries-related factors.* ***Study design***: *Collected supra gingival plaque from 140 Thai children aged 2-6 years old (S-ECC = 68, caries-free=72). Recorded plaque and gingival indices, dmft score, salivary mutans streptococci level, pH and buffer capacity. Firstly, the overnight plaque was collected, then, 4 hrs. after a thorough prophylaxis, the initial plaque was collected. Accessed parent's attitude and behavior in children's oral hygiene care and diet practice using a questionnaire. A quantitative real-time PCR was performed.* ***Results:*** *For initial plaque, S. sanguinis was higher in caries-free. S. mutans (0.011) and S. mutans/S. sanguinis ratio (0.005) were higher in S-ECC. S. sanguinis amount was inverse correlated with dmft (0.00), gingival index (0.044), and plaque index (0.011). For overnight plaque, S. mutans (0.00) and S. mutans/S. sanguinis ratio (0.005) were also higher in S-ECC. S. mutans, S. mutans/S. sanguinis ratio were positive correlated to dmft (0.00). Parent education levels (0.004) and bottle feeding (0.011) between 2 groups were different.* ***Conclusion:*** *S. sanguinis, S. mutans and their ratio in initial and overnight plaque, low family income and bottle feeding are strongly associated with S-ECC.*

Key Words:, *dental plaque, Streptococcus mutans, Streptococcus sanguinis, severe early childhood caries, PCR*

INTRODUCTION

Severe Early Childhood Caries (S-ECC) is still a high prevalence oral disease in children [1]. Dental plaque or oral biofilm are complex microbial communities found on tooth and mucosal surfaces[2]. Approximately 20% of the oral bacteria are streptococci, which the pioneer group found in the initial plaque [2]. Oral streptococci have a specific temporal and spatial distribution that is crucial for the development of oral biofilms [2].

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Streptococcus mutans plays a crucial role in caries initiation and progression. It was detected higher in S-ECC as compared with caries-free children [3-7]. Moreover, its level was associated with caries status and is used as one of the microbial parameters for assessing children's caries risk [8-10]. According to the ecological plaque hypothesis, caries is a consequence of alteration in the oral environment. Several studies reported that *Streptococcus sanguinis* was detected higher in caries-free children [8,11]. Previous studies demonstrated the antagonistic relationship between *S. sanguinis* and *S. mutans* and suggested that *S. sanguinis* might delay the colonization of *S. mutans* in oral cavities [12,13]. Ge and colleagues suggested that caries-free children were colonized by high amounts of *S. sanguinis* at a much higher level than *S. mutans* and they also found that the interaction between *S. sanguinis* and *S. mutans* was significantly associated with caries outcomes [8]. This finding not only supports the hypothesis that the presence of *S. mutans* alone may not be the only indicator for increased caries risk but also suggests that the interactive effect between *S. mutans* and *S. sanguinis* may play an important role in the process of children's caries [8].

Simon-Soro and colleagues reported that the microbial composition at the initial, enamel-affecting stage of caries is significantly different from that found at subsequent stages [14]. Although the relative proportion of *S. mutans* increased from 0.12% in dental

plaque to 0.72% in enamel caries, *S. mitis* and *S. sanguinis* were the dominant streptococci in these lesions. Their results supported a scenario in which pH and diet are determinants of the disease during the degradation of enamel, but in dentin caries lesions not only acidogenic but also proteolytic bacteria are involved. From the study of the initial colonized microorganisms on the enamel in adult subjects, *S. sanguinis* presented in 4 hrs. plaque and was not retained at 8 hrs. in some subjects [15]. From our preliminary studies, we used a conventional PCR method to detect *S. sanguinis* in initial plaque (less than 4 hrs. formed) obtained from S-ECC and caries-free groups of Thai children aged 1-6 years old. Results showed that the amount of *S. sanguinis* was detected higher in the initial plaque as compared with overnight plaque (unpublished data). Even though the relationship between *S. mutans* and *S. sanguinis* has been demonstrated in several studies, none of them provided information in different stage of plaque formation. Because of oral biofilm is dynamic, knowing this information might help us understand more about the role of these bacteria in caries process.

Although conventional PCR gives acceptable results, it lacks the ability for precise quantification. Quantitative real-time PCR with species-specific primers can provide an accurate and sensitive method for detection and quantification of individual species and bacterial populations [16]. The ability to quantify the bacteria in a sample has advantages to previous approaches in that it not only identifies a presence or absence but also the amount of bacteria that could be related to clinical conditions [15].

This study aimed to quantitatively detect *S. mutans* and *S. sanguinis* in initial (less than 4 hrs. formed) and overnight plaque samples using quantitative real-time PCR from S-ECC and caries-free groups of Thai children aged from 2 to 6 years old and to analyze the association between the amount of these bacteria, caries status and caries-associated factors between 2 groups. The hypothesis is that the quantities of *S. mutans* and *S. sanguinis* in both initial and overnight plaque samples from S-ECC and caries-free groups should be different.

MATERIALS AND METHOD

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 2013/ 027.2606) . A statistician consultation had been done before a sample size calculation that based on previous studies with α=0.05 and power of 80%, using the software package Primer of Biostatistics (McGraw-Hill, NY, USA). A minimum of 27 children was required to achieve the statistically different [17].

Subject selection

Total subjects were 140 (caries-free=72, S-ECC=68) Thai children aged 2 to 6 years old. All subjects were randomly selected from 30 public childcare centers in Suphanburi province, Thailand. 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 (WT and AS). They were calibrated for clinical examination (kappa co-efficiency=0.83). The diagnosis of S-ECC was based on the AAPD 2012-2013 definition which states that in children <3 years old, any sign of smooth-surface caries is indicative of S-ECC [10]. From ages 3–5, 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 ≥4 (age 3), ≥5 (age 4), or ≥6 (age 5) surfaces also constitutes S-ECC. For the caries-free group, subjects must have no caries nor existing restorations (dmft = 0). Subject who had any systemic disease(s), taking 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 using modified debris index of simplified oral hygiene index for deciduous dentition [18-20]. The six index teeth (surface) were 55 (B), 51 (La), 65 (B), 75 (Li), 71 (La) and 85 (Li). In the absence of either of these anterior teeth, the opposite side of the primary central incisors were substituted. In the absence of either of the second primary molar, the first primary molar in the same quadrant was substituted. Each area of each tooth was assigned a score from 0–3. A score of 0 indicates no debris or stain present, 1 indicates soft debris covering not more than one third of the tooth surface, or the presence of extrinsic stains without other debris regardless of surface area covered, 2 indicates soft debris covering more than one and 3 indicates soft debris covering more than two-thirds of the exposed tooth surface [19].

To determine total individual plaque index, scores of each tooth were sum and divided by the number of teeth examined. Gingival inflammation was recorded on a 0–4 scale following the modified gingival index [20]. A score of 0 indicates absence of inflammation, 1 indicates mild inflammation; slight change in color, little change in texture of any portion but not the entire marginal or papillary gingival unit, 2 indicates mild inflammation; criteria as above but involving the entire marginal or papillary gingival unit, 3 indicates moderate inflammation; glazing, redness, edema and/or hypertrophy of the marginal or papillary gingival unit, 4 indicates severe inflammation; marked redness, edema and/or hypertrophy of the marginal or papillary gingival unit, spontaneous bleeding, congestion, or ulceration. The six index teeth which were same as for plaque index examined at mesial, distal, labial (or buccal) and lingual (or palatal) surfaces. Each of four gingival areas of teeth were given a score from 0–4 and this is the area of the gingival index [20]. The scores from the four areas of the teeth were added and divided by four to give the gingival index for that tooth. The scores for individual teeth were grouped to represent the gingival index for the group of teeth. Finally, by adding the indices for the teeth and dividing by the total number of teeth examined, the gingival index for the individual was obtained.

The questionnaire

All participants' parents or caretakers were asked to complete the questionnaire. All questions are close-end answers. Besides parents' general information (age, career, education level, monthly income, and self-oral hygiene care), 4 categories were asked (Table 1).

Salivary pH and buffer capacity

Using Saliva-Check Buffer (GC Corporation, Japan) by following the company booklet instructions [23]. Children were instructed to refrain from food or drink, tooth brushing or use of a mouth wash at least 1 hour prior to test. They were asked to expectorate saliva into a cup. Placed a pH strip into a saliva sample for 10 seconds and checked the color of the strip. One of 3 categories of pH plaque were obtained (red = highly acidic, yellow = moderately acidic, green = healthy saliva). Drop saliva sample onto test pads

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was dispensed and waited for 2 minutes. Checked the color of a test pad (green = 4 points, green ⁄ blue = 3 points, blue = 2 points, blue ⁄red =1 point, red = 0 point). Finally, calculated the total points to obtain saliva buffering capacity (very low = 0-5 points, low = 6-9 points, normal = 10-12 points).

Salivary Mutans streptococci level

Using Saliva-Check Mutans (GC Corporation, Japan). Collected saliva sample in the mixing container as recommended by the company. Added 1 drop of reagent#1 to saliva and tapped mixing container 15 times. Added 4 drops of reagent#2 and tapped mixing container for several seconds until the color of saliva sample changed to light green. Dropped saliva sample to the test device window and left for 15 minutes at room temperature. Checked a red thick line in the control (C) window of the test device which indicating the device was working properly. The test (T) window indicating (MS) level (no line = low level of MS, thin red line = MS level >5 x 10^5 CFU/ml [24].

Plaque sample collection

Collected plaque samples from buccogingival surfaces of all teeth using a sterile toothpick and released in 1ml of TE buffer. All samples were immediately transported to the Oral Biology Laboratory on ice and stored at -20°C until the DNA extraction process.

DNA extraction

DNA was extracted base on the enzymatic lysis using a commercial kit (Flavogen, Taiwan) as previously described [17]. By following the company recommendation, we added 20µl of Proteinase K, 400µl of FABG buffer and 20µl of a lysozyme mixture (lysozyme 20mg/ml and mutanolysin (Sigma Aldrich, USA) in 1:10 proteinase K) and vortex. Incubated at 60°C for 1 hour. Added 200µl ethanol and centrifuged at 11,000 x rpm for 30 seconds. Transferred the solution into a spin column and centrifuged for 1 minute. Discarded the supernatant. Added 500µl of W1 buffer and centrifuged for 1 minute. Discarded the supernatant. Added 750µl of wash buffer and centrifuged for 1 minute. Added 50µl of elution buffer and left at room temperature for 3 minutes before final centrifugation for 2 minutes. Measured extracted DNA concentration and purity using spectrophotometer at 260nm/280nm (Nanodrop 2000C® Thermo Scientific, Delaware, USA).

Culture condition and standard strains

S. mutans ATCC 25175 and *S. sanguinis* OMZ 2176 strains were cultured on Brain Heart Infusion agar and broth. Genomic DNA was extracted from the overnight culture as described above. Ten-fold serial dilution starting from 10^8–10^2 CFU/ml was performed.

Conventional PCR

All extracted DNA samples were confirmed with 16srRNA universal primers (27F: 5'-AGAGTTTGATCMTGGCTCAG-3', 1492R: 5'-TACGGYTACCTTGTTACGACTT-3') [25]. Reaction mixture and components as described before [17].

Quantitative Real-time PCR of S. mutans and S. sanguinis

The standard curve was generated by 10-fold serial dilutions of *S. sanguinis* and *S. mutans* using specific primers (*S. sanguinis* MKP-F: 5'-GGATAGTGGCTCAGGGCAGCCAGTT-3', MKP-R: 5'-GAACAGTTGCTGGACTTGCTTGTC-3', *S. mutans* Sm1: 5'-GGTCAGGAAAGTCTGGAGTAAAAGGCTT-3', Sm2: 5'-GCGGTAGCTCCGGCACTAAGCC-3') [25, 26]. Reaction mixture (total volume was 20µl) contained 8.2µl of nuclease-free water, 10µl of 2X KAPA SYBR® FAST qPCR Master Mix (KAPA Biosystems, USA), 0.4µl of 10 µM forward and reverse primer, and 1µl standard bacteria DNA. The reaction for DNA plaque samples from plaque samples was similar to the standard strains. Set the thermocycler (C1000™ Thermal cycler and CFX 96 Real-time System) for 40 cycles. Each cycle consisted of enzyme activation 95°C for 3 minutes, denaturing at 95°C for 3 seconds, annealing and extension for 30 seconds at 61.5°C and 20 seconds at 60°C for *S. sanguinis* and *S. mutans*, respectively. Melting curves were generated from 60°C to 95°C and read every 0.5°C for 5 seconds.

Agarose gel electrophoresis

Amplified PCR products from conventional PCR and quantitative real-time PCR were checked on 2% and 1.5 % agarose gel (Broad Separation Range for DNA/RNA agarose, Fisher Scientific,

Table 1: Questionnaire use in this study to assess the diet and oral hygiene care

Categories	Questions
1. Child's general information	1. Child's age 2. Child's major caretaker 3. What is your child's dental insurance coverage? [21]
2. Parent attitude towards child's diet	1. Is your child still bottle feeding? 2. Did your child ever have breast and/or bottle feeding *ad lib*? 3. Did your child breast and/or bottle feed (ad lib) and fall asleep? 4. Did you always give your child water after breast or bottle feeding? 5. What type of snacks does your child have per day? 5.1-5.9; Type and frequency of snacks 6. Does your child always have snacks while watching television? [22]
3. Parent attitude and behavior in child's oral hygiene care	7. How many times per day do you brush your child's teeth? 8. When did you last take your child to the dentist?
4. Child's pre-natal and infancy medical history	9. Was your child born with a low birth weight (<2,500 grams)? 10. Was your child a pre-term baby? (<37 weeks) [21].

behavior of the parents or caretakers of all participants

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UK) , respectively. Using 1xTris–borate EDTA buffer (100 mM Tris, 90 mM borate; 1 mM EDTA, pH 8.4). Gels were stained with ethidium bromide. Image results were captured with a digital imaging system (Molecular Imager ®Gel docTM Systems, Bio-Rad Laboratories Inc., CA, USA).

Statistical analysis

All data were recorded and analyzed by SPSS 16.0 software (Microsoft Corporation, USA). Data distribution was tested by Kolmogorov-Smirnov and Shapiro-wink test ($p<0.001$). Analyzed the different amounts of *S. mutans* and *S. sanguinis* between 2 groups using a Mann-Whitney U test for non-parametric data (p ≤≤0.05). Analyzed the correlation between amount of *S. mutans* and *S. sanguinis* and dmft score, age, gingival and plaque indices, salivary pH, and buffer capacity using Spearman's correlation test at p≤≤0.05. The association between caries status and demographic, socioeconomic, diet, and other factors were analyzed by Pearson's Chi-Squared test (p≤≤0.05).

RESULTS

Total subjects were 140 with a dropout rate of 1.42% (mean age = 3.43±0.56 years old). Mean ages in caries-free and S-ECC groups were 3.39±0.64 and 3.46±0.07 years old, respectively. A total of 136 questionnaires were answered by the parents. Table 2 showed that most of parents were employees and merchants. There was a difference in parent education levels ($p=0.004$, Chi square test at $p<0.05$) and history of bottle or breast feeding ($p=0.011$, Fisher's exact test at $p<0.05$) between 2 groups (Figure 2). Monthly family income was higher in caries-free group as compared with S-ECC group. Further analysis using a multivariate analysis found that children who have *S. sanguinis* in the initial plaque $<10^5$ (cfu/ml) have a 5.27 chance of children who have *S. sanguinis*$>10^5$ in developing S-ECC. Children who came from a low income family (<10,000 baht/month) have a 2.885 chance of developing S-ECC than children who came from a family with income >10,000 and children who were bottle feeding have a 2.601 higher chance in developing S-ECC than children who were not. Figure 3 showed that most subjects in S-ECC groups had a higher between meal consumption of sweet drinks, sweets and candies, sugar coated cereals and grains and sugar coated starch as compared with caries-free group.

Mean dmft score in S-ECC was 8.31±0.67. Table 3 showed that mean and median of plaque and gingival indices in caries-free group were lower than in S-ECC group. Mean and median of salivary pH and buffer capacity in caries-free and S-ECC were in the range of normal and low capacity, respectively. Plaque and gingival scores were significantly different between 2 groups ($p=0.001$, Mann-Whitney U test at $p<0.05$).

Table 2: Demographic characteristics of subjects in S-ECC and caries-free groups.

Variables	Caries-free	S-ECC	p-*value*
	n (%)	n (%)	
Child's gender			
Male	35 (48.6)	32 (50.8)	0.870
Female	37 (51.4)	31 (49.2)	
Parent's education levels			
Primary school	18 (27.7)	26 (40.6)	**0.004***
High school or diploma	35 (53.8)	37 (57.8)	
≥ Bachelor Degree	12 (18.5)	1 (1.6)	
Parent's career			
Government or private company employee	10 (14.3)	7 (10.8)	0.359
Merchant	13 (18.6)	15 (23.1)	
General employee	33 (47.1)	32 (49.2)	
Housekeeper or jobless	9 (12.9)	7 (10.8)	
Agriculturist	5 (7.1)	4 (6.2)	
Monthly family income			
< 10,000 baht	27 (39.1)	38 (56.7)	0.121
10,001-20,000 baht	30 (43.5)	21 (31.3)	
≥ 20,000 baht	12 (17.4)	8 (11.9)	
Parent's tooth brushing frequency			
> 1 times/day	48 (66.7)	44 (66.7)	1.000
1 times/day	13 (18.1)	12 (18.2)	
1 times/ 2 days	11 (15.3)	10 (15.2)	
Major caregiver			
Father or mother	47 (67.1)	43 (66.2)	0.903
Grandparents or others	23 (32.9)	22 (33.8)	
Method of dental service payment			
Universal coverage scheme or Civil	(93.6)	57 (85.1)	0.096
Servant Medical Benefit Scheme	4 (6.1)	10 (14.9)	
Self-payment or health insurance			

* Pearson chi-square test at the significant level of $p<0.05$

Table 3: Clinical parameters in S-ECC and caries-free groups.

Variables	Caries-free		S-ECC		*p*-value
	Mean ±SD	Median	Mean ±SD	Median	
Plaque score	1.46 ± 0.045	1.50	1.58 ± 0.058	1.66	**0.001***
Gingival score	0.32 ± 0.026	0.33	0.47 ± 0.35	0.41	**0.001***
Salivary pH	7.29 ± 0.05	7.40	7.37 ± 0.07	7.60	0.095
Salivary buffer	6.21 ± 0.27	6.00	6.60 ± 0.38	7.00	0.442

* Nonparametric Mann-Whitney U test. * $p<0.05$

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Figure 1: Analysis of the association between inappropriate bottle or breast feeding and caries status by Fisher's exact test at p<0.05.

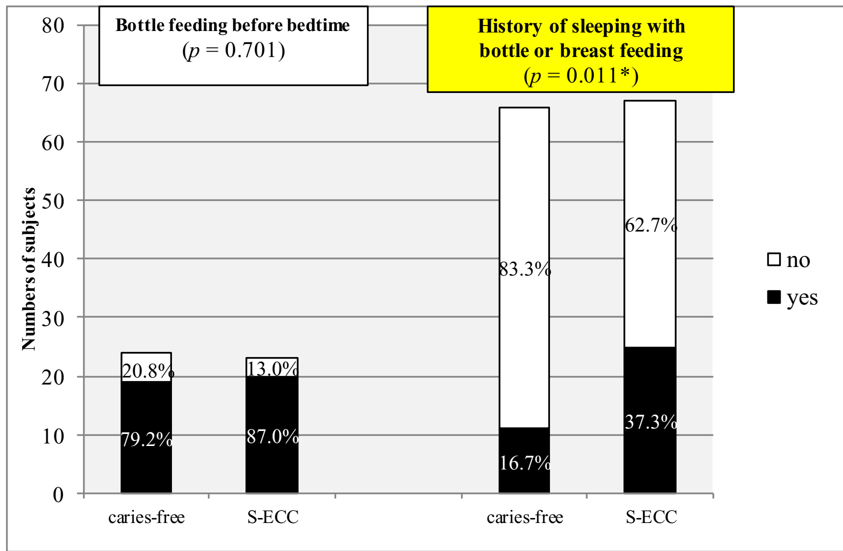
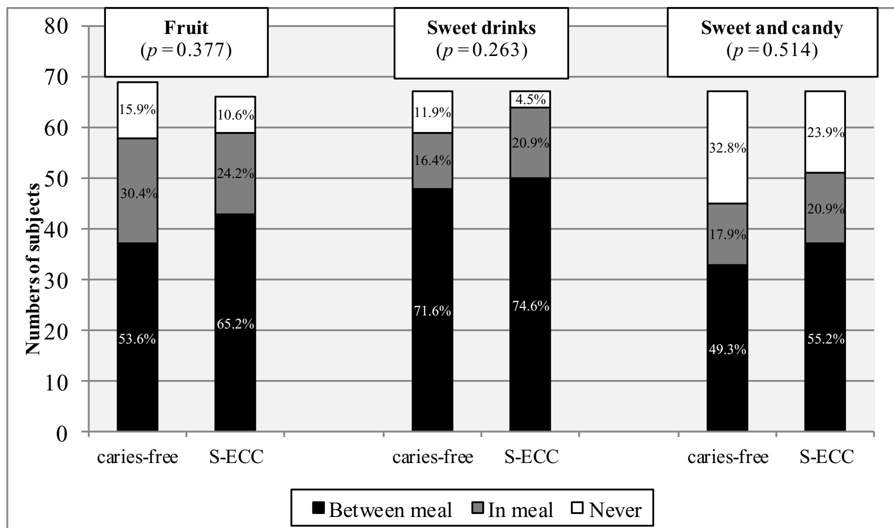
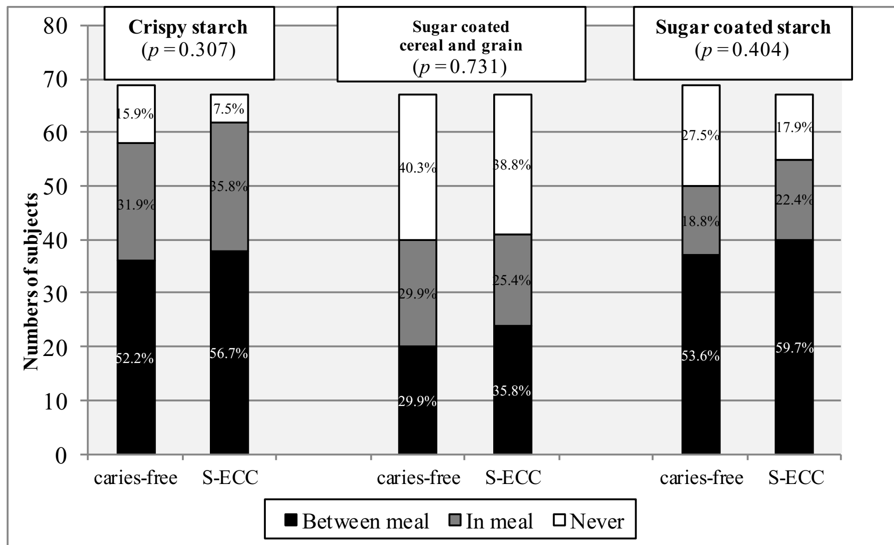


Figure 2: Analysis of the association between dietary habit and caries status by Pearson chi-square test at p<0.05. A: Fruit, juice, sweet and candy B: Crispy starch, sugar coated grain and sugar coated starch



(A)



(B)

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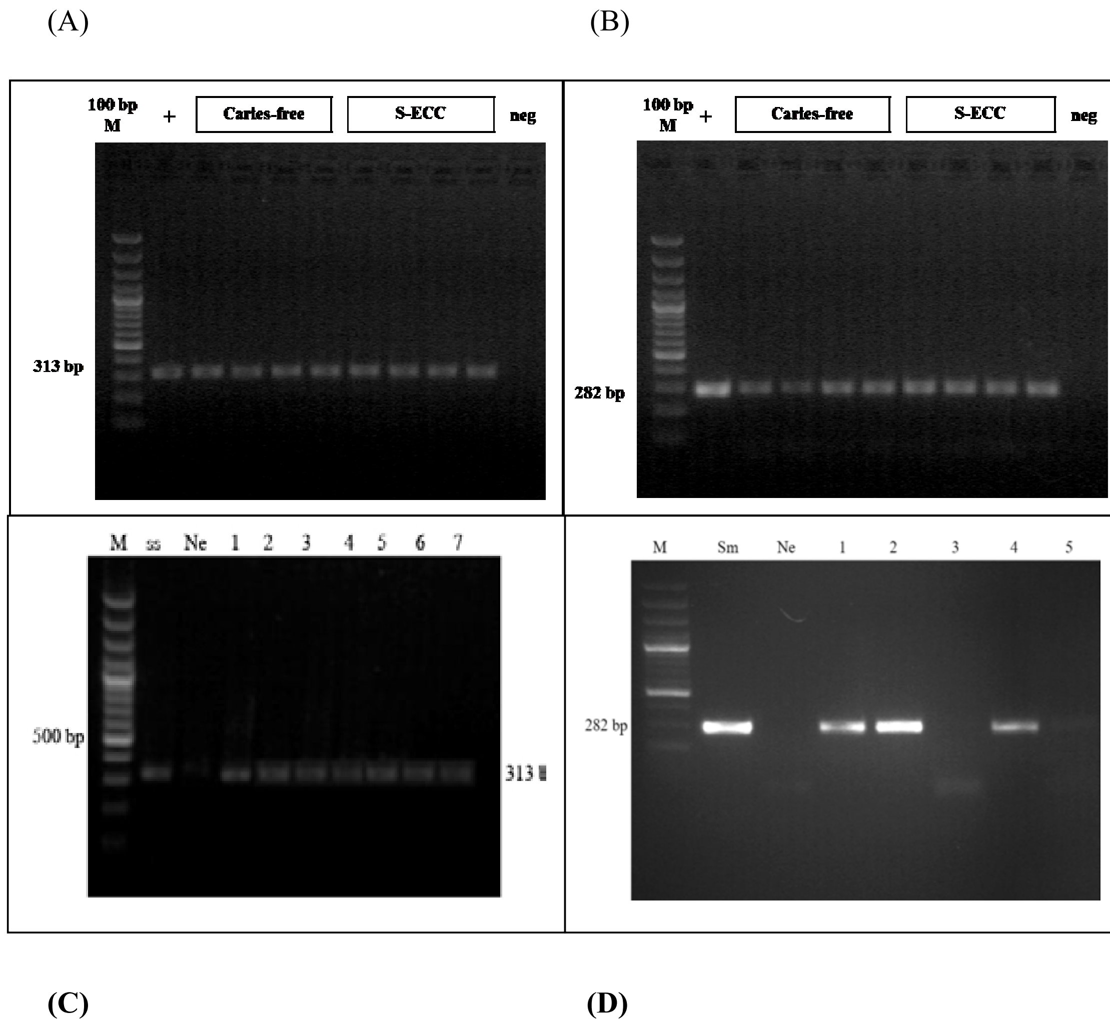
Figure 3: Agarose gel electrophoresis of some real-time PCR products from mature plaque using specific primers of (A) *S. sanguinis* and (B) *S. mutans*. Lane 1: 100 bp molecular marker (Geneaid Biotech Ltd, Taiwan), Lane 2: Positive control, Lane 3-6: Caries-free samples, Lane 7-10: S-ECC samples; Lane 11: Negative control and initial plaque using specific primers of (C) *S. sanguinis*, Lane 1: 100 bp marker, Lane 2: Positive control, Lane 3: Negative control, Lane 4-10: *S. sanguinis* DNA positive samples and (D) *S. mutans*, Lane 1: 100 bp marker, Lane 2: Positive control, Lane 3: Negative control, Lane 4-5,7: *S.mutans* DNA positive samples, Lane 6,8: *S. mutans* DNA negative samples



Conventional PCR and Quantification real-time PCR of S. sanguinis and S. mutans

There was a 100% detection rate by the universal primers. For real-time PCR, the detection limit of MKP primers were 10^1 and SM primer were 10^5. The quantities of bacteria were identified by comparing threshold cycles of DNA samples with those in the standard curve that was generated from known quantities of bacteria. Table 3 showed that there were significant differences in *S. mutans* and *S. mutans*/*S. sanguinis* ratio (p=0.005, Mann-Whitney U test at p<0.05) in the initial plaque between 2 groups. There was a significant difference in *S. mutans*/*S. sanguinis* ratio (p=0.005, Mann-Whitney U test at p<0.05) in mature plaque between 2 groups. Figure 6 showed the comparison of median of *S. sanguinis* levels in initial plaque samples between low and high salivary MS group. There was a significantly difference in the high salivary MS group (p=0.013, Mann-Whitney U test at p<0.05). This result showed that the *S. sanguinis* level was higher in the caries-free group compared with S-ECC in both high and low salivary MS subjects. However, a significant difference was found only in the high salivary MS group.

In Table 5, for initial plaque, *S. sanguinis* was higher in caries-free. *S. mutans*/*S. sanguinis* ratio (0.005, Mann-Whitney U test at p<0.05) were higher in S-ECC. The *S. sanguinis* amount was inverse correlated with dmft (0.001), gingival index (0.013), and plaque index (0.048, Spearman correlation test at p<0.05). For mature plaque, *S. mutans* (0.00) and *S. mutans*/*S. sanguinis* ratio (0.005, Mann-Whitney U test at p<0.05) were higher in S-ECC. When determining the correlations between bacterial levels and dmft score, child's age, plaque index, gingival index, salivary pH and buffer capacity, results showed that the *S. mutans* (p=0.001) and *S. mutans/S. sanguinis* ratio (p=0.009) were significantly positive correlated with dmft score (Spearman correlation test at p<0.05).

DISCUSSION

This was the first study which determined the amount of *S. sanguinis* and *S. mutans* in 2 different stages of plaque formation using quantitative real-time PCR. Results showed that *S. sanguinis* in the initial plaque was similar to the detection using checkerboard DNA–DNA hybridization [4]. *S. sanguinis* levels between 2 groups

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Table 4: Amount of bacteria in initial and mature plaques

Species in initial plaque	Mean±SD		*p-value*
	CF	S-ECC	
S. mutans	$1.8x10^4±7.3x10^3$	$7.9x10^5±5.4x10^5$	**0.000***
S. sanguinis	$1.8x10^5±2.5x10^4$	$2.9x10^5±4.5x10^4$	0.225
S. mutans/ S. sanguinis	0.49±0.25	$2.0x10^2±1.8x10^2$	0.005*
Species in mature plaque			
S. mutans	$3.51x10^9±2.67x10^{10}$	$2.11x10^8±1.24x10^9$	0.607
S. sanguinis	$2.4x10^5±5.96x10^4$	$1.88x10^7±1.88x10^7$	0.225
S. mutans/ S. sanguinis	$8.85x10^4±1.14x10^4$	$2.66x10^4±1.26x10^4$	0.005*

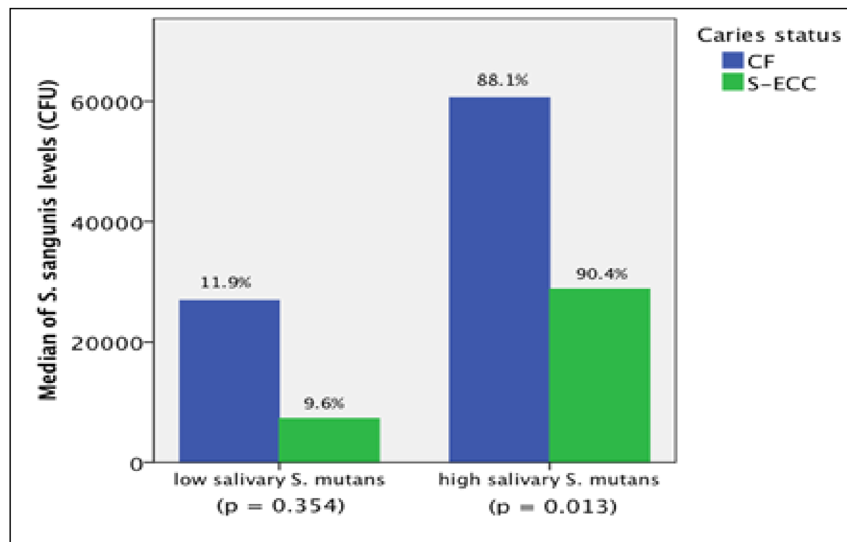
* Mann-Whitney U Test at p<0.05

Table 5: Correlation between bacterial levels in mature plaque samples and dmft score, child's age, plaque index, gingival index, salivary pH and buffer capacity

Clinical parameters in mature plaque	S. sanguinis		S. mutans		S. mutans/S. sanguinis	
	Correlation coefficient	*p*-value[1]	Correlation coefficient	*p*-value[1]	Correlation coefficient	*p*-value
dmft	0.001	0.495	0.447	**0.001***	0.335	**0.009***
Child's age	-0.030	0.365	-0.149	0.151	-0.017	0.453
Plaque index	-0.091	0.143	-0.079	0.292	-0.038	0.395
Gingival index	-0.123	0.075	0.03	0.418	0.035	0.404
Salivary pH	0.084	0.184	-0.086	0.297	-0.032	0.421
Buffer capacity	0.034	0.357	0.041	0.399	-0.090	0.289
Clinical parameters in initial plaque	**Correlation coefficient**	*p*-value[1]	**Correlation coefficient**	*p*-value[1]	**Correlation coefficient**	*p*-value
dmft	**-0.254**	**0.001***	-0.149	0.197	-0.004	0.491
Age	0.020	0.410	-0.029	0.412	0.122	0.243
Time of initial plaque collection	-0.017	0.421	-0.128	0.231	-0.136	0.218
PI	**-0.143**	**0.048**	0.046	0.397	0.001	0.498
GI	**-0.190**	**0.013**	-0.159	0.181	-0.066	0.353
Salivary pH	-0.149	0.054	-0.250	0.088	-0.059	0.376
Salivary buffer	0.006	0.478	-0.073	0.348	-0.125	0.251

*Spearman's correlation at the significant level of *p*<0.05

Figure 4: Comparison in median of *S. sanguinis* levels in initial plaque samples between low and high salivary *S. mutans* groups. The significantly difference of *S. sanguinis* level in caries-free was found in the high salivary *S. mutans* group.



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were significantly different and was higher in caries-free children as compared with S-ECC. Moreover, *S. sanguinis* level was inverse correlated with dmft scores. Previous study which used a cultural method showed that *S. sanguinis* level was also higher in caries-free but its levels was not related to caries status [8]. This study was corresponded with a study by Becker and colleagues using reverse capture checkerboard hybridization which showed that *S. sanguinis* was associated with good oral health and another study using PCR-DGGE technique found that the prevalence of *S. sanguinis* was higher in healthy children as compared with ECC [5, 11].

For mature plaque, previous studies found the association between *S. mutans* and dental caries in children and several of them used a quantitative real-time PCR method [3, 6-8, 11, 21, 27, 28]. Hata and colleagues found that the ratio of *S. mutans* to total bacteria from carious teeth was higher than those of sound teeth [16]. Choi and colleagues reported that *S. mutans* was higher in S-ECC as compared with caries-free and was positive correlated with dmft score [29]. Our study gave similar results to these previous studies. There are two possible reasons that *S. sanguinis* was not different in mature plaque between caries-free and S-ECC group. The first reason is that *S. sanguinis* and *S. mutans* have an antagonistic interaction. Bacteriocin of *S. mutans* can inhibit *S. sanguinis* and *S. sanguinis* uses H_2O_2 to compete with *S. mutans* [13, 30]. The production of H_2O_2 depends on oxygen. In this study, the overnight plaque had numerous colonizing bacteria on tooth. High cell density of *S. mutans* might induce high production of bacteriocin which could inhibit growth of *S. sanguinis*. Thus, *S. sanguinis* might die especially in S-ECC children. So, there were both live and dead *S. sanguinis* in dental plaque. Caries-free children might have more lived *S. sanguinis* than S-ECC children, but quantitative real-time PCR method could not differentiate live and dead bacteria. The second reason is that most *S. sanguinis* might not retain in mature plaque. From the study by Diaz and colleagues, they could not detect *S. sanguinis* on the enamel at 8 hrs. in some subjects [15]. In this study, mature plaque was collected 12-14 hrs. of plaque formation. At this stage, most *S. sanguinis* might not retain in biofilm.

Interestingly, this study found positive correlation between *S. mutans/S. sanguinis* ratio and dmft score. Thus, their ratio might be more interesting and might related to caries prediction that the amount of *S. sanguinis* alone. This result was similar to previous studies which found that *S. mutans/S. sanguinis* ratio related to caries risk [28, 30-33].

In this study, plaque score and gingival score was higher in S-ECC than caries-free. However, there was a statistically difference only gingival score which similar to previous studies [6, 20]. Higher gingival score resulted from plaque accumulation continuously which reflected poor oral hygiene care in S-ECC children. Additionally, *S. sanguinis* was inverse correlated with plaque score and gingival score. These results similar to a study by Loesche and colleagues which found that the detection of *S. sanguinis* decreased when plaque and gingival score increased [32]. Furthermore, Haffajee and colleagues demonstrated that *S. sanguinis* was found significantly higher in site without sign of bleeding on probing [32].

In this study, we found that salivary buffer in both groups were low and there was no significantly difference between 2 groups. Also, salivary pH in both group were in normal range with no statistical difference. This results was different from previous study which could resulted from using unstimulated saliva for eliminate risk of choking when chewing paraffin wax in young children. Because the concentration of the bicarbonate and phosphate ions in unstimulated saliva was lower than in stimulated saliva [33, 34]. Therefore, the salivary buffer values in both groups fell into low capacity.

Socioeconomic factors of children could be assessed from data of family income and education level of caregiver. The significant association of low family income and caries rate in children was presented in other studies [35-37]. On the other hand, Palmer *et al.* could not found the significant relation between them [3]. In this study, the association of household income and caries status was not statistical significant, but caries-free children had greater proportion of high family income than S-ECC group. Furthermore, we also found significant association of caregiver's education and caries status. Caries-free children had caregivers who had more bachelor's degree than S-ECC children. This result is in agreement with other studies [36, 37]. Higher income guardian could support better dental care of child [37].

In this study, S-ECC children had relatively high between-meal consuming of most snacks including sweet snack, starch, sugar coated starch or protein, sweet drink and fruit. Previous study reported that some kinds of fruit are sweets such as banana were moderate cariogenic food. The minimal pH from consumption of them was 4.5-5.5 [39]. Regarding sugar, it is known as substrate for cariogenic bacteria [2]. Many studies showed the relation of consumption of sugar-containing food and juice with caries [40, 41]. Besides, protein itself was non-cariogenic or low cariogenic food. But when it was coated with sugar like crispy sugared nut, cariogenic potential was increased to high or very high cariogenicity [42]. With regard to non-sweet starch such as chip and cracker, S-ECC children likely consumed these snacks more than caries-free group in this study. There was finding that carbohydrate food associated with MS. Starch-containing snacks which contained few or no sugar like non-sweet bread and chip could reduce pH as sucrose did [42]. High starch snack retained in oral cavity in higher amount and longer duration than little starch snack [42]. Previous studies found that S-ECC children consumed more solid retentive starch-containing snacks such as chip, bread and cracker than caries-free children [3, 20]. Moreover, there was finding that starch which was heated and turned into gelatinization could decrease pH more than raw starch [42]. However, they suggested that consumption of starch in three regular meals did not particularly increase caries [42].

CONCLUSION

The amount of *S. sanguinis* in the initial plaque, low family income and sleeping with a bottle are important factors in determining risk of S-ECC. The *S. mutans* to *S. sanguinis* ratio in mature plaque was also significantly associated with S-ECC. It might be useful to use the *S. mutans* to *S. sanguinis* ratio as one of the S-ECC risk indicators in addition to *S. mutans.*

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