



ORIGINAL ARTICLE

Caries status and quantification of four bacteria in saliva of Chinese preschool children: A cross-sectional study



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Abstract *Background/purpose:* Mutans streptococci, bifidobacteria, and lactobacilli are acidogenic and aciduric bacteria which may be associated with early childhood caries (ECC). This study aimed to analyze the bacterial composition of four cariogenic microorganisms, namely *Streptococcus mutans*, *Streptococcus sobrinus*, *Bifidobacterium* spp., and *Lactobacillus* spp. in the saliva of Chinese preschool children and to assess the association of the bacterial burden with the caries status.

Materials and methods: A total of 109 saliva samples were collected from 36 children with ECC and 73 caries-free (CF) children aged 2–4 years. A questionnaire was used to evaluate other caries risk factors such as dietary habits and oral hygiene practice. A quantitative real-time polymerase chain reaction was performed to determine the presence and loads of the four microorganisms. Pearson's correlation test was conducted to evaluate the relationship between the World Health Organization caries diagnostic criteria dmft/dmfs index scores and saliva levels of the selected bacteria.

Results: The prevalence of *S. mutans* and *S. sobrinus* in children with ECC was 88.9% and 19.4%, respectively, whereas the prevalence in CF children was 76.7% and 4.1%, respectively. The saliva levels of *S. mutans* and *S. sobrinus* in children with ECC were significantly higher than in CF children ($P = 0.004$ and $P = 0.007$, respectively) and positively correlated with

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dmft/dmfs index scores ($r = 0.321/0.316$ and $r = 0.271/0.245$, respectively). An association was found between children with caries-active lesions and the frequency of sugar consumption ($P = 0.004$). No significant difference in the presence and load of *Bifidobacterium* spp. or *Lactobacillus* spp. were found between the two groups.

Conclusion: *S. mutans*, *S. sobrinus*, and sugar consumption appear to be associated with caries status in the studied population, which might be useful for caries screening in Chinese preschool children.

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Introduction

Early childhood caries (ECC), dental caries in the primary dentition, is a major health problem worldwide and affects 38% of children in the USA and over 40% of Chinese children.^{1,2} For many children, ECC is a source of pain, chewing difficulties, speech problems, psychological problems, and a lowered quality of life. Children experiencing caries in their primary dentition are likely to have more caries later in life, affecting the permanent dentition.³

Despite its public health significance, the etiological factors involved in ECC are still incompletely understood. Several epidemiological studies have shown that mutans streptococci (MS, mostly *Streptococcus mutans* and *Streptococcus sobrinus*) are associated with ECC.^{4,5} A number of studies, however, have reported that the mere presence of *S. mutans* or *S. sobrinus* is not sufficient to predict the occurrence of dental caries in children.^{6,7} In addition, due to their acidogenicity and aciduricity, bifidobacteria⁸ and lactobacilli⁹ have been reported to be associated with ECC by some workers. Bifidobacteria¹⁰ and lactobacilli¹¹ have also been introduced as probiotics in caries prevention. Based on these controversial observations and the fact that MS, bifidobacteria, and lactobacilli are the most frequent caries-associated bacteria,^{8,12} four microorganisms, namely *S. mutans*, *S. sobrinus*, *Bifidobacterium* spp., and *Lactobacillus* spp., were selected as target microorganisms in the present study.

Several studies have qualitatively and quantitatively described the microbiology of dental caries in various populations.^{13–15} However, culture-independent studies describing the amount of distinct microorganisms in the saliva of Chinese preschool children with ECC have not so far been published. This study aimed to analyze the bacterial composition of these four microorganisms in the saliva of Chinese preschool children using a quantitative real-time polymerase chain reaction (qPCR). The study hypothesized that all four targeted microorganisms would be more abundant in the saliva of children with active caries than in caries-free (CF) children, and that the saliva levels of the selected bacteria would be associated with the severity of ECC.

Materials and methods

Participant population

Approval for this study was obtained from the Human Ethics Research Committee of the Hospital of Stomatology, Wuhan

University, Wuhan, China. A total of 109 participants were randomly recruited from two kindergartens in Wuhan, China. Informed consent was obtained from the parents of all the children. The children ranged in age from 24 months to 48 months. Thirty-six children with active caries were diagnosed as having ECC and the remaining 73 children without caries belonged to the CF group. The exclusion criteria included: the use of systemic drugs; the use of systemic antibiotic drugs or an antibacterial mouth rinse 2 months prior to the commencement of the study; or the local application of fluoride or antibacterial agents 3 months prior to start of the study.

Clinical examination and questionnaires

The children were examined clinically in the dental clinic by one calibrated dental epidemiologist. The agreement for intra-examiner results was assessed for assurance of the quality of the data obtained (>0.85). The clinical oral health status was measured using the decayed (d), missing due to caries (m), and filled (f) tooth (t) or surface (s) (dmft or dmfs) World Health Organization caries diagnostic criteria. Data, including oral hygiene practice and frequency of sugar consumption of each child, were collected by questionnaire after the examination.

Sampling and extraction of genomic DNA

Sampling was performed in the morning and all the children were asked to avoid eating or drinking for at least 1 hour prior to oral sampling. Nonstimulated whole saliva samples (at least 2 mL) were collected from the children's mouths around the sublingual and mucosal surfaces with a sterile syringe according to a previously published method.¹⁶

Total bacterial genomic DNA was extracted from each saliva sample using a modification of the Epicentre method (Epicentre, Madison, WI, USA) based on a published protocol.¹⁷ A standard concentration of 10 ng/μL was prepared for each individual sample for all qPCR assays.

Bacterial strains

Four oral bacterial strains comprising four species were used in this study for construction of the quantification standard curves. *S. mutans* strain UA159 and *S. sobrinus* strain 6715 were supplied by the China Center for Type Culture Collection (CCTCC, Wuhan, China). *Bifidobacterium dentium* strain ATCC 27534 and *Lactobacillus*

Table 1 Bacteria specific primers for quantitative real-time polymerase chain reaction.

Targeted bacteria	Primers (5'–3')	Amplicon size (bp)	Target	Refs
<i>Streptococcus mutans</i>	GCCTACAGCTCAGAGATGCTATTCT GCCATACACCACTCATGAATTGA	114	<i>gtfB</i>	22
<i>Streptococcus sobrinus</i>	AAAACATTGGGTTACGATTGCG CGTCATTGGTAGTAGCCTGA	156	<i>gtfU</i>	4
<i>Bifidobacterium</i> spp.	GGGTGGTAATGCCGGATG CCACCGTTACCCGGGAA	498	16S rDNA	27
<i>Lactobacillus</i> spp.	TGGAAACAGATGCTAATACCG CGTCCATTGTGGTAGATTCCCT	223	16S rDNA	23

acidophilus strain ATCC 4356 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Bacterial genomic DNA was extracted and purified as described in this paper.

qPCR assays

Table 1 list the specific primers for qPCR used in this study. The qPCR assay was carried out in a total volume of 20 μ L consisting of 10 μ L 2 $\times$ SYBR Premix DimerEraser (Takara, Shiga, Japan), 0.4 μ M of each forward and reverse primer, 0.4 μ L 50 $\times$ ROX (Takara, Shiga, Japan) and 2.5 μ L of template DNA. Each sample was tested twice. Thermal cycling conditions for all qPCR assays was set as follows: 1 cycle at 95°C for 2 minutes followed by 40 cycles of denaturation for 5 seconds at 95°C, primer annealing for 30 seconds at 60°C for *S. mutans* and *Lactobacillus* spp., at 57.5°C for *S. sobrinus*, at 58°C for *Bifidobacterium* spp., and extension for 30 seconds at 60°C for *S. mutans* and *Lactobacillus* spp., at 72°C for *S. sobrinus* (30 seconds) and *Bifidobacterium* spp. (60 seconds).

For the construction of quantification standards curves, the qPCR reaction mixture contained a total volume of 20 μ L, which consisted of 10 μ L 2 $\times$ SYBR Premix DimerEraser (Takara, Shiga, Japan), 0.4 μ M of each forward and reverse primer, 0.4 μ L 50 $\times$ ROX and 2.5 μ L of template genomic DNA from the above four bacteria strains (concentration range from 10 ng/ μ L to 10 fg/ μ L). The result was

represented as the number of cells per mL following the calculation methods described elsewhere.¹²

Statistical analysis

The qPCR results were organized and analyzed using the SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Descriptive statistics including prevalence, mean, range, and standard deviation were examined. The nonparametric Mann–Whitney U test was used to examine the significance of the distribution among continuous variables including age, oral hygiene practice, sugar consumption, and bacteria loads. Fisher's exact test was used to examine the significance of the distribution among categorical variables such as sex and prevalence. The Spearman correlation test was used to examine the coefficients between saliva levels of bacteria and caries index. $P < 0.05$ was considered statistically significant.

Results

General results

Table 2 gives the demographic and clinical characteristics of the study population. Age, sex, and oral hygiene practice per day were not statistically different between the children with ECC and CF children ($P > 0.05$, **Table 2**).

Table 2 Demographic and clinical characteristics of the study population.

	ECC (n = 36)	CF (n = 73)	P
General characteristics			
Age (mo) ^a	42.2 $\pm$ 4.9	42.8 $\pm$ 4.8	0.338
Female sex ^b	47.2	42.3	0.685
Clinical characteristics			
dmft	4.0 $\pm$ 3.8	0	
dmfs	8.7 $\pm$ 10.5	0	
Survey characteristics			
No. of times oral hygiene practised per day ^a	2.2 $\pm$ 0.8	2.3 $\pm$ 0.8	0.422
No. of times sugar consumed per day ^a	1.8 $\pm$ 0.7	1.4 $\pm$ 0.5	0.004*

CF = caries-free children; dmft/dmfs = decayed, missing, and filled teeth/surfaces; ECC = children with early childhood caries.

* $P < 0.01$.

Data are presented as mean $\pm$ SD or %.

^a Nonparametric Mann–Whitney U test.

^b Fisher's exact test.

Table 3 Frequencies of identification of *Streptococcus mutans* and *Streptococcus sobrinus* from saliva in children with early childhood caries and caries-free children.

Microorganism		ECC (n = 36)	CF (n = 73)	P
<i>Streptococcus mutans</i>	<i>Streptococcus sobrinus</i>	Frequency (%)	Frequency (%)	
+	—	25 (69.5)	53 (72.6)	0.83
—	+	0	0	1
+	+	7 (19.4)	3 (4.1)	0.01*
—	—	4 (11.1)	17 (23.2)	0.19

Fisher's exact test.

*P < 0.01.

CF = caries-free children; ECC = children with early childhood caries.

Table 4 Comparison of the mean DNA levels log₁₀ (cells/mL + 1) of targeted microorganisms between children with early dental caries and caries-free children.

Microorganism	Cells/mL		
	ECC (n = 36)	CF (n = 73)	P
<i>Streptococcus mutans</i>	4.1 ± 1.7	3.2 ± 1.9	0.004*
<i>Streptococcus sobrinus</i>	0.5 ± 1.1	0.1 ± 0.5	0.007*
<i>Bifidobacterium</i> spp.	5.2 ± 0.6	5.1 ± 0.5	0.421
<i>Lactobacillus</i> spp.	6.1 ± 0.6	6.2 ± 0.5	0.260

Data are presented as mean ± SD values.

Nonparametric Mann–Whitney U test.

*P < 0.01.

CF = caries-free children; ECC = children with early childhood caries.

However, an association was found between children with caries-active lesions and the frequency of sugar consumption, namely the frequency of sugar consumption was significantly higher in children with ECC than in CF children (P = 0.004, Table 2).

Prevalence of bacteria in children with ECC and CF children

S. mutans was detected in 88.9% of children with ECC and 76.7% in CF children with no statistically significant

difference (P > 0.05, Table 3). *S. sobrinus* was detected less frequently than *S. mutans*; however, the prevalence of *S. sobrinus* was significantly higher in children with ECC (19.4%) than in CF children (4.1%) (P < 0.05, Table 3). *Bifidobacterium* spp. and *Lactobacilli* spp. were positive for all children regardless of their caries status.

Saliva level of the four targeted microorganisms in the two groups

The saliva levels of the four microorganisms in children with ECC and CF children were evaluated. As shown in Table 4, except for *Lactobacillus* spp., the saliva levels of the three other bacteria were higher in children with ECC than in CF children. However, only the loads of *S. mutans* and *S. sobrinus* in saliva were significantly higher in the caries-active group compared with the caries-free group (P = 0.004 and P = 0.007, respectively, Table 4).

Correlation of microorganisms and caries index

The mean ± standard deviation value of dmft was 4.0 ± 3.8 (range 1–16) in children with ECC. The mean dmfs index for children with ECC was 8.7 ± 10.5 (range 1–43). Saliva levels of *S. mutans* and *S. sobrinus* were significantly correlated with *Bifidobacterium* spp. ($r = 0.345$ and $r = 0.263$, respectively), dmft ($r = 0.321$ and $r = 0.271$, respectively), and dmfs ($r = 0.316$ and $r = 0.245$, respectively). The Spearman correlation coefficients between the saliva level of *Lactobacillus* spp. and dmft/dmfs were −0.106/0.114 (Table 5).

Discussion

ECC is prevalent and consequential. Investigation of the composition of the oral microbial ecosystem is essential in better understanding the etiology of ECC. A few previous studies have described the microbiota in various populations of children with ECC, most focusing on plaque, not saliva.^{4,18,19} However, saliva is easy to access and is considered the most suitable sample for providing information on the microbial population in the entire oral cavity.²⁰ Therefore we analyzed the bacterial composition of four putative cariogenic bacteria in the saliva of Chinese preschool children with or without caries using a qPCR assay.

Table 5 Spearman correlation coefficient between saliva levels of bacteria (cells/mL) and caries indices.

	Spearman correlation coefficients			
	<i>S. mutans</i>	<i>S. sobrinus</i>	<i>Bifidobacterium</i> spp.	<i>Lactobacillus</i> spp.
<i>Streptococcus mutans</i>	—	—	—	—
<i>Streptococcus sobrinus</i>	0.204*	—	—	—
<i>Bifidobacterium</i> spp.	0.345**	0.263**	—	—
<i>Lactobacillus</i> spp.	0.124	−0.50	0.126	—
dmft	0.321**	0.271**	0.127	−0.106
dmfs	0.316**	0.245**	0.107	−0.114

*P < 0.05.

**P < 0.01.

MS (mostly *S. mutans* and *S. sobrinus*) have been the most commonly investigated cariogenic bacteria over the past few years. In this study, the prevalence of *S. mutans* was 88.9% in children with ECC and 76.7% in CF children. The difference between children with ECC and CF children was not statistically significant ($P > 0.05$). In 2008, Ge et al²¹ found that the prevalence of *S. mutans* was significantly higher in severe children with ECC (100%) than in CF children (37.5%) using a culture-based counting assay. It is accepted that qPCR is much more sensitive than standard PCR or culture-based counting,²² which may account for the difference in the detection rate between qPCR and culture-based counting methods. Furthermore, we found that it was five times more likely to detect both *S. mutans* and *S. sobrinus* in caries-active samples than in caries-free samples (Table 3), which is in accord with previous studies.¹³ These findings not only indicate that *S. sobrinus* may play a more important role in the caries status of children if it exists with *S. mutans*, but also suggest that additional species combinations could be valuable in the risk assessment for ECC, which is supported by Tanner et al.¹⁹

In this study, the loads of *S. mutans* and *S. sobrinus* were significantly higher in children with ECC than in CF children (Table 4), which is in line with previous studies⁴ and verified our first study hypothesis. We also found that the severity of ECC measured by the dmft/dmfs index scores was positively correlated with high levels of *S. mutans* and *S. sobrinus* (Table 5). These results suggest that *S. mutans* and *S. sobrinus* are significantly associated with the caries status in the preschool population sampled, which might be considered as meaningful indicators in the screening test for ECC. In 2009, a study by Choi et al⁴ retained strengths similar to those in our work. However, by the use of saliva samples and qPCR for analysis, Nurelhuda et al¹³ found that the amount of *S. mutans* in the saliva of Sudanese school children was not associated with their caries status, suggesting that various populations or demographic factors may correspond to different results.

Bifidobacteria have been verified to be related to occlusion carious lesions in children⁸ and older adults,²³ and root carious lesions in elderly patients.²⁴ However, most previous studies were performed using culture or culture-based methods. In this study using qPCR, we observed that the saliva level of *Bifidobacterium* spp. was higher in children with ECC than in CF children, but differences in the presence and bacterial load between children with ECC and CF children were not statistically significant, which was in disaccord with previous studies.⁸ These contradicting results might be partly ascribed to culture bias. We speculate that some species of *Bifidobacteria* are nonculturable microorganisms that cannot be detected by conventional methods. Although no significant difference was found in *Bifidobacterium* spp. between two groups, it should be noted that the saliva level of *Bifidobacterium* spp. was positively associated with *S. mutans* and *S. sobrinus* (Table 5). Similarly, Kaur et al⁸ reported that the saliva level of bifidobacteria was significantly associated with MS. All these findings imply that an interactive effect of bifidobacteria and MS (*S. mutans* and *S. sobrinus*) may play a role in children's caries status.

Among the four microorganisms analyzed in this study, *Lactobacillus* spp. showed the highest saliva level in

children with ECC and in CF children. *Lactobacillus* was reported to be associated with ECC by a few previous studies.^{9,25} However, Ge et al²¹ reported that the presence and amounts of total oral *Lactobacillus* were not significantly different between children with ECC and CF children. Our results confirmed that the presence and load of *Lactobacillus* spp. was not correlated with caries status in the population studied here. In addition, we found that the coefficients between saliva level of *Lactobacillus* spp. and dmft and dmfs were -0.106 and -0.114 , respectively (Table 5). These findings highlight the probiotic role of *Lactobacillus* spp., as supported by previous studies.^{11,26} Nevertheless, the limited information on *Lactobacillus* spp. could not verify the conclusions.

The qPCR provided a sensitive, rapid, reliable, and simple method for quantifying bacteria.²⁷ Although specific primers were eligible for the identification of the targeted microorganisms, primer bias still exists. For example, *Bifidobacterium* spp. not only covers the targeted genera, but also recognizes other oral Bifidobacteriaceae, which may have resulted in overestimating the quantification data. Another limitation of this study is that only four microorganisms were selected, which may not completely represent the relationship between all microorganisms and ECC. Although our data are valid and substantial enough to meet the aim of our study, further studies referring to more taxons and species are needed and recommended.

In conclusion, we demonstrated bacterial composition of four bacteria in the saliva of Chinese preschool children with or without caries using a qPCR assay. The two study hypotheses were only confirmed for *S. mutans* and *S. sobrinus*. An association was also found between children with caries-active lesions and the frequency of sugar consumption. Hence dietary habits, combined with the microbiological indicator (*S. mutans* and *S. sobrinus*), should be utilized in designing a caries screening program to enhance oral health promotion in the studied population.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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