DOI: 10.1111/j.1365-263X.2009.01003.x

INTERNATIONAL JOURNAL OF PAEDIATRIC DENTISTRY

# Oral *Streptococcus* species in pre-term and full-term children – a longitudinal study

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*International Journal of Paediatric Dentistry 2009; 19: 406–411*

**Background.** Despite high clinical significance, the microbiology of the dental biofilm in young children remains poorly understood.

**Aim.** The aim of this longitudinal study was to investigate five *Streptoccocus* species commonly found in the oral biofilm of children, namely *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus mitis*, *Streptococcus sanguinis*, and *Streptococcus salivarius* to determine their relative numbers in caries-free pre-term children, and age-matched full-term controls.

**Design.** Plaque and saliva samples were obtained from 15 pre-term children and 15 age-matched controls at ages 3, 6, 12, 18, and 24 months. A quantitative real-time PCR technique was used to determine the numbers of five species of *Streptococcus* using probes and primers specific for each bacterial species.

**Results.** All species of *Streptococcus* generally increased from ages 3 to 24 months. The relative ratios of the bacteria remained fairly constant at all ages studied ($P > 0.1$). There were no significant differences in numbers of all *Streptococcus* species between pre-term children and full-term controls at all the ages investigated between.

**Conclusion.** The results show that the relative numbers of *S. mutans*, *S. sobrinus*, *S. mitis*, *S. sanguinis*, *and S. salivarius* remain relatively constant from 3 to 24 months of age in caries-free pre- and full-term children.

## Introduction

Early childhood caries, a form of rampant caries responsible for a majority of toothaches and dental abscesses in young children, is highly prevalent in socially disadvantaged groups[1]. Despite its public health significance, the aetiological factors involved in early childhood caries are still incompletely understood[2]. In particular, the microbiology of the condition is still unclear. Although most investigations implicate *Streptococcus mutans* and *Streptococcus sobrinus* as the main microbial agents involved in early childhood caries[1,3–6], there is also evidence to suggest that other organisms of the streptococcal viridians group may have significant roles[7,8]. In health, the relative numbers of these commensal species are thought to be fairly constant, and shifts in their proportions are likely to indicate disease. For example, increases in the relative ratios of *S. mutans* and *S. sobrinus* have been associated with caries[5,9]. On the other hand, *Streptococcus sanguinis* has been reported to be in relatively high numbers when the numbers of *S. mutans* are low, suggesting that the presence of high numbers of *S. sanguinis* is associated with low caries risk[7,10].

The roles of other common oral *Streptococcus* species in childhood caries such as *Streptococcus mitis* and *Streptococcus salivarius* remain unclear. Although these bacteria are not likely to be the primary causative organisms, it is possible that they may have contributory roles in the caries process due to their ecological position or pathogenic potential in the biofilm. Thus, shifts in their relative proportions in the dental biofilm may indicate changes in caries risk. Yet, little is known regarding the relative proportions of the *Streptococcus* species in relation to oral health and disease. Furthermore, in children, changes in relative proportions of the bacterial species

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may occur as a result of maturation of the biofilm ecology, yet there is little information available regarding the normal proportions of the various *Streptococcal* species at the various ages. Knowledge of the relative number of the various bacteria in the dental biofilm in a healthy mouth would help in the understanding of how caries risk can change in relation to dietary, oral hygiene practices, and the effects of systemic antibiotics and oral antiseptics.

As pre-term children have been shown to have higher caries risk compared to full-term children[11], it is possible that this is associated with altered proportions of the microbial species, but there is a paucity of information regarding the oral microbiological changes in pre-term children. Therefore, the aim of this study was to examine the longitudinal changes regarding the relative proportions of *S. mutans*, *S. sobrinus*, *S. mitis*, *S. salivarius*, and *S. sanguinis* in caries-free, healthy pre-term children compared to caries-free full-term children from ages 3 to 24 months.

## Materials and methods

Ethical approval for the investigations was obtained from the relevant institutions. As part of a longitudinal study comparing the oral colonization of *S. mutans* in pre- and full-term children, microbiological samples were obtained from children at ages 3, 6, 9, 12, and 18 months respectively to determine the presence of five oral streptococci[12,13]. Dietary and toothbrushing histories were obtained from the parents and the children examined for dental caries at these time points. For this pilot study, 15 pre-term and 15 full-term children were randomly selected to compare the relative numbers of the five oral streptococci at various time points.

Plaque and saliva samples were obtained by swabbing all tooth and soft tissue surfaces using a sterile cotton bud, which was saturated with saliva. The cotton buds were placed in vials containing sterile phosphate buffer saline and stored at −80 °C. Bacterial numbers were determined as numbers of colony-forming units per mg total protein (CFUs/mg protein). Protein quantities were assayed using BCA Protein Assay (Co 23225/7; Pierce, IL, USA).

Standard bacterial controls used were *S. mutans* NCTC10449, *S. mitis* NCTC 12261, *S. salivarius* ATCC 25975, *S. sanguinis* NCTC 7863, and *S. sobrinus* strain 6715. Stock strains were revived from freeze-dried vials, cultivated on Columbia Agar enriched with 5% defibrinated sheep blood (CBA) and incubated in 5% carbon dioxide in air at 37 °C. Bacteria identification was verified using API ID32 Strept® identification test strips (Biomerieux, Sydney, Australia).

Quantification of *Streptococcus* species were determined by cultivation in brain heart infusion broth. Viable numbers of exponential growing culture (24 h) were determined using a plate-dilution method on CBA plates from triplicate plates. Final concentrations of each *Streptococcus* species were determined before storage at −20 °C.

The template-extraction method employed was developed in Dr Diane Citron laboratory, RM Alden Research Laboratories, Santa Monica, UCLA (personal communication). Samples were vortexed to remove bacteria from swabs. The swabs were wrung out on the sides of the microfuge tubes, then discarded, and vials were centifuged at 8000 × ***g***. The pellets were re-suspended in 160 μL of enzymatic lysis buffer (20 mM Tris–HCL, pH 8.0; 2 mM EDTA; 1.2% Triton X-100), 20μL of 180mg/mL lysozyme, and 20μL of 2500 U/mL achromopeptidase. Standards and blanks were processed using the same extraction procedure. Total DNA was purified using the DNeasy blood and tissue kit (QIAGEN, Austin, TX, USA) Briefly, vials were then incubated for 60 min at 37 °C after which 25 μL of proteinase K and 200 μL of AL buffer was added and vortexed. The vials were incubated at 55 °C overnight (18 h), followed by the addition of 200 μL of 90% ethanol, and the mixture pipetted into the DNeasy mini-column-collection tube set according to manufacturer's instructions.

After DNA extraction, the total DNA : Protein (A260 : A280 nm) per sample was determined using a spectrophotometer (Nanodrop®) and employing MillliQ reagent water as a blank. All samples were processed in triplicates

**Table 1. List of *Streptococcus* species investigated.**

| Species | GenBank accession no. | Target | Accession code |
|---|---|---|---|
| *Streptococcus mutans* | AY966490 | *Gtf*B | 25175 |
| *Streptococcus salivarius* | Z17279 | *Gtf*P | 25975 |
| *Streptococcus sanguinus* | AB056712 | *Tnp*A | 10556 |
| *Streptococcus sobrinus* | AF204255 | *Gtf*T | 33478 |
| *Streptococcus mitis* | AJ582646 | *Mer*A | 49456 |

*Gft*, gycosyltransferase; *Tnp*, transposase; *Mer*A, mercuric reductase.

(Table 1). The nucleotide sequences of *S. mutans* and *S. sobrinus* of Suzuki and co-workers were used[14]. *Streptococcus salivarius*, *S. mitis* and *S. sanguinis* sequences were obtained from Gene Bank and probes and primers designed (Table 2). The specificity and sensitivity of probes and primers to all five streptococci in this study have been previously determined in our laboratory. Real-time PCR was conducted using Applied Biosystems (AB) 7900 and TaqMan® Chemistry. The Eppendorf Robotics system in the 384-well format was employed to aliquot the samples. Optimization of the primer/probe concentrations was confirmed before samples were assayed. DNA templates were diluted 1 : 10 in distilled water. All samples were run in triplicate with duplicate standards on each plate using the following protocol: 2 min at 50 °C, 10 min at 90 °C, then 15 s at 95 °C, and 1 min at 60 °C for 45 cycles.

**Table 2. Sequence of primers and probes used for *S. mutans, S. salivarius, S. sanguinus and S. sobrinus.***

| Species | Sequence (5′-3′) | Primer/Probe |
|---|---|---|
| *S. mutans* | GCCTACAGCTCAGAGATGCTATTCT | Forward Primer |
| | GCCATACACCACTCATGAATTGA | Reverse Primer |
| | TGGAAATGACGGTCGCCGTTATGAA | Probe |
| *S. salivarius* | CACGCCATGCTGGAAGTG | Forward Primer |
| | GCGATGAGCCAAGCTGAAG | Reverse Primer |
| | TTAGCTGCTGCGTAGACTTCGTCT | Probe |
| *S. sanguinus* | CAAAATTGTTGCAAATCCAAAGG | Forward Primer |
| | GCTATCGCTCCCTGTCTTTGA | Reverse Primer |
| | AAAGAAAGATCGCTTGCCAGAACCGG | Probe |
| *S. sobrinus* | TTCAAAGCCAAGACCAAGCTAGT | Forward Primer |
| | CCAGCCTGAGATTCAGCTTGT | Reverse Primer |
| | CCTGCTCCAGCGACAAAGGCAGC | Probe |
| *S. mitis* | GCCATTGAAGCGGTTACTTTG | Forward Primer |
| | CATCCGACATTAACGCAAGTTC | Reverse Primer |
| | ATGATTGAGCGTGGAACGGTGGGT | Probe |

### *Statistical analysis*

The data were analysed using the analysis of variance and the student's *t*-test, with alpha-level set at $P < 0.05$.

## Results

The 15 pre-term children had a mean birthweight of 1.9 ± 0.7 kg and gestational age 32.7 ± 3.6 weeks, and the 15 control full-term children had mean birthweight 3.6 ± 0.5 kg and gestational age 39.8 ± 1.0 weeks. None of the children had dental caries and there were no significant differences in their toothbrushing and feeding habits as well as daily sugar consumption frequencies (data not shown).

Comparison of the relative concentrations of *S. mutans*, *S. mitis*, *S. salivarius*, *S. sanguinis*, and *S. sobrinus* at ages 3, 6, 12, 18, and 24 months respectively are shown in Table 3. There were no significant differences between pre- and full-term children in the relative numbers of all five species at the various ages ($P > 0.1$; Table 3).

When the numbers of the five *Streptococcus* species present at the various ages in full-term children were compared, the results suggest a general increase in bacterial numbers with increasing age. There were, however, large variations in numbers among all *Streptococcus* species at ages of 6, 12, and 18 months, and no significant differences in numbers of bacteria at the various ages for all the *Streptococcus* species tested could be detected ($P > 0.1$; Fig. 1).

Similar findings were noted in the numbers of the five *Streptococcus* species at the various ages in pre-term children. The data shown in Fig. 2 reveal that as with the full-term children, there was a general increase of all the five *Streptococcus* species from 3 to 24 months. There were, however, large variations within the bacterial species at each age period and there were no significant differences between the species.

## Discussion

The normal ecology of the dental biofilm in health is maintained through balanced

**Table 3. Relative numbers of *Streptococcus mutans*, *Streptococcus mitis*, *Streptococcus salivarius*, *Streptococcus sanguinis*, and *Streptococcus sobrinus* at ages 3–24 months.**

| Age (months) | Log CFU/mg protein | | | | Pre-term versus term |
|---|---|---|---|---|---|
| | Pre-term | SD | Term | SD | |
| *S. mutans* | | | | | |
| 3 | 1.95E+04 | 6.67E+04 | 1.23E+03 | 1.04E+03 | All NS |
| 6 | 6.65E+03 | 1.19E+04 | 1.52E+03 | 2.15E+03 | |
| 12 | 3.76E+03 | 8.00E+03 | 2.51E+03 | 2.75E+03 | |
| 18 | 9.51E+02 | 1.60E+03 | 1.06E+04 | 3.33E+04 | |
| 24 | 5.24E+06 | 1.94E+07 | 1.87E+03 | 3.11E+03 | |
| *S. mitis* | | | | | |
| 3 | 4.17E+03 | 9.38E+03 | 1.31E+04 | 2.40E+04 | All NS |
| 6 | 1.74E+06 | 4.30E+06 | 2.43E+06 | 7.13E+06 | |
| 12 | 3.99E+06 | 7.30E+06 | 1.63E+06 | 5.13E+06 | |
| 18 | 3.91E+06 | 7.48E+06 | 2.03E+06 | 5.96E+06 | |
| 24 | 4.19E+06 | 7.84E+06 | 2.22E+06 | 6.26E+06 | |
| *S. salivarius* | | | | | |
| 3 | 4.33E+01 | 2.73E+03 | 1.13E+04 | 2.94E+04 | All NS |
| 6 | 3.19E+04 | 1.01E+05 | 6.33E+03 | 1.14E+04 | |
| 12 | 4.33E+01 | 8.84E+04 | 6.74E+03 | 1.19E+04 | |
| 18 | 7.15E+04 | 2.37E+05 | 7.98E+04 | 2.06E+05 | |
| 24 | 7.70E+07 | 2.88E+08 | 3.50E+04 | 6.92E+04 | |
| *S. sanguinis* | | | | | |
| 3 | 2.41E+01 | 5.79E+01 | 2.72E+00 | 7.76E+00 | All NS |
| 6 | 4.92E+03 | 1.36E+04 | 1.47E+05 | 5.49E+05 | |
| 12 | 3.12E+04 | 7.84E+04 | 5.12E+03 | 1.38E+04 | |
| 18 | 6.26E+05 | 2.24E+06 | 4.23E+03 | 6.85E+03 | |
| 24 | 2.61E+06 | 7.06E+06 | 2.61E+05 | 7.15E+05 | |
| *S. sobrinus* | | | | | |
| 3 | 3.68E+01 | 1.47E+02 | 3.05E+00 | 1.14E+01 | All NS |
| 6 | 2.16E+04 | 4.89E+04 | 1.33E+04 | 3.78E+04 | |
| 12 | 2.65E+04 | 5.20E+04 | 1.14E+04 | 3.17E+04 | |
| 18 | 1.08E+06 | 4.25E+06 | 1.48E+04 | 4.48E+04 | |
| 24 | 7.81E+04 | 1.95E+05 | 2.21E+04 | 6.93E+04 | |

proportions of the various commensal bacterial species[15]. Although *Streptococcus* species comprise approximately 10% of the total counts of plaques[3], they have important roles in the aetiology of dental caries. *Streptococcus mutans* and *S. sobrinus* are considered the main aetiological agents in early childhood caries, whereas other bacterial species may have contributory roles[4–6,16,17]. Based on the observation that *S. sanguinis* is usually found in large numbers in caries-free individuals, high *S. sanguinis* to *S. mutans* ratios are thought to indicate low caries risk. Conversely, authors have proposed that relatively high concentrations of *S. mutans* to *S. sanguinis* suggest high caries risk[7,16]. This hypothesis has been supported in recent clinical studies of children with early childhood caries and caries-free subjects, as well as investigations which demonstrated that early colonization with *S. sanguinis* is associated with later acquisition of *S. mutans*[7,10,18]. On the other hand, increased numbers of *S. mitis* is thought to promote caries, although the mechanisms involved have not been well defined[7]. Also, *S. salivarius* have been reported to increase proportionately in plaque in children with caries, although its roles in the aetiology of caries are still unclear[7].

Thus, the relative concentrations of the *Streptococcus* species can indicate states of health and disease in the oral environment. In order to predict the disease, such as caries, knowledge of the normal relative proportions of the endogenous species in the mouth at various ages is essential. The results of this study show that there are no significant differences in the relative numbers of *S. mutans*, *S. sobrinus*, *S. mitis*, *S. salivarius*, and *S. sanguinis* in pre-term children compared to full-term controls at ages of 3, 6, 12, 18, and 24 months respectively. As

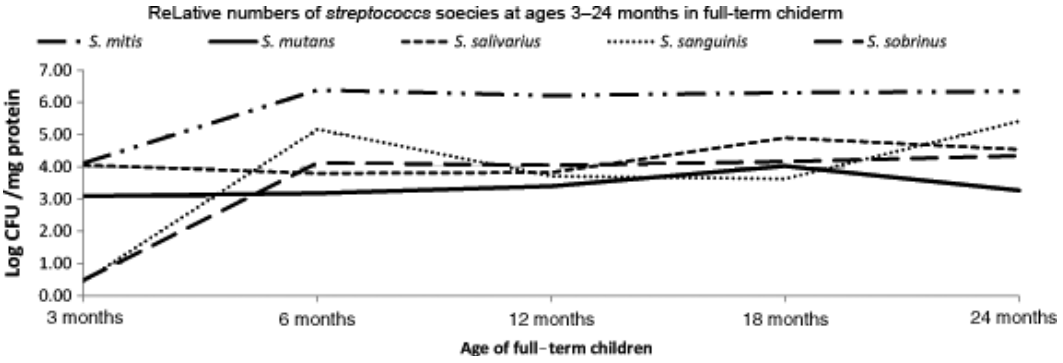


**Fig. 1.** Relative numbers of *Streptococcus* species detected by PCR at ages 3–24 months in full-term children.

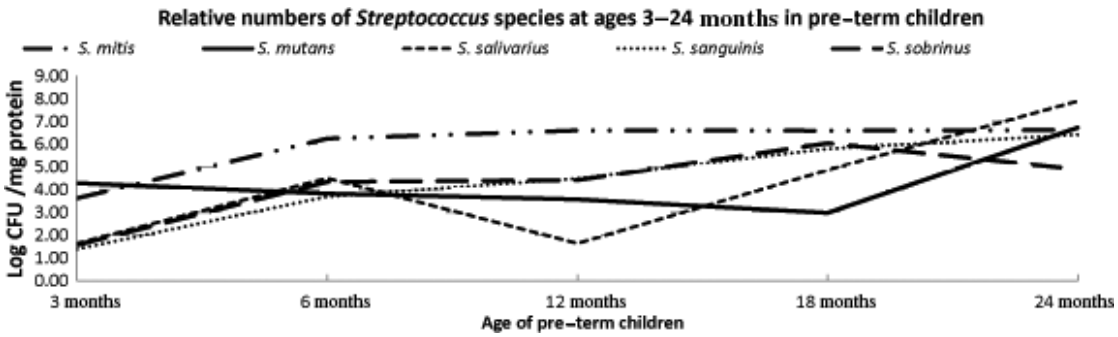


**Fig. 2.** Relative numbers of *Streptococcus* species detected by PCR at ages 3–24 months in pre-term children.

microbial numbers reflect host immunocompetence, the data suggest that at both predentate and post-eruption stages, pre-term children have achieved a level of immunocompetence, which is associated with similar microbial status compared to full-term children. This observation thus supports our earlier findings that pre-term children have similar levels of salivary immunoglobulins compared to full-term children for the study duration of 0–18 months[19].

In addition, the lack of difference in the relative quantities of the *Streptococcus* species between the two groups could also be related to their similar dietary and oral hygiene habits. Both groups of children were Australian-born, Caucasian in race, and of middle-class socioeconomic status. Although frequent consumption of sugary snacks, night-time feeding with a nursing bottle containing sweet fluids, and lack of toothbrushing are well-reported factors associated with an increased numbers of *S. mutans*[1], the children in this study groups did not take a nursing bottle to bed, had a mean daily sugar frequency of approximately three times, and all reported to have brushed their teeth daily[12,13,20].

The present data of caries-free, low-risk children also show that in the absence of disease, the relative numbers of the five *Streptococcus* species remain fairly constant for over the period of 3–24 months. This observation supports the hypothesis that, in healthy states, microbial balance is maintained. Although further longitudinal investigations are necessary to explore the microbial changes involved in the development of dental caries, previous authors have reported that increased numbers of *S. mutans* and *S. sobrinus* are associated with clinical signs of caries[1,7,10]. The results of this study may thus be usefully applied as baseline data to examine how the main *Streptococcus* species change with the development of dental caries and other conditions, as well as the effects of prevention and intervention. Due to the relatively small numbers of children in this study, further studies are necessary to confirm these initial baseline results. Additionally, longitudinal studies that examine changes in relative numbers of other bacteria in the oral biofilm will also help to shed further light on the relationships of the various microbial species in health and disease.

**What this paper adds**

- Pilot information regarding the longitudinal development of the dental biofilm and the relative ratios of five oral *Streptococcus* species in full- and pre-term caries-free children from ages 3 to 24 months.

**Why this paper is important to paediatric dentists**

- This study may have implications for the prevention of early childhood caries as the *Streptococcus* species in the study are important in caries development.
- This pilot data will help guide future clinical studies of caries development in children.

## Acknowledgements

This study was supported by the Australian Dental Research Foundation. The authors thank Mr Bob Simpson, Department of Biochemistry, The University of Queensland, for his excellent assistance with the qRT-PCR, and Dr Derek Harty, Institute of Dental

Research, Westmead Centre for Oral Health, Wentworthville, NSW, and Dr Stuart Daspher, Cooperative Research Centre for Oral Health Science, The School of Dental Science, The University of Melbourne, for providing the *Streptococcus* species for this study.

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