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Distribution of selected cariogenic bacteria in five different intra-oral habitats in young children

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Background. Knowledge of the colonization patterns and composition of the oral microbiota can lead to a better understanding of disease initiation.
Aim. The aim of this study was to examine the distribution of selected cariogenic bacteria in samples from five different oral habitats in young Greek children.
Design. Ninety-three children 3–12 years old (mean + SD 7.9 ± 2.5) (60.2% male, 39.8% female) participated and split into three different age groups: primary (3–6 years), early mixed (6–9 years), and mixed dentition (9–12 years). Samples for bacterial enumeration were taken from saliva, supragingival and subgingival plaque, tongue dorsum, and soft tissues from each child, and were further analysed using checkerboard DNA–DNA hybridization.
Results. Mean counts and proportions of all the test bacteria differed significantly among sample locations. Cariogenic bacteria were present in almost all healthy children. Mean proportions of *Streptococcus mutans* isolated from soft tissue and *Streptococcus sanguinis* from soft tissue, subgingival and saliva samples increased significantly with age, whereas the opposite was seen for *Lactobacillus acidophilus*.
Conclusions. Cariogenic bacteria were present in almost all young children. Soft tissues, saliva, and tongue were more often colonized by cariogenic streptococcal species than teeth. These surfaces may serve as reservoirs for oral pathogens, requiring attention during preventive interventions.

Introduction

Both dental caries and periodontal disease comprise multi-factorial diseases in which the oral microorganisms play a key role. In caries, the major suspect organisms are mainly *Streptococcus mutans*, *Streptococcus sobrinus*, and lactobacilli species[1–3]. These diseases require colonization by aetiological pathogens before their occurrence. *Streptococcus mutans* as well as periodontal pathogens such as *Porphyromonas gingivalis* and *Tannerella forsythia* have been found even in subjects under 18 months of age[4]. Knowledge of the age and possible changes in colonization patterns and composition of the oral microbiota can lead to a better understanding of disease initiation. Furthermore, this knowledge can be the basis of a primary preventive programme.

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Most of our knowledge regarding the microbial composition of dental plaque comes from studies using culture, as well as more rapid and precise bacterial identification methods such as DNA hybridization and PCR. Studies have shown that marked differences exist in the composition of biofilms from person to person and from one type of intra-oral location to another and within the same individual[5].

Little is known about the microbiota colonizing the oral soft tissues that comprise about 80% of the surface area of the oral cavity. Findings from recent studies suggest that soft tissues may act as reservoirs for initial infection or reinfection of the periodontium, and may deserve therapeutic attention[6]. Mager and coworkers [7] found that the microbiotas of different oral soft tissues were more similar to each other than the bacterial composition of supra- and subgingival biofilms colonizing the teeth. Few studies have examined a broad range of bacterial species in samples taken from both

the hard and soft tissues of the oral cavity. Even fewer studies have examined the species associated with caries in biofilms on the different oral surfaces in different age groups of children.

The aim of this study was to examine the distribution of selected cariogenic bacteria in samples from five different oral habitats in young Greek children with good general health. In addition, changes in the levels and proportions of the cariogenic microbiota with increasing age were also examined.

Materials and methods

Study subjects

Ninety-three children aged between 3 and 12 years (mean ± SD: 7.9 ± 2.5) who were patients attending the Postgraduate Paediatric Dental Clinic of the University of Athens participated in the study (60.2% male, 39.8% female). All children were in good general health and had not received any antibiotics or prophylactic dental cleaning in the previous 3 months. Children were recruited consecutively and were allocated to three different age groups based on their dentition: primary (GR1: 3–6 years), early mixed (GR2: 6–9 years), and mixed dentition (GR3: 9–12 years). Each group comprised 31 children. Approval for the study was provided by the Committee for Ethics and Research of the Athens Dental School, in accordance with the Helsinki Declaration. Parental informed consent was obtained for all children in the study.

Clinical examination

Children were clinically examined in the dental clinic by the same investigator. Evaluation of dental caries was carried out using a mouth mirror and a blunt probe. Lesions at the cavitation stage in dentine were considered caries[8]. Initial lesions were not included. Radiographs were not used in the caries evaluation. The absence or presence of plaque was assessed on all tooth surfaces using the Simplified Plaque Index. Intra-examiner reliability was determined, and agreement was achieved with *k*-scores above 80%.

Collection of samples

Samples for bacterial enumeration were taken from five different oral habitats from each child: saliva, supragingival and subgingival plaque, tongue dorsum, and soft tissues as described below.

Saliva sample. Patients expectorated a sample of whole unstimulated saliva into sterile tubes. A 0.2 mL aliquot of the sample was vortexed with 0.15 mL sterile, filtered Tris–EDTA buffer (TE: 10 mM Tris–HCl, 1 mM EDTA, pH 7.6). A 0.2 mL sample of this mixture was then taken, and 0.1 mL of 0.5 M NaOH was added[7].

Subgingival plaque sample. Samples of subgingival plaque were collected using a sterile curette from the mesial–buccal aspect of either one of the second primary molars of the younger patients or one of the first permanent molars in children older than 5 years. After removal of supragingival plaque, the sample sites were isolated, dried, and sampled using a sterile curette. Each sample was placed into an individual Eppendorf tube containing 0.15 mL of TE buffer to which 0.1 mL of 0.5 M NaOH was then added[7].

Tongue and soft tissue samples. Samples were obtained using a MasterAmp buccal swab brush (Epicentre Technologies, Madison, WI, USA). The tongue sample was obtained by brushing 1 cm^2 of the centre of the dorsum of the tongue for 5 s. The buccal mucosa, hard palate, anterior vestibule, and mucosa of the maxillary and mandibular lips were sampled using a swab brush. The swab brushes were swirled, to remove adhering bacteria, in individual Eppendorf tube containing 0.15 mL of TE buffer. Then, 0.1 mL of 0.5 M NaOH was added to each tube[7].

Total supragingival plaque samples. Supragingival plaque samples were collected from all erupted teeth by brushing for 1 min with a swab brush[9]. The plaque was removed from the swab brush by washing, and diluting, in a tube with 0.15 mL TE buffer. From this, 0.2 mL of the solution was removed and placed into an individual Eppendorf tube, and 0.1 mL of 0.5 M NaOH was added[7].

All final samples were stored in –60 °C until transportation to The Forsyth Institute for microbiological assessment using checkerboard DNA–DNA hybridization.

Microbiological assessment

Samples were evaluated using a modification of the checkerboard DNA–DNA hybridization technique[10]. More detailed information about the microbiological assessment is provided in Papaioannou *et al.*[11].

Statistical analysis

Microbiological data consisted of the counts and proportions of 20 bacterial species in samples from five different oral locations: supra- and subgingival plaque, tongue dorsum, saliva, and the soft tissues in 93 children. Proportions were computed by determining the percentage that each species comprised of the total DNA probe count in each sample. Counts and proportions were averaged across subjects for the five different sample locations separately. The significance of differences in mean counts and proportions of the test bacterial species in the five different sample locations, and among the three age groups, was determined using Kruskal–Wallis test and adjusted for 20 comparisons[12]. The level of significance was set at $P = 0.05$.

Results

Oral health condition of study subjects

Dental condition. Twenty-four of 93 children were caries free. More than one-third of the 3- to 6-year-old children and 16.13% of the oldest children were caries free (Table 1). Significant differences among the three age groups were found for the decayed, missing, or filled surfaces–permanent teeth (DMFS) index. Children aged between 9 and 12 years had the highest DMFS score compared with the younger groups (Table 1).

In the permanent teeth (older children), decayed tooth surfaces comprised the largest component of the DMFS index. In the primary dentition (younger children), filled teeth comprised the largest component followed by decayed surfaces. The missing component of the index was low in both dentitions (Fig. 1).

Table 1. Dental status of children according to their age.

Age group	dmfs mean (SD)	DMFS* mean (SD)	Caries free (%)
3–6 years (GR1)	11.03 (12.93)	1.33 (3.27)	35.48
6–9 years (GR2)	10.38 (1.19)	1.19 (2.60)	25.81
9–12 years (GR3)	7.54 (7.49)	3.40 (3.40)	16.13

*Kruskal–Wallis test $P = 0.006$ (significant difference in DMFS mean score between the age groups).
dmfs, decayed, missing, or filled surfaces–primary teeth; DMFS, decayed, missing, or filled surfaces–permanent teeth.

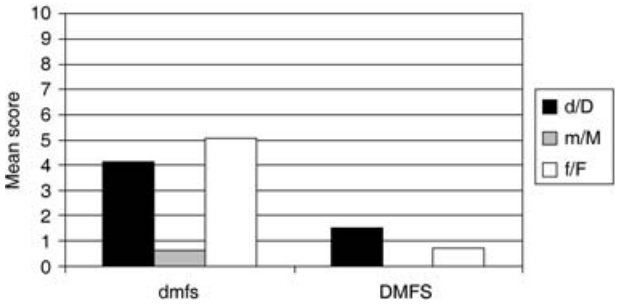

Fig. 1. Caries experience in the primary [decayed, missing, or filled surfaces–primary teeth (dmfs)] and permanent dentition [decayed, missing, or filled surfaces–permanent teeth (DMFS)] of the study subjects.

Oral hygiene. Overall, approximately 70% of the tooth surfaces were free of plaque. However, the mean percentage of sites with plaque increased significantly with age. The youngest age group (GR1) had the best oral hygiene with an average score of 18% of tooth surfaces with plaque, whereas the values for GR2 and GR3 were 28% and 38%, respectively ($P = 0.002$).

Cariogenic microbiota in the different oral habitats

The microbiological examination focused primarily on three specific cariogenic groups of bacteria: streptococci, lactobacilli, and actinomyces. The results showed statistically significant differences in the mean counts and mean proportions of all the test bacteria among the

different sample locations (Fig. 2a,b). Streptococci made up a large proportion of the total DNA bacterial counts, for the soft tissues as well as saliva, tongue, and supragingival samples (54.3%, 32.5%, 29.4%, and 24.9% of the total DNA probe counts, respectively). Specifically, *Streptococcus mitis* (18.7%) and *Streptococcus oralis* (9.5%) were found in high proportions in the soft tissue samples, whereas *Streptococcus salivarius* (9.5%) was found in high proportions in saliva, soft tissue, and tongue samples. *Actinomyces* species were found most frequently in supra- and subgingival samples with proportions of 20.1% and 13.4%, respectively. *Lactobacilus acidophilus* was found in low proportions in all oral habitats, but reached close to 5% in tongue dorsum samples. Other bacteria such as *Veillonella parvula* and *Neisseria mucosa*, common inhabitants of the mouth, were found, on average, in all locations in relatively high proportions.

Proportions and detection frequency of specific cariogenic bacteria at different oral habitats. More specifically, *S. mutans*, *S. sobrinus*, and *L. acidophilus*, the most well-known cariogenic bacteria, were found in almost all children. More than 80% of locations sampled had these cariogenic bacteria, whereas the lowest detection frequency was seen in subgingival samples especially for *S. mutans* (77.0%) (Table 2). The proportions of DNA probe counts of *S. sobrinus* were higher than *S. mutans* in all habitats (Fig. 3).

Changes in the microbiota with age. Mean proportions of many species increased with age including *S. mutans* and *S. sobrinus* (Fig. 4a,b,c).

Table 2. Detection frequencies of *Streptococcus mutans*, *Streptococcus sobrinus*, and *Lactobacillus acidophilus* in all sample locations.

	S. mutans (%)*	*S. sobrinus* (%)*	*L. acidophilus* (%)*
Supragingival	88.51	89.66	89.66
Subgingival	77.01	82.56	87.36
Saliva	87.36	89.66	88.51
Soft tissues	85.06	88.51	88.51
Tongue	88.51	89.66	89.66

*%: percentage of sampling sites positive to the bacteria.

Nevertheless, the only significant difference among age groups was seen for the proportions of *S. mutans* from soft samples, and *Streptococcus sanguinis* isolated from soft tissue as well as saliva and subgingival samples. In contrast, the mean proportions of *L. acidophilus* decreased with age, and this trend was significantly different among age groups for all sampled sites (Fig. 5).

Discussion

The goal of the present investigation was to determine the oral colonization patterns of selected oral species, particularly those associated with dental caries, in healthy children aged 3–12 years. Specific species of streptococci, actinomyces, and lactobacilli were detected in almost all children and in all habitats sampled. Streptococci comprised a large proportion of the total DNA probe count in all oral habitats sampled in the three age groups examined. This was expected because many of the streptococci are known to be early colonizers of the mouth, particularly, *S. mitis*, *S. oralis*, *S. salivarius*, *S. sanguinis*, and *Streptococcus gordonii*. These species have been detected even in young infants and pre-dentate children[4,13,14]. On the contrary, in the study by Aas and co-workers[15], bacteria commonly thought to be involved in the dental caries and deep dentine caries such as *S. mutans* and *Lactobacillus* spp., were not detected in supragingival and subgingival samples, but from older caries-free individuals. In this study, the cariogenic streptococci, *S. mutans* and *S. sobrinus*, were detected in almost all children despite their young age, with *S. sobrinus* detected more frequently than *S. mutans*. This is in contrast to studies in the literature that have shown that *S. mutans* has been strongly associated with the initiation of caries, whereas *S. sobrinus* was not always detected in association with caries[16]. On the other hand, *S. sobrinus* has been identified as an important additional risk factor for dental caries due to its higher cariogenic potential[17]. Potential differences in colonization or antigenic composition of pathogens between patient populations from different geographical regions have been proposed as factors that might explain differences in the pathogens for early onset periodontitis between United States

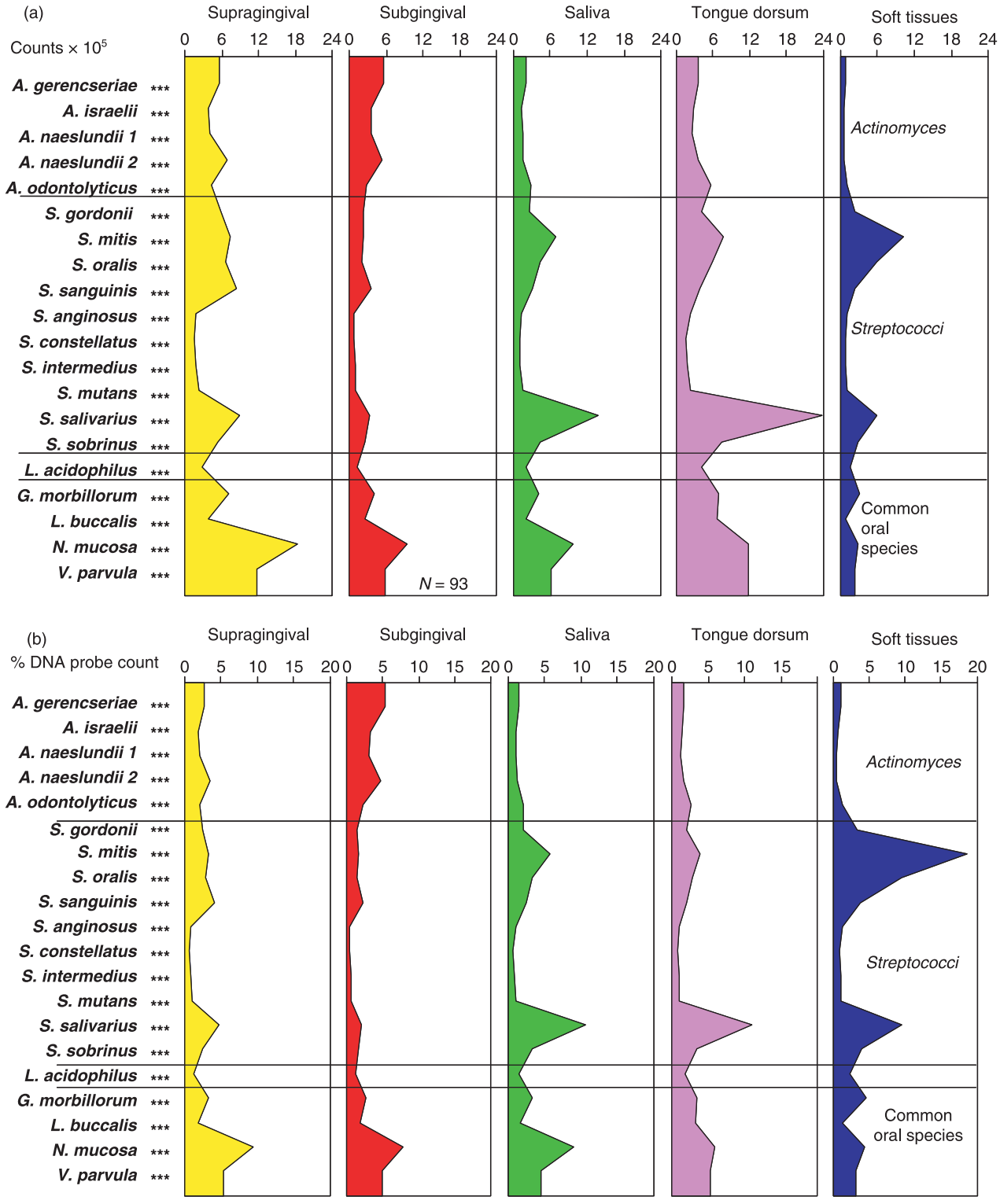

Fig. 2. Composition of the microbiota in five oral habitats of young Greek schoolchildren according to the (a) counts and (b) proportions of DNA probe counts of 20 bacterial species. Significant differences between sampled sites were determined by the Kruskal–Wallis test adjusted for multiple comparisons: $*P \leq 0.05$, $**P \leq 0.005$, $***P \leq 0001$.

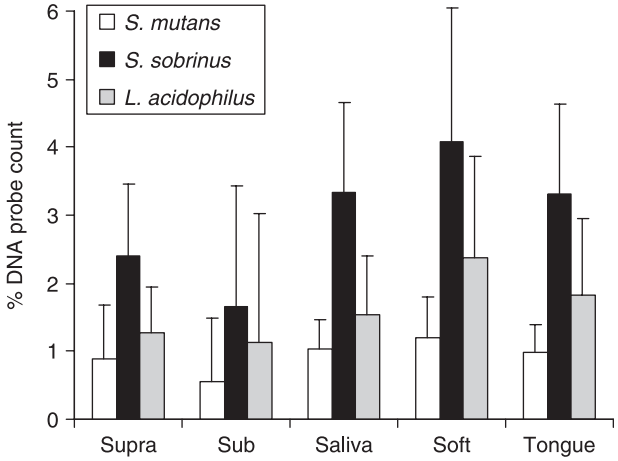

Fig. 3. The mean proportions of DNA probe counts of *Streptococcus mutans*, *Streptococcus sobrinus*, and *Lactobacillus acidophilus* in samples from the five habitats.

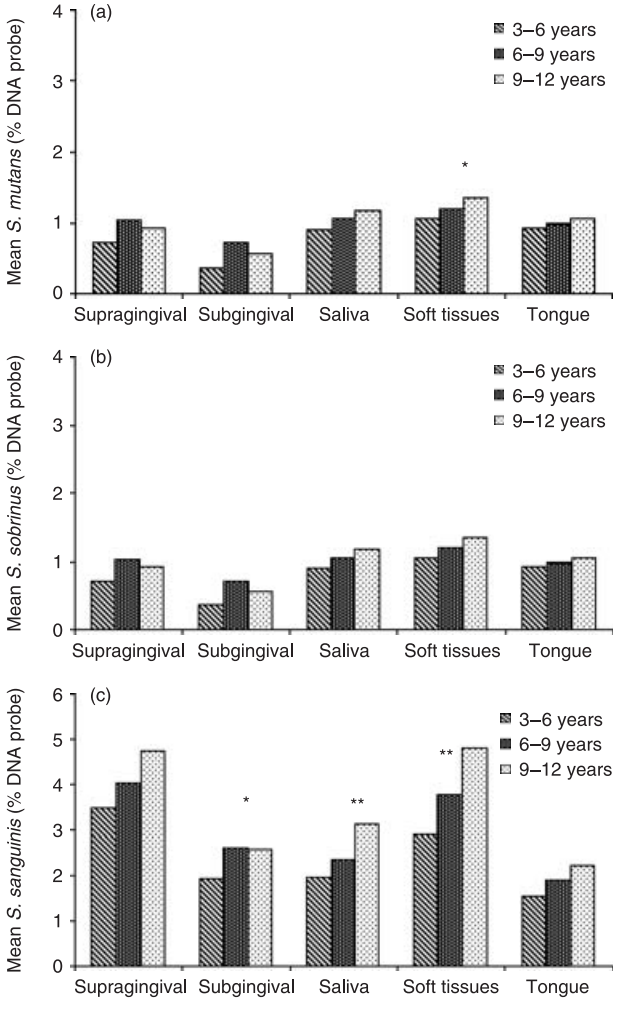

Fig. 4. Proportions of DNA probe counts of (a) *Streptococcus mutans*, (b) *Streptococcus sobrinus*, and (c) *Streptococcus sanguinis* in five oral habitats according to the age of the study subjects. Significance of differences between age groups are denoted: $*P \leq 0.05$, $**P \leq 0.005$, $***P \leq 0001$.

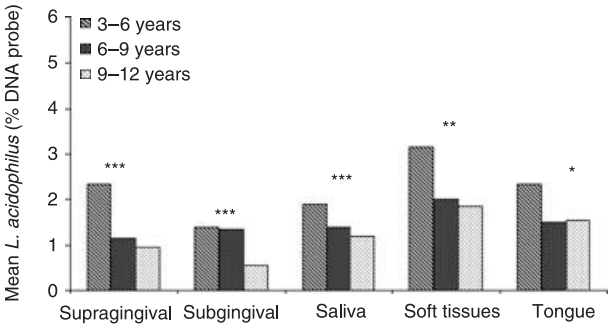

Fig. 5. Proportions of DNA probe counts of *Lactobacillus acidophilus* in all oral habitats tested according to the age of the study subjects. Significance of differences between age groups is denoted: $*P \leq 0.05$, $**P \leq 0.005$, $***P \leq 0001$.

of America and Turkey[18]. Such factors might also explain the high detection frequency of *S. sobrinus* in the current investigation. To our knowledge, this is the first study to comprehensively examine the oral distribution of streptococcal species in Greek children.

Other streptococci including *Streptococcus anginosus* and *Streptococcus intermedius* have been isolated from the soft tissues and tongue samples. This is in accord with other studies that have also found these species in the oral cavities of children[19]. *Streptococcus anginosus* has been found also in the mouths of some bone marrow transplant children[20].

Actinomyces spp. were found in all sample locations, particularly in supra- and subgingival biofilm samples. In accordance with the current investigation, studies in the literature have shown that several strains of actinomyces preferentially colonize hard tissues[21]. Studies have indicated the association of the *Actinomyces* spp. together with lactobacilli and *S. mutans* with caries initiation in 4- to 9-year-old children[2] as well as with caries development in school-aged children[22]. Lactobacilli have been found mostly together with *S. mutans* in plaque and saliva because they have poor adhesive capability and similar dietary needs as *S. mutans*[23,24]. Lactobacilli have been also implicated with the persons having a diet rich in sugar and deep caries and/or large restorations[3]. Therefore, it is not surprising that the children in this study had *L. acidophilus*, on average, in all sample locations, although in somewhat low proportions, because more than two-thirds of them had carious lesions or restored caries.

The detection of streptococci, lactobacilli, and actinomyces in the children at an early age could be important in caries development/progression based on the ecological plaque hypothesis[25]. Based on this theory, potentially cariogenic bacteria may be found in dental plaque at clinically insignificant levels as long as no major disturbances to the local habitat occur. Once normal homeostatic mechanisms are disrupted, such as an increase in the frequency of sugar consumption, greater numbers of bacteria in plaque such as mutans streptococci and lactobacilli would result in the production of more acid, a decrease in pH below 5.5, which could lead to enamel demineralization[25]. In this study, although the detection frequency of *S. mutans*, *S. sobrinus*, and *L. acidophilus*, the three known major cariogenic species, was high in all habitats, their counts were low, especially for *S. mutans*.

Here, it was reported that the highest bacterial proportions and detection frequency were found in soft tissue, saliva, and tongue samples. The tongue was the habitat most often and most heavily colonized by the cariogenic species. This is in agreement with the findings of Tanner and co-workers [4] where higher species detection frequency in young children was found on the tongue compared with tooth samples. The authors suggested that the tongue serves as a reservoir for tooth-associated species. The findings that oral habitats other than the teeth were more frequently colonized by cariogenic bacteria suggest that the entire mouth should be taken into consideration when a preventive approach is designed.

With the increase in age, there was an increase in the levels and proportions of several of the test species on the different oral surfaces. This was particularly noticeable for *S. sanguinis*. The role of this species in the initiation of biofilm formation may explain the fact that these streptococcal species are increasing with age when a more mature biofilm is formed. Colonization of *S. sanguinis* is tooth dependent, and its proportion in saliva increases as new teeth erupt[26]. Caufield and co-workers [27] have found that children who do not harbour detectable levels of *S. mutans* have significantly higher levels of *S. sanguinis* in their saliva than do children colonized with *S. mutans*. According to the authors, this finding suggests that colonization of *S. sanguinis* may influence the subsequent colonization of *S. mutans* and may impact the design of preventive approaches for caries. A similar picture was seen in the results of this study. Proportions of *S. mutans* and *S. sobrinus* also increased as the children became older. In addition, their caries experience was higher and their oral hygiene became worse. Nevertheless, these differences were not statistically significant. On the other hand, levels and proportions of *L. acidophilus* were significantly decreased in older children compared with younger children. More controlled dietary habits such as frequency of sugar consumption in older children could probably be involved in these results.

We can conclude that:

1) Cariogenic bacteria were present in almost all healthy young children.
2) Differences were found in the proportions of the bacteria colonizing the different oral habitats sampled.
3) Soft tissues, saliva, and tongue were more often colonized by cariogenic streptococcal species than teeth. Therefore, these surfaces may serve as reservoirs for oral pathogens and require attention during therapeutic and preventive interventions.
4) The proportions of *S. sobrinus* were higher than those of *S. mutans* in all habitats.
5) *Streptococcus sanguinis* increased with age, whereas *S. mutans* and *S. sobrinus* showed no change with age. In contrast, *L. acidophilus* decreased with age.

What this paper adds

- Almost all children were colonized by cariogenic bacteria from an early age.
- *Streptococcus mutans* and *S. sobrinus* were found with high detection frequencies in other habitats besides teeth.

Why this paper is important to paediatric dentists

- The detection of streptococci, lactobacilli, and actinomyces in the children at an early age could be important in caries development/progression, fortifying the concept that prevention is important from early in life.
- The fact that soft tissues, saliva, and tongue were more often colonized by cariogenic bacteria than teeth indicates that these surfaces should also be the target of every preventive programme.

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