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Microbial Indicators of Caries Activity in Saliva of Children Living in Greece

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Abstract

Dental caries is one of the most prevalent infectious diseases affecting children worldwide. Dynamic interactions between microbes, host and diet lead to the establishment of a highly cariogenic oral microbiota. *Streptococcus mutans, Streptococcus sobrinus* and *Lactobacillus spp*., have been associated with dental caries in children, whereas *Rothia dentocariosa* in adults. The aim of the present study is to look at the occurrence of *S. mutans, S. sobrinus, Rothia spp*. and Lactobacillus spp. in saliva from caries free and caries active children and assess their potentiality as biomarkers of caries activity. Saliva samples were collected and cultured onto selective media. Colonies were enumerated with the help of a stereomicroscope. In addition, a rapid test for sucrose catabolism velocity was performed and correlated with caries activity. In a total of 95 children, 77%, 13%, 100%, 69% and 95% harbored *S.mutans, S. sobrinus*, *Rothia spp*., *Lactobacillus spp*. and aciduric microbes respectively. The presence of studied microbes did not significantly differ either between boys and girls, or with age. However, *Rothia spp., Lactobacillus spp*. and facultatively aciduric microbes were less commonly found in younger children and *S. sobrinus* appeared more in older ages. Mean response times at the sucrose catabolism test were significantly lower in the caries active group. Caries activity was correlated with the presence and abundance of *Streptococci spp., Lactobacilli spp*. and total aciduric microbes. *Rothia spp*. appears as an unsuitable biomarker of caries activity.

Keywords: Caries; Saliva; Microbes; Acidogenicity; Rothia; Sucrose

Abbreviations: CFU: Colony Forming Units; NS: Not Significant; SCV: Sugar Catabolism Velocity; *S. Mutans: Streptococcus Mutans; S. Sobrinus: Streptococcus Sobrinus; R. Dentocariosa: Rothia Dentocariosa*

Introduction

Dental caries is the most common chronic disease of childhood [1] and is about five times more common than the next most prevalent chronic disease among children and adolescents, asthma [2]. If not treated in time, it can affect not only the mastication function but also the speech, smile and psychosocial environment and the quality of life of the child and the family [3,4]. The incidence of dental caries for children from lower socio-economic families is higher. In Greece, the prevalence of dental caries in the 5-year-old age group is 42.8% and it increased to 62,9% among the 12-year-old children [5]. Dental rehabilitation is often expensive, and children suffering from dental caries are highly predisposed to greater caries incidence in later years [6]. The most recent hypothesis for the etiology of dental caries is the ecological plaque hypothesis and states that the dental plaque biofilm becomes pathogenic when external challenges drive it towards a state with a high proportion of acid-producing bacteria [7]. However, only a limited number of bacteria are consistently recovered from caries lesions and have thus been recognized to be specifically associated with dental caries. The ecological concept of caries was subsequently extended by Takahashi and Nyvad [8,9], and maintains that ecological phenomena, e.g. bacterial adaptation to acidic environments (increases in bacterial acidogenicity and acidurance) and bacterial shifts to a more acidogenic and aciduric microbiota (increases in the proportion of acidogenic and aciduric bacteria), are induced by frequent and prolonged acidification.

Mutans streptococci, particularly *S. mutans* and *S. sobrinus*, have a well-established relationship with dental caries [10-13], and several studies have shown that patients harboring both species have a significantly higher incidence of dental caries than those with *S. mutans* alone [14-16]. Nevertheless, *S. mutans* can be detected in plaque samples from some caries-free children, whereas children with caries do not have any detectable *S. mutans* [17], suggesting that *S. mutans* is not the only cariogenic bacteria. *Lactobacillus spp.* have also the ability to ferment a variety of carbohydrates and to survive in a low pH environment. They were among the first bacteria suggested to cause the disease and are regarded as good markers [18-20]. *Rothia dentocariosa* has been detected consistently in people with dental caries only with PCR assays [21,22]. In addition, *R. dentocariosa* was found to be more abundant in the saliva of caries-affected children suggesting that it

might be associated with dental caries and a potential biomarker of childhood caries in salivary flora [23]. The purpose of the present study is to examine the occurrence of *S. mutans*, *S. sobrinus*, *Rothia spp.* and *Lactobacillus spp.* and facultatively aciduric bacteria in saliva from caries free and caries active children and examine those bacteria as potential biomarkers of caries activity. In addition, since the oral environmental acidification is affected by the frequency that carbohydrates are supplied, we studied the velocity of sucrose catabolism by saliva samples by a rapid test and its correlation to caries activity.

Materials and Methods

Subject Recruitment and Oral Examination

Ethical approval for this study was obtained from the Ethics Committee of the Dental school of Aristotle University of Thessaloniki. Children aged 5 to 16 years old, who visited the post-graduate clinic of Pediatric Dentistry of Aristotle University of Thessaloniki in Greece, participated in the study. Parents and / or legal guardians of the children were informed of the content of the study and gave written consent. Children with serious health condition, under regular medication or that had received antibiotics during a three-month period prior to their examination, and those that refused to cooperate in dental examination or saliva collection were excluded from this study. The tooth status of each child was assessed in the dental chair using artificial light and a dental mirror. The visual inspection was aided by bite-wing radiographs, using a digital technique, when the health status of the proximal tooth surfaces could not be ascertained by clinical examination. Decayed teeth were detected at the cavitation level. A trained and calibrated dentist performed all dental examinations. The children's caries experience was expressed by the dmft/+DMFT index and they were divided into two groups according to their clinical and radiographical assessment of caries incidence: the caries-free group (dmft/+DMFT = 0) and the caries active (ds/+DS≥1).

Saliva Collection

Prior to saliva collection, the children were asked to refrain from any food, drinks and tooth brushing for 2 hours. Samples of stimulated whole saliva were collected [24]. Each child was given a piece of paraffin wax and was instructed to chew on it and swallow. A minute later they would chew on it and spit the saliva on a clean plastic container for 5 minutes or until 5 ml of stimulated saliva were collected. For microbiological analysis, each saliva sample was mixed and 1 ml of it was transferred to a sterile tube with 3 ml of phosphate saline buffer (PBS), pH 7.0-7.2. All tubes were transported to the laboratory and the samples were processed for cultures within two hours.

Microbiological Analysis

The four selective media, MS-MUTV, MS-SOB, RDSM and Rogosa agar were prepared as described [25-27] and used for the detection of *S. mutans*, *S. sobrinus, Rothia spp.,* and *Lactobacillus spp.,* respectively. Trypticase Soy Agar with pH 7.0 (TSA) was used to culture the total facultatively anaerobic microbes while acidic Trypticase Soy Agar (a TSA) with the pH adjusted to 5.5 by addition of lactate acid was employed for the detection of aciduric bacteria. The saliva samples were serially diluted with PBS, from appropriate dilutions aliquots of 0.05 ml were inoculated onto the above agar media and incubated at 37 °C for 48h-72h in 5% CO_2. The cultures were examined using a stereomicroscope (magnification 20X) and the number of colonies were counted. The number of colony - forming units (CFU) per ml saliva was then calculated for each group of bacteria. The theoretical detection limit was ≤ 50 CFU/ml.

Sucrose Catabolism Velocity (SCV) Test

To an aliquot of 0.5 ml of stimulated saliva, sucrose and the pH indicator bromcresol blue were added to final concentrations of 5% w/v and 0.01% w/v, respectively. The mixture was immediately incubated at 37 °C in an Eppendorf tube for up to 24 hours. The samples were visually inspected after 30', 1 hour, 2 hours, 3 hours and 24 hours for any colour change. The colour changes of the pH indicator were registered as purple-blue, green-light green or yellow.

Statistical Analysis

Obtained bacterial counts were transformed to approximate normality by taking their logarithms. Data are presented in the form of absolute and relative frequencies (%), median values, means ± standard deviation (SD), and correlations (Spearman's rho). The statistical analysis included the Kolmogorov – Smirnov test for evaluating the normality and homogeneity of the data. In all parametric statistical tests, the observed significance level (P value) was computed either by independent samples t-test or by the paired samples t-test. The Mann Whitney test was used to compare the time for positive response in the sucrose catabolic test between caries - free and caries - active groups. In all hypotheses testing procedures the significance level was predetermined at P =0.05. Statistical analyses were done using the software SPSS v.22.0 (SPSS Inc, Chicago, Illinois, USA).

Results

A total of 95 children, 59 boys and 36 girls, were examined after division into two groups, the caries free (n=46) and the caries active (n=49). The group characteristics are given in Table 1. Stimulated saliva samples were collected from all participants. Based on the results of the cultivation method, the isolation frequencies of *S. mutans*, *S. sobrinus*, *Rothia spp.*, *Lactobacillus spp.* and aciduric bacteria are presented in Table 2. In total, *S. mutans* was detected in 77% of the children, S. sobrinus in 13%, Rothia spp. in 100%, Lactobacillus in 69% and aciduric microbes in 95%. Figure 1 pictures the isolation frequencies of the tested microbes between the caries active and caries free children Figure 2. For the caries active group 12 out of 49 children (24%) harboured

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all tested microbes. *S. sobrinus* was never detected alone nor was found in the saliva of caries free children. Mean values of the specific microbes detected in saliva in both groups are presented in Table 3. Significantly lower mean values of *S. mutans, S. sobrinus, Lactobacillus spp.* and aciduric microbes were detected in the saliva of caries free group compared to caries active group (Table 3). In the caries active children there was a positive correlation between the levels of *S. mutans*, *Lactobacillus spp.* and aciduric microbes (P <0.05), whereas no correlation was found for *Rothia spp.* (P<0.05) (Table 4).

Table 1: Characteristics of the study groups.

Characteristic	Caries free group	Caries active group
Total number of children	46	49
Boys	30	29
Girls	16	20
Age in years		
Mean ± SD	10.1 ± 2.9	8.7 ± 2.2
Median (min-max)	8.8 (5.1-15,9)	10.8 (4.7-16.5)
Caries status (Mean ± SD)		
dmfs/DMFS	0 (0)	14.7 (9.5)
ds/DS	0 (0)	11.0 (6.9)
ms/MS	0 (0)	1.4 (3.9)
fs/FS	0.5 (1.4)	2.3 (5.2)

Table 2: Isolation frequencies of *S. mutans, S. sobrinus, Rothia spp., Lactobacillus spp.* and aciduric microbes in saliva. The frequency is given in absolute numbers and as a percentage (in parenthesis) of children harbouring the microbes within each group.

Microbe	Caries free	Caries active
S. mutans	26 (56.5)	47 (95.9)
S. sobrinus	0(0)	12(26)
Rothia spp.	46 (100)	49 (100)
Lactobacillus spp.	20 (43.5)	46 (93.9)
Aciduric microbes	41 (89.1)	49 (100)

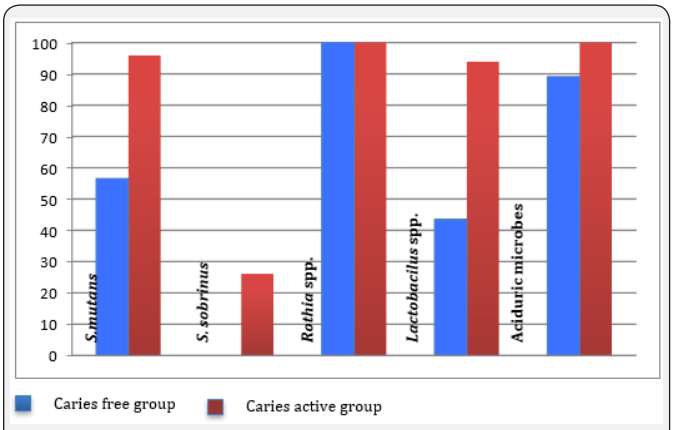

Figure 1: Isolation frequencies (%) of *S. mutans, S. sobrinus, Rothia spp., Lactobacillus spp.* and aciduric microbes in saliva of children with dmfs/DMFS = 0 (Caries free group) and ds/DS ≥ 1 (Caries active group).

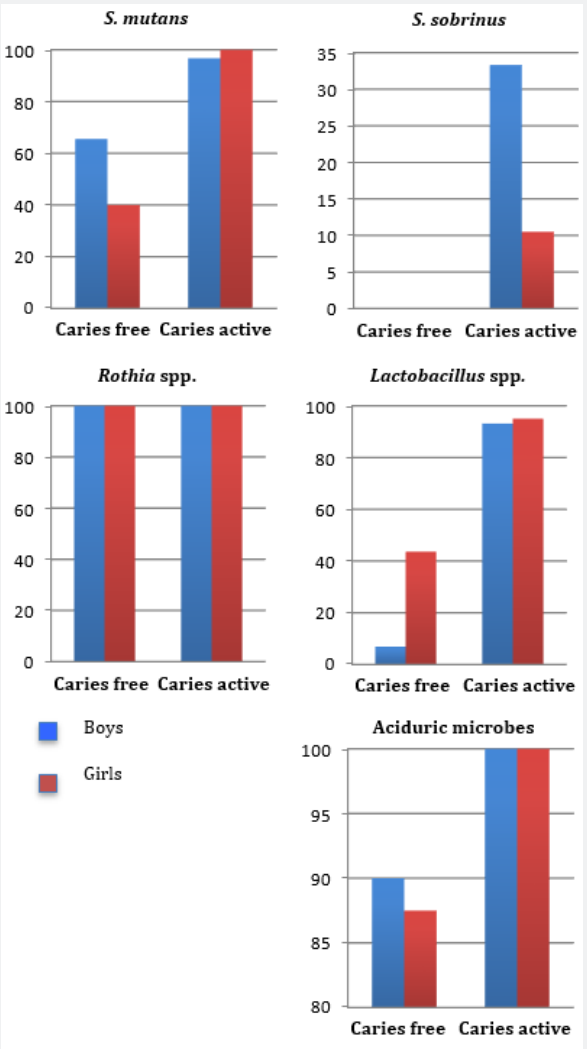

Figure 2: Isolation frequencies (%) of *S. mutans, S. sobrinus, Rothia spp., Lactobacillus spp.* and aciduric microbes in the saliva of children in relation with gender (blue: boys and red: girls) and caries activity (Caries free group: dmfs/DMFS = 0 and Caries active group: ds/DS ≥ 1).

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Table 3: Numbers of *S. mutans, S. sobrinus, Rothia spp, Lactobacillus spp*. aciduric microbes/ml of saliva in saliva samples of children with dmfs/DMFS = 0 (Caries free group) and ds/DS ≥ 1 (Caries active group).

Microbe (CFU/ml)	Caries free	Caries active
S. mutans		
Mean ± SD	$3.7 \times 10^5 \pm 5.8 \times 10^5$	$1.7 \times 10^6 \pm 3.2 \times 10^6$
Median	2.1×10^5	$3,2 \times 10^5$
[min-max]	[266 – 2.9×10^6]	[799 – 1.6×10^7]
S. sobrinus		
Mean ± SD	<5×10^1*	$1.1 \times 10^5 \pm 1.4 \times 10^5$
Median	<5×10^1*	4.8×10^4
[min-max]		[932 – 3.8×10^5]
Rothia spp.		
Mean ± SD	$1.8 \times 10^6 \pm 3.9 \times 10^6$	$2 \times 10^6 \pm 2.5 \times 10^6$
Median	7.3×10^5	1.1×10^3
[min-max]	[1.1×10^4 – 2.6×10^7]	[5.3×10^3 – 1.3×10^7]
Lactobacillus spp.		
Mean ± SD	$2.7 \times 10^4 \pm 5.3 \times 10^4$	$5.4 \times 10^5 \pm 1.1 \times 10^6$
Median	1.4×10^3	6.6×10^4
[min-max]	[133 – 2×10^5]	[133 – 4.8×10^6]
Aciduric microbes		
Mean ± SD	$7.6 \times 10^7 \pm 10^8$	$1.8 \times 10^8 \pm 2.7 \times 10^8$
Median	3.7×10^7	6.9×10^7
[min-max]	[1.3×10^6 – 4.2×10^8]	[1.3×10^5 – 1.2×10^9]

Table 4: Correlations between caries activity (caries free vs caries active group) and microbial numbers for each microbe in saliva.

Microbial population	P
S. mutans	0.045
S. sobrinus	0.131 (NS)
Rothia spp.	0.2 (NS)
Lactobacillus spp.	0.001
Aciduric microbes	0.032

P: level of significance, NS: not significant

The presence of oral microbes did not significantly differ either between boys and girls (in all Chi-Square tests P>0.05), or age (Spearman's RHO tests P>0.05); however *Rothia spp., Lactobacillus spp.* and facultatively aciduric microbes were less common in younger children and *S. sobrinus* appeared more in older ages (Figure 3). Figure 3 demonstrates the appearance of the tested microbes in relation with the gender and the caries activity. Figure 4 demonstrates how fast the saliva samples from caries free and caries active groups responded positively to the SCV test. The relation between the response time detected by SCV and caries activity is portrayed in Figure 5. Mean response times of caries active children were significantly faster than caries free group (P<0.05).

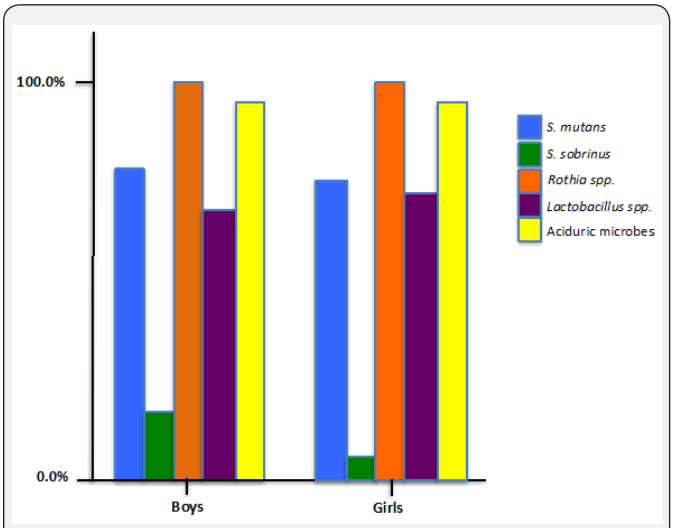

Figure 3: Isolation frequencies of tested microbes (in percentages of children harbouring *S. mutans, S. sobrinus, Rothia spp., Lactobacillus spp*. and facultatively aciduric microbes in their saliva) in relation with gender.

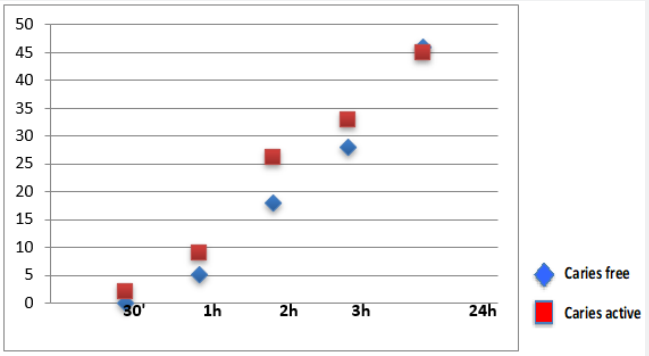

Figure 4: Distribution of positive responses to the sucrose catabolic test at specific time intervals (30', 1h, 2h, 3h, 24h).

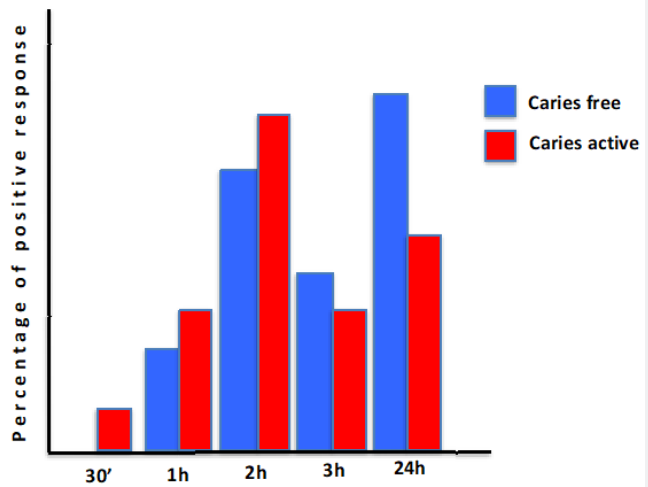

Figure 5: Correlation of positive response times between caries free and caries active children.

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Discussion

This study reports detection frequencies as well as mean levels of *S. mutans*, *S. sobrinus*, *Rothia spp.*, *Lactobacillus spp.* and facultatively aciduric microbes in saliva samples of children living in Greece, and the efficacy of SCV, a semi-quantitative test detecting the velocity of sucrose catabolism by bacteria in saliva, in assessing caries activity. Only two studies have reported detection frequencies of the two most common cariogenic bacteria *S. mutans*, *S. sobrinus* in Greek children but the results varied, and no safe conclusion could be drawn. In the most recent study [28], the authors concluded that 66% and 11% of the 97 children harbored *S. mutans* and *S. sobrinus* respectively. Detection and identification of the bacteria were performed by cultivation in selective media and PCR analysis of isolates. In the other study [29], *S. mutans* and *S. sobrinus* where found in nearly all children (87% and 90% respectively), by using checkerboard DNA-DNA hybridization detection. Moreover, the proportions of *S. sobrinus* were higher than those of *S. mutans*. Possibly, the diverse results are due to differentiate differentiate between closely related species [30]as are species of oral streptococci that are very close phylogenetically [31,32].

The present study indicates that 77% and 13% of the 95 children harboured *S. mutans* and *S. sobrinus*, respectively. These findings are in agreement with other studies coming from various continents across the world [33-35] as well as with the study of Fragkou et al. [28]. These results are also in accordance with findings in studies using PCR as identification method [15], [36-38]. In some studies *S. mutans* was detected more frequently [15], [39]. The contrusting results may be due to thd different ethnicity of the participants [40]. *S. sobrinus* was found in lower frequencies than *S. mutans* but correlated significantly ($P<0.05$) with caries activity. Moreover, *S. sobrinus* was not detected in the caries free group, and it was always found in mixed colonization with *S. mutans*. These results are in accordance with the literature [11,15,41] and support the close association of *S. sobrinus* with high caries activity.

Rothia spp. were detected in 100% of the children, which is in line with the literature [21,42], The mean concentration of the bacteria was 1.9 X 106 bacteria/ml of saliva, which is in accordance with previous studies [26]. The medium RDSM was developed to selectively allow growth of *R. dentocariosa* [26]. Using stereomicroscope, at least two types of colonies with dinstict morphology were found to grow on this medium. One type was smooth, mucoid, low convex, round with diameter >2mm while the other type had irregular high convex appearence with a diameter <1.5mm. When isolated on TSA, the colonies resembled those of Rothia and Gram-stained smears of these colonies showed the presence of Gram-positive pleomorphic rods and cocoid cells, which is consistent with the cell appearence of *Rothia spp.* No further attempt was made to identify the various isolates since no correlation could be established between the caries activity and the presence of the different colonies. Neither was a significant correlation between the proportion of *Rothia spp.* in the saliva samples and the caries activity of the children. Thus, this bacterium appears as an unsuitable biomarker of caries activity.

The mean amount of Lactobacillus spp. detected in the saliva of children with dental caries (5.4 x 105 CFU/ml) and without caries (2.7 x 10 CFU4/ml) is significantly higher those reported in the literature [43], whereas the prevalence (66%) is in agreement with earlier results [44]. High lactobacilli proportions have been positively correlated with carbohydrate intake, suggesting that they can represent a useful marker in assessing a cariogenic diet [45,18]. Caries activity was correlated with the presence and abundance of all studied bacteria, but statistically significant with streptococci, lactobacilli and total aciduric microbes. The positive correlation between caries activity and total cultivable aciduric microbial counts in combination with the large percentage of children in the caries free group that harbored *S. mutans* and the fact that in some caries-active children *S. mutans* was not detected, agree with views that high levels of *S. mutans*, do not always predict high caries activity [46,36].

The homeostasis of the oral ecosystem is affected by several genetic and environmental factors. Absence of a pathogen in some caries affected patients suggests a shift to an environmental change that does not promote the certain bacteria increase. This view is in accordance with the extended plaque hypothesis that suggests that there is a relation between demineralization/remineralization balance of the caries process and dynamic changes in the phenotypic/genotypic properties of bacteria involved. When sugar is frequently supplied, acidification becomes moderate and frequent. This may enhance the acidogenicity and acidurance of several bacteria adaptively. In addition, more aciduric strains, such as 'low-pH' non-Mutans streptococci, may increase selectively. Once the acidic environment has been established, Mutans streptococci and other aciduric bacteria may increase and promote lesion development by sustaining an environment characterized by 'net mineral loss' (aciduric stage). At this stage, *S. mutans*, *Lactobacillus spp.* as well as aciduric strains of non-Mutans streptococci may become dominant.

Environmental acidification is the main determinant of the phenotypic and genotypic changes that occur in the microflora during caries [8]. Given the fact that *Rothia spp.* are always found in both caries free and caries active environments, we assume that a phenotypic alteration to an enhanced cariogenic potential (pathogenesis) of the microbe may take place, influenced by environmental changes like carbohydrate rich diet [47]. The caries activity was positively correlated with response times in SCV test, but no correlation could be observed of this test with the presence and abundance of the studied microbes. SVC is an easy, non-invasive and fast test, and further studies are necessary to evaluate the efficacy and accuracy of it in assessing caries risk. The main limitation of the present study is the small sample size that did not allow to reach saturation of the curves studying microbial presence and abundance in correlation with caries activity and

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response times in SCV test. Nevertheless, the findings are not discrepant with those previously reported worldwide and they are also in line with modern aspects of caries etiopathology as depicted in the ecological plaque hypothesis and its modifications.

Conclusion

The significant differences in presence and abundance of *S. mutans*, *S. sobrinus*, *Lactobacillus spp.* and aciduric microbes in saliva between caries active and caries free children support the importance of all these microbes in the caries process. SCV test appears to be indicative of caries activity but not of the presence and/or abundance of the studied microbes.

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