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Salivary microbiota and caries occurrence in Mutans Streptococci–positive school children

ABSTRACT

Aim *To compare the composition of the salivary microbiota in caries-affected vs. caries-free mutans streptococci (MS)-positive children with mixed dentition.*
Methods *Twenty eight healthy, 11-12-year-old schoolchildren with high MS counts (>10^5CFU/mL) were included in this study. The children were screened with the Dentocult® SM Strip Mutans test (Orion Diagnostica, Espoo, Finland) and examined using the International Caries Detection and Assessment System (ICDAS). The microbial composition of the saliva was assessed using the Human Oral Microbe Identification Microarray (HOMIM). Microbial differences between caries-affected (n=18) and caries-free children (n=10) were compared by Mann-Whitney analysis.*
Results *The microbiota of the caries-affected vs. caries-free children was rather similar. Abiotrophia defectiva and Actinomyces meyeri/A. odontolyticus were significantly higher in caries-affected than in caries-free children (p=0.006, 0.046, respectively). Shuttleworthia satelles was significantly higher in caries-free compared to caries-affected children (p=0.031). A. defectiva and A. meyeri/A. odontolyticus correlated positively with caries severity measured by ICDAS Caries Index (p = 0.494, 0.454, 0.400 respectively) while S. satelles was negatively correlated with caries severity (p= -0.489).*

Conclusions *Salivary A. defectiva and A. meyeri/A. odontolyticus and are associated with caries occurrence in MS-positive children with mixed dentition.*

Keywords *Caries prevalence; Children with mixed dentition; Salivary microbiota.*

Introduction

Dental caries is a localised and transmissible infectious process that results in destruction of dental hard tissues [Loesche, 1986]. The localised chemical dissolution of tooth surface results from metabolic events taking place in the dental plaque. Although the role of bacteria in the development of a caries lesion is crucial, dental caries can be considered to be a multifactorial, sugar and plaque-associated disease. Dental plaque composition, salivary secretion, fluoride exposure, diet, and daily hygiene habits are all factors that affect caries occurrence [Takahashi and Nyvad, 2011].

Early acquisition of Streptococcus mutans has been associated with a considerable increase in caries risk in children [Thenisch et al., 2006]. Other species associated with caries include non-mutans streptococci, Lactobacillus, Actinomyces, Bifidobacterium, and Veillonella species [Beighton et al., 2004; Beighton, 2005]. As caries is considered a plaque-associated disease, contributors in plaque formation can also play an important role in caries formation even though they may not produce acids and dissolve tooth substance. Over the years, microbiological studies based on cultural and molecular methods have identified over 700 bacterial species in the human oral cavity of which 35% are not yet cultivable [Aas et al., 2005]. Furthermore, substantial intra/inter-individual microbial diversity has also been observed [Aas et al., 2005; Aas et al., 2008; de Paula et al., 2011]. Although most of these organisms are commensal, several bacterial species, including those that cannot be grown *in vitro*, can contribute in caries formation and their role is still not well known.

Different high-throughput techniques provide the possibility of surveying the oral microbial community structure at high resolution [Ahn et al., 2011; Nyvad et al., 2013]. These techniques have been used previously for analysis of bacterial composition in plaque and saliva [Aas et al., 2008; Crielaard et al., 2011; Birsan, 2014; Gomar-Vercher et al., 2014; Soderling et al., 2014]. The Human Oral Microbe Identification Microarray (HOMIM) is a phylogenetic approach based on 16S rRNA genes (rDNA) and has been applied to investigate the diversity of selected cultivable and non-cultivable species in the human oral cavity [Ahn et al., 2011].

The aim of this study was to examine the association between the composition of the salivary microbiota and

caries occurrence in children by comparing the salivary microbiota in caries-affected and caries-free mutans streptococci (MS)-positive subjects using the HOMIM method.

Materials and Methods

Participants and study design

A total of 122 children aged 11-12 years at Jabriya Intermediate School for Boys in Kuwait volunteered for the study and were clinically examined and screened for MS with the Dentocult® SM Strip Mutans test (Orion Diagnostica, Espoo, Finland). Seventy-three children met the inclusion criteria: 1) good general health; 2) willingness to participate; and 3) high MS counts (>10^5 CFU/ml) in either saliva or plaque (mutans streptococci-positive children). The subjects were participants of a xylitol intervention study [Söderling et al., 2014]. Of the baseline saliva samples, thirty were randomly selected for HOMIM analysis. Twenty eight samples were successfully analysed.

The study protocol was approved by the Joint Committee for Protection of Human Subjects in Research, Kuwait (project number DD02/10) with written informed consent from parents/guardians of every child, in full accordance with the Helsinki Declaration and following STROBE Guidelines. The ClinicalTrials.gov identifier of the study is NCT01528969.

Clinical examination

Before the clinical examination, students were asked to brush their teeth. All the clinical examinations were conducted in a mobile dental chair, using an artificial spot light and a mobile dental unit at the school clinic. The clinical examinations were conducted by one examiner (EH) using the International Caries Detection and Assessment System (ICDAS) criteria [Ismail et al., 2007]. The examiner had training and experience in the use of ICDAS from an earlier study with high consistency (kappa > 0.9)[Runnel et al., 2013].

The following caries indices were calculated from the data to evaluate caries experience and severity:

- ICDAS Caries Index (ICDAS CI): calculated by counting all ICDAS caries scores (1-6) of all surfaces divided by total number of carious teeth [ElSalhy et al., 2013].
- DMFT/dmft: total decayed (dentine caries ICDAS caries scores 4-6), missing (due to caries) and/or filled teeth (D4-6MFT/d4-6mft).
- Total number of enamel carious surfaces (D1-3S; ICDAS caries scores 1-3).
- Total number of dentine carious surfaces (D4-6S; ICDAS caries scores 4-6).

Saliva collection

For saliva collection day, all students were asked to refrain from oral hygiene for approximately 24 h before the saliva collection. Saliva collection was scheduled at the beginning of the day and took place for all subjects at the same time in the school gymnasium under the supervision. After swallowing pre-existing saliva, each student was asked to chew a standard piece of paraffin wax and 2 ml of stimulated saliva was collected in sterile plastic tubes (Corning®, Tewksbury, MA, USA). The saliva samples were stored in ice before allocating them for analysis. At baseline, saliva collection and clinical examination was done in the same day.

For the HOMIM analyses performed in the Forsyth Institute, Boston; a 1 mL sample of the saliva was pipetted onto 10 µL TE-buffer (Sigma-Aldrich, St. Louis, MO, USA) and stored at -70 °C before shipment on dry ice. Once in the USA, genomic DNA was isolated using the MasterPure Gram Positive DNA Purification kit (Epicentre Biotechnologies, Madison, WI, USA), with modifications (http://mim.forsyth.org).

Human oral microbe identification microarray analyses

Microbial profiles were generated from image files of scanned HOMIM microarrays (http://bioinformatics.forsyth.org/homim/). In brief, concentration levels of approximately 300 oral taxa were determined by microarray hybridisation using a fluorescent readout reverse-capture method. Fluorescently-labelled PCR products from DNA samples were captured by 16S rRNA-based probes attached to glass slides. The fluorescent intensity for each probe was scanned, normalized, and scaled as previously reported. Signals of < 2 x background were considered to be negative and assigned a HOMIM level score of 0. Positive hybridisation signals were categorised into 5 levels, with 1 indicating a signal that was just detectable and 5 indicating maximum signal intensity.

Outcome measures

The primary outcomes were the relative levels of bacteria in stimulated saliva measured by HOMIM. The relative level of each taxa was compared between caries-affected (ICDAS CI >0, n=18) and caries-free (ICDAS CI=0, n=10) children. Their levels were correlated with the caries severity, caries experience, number of enamel and dentine surfaces affected by caries.

Statistical Analyses

Data were analysed using SPSS 21.0 software (SPSS Inc., Chicago, Ill., USA). Relative levels of taxa were analysed by comparing and caries-affected and caries-free subjects. Data normality was tested by Shapiro-Wilk test. As data was not normally distributed, Mann-Whitney analysis was used to determine statistical significance of the differences in bacterial levels between the study groups. Correlations between the proportions of bacterial taxa and ICDAS CI, DMFT/dmft, D1-3S, D4-6S indices were analysed by the Spearman correlation coefficient (ρ). P-value of less

than 0.05 was considered significant. Results were also adjusted for multiple comparisons by the false-discovery rate ($\alpha = 0.05$).

Results

Caries lesions were detected in 64% (18) of the 28 children who participated in this study. The median ICDAS CI was 3.2 (25th percentile = 0.5; 75th percentile = 4.7; range = 0-15) and the median DMFT/dmft was 2 (0, 2.75; 0-10). The median D1-3S was 2.0 (0, 3.75; 0-8) and median was D4-6S 1.0 (0, 2.75; 0-24).

Ninety eight bacterial species and 9 bacterial clusters were detected. Out of them, 15 species of Streptococcus were detected. The microbiota of the caries-affected vs. caries-free children was rather similar (Fig. 1) and showed significant differences only for three microbial taxa. Mean levels of Abiotrophia defectiva and Actinomyces meyeri/A. odontolyticus were significantly higher in the caries-affected than in the caries-free group ($p=0.006$, 0.046, respectively). Shuttleworthia satelles was significantly higher in the caries-free group compared to the caries-affected group ($p=0.031$) (Fig. 1).

A. defectiva and A. meyeri/A. odontolyticus were positively correlated with ICDAS CI, DMFT/dmft, and D4-6S ($p<0.05$) (Table 1) while S. satelles was negatively correlated with ICDAS CI, andD1-3S. The other bacterial species having significant correlations with caries measures are shown in Table 1.

Discussion

We found that the microbiota of the caries-affected children was associated with higher levels of A. defectiva and A. meyeri/A. odontolyticus compared with caries-free children. A. defectiva has been associated with bacteraemia and endocarditis [Senn et al., 2006]. It is a nutritional variant of Streptococcus and has been previously detected at higher levels in plaque of caries-free than caries-affected children [Becker et al., 2002; Corby et al., 2005]. It has been detected in plaque of 63% of caries-free children compared to 23% of children with early childhood caries [Kanasi et al., 2010a]. However, higher salivary proportions of A. defectiva were associated with higher DMFT/dmft, and more prevalent dentine caries in the present study, but they were not associated with the number of enamel caries lesions. A. defectiva was not significant in the previous HOMIM saliva study analysing expectorated whole saliva in caries active children (DMFT/dmft >8) [Luo et al., 2012]. This discrepancy in results can be due to different sample types used in different populations and would be worth further investigation.

In our study, Actinomyces species were significantly higher in caries-affected children than in caries-free children. The levels also correlated with caries severity and the number of dentine caries lesions. A. israelii and A. odontolyticus have been detected more frequently in caries-affected subjects [Tanner et al., 2002; Kanasi et al., 2010b]. Actinomyces species have also been detected at high levels in caries initiation compared to those of cavitated and dentine plaque samples [Ahn et al., 2011]. A previous HOMIM analysis of whole saliva found the Actinomyces cluster to be present only in caries-affected subjects which also supports our results [Luo et al., 2012]. Actinomyces species are major participants in oral co-aggregation interactions and have been shown to be associated with root caries which, however, is not a concern in children [Mc Crory, 2013].

Levels of Lachnospiraceae sp., Capnocytophaga granulosa, Campylobacter concisus, C. rectus, Prevotella histicola, and P. melaninogenica were correlated with the number of enamel caries lesions. Luo et al. found out that Lachnospiraceae sp. were detectable only in the caries active-group [Luo et al., 2012]. C. granulosa was one of the most detectable bacterial taxa in the study by Kanasi et al. with a significant difference between early childhood caries group and caries-free groups [Kanasi et al., 2010a]. P. melaninogenica were also detected only in caries-active group in a Luo et al. [2012] study.

In the present study, ICDAS was used as the main measure of caries and in categorising the children into caries-free and caries-affected groups instead of DMFT/dmft. The DMFT/dmft includes both filled and extracted teeth in addition to caries and the total number does not reflect caries only. Filling or extracting carious teeth affect the oral environment and its flora. In addition, using D1-3S can be a measure of caries activity as it reflects the number of enamel caries lesions present in subjects. However, D1-3S also includes the non-active, arrested enamel caries lesions.

We found that the salivary microbiota of caries-free children was significantly associated with higher levels of S. satelles compared to caries-affected children. In addition, S. satelles was also associated with lower ICDAS CI and lower number of enamel caries lesions with no correlations with DMFT or D4-6S. This bacterial species have been isolated from endocarditis cases but nothing is known about its role in caries [Marsh, 2010].

S. mutans and lactobacilli are the most studied bacteria in association with caries. In the present study, all children were originally selected to the study based on high mutans streptococci levels in either plaque or saliva, thus an association could not be studied. Previous HOMIM analyses of expectorated whole saliva in children with mixed dentition showed no association between S. mutans and caries [Luo et al., 2012]. Low proportions of S. mutans in caries-affected children were reported in a cultural analysis of saliva of the children with extensive caries as well as by molecular analysis in childhood caries, advanced carious lesions and root caries [Aas et al., 2008; Kanasi et al., 2010a; Luo et al., 2012; Mitrakul et al., 2013].

The results are similar for lactobacilli which were almost absent in the present study as well as previous studies and could not be correlated with caries [Yang et al., 2012; Mitrakul et al., 2013]. On the other hand, some species of lactobacilli as well as S. mutans and S. sobrinus in plaque have been shown to be positively associated with caries using a checkerboard assay in addition to culture based microbial studies [Rupf et al., 2006; Kanasi et al., 2010b]. A major concern with molecular detection of bacteria is the bias in detection, as the detection is based on primers

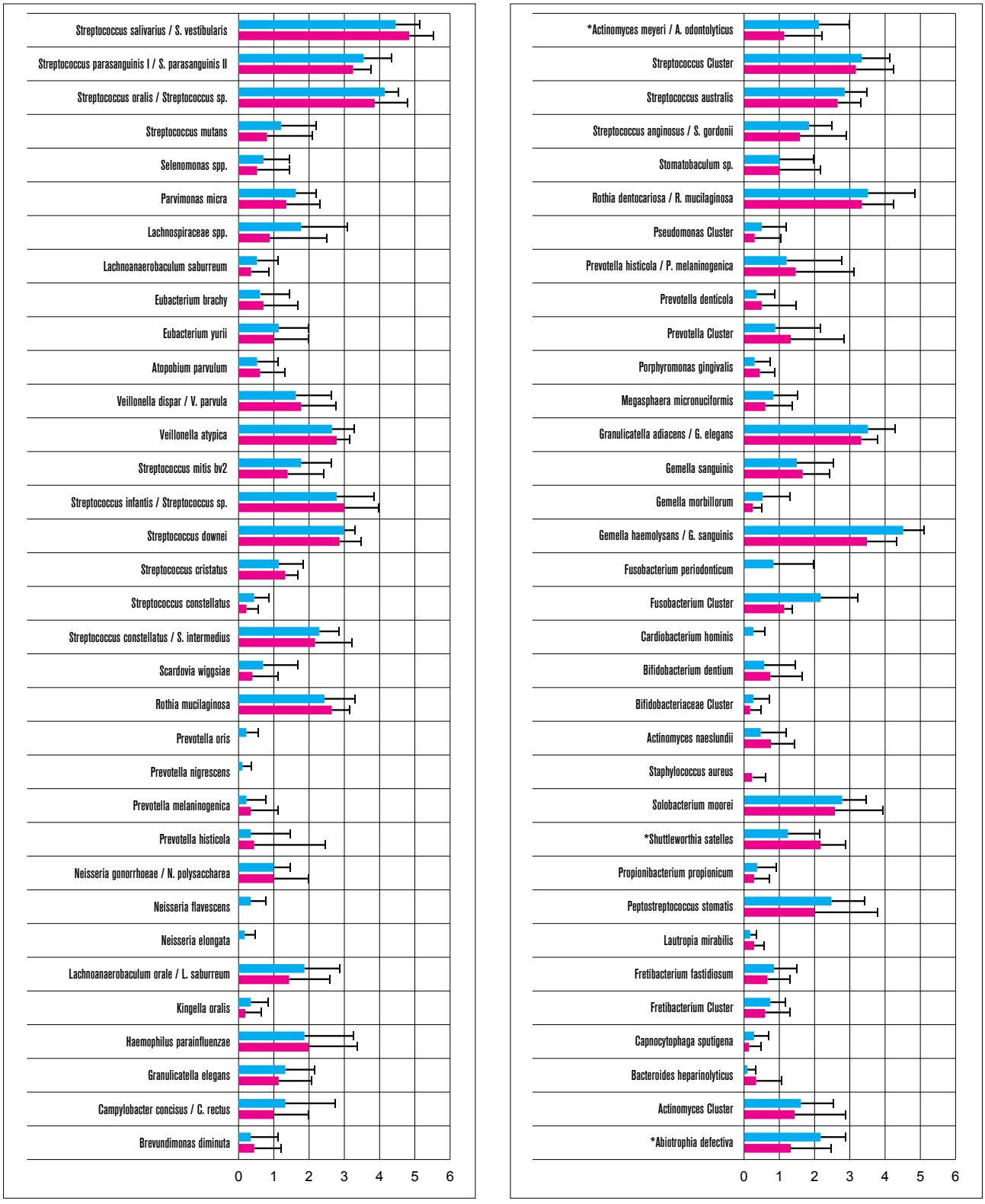

FIG. 1 HOMIM Signals Intensity Level.

	ICDAS CI	DMFT/dmft	D1-3S	D4-6S
Abiotrophia defectiva	0.495*	0.471*		0.408*
Actinomyces meyeri/A. odontolyticus	0.400*	0.492*		0.462*
Shuttleworthia satelles	-0.489*		-0.390*	
Prevotella histicola/P. melaninogenica			0.391*	
Campylobacter concisus/C. rectus			0.412*	
Capnocytophaga granulosa			0.393*	
Lachnospiraceae sp.			0.512**	0.411*
* p < 0.05 FDR level (two-tailed)				

TABLE 1 Correlation coefficients of different caries indices and salivary bacteria species. Only bacteria with significant correlations are shown.

or probes chosen and not an open-end detection.

All participants in this study were male students as the study was done in a boys' school. The main reason for single gender is that there are no mixed gender government schools in Kuwait. However, having only male participants should not affect the results of this study, because previous microbial studies on oral microbiota showed no effect of gender on the microflora [Tanner et al., 2002; Luo et al., 2012]. In addition, there are no major differences in use of sugar products between male and female school children in Kuwait, although the difference in oral hygiene habits is the same as in other countries [Honkala et al., 2006]. The diet and hygiene habits of the children and their relationship to caries were previously evaluated [ElSalhy et al., 2013]. The small number of subjects is the main limitation in the present study. It was an explanatory study to identify additional members in oral microflora which warrant further investigation to clarify their contribution in the caries process.

In summary, the complexity of the caries process requires information about all bacterial species that may participate in its formation. In this study, A. defectiva and A. meyeri/A. odontolyticus in stimulated saliva were associated with caries occurrence.

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